Second Edition

Handbook of Plant-Based Fermented Food and Beverage Technology

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Handbook of Plant-Based Fermented Food and Beverage Technology

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Preface

Fermented food is a very interesting category of food products. Not only can it be produced with inexpensive ingredients and simple techniques, but it also makes a significant contribution to human diet, especially in rural households and village communities in many areas of the world. In every ethnic group in the world, there are fermented foods produced from recipes handed down from generation to generation. Such food products play an important role in cultural identity, local economy, and gastronomical delight. With progresses in biological and microbiological sciences, the manufacture of some of the more popular fermented food products has been commercialized, while others are still produced at home or in small-scale industries using traditional methods.

Fermentation changes the initial characteristics of a food into a product that is significantly different but highly acceptable by consumers. Of course, consumer preference for fermented food varies within and between cultures. For example, within the United States, many consumers like sauerkraut, although some do not. The trend in North America is toward acceptance and preference of foreign fermented non-European food products. You can find Chinese fermented black beans and Korean Kim Chee in most Asian markets and some major grocery chains in North America.

Although reference books on fermented foods have been in existence for at least 50 years, those with details on the science, technology, and engineering of food fermentation began to appear after 1980. Scientific literature in the past decade has been flooded with new applications of genetic engineering in the fermentation of food products, especially in the breeding of plants.

This book provides an up-to-date reference for fermented foods and beverages derived from plant products. Almost every book on food fermentation has something not found in others. The *Handbook of Plant-Based Fermented Food and Beverage Technology* provides a detailed background of history, microorganisms, quality assurance, and the manufacture of general fermented food products and discusses the production of six categories of fermented foods and beverages:

- Soy: soy sauce, tofu
- Fruit: wine, olive
- Vegetables: red beet juice, pickles
- Cereals: sourdough, beer
- Specialties: tea, coffee
- Ingredients: enzymes, probiotic bacteria

Traditional fermented products are discussed, including wine, sourdough, and olive. We also present details of the manufacture and quality characteristics of some fermented foods that are less common in other books on fermentation in the English language: fermented products from East Asia, for example, soy sauce, and fermented cereal products from Turkey, for example, boza. Although this book has covered a variety of products, many topics are omitted for different reasons including space limitation, product selection, and the contributors' areas of expertise.

This book is unique in several aspects: it is an updated and comprehensive reference source, it contains topics not covered in similar books, and its contributors include experts from government, industry, and academia worldwide. The book has 47 chapters and is divided into seven parts. It is the cooperative effort of more than 60 contributors from more than 20 countries (Argentina, Brazil, Canada, China, Denmark, Germany, Himalaya, Hungary, India, Indonesia, Italy, Japan, Malaysia, Mexico, Serbia, Singapore, Spain, Thailand, Turkey, and the United States) with expertise in one or more fermented products, led by an editorial team of nine academicians from seven countries (Brazil, Canada, Denmark, Mexico, Serbia,

Singapore, Spain, Turkey, and the United States). The seven associate editors of this team are all experts in their areas of specialization as indicated below:

- Dr. Francisco Noé Arroyo-López is a research scientist with the Department of Food Biotechnology, Instituto de la Grasa (CSIC), Seville, Spain. His expertise focuses on table olives and fermented vegetables, as supported by his published work in journals and book chapters.
- Dr. Lihua Fan is a research scientist with Agriculture and Agri-Food Canada. Food fermentation and microbial safety of fruits and vegetables are her major research interests, among others, as documented by her numerous published journal articles, book chapters, and other professional works.
- Professor Åse Solvej Hansen is with the Department of Food Science, University of Copenhagen. Her major research has focused on rye and wheat baking technology and microbiology, including the influence of sourdough fermentation on bread quality, as documented by her extensive publications.
- Dr. M. Eugenia Jaramillo-Flores is with the Department of Food Science, National Polytechnic Institute, Mexico. With an expertise in food biochemistry and biotechnology, she focuses her research on nutraceuticals and functional foods, as confirmed by her published work in peer-reviewed journals and other professional publications that are nationally and internationally recognized.
- Dr. Marica Rakin is with the Department of Biochemical Engineering and Biotechnology, University of Belgrade, Serbia and Montenegro. She has published extensively on fermentation, and her research focuses on the production of bio-fermented vegetables and drinks and the production of bio-ethanol related to renewable biomass.
- Dr. Rosane Freitas Schwan is an agronomist at Federal University of Lavras, Minas Gerais, Brazil, where she is mostly involved in research on microbiology of several fermented foods including cocoa and coffee. In addition, she is studying the development of culture starters to improve the quality of fermentative processes.
- Dr. Weibiao Zhou is the director of the Food Science and Technology Program at the National University of Singapore. Dr. Zhou's research interests and expertise are in food engineering and food processing, particularly bakery products, as supported by his numerous publications.

In sum, the approach for this book makes it an essential reference on plant-based fermented foods and beverages. The editorial team thanks all the contributors for sharing their experience in their fields of expertise. They are the people who made this book possible. We hope you enjoy and benefit from the fruits of their labor.

We know how hard it is to develop the content of a book. However, we believe that the production of a professional book of this nature is even more difficult. We thank the production team at CRC Press. You are the best judge of the quality of this book.

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Editors

Y. H. Hui received his PhD from the University of California at Berkeley. He is currently a senior scientist with the consulting firm of Science Technology System at West Sacramento, California. He has authored, coauthored, edited, and coedited more than 30 books in food science, food technology, food engineering, and food laws, including *Handbook of Food Product Manufacturing* (2 volumes, Wiley, 2007) and *Handbook of Food Science, Technology, and Engineering* (4 volumes, CRC Press, 2006). He has guest-lectured in universities in the Americas (North, Central, and South), Europe, and Asia and is currently a consultant for the food industries, with an emphasis on food sanitation and food safety.

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Associate Editors

Francisco Noé Arroyo-López received a degree in biology from the University of Jaen, Spain (2001), where he also received his PhD. His doctoral thesis at the University of Seville (Spain) was about table olive fermentations (2007). Currently, he is a postdoctoral researcher at the Department of Food Biotechnology from the Instituto de la Grasa (CSIC). His areas of research are mainly application of mathematical models to predict microorganism growth/survival in table olive processing and the use of molecular methods for identification of lactic acid bacteria, yeasts, and *Enterobacteriaceae* related to this fermented vegetable. He has more than 40 international publications in peer-reviewed SCI journals as well as 6 chapter books. He is a professor in diverse international table olive–related courses.

Lihua Fan is a research scientist working at the Atlantic Food and Horticulture Research Centre, Agriculture and Agri-Food Canada, since 1998. She received her MS and PhD in food science from China Agricultural University in 1989 and 1991, respectively. Her research interests include natural antimicrobials on safety and quality of whole and fresh-cut fruit and vegetables, bioactive compounds and biopreservatives, food fermentation, and disease control of postharvest horticultural produce. Fan is a member of the International Association for Food Protection and a member and subpanel member of the Food Microbiology Division of the Institute of Food Technologists. She is a committee member of the Multi-State Research Project S-294 for the National Information Management Support System, USA, and a Graduate Supervisory Committee member in Food Program of Dalhousie University, Canada. In recognition of her supervising efforts, she recently received the Canadian Forces Liaison Council's Nova Scotia Provincial Award for Best Practices in Employer Support.

Åse Solvej Hansen is an associate professor of the Department of Food Science, University of Copenhagen, Denmark. She received her MSc in food science and technology from the Royal Veterinary and Agricultural University, Denmark, and has worked with quality control and product development for several years in a Danish industrial bakery. With a background in practical problems from the baking industry, she has worked with cereal research focusing on wheat and rye baking technology, including the influence of sourdough fermentation on bread quality. Her bread quality research has also included the formation of flavor compounds, phytate degradation, and microbial stability of sourdoughs.

M. Eugenia Jaramillo-Flores received her BS in biochemical engineering and MS and PhD in food at the National School of Biological Sciences, National Polytechnic Institute, Mexico. She is currently a professor of food biochemistry and the chair of the PhD program in biotechnology. She has directed undergraduate and graduate theses on food science and food biotechnology. She belongs to different international scientific associations of chemical, food, and pigments (Institute of Food Technologists, American Chemical Society, and International Carotenoids Society). She has participated in countless international conferences and has published articles in both national and international journals. She works as a researcher in the area of nutraceuticals and functional foods, particularly in the evaluation of the effect of the process.

Marica Rakin is an associate professor since December 2011 at the Department of Biochemical Engineering and Biotechnology, Faculty of Technology and Metallurgy, University of Belgrade, Belgrade, Serbia, where she received PhD. Her thesis was titled "The Obtaining of Bioactive Materials on Basis Brewer's Yeast and Healing Vegetables." She has extensive experience in the application and development of novel by-products of food in agroindustry, with an emphasis on the technological and microbiological aspects of the production of biofermented vegetables and drinks and the bioethanol production on renewable biomass. She has special expertise in yeast (*Saccharomyces cerevisiae*) fermentation and lactic acid bacteria with an emphasis on their applications.

Rosane Freitas Schwan is an agronomist and associate professor in microbiology and has been working for more than 15 years at the Federal University of Lavras, Minas Gerais, Brazil, where she is mostly involved in research on microbiology of several fermented foods. She worked for 12 years at Cocoa Research Centre in Itabuna, Bahia, Brazil, where she developed a microbial cocktail for cocoa fermentation. Her research interest includes biodiversity and phylogeny of bacteria, yeasts, and filamentous fungi involved in cocoa, coffee, and other tropical fermentations and biodiversity of yeasts in agrofood ecosystems and development of culture starters to improve the quality of fermentative processes.

Weibiao Zhou is a full professor and the director of the Food Science and Technology Program at the National University of Singapore. He is a board member of the Agri-food and Veterinary Authority of Singapore, a fellow of the Australian Institute of Food Science and Technology, a council member of the Singapore Institute of Food Science and Technology, a member of the Singapore Food Standards Committee, and a member of the editorial boards of five international peer-refereed journals in the area of food engineering and food processing. Educated in China and Australia, he has held various professional positions in a number of organizations in Australia, Canada, China, France, and the United States. With more than 180 scientific publications, Zhou's research interests and expertise are in food engineering and food processing, particularly baking processes, dairy processes, drying, functional foods, and process modeling, optimization, and control.

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Part I

Introduction

1

*Fermented Plant Products and Their Manufacturing**

Y. H. Hui

CONTENTS

1.1 Introduction

The availability of fermented foods has a long history among different cultures. The acceptability of fermented foods also differ because of cultural habits. A product highly acceptable in one culture may not be so acceptable by consumers in another culture. The number of fermented food products is countless. The manufacturing processes of fermented products vary considerably owing to variables such as food groups, form and characteristics of the final products, kind of ingredients used, and cultural diversity. It is beyond the scope of this chapter to address all the manufacturing processes used to produce fermented

foods. Instead, this chapter is organized to address fermented food products based on plant food groups such as cereal, soy, and vegetables. Within each food group, the manufacturing processes of typical products are addressed. This chapter is only an introduction to the manufacturing processes of selected fermented food products. Readers should consult the references and other available literature for more detailed information.

1.2 Fermented Cereal Products (Breads and Related Products)

1.2.1 Kinds of Products and Ingredients

In wheat-producing countries or areas, baked yeast bread is a major staple in the people's diet. This is common in more developed countries; elsewhere, other forms of bread may be the major staple. Baked bread may come in different forms such as regular yeast breads, flat breads, and specialty breads. Today, even retarded (chilled or frozen) doughs are available to meet consumers' preference for a semblance of home-cooked food. For countries or areas with less available energy, other forms of bread such as steamed bread and boiled breads are available. Fried breads are consumed mainly as breakfast or snack items. Table 1.1 lists some examples of different types of breads (Cauvain and Young 1998; Groff and Steinbaecher 1995; Huang 1999; Pyler 1988; Quail 1998; Quarooni 1996).

Today, as a result of centuries of breeding selection, there are different types of wheat available to suit production environments in various regions with diverse climatic conditions. Wheat used for making bread includes hard wheat, soft wheat, or a combination of both, to meet product specifications. Wheat kernels are milled by the removal of the bran and germ and further processed into wheat flour. Traditionally, this flour is the major ingredient for baking bread. For some health-conscious consumers, whole wheat flour is the flour of choice for making bread nowadays. Wheat bran is also added to increase the fiber content of the product. Table 1.2 lists the proximate composition of wheat and some of their common wheat products (Cauvain and Young 1998; Groff and Steinbaecher 1995; Pyler 1988).

In the manufacture of various wheat-based breads and related products, the major ingredients are wheat flour, yeast, sourdough bacteria (optional), salt, and water. Other ingredients vary considerably with the types of product produced. These may be grossly classified as optional ingredients, additives, or processing aids. Each country has its own regulations and requirements. Table 1.3 lists the basic ingredients, optional ingredients, additives, and processing aids used in the manufacturing of bread and related products (Cauvain and Young 1998; Groff and Steinbaecher 1995; Pyler 1988).

TABLE 1.1

Types of Bread and Related Products

Type	Examples
Baked breads	
Regular yeast breads	Bread (white, whole wheat, or multigrain)
Flat (layered) breads	Pocket bread, croissants
Specialty breads	Sourdough bread, rye bread, hamburger bun, part-baked bread, Danish pastry, stuffed bun
Chilled or frozen doughs	Ready-to-bake doughs, retarded pizza doughs, frozen proved dough
Steamed breads	Chinese steamed bread (mantou), steamed stuffed bun
Fried breads	Doughnuts
Boiled breads	Pretzels

Sources: Cauvain, S. P., and L. S. Young, *Technology of breadmaking*, Blackie Academic & Professional, London, 1998. With permission. Groff, E. T., and M. A. Steinbaecher, *Cereal Foods World*, 40(8), 524–526, 1995. With permission. Huang, S. D., in *Asian foods: science and technology*, edited by Ang, C. Y. W., K. S. Liu, and Y. W. Huang, 71–109, Technomics Publishing, Lancaster, PA, 1999. With permission. Pyler, E. J., *Baking science and technology*, 3rd ed., Vols. 1 and 2, Sosland Publishing, Merriam, KS, 1988. With permission. Qarooni, J., *Flat bread technology*, Chapman & Hall, New York, 1996. With permission. Quail, K. J., *Arabic bread production*, American Association of Cereal Chemists, St. Paul, MN, 1998. With permission.

TABLE 1.2

Composition of Wheat, Flour, and Germ

Material	Moisture (%)	Protein (%)	Fat (%)	Total CHO (%)	Fiber (%)	Ash (%)
Wheat						
Hard red spring	13	14	2.2	69.1	2.3	1.7
Hard red winter	12.5	12.3	1.8	71.7	2.3	1.7
Soft red winter	14	10.2	2	72.1	2.3	1.7
White	11.5	9.4	2	75.4	1.9	1.7
Durum	13	12.7	2.5	70.1	1.8	1.7
Flour, straight						
Hard wheat	12	11.8	1.2	74.5	0.4	0.46
Soft wheat	12	9.7	1	76.9	0.4	0.42
Flour, patent						
Bread	12	11.8	1.1	74.7	0.3	0.44
Germ	11	25.2	10	49.5	2.5	4.3

Sources: Cauvain, S. P., and L. S. Young, *Technology of breadmaking*, Blackie Academic & Professional, London, 1998. With permission. Groff, E. T., and M. A. Steinbaecher, *Cereal Foods World*, 40(8), 524–526, 1995. With permission. Pyler, E. J., *Baking science and technology*, 3rd ed., Vols. 1 and 2, Sosland Publishing, Merriam, KS, 1988. With permission.

TABLE 1.3

Bread-Making Functional Ingredients

Kind	Examples
Basic ingredient	
Wheat flour	Bread flour, whole wheat flour
Yeast	Compressed yeast, granular yeast, cream yeast, dried yeast, instant yeast, encapsulated yeast, frozen yeast, pizza yeast, deactivated yeast
	Saccharomyces cerevisiae, *S. carlsburgenis*, *S. exisguus*
Salt	
Water	
Optional ingredients	Whole wheat flour, gluten, soya flour, wheat bran, other cereals or seeds, milk powder, fat, malt flour, egg, dried fruit, vitamins
	Sourdough bacteria:
	Lactobacillus plantarum, *L. brevis*, *L. fermentum*, *L. sanfrancisco*
	Other yeasts
Additives	
Emulsifier	Diacetylated tartaric acid esters of monoglycerides and diglycerides of fatty acids (DATA esters), sodium stearyl-2-lactylate (SSL), distilled monoglyceride, Lecithin
Flour treatment agents	Ascorbic acid, L-cysteine, potassium bromate, potassium iodate, azodicarbonamide
Preservatives	Acetic acid, potassium acetate, sodium diacetate, sorbic acid, potassium sorbate, calcium sorbate, propionic acid, sodium propionate, calcium propionate, potassium propionate
Processing aids	Alpha-amylase, hemicellulose, proteinase, novel enzyme systems (lipases, oxidases, peroxidases)

Sources: Cauvain, S. P., and L. S. Young, *Technology of breadmaking*, Blackie Academic & Professional, London, 1998. With permission. Groff, E. T., and M. A. Steinbaecher, *Cereal Foods World*, 40(8), 524–526, 1995. With permission. Pyler, E. J., *Baking science and technology*, 3rd ed., Vols. 1 and 2, Sosland Publishing, Merriam, KS, 1988. With permission.

1.2.2 Regular Bread

Table 1.4 lists the basic steps in bread manufacturing (Cauvain and Young 1998; Groff and Steinbaecher 1995; Pyler 1988).

There are three basic processes in commercial bread-making: straight dough, sponge-and-dough, and continuous baking processes. The process to be used is determined by the manufacturer and the equipment available in the baking plant. Table 1.5 lists the basic steps in the different processes. The major difference is in the way the dough is prepared and handled (Cauvain and Young 1998; Groff and Steinbaecher 1995; Pyler 1988).

Because the dough may be prepared in various ways, the amounts of ingredients used differ accordingly. Table 1.6 lists two formulations comparing the differences in ingredients that arise from differences in the dough preparation processes (Cauvain and Young 1998; Groff and Steinbaecher 1995; Pyler 1988).

1.2.3 Retarded Dough

As indicated previously, retarded dough is also available to some consumers. This type of dough is more accessible in developed countries where refrigerators and freezers are more common. Dough is prepared so that the fermentation is carefully controlled and the dough is packed inside the container. Storage of this package is also carefully controlled. When the package is open, consumers can just follow the instructions on the package to bake their own bread. The technology is proprietary to the manufacturers, but there are some guidelines available (Table 1.7; Cauvain and Young 1998; Groff and Steinbaecher 1995; Pyler 1988).

1.2.4 Flat (Layered) Bread

Flat bread is a general term for bread products that do not rise to the same extent as regular bread. Flat breads are common commodities in Middle Eastern countries and in countries or areas with less accessible energy. In developed countries, flat breads are considered specialty breads. The making of the dough is similar to that of regular bread. However, the dough is flattened and sometimes layered before it is baked directly inside the hearth or in an oven. Table 1.8 lists the general production scheme for flat breads (Quail 1998; Qarooni 1996).

TABLE 1.4

Basic Steps in Regular or Common Bread-Making

Preparation of basic and optional ingredients
Preparation of yeast or sourdough for inoculation
Mixing of proper ingredients to make dough
Fermentation
Remixing of dough (optional)
Sheeting
Molding and panning
Proofing in a temperature- and relative humidity–controlled chamber
Decorative cutting of dough surface (optional)
Baking, steaming, frying, or boiling
Cooling
Packaging
Storage

Sources: Cauvain, S. P., and L. S. Young, *Technology of breadmaking*, Blackie Academic & Professional, London, 1998. With permission. Groff, E. T., and M. A. Steinbaecher, *Cereal Foods World*, 40(8), 524–526, 1995. With permission. Pyler, E. J., *Baking science and technology*, 3rd ed., Vols. 1 and 2, Sosland Publishing, Merriam, KS, 1988. With permission.

TABLE 1.5

Various Bread-Making Processes

Straight Dough Baking Process
Weigh out all ingredients
Add all ingredients to mixing bowl
Mix to optimum development
First fermentation: 100 minutes at room temperature, or at 27°C for 1.5 hours
Punch
Second fermentation: 55 minutes at room temperature, or at 27°C for 1.5 hours
Divide
Intermediate proofing: 25 minutes at 30°C to 35°C, 85% RH
Mold and pan
Final proofing: 55 minutes at 30°C to 35°C, 85% RH
Bake at 191°C to 232°C for 18 to 35 minutes to approximately 100°C internal temperature
Sponge-and-Dough Baking Process
Weigh out all ingredients
Mix part of flour, part of water, yeast and yeast food to a loose dough (not developed)
Ferment 3 to 5 hours at room temperature, or at 21°C for 12 to 16 hours
Add other ingredients and mix to optimum development
Fermentation (floor time), 40 minutes
Divide
Intermediate proofing: 20 minutes at 30°C to 35°C, 85% RH, or at 27°C for 30 minutes
Mold and pan
Final proofing: 55 minutes at 30°C to 35°C, 85% RH
Baking at 191°C to 232°C for 18 to 35 minutes to approximately 100°C internal temperature
Continuous Baking Process
Weigh out all ingredients
Mixing of yeast, water, and maybe part of the flour to form a liquid sponge
Addition of remaining flour and other dry ingredients
Mixing in dough incorporator
Fermentation, 2 to 4 hours at 27°C
Pumping of dough to development chamber
Dough development under pressure at 80 psi
Extrusion within 1 minute with 14.5°C and panning
Proofing for 90 minutes
Baking at 191°C to 232°C for 18 to 35 minutes to approximately 100°C internal temperature

Sources: Cauvain, S. P., and L. S. Young, *Technology of breadmaking*, Blackie Academic & Professional, London, 1998. With permission. Groff, E. T., and M. A. Steinbaecher, *Cereal Foods World*, 40(8), 524–526, 1995. With permission. Pyler, E. J., *Baking science and technology*, 3rd ed., Vols. 1 and 2, Sosland Publishing, Merriam, KS, 1988. With permission.

Note: RH, relative humidity.

1.2.5 Croissant and Danish Pastries

Croissant and Danish pastries can be considered as products that result from a modification of the basic bread-making process. The dough preparation steps are similar but their ingredients are different. Table 1.9 compares the ingredients used in making croissant and Danish pastries. From this table, it is clear that even within each group, the ingredient formulation can vary considerably, producing a wide variety of products that are available in the market (Cauvain and Young 1998; Groff and Steinbaecher 1995; Pyler 1988).

TABLE 1.6

Sample Bread Recipes

White Pan Bread (Bulk Fermentation or Straight Dough Process)	
Ingredients	*Percent (on flour weight)*
Flour	100.0
Yeast	1.0
Salt	2.0
Water	57.0
Optional dough improving ingredients	
Fat	0.7
Soya flour	0.7
Malt flour	0.2
White Pan Bread (Sponge and Dough Processes)	
Sponge ingredients	*Percent (of total flour)*
Flour	25.0
Yeast	0.7
Salt	0.5
Water	14.0
Dough ingredients	*Percent (of total flour)*
Flour	75.0
Yeast	2.0
Salt	1.5
Water	44.0
Optional improving ingredients	
Fat	0.7
Soya flour	0.7
Malt flour	0.2

Sources: Cauvain, S. P., and L. S. Young, *Technology of breadmaking*, Blackie Academic & Professional, London, 1998. With permission. Groff, E. T., and M. A. Steinbaecher, *Cereal Foods World*, 40(8), 524–526, 1995. With permission. Pyler, E. J., *Baking science and technology*, 3rd ed., Vols. 1 and 2, Sosland Publishing, Merriam, KS, 1988. With permission.

1.2.6 Steamed Bread (Mantou)

Steamed bread is common in the Chinese community. Plain steamed bread is consumed as the major staple in the northern provinces of China. However, stuffed steamed breads are consumed as specialty items in various parts of China. The manufacture of steamed bread differs from that of regular bread mainly in the dough solidification process. Regular bread uses a baking process, whereas in steamed bread, steaming is used instead of baking. Consequently, in steamed bread, there is no brown crust on the breads' surface because the temperature used is not high enough to cause the browning reaction. Steamed bread is always consumed hot or held in a steamer because the bread is soft at this temperature. Sometimes, steamed bread is deep-fried before consumption. Steamed bread hardens when it cools down, making it less palatable. Various procedures are available for the production of steamed bread. Table 1.10 lists the basic steps in steamed bread processing in China (Huang 1999).

1.3 Fermented Soy Products

1.3.1 Kinds of Products and Ingredients

Soybeans have been available to the Chinese for centuries, and various fermented soy products have been developed and spread to its neighboring countries. These countries further developed their own fermented

TABLE 1.7

General Guidelines for Retarded Dough Production

Reduce yeast levels as storage times increase
Keep yeast levels constant when using separate retarders and provers
Reduce yeast levels as the dough radius increases
Reduce yeast levels with higher storage temperatures
The lower the yeast level used, the longer the proof time will be for a given dough piece volume
Yeast levels should not normally be less than 50% of the level used in scratch production
For doughs stored below –5°C, the yeast level may need to be increased
Reduce the storage temperature to reduce expansion and weight loss from all dough pieces
Lower the yeast levels to reduce expansion and weight losses at all storage temperatures
Dough pieces of large radius are more susceptible to the effects of storage temperature
The lower freezing rate achieved in most retarder-provers combined with the poor thermal conductivity of dough could cause losses in quality
Prove dough pieces of large radius at a lower temperature than those of small radius
Lower the yeast level in the dough to lengthen the final proof time and to help minimize temperature differentials
Maintain a high relative humidity in proof to prevent skinning

Sources: Cauvain, S. P., and L. S. Young, *Technology of breadmaking*, Blackie Academic & Professional, London, 1998. With permission. Pyler, E. J., *Baking science and technology*, 3rd ed., Vols. 1 and 2, Sosland Publishing, Merriam, KS, 1988. With permission. Groff, E. T., and M. A. Steinbaecher, *Cereal Foods World*, 40(8), 524–526, 1995. With permission.

TABLE 1.8

General Production Scheme for Flat Bread

Ingredient preparation
Mixing of ingredients (dough formation)
Fermentation
Dough cutting and rounding
Extrusion and sheeting (optional)
First proofing
Flattening and layering
Second proofing
Second pressing (optional)
Baking or steaming
Cooling
Packaging and distribution

Sources: Quail, K. J., *Arabic bread production*, American Association of Cereal Chemists, St. Paul, MN, 1998. With permission. Qarooni, J., *Flat bread technology*, Chapman & Hall, New York, 1996. With permission.

soy products. Soy sauce originating in China is probably the most famous and the most widely accepted fermented soy product. The credit for this wide acceptance also goes to the Kikkoman Corporation from Japan, which has helped the availability of soy sauce worldwide through their marketing strategy. Fermented whole soybeans such as ordinary natto, salted soybeans (e.g., Japanese Hama-natto and Chinese dou-chi), and tempe (Indonesia); fermented soy pastes (e.g., Japanese miso and Chinese dou-pan-chiang); and fermented tofus (e.g., sufu and stinky tofu or chao-tofu of Chinese origin) are more acceptable to ethnic groups. Consumers worldwide are gradually accepting these products through cultural exchange activities. The manufacturing of these products varies widely. Table 1.11 summarizes the ingredients needed for the manufacture of common fermented soy products (Ebine 1986; Liu 1986, 1997, 1999; Luh 1995; Muramatsu et al. 1993; Steinkraus 1996; Sugiyama 1986; Teng et al. 2004; Winarno 1986; Yoneya 2003).

TABLE 1.9

Formulations for Croissant and Danish Pastries

Flour (100%)	Croissant	Danish Pastries
Flour	100	100
Salt	1.8–2.0	1.1–1.56
Water	52–55.4	43.6–52
Yeast (compressed)	4–5.5	6–7.6
Shortening	2–9.7	6.3–12.5
Sugar	2–10	9.2–25
Egg	0–24	5–25
Skimmed milk powder	3–6.5	4–6.25
Laminating margarine/butter	32–57	50–64

Sources: Cauvain, S. P., and L. S. Young, *Technology of breadmaking*, London: Blackie Academic & Professional, 1998. With permission. Groff, E. T., and M. A. Steinbaecher, *Cereal Foods World*, 40(8), 524–526, 1995. With permission. Pyler, E. J., *Baking science and technology*, 3rd ed., Vols. 1 and 2, Merriam, KS: Sosland Publishing, 1988. With permission.

TABLE 1.10

Basic Steps in Steamed Bread Processing

Selection of flour and ingredients such as milk, powder, and sugar (optional)
Mixing of dough
Fermentation:
Full fermentation—1 to 3 hours
Partial fermentation—0.5 to 1.5 hours
No-time fermentation—0 hours
Remixed fermentation dough—remixing of fully fermented dough with up to 40% of flour by weight
Neutralization with 40% sodium bicarbonate and remixing
Molding
Proofing at 40°C for 30 to 40 minutes (no-time dough)
Steaming for approximately 20 minutes
Steamed bread is maintained at least warm to preserve quality

Source: Huang, S. D., in *Asian foods: science and technology*, edited by Ang, C. Y. W., K. S. Liu, Y. W. Huang, 71–109, Technomics Publishing Co., Inc., Lancaster, PA, 1999. With permission.

1.3.2 Soy Sauce

There are many types of soy sauce depending on the ratio of ingredients (wheat and soybeans), the fermentation and extraction procedures, and the flavoring ingredients (caramel and others) used. However, the procedures for manufacturing are similar. Basically, soy sauce is made by fermenting cooked soybeans in salt or brine under controlled conditions to hydrolyze the soy proteins and starches into smaller flavoring components. The soy sauce is then extracted from the fermented soybeans for standardization and packaging. Table 1.12 lists a generalized scheme for the manufacture of soy sauce. More detailed information is presented in the references listed in this chapter and in literature available elsewhere (Ebine 1986; Liu 1986, 1997, 1999; Sugiyama 1986; Yoneya 2003).

TABLE 1.11

Raw Ingredients for Fermented Soy Products

Ingredient	Soy Sauce	Natto	Soy Nuggets	Soy Paste	Tempe	Soy Cheese	Stinky Tofu
Major Ingredients							
Soy							
Soy bean	Yes	Yes	Yes	Optional	Yes	Yes	Yes
Soybean flour	Optional	No	No	Yes	No	Optional	Optional
Salt	Yes	Yes	Yes	Yes	No	Yes	No
Wheat	Optional	No	No	No	No	No	No
Rice flour	No	No	No	Optional	No	No	No
Major Microorganism(s)							
Mold							
Aspergillus oryzae	Yes	No	Yes	Yes	No	Optional	No
A. sojae	No	No	No	Optional	No	No	No
Mucor hiemalis, M. silivaticus	No	No	No	No	No	Yes	No
M. piaini	No	No	No	No	No	Yes	No
Actinomucor elegans	No	No	No	No	No	Yes	No
A. repens, A. taiwanensis	No	No	No	No	No	Yes	No
Rhizopus oligosporus	No	No	No	No	Yes	No	No
R. chinesis var. chungyuen	No	No	No	No	No	Yes	No
Bacteria							
Bacillus natto	No	Yes	No	No	No	No	No
Klebsiella pneumoniae	No	No	No	No	Yes	No	No
Bacillus sp.	No	No	No	No	No	No	Yes
Streptococcus sp.	No	No	No	No	No	No	Yes
Enterococcus sp.	No	No	No	No	No	No	Yes
Lactobacillus sp.	No	No	No	No	No	No	Yes
Halophilic yeasts							
Saccharomyces rouxii	Yes	No	Yes	Yes	No	No	No
Torulopsis versatilis	Yes	No	Yes	Yes	No	No	No
Halophilic lactic bacteria							
Pediococcus halophilus	Yes	No	Yes	Yes	No	No	No
Bacillus subtilus	Yes	No	Yes	Yes	No	No	No
Additional flavor added	Optional	No	No	No	No	Optional	No
Preservative added	Optional	No	No	No	No	No	No

Sources: Ebine, H., in *Food uses of whole oil and protein seeds*, edited by Lusas, E. W., D. R. Erickson, and W. K. Nip, 131–147, American Oil Chemists' Society, Champaign, IL, 1986. With permission. Liu, K. S., in *Food uses of whole oil and protein seeds*, edited by Lusas, E. W., D. R. Erickson, and W. K. Nip, 148–158, American Oil Chemists' Society, Champaign, IL, 1986. Liu, K. S., *Soybean: chemistry, technology and utilization*, Chapman & Hall, New York, 1997. Liu, K. S., in *Asian foods: science and technology*, edited by Ang, C. Y. W., K. S. Liu, and Y. W. Huang, 139–199, Technomic Publishing, Lancaster, PA, 1999. With permission. Steinkraus, K. H., *Handbook of indigenous fermented foods*, 2nd ed. (revised and expanded), Marcel Dekker, New York, 1996. With permission. Teng, D. F. et al., in *Handbook of food and beverage fermentation technology*, edited by Hui, Y. H., L. M. Goddik, A. S. Hansen, J. Josephsen, W. K. Nip, P. S. Stanfield, and F. Toldra, 533–570, Marcel Dekker, New York, 2004. With permission. Winarno, F. G., in *Food uses of whole oil and protein seeds*, edited by Lusas, E. W., D. R. Drickson, and W. K. Nip, 102–130, American Oil Chemists' Society, Champaign, IL, 1986. With permission. Yoneya, T., in *Handbook of vegetable preservation and processing*, edited by Hui, Y. H., S. Ghazala, D. M. Graham, K. D. Murrell, and W. K. Nip, 251–272, Marcel Dekker, New York, 2003. With permission.

TABLE 1.12

Production Scheme for Soy Sauce

Selection and soaking of beans
Cooking of clean or defatted soybean, pressurized steam cooking at 1.8 kg/cm^2 for 5 minutes
Cooling of cooked beans to 40°C
Roasting and crushing of wheat
Mixing of prepared soybeans and wheat
Inoculation with *Aspergillus oryzae* or *sojae*
Incubation of mixture to make starter koji at 28°C to 40°C
Addition of brine (23% salt water) to make moromi (mash)
Inoculation with halophilic yeasts and lactic acid bacteria (optional)
Brine fermentation at 15°C to 28°C
Addition of saccharified rice koji (optional)
Aging of moromi (optional)
Separation of raw soy sauce by pressing or natural gravity
Refining
Addition of preservative and caramel (option)
Packaging and storage

Sources: Ebine, H., in *Food uses of whole oil and protein seeds*, edited by Lusas, E. W., D. R. Erickson, and W. K. Nip, 131–147, American Oil Chemists' Society, Champaign, IL, 1986. With permission. Liu, K. S., in *Food uses of whole oil and protein seeds*, edited by Lusas, E. W., D. R. Erickson, and W. K. Nip, 148–158, American Oil Chemists' Society, Champaign, IL, 1986. Liu, K. S., *Soybean: chemistry, technology and utilization*, Chapman & Hall, New York, 1997. Liu, K. S., in *Asian foods: science and technology*, edited by Ang, C. Y. W., K. S. Liu, and Y. W. Huang, 139–199, Technomic Publishing, Lancaster, PA, 1999. With permission. Steinkraus, K. H., *Handbook of indigenous fermented foods*, 2nd ed. (revised and expanded), Marcel Dekker, New York, 1996. With permission. Sugiyama, S., in *Food uses of whole oil and protein seeds*, edited by Lusas, E. W., D. R. Erickson, and W. K. Nip, 118–130, American Oil Chemists' Society, Champaign, IL, 1986. With permission. Yoneya, T., in *Handbook of vegetable preservation and processing*, edited by Hui, Y. H., S. Ghazala, D. M. Graham, K. D. Murrell, and W. K. Nip, 251–272, Marcel Dekker, New York, 2003. With permission.

1.3.3 Fermented Whole Soybeans

1.3.3.1 Ordinary (Itohiki) Natto

Ordinary natto is a typical Japanese fermented whole soybean product. The sticky mucilageous substance on the surface of soybeans is its characteristic and is now considered to have health benefits. It is produced by a brief fermentation of cooked soybeans with *Bacillus natto*, and it has a short shelf life. Table 1.13 lists the basic steps in the manufacture of ordinary natto. For detailed information on ordinary natto, please check the references in this chapter (Liu 1997, 1999; Yoneya 2003).

1.3.3.2 Hama-natto and Dou-chi

Hama-natto is fermented whole soybeans produced in the Hamamatsu area of Japan. Similar products are produced in Japan prefixed with different names taken from the production location. A very similar product in the Chinese culture is "tou-chi" or "dou-chi." It is produced by fermenting the cooked soybeans in salt, brine, or soy sauce, and then drying them as individual beans. Hama-natto includes ginger in the flavoring, whereas the inclusion of ginger flavoring is optional in dou-chi. Table 1.14 lists the basic steps in the production of Hama-natto and dou-chi. For further information, readers should check the references in this chapter and other available literature (Liu 1986, 1997, 1999; Yoneya 2003).

TABLE 1.13

Production Scheme for Itohiki (Ordinary) Natto

Clean whole soybean
Washing and soaking at 21°C to 25°C for 10 to 30 hours
Pressurized steam cooking of soybean at 1 to 1.5 kg/cm^2 for 20 to 30 minutes
Draining and cooling of soybean at 80°C
Inoculation with *Bacillus natto*
Mixing and packaging in small packages
Incubation
40°C to 43°C for 12 to 20 hours, or
38°C for 20 hours plus 5°C for 24 hours
Final product
Refrigeration to prolong shelf life

Sources: Liu, K. S., *Soybean: chemistry, technology and utilization*, Chapman & Hall, New York, 1997. Liu, K. S., in *Asian foods: science and technology*, edited by Ang, C. Y. W., K. S. Liu, and Y. W. Huang, 139–199, Technomic Publishing, Lancaster, PA, 1999. With permission. Yoneya, T., in *Handbook of vegetable preservation and processing*, edited by Hui, Y. H., S. Ghazala, D. M. Graham, K. D. Murrell, and W. K. Nip, 251–272, Marcel Dekker, New York, 2003. With permission.

TABLE 1.14

Production Scheme for Soy Nuggets (Hama-natto and Dou-chi)

Clean whole soybean
Washing and soaking for 3 to 4 hours at 20°C
Steam-cooking of soybean at ambient pressure for 5 to 6 hours, or at 0.81.0 kg/cm^2 for 30 to 40 minutes
Draining and cooling of soybean to 40°C
Addition of alum (optional for dou-chi)
Mixing with wheat flour (optional for Hama-natto)
Inoculation with *Aspergillus oryzae*
Procedure 1 (Hama-natto)
Incubation for 50 hours at 30°C to 33°C
Soaking of inoculated soybean in flavoring solution for 8 months
Incubation under slight pressure in closed containers
Procedure 2 (Dou-chi)
Incubation at 35°C to 40°C for 5 days
Washing
Incubation for 5 to 6 days at 35°C
Beans removed from liquid for drying
Mixing with ginger soaked in soy sauce (Hama-natto only)
Final product (soy nuggets)
Packaging
Refrigeration to prolong shelf life (optional)

Sources: Liu, K. S., in *Food uses of whole oil and protein seeds*, edited by Lusas, E. W., D. R. Erickson, and W. K. Nip, 148–158, American Oil Chemists' Society, Champaign, IL, 1986. Liu, K. S., *Soybean: chemistry, technology and utilization*, Chapman & Hall, New York, 1997. Liu, K. S., in *Asian foods: science and technology*, edited by Ang, C. Y. W., K. S. Liu, and Y. W. Huang, 139–199, Technomic Publishing, Lancaster, PA, 1999. With permission. Yoneya, T., in *Handbook of vegetable preservation and processing*, edited by Hui, Y. H., S. Ghazala, D. M. Graham, K. D. Murrell, and W. K. Nip, 251–272, Marcel Dekker, New York, 2003. With permission.

1.3.4 Fermented Soy Pastes

Both the Chinese and Japanese have fermented soy pastes available in their cultures and they are made in a similar manner. However, the usage for these two products is quite different. The Japanese use their fermented soy paste, miso, in making miso soup, and to a lesser extent, for example, in marinating/flavoring of fish. Miso soup is common in traditional Japanese meals. The Chinese use their fermented soy paste, dou-pan-chiang, mainly as a condiment in food preparation. Dou-pan-chiang can also be made from wing beans (however, this is beyond the scope of this chapter). Table 1.15 lists the basic steps in the manufacture of miso. For more detailed information on miso and dou-pan-chiang, readers should consult the references for this chapter and other literature available elsewhere (Ebine 1986; Liu 1986, 1997, 1999; Steinkraus 1996; Sugiyama 1986; Yoneya 2003).

1.3.5 Fermented Tofu

1.3.5.1 Sufu or Fermented Soy Cheese

Sufu or fermented soy cheese is produced by fermenting tofu that is made by coagulating the soy protein in soymilk with calcium or magnesium sulfate (or both). It is similar to feta cheese in its fermentation process. Both products are matured in brine in sealed containers. Some packed sufu contains flavoring ingredients. Table 1.16 lists the basic steps in the manufacture of sufu. For more detailed information, readers should check the list of references in this chapter and the other available literature (Liu 1986, 1997, 1999; Teng et al. 2004).

TABLE 1.15

Production Scheme of Fermented Soybean Pastes (Miso)

Whole, clean soybean
Washing and soaking at 15°C for 8 hours
Cooking at 121°C for 45 to 50 minutes or equivalent
Cooling and mashing the soybeans
Preparation of soaked, cooked, and cooled rice (optional)
Preparation of parched barley (optional)
Inoculation of rice or barley with *Aspergillus oryzae* (tane-koji, optional)
Mixing of koji and rice or barley mixture
Addition and mixing of salt to koji and rice or barley mixture
Inoculation of halophilic yeasts and lactic acid bacteria (optional)
Packing of mixture (mashed soybean and koji) into fermenting vat with 20% to 21% salt brine
Fermentation at 25°C to 30°C for 50 to 70 days
Blending and crashing of ripened miso
Addition of preservative and colorant (optional)
Pasteurization (optional)
Packaging and storage

Sources: Ebine, H., in *Food uses of whole oil and protein seeds*, edited by Lusas, E. W., D. R. Erickson, and W. K. Nip, 131–147, American Oil Chemists' Society, Champaign, IL, 1986. With permission. Liu, K. S., in *Food uses of whole oil and protein seeds*, edited by Lusas, E. W., D. R. Erickson, and W. K. Nip, 148–158, American Oil Chemists' Society, Champaign, IL, 1986. Liu, K. S., *Soybean: chemistry, technology and utilization*, Chapman & Hall, New York, 1997. Liu, K. S., in *Asian foods: science and technology*, edited by Ang, C. Y. W., K. S. Liu, and Y. W. Huang, 139–199, Technomic Publishing, Lancaster, PA, 1999. With permission. Steinkraus, K. H., *Handbook of indigenous fermented foods*, 2nd ed. (revised and expanded), Marcel Dekker, New York, 1996. With permission. Sugiyama, S., in *Food uses of whole oil and protein seeds*, edited by Lusas, E. W., D. R. Erickson, and W. K. Nip, 118–130, American Oil Chemists' Society, Champaign, IL, 1986. With permission. Yoneya, T., in *Handbook of vegetable preservation and processing*, edited by Hui, Y. H., S. Ghazala, D. M. Graham, K. D. Murrell, and W. K. Nip, 251–272, Marcel Dekker, New York, 2003. With permission.

TABLE 1.16

Production Scheme for Sufu (Chinese Soy Cheese)

Cleaning of whole soybean
Soaking
Grinding with water
Straining through cheesecloth to recover soymilk
Heated to boiling and then cooled
Coagulation of soymilk with calcium and magnesium sulfate (or both)
Cooled to 50°C
Pressed to remove water (formation of tofu)
Sterilized at 100°C for 10 minutes in hot-air oven
Inoculation with *Mucor*, *Actinomucor*, and *Rhizopus* sp.
Procedure 1
Incubation in dry form for 2 to 7 days depending on inocula
Incubation (fermentation at 25°C in 30% salt brine) for 1 month or longer
Brined and aged in small containers with or without the addition of alcohol or other flavoring ingredients
Procedure 2
Incubation at 35°C for 7 days until covered with yellow mold
Packed in closed container with 8% brine and 3% alcohol
Fermentation at room temperature for 6 to 12 months
Final product (sufu or Chinese soy cheese)

Sources: Liu, K. S., in *Food uses of whole oil and protein seeds*, edited by Lusas, E. W., D. R. Erickson, and W. K. Nip, 148–158, American Oil Chemists' Society, Champaign, IL, 1986. Liu, K. S., *Soybean: chemistry, technology and utilization*, Chapman & Hall, New York, 1997. Liu, K. S., in *Asian foods: science and technology*, edited by Ang, C. Y. W., K. S. Liu, and Y. W. Huang, 139–199, Technomic Publishing, Lancaster, PA, 1999. With permission. Teng, D. F. et al., in *Handbook of food and beverage fermentation technology*, edited by Hui, Y. H., L. M. Goddik, A. S. Hansen, J. Josephsen, W. K. Nip, P. S. Stanfield, and F. Toldra, 533–570, Marcel Dekker, New York, 2004. With permission.

1.3.5.2 Stinky Tofu

Stinky tofu is a traditional Chinese food made by fermenting tofu briefly in "stinky brine" produced by fermenting vegetables under special conditions. The tofu is hydrolyzed slightly during this brief fermentation and develops its characteristic flavoring compounds. When this raw stinky tofu is deep-fried, these compounds volatilize and produce the characteristic stinky odor, thus the name of "stinky tofu." It is usually consumed with chili and soy sauces. Stinky tofu is also steamed with condiments for consumption. Table 1.17 lists the basic steps in the manufacture of stinky tofu. Readers should consult the references in this chapter for further reading (Liu 1986, 1997, 1999; Teng et al. 2004).

1.3.6 Tempe (Tempeh)

Tempe is a traditional Indonesian food commonly consumed by its people. It is made by fermenting cooked soybeans wrapped in wilted banana leaves or plastic wraps. The mold *Rhizopus oligosporus* produces its mycelia and these mycelia penetrate into the block of soybeans. The mold mycelia also surround the block. This is similar to the fermentation of molded cheese. Tempe is gradually being accepted by vegetarians in the West as a nutritious and healthy food. It is generally consumed as a deep-fried product. Table 1.18 lists the basic steps in the production of tempe (Liu 1997, 1999; Winarno 1986; Yoneya 2003).

TABLE 1.17

Production Scheme for Stinky Tofu

Cleaning of whole soybean
Soaking
Grind with water
Straining through cheesecloth to recover soymilk
Heated to boiling and then cooled
Coagulation of soymilk with calcium and magnesium sulfate (or both)
Cooled to 50°C
Pressed to remove water (formation of tofu)
Pressing to remove additional water
Soaking in fermentation liquid for 4 to 20 hours at 5°C to 30°C
Fresh stinky tofu ready for frying or steaming
Refrigeration to prolong shelf life

Sources: Liu, K. S., in *Food uses of whole oil and protein seeds*, edited by Lusas, E. W., D. R. Erickson, and W. K. Nip, 148–158, American Oil Chemists' Society, Champaign, IL, 1986. Liu, K. S., *Soybean: chemistry, technology and utilization*, Chapman & Hall, New York, 1997. Liu, K. S., in *Asian foods: science and technology*, edited by Ang, C. Y. W., K. S. Liu, and Y. W. Huang, 139–199, Technomic Publishing, Lancaster, PA, 1999. With permission. Teng, D. F. et al., in *Handbook of food and beverage fermentation technology*, edited by Hui, Y. H., L. M. Goddik, A. S. Hansen, J. Josephsen, W. K. Nip, P. S. Stanfield, and F. Toldra, 533–570, Marcel Dekker, New York, 2004. With permission.

TABLE 1.18

Production Scheme for Tempe

Whole, clean soybeans
Rehydration in hot water at 93°C for 10 minutes
Dehulling
Soaking with or without lactic acid overnight
Boiling for 68 minutes
Draining and cooling to 38°C
Inoculation with *Rhizopus oligosporus* without *Klebsiella pneumonia*
Incubation on trays at 35°C to 38°C, 75% to 78% RH for 18 hours
Dehydration
Wrapping

Sources: Liu, K. S., *Soybean: chemistry, technology and utilization*, Chapman & Hall, New York, 1997. Liu, K. S., in *Asian foods: science and technology*, edited by Ang, C. Y. W., K. S. Liu, and Y. W. Huang, 139–199, Technomic Publishing, Lancaster, PA, 1999. With permission. Winarno, F. G., in *Food uses of whole oil and protein seeds*, edited by Lusas E. W., D. R. Drickson, and W. K. Nip, 102–130, American Oil Chemists' Society, Champaign, IL, 1986. With permission. Yoneya, T., in *Handbook of vegetable preservation and processing*, edited by Hui, Y. H., S. Ghazala, D. M. Graham, K. D. Murrell, and W. K. Nip, 251–272, Marcel Dekker, New York, 2003. With permission.

1.4 Fermented Vegetables

1.4.1 Kinds of Products and Ingredients

Fermented vegetables were produced in different cultures in the old days to preserve harvested vegetables when they are not available or because of climatic limitations. Some of these products started as traditional cultural foods but became widely accepted in other cultures. It is interesting that most of these processes are similar. Salt is used in the production of these fermented vegetables or in a salt stock. Natural lactic acid fermentation, to produce enough lactic acid to lower the pH, is the major microbial

activity in these processes. With the amount of salt added and lactic acid produced, these two ingredients create an environment that can inhibit the growth of other spoilage microorganisms. Available leafy vegetables, fruits (commonly used as vegetables), and roots are used as the raw materials. Starter cultures are used occasionally. Vinegar is used to preserve some vegetable products whereas chili pepper and other spices are used in others. Preservatives may also be used to extend shelf life after the package is opened. Table 1.19 compares the ingredients used in different fermented vegetable products (Anonymous 1991; Beck 1991; Brady 1994; Chiou 2003; Desrosier 1977; Duncan 1987; Fleming et al. 1984; Hang 2003; Lee 2003; Park and Cheigh 2003).

1.4.2 Sauerkraut

The term *sauerkraut* literally means sour (sauer) cabbage (kraut). It is a traditional German fermented vegetable product that has spread to other cultures, and it can be used on its own or in food preparations. Its sequential growth of lactic acid bacteria has long been recognized. Each lactic acid bacterium dominates the fermentation until its end product becomes inhibitory for its own development and creates an environment suitable for another lactic acid bacterium to take over. The fermentation continues until most of the available fermentable sugars are exhausted. The production of sauerkraut is not risk-free and sanitary precautions must be taken to avoid spoilage. Table 1.20 presents the basic steps in sauerkraut processing (Anonymous 1991; Desrosier 1977; Fleming et al. 1984; Hang 2003).

TABLE 1.19

Raw Ingredients for Fermented Vegetables

Ingredient	Sauerkraut	Western Pickles	Jalapeno Peppers	Kimchi	Oriental Vegetables
Vegetable					
Head cabbage	Yes	No	No	Optional	Optional
Chinese cabbage	No	No	No	Major	Optional
Mustard green	No	No	No	Optional	Optional
Turnip	No	No	No	Optional	Optional
Jalapeno pepper	No	No	Yes	Optional	Optional
Chili pepper	No	No	No	Yes	Optional
Pickle/cucumber	No	Yes	No	Optional	Optional
Salt	Yes	Yes	Yes	Yes	Yes
Starter culture (lactic acid bacteria)	Optional	Optional	Optional	No	No
Added vinegar	No	Yes	Yes	No	Optional
Added spices	No	Optional	Optional	Optional	Optional
Other added flavors	No	Yes	No	Optional	Optional
Preservative(s)	No	Optional	Optional	Optional	Optional

Sources: Anonymous, *Let's preserve pickles*, Cooperative Extension Service, Purdue University, West Lafayette, IN, 1991. With permission. Beck, P., *Making pickled products*, Extension Service, North Dakota State University, Fargo, ND, 1991. With permission. Brady, P. L., *Making brined pickles and sauerkraut*, Cooperative Extension Service, University of Arkansas, Little Rock, AR, 1994. With permission. Chiou, Y. Y., in *Handbook of vegetable preservation and processing*, edited by Hui, Y. H., S. Ghazala, K. D. Murrell, D. M. Graham, and W. K. Nip, 169–178, Marcel Dekker, New York, 2003. With permission. Desroiser, N. W., *Elements of food technology*, AVI Publishing, Westport, CT, 1977. With permission. Duncan, A., *Oregon's Agric Prog* 33(2/3), 6–9, 1987. With permission. Fleming, H. P. et al., in *Compendium of methods for the microbiological examination of foods*, edited by Speck, M. L., 663–681, APHA Technical Committee on Microbiological Methods of Foods, 1984. With permission. Hang, D. Y., in *Handbook of vegetable preservation and processing*, edited by Hui, Y. H., S. Ghazala, K. D. Murrell, D. M. Graham, and W. K. Nip, 223–230, Marcel Dekker, New York, 2003. With permission. Lee, K. Y., in *Handbook of vegetable preservation and processing*, edited by Hui, Y. H., S. Ghazala, K. D. Murrell, D. M. Graham, and W. K. Nip, 155–168, Marcel Dekker, New York, 2003. With permission. Park, K. Y., and H. S. Cheigh, in *Handbook of vegetable preservation and processing*, edited by Hui, Y. H., S. Ghazala, and K. D. Murrell, D. M. Graham, and W. K. Nip, 189–222, Marcel Dekker, New York, 2003. With permission.

TABLE 1.20

Basic Steps in Sauerkraut Processing

Selection and trimming of white head cabbage
Coring and shredding of head cabbage to 1/8 in. thick
Salting with 2.25% to 2.50% salt by weight with thorough mixing
Storage of salted cabbage in vats with plastic cover weighed with water to displace air from the cabbage
Fermentation at 7°C to 23°C for 2 to 3 months or longer to achieve an acidity of 2.0% (lactic)
Heating of kraut to 73.9°C before filling the cans or jars followed by exhausting, sealing, and cooling
Storage and distribution

Sources: Brady, P. L., *Making brined pickles and sauerkraut*, Cooperative Extension Service, University of Arkansas, Little Rock, AR, 1994. With permission. Desrosier, N. W., *Elements of food technology*, AVI Publishing, Westport, CT, 1977. With permission. Fleming, H. P. et al., in *Compendium of methods for the microbiological examination of foods*, edited by Speck, M. L., 663–681, APHA Technical Committee on Microbiological Methods of Foods, 1984. With permission. Hang, D. Y., in *Handbook of vegetable preservation and processing*, edited by Hui, Y. H., S. Ghazala, K. D. Murrell, D. M. Graham, and W. K. Nip, 223–230, Marcel Dekker, New York, 2003. With permission.

1.4.3 Pickles

Western-style pickles are produced by salting the pickling cucumbers in vats in salt stocks for long-term storage, followed by desalting and bottling in sugar and vinegar with or without spices. The fermentation is still through lactic acid fermentation. However, it is more susceptible to spoilage because air may be trapped inside the slightly wax-coated cucumbers. In the salt curing of cucumbers, spoilage can occur and precautions should be taken to avoid its occurrence. Because of their high acidity and low pH, as well as the high salt content, Western-style pickles are generally mildly heat-treated to sterilize or pasteurize the product. Table 1.21 lists the basic steps in the production of Western-style pickles (Anonymous 1991; Beck 1991; Brady 1994; Desrosier 1977; Duncan 1987; Fleming et al. 1984).

1.4.4 Kimchi

Kimchi is a traditional Korean fermented vegetable. Most kimchi is characterized by its hot taste because of the fairly high amount of chili pepper used in the product and its visibility. However, some kimchi are

TABLE 1.21

Basic Steps in Fermented Pickles Processing

Sizing and cleaning of cucumbers
Preparation of 5% (low salt) or 10% brine (salt stock)
Curing (fermenting) of cucumbers in brine for 1 to 6 weeks to 0.7% to 1.0% acidity (lactic) and pH of 3.4 to 3.6, dependent on temperature, with salinity maintained at a desirable level (15% for salt stock). Addition of sugar, starter culture, and spices are optional
Recovery of pickles from brine followed by rinsing or desalting (salt stock)
Grading
Packing of pickles into jars and filled with vinegar, sugar, spices, and alum depending on the formulation
Pasteurization at 74°C for 15 minutes followed by refrigerated storage, or exhausting to 74°C at cold point followed by sealing and cooling, or vacuum pack followed by heating at 74°C (cold point) for 15 minutes and then cooling
Storage and distribution

Sources: Anonymous, *Let's preserve pickles*, Cooperative Extension Service, Purdue University, West Lafayette, IN, 1991. With permission. Brady, P. L., *Making brined pickles and sauerkraut*, Cooperative Extension Service, University of Arkansas, Little Rock, AR, 1994. With permission. Beck, P., *Making pickled products*, Extension Service, North Dakota State University, Fargo, ND, 1991. With permission. Desrosier, N. W., *Elements of food technology*, AVI Publishing, Westport, CT, 1977. With permission. Duncan, A., *Oregon's Agric Prog* 33(2/3), 6–9, 1987. With permission. Fleming, H. P. et al., in *Compendium of methods for the microbiological examination of foods*, edited by Speck, M. L., 663–681, APHA Technical Committee on Microbiological Methods of Foods, 1984. With permission.

TABLE 1.22

Basic Steps in Kimchi Processing

Selection of vegetables (Chinese cabbage, radish, cucumber, or others)
Washing of vegetables
Cutting of vegetables, if necessary
Preparation of 8% to 15% brine
Immersion of vegetables in brine for 2 to 7 hours to achieve 2% to 4% salt in vegetable
Rinsing and draining briefly
Addition of seasoning
Fermentation at 0°C to room temperature for approximately 3 days
Package (can also be conducted before fermentation)
Storage at 3°C to 4°C

Sources: Lee, K. Y., in *Handbook of vegetable preservation and processing*, edited by Hui, Y. H., S. Ghazala, K. D. Murrell, D. M. Graham, and W. K. Nip, 155–168, Marcel Dekker, New York, 2003. With permission. Park, K. Y., and H. S. Cheigh, in *Handbook of vegetable preservation and processing*, edited by Hui, Y. H., S. Ghazala, and K. D. Murrell, D. M. Graham, and W. K. Nip, 189–222, Marcel Dekker, New York, 2003. With permission.

made without chili pepper, but with garlic and ginger as well as vegetables and other ingredients. This kind of kimchi still has the hot taste but does not have the red color. Vegetables used in making kimchi vary with its formulations: Chinese cabbage, cucumber, and large turnip are more common. Either chili pepper or garlic and ginger can be used to provide a hot sensation. Other ingredients may also be added to provide the typical flavor. The fermentation is still through lactic acid fermentation. Traditionally, kimchi was made in every household in rural areas in Korea to provide vegetables for the winter when other fresh vegetables are not readily available. Today, it is a big industry in Korea, and kimchi is available all-year-round. Even small South Korean–made kimchi refrigerators are now available to meet the demands of

TABLE 1.23

Basic Steps in Fermented Chinese Vegetables

Selection and cleaning of vegetables
Cutting of vegetables (optional)
Procedure 1
Wilting of vegetables for 1 to 2 days to remove moisture
Dry salting of vegetables in layers with weights on top (5%–7.5% salt)
Fermentation for 3 to 10 days
Washing
Drying or pressing of fermented vegetables (optional)
Addition of spices and flavoring compounds
Packaging
Sterilization (optional)
Procedure 2
Wilting of cut vegetables
Hot water rinsing of fermentation container
Fill the container with cut vegetables
Addition of 2% to 3% brine and other flavoring compounds (optional)
Ferment at 20°C to 25°C for 2 to 3 days
Ready for direct consumption or packaging and cool storage

Sources: Chiou, Y. Y., in *Handbook of vegetable preservation and processing*, edited by Hui, Y. H., S. Ghazala, K. D. Murrell, D. M. Graham, and W. K. Nip, 169–178, Marcel Dekker, New York, 2003. With permission. Lee, K. Y., in *Handbook of vegetable preservation and processing*, edited by Hui, Y. H., S. Ghazala, K. D. Murrell, D. M. Graham, and W. K. Nip, 155–168, Marcel Dekker, New York, 2003. With permission.

consumers living in cities in South Korea and elsewhere. In other parts of the world where Koreans are residents, kimchi is available either as a household item or as a commercial product. Kimchi is usually not heat-sterilized after packaging in jars. Pasteurization is optional. It is considered perishable and is stored refrigerated. Kimchi is now gradually being accepted in other cultures and Koreans are attempting to develop standards for their kimchi. Today, China also exports considerable amounts of kimchi to South Korea. Table 1.22 lists the basic steps in the manufacture of kimchi (Lee 2003; Park and Chiegh 2003).

1.4.5 Chinese Pickled Vegetables

The Chinese also manufacture a wide range of pickled vegetables. Various kinds of vegetables are used as raw materials. The fermentation can be either a dry-salting or a brining process, depending on the product to be manufactured. However, the fermentation is still through lactic acid fermentation. The major difference between Chinese-style pickled vegetable products and the Western-style pickles is that desalting is usually not practiced in the manufacture of Chinese-style pickled vegetables. The desalting process is left to the consumers, if needed. Also, some Chinese-style vegetables are made into intermediate moisture products that are not practiced in their Western-style counterparts. Table 1.23 lists some of the basic steps in the manufacturing of selected Chinese pickled vegetables (Chiou 2003; Lee 2003).

REFERENCES

Anonymous. 1991. *Let's preserve pickles*. West Lafayette, IN: Cooperative Extension Service, Purdue University.

Beck P. 1991. *Making pickled products*. Fargo, ND: Extension Service, North Dakota State University.

Brady PL. 1994. *Making brined pickles and sauerkraut*. Little Rock, AR: Cooperative Extension Service, University of Arkansas.

Cauvain SP, Young LS. 1998. *Technology of breadmaking*. London: Blackie Academic & Professional.

Chiou YY. 2003. Leaf mustard pickles and derived products. In: Hui YH, Ghazala S, Murrell KD, Graham DM, Nip WK, editors. *Handbook of vegetable preservation and processing*. New York: Marcel Dekker. p. 169–78.

Desrosier NW. 1977. *Elements of food technology*. Westport, CT: AVI Publishing.

Duncan A. 1987. Perfecting the pickle. *Oregon's Agric Prog* 33(2/3):6–9.

Ebine H. 1986. Miso preparation and use (also hot and sweet pastes). In: Lusas EW, Erickson DR, Nip WK, editors. *Food uses of whole oil and protein seeds*. Champaign, IL: American Oil Chemists' Society. p. 131–47.

Fleming HP, McFeeters RF, Ethchells JL, Bell TA. 1984. Pickled vegetables. In: Speck ML, editor. *Compendium of methods for the microbiological examination of foods*. APHA Technical Committee on Microbiological Methods of Foods. Washington, DC. p. 663–81.

Groff ET, Steinbaecher MA. 1995. Baking technology. *Cereal Foods World* 40(8):524–6.

Hang DY. 2003. Western fermented vegetables: sauerkraut. In Hui YH, Ghazala S., Murrell KD, Graham DM, Nip WK, editors. *Handbook of vegetable preservation and processing.* New York: Marcel Dekker. p. 223–30.

Huang SD. 1999. Wheat products: 2. breads, cakes, cookies, pastries and dumplings. In: Ang CYW, Liu KS, Huang YW, editors. *Asian foods: science and technology*. Lancaster, PA: Technomics Publishing. p. 71–109.

Lee KY. 2003. Fermentation: principles and microorganisms. In: Hui YH, Ghazala S, Murrell KD, Graham DM, Nip WK, editors. *Handbook of vegetable preservation and processing*. New York: Marcel Dekker. p. 155–168.

Liu KS. 1986. Food uses of soybeans. In: Lusas EW, Erickson DR, Nip WK, editors. *Food uses of whole oil and protein seeds*. Champaign, IL: American Oil Chemists' Society. p. 148–158.

Liu KS. 1997. *Soybean: Chemistry, technology and utilization*. New York: Chapman & Hall.

Liu KS. 1999. Oriental soyfoods. In: Ang CYW, Liu KS, Huang YW, editors. *Asian foods: Science and technology*. Lancaster, PA: Technomic Publishing. p. 139–199.

Luh BS. 1995. Industrial production of soy sauce. *J Indust Microbiol* 14(6):467–471.

Muramatsu S, Sano Y, Uzuka Y. 1993. Rapid fermentation of soy sauce. application of preparation of soy sauce low in sodium chloride. In: Spanier AM, Okai H, Tamura M, editors. *Food flavor and safety: molecular analysis and design. ACS Symposium 528*. Columbus, OH: American Chemical Society. p. 200–210.

Park KY, Cheigh HS. 2003. Fermented Korean vegetables (kimchi). In: Hui YH, Ghazala S, Murrell KD, Graham DM, Nip WK, editors. *Handbook of vegetable preservation and processing*. New York: Marcel Dekker. p. 189–222.

Pyler EJ. 1988. *Baking science and technology*. 3rd ed. Vols. 1 and 2. Merriam, KS: Sosland Publishing.

Qarooni J. 1996. *Flat bread technology*. New York: Chapman & Hall.

Quail KJ. 1998. *Arabic bread production*. St. Paul, MN: American Association of Cereal Chemists.

Steinkraus KH. 1996. *Handbook of indigenous fermented foods*. 2nd ed. (revised and expanded). New York: Marcel Dekker.

Sugiyama S. 1986. Production and uses of soybean sauces. In: Lusas EW, Erickson DR, Nip WK, editors. *Food uses of whole oil and protein seeds*. Champaign, IL: American Oil Chemists' Society. p. 118–130.

Teng DF, Lin CS, Hsieh PC. 2004. Fermented tofu: Sufu and stinky tofu. In: Hui YH, Goddik LM, Hansen AS, Josephsen J, Nip WK, Stanfield PS, Toldra F, editors. *Handbook of food and beverage fermentation technology*. New York: Marcel Dekker. p. 533–570.

Winarno FG. 1986. Production and uses of soybean tempe. In: Lusas EW, Drickson DR, Nip WK, editors. *Food uses of whole oil and protein seeds*. Champaign, IL: American Oil Chemists' Society. p. 102–130.

Yoneya T. 2003. Fermented soy products: tempe, nattos, miso and soy sauce. In: Hui YH, Ghazala S, Graham DM, Murrell KD, Nip WK, editors. *Handbook of vegetable preservation and processing*. New York: Marcel Dekker. p. 251–272.

2

Flavors and Food Fermentation

Shao Quan Liu

CONTENTS

2.1 Introduction

Fermentation is one of the most ancient food processing and preservation methods used in the food industry. Raw food materials are transformed through fermentation from perishable, sometimes nonfunctional forms into more stable, functional forms such as milk to cheese and yogurt, grape juice to wine, wheat to bread, barley/malt to beer, and soybean to soy sauce. The fundamental roles of food fermentation include preservation, enhancement of sensory characteristics (flavor, texture, or color), and transformation of nutrients (improvement of digestibility and biofortification) (Cogan and Accolas 1996; Leroy and De Vuyst 2004; Leroy et al. 2006).

Flavor is one sensory attribute that is most transformed by fermentation. Food substrates (carbohydrates, proteins, lipids, organic acids, amino acids, phenolic compounds, glycosides, etc.) are transformed into nonvolatile and volatile flavor compounds that affect not only taste but also aroma, and thus, food acceptance. These flavor compounds impart a range of sensory properties such as sweetness (e.g., mannitol), sourness (e.g., lactic acid), savoriness or umami (e.g., L-glutamic acid), bitterness (e.g., hydrophobic peptides), fruitiness (esters), and sulfurous notes (volatile sulfur compounds). The desirability or undesirability of the flavor compounds is dependent on their concentrations and the food matrices in which they are produced.

Consumer acceptance and choice of foods depend largely on flavor (aroma and taste). Flavor consistency is of critical importance to food quality in modern food manufacture. In addition, flavor diversification is an emerging trend because of consumer demand for novel and diverse flavors. A better understanding and knowledge of flavor formation during fermentation is a prerequisite for ensuring flavor consistency and for creating flavor diversity. The aim of this chapter is to give an overview of flavor formation in relation to food fermentation. Focus is placed on *Saccharomyces cerevisiae* yeasts and lactic acid bacteria because they are the most common and the best-studied microorganisms used in food fermentation. Detailed information on flavor formation in specific fermented food products such as

cheese and wine is available elsewhere (Smit et al. 2004; Weimer 2007; Bartowsky and Pretorius 2009; Ugliano and Henschke 2009; Sumby et al. 2010).

2.2 Type of Fermentation

Food fermentation can be classified into several types according to the microorganisms involved, main product produced, pH (acidity or alkalinity) changes, and moisture content of the food matrices, which are summarized below. Detailed information is available elsewhere (Hansen 2002; Hui et al. 2004; Hutkins 2006).

Fermented foods can be produced by relying on the microflora that are naturally present on or in the raw materials (the so-called natural, spontaneous, or wild fermentations), for example, raw milk cheeses and fermented leafy vegetables (e.g., sauerkraut and kimchi). Backslopping is a common practice in household operations or in the preparations of some traditional fermented foods (e.g., bread and fermented milks) in which the natural microflora in the fermenting materials ("mother cultures") are transferred to the new starting materials. Nowadays, industrial production of fermented foods entails the inoculation of raw materials with starter cultures to ensure quality consistency and better control.

Microorganisms involved in food fermentation include bacteria, yeasts, and fungi (molds). As such, there are four main types of food fermentation on the basis of the key microorganisms responsible: bacterial fermentation, yeast fermentation, fungal fermentation, and mixed culture fermentation. Bacterial fermentation results in the production of a range of fermented foods including many types of cheeses, fermented milks, fermented vegetables, natto, and fermented sausages. Yeast fermentation commonly transforms flour into bread or sugar-rich raw materials into alcoholic beverages. True fungi-fermented foods are few and the major examples are some fermented soybean products (tempeh and fermented soybean curd or sufu) in which other microflora may be involved to some extent. Most fermented foods, especially those that are traditional or indigenous, are produced using mixed cultures of bacteria, yeasts, or fungi. There are many examples of fermented foods produced by mixed cultures: mold-ripened cheeses (molds and lactic acid bacteria), smear-ripened cheeses (bacteria and yeasts), kefir and koumiss (lactic acid bacteria and yeasts), Swiss cheese (lactic acid bacteria and propionic acid bacteria), soy sauce (molds, lactic acid bacteria and yeasts), wine (yeasts and lactic acid bacteria), and sourdough bread (yeasts and lactic acid bacteria).

The process of food fermentation can also be categorized into alcoholic, acidic, and alkaline fermentations based on the main products formed or pH changes. Alcoholic beverages, in which ethanol is the main product of sugar metabolism, clearly fall into the category of alcoholic fermentation carried out by yeasts. Lactic fermentation involving lactic acid bacteria belongs to the category of acid fermentation (pH reduction), in which lactic acid is the main product of sugar transformation (e.g., fermented milks and vegetables). Alkaline fermentation typically involves ammonia production due to amino acid deamination (pH increase), for instance, during fermentation of some fermented soybean products (e.g., tempeh, natto, stinky tofu/fermented soy bean curd). This differs from the pH increase in smear-ripened cheeses due to lactic acid catabolism by yeasts. It should be pointed out that there is an initial pH reduction due to acidification by lactic acid bacteria before pH increase due to ammonia formation or lactic acid degradation.

The fermentation of foods can be performed in liquid state ("submerged culture"), solid state (little or no free water), or mixed state. Liquid state food fermentations are often seen in the production of alcoholic beverages such as beer and wine, fermented milks, and yogurts. Examples of solid state food fermentations are bread, cocoa and coffee bean fermentation, and some fermented soy bean products (e.g., tempeh and fermented whole soy beans). Some food fermentation involves phase transformation from liquid state to solid state (e.g., milk to cheese) or from solid state to liquid state (soybean to soy sauce).

2.3 Microorganisms and Starter Cultures Involved in Food Fermentations

Bacteria, yeasts, and fungi are the microorganisms that are responsible for food fermentation and flavor formation in the form of pure, defined, or undefined mixed cultures. The specific type(s) and functions of

microorganisms in food fermentation vary with food substrates as well as intrinsic and extrinsic factors such as pH, temperature, and water activity. The following is a summary of microorganisms and starter cultures involved in food fermentation, and detailed information can be obtained elsewhere (Stiles and Holzapfel 1997; Grappin et al. 1999; Wouters et al. 2002; Romano et al. 2003; Bockelmann et al. 2005; Fleet 2008).

The main bacteria involved in food fermentation include acetic acid bacteria (e.g., vinegar), bacilli (e.g., some fermented soy products—natto and stinky tofu), lactic acid bacteria (e.g., fermented dairy products, sourdough bread, fermented sausages, and vegetables), propionic acid bacteria (Swiss cheese), smear bacteria (smear-ripened cheese), staphylococci and micrococci (fermented sausages), and *Zymomonas* sp. (e.g., some fermented beverages—palm wine). These bacteria contribute to flavor formation in fermented foods by way of acid production, proteolytic and lipolytical activities, as well as transformation of amino acids.

Yeasts are responsible for the fermentation of bread, soy sauce, smear-ripened cheeses, and alcoholic beverages such as beer, wine, and sake. *S. cerevisiae* is the main species of yeast used in food fermentation, although the properties of *S. cerevisiae* yeast strains used in bread, beer, and wine fermentation vary tremendously. However, non-*Saccharomyces* yeasts also play a role in the fermentation of certain foods such as smear-ripened cheeses, kefir and koumiss, soy sauce, fermented sausages, and sourdough bread. These non-*Saccharomyces* yeasts include the so-called dairy yeasts (*Candida kefyr*, *Debaryomyces hansenii*, *Geotrichum candidum*, *Kluyveromyces lactis* and *K. marxianus*, and *Yarrowia lipolytica*), sourdough yeast (e.g., *Candida milleri*), soy sauce yeasts (e.g., *Candida versatilis* and *Zygosaccharomyces rouxii*), etc. These yeasts contribute to flavor through the generation of alcohols, esters, carboxylic acids, lactones, aldehydes, volatile sulfur compounds, and volatile phenols by transforming sugars, amino acids, glycosides, and phenolic acids during fermentation.

Fungi (molds) are also an essential part of the microflora in the fermentation of several types of fermented foods. Mold-ripened cheeses are made using *Penicillium camembert* (e.g., Camembert and Brie cheeses) and *Penicillium roqueforti* (e.g., blue cheeses such as Roquefort and Danablu). Several types of sausages and hams are fermented involving *P. camembert*, *Penicillium nalgiovense*, and *Penicillium chrysogenum*. The fungal cultures for the production of fermented soy products include *Aspergillus oryzae* (soy sauce, miso, soy bean paste, and fermented whole soybeans), *Aspergillus sojae* (soy sauce), *Mucor* sp. and *Actinomucor* sp. (sufu or fermented soybean curd/tofu), *Monascus* sp. (red yeast rice), and *Rhizopus oligosporus* (tempeh). *A. oryzae* is used almost universally in the making of grain-based alcoholic beverages such as rice wine (e.g., sake). These fungi produce enzymes that break down substrates such as proteins, lipids, and polysaccharides in the raw materials during the solid-state stage of fermentation. The substances produced by fungi serve as direct flavor compounds (e.g., methylketones in mold-ripened cheeses) or act as flavor precursors during subsequent yeast or bacterial fermentations (e.g., fermented soy products and grain-based alcoholic beverages).

2.4 General Flavors Associated with Type of Food Fermentations

The flavor of fermented foods is derived not only from microbial fermentation but also from raw materials and manufacturing processes (e.g., Maillard reaction) due to chemical and biochemical transformations. This section is intended to give a brief introduction to flavors associated with food fermentation, and the interested reader is referred to detailed information available elsewhere (Piggot and Paterson 1994; Cheetham 2002; Berger 2007; Weimer 2007; Moreno-Arribas and Polo 2009).

Fermented dairy products consist of a diverse group of foods such as fermented milks, yogurt, cheese, and traditional lactic butter. The flavor of fermented dairy products varies with the product type. The characteristic aroma of traditional lactic butter is predominantly due to diacetyl, whereas the typical flavor of natural plain yogurt is largely determined by both lactic acid (sour taste) and acetaldehyde ("fresh" aroma). Cheese flavor is complex and diverse, and depends on the cheese variety. The peptides generated from protein breakdown cause bitterness or may bring about savoriness. On the other hand, the amino acids from proteolysis and peptidolysis contribute to the basic tastes of cheeses, especially glutamate (savory or umami taste). Short-chain fatty acids (e.g., butyric acid) from lipolysis result in the

"cheesy," lipolytic odor. Blue cheese flavor is characterized by high levels of methylketones, whereas the smear-ripened cheese aroma is caused mainly by volatile sulfur flavor compounds. Esters impart the fruitiness associated with Italian-type cheeses, whereas Swiss cheeses owe their typical flavor to propionic and acetic acids. The flavor of cheddar-type cheeses is due to a diverse range of compounds, especially volatile sulfur flavor compounds according to the balance component theory, rather than some character-impact compounds.

Fermented meat products include mainly fermented sausages and ham. Lactic acid, fatty acids, branched-chain aldehydes and acids, ketones (acetoin), and amino acids contribute to the flavor of these products (Ordonez et al. 1999; Blank et al. 2001). Fish sauce is a common fermented fish product in Southeast Asia. The flavor of fish sauce is due to lactic acid produced by halophilic lactic acid bacteria (Françoise 2010) as well as aldehydes, ammonia, hydrocarbons (decane and dodecane), and sulfur compounds (Beddows 1998).

There is a range of fermented soy bean products, for instance, soy sauce, Japanese miso, soy bean paste (doujiang in Chinese), Japanese natto and Korean cheonggukjang (similar products), fermented whole black beans (douchi in Chinese), and fermented soy bean curd or tofu (sufu or soy cheese) (Liu 2004). The natto odor is attributed to branched-chain fatty acids such as 2- and 3-methylbutanoic acids. The flavor of soy sauce is mainly composed of glutamic acid (umami taste) as well as alcohols, 4-hydroxyfuranones, methionol, and volatile phenolic compounds which, together, impart a savory, sweet, and spicy flavor to soy sauce (van der Sluis et al. 2001). Volatile sulfur compounds are common in fermented soybean products (Ong and Liu 2011).

Many fermentation-derived volatiles contribute to the flavor of fermented alcoholic beverages such as beer, wine, and grain-based spirits: alcohols, carboxylic acids, carbonyls, esters, and even volatile sulfur compounds (e.g., some thiols). Yeast-derived esters are undoubtedly the most important volatile compounds, imparting fruitiness. Typical esters include branched-chain esters (e.g., isoamyl acetate, banana aroma), ethyl esters of fatty acids (e.g., ethyl octanoate, fruity), and 2-phenylethyl acetate (rose-like, floral aroma).

The sharp, sour taste of fermented vegetables, such as sauerkraut and kimchi, is imparted by lactic acid formed by lactic acid bacterial fermentation, in addition to the acetic acid, ethanol, and succinic acid produced.

The main flavor compounds of bread and sourdough bread are produced by fermentation, including alcohols (typically ethanol and branched-chain alcohols), aldehydes (especially branched-chain aldehydes), and esters. These yeast-derived volatiles contribute to the yeasty, sweet, and malty flavor of bread. The sourness of sourdough bread is caused by the lactic and acetic acids formed by lactic acid bacteria.

Acetic acid is known to be the key flavor compound of vinegar. However, the flavor of vinegar is more than just acetic acid. There are significant differences in the composition of volatile compounds among different vinegar varieties. Vinegar flavor is strongly influenced by volatile compounds carried over from the raw ethanolic materials such as cider, wine, rice wine, and malt mash, as well as other volatiles (e.g., aldehydes, carboxylic acids, and esters) produced during vinegar fermentation (Hutkins 2006).

2.5 Carbohydrate-Derived Flavors

Carbohydrates (typically monosaccharides and disaccharides) are the growth and energy sources for microorganisms. As such, a number of nonvolatile and volatile flavor compounds can be produced such as acids, alcohols, and esters (Figure 2.1). The type and concentration of flavor compounds produced vary with the microorganisms involved.

Lactic acid bacteria use hexoses via the homofermentative or heterofermentative pathways; the former pathway results in the production of lactic acid as the major end product, whereas the latter pathway produces lactic acid, acetic acid, ethanol, and carbon dioxide (Figure 2.1) (Axelsson 1993). Some lactic acid bacteria convert pentoses to lactic and acetic acids. Heterofermentative lactic acid bacteria can use fructose as an electron acceptor and reduce fructose to mannitol, besides producing lactic acid, acetic

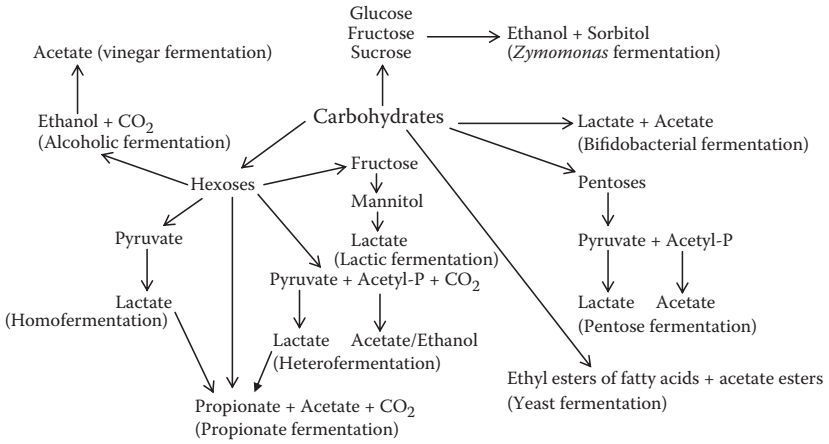

FIGURE 2.1 A simplified scheme of flavor compound formation from carbohydrates via microbial fermentation.

acid, and carbon dioxide (Figure 2.1). Mannitol is a sugar alcohol with a range of applications such as sweetening (reduced calorie) and texturizing (Saha and Racine 2010). Mannitol can be produced in fruit and vegetable fermentations in which fructose and sucrose are available abundantly and may contribute to sweetness if it is produced at sufficient levels. On the other hand, mannitol can be catabolized to lactic acid by facultative *Lactobacillus plantarum* (Liu 2003). The production of mannitol by *Leuconostoc mesenteroides* and the use of mannitol by *L. plantarum* may facilitate microbial succession and contribute to the acidification of fermented vegetables such as sauerkraut and kimchi.

Propionic acid bacteria metabolize sugars and lactic acid derived from sugars by lactic acid bacteria to propionic acid, acetic acid, and carbon dioxide, which are key compounds for Swiss-type cheese flavor and texture (Grappin et al. 1999). Bifidobacterial fermentation of sugars results in the formation of lactic and acetic acids; the latter accounts for the vinegary odor in fermented milks made with bifidobacteria. Bacteria from the *Zymomonas* genus convert glucose to ethanol or a mixture of glucose and fructose (or sucrose) to ethanol and sorbitol (a sweetener) (Hardman et al. 1992). *Zymomonas* bacteria can also produce excessive levels of acetaldehyde from sugar fermentation, causing spoilage of beer and cider.

Sugar fermentation by yeasts (i.e., glycolysis or alcoholic fermentation) produces more than just ethanol. A wide variety of nonvolatile and volatile flavor compounds can be produced from sugars by yeasts, for example, glycerol (sweet), higher alcohols (e.g., 3-methylbutanol), carboxylic acids (e.g., octanoic acid), ethyl esters of fatty acids (e.g., ethyl octanoate), and carbonyls (e.g., acetaldehyde) (Ugliano and Henschke 2009).

The production of vinegar involves two-stage fermentation of sugars: alcoholic fermentation of sugar-rich substrates by yeasts to produce ethanol, followed by the conversion of ethanol to acetic acid by acetic acid bacteria (real vinegar fermentation) (Hutkins 2006).

2.6 Protein-Derived Flavors

Proteolysis and peptidolysis associated with microbial transformation generate peptides and amino acids, which impart basic tastes to fermented, protein-rich foods such as cheeses and fermented soy products (Christensen et al. 1999; McSweeney and Sousa 2000; Smit et al. 2005). Glutamate-rich peptides generally give umami taste sensations, whereas hydrophobic peptides (high in hydrophobic amino acids such as proline) cause bitterness, especially in cheeses. The basic tastes of amino acids include sourness (e.g., aspartic acid), sweetness (e.g., alanine), bitterness (e.g., arginine), and umami (glutamic acid). Protein-derived glutamic acid is the key savory taste compound in cheeses (especially ripened ones) and fermented soy products (soy sauce in particular).

2.7 Amino Acid–Derived Flavors

Amino acids, derived from proteolysis or present in free form in food materials, are precursors to numerous aroma compounds, which can be produced by bacteria, yeasts, and fungi through various pathways (Figure 2.2). The specific types of aroma compounds produced vary with the amino acid precursors and the microbes, which include acid, alcohols, aldehydes, and esters (Figure 2.2).

Branched-chain amino acids (leucine, isoleucine, and valine) and aromatic amino acids (phenylalanine, tryptophan, and tyrosine) are the major aroma precursors for branched-chain alcohols (fusel oil or higher alcohols) and aromatic alcohols produced by yeasts following the Ehrlich pathway (Hazelwood et al. 2008). Lactic acid bacteria can similarly catabolize these amino acids but generally require the supply of an amino acceptor such as α-ketoglutaric acid (Christensen et al. 1999; Yvon and Rijnen 2001; Smit et al. 2004, 2005; Ardö 2006; Ziadi et al. 2010). In addition to branched-chain and aromatic alcohols, aldehydes, carboxylic acids, and esters can be produced microbially from these amino acids. Most of the volatiles have a positive effect on the flavor of fermented foods, imparting attributes such as maltiness (aldehydes), fruitiness (esters), and floralness (2-phenylethanol and 2-phenylethyl acetate). However, volatiles derived from tryptophan and tyrosine (e.g., tryptophol and tyrosol) can affect flavor negatively when produced at higher levels, giving off an unclean odor (Christensen et al. 1999; McSweeney and Sousa 2000).

Other amino acids can also be catabolized to produce various compounds that affect flavor, for instance, transamination of aspartic acid to diacetyl and acetoin by *Lactococcus lactis*, degradation of serine to acetoin by *L. plantarum*, and cleavage of threonine to acetaldehyde and glycine by yogurt cultures (Liu et al. 2003; Le Bars and Yvon 2008). Interestingly, a soy sauce lactic acid bacterium, *Tetragenococcus halophilus*, can decarboxylate the sour-tasting aspartic acid to the sweet-tasting alanine, which may potentially affect the taste of soy sauce (Higuchi et al. 1998).

It is worth noting that propionibacteria used in Swiss cheese fermentation produce aroma compounds such as carboxylic acids from branched-chain and aromatic amino acids in a similar way to that of lactic acid bacteria via transamination in the presence of an amino acceptor such as α-ketoglutaric acid (Thierry and Maillard 2002; Thierry et al. 2002, 2005). Propionibacteria also convert aspartate to succinate, acetate, ammonia, and carbon dioxide (Crow 1986).

Sausage starter cultures, *Staphylococcus carnosus* and *Staphylococcus xylosus*, also catalyze the transamination of branched-chain amino acids into corresponding aldehydes and acids, requiring the presence of an amino acceptor such as α-ketoglutaric acid (Larrouture et al. 2000; Beck et al. 2004).

Volatile sulfur compounds represent a unique group of flavor compounds that can have a positive or negative impact on the organoleptic properties of fermented foods. Although volatile sulfur compounds

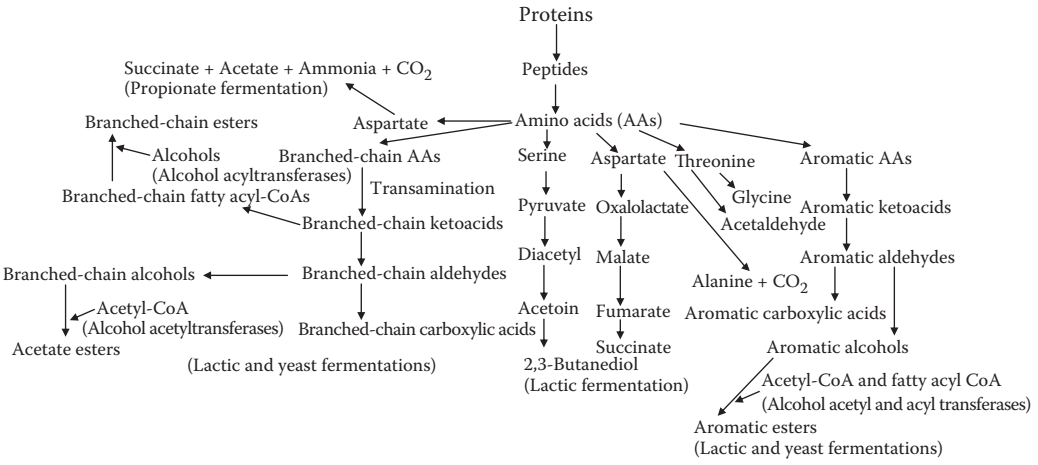

FIGURE 2.2 A simplified scheme of flavor compound formation from proteins, peptides, and amino acids via microbial fermentation.

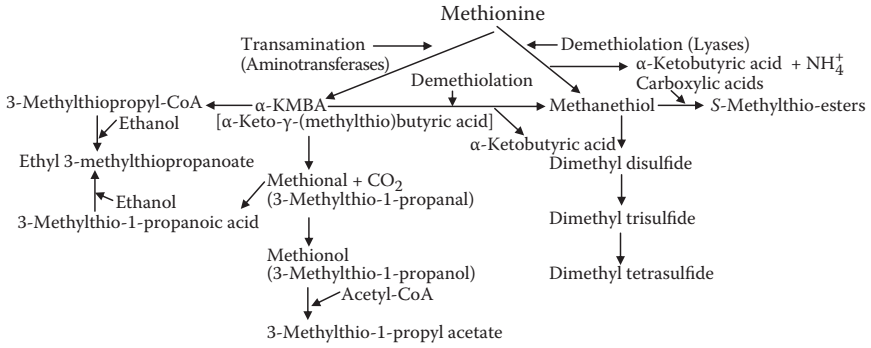

FIGURE 2.3 A simplified scheme of flavor compound formation from L-methionine via microbial fermentation.

(e.g., methional and methionol) are central to the flavor of cheeses and fermented soy products, they can have a detrimental effect on the flavor of beer and wine (Landaud et al. 2008; Rauhut 2009; Ugliano and Henschke 2009; Ong and Liu 2011). The exception is some volatile thiols (4-mercapto-4-methyl-pentan-2-one, 3-mercaptohexanol-1-ol, and 3-mercaptohexyl acetate), which can add fruitiness to some wine varieties (Swiegers and Pretorius 2007).

Many of these sulfur compounds have direct or indirect microbial origins such as methionol, methional, thioesters, thiols, and sulfides (Figure 2.3). Methionine is the main precursor to volatile sulfur compounds. Methionol is mainly produced by yeasts from methionine through the Ehrlich pathway, although demethiolation of methionine is an alternative route with the formation of methanethiol (Hazelwood et al. 2008). On the other hand, methionine demethiolation (elimination by lyases) seems to be the major pathway operating in lactic acid bacteria and brevibacteria (Weimer et al. 1999). Methional and methionine-derived esters (ethyl 3-methylthio-1-propanoate and 3-methylthio-1-propyl acetate) can be produced by *Saccharomyces* and non-*Saccharomyces* yeasts (Liu and Crow 2010; Seow et al. 2010). Volatile thiols are released from nonvolatile, cysteine-bound conjugates by yeasts (Swiegers and Pretorius 2007).

2.8 Lipid-Derived Flavors

Lipids include fats and oils. Microbial metabolism of lipids plays a central role in the flavor of some fermented foods, especially cheeses, fermented meats, and fermented soy products. A large number of flavor compounds can be generated from lipids because of microbial catabolism, including fatty acids, secondary alcohols, methylketones, esters, and lactones (Figure 2.4) (McSweeney and Sousa

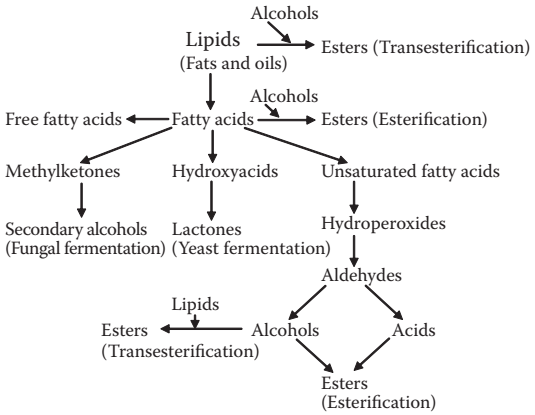

FIGURE 2.4 A simplified scheme of flavor compound formation from lipids via microbial fermentation.

2000; Holland et al. 2005). The type and relative concentrations of flavor compounds formed are dependent on the lipid composition of the food materials and microbes involved. The release of short-chain fatty acids is essential to the flavor of cheeses that contain milk fats high in such fatty acids. The fungal conversion of fatty acids to methylketones is necessary for the development of blue cheese flavors.

Fruitiness due to esters is an attribute of Italian-type cheeses, but is a flavor defect of cheddar-type cheeses. It has been demonstrated that lactic acid bacteria catalyze the synthesis of esters from alcohols and milk fat by way of alcoholysis (transesterification) in dairy systems (Liu et al. 2004). Recent evidence also shows that the addition of ethanol to Swiss cheese enhances the formation of ethyl esters, suggesting a similar mechanism of ester biosynthesis in propionibacteria (Thierry et al. 2006; Richoux et al. 2008).

2.9 Organic Acid–Derived Flavors

Malic and citric acids are two common organic acids that are susceptible to microbial attack during fermentation, affecting sensory properties. Malic acid can be converted to ethanol and carbon dioxide by yeasts (the so-called maloethanolic fermentation; Figure 2.5), especially *Schizosaccharomyces pombe* and *Schizosaccharomyces malidevorans*, but *S. cerevisiae* yeasts have a limited capacity to do so (Radler 1993). On the other hand, malic acid can be degraded to form lactic acid and carbon dioxide (the so-called malolactic fermentation) by lactic acid bacteria (Figure 2.5), especially *Oenococcus oeni* (Liu 2002). The transformation of malic acid to less acidic lactic acid or ethanol is beneficial to high-acid wines produced in cool climate regions, reducing tartness and improving mouthfeel.

Citric acid is a precursor to several flavor compounds including acetic acid, diacetyl, acetoin, and succinic acid, aside from lactic acid (Figure 2.5). This organic acid is typically degraded by lactic acid bacteria, for example, *L. mesenteroides* subsp. *cremoris* and *Lactococcus lactis* subsp. *lactis* var. *diacetyllactis* in dairy fermentation, *O. oeni* in wine fermentation, and spoilage lactic acid bacteria in beer fermentation (Cogan 1995; Liu 2002). Acetic acid is undesirable in alcoholic beverages, imparting a vinegary off-flavor, although it is part of the aroma array of other fermented foods such as cheese, fermented vegetables, and in particular, sourdough bread. Diacetyl is an important aroma compound of butter, cheese, and yogurt. At low levels, it adds complexity to wine flavor (buttery aroma). However, diacetyl is highly undesirable in beer, causing an off-odor due to its very low detection threshold. Acetoin, although possessing a buttery odor, has a much higher detection threshold than diacetyl and, therefore, generally its concentration is not high enough to impart an odor to fermented foods and beverages. Some lactobacilli can transform citric acid to succinic acid, which is a taste modifier (giving a broth-like taste).

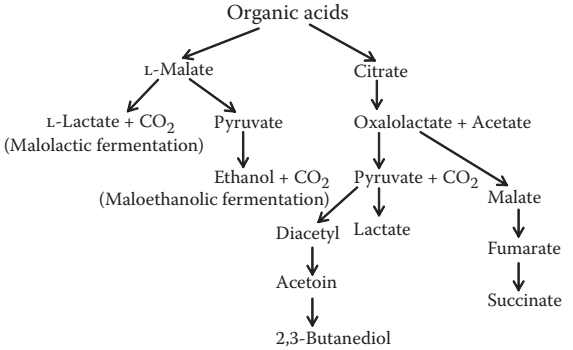

FIGURE 2.5 A simplified scheme of flavor compound formation from organic acids via microbial fermentation.

2.10 Glycoside-Derived Flavors

Aroma compounds in some plant-based matrices such as fruit juices exist in free forms or bound, nonvolatile, and odorless forms (known as glycosides or glycoconjugates). The aglycones of the glycosides typically include oxygenated terpenes, C13-norisoprenoids, benzene derivatives (volatile phenolics), and aliphatic alcohols. These glycosides serve as aroma precursors and can release aroma compounds during fermentation due to chemical and enzymatic hydrolysis (Pogorzelski and Wilkowska 2007). There is evidence that not only *S. cerevisiae* yeasts but also lactic acid bacteria *O. oeni* possess glycosidases, which can hydrolyze glycosidically bound aroma compounds such as linalool, citronellol, and hexanol during alcoholic and malolactic fermentations (Ugliano et al. 2003, 2006). The increased concentrations of oxygenated terpenes (above threshold levels) enhance the fruity and floral notes of fruit-based alcoholic beverages. The liberation of aroma compounds on hydrolysis of sugar-bound aroma precursors is influenced by a number of factors such as yeast strains, malolactic bacterial strains, and fermentation conditions (pH, temperature, free sugars, and ethanol) (Grimaldi et al. 2005; Pogorzelski and Wilkowska 2007).

2.11 Phenolic Acid–Derived Flavors

Ferulic and *p*-coumaric acids are the main constituents of phenolic acids which are usually bound to plant cell walls. Free phenolic acids can be liberated by certain microbial cinnamoyl esterase activities during alcoholic beverage fermentations such as beer and wine. These free phenolic acids can be further metabolized by *S. cerevisiae* yeast strains and lactic acid bacteria through decarboxylation to form various volatile phenolics (Barthelmebs et al. 2001; Smit et al. 2003; Coghe et al. 2004). Ferulic and *p*-coumaric acids can be decarboxylated to form 4-vinylguaiacol and 4-vinylphenol, respectively, followed by reduction to corresponding 4-ethylguaiacol and 4-ethylphenol (Figure 2.6). The effect of these volatile phenolics on flavor is dependent on concentration and food type. At low levels, they may contribute positively to flavor by adding complexity and uniqueness (clove-like, spicy, woody, or smoky flavor notes). However, high levels of these volatile phenolics cause a medicinal phenolic off-flavor in alcoholic beverages. In soy sauce fermentation, *Candida versatilis* (not *Z. rouxii*) is the yeast that produces these important flavor compounds from ferulic and *p*-coumaric acids (van der Sluis et al. 2001).

2.12 Conclusions and Future Prospects

Fermentation is central to the generation of flavor compounds that impart sensory characteristics to fermented foods and beverages. A deeper understanding of microbial physiology and metabolism will enable better control of microbial flavor formation so as to accentuate, diversify, or minimize the formation of certain flavor compounds (desirable or undesirable). This can be achieved using molecular tools such as metabolic engineering and genomics approaches. Examples of metabolically engineered microorganisms are yeast strains of *S. cerevisiae* with altered enzyme activities for ester biosynthesis in alcoholic beverage fermentations such as beer, sake, and wine (Fukuda et al. 1998; Lilly et al. 2006; Saerens et al. 2008). On the other hand, many food fermentations are performed using mixed cultures

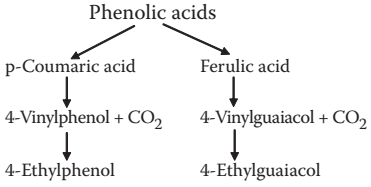

FIGURE 2.6 A simplified scheme of flavor compound formation from phenolic acids via microbial fermentation.

and, as such, microbial interactions that affect flavor formation would occur. Genomics tools may help us to understand the microbial interactions and the consequent flavor impact (Sieuwerts et al. 2008). These molecular approaches are expected to play a significant part in future food fermentations in terms of flavor modulation, consumer acceptance, and application of genetically modified microorganisms in food production.

REFERENCES

Ardö Y. 2006. Flavour formation by amino acid catabolism. *Biotechnol Adv* 24:238–42.

Axelsson LT. 1993. Lactic acid bacteria: classification and physiology. In: Salminen S, von Wright A, editors. *Lactic acid bacteria*. New York: Marcel Dekker. p. 1–63.

Barthelmebs L, Divies C, Cavin J-F. 2001. Molecular characterization of the phenolic acid metabolism in the lactic acid bacteria *Lactobacillus plantarum*. *Lait* 81:161–71.

Bartowsky EJ, Pretorius IS. 2009. Microbial formation and modification of flavour and off-flavor compounds in wine. In: König H, Uden G, Frohlich J, editors. *Biology of microorganisms on grapes, in must and in wine*. Berlin: Springer-Verlag. p. 209–31.

Beck HC, Hansen AM, Lauritsen FR. 2004. Catabolism of leucine to branched-chain fatty acids in *Staphylococcus xylosus*. *J Appl Microbiol* 96:1185–93.

Beddows CG. 1998. Fermented fish and fish products. In: Wood BJB, editor. *Microbiology of fermented foods*. Vol. 1, 2nd ed. London: Blackie Academic and Professional. p. 416–40.

Berger RG. 2007. *Flavours and Fragrances: Chemistry, Bioprocessing and Sustainability*. Berlin: Springer.

Blank I, Devaud S, Fay LB, Cerny C, Steiner M, Zurbriggen B. 2001. Odor-active compounds of dry-cured meat: Italian-type salami and ham. In: Takeoka GR, Guntert M, Engel K-H, editors. *Aroma active compounds in foods. Chemistry and sensory properties. ACS Symposium Series 794*. Washington, DC: American Chemical Society. p. 9–20.

Bockelmann W, Willems KP, Neve H, Heller KH. 2005. Cultures for the ripening of smear cheeses. *Int Dairy J* 15:719–32.

Cheetham PSJ. 2002. Plant-derived natural sources of flavours. In: Taylor A, editor. *Food flavor technology*. New Jersey: Wiley-Blackwell. p. 105–52.

Christensen JE, Dudley EG, Pederson JA, Steele JL. 1999. Peptidases and amino acid catabolism in lactic acid bacteria. *Antonie von Leeuwenhoek* 76:217–46.

Cogan TM 1995. Flavour production by dairy starter cultures. *J Appl Bacteriol - Symp Suppl* 79:49S–64S.

Cogan TM, Accolas J-P. 1996. *Dairy starter cultures*. New York: VCH Publishers.

Coghe S, Benoot K, Delvaux F, Vanderhaegen B, Delvaux FR. 2004. Ferulic acid release and 4-vinylguaiacol formation during brewing and fermentation: indications for feruloyl esterase activity in *Saccharomyces cerevisiae*. *J Agric Food Chem* 52:602–8.

Crow VL. 1986. Metabolism of aspartate by *Propionibacterium freudenreichii* subsp. *shermanii*: effect on lactate fermentation. *Appl Environ Microbiol* 52:359–65.

Fleet GH. 2008. Wine yeasts for the future. *FEMS Yeast Res* 8:979–95.

Françoise L. 2010. Occurrence and role of lactic acid bacteria in seafood products. *Food Microbiol* 27: 698–709.

Fukuda K, Yamamoto N, Kiyokawa Y, Yanagiuchi T, Wakai Y, Kitamoto K, Inoue Y, Kimura A. 1998. Balance of activities of alcohol acetyltransferase and esterase in *Saccharomyces cerevisiae* is important for production of isoamyl acetate. *Appl Environ Microbiol* 64(10):4076–8.

Grappin R, Beuvier E, Bouton Y, Pochet S. 1999. Advances in the biochemistry and microbiology of Swiss-type cheeses. *Lait* 79:3–22.

Grimaldi A, Bartowsky E, Jiranek V. 2005. A survey of glycosidase activities of commercial wine strains of *Oenococcus oeni*. *Int J Food Microbiol* 105:233–44.

Hansen EB. 2002. Commercial bacterial starter cultures for fermented foods of the future. *Int J Food Microbiol* 78:119–31.

Hardman MJ, Tsao N, Scopes RK. 1992. Changes in the fluorescence of bound nucleotide during the reaction catalysed by glucose-fructose oxidoreductase from *Zymomonas mobilis*. *Eur J Biochem* 205:715–20.

Hazelwood LA, Daran J-M, van Maris AJA, Pronk JT, Dickinson JR. 2008. The Ehrlich pathway for fusel alcohol production: a century of research on *Saccharomyces cerevisiae* metabolism. *Appl Environ Microbiol* 74(8):2259–66.

Higuchi T, Uchida K, Abe K. 1998. Aspartate decarboxylation encoded on the plasmid in the soy sauce lactic acid bacteria, *Tetragenococcus halophilus* D10. *Biosci Biotechnol Biochem* 62:1601–3.

Holland R, Liu S-Q, Crow VL, Delabre M-L, Lubbers M, Bennett M, Norris G. 2005. Esterases of lactic acid bacteria and cheese flavor: milk fat hydrolysis, alcoholysis and esterification. *Int Dairy J* 15:711–8.

Hui YH, Meunier-Goddik L, Hansen AS, Josephsen J, Nip W-K, Stanfield PS, Toldra F. 2004. *Handbook of food and beverage fermentation technology*. New York: Marcel Dekker.

Hutkins RW. 2006. *Microbiology and technology of fermented foods*. Oxford, UK: Blackwell Publishing.

Landaud S, Helinck S, Bonnarme P. 2008. Formation of volatile sulfur compounds and metabolism of methionine and other sulphur compounds in fermented foods. *Appl Microbiol Biotechnol* 77:1191–205.

Larrouture C, Ardaillon V, Pepin M, Montel MC. 2000. Ability of meat starter cultures to catabolize leucine and evaluation of the degradation products by using an HPLC method. *Food Microbiol* 17:563–70.

Le Bars D, Yvon M. 2008. Formation of diacetyl and acetoin by *Lactococcus lactis* via aspartate catabolism. *J Appl Microbiol* 104:171–7.

Leroy F, De Vuyst L. 2004. Lactic acid bacteria as functional starter cultures for the food fermentation industry. *Trends Food Sci Technol* 15:67–78.

Leroy F, Verluyten J, De Vuyst L. 2006. Functional meat starter cultures for improved sausage fermentation. *Int J Food Microbiol* 106:270–85.

Lilly M, Bauer FF, Lambrechts MG, Swiegers JH, Cozzolino D, Pretorius IS. 2006. The effect of increased yeast acetyltransferase and esterase activity on the flavour profile of wine and distillates. *Yeast* 23:641–59.

Liu K. 2004. Fermented soy foods: an overview. In: Hui YH, Meunier-Goddik L, Hansen AS, Josephsen J, Nip W-K, Stanfield PS, Toldra F, editors. *Handbook of food and beverage fermentation technology*. New York: Marcel Dekker. p. 481–96.

Liu S-Q. 2002. A review: Malolactic fermentation in wine—beyond deacidification. *J Appl Microbiol* 92: 589–601.

Liu S-Q. 2003. Review article: Practical implications of lactate and pyruvate metabolism by lactic acid bacteria in food and beverage fermentation. *Int J Food Microbiol* 83:115–31.

Liu S-Q, Crow VL. 2010. Production of dairy-based, natural sulphur flavour concentrate by yeast fermentation. *Food Biotechnol* 24:62–77.

Liu S-Q, Holland R, Crow VL. 2004. Esters and their biosynthesis in fermented dairy products: a review. *Int Dairy J* 14:923–45.

Liu S-Q, Holland R, McJarrow PJ, Crow VL. 2003. Serine metabolism in *Lactobacillus plantarum*. *Int J Food Microbiol* 89:265–73.

McSweeney PLH, Sousa MA. 2000. Biochemical pathways for the production of flavour compounds in cheeses during ripening: a review. *Lait* 80:293–324.

Moreno-Arribas MV, Polo MC. 2009. *Wine chemistry and biochemistry*. New York: Springer Science+Business Media.

Ong PKC, Liu S-Q. 2011. Flavor and sensory characteristics of vegetables. In: Sinha NK, editor. *Handbook of vegetables and vegetable processing*. New Jersey: Blackwell Publishing. p. 59–82.

Ordonez JA, Hierro EM, Bruna JM, de la Hoz L. 1999. Changes in the components of dry-fermented sausages during ripening. *Crit Rev Food Sci Nutri* 39:329–67.

Piggott JR, Paterson A. 1994. *Understanding natural flavor*. London: Blackie Academic and Professional.

Pogorzelski W, Wilkowska A. 2007. Flavor enhancement through the enzymatic hydrolysis of glycosidic aroma precursors in juices and wine beverages: a review. *Flav Fragrance J* 22:251–4.

Radler F. 1993. Yeast: metabolism of organic acids. In: Fleet GH, editor. *Wine microbiology and biotechnology*. Chur, Switzerland: Harwood Academic. p. 165–82.

Rauhut D. 2009. Usage and formation of sulphur compounds. In: König H, Uden G, Frohlich J, editors. *Biology of microorganisms on grapes, in must and in wine*. Berlin: Springer-Verlag. p. 181–207.

Richoux R, Maillard M-B, Kerjean J-R, Lortal S, Thierry A. 2008. Enhance of ethyl ester formation in Swiss cheese by ethanol addition. *Int Dairy J* 18:1140–5.

Romano P, Fiore C, Paraggio M, Caruso M, Capece A. 2003. Functions of yeast species and strains in wine flavor. *Int J Food Microbiol* 86:169–80.

Saerens SMG, Delvaux F, Verstrepen KJ, van Dijck P, Thevelein JM, Delvaux FR. 2008. Parameters affecting ethyl ester production by *Saccharomyces cerevisiae* during fermentation. *Appl Environ Microbiol* 74(2):454–61.

Saha BC, Racine FM. 2010. Biotechnological production of mannitol and its applications. *Appl Microbiol Biotechnol* 89:879–91.

Seow YX, Ong PKC, Liu S-Q. 2010. Production of flavor-active methionol from methionine metabolism by yeasts in coconut cream. *Int J Food Microbiol* 143:235–40.

Sieuwerts S, de Bok FAM, Hugenholtz J, van Hyckama Vlig JET. 2008. Unraveling microbial interactions in food fermentations: from classical to genomics approaches. *Appl Environ Microbiol* 74(16):4997–5007.

Smit A, Cordero Otero RR, Lambrechts MG, Pretorius IS, van Renburg P. 2003. Enhancing volatile phenol concentrations in wine by expressing various phenolic acid decarboxylase genes in *Saccharomyces cerevisiae*. *J Agric Food Chem* 51:4909–15.

Smit G, Smit BA, Engels WJM. 2005. Flavour formation by lactic acid bacteria and biochemical flavor profiling of cheese products. *FEMS Microbiol Rev* 29:591–610.

Smit G, Wouters JTM, Meijer WC. 2004. Quality and flavor of fermented products. In: Hui YH, Meunier-Goddik L, Hansen AS, Josephsen J, Nip W-K, Stanfield PS, Toldra F, editors. *Handbook of food and beverage fermentation technology*. New York: Marcel Dekker. p. 89–112.

Stiles M, Holzapfel WH. 1997. Lactic acid bacteria of foods and their taxonomy. *Int J Food Microbiol* 36:1–29.

Sumby KM, Grbin PR, Jiranek V. 2010. Microbial modulation of aromatic esters in wine: current knowledge and future prospects. *Food Chem* 121:1–16.

Swiegers JH, Pretorius IS. 2007. Modulation of volatile sulphur compounds by wine yeasts. *Appl Microbiol Biotechnol* 74:954–60.

Thierry A, Maillard M-B. 2002. Production of cheese flavor compounds derived from amino acid catabolism by *Propionibacterium freudenreichii*. *Lait* 82:17–32.

Thierry A, Maillard M-B, Richoux R, Kerjean J-R, Lortal S. 2005. *Propionibacterium freudenreichii* strains quantitatively affect production of volatile compounds in Swiss cheese. *Lait* 85:57–74.

Thierry A, Maillard M-B, Richoux R, Lortal S. 2006. Ethyl ester formation is enhanced by ethanol addition in mini Swiss cheese with and without added propionibacteria. *J Agric Food Chem* 54:6819–24.

Thierry A, Maillard M-B, Yvon M. 2002. Conversion of L-leucine to isovaleric acid by *Propionibacterium freudenreichii* TL 34 and ITGP23. *Appl Environ Microbiol* 68(2):608–15.

Ugliano M, Bartowsky EJ, McCarthy J, Moio L, Henschke PA. 2006. Hydrolysis and transformation of grape glycosidically bound volatile compounds during fermentation with three *Saccharomyces* yeast strains. *J Agric Food Chem* 54:6322–31.

Ugliano M, Genovese A, Moio L. 2003. Hydrolysis of wine aroma precursors during malolactic fermentation with four commercial starter cultures of *Oenococcus oeni*. *J Agric Food Chem* 51:5073–8.

Ugliano M, Henschke PA. 2009. Yeasts and wine flavor. In: Moreno-Arribas MV, Polo MC, editors. *Wine chemistry and biochemistry*. New York: Springer Science+Business Media. p. 313–92.

van der Sluis C, Tramper J, Wijffels RH. 2001. Enhancing and accelerating flavor formation by salt-tolerant yeasts in Japanese soy-sauce process. *Trends Food Sci Technol* 12:322–7.

Weimer B, Seefeldt K, Dias B. 1999. Sulphur metabolism in bacteria associated with cheese. *Antonie von Leeuwenhoek* 76:247–61.

Weimer BC. 2007. *Improving the flavor of cheese*. Sawston, UK: Woodhead Publishing.

Wouters JTM, Ayad EHE, Hugenholtz J, Smit G. 2002. Microbes from raw milk for fermented dairy products. *Int Dairy J* 12:91–109.

Yvon M, Rijnen L. 2001. Cheese flavor formation by amino acid catabolism. *Int Dairy J* 11:185–201.

Ziadi M, Bergot G, Courtin P, Chambellon E, Hamdi M, Yvon M. 2010. Amino acid catabolism by *Lactococcus lactis* during milk fermentation. *Int Dairy J* 20:25–31.

3

Fermentation and Biopreservation of Plant-Based Foods with Lactic Acid Bacteria

Lihua Fan and Lisbeth Truelstrup Hansen

CONTENTS

3.1 Introduction

Lactic acid bacteria (LAB) is the term used for a large and rather diverse group of prokaryotes, which have in common the production of lactic acid as their main metabolic end product (Klaenhammer et al. 2005). Traditionally, LAB have been characterized as aerotolerant anaerobes belonging to the Gram-positive bacterial phylum. In the new molecular taxonomy in which organisms are clustered according to their genetic relatedness, the LAB clade is grouped with the firmicutes together with other low %G+C Gram-positive bacteria such as *Clostridium*, *Bacillus*, *Staphylococcus*, *Listeria*, etc. (Teuber and Geis 2006). Use of a combination of traditional and molecular techniques has divided bacteria belonging to the LAB family up into the following genera: *Lactobacillus*, *Streptococcus*, *Lactococcus*, *Enterococcus*, *Melissococcus*, *Tetragenococcus*, *Vagococcus*, *Trichococcus*, *Carnobacterium*, *Desemzia*, *Leuconostoc*, *Weisella*, and *Pediococcus* (Hammes and Hertel 2006).

It has been generally recognized that the tolerance of oxygen varies among strains and that, strictly speaking, LAB have no use for oxygen as they do not carry out oxidative phosphorylation but only use substrate level phosphorylation or fermentation to create energy (Björkroth and Holzapfel 2006). Lately, however, evidence has appeared that some members of LAB including *Lactobacillus sanfranciscensis*

may be using NADH-oxidases or Mn(II) to allow the use of oxygen as an external electron acceptor which in turn significantly increases the energy yield (Jänsch et al. 2011).

LAB naturally occurs in a large variety of natural environments in which soluble carbohydrates, protein breakdown products, and low oxygen tension are found. The production of extracellular polysaccharides by many LAB allow them to adhere well to surfaces and making them excellent biofilm formers (Ruas-Madiedo et al. 2009). In general, LAB are resistant to low pH conditions and also have some resistance to higher osmotic pressures. These characteristics are important when it comes to the industrial use of LAB, in which bacteria play an important role in the preservation and microbial safety of many fermented foods. The basis of LAB's protection of foods is mainly due to their production of organic acids, carbon dioxide, ethanol, hydrogen peroxide, diacetyl, antifungal compounds such as free fatty acids or phenyllactic acid, bacteriocins, and antibiotics such as reutericyclin (Höltzel et al. 2000). Bacteriocins are ribosomally synthesized, extracellularly released low molecular mass peptides which have a bactericidal or bacteriostatic effect on bacteria, either from the same species or across genera (Klaenhammer 1988; Cotter et al. 2005). Although nisin is the only purified bacteriocin used thus far in industrially processed foods, many bacteriocins produced by various LAB may have potential applications in food products (Helander et al. 1997; Cleveland et al. 2001; Settanni and Corsetti 2008). Increasingly, it is also recognized that LAB have antifungal properties and may be used to eliminate fungal spoilage or the formation of mycotoxins (Rouse et al. 2008; Dalié et al. 2010). Also, through the manipulation of the intrinsic and extrinsic environment, LAB can naturally outcompete unwanted microorganisms such as *Staphylococcus aureus* (Charlier et al. 2009). An example of this outcompetition is seen in the production of sauerkraut, in which one of the production steps consists of the addition of salt to decrease water activity and at the same time to extract moisture and nutrients from the shredded cabbage. The growth of LAB is now selected over the growth of endogenous Gram-negative spoilage bacteria. The subsequent decrease in pH caused by the production of lactic (and acetic) acid by LAB further causes Gram-negative bacteria to stop growing and slowly die off (Adams and Nicolaides 1997). Furthermore, studies have shown that LAB, during the fermentation of plant material such as carrot juice, degrade oxalate, phytate, and tannins, which in combination with the decrease in pH, increase the solubility (bioavailability) of essential metals such as iron, manganese, and zinc but not copper (Bjergqvist et al. 2005).

3.2 Plant-Associated LAB

LABs are found naturally occurring in a wide range of environments, including the surface of growing and postharvest plant materials. Naturally, selection means that successful LAB have become adapted to their particular environment. This adaptation can be expressed in different ways, which includes the ability to use the available nutrients, tolerance of the pertinent environmental conditions (intrinsic and extrinsic factors), and possibly the production of antimicrobial compounds to inhibit competitors and secure survival. In choosing a suitable LAB for biopreservation and fermentation purposes, it is most obvious to look for strains/species which are already adapted to grow well under the given plant nutritive conditions.

Therefore, which LAB are regularly found in fermented and nonfermented fruit and vegetable products? Literature reports using both culture-dependent and -independent methods mainly associate members of the LAB genera *Leuconostoc, Lactobacillus, Weissella, Pediococcus, Enterococcus*, and *Lactococcus* with fruits and vegetable products (see recent reviews by Björkroth and Holzapfel 2006; Devriese et al. 2006; Hammes and Hertel 2006; and Teuber and Geis 2006). In the past, culture methods have identified homofermentative strains such as *Lactobacillus plantarum* as being very common. Of the heterofermentative LAB, species such as *Leuconostoc mesenteroides* and *Leuconostoc citreum* dominate. However, this view on the plant-associated LAB flora is bound to change as more studies using molecular biology methods investigate the microbial ecology of products.

In one such recent study conducted by Plengvidhya et al. (2007), an rRNA gene intergenic transcribed spacer–polymerase chain reaction method combined with 16S rRNA sequencing was used to identify 686 LAB isolated from four sauerkraut fermentations. Strains normally identified by traditional biochemical

methods including *Leuco. mesenteroides*, *Lb. plantarum*, and *Lactobacillus curvatus* were abundant. However, unexpectedly, they found low numbers of *Pediococcus pentosaceus* and *Lactobacillus brevis*, which are LABs normally expected to play a major role in fermentation. Use of the molecular method revealed the presence of strains such as *Leuco. citreum*, *Leuconostoc argentinum*, *Lactobacillus paraplantarum*, *Lactobacillus coryniformis*, *Weissella* sp., and the newly described *Leuconostoc fallax*, indicating that the LAB flora developing during fermentation of sauerkraut is much more diverse than previously thought. When using 16S rRNA sequencing to analyze the identity of 120 LAB strains isolated during kimchi fermentation, *Leuco. citreum* was found to dominate in the early phase of fermentation whereas *Lactobacillus sakei/Lb. curvatus* and *Lb. brevis* took over in the latter phases (Choi et al. 2003). They also isolated a few *Weissella confusa* strains. A more recent investigation of kimchi fermentations confirmed the presence of these LAB species as well as *Weisella koreensis* and in the overripening stage also *Leuconostoc gelidum* and *Lb. brevis* (Park et al. 2010).

3.3 Fermentation of Plant Material

The fermentation of plant material is an ancient preservation method, which likely originated in Asia. Nowadays, the traditional Korean product, kimchi, is the most popular fermented vegetable food in many Asian countries (Cheigh and Park 1994; Lee 1997; Kwon and Kim 2007). The most common fermented vegetable products in Europe and North America (Canada and the United States) are sauerkraut, cucumbers, and olives (Niksic et al. 2005; Mäki 2004; Settanni and Corsetti 2008). Before the advent of modern food preservation technologies such as refrigeration, controlled atmosphere storage, and freezing, the consumption of fermented vegetable products ensured continuous access to essential vitamins including vitamin C, minerals, and dietary fibers for people in Northern climates with just one annual growth season.

3.3.1 Kimchi

3.3.1.1 Introduction

Kimchi is a traditional Korean fermented vegetable product, which is most commonly prepared with Chinese cabbage as the main ingredient in combination with many minor ingredients such as red pepper powder, ginger, garlic, radish, and fish sauce (Cheigh and Park 1994; Kim et al. 2008). Kimchi preparation methods differ depending on the variety of kimchi and the ingredients used, as well as on whether the production takes place domestically or industrially. Also, the types of kimchi differ from region to region because of differences in harvest and weather conditions. The final flavor profile is determined by the ingredients, condiments, the addition of salt and spices, and fermentation. According to a review by Lee (1997), approximately 190 different types of kimchi are produced in Korean households. In general, kimchi tastes best when it is fermented for 2 to 3 weeks at 2°C to 7°C, to reach a pH level of 4.2 and a titratable acidity of 0.6% to 0.8% (Kim et al. 2008).

3.3.1.2 Fermentation Process

The principal process consists of pretreatment, brining, blending of ingredients, and fermentation steps. For example, the preparation method for Baechu kimchi (cut Chinese cabbage kimchi) is as follows: pretreated Chinese cabbage is cut into cubes of 3 to 5 cm, brined in a salt solution (8–15% w/v) for 2 to 7 hours, rinsed with fresh water, and drained. In a separate process, sliced Oriental radish, carrots, green onion, onion, chopped garlic, ginger, red pepper, salt-pickled seafood, other minor ingredients, and dry salt are combined to make a premixture according to the specific recipe. This premixture is blended well with the treated cabbage. This mixture is then placed in a fermentation vessel sealed with a lid and fermented either for 1 to 3 weeks at low temperatures of 2°C to 10°C or for 1 to 3 days at 20°C to 25°C. After fermentation, kimchi is usually stored at temperatures between 0°C and 8°C and served cold (Cheigh and Park 1994).

The fermentation occurs mainly due to LAB and yeast strains, which are naturally present in the diverse microflora of the raw material. The fermentation of kimchi is initiated by *Leuco. mesenteroides* under anaerobic conditions (Cheigh and Park 1994; Lee 1997). This organism differs from other LAB species in that it can tolerate fairly high concentrations of salt and sugar. *Leuco. mesenteroides* initiates growth in vegetables more rapidly over a wide range of temperatures and salt concentrations than any other LAB. Its heterofermentative metabolism produces carbon dioxide and acids which rapidly lower the pH and inhibit the development of undesirable microorganisms. The carbon dioxide produced replaces the oxygen, making the environment anaerobic and suitable for the growth of subsequent species of lactobacillus. Removal of oxygen also helps to preserve the color of fermented vegetables and stabilizes the ascorbic acid (vitamin C) that is present in the vegetables. As the pH drops to between 4.6 and 4.9, *Leuco. mesenteroides* becomes relatively inhibited, but the fermentation continues with other LAB such as *Streptococcus* (now classified as *Enterococcus*) *faecalis*, *Lb. brevis*, *Pediococcus cerevisiae*, and *Lb. plantarum*. Lee et al. (2005) analyzed the kimchi microflora using denaturing gradient gel electrophoresis, a 16S rRNA gene–based culture-independent molecular technique, and found that *W. confusa*, *Leuco. citreum*, *Lb. sakei*, and *Lb. curvatus* were the main microorganisms responsible for kimchi fermentation. Other techniques, which have been used to analyze the microbial community dynamics during kimchi fermentation, include genome-probing microarray for quantitative, high-throughput monitoring (Bae et al. 2005), polymerase chain reaction–enzyme-linked immunosorbent assay for the screening of many bacterial isolates from fermented vegetables (Tamminen et al. 2004) and sodium dodecyl sulfate–polyacrylamide gel electrophoresis profiles of whole cell proteins for the identification of LAB in kimchi (Tae-Woon et al. 2003).

The most important factors that affect kimchi fermentation are the endogenous plant microorganisms, salt concentration, fermentable carbohydrates, other available nutrients, presence of inhibitory compounds as well as oxygen, pH, and temperature. The effect of salt concentration, temperature (Lee et al. 2008), and pH is mostly due to the influence of these factors on the rate and extent of lactic acid fermentation. The optimum fermentation time to obtain the most acceptable quality of fermented kimchi with different salt concentration and temperature combinations has been investigated by Mheen and Kwon (1984). They found that the optimum period of fermentation for Baechu kimchi is 1 to 2 days at 30°C with a 3.5% salt concentration, and 2 days with a 5% salt concentration and the same temperature. At 14°C, 5 to 12 days are required if combined with 3.5% salt, and this increases to 10 to 18 days if 5.0% salt is used.

The use of well-defined starter cultures is of considerable commercial interest as it can improve the control and predictability of the fermentation process (Chang and Chang 2010). Choi et al. (2003) isolated the dominant *Leuco. citreum* IH22 in their polyphasic study of the LAB community in kimchi fermenting at 15°C and successfully used this strain to obtain a faster and more consistent fermentation to yield a product with better organoleptic properties than the control kimchi.

3.3.1.3 Microbiological Characteristics

The kimchi fermentation pattern is similar to that found in other vegetable lactic acid fermentations; however, due to the many types of kimchi products, comparatively greater variance is found in the associated LAB flora than in other products. Also, because the raw ingredients for kimchi are traditionally not blanched before use, the epiphytic LAB as well as the rest of the other microflora associated with the plant material in the field will be retained to possibly influence the fermentation.

As with other vegetable fermentations, the brining step is very important for kimchi fermentation. Brining extracts the water and nutrients from the raw materials by osmotic activity and suppresses the growth of some undesirable bacteria that may spoil the kimchi. At the same time, it makes conditions relatively favorable for LAB by increasing the salt content in the Chinese cabbage or radish. The LAB count increases approximately fourfold after brining of the Chinese cabbage (Kim et al. 1987; Cheigh and Park 1994). Also, after the brining and rinsing/washing treatments, the counts of bacteria, yeasts, and molds are greatly decreased.

The fermentation containers are usually covered with lids to provide anaerobic conditions and thus minimize the growth of aerobic microorganisms. *Leuco. mesenteroides* is the predominant LAB in the

early fermentation stages but, because of the decrease in pH, is gradually replaced by other LAB such as *E. faecalis*, *Lb. brevis*, *P. cerevisiae*, and *Lb. plantarum*. There is a considerable overlap of LAB species. The growth of each species depends on its initial number in the raw material (Chinese cabbage and other ingredients), the concentrations of salt and sugar, the absence of oxygen, and the fermentation temperature. *Lb. plantarum* is commonly present in the greatest numbers after the initial fermentation and results in maximum acidity at the later stages. Aerobic yeasts and molds may appear on the surface of improperly covered vegetables at the later fermentation stages. The appearance of undesirable and spoiled kimchi, which is characterized by off-flavors and softened texture, is possibly due to excessive aerobic growth of molds and film-forming yeasts (Cheigh and Park 1994). Softening and excessive acidification of kimchi at the overripening stages during fermentation and storage are the most serious problems. It should be pointed out that once prepared, kimchi is ready-to-eat at any stage of the fermentation depending on the individual consumer's preferential taste. Because there is no sterilizing process during kimchi processing, microbial quality and safety are very important issues for the shelf life of kimchi (Kwon and Kim 2007).

3.3.2 Sauerkraut

3.3.2.1 Characteristics of LAB in Sauerkraut Fermentation

The LAB that dominate Western vegetable fermentations, such as sauerkraut, mainly belong to the genera *Lactobacillus*, *Leuconostoc*, and *Pediococcus*. Homofermentative strains of lactobacilli produce 85% lactic acid from glucose, whereas heterofermentative strains produce lactic acid, carbon dioxide, ethanol, and acetic acid in equimolar amounts. The strains of *Lb. sakei* and *Lb. curvatus* are phenotypically closely related, and they are considered beneficial in sauerkraut fermentation (Mäki 2004).

Previous studies using traditional biochemical identification methods to study the ecology of commercial sauerkraut fermentations revealed that the obligate heterofermenter, *Leuco. mesenteroides*; the facultative homofermenter, *Lb. plantarum*; the homofermenter, *Ped. pentosaceus*; and the obligate heterofermenter, *Lb. brevis* were the primary microorganisms responsible for fermentation. However, as previously mentioned, using DNA fingerprinting techniques, Plengvidhya et al. (2007) identified more species of LAB to be present in sauerkraut fermentation including *Leuco. citreum*, *Leuco. argentinum*, *Leuco. fallax*, *Lb. paraplantarum*, *Lb. coryniformis*, and *Weissella* sp.

In addition to the desirable LAB, there are undesirable microorganisms present on cabbage which can interfere with the sauerkraut production process. The quality of the final product depends largely on how well the undesirable organisms are controlled during the fermentation process. Some spoilage organisms use the protein as an energy source, producing unpleasant odors and flavors.

3.3.2.2 Fermentation Process

Shredded cabbage is placed in a container and salt is added. Mechanical pressure can be applied to expel the cabbage juice, which contains fermentable sugars and other nutrients suitable for LAB activity. The heterofermentative gas-producing cocci (*Leuco. mesenteroides*) start the initial fermentation. When the acidity (lactic acid) reaches 0.25% to 0.3%, the activity of this group of LAB slows down, although their enzymes continue to function. The fermentation is subsequently continued by the lactobacilli (*Lb. plantarum*, *Lb. cucumeris*, and *Lb. brevis*) until the acidity level increases to 1.5% to 2%. The high salt concentration and low temperature inhibit these bacteria to some extent. Finally, *Lb. pentoaceticus* continues the fermentation, bringing the acidity to 2% to 2.5% to complete the fermentation (Vaughn 1985; Daeschel et al. 1987).

The end products from the sauerkraut fermentation are lactic acid along with smaller amounts of acetic and propionic acids, a mixture of gases (of which carbon dioxide is the principal gas), small amounts of alcohol, and a mixture of aromatic esters. The acids, in combination with alcohol, form esters which contribute to the characteristic flavor of sauerkraut. The acidity helps in controlling the growth of spoilage organisms and contributes to the extended shelf life of the product.

3.3.2.3 Temperature and Salt

Temperature control is one of the most important factors in the sauerkraut production process. The optimum temperature for sauerkraut fermentation is approximately 21°C. A temperature range of 18°C to 22°C is most desirable for initiating fermentation due to the growth and metabolism of *Leuco. mesenteroides*. In contrast, temperatures higher than 22°C favor the growth of *Lactobacillus* species.

Salt also plays an important role in initiating the sauerkraut production process and affects the quality of the final product (Viander et al. 2003; Holzapfel et al. 2003). Salt is added to withdraw juice from the cabbage, thus creating a more favorable environment for the development of the desired LAB flora. Generally, salt is added to a final concentration of 2.0% to 2.5%. At this concentration, lactobacilli are slightly inhibited, but cocci are not affected. It is essential to use pure sodium chloride because salts with added alkali may neutralize the acid.

3.3.2.4 Starter Cultures

Instead of relying on the endogenous LAB flora and inherent variability between different harvest areas, cultivars, processing environments, etc., the use of starter cultures can improve the fermentation process to ensure the safety and health aspects of the products. More importantly, the use of starter cultures results in a more controllable and consistent quality sauerkraut and helps to speed up the fermentation process. Starter cultures also helps ensure that the correct level of acidity is reached in the fermented product such that undesirable (Gram-negative) microorganisms are inhibited. It is possible to add starter culture such as *Lactococcus lactis* without adverse effects on final product quality. These starters only survive for a short time but initiate the acidification process; therefore, they do not disturb the natural sequence of microorganisms. The racemase-deficient *Lactobacillus bavaricus* strains are also used as starter cultures in sauerkraut (Mäki 2004). Johanningsmeier and colleagues (2005) successfully used *Leuco. mesenteroides* with or without malo-lactic activity to ferment sauerkraut. However, although the addition of *Leuco. mesenteroides* in the early stages of fermentation can provide attractive flavors to the final product (Breidt 2004), the sequence of subsequent LAB growth may become altered to possibly result in a final product that is incompletely fermented. The correct sequence of organisms is essential in achieving a stable product with typical sauerkraut flavor and aroma. It should be noted that the juice from previous sauerkraut fermentations was traditionally used as a starter culture for subsequent fermentations. The efficacy of using old spent juice depends largely on the types of organisms present in the juice and its acidity (Battcock and Azam-Ali 1998).

3.3.2.5 Spoilage and Defects in the Sauerkraut Process

Most spoilage of sauerkraut is due to aerobic soil microorganisms which break down the protein to produce undesirable flavor and texture changes. The growth of these aerobes can be controlled by the fermentation rate and control of temperature and salt concentrations.

Whenever the normal sequence of bacterial growth is altered, it usually results in a soft product. *Lactobacillus* sp. seem to have a greater ability than the cocci to break down cabbage tissue, thereby causing the softening. High fermentation temperatures and reduced salt concentrations favor the growth of *Lactobacillus* sp. If the salt concentration is too low initially, the *Lactobacillus* sp. will grow rapidly at the beginning and affect the normal sequence of fermentation.

Several factors can favor the growth of spoilage organisms. For example, an uneven distribution of salt and an insufficient level of cabbage juice to cover the sauerkraut during the fermentation can allow undesirable aerobic bacteria and yeasts to grow on the surface of the sauerkraut, causing production of off-flavors and discoloration. In addition, if the fermentation temperature is too high, growth of undesirable pigmented microorganisms may result in the development of dark spots (Battcock and Azam-Ali 1998).

Pink sauerkraut is caused by a group of yeasts which produce an intense red pigment in the juice and on the surface of the cabbage (Battcock and Azam-Ali 1998) if the salt is unevenly distributed or added to an excessive concentration, allowing yeast to grow while inhibiting the LAB. Savard et al. (2002) isolated and identified two spoilage yeasts, *Saccharomyces bayanus* and *Saccharomyces unisporus*, from

spoiled fermented vegetables. In addition, the shelf life of unpasteurized fermented vegetables is influenced by the presence of fermentative yeasts which may grow during storage if the sugar catabolism by LAB is incomplete (Fleming et al. 1985).

3.3.3 Comparison of Kimchi and Sauerkraut

Although the Western and Eastern fermented vegetable products seem to share many commonalities, there are some differences. For example, the preferred end point of fermentation is different for kimchi and sauerkraut (Lee 1997). In the Oriental tradition, the best-tasting kimchi is attained before overgrowth of *Lb. brevis* and *Lb. plantarum* happens, with an optimal product pH of 4.5. The overgrowth of *Lb. brevis* and *Lb. plantarum* affects the quality of kimchi due to their acidification of product pH values to lower than 4.5. In contrast, sauerkraut production depends on these organisms and the fermentation is specifically manipulated to promote their growth by using elective salt and temperature combinations. The optimal range of salt concentration of sauerkraut is 0.7% to 3.0%, whereas it is 3.0% to 5.0% for kimchi (Lee 1997; Holzapfel et al. 2003).

Several strains of bacteriocin-producing microorganisms have been isolated from kimchi and sauerkraut (Choi et al. 1999; Ohmomo et al. 2000; Settanni and Corsetti 2008). For example, *Enterococcus faecium* strains found in kimchi have been reported to have a broad spectrum of bacteriocin activities, and several *Lactobacillus* spp. have also been shown to have an antimicrobial effect. The organic acids, bacteriocin(s) produced during fermentation, and the antimicrobial activity of the ingredients together contribute to the control of undesirable microflora, including pathogenic microorganisms, without costly preservation treatments and packaging (Lee 1997).

3.4 Biopreservation of Vegetable Products

The idea of using biopreservation with innocuous commensal bacteria to inhibit foodborne microbial pathogens and spoilage organisms is not new but has mainly been explored in meat and fish products (Calo-Mata et al. 2008; Castellano et al. 2008; Lücke 2000). This purposeful use of microorganisms and their metabolites aims to extend the shelf life and improve the safety of foods (Ross et al. 2002). It is, in particular, biopreservation by use of active LAB cultures and their metabolites including bacteriocins that has gained considerable interest both in terms of their antibacterial (Chen and Hoover 2003; Bhattacharyya and Bhattacharjee 2007) and antifungal effects (Schnürer and Magnusson 2005).

As fresh-cut, ready-to-eat fruit and vegetable products are becoming more popular with consumers, the food industry needs to seek out ways of managing the associated microbial hazards and quality problems. Biopreservation with LAB of plant origin may present an attractive alternative to chemical washes and preservatives (Schnürer and Magnusson 2005; Allende et al. 2007; Settanni and Corsetti 2008). It is generally recognized that LAB best suitable for biopreservation should originate from the same type of food material as they are intended for use in (Vescovo et al. 1995, 1996). In a search for plant LABs with biopreservative properties, Trias and colleagues (2008) isolated 523 LAB strains from more than 700 samples of fruits and vegetables. After an initial screening, they selected 18 antagonistic strains for further characterization. Challenge experiments with *Leuco. mesenteroides*, *Leuco. citreum*, *Lb. plantarum*, *Lact. lactis*, and *Weisella cibaria* on apples and lettuce showed that four *Leuconostoc* sp. strains completely inhibited *Listeria monocytogenes* and reduced the growth of *Escherichia coli* and *Salmonella typhimurium*. Rouse and colleagues (2008) investigated the antifungal properties of plant-derived *W. confusa*, *W. cibaria*, *Lb. plantarum*, and *Ped. pentosaceus* strains and found activity against a range of spoilage fungi. As their work was performed in Spain and Ireland, respectively, we were wondering if antagonistic LAB could also be isolated from local produce from our area in Atlantic Canada and used for biopreservation.

3.4.1 Case Study

As biopreservation of fresh or fresh-cut products using bacteriocinogenic LAB isolated from vegetables presents an innovative approach to improve the safety and quality of the products, we examined fourteen

different local vegetable products for content of native LAB (Sharpe 2009). After isolation on LAB-elective agars and tentative identification, cell-free supernatants (CFS) from 92 isolates were screened for antimicrobial activity using the agar diffusion bioassay with minor modifications (Herreros et al. 2005). This resulted in the isolation of eight bacteriocinogenic LAB isolates, which retained inhibitory activity against both indicator bacteria, *Listeria innocua* (ATCC 33090) and *Lb. sakei* subsp. *sakei* (ATCC 15521), after neutralization of pH and elimination of H_2O_2 in CFS. The proteinaceous nature of the bacteriocin(s) was further confirmed by incubating the CFS with proteolytic enzymes (1 mg/mL α-chymotrypsin [C4129], protease [P4630], or trypsin [T8802] from Sigma-Aldrich Corporation, St. Louis, MO) at 37°C for 2 hours as this treatment led to the disappearance of the inhibition zones. The eight strains were later identified using 16S rRNA gene sequencing following the method of Abnous et al. (2009) as one *Lact. lactis* ssp. *lactis* and seven *E. faecium* strains (Table 3.1).

We further tested the eight strains for their antibacterial effectiveness against common produce pathogens and spoilage organisms at 5°C and 20°C using agar diffusion bioassays as well as the antifungal effect against fungal spore growth using a microdilution plate method slightly modified from work done by Lavermicocca et al. (2003). We found that the bacteriocin-like substances (BLS) produced by the eight strains showed a significant antimicrobial effect against *Li. innocua* (Figure 3.1), however, only their production of organic acids and H_2O_2 showed strong antimicrobial effects against *Pseudomonas fluorescens* (A7B), *Erwinia carotovora* (ATCC 15713), *Bacillus cereus* (ATCC 14579), *Penicillium expansum* (Pex 03–10.1), *Botrytis cinerea* (B94-b), *Monilinia fructicola* (Mof 03-25), and weak effects against *Leuco. mesenteroides*. The antifungal effect of *E. faecium* and *Lact. lactis* on *Pen. expansum*, *Bo. cinerea*, and *M. fructicola* during incubation at 5°C and 20°C was mainly due to the content of organic acids and H_2O_2 in the LAB CFS, as shown in Figures 3.2 and 3.3.

Furthermore, we applied *E. faecium* (13.2) and *Lact. lactis* (7.17) onto fresh-cut salads to investigate their effect on the growth of the natural microflora and *Li. innocua* inoculated on salads. Results showed that the addition of the LAB isolates significantly reduced the loads of naturally occurring *Listeria* sp. ($P = 0.033$), yeasts ($P = 0.011$), *Pseudomonas* sp. ($P = 0.010$), and coliforms ($P = 0.011$) in comparison with the controls. *E. faecium* and *Lact. lactis* also significantly reduced the growth of *Li. innocua* on fresh-cut salads ($P = 0.005$) during a 10-day storage trial at 5°C. Scanning electron microscopy revealed a significantly reduced presence of *Li. innocua* on the surface of fresh-cut salads after the addition of the bacteriocinogenic LAB (Figure 3.4).

In conclusion, our results showed that it is possible to readily isolate antagonistic LAB from vegetable products from Nova Scotia. The BLS producing *E. faecium* and *Lact. lactis* were demonstrated to have the potential to be used as protective cultures in fresh-cut produce to control spoilage and pathogenic microorganisms, thereby improving product shelf life and safety. Although the production of organic acids and H_2O_2 by the LAB was important for the inhibition of all test organisms, *Li. innocua* was inhibited solely by the BLS compounds produced by the LAB strains. Future characterization of the bacteriocins produced by the LAB strains and their mechanism of action may result in a better understanding of their implications in food systems, perhaps allowing for more targeted use to inhibit specific

TABLE 3.1

Origin and Identification of the Eight BLS Producing LAB Using 16S rRNA Gene Sequencing

Isolates	Ribosomal Database Project Identification	Accession No.	Similarity (%)	Source
7.12	*E. faecium*	GQ370527	99.2	Mung bean sprouts
7.14	*E. faecium*	DQ471797	98.6	Mung bean sprouts
7.17	*Lact. lactis* subsp. *lactis*	FJ749490	99.0	Mung bean sprouts
12.6	*E. faecium*	DQ471797	99.4	Swiss chard
12.8	*E. faecium*	DQ471797	97.8	Swiss chard
12.9	*E. faecium*	DQ471797	100	Swiss chard
13.1	*E. faecium*	DQ471797	98.8	Mini seedless cucumbers
13.2	*E. faecium*	DQ672262	99.2	Mini seedless cucumbers

Source: http://rdp.cme.msu.edu/.

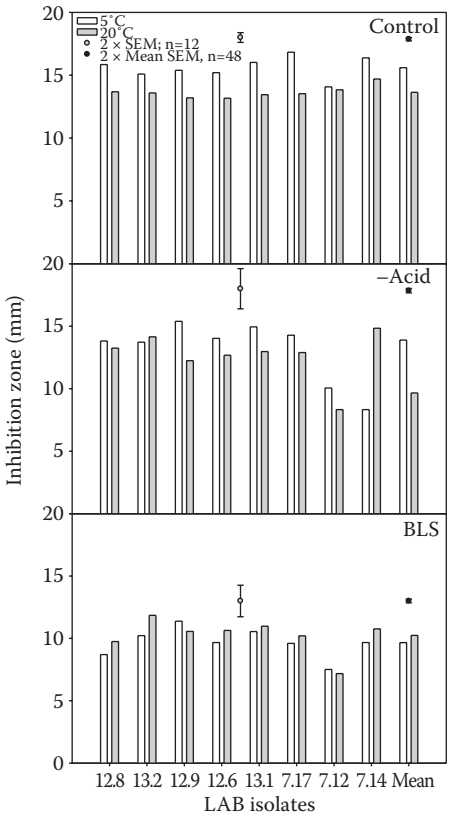

FIGURE 3.1 Inhibitory effect of the eight LAB isolates on *Li. innocua* at 5°C and 20°C using the agar diffusion bioassay. Control, CFS without any treatment; –Acid, CFS with pH neutralized to 6.0; and BLS, CFS with pH neutralized to 6.0 and H_2O_2 eliminated with catalase treatment. The vertical bar represents the standard error of the mean for comparison of means within the figure.

food pathogens such as *Li. monocytogenes*. Finally, because of the virulence of some *Enterococcus* sp. (Devriese et al. 2006), it will be necessary to ascertain that these plant *E. faecium* strains are not virulent before being used in biopreservation or food fermentations (Yoon et al. 2008).

3.5 Conclusions and Future Research

The use of naturally occurring LAB to ferment vegetable-based foods is a very old tradition with roots in many cultures worldwide (Steinkraus 1997). Previous times' need for extending the shelf life of vegetables through fermentation has largely disappeared because of the arrival of modern food storage technologies; however, the popularity of fermented vegetable products remains (Liu et al. 2011).

With the advent of DNA-based community analysis techniques, our view of the microbial dynamics during fermentation and biopreservation is likely to change as more research groups apply these techniques to different products from different geographical areas. We expect that further investigations using deep sequencing techniques or pyrosequencing, as exemplified by the recent publication of Sakamoto and colleagues (2011), will provide new ecological insights allowing us to better understand and control the microflora. Also, better knowledge of the microbial ecology should enable us to select better starter cultures, which produce antimicrobial compounds with antagonistic activities against yeasts and spoilage bacteria (Choi et al. 1999), and refine the technology for controlled fermentation and preservation of commercially fermented kimchi and sauerkraut products.

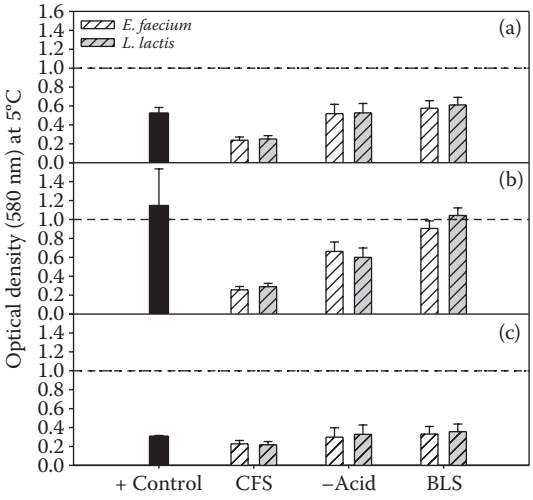

FIGURE 3.2 The growth of fungal spores (a, *Bo. cinerea*; b, *Pen. expansum*; c, *M. fructicola*) after incubation at 5°C for 96 hours without or with LAB CFS from *E. faecium* and *Lact. lactis*. Positive control, fungal spores in de Man Rogosa Sharpe (MRS) broth without the addition of LAB CFS. CFS (control), fungal spores incubated with untreated CFS. –Acid, fungal spores in CFS after pH neutralization. BLS, fungal spores in CFS after pH neutralization and H_2O_2 elimination. The negative control $OD_{580\,nm}$ values (MRS with dead fungal spores) ranged from 0.2 to 0.3.

Flavor is an important aspect of the organoleptic quality of kimchi and sauerkraut, and yet the flavor compounds and their relationship to individual LAB strains have not been well elucidated. Research in this area should look at both the flavor chemistry as well as the functional genomics related to the dominant LAB strains during fermentation. The role of the microorganisms and enzymes involved in the softening of tissue of fermented products should also be investigated.

A better understanding of the microbiota may aid in the development of low salt fermentations, which may have altered microbial and sensory characteristics (Breidt 2004; Plengvidhya et al. 2007). However,

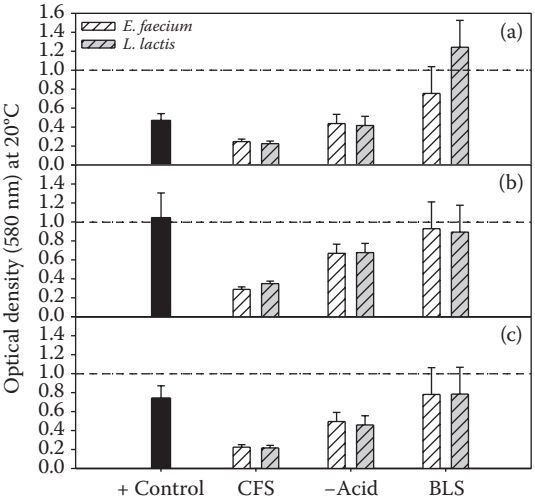

FIGURE 3.3 The growth of fungal spores (a, *Bo. cinerea*; b, *Pen. expansum*; c, *M. fructicola*) after incubation at 20°C for 40 hours without or with LAB CFS from *E. faecium* and *Lact. lactis*. Positive control, fungal spores in MRS broth without the addition of LAB CFS. CFS (control), fungal spores incubated with untreated CFS. –Acid, fungal spores in CFS after pH neutralization. BLS, fungal spores in CFS after pH neutralization and H_2O_2 elimination. The negative control (MRS with dead fungal spores) ranged in $OD_{580\,nm}$ values from 0.2 to 0.3.

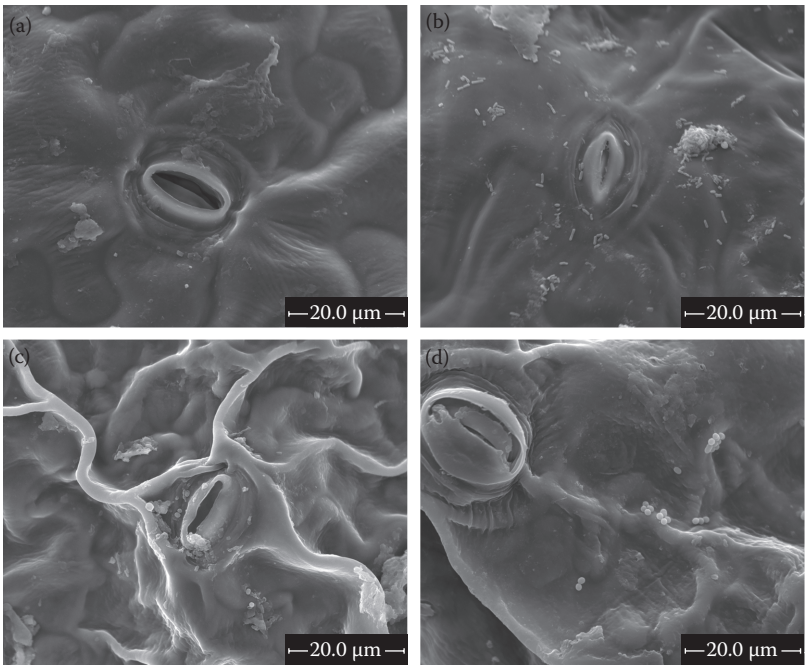

FIGURE 3.4 Scanning electron micrographs of lettuce leaves from fresh-cut salad samples in the *Listeria* challenge test. Salad samples were observed after storage for 10 days at 5°C. (a) Control sample. (b) Samples inoculated with *Li. innocua* at 5.0×10^3 cfu/g and no LAB inoculation. (c) *Lact. lactis* and *Li. innocua*–inoculated salad sample. (d) *E. faecium* and *Li. innocua*–inoculated salad sample.

more importantly, it is necessary to assure that the safety of the products is not jeopardized in fermented products with low salt contents or biopreserved products with no salt. The removal of salt may allow for the growth of Gram-negative and Gram-positive pathogens unless the LAB cultures effectively inhibit these.

The properties of the fermenting or bioprotective LAB cultures also need to be investigated. One could envision that cultures be genome sequenced to (a) ascertain the absence of virulence genes or pathogenicity islands, something which is particularly relevant for *Enterococcus* strains; and (b) to affirm particular functional characteristics such as extracellular polysaccharide formation, degradation of specific carbohydrates, bacteriocin production, or probiotic activities. This characterization may allow us to better explore probiotic and anticarcinogenic activities, improve the nutritive properties of kimchi and sauerkraut products, and inhibit foodborne pathogens.

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REFERENCES

Abnous K, Brooks S, Kwan J, Matias F, Green-Johnson J, Selinger LB, Thomas M, Kalmokoff M. 2009. Diets enriched in oat bran or wheat bran temporally and differentially alter the composition of the fecal community of rats. *J Nutri* 139:2024–31.

Adams MR, Nicolaides L. 1997. Review of the sensitivity of different foodborne pathogens to fermentation. *Food Control* 8(5/6):227–39.

Allende A, Martínez B, Selma V, Gil MI, Suárez JE, Rodríguez A. 2007. Growth and bacteriocin production by lactic acid bacteria in vegetable broth and their effectiveness at reducing *Listeria monocytogenes* in vitro and in fresh-cut lettuce. *Food Microbiol* 24:759–66.

Battcock M, Azam-Ali S. 1998. Fermented fruits and vegetables—A global perspective. FAO Agricultural Services Bulletin No. 134.

Bae JW, Rhee SK, Park JR, Chung WH, Nam YD, Lee I, Kim H, Park YH. 2005. Development and evaluation of genome-probing microarrays for monitoring lactic acid bacteria. *Appl Environ Microbiol* 71(12):8825–35.

Bhattacharyya BK, Bhattacharjee D. 2007. Bacteriocin: A biological food preservative. *J Food Sci Technol* 44:459–64.

Bjergqvist SW, Sandberg AS, Carlsson NG, Andlid T. 2005. Improved iron solubility in carrot juice fermented by homo- and hetero-fermentative lactic acid bacteria. *Food Microbiol* 22:53–62.

Björkroth J, Holzapfel W. 2006. Genera *Leuconostoc*, *Oenococcus* and *Weisella*. *Prokaryotes* 4:267–319.

Breidt F. 2004. A genomic study of *Leuconostoc mesenteroides* and the molecular ecology of sauerkraut fermentations. *J Food Sci* 69(1):30–2.

Calo-Mata P, Arlindo S, Boehme K, de Miguel T, Pascoal A, Barros-Velazquez J. 2008. Current applications and future trends of lactic acid bacteria and their bacteriocins for the biopreservation of aquatic food products. *Food Bioprocess Technol* 1:43–63.

Castellano P, Belfiore C, Fadda S, Vignolo G. 2008. A review of bacteriocinogenic lactic acid bacteria used as bioprotective cultures in fresh meat produced in Argentina. *Meat Sci* 79(3):483–99.

Chang JY, Chang HC. 2010. Improvements in the quality and shelf life of kimchi by fermentation with the induced bacteriocin-producing strain, *Leuconostoc citreum* GJ7 as a starter. *J Food Sci* 75(2): M103–10.

Charlier C, Cretenet M, Even S, Le Loir Y. 2009. Interactions between *Staphylococcus aureus* and lactic acid bacteria: An old story with new perspectives. *Int J Food Microbiol* 131:30–9.

Cheigh HS, Park KY. 1994. Biochemical, microbiological, and nutritional aspects of Kimchi (Korean fermented vegetable products). *Crit Rev Food Sci Nutri* 34(2):175–203.

Chen H, Hoover DG. 2003. Bacteriocins and their food applications. *Comprehensive Rev Food Sci Food Safety* 2:82–100.

Choi HJ, Lee HS, Her S, Oh DH, Yoon SS. 1999. Partial characterization and cloning of leuconocin J, a bacteriocin produced by *Leuconostoc* sp. J2 isolated from the Korean fermented vegetable Kimchi. *J Appl Microbiol* 86:175–81.

Choi IK, Jung SH, Kim BJ, Park SY, Kim J, Han HU. 2003. Novel *Leuconostoc citreum* starter culture system for the fermentation of kimchi, a fermented cabbage product. *Antonie van Leeuwenhoek* 84:247–53.

Cleveland J, Montville TJ, Nes IF, Chikindas ML. 2001. Bacteriocins: safe, natural antimicrobials for food preservation. *Int J Food Microbiol* 71:1–20.

Cotter PD, Hill C, Ross RP. 2005. Bacteriocins: Developing innate immunity for food. *Nature Rev* 3:777–88.

Daeschel MA, Andersson RE, Fleming HP. 1987. Microbial ecology of fermenting plant materials. *FEMS Microbiol Rev* 46:357–67.

Dalié DKD, Deschamp AM, Richard-Forget F. 2010. Lactic acid bacteria—Potential for control of mould growth and mycotoxins: A review. *Food Control* 21:370–80.

Devriese L, Baele M, Buaye P. 2006. The genus *Enterococcus*: Taxonomy. *Prokaryotes* 4:163–74.

Fleming HP, McFeeters RF, Daeschel MA. 1985. The lactobacilli, pediococci, and leuconostocs: Vegetable products. In: Gilliland SE, editor. *Bacterial Starter Cultures for Foods*. Boca Raton, FL: CRC Press. p 97–118.

Hammes WP, Hertel C. 2006. The genera *Lactobacillus* and *Carnobacterium*. *Prokaryotes* 4:320–403.

Helander IM, von Wright A, Mattila-Sandholm TM. 1997. Potential of lactic acid bacteria and novel antimicrobials against Gram-negative bacteria. *Trends Food Sci Technol* 8:146–50.

Herreros MA, Sandoval H, Gonzalez L, Castro JM, Fresno JM, Tornadijo ME. 2005. Antimicrobial activity and antibiotic resistance of lactic acid bacteria isolated from Armada cheese (a Spanish goats' milk cheese). *Food Microbiol* 22:455–9.

Holzapfel W, Schillinger U, Buckenhuskes HJ. 2003. Sauerkraut. In: Farnworth ER, editor. *Handbook of Fermented Functional Foods*. Boca Raton, FL: CRC Press. p 343–59.

Höltzel A, Gänzle MG, Nicholson GJ, Hammes WP, Jung G. 2000. The first low molecular weight antibiotic from lactic acid bacteria: Reutericyclin, a new tetrameric acid. *Angew Chem Int Ed* 39:2766–8.

Jänsch A, Freiding S, Behr J, Vogel RF. 2011. Contribution of the NADH-oxidase (Nox) to the aerobic life of *Lactobacillus sanfranciscensis* DSM20451^T. *Food-Microbiol* 28:29–37.

Johanningsmeier SD, Felming HP, Thompson RL, McFeeters RF. 2005. Chemical and sensory properties of sauerkraut produced with *Leuconostoc mesenteroides* starter cultures of differing malolactic phenotypes. *J Food Sci* 70(5):S343–9.

Kim JM, Kim IS, Yang HC. 1987. Storage of salted Chinese cabbages for kimchi. I. Physico-chemical and microbial changes during salting of Chinese cabbage. *J Korean Soc Food Sci Nutri* 16(2):75.

Kim TW, Jung SH, Lee JY, Cho SK, Park SH, Jo JS, Kim HY. 2003. Identification of lactic acid bacteria in kimchi using SDS-PAGE profiles of whole cell proteins. *J Microbiol Biotechnol* 13(1):119–24.

Kim YS, Zheng ZB, Shin DH. 2008. Growth inhibitory effects of Kimchi (Korean traditional fermented vegetable product) against *Bacillus cereus*, *Listeria monocytogenes*, and *Staphylococcus aureus*. *J Food Protect* 71(2):325–32.

Klaenhammer TR. 1988. Bacteriocins of lactic acid bacteria. *Biochimie* 70:337–49.

Klaenhammer TR, Barrangou R, Buck BL, Azcrate-Peril MA, Altermann E. 2005. Genomic features of lactic acid bacteria effecting bioprocessing and health. *FEMS Microbiol Rev* 29:393–409.

Kwon EA, Kim M. 2007. Microbial evaluation of commercially packed kimchi products. *Food Science and Biotechnology* 16(4):615–20.

Lavermicocca P, Valerio F, Visconti A. 2003. Antifungal activity of phenyllactic acid against molds isolated from bakery products. *Appl Environ Microbiol* 69:634–40.

Lee CH. 1997. Lactic acid fermented foods and their benefits in Asia. *Food Control* 8(5/6):259–69.

Lee D, Kim S, Cho J, Kim J. 2008. Microbial population dynamics and temperature changes during fermentation of Kimjang kimchi. *J Microbiol* 46(5):590–3.

Lee JS, Heo GY, Lee JW, Oh YJ, Park JA, Park YH, Pyun YR, Ahn JS. 2005. Analysis of kimchi microflora using denaturing gradient gel electrophoresis. *Int J Food Microbiol* 102:143–50.

Liu S, Han Y, Zhou Z. 2011. Lactic acid bacteria in traditional fermented Chinese foods. *Food Res Int* 44(3):643–51. doi: 10.1016/j.foodres.2010.12.034.

Lücke FK. 2000. Utilization of microbes to process and preserve meat. *Meat Sci* 56(2):105–15.

Mäki M. 2004. Lactic acid bacteria in vegetable fermentations. In: Salminen S, von Wright A, Ouwehand A, editors. *Lactic Acid Bacteria: Microbiological and Functional Aspects*. 3rd ed. New York: Marcel Dekker. p 419–30.

Mheen TI, Kwon TW. 1984. Effect of temperature and salt concentration on kimchi fermentation. *Korean J Food Sci Technol* 16(4):443.

Niksic M, Niebuhr SE, Dickson JS, Mendonca AF, Koziczkowski JJ, Ellingson JLE. 2005. Survival of *Listeria monocytogenes* and *Escherichia coli* O157:H7 during sauerkraut fermentation. *J Food Protect* 68(7):1367–74.

Ohmomo S, Murata S, Katayama N, Nitisinprasart S, Kobayashi M, Nakajima T, Yajima M, Nakanishi K. 2000. Purification and some characteristics of enterocin ON-157, a bacteriocin produced by *Enterococcus faecium* N1A1 157. *J Appl Microbiol* 88:81–9.

Park JM, Shin JH, Lee DW, Song JC, Suh HJ. 2010. Identification of the lactic acid bacteria in kimchi according to initial and over-ripened fermentation using PCR and 16S rRNA gene sequence analysis. *Food Sci Biotechnol* 19(2):541–6.

Plengvidhya V, Breidt F, Lu Z, Fleming HP. 2007. DNA fingerprinting of lactic acid bacteria in sauerkraut fermentations. *Appl Environ Microbiol* 73(23):7697–702.

Ross RP, Morgan S, Hill C. 2002. Preservation and fermentation: Past, present and future. *Int J Food Microbiol* 79:3–16.

Rouse S, Harnett D, Vaughan A, van Sinderen D. 2008. Lactic acid bacteria with potential to eliminate fungal spoilage in foods. *J Appl Microbiol* 104:915–23.

Ruas-Madiedo P, Salazar N, de los Reyes-Gavilan CG. 2009. Exopolysaccharides produced by lactic acid bacteria in food and probiotic applications. Chapter 45. In: *Microbial Glycobiology*. Editor-in-chief, Anthony P. Moran; editors, Otto Holst, Patrick J. Brennan, Mark von Itzstein, Amsterdam, The Netherlands: Elsevier. p 887–920.

Sakamoto N, Tanaka S, Sonomoto K, Nakayama J. 2011. 16S rRNA pyrosequencing-based investigation of the bacterial community in nukadoko, a pickling bed of fermented rice bran. *Int J Food Microbiol* 144:352–9.

Savard T, Beaulieu C, Boucher I, Champagne C. 2002. Antimicrobial action of hydrolyzed chitosan against spoilage yeasts and lactic acid bacteria of fermented vegetables. *J Food Protect* 65(5):828–33.

Schnürer J, Magnusson J. 2005. Antifungal lactic acid bacteria as biopreservatives. *Trends Food Sci Technol* 16:70–8.

Settanni L, Corsetti A. 2008. Application of bacteriocins in vegetable food biopreservation. *Int J Food Microbiol* 121:123–38.

Sharpe DV. 2009. Biopreservation of fresh-cut salads using bacteriocinogenic lactic acid bacteria isolated from commercial produce. [MSc thesis]. Halifax, Nova Scotia, Canada: Dalhousie Univ. 111 pp.

Steinkraus KH. 1997. Classification of fermented foods: Worldwide review of household fermentation techniques. *Food Control* 8(5/6):311–7.

Tamminen M, Joutsjoki T, Sjöblom M, Joutsen M, Palva A, Ryhänen EL. 2004. Screening of lactic acid bacteria from fermented vegetables by carbohydrate profiling and PCR-ELISA. *Lett Appl Microbiol* 39:439–44.

Teuber M, Geis A. 2006. The genus *Lactococcus*. *Prokaryotes* 4:205–28.

Trias R, Bañeras L, Badosa E, Montesinos E. 2008. Bioprotection of golden delicious apples and iceberg lettuce against foodborne bacterial pathogens by lactic acid bacteria. *Int J Food Microbiol* 123:50–60.

Vaughn RH. 1985. The microbiology of vegetable fermentations. In: Wood BJB, editor. *Microbiology of Fermented Foods*. Vol. 2. New York: Elsevier. p 49–109.

Vescovo M, Orsi C, Scolari G, Torriani S. 1995. Inhibitory effect of selected lactic acid bacteria on microflora associated with ready-to-eat vegetables. *Lett Appl Microbiol* 21:121–5.

Vescovo M, Torriani S, Orsi C, Macchiarolo F, Scolari G. 1996. Application of antimicrobial-producing lactic acid bacteria to control pathogens in ready-to-use vegetables. *J Appl Bacteriol* 81:113–9.

Viander B, Mäki M, Palva A. 2003. Impact of low salt concentration, salt quality on natural large-scale sauerkraut fermentation. *Food Microbiol* 20:391–5.

Yoon MY, Kim YJ, Hwang HJ. 2008. Properties and safety aspects of *Enterococcus faecium* strains isolated from Chungkukjang, a fermented soy product. *LWT - Food Sci Technol* 41:925–33.

4

Plant-Based Fermented Foods and Beverages of Asia

Jyoti Prakash Tamang

CONTENTS

4.1 Introduction

There are several definitions for fermented foods in the literature. The most recent definition of fermented foods and beverages is given by Tamang (2010b). Ethnic fermented foods are defined as foods produced by ethnic people using their native knowledge from locally available raw materials of plant or animal sources either naturally or by adding starter culture(s) containing functional microorganisms which modify the substrates biochemically and organoleptically into edible products that are culturally and socially acceptable to the consumers (Tamang 2010a). Asian people consume vegetables, both domesticated and wild, in a variety of recipes. Perishable and seasonal leafy vegetables, radish, cucumbers, including young edible tender bamboo shoots, are traditionally fermented into edible products using the indigenous knowledge of biopreservation (Table 4.1). The knowledge of the art of pickling vegetables, which is basically fermentation, might have developed in Asia (Pederson 1979). Soybeans are the most common among the legumes used for fermentation of soybean foods in the South East, Far Asia, and the Himalayan regions of India, Nepal, and Bhutan. Cooked rice is a staple food in Asia. Wheat and barley are also important staple diets in the Indian subcontinent and in western and middle eastern Asia. Production of ethnic fermented cereal products is mostly restricted to the Indian subcontinent. Comparable to the African and European dietary cultures, Asia has a limited number of ethnic nonalcoholic fermented cereal products. Cereals are commonly fermented into several ethnic alcoholic drinks in Asia. Tea, the second most popular beverage in the world after water, originated in China and two common species of tea are *Camellia sinensis* var. *sinensis* and *C. sinensis* var. *assamica* (Schillinger et al. 2010). Although normal black tea is drunk everywhere, some ethnic Asian communities, however, have special fermented teas such as *miang* from Thailand, *puer* tea and *fuzhuan brick* and *kombucha* from China.

In addition to being a recreational beverage, ethnic alcoholic drinks have strong ritualistic importance among the ethnic people in Asia where social activities require the provision and drinking of alcohol. Drinking of alcohol in India has been mentioned in the *Ramayana* during 300 to 75 BC (Prakash 1961). Interestingly, the malting process for alcohol production is rare or unknown in Asia. Wine making is not a tradition in Asia because fruits are eaten directly without extracting into juice or fermenting into wines. Dry mixed starters containing a consortia of microorganisms are traditionally used in the fermentation of alcoholic beverages in many countries in Asia (Table 4.2). In general, three types of amylolytic mixed cultures or inocula are traditionally used in Asia as a starter to convert cereal starch to sugars and, subsequently, to alcohol and organic acids (Tamang and Fleet 2009):

Type I: A consortium of mycelial or filamentous molds, amylolytic, and alcohol-producing yeasts, and lactic acid bacteria (LAB) with rice or wheat as the base in the form of dry, flattened, or round balls of various sizes. The starter is inoculated with material from a previous batch. This mixed flora is allowed to develop for a short time, then dried and used to make either alcohol or fermented foods from starchy materials. Ethnic starters, which are used for a number of fermentations based on rice and cassava or other cereals in Asia, have different vernacular names such as *marcha* in India and Nepal, *ragi* in Indonesia, *bubod* in the Philippines, *chiu/chu* in China and Taiwan, *loogpang* in Thailand, *nuruk* in Korea, and *men* in Vietnam (Tamang 2010c).

Type II: A combination of *Aspergillus oryzae* and *Aspergillus sojae* is used in the form of a starter called *koji* in Japan to produce alcoholic beverages, including *saké*. Koji also produces amylases that convert starch to fermentable sugars, which are then used for the second stage of yeast fermentation to make a nonalcoholic fermented soybean product called *miso* and *shoyu*, whereas proteases are formed to break down the soybean protein.

Type III: Whole wheat flour is moistened and made into large compact cakes that are incubated in culture yeasts and filamentous molds and used to ferment starchy material to produce alcohol. This type is mainly used in China for alcohol production.

The Indian and Chinese communities have the most diverse variety of ethnic fermented foods and alcoholic beverages among Asians (Tamang 2010b). Plant-based fermented foods from Asia are categorized into fermented vegetables, fermented bamboo shoots, fermented legumes, fermented cereal, fermented tea, and fermented beverages.

TABLE 4.1

Plant-Based Fermented Foods of Asia

Country	Plant Source	Fermented Food	Nature and Use
Afghanistan	Wheat flour	*Nan*	Leaved bread, baked; staple
Bangladesh	Fruits, vegetables	*Chatney*	Acidic, hot and sour; pickles
Bhutan	Leafy vegetable	*Gundruk*	Acidic, sour, dry; soup, pickle
Bhutan	Radish taproot	*Sinki*	Acidic, sour, dry; soup, pickle
Bhutan	Soybean	*Kinema*	Alkaline, sticky; curry, soup
Bhutan	Rice-wheat flour	*Selroti*	Pretzel-like, deep fried; staple
China	Vegetables	*Suan-cai*	Acidic, sour, wet; pickle
China	Vegetables	*Paocai*	Acidic, sour, wet; pickle
China	Soybean	*Chee-fan*	Cheese-like, solid; salad
China	Soybean	*Chiang*	Alkaline, paste; soup
China	Soybean	*Dauchi*	Alkaline, paste; condiment, soup
China	Soybean	*Furu*	Mild acidic; savory
China	Soybean	*Meitauza*	Liquid drink
China	Soybean	*Sufu*	Mild-acidic, soft; side dish
China	Soybean	*Tofu si*	Alkaline, liquid; seasoning
China	Rice	*Ang-kak*	Powder colorant
China	Wheat gluten	*Minchin*	Solid; condiment
China	Tea liquor	*Kombucha*	Flavored; drink
China	Tea	*Fuzhuan brick*	Fermented tea; drink
China	Tea	*Puer*	Fermented tea; drink
Cambodia	Soybean	*Sieng*	Alkaline, sticky; side dish
India	Taro leaves	*Anishi*	Acidic, wet; curry
India	Bamboo shoot	*Bastanga*	Acidic, soft; curry
India	Bamboo shoot	*Ekung*	Acidic, sour, soft; soup
India	Bamboo shoot	*Eup*	Acidic, sour, dry; curry, soup
India	Bamboo shoot	*Hirring*	Acidic, sour, wet; curry, soup
India	Bamboo shoot	*Mesu*	Acidic, sour, wet; pickle
India	Bamboo shoot	*Lung-siej*	Sour-acidic, soft; curry
India	Bamboo shoot	*Soibum*	Acidic, sour, soft; curry
India	Bamboo shoot	*Soidon*	Acidic, sour, soft; curry
India	Leafy vegetable	*Gundruk*	Acidic, sour, dry; soup, pickle
India	Wild vegetable	*Goyang*	Acidic, sour, wet; condiment, soup
India	Mustard leaves	*Inziang-sang*	Acidic, sour, dry; curry, soup
India	Mustard leaves	*Inziang-dui*	Acidic, sour, liquid; condiment
India	Cucumber	*Khalpi*	Acidic, sour, wet; pickle
India	Radish taproot	*Sinki*	Acidic, sour, dry; soup, pickle
India	Soybean	*Kinema*	Alkaline, sticky; curry, soup
India	Soybean	*Hawaijar*	Alkaline, sticky; side dish
India	Soybean	*Aakhone*	Alkaline, sticky, paste; side dish
India	Soybean	*Tungrymbai*	Alkaline, sticky; curry, soup
India	Soybean	*Bekang*	Alkaline, sticky, paste; side dish
India	Soybean	*Peruyaan*	Alkaline, sticky; side dish
India	Bengal gram	*Dhokla*	Mild acidic, spongy; snack
India	Black gram	*Bhallae*	Mild acidic, fried patties; snack
India	Bengal gram	*Khaman*	Mild acidic, spongy; breakfast food
India	Black gram	*Vadai*	Paste, fried patties; snack
India	Black gram	*Wari*	Ball-like, brittle; condiment
India	Rice and black gram	*Idli*	Mild-acidic, soft, moist, spongy; breakfast food

(*continued*)

TABLE 4.1 (Continued)

Plant-Based Fermented Foods of Asia

Country	Plant Source	Fermented Food	Nature and Use
India	Rice and black gram	*Dosa*	Thin, crisp pancake; shallow-fried, staple
India	Maize, Bengal gram	*Puda/Pudla*	Solid food, pancake; snack food
India	Wheat flour	*Bahtura*	Bread; deep-fried bread
India	Wheat flour	*Nan*	Leaved bread, baked; staple
India	Wheat, barley, buckwheat	*Chilra*	Like *dosa;* staple
India	Wheat flour	*Marchu*	Baked bread; staple
India	Wheat flour	*Jalebi*	Crispy sweet, doughnut-like, deep-fried; snacks
India	Millet, rice	*Ambali*	Acidic, pan cake shallow-fried; staple
India	Black gram	*Maseura*	Dry, ball-like, brittle; condiment
India	Black gram	*Papad*	Circular wafers; snack
India	Cereals, pulses, milk	*Rabadi*	Mild-acidic, thick slurry-like product; drink
India	Wheat grains	*Seera*	Dried; sweet dish
India	Rice-wheat	*Selroti*	Pretzel-like, deep fried; staple
India	Wheat flour, opium seeds	*Siddu*	Steamed bread, oval-shaped; staple
India	Fruits, vegetables, oil, salt	*Achar/chatney*	Acidic, hot and sour; pickles
India	Fruits	*Chuk*	Sour, dark-brown paste; therapeutic uses
India	Rice	*Hakua*	Strong off-flavor; therapeutic uses
India	Tea liquor	*Kombucha*	Flavored; drink
Indonesia	Soybean, wheat	*Kecap*	Liquid; condiment, seasoning
Indonesia	Soybean (black)	*Ketjap*	Syrup; seasoning agent
Indonesia	Mustard leaves, cabbage	*Sayur asin*	Acidic, sour, wet; salad, side dish
Indonesia	Peanut	*Ontjom*	Alkaline, solid cake, roasted or fried; snack
Indonesia	Soybean	*Tauco*	Alkaline, paste; soup
Indonesia	Soybean	*Tempe*	Alkaline, solid; fried cake; snack
Indonesia	Coconut	*Bongkrek*	Solid, roasted or fried in oil; snack
Indonesia	Cassava roots	*Peujeum*	Acidic, solid; snack
Japan	Soybean	*Natto*	Alkaline, sticky; breakfast
Japan	Soybean	*Miso*	Alkaline, paste; soup
Japan	Soybean	*Shoyu*	Alkaline, liquid; seasoning
Japan	Turnip	*Sunki*	Acidic, sour, wet; pickle
Korea	Rice	*Kichudok*	Steamed cake; side dish
Korea	Cabbage, radish	*Kimchi*	Acidic, mild-sour, wet; salad
Korea	Soybean	*Doenjang*	Alkaline, paste; soup
Korea	Soybean	*Chungkokjang*	Alkaline, sticky; condiment, soup
Korea	Soybean, rice	*Kochujang*	Alkaline, paste; condiment, soup
Korea	Soybean	*Kanjang*	Alkaline, paste; condiment
Korea	Soybean	*Meju*	Alkaline, paste; seasoning
Laos	Soybean	*Sieng*	Alkaline, sticky; side dish
Malaysia	Fruits, vegetables	*Jeruk*	Acidic, wet; salad
Myanmar	Soybean	*Pepok*	Alkaline, sticky; side dish
Nepal	Leafy vegetable	*Gundruk*	Acidic, sour, dry; soup, pickle
Nepal	Wild vegetable	*Goyang*	Acidic, sour, wet; condiment, soup
Nepal	Cucumber	*Khalpi*	Acidic, sour, wet; pickle
Nepal	Radish	*Sinnamani*	Acidic, sour, wet; pickle

(*continued*)

TABLE 4.1 (Continued)

Plant-Based Fermented Foods of Asia

Country	Plant Source	Fermented Food	Nature and Use
Nepal	Radish taproot	*Sinki*	Acidic, sour, dry; soup, pickle
Nepal	Bamboo shoot	*Mesu*	Acidic, sour, wet; pickle
Nepal	Soybean	*Kinema*	Alkaline, sticky; curry, soup
Nepal	Black gram	*Maseura*	Dry, ball-like, brittle; condiment
Nepal	Rice	*Hakua*	Strong off-flavor; therapeutic uses
Nepal	Fruits, vegetables, oil, salt	*Achar*	Acidic, hot and sour; pickles
Nepal	Rice-wheat	*Selroti*	Pretzel-like, deep fried bread, staple
Pakistan	Wheat flour	*Marchu*	Baked bread; staple
Pakistan	Wheat flour	*Nan*	Leaved bread, baked; staple
Pakistan	cereals, pulses-milk	*Rabadi*	Mild-acidic, thick slurry-like product; drink
Pakistan	Wheat grains	*Seera*	Dried; sweet dish
Pakistan	Fruits, vegetables, oil, salt	*Chatney*	Acidic, hot and sour; pickles
Philippines	Rice	*Puto*	Steamed cake; breakfast or snack
Philippines	Mustard	*Burong mustasa*	Acidic, wet; salad
Philippines	Coconut water or coconut skim milk	*Nata de coco*	Thick white or cream-colored, candied; Ice cream, fruit salads
Philippines	Juice from pineapple	*Nata de piña*	Thick white or cream-colored, insoluble gelatinous film of polysaccharides; ice cream, fruit salads
Taiwan	Mustard	*Suan-tsai*	Acidic, sour, dry; soup, stew
Taiwan	Mustard	*Fu-tsai*	Acidic, sour; soup, stew
Taiwan	Soybean	*Meitauza*	Liquid; drink
Taiwan	Soybean curd	*Sufu*	Mild-acidic, soft; side dish
Taiwan	Bamboo shoots	*Jiang-sun*	Acidic, sour; side dish
Thailand	Mustard leaf	*Dakguadong*	Acidic, wet; salad
Thailand	Bamboo shoots	*Naw-mai-dong*	Acidic, wet; side dish
Thailand	Leafy vegetable	*Pak-gard-dong*	Acidic, wet; side dish
Thailand	Leaves of Gynandropis pentaphylla	*Pak-sian-dong*	Acidic, wet; side dish
Thailand	Soybean	*Thua nao*	Alkaline, paste, dry; soup
Thailand	Rice	*Khanom-jeen*	Noodle; staple
Thailand	Tea	*Miang*	Fermented tea, flavored; drink
Sri Lanka	Rice and black gram	*Dosa*	Thin, crisp pancake, shallow-fried; staple
Sri Lanka	Rice and black gram	*Idli*	Mild-acidic, soft, moist, spongy; beakfast food
Sri Lanka	Rice, coconut water	*Hopper*	Steak-baked, pancake; staple
Vietnam	Cabbage	*Dhamuoi*	Acidic, wet; salad
Vietnam	Rice	*Me*	Acidic, sour; condiment
Southeast Asia	Soybean	Soy sauce	Alkaline, liquid; seasoning
Southeast Asia	Sugar-containing substrates	Vinegar	Acetic acid flavored, liquid; condiment, seasoning

TABLE 4.2

Plant-Based Fermented Beverages of Asia

Country	Plant Source	Mixed Starter	Beverage	Nature and Use
Bhutan	Finger millet/ barley	*Poo*	*Chyang*	Mild alcoholic, slightly, sweet-acidic
Bhutan	Barley, millet	*Poo*	*Aarak*	Distilled from *chyang*, clear liquor
China	Sorghum	*Daqu*	*Maotai*	Distilled, clear liquor (national drink)
China	Finger millet/ barley	*Phab*	*Chyang*	Mild alcoholic, slightly sweet-acidic
China	Barley, millet	*Phab*	*Aarak*	Distilled from *chyang*, clear liquor
China	Rice	*Chiu yueh*	*Lao chao*	Sweet-sour, mild alcoholic paste
China	Rice	*Chiu-yueh*	*Tien-chiu-niang*	Mild alcoholic, sweet
India	Finger millet	*Marcha*	*Kodo ko jaanr*	Mild alcoholic, sweet-acidic
India	Rice	*Marcha*	*Bhaati jaanr*	Mild alcoholic, sweet-sour, paste
India	Rice	*Hamei*	*Atingba*	Mild alcoholic, sweet-sour
India	Rice	*Humao*	*Judima*	Mild alcoholic, sweet-sour
India	Maize-rice/barley	*Phab*	*Bhang-chyang*	Extract of *mingri*
India	Rice	*Phab*	*Apong*	Mild alcoholic
India	Barley	*Phab*	*Buza*	Thick liquor
India	Apricot	Yeast	*Chulli*	Filtrate, clear
India	Buck wheat	*Marcha*	*Faapar ko jaanr*	Mild acidic, alcoholic
India	Cereal	Yeast, LAB	*Daru*	Alcoholic beverages; filtrate
India	Red rice	Yeast, LAB	*Duizou*	Fermented rice beverage
India	Rice, paddy husk	Yeast, LAB	Ennog	Black rice beverage
India	Wheat	*Marcha*	*Gahoon ko jaanr*	Mild acidic, alcoholic
India	Rice	*Thiat*	*Kiad lieh*	Distilled liquor, clear
India	Barley	*Marcha*	*Jao ko jaanr*	Mild acidic, alcoholic
India	Rice	Yeasts, LAB	Jou	Mild alcoholic beverage
India	Carrot/beetroots	*Torani* (starter)	*Kanji*	Strong flavored
India	Finger millet/ barley	*Phab*	*Chyang/Chee*	Mild alcoholic, slightly sweet-acidic
India	Maize-rice/barley	*Phab*	*Mingri*	Sweet, mild alcoholic, thick
India	Coconut palm	Yeasts, LAB	*Nareli*	Sweet, milky, effervescent, mild alcoholic
India	Red rice	*Khekhrii*	*Nchiangne*	Distilled liquor
India	Rice-millet	Unknown	Oh	Soft, mild alcoholic beverage
India	Palmyra and date palm sap	Yeasts, LAB	*Tari*	Sweet, effervescent, and mild alcoholic
India	Date palm	Yeasts, LAB	*Tari*	Sweet, alcoholic beverage
India	Finger millet, barley	Molds, yeasts	*Themsing*	Mild alcoholic, sweet
India	Rice	*Hamei*	*Yu*	Distilled from liquor from *atingba*, alcoholic drink
India	Barley	Yeasts	*Sing sing*	Beverage
India	Cassava tuber	*Marcha*	*Simal tarul ko jaanr*	Mild alcoholic, sweet-sour
India	Rice	Yeasts, LAB	*Zu*	Distilled from fermented rice; clear liquor
India	Rice	*Khekhrii*	Zutho	Milky white, sweet-sour, mild alcoholic
India	Finger millet	*Dhehli*	*Sura*	Alcoholic
India	Maize-rice, barley	Unknown	*Lohpani*	Alcoholic liquor

(*continued*)

TABLE 4.2 (Continued)

Plant-Based Fermented Beverages of Asia

Country	Plant Source	Mixed Starter	Beverage	Nature and Use
India	Barley	*Phab*	*Lugri*	Sweet-sour, mild alcoholic, thick liquid
India	Rice	Yeast, mold	*Madhu*	Distilled liquor
India	Maize	*Marcha*	*Makai ko jaanr*	Mild alcoholic, sweet-sour
India	Rice	Molds, yeast, LAB	*Pona*	Mild alcoholic, sweet-sour, paste
India	Cereals	*Marcha*	*Raksi*	Clear distilled liquor
India	Rice	Yeasts	*Ruhi*	Distilled liquor
Indonesia	Rice	*Ragi*	*Brem*	Dried, sweet-sour, mild alcoholic product
Indonesia	Rice, cassava, maize, millet	*Ragi*	*Tapé kekan*	Sweet-sour alcoholic paste
Japan	Rice	*Koji*	*Saké*	Nondistilled, clarified and filtered liquor (national drink)
Japan	Rice	*Koji*	*Shochu*	Distilled spirit
Japan	Sugar cane	*Koji*	*Shoto saké*	Liquor
Korea	Rice	*Nuruk*	*Soju*	Distilled liquor
Korea	Rice	*Nuruk* or commercial starter	*Makgeolli*	Sweet-acidic, mild alcoholic drink (national drink)
Korea	Rice, wheat, barley, maize	*Nuruk*	*Takju*	Alcoholic
Korea	Rice	*Nuruk*	*Ewhaju*	Nondistilled, filtered, and clarified, clear liquor
Korea	Rice, wheat, barley, maize	*Nuruk*	*Yakju*	Alcoholic
Korea	Rice	*Nuruk*	*Soju*	Distilled liquor
Malaysia	Rice	*Ragi* or *jui-piang*	*Tapai pulut*	Sweet, sour, mild alcoholic
Malaysia	Cassava	*Ragi* or *jui-piang*	*Tapai ubi*	Sweet, sour, mild alcoholic
Mongolia	Millet	LAB, yeasts	*Darassun*	Liquid
Nepal	Rice	*Marcha*	*Bhaati jaanr*	Mild alcoholic, sweet-sour, paste
Nepal	Buck wheat	*Marcha*	*Faapar ko jaanr*	Mild acidic, alcoholic
Nepal	Wheat	*Marcha*	*Gahoon ko jaanr*	Mild acidic, alcoholic
Nepal	Barley	*Marcha*	*Jao ko jaanr*	Mild acidic, alcoholic
Nepal	Maize	*Marcha*	*Makai ko jaanr*	Mild alcoholic, sweet-sour
Nepal	Finger millet	*Marcha*	*Kodo ko jaanr*	Mild alcoholic, sweet-acidic
Nepal	Rice	*Manapu*	*Poko*	Sweet-acidic, mild-alcoholic
Nepal	Cereals	*Marcha*	*Raksi*	Clear distilled liquor
Nepal	Cassava tuber	*Marcha*	*Simal tarul ko jaanr*	Mild-alcoholic, sweet-sour
Philippines	Sugar cane	*Bubod, binubudan*	*Basi*	Clear or cloudy liquid
Taiwan	Rice	*Chiu-yueh*	*Tien-chiu-niang*	Mild-alcoholic, sweet
Thailand	Rice	*Loogpang*	*Ou*	Distilled liquor
Thailand	Rice	*Loogpang*	*Khao maak*	Juicy, white colored, sweet taste, mild alcoholic
Thailand	Rice	*Loogpang*	*Nam khao*	Distilled liquor
Thailand	Rice	*Loogpang*	*Krachae*	Nondistilled and filtered liquor
Thailand	Rice	*Loogpang*	*Sato*	Distilled liquor
Philippines	Rice	*Bobod*	*Tapuy*	Sweet, sour, mild alcoholic
Vietnam	Rice	*Men*	Ruou de	Distilled liquor, clear
Vietnam	Rice	*Men*	Ruou nep	Distilled liquor, clear
Vietnam	Rice (purple)	*Men*	Ruou nep than	Nondistilled, viscous, thick
Vietnam	Rice, maize, cassava	*Men*	Ruou nep chan	Nondistilled, viscous, thick; or distilled

4.2 Fermented Vegetables

4.2.1 Gundruk

Gundruk is an ethnic fermented vegetable of the Himalayan regions of India, Nepal, and Bhutan. Daily per capita consumption of gundruk is 1.4 g with an annual production of 3.2 kg/house in the Indian state of Sikkim (Tamang et al. 2007a). Gundruk is prepared from the fresh leaves of a local vegetable called *rayo-sag* (*Brasicca rapa* subspecies *campestris* variety *cuneifolia*), mustard, and cauliflower. This mixture is wilted and shredded, crushed mildly, and pressed into an airtight earthen jar or container. The container is kept in a warm place and allowed to ferment naturally for approximately 7 to 10 days. Unlike kimchi and sauerkraut, freshly fermented gundruk is sun-dried for 3 to 4 days before consumption whereas dried gundruk can be preserved for more than 2 years at room temperature. Gundruk is eaten as a soup or pickle.

Lactobacillus fermentum, *Lactobacillus plantarum*, *Lactobacillus casei*, *Lactobacillus casei* subsp. *pseudoplantarum*, and *Pediococcus pentosaceus* have been isolated from gundruk (Tamang et al. 2005). Gundruk fermentation is initiated by *Lb. fermentum*, followed by *P. pentosaceus* and, finally, by *Lb. plantarum*, *Lb. casei*, and *Lb. casei* subsp. *pseudoplantarum* (Karki et al. 1983d; Tamang and Tamang 2010). *Lb. plantarum* MTCC 9483 and *P. pentosaceus* MTCC 9484 have been selected as starters for the production of gundruk (Tamang and Tamang 2010). Some LAB isolated from gundruk showed strong acidification, antimicrobial properties, and the ability to degrade antinutritive factors and probiotic character (Tamang et al. 2009a). Gundruk contains organic acids such as lactic, acetic, citric, malic, and acetic acids (Karki 1986), and is considered as a good appetizer (Tamang 2010a). Cyanides and isothiocyanates are the main flavor components, followed by alcohols, esters, and phenyl acetaldehyde in gundruk (Karki et al. 1983a). Increases of palmitic, oleic, linoleic, and linolenic acids and free amino acids, mostly glutamic acid, alanine, leucine, lysine, and threonine have been observed in gundruk (Karki et al. 1983b,c).

4.2.2 Kimchi

Kimchi symbolizes the rich food culture of Korea, and is one of the most popular ethnic fermented vegetables in Korea. Kimchi is a generic term used to denote a group of fermented cabbage, radish, and garlic foods in Korea. Per capita consumption of kimchi is 50 to 200 g per day (Kim and Chun 2005). More than 106 tons of kimchi is consumed annually in South Korea using not less than100 types of vegetables to prepare kimchi (Wacher et al. 2010). Chinese cabbage, radish, cucumber, a mixture of red pepper powder, garlic, ginger, and green onion are the ingredients for the production of kimchi (Nam et al. 2009). During the traditional method of preparation of kimchi, cabbage and radish are cut into small chunks, salted, mixed with chopped hot red pepper, garlic, dropwort, leaf mustards, and some seaweed. Water is added to the mixture which is kept in a closed earthen pot until it is fermented. The freshly fermented product, kimchi, is taken out and is eaten directly as a side dish. Three major types of kimchi are commonly prepared in Korea—whole cabbage kimchi (*jeotgukji*), diced-radish kimchi (*kakdug*i), and water kimchi (*yeolmu*).

Several species of LAB have been identified from kimchi, including *Leuconostoc kimchii*, *Leuconostoc mesenteroides*, *Leuconostoc pseudomesenteroides*, *Leuconostoc lactic*, *Lactobacillus brevis*, *Lactobacillus kimchii*, *Lb. plantarum*, *Lactococcus inhae*, *Weisella kimchi*, *Weisella koreensis*, *Tetragenococcus koreensis* (Yoon et al. 2000; Lee et al. 2002, 2005; Choy et al. 2002; Kim et al. 2003; Kim and Chun 2005). Several species of non-LAB and yeasts have also been reported from kimchi—*Halococcus*, *Natronococcus*, *Natrialba*, *Haloterrigena*, *Lodderomyces*, *Trichosporon*, *Candida*, *Saccharomyces*, *Pichia*, *Sporisorium*, and *Kluyveromyces* (Chang et al. 2008). Kimchi has several health-promoting benefits and is considered as a functional food. It has an antiobesity effect (Kong et al. 2008), probiotic properties (Lee and Lee 2006), and antioxidants (Sim and Han 2008; Sun et al. 2009). Consumption of kimchi prevents constipation, colon cancer, reduces serum cholesterol (Park et al. 2006), inhibits atherosclerosis (Kim et al. 2008), exerts antistress principles (Lee and Lee 2009), and helps in the reduction of depression, osteoarthritis, and liver disease (Lee et al. 2008). Nowadays, kimchi is no longer limited to the Korean diet and has been widely accepted by non-Korean consumers throughout the world as a popular salad due to its taste and functionality.

4.2.3 Sinki

Sinki is an ethnic fermented radish taproot commonly prepared in the Himalayan regions of India, Nepal, and Bhutan by pit fermentation. Pit fermentation of a *sinki* preparation is a unique type of biopreservation of perishable radish using lactic acid fermentation in the Himalayas (Tamang 2010a). A pit approximately 2 to 3 ft. with the same diameter is dug in a dry field, cleaned, plastered with mud, and warmed by burning. After removing the ashes, the pit is lined with bamboo sheaths and paddy straw. Radish taproots are wilted for 2 to 3 days, crushed, squeezed, and pressed tightly into the pit, then covered with dry leaves and weighted down by heavy planks or stones. The top of the pit is plastered with mud and left to ferment naturally for 22 to 30 days. Freshly fermented sinki is removed, cut into small pieces, sun-dried for 3 to 5 days, and stored at room temperature for future consumption. Dry sinki can be kept for several years in an airtight container. It is eaten as a soup or pickle. *Lb. plantarum*, *Lb. brevis*, *Lb. casei* and *Leuconostoc fallax* have been isolated from sinki (Tamang and Sarkar 1993; Tamang et al. 2005).

4.2.4 Sunki

Sunki is an ethnic nonsalted and fermented vegetable product of Japan prepared from the leaves and stems of red turnip (Wacher et al. 2010). Turnip is boiled, inoculated with *zumi* (a small, wild apple), and dried *sunki* from the previous year and allowed to ferment for 1 to 2 months. *Sunki* is eaten with rice and miso soup. *Lb. plantarum*, *Lb. brevis*, *Lactobacillus buchneri*, *Lactobacillus kisonensis*, *Lactobacillus otakiensis*, *Lactobacillus rapi*, *Lactobacillus sunkii*, *Enterococcus faecalis*, *Bacillus coagulans*, and *P. pentosaceus* have been isolated from *sunki* (Itabashi 1986; Watanabe et al. 2009).

4.2.5 Khalpi

Khalpi is an ethnic fermented cucumber product of Nepal and India. Ripened cucumber is cut into pieces and sun-dried for 2 days, then put into a closed bamboo vessel and fermented naturally at room temperature for 3 to 5 days. Khalpi is consumed as a pickle. *Leuc. fallax*, *P. pentosaceus*, *Lb. brevis*, and *Lb. plantarum* are the dominant LAB in khalpi fermentation (Tamang et al. 2005). *Lb. plantarum* MTCC 9485, *Lb. brevis* MTCC 9486, and *Leuc. fallax* MTCC 9487 were selected as a mixed starter for the commercial production of khalpi (Tamang and Tamang 2010).

4.2.6 Goyang

Goyang is an ethnic fermented wild plant food, generally prepared by the Sherpa women of high mountains in the Himalayan regions of India and Nepal (Tamang and Tamang 2007). The leaves of the wild edible plant (*Cardamine macrophylla* Willd), are collected, washed and cut into pieces, squeezed to drain off excess water, and tightly pressed into bamboo baskets lined with two to three layers of leaves from fig plants. The top of the baskets are then covered with fig plant leaves, and fermented naturally at room temperature for 25 to 30 days. Freshly fermented goyang is transferred into an airtight container which can be stored for 2 to 3 months. The shelf life of *goyang* can be prolonged by making the freshly fermented goyang into balls that are sun-dried for 2 to 3 days before being stored for several months. Goyang is eaten as a soup. *Lb. plantarum*, *Lb. brevis*, *Lactococcus lactis*, *Enterococcus faecium*, *P. pentosaceus*, and yeasts from *Candida* spp. have been isolated from goyang (Tamang and Tamang 2007).

4.2.7 Pao Cai and Suan Cai

Pao cai is an ethnic fermented cabbage from China, similar to Korean kimchi. It is most common in northern and western China. A similar form of pao cai, called *suan cai*, is prepared and eaten in northeast China. There are more than 11 major types of fermented vegetables or pickled vegetables in China. Pao cai is sweet and sour rather than spicy, and is eaten with congee as a breakfast food. The flavor and method of production of pao cai vary greatly across China. During production, vegetables are selected, cleaned, drained, cut into pieces, and seasonings (sugar, salt, vinegar, a little alcohol) are added before

being sealed in jars that are filled up with water and fermented for a week or more. The total annual production of pao cai in China is 2 million tons, fetching 150 million Chinese yuan annually (Lu 2010). *Lactobacillus pentosus*, *Lb. plantarum*, *Lb. brevis*, *Lb. lactis*, *Lb. fermentum*, *Leuc. mesenteroides*, and *P. pentosaceus* are the functional LAB in pao cai (Yan et al. 2008; Huang et al. 2009). *Lb. harbinensis* was isolated from suan cai (Miyamoto et al. 2005).

4.2.8 Fu-tsai and Suan-tsai

Fu-tsai and *suan-tsai* are ethnic fermented mustard products from Taiwan prepared by the Hakka tribes (Chao et al. 2009). Freshly harvested mustard is wilted, placed in a bucket in layers by alternating with 4% salt, sealed airtight, and left to ferment naturally for 7 days to 2 months at ambient temperature. If fermentation is continued for approximately 2 months, the fermented product is called suan-tsai. However, for the production of fu-tsai, partly fermented mustard is removed from the bucket after 7 days, and cleaned with water, sun-dried for 1 to 2 days, and then again fermented for at least 2 days. This step of sun-drying in the daytime and fermentation at night is repeated two or three times. The partly dried mustard is then divided into pieces and packed tightly into glass bottles or earthenware pots or plastic containers, before being sealed, and placed upside down for maturation for 3 months. The resulting product is called *fu-tsai*. Fu-tsai and suan-tsai are eaten as a soup, fried with shredded meat, or stewed with meat. LAB isolated from fu-tsai include *P. pentosaceus* and *Tetragenococcus halophilus* (Chen et al. 2006), *Lactobacillus farciminis*, *Leuc. mesenteroides*, *Leuc. pseudomesenteroides*, *Weisella cibaria*, and *Weisella paramesenteroides* (Chao et al. 2009).

4.2.9 Jeruk

Jeruk is an ethnic fermented vegetable from Malaysia, which is prepared from common fruits and vegetables (Merican 1996). Pickled vegetables are prepared like fresh vegetables; pickled fruit is eaten as a relish, especially by children and expectant mothers, because of the sweet-sour flavor. Species of *Leuconostoc*, *Lactobacillus*, *Pediococcus*, and *Enterococcus* are present in jeruk (Merican 1996).

4.2.10 Pak-gard-dong

Pak-gard-dong is an ethnic fermented vegetable product of Thailand prepared from mustard leaves (Boon-Long 1986). Black mustard leaves are collected (defective leaves are removed), washed, and wilted in the sun, and 2.5% salt is added to the wilted leaves which are then packed into a container and left for 12 hours. After removing the water, 3% sugar is added and fermented at room temperature for 3 to 5 days. *P. pentosaceus*, *Lb. brevis*, and *Lb. plantarum* as the major LAB involved in the fermentation of pak-gard-dong (Mingmuang 1974).

4.2.11 Pak-sian-dong

Pak-sian-dong is an ethnic fermented leafy vegetable product of Thailand (Dhavises 1972). It is prepared from the leaves of *pak-sian* (*Gynandropis pentaphylla* DC). Fresh vegetables are thoroughly cleaned with water and then spread out in the air or under the sun to dehydrate until the sample is distinctly flaccid. It is then mixed with water, salt, and sugar and kept in a tightly covered container for 2 to 3 days. *Leuc. mesenteroides*, *Lb. fermentum*, *Lb. buchneri*, *Lb. plantarum*, *Lb. brevis*, and *P. pentosaceus* are present in pak-sian-dong (Dhavises 1972).

4.2.12 Sayur Asin

Sayur asin is an ethnic, fermented mustard cabbage product from Indonesia (Puspito and Fleet 1985). Mustard cabbage leaves are wilted and rubbed or squeezed with 2.5% to 5% salt. Liquid from boiled rice is added to provide fermentable carbohydrate to assure that sufficient acid is produced during fermentation. Fermentation is initiated by *Leuc. mesenteroides* and *Lactobacillus confusus* and later dominated by *Lb. plantarum* and *P. pentosaceus* (Puspito and Fleet 1985).

4.3 Fermented Bamboo Shoots

4.3.1 Mesu

Mesu is an ethnic fermented bamboo shoot product of India, Nepal, and Bhutan. Young shoots of species of bamboo such as *Dendrocalamus sikkimensis*, *Dendrocalamus hamiltonii*, and *Bambusa tulda* are defoliated, finely chopped, and pressed tightly into a hollow, green bamboo stem. Any openings in the vessel are covered tightly with bamboo leaves and it is left to ferment under natural anaerobic conditions for 7 to 15 days. Mesu is eaten as a pickle. *Lb. plantarum*, *Lb. brevis*, *Lactobacillus curvatus*, *Leuconostoc citreum*, and *P. pentosaceus* have been isolated from mesu (Tamang and Sarkar 1996; Tamang et al. 2008).

4.3.2 Soibum

Soibum is an ethnic fermented bamboo shoot dish from India. Thin slices of young bamboo shoots are packed into this chamber, the upper surface is sealed with a polytethylene sheet, and weights are then put on top for proper pressing. The bottom of the chamber is perforated for draining any acidic juices before being left for 6 to 12 months for fermentation. After fermentation, soibum can be stored for 10 to 12 months. In Manipur, India, different dishes are prepared from soibum such as *ironba*, *athongba*, *kangou*, and *chagempomba* (Tamang 2010a). *Lb. plantarum*, *Lb. brevis*, *Leuc. fallax*, *Leuc. mesenteroides*, *Leuc. lactis*, and *Enterococcus durans* are present in soibum (Tamang et al. 2008). *Bacillus subtilis*, *Bacillus licheniformis*, *B. coagulans*, and *Micrococcus luteus* were also isolated from soibum (Sarangthem and Singh 2003). An increase in free amino acids has been observed during the fermentation of soibum (Giri and Janmejay 2000).

4.3.3 Soidon

Soidon is the fermented tip of mature bamboo shoot products commonly consumed in Manipur, India. The outer casings and lower portions of mature bamboo shoots are removed; entire tips are submerged in water in an earthen pot. Sour liquid from a previous batch is added as a starter in a 1:1 dilution. This mixture is covered and fermented for 3 to 7 days at room temperature. Leaves of *Garcinia pedunculata* Roxb., locally called *heibung*, may be added in the fermenting vessel during fermentation to enhance the flavor of soidon. After fermentation, soidon is removed from the pot and can be stored in a closed container at room temperature for 1 year. It is consumed as a curry as well as a pickle with steamed rice. *Lb. brevis*, *Leuc. fallax*, and *Leuc. lactis* have been isolated from soidon (Tamang et al. 2008).

4.3.4 Ekung

Ekung is an ethnic fermented bamboo shoot product of India, prepared in Arunachal Pradesh. A pit approximately 3 to 4 ft. is dug in the forest, bamboo baskets are laid into the pit and lined with leaves, and pieces of chopped bamboo shoots are put into the basket. When the basket is full, it is covered with leaves and then sealed. Heavy stones are used to weight and drain excess water from the bamboo shoots, which are then fermented for 1 to 3 months. Ekung can be kept for a year in an airtight container at room temperature. It is consumed raw or cooked with meat, fish, or vegetables. *Lb. plantarum*, *Lb. brevis*, *Lb. casei*, and *T. halophilus* have been isolated from ekung (Tamang and Tamang 2009).

4.3.5 Eup

Eup is a dry, fermented tender bamboo shoot food commonly prepared and consumed by different tribes from India. Bamboo shoots are chopped into small pieces and fermented in a similar manner to ekung. Fermentation is completed within 1 to 3 months. After fermentation, the product, now called *eup*, is again cut into smaller pieces and then dried in the sun for 5 to 10 days until its color changes from whitish

to chocolate brown. Eup is consumed as a side dish with steamed rice, meat, fish, or vegetables. *Lb. plantarum* and *Lb. fermentum* have been isolated from eup (Tamang and Tamang 2009).

4.3.6 Hirring

Hirring is the fermented topmost portion of the whole bamboo shoot product, commonly prepared in Arunachal Pradesh, India. The outer leaf sheaths of tender bamboo shoots are removed and the topmost tender edible portions are either cut longitudinally into two to three pieces or whole shoots are flattened by crushing before being put into bamboo baskets lined with leaves. The baskets are placed into the pit, covered with leaves, sealed and weighted down with heavy stones, and fermented for 1 to 3 months. Baskets are taken out from the pits after fermentation upon which time hirring is ready for consumption. Hirring can be kept for 2 to 3 months at room temperature. It is consumed as a side dish mixed with vegetables, meat, and fish along with steamed rice. *Lb. plantarum* and *Lc. lactis* are the functional LAB in hirring (Tamang and Tamang 2009).

4.3.7 Naw-mai-dong

Naw-mai-dong is an ethnic fermented bamboo shoot product of Thailand (Dhavises 1972). During preparation, young bamboo shoots are harvested, woody and defective portions are removed from the shoots by trimming. After washing, shoots are sliced and mixed with salt at 2% level. The bamboo shoots are boiled in water and the bitter liquor is discarded. These are then packed into earthen jars, covered with a plastic sheet, and weighted on top. Fermentation is complete in 3 to 4 weeks at room temperature. *Leuc. mesenteroides*, *Lb. fermentum*, *Lb. buchneri*, *Lb. brevis*, and *P. pentosaceus* are present in naw-mai-dong (Dhavises 1972; Phithakpol et al. 1995).

4.4 Fermented Legumes

4.4.1 Kinema

Kinema is an ethnic fermented soybean food from India, Nepal, and Bhutan, and is one of the oldest cultural foods from Asia (Tamang 2010a). Daily per capita consumption of kinema in Sikkim, India is 2.3 g (Tamang et al. 2007a). Soybeans are washed, soaked overnight, and placed into containers with fresh water and boiled for 2 to 3 hours until soft. Excess water is drained off and the cooked soybean seeds are placed into a wooden mortar and cracked lightly with a wooden pestle to split the cotyledons. This practice of cracking cooked soybean seeds has been observed only in kinema production, unlike natto and chungkokjang, probably to increase the surface area for speedy fermentation by aerobic spore-forming bacteria (Tamang 2001). Approximately 1% firewood ash is added directly to the cooked soybeans and mixed thoroughly to maintain the alkaline condition of the product. Soybean grits are placed in a bamboo basket lined with locally grown fresh fern (*Glaphylopteriolopsis erubescens*) or ficus (fig plant) and banana leaves. The basket is covered in a jute bag and left to ferment naturally at ambient temperatures (25–40°C) for 1 to 3 days above an earthen oven kitchen. During the summer, the required fermentation time may be 1 to 2 days, whereas in the winter, 2 to 3 days might be needed. Freshly prepared kinema remains viable for 2 to 3 days in the summer and for a maximum of 1 week in winter without refrigeration. Shelf life may be prolonged by drying in the sun for 2 to 3 days. Dried kinema can be stored for several months at room temperature.

A number of *Bacillus* species have been isolated from kinema, including *B. subtilis*, *B. licheniformis*, *Bacillus cereus*, *Bacillus circulans*, *Bacillus thuringiensis*, and *Bacillus sphaericus* (Sarkar et al. 1994, 2002). However, *B. subtilis* is the dominant functional bacterium in kinema (Sarkar and Tamang 1994; Tamang and Nikkuni 1996). Besides bacilli, *E. faecium* and two types of yeasts, *Candida parapsilosis* and *Geotrichum candidum*, were also isolated from kinema samples (Sarkar et al. 1994). It has been observed that rich microbial diversity from various sources, particularly the soybean, equipment, and

leaves as wrapping materials, harness microbiota for the spontaneous fermentation of kinema (Tamang 2003). With the decrease in protein nitrogen content, the nonprotein and soluble nitrogen contents increase during kinema fermentation (Sarkar and Tamang 1995). Tamang and Nikkuni (1998) found a significant increase in the relative viscosity of kinema during maturation at 5°C and 10°C. Keeping freshly prepared kinema below 10°C for 1 day stabilizes the quality of the product by preventing further biological activity of microorganisms and showing improved stickiness, which is a very important sensory property of kinema (Tamang and Nikkuni 1998). Organoleptically, the monoculture fermentation of soybeans by *B. subtilis* MTCC 2756 produces the best kinema because of a pleasant nutty flavor and highly sticky texture, minimizing the conventional fermentation time, maintaining better hygienic conditions, consistency, and increasing the levels of soluble protein (Tamang 1999).

Instead of discarding the soybean extract after autoclaving, an inexpensive soybean extract broth can be prepared, after adjusting the pH to 7, as a medium for the enrichment of *B. subtilis* spores (Tamang 1999). Moreover, the nutrient broth, conventionally used for the enrichment of *B. subtilis* spores, is composed of expensive beef extract, which is not acceptable to most of the Hindi population in the Himalayas. Kinema prepared by using the *B. subtilis* KK2:B10 strain, which is harvested from soybean extract broth, is dried in an oven at 70°C for 10 hours and ground aseptically to make a pulverized starter. Instead of *B. subtilis*, 1% of the pulverized starter is added aseptically to the autoclaved soybeans and fermented to obtain kinema. Consumers' preference trials show that kinema prepared by using pulverized starter under optimized conditions is more acceptable than kinema bought from local market (Tamang 1999). Water-soluble nitrogen and formol nitrogen contents are higher in kinema prepared by using the pulverized starter compared with store-bought kinema (Tamang 1999). Increased water-soluble nitrogen levels in kinema helps in digestibility, and high amounts of formol nitrogen, which contain free amino acid supplements, impart a better taste to kinema (Nikkuni et al. 1995). The application of ready-to-use pulverized starter seems appropriate in kinema production for marginal kinema producers in the Himalayas because it is cost-effective and easy to handle (Tamang 2010a).

Kinema has many health-promoting benefits, including having antioxidant activity, increased protein digestibility, essential amino acids, vitamin B complex, low cholesterol content, etc. (Tamang 2010a); therefore, kinema can be considered as a functional food. Kinema is the cheapest source of plant protein compared with milk and animal products on the basis of protein cost per kilogram. It contains all the essential amino acids (Sarkar et al. 1997), and is rich in linoleic acid, an essential fatty acid (Sarkar et al. 1996). Total amino acids, free amino acids, and mineral contents are increased during kinema fermentation (Sarkar and Tamang 1995; Nikkuni et al. 1995; Tamang and Nikkuni 1998). Phytosterols (cholesterol-lowering effect) are increased during kinema fermentation (Sarkar et al. 1996). Riboflavin and niacin levels increase in kinema during fermentation (Sarkar et al. 1998). Kinema has antioxidant activities (Tamang et al. 2009b). Because it contains large amounts of Group B saponins, kinema reportedly has health-promoting benefits (Omizu et al. 2011).

Many kinema-like sticky bacilli–fermented soybeans are consumed by different ethnic communities living in the northeastern part of India bordering Bhutan, China, and Myanmar, which include *hawaijar* in Manipur, *bekang* in Mizoram, *peruyaan* in Arunachal Pradesh, *aakhone* in Nagaland, and *tungrymbai* in Meghalaya (Tamang et al. 2009b).

4.4.1.1 KNT Triangle Hypothesis

Nonsalted fermented soybean foods are concentrated in a triangle with three vertices: Japan (natto), India and Nepal (kinema), and Indonesia (tempe). Nakao (1972) named this the *natto triangle* and included both bacilli and mold-fermented soybean products, including tempe, and extended the triangle up to Indonesia. Tamang (2010a) renamed this hypothetical triangle the *kinema-natto-thua nao triangle* (KNT triangle), and included only nonsalty, bacilli-fermented soybean foods with three vertices in India and Nepal (kinema), Thailand (thua nao), and Japan (natto). Within the proposed triangle-bound countries, many fermented sticky, nonsalty soybean foods were consumed by different ethnic groups of people such as kinema (India, Nepal, and Bhutan), natto (Japan), tungrymbai, bekang, hawaijar, aakhone, and peruyaan (India), thua nao (Thailand), chungkokjang (Korea), pepok (Myanmar), and sieng (Cambodia and Laos). Beyond this hypothetical KNT triangle, there is no report of kinema-like

products with sticky and ammonia-flavored fermented soybean foods and the proposed KNT triangle does not include salted, nonsticky, and non–bacilli-fermented soybean products such as tempe, miso, sufu, soy sauce, etc. (Tamang 2010a). Although the method of production and culinary practices vary from product to product, all bacilli-fermented Asian soybean foods have a characteristic stickiness and typical flavor. Hara et al. (1995) reported that the plasmid of *B. subtilis* (*natto*) strain resembles that of *B. subtilis* isolated from thua nao and kinema. The phylogenetic relationships among bacilli isolated from kinema (India), chungkokjang (Korea), natto (Japan), and similar fermented sticky soybean foods from Asia reveal that all bacilli strains belonged to *B. subtilis* (Tamang et al. 2002). This suggests that *B. subtilis* strains responsible for the fermentation of sticky soybean food in Asia might have originated from the same stock. Another theory was proposed in which nonsalted fermented soybean foods originated in Yunnan Province of China, which was the center of the hypothetical triangle (Nagai and Tamang 2010).

4.4.2 Natto

Natto is an ethnic fermented soybean food from Japan, similar to kinema. In the traditional method of natto preparation, soybeans are soaked in water overnight, boiled, water is discarded, and the boiled beans are wrapped with tied rice straws which have been soaked in boiling water to sterilize the microorganisms, other than the heat-tolerant spores of *B. subtilis* (*natto*), which inhabit the surface of straws (Ohta 1986). A modern method to prepare natto is based on the classical method. After boiling, the soybeans are sprayed with a suspension of spores of *B. subtilis* (*natto*) and weighed out by 30 to 100 g in polystyrene paper packages instead of wrapping in rice straws. The packages are transferred into incubators. After 16 hours at approximately 40°C, the packages are cooled for 6 to 8 hours for maturation. Natto is eaten directly with boiled rice, without frying or cooking.

Bacillus natto was isolated from homemade natto by Sawamura (1906). The bacterium is different from *B. subtilis* at the points of biotin requirement, production of polyglutamate, possession of 5.7 and 60 kb plasmids (Hara et al. 1983; Nagai et al. 1997), and insertion sequences (Nagai et al. 2000; Kimura and Itoh 2007). *B. subtilis* (*natto*) produces nattokinase, which has a high fibrinolytic activity equal to those of urokinase and plasmin (Sumi et al. 1987). Actually, capsulated nattokinase showed a significant enhancement of fibrinolytic activity in plasma after being given to adults (Sumi et al. 1990). Gly m Bd 30K of raw soybeans was degraded by *B. subtilis* (*natto*) during fermentation (Yamanishi et al. 1995). Therefore, natto is a suitable soybean food for patients allergic to raw soybeans. Pyrazines are found to be the major flavor components contributing to the characteristic natto flavor (Sugawara et al. 1985).

4.4.3 Thua Nao

Thua nao is an ethnic fermented, nonsalty soybean food from Thailand. It is generally available as a dried paste, and is used as a flavoring agent in vegetable dishes. Dry, whole soybeans are washed, boiled for 3 to 4 hours, and the cooked beans are transferred to a bamboo basket lined with banana leaves. The basket is covered with banana leaves, left at room temperature for 3 to 4 days to undergo natural fermentation (Sundhagul et al. 1972). After fermentation, thua nao is mashed lightly into a paste and salt, garlic, onion, and red pepper are added. The resulting paste is wrapped in banana leaves and cooked by steaming before eating. Thua nao is used as a seasoning and as an ingredient in soups, curry, and stir-fried vegetables (Okada 2008). *B. subtilis* is responsible for the fermentation of thua nao (Sundhagul et al. 1972). *B. subtilis* produces protease, amylase, subtilisin nattokinase, and γ-polyglutamic acid (PGA) in thua nao (Inatsu et al. 2006). Visessanguan et al. (2005) reported that *B. subtilis*–inoculated thua nao showed an increased proteolysis of soybean. Enzyme γ-glutamyl hydrolase (28 kDa) purified from thua nao degraded PGA to a hydrolysate of only approximately 20 kDa (with D-glutamic and L-glutamic acid in a ratio of 70:30), suggesting that the enzyme cleaves the γ-glutamyl linkage between L- and L-glutamic acid of PGA (Chunhachart et al. 2006). PGA-producing *Bacillus* strains isolated from thua nao, including *B. subtilis* IFO 3022, does not require biotin for growth (Hara et al. 1986).

4.4.4 Chungkokjang

Chungkokjang (or *jeonkukjang*, *cheonggukjang*) is an ethnic fermented soybean food from Korea. Cooked soybeans covered with matting made of straws are put on an ondol (Korean under-floor heating). After 3 days of fermentation, the soybeans are broken, mixed with soybean powder, ground with salt and dried in the sun. In the modern method for preparation of chungkokjang, a starter of *Bacillus* is used (Lee 2008). It is eaten as soup. *B. subitilis* isolated from chungkokjang produce viscous polyglutamate and fibrinolytic activity (Lee et al. 1991). *B. licheniformis* B1, which was isolated from soil in Korea, is known to produce chungkokjang with a good quality (Lee et al. 1999; Choy 2007). Super-high molecular weight PGA has been reported to be synthesized by *B. subtilis* subsp. *chungkokjang*, isolated from chungkokjang (Park et al. 2005). An increase in total phenol content has also been reported in chungkokjang (Shon et al. 2007). *Bacillus megaterium* SMY-212 is a suitable fermenting strain to promote the antioxidant and free-radical scavenging activities in chungkokjang (Shon et al. 2007). Fibrinolytic enzyme purified from *Bacillus* sp. strain CK 11-4 shows thermophillic, hydrophilic, and strong fibrinolytic activity (Kim et al. 1996).

4.4.5 Pepok

Pepok is an ethnic, fermented soybean food from northern Myanmar (Tanaka 2008a). Soybeans are soaked in water overnight, dewatered, boiled, and wrapped in leaves. After fermentation for 2 to 4 days, fermented beans are mashed up with salt and hot pepper, rolled out into disks, and dried under the sun. Pepok is used as a seasoning or eaten after roasting.

4.4.6 Sieng

Sieng is a traditional fermented soybean food from Cambodia (Tanaka 2008b). After boiling, soybeans are spread on bamboo baskets and fermented naturally by bacteria adhering to the baskets or suspended in the atmosphere. The soybeans are soaked in saltwater for 5 to 7 days immediately after a 2-day fermentation. Tree sap or enzymes are occasionally added to the saltwater. Sieng is used as a seasoning with salt and spices.

4.4.7 Tempe

Tempe is an ethnic, mold-fermented soybean food from Indonesia. Soybeans are soaked in water, where the pH of the soybeans is lowered by LAB inhabiting the water (Nagai and Tamang 2010). After being dehulled and drained, the soybeans are inoculated with tempe starter, locally called *usar* (*Rhizopus* spores developed on hibiscus leaves) or *ragi tempe* (*Rhizopus* inoculated on starch pulp). The inoculated soybeans are packed in leaves of banana or, recently, plastic bags, and fermented in 30°C for 24 hours. In modern procedures, the acidification process by LAB is replaced by the addition of organic acid (lactic acid, acetic acid, or vinegar or malic acid). Pure culture of *Rhizopus* starter is used in some tempe factories. Tempe is fried and is eaten as a snack.

Rhizopus microsporus variety *oligosporus* is a major fungus for the fermentation of tempe, although some bacteria were isolated in natural tempe (Nagai and Tamang 2010). *Klebsiella pneumoniae*, *K. pneumoniae* subsp. *ozaenae*, *Enterobacter cloacae*, and Gram-positive bacteria were also isolated as vitamin B_{12}–producing bacteria in tempe (Okada et al. 1985). In homemade tempe in Indonesia, the second most important microorganisms are LAB. The role of the bacteria is in lowering the pH of soybeans, both to prevent contaminated bacteria from growing dominantly and to provide *R. oligosporus* with suitable environments to grow. *Lb. plantarum* and *Lb. lactis* were isolated from water in which soybeans were soaked. Tempe possesses several functional properties such as thrombolytic activity, contains γ-amino butyric acid that retards the elevation of blood pressure, contains dietary fiber, saponins, isoflavones, and superoxide dismutase which eliminates active oxygen, and has an anticarcinogenic effect (Sumi and Okamoto 2003; Aoki et al. 2003).

4.4.8 Douchi

Douchi is an ethnic fermented soybean product of China. Four types of douchi are produced in China: *Mucor*-fermented douchi, *Aspergillus*-fermented douchi, *Rhizopus*-fermented douchi, and *Bacillus*-fermented douchi. Among them, *Aspergillus*-type douchi is the earliest known, and has been the most popular type in China (Bao 1985). Douchi is prepared from soybeans by pretreatment in a two-step fermentation process (primary and secondary fermentation) as described by Kang (2001). Soybeans are soaked in water and boiled for approximately 1 hour, but for bacterial-type douchi, the beans only need to be boiled for 30 to 40 minutes. The second step is *qu*-making, in which the boiled beans are inoculated with *A. oryzae* (0.3%) or *Mucor* strain spores (0.5%), or simply incubated at high temperature (>25°C) for 3 to 4 days to harvest matured *qu*. In *Aspergillus*-type douchi preparations, koji is washed with water to remove the spores, mycelium, and part of the enzymes to avoid an astringent flavor in the final product (Zhang et al. 2007). Then, 18% (w/w, soybean base) salt, a little sugar, and flavoring such as capsicum paste are mixed with *qu* to obtain a desirable flavor. The mixture is packed into jars and sealed with plastic film. The *Aspergillus*-type douchi is fermented at 30°C to 35°C for 7 to 40 days, whereas *Mucor*-type douchi is fermented at approximately 20°C for 10 to 12 months. Douchi is consumed as a soup or side dish.

The presence of two biogenic amines, that is, cadaverine and putrescine, leads to histamine toxicity in douchi by inhibiting histamine-metabolizing enzymes such as diamine oxidase and histamine methyltransferase (Arnold and Brown 1978; Lehane and Olley 2000). Douchi produces angiotensin I–converting enzyme inhibitors, which lower the blood pressure (Zhang et al. 2006). It also shows antioxidative activity (Wang et al. 2007).

4.4.9 Sufu

Sufu is an ethnic fermented soybean food from China and Taiwan. It is a cheese-like product with a spreadable creamy consistency and a strong flavor (Han et al. 2001a). Soybeans are washed, soaked overnight, ground into a slurry, and pressed to obtain soymilk. It is then salted to coagulate, the excess water is removed by pressing with stones or wooden planks, and finally, a soft and firm cake-like *tofu* results (Steinkraus 1996). In the next step, *Pehtze* (*pizi*) is prepared from fresh bean curd overgrown with mold mycelia by natural fermentation. Cubes of *tofu* are placed in wooden trays made up of loosely woven bamboo strips and surrounded with straw for natural inoculation and fermentation at a temperature of 15°C to 20°C for 5 to 15 days. The prepared pehtze has white or light yellow-white mycelium for an attractive appearance. It is then placed into a big earthen jar for salting, in which salt is spread between the layers of pehtze for 6 to 12 days. Afterward, the pehtze is removed from the jar, washed, and salted. Various dressing mixtures are then added for different types of sufu. The most common dressing mixture used consists of *angkak*, alcoholic beverage, salt, sugar, bean paste, and spices and sometimes even essence for flavor. For ripening, alternate layers of pehtze and dressing mixture are packed into jars in a 2:1 ratio. The mouth of the jar is wrapped with sheath leaves of bamboo and sealed with clay and aged for 6 months for further maturation. Three types of sufu—red, white, and yellow are prepared in Taiwan (Yuan 1994). Sufu is consumed as a side dish.

Pure culture starters consisting mainly of molds—mucoraceae (*Actinomucor*, *Mucor*, and *Rhizopus*) or bacteria—*Micrococcus* and *Bacillus* spp. are used for the production of sufu. Most sufu contains considerable levels of antimicrobial NaCl (5–15%) and ethanol (1–7%) that could prevent the survival and growth of pathogens; it is also known that the endospore-forming rods such as *Bacillus* spp. and *Clostridium* spp. vary greatly in their salt tolerance (Brewer 2000). Chou and Hwan (1994) reported that the addition of ethanol to the brine solution for aging resulted in free fatty acids in the sufu product. *Actinomucor repens*, *Actinomucor taiwanensis*, *Mucor circinelloides*, *Mucor hiemalis*, *Mucor racemosus*, and *R. microsporus* variety *microsporus* have been isolated from starter cultures used in commercial pehtze fermentation for sufu production (Han et al. 2001b). The diversity of LAB was reported in fermented brines used to ferment *tofu* into sufu, which included *Enterococcus hermanniensis*, *Lactobacillus agilis*, *Lb. brevis*, *Lb. buchneri*, *Lactobacillus crispatus*, *Lb. curvatus*, *Lactobacillus*

delbrueckii, *Lactobacillus farciminis*, *Lactobacillus fermentum*, *Lactobacillus pantheris*, *Lactobacillus salivarius*, *Lactobacillus vaccinostercus*, *Lc. lactis* subsp. *lactis*, *Lc. lactis* subsp. *cremoris*, *Leuc. carnosum*, *Leuc. citreum*, *Leuc. fallax*, *Leuc. lactis*, *Leuc. mesenteroides*, *Leuc. pseudomesenteroides*, *P. acidilactici*, *Streptococcus bovis*, *S. macedonicus*, *W. cibaria*, *Weisella confusa*, *W. paramesenteroides*, and *Weisella soli* (Chao et al. 2008).

4.4.10 Miso

Miso is a semisolid fermented food made from soybeans, rice, or barley. Salt is mainly used for preparing miso soup in Japan. The original meaning of miso in Japanese is *immature shoyu* (Sugawara 2010). There are various types of miso in Japan: rice miso, barley miso, and soybean miso depending on the kind of koji used. The most popular miso in Japan is rice miso, which is prepared by mixing cooked soybeans with koji (steamed rice on which *A. oryzae* is cultured); salt and a small amount of water is added to control the moisture level (Ebine 1989). Miso koji is made from a single cereal or soybean, that is, steamed rice, barley, or soybean itself, by inoculating with a koji starter of *A. oryzae*. The main product is a red, salty rice miso (*akakei-karakuti-kome* miso), whose typical composition at pH 5.0 is as follows: NaCl content, 12 to 13 g/100 g; total nitrogen, 1.6 to 2.0 g/100 g; total sugar, 13 to 14 g/100 mL; alcohol, approximately 1.0 mL/100 g (Sugawara 2010). The manufacturing process basically consists of three stages: koji-making; mixing of koji, steamed soybean, and salt; and fermentation. In the first process, steamed rice is inoculated with a starter culture of *A. oryzae*. After 40 hours, a fungus grows over the culture mixture, which is called *rice koji*. In the second process, miso is prepared by mixing koji, steamed soybean, salt, and water, and then the halo-tolerant yeast, *Zygosaccharomyces rouxii* or *Candida versatilis* is added to the mixture. Salt is then added to facilitate the fermentation by yeast and LAB, and to preclude any undesirable fermentation. In the third process, the mixture is fermented for a longer period of 3 to 12 months. During this time, starch is changed to lactic acid, alcohol, and carbon dioxide by the conversion of simple sugars from starch as well as by enzymatic hydrolysis of protein to peptides and amino acids by yeast and LAB. Microbial composition in miso is almost the same as that for shoyu; however, the degree of hydrolysis is much lower in miso than in shoyu, and the product is a solid paste. At the same time, various kinds of volatile compounds are formed (Sugawara 2010).

4.4.11 Doenjang

Doenjang is a naturally fermented soybean paste of Korea similar to Japanese miso (Min and Kim 1990). Chinese *jiang* is believed to be the oldest form of doenjang. During the preparation of doenjang, cooked soybeans are pounded and mashed in a mortar, shaped into balls, wrapped in rice straw, and hung under the rafters until each ball is covered with a white bloom of natural mold. Next, the balls are crushed and mixed with salt and water to form *meju*, sometimes with the addition of sesame seeds or leaves, and placed in an earthenware container of 1- to 10-gallon capacity. The fermentation period generally lasts for 6 months. It is used as a seasoning or consumed as soup. *Leuc. mesenteroides*, *T. halophilus*, *E. faecium*, *B. subtilis* and *B. licheniformis*, *Mucor plumbeus*, *A. oryzae*, and *Debaryomyces hansenii* have been reported from doenjang (Kim et al. 2009). Doenjang has antiobesity and antiatherogenesis properties (Back et al. 2010).

4.4.12 Shoyu

Shoyu or soy (soya) sauce is a fermented soybean liquid from Japan made from fungal fermentation and is commonly used as a seasoning or condiment in Asian countries, except in the Indian continent and in middle east Asia (Sasaki and Nunomura 2003). There are many types of shoyu in Japan, although the main product is *koikuchi-shoyu*, a deep brown–colored shoyu, whose typical composition at pH 4.7 is as follows: NaCl content, 16.9 g/100 mL; total nitrogen, 1.57 g/100 mL; reducing sugar, 3.0 g/100 mL; alcohol, 2 to 3 mL/100 mL (Mizunuma 1991). The manufacturing process basically consists of three stages: koji-making, brine fermentation, and refining. In the first process, a mixture of steamed soybean

and roasted wheat is inoculated with a starter culture of *A. oryzae* or *A. sojae*. After 3 or 4 days, a fungus, which is called *koji*, grows over the culture mixture. Brine is then added to facilitate the fermentation by the halo-tolerant yeast, *Z. rouxii* and *C. versatilis* and LAB, and to preclude any undesirable fermentation. This fermentation is continued for 6 to 8 months, during which starch is changed to alcohol, lactic acid, and carbon dioxide by the conversion of simple sugars from starch as well as by enzymatic hydrolysis of protein to peptides and amino acids (Sugawara 2010). The two starting materials, soybean and wheat, are generally used in equal amounts in the mixture. Therefore, the average amino acid composition of these two should correspond to that of shoyu if there are no chemical changes to amino acids during the fermentation process to hydrolyze the protein into water-soluble peptides and amino acids. The palatability of shoyu is greatly enhanced by a high concentration (~7.5%) of amino acids and particularly by the presence of glutamic acid (Yamaguchi 1987). During the fermentation process, different types of volatile compounds are formed as fermented products. The refining process includes filtration and pasteurization, the latter involving heating to 70°C to 80°C to deve1op the flavor of shoyu (Sugawara 2010).

4.4.13 Tauco

Tauco is an ethnic fermented soybean product of Indonesia, similar to miso. It is a yellow-colored saline paste with a meat-like flavor. Tauco is prepared by mold fermentation for 3 to 5 days followed by brine (20%) fermentation for 20 to 30 days. The first stage of preparation consists of inoculating soybean with *R. oligosporus*, *R. oryzae*, and *A. oryzae.* Microorganisms which are active during brine fermentation are *Lb. delbruekii* and *Hansenula* sp. After the second phase of fermentation is completed, the brine is drained; palm sugar (25%) is added and the mixture is cooked and stored for 24 hours or placed directly into bottles. Tauco is available in viscous liquid form. It is also available in semisolid form which is obtained by sun-drying the liquid product to a final moisture content of 25%. Tauco is used as a flavoring agent. Microorganisms present in tauco are *A. oryzae*, *R. oligosporus*, *R. oryzae*, *Hansenula* sp., *Zygosaccharomyces soyae*, and *Lb. delbruckii* (Winarno et al. 1973).

4.4.14 Maseura

Maseura is an ethnic fermented black or green gram product of India and Nepal (Tamang 2010a). Dry seeds of black or green gram are cleaned, washed, and soaked overnight. Soaked seeds are split by pressing through the hands and the hulls are blown off, the beans are ground into a thick paste, water is added until the paste becomes sticky, and is hand-molded into small balls or cones. If rice bean is used, then boiled potato, squash, or yam is mixed with the paste to make it sticky. The mixture is placed on a bamboo mat and left for natural fermentation for 2 to 3 days, and then sun-dried for 3 to 5 days depending on the weather conditions. Maseura can be stored in a dry container at room temperature for a year or more (Dahal et al. 2003). It is commonly used as a condiment or adjunct to vegetables. Species of bacteria present in maseura include *Lb. fermentum*, *Lb. salivarius*, *Pediococcus pantosaceous*, *P. acidilactici*, *E. durans*, *B. subtilis*, *B. mycoides*, *B. pumilus*, and *B. laterosporous*; yeasts include *Saccharomyces cerevisiae*, *Pichia burtonii*, *Candida castellii*, and *C. versatilis*; and molds include species of *Cladosporium*, *Penicillium*, and *Aspergillus niger* (Dahal et at. 2003; Chettri and Tamang 2008). An increase in soluble protein, amino nitrogen, nonprotein nitrogen, thiamine, and riboflavin have been observed in maseura (Dahal et al. 2003).

4.4.15 Wari

Wari is an ethnic fermented black gram product of India and Pakistan, similar to maseura. It is a dry, hollow, brittle, spicy, and friable ball of 3 to 8 cm in diameter and 15 to 40 g in weight (Batra 1986). Seeds of black gram are soaked in water for 6 to 12 hours, dewatered, dehulled, and ground into a smooth, mucilaginous paste. The dough is mixed with inoculum from a previous batch, salt and spices including asafetida, caraway, cardamom, clove, fenugreek, ginger, and red pepper. The mixture is allowed to ferment at room temperature for 1 to 3 days and hand-molded into balls, and dried for 2 to 8 days on bamboo

or palm mats (Batra and Millner 1976). Wari is used as a condiment and is mixed with vegetables as a side dish. Yeasts species of *Candida krusei*, *C. vartiovaarai*, *Kluyveromyces marxianus*, *Trichosporon beigelii*, *Hansenula anomala*, *S. cerevisiae*, *Leuc. mesenteroides*, and *Lb. fermentum* have been isolated from wari (Batra and Millner 1974, 1976; Batra 1981, 1986; Sandhu and Soni 1989). An increase in total acids, soluble nitrogen, free amino acids, thiamine, riboflavin, and cyanocobalamine has been observed during wari fermentation (Soni and Sandhu 1990).

4.4.16 Dhokla and Khaman

Dhokla is an ethnic fermented spongy-textured product of India prepared from Bengal gram and rice product, and is similar to idli except that dehulled Bengal gram dhal is used in place of black gram. *Khaman*, similar to dhokla, is also an ethnic fermented spongy-textured product of Gujarat in India and is made solely from the seeds of Bengal gram. Dry seeds of Bengal gram and white polished rice are washed, soaked, and ground. Salt and water are then added to make a thick paste. The slurries are left for natural fermentation in a warm place (30–32°C) for 8 to 10 hours. Once the product develops a spongy texture, the dhokla is ready. It is steamed for 10 to 15 minutes and is eaten as a snack. *Leuc. mesenteroides* and *E. faecalis* are essential and responsible for leavening the batter and acid-producing microorganisms during dhokla fermentation (Joshi et al. 1989). Acetoin and volatile fatty acids, at their optimum concentrations, impart a characteristic flavor to dhokla (Joshi et al. 1989).

4.4.17 Oncom

Oncom is an ethnic fermented peanut or groundnut cake-like product of Indonesia. Peanut or groundnut seeds are soaked, and press-cakes, as the major ingredients, are cooked along with solid wastes of tapioca and tahu, using a mixed culture of microorganisms with *Rhizopus* or *Neurospora* species (Winarno et al. 1973). Traditionally, two kinds of oncom are produced in Indonesia, *oncom hitam* (black oncom) and *oncom merah* (orange oncom). When fermentation is carried out by strains of mold belonging to the genus *Neurospora*, the product is called red oncom; if *R. oligosporus* is used, the resulting product is called black oncom. *Neurospora intermedia*, *Neurospora crassa*, and *Neurospora sitophila* have been reported from oncom (Ho 1986).

4.5 Fermented Cereals

4.5.1 Idli

Idli is an ethnic fermented rice/black gram food from India and Sri Lanka. It is an acid-leavened and steamed cake made by natural fermentation of a thick batter made from coarsely ground rice and dehulled black gram. Idli cakes are soft, moist, and spongy, have a sour flavor, and are eaten for breakfast in South India. The predominant bacteria responsible for souring as well as the production of gas in idli fermentation is *Leuc. mesenteroides* (Mukherjee et al. 1965). Yeasts have also been reported in idli, mostly *S. cerevisiae*, *D. hansenii*, *H. anomala*, *Torulopsis candida*, and *T. beigelii* (Soni and Sandhu 1989b, 1991; Thyagaraja et al. 1992). Yeasts in idli fermentation contribute to leavening and flavor development and results in enhanced contents of thiamine and riboflavin (Soni and Sandhu 1989a). Idli batter obtained from hotels and restaurants showed yeasts' participation in the leavening process in addition to *Leuc. mesenteroides* (Venkatasubbaiah et al. 1984). Idli makes an important contribution to the diet as a source of protein, calories, and B complex vitamins compared with raw unfermented ingredients (Steinkraus et al. 1967; Reddy et al. 1981).

4.5.2 Dosa

Dosa is also an ethnic fermented rice/black gram food from India and Sri Lanka. It is a light, shallow-fried, thin crisp pancake, made from finely grounded rice and dehulled black gram (Steinkraus 1983).

Dosa batter is very similar to idli batter, except that both the rice and black gram are finely ground. Bacteria alone or in combination with yeasts were found to be responsible for the fermentation of dosa and, ordinarily, the microorganisms developed during the initial soak and fermentation are sufficient to bring about fermentation (Soni et al. 1986). Overall increase in batter volume, microbial load, total nitrogen, soluble proteins, reducing sugar, and decrease of pH have been noted after 30 hours of fermentation of dosa (Soni et al. 1985). The combination of *S. cerevisiae* and natural bacterial flora was found to be the best microbial factor for standardizing the dosa fermentation (Soni and Sandhu 1989a).

4.5.3 Selroti

Selroti is an ethnic fermented rice food from the Himalayan regions of India, Nepal, and Bhutan (Yonzan and Tamang 2009). Rice is soaked overnight, pounded into coarse powder, and larger particles of pounded rice flour are separated from the rest by winnowing in a bamboo tray. Then, the rice flour is mixed with nearly 25% refined wheat flour, 25% sugar, 10% butter or fresh cream, and 2.5% spices/condiments containing large cardamom, cloves, fennel, nutmeg, and cinnamon are added to the rice flour and mixed thoroughly. Milk (boiled/unboiled) or water is added, kneaded into a soft dough, and finally into an easy-flowing batter. The batter is left to ferment naturally at ambient temperature (20–28°C) for 2 to 4 hours during summer and at 10°C to 18°C for 6 to 8 hours during winter. Oil is heated in a cast iron frying pan, the fermented batter is squeezed by hand, deposited as continuous rings onto hot edible oil, fried until golden brown, and drained out from the hot oil with a poker. Selroti is served as a confectionary bread. It can be served hot or cold. *Leuc. mesenteroides*, *E. faecium*, *P. pentosaceus* and *Lb. curvatus*, *S. cerevisiae*, *Saccharomyces kluyveri*, *D. hansenii*, *P. burtonii*, and *Z. rouxii* have been isolated from selroti (Yonzan and Tamang 2010). Yeasts in selroti show high activity of phosphatase, advocating their phytic acid–degrading abilities in the product (Yonzan and Tamang 2010).

4.5.4 Rabadi

Rabadi is an ethnic fermented cereal-based food from India. It is prepared by mixing flour from wheat, barley, pearl millet, or maize with buttermilk in an earthen or metallic vessel, and then allowing the mixture to ferment in the open sun for 5 to 6 hours in the hot summer. After fermentation, it is boiled, salted to taste, and consumed (Gupta et al. 1992a). *P. acidilactici*, *Bacillus* sp., and *Micrococcus* sp. were reported from rabadi (Ramakrishnan 1979). Phytic acid content decreased during rabadi fermentation (Mahajan and Chauhan 1987; Gupta et al. 1992b). Single as well as mixed culture fermentation of pearl millet with yeast (*S. cerevisiae* or *S. diastaticus*) and LAB (*Lb. brevis* or *Lb. fermentum*) were developed to prepare rabadi using pearl millet (Khetarpaul and Chauhan 1990a,b). Pearl millet–based rabadi prepared with developed starters has been found to increase the bioavailability of minerals (Khetarpaul and Chauhan 1989), improve starch and protein digestibility (Khetarpaul and Chauhan 1990a), increase total soluble sugar with a decrease in starch (Khetarpaul and Chauhan 1990b), and degrade antinutrients (Khetarpaul and Chauhan 1991).

4.5.5 Jalebi

Jalebi is one of the most popular sweet dishes of India, Bangladesh, and Nepal. It is a crispy sweet, deep-fried pretzel made from wheat flour, and is eaten as a snack food (Chitale 2000). Jalebi is prepared by mixing wheat flour with *dahi* (curd), adding water, and leaving this mixture overnight at room temperature. The thick leavened batter is squeezed through an embroidered hole (~4 mm in diameter) on a thick and durable cotton cloth, deposited as continuous spirals into hot edible oil, and fried on both sides until golden crisp. After about a minute, these are removed from the fat with a sieved spatula and submerged for several seconds in hot sugar saffron–scented syrup, which saturates their hollow insides, although skill is required to master the uniform shapes of jalebi. Often, rose or *kewda* (*Pandanus tectorius*) water and orange food color are added to the syrup (Ramakrishnan 1979; Batra 1981). The rheological parameters of jalebi batter can be controlled by adjusting the moisture content of the batter system (Chakkaravarthi et al. 2009). It is eaten as a snack when hot. *Lb. fermentum*, *Lb. buchneri*, *Lactobacillus*

bulgaricus, *Streptococcus lactis*, *E. faecalis*, *S. thermophilus*, and yeasts *Saccharomyces bayanus*, *S. cerevisiae* and *H. anomala* have been isolated from Jalebi (Batra and Millner 1974, 1976; Soni and Sandhu 1990b).

4.5.6 Nan

Nan is an ethnic leavened bread of India, Pakistan, and Afghanistan, and is made from wheat flour. Wheat flour is thoroughly mixed with butter, baking powder, *dahi* (curd), milk, salt, sugar, and water are added to make a thick dough. The dough is fermented for 3 to 5 hours at room temperature. Fermented dough is sheeted between the palms of the hand to approximately 2 to 3 mm thickness, slightly wetted, and pasted on the inner wall of the tandoori oven. The baked product, called *nan*, has a typical soft texture and flavor. Nan is baked in a specially designed oven known as a *tandoori* with a temperature range of 300°C to 350°C. It is also baked over live coals or a flame for a short time. Nan is eaten as a staple food with vegetable or *dal* (legume soup), and meats. *S. kluyveri* is the dominant yeast in nan (Batra 1986).

4.6 Fermented Tea

4.6.1 Miang

Miang is an ethnic fermented tea from northern Thailand. Young tea leaves are collected, tied into small bundles, and steamed for 2 hours or more to inactivate the polyphenol oxidases (Phithakpol et al. 1995). After cooling, the leaf bundles are carefully packed in a cement tank lined with banana leaves, and covered with banana leaves or a plastic sheet. The leaves are pressed tightly and weighted down to remove oxygen and thus to discourage the growth of yeasts and aerobic spoilage bacteria (Phithakpol et al. 1995). Fermentation takes approximately 3 or 4 months. Miang is consumed as a snack with salt or with condiments such as roasted coconut or shredded ginger. Miang is traditionally used during religious ceremonies and funerals in Thailand (Sasaki et al. 2007). *Lactobacillus thailandensis*, *Lb. camelliae*, *Lb. plantarum*, *Lb. pentosus*, *Lb. vaccinostercus*, *Lb. pantheris*, *Lb. fermentum*, *Lactobacillus suebicus*, *Pediococcus siamensis*, *Enterococcus casseliflavus*, and *Enterococcus camelliae* are involved in the fermentation of miang production (Tanasupawat et al. 2007; Sukontasing et al. 2007).

4.6.2 Puer Tea

Puer tea is an ethnic fermented tea from China, mainly produced in Yunnan Province. Fresh leaves are heat-treated to inactivate polyphenol oxidases (Mo et al. 2008). There are two types of puer tea: raw puer tea and cooked puer tea. During the production of raw puer tea, the leaves are simply softened by steaming and compressed into different sizes before the natural fermentation starts. The production of cooked puer tea is a more complex and relatively recent process developed in the early 1970s. In this case, the fermentation is initiated artificially. The dry black leaves are laid out in thick piles in a well-heated room and are sprayed with water and covered with a canvas or tarpaulin for a few weeks. During this fermentation period, the tea polyphenols are more intensively oxidized by the action of microorganisms and environmental oxygen than in the black tea fermentation process (Abe et al. 2008; Mo et al. 2008). The color turns from green to brown or brownish red, and a particular fragrance is produced. Puer tea acquires a characteristic flavor and numerous health-beneficial properties. The aging of puer tea is an essential process allowing the tea's aromatic bouquet to develop while mellowing the tannins and reducing the astringency. *A. niger* was identified as the predominating microorganism; *Aspergillus glaucus*, species of *Penicillium*, and *Rhizopus*, *Blastobotrys adeninivorans*, and *Saccharomyces* were also isolated (Mo et al. 2005; Jeng et al. 2007; Abe et al. 2008). Bacterial strains belonging to species of *Actinoplanes* and *Streptomyces* were also isolated from puer tea (Jeng et al. 2007). Puer tea has hypolipidemic effects

(Sano et al. 1986) and antioxidative properties (Jie et al. 2006). The antimutagenic and antimicrobial activities of puer tea have also been reported (Wu et al. 2007).

4.6.3 Fuzhuan Brick Tea

Fuzhuan brick tea is an ethnic fermented tea from China. *Aspergillus*, *Penicillium*, and *Eurotium* are the major fungi for fermentation of fuzhuan brick tea (Mo et al. 2008).

4.6.4 Kombucha

Kombucha is a slightly carbonated tea beverage, consumed worldwide, but historically originated from China (Schillinger et al. 2010). It is typically prepared by fermenting black tea which has been sweetened with sugar. In general, black tea leaves are infused in boiling water sweetened with sucrose (50–150 g/L) for approximately 10 minutes. After removing the tea leaves, the tea is poured into a wide-mouthed clean vessel and is acidified by the addition of vinegar or the so-called tea fungus, which is actually a floating cellulose mat (cellulosic pellicle) formed by a symbiotic association of yeasts and acetic acid bacteria from a previous fermentation. The jar is carefully covered with a clean cloth and the preparation is allowed to incubate at room temperature for 10 to 12 days. The final product is composed of organic acids, vitamins, minerals, and tea components resembling the taste of cider (Greenwalt et al. 2000). The acetic acid bacteria and the yeasts present in kombucha form a powerful symbiosis that is able to inhibit the growth of potential contaminating bacteria (Dufresne and Farnworth 2000). *Acetobacter xylinum* has been shown to be the primary bacterium in the mat, which is able to produce acetic acid and gluconic acid. It also synthesizes the floating cellulose network which is the prerequisite for the close association with the yeasts. *Zygosaccharomyces kombuchaensis*, *Zygosaccharomyces bailii*, *Zygosaccharomyces bisporus*, *Zygosaccharomyces microellipsoides*, *Brettanomyces*, *Saccharomyces*, *Schizosaccharomyces pombe*, *Torulaspora delbrueckii*, *Rhodotorula mucilaginosa*, *Candida stellata*, and *Brettanomyces bruxellensis* have been reported from kombucha (Mayser et al. 1995; Kurtzman et al. 2001; Teoh et al. 2004). Kombucha has gained popularity because of the apparent health benefits resulting from regular consumption. Stimulation of the immune system, digestion and liver function improvement, some detoxification activity and reduction of obesity are some of the health claims reported (Schillinger et al. 2010).

4.7 Vinegar

Vinegar has been used as a condiment, preservative, and medicine since ancient times in Asia. Vinegar is prepared from any sugar-containing substrate and hydrolyzes starchy materials through alcoholic fermentation followed by acetic acid fermentation (Yokotsuka 1991). The typical vinegar fermented with koji mold is rice vinegar, which is popular in China, Japan, Thailand, and other oriental countries where rice wine is produced with koji mold. *Acetobacter pasteurianus*, *Acetobacter aceti*, *A. xylinum*, and *Acetobacter polyxygenes* are the dominant bacterium for vinegar fermentation (Entani and Mashai 1985). *Lactobacillus fructivorans*, *Lactobacillus acetotolerans*, and *Moniliella acetobutans* also supplement fermentation (Entani and Mashai 1985). Yeast species involved in the fermentation of vinegar are *Z. bailii*, *Z. rouxii*, *Zygosaccharomyces pseudorouxii*, *Zygosaccharomyces mellis*, *Z. bisporus*, *Zygosaccharomyces lentus*, *Hanseniaspora valbyensis*, *Hanseniaspora osmophila*, *Candida lactis-condensi*, *C. stellata*, *Saccharomycodes ludwigii*, and *S. cerevisiae* (Solieri and Giudici 2008). The fungal denaturing gradient gel electrophoresis profile indicated that the transition from *A. oryzae* to *Saccharomyces* sp. took place at the initial stage of vinegar fermentation during which alcohol production was observed (Haruta et al. 2006). The early stage was characterized by the coexistence of *Saccharomyces* sp. and LAB, and almost all of the LAB denaturing gradient gel electrophoresis bands were replaced by bands derived from *Lactobacillus acetotolerance* and *A. pasteurianus* at the stage during which acetic acid started to accumulate (Haruta et al. 2006).

4.8 Nata

Nata is the thick white or cream-colored, insoluble gelatinous film of cells and polysaccharides which is a delicacy of the Philippines (Steinkraus 1996). Fermentation is due to *A. xylinum*, which forms on the surface of an acidified medium containing sugar, ethyl alcohol, and other nutrients (Kozaki 1976). Two types of *nata* are well known: *nata de piña*, produced from the juice of pineapple trimmings, and *nata de coco*, produced from coconut water or coconut skimmed milk. It is often eaten with ice cream and in fruit salads. The process of cellulose formation in a nata de coco culture system has been investigated and it has been found that cellulose formation or the consumption of glucose is controlled by the diffusion of atmospheric oxygen (Budhiono et al. 1999).

4.9 Amylolytic Mixed Cultures

4.9.1 Koji

Koji is a mold culture and is prepared from steamed/cooked cereal in Japan. The term *koji* is Japanese meaning naturally, spontaneously, or artificially molded cereals and beans (Yokotsuka 1991). The Chinese name for koji is *chu*, *shi*, or *qu*. Rice is steamed, cooled on bamboo-made trays, stacked with gaps of approximately 10 cm in between to allow air circulation, inoculated with 0.1% mold spores (locally called *tane-koji*), and incubated at 23°C to 25°C. The increase in temperature due to the growth of mold is kept within the range of 35°C to 45°C by stirring and turning the koji from top to bottom on trays after approximately 20 to 40 hours, normally fermented for 3 days, when mold mycelium has spread throughout the mass, and before sporulation (Lotong 1985). *A. oryzae*, *A. sojae*, *Aspergillus kawachii*, *Aspergillus shirousamii*, and *Aspergillus awamori* have been widely used as starters in the preparation of *koji* in Japan for the production of saké, shoyu, miso, and shochu (Kitamoto 2002; Matsushita et al. 2009). Among these molds, *A. oryzae* is the most important and popular in Japan, and has been used as yellow koji (Suganuma et al. 2007). *A. oryzae* is used for starch saccharification in saké manufacture (Inoue et al. 1992). Because koji is not cultivated in a closed system, it is a mixture of several microorganisms. At an early stage of cultivation, yeast grows on steamed rice grain and, approximately 20 hours after inoculation of seed koji, mold begins to grow. Koji, besides being a saccharifying and diastatic agent, also contributes to the color, flavor, and aroma of the fermented foods that are important for their overall attributes (Kitamoto 2002). Antioxidant activity is observed in koji prepared with *A. awamori* (Lee et al. 2007) and with *Aspergillus candidus* (Yen et al. 2003).

4.9.2 Marcha

Marcha is an ethnic amylolytic starter to produce fermented beverages and alcoholic drinks in the Himalayan regions of India, Nepal, Bhutan, and Tibet in China (Tamang 2010a). Marcha is a dry, round to flattened, creamy white to dusty white, solid ball–like structure. Glutinous rice is soaked, crushed, ground, mixed with the roots of *Plumbago zeylanica*, the leaves of *Buddleja asiatica*, the flowers of *Vernonia cinerea*, ginger, red dry chili, and 1% to 2% of previously prepared marcha. The mixture is made into a paste by adding water and kneaded into flat cakes of varying sizes and shapes. This is then placed individually on the ceiling-floor, above the kitchen, made up of bamboo strips inlaid with fresh fronds of ferns (*G. erubescens*), covered with dry ferns and jute bags, and left to ferment for 1 to 3 days. A distinct alcoholic and ester aroma and puffy/swollen appearance of marcha indicate the completion of fermentation, and fresh cakes of marcha are sun-dried for 2 to 3 days (Tamang et al. 1996). Marcha is stored at room temperature and can be kept in a dry place for more than a year. Marcha is a Nepali word. The different ethnic communities of the Himalayas call it by their vernacular names, such as *phab* (Tibetan), *poo* (Drukpa), *khesung* (Limboo), *bharama* (Tamang), *bopkha* or *khabed* (Rai), *buth* or *thanbum* (Lepcha), *manapu* (Newar), *hamei* (Meitei), *thiat* (Khasi),

humao (Dimasa), *pham* and *ipoh* (Apatani), *bakhar* (people of Himachal Pradesh in India), and *balan* (people of Uttarakhand in India). Marcha-making technology reflects a native skill of ethnic people on subculturing of desirable inocula (microorganisms consisting of filamentous molds, amylolytic and alcohol-producing yeasts, and species of LAB) from a previous batch to a new culture using rice or wheat as a starchy base or medium. This indigenous technique of "microbiology" preserves the functional microorganisms necessary for the fermentation of starchy substrates to alcoholic beverages in the Himalayas (Tamang 2010a).

Species of mycelial molds present in marcha are *M. circinelloides* forma *circinelloides*, *Mucor* sp. close to *M. hiemalis*, *R. chinensis*, and *R. stolonifer* variety *lyococcus* (Tamang et al. 1988). Amylolytic and alcohol-producing yeasts isolated from marcha are *Saccharomycopsis fibuligera*, *Saccharomycopsis capsularis*, *S. cerevisiae*, *S. bayanus*, *Pichia anomala*, *P. burtonii*, and *Candida glabrata* (Tsuyoshi et al. 2005). *Sm. fibuligera* is the most dominant yeast in marcha (Tamang and Sarkar 1995). Saccharifying activities are mostly shown by *Rhizopus* spp. and *Sm. fibuligera*, whereas liquefying activities are shown by *Sm. fibuligera* and *S. cerevisiae* (Thapa and Tamang 2004). Among LAB, *P. pentosaceus*, *Lb. bifermentans*, and *Lb. brevis* are present in marcha (Tamang and Sarkar 1995; Tamang et al. 2007b). LAB imparts flavor, antagonism, and acidification of the substrates in marcha (Tamang et al. 2007a,b). Hesseltine et al. (1988) isolated *Mucor* and *Rhizopus* spp. in marcha samples from Nepal. Uchimura et al. (1990) reported yeast, *Saccharomycopsis*, and molds, *Penicillium* sp. and *Aspergillus* sp., in *poo* or *phab* (marcha of Bhutan).

4.9.3 Mana

Mana is a unique granular type of ethnic starter of Nepal prepared from wheat (Tamang 2010a). Wheat grains are soaked in water overnight, steamed for 30 minutes and transferred to a bamboo basket, drained, and ground into a lump. The floor is cleaned, straw is spread on the ground, and the wheat lump is placed over it, covered with paddy straw or straw mat, and fermented for 6 to 7 days. After 7 days, green mold appears on the wheat grains and is dried in the sun to obtain mana that is then stored. *A. oryzae* and *Rhizopus* spp. are present in *mana* (Nikkuni et al. 1996; Shrestha et al. 2002).

4.9.4 Ragi

Ragi is an amylolytic starter from Indonesia in the form of dry and flat cakes. Rice or millet or cassava or other starchy bases are milled, mixed with herbs and spices, roasted together, and then sieved. Water is added to the mixture along with 2% to 4% powder of old ragi, and then it is mixed thoroughly and shaped into balls, and fermented at 25°C to 30°C for 72 hours, sun-dried, and used as a starter for the production of alcoholic beverages and drinks in Indonesia. Microorganisms associated with ragi are species of *Rhizopus*, *Mucor*, *Amylomyces rouxii*, *Aspergillus*, *Saccharomycopsis*, *C. parapsilosis*, *C. melinii*, *C. lactosa*, *C. pelliculosa*, *S. cerevisiae*, *Hansenula subpelliculosa*, *H. anomala*, and *H. malanga*; *E. faecalis*, *Lb. Plantarum*, and *P. pentosaceus; B. coagulans*, *B. brevis*, and *Bacillus stearothermophilus* are associated with ragi (Dwidjoseputro and Wolf 1970; Saono et al. 1974; Hadisepoetro et al. 1979; Hesseltine et al. 1988; Hesseltine and Ray 1988; Ardhana and Fleet 1989; Yokotsuka 1991). Elegado and Fujio (1993) isolated two polygalacturonase-producing strains of *Rhizopus* spp. from ragi and studied the enzymes' stability in a wide range of pH values from 2 to 11, and tolerance at 50°C for 20 minutes. Uchimura et al. (1991b) reported that there is a higher variability rate of *P. pentosaceus* in older ragi than in younger ones and the results suggest that rod-shaped bacteria cannot survive for a long time under dry conditions in ragi.

4.9.5 Bubod

Bubod is an ethnic amylolytic starter from the Philippines. Rice and ginger are powdered, and mixed thoroughly with enough water to have a consistency that permits rolling the material into a ball and

flattening it (Tanimura et al. 1977). The discs are coated with 1- to 3-month-old bubod and incubated in rice straw for 36 hours at room temperature and sun-dried. *M. circinelloides*, *Mucor grisecyanus*, *Rhizopus cohnii*, *S. cerevisiae*, and *Sm. fibuligera* have been reported from bubod (Kozaki and Uchimura 1990); however, *Sm. fibuligera* is the dominant amylolytic yeast in bubod (Hesseltine and Kurtzman 1990). The activated starter for the production of *basi*, a mild alcoholic food beverage in the Philippines is called *binubudan* (Tanimura et al. 1978). Rice is cooked with water so that the grains remain separate, any lumps are broken down, and the rice is cooled to 40°C to 45°C. Then, it is inoculated with 300 g of powdered bubod for 4 kg rice. The rice and bubod are mixed in a clean basin and covered with banana leaves and then a clean cheese cloth. Fermentation continues for 24 hours to obtain a fresh, activated starter called binubudan.

4.9.6 Nuruk

Nuruk is an ethnic amylolytic starter from Korea. Traditionally, nuruk is prepared by moistening wheat flour, kneaded and molded into a ball, and fermented for 17 days at 30°C to 45°C, dried for 2 weeks, and cured for 1 to 2 months at room temperature (Park et al. 1977). *A. oryzae*, *A. niger*, *Rhizopus* sp., along with a few bacteria and yeast species have been isolated from nuruk (Kim 1968). Generally, nuruk is prepared by natural inoculation of molds, bacteria, and yeasts; however, it can be prepared by inoculation with *Aspergillus usamii* (Park et al. 1977).

4.9.7 Loogpang

Loogpang is an ethnic amylolytic starter from Thailand, which is commonly used to prepare alcoholic drinks and vinegar. The main ingredient of loogpang is rice flour with the addition of different types of spices and microorganisms from previous batches of loogpang (Vachanavinich et al. 1994). Species of molds present in loogpang are *Amylomyces*, *Rhizopus*, *Aspergillus*, *Mucor*, and *Absidia* (Pichyangkura and Kulprecha 1977); and yeasts and LAB including *Sm. fibuligera*, *Hansenula*, *Saccharomyces*, and *Pediococcus* (Dhamcharee 1982; Uchimura et al. 1991a). *Sm. fibuligera* of loogpang showed high glucoamylase activity (Sukhumavasi et al. 1975).

4.9.8 Men

Men or *banh men* is the ethnic amylolytic starter from Vietnam, which is used to produce traditional alcoholic beverages and drinks called *ruou* (Dung 2004). Rice flour is mixed with local herbs and spices and moistened with a small amount of water to form a dough, which is then made into small balls or flattened discs. The dough is spread on a bamboo tray and mixed with powdered men, and fermented at room temperature for a few days. Molds *Am. rouxii* and *Rhizopus* spp.; yeasts, *Saccharomycopsis fibuliger*, *Hyphopichia burtonii*, and *S. cerevisiae*; and LAB, have been found in men (Dung et al. 2005, 2006, 2007). *S. bayanus* has not been isolated from men, but the closely related species of *S. cerevisiae* has been isolated from banh men (Lee and Fujio 1999). *Rhizopus oryzae*, *R. microsporus*, *Absidia corymbifera*, *Amylomyces* sp., *Sm. fibuligera*, *S. cerevisiae*, *Issatchenkia* sp., *Pichia anomala*, *P. ranongensis*, *Candida tropicalis*, *Clavispora lusitaniae*, *P. pentosaceus*, *Lb. plantarum*, *Lb. brevis*, *W. confusa*, *W. paramesenteroides*, *B. subtilis*, *B. circulans*, *B. amyloliquefaciens*, *B. sporothermodurans*, *Acetobacter orientalis*, and *A. pasteurianus* have been isolated from banh men (Thanh et al. 2008).

4.9.9 Chiu-yueh

Chiu-yueh or *peh-yueh* is an ethnic Chinese amylolytic starter for *lao-chao*, a fermented rice product (Wang and Hesseltine 1970). It is a gray-white ball containing yeasts and fungi grown on rice flour, which is closely related to Indonesian ragi. Species of *Rhizopus*, *Amylomyces*, *Torulopsis*, and *Hansenula* are present in *chiu-yueh* (Wei and Jong 1983).

4.10 Fermented Beverages and Alcoholic Drinks

4.10.1 Saké

Saké is the most popular, ethnic nondistilled alcoholic drink of Japan. It is prepared from rice using koji and is clear, pale yellow, containing 15% to 20% alcohol (Tamang 2010c). Polished rice is washed, steeped in water, and steamed for 30 to 60 minutes, and then cooled, mixed with koji, water, and a selected yeast starter culture for alcoholic fermentation. Main fermentation takes place in open tanks in cool conditions, starting at approximately 10°C, increasing to approximately 15°C. After fermentation, the liquid material called *moromi* is separated from the solids to produce a clarified saké, which is settled, refiltered, pasteurized, and blended and diluted with water before bottling (Yoshizawa and Ishikawa 1989). Unique strains of *S. cerevisiae* have evolved to carry out those fermentations, generating products with high ethanol content (12–20%), attractive flavor and aroma, and odor (Kodama 1993). The first organisms developed in the mash, under traditional fermentation conditions, are nitrate-reducing bacteria such as *Pseudomonas*, *Achromobacter*, *Flavobacterium*, or *Micrococcus* spp. (Murakami 1972). These are followed by *Leuc. mesenteroides* variety *saké* and *Lb. saké* and yeasts (Kodama and Yoshizawa 1977). The highly refined saké brewed by the most skillful brewers using very highly polished rice at low temperatures of 9°C to 11°C for 25 to 30 days is known as *gonjoshu* (Kodama and Yoshizawa 1977). The difference in responses to osmotic stress between the laboratory and saké-brewing strains of *S. cerevisiae* at the translational level was compared and it was found that enhancement of glycerol formation due to enhancement of the translation of proteins Hor2p, is required for the growth of *S. cerevisiae* under high osmotic pressure conditions (Hirasawa et al. 2009). *S. cerevisiae* strains with disrupted ubiquitin-related genes produced more ethanol than the parental strain during saké brewing (Wu et al. 2009). Several researchers have reported on the industrial scale use of improved strains of *A. oryzae* for saké production (Hirooka et al. 2005; Kotaka et al. 2008; Hirasawa et al. 2009).

4.10.2 Kodo ko Jaanr

Kodo ko jaanr or *chyang* is one of the most popular ethnic fermented finger millet (*Eleusine coracana*) beverages of the Himalayan regions of India, Nepal, Bhutan, and China (Tibet) with a mild alcoholic (4.8%) and sweet taste (Tamang 2010c). Kodo ko jaanr has several synonyms as used by different ethnic groups of the Himalayan people such as *chyang* (Tibetan, Ladakhi, and Drupka), *mandokpenaa thee* (Limboo), and *mong chee* (Lepcha). During its production, finger millet seeds are cleaned, washed, and cooked for approximately 30 minutes, excess water is drained off and cooked millets are spread on a bamboo mat for cooling. Approximately 1% to 2% of powdered marcha is sprinkled over the cooked seeds, mixed thoroughly and packed in a bamboo basket lined with fresh fern (*Thelypteris erubescens*) and then covered with sackcloths, and fermented at room temperature for 2 to 4 days. The saccharified mass is transferred into an earthen pot or bamboo basket, made airtight, and fermented for 3 to 4 days during summer and 5 to 7 days in winter at room temperature for alcohol production. Freshly fermented kodo ko jaanr is filled into a bamboo-made vessel locally called *toongbaa*, and lukewarm water is added up to its edge and it is left for 10 to 15 minutes. Then, the milky white extract of jaanr is sipped through a narrow bamboo straw called *pipsing* which has a hole in a side near the bottom to avoid passing of grits. Water is added twice or thrice after sipping of the extract. Consumption of fermented finger millet beverages in exclusively decorated bamboo or wood-made vessels called *toongbaa* is unique in the Himalayas (Tamang 2010a). Kodo ko jaanr liquor is believed to be a good tonic for ailing persons and postnatal women. After consumption, residual or grits of kodo ko jaanr are used as fodder for pigs and cattle. This is a good example of total utilization of the substrate as food and fodder; the discarded grits also contain nutrient used as animal feed.

Marcha, used as an amylolytic starter, supplements all functional microorganisms in kodo ko jaanr fermentation (Thapa and Tamang 2004). Mycelial molds have roles only in the initial phase of fermentation, mostly in saccharification of the substrates. Yeasts *Pichia anomala*, *S. cerevisiae*, *Candida glabrata*, *Sm. fibuligera*, and LAB *P. pentosaceus* and *Lactobacillus bifermentans* have been recovered in kodo

ko jaanr samples. The population of filamentous molds, which originated from marcha, declines daily during *in situ* fermentation of kodo ko jaanr and finally disappears after the fifth day (Thapa and Tamang 2006). *Sm. fibuligera* and *R. chinensis* saccharify and liquefy millet starch into glucose and produce alcohol in *in situ* fermentation of kodo ko jaanr. Fermentation of finger millet enhances bioenrichment of minerals such as calcium, magnesium, manganese, iron, potassium, and phosphorus, contributing to mineral intake in the daily diet of the Himalayan rural people (Thapa and Tamang 2004). Because of the high calorie content, ailing persons and postnatal women consume the extract of kodo ko jaanr to regain strength. Chyang contains more riboflavin, niacin, and pantothenic acid and folic acid than the substrate (Basappa 2002). The essential amino acids also increase in chyang (Bassapa et al. 1997).

4.10.3 Bhaati Jaanr

Bhaati jaanr is the Himalayan sweet-sour, mild alcoholic food beverage paste prepared from rice and consumed as a staple food (Tamang 2010a). Rice is cooked, spread on a bamboo mat for cooling, 2% to 4% of powdered marcha is sprinkled over the cooked rice, mixed well, and kept in a vessel or an earthen pot for 1 to 2 days at room temperature. After saccharification, the vessel is made airtight and fermented for 2 to 3 days in the summer and 7 to 8 days in the winter. Bhaati jaanr is made into a thick paste by stirring the fermented mass with the help of a hand-driven wooden or bamboo stirrer. It is consumed directly as a food beverage. A similar product from Nepal is called *poko*. Occasionally, bhaati jaanr is stored in an earthenware crock for 6 to 9 days and thick yellowish-white supernatant liquor locally called *nigaar* is collected at the bottom of the earthenware crock. Nigaar is drunk directly with or without the addition of water. Alcohol content of bhaati jaanr is 5.9% (Tamang 2010a). Bhaati jaanr is an inexpensive high-calorie staple food/beverage for postnatal women and ailing old persons in the villages, who believe that it helps to regain their strength. Maximum activities of saccharification and liquefaction of rice are observed on the third day of fermentation (Tamang and Thapa 2006). *Sm. fibuligera*, *Rhizopus* ssp. and *Mucor* spp. contribute in saccharification and liquefaction of glutinous rice, breaking the starch of substrates into glucose for alcohol production and also in aroma formation in bhaati jaanr preparation. An increase in the mineral contents, mostly calcium, iron, sodium, potassium and phosphorus is also observed in bhaati jaanr due to fermentation (Tamang and Thapa 2006).

4.10.4 Lao-chao

Lao-chao is a popular ethnic fermented rice food of the Cantonese in China. It has a sweet taste and mild alcoholic flavor with a fruity aroma, made from rice by using *chiu-yueh* or *peh-yueh* as amylolytic starters (Wang and Hesseltine 1970). It is served as a dessert and is also a traditional diet for new mothers who believe that it helps them regain their strength (Wei and Jong 1983). The process of production is similar to Indonesian *tapé keatan*. *Rhizopus*, *Amylomyces*, *Torulopsis*, and *Hansenula* are found in lao-chao (Wei and Jong 1983). Pure culture fermentation of lao-chao was developed by Wang and Hesseltine (1970) and showed that good fermented rice was made when a mold, *Rhizopus chinensis* NRRL 3671, and yeast, *Saccharomycopsis* sp. NRRL Y7067, were used as inocula instead of a commercial starter.

4.10.5 Tapé

Tapé is a sweet and sour paste with an alcoholic flavor, prepared from glutinous rice or cassava or other cereals by using starter ragi in Indonesia (Campbell-Platt 1994). It is eaten as dessert or delicacy before meals in Indonesia. There are various starchy substrates used to prepare tapé, such as cassava (*tapé ketala*), glutinous rice (*tapé ketan*), maize (*tapé jagung*), and millet (*tapé cantel*). Rice is washed, soaked, steamed, cooled to room temperature on a woven bamboo tray, sprinkled with powdered ragi, packed in small banana leaves, and fermented for 2 to 3 days at room temperature and a soft juicy mass of tapé is produced (Saono et al. 1977). Tapé ketala is deep-fried in coconut oil before consumption. It is sun-dried and used later in soups (Ardhana and Fleet 1989). Ethanol content ranged from 3% to 8.5% v/v (Cronk et

al. 1977). Rice lipids are hydrolyzed during tapé ketan fermentation (Cronk et al. 1979). Mixed cultures of *Streptococcus*, *Rhizopus*, and *Saccharomycopsis* produce aroma in tapé, whereas *Sm. fibuligera* produces α-amylase, and *Rhizopus* sp. produces glucoamylase (Suprianto et al. 1989).

4.10.6 Tapai

Tapai is the Malaysian fermented food/beverage commonly consumed as a dessert (Merican and Yeoh 1989). There are two main types of tapai, that is, *tapai pulut*, prepared from fermented glutinous rice, and *tapai ubi*, prepared from tapioca or cassava. Tapai tastes sweet, slightly alcoholic with a pleasant and fragrant aroma. Amylolytic starter used for production of tapai are *ragi tapai* or *jui-paing*, which originated from Indonesia and is mainly for dessert tapai, and *ragi samsu*, which originated from China and is mainly for the alcoholic drink tapai. Rice is washed, soaked, cooked, and cooled, powdered ragi tapai or jui-paing is mixed on a woven bamboo tray and is covered in banana leaves and allowed to ferment at room temperature for 1 to 3 days. The fermenting mass is stirred at least once a day to keep the surface moist. The resulting liquid becomes clear yellow and very sweet. *Candida* spp., *Sm. fibuligera*, *Am. rouxii*, *M. circinelloides*, *M. javanicus*, *Hansenula* spp., *Rhizopus arrhizus*, *R. oryzae*, and *R. chinensis* are found in tapai ubi and tapai pulput (Wang and Hesseltine 1970; Ko 1972; Merican and Yeoh 1982). *Lb. casei* is present in tapai (Mohd Adnan and Tan 2007). Protein content of rice doubles to approximately 16% after fermentation because of losses of total solids and synthesis of proteins by the microorganisms (Cronk et al. 1977). Microbial consortia to produce a good tapai pulut consists of a mixture of *A. rouxii*, *Sm. fibuligera*, and *H. anomala*, and for the production of good quality tapai ubi, the consortia include *A. rouxii*, and *Sm. fibuligera* (Merican and Norrijah 1983, 1985).

Tapai is also used as an alcoholic drink in Malaysia. After 1 week of fermentation, if continued, wine or brandy is added to the mash as a preservative and it is allowed to ferment for another 25 days. The alcoholic drink tapai, which is red to pink, is collected by immersing a strainer-like collection vessel into the mash (Wong and Jackson 1977).

4.10.7 Tapuy

Tapuy is an ethnic mild alcoholic beverage of the Philippines prepared from rice with sweet and sour taste. The rice is washed, cooked, placed in a clay pot, and mixed with pulverized bubod. The pot is covered with cheesecloth and incubated in a cool place for 2 weeks (Tanimura et al. 1977). *Sm. fibuligera*, *Rhodotorula glutinis*, *D. hansenii*, *C. parapsilosis*, *Trichosporon fennicum*, and *Leuconostoc* constitute the microbial consortia in *tapuy* (Uyenco and Gacutan 1977) mainly supplemented by bubod. Alcohol content of *tapuy* is 13.5% to 19.1% (Tanimura et al. 1977).

4.10.8 Basi

Basi is an ethnic, nondistilled alcoholic drink from the Philippines made by fermenting boiled, freshly extracted sugarcane juice (Tanimura et al. 1978). Sugarcane is extracted into juice and is boiled with tree barks to provide color and flavor. After 2 to 3 hours of boiling, the concentrated juice is filtered and poured into earthenware jars and allowed to cool, and inoculated within 24 hours with a binubudan starter, covered with a lid, and fermented for a week before bubod is added to speed up the fermentation. After 1 month of fermentation, the earthenware jar is covered with a clean cloth or absorbent paper and is sealed. The product, basi, is then allowed to age for 1 year before consumption. In another method of preparation, the sugarcane juice is inoculated with organisms present on 1-year-old dried fruit, leaves, and leaves or bark of *samac* (*Macharanga tanarius*, *M. gradifolia*; Tanimura et al. 1978). In this process, fresh cane juice is boiled for 2 hours to approximately three-quarters volume, at which point the concentrate is transferred to an earthenware jug and cooled overnight. Milled rice, dried samac leaves, bark, and dried fruit are added and mixed thoroughly. The mouth of the jug is covered with banana leaves and fermentation continues for 3 months, and is aged for 1 year. The dominant yeasts in basi are *S. cerevisiae*

as well as *Saccharomycopsis* spp. (Kozaki 1976; Sakai and Caldo 1985). An improved method for the preparation of basi has been developed which is more acceptable to consumers (Sanchez and Kozaki 1984).

4.10.9 Yakju and Takju

Yakju and *takju* are the ethnic Korean alcoholic beverages made from rice by using nuruk (Park et al. 1977). The lower or diluted concentration of yakju is known as *takju*. During yakju preparation, steamed rice is cooled and then mixed with nuruk and fermented, the liquid pressed from the fermenting mass is filtered under pressure, and is aged and bottled. Yeasts, *Bacillus* spp., *Lactobacillus* sp., and *Leuconostoc* spp., are present in these Korean beverages (Shin and Cho 1970; Kim 1970; Lee and Rhee 1970). *S. cerevisiae* is the most important organism in alcohol production, whereas *Hansenula* spp. plays an important role in flavor development (Kim and Lee 1970; Kim and Kim 1993). An increase in thiamine during the fermentation of yakju and takju has been observed (Kim and Choi 1970). High-value yakju possessing the pharmaceutical functionality of *Ganoderma lucidum* was developed with antihypertensive properties (Kim et al. 2004).

4.10.10 Brem

Brem is a nondistilled ethnic alcoholic drink from Indonesia which is prepared from rice. It is a dried, starchy, sweet-sour rice extract, and is eaten as a snack. Brem is of three types: *brem madiun*, which is yellowish-white, sweet-sour, and is prepared in blocks of 0.5 × 5 to 7 cm; *brem wonogiri*, which is sweet, very soluble, white, and thin circular blocks of 5 cm diameter; and *brem bali*, which is a famous alcoholic liquor produced in Bali, Indonesia (Basuki 1977). All three types of brem are made from the liquid portions of tapé ketan. During brem production, the filtrate of tapé ketan is boiled down, poured onto a table, covered with banana leaves, and left to cool at ambient temperature over 8 to 12 hours (brem madiun) or sun-dried for 1 day to produce brem wonogiri (Campbell-Platt 1987). The liquid portion of tapé ketan is aged for 7 months, during which solids precipitate, leaving a clarified brem, known as brem bali, and is decanted and bottled (Basuki 1977). Alcohol content of brem is 6.1% (Winarno 1986). Brem with improved ragi is produced which has more desirable flavor than conventionally made brem (Saono et al. 1984).

4.10.11 Krachae

Krachae or *nam-khaao* or *sato* is a nondistilled ethnic alcoholic drink from Thailand prepared from Thai rice using loogpang as starter (Vachanavinich et al. 1994). During krachae production, a local rice variety of Thailand is washed, soaked, cooked, and cooled and then sprinkled with loogpang powder and mixed well. The mixture is placed in an earthenware jar, and fermented at 30°C to 35°C for 2 days, and water is added. The jar is again incubated for 3 to 4 days. The fermented mass is filtered and only clarified supernatant is collected, which is a yellowish white color and effervescent and is now called *krachae*. Microorganisms in krachae are supplemented from the ethnic starter *loogpang*. Molds play an important role at the initial stage of fermentation for the cleavage of sugar polymers present in rice to substantial sugars which can be used for substrates for simultaneous fermentation by yeast and LAB (Lotong 1985). Molds produce sugars from starch, and subsequently, yeast converts sugars to alcohol (Ko 1982), and LAB helps in the formation of flavor and taste in krachae (Lim 1991).

4.10.12 Raksi

Raksi is an ethnic alcoholic drink from the Himalayas with a characteristic aroma, and is distilled from traditional fermented cereal beverages (Kozaki et al. 2000). Raksi is a common term in Nepali meaning alcoholic drink. Bhaati jaanr, poko, makai ko jaanr, kodo ko jaanr, gahoon ko jaanr, fermented masses of buckwheat, potato, canna, and cassava roots are distilled in a large cylindrical metallic vessel measuring

40 cm × 30 cm × 25 cm for 2 to 3 hours continuously over firewood in an earthen oven (Tamang 2005). A perforated container called *phunga* is placed above the main cylindrical vessel, inside of which is a small metallic collector called *poini*, that is kept on an iron tripod called *odhan* to collect the distillate called *raksi*. Another metallic vessel with cold water is placed above the phunga as condenser. The bottom of the condenser vessel is plastered with mud along with the tip of the phunga to prevent excess ventilation during distillation. Water is replaced three to five times after it is heated. Condensed raksi is collected in a small collecting metallic vessel called *poini*. Raksi obtained after replacing the condensing water thrice is known as *theen pani raksi*; this contains a high amount of alcohol and is traditionally prepared for religious purposes. Raksi prepared after replacing the condensing water five times is known as *panch pani raksi*, which is a common alcoholic drink. The traditional distillation apparatus can distil 2 to 4 kg of jaanr to obtain 1 to 2 liters of raksi after replacing the condensing water thrice. Raksi is usually stored in bottles capped with a piece of dry corncob. Sometimes, petals of *Rhododendron* spp. are mixed in during distillation to provide a distinct aroma to raksi. This type of raksi is commonly prepared in *Rhododendron*-growing regions of the Himalayas (Tamang et al. 1996). The alcohol content of raksi is 22% to 27% v/v (Tamang 2005). Raksi is drunk directly without the addition of water along with fried meat or any other curry. Raksi is commonly available in local liquor shops, restaurants, and hotels.

4.10.13 Toddy or Tari

Toddy or *tari* is an ethnic alcoholic drink from India prepared from palm juice. There are three types of toddy (Batra and Millner 1974): (1) *sendi*, from the palm; (2) *tari*, from the *palmyra* and date palms; and (3) *nareli*, from the coconut palm. *Geotrichum*, *Saccharomyces*, and *Schizosacchoramyces* spp. of yeast are responsible for fermentation (Batra and Millner 1974). Microorganisms that are responsible in fermenting toddy are *S. cerevisiae*, *Sch. pombe*, *A. aceti*, *A. rancens*, *A. suboxydans*, *Leuconostoc dextranicum*, *Micrococcus* sp., *Pediococcus* sp., *Bacillus* sp., and *Sarcina* sp. (Shamala and Sreekantiah 1988).

4.10.14 Kanji

Kanji is an ethnic Indian strong-flavored, mild alcoholic beverage prepared from beetroot and carrot by natural fermentation (Batra and Millner 1974). It is drunk as a mild alcoholic refreshing drink in India. The alcohol content of kanji is 2.5% and pH is 4.0; hence, the product's mild-alcoholic and acidic taste (Sura et al. 2001). During its preparation, carrots or beets are washed, shredded and mixed with salt and mustard seeds, and placed in earthen pots and allowed to ferment naturally at 26°C to 34°C for 4 to 7 days. Sometimes, the mixture is inoculated with a portion of a previous batch of kanji. After fermentation, pink alcoholic liquor is drained off and bottled or drunk directly. In northern India, it is prepared with purple or occasionally orange cultivars of carrots plus beets and spices, whereas in southern India, *torami*, a yeast-containing fermented rice gruel is used as a starter for kanji production. *Hansenlu anomala*, *Candida guilliermondii*, *C. tropicalis*, and *G. candidum* are involved in kanji fermentation (Batra and Millner 1974). *Leuc. mesenteroides*, *Pediococcus* spp., and *Lactobacillus dextranicum* have been isolated from kanji fermentation (Sura et al. 2001). Recently, Kingston et al. (2010) reported *Lb. paraplantarum* and *Lb. pentosus* from kanji based on rep-PCR identification method.

4.11 Conclusions

The diversity of Asian fermented foods is related to the diversity of ethnicity and unparalleled food culture of each community. The diversity of microorganisms ranges from mycelia fungi to enzyme-producing and alcohol-producing yeasts, to Gram-positive and a few Gram-negative bacteria with several functional properties. There is a relationship between human life and microorganisms. Asian ethnic fermented foods and beverages have biological functions enhancing several health-promoting benefits to the consumers because of the functional microorganisms associated with them, such as the biopreservation of perishable foods, bioenrichment of nutritional value, protective properties, bioavailability of

minerals, production of antioxidants and omega-3-polyunsaturated fatty acids, therapeutic values, and immunological effects. Some Asian ethnic fermented foods, such as tempe, natta, natto, shoyu, kimchi, etc., have been commercialized and marketed globally as health foods or functional foods.

It has been noticed that the consumption of several uncommon ethnic foods is declining in many Asian countries because of lifestyle changes; shifting from cultural food habits to commercial foods and fast foods, drastically affecting traditional culinary practices, and also because of climate change in some places. The effect of climate change is mostly on the production of indigenous crops, vegetables, legumes, seeds, etc. Many minor, but culturally important, ethnic foods are not seen in local markets of many Asian countries, and most of the young generation do not know ethnic foods, their culinary practices, and processing methods. Native microorganisms with vast biological importance and potential genetic resources, which are associated with ethnic fermented foods, have disappeared. Surveys on the consumption and production of ethnic fermented foods and beverages in every country, and the calculation of per capita consumption, needs to be urgently addressed by the food policy-makers of respective ministries or governments. Introduction of global ethnic fermented foods in the syllabus at the master's level in microbiology and food sciences courses of all universities may be initiated. The native skill of making ethnic fermented foods has been passed from mothers to daughters, fathers to sons, for generation to generation. Such family tradition of culinary skills using important indigenous knowledge of food fermentation is, nowadays, unnoticed because of the increased incidence of nuclear families, separation from elder people, a fast and busy lifestyle, the popularity of fast commercial foods, and a lack of interest among the educated urban mass to interact with elderly people from the villages to explore their vast indigenous knowledge. Everyday throughout the world, the indigenous knowledge of many elderly people has disappeared with their death. As a result, many important ancient and ethnic foods have become extinct worldwide. This is a serious phenomenon of the 21st century related to cultural foods.

REFERENCES

Abe M, Takaoka N, Idemoto Y, Takagi C, Imai T, Nakasaki K. 2008. Characteristic fungi observed in the fermentation process for Puer tea. *Int J Food Microbiol* 124:199–203.

Aoki H, Uda I, Tagami K, Furuta Y, Endo Y, Fujimoto K. 2003. The production of a new tempeh-like fermented soybean containing a high level of γ-aminobutyric acid by anaerobic incubation with *Rhizopus*. *Biosci, Biotechnol Biochem* 67:1018–23.

Ardhana MM, Fleet GH. 1989. The microbial ecology of tapé ketan fermentation. *Int J Food Microbiol* 9:157–65.

Arnold SH, Brown WD. 1978. Histamine toxicity from fish products. *Adv Food Res* 24:113–54.

Back HI, Yang JA, Kim SR, Kim MG, Jeon JY, Kim SY, Cha YS, Chae SW, editors. Clinical functionality of Korean fermented foods: Antiobesity and antiatherogenesis effect of Korean traditional soybean fermented foods (*Chungkookjang*, *Doenjang* and *Kochujang*) supplementation in overweight/obese Korean subjects. *Proceeding of the International Symposium on Fermented Foods and their Bright Future*; 2010 November 18; Seoul, South Korea: Ministry of Food, Agriculture, Forestry and Fisheries. 2010. p 55–72.

Bao QA. 1985. The headsteam and technology of douchi. *China Brew* 2:9–14.

Basappa SC. 2002. Investigations on *Chhang* from finger millet (*Eleucine coracana* Gaertn.) and its commercial prospects. *Ind Food Ind* 21(1):46–51.

Basappa SC, Somashekar D, Agrawal R, Suma K, Bharathi K. 1997. Nutritional composition of fermented ragi (*chhang*) by *phab* and defined starter cultures as compared to unfermented ragi (*Eleucine coracana* G.). *Int J Food Sci Nutri* 48:313–9.

Basuki T, editor. The less well-known fermented foodstuffs of Indonesia. *Proceedings and Symposium on Indigenous Fermented Foods*; 1977 November 21–27. Bangkok: GIAMI. 2007.

Batra LR. 1981. Fermented cereals and grain legumes of India and vicinity. In: Moo-Young M, Robinson CW, editors. *Advances in biotechnology*, vol 2. New York: Pergamon Press. p 547–53.

Batra LR. 1986. Microbiology of some fermented cereals and grains legumes of India and vicinity. In: Hesseltine CW, Wang HL, editors. *Indigenous fermented food of non-western origin*. Berlin: J. Cramer. p 85–104.

Batra LR, Millner PD. 1974. Some Asian fermented foods and beverages and associated fungi. *Mycologia* 66:942–50.

Batra LR, Millner PD. 1976. Asian fermented foods and beverages. *Dev Ind Microbiol* 17:117–28.

Boon-Long N. 1986. Traditional technologies of Thailand: Traditional fermented food products. In: *Traditional foods: Some products and technologies*. p 114–33. Mysore: Central Food Technological Research Institute.

Brewer MS. 2000. Traditional preservatives—sodium chloride. In: Robinson RK, Batt CA, Patels PD, editors. *Encyclopedia of food microbiology*. New York: Academic Press. p 1723–8.

Budhiono A, Rosidi B, Taher H, Iguchi M. 1999. Kinetic aspects of bacterial cellulose formation in *nata-de-coco* culture system. *Carbohydrate Polym* 40:137–43.

Campbell-Platt G. 1994. Fermented foods—A world perspective. *Food Res Int* 27:253–7.

Chakkaravarthi A, Punil Kumar HN, Bhattacharya S. 2009. *Jilebi*: 1. Effect of moisture content, curd addition and fermentation time on the rheological properties of dispersions. *J Food Sci Technol* 46(6):543–8.

Chang JY, Lee HJ, Chang HC. 2008. Identification of the agent from *Lactobacillus plantarum* KFRI464 that enhances bacteriocin production by *Leuconostoc citreum* GJ7. *J Appl Microbiol* 103(6):2504–15.

Chao S-H, Tomii Y, Watanabe K, Tsai Y-C. 2008. Diversity of lactic acid bacteria in fermented brines used to make stinky tofu. *Int J Food Microbiol* 123:134–41.

Chao S-H, Wu R-J, Watanabe K, Tsai Y-C. 2009. Diversity of lactic acid bacteria in *suan-tsai* and *fu-tsai*, traditional fermented mustard products of Taiwan. *Int J Food Microbiol* 135:203–10.

Chen YS, Yanagida F, Hsu JS. 2006. Isolation and characterization of lactic acid bacteria from *suan-tsai* (fermented mustard), a traditional fermented food in Taiwan. *J Appl Microbiol* 101:125–30.

Chettri R, Tamang JP. 2008. Microbiological evaluation of maseura, an ethnic fermented legume-based condiment of Sikkim. *J Hill Res* 21:1–7.

Chitale SR, editor. Commercialization of Indian traditional foods: *jeelebi*, *laddoo* and *bakervadi. Proceedings of the International Conference on Traditional Foods*, ed. Director, CFTRI, 331; 1997 March 6–8; Mysore: Central Food Technological Research Institute. 2000.

Choi J, Cheigh CI, Kim SB, Lee JC, Lee DW, Choi SW, Park JM, Pyun YR. 2002. *Weissella kimchii* sp. nov., a novel lactic acid bacterium from kimchi. *Int J Syst Evolution Microbiol* 52:507–11.

Choi U-K, Kim M-H, Lee N-H. 2007. The characteristics of cheonggukjang, a fermented soybean product, by the degree of germination of raw soybeans. *Food Sci Biotechnol* 16:734–9.

Chou C-C, Hwan C-H. 1994. Effect of ethanol on the hydrolysis of protein and lipid during the ageing of a Chinese fermented soy bean curd-sufu. *J Sci Food Agric* 66:393–8.

Chunhachart O, Itoh T, Sukchotiratana M, Tanimoto H, Tahara Y. 2006. Characterization of γ-glutamyl hydrolase produced by *Bacillus* sp. isolated from Thai thua-nao. *Biosci, Biotechnol Biochem* 70(11): 2779–82.

Cronk TC, Mattick LR, Steinkraus KH, Hackler LR. 1979. Production of higher alcohol during the Indonesian tapé ketan fermentation. *Appl Environ Microbiol* 37:892–6.

Cronk TC, Steinkraus KH, Hackler LR, Mattick LR. 1977. Indonesia *tapé ketan* fermentation. *Appl Environ Microbiol* 33:1067–73.

Dahal N, Rao ER, Swamylingappa B. 2003. Biochemical and nutritional evaluation of masyaura—a legume based traditional savoury of Nepal. *J Food Sci Technol* 40:17–22.

Dhamcharee B. 1982. Traditional fermented food in Thailand. In: Saono S, Winarno WJ, Karjarki D, editors. *Traditional food fermentation as industrial resources in ASCA countries*. p 85–90. Jakarta: The Indonesian Institute of Science (LIPI).

Dhavises G. 1972. Microbial studies during the pickling of the shoot of bamboo, *Bambusa arundinacea* Willd, and of pak sian, *Gynandropis pentaphylla DC*. [MS thesis]. Bangkok: Kasetsart Univ.

Dufresne C, Farnworth E. 2000. Tea, kombucha, and health: a review. *Food Res Int* 33:409–21.

Dung NTP. 2004. Defined fungal starter granules for purplae glutinous rice wine. [DPhil thesis]. Wageningen, The Netherlands: Wageningen Univ.

Dung NTP, Rombouts FM, Nout MJR. 2005. Development of defined mixed-culture fungal fermentation starter granules for controlled production of rice wine. *Innov Food Sci Emerg Technol* 6:429–41.

Dung NTP, Rombouts FM, Nout MJR. 2006. Functionality of selected strains of moulds and yeasts from Vietnamese rice wine starters. *Food Microbiol* 23:331–40.

Dung NTP, Rombouts FM, Nout MJR. 2007. Characteristics of some traditional Vietnamese starch-based rice wine starters (*Men*). *LWT - Food Sci Technol* 40:130–5.

Dwidjoseputro D, Wolf FT. 1970. Microbiological studies of Indonesian fermented foodstuffs. *Mycopathol Mycol Appl* 41:211–22.

Ebine H. 1989. Industrialization of Japanese *miso* fermentation. In: Steinkraus KH, editor. *Industrialization of indigenous fermented foods*. New York: Marcel Dekker. p 89–126.

Elegado FB, Fujio Y. 1993. Polygalacturonase production by *Rhizopus* spp. *J Gen Appl Microbiol* 39:409–18.
Entani E, Masai H, editors. Morphology and prevention of acetic acid tolerant microbes in vinegar fermentation. *Proceedings of the 17th Symposium on Brewing;* Tokyo: Brewing Society of Japan. 1985. p 19–23.
Giri SS, Janmejay LS. 1994. Changes in soluble sugars and other constituents of bamboo shoots in soibum fermentation. *J Food Sci Technol* 31(6):500–2.
Greenwalt CJ, Ledford RA, Steinkraus KH. 1998. Determination and characterization of the antimicrobial activity of the fermented tea Kombucha. *Lebensmittel-Wissenschaften und Technologie* 31:291–6.
Gupta M, Khetarpaul N, Chauhan BM. 1992a. Preparation, nutritional value and acceptability of barley *rabadi*—An indigenous fermented food of India. *Plant Foods Human Nutri* 42:351–8.
Gupta M, Khetarpaul N, Chauhan BM. 1992b. Rabadi fermentation of wheat: changes in phytic acid content and in vitro digestibility. *Plant Foods Human Nutri* 42:109–16.
Hadisepoetro ESS, Takada N, Oshima Y. 1979. Microflora in ragi and usar. *J Ferment Technol* 57:251–9.
Han B-Z, Beumer RR, Rombouts FM, Nout MJR. 2001b. Microbiological safety and quality of commercial sufu—a Chinese fermented soybean food. *Food Control* 12:541–7.
Han BZ, Rombouts FM, Nout MJR. 2001a. A Chinese fermented soybean food. *Int J Food Microbiol* 65:1–10.
Hara T, Chetanachit C, Fujio Y, Ueda S. 1986. Distribution of plasmids in polyglutamate-producing *Bacillus* strains isolated from 'natto'-like fermented soybeans, 'thua nao,' in Thailand. *J Gen Appl Microbiol* 32:241–9.
Hara T, Hiroyuki S, Nobuhide I, Shinji K. 1995. Plasmid analysis in polyglutamate-producing *Bacillus* strain isolated from non-salty fermented soybean food, '*kinema*,' in Nepal. *J Gen Appl Microbiol* 41:3–9.
Hara T, Zhang J-R, Ueda S. 1983. Identification of plasmids linked with polyglutamate production in *Bacillus subtilis* (*natto*). *J Gen Appl Microbiol* 29:345–54.
Haruta S, Ueno S, Egawa I, Hashiguchi K, Fujii A, Nagano M, Ishii M, Igarashi Y. 2006. Succession of bacterial and fungal communities during a traditional pot fermentation of rice vinegar assessed by PCR-mediated denaturing gradient gel electrophoresis. *Int J Food Microbiol* 109:79–87.
Hesseltine CW, Kurtzman CP. 1990. Yeasts in amylolytic food starters. Anales del Instituto de Biologıa, Universidad Nacional. Autónoma de México. *Serie Botánica* 60:1–7.
Hesseltine CW, Ray ML. 1988. Lactic acid bacteria in murcha and ragi. *J Appl Bacteriol* 64:395–401.
Hesseltine CW, Rogers R, Winarno FG. 1988. Microbiological studies on amylolytic Oriental fermentation starters. *Mycopathologia* 101:141–55.
Hirasawa T, Yamada K, Nagahisa K, Dinh TN, Furusawa C, Katakura Y, Shioya S, Shimizu H. 2009. Proteomic analysis of responses to osmotic stress in laboratory and sake-brewing strains of *Saccharomyces cerevisiae*. *Process Biochemistry* 44:647–53.
Hirooka K, Yamamoto Y, Tsutsui N, Tanaka T. 2005. Improved production of isoamyl acetate by a sake yeast mutant resistant to an isoprenoid analog and its dependence on alcohol acetyltransferase activity, but not on isoamyl alcohol production. *J Biosci Bioeng* 99:125–9.
Ho CC. 1986. Identity and characteristics of *Neurospora intermedia* responsible for *oncom* fermentation in Indonesia. *Food Microbiol* 3:115–32.
Huang Y, Luo Y, Zhai Z, Zhang H, Yang C, Tian H, Li Z, Feng J, Liu H, Hao Y. 2009. Characterization and application of an anti-*Listeria* bacteriocin produced by *P. pentosaceus* 05–10 isolated from Sichuan Pickle, a traditionally fermented vegetable product from China. *Food Control* 20(11):1030–5.
Inatsu Y, Nakamura N, Yuriko Y, Fushimi T, Watanasiritum L, Kawamoto S. 2006. Characterization of *Bacillus subtilis* strains in thua nao, a traditional fermented soybean food in northern Thailand. *Lett Appl Microbiol* 43:237.
Inoue T, Tanaka J, Mitsui S. 1992. *Recent advances in Japanese brewing technology*, vol. 2(1). Tokyo: Gordon and Breach Science Publishers.
Itabashi M. 1986. Sunki—pickles prepared by single species of lactic acid bacteria. *Nippon Shokuhin Kogyo Gakkaishi* 34(6):356–61.
Jeng K-C, Chen C-S, Fang Y-P, Hou RC-W, Chen Y-S. 2007. Effect of microbial fermentation on content of statin, GABA, and polyphenols in Pu-Erh tea. *J Agric Food Chem* 55:8787–92.
Jie G, Lin Z, Zhang L, Lv H, He P, Zhao B. 2006. Free radical scavenging effect of Pu-erh tea extracts and their protective effect on oxidative damage in human fibroblast cells. *J Agric Food Chem* 54:8058–64.
Joshi N, Godbole SH, Kanekar P. 1989. Microbial and biochemical changes during *dhokla* fermentation with special reference to flavor compounds. *J Food Sci Technol* 26(2):113–5.

Kang MG. 2001. In: *Handbook of technology for chinese and foreign fermented foods*. Beijing: Chemical Industrial Press. p 99–111.

Karki T. 1986. Some Nepalese fermented foods and beverages. In: *Traditional foods: Some products and technologies,* Mysore: Central Food Technological Research Institute. p 84–96.

Karki T, Itoh H, Hayashi K, Kozaki M. 1983b. Chemical changes occurring during gundruk fermentation Part II-1 Amino acids. *Lebensmittel-Wissenschaft und-Technologie* 16:180–3.

Karki T, Itoh H, Kiuchi K, Ebine H, Kozaki M. 1983c. Lipids in gundruk and takana fermented vegetables. *Lebensmittel-Wissenschaft und-Technologie* 16:167–71.

Karki T, Itoh H, Kozaki M. 1983a. Chemical changes occurring during gundruk fermentation Part II-2 Flavor components. *Lebensmittel-Wissenschaft und-Technologie* 16:203–8.

Karki T, Okada S, Baba T, Itoh H, Kozaki M. 1983d. Studies on the microflora of Nepalese pickles gundruk. *Nippon Shokuhin Kogyo Gakkaishi* 30:357–67.

Khetarpaul N, Chauhan BM. 1989. Effect of fermentation by pure cultures of yeasts and lactobacilli on phytic acid and polyphenol content of pearl millet. *J Food Sci* 54:780–1.

Khetarpaul N, Chauhan BM. 1990a. Fermentation of pearl millet flour with yeasts and lactobacilli: In vitro digestibility and utilization of fermented flour for weaning mixtures. *Plant Foods Human Nutr* 40:167–73.

Khetarpaul N, Chauhan BM. 1990b. Effect of fermentation by pure cultures of yeasts and lactobacilli on the available carbohydrate content of pearl millet. *Food Chem* 36:287–93.

Khetarpaul N, Chauhan BM. 1991. Effect of pure sequential culture fermentation by yeasts and lactobacilli on HCl-extractability of minerals from pearl millet. *Food Chem* 39:347–55.

Kim B, Lee J, Jang J, Kim J, Han H. 2003. *Leuconostoc inhae*, sp. nov., a lactic bacterium isolated from kimchi. *Int J Syst Evol Microbiol* 53:1123–6.

Kim CJ. 1968. Microbiological and enzymological studies on Takju brewing. *J Korean Agric Chem Soc* 10: 69–9.

Kim CJ. 1970. Studies on the differentiation and the population changes of Takju yeasts by the TTC-agar overlay techniques. *Korean J Microbiol* 8:69–76.

Kim CJ, Choi WY. 1970. Studies on the quantitative changes of thiamine during Takju brewing. *J Korean Agric Chem Soc* 13:105–9.

Kim HJ, Lee JS, Chung HY, Song SH, Suh H, Noh JS, Song YO. 2008. 3-(4′-hydroxyl-3′,5′-dimethoxyphenyl) propionic acid, an active principle of kimchi, inhibits development of atherosclerosis in rabbits. *J Agric Food Chem* 55(25):10486–92.

Kim JH, Lee DH, Lee SH, Choi SY, Lee JS. 2004. Effect of *Ganoderma lucidum* on the quality and functionality of Korean traditional rice wine, yakju. *J Biosci Bioeng* 97:24–8.

Kim JO, Kim JG. 1993. Microbial and enzymatic properties related to brewing of traditional ewhaju. *Korean J Soc Food Sci* 9(4):266–71.

Kim JO, Lee BH. 1970. Taxonomical studies of yeasts in Korea. I. On yeasts isolated from Takju. *Korean J Microbiol* 8:77–84.

Kim M, Chun J. 2005. Bacterial community structure in kimchi, a Korean fermented vegetable food, as revealed by 16S rRNA gene analysis. *Int J Food Microbiol* 103(1):91–6.

Kim T-W, Lee J-W, Kim S-E, Park M-H, Chang HC, Kim H-Y. 2009. Analysis of microbial communities in *doenjang*, a Korean fermented soybean paste, using nested PCR-denaturing gradient gel electrophoresis. *Int J Food Microbiol* 131:265–71.

Kim W, Choi K, Kim Y, Park H, Choi J, Lee Y, Oh H, Kwon I, Lee S. 1996. Purification and characterization of a fibrinolytic enzyme produced from *Bacillus* sp. strain CK 11–4 screened from Chungkook-Jang. *Appl Environ Microbiol* 2482–8.

Kimura K, Itoh Y. 2007. Determination and characterization of IS*4Bsu1*-insertion loci and identification of a new insertion sequence element of the IS*256* family in a natto starter. *Biosci, Biotechnol, Biochem* 71:2458–64.

Kingston JJ, Radhika M, Roshini PT, Raksha MA, Murali HS, Batra HV. 2010. Molecular characterization of lactic acid bacteria recovered from natural fermentation of beet root and carrot Kanji. *Ind J Microbiol* 50:292–8.

Kitamoto K. 2002. Molecular biology of the *Koji* molds. *Adv Appl Microbiol* 51:129–53.

Ko SD. 1972. Tapé fermentation. *Appl Microbiol* 23:976–8.

Ko SD. 1982. Indigenous fermented food. In: Rose AH, editor. *Economic microbiology fermented foods*. vol. 7. London: Academic Press. p 16–37.

Kodama K. 1993. Saké brewing yeasts. In: Rose AH, Harrison JS, editors. *The yeast: Yeast technology*, vol. V, 2nd ed. London: Academic Press. p 129–68.

Kodama K, Yoshizawa K. 1977. Saké. In: Rose AH, editor. *Economic microbiology*, vol. 1, New York: Academic Press. p 432–75.

Kong Y-H, Cheigh H-S, Song Y-O, Jo Y-O, Choi SY. 2008. Anti-obesity effects of kimchi tablet composition in rats fed high-fat diet. *J Korean Soc Food Sci Nutri* 36(12):1529–36.

Kotaka A, Bando H, Kaya M, Kato-Murai M, Kuroda K, Sahara H, Hata Y, Kondo A, Ueda M. 2008. Direct ethanol production from barley β-glucan by sake yeast displaying *Aspergillus oryzae* β-glucosidase and endoglucanase. *J Biosci Bioeng* 105:622–7.

Kozaki M. 1976. Fermented foods and related microorganisms in Southeast Asia. *Proc Jpn Assoc Mycotoxicol* 2:1–9.

Kozaki M, Tamang JP, Kataoka J, Yamanaka S, Yoshida S. 2000. Cereal wine (*jaanr*) and distilled wine (*raksi*) in Sikkim. *J Brew Soc Jpn* 95(2):115–22.

Kozaki M, Uchimura T. 1990. Microorganisms in Chinese starter '*bubod*' and rice wine 'tapuy' in the Philippines. *J Brew Soc Jpn* 85(11):818–24.

Kurtzman CP, Robnett CJ, Basehoar-Powers E. 2001. *Zygosaccharomyces kombuchaensis*, a new ascosporogenous yeast from 'Kombucha tea.' *FEMS Yeast Res* 1:133–8.

Lee AC, Fujio Y. 1999. Microflora of *banh men*, a fermentation starter from Vietnam. *World J Microbiol Biotechnol* 15:51–5.

Lee B-Y, Kim D-M, Kim K-H. 1991. Physico-chemical properties of viscous substance extracted from Chungkook-Jang. *Korean J Food Sci Technol* 23:599–604 (in Korean).

Lee C-H. 2008. Chongkukjang. In: Kiuchi K, Nagai T, Kimura K, editors. *Advanced science on Natto*. Tokyo: Kenpakusha (in Japanese). p 224–6.

Lee HM, Lee Y. 2006. Isolation of *Lactobacillus plantarum* from kimchi and its inhibitory activity on the adherence and growth of *Helicobacter pylori*. *J Microbiol Biotechnol* 16(10):1513–7.

Lee HR, Lee JM. 2009. Anti-stress effects of kimchi. *Food Sci Biotechnol* 18(1):25–30.

Lee I-H, Hung Y-H, Chou CC. 2007. Total phenolic and anthocyanin contents, as well as antioxidant activity, of black bean *koji* fermented by *Aspergillus awamori* under different culture conditions. *Food Chem* 3:936–42.

Lee J-J, Lee D-S, Kim H-B. 1999. Fermentation patterns of chungkookjang and kanjang by *Bacillus licheniformis* B1. *Korean J Microbiol* 35: 296–301 (in Korean).

Lee JS, Lee KC, Ahn JS, Mheen TI, Pyun YR, Park YH. 2002. *Weissella koreensis* sp. nov., isolated from kimchi. *Int J Syst Evol Microbiol* 52:1257–61.

Lee M, Kim MK, Vancanneyt M, Swings J, Kim S-H, Kang MS, Lee S-T. 2005. *Tetragenococcus koreensis* sp. nov., a novel rhamnolipid-producing bacterium. *Int J Syst Evol Microbiol* 55:1409–13.

Lee Y, Lee HJ, Lee H-S, Jang Y-A, Kim C-I. 2008. Analytical dietary fiber database for the National Health and Nutrition Survey in Korea. *J Food Compos Anal* 21:S35–42.

Lee ZS, Rhee TW. 1970. Studies on the microflora of Takju brewing. *Korean J Microbiol* 8:116–33.

Lehane L, Olley J. 2000. Histamine fish poisoning revisited. *Int J Syst Microbiol* 58:1–37.

Lim G. 1991. Indigenous fermented food in South East Asia. *ASEAN Food J* 6:83–101.

Lotong N. 1985. Koji. In: Wood BJB, editor. *Microbiology of fermented food*, vol. 2. London: Elsevier Applied Science Publishers. p 237–70.

Lu F, editor. Chinese fermented condiments and their industry situation. *The Proceedings of International Symposium on Fermented Foods and their Bright Future*, 99–123. Ministry of Food, Agriculture, Forestry and Fisheries; 2010 November 18; Seoul, South Korea. 2010.

Mahajan S, Chauhan BM. 1987. Phytic acid and extractable phosphorus of pearl millet flour as affected by natural lactic acid fermentation. *J Sci Food Agric* 41:381–6.

Matsushita M, Tada S, Suzuki S, Kusumoto K, Kashiwagi Y. 2009. Deletion analysis of the promoter of *Aspergillus oryzae* gene encoding heat shock protein 30. *J Biosci Bioeng* 107(4):345–51.

Mayser P, Fromme S, Leitzmann C, Grunder K. 1995. The yeast spectrum of the tea fungus *Kombucha. Mycoses* 38:289–95.

Merican Z. 1996. Malaysian pickles. In: Steinkraus KH, editor. *Handbook of indigenous fermented food*. 2nd ed. New York: Marcel Dekker. p 138–41.

Merican Z, Norrijah O. 1983. *Innovations in traditional food fermentation—tapai.* Paper presented at the Regional Conference on Impact of Microbiology in Tropical Agriculture, Serang, Malaysia.

Merican Z, Norrijah O. 1985. Adapting modern technology for traditional fermented food: The tapai experience. In: *The seminar proceedings 1984*. Food Technology Division MARDI. p 339–46.

Merican Z, Yeoh QL. 1982. Penyediaan tapai secara modern. *Teknologi Makanan Jld. Bil*. 1:10–12.

Merican Z, Yeoh QL. 1989. Tapai proceeding in Malaysia: A technology in transition. In: Steinkraus KH, editor. *Industrialization of indigenous fermented foods*. New York: Marcel Dekker. p 169–89.

Min YK, Kim Z-U. 1990. Making of improved doenjang by natto mixing. *Seoul Natl Univ J Agric Sci* 15:105–9.

Mingmuang M. 1974. Microbial study during the pickling of leaves of mustard (Brassica juncea L.) by fermentation process. [MSc thesis]. Bangkok: Kasetsart Univ.

Miyamoto M, Seto Y, Hao DH, Teshima T, Sun YB, Kabuki T, Yao LB, Nakajima H. 2005. *Lactobacillus harbinensis* sp. nov., consisted of strains isolated from traditional fermented vegetables 'Suan cai' in Harbin, Northeastern China and *Lactobacillus perolens* DSM 12745. *Syst Appl Microbiol* 28(8):688–94.

Mizunuma T. 1991. Shoyu. In: Noshiro K, Yoshizawa K, Kamata K, Mizunuma T, Tadenuma M, editors. *Encyclopedia of brewing*. 2nd ed. Tokyo: Asakura Shoten. p 398–440.

Mo H, Zhu Y, Chen Z. 2008. Review. Microbial fermented tea—a potential source of natural food preservatives. *Trends Food Sci Technol* 19:124–30.

Mo HZ, Xu XQ, Yan MC, Zhu Y. 2005. Microbiological analysis and antibacterial effects of the indigenous fermented Puer tea. *Agro Food Ind Hi-Tech* 16:16–8.

Mohd Adnan AF, Tan IKP. 2007. Isolation of lactic acid bacteria from Malaysian foods and assessment of the slates for industrial potential. *Biores Technol* 98:1380–5.

Mukherjee SK, Albury CS, Pederson AG, Steinkraus KH. 1965. Role of Leuconostoc mesenteroides in leavening the batter of idli, a fermented food of India. *Appl Microbiol* 13(2):227–31.

Murakami H. Some problems on saké brewing. In: Terui G, editor. *Fermentation Technology, Proceedings of the 4th International Fermentations Symposium*; 1972; Kyoto, Japan. p 639–43. 1972.

Nagai T, Koguchi K, Itoh Y. 1997. Chemical analysis of poly-γ-glutamic acid produced by plasmid-free *Bacillus subtilis* (*natto*): evidence that plasmids are not involved in poly-γ-glutamic acid production. *J Gen Appl Microbiol* 43:139–43.

Nagai T, Tamang JP. 2010. Fermented soybeans and non-soybeans legume foods. In: Tamang JP, Kailasapathy K, editors. *Fermented foods and beverages of the world*. New York: CRC Press, Taylor & Francis. p 191–224.

Nagai T, Tran L-S, Inatsu Y, Itoh Y. 2000. A new IS*4* family insertion sequence, IS*4Bsu1*, responsible for genetic instability of poly-γ-glutamic acid production in *Bacillus subtilis*. *J Bacteriol* 182:2387–92.

Nakao S. 1972. Mame no ryori. In: *Ryori no kigen* (in Japanese). Tokyo: Japan Broadcast Publishing. p 115–26.

Nam YD, Kim HW, Chang KH, Roh SW, Bae JW. 2009. Metatranscriptome analysis of lactic acid bacteria during kimchi fermentation with genome-probing microarrays. *Int J Food Microbiol* 130(2):140–6.

Nikkuni S, Karki TB, Terao T, Suzuki C. 1996. Microflora of mana, a Nepalese rice koji. *J Ferment Bioeng* 81(2):168–70.

Nikkuni S, Karki TB, Vilku KS, Suzuki T, Shindoh K, Suzuki C, Okada N. 1995. Mineral and amino acid contents of *kinema*, a fermented soybean food prepared in Nepal. *Food Sci Technol Int* 1(2):107–11.

Ohta T. 1986. Natto. In: Reddy NR, Pierson MD, Salunkhe DK, editors. *Legume-based fermented foods*. Florida: CRC Press. p 85–95.

Okada N. 2008. Toa-nao. In: Kiuchi K, Nagai T, Kimura K, editors. *Advanced science on Natto*. Tokyo: Kenpakusha. p 212–6.

Okada N, Hariantono J, Hadioetomo RS, Nikkuni S, Itoh H. 1985. Survey of vitamin B_{12}–producing bacteria isolated from Indonesian tempeh. *Rep Nati Food Res Inst* 47:49–56.

Omizu Y, Tsukamoto C, Chettri R, Tamang JP. 2011. Determination of saponin contents in raw soybean and fermented soybean foods of India. *J Sci Ind Res* 70:533–538.

Park C, Choi J-C, Choi Y-H, Nakamurab H, Shimanouchi K, Horiuchi T, Misono H, Sewaki T, Soda K, Ashiuchi M, Sunga M-H. 2005. Synthesis of super-high-molecular-weight poly-γ-glutamic acid by *Bacillus subtilis* subsp. *chungkookjang*. *J Mol Catal B: Enzymatic* 35:128–33.

Park KI, Mheen TI, Lee KH, Chang CH, Lee SR, Kwon TW, editors. Korean yakju and takju. *The Proceeding and Symposium on Indigenous Fermented Foods*; 1977 November 21–27; Bangkok: GIAMI. 1977.

Park K-Y, Kil J-H, Jung KO, Kong CS, Lee J. 2006. Functional properties of kimchi (Korean fermented vegetables). *Acta Horticulturae* 706:167–72.

Pederson CS. 1979. *Microbiology of food fermentations*, 2nd ed. Westport: AVI Publishing Company.

Phithakpol B, Varanyanond W, Reungmaneepaitoon S, Wood H. 1995. *The traditional fermented foods of Thailand.* Kuala Lumpur: ASEAN Food Handling Bureau.

Pichyangkura S, Kulprecha S, editors. Survey of mycelial moulds in loogpang from various sources in Thailand. *The Proceeding and Symposium on Indigenous Fermented Foods*; 1977 November 21–27; Bangkok: GIAMI. 1977.

Prakash O. 1961. *Food and drinks in ancient India*. Delhi: Munshi Ram Monoharlal Publ.

Puspito H, Fleet GH. 1985. Microbiology of sayur asin fermentation. *Appl Microbiol Biotechnol* 22:442–5.

Ramakrishnan CV. 1979. Studies on Indian fermented foods. *Baroda J Nutri* 6:1–54.

Reddy NR, Sathe SK, Pierson MD, Salunkhe DK. 1981. Idli, an Indian fermented food: a review. *J Food Qual* 5:89–101.

Sakai H, Caldo GA. 1985. Microbiological studies on *Bubod*, a fermentation starter in the Philippines. Isolation and identification of yeasts. *Phil Agricult* 68:139–44.

Sanchez PC, Kozaki M. 1984. Improved production of Basi (sugarcane wine). *Phil Agricult* 67:391–405.

Sandhu DK, Soni SK. 1989. Microflora associated with Indian Punjabi wari fermentation. *J Food Sci Technol* 26:21–5.

Sano M, Takenaka Y, Kojima R, Saito S, Tomita I, Katou M, Shibuya S. 1986. Effects of pu-erh tea on lipid metabolism in rats. *Chem Pharmaceut Bull* 34:221–8.

Saono S, Basuki T, Sastraatmadja DD, editors. Indonesian ragi. *The Proceeding and Symposium on Indigenous Fermented Foods*; 1977 November 21–27; Bangkok: GIAMI. 1977.

Saono S, Gandjar I, Basuki T, Karsono H. 1974. Mycoflora of ragi and some other traditional fermented foods of Indonesia. *Ann Bogor* V(4):187–204.

Saono S, Hosono A, Tomomatsu A, Matsuyama A, Kozaki M, Baba T, editors. 1984. The preparation of brem ragi—an improved method. *Proceedings of IPB–JICA*; 1984 July 31–August 2. p 152–8.

Sarangthem K, Singh TN. 2003. Microbial bioconversion of metabolites from fermented succulent bamboo shoots into phytosterols. *Curr Sci* 84(12):1544–7.

Sarkar PK, Hasenack B, Nout MJR. 2002. Diversity and functionality of *Bacillus* and related genera isolated from spontaneously fermented soybeans (Indian Kinema) and locust beans (African Soumbala). *Int J Food Microbiol* 77:175–86.

Sarkar PK, Jones LJ, Craven GS, Somerset SM, Palmer C. 1997. Amino acid profiles of kinema, a soybean-fermented food. *Food Chem* 59(1):69–75.

Sarkar PK, Jones LJ, Gore W, Craven GS. 1996. Changes in soya bean lipid profiles during kinema production. *J Sci Food Agric* 71:321–8.

Sarkar PK, Morrison E, Tingii U, Somerset SM, Craven GS. 1998. B-group vitamin and mineral contents of soybeans during kinema production. *J Sci Food Agric* 78:498–502.

Sarkar PK, Tamang JP. 1994. The influence of process variables and inoculum composition on the sensory quality of kinema. *Food Microbiol* 11:317–25.

Sarkar PK, Tamang JP. 1995. Changes in the microbial profile and proximate composition during natural and controlled fermentations of soybeans to produce kinema. *Food Microbiol* 12:317–25.

Sarkar PK, Tamang JP, Cook PE, Owens JD. 1994. Kinema—a traditional soybean fermented food: proximate composition and microflora. *Food Microbiol* 11:47–55.

Sasaki M, Nunomura N. 2003. Soy (soya) sauce. In: Caballero B, Trugo LC, Finglas PM, editors. *Encyclopedia of food sciences and nutrition*. 2nd ed. London: Academic Press. p 2359–69.

Sasaki A, Takeda K, Kanzaki M, Ohta S, Preechapanya P. 2007. Population dynamics and land-use changes in a *Miang* (chewing tea) village, northern Thailand. *Tropics* 16:75–85.

Sawamura S. 1906. On the micro-organisms of natto. *Bull Coll Agric, Tokyo Imperial Univ* 7:107–10.

Schillinger U, Ban-Koffi L, Franz CMAP. 2010. Tea, coffee and cacao. In: Tamang JP, Kailasapathy K, editors. *Fermented foods and beverages of the world*. New York: CRC Press, Taylor & Francis Group. p 353–75.

Shamala TR, Sreekantiah KR. 1988. Microbiological and biochemical studies on traditional Indian palm wine fermentation. *Food Microbiol* 5:157–62.

Shin YD, Cho DH. 1970. A study on the microflora changes during Takju brewing. *Korean J Microbiol* 8:53–4.

Shon M-Y, Lee J, Choi J-H, Choi S-Y, Nam S-H, Seo K-I, Lee S-W, Sung N-J, Park S-K. 2007. Antioxidant and free radical scavenging activity of methanol extract of chungkukjang. *J Food Compos Anal* 20:113–8.

Shrestha H, Nand K, Rati ER. 2002. Microbiological profile of *murcha* starters and physico-chemical characteristics of *poko*, a rice based traditional food products of Nepal. *Food Biotechnol* 16:1–15.

Sim KH, Han YS. 2008. Effect of red pepper seed on kimchi antioxidant activity during fermentation. *Food Sci Biotechnol* 17(2):295–301.

Solieri L, Giudici P. 2008. Yeasts associated to traditional balsamic vinegar: Ecological and technological features. *Int J Food Microbiol* 125:36–45.

Soni SK, Sandhu DK. 1989a. Nutritional improvement of Indian Dosa batter by yeast enrichment and black gram replacement. *J Ferment Bioeng* 68(1):1–4.

Soni SK, Sandhu DK. 1989b. Fermentation of idli: Effects of changes in raw materials and physico-chemical conditions. *J Cereal Sci* 10:227–38.

Soni SK, Sandhu DK. 1990. Biochemical and nutritional changes associated with Indian Punjab *Wari* fermentation. *J Food Sci Technol* 27:82–5.

Soni SK, Sandhu DK. 1991. Role of yeast domination in Indian idli batter fermentation. *World J Microbiol Biotechnol* 7:505–7.

Soni SK, Sandhu DK, Vilkhu KS. 1985. Studies on Dosa—an indigenous Indian fermented food: some biochemical changes accompanying fermentation. *Food Microbiol* 2:175–81.

Soni SK, Sandhu DK, Vilkhu KS, Kamra N. 1986. Microbiological studies on Dosa fermentation. *Food Microbiol* 3:45–53.

Steinkraus KH. 1983. Lactic acid fermentation in the production of foods from vegetables, cereals and legumes. *Antonie van Leeuwenhoek* 49:337–48.

Steinkraus KH. 1996. *Handbook of indigenous fermented food*, 2nd ed. New York: Marcel Dekker.

Steinkraus KH, van Veer AG, Thiebeau DB. 1967. Studies on idli—an Indian fermented black gram-rice food. *Food Technol* 21(6):110–3.

Suganuma T, Fujita K, Kitahara K. 2007. Some distinguishable properties between acid-stable and neutral types of α-amylases from acid-producing *koji*. *J Biosci Bioeng* 104:353–62.

Sugawara E. 2010. Fermented soybean pastes miso and *shoyu* with reference to aroma. In: Tamang JP, Kailasapathy K, editors. *Fermented foods and beverages of the world*. New York: CRC Press, Taylor & Francis Group. p 225–45.

Sugawara E, Itoh T, Odagiri S, Kubota K, Kobayashi A. 1985. Comparison of compositions of natto and cooked soybeans. *Agric Biol Chem* 49:311–7.

Sukhumavasi J, Kato K, Harada T. 1975. Glucoamylase of a strain of *Endomycopsis fibuligera* isolated from mould bran (look pang) of Thailand. *J Ferment Technol* 53(8):559–65.

Sukontasing S, Tanasupawat S, Noonmangmee S, Lee J-S, Suzuki K. 2007. *Enterococcus camelliae* sp. nov., isolated from fermented tea leaves in Thailand. *Int J Syst Evol Microbiol* 57:2151–4.

Sumi H, Hamada H, Nakanishi N, Hiratani H. 1990. Enhancement of fibrinolytic activity in plasma by oral administration of nattokinase. *Acta Haematol* 84:139–43.

Sumi H, Hamada H, Tsushima S, Mihara H, Muraki H. 1987. A novel fibrinolytic enzyme (nattokinase) in the vegetable cheese natto, a typical and popular soybean food of the Japanese diet. *Experientia* 43:1110–1.

Sumi H, Okamoto T. 2003. Thrombolytic activity of an aqueous extract of *tempe*. *J Home Econom Jpn* 54:337–42.

Sun YP, Chou CC, Yu RC. 2009. Antioxidant activity of lactic-fermented Chinese cabbage. *Food Chem* 115(3): 912–7.

Sundhagul M, Smanmathuroj P, Bhodachareon W. 1972. Thua-nao: A fermented soybean food of northern Thailand. I: Traditional processing method. *Thai J Agric Sci* 5:43–56.

Suprianto OR, Koga T, Ueda S. 1989. Liquefaction of glutinous rice and aroma formation in tapé preparation by ragi. *J Ferment Bioeng* 64(4):249–52.

Sura K, Garg S, Garg FC. 2001. Microbiological and biochemical changes during fermentation of *kanji*. *J Food Sci Technol* 38:165–7.

Tamang B, Tamang JP. 2007. Role of lactic acid bacteria and their functional properties in *Goyang*, a fermented leafy vegetable product of the Sherpas. *J Hill Res* 20(20):53–61.

Tamang B, Tamang JP. 2009. Lactic acid bacteria isolated from indigenous fermented bamboo products of Arunachal Pradesh in India and their functionality. *Food Biotechnol* 23:133–47.

Tamang B, Tamang JP. 2010. *In situ* fermentation dynamics during production of *gundruk* and *khalpi*, ethnic fermented vegetables products of the Himalayas. *Indian J Microbiol* 50(Supplement 1):93–8.

Tamang B, Tamang JP, Schillinger U, Franz CMAP, Gores M, Holzapfel WH. 2008. Phenotypic and genotypic identification of lactic acid bacteria isolated from ethnic fermented tender bamboo shoots of North East India. *Int J Food Microbiol* 121:35–40.

Tamang JP. 1999. Development of pulverised starter for kinema production. *J Food Sci Technol* 36:475–8.

Tamang JP. 2001. Kinema. *Food Culture* 3:11–4.

Tamang JP. 2003. Native microorganisms in fermentation of kinema. *Indian J Microbiol* 43(2):127–30.
Tamang JP. 2005. Food culture of Sikkim, Sikkim Study Series, vol. 4. Gangtok: Information and Public Relations Department. Government of Sikkim.
Tamang JP. 2010a. *Himalayan fermented foods: microbiology, nutrition, and ethnic values*. New York: CRC Press, Taylor & Francis Group.
Tamang JP. 2010b. Diversity of fermented foods. In: Tamang JP, Kailasapathy K, editors. *Fermented foods and beverages of the world*. New York: CRC Press, Taylor & Francis Group. p 41–84.
Tamang JP. 2010c. Diversity of fermented beverages. In: Tamang JP, Kailasapathy K, editors. *Fermented foods and beverages of the world*. New York: CRC Press, Taylor & Francis Group. p 85–125.
Tamang JP, Chettri R, Sharma RM. 2009b. Indigenous knowledge of Northeast women on production of ethnic fermented soybean foods. *Indian J Traditional Knowl* 8(1):122–6.
Tamang JP, Dewan S, Tamang B, Rai A, Schillinger U, Holzapfel WH. 2007b. Lactic acid bacteria in *Hamei* and *Marcha* of North East India. *Indian J Microbiol* 47(2):119–25.
Tamang JP, Fleet GH. 2009. Yeasts diversity in fermented foods and beverages. In: Satyanarayana T, Kunze G, editors. *Yeasts biotechnology: Diversity and applications*. New York: Springer. p 169–98.
Tamang JP, Nikkuni S. 1996. Selection of starter cultures for the production of kinema, a fermented soybean food of the Himalaya. *World J Microbiol Biotechnol* 12:629–35.
Tamang JP, Nikkuni S. 1998. Effect of temperatures during pure culture fermentation of kinema. *World J Microbiol Biotechnol* 14:847–50.
Tamang JP, Sarkar PK. 1993. Sinki—a traditional lactic acid fermented radish tap root product. *J Gen Appl Microbiol* 39:395–408.
Tamang JP, Sarkar PK. 1995. Microflora of murcha: an amylolytic fermentation starter. *Microbios* 81:115–22.
Tamang JP, Sarkar PK. 1996. Microbiology of mesu, a traditional fermented bamboo shoot product. *Int J Food Microbiol* 29:49–58.
Tamang JP, Sarkar PK, Hesseltine CW. 1988. Traditional fermented foods and beverages of Darjeeling and Sikkim—a review. *J Sci Food Agric* 44:375–85.
Tamang JP, Tamang B, Schillinger U, Franz CMAP, Gores M, Holzapfel WH. 2005. Identification of predominant lactic acid bacteria isolated from traditional fermented vegetable products of the Eastern Himalayas. *Int J Food Microbiol* 105(3):347–56.
Tamang JP, Tamang B, Schillinger U, Guigas C, Holzapfel WH. 2009a. Functional properties of lactic acid bacteria isolated from ethnic fermented vegetables of the Himalayas. *Int J Food Microbiol* 135:28–33.
Tamang JP, Thapa N, Rai B, Thapa S, Yonzan H, Dewan S, Tamang B, Sharma RM, Rai AK, Chettri R, Mukhopadhyay B, Pal B. 2007a. Food consumption in Sikkim with special reference to traditional fermented foods and beverages: A micro-level survey. *J Hill Res, Supplementary Issue* 20(1):1–37.
Tamang JP, Thapa S. 2006. Fermentation dynamics during production of bhaati jaanr, a traditional fermented rice beverage of the Eastern Himalayas. *Food Biotechnol* 20(3):251–61.
Tamang JP, Thapa S, Dewan S, Jojima Y, Fudou R, Yamanaka S. 2002. Phylogenetic analysis of Bacillus strains isolated from fermented soybean foods of Asia: Kinema, chungkokjang and natto. *J Hill Res* 15:56–62.
Tamang JP, Thapa S, Tamang N, Rai B. 1996. Indigenous fermented food beverages of the Darjeeling hills and Sikkim: process and product characterization. *J Hill Res* 9(2):401–11.
Tanaka T. 2008a. Pepok. In: Kiuchi K, Nagai T, Kimura K, editors. *Advanced science on Natto (in Japanese)*. Tokyo: Kenpakusha. p 218–21.
Tanaka T. 2008b. Sieng. In: Kiuchi K, Nagai T, Kimura K, editors. *Advanced science on Natto (in Japanese)*. Tokyo: Kenpakusha. p 221–4.
Tanasupawat S, Pakdeeto A, Thawai C, Yukphan P, Okada S. 2007. Identification of lactic acid bacteria from fermented tea leaves (miang) in Thailand and proposals of *Lactobacillus thailandensis* sp. nov., *Lactobacillus camelliae* sp. nov., and *Pediococcus siamensis* sp. nov. *J Gen Appl Microbiol* 53:7–15.
Tanimura W, Sanchez PC, Kozaki M. 1977. The fermented food in the Philippines (Part 1). Tapuy (rice wine). *J Agric Sci Tokyo Univ Agric* 22(1):118–34.
Tanimura W, Sanchez PC, Kozaki M. 1978. The fermented foods in the Philippines (Part II). Basi (Sugarcane wine). *J Agric Soc (Japan)* 22:118–33.
Teoh AL, Heard G, Cox J. 2004. Yeasts ecology of Kombucha fermentation. *Int J Food Microbiol* 95:119–26.
Thanh VN, Mai LT, Tuan DA. 2008. Microbial diversity of traditional Vietnamese alcohol fermentation starters (*banh men*) as determined by PCR-mediated DGGE. *Int J Food Microbiol* 128:268–73.

Thapa S, Tamang JP. 2004. Product characterization of kodo ko jaanr: fermented finger millet beverage of the Himalayas. *Food Microbiol* 21:617–22.

Thapa S, Tamang JP. 2006. Microbiological and physico-chemical changes during fermentation of kodo ko jaanr, a traditional alcoholic beverage of the Darjeeling hills and Sikkim. *Indian J Microbiol* 46(4):333–41.

Thyagaraja N, Otani H, Hosono A. 1992. Studies on microbiological changes during the fermentation of 'idly.' *Lebensmittel-Wissenschaft und-Technologie* 25:77–9.

Tsuyoshi N, Fudou R, Yamanaka S, Kozaki M, Tamang N, Thapa S, Tamang JP. 2005. Identification of yeast strains isolated from marcha in Sikkim, a microbial starter for amylolytic fermentation. *Int J Food Microbiol* 99(2):135–46.

Uchimura T, Kojima Y, Kozaki M. 1990. Studies on the main saccharifying microorganism in the Chinese starter of Bhutan 'Chang poo. *J Brew Soc Jpn* 85(12):881–7.

Uchimura T, Niimura Y, Ohara N, Kozaki M. 1991a. Microorganisms in luck pang used for vinegar production in Thailand. *J Brew Soc Jpn* 86(1):62–7.

Uchimura T, Okada S, Kozaki M. 1991b. Identification of lactic acid bacteria isolated from Indonesian Chinese starter, 'ragi.' Microorganisms in Chinese Starters from Asia (Part 4). *J Brew Soc Jpn* 86(1):55–61.

Uyenco FR, Gacutan RQ, editors. Philippine tapuy. *The Proceeding and Symposium on Indigenous Fermented Foods*; 1977 November 21–27; Bangkok: GIAMI. 1977.

Vachanavinich K, Kim WJ, Park YI. 1994. Microbial study on krachae, Thai rice wine. In: Lee CH, Adler-Nissen J, Bärwald G, editors. *Lactic acid fermentation of non-alcoholic dairy food and beverages*. p 233–46. Seoul: Ham Lim Won.

Venkatasubbaiah PC, Dwarakanath T, Sreenivasa Murthy V. 1984. Microbioligical and physico-chemical changes in idli batter during fermentation. *J Food Sci Technol* 21:59–62.

Visessanguan W, Benjakul S, Potachareon W, Panya A, Riebroy S. 2005. Accelerated proteolysis of soy proteins during fermentation of Thua-nao inoculated with *Bacillus subtilis*. *J Food Biochem* 29(4):349–66.

Wacher C, Díaz-Ruiz G, Tamang JP. 2010. Fermented vegetable products. In: Tamang JP, Kailasapathy K, editors. *Fermented foods and beverages of the world*. New York: CRC Press, Taylor & Francis Group. p 149–90.

Wang HL, Hesseltine CW. 1970. Sufu and lao-chao. *J Agric Food Chem* 18:572–5.

Wang L-J, Li D, Zou L, Chen XD, Cheng Y-Q, Yamaki K, Li L-T. 2007. Antioxidative activity of douchi (a Chinese traditional salt-fermented soybean food) extracts during its processing. *Int J Food Properties* 10:1–12.

Watanabe K, Fujimoto J, Tomii Y, Sasamoto M, Makino H, Kudo Y, Okada S. 2009. *Lactobacillus kisonensis* sp. nov., *Lactobacillus otakiensis* sp. nov., *Lactobacillus* rapi sp. nov. and *Lactobacillus sunkii* sp. nov., heterofermentative species isolated from sunki, a traditional Japanese pickle. *Int J Syst Evol Microbiol* 59:754–60.

Wei D, Jong S. 1983. Chinese rice pudding fermentation: fungal flora of starter cultures and biochemical changes during fermentation. *J Ferment Technol* 61(6):573–9.

Winarno FG. 1986. Traditional technologies of Indonesia with special attention to fermented foods. In: *Traditional foods: Some products and technologies*. Mysore: CFTRI. p 136–47.

Winarno FG, Fardiaz S, Daulay D. 1973. Indonesian fermented foods, Bogor Indonesia: Department of Agricultural Product Technology. Fatema, Bogor Agricultural Univ.

Wong PW, Jackson H, editors. Fermented foods of Sabah (Malaysia). *The Proceeding and Symposium on Indigenous Fermented Foods*; 1977 November 21–27; Bangkok: GIAMI. 1977.

Wu H, Watanabe T, Araki Y, Kitagaki H, Akao T, Takagi H, Shimoi H. 2009. Disruption of ubiquitin-related genes in laboratory yeast strains enhances ethanol production during sake brewing. *J Biosci Bioeng* 107:636–40.

Wu S-C, Yen G-C, Wang B-S, Chiu C-K, Yen W-J, Chang L-W, Duh P-D. 2007. Antimutagenic and antimicrobial activities of pu-erh tea. *LWT - Food Sci Technol* 40:506–12.

Yamaguchi S. 1987. Fundamental properties of umami in human taste. In: Kawamura Y, Kase MR, editors. *Umami: a basic taste*. New York: Marcel Dekker. p 41–73.

Yamanishi R, Huang T, Tsuji H, Bando N, Ogawa T. 1995. Reduction of the soybean allergenicity by the fermentation with *Bacillus natto*. *Food Sci Technol Int* 1:14–7.

Yan PM, Xue WT, Tan SS, Zhang H, Chang XH. 2008. Effect of inoculating lactic acid bacteria starter cultures on the nitrite concentration of fermenting Chinese paocai. *Food Control* 19:50–5.

Yen G-C, Chang Y-C, Su S-W. 2003. Antioxidant activity and active compounds of rice koji fermented with *Aspergillus candidus*. *Food Chem* 83:49–54.

Yokotsuka T. 1991. Nonproteinaceous fermented foods and condiments prepared with koji molds. In: Arora DK, Mukerji KG, Marth EH, editors. *Handbook of applied mycology*, vol. 3. New York: Marcel Dekker. p 293–328.

Yonzan H, Tamang JP. 2009. Traditional processing of *Selroti*—a cereal-based ethnic fermented food of the Nepalis. *Indian J Traditional Knowl* 8(1):110–4.

Yonzan H, Tamang JP. 2010. Microbiology and nutritional value of *selroti*, an ethnic fermented cereal food of the Himalayas. *Food Biotechnol* 24(3):227–47.

Yoon JH, Kang SS, Mheen TI, Ahn JS, Lee HJ, Kim TK, Park CS, Kho YH, Kang KH, Park YH. 2000. *Lactobacillus kimchii* sp. nov., a new species from kimchi. *Int J Syst Evol Microbiol* 50:1789–95.

Yoshizawa K, Ishikawa T. 1989. Industrialization of saké manufacture. In: Steinkraus KH, editor. *Industrialization of indigenous fermented foods*, vol. 3. New York: Marcel Dekker. p 127–68.

Yuan G-F. 1994. Isolation and identification of the microorganisms using for fermented soy cheese (Sufu) in Taiwan. In: Komogata K, Yoshida T, Nakase T, Osada H, editors. *The Proceedings of the International Workshop on Application and Control of Microorganisms in Asia*. Tokyo: The Institute of Physical and Chemical Research and Japan International Science and Technology Exchange Center. p 189–95.

Zhang J-H, Tatsumi E, Ding C-H, Li L-T. 2006. Angiotensin I-converting enzyme inhibitory peptides in douche, a Chinese traditional fermented soybean product. *Food Chem* 98:551–7.

Zhang J-H, Tatsumi E, Fan J-F, Li L-T. 2007. Chemical components of *Aspergillus*-type Douchi, a Chinese traditional fermented soybean product, change during the fermentation process. *Int J Food Sci Technol* 42:263.

Part II

Soy Products

5

Soy Sauce: Typical Aspects of Japanese Shoyu and Indonesian Kecap

Hanifah Nuryani Lioe, Anton Apriyantono, and Masaaki Yasuda

CONTENTS

5.1 Introduction

Soy sauce is a typical condiment and seasoning made from soybeans in a two-step fermentation procedure. The first step is a solid fermentation by fungi, followed by liquid fermentation in a high-concentration brine solution of osmophilic lactic acid bacteria and yeasts. These two fermentation steps involve three microbial groups that give the product a desirable taste, flavor, and color, serving as a basic condiment and seasoning for the cuisines of several Asian countries. The soybeans used for its production can be from yellow or black soybean varieties.

Soy sauce originated from China and has, over the centuries, spread to Japan and Southeast Asia including Indonesia. In Japan, it is called *shoyu*, whereas in Indonesia, it is called *kecap.* Shoyu is made mostly from yellow soybeans; on the other hand, kecap is traditionally made from black soybeans. Both soy sauce products have different chemical and sensorial characteristics, primarily because of the different molds, bacteria, and yeasts as well as the different raw material compositions used in their production. In addition, kecap typically has a deeper brown color and a thicker viscosity than that of common soy sauce because of the sugar added in the final process of production.

There are several types of shoyu and kecap, which will be described subsequently. Generally, in todays' market, soy sauce is sold in three different categories, that is, fermented soy sauce, chemical or artificial soy sauce, and half-fermented soy sauce with a combination of the fermentation process and the addition of chemical soy sauce. The chemical soy sauce is made by rapid acid hydrolysis of soybean proteins at temperatures of 80°C or higher for a couple of hours, whereas the fermented variety needs several months of fermentation at room temperature between 25°C and 35°C to have complete protein

hydrolysis (Hesseltine and Wang 1980; Fukushima 1981). The two categories have wide differences in flavor and aroma characteristics. This chapter only deals with the fermented category.

5.2 Japanese Shoyu

5.2.1 History of Shoyu

Soy sauce originated from China approximately 2000 years ago and was formerly known as *chiang* and *shih*. Then, in the 16th century, it was produced as *chiang-yu*. Chiang and shih were introduced into Japan before AD 700, and since the 16th century, it has been produced as *shoyu* (which has the same Chinese characters as chiang-yu; Yokotsuka 1986). Chiang was produced using soybeans and wheat as raw materials at a ratio of 3:2. This ratio is close to a type of shoyu called *koikuchi shoyu*. On the other hand, shih uses a high proportion of soybeans with or without a small proportion of wheat, and is known as the origin of *tamari shoyu* (Fukushima 1985, 1989).

The basic procedure for the mass production of shoyu in Japan was formulated in the 17th century. It has been exported to India, Southeast Asia, and even to Europe according to a record of the Dutch East India Company stored in the archives in the Hague, Holland (Fukushima 1989). According to those records, the soy sauce products were thermally sterilized and transported in iron or ceramic containers with stoppers.

5.2.2 Types of Shoyu

There are five types of shoyu based on the different characteristics of color, viscosity, taste, and aroma. These are koikuchi shoyu, usukuchi shoyu, tamari shoyu, saishikomi shoyu, and shiro shoyu (Fukushima 1981, 1985, 1989; Yokotsuka 1981, 1986; Flegel 1988). The regular type for daily use in Japan is koikuchi shoyu. The different characteristics are yielded primarily due to the different raw material composition of soybeans and wheat, and the fermentation process used.

Koikuchi, usukuchi, and saishikomi shoyu use the same composition of raw materials in their production, that is, an equal amount of soybeans and wheat; however, they undergo different fermentation processes. Koikuchi shoyu has a strong aroma and a deep reddish-brown color because a relatively long brine fermentation process is applied. It is known that koikuchi production reaches 80% or more of the total shoyu production in Japan. Usukuchi shoyu has a lighter color and milder aroma as compared with koikuchi shoyu. These properties are achieved because of a shorter brine fermentation process in which the color and aroma development is reduced. Saishikomi shoyu has a specific full aroma and taste due to the use of raw soy sauce (without pasteurization) instead of the salt solution for the brine fermentation (Yokotsuka 1960, 1981, 1983, 1986; Whitaker 1978; Fukushima 1981, 1985, 1989).

Tamari shoyu uses a high proportion of soybeans with 10% or less of wheat in its production. This type of shoyu has a milder aroma, but has a greater viscosity and a darker color than that of koikuchi shoyu. With the contrast in composition of raw materials compared with tamari shoyu, that is, a very high proportion of wheat to soybeans, shiro shoyu has a bright yellowish color due to the fermentation conditions, which prevent color development (Yokotsuka 1960, 1981, 1986; Fukushima 1981, 1985, 1989).

5.2.3 Shoyu Manufacturing Process

In general, shoyu is produced in two main steps of fermentation: the first step is to make koji, and the second step is fermentation to yield moromi. The two steps belong to solid and liquid fermentation, respectively. After the fermentation process, there is a refining process to filter or extract the last fermentation product and pasteurize it.

Some aspects of shoyu production, which are different from those of oriental soy sauce or kecap, are described as follows: (1) the use of wheat at relatively greater amounts as a raw material in addition to soybeans; (2) protein in the raw materials is almost completely degraded by enzymes from koji; (3) in the moromi process, extensive lactic and alcoholic fermentations occur; and (4) it is subjected to

pasteurization to give a strong aroma, flavor, and color to the final product (Whitaker 1978; Hesseltine and Wang 1980; Yokotsuka 1981, 1986; Fukushima 1985, 1989). The production scheme of Japanese soy sauce or shoyu is shown in Figure 5.1.

5.2.3.1 Koji Preparation

The traditional koji preparation in Japan has now been replaced by a fully equipped process. This achievement allows the automatic koji preparation using a continuous soybean cooker, a continuous wheat roaster, automatic inoculators, an automatic mixer, large perforated shallow vats equipped with forced air devices, a temperature control device, and a mechanical device for turning the koji during incubation (Fukushima 1985).

Koji is prepared from cooked soybeans or defatted soybean flakes or grits mixed with roasted wheat. The soybeans are cooked in a continuous cooker at a high temperature and pressure but only for a short time, whereas wheat is roasted on a continuous roaster at 170°C to 180°C for a few minutes and then crushed slightly. In koikuchi, usukuchi, and saishikomi shoyu production, the same proportion of cooked soybeans and roasted wheat is mixed and then inoculated with a pure koji starter of *Aspergillus oryzae* or *Aspergillus sojae*. The raw material proportion for tamari shoyu production is 90% cooked soybeans and 10% roasted wheat, in contrast with shiro shoyu production, in which the roasted wheat may reach 90% of the total mix. Certain species of Aspergilli are also used in the production of usukuchi and shiro shoyu to prevent a deep color development in the next step of fermentation.

In contrast to the three soy sauces, tamari shoyu uses *Aspergillus tamarii* in addition to the two aspergillus species previously mentioned to obtain a full-bodied taste (Yokotsuka 1986; Flegel 1988). Koji preparation lasts for 3 days at temperatures of 25°C to 35°C to yield koji, which has a green-yellow color; the color comes from the spores on the material surface and is produced by molds (Yokotsuka 1960,

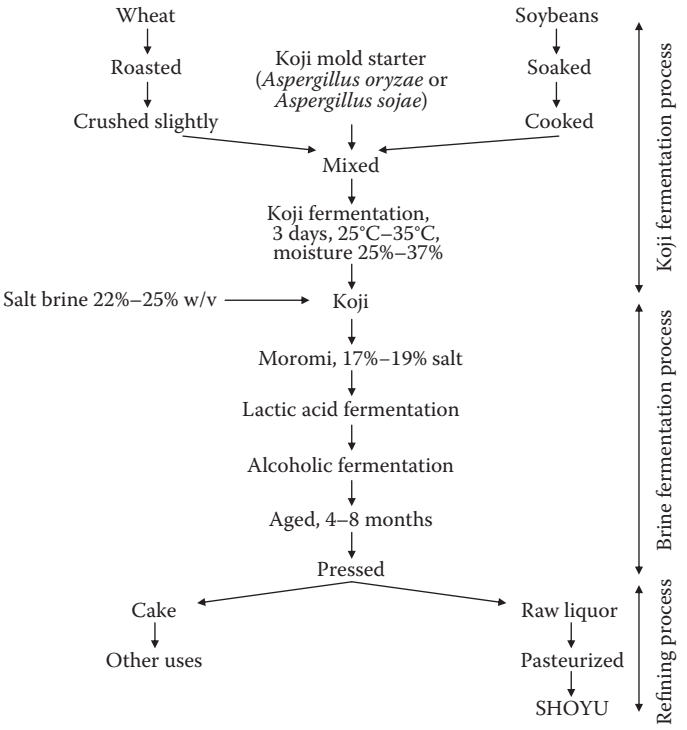

FIGURE 5.1 Scheme of *shoyu* manufacturing process. (From Hesseltine, C.W., and H.L. Wang, in *Soybeans: Chemistry and Technology*, Vol. 1, Proteins, 2nd ed., edited by Smith A.K., S.J. Circle, 389–419, AVI Publishing, Westport, CT, 1980. With permission. Fukushima, D., *Food Reviews International*, 1, 149–209, 1985. With permission.)

1981, 1983, 1986; Yong and Wood 1977; Whitaker 1978; Hesseltine and Wang 1980; Fukushima 1981, 1985, 1989; Flegel 1988; Chou and Ling 1998).

5.2.3.2 Moromi Process

This process is initially prepared by mixing koji with a brine solution at a sodium salt concentration range of 22% to 25% w/v in deep fermentation tanks (Fukushima 1985). The mix is called moromi or moromi mash. The mixing ratio is determined based on the final sodium salt concentration desired in the mix. A ratio of 1.2 to 1.3 parts of brine solution to 1 part of koji is commonly applied. The final salt concentration desired ranges from 17% to 19% to effectively prevent the growth of undesirable microorganisms (Hesseltine and Wang 1980; Fukushima 1985). An exception is found in saishikomi shoyu production, which uses raw soy sauce liquor instead of a fresh brine solution.

In the moromi process, osmophilic lactic acid bacteria and yeasts produce a characteristic taste, aroma, and color. High amounts of proteins and carbohydrates from the raw materials are degraded extensively by enzymes from koji (Yong and Wood 1977; Whitaker 1978) and by the microbial action (Noda et al. 1980). The pure cultures of osmophilic lactic acid bacterium *Pediococcus halophilus* and osmophilic yeast *Saccharomyces rouxii* are added to obtain a degree of desirable flavor and color (Fukushima 1985, 1989; Yokotsuka 1986). The length of the moromi process ranges from 4 to 8 months, depending on its temperature, agitation, and air supply by an air compressor (Yokotsuka 1986). The moromi mash is occasionally stirred and aerated to provide homogeneous conditions and to stimulate microbial growth.

The first stage of the moromi process is dominated by lactic acid fermentation, which results in a decrease in moromi pH, followed by alcoholic fermentation with osmophilic yeasts which provide a lower moromi pH and a substantial release of alcohol. In koikuchi and usukuchi shoyu production, *S. rouxii* gives a vigorous alcoholic fermentation due to the availability of starch from wheat. On the other hand, in tamari shoyu production, the alcoholic fermentation is much reduced due to the shortage of carbohydrates from its raw materials.

5.2.3.3 Refining Process

The refining process consists of filtration of the moromi mash, pasteurization, and sedimentation. The filtration is usually done by using cloth under an increasing hydraulic pressure until the residue has 25% or less of water. The resulting filtrate is called raw soy sauce. This raw liquor is pasteurized at 70°C to 80°C by a plate heater. This last process is very important for the development of strong flavor and reddish-brown color. It is also necessary to inactivate the enzymes and to reduce undesirable microorganisms such as film-forming yeasts (Hesseltine and Wang 1980; Fukushima 1985, 1989; Yokotsuka 1986).

After sedimentation of the pasteurized liquor, the supernatant is bottled, packed, and marketed. To minimize the strong flavor of usukuchi shoyu, a saccharification autolysis procedure is applied by adding a digested rice koji to the moromi mash before filtration to lower the number of reducing carbohydrates which could cause a reduction of flavor development during heating in the pasteurization process (Fukushima 1989).

5.2.4 Microbiology, Chemistry, Biochemistry, and Sensory Characteristics of Shoyu

In koji preparation, the release of extracellular enzymes from molds was observed in an experiment performed by Yong and Wood (1977). The enzymes consist of protease and carbohydrase complexes. These enzymes play an important role in the subsequent brine fermentation. Protease complexes, including glutaminase, hydrolyze proteins into small molecular weight peptides and amino acids, whereas carbohydrase complexes hydrolyze carbohydrates into sugars (Kuroshima et al. 1969; Yong and Wood 1977; Whitaker 1978; Hesseltine and Wang 1980; Fukushima 1981, 1985, 1989; Yokotsuka 1983, 1986; Flegel 1988). The main free amino acids released by the enzymes' action are glutamine and glutamic acid (Flegel 1988), which are known as the major amino acid residues in soybean and wheat proteins

(Fujiwara et al. 1962a; Kuroshima et al. 1969; Fukushima 1991). The release of glutamic acid is related to the umami taste of shoyu (Nishimura and Kato 1988; Lioe et al. 2010).

In the second step of shoyu fermentation, the sodium salt concentration of moromi mash, averaged at 18%, limits the growth of microorganisms. The microbes in this fermentation belong to halophilic bacteria and osmophilic yeasts. At the earlier stages, lactic acid fermentation occurring because of *P. halophilus* resulted in a pH decrease of 6.5 to 7.0, to lower than 5. The next stage is alcoholic fermentation by *Zygosaccharomyces rouxii* or *S. rouxii*. Then, at the last stage, *Torulopsis* sp. or *Candida* sp. grow to produce phenolic and aromatic compounds (Yokotsuka 1960, 1983, 1986; Fujiwara et al. 1962a; Kuroshima et al. 1969; Whitaker 1978; Hesseltine and Wang 1980; Noda et al. 1980; Fukushima 1981, 1985, 1989; Inamori et al. 1984; Chou and Ling 1998; Kinoshita et al. 1998).

The chemical compositions of five types of shoyu are presented in Table 5.1. The ratio of inorganic to organic constituents is nearly 1:1. The inorganic components are primarily sodium and chlorine, which comprise 46% of the total soluble solid, whereas the organic components consist of approximately 25% free amino acids and small peptides, 13% sugars, 3% organic acids, and 5% alcohols (Yokotsuka 1986). The ratio of free amino acids to small peptides is approximately 1:1 (Yokotsuka 1960, 1983, 1986; Whitaker 1978; Fukushima 1985, 1989). Glutamic acid and aspartic acid are the major amino acids found in shoyu (Fujiwara et al. 1962b, 1962c; Kaneko et al. 1994; Chou and Ling 1998). Lactic acid and glucose are the main organic acid and sugar, respectively. It has been proved that the presence of lactic acid can be used to distinguish the fermented soy sauce from the chemical one (Fukushima 1981, 1985, 1989; Kaneko et al. 1994).

The delicious taste of shoyu primarily comes from its nitrogenous compounds, that is, free amino acids and small peptides that are found in their low molecular weight fractions of less than 500 Da, which have the highest intensity of umami taste compared with the fractions higher than 500 Da (Lioe et al. 2007). It is already known that free amino acids and some peptides contain some flavor (Solms 1969; Yamasaki and Maekawa 1978; Nishimura and Kato 1988; Nakata et al. 1995). The small nitrogenous compounds as well as the sugars and organic acids formed during brine fermentation together make up soy sauce taste and quality (Yokotsuka 1960; Noda et al. 1980; Lioe et al. 2007, 2010).

Glutamic acid has a pure umami taste in the presence of sodium salt (Ikeda 1908; Fuke and Shimidzu 1993; Lioe et al. 2005). In shoyu, it has been revealed that free amino acids consist of umami amino acids such as glutamic acid together with sweet amino acids such as alanine, serine, and glycine giving a strong delicious taste to koikuchi shoyu, whereas the taste of tamari shoyu is mainly contributed by glutamic acid and aspartic acid (Lioe et al. 2006).

The small peptides contained in koikuchi shoyu and tamari shoyu were found in half of the total free amino acid content in their umami fractions, most of which have glutamyl residues. After further elucidation, the peptides contributing directly to the intense umami taste were considered to be negligible; however, an interaction between these peptides and free amino acids may have occurred (Lioe et al. 2006). Takeuchi et al. (1962) have reported that small peptides present in tamari shoyu comprise 19% of the total nitrogenous compounds, about twice of that in koikuchi shoyu. The length of the peptides in

TABLE 5.1

Chemical Composition of Shoyu Recognized by the Japanese Government

Type of Shoyu	NaCl (% w/v)	Total N (% w/v)	Sugars (% w/v)	Alcohol (% v/v)	pH
Koikuchi shoyu	17.6	1.6	3.8	2.2	4.7
Usukuchi shoyu	19.2	1.8	5.5	0.6	4.8
Tamari shoyu	19.0	2.6	5.3	0.1	4.8
Saishikomi shoyu	18.6	2.4	7.5	Trace	4.8
Shiro shoyu	19.0	0.5	20.2	Trace	4.6

Source: Fukushima, D., *Journal of the American Oil Chemists' Society*, 58, 346–354, 1981. With permission.

tamari shoyu are approximately 3 to 4, whereas in koikuchi shoyu, it is 2 to 3. Other researchers found three glucodipeptides and eight dipeptides in a neutral fraction of the Japanese soy sauce which had no flavor (Oka and Nagata 1974a), whereas four peptides, ten glycodipeptides, and two tripeptides found in an acidic fraction had a slight umami taste, considered less than 12% of the monosodium glutamate taste (Oka and Nagata 1974b). Moreover, the other peptides isolated by Kirimura et al. (1969) had bitter and astringent tastes.

Approximately 150 flavor volatiles or aroma compounds were identified in shoyu, including organic acids, alcohols, esters, carbonyls and related compounds, phenolic compounds, lactones, pyrazines, sulfur-containing compounds, and terpenes (Yokotsuka 1986). These are formed during wheat roasting, koji and moromi fermentation, and pasteurization. None of the known compounds affect the character of shoyu aroma. Among the flavor volatiles, 4-hydroxy-2(or 5)-ethyl-5(or 2)-methyl-3-(2*H*)-furanone (HEMF) is the most abundant in shoyu, and is formed through Maillard browning reaction during shoyu processing (Yokotsuka 1986). HEMF can be present at concentrations of 100 to 300 ppm (Yokotsuka 1986; Fukushima 1989; van der Sluis et al. 2001). Interestingly, HEMF, which is actually less volatile, may give a full-bodied taste, recognized as a sweet and caramel-like taste, especially to koikuchi shoyu. However, the compound is rarely found in tamari shoyu, which uses very little or no wheat in its production.

Half of the color development of shoyu occurs during the roasting of wheat, shoyu fermentation, and moromi aging, whereas the rest of the color development occurs during pasteurization as observed from the color intensity scores observed during the shoyu processing procedure (Whitaker 1978; Yokotsuka 1986). This color development is most likely due to Maillard browning reaction between amino compounds and reducing sugars (Whitaker 1978; Yokotsuka 1986; Fukushima 1989; Villamiel et al. 2006).

5.2.5 Shoyu Manufacturers in Japan

According to a literature review by Fukushima (1989), there are five large shoyu manufacturers in Japan. These are Kikkoman, Yamasa, Higashimaru, Higeta, and Marukin companies, whose market shares total more than 50%. The total shoyu production of these companies was reported to be as high as 1.2 million kiloliters per year.

5.3 Indonesian Kecap

5.3.1 A Brief History of Kecap

Until two decades ago, kecap was widely produced in Indonesia by small-scale operators (Röling et al. 1996; Apriyantono et al. 1999) using only black soybeans as a raw material (Judoamidjojo 1987; Röling et al. 1996; Apriyantono et al. 1999). This traditional product was introduced several centuries ago. However, in the last two decades, the kecap available in the markets has been dominantly produced by several soy sauce manufacturers using yellow soybeans and wheat as raw materials and applying modern Japanese technology for its fermentation processes (Röling et al. 1996; Apriyantono et al. 2004).

The kecap produced by either small-scale producers or manufacturers has the same types, these are *kecap asin* (salty soy sauce) and *kecap manis* (sweet thick soy sauce). Kecap asin has similar physical and taste characteristics as well as similar production steps as tamari shoyu and common soy sauce. In contrast, kecap manis has a sweet and darker color with a typical aroma due to further heat processing for 1 hour with the addition of brown sugar to approximately 50% w/v, along with spices such as fennel and star anise (Apriyantono et al. 1999). The difference between kecap asin and kecap manis is the heating process after fermentation. Therefore, the flavor formed during the heating process in kecap manis is much more important than that formed by fermentation. Kecap manis is actually much more popular than kecap asin because of its sweet and *gurih* (tasty, umami) taste; thus, the term kecap in Indonesia can be directly attributed to kecap manis.

5.3.2 Basic Kecap Production

The same principle of shoyu fermentation is applied in kecap, that is, the process consists of two steps of fermentation, koji making or mold fermentation called *bungkil* fermentation in Indonesia, and brine fermentation to yield moromi called *baceman* fermentation (Judoamidjojo 1987; Röling et al. 1994; Apriyantono et al. 1999, 2004). The molds used in bungkil fermentation are a mix of *Aspergillus* sp. and *Rhizopus* sp. The length of fermentation is several days to 1 to 2 weeks at room temperature. The bungkil is then dried under the sun, and blown to remove any spores on its surface. Baceman fermentation is started by mixing dried bungkil with three to four times the amount of brine solution at a salt concentration range of 17.5% to 20% w/v (Röling et al. 1994; Apriyantono et al. 1999, 2004). In traditional baceman fermentation, spontaneous fermentation is allowed for 4 months or less and is done under the sun. The evaporation of some water on the surface of the baceman mix under the sun may avoid the formation of a microbial layer which can spoil the mix.

During traditional baceman fermentation, total nitrogenous compounds consisting of amino acids and small peptides substantially increased within 2 weeks of fermentation (Röling et al. 1994; Apriyantono et al. 2004) primarily due to the enzymes of bungkil. The growth of lactic acid bacterium *P. halophilus* (or *Tetragenococcus halophila*) was observed during the baceman process; however, the growth of yeasts was almost not detected because of the fewer carbohydrate sources (Röling et al. 1996; Apriyantono et al. 1999; Lioe et al. 2010). After the baceman process, the result of filtration is boiled with the addition of palm sugar (brown sugar) and spices for at least 1 hour to yield kecap (kecap manis) which has a relatively higher viscosity. Unlike traditional kecap, in industrial kecap fermentation, yeast fermentation is obviously present due to the use of wheat as one of the raw materials (Röling et al. 1996). Recently, some pure microbial starters have been used in kecap production as found in the shoyu fermentation process.

5.3.3 Flavor and Sensory Characteristics of Kecap

The flavor volatiles composition of kecap has been observed in kecap manis, extracted by a Likens–Nickerson apparatus with diethyl ether as the extraction solvent. The 98 volatiles found consisted of 16 acids, 4 aliphatic aldehydes, 4 aliphatic alcohols, 1 phenol, 6 aliphatic ketones and lactone, 21 furans, 10 pyrazines, 5 esters, 1 pyran, 1 pyridine, 3 pyrroles, 1 thiazole, 4 alicyclic hydrocarbons, 11 benzene derivatives, and 11 unknowns (Apriyantono et al. 1999). These compounds are mainly associated with the Maillard reaction that occurred during the heating process and in the presence of sugars. Some acids such as malic and citric acids derived from palm sugar were observed in the investigation. These acids may contribute to the typical taste of kecap.

The delicious taste of kecap is dominantly from nitrogenous compounds with molecular weights of less than 500 Da (Apriyantono et al. 2004; Lioe et al. 2004a,b). In this case, free acidic amino acids such as glutamic acid and aspartic acid, with sweet amino acid alanine and bitter amino acid phenylalanine in the presence of sodium salt could give the intense umami taste of kecap (Lioe et al. 2004b). Kecap or kecap manis is commonly marked by a sweet and umami taste (Apriyantono et al. 1999).

REFERENCES

Apriyantono A, Husain H, Lie L, Jodoamidjojo M, Puspitasari-Nienaber NL. 1999. Flavor characteristics of Indonesian soy sauce (kecap manis). In: Shahidi F, Ho C-T, editors. *Flavor Chemistry of Ethnic Foods*. p 15–31. New York: Kluwer Academic/Plenum Publishers.

Apriyantono A, Setyaningsih D, Hariyadi P, Nuraida L. 2004. Sensory and peptides characteristics of soy sauce fractions obtained by ultrafiltration. In: Shahidi F, Spanier AM, Ho C-T, Braggins T, editors. *Quality of Fresh and Processed Foods*. New York: Kluwer Academic/Plenum Publishers. p 213–26.

Chou C-C, Ling M-Y. 1998. Biochemical changes in soy sauce prepared with extruded and traditional raw materials. *Food Res Int* 31:487–92.

Flegel TW. 1988. Yellow-green *Aspergillus* strains used in Asian soybean fermentations. *ASEAN Food J* 4:14–30.

Fujiwara K, Ishikawa H, Nanba A. 1962a. Studies on amino acids in Japanese soy sauce. (III) Changes in the amounts of the main amino acids during the mass process accelerated with elevating temperature. *Hakko Kogaku Zasshi* (in Japanese) 40:384–9.

Fujiwara K, Tanaka S, Fujita E. 1962b. Studies on amino acids in Japanese soy sauce. (I) The contents of amino acids in soy sauces of various types. *Hakko Kogaku Zasshi* (in Japanese) 40:321–7.

Fujiwara K, Tokuda M, Nanba A. 1962c. Studies on amino acids in Japanese soy sauce. (II) Changes in the amounts of the main amino acids during ordinary mass process. *Hakko Kogaku Zasshi* (in Japanese) 40:327–32.

Fuke S, Shimizu T. 1993. Sensory and preference aspects of umami. *Trends Food Sci Technol* 7:407–11.

Fukushima D. 1981. Soy proteins for foods centering around soy sauce and tofu. *J Amer Oil Chem Soc* 58:346–54.

Fukushima D. 1985. Fermented vegetable protein and related foods of Japan and China. *Food Rev Int* 1: 149–209.

Fukushima D. 1989. Industrialization of fermented soy sauce production centering around Japanese shoyu. In: Steinkraus KH, editor. *Industrialization of indigenous fermented foods*. New York and Basel: Marcel Dekker. p 1–88.

Fukushima D. 1991. Recent progress of soybean protein foods: chemistry, technology, and nutrition. *Food Rev Int* 7:323–51.

Hesseltine CW, Wang HL. 1980. Fermented soybean food products. In: Smith AK, Circle SJ, editors. *Soybeans: Chemistry and Technology*. Vol. 1, Proteins, 2nd ed. Westport, CT: AVI Publishing. p 389–419.

Ikeda K. 1908. On a new seasoning. *J Tokyo Chem Soc* (in Japanese) 30:820–6.

Inamori K, Miyauchi K, Uchida K, Yoshino H. 1984. Interaction between *Pediococcus halophilus* and *Saccharomyces rouxii* (microorganisms involved in shoyu moromi fermentation. Part I). *Nippon Nogeikagaku Kaishi* (in Japanese) 58:771–7.

Judoamidjojo M. 1987. The studies on kecap—indigenous seasoning of Indonesia. *Memoirs of the Tokyo Univ Agric* 28:100–59.

Kaneko K, Tsuji K, Kim CH, Otoguro C, Sumino T, Aida K, Sahara K, Kaneda T. 1994. Contents and compositions of free sugars, organic acids, free amino acids and oligopeptides in soy sauce and soy paste produced in Korea and Japan. *Nippon Shokuhin Kogyo Gakkaishi* (in Japanese) 41:148–56.

Kato H, Rhue MR, Nishimura T. 1989. Role of free amino acids and peptides in food taste. In: Teranishi R, Buttery RG, Shahidi F, editors, *Flavor Chemistry: Trends and Developments*. Washington, DC: American Chemical Society. p 158–74.

Kawai M, Okiyama A, Ueda Y. 2002. Taste enhancements between various amino acids and IMP. *Chem Sens* 27:739–45.

Kinoshita E, Sugimoto T, Ozawa Y, Aishima T. 1998. Differentiation of soy sauce produced from whole soybeans and defatted soybeans by pattern recognition analysis of HPLC profiles. *J Agric Food Chem* 48:877–83.

Kirimura J, Shimizu A, Kimizuka A, Ninomiya T, Katsuya N. 1969. The contribution of peptides and amino acids to the taste of foodstuffs. *J Agric Food Chem* 17:689–95.

Kuroshima E, Oyama Y, Matsuo T, Shugimori T. 1969. Biosynthesis and degradation of glutamic acid in microorganisms relating to the soy sauce brewing. (III) Some factors affecting the glutamic acid and its related substances formation in soy sauce brewing. *Hakko Kogaku Zasshi* (in Japanese) 47:693–700.

Lioe HN, Apriyantono A, Fardiaz D, Satiawihardja B, Ames JM, Inns EL. 2004a. Savory peptides present in moromi obtained from soy sauce fermentation of yellow soybean. In: Hofmann T, Ho C-T, Pickenhagen W, editors. *Challenges in Taste Chemistry and Biology, ACS Symposium Series 867.* Washington, DC, USA: American Chemical Society. p 180–94.

Lioe HN, Apriyantono A, Takara K, Wada K, Naoki H, Yasuda M. 2004b. Low molecular weight compounds responsible for savory taste of Indonesian soy sauce. *J Agric Food Chem* 52:5950–6.

Lioe HN, Apriyantono A, Takara K, Wada K, Yasuda M. 2005. Umami taste enhancement of MSG/NaCl mixtures by subthreshold L-α-aromatic amino acids. *J Food Sci* 70:401–5.

Lioe HN, Selamat J, Yasuda M. 2010. Soy sauce and its umami taste: a link from the past to current situation. *J Food Sci* 75:71–6.

Lioe HN, Takara K, Yasuda M. 2006. Evaluation of peptide contribution to the intense umami taste of Japanese soy sauces. *J Food Sci* 71:277–83.

Lioe HN, Wada K, Aoki T, Yasuda M. 2007. Chemical and sensory characteristics of low molecular weight fractions obtained from three types of Japanese soy sauce (shoyu)—koikuchi, tamari and shiro shoyu. *Food Chem* 100:1669–77.

Nakata T, Takahashi M, Nakatani M, Kuramitsu R, Tamura M, Okai H. 1995. Role of basic and acidic fragments in delicious peptides (Lys-Gly-Asp-Glu-Glu-Ser-Leu-Ala) and the taste behavior of sodium and potassium salts in acidic oligopeptides. *Biosci, Biotechnol, Biochem* 59:689–93.

Nishimura T, Kato H. 1988. Taste of free amino acids and peptides. *Food Rev Int* 4:175–94.

Noda F, Hayashi K, Mizunuma T. 1980. Antagonism between osmophilic lactic acid bacteria and yeasts in brine fermentation of soy sauce. *Appl Environ Microbiol* 40:452–7.

Oka S, Nagata K. 1974a. Isolation and characterization of neutral peptides in soy sauce. *Agric Biol Chem* 38:1185–94.

Oka S, Nagata K. 1974b. Isolation and characterization of acidic peptides in soy sauce. *Agric Biol Chem* 38:1195–202.

Röling WFM, Apriyantono A, van Verseveld HW. 1996. Comparison between traditional and industrial soy sauce (kecap) fermentation in Indonesia. *J Ferment Bioeng* 81:275–8.

Röling WFM, Timotius KH, Prasetyo AB, Stouthamer AH, van Verseveld HW. 1994. Changes in microflora and biochemical composition during the baceman stage of traditional Indonesian kecap (soy sauce) production. *J Ferment Bioeng* 77:62–70.

Solms J. 1969. The taste of amino acids, peptides and proteins. *J Agric Food Chem* 17:686–8.

Takeuchi T, Kato H, Yoshii H. 1962. Studies on the peptides in miso and soy sauce. (II) On the peptide composition of soy sauce. *Hakko Kogaku Zasshi* (in Japanese) 40:379–84.

Tanaka T, Saito N, Okuhara A, Yokotsuka T. 1969. Studies on the taste of α-amino acids. II. Ternary synergism of palatable taste of α-amino acids (1). Nippon Nogeikagaku Kaishi (in Japanese) 43:171–6.

Whitaker JR. 1978. Biochemical changes occurring during the fermentation of high-protein foods. *J Food Technol* 32:175–80.

Yamasaki Y, Maekawa K. 1978. A peptide with delicious taste. *Agric Biol Chem* 42:1761–5.

Yokotsuka T. 1960. Aroma and flavor of Japanese soy sauce. *Adv Food Res* 10:75–134.

Yokotsuka T. 1981. Recent advances in shoyu research. In: Charalambous G, Inglett G, editors, *The Quality of Foods and Beverages*, Vol. 2. New York: Academic Press. p 171–96.

Yokotsuka T. 1983. Soy sauces. In: Steinkraus KH, editor. *Handbook of indigenous fermented foods*, Vol. 9. New York: Marcel Dekker. p 437–51.

Yokotsuka T. 1986. Soy sauce biochemistry. *Adv Food Res* 30:195–329.

Yong FM, Wood BJB. 1977. Biochemical changes in experimental soy sauce koji. *J Food Technol* 12:163–75.

6

Synbiotic Soy Beverages: Principles and Sensory Attributes

Olga Lucía Mondragón-Bernal, Francisco Maugeri Filho, José Guilherme Lembi Ferreira Alves, and Maria Isabel Rodrigues

CONTENTS

6.1 Introduction

The incidence of problems such as intestinal dysbiosis, that is, imbalances in the intestinal flora caused by many aspects of modern life, have been growing in recent years. There is a worldwide trend in recognizing intestinal dysbiosis as a disease, and the conscientious consumers of the importance of healthful diets have been searching for foods that can improve this balance. Hydrosoluble soy extract is a raw material with great potential in the development of food with a healthy appeal such as functional synbiotic and fermented drinks (with probiotic and prebiotic substances). Synbiotic soyfoods need to be carefully developed, mainly with regard to sensory characteristics, because it already presents good functional characteristics; however, if the product's sensorial characteristics are unsatisfactory, this will not be well accepted by the consumers. During storage, it is common to observe that fermented products have problems such as syneresis, changes in sensorial characteristics such as color, taste, viscosity, and

other changes caused by high bacterial activity. For these problems, the trends of the new researches in this field are the optimization of the most important variables of the fermentative process, sensorial optimization of the functional soymilk beverages, shelf-life analysis, effects of several factors involved on product stability, determination of functional probiotic effects such as resistance to simulated conditions in the gastrointestinal tract, development of packs, and others. This chapter shows various methodologies and aspects of the development of functional and/or synbiotic fermented foods, specifically for soymilk-based products.

6.2 Definitions

6.2.1 Functional Foods

Functional foods could be naturally occurring foods, not just enriched or modified foods. The Institute of Medicine of the National Academy of Sciences limits functional foods to those in which the concentrations of one or more ingredients have been manipulated or modified to enhance their contribution to a healthful diet. The International Life Sciences Institute of North America defines functional foods as foodstuffs that, by virtue of physiologically active food components, proved health benefits beyond basic nutrition (ADA Reports 2004). Probiotics and prebiotics are functional foods because they offer benefits such as improving digestion (Guarner et al. 2005).

Only recently, great advances have been made in the development of the probiotic products (Lourens-Hattingh and Viljoen 2001) and synbiotic products (Morelli 2007; Morelli et al. 2003; Crittiden et al. 2001).

Probiotics, prebiotics (such as oligosaccharides), and synbiotics alter the composition of the intestinal microflora, restoring microbial balance; consequently, they have potential health benefits and are therefore regarded as functional foods (Mattila-Sandholm et al. 2002; Roberfroid et al. 1998). Synbiotic are food products that contain both probiotic and prebiotic ingredients (Crittiden et al. 2001; Gibson et al. 2004; Crittiden and Playne 1996; Gibson and Roberfroid 1995).

6.2.2 Probiotics

Probiotics are food supplements that present a high concentration of live microorganisms which are active all along the gastrointestinal tract, beneficially affecting the host by improving the balance of intestinal microbial flora (Guarner 1998, 2005; Crittiden and Playne 1996; Fuller 1992). Recently, there has been great progress in the development of so-called probiotic products (Tamime et al. 1995).

Microorganisms from the *Lactobacillus* genus and the bifidogenic group form part of the normal human intestinal microbiota (Crittenden et al. 2001; Tannock et al. 2000; Mitsuoka 1982). The reintroduction of these microbial groups into the host is done by the administration of selected species, which will have to be in high and viable numbers at the moment of consumption (Roberfroid 2000). An alternative to the increase of the bifidobacteria group in the gastrointestinal tract is the use of prebiotics (Gibson et al. 1995; Crittenden et al. 2001).

6.2.3 Prebiotics

Prebiotic substances are nondigestible food ingredients, fermented by a selected group of beneficial bacteria (Yun 1996) and are food ingredients that beneficially affect the host by selectively stimulating the growth and activity of one or a limited number or bacterial species already resident in the colon (Ferreira 2000).

Food ingredients classified as prebiotics must not be hydrolyzed or absorbed in the upper gastrointestinal tract (Ziemer and Gibson 1998), and when arriving at the large intestine, they are selectively used as a carbon source for the bifidobacteria present there (Gibson et al. 2004; Yun 1996). The consumption of these nondigestible ingredients has been shown to alter intestinal bacterial populations (Crittenden et al. 2001; Van Loo et al. 1999; Roberfroid et al. 1998; Fuller and Gibson 1997). The main

objective of providing prebiotics is to favor the development of the *Bifidobacterium* genus (Crittenden and Playne 1996).

The dietary components that fulfill those requirements, thus far, are oligosaccharides such as fructo-oligosaccharides, inulin, and lactulose, among others. Fructooligosaccharides are nondigestible sugars (for the human organism) that selectively stimulate the growth and activity of indigenous beneficial bacteria in the intestinal tract (Pinheiro 2002; Van Loo et al. 1999).

Oligosaccharides are sugars with three to ten monomeric units chained for α(1,2) links between fructose terminals and glucose, which may be either linear or branched, and are characterized by number, type, and sequence of its monosaccharide moieties (Roberfroid et al. 1998; Tamime et al. 1995). Synthetic oligosaccharides have been extensively studied and several patents (in Japan, Brazil, and Europe) already exist (Hernalsteens 2006).

The α-galactosides, raffinose and stachyose, are oligosaccharides present in some vegetables such as leguminous vegetables, they are characterized as nondigestives for the human intestine, having prebiotic effects, and as the cause of digestive problems such as flatulence (Scalabrini et al. 1998). Most mammals do not express the pancreatic α-galactosidase necessary to hydrolyze the α-1,6 linkages of these sugars. Soy products have an excellent nutritional status based on their high protein content; however, the presence of nondigestible oligosaccharides (raffinose and stachyose) in soybeans is not eliminated by the usual soy processing methods (Table 6.1). In soybeans, α-galactosides represent 4% to 6% of the dry mass (LeBlanc et al. 2004).

Mackellar and Modler (1989) verified that bifidobacteria possessed hydrolytic enzymes known as inulinase (β-2,1-D-fructano-fructanohidrolyse), which hydrolyze fructooligosaccharides, in contrast with digestive enzymes in humans and animals.

Bifidobacterium strains possess high-activity α-galactosidase and β-galactosidase (Hou et al. 2000). Scalabrini et al. (1998) showed that bifidobacteria can be used for biotechnological processes that utilize soymilk as a substrate; using products with low levels of α-D-galactosyl oligosaccharides and alkylic aldehydes, such as n-hexanal and pentanal, an unpleasant off-flavor, may be obtained. Hou et al. (2000) observed a reduction of raffinose and stachyose and an increase in the contents of monosaccharides when soymilk was fermented with *Bifidobacterium infantis* CCRC and *Bifidobacterium longum* B6. This can be attributed to the hydrolytic reaction catalyzed by the α- and β-D-galactosidase produced by bifidobacteria and may be a practical approach to overcoming the flatulence factor in soybeans.

According to Chou and Hou (2000), it is difficult to manipulate bifidobacteria because it is anaerobic and does not tolerate acid environments well. As an alternative, prebiotics may be used to increase bifidobacterial counts in gastrointestinal treatment (Gibson 1994). According to Pineiro et al. (2008), prebiotics were originally defined by Gibson and Roberfroid (1995) as "healthful ingredients, nondigestives that beneficially affect the host when stimulating selectively the growth or a limited number of bacteria in the colon and improving the health of the host." A more recent definition establishes that "a prebiotic is an ingredient fermented selectively that allows specific changes in the composition and activity of gastrointestinal microbiotics and confers benefits to the well-being and health of the host" (Gibson et al. 2004).

Other authors explain that prebiotics are not metabolized substances in the small intestine, and that, arriving at the large intestine, are selectively used as a carbon source for the bifidobacteria currently present there. For one alimentary ingredient to be considered as a prebiotic, it must not be hydrolyzed or absorbed in the upper gastrointestinal tract, it must offer a beneficial effect to the host, it must selectively promote growth and stimulate the metabolic activity of a limited number of indigenous bacteria promoters of health (and not of any other kinds of bacteria), and it must modify colonic microbiota for a more healthful composition (Yun 1996; Gibson and Roberfroid 1995; Organização Mundial de Gastroenterologia 2009). The main objective for offering prebiotics is to encourage the development of the *Bifidobacterium* genus (Crittenden and Playne 1996).

In 2008, a report of the "Technician Meeting of the FAO on Prebiotics" was published and considered a more detailed definition that includes new prebiotics and shows, in more precise terms, the current understanding about the ecology of human microbiotics. The following is the revised definition: "A prebiotic is a not viable alimentary component that confers benefit to the health of the associated host with the modulation of its microbiotic" (Pineiro 2008). The foodstuff ingredients that satisfy this definition, at present, are the oligosaccharides (Pinheiro 2002; Gibson 1994). Oligosaccharides, such

TABLE 6.1

Chemical Composition of Soymilk and Cow's Milk (g/100 g)

	Milk	
Compound	**Soy**	**Cow**
Protein	3.6	3.3
Amino acids[a]		
Isoleucine	5.1	7.5
Leucine	8.3	11.0
Lysine	6.2	8.7
Methionine	1.4	3.2
Cysteine	1.7	1.0
AA total sulfuredes	3.1	4.2
Phenylalanine	–	–
Tyrosine	–	–
AA total aromatics	9	11.5
Threonine	3.8	4.7
Tryptophan	1.3	1.5
Valine	4.9	7.0
Fats	1.9	3.9
Carbohydrates	2.8	4.7
Sugars[b]		
Sucrose	4928.2	
Fructose	133.0	
Glucose + galactose	189.0	
Raffinose[c]	695.5	
Stachyose[c]	391.1	
Vitamins: B_2 complex		
Niacin	7.45	
Riboflavin	7.36	
Thiamine	0.33	
Ash	0.4	0.7
Water	91.3	87.4

Source: Mondragón-Bernal, O. L., *Desenvolvimento de uma bebida fermentada de soja contendo agentes probióticos e probióticos*, Thesis (master's degree in food engineering), Food Engineering Faculty, University of Campinas, Campinas, 2004; adapted from Tamime, Y., and R. Robinson, *Yogurt Science and Technology*, Woodhead Publishing Limited, Cambridge, 2001.

Note: Amino acids (g/16 g de N), sugars (mg/L), vitamins (mg/100 mL).

[a] Zangelmi, A. B. C., and M. A. Tagliolatto, Produtos de soja, leite, farinha e outros: Serie Tecnologia Agroindustrial. São Paulo: Governo do Estado de São Paulo, Secretaria da Indústria, Comércio, Ciência e Tecnologia, Coord. da indústria e comércio, p 97–132, 1982.

[b] Hou, J.-W., R.-C. Yu, and C.-C. Chou, Changes in some components of soymilk during fermentation with bifidobacteria, Food Res Int, 33, 393–7, 2000.

[c] Soy oligosaccharides.

as the fructooligosaccharides (Figure 6.1a), β-D-fructans, galactoligosaccharides, transgalact oligosaccharides, isomalt oligosaccharides, mananoligosaccharides (present in the cellular wall of yeasts), and inulin fulfill the conditions of prebiotics (Fooks et al. 1999). A prebiotic can be a fiber, but a fiber is not necessarily a prebiotic (Pineiro et al. 2008). The synthesis of oligosaccharides has been extensively studied and Japanese, Brazilian, and European patents exist for its production and use. Food-grade oligosaccharides can be produced enzymatically or chemically, as the enzymatic synthesis from simple sugars for the transglycosilation reaction of plants or of microbiological origin (Ekhart and

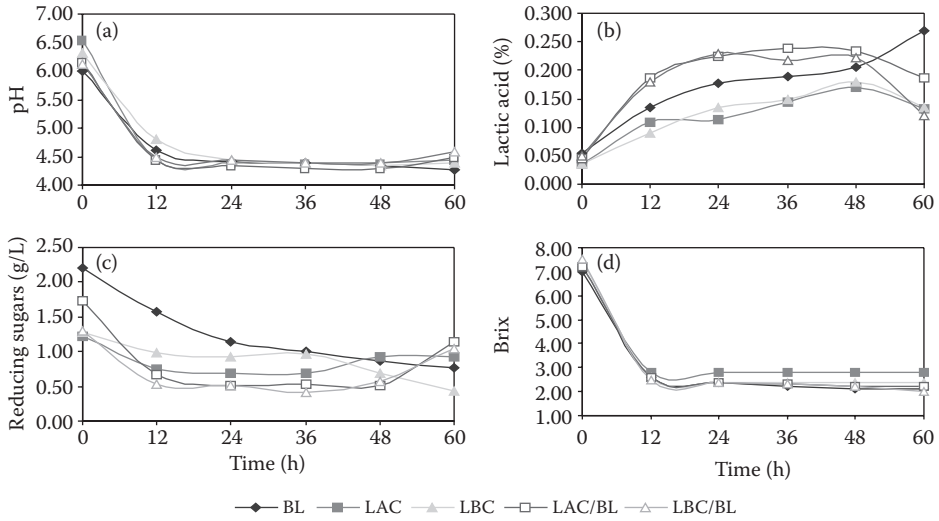

FIGURE 6.1 Fermentation kinetics for pH (a), titratable acidity (b), reducing sugars (*RS*) (c), and Brix (d) during individual and mixture of *B. longum* (*BL*), *L. acidophilus* (*LAC*), *L. paracasei* subsp. *paracasei* (*LBC*) fermentation in water-extracted soybeans at 37°C (Mondragón-Bernal 2004).

Timmermans 1996). In the colon, the fructooligosaccharides are quickly fermented by beneficial microbiotics, mainly bifidobacteria. These, in turn, inhibit the growth of harmful bacteria, such as *Clostridi*a, and such alteration in the composition of intestinal microbiotics can be beneficial for intestinal health and the sensation of well-being (Yun 1996). It also has an emergent variety of new prebiotics that include pectinoligosaccharides, lactosucrose, alcohol sugars, glucoligosaccharides, levanes, resistant starches, xiloligosaccharides, and soy oligosaccharides. These substances are being studied *in vitro* in varying degrees (in animal feeding studies, but rarely in studies being administered to human beings). New composites for the human diet that, if within the category of the European regulation of "novel foods," had previously required legislation to evaluate the toxicological levels and safety are now being included as food products (Pineiro et al. 2008). The oligosaccharides contained in some legumes, such as soybeans, include raffinose and stachyose and are characterized as being nondigestive for humans and to have prebiotic effect (Scalabrini et al. 1998).

Recently, a prebiotic effect has also been attributed to polydextrose. This substance is a polysaccharide synthesized for the randomized polymerization of glucose, in the presence of small amounts of sorbitol and one adequate acid catalyzer, under high temperature, and partial vacuum. Polydextrose is partially fermented in the large intestine, but it is not digested or absorbed in the small intestine, and a large proportion of it is excreted, having had a similar physiological effect as dietary fibers: it is conducive to the growth of beneficial microbiotics, reduces putrefactive microbiotics, produces short-chain fatty acids, and suppresses the production of carcinogenic metabolites (Jie et al. 2000).

6.2.4 Synbiotics

The positive influence of prebiotic substances, considered as soluble fibers, on intestinal flora has been tested in several studies, in which the utilization of probiotic species in combination with prebiotic substances provides a combined effect called "synbiotic" (Mattila-Sandholm et al. 2002; Crittenden et al. 2001). Therefore, synbiotic functional foods are those that supply both prebiotics and probiotics.

Given the potential synergy between probiotics and prebiotics, the foods that contains a combination of these ingredients are frequently called synbiotics, in which *syn* means synergy and *biotic* means life. The original term used in English was "symbiotic" (Mattila-Sandholm et al. 2002).

A functional product must contain at least 10^7 colony-forming units (CFU)/mL of viable probiotic bacteria for optimum therapeutic effects; however, the National Health Surveillance Agency of Brazil

(ANVISA 2002) allows these products, within their validity term, to have a minimum of 10^6 CFU/mL and a content of prebiotics which supplements the daily requirement of 8 g/day (Yun 1996; Modler 1994).

These synbiotic foods must contain at least 10^7 CFU/mL of viable probiotic bacteria for optimum therapeutic effects, a minimum of 10^6 CFU/mL within the validity date (ANVISA 2002), and prebiotics that supplement the daily requirement of 8 g/day (Yun 1996; Modler 1994). With regard to food fiber, a food claim for "fiber source" ought to contain at least 3.0 g/100 g for solid food and 1.5 g/100 mL for liquid food; whereas with regard to a claim for "high fiber content" solid foods should contain a minimum of 6.0 g/100 g whereas liquid foods should have 3.0 g/100 mL.

For instance, because the concept of synbiotics is relatively new, there are few specific studies of the interaction between probiotics and prebiotics (Mondragón-Bernal 2009). In the development of synbiotics, the selection of strains of microorganisms with a better capacity for the determined use of one prebiotic is necessary to obtain a synergistic effect in the implantation and proliferation of desirable bacteria (Ferreira and Teshima 2000). Mattila-Sandholm and collaborators (2002) explain that, despite the development of integrated synbiotics, beyond studies involving specific synergies between probiotic and prebiotic ingredients in the gastrointestinal tract, the need for studies of prebiotics that protect probiotics during the manufacture, storage, formulation, and gastrointestinal transit, that control the release of probiotics in specific places in the gastrointestinal tract and, moreover, that promote a slow fermentation with the production of short-chain fatty acids in the distal colon. The fermentation of carbohydrates by anaerobic bacteria such as bifidobacteria, lactobacilli, streptococci, and enterobacteria produces short-chain fatty acids; these substances reduce the pH in the distal colon and inhibit the growth of pathogenic bacteria and, as a consequence, the reduction of the putrefactive fermentation caused by these bacteria.

The addition of bifidogenic factors in the human diet normally favors an increase in the occurrence in the number of bifidobacteria alone in the fecal material, suggesting that supplementation in the diet would have to be implemented (Crittenden et al. 1996).

Hidaka and collaborators (1989) verified that supplying 8.8 g/day of acid fructooligosaccharides would increase the production of fatty acids. Mackellar and Modler (1989) showed that bifidobacteria possess hydrolytic enzymes known as inulinase (β-2,1-D-frutano-frutanohidrolase) that hydrolyze fructooligosaccharides, in contrast with the digestive enzymes of human and animals.

Studies performed *in vitro* have demonstrated that *B. longum* has difficulty in metabolizing long-chain polymers such as the inulina. *Bifidobacterium adolescentis* metabolized the inulina with more efficiency than *B. longum*, but *Bifidobacterium thermophilum* presented better growth and inulinase activity. *Bifidobacterium infantile* is capable of using short-chain fructooligosaccharides; however, they are incapable of metabolizing long-chain polymers with a degree of polymerization bigger than 25. Modler (1994) verified that the addition of fructooligosaccharides to the human diet (15 g/day), caused a 10-fold increase in the population of bifidobacteria in the large intestine, had a reduction of 0.3 units in the intestinal value of pH, and a decrease in the number of enterobacteria. Chou and Hou (2000) verified that during the addition of different saccharides and oligosaccharides in the fermentation of soymilk, the isomaltooligosaccharide (bifitose) only received a stimulant effect to the growth of *B. longum*, the bacterial count increased by 8.50 to 8.86 (log CFU/mL) after 48 hours of fermentation. Mondragón-Bernal (2004) obtained bacterial counts of approximately 10 log CFU/mL in 20 hours of fermentation for *B. longum*, in the presence of *Lactobacillus acidophilus* and *Lactobacillus paracasei* subsp. *paracasei*, with the addition of fructooligosaccharides at 2.5 g/L and soy oligosaccharides when the inoculum was 6 log CFU/mL. The probiotics, *B. longum* and *L. paracasei* subsp. *paracasei* pure, and the associated bacteria, *L. acidophilus* and *L. paracasei* subsp. *paracasei* and *bifidobacteria*, have acceptable growth in soy extract. Symbiotic relations were observed because of the protein availability and the reduction of acidity in the broth for *L. acidophilus*, and the availability of sugars for the bifidobacteria due to its β-galactosidase activity, fortifying the growth of *L. acidophilus*.

6.3 Soymilk Characterization

The hydrosoluble soy extract more recognized as "soymilk" shows good potential for the development of new products with health appeal, as well as being an alternative for people with allergies/eating

disorders from the ingestion of human or cow's milk, besides naturally containing oligosaccharides regarded as prebiotics to stimulate the growth of probiotics (Hou et al. 2000). Recent studies have indicated the beneficial health effects of consuming soy compounds. It is ranked as a functional food because it includes substances that reduce the chances of certain diseases from occurring. Soy may be used in both preventive and therapeutic manners in the treatment of cardiovascular diseases, cancer, osteoporosis, and menopausal symptoms (Donkor and Shah 2008; Chiarello 2002; Hasler 1998). Chou and Hou (2000), Hou et al. (2000), Scalabrini et al. (1998), and Tamime et al. (1995) have associated the presence of bifidogenic factors in soybean fat–free water extract as oligosaccharides, raffinose, and stachyose, aside from some others sugars such as sucrose, fructose, glucose, and galactose, as well as B complex vitamins and as a source of protein's nitrogen, make soymilk a complex medium and optimal substrate for the growth of probiotics such as *B. longum* and *L. paracasei* in pure and mixed cultures. However, it is frequently rejected because of its unfavorable leguminous flavor, mainly caused by the presence of n-hexanal (Hou et al. 2000; Tamime and Robinson 2001), a compound derived from the action of lipoxygenases (Ciabotti et al. 2007; Evangelista and Regitano-D'arce 1997; Murti et al. 1993; Okubo et al. 1992).

The nutritional facts of soymilk are similar in protein values (3–4%) in relation to cow's milk, have a deficient amount of sulfured amino acids (methionine and cysteine), is a good source of vitamin B complex (except for vitamin B_{12}), the calcium content is just 0.76% (18.5% of the calcium content of cow's milk), and is a source of raffinose and stachyose oligosaccharides, as observed in Table 6.1.

6.3.1 Description of Soymilk as a Substrate Used in the Development of Fermented Synbiotic Foods

The changes in some carbohydrates and organic acid production during fermentation of soybean water extract with probiotic bacteria (*L. acidophilus*, *L. paracasei* subsp. *paracasei*, and *B. longum*) in pure or mixed cultures was studied by Mondragón-Bernal (2004) and some results are shown in Figure 6.1. Data regarding the analytical assays of different soymilk-fermented beverages by probiotics are shown in Table 6.2. In Figures 6.2 and 6.3, the qualitative data for some sugars and oligosaccharides in extracts of soy with fermentation are shown. The presence of monosaccharides,

TABLE 6.2

pH, Reducing Sugars, and Total Reducing Sugars in the Fermentation of Soymilk by Probiotic Bacteria with and without the Addition of Sugar Syrup

Sample[a]	pH	RS (g/L)	TRS (g/L)
Soymilk fat–free, 7.5% (w/v) without fermentation	6.81	0.4	38.9
Soymilk fat–free + 25 g/L of syrup with fructooligosaccharides, 12.3% (w/v)	6.81	15.7	94.2
Fermented with *B. longum* for 24 hours	4.96	0.8	37.4
Fermented with *L. paracasei* subsp. *paracasei* for 20 hours	4.47	0.9	15.9
Fermented with *L. paracasei* subsp. *paracasei/B. longum* for 16 hours	4.92	1.8	24.4
Fermented with *L. acidophilus/B. longum* for 24 hours	4.92	0.1	23.2
Fermented with *L. acidophilus/L. paracasei* subsp. *paracasei/B. longum* for 24 hours	4.46	0.9	23.5
Fermented with *L. acidophilus/L. paracasei* subsp. *paracasei/B. longum* + 25 g/L of syrup with fructooligosaccharides, 12.3% (w/v) for 20 hours	4.01	9.3	69.3

Sources: Maugeri, F., O. Mondragón, and J. Mukuno, inventors; University of Campinas, assignee, Alimento funcional, composição probiótica, composição alimentícia e processo de produção de alimento funcional fermentado à base de extrato de soja, contendo agentes probióticos e prebióticos, BR Innovation Patent PI0504056-6, September 26, 2006. With permission; Mondragón-Bernal, O. L., *Desenvolvimento de uma bebida fermentada de soja contendo agentes probióticos e probióticos*, thesis (master's degree in food engineering), Food Engineering Faculty, University of Campinas, Campinas, 2004.

Note: RS, reducing sugars and TRS, total reducing sugars.

[a] Soymilk fat–free 7.5% (w/v) fermented at 37°C. Sample data corresponds to time when the counts of probiotic bacteria were greater than 1010 CFU/mL.

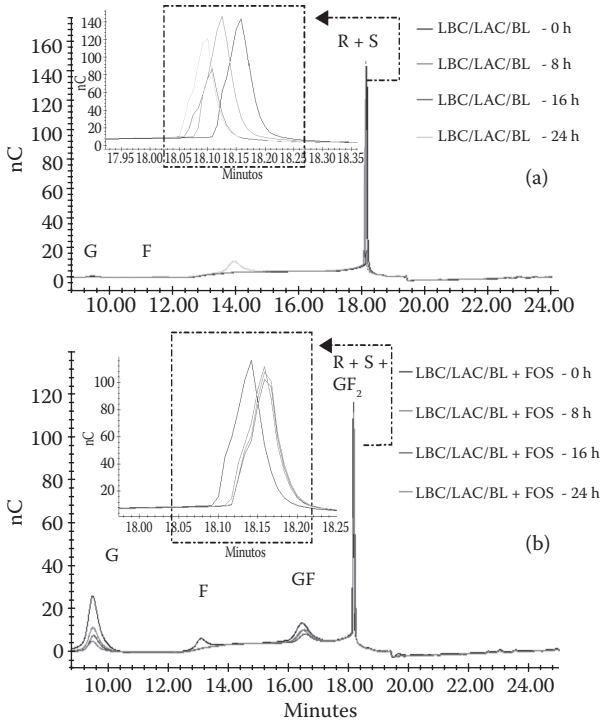

FIGURE 6.2 Carbohydrates of soybean fat–free water extract 7.5% during fermentation (a) without and (b) with the addition of fructooligosaccharides, during fermentation with mixture of *B. longum* (BL), *L. paracasei* subsp. *paracasei* (LBC), and *L. acidophilus* (LAC). Sample chromatograms of fermentation between 0 and 24 hours had overlapped. G, glucose; F, fructose; GF, sucrose; R, raffinose; S, stachyose; and GF_2, kestose (Mondragón-Bernal 2004).

disaccharides, and oligosaccharides such as glucose, fructose, sucrose, galactose, raffinose, and stachyose in water-extracted soybeans can be verified.

It is possible to appreciate, qualitatively, that the nonreducing sugar concentrations (sucrose and oligosaccharides) changed during fermentation, being hydrolyzed to reducing sugars and used, at some period, by the microorganisms as a carbon source (Table 6.2).

Analyses of carbohydrates by ion chromatography and high performance liquid chromatography (HPLC) have proved difficult because of the requirement for different columns to separate the carbohydrates. Using HPLC with pulsed amperometric detection (HPLC-PAD) chromatograph and column PA100, the separation of fructose and sucrose is possible; however, the fructooligosaccharides and

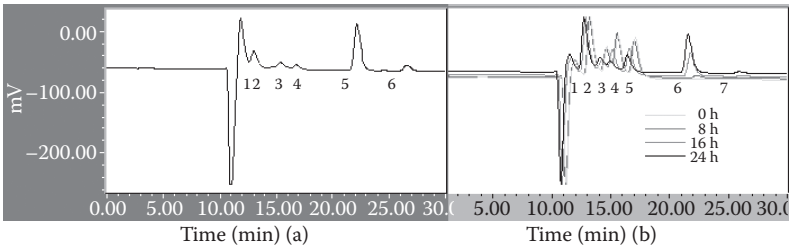

FIGURE 6.3 (a) Chromatogram of carbohydrates and organic acids of soybean fat–free water extract 7.5% fermented with *B. longum* at 4 and 24 hours. (b) Kinetic *L. acidophilus*/*L. paracasei*/*B. longum* in soybean fat–free water extract 7.5% + syrup with fructooligosaccharides (25 g/L). HPLC Column HPX 87H. (From Mondragón-Bernal, O. L. *Desenvolvimento de uma bebida fermentada de soja contendo agentes probióticos e probióticos*. Thesis (Master's in Food Engineering). Food Engineering Faculty, University of Campinas, Campinas. 2004.)

the sugars, raffinose and stachyose, overlapped, whereas the glucose and galactose presented the same retention times. Thus, column PA10 can be used for the separation of galactose, glucose, raffinose, and stachyose. On the other hand, sucrose and fructose overlapped and the fructooligosaccharides were not separated. Using HPLC equipped with refractive index detector and HPX 87H column, glucose, fructose, and galactose can be separated, but sucrose and raffinose overlap; stachyose and fructooligosaccharides cannot be separated chromatographically.

6.4 Fermentation Theory

6.4.1 Lactic Fermentation Theory

García et al. (1993) explains that all lactic acid bacteria (LAB) are saccharolytic and deficient in various metabolic ways. They have limited biosynthetic ability, having evolved in environments that are rich in amino acids, vitamins, purines, and pyrimidines; therefore, they must be cultivated in complex media that fulfill all their nutritional requirements, such as in the intestinal tract or in milk (Chou and Hou 2000). These strains are anaerobic but essentially aerotolerant. There are three metabolic pathways for carbohydrate degradation to lactic acid. The homofermentative pathway that produces lactate as a single product with a yield of 2 mol for each mole of glucose, and thus, its production is so interesting. The heterofermentative pathway results in the production of ethanol and carbon dioxide (and in some cases, acetic acid) in equimolar quantities to lactic acid. This transformation occurs through the metabolic pathway of pentoses. Finally, *Bifidobacterium* produces 1.5 mol of acetate and 1 mol of lactate for each mole of glucose in the bifidum pathway (García et al. 1993). Lactobacilli release lactic acid and some aldehydes (Tamime et al. 1995).

From the sensory point of view, lactic fermentation of the soy extract by *L. acidophilus* and *L. casei* contributes to the improvement not only of the product's flavor, but also of the aroma. The production of lactic acid and the proteolytic activity of these microorganisms, mainly of *L. casei*, accounts for the formation of aroma and flavor precursors, contributing to the elimination of undesirable taste, aromas, and flavors (Murti et al. 1993; Evangelista and Regitano-D'arce 1997). However, *Bifidobacterium* also produce acetic acid, which is unfavorable in the final product, representing an off-flavor (Mondragón-Bernal 2004; Lourens-Hattingh and Viljoen 2001; Cravero 2000; Chou and Hou 2000; Wang et al. 1994). This effect diminishes with fermentation using starter cultures in mixtures, such as *Lactobacilli* and bifidobacteria, producing better results in the balance of acidity and providing a more sensorially acceptable product. With starters from the fermentation of milks and other substrates such as soymilk by probiotic lactic bacteria (*Lactobacilli* and bifidobacteria), and with the presence of selected prebiotic agents, a "synbiotic" product is obtained as a functional food alternative (Mondragón-Bernal 2004).

6.4.2 Starter Technology

The starter cultures ferment sugars to produce lactic acid, which serve to acidify the product, preserve it, and give it flavor. They also hydrolyze proteins, changing the texture of the product (Batt 1999) and some have the capacity to produce exopolysaccharides (EPS) when fermented in a suitable medium (Tuinier et al. 2000; Gorret et al. 2003; Champagne et al. 2006; Grattepanche et al. 2007).

Soy-fermented beverages containing probiotics and prebiotics (symbiotic food), after fermentation by lactic bacteria, display a drop in pH that gives them sensorial characteristics such as viscosity and acidity. The fermented products must maintain their sensorial characteristics throughout storage, but it has been noted that pH continues to drop, with consequent syneresis (Mondragón-Bernal 2009).

For the reasons set out previously, this work studied some EPS-producing microorganisms, and a selection was made to choose the microorganism with the best characteristics of EPS production in the soy extract medium enriched with prebiotic agents and sweetened or not with saccharose. The objective is to obtain symbiotic products more stable during storage without requiring the use of chemical additives to this end. With the strain chosen, kinetic studies of fermentation and stability during storage were performed on symbiotic products based on water-soluble soy extract.

6.4.3 Exopolysaccharides Produced by Lactic Acid Bacteria

Starter cultures ferment sugars to produce lactic acid, which serves to acidify, preserve, and give flavor to the product. They also hydrolyze proteins, changing the product's texture (Batt 1999), and some of them have the capacity to produce EPS when fermented in the appropriate medium (Tuinier et al. 2000; Gorret et al. 2003; Champagne et al. 2006; Grattepanche et al. 2007).

Beneficial health effects have been attributed to several EPS-producing LAB (Chabot et al. 2001). *Lactobacillus rhamnosus* ssp. is an EPS-producing species with probiotic potential (Dupont et al. 2000; Macedo et al. 2002; Champagne et al. 2006).

Several authors have reported that species producing EPS are used in the production of fermented milks to enhance their rheological properties (de Vuyst et al. 1999; Ruas-Madiedo et al. 2006; Shihata and Shah 2002; Champagne et al. 2006; Grattepanche et al. 2007). In many cases, the polysaccharides released outside the cells by lactic bacteria may offer advantages in a variety of fermented food products (Cerning 1990). In actual fact, rather than using additives as enhancers of texture, stabilizers, emulsifiers, gellifiers, or antisyneresis agents in fermented foods, it may be convenient to use lactic bacteria that produce EPS as starter cultures (Smitinont et al. 1999).

Lactobacilli are often used as starters in the manufacture of lacteal products, as is the case of *L. rhamnosus* ssp., which is an ESP-producing species with probiotic potential (Dupont et al. 2000; Macedo et al. 2002; Champagne et al. 2006). Other species with potential for EPS production are *Lactococcus lactis* spp. (Turnier et al. 2000; Champagne et al. 2006; Grattepanche et al. 2007); *Leuconostoc* spp., *Leuconostoc fructosum*, *Leuconostoc mesenteroides* spp. (Hernalsteens 2002; Champagne et al. 2006); *Pediococcus acidilactici* var. (Smitinont et al. 1999), and *Proponibacterium* such as *Propionibacterium freudenreichii* spp. (Jan et al. 2000; Gorret et al. 2003; Tharmaraj and Shah 2004; Leverrier et al. 2005), and there is growing interest in the latter given its stimulant effects on probiotics, and also as these are producers of vitamin B_{12} (Tharmaraj and Shah 2004).

According to Grattepanche et al. (2007), the production of EPS by cultures in mixtures of lactobacilli was three times lower than in a pure culture of *L. rhamnosus* RW-9595M. The EPS interacts with the casein micella, improving the texture of the yogurt, increasing viscosity, and reducing susceptibility to syneresis (Hess et al. 1997; Grattepanche et al. 2007). Some lactic bacteria that produce EPS have been attributed a beneficial health effect (Chabot et al. 2002). *L. rhamnosus* ssp. is an EPS-producing species with probiotic potential (Dupont et al. 2000; Macedo et al. 2002; Champagne et al. 2006).

In the presence of polysaccharides, the denaturation temperature of some proteins is changed or the capacity for forming gels is modified. In solution, the proteins may attract and repel the polysaccharides depending on their origin, the pH, ionic force, the temperature, concentration or the shearing to which they are submitted (Braga 2006).

The properties of a protein gel, such as texture and syneresis, may be altered not only by the addition of polysaccharides, but also by variations in the ionic force and pH of the system. The formation of a protein network is the outcome of aggregation of the protein molecules, based on their prior denaturation and the acidification of the system, for example, through the action of bacterial culture. Utsumi and Kinsella (1985) report that the molecular forces involved in the formation of thermal gels from isolated soy proteins are hydrogen bridges and hydrophobic interactions, whereas the gel is maintained by bridges of hydrogen and disulfide.

During the fermentation of complex media (such as soy extract and milk) by LAB, the lactic acid produced causes an aggregation of the particles of proteins (globulins), leading to the formation of a fragile gel. The gel in which the bacterial cells, lactose, and other lesser components are retained is a structure of a highly complex network of proteins and EPS (Goh et al. 2005). The careful separation of the EPS from the non-EPS components, particularly proteins, lactose, and cells is necessary (Cerning 1990) because the inclusion of each one of these components may influence the results of the chemical methods used for the total determination of carbohydrates. Additionally, some methods do not take into consideration the contribution of the total of carbohydrates for the control samples (blanks). Moreover, the centrifuge speeds applied in various studies may not be suitable. The significant factors in each phase of a procedure for the production of EPS were studied by Goh et al. (2005) and include the use of enzymes in the hydrolysis of proteins in culture media based on milk or other complex proteins, the percentage of ethanol for

the precipitation of EPS, with 70% of the optimum to solubilize the lactose, and the implementation of an optimum centrifuge regime for the effective isolation of EPS precipitation.

In many cases, polysaccharides released extracellularly by lactic bacteria are advantageous in a variety of fermented food products (Cerning 1990). Actually, instead of using additives such as texture improvers, stabilizers, emulsifiers, jellifiers, or antisyneresis agents in fermented foods, it may be convenient to use EPS-producing lactic bacteria as starter cultures (Smitinont et al. 1999). EPS produced by LAB species, such as *L. rhamnosus*, are used in the production of fermented products to improve their rheological properties (de Vuyst et al. 1999; Ruas-Madiedo et al. 2006; Shihata and Shah 2002; Champagne et al. 2006; Grattepanche et al. 2007).

The LAB *L. rhamnosus* sp. was selected from several LAB lineages because of its growth in soy extract with high sucrose concentrations (12%) and its capacity to produce EPS. It has been observed that EPS produced by *L. rhamnosus* decreased syneresis in soy-based fermented synbiotic products and had a good association with standard inoculum containing *L. acidophilus*, *L. paracasei* subsp. *paracasei*, and *B. longum* probiotics. However, it was observed that EPS from *L. rhamnosus* produced less viscous fermented products (Mondragón-Bernal et al. 2007).

According to Braga (2006), the properties of a protein gel for texture and syneresis can be altered not only by the addition of polysaccharides but also through changes in the system's ionic force and pH. The formation of a protein network results from protein molecule aggregation from their previous denaturation or system acidification, which occurs during lactic fermentation by bacterial cultures. Utsumi and Kinsella (1985) explain that the molecular forces involved in the formation of thermal gels from soy protein isolates are hydrogen bridges and hydrophobic interactions, whereas gel is maintained by hydrogen and disulfide bridges.

During fermentation of complex media (such as soy extract and milk) by LAB, the produced lactic acid causes the aggregation of protein particles (globulins), leading to the formation of a fragile gel. The gel in which bacterial cells, sugars, and other smaller components are retained is a structure of a highly complex network of proteins and EPS (Goh et al. 2005).

According to Braga (2006), in the presence of polysaccharides, the denaturation temperature of some proteins is changed or the gel formation capacity is modified. In solution, proteins can attract and repel polysaccharides, depending on their origin, pH, ionic force, temperature, concentration, or the shear they are submitted to. Complex interactions between proteins and polysaccharides are the result of the physical properties of each pure biopolymer in solution. The physical properties of biopolymer solutions seem to be highly dependent on the presence of a co-solute such as sucrose and on thermal processing, which can also change the gel characteristics, being related to molecular conformation in solution, such as the formation of double helixes, ultra-aggregates, and molecular disorder. Braga (2006) found, in his studies, that the addition of sucrose in thermally treated solutions in the presence of some polysaccharides like xanthan gum affected the rheological properties of anisotropic or biphase solutions (mixed).

6.5 Scientific Tools to Novel Developments

Several scientific knowledge and statistical resources can be used in the development of novel foods with health claims, mainly functional soy foods such as synbiotics. These kinds of functional foods involve a lot of variables that influence its sensorial, functional, and physicochemical characteristics in different stages such as manufacturing, storage, and final consumption. Some of these scientific tools are kinetics studies, sensorial evaluations (including affective tests, just-about-right scales, magnitude scales to sweeteners, internal map of preferences, descriptive and quantitative analyses, and others), shelf life studies, rheological behaviors, experimental designs (a screening design and central composite rotatable design; CCRD), *in vitro* and *in vivo* functional and therapeutic studies such as survival, persistence of probiotic strains, and its effect on indigenous flora, among others.

A variety of technologies have been developed and aimed at producing soy extracts and derivatives with a lower intensity of flavor (Wang et al. 1994). It is possible to improve a product to meet consumer expectations by using sensory techniques that adjust to the fundamental characteristics of each type of food (Behrens et al. 2004).

6.5.1 Development Technologies of Synbiotic Food Production

6.5.1.1 Kinetics Studies

Kinetics studies are necessary to carry out and challenge with the aim of acquiring a profound knowledge of the development and growth of probiotic or other lactic bacteria as pure strains or their combinations. Then, it is possible to understand the interaction with the microorganisms, the synergies and symbiotic relations between the cells and prebiotic compounds, and other factors that affect the growth of probiotic bacteria in the soy extract broth medium.

Some assays of substrates and products change during the fermentation of soymilk with pure cultures and mixtures of probiotics, showing fast substrate consumption and the generation of metabolics, basically organic acids. Sugars in the soymilk were usually consumed in the first 12 hours of fermentation, and converted into titratable acids, causing a reduction of pH (Figure 6.1a–d). The pH and Brix values presented similar behaviors in each of the fermentations. *B. longum*, in pure culture, was the species that better acidified the medium after 48 hours of fermentation. The other microorganisms and mixtures reduced the pH, although they presented a light increment of pH after 60 hours of fermentation, probably caused by the autolysis of the dead cells (Mondragón-Bernal 2004).

6.5.1.1.1 Soymilk Fermentation by Pure Cultures of Probiotics

Analyzing a range of fermentation times from 0 to 24 hours, it can be observed that the strains showed different behaviors in relation to pH and sugar consumption (Table 6.3). Probiotics such as *L. paracasei* subsp. *paracasei* and *B. longum*, in pure culture, presented excellent growth in soymilk, reaching counts of 12 to 13 log CFU/mL between 20 and 24 hours of fermentation, the soy proteins were denatured when the pH decreased, forming a gel with a consistency similar to yogurt, whereas pure *L. acidophilus* presented weak growth in soy (as could be seen from the low counts), to modest changes in pH throughout the 24 hours of fermentation, keeping the soy proteins in suspension with very little denaturation. Some kinetics values are shown in Table 6.3.

The majority of fermentations presented a phase lag between 0 and 4 hours, the exponential phase goes up to 24 hours in six to seven log cycles. For *L. acidophilus*, this goes up to 16 hours with four to five growth logarithmic cycles, parting of inoculums between five and six log CFU/mL (Mondragón-Bernal 2004). Chou et al. (2000) found a maximum of 7.11 log CFU/mL after 48 hours of soymilk fermentation by *B. longum*, when the inoculum was 4.06 log CFU/mL. The level of inoculums cannot be so low by two motives: to reduce the time of fermentation and to obtain high final counts (>8 log CFU/mL). With *L. acidophilus*, the inoculum was 3.7 log CFU/mL and reached only 8.5 log CFU/mL.

The nonreducing sugars (saccharose and oligosaccharídes) showed changes throughout fermentation, sometimes being hydrolyzed into reducing sugars and, at other times, they are exploited by the microorganisms as a carbon source, mainly for *L. paracasei* subsp. *paracasei*.

6.5.1.1.2 Soymilk Fermentation by Mixtures of Probiotics

Some data about cellular growth, pH, and carbohydrates during soymilk fermentation by mixtures of *L. paracasei* subsp. *paracasei* and *B. longum*, *L. acidophilus*, *B. longum*, and the three strains in soymilk are illustrated in Table 6.4. The phase lags among the different mixtures take place in 0 to 4 hours, and the exponential phase extends to 20 hours. *L. acidophilus* regulates the consumption of sugars, controlling the decrease of pH through proteolysis (Tamime et al. 1995), allowing for better individual development. Simultaneously, *B. longum* hydrolyzes sugars, such as raffinose and stachyose, because of its α-galactosidase activity (Scalabrini et al. 1998), making them available for lactobacilli and facilitating their development. Thus, it is a method by which to verify the probable symbiosis between the probiotic strains and genus. Mondragón-Bernal (2004) showed the symbiosis between *B. longum* and *L. acidophilus* because they observed high populations when they had been mixed, this was observed especially for the increment in the individual counts of *L. acidophilus*, reaching 12 log CFU/mL after 24 hours of fermentation. The quick consumption of sugars for these strains occurred together with a decrease in pH, but with the time it cause inhibition of the cellular growth else, as presented in the Table 6.5.

TABLE 6.3

Probiotic Growth in Pure Cultures of *L. paracasei* subsp. *paracasei*, *L. acidophilus*, and *B. longum* in Soymilk 7.5% (w/v) at 37°C, pH, Reducing Sugars, and Total Reducing Sugars

	Fermentation Time (hours)	Counts, Log CFU/mL	pH	RS (g/L)	TRS (g/L)
B. longum					
	0.02	6.5	6.91	0.4	31.6
	4	6.7	6.86	0.3	35.3
	8	7.1	6.74	0.4	32.2
	12	8.2	6.32	0.7	31.3
	16	10.2	5.52	1.1	32.0
	20	12.1	5.15	1.2	27.3
	24	12.8	4.96	0.8	37.4
L. acidophilus					
	0.02	3.7	6.91	0.2	46.1
	4	3.7	6.89	0.5	34.5
	8	6.0	6.85	0.4	40.3
	12	7.6	6.64	1.1	35.5
	16	8.5	6.75	1.7	36.2
	20	8.3	6.55	1.3	28.7
	24	7.1	6.17	0.9	24.9
L. paracasei subsp. paracasei					
	0.02	7.0	6.81	0.9	34.1
	4	7.2	6.12	0.6	31.9
	8	9.6	5.52	0.8	39.1
	12	10.2	5.28	0.8	20.1
	16	12.7	4.64	0.9	18.8
	20	13.3	4.47	0.9	15.9
	24	13	4.40	0.3	17.6

Source: Mondragón-Bernal, O. L., *Desenvolvimento de uma bebida fermentada de soja contendo agentes probióticos e probióticos*, thesis (master's degree in food engineering), Food Engineering Faculty, University of Campinas, Campinas, 2004.

Note: RS, reducing sugars and TRS, total reducing sugars.

The mixture of *L. paracasei* subsp. *paracasei*, *L. acidophilus*, and *B. longum* with the addition of fructooligosaccharides (Table 6.5) achieved counts of 10 log CFU/mL in 20 hours of fermentation, reaching the maximum counts for *B. longum*; however, *L. paracasei* spp. and *L. acidophilus* reached maximum counts of only 9 CFU/mL in 24 hours under the same conditions.

The individual probiotics and mixtures of probiotics consume mono-, di-, and oligosaccharides of the soymilk along with the fructooligosaccharides added, producing mainly lactic acid (L) with a small amount of acetic acid (A) (because the rate of lactic acid/acetic acid (LA) was higher than 1; LA > 1). This is a positive sensorial fact, as much as for the reduction of the original taste and the acetic taste of soy, as well as for the improvement in digestibility (Mondragón-Bernal 2004; Hou et al. 2000).

Probiotics showed good consumption of the soy nutrients, and were even better when lactobacilli were associated with bifidobacteria, generating synbiotic relations as protein availability and acidity reduction in the medium by *L. acidophilus* (proteolithic activity), and the availability of sugars by bifidobacteria (because of its β-galactosidase activity) to *L. acidophilus* reducing its inhibition and fortifying its growth (Hou et al. 2000).

TABLE 6.4

Growth of the Mixture of Probiotics *L. paracasei* subsp. *paracasei*/*B. longum* and *L. acidophilus*/*B. longum* in Soymilk and Changes in pH, RS, and TRS

Time (hours)	*Lactobacilli* (log CFU/mL)	*Bifidobacterium*[a] (log CFU/mL)	Total Counts (log CFU/mL)	pH	RS (g/L)	TRS (g/L)
L. paracasei subsp. paracasei/B. longum						
0.02	7.27	6.30	7.32	6.86	0.4	34.2
4	7.39	6.43	7.44	6.81	0.3	35.0
8	8.65	8.52	8.89	6.39	0.5	30.60
12	8.92	8.33	9.02	5.79	2.0	31.7
16	10.03	9.07	10.08	4.92	1.8	24.4
20	9.38	8.90	9.38	4.59	1.4	16.1
24	9.60	8.78	9.66	5.43	1.3	23.6
L. acidophilus/B. longum						
0.02	6.67	5.85	6.73	6.86	0.3	33.6
3	5.00	6.47	6.49	6.79	0.4	36.1
7	5.78	7.06	7.09	6.72	0.4	33.5
11	8.04	8.12	8.38	6.46	0.6	34.1
16	7.70	8.53	8.59	5.60	1.6	32.3
20	10.81	8.93	10.81	5.15	1.3	24.9
24	12.00	8.48	12.00	4.92	0.1	23.2

Note: Counts of *B. longum* (CFU/mL) = total count – lactobacilli count. RS, reducing sugars and TRS, total reducing sugars.

[a] Soymilk 7.5% (w/v) at 37°C.

6.5.1.1.3 Sugars, Oligosaccharides, and Fructooligosaccharides during Probiotic Growth in Soymilk

The natural presence of sucrose, fructose, galactose, raffinose, and stachyose in soybean fat–free water extract was verified. In Figures 6.2 and 6.3, it can be observed that monosaccharides and disaccharides are the first ones to be consumed by probiotic bacteria, whereas raffinose and stachyose are only partially used during fermentation. During the first 8 hours of fermentation, *B. longum* and *L. acidophilus* consume part of the oligosaccharides and release small fractions of sucrose (Figure 6.2). Scalabrini (1998) verified that raffinose is completely metabolized, whereas consumption of stachyose is between 35% and 68% by different species of bifidobacteria.

TABLE 6.5

Growth of the Mixture of Probiotics in Soymilk Plus Fructooligosaccharides and Changes in pH, Reducing Sugars, and Total Reducing Sugars

Time (hours)	Log CFU/mL LAC	Log CFU/mL LBC	Log CFU/mL LAC + LBC	Log CFU/mL BL	Log CFU/mL Total	pH	RS (g/L)	TRS (g/L)
0.02	5.59	6.37	6.44	6.04	6.58	6.72	17.3	89.2
4	6.35	6.82	6.95	6.32	7.04	6.67	17.1	85.5
8	7.01	7.77	7.84	8.00	8.22	6.24	18.1	86.3
12	8.48	9.18	9.26	9.57	9.74	5.05	15.8	84.4
16	8.08	9.44	9.46	8.71	9.53	4.33	12.5	80.2
20	8.32	9.76	9.77	10.05	10.23	4.01	9.3	69.3
24	8.83	9.85	9.89	9.64	10.08	3.85	5.7	65.1

Note: Soymilk, 7.5% (w/v) at 37°C; fructooligosaccharides, 25 g/L. LBC, *L. paracasei* subsp. *paracasei*; LAC, *L. acidophilus*; and BL, *B. longum;* RS, reducing sugars, and TRS, total reducing sugars.

Figure 6.2 shows chromatograms of the carbohydrate consumption during 24 hours of soybean fat–free water extract plus fructooligosaccharides or without fructooligosaccharide fermentation by mixture of *B. longum*, *L. paracasei* subsp. *paracasei*, and *L. acidophilus*.

It was verified that, in the soybean fat–free water extract, 7.5% without fructooligosaccharides, the contents of monosaccharides (RS, reducing sugars) and disaccharides (TRS-RS, nonreducing sugars) were low whereas the content of raffinose and stachyose (TRS-RS) is higher (Figure 6.2a). Oligosaccharides from the soybean extract are carbon sources for probiotic bacteria during the fermentation process. Between 0 and 16 hours of fermentation, there was a consumption of these oligosaccharides; however, at 24 hours of fermentation, an increment of sucrose was detected. It can be presumed that raffinose and stachyose were partially hydrolyzed into sucrose and galactose, and galactose was consumed afterward (Figure 6.2).

In Figure 6.2b, it can be seen that glucose and fructose, presented in the syrup with fructooligosaccharides, are consumed along the fermentation, whereas oligosaccharides were partially hydrolyzed and sucrose was released. Fructose and galactose were probably released by the action of the probiotic enzymes, and were consumed by the cells. Sucrose diminished between 0 and 16 hours, however, showed an increase after 24 hours, probably as a product of the oligosaccharide hydrolysis.

6.5.1.1.4 Lactic and Acetic Acids during Fermentation of Soymilk by Probiotics

Mondragón-Bernal (2004) observed that the reduction in the sugar content was related directly with the production of organic acid. Bifidobacteria is a high producer of acetic acid from glucose, at a ratio of 1.0:1.5 (lactic acid mol/acetic acid mol, LA < 1) (García et al. 1993; Scalabrini et al. 1998; Hou et al. 2000), causing off-flavor in the fermented drinks. In the conditions used for this work, *B. longum* (Figure 6.3a) and its lactobacilli mixtures (Figure 6.3b) showed low acetate production with high lactate production (LA > 1) during 24 hours of fermentation. Scalabrini et al. (1998) found that some species of *B. longum* had produced more lactic acid than acetic acid during fermentations of soymilk.

Thus, *B. longum*, *L. paracasei* subsp. *paracasei*, and *L. acidophilus*, alone and in mixtures, use the nutrients of the soybean extract better, generating synbiotic relations. They consume the medium monosaccharides and disaccharides, resting a residual of oligosaccharides.

The undesirable flavor caused by the production of acetic acid during the fermentation of soybean water extract by *Bifidobacterium* was decreased with the lactobacilli and bifidobacteria mixtures because of the better balance in acidity, as a result, giving a sensorially more acceptable product. In this study, it was qualitatively verified that the ratio of lactic acid to acetic acid was higher than 1 (LA > 1) with *B. longum* or in mixtures with lactobacilli through 24 hours of fermentation in soymilk.

Other studies reviewed by Mondragón-Bernal et al. (2007) showed that *L. rhamnosus* sp. was the LAB strain chosen with the best characteristics of EPS production and growth in the soymilk enriched with high concentrations of sucrose (120 g/L), in addition, it possesses probiotic potential. There was evidence of symbiosis between *L. rhamnosus* and the standard mixture of probiotics *L. acidophilus*, *L. paracasei* subsp. *paracasei*, and *B. longum* because the counts of *L. rhamnosus* increased significantly in the products in a mixture. The EPS produced by *L. rhamnosus* tends to retain more free water from the fermented broth and, therefore, causes a reduction in syneresis. However, the products have a poor consistency compared with that of other similar products made with a standard mixture of probiotics which, in spite of containing more EPS, show little retention of liquids. When the pH value of the medium is approximately 4.0, the product increases consistency and apparent viscosity. The presence of sucrose and *L. rhamnosus* contributed to the reduction of syneresis. Products with sucrose displayed Herschel–Bulkley fluid behaviors, and without sucrose of the pseudo-plastic type. After 16 hours of fermentation, it was noted that *L. rhamnosus*, in the presence of a mixture of products, obtained the best apparent viscosity.

6.5.1.2 Sensory Attributes

Sensory evaluation delivers complete and prompt information about product quality, determines acceptance on behalf of the consumer and, consequently, the purchasing intent for that product (Moraes 1993).

On the basis of early studies from Green et al. (1993), Schutz and Cardello (2001) developed a labeled affective magnitude scale for assessing food liking/disliking, in an attempt to develop a scale showing the advantages provided by magnitude estimation, but one that was easy-to-use. The hedonic unstructured scale or self-adjusting scale (Villanueva and Da Silva 2009) is one of the sensory methods available, mostly used to measure consumer acceptance of a product due to the reliability and validity of its results, as well as its simplicity for use by the tasters (Schutz and Cardello 2001; Stone and Sidel 2004). The unstructured scale contains verbal affective labels in the extreme regions to make it more consumer-friendly. The capability of the labeled affective magnitude scale to minimize heteroscedasticity problems associated with analysis of variance (ANOVA) models is not restricted to a limited number of categories, allowing the panelist to use any part of the scale to attribute their hedonic values, it reduces the psychological error of habituation, encouraging the panelist to make their evaluation in the most conscientious and discerning way, it reduces or eliminates numerical and contextual effects (Villanueva and Da Silva 2009).

Different researchers have reported on studies concerning the sensory features of traditional cow's milk yogurts (Santana et al. 2006; Fox et al. 2004; Saint-Eve et al. 2004; Sigman-Grant et al. 2003; Ward et al. 1999; Penna et al. 1997). Shelef et al. (1998) stated that the soymilk yogurt obtained using traditional starter cultures plus 4% sucrose had an improved aroma and flavor, and that the addition of 10% sucrose plus a flavoring agent enhanced preference by the tasters. Wang et al. (1994) studied soy yogurts supplemented with 2% sucrose, 2% lactose, or 2% sucrose + lactose (1:1) and fermented by *L. acidophilus* + *Streptococcus thermophilus.* They showed that yogurt supplemented with 2% sucrose was the one preferred in the pilot test. Behrens et al. (2004) studied the acceptance of different flavored soy beverages, fermented by *L. casei*, *L. acidophilus* or a commercial mixture of *S. thermophilus* + *L. acidophilus* + *Bifidobacterium lactis* and supplemented with sucrose and lactose. Their study showed that the nutritional and health features might well be used in promoting a new product, enhancing its potential success on the consumer market. Behrens (2002) evaluated consumer beliefs and attitudes with respect to soy-derived products, showing that the consumption of soy and soy-derived products was still low, and that soy yogurt was practically unknown. A certain proportion of the interviewees assumed that soy yogurt would have a bad flavor, but the majority showed a neutral attitude concerning this question, thus indicating a market opportunity. Previous studies have reported on sensory evaluations carried out with samples of milk yogurt and soy yogurt prepared using traditional lactic cultures, but few have reported on synbiotic-fermented soy beverages. Mondragón-Bernal and collaborators (2010) optimized a synbiotic fermented soy beverage containing a mixture of *L. acidophilus* + *L. paracasei* sp. *paracasei* + *B. longum*, and supplemented this with prebiotic agents using combined techniques of sensory analyses and experimental design.

The use of the just-about-right scale in affective analyses is one of the most applied methods because of the reliability of its results, and because it is used to measure the ideal amount of a determined compound that should be added to promote greater acceptance and preference according to the taste panel. Just-about-right scales measure the appropriateness of the level of a specific attribute, and is used to determine the optimum levels of attributes in a product: is it too high, too low, or just-about-right? In this analysis, the tasters evaluate the samples and register their answers on a specific scale, according to how close the samples are to the ideal for the particular attribute under evaluation (sweetness, flavor, or others), according to Vickers' method (1998) (Cardoso et al. 2004).

Although there are many variations of just-about-right scales, such scales typically consist of five or seven points, ranging from too little to too much for a given characteristic (Gacula et al. 2009; Meullenet et al. 2007; Gacula 1993). One end point is labeled as "much too little," the other end point as "much too much," and the middle point as "just right" or "just about right." However, an unstructured line scale anchored with the attributes was used to optimize sweetness in fruit juice (Vickers 1988).

The use of the just-about-right scale in sensory tests measures the ideal amount of a determined compound that should be added to promote greater acceptance and preference according to the taste panel. Thus, some studies aimed to optimize ideal characteristics such as consistency, sweetness, color, and flavor into others of a fermented soy beverage. In these cases, it would be convenient to use the combination of an experimental design and sensory analysis. An example would be to improve the sweetness for

a product sweetened with sucrose, applying the ideal scale and determining the models to predict the acceptance and purchasing intention on behalf of potential consumers through a complete experimental rotational design (Mondragón-Bernal 2011).

Thus, with the aim of optimizing the ideal qualities of a synbiotically fermented soy beverage, the combination of an experimental design and sensory analysis techniques help to optimize the formulae, and to process and obtain predictive models such as the acceptance or purchasing intention on behalf of potential consumers (Mondragón-Bernal 2011). Given that the sensory tests for the ideal choice are carried out in a linear manner and with one variable at a time, Mondragón-Bernal et al. (2010) applied the just-about-right scale, simultaneously evaluating two aspects of the ideal (sweetness and flavor), with a CCRD experimental design as described by Rodrigues and Iemma (2009), and using the response surface methodology applied in the optimization of soymilk's symbiotic food. Predictive models for responses could be obtained to different sensorial optimizations in food researches.

6.5.1.2.1 Equi-Sweetness to Sweeteners and Sucrose in the Symbiotic Fermented Food Based in Soymilk

Each sweetener possesses specific characteristics of intensity and persistence of the sweet taste and the presence or not of residual taste. These factors determine the acceptance, preference, and choice for the consumers (Higginbotham 1983). However, the established theoretical sweetness power for each substance, almost not adjusted to reality, given that the sweet sensation of each one, in relation to sucrose, depends on diverse factors such as the food composition that carries it, temperature, pH, and sweetener content, as was proven by Tunaley et al. (1987). These authors evaluated the equivalent sweetness of nine different sweeteners, comparing them with a glucose standard solution at 5% (w/v) and obtained a great variation in the data, in relation to the panelists. The authors concluded that this variation is dependent of the type of sweetener, therefore, the more complex the perception total, the bigger the variation in the results.

Foods or beverages with sweeteners can be labeled as "light," "sugar-free," "low-calorie," to "reduced-calorie" (Nabors 2002). For its development, it is necessary to conduct studies that allow previous knowledge of the concentrations of sweeteners to be used and its equivalent sweetness in sucrose. The method of estimating the magnitude of sweetness is thoroughly applied in these cases. Through the graphical representation of the normalized results and Stevens' power law, sweetness power models are obtained for different substances in relation to sucrose (Cardoso et al. 2004). In the method of estimating magnitude described by Stone and Oliver (1969), the selected panelists must receive a sample reference with an intensity assigned to an arbitrary value, followed by a series of samples in causal sequence, with bigger or smaller intensities than that of the reference. The panelists will have to estimate the sweetness power of the unknown sample and to attribute to notes they, in relation to the reference, if not able to attribute value zero to no sample. The double of one determined concentration of sucrose or sodium chloride, normally does not produce the double perception. Thus, it is necessary to quantify this perception sensorially. The values obtained from the panelists and the values of the concentrations evaluated are normalized, the logarithms of these results are calculated and placed in a graph in logarithmic coordinates. For the sweetener (or another composition), a straight line is obtained, which follows Stevens' law or the "power function" $S = aC^n$, where S is the stimulus perceived, C is the stimulus concentration, a is the antilog of the value of Y in the intersection, and n is the angular coefficient of the straight line. Regions of the straight lines of the sweeteners that are in the same level, parallel to the axle of the abscissa, possess equi-sweetness power (Moskowitz 1970).

Thus, using previous methodologies, Mondragón-Bernal (2009) determined the power function for equi-sweetness of the mixture acesulfame-k/sucralose in relation to the sucrose in a fermented soymilk beverage. Its power function was $S = 69.37C^{(1,14)}$. In the same product, the model for sensation perceived for sucrose was obtained as $S = 0.042.C^{(1,28)}$ power function. Obtaining the same sensation in 12% of saccharose ($S = 1.011$), 0.024% (25:75 w/w) is the equi-sweetness for a mixture of acesulfame-k/sucralose. Despite this association of used edulcorantes, was 490.01 times sweeter than sucrose in the product studied. Power is defined as the number of times in which a substance or mixture of substances is sweeter than sucrose, based on its equivalent sweetness.

6.5.1.2.2 Expectations of Acceptance of Soymilk and Derivatives

The quality, diversity, and choices of the products from the consumers will direct the expansion of the light, diet, and functional markets (Kapsak et al. 2011). Attention must be given so that low-carb diets (diets with low carbohydrate contents), and its marketing potential, will create a new segment and new chances. Moreover, there is a worldwide trend for the consumption of foods for wellness or healthful drinks by consumers with good educational levels and those conscientious of the importance of healthful diets (Benhrens et al. 2004). Soymilk is limited by its astringent taste which is caused by the volatile composites of low molecular mass such as n-hexanal and the off-flavor leguminous product of lipoxygenase action (Murti et al. 1993; Hou et al. 2000) which, after thermal treatment, can be inactivated. The undesirable antitripsina and hemaglutinina factors are thermosensible, being easily eliminated by thermal treatment. The problem with possessing high α-D-galactosil-oligosaccharide contents, indigestible pseudo-sugars for the human organism causing flatulence, can be solved through diverse techniques of processing or fermentation (Hou et al. 2000). Fermentation has been extensively used to develop some products and to surpass these limitations, for example, in the production of soy cheese and types of yogurt (Chou and Hou 2000). From a sensorial point of view, the fermentation of soymilk by *L. acidophilus* and *L. casei* not only contributes to the improvement of flavor, but also to the aroma of the products. The proteolytic activity of these microorganisms, mainly *L. casei*, is responsible for the formation of precursors of the aroma and flavor, contributing to the elimination of the off-flavor. Behrens (2002) studied the acceptance from an internal map of preference, for fermented drinks of soymilk with *L. casei* or *L. acidophilus*, or a commercial mixture containing *S. thermophilus*, *L. acidophilus*, and *B. lactis*, supplemented with sucrose and lactose. The commercial mixture with sucrose received the best results in terms of acceptance. The product combined low acidity with a set of aromatic candied notes. When pineapple and guava flavors were used, a high consumer acceptance was achieved, therefore showing high marketing potential. The flavors of strawberry, coconut, and kiwi also received good acceptance, whereas the hazelnut-flavored product was disapproved. This study demonstrated that it is possible to improve sensorial quality beyond the nutritional and health aspects, increasing the potential for success in the consumer market. In this work, the beliefs and the attitude of the consumer in relation to the products derived from the soy were evaluated, indicating that the consumption of soy and derived products is still low; that the "yogurt" from soy is practically unknown; and that a small proportion of the interviewees found that the "yogurt" of soy would have a tasteless flavor, whereas most demonstrated a neutral attitude with regard to this affirmation. This indicates a market opportunity; besides, it must be considered that the nutritional and health characteristics claims generate high expectations and intent to purchase among consumers because the biggest percentage of consumers are positively influenced by the claims increasing the acceptance of the product in relation to the blind test.

6.5.1.3 Experimental Design

A precise and reliable inference can be reached only through the use of an experimental design for random sampling and data collection (Gacula et al. 2009). The factorial design methodology, associated with the analysis of response surfaces, is a tool based on a statistical theory, which conveys safe information about the process, minimizing the empiricism involved in trial and error techniques (Rodrigues and Iemma 2005; Box et al. 1978). More than one response may be optimized at the same time and it is able to maximize or minimize responses such as yield, acceptance, or cost, individually or simultaneously. It allows for the evaluation of the experimental error to specify the statistical reliability level, which is useful in estimating the reproducibility of the desired result (Rodrigues and Iemma 2009; Gacula 1993).

Commonly, a second-order model was adopted to fit the experimental responses to the six parameters: $y = \beta_0 + \beta_1 x_1 + \beta_2 x_2 + \beta_{12} x_1 x_2 + \beta_{11} x_1^2 + \beta_{22} x_2^2 + e$; where x_1 and x_2 stand for the coded variables and β_0, β_1, β_2, and β_{12} stand for the regression model parameters, estimated by the least squares method (Rodrigues and Iemma 2009; Gacula et al. 2009).

In proofs that involve more than one product or variable, other statistical analyses such as the Student's *t* test, ANOVA, and surface response analyses are used in the data analyses (MacFie and Thomson 1988; Rodrigues and Iemma 2009).

Mondragón-Bernal and collaborators (2010), using CCRD predictive models for responses with respect to the ideal flavor and sweetness, achieved overall acceptance and purchasing attitude of a fermented functional food (synbiotic) from milk with condensed milk flavor. The models were validated by ANOVA and verified by the Tukey test with a just-about-right scale ($P < 0.05$), as it was possible to predict the acceptance and purchasing attitude according to the product formulation. With respect to the totality of the responses, the sucrose concentration presented the highest significant effect ($P < 0.05$). Greater acceptance and purchasing intent by potential consumers were found for higher sucrose concentrations, within the boundaries of this study. These responses have concordance with other preliminary tests, in which it was observed that regardless of the flavoring brand, the synbiotic soy fermented product showed the same level of acceptance among potential consumers, and that they preferred a product with more intense sweetness. Products elaborated with higher sucrose contents of between 9.0 and 11.2 g/100 mL and 0.08 and 0.16 g/100 mL of flavoring presented ideal sweetness, whereas for flavor, 11 g/100 mL of sucrose content or higher with any amount of flavoring was considered ideal (within the boundaries of the study). As confirmed by the Tukey test, the product elaborated with 12 g/100 mL of sucrose and the lowest flavor content (0.09 g/100 mL), fulfilled both responses for ideal sweetness and flavor. The study showed that a fermented synbiotic beverage obtained from soymilk improved its sensory features after optimization of the sucrose and flavoring concentrations. Products in which acceptance and purchasing intent improved by more than 70% were obtained on behalf of the target public by applying the combined techniques of experimental design and a sensory affective analysis such as the just-about-right scale.

The design scheme proposed by Plackett and Burman in 1946, *Screening Design*, based on the factorial design methodology, is a very useful statistical tool for the evaluation of a previous process when one has a great number of variables. As an advantage, one has a reduced number of tests to perform, it allows for estimating the main effects and identifying the most relevant variables that must be chosen to make a complete planning, but it is necessary to make a greater number of tests than the number of studied variables; it has the disadvantage of not allowing the optimization of the process (Rodrigues and Iemma 2009; Silva et al. 2011).

Through a Plackett and Burman experimental design, there was a search for prototypes of soy-based synbiotic food with functional, physicochemical, and sensory characteristics that remained more stable during storage. Therefore, the effects of the different variables can be studied, such as the kind, quantity, or proportion of probiotic bacteria strains or starters and specific ingredients (i.e., sweetener contents, soymilk percentage, acidity regulator, quantity of prebiotics, fibers, flavor compounds, and others). Any responses can be obtained and analyzed from the experimental design such as syneresis reduction, apparent viscosity, stability characteristics during storage, EPS production by LAB, total counts during shelf life, quantification of sugars, pH, acidity total, and others. Affective sensory tests can also be performed and directed to various goals such as intent to purchase and acceptance (Mondragón-Bernal 2011). The correlation into all variables and responses can be analyzed and discussed to select the variables affecting the process or in the product characteristics (Silva et al. 2011).

6.5.1.4 Shelf Life Studies

Soy-fermented products containing prebiotics and probiotics (synbiotic food) after fermentation by LAB show a decrease in pH, which gives them sensory characteristics such as viscosity and acidity with consequent syneresis and changes in consistency and other characteristics (Mondragón-Bernal 2004). As a consequence, it is necessary that fermented products conserve their sensory characteristics during storage by means of studies and optimization of the process variables.

In searching for a solution to this problem, lactic bacteria with the capacity of producing EPS have been applied. In many cases, EPS released extracellularly by lactic bacteria are advantageous and are used for fermented milk production to improve its rheological properties. The variables involved in the growth of *L. rhamnosus* in the presence of a standard mixture of probiotics containing lactobacilli and bifidobacteria were studied by Mondragón-Bernal (2009). In this study, through a Plackett and Burman experimental design, there was a search for prototypes of soy-based synbiotic food with functional, physicochemical, and sensory characteristics that remained more stable during 30 days of storage. Therefore,

the effects of the independent variables were studied: the proportion of *L. rhamnosus*, sucrose contents, soymilk, acidity regulator (calcium lactate), fructooligosaccharides, and polydextrose in the syneresis reduction, apparent viscosity, and total counts during storage.

A number greater than 10^7 CFU/mL of probiotics is estimated to provide optimum therapeutic effect (Rybka and Kailasapathy 1995); at least 10^6 CFU/mL during storage (ANVISA 2002), or according to Ouwehand et al. (1999), through consumption of a 10^9 CFU daily serving.

In the studies by Mondragón-Bernal (2009), the counts of biobacteria remained practically constant and with values 7.0 log CFU/mL or greater and the other probiotic counts remained at high levels after 30 days on the shelf. Sucrose and soymilk had a significant positive effect in most stability responses during storage. These variables favor the maintenance of probiotics and rheological characteristics. The calcium lactate buffer showed the greatest significant positive effect on the syneresis response: tests with a lower percentage of syneresis did not. Lactate highly affects stability in relation to syneresis, counts, and rheologic properties, but samples had a small improvement in pH stability during storage and bifidobacteria counts. Other factors with significant negative effects on syneresis were sucrose and polydextrose, indicating that their presence diminishes it in the products. The best tests for the stability study during the 30-day shelf life for the synbiotic soymilk products were characterized by not containing calcium lactate and containing maximum quantities of sucrose (>12%) and soy extract (>10%). Counts of probiotics had little changes, keeping excellent concentrations and assuring the product's functionality. Variables with the best effect on stability were soy extracts, sucrose, and the fructooligosaccharide and polydextrose prebiotics. These ingredients could be used for the preparation of light or sucrose-free products with high fiber values.

6.5.1.5 Therapeutic and Functional Tests

To become effective, probiotic microorganisms should accomplish certain requirements, such as having a large number of those microorganisms that will act along the entire extension of the gastrointestinal tract. In addition, they should be capable of surpassing various barriers until reaching their site of action. Among some of the criteria are resistance to gastric juice, resistance to bile, resistance to lysozymes, and persistence within the human gastrointestinal tract.

Among the main benefits and therapeutic effects attributed to symbiotic consumption are the maintenance of intestinal microflora balance, antagonism to pathogenic growth, stimulation of the immune system, and reduction of lactose intolerance, thus benefitting health by increasing protection against intestinal infection and preventing many diseases, among other benefits (Mattila-Sandholm et al. 2002).

The label claims for functional food concerning benefits for human health, generate expectancy which might lead the consumer to purchase the product. Legislation has become much stricter with regard to the use of advertisements, which is why functional products and wellness drinks ought to be developed on well-founded research (ANVISA 1999; Behrens 2002).

Thus, it is of capital importance that once the functional property of a food is validated, this information should be carried out ethically and objectively, not only to promote product sales but also, and more importantly, to respect and benefit the consumer (Behrens 2002).

According to Morelli (2007), the selection of new probiotic bacteria by *in vitro* testing constitutes a new challenge for scientists, as well as for the companies that produce functional beverages. Nowadays, most probiotics are analyzed by using tests focused on their ability to survive within the gastrointestinal atmosphere as well as subsequent colonization.

A great number of new lactobacilli and bifidobacteria species have previously been isolated from neonatal fecal samples and from different age groups, genetically identified and evaluated either *in vivo* or *in vitro*, concerning some *Lactobacilli* spp. and *Bifidobacterium* in regards to their application as probiotics. The reintroduction of these microbial groups into the host is done by administering selected species, which should be done in large numbers and with viable microorganisms at the moment of consumption. A broad-spectrum probiotic ought to contain microorganisms that will act along the entire extent of the gastrointestinal tract. To accomplish their probiotic effect, these bacteria should be able to adhere to the mucosal surface of the gastrointestinal tract (Ouwehand et al. 1999; Fooks et al. 1999; Stanton et al. 2001).

To be considered a probiotic food, the microorganisms should be capable of surpassing various barriers until reaching the site on which they will act. Several authors (Tamime et al. 1995; Jan et al. 2002; Morelli et al. 2003; Leverrier et al. 2005; Mishra and Prasad 2005; Morelli 2007) suggest that a probiotic strain could be analyzed and selected according to the following criteria: (i) must be of human origin; (ii) must be able to survive transit through the stomach, resistance to gastric juice; (iii) must be tolerant to biliary salts, resistance to bile; (iv) must be resistant to lysozyme; (v) must be adhesive to intestinal epithelial tissue, and (vi) must have persistence within the human intestinal tract.

Mattila-Sandholm et al. (2002) point out that the evolution in the development of integrated synbiotics call for studies involving specific synergies between probiotic and prebiotic ingredients in the gastrointestinal tract, and prebiotics that additionally protect the probiotics during manufacture, storage, formulation, and gastrointestinal transit for probiotic control and release at specific sites in the gastrointestinal tract and, aside from this, slower fermentable prebiotics plus the production of short-chain fatty acids in the distal colon. Some exopolysaccharides, produced *in situ* by lactic bacteria, could partially fulfill these functions (Dupont et al. 2002).

A functional product must contain at least 10^7 CFU/mL of viable probiotic bacteria for optimum therapeutic effects; however, ANVISA (2002) allows these products, within their validity term, to have a minimum of 10^6 CFU/mL and a content of prebiotics which supplements the daily requirement of 8 g/day (Yun 1996; Modler 1994).

According to ANVISA (1999), in relation to food containing edible fibers, the following nutritional statements can be added: "source of fibers," when the content is 3.0 g/100 g in case of solid foods and 1.5 g/100 mL for liquids, or "high fiber content," when containing 6.0 g/100 g in case of solids or 3.0 g/100 mL for liquids.

6.5.1.5.1 Survival during Transit through the Stomach

To reach the intestine, the probiotics have to first pass through the stomach, which constitutes a powerful barrier for entering the intestine. The first studies on survival of lactic bacteria in the gastrointestinal tract atmosphere focused on the identification of strains which were able to survive, among many other species. Conway et al. (1987) used gastric juice from human volunteers to demonstrate that *S. thermophilus* and *L. bulgaricus* (*L. delbrueckii* subsp. *bulgaricus*) species are extremely sensitive, whereas the majority of enteric lactobacillus species are more resistant, with significant variations among strains.

Recent publications have shown that the sensitivity of yogurt cultures to acid conditions can reduce the recovery of these bacteria in consumers' fecal samples; however, there were a number of cells capable of remaining viable (Elli et al. 2006; Mater et al. 2005) and this has led some researchers to suggest that fermented milk containing viable cultures could have a real probiotic action (Guarner et al. 2005). Strains pertaining to species which regularly inhabit the human intestine have shown an improved *in vitro* behavior when their resistance to low pHs, or to simulated gastric juice, was analyzed (Conway et al. 1987). However, these enteric lactobacilli species, documented strains with a good ability to colonize in the human intestine, received bad evaluations for *in vitro* assays from some authors (Charteris et al. 1998; Mishra and Prasad 2005); this discrepancy between *in vitro* and *in vivo* results point out the need for improving these types of tests. For example, *in vitro* strains of *L. paracasei* demonstrated a limited tolerance to acid; however, the same species showed excellent *in vivo* results (Ohlson et al. 2002). Morelli (2007) analyzes that *in vitro* evaluation of probiotics' tolerance to acid seems to be a weak *in vivo* behavioral model for these strains. The strains that are extremely sensitive to *in vitro* acidity seem to be the only ones incapable of surviving to intestinal transit because a moderate resistance to low pH is necessary. However, it has yet to be proven that the high resistances evaluated *in vitro* correspond to better abilities for colonization.

6.5.1.5.2 Tolerance to Bile Salts

The ability to survive the action of bile salts is usually included in the criteria used to select potential probiotic strains. In a pioneer study, Gilliland et al. (1984) showed that the resistance to bile may differ among strains of the same enteric lactobacilli species and that this may account for the differences in the ability to colonize calves intestinal tract. It is worth emphasizing that the bile formula used by Gilliland

et al. (1984) for *in vitro* tests was "*oxgall*," a product derived from bovine bile. The same bile formulation was used by many authors to evaluate the probiotic bacteria, lactobacillus or bifidobacterium resistance to bile. There isn't much data available concerning strain behavior when tested with human bile or with bile from other sources. However, no data allows for the evaluation of a possible correspondence between the bacteria's original host animal and the resistance of these bacteria to that bile. Morelli (2007) discussed the possibility, for example, of a *Lactobacillus* isolated from pork being more resistant to pork bile than another strain of the same species, however isolated from calves. Thus, as recently tested, not only with oxgall, but also with commercially available pork bile, 10 *L. johnsonii* strains (three strains from human isolates, five isolates from calves, as well as one isolate each from pork and fowl) and five *L. acidophilus* strains (two from human isolates and three from pork) were also included in this study. The strains were plated on MRS Agar containing bile formulation and counts were compared with plates containing MRS Agar at the same dilution, however, without containing bile. The results showed that the pork bile displayed a greater inhibitory power than the bovine bile; thus, the role of the species remained farther from being relevant than the ecological origin; a specific behavior was observed for each strain.

Many studies concerning probiotic tolerance to gastrointestinal conditions carry out these tests separately, exposing the strains under study, to stomach acid or to biliary salts, sometimes altering the pH on a preestablished range or bile concentration.

The study by Mondragón-Bernal (2009) simulated *in vitro* gastrointestinal conditions, as close as possible to human conditions, accounting for the following features: the series of exposures to digestive fluids, the times of exposure, as well as the chemical and enzymatic composition, and the pH of each one of the digestive juices to which probiotics would come in contact with, when consumed by the human organism. Therapeutic tests regarding the resistance to *in vitro*–simulated gastrointestinal tract conditions were carried out with two commercial brands of probiotic and synbiotic soymilk beverages selected with the best features for stability during storage. The samples selected for survival tests to gastrointestinal conditions were some of the most stable during storage, concerning the different aspects studied as well as those which maintained counts higher than 10 log CFU/mL during 30 days of storage. This symbiotic beverage contains four strains of probiotic bacteria (*L. rhamnosus*, *L. acidophilus*, *L. paracasei* spp., and *B. longum*) and two prebiotic agents (fructooligosaccharides and polydextrose) sweetened or not with sucrose. Although the commercial brands based on cow's milk contained one or two probiotic bacteria (*L. acidophilus* or *B. lactis*/*Bifidobacterium animalis*) and the common yogurt cultures (*S. thermophilus* and *L. bulgaricus*), sweetened with sucrose and glucose, fibers, and jellifiers (inulin, resistant starches, xanthan gum, and others) are added. *In vitro* simulated digestive juice resistance tests were carried out: saliva at pH 6.0, gastric juice at pH 1.2, intestinal juice at pH 7.0, and pancreatic juice at pH 7.0. The treatments underwent digestion, the samples were retrieved at different exposure times and plate counts carried out, sugar analysis by DNS method (Miller 1959) as well as polysaccharides by the Antrona method (Silva 2003) were carried out after alcohol extraction. In all digestions, a high transformation activity of sugars and polysaccharides was observed. One sample without sucrose containing polydextrose and fructooligosaccharides showed the best results for resistance under *in vitro* simulated conditions of the intestinal tract, denoting a diet product in sucrose, and at the end of digestion, it presented low values of total and reducing sugars, a constant EPS value (13 g/L), indicating high residual soluble fiber content, high total count, and high *Bifidobacterium* survival rate (8.8 log CFU/mL, <7% in relation to the initial count). Other samples containing sucrose and polydextrose, and those containing sucrose plus fructooligosaccharides, showed good results, with a reduction of less than 35% regarding initial counts. One more sample containing sucrose, polydextrose, and a lower soy concentrate, and another sample with sucrose alone resulted in a loss of less than 40%. The commercial samples presented the lowest tolerance, with a 69% of total count loss and without soluble fiber content. It has been proved that Mg^{2+} and Mn^{2+} are essential factors for *Lactobacillus* growth and that they improve EPS production by at least promoting growth. Mg^{2+}, for example, influences the enzyme activity of phosphoglucomutase which takes part in EPS biosynthesis, catalyzing the transference of phosphate groups between glucose carbon C_1 and C_6 (Macedo et al. 2002). Thus, because the medium used within this study (10% soymilk) is complex, rich in amino acids, vitamins, and mineral salts [~216 mg/L of Mg^{2+} and ~2.68 mg/L of Mn^{2+}; Núcleo de Estudos e Pesquisas em Alimentação (NEPA) 2006], this may account for the high EPS results obtained. On the other hand, whole bovine milk contains 100 mg/L of Mg^{2+} and traces of Mn^{2+} (NEPA

2006). Therefore, partially demonstrating that the best results concerning tolerance to gastrointestinal conditions for probiotics conveyed in soymilk in relation to bovine milk are due to the existence of an EPS produced *in situ* by the LAB applied. The interaction of EPS with the prebiotics present (fructooligosaccharides and polydextrose) and the soy protein shows a protector effect for probiotic bacteria as well as featuring as a soluble food fiber due to its low digestibility.

6.6 Conclusions

To be successful with novel developments, it is necessary to apply knowledge from different areas. The functional foods involved countless variables that influence its fundamental characteristics as sensorial, functional, nutritional, and physicochemical aspects in all steps of the development and production chain. The raw material, process variables (i.e., temperature, pH, oxide reduction potential, osmotic pressure, solvent solids concentration, and others), kind and number of probiotic strains involved, the mutual relations such as synergy, synbiosis, and symbiosis between the strains and the strains/ingredients (as the prebiotic agents and other foodstuffs) in the different states such as manufacturing, storage, and final consumption affect the final results. All of these aspects must be taken into consideration to begin studies involving functional foods such as synbiotics. Thus, the scientific tools such as kinetic studies, sensorial evaluations (including affective tests, just-about-right scales, magnitude scales for sweeteners, internal map of preferences, descriptive and quantitative analyses, and others), the shelf life studies, the rheological behaviors, the experimental designs (as screening design and CCRD, factorial designs, and Plackett and Burman designs), *in vitro* and *in vivo* functional and therapeutic studies as survival persistence of probiotic strains and its effect on indigenous flora, among others. These studies must be carried out on theories and scientific fundamentals and attending to the laws of each country to be successful with these novel developments. It is possible to improve functional foods to meet consumer expectations by using sensory techniques that adjust to the fundamental characteristics of each type of food.

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REFERENCES

ADA Reports. 2004. Position of the American Dietetic Association: Functional foods. *J Amer Diet Assoc* 104(5):814–26.

Agência Nacional De Vigilância Sanitária (ANVISA) 1999. Diretrizes básicas para análise e comprovação de propriedades funcionais e ou de saúde alegadas em rotulagem de alimentos. Resolução RDC No. no. 18, de 30 de abril de 1999.

Agência Nacional De Vigilância Sanitária (ANVISA) 2002. Regulamento Técnico de Substâncias Bioativas e Probióticos Isolados com Alegação de Propriedades Funcionais ou de Saúde. Resolução RDC No. 2, 7 de janeiro de 2002.

Behrens JH. 2002. Aceitação, Atitude e expectativa do consumidor em relação a uma nova bebida fermentada à base de extrato hidrossolúvel de soja (Glycine Max L-Merril). Tese de Doutorado em Engenharia de Alimentos. Faculdade de Engenharia de Alimentos. Campinas: Universidade Estadual de Campinas.

Behrens JH, Roig SM, Silva MAAP. 2004. Fermentation of soymilk by commercial lactic cultures: Development of a product with market potential. *Acta Alimentaria* 33(2):101–9.

Box GEP, Hunter WG, Hunter JS. 1978. *Statistics for experimenters: An introduction to designs, data analysis and model building*. New York: Wiley.

Braga ALM. 2006. Formação, estrutura e propriedades reológicas de sistemas biopoliméricos. Tese de Doutorado em Engenharia de Alimentos. Faculdade de Engenharia de Alimentos. Campinas: Univesidade Estadual de Campinas.

Cardoso JMP, Battochio JR, Bolini-Cardello HMAB. 2004. Equivalência de dulçor e poder edulcorante de edulcorantes em função da temperatura de consumo em bebidas preparadas com chá-mate em pó solúvel. *Ciência e Tecnologia de Alimentos* 24(3):448–52.

Cerning J. 1990. Exocellular polysaccharides produced by lactic acid bacteria. *FEMS Microbiol* 87:113–30.

Chabot S, Yu HL, De Le´Se´Leuc L, Coutier D, van Calsteren MR, Lessard M, et al. 2001. Exopolysaccharides from *Lactobacillus rhamnosus* RW-9595M stimulate TNF, IL-6, and IL-12 in human and mouse cultured immunocompetent cells, and IFN-c in mouse splenocytes. *Lait* 81:683–97.

Champagne CP, Barrete J, Roy D, Rodrigues N. 2006. Fresh-cheesemilk formulation fermented by a combination of freeze-dried citrate-positive cultures and exopolysaccharide-producing lactobacilli with liquid lactococcal starters. *Food Res Int* 39:651–9.

Charteris WP, Kelly PM, Morelli L, Collins JK. 1998. Development and application of an in vitro methodology to determine the transit tolerance of potentially probiotic Lactobacillus and Bifidobacterium species in the upper human gastrointestinal tract. *J Appl Microbiol* 84:759–68.

Chiarello MD. 2002. Soja e os alimentos funcionais. Encontro francobrasileiro de biosciência e biotecnologia. In: de Sousa JM, Reis L, editors. *Alimentos Funcionais e nutracêuticos*. Embrapa—Documento 85. Brasília. 16 p.

Chou C, Hou J. 2000. Growth of bifidobacteria in soymilk and their survival in the fermented soymilk drink during storage. *Int J Food Microbiol* 56:113–21.

Ciabotti S, Píccolo MFB, Pinheiro ACM, Clemente PR, Lima MAC. 2007. Características sensoriais e físicas de extratos e tofus de soja comum processada termicamente e livre de lipoxigenase. *Ciência e Tecnologia de Alimentos* 27(3):643–8.

Conway PL, Gorbach SL, Goldin BR. 1987. Survival of lactic acid bacteria in the human stomach and adhesion to intestinal cells. *J Dairy Sci* 70:1–12.

Cravero RA, inventors; Sancor Cooperativas Unidas Limitada., assignee. 2000. Process for manufacturing a biologically active fermented milk product and product obtained by the process. U.S. patent 6,033,691, September 17, 1998–March 7, 2000.

Crittenden RG, Morris LF, Harvey ML, Tran LT, Mitchell HL, Playne MJ. 2001. Selection of a Bifidobacterium strain to complement resistant starch in a synbiotic yogurt. *J Appl Microbiol* 90:268–78.

Crittenden RG, Playne MJ. 1996. Production, properties and applications or food-grade oligosaccharides. *Trends Food Sci Technol* 7:353–261.

Donkor ON, Shah NP. 2008. Production of β-glucosidase and hydrolysis of isoflavone phytoestrogens by *Lactobacillus acidophilus*, *Bifidobacterium lactis*, and *Lactobacillus casei* in soymilk. *J Food Sci* 73(1):M15–20.

Dupont I, Roy D, Lapointe G. 2000. Comparison of exopolysaccharide production by strains of *Lactobacillus rhamnosus* and *Lactobacillus paracasei* grown in chemically defined medium and milk. *J Ind Microbiol Biotechnol* 24:251–5.

de Vuyst L, Degeest B. 1999. Heteropolysaccharides from lactic acid bacteria. *FEMS Microbiol Rev* 23:153–77.

Ekhart PF, Timmermans E. 1996. Techniques for the production of transgalactosylated oligosaccharides (TOS). *Bull IDF* 313:59–64.

Elli M, Callegari ML, Ferrari S, Bessi E, Cattivelli D, Soldi S, et al. 2006. Assessing the survival of yogurt bacteria in human gut. *Appl Environ Microbiol* 72:5113–7.

Evangelista CM, Regitano-D'arce MAB. 1997. Spectrophotometric analysis of the lipoxygenase action on soybeans soaked at different temperatures. *Ciência e Tecnologia de Alimentos* 17(3):270–4.

Ferreira CLL, Teshima E. 2000. Prebióticos, estratégia dietética para a manutenção da microbiota colônica desejável. Revista Biotecnologia Ciência & Desenvolvimento. ano III, no. 16, set./out., 22–25.

Fooks LJ, Fuller R, Gibson GR. 1999. Prebiotics, probiotics and human gut microbiology. *Int Dairy J* 9:53–61.

Fox M, Pac S, Devaney B, Jankowski L. 2004. Feeding infants and toddlers study: What foods are infants and toddlers eating? *J Amer Diet Assoc* 104(2):22–30.

Fuller R, Gibson GR. 1997. Modification of the intestinal flora using probiotics and prebiotics. *Scandinav J Gastroenterol* 32 (suppl. 222):28–31.

Gacula M, Jr. 1993. *Design and analysis of sensory optimization*, Trumbull, CT: Food & Nutrition Press.

Gacula M, Jr. Singh J. 2009. *Statistical methods in food and consumer research*, 2nd ed. San Diego, CA: Academic Press Inc.

García GM, Quintero RM, López-Munguía CA. 1993. *Biotecnologia alimentaria*. México D.F.: Editorial Limusa. 636 p.

Gibson GR. 1994. Nondigestible oligosaccharides and bifidobacteria—implications for health. *Int Sugar J* 96(1150):381–7.

Gibson GR, Probert HM, van Loo J, Rastall RA, Roberfroid M, 2004. Dietary modulation of the human colonic microbiota: Updating the concept of prebiotics. *Nutri Res Rev* 17:259–75.

Gibson GR, Roberfroid MB. 1995. Dietary and modulation of human colonic microbiota: Introducing the concept of prebiotics. *J Nutri* 125:1401–12.

Gilliland SE, Staley TE, Bush LJ. 1984. Importance of bile tolerance of *Lactobacillus acidophilus* used as dietary adjunct. *J Dairy Sci* 67:3045–51.

Goh KT, Haisman DR, Archer RH, Singh H. 2005. Evaluation and modification of existing methods for the quantification of exopolysaccharides in milk-based media. *Food Res Int* 38:605–13.

Gorret N, Renard CMGC, Famelan MH, Maubois JL, Doublier JL. 2003. Rheological characterization of de EPS produced by *P. acidici-propionici* on milk microfiltrate. *Carbohydrate Polym* 51:149–58.

Grattepanche F, Audet P, Lacroix C. 2007. Milk fermentation by functional mixed culture producing nisin Z and exopolysaccharides in a fresh cheese model. *Int Dairy J* 17(2):123–32.

Green BG, Shaffer GS, Gilmore MM, 1993. Derivation and evaluation of a semantic scale of oral sensation magnitude with apparent ratio properties. *Chem Sens* 18:683–702.

Guarner F, Perdigon G, Corthier G, Salminen S, Koletzko B, Morelli L. 2005. Should yogurt cultures be considered probiotic? *Br J Nutri* 93:783–6.

Guarner F, Schaafsma GJ. 1998. Probiotics. *Int J Food Microbiol* 39:237–8.

Hasler CM. 1998. Functional foods: Their role in disease prevention and health promotion. *Food Technol* 52(11):63–70.

Hernalsteens S. 2006. Isolamento e identificação de leveduras produtoras de oligossacarídeos coletadas em diferentes regiões brasileiras. Campinas: Tese de Doutorado—Faculdade de Engenharia de Alimentos, Universidade Estadual de Campinas.

Hernalsteens S. 2002. Obtenção de dextrana clínica, oligossacarídeos e frutose por via enzimática. [DPhil dissertation]. Campinas, SP: Faculdade de Engenharia de Alimentos Unicamp.

Hess SJ, Roberts RF, Ziegler GR. 1997. Rheological properties of nonfat yogurt stabilized using *Lactobacillus delbrueckii* ssp *bulgaricus* producing exopolysaccharide or using commercial stabilizer systems. *J Dairy Sci* 80:252–65.

Hidaka H, Hirayama M, Sumi N. 1989. A fructoligosaccharides producing enzyme from *Aspergillus niger* ATCC 20611. *Agric Biol Chem* 52(5):1181–7.

Higginbotham JD. 1983. Recent developments in non-nutritive sweeteners. In: Gremby TH, Parker KJ, Lindley MG, editors. *Developments in sweeteners-2*. London: Applied Science. p 119–55.

Hou J-W, Yu R-C, Chou C-C, 2000. Changes in some components of soymilk during fermentation with bifidobacteria. *Food Res Int* 33:393–7.

Jan G, Leverrier P, Roland N. 2002. Survival and beneficial effects of propionibacteria in the human gut: *In vivo* and *in vitro* investigations. *Lait* 82:31–144.

Jie Z, Bang-Yao L, Ming-Jie X, Hai-Wei L, Zu-Kang Z, Ting-Song W, Craig SA. 2000. Estudo sobre os efeitos da ingestão de polidextrose sobre as funções fisiológicas em chineses. *Amer J Clin Nutri* 72:1503–9.

Kapsak WR, Ravi EB, Childs NM, White C. 2011. Functional foods: Consumer attitudes, perceptions, and behaviors in a growing market. *J Am Diet Assoc* 111(6):804–10.

Leblanc JG, Silvestroni A, Connes C, Juillard V, Savoy de Giori G, Piard J-CH, Sesma F. 2004. Reduction of non-digestible oligosaccharides in soymilk: Application of engineered lactic acid bacteria that produce α-galactosidase. *Gen Mol Res* 3(3):432–40.

Leverrier P, Fremont Y, Rouault A, Boyaval P, Jan G. 2005. *In vitro* tolerance to digestive stresses of propionibacteria: influence of food matrices. *Food Microbiol* 22:11–8.

Lourens-Hattingh A, Viljoen BC. 2001. Yogurt as probiotic carrier food. *Int Dairy J* 11:1–17.

Macedo MG, Lacroix C, Champagne CP. 2002. Combined effects of temperature and medium composition on exopolysaccharide production by *Lactobacillus rhamnosus* RW-9595M in a whey permeate–based medium. *Biotechnol Progr* 18:167–73.

MacFie HJH, Thomson, DMH. 1988. Preference mapping and multidimensional scaling. In: JR Piggott, *Sensory Analysis of Foods*. New York: Elsevier Applied Science.

Mackellar RC, Modler HW. 1989. Metabolism of frutooligosaccharides by Bifidobacterium sp. *Appl Microbiol Biotechnol* 31:537–41.

Mater DD, Bretigny L, Firmesse O, Flores MJ, Mogenet A, Bresson JL. 2005. *Streptococcus thermophilus* and *Lactobacillus delbrueckii* subsp. *bulgaricus* survive gastrointestinal transit of healthy volunteers consuming yogurt. *FEMS Microbiol Lett* 250:85–187.

Mattila-Sandholm T, Myllärinen P, Crittenden R, Mogensen G, Fondén R, Saarela M. 2002. Technological challenges for future probiotic foods. *Int Dairy J* 12:173–82.

Maugeri F, Mondragón O, Mukuno J, inventors; Campinas: University of Campinas, assignee. 2006 Sept 26. Alimento funcional, composição probiótica, composição alimentícia e processo de produção de alimento funcional fermentado à base de extrato de soja, contendo agentes probióticos e prebióticos. BR Innovation PatentPI0504056–6.

Meullenet JF, Xiong R, Findlay C. 2007. *Multivariate and probabilistic analyses of sensory science problems*. Ames, IA: IFT Press, Blackwell Publishing.

Miller GL. 1959. Use of dinitrosalicylic acid reagent for determination of reducing sugar. *Anal Chem* 31(3):426–8.

Mishra V, Prasad DN. 2005. Application of in vitro methods for selection of *Lactobacillus casei* strains as potential probiotics. *Int J Food Microbiol* 15:109–15.

Mitsuoka T. 1982. Recent trends in research on intestinal flora. *Bifidobacteria Microflora* 1:13–24.

Modler HW. 1994. Bifidogenic factors—sources, metabolism and applications. *Int Dairy* J 4:383–407.

Mondragón-Bernal OL. 2004. Desenvolvimento de uma bebida fermentada de soja contendo agentes probióticos e probióticos. [MSc thesis]. Campinas: Food Engineering Faculty, Univ Campinas.

Mondragón-Bernal OL. 2009. Desenvolvimento de alimento simbiótico fermentado de soja, 192 p. [PhD thesis]. Campinas: Food Engineering Faculty, Univ Campinas.

Mondragón-Bernal O, Horita J, Costa F, Maugeri F. 2007. *Lactobacillus rhamnosus* exopolysaccharides (EPS) production and growth in soybeans water extract synbiotic beverage. *J Biotechnol* 131S:S133–87.

Mondragón-Bernal O, Rodrigues MI, Bolini MA, Maugeri F. 2010. Optimization of synbiotic fermented food from hydrosoluble soy extract applying experimental design and sensory analysis techniques. *J Sens Stud* 25(3):371–89.

Mondragón-Bernal OL, Rodrigues MI, Bolini HMA, Maugeri F. 2011. Desenvolvimento e avaliação de um produto simbiótico. Chapter 27. p. 649–669. In: Saad SMI, Cruz AG, Faria JAF, editors. *Probióticos e Prebióticos em Alimentos: Fundamentos e Aplicaçóes Tecnológicas*. 1st ed. São Paulo: Livraria Varela, 671 p.

Moraes MA. 1993. *Métodos para avaliação sensorial dos Alimentos*. Campinas, Campinas, Brazil: University of Campinas.

Morelli L. 2007. *In vitro* assessment of probiotic bacteria: From survival to functionality. *Int Dairy J* 17:1278–83.

Morelli L, Zonenschain D, Callegari ML, Grossi E, Maisano F, Fusillo M. 2003. Assessment of a new synbiotic preparation in healthy volunteers: survival, persistence of probiotic strains and its effect on the indigenous flora. *Nutri J* 2:6–11.

Moskowitz HR. 1970. Ratio scales of sugar sweetness. *Percep Psychophys* 7:315–20.

Murti TW, Lamberet G, Bouillanne C, Desmazeaud MJ, Landon M. 1993. Croissance des lactobacilles dans l'extrait de soja. *Sciences des Aliments* 13(3):491–500.

Núcleo de Estudos e Pesquisas em Alimentação (NEPA). 2006. Tabela Brasileira de Composição de Alimentos—TACO Versão 2—Segunda Edição. Universidade Estadual de Campinas–UNICAMP, Campinas, 114 p.

Ohlson K, Bjorneholm S, Fonden R, Svensson U. 2002. Lactobacillus F19: a probiotic strain suitable for consumer products. *Microbial Ecol Health Dis* 3(Suppl):27–32.

Okubo K, Iijima M, Kobayashi Y, Yoshikoshi M, Uchida T, Kudou S. 1992. Components responsible for the undesirable taste of soybean seeds. *Biosci Biotechnol Biochem* 56(1):99–103.

Organização Mundial de Gastroenterologia. 2009. Probióticos e Prebióticos. Guias Práticas da OMGE. Disponível em (http://www.worldgastroenterology.org/assets/downloads/pt/pdf/guidelines/19_probiotics_prebiotics_pt.pdf). Acessado em Outubro de 2009.

Ouwehand AC, Kirjavainen PV, Shortt C, Salminen S. 1999. Probiotics: Mechanisms and established effects. *Int Dairy J* 9:43–52.

Penna ALB, Oliveira M, Baruffaldi R. 1997. Análise de consistência de iogurte: Correlação entre medida sensorial e instrumental. *Ciência e Tecnologia de Alimentos* 17(2):98–101.

Pineiro M, Asp NG, Reid G, MacFarlane S, Morelli L, Brunser O, Tuohy K. 2008. FAO Technical Meeting on Prebiotics. *J Clin Gastroenterol* 42(Pt 2; Suppl 3):S156–9.

Pinheiro AS. 2002. Síntese de oligossacarídeos por inulinase de Kluyveromyces marxianus var. bulgaricus. [MSc thesis]. Campinas: Food Engineering Faculty, University of Campinas.

Plackett RL, Burman JP. 1946. The design of optimal multifactorial experiments. *Biometrika* 33(4,4):305–25.

Rastall RA, Maitin V. 2002. Prebiotics and synbiotics: Towards the next generation. *Curr Opin Biotechnol* 13:490–6.

Roberfroid M. 2000. Prebiotics and probiotics: are they functional foods? *Amer J Clin Nutri* 71(suppl):1682S-7S.

Roberfroid MB, Van Loo JAE, Gibson GR. 1998. The bifidogenic nature of chicory inulin and its hydrolysis products. *J Nutri* 128:11–9.

Rodrigues MI, Iemma AF. 2009. *Planejamento de experimentos e otimização de processos*, 2nd ed. Campinas, Brazil: Casa do Espírito Amigo Fraternidade Fé e amor. 358 p.

Ruas-Madiedo P, Gueimonde M, de los Reyes-Gavilan CG, Salminen S. 2006. Short communication: Effect of exopolysaccharide isolated from 'villi' on the adhesion of probiotics and pathogens to intestinal mucus. *J Dairy Sci* 89:2355–8.

Rybka S, Kailasapathy K. 1995. The survival of culture bacteria in fresh and freeze-dried AB yogurts. *Aust J Dairy Technol* 50(2):51–7.

Saint-Eve A, Paçi Kora E, Martin N. 2004. Impact of the olfactory quality and chemical complexity of the flavoring agent on the texture of low fat stirred yogurts assessed by three different sensory methodologies. *Food Qual Pref* 15(3):655–68.

Santana LRR, Santos LCS, Natalicio MA, Mondragón-Bernal OL, Elias EM, Silva CB, Zepka LQ, Martins ISL, Vernaza MG, Castillo-Pizarro C, et al. 2006. Perfil Sensorial de Iogurte Light, Sabor Pêssego. *Ciência e Tecnologia de Alimentos* 26(3):619–25.

Savadogo A, Ouattara CAT, Savadogo PW, Barro N, Ouattara AS, Traore AS. 2004. Identification of exopolysaccharides-producing lactic acid bacteria from Burkina Faso fermented milk samples. *African J Biotechnol* 3(3):189–94.

Scalabrini P, Rossi M, Spettoli P, Matteuzzi D. 1998. Characterization of Bifidobacterium stains for use in soymilk fermentation. *Int J Food Microbiol* 39:213–9.

Schutz HG, Cardello AV. 2001. A labeled affective magnitude (LAM) scale for assessing food liking/disliking. *J Sens Stud* 16:117–59.

Shelef LA, Bahnmiller KR, Zemel MEB, Monte LM. 1998. Fermentation of soymilk with commercial freeze-dried starter lactic cultures. *J Food Process Preserv* 12(3):187–95.

Shihata A, Shah NP. 2002. Influence of addition of proteolytic strains of *Lactobacillus delbrueckii* ssp. *bulgaricus* to commercial ABT starter cultures on texture of yogurt, exopolysaccharide production and survival of bacteria. *Int Dairy J* 12:765–72.

Sigman-Grant M, Warland R, Hsieh G. 2003. Selected lower-fat foods positively impact nutrient quality in diet so free-living Americans. *J Amer Diet Assoc* 103(1):570–6.

Silva CF, Arcuri SL, Campos CR, Vilela DM, Alves JGLF, Schwan RF. 2011. Using the residue of spirit production and bio-ethanol for protein production by yeasts. *Waste Manage* 31:108–14.

Silva RN, Monteiro VN, Alcanfor JDX, Assis EM, Asquieri ER. 2003. Comparação de métodos para a determinação de açúcares redutores e totais em mel. *Ciência e Tecnologia de Alimentos* 23(3):337–41.

Smitinont T, Tansakul C, Tanasupawat S, Keeratipibul S, Navarini L. 1999. Exopolysaccharide-producing lactic acid bacteria strains from traditional Thai fermented foods: Isolation, identification and exopolysaccharide characterization. *Int J Food Microbiol* 51:105–11.

Stanton C, Gardiner G, Meehan H, Collins K, Fitzgerald G, Lynch PB, Ross RP. 2001. Market potential for probiotics. *Amer J Clin Nutri* 73:476–83.

Stone H, Oliver SM. 1969. Measurement of the relative sweetness of selected sweeteners and sweetener mixtures. *J Food Sci* 34:215–22.

Stone H. Sidel JL. 2004. Descriptive analysis. In: Stone H, Sidel JL, editors. *Sensory evaluation practices*. London, UK: Academic Press. 377 p.

Tamime A, Marshall V, Robinson R. 1995, Microbiological and technological aspects of milks fermented by bifidobacteria. *J Dairy Res* 62:151–87.

Tamime Y, Robinson R. 2001. *Yogurt science and technology*, 2nd ed. Cambridge: Woodhead Publishing Limited. 368 p.

Tannock GW, Munro K, Harmsen HJ, Welling GW, Smart J, Gopal PK. 2000. Analysis of the fecal microbiota of human subjects consuming a probiotic product containing *Lactobacillus rhamnosus* DR20. *Appl Environ Microbiol* 66:2578–88.

Tharmaraj N, Shah NP. 2004. Survival of *Lactobacillus acidophilus, Lactobacillus paracasei* subsp. *paracasei*, *Lactobacillus rhamnosus, Bifidobacterium animalis* and *Propionibacterium* in cheese-based dips and the suitability of dips as effective carriers of probiotic bacteria. *Int Dairy J* 14:1055–66.

Tuinier R, Oomen CJ, Zoon P, Cohen MAS, Kruif CG. 2000. Viscoelastic properties of an exocellular polysaccharide produced by a *Lactococcus lactis*. *Biomacromolecules* 1:219–23.

Tunaley A, Thomson DMH, McEwan JA. 1987. Determination of equi-sweet concentrations of nine sweeteners using a relative rating technique. *Int J Food Sci Technol* 22:627–35.

Utsumi S, Kinsella JE. 1985. Forces involved in soy protein gelation: Effects of various reagents on the formation, hardness and solubility of heat-induced gels made from 7S, 11S and soy isolate. *J Food Sci* 50:1278–82.

Van Loo J, Cummings J, Delzenne N, Englyst H, Franck A, Hopkins M, Kok N, MacFarlane G, Newton D, Quigley M, Roberfroid M, van Vliet T, van den Heuvel E. 1999. Functional food properties of non-digestible oligosaccharides: a consensus report from ENDO project (DGXII AIRII-CT94–1095). *Br J Nutrit* 81:121–32.

Vickers Z. 1988. Sensory specific satiety in lemonade using a just right scale for sweetness. *J Sens Stud* 3(1):1–8.

Villanueva NDM, Da Silva MAAP. 2009. Comparative performance of the nine-point hedonic, hybrid and self-adjusting scales in the generation of internal preference maps. *Food Qual Pref* 20:1–12.

Wang SH, Marinho CS, Carvalho EP, 1994. Produção de iogurte de soja com diferentes associações de bactérias lácticas. *Pesqui Agropecu Brás* 29(10):1593–601.

Ward CDW, Stampanoni-Koeferli C, Piccinalischwegler P, Schaeppi D, Plemmons LE. 1999. European strawberry yogurt market analysis with a case study on acceptance drivers for children in Spain using principal component analysis and partial least squares regression. *Food Qual Pref* 10(4):387–400.

Yun JW. 1996. Fructooligosaccharides—Occurrence, preparations, and application. *Enzyme Microb Technol* 19:107–17.

Zangelmi ABC, Tagliolatto MA. 1982. Produtos de soja, leite, farinha e outros: Serie Tecnologia Agroindustrial. São Paulo: Governo do Estado de São Paulo. Secretaria da Indústria, Comércio, Ciência e Tecnologia. Coord. da indústria e comércio. p 97–132.

Ziemer CJ, Gibson GR. 1998. An overview of probiotics, prebiotics, and synbiotics, in the functional food concept—perspectives and future strategies. *Int Dairy J* 8:473–9.

7

Thua Nao: A Traditional Thai Fermented Soy Product

Ekachai Chukeatirote, Katekan Dajanta, and Arunee Apichartsrangkoon

CONTENTS

7.1 Introduction

Fermented soybeans constitute a key part of the diet in many parts of the world, especially in Asia and Africa. Although the main raw materials (i.e., soybeans) and fermentation processes are very similar, they come in various forms and names. The widely known examples of food products in this group are the Japanese *natto*, the Korean *chungkukjang*, and the Indian *kinema* (Steinkraus 1996). The major microorganisms responsible for fermentation are the *Bacillus* bacteria, in which their proteases cause protein hydrolysis and ammonia liberation. The release of ammonia provides an alkaline condition; hence, these products are often termed *alkaline-fermented soybeans*. Among these, *natto* is probably best characterized with comprehensive scientific data. In contrast, there is little scientific information on *thua nao*. This chapter is therefore introduced to document the current status of *thua nao* in terms of manufacture, fermenting microbes, and nutritive quality.

7.2 *Thua Nao* and Related Products

Thua nao is a traditional fermented soybean food widely consumed in Northern Thailand (i.e., Chiang Mai, Chiang Rai, Mae Hong Son, Lam Phun, and Lam Pang). It is not only an important food/condiment but also serves as a low cost protein supplement for the local people. Traditional *thua nao* has a strong unpleasant ammoniacal smell, dark grayish or brownish color, and slightly slime matter (Figure 7.1). This is different from *natto*, which are a white mucous substance that has a unique flavor and a palatably soft texture, are light yellow, and are able to generate silky and sticky mass (Wei et al. 2001). Some features of *thua nao* and other traditional *Bacillus*-fermented soybeans are summarized in Table 7.1.

FIGURE 7.1 *Thua nao* products in the local market of Chiang Rai. Left, dried form known as *thua nao kab*; right, roasted *thua nao* covered in banana leaf. (Courtesy of Mr. Pichitchai Nantarat.)

TABLE 7.1

Some Features of Traditional *Bacillus*-Fermented Soybeans

Product	Country	Uses	Reference
Thua nao	Thailand	Flavor enhancer in local dishes	Chukeatirote et al. (2010)
		Main dish: mashed fresh *thua nao* in combination with salt, chili, lemongrass, onion, and garlic, packed in banana leaves into small pocket and cooked by steaming or roasting, eaten with sticky rice	
Natto	Japan	Eaten directly with rice	Kiuchi and Watanabe (2004)
		Flavor enhancer in local dishes, i.e., miso soup and sushi	
		Accompaniments to drinks, i.e., sake and tea	
		Main dish: mixed with miso, salt, sugar, garlic, and other ingredients, mashed together and stored in chinaware for few days to produce a pickled dish	
Kinema	India	Fried in vegetable oil for 3 to 5 minutes and tomato, onion, spices, salt, and a little amount of water are added to produce a thick curry	Tamang et al. (1988); Nout et al. (1998)
Dawadawa	Nigeria and Ghana	Flavor enhancer in local dishes	Omafuvbe et al. (2002)
Chungkukjang	Korea	Flavor enhancer in local dishes	Kwak et al. (2007)

7.3 *Thua Nao* Fermentation Process

Three major raw ingredients for *thua nao* production are as follows: soybeans, water, and mixed natural bacterial culture. In general, four major steps are involved in the conventional *thua nao* manufacturing process consisting of soaking, boiling, fermenting, and incubating (Chantawannakul et al. 2002; Chukeatirote et al. 2010). For detailed descriptions, it typically begins with soaking of soybean for overnight and then boiling of soybean seeds for 3 to 4 hours until soft. The cooked soybeans are then packed in bamboo baskets lined and covered with banana leaves and allowed to undergo spontaneous fermentation at ambient temperature for 2 to 3 days. However, there are differences in the procedures and equipment used in different areas and communities. For example, in the production of *thua nao* at Fang

District in Chiang Mai, dry soybeans are cooked in boiling water for 7 hours after being washed twice with clean water, fermented in woven bamboo baskets lined with fern leaves, and then covered with the plastic bags and placed outside the building exposed to sunlight for 3 days (Leejeerajumnean et al. 2001). According to the production process of Muang Chiang Mai District, soybean seeds are soaked overnight (12–14 hours) in water before being boiled for a shorter time (4–6 hours). Besides, the fermentation conditions are also different, as cooked seeds are packed in bamboo basket lined and covered with banana leaves and left to stand at ambient temperature in the household for 2 to 3 days. The production of *thua nao* in Lam Phun and Lam Pang follows closely the method of Fang District, except for omitting the soaking step of the seeds; instead, dry soybean seeds are immediately cooked by boiling in water for 3 to 4 h, and the banana leaves are used to enclose the cooked soybeans in bamboo baskets and allowed to spontaneously ferment at ambient temperature in the household for 3 to 4 days (Sundhagul et al. 1972). The diversity of soaking and cooking steps including the variation of equipment and fermentation conditions leads to the inconsistency and safety qualities of the product. The complete fermented soybean is indicated by the grayish brown color of soybean seeds and the appearance of a slight mucilaginous substance and is dominated by a strong ammonia-like odor. *Thua nao* is regarded as spoiled when they liberate putrid or rancid smell or appeared contaminated with mold or yellow-pigmented slimy material on the beans. A major problem of cooked *thua nao* is a short shelf life, approximately 2 days, if stored under ambient conditions. Hence, to extend the shelf life of this product, a sun-dried process of *thua nao* is developed and widely known among the locals as *thua nao kab* (see Figure 7.1), which could be kept for several months.

7.4 *Bacillus* Species as Predominant in *Thua Nao* Fermentation

As mentioned earlier, *thua nao* is still produced conventionally by natural microflora. It should be noted, however, that *Bacillus* species are always present in high numbers. According to Chukeatirote et al. (2006), *Bacillus* species, especially *Bacillus subtilis*, predominated from the beginning to the end of the fermentation. The occurrence of *B. subtilis* as the predominant species in *thua nao* product was also reported by Chantawannakul et al. (2002) and Leejeerajumnean (2000). Other *Bacillus* species were also found including *Bacillus cereus*, *Bacillus licheniformis*, *Bacillus megaterium*, and *Bacillus pumilus* (Chukeatirote et al. 2006; Leejeerajumnean 2000). Apart from dominant *Bacillus* species, a few bacterial groups (i.e., lactic acid bacteria and unidentified Gram-positive cocci) were also identified only at the onset, and thus their involvement in fermentation is possibly limited (Boontim et al. 2003; Chukeatirote et al. 2006; Table 7.2).

TABLE 7.2

Predominant Bacteria Involved in Alkaline-Fermented Soybeans

Product	Microorganisms Involved	References
Thua Nao	*B. subtilis*, *B. licheniformis*, *B. pumilus*, *B. megaterium*, *B. cereus*, *Lactobacillus* sp., Gram-positive cocci	Chukeatirote et al. (2006); Sundhagul et al. (1972); Leejeerajumnean (2003)
Kinema	*B. subtilis*, *B. licheniformis*, *B. cereus*, *Bacillus circulans*, *B. thuringensis*, *B. sphaericus*, *Enterococcus faecum*, *Candida parapsilosis*, *Geotrichum candidum*, *Escherichia coli*, *coliform*, and *Enterobaceriaceae*	Tamang (1993); Sarkar et al. (1994, 2002); Nout et al. (1998)
Daddawa	*B. subtilis*, *B. licheniformis*, *B. pumilus*, *B. cereus*, *B. firmus*, *B. circulans*, *Bacillus metaterium*, *Staphylococcus saprophysicus*, *Staphylococcus epidermidis*, *Micrococcus luteus*, *Pseudomonas aeroginosa*	Omafuvbe et al. (2000); Dakwa et al. (2005); Dike and Odunfa (2003); Jideani and Okeke (1991)
Chungkukjang	*B. subtilis*	Kwak et al. (2007)
Natto	*B. subtilis (natto)*	Kiuchi and Watanabe (2004)

TABLE 7.3

Proximate Composition of *Bacillus*-Fermented Soybeans

Chemical Composition	*Kinema*[a]	*Thua Nao*[b]	*Dawadawa*[c,d]	*Chungkukjang*[e]	*Natto*[f]
Protein (%)	48	38.76	31.25	15.70	42.51
Fat (%)	17	16.97	19.90	9.0	21.11
Ash (%)	7	5.73	1.72	5.2	4.38
Carbohydrate (%)	28	26.38	22.86	16.1	30.80
Fiber (%)	–	11.93	3.47	–	–
Reducing sugar (%)	–	–	–	–	8.20
Ammonia (mg/100 g)	–	–	–	–	240.49
Moisture (%)	62	–	59.40	54.00	59.30
pH	7.89	8.0–8.6	8.2–8.9	8.49	6.83

Note: –, data not available.

[a] *Source:* Sarkar, P. K. et al., *Food Microbiol*, 11, 47–55, 1994. With permission.

[b] *Source:* Sundhagul, M. et al., *Thai J Agric Sci*, 5, 43–56, 1972. With permission.

[c] *Source:* Jeff-Agboola, Y. A., O. S. Oguntuase, *J Nutr*, 5, 606–7, 2006. With permission.

[d] *Source:* Omafuvbe, B. O. et al., *Food Microbiol*, 19, 561–6, 2002. With permission.

[e] Lee, M. Y., et al., *J Food Sci*, 70, M191–6, 2005. With permission.

[f] *Source:* Wei, Q., and S. K. C. Chang, *J Food Process Preserv*, 28, 251–73, 2004. With permission.

7.5 Proximate Composition of *Thua Nao*

It is expected that there exist some differences of these proximate values between *thua nao* product and raw soybean because of biochemical changes during fermentation. As described by Chukeatirote and Thakang (2006), the content of protein, fat, and reducing sugar of fresh *thua nao* appeared to be slightly higher than unfermented soybean. These changes are possibly caused by several extracellular enzymes produced during fermentation (Chukeatirote et al. 2006). The overall data of proximate analysis of *thua nao* products are as follows: 64.91% moisture, 1.65% ash, 7.69% crude fiber, 14.33% crude protein, and 1.83% fat, whereas those features of *thua nao kab* were 11.88%, 4.94%, 13.46%, 36.43%, and 17.96%, respectively (Chukeatirote and Thakang 2006). Table 7.3 summarizes proximate composition data of *thua nao* compared with other fermented soybeans. Please note that the data of *thua nao* in Table 7.3 are from Sundhagul et al. (1972) in which some variations in these values are also observed compared with the data mentioned previously.

7.6 Amino Acid Profiles of *Thua Nao*

Thua nao products seem to be a good protein source based on the amino acid profiles. Available data show that all amino acids are present in *thua nao* with quantities of total free amino acids ranging from 11.03 to 61.23 g/kg, as dry basis. The major amino acids are Trp, followed by Glu, Cys, Lys, and Leu. All essential amino acids are also present in considerable amounts. It should be noted interestingly that there exist a large proportion of bitter amino acids accounting for more than 50% of total free amino acids (Dajanta et al. 2011a,c).

7.7 Isoflavones of *Thua Nao*

Isoflavones, a type of phenolic compounds, exhibit estrogen-like activity, which has been reported to exhibit several health beneficial effects (Wiseman 2006). Similar to other fermented soybeans, *thua nao* products contain high amounts of isoflavone compounds. Besides, the use of pure starter culture to prepare *thua nao* has showed to increase the content of aglycone isoflavones (Dajanta et al. 2009). Aglycone compounds are of great interest because of their bioavailability, high rate of absorption in animals and

humans (Izumi et al. 2000; Kano et al. 2006), and high antiproliferative activity on human cancer cells (Peterson et al. 1998).

7.8 Aroma and Flavoring Agents of *Thua Nao*

The microbial fermentation of soybeans causes biochemical changes including organoleptic properties. Such changes affect texture, odor, and flavors of the products. Traditionally produced *thua nao* is typically described as a fish sauce-like product due to a strong ammoniacal smell. In contrast, *natto* produced by a pure starter culture has a fruity/nutty aroma. Leejeerajumnean et al. (2001) reported that *thua nao* products contain aldehydes, aliphatic acids, esters, and sulfur compounds, whereas *natto* condiments are devoid of these chemicals. Besides, several pyrazine compounds found in *natto* are proposed to be the main contributors to *natto* odor characteristics (Sugawara 1985; Owens et al. 1997).

As part of the program to improve *thua nao* quality, the use of pure starter culture of *B. subtilis* strain TN51 isolated from commercial *thua nao* product has been shown to improve the organoleptic quality of the product (Dajanta et al. 2011b). The profile of volatile compounds responsible for the aroma of *thua nao* produced with pure and mixed cultures was determined using gas chromatography–mass spectrometry. In total, 85 volatile compounds were identified as follows: pyrazines (10), aldyhydes (6), ketones (17), esters (8), alcohols (15), acids (4), aromatics (4), furans (5), sulfur-containing compounds (2), and other compounds (14). Generally, the predominant volatiles in fermented *thua nao* products were 2-methylbutanoic acid, 2,3,5-trimethylpyrazine, 2-methylpropanoic acid, acetic acid, 2,5-dimethylpyrazine, benzaldehyde, 5-methyl-3-hexanone, 2-butanone, and 3-methyl-2-pentanone, whereas those in cooked nonfermented products were ethanol, 2,3-butanedione, 3-hydroxy-2-butanone, acetone, benzaldehyde, and 2,5-dimethylpyrazine. It is therefore evident that there was a shift in volatile profiles from the cooked nonfermented to fermented products. Besides, a different pattern of the volatile compounds was also observed in the *thua nao* samples prepared with pure and mixed starter culture (Dajanta et al. 2011b).

7.9 Conclusion

Thua nao is an indigenous alkaline-fermented soybean of Thailand. This condiment plays a key role as dietary culture of local Thai people. In terms of nutrition, the product is acceptable as a good source of protein supplement in which other health benefits must be further explored. At present, scientific data of Thai *thua nao* are increasing. This chapter, which is a focus of *thua nao*, is our attempt to update its information currently available with an anticipation to attract researcher's attention on this local food product. Several issues are open for further experiment to study and to improve the product's quality. For instance, the use of pure starter culture is a good start and potential for product improvement. An alternative research relevant to nutritional quality (i.e., enhancement of amino acids and flavoring agents), health benefits (i.e., anticancer, antimicrobial, and antioxidant activities), and fermentation process would provide a new image of this indigenous food to the Thai and world community.

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REFERENCES

Boontim N, Lumyong S, Moriguchi M. 2003. Characterisation of thai fermented foods by organic acid contents. *ScienceAsia* 29:301–6.

Chantawannakul P, Oncharoen A, Klanbut K, Chukeatirote E, Lumyong S. 2002. Characterisation of proteases of *Bacillus subtilis* strain 38 isolated from traditionally fermented soybean in Northern Thailand. *ScienceAsia* 28:241–5.

Chukeatirote E, Chainun C, Siengsubchart A, Moukamnerd C, Chantawannakul P, Lumyong S, Boontim N, Thakang P. 2006. Microbiological and biochemical changes in *thua nao* fermentation. *Res J Microbiol* 1:38–44.

Chukeatirote E, Dajanta K, Apichartsrangkoon A. 2010. Thua nao, indigenous thai fermented soybean: A review. *J Biol Sci* 10:581–3.

Chukeatirote E, Thakang P. 2006. Chemical composition of *thua nao*—A fermented soybean food of northern Thailand. *Chiang Mai J Sci* 33:243–5.

Dajanta K, Apichartsrangkoon A, Chukeatirote E. 2011a. Free-amino acid profiles of thua nao, a Thai femented soybean. *Food Chem* 125:342–7.

Dajanta K, Apichartsrangkoon A, Chukeatirote E. 2011b. Volatile profiles of thua nao, a Thai fermented soy product. *Food Chem* 125:464–70.

Dajanta K, Chukeatirote E, Apichartsrangkoon A. 2011c. Analysis and characterisation of amino acid contents of *thua nao*, a traditionally fermented soybean food of Northern Thailand. *Int Food Res J* 18:588–92.

Dajanta K, Chukeatirote E, Apichartsrangkoon A, Frazier RA. 2009. Enhanced aglycone production of fermented soybean products by *Bacillus* species. *Acta Biol Szeged* 53:93–8.

Dakwa S, Sakyi-Dawson E, Diako C, Annan NT, Amoa-Awua WK. 2005. Effect of Boiling and Roasting on the Fermentation of Soybeans into Dawadawa (soy-dawadawa). *Int J Food Microbiol* 104:69–82.

Dike EN, Odunfa SA. 2003. Microbiological and biochemical evaluation of a fermented soyabean product—Soy-dadawadwa. *J Food Sci Technol* 40:606–10.

Izumi T, Piskula MK, Osawa S, Obata A, Tobe K, Saito M, Kataoka S, Kubota Y, Kikuchi M. 2000. Soy isoflavone aglycones are absorbed faster and in higher amounts than their glucosides in humans. *J Nutri* 130:1695–9.

Jeff-Agboola YA, Oguntuase OS. 2006. Effect of *Bacillus sphaericus* on proximate composition of soybean (Glycine max) for the production of soy iru. *Pakistan J Nutri* 5:606–7.

Jideani IAO, Okeke CR. 1991. Comparative-study of microorganisms and sensory attributes of condiments from the fermentation of different seeds. *Plant Foods Human Nutri* 41:27–34.

Kano M, Takayanagi T, Harada K, Sawada S, Ishikawa F. 2006. Bioavailability of isoflavones after ingestion of soy beverages in healthy adults. *J Nutri* 136:2291–6.

Kiuchi K, Watanabe S. 2004. Industrialization of japanese natto. In: Steinkraus KH, editor. *Industrialization of indigenous fermented foods*. 2nd ed. New York: Marcel Dekker. p 193–246.

Kwak CS, Lee MS, Park SC. 2007. Higher antioxidant properties of chungkukjang, a fermented soybean paste, may be due to increased aglycone and malonylglycoside isoflavone during fermentation. *Nutri Res* 27:719–27.

Lee MY, Park SY, Jung KO, Park KY, Kim SD. 2005. Quality and functional characteristics of Chungkukjang prepared with various *Bacillus* sp. isolated from traditional Chungkukjang. *J Food Sci* 70:M191–6.

Leejeerajumnean A. 2000. Bacillus fermentation of soybeans: Characterisation of traditional thua nao manufacture. [DPhil thesis]. Reading, UK: The University of Reading.

Leejeerajumnean A. 2003. Thua nao: Alkali fermented soybean from *Bacillus subtilis*. *Silpakorn Univ Int J* 3:277–92.

Leejeerajumnean A, Duckham SC, Owens JD, Ames JM. 2001. Volatile Compounds in *Bacillus*-fermented soybeans. *J Sci Food Agric* 81:525–9.

Nout MJR, Bakshi D, Sarkar PK. 1998. Microbiological safety of kinema, a fermented soya bean food. *Food Control* 9:357–62.

Omafuvbe BO, Abiose SH, Shonukan OO. 2002. Fermentation of soybean (Glycine max) for soy-daddawa production by starter cultures of *Bacillus*. *Food Microbiol* 19:561–6.

Omafuvbe BO, Shonukan OO, Abiose SH. 2000. Microbiological and biochemical changes in the traditional fermentation of soybean for soydaddawa—Nigerian food condiment. *Food Microbiol* 17:465–74.

Owens JD, Allagheny N, Kipping G, Ames JM. 1997. Formation of volatile compounds during *Bacillus subtilis* fermentation of soya beans. *J Sci Food Agric* 74:132–40.

Peterson TG, Ji GP, Kirk M, Coward L, Falany CN, Barnes S. 1998. Metabolism of the isoflavones genistein and dichanin a in human breast cancer cell lines. *Amer J Clin Nutri* 68:1505–11.

Sarkar PK, Hasenack B, Nout MJR. 2002. Diversity and functionality of bacillus and related genera isolated from spontaneously fermented soybeans Indian kinema, and locust beans African Soumbala. *Int J Food Microbiol* 77:175–86.

Sarkar PK, Tamang JP, Cook PE, Owens JD. 1994. Kinema—A traditional soybean fermented food: Proximate composition and microflora. *Food Microbiol* 11:47–55.

Steinkraus KH. 1996. *Handbook of indigenous fermented foods*. 2nd ed. New York: Marcel Dekker.

Sugawara E, Ito T, Odagiri S, Kubota K, Kobayashi A. 1985. Comparison of compositions of odour components of *natto* and cooked soybeans. *Agric Biol Chem* 49:311–7.

Sundhagul M, Smanmathuroj P, Bhodacharoen W. 1972. Thua-Nao: A fermented soybean food of Northern Thailand: I. Traditional processing method. *Thai J Agric Sci* 5:43–56.

Tamang JP. 1993. Studies on the microflora of some traditional fermented foods of darjeeling hills and sikkim. [DPhil thesis]. Siliguri: University of North Bengal, India.

Tamang JP, Sarkar PK, Hesseltine CW. 1988. Traditional fermented foods and beverages of darjeeling and sikkim—A review. *J Sci Food Agric* 44:375–85.

Wei Q, Chang SKC. 2004. Characteristics of fermented natto products as affected by soybean cultivars. *J Food Process Preserv* 28:251–73.

Wei Q, Wolf-Hall C, Chang KC. 2001. Natto characteristics as affected by steaming time, *bacillus* strains, and fermentation time. *J Food Sci* 66:167–73.

Wiseman H. 2006. Isoflavonoids and human health. In: Andersen OM, Markham KR, editors. *Flavonoids, chemistry, biochemistry and applications*. Florida: CRC Press. p 371–96.

8

Soymilk and Tofu Manufacturing

Sam K. C. Chang and Zhisheng Liu

CONTENTS

8.1 Introduction

The availability of soymilk and tofu has a long history, since the Han Dynasty in China approximately 2000 years ago. It has been an integral part of the Chinese food culture and has also spread to Korea, Japan, and several Eastern Asian countries. It is generally believed that the absence of prolonged malnutrition in China may be due to the regular consumption of soymilk, tofu, and other soy foods in their diet. The consumption of tofu has gradually gained acceptance in Western culture, as some of its nutritional and health benefits are now known. It is also popular among vegetarian consumers.

The main components in soymilk and tofu are water, protein, fat, and minerals. Its protein profile is not as good as milk and egg proteins but is probably the best among foods of plant origin. Soymilk is often referred to as *poor man's milk*. Dieticians also consider tofu made with calcium salts as a good source of calcium. The U.S. Food and Drug Administration approved a health claim for processed foods containing soy proteins that states "consumption of 25 g soy proteins per day in conjunction with a low cholesterol diet would reduce the risk of heart disease."

The availability of tofu in various parts of the world is credited to the migration of East Asians to different countries and also to the development of semiautomatic to fully automatic machines to make tofu by the Japanese. Many people can claim they can make tofu. However, making tofu with consistent good quality is not that easy, although tofu making consists of only two basic steps: the preparation of soymilk and the coagulation of soymilk to form the curd, which is then made into various types of tofu. Many processing factors are involved in the preparation of tofu, and the raw bean components are also determinant factors on the quality of tofu.

Four books contain information on soymilk and tofu making, including the *Chemistry, Texture and Flavor of Soy* (Cadwallader and Chang 2010), *The Science of Tofu* (Watanabe 1997), the *Tofu & Soymilk Production, the Book of Tofu, Volume 2* (Shurtleff and Aoyagi 1990), and *Soybeans: Chemistry, Technology and Utilization* (Liu 1997). Two recent reviews on the science and technology of tofu making are also available (Chang and Hou 2004; Chang 2006). Soymilk is an intermediate product for tofu making. The technology for making soymilk and for tofu-making is similar to that for making unflavored plain soymilk products (Chen 1989). This chapter will focus on the basics in making plain soymilk, which can be used as the base for flavored soymilk products, and on tofu and the factors affecting tofu production and quality from a different approach.

8.2 Manufacturing of Soymilk and Tofu

There are many methods for producing soymilk (Chen 1989). Several types of tofu are produced nowadays, namely, *momen* (firm or extra firm) tofu, soft tofu, silken (Kinugoshi) tofu, and fill-packed silken tofu. These tofu products are usually packed in trays with water, pasteurized, and kept refrigerated in display chambers in retail stores for selling in developed countries. They have a shelf life of 3 to 4 weeks under proper refrigeration. However, in some countries, they may be sold as fresh tofu in plastic bags or containers with water, or even just cut into smaller blocks as ordered by the consumers. These types of tofu have a short shelf life, 1 to 3 days under proper refrigeration. Filled silken tofu has been sterilized and is shelf-stable for 6 months or longer under refrigeration.

Figure 8.1 presents a generalized flowchart in the preparation of soymilk, and Figures 8.2, 8.3, and 8.4 are schematic diagrams on the manufacture of various types of tofu from soymilk.

8.2.1 Preparation of Soymilk

8.2.1.1 Cleaning and Soaking

Raw soybeans are first cleaned to remove all the foreign matters, followed by soaking the cleaned beans in water to allow the dry beans to absorb enough water before grinding. Soaking time is dependent on soaking temperature and raw material characteristics as affected by cultivar and storage. Usually, this soaking process takes 8 to 10 hours at 15°C to 20°C or 12 to 16 hours at 10°C to 15°C. This difference

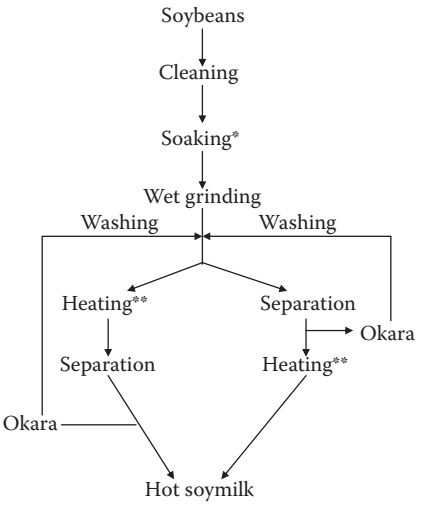

FIGURE 8.1 Preparation of soymilk for tofu making. *15°C to 20°C for 8 to 10 hours or 10°C to 15°C for 12 to 16 hours. **98 to 105°C for 2 to 5 minutes.

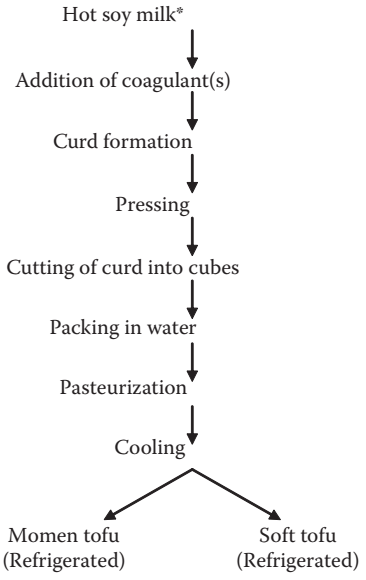

FIGURE 8.2 Schematic diagram for production of momen (firm or extra firm) tofu and soft (silken) tofu. *9 to 10°Brix soymilk solid for momen tofu production and 10 to 12°Brix soymilk for soft tofu production.

is due to ambient temperature difference at various locations of the manufacturing plant. Soaking time can be shortened to 3 to 6 hours if temperature is higher than 20°C. On the other hand, soaking could be performed at a low temperature (2°C–4°C) to prevent off-flavor development during soaking and grinding. After soaking, the beans weigh approximately 2.2 to 2.3 times their initial weight. When water is absorbed, it rehydrates the various components in the dry beans, making it easy to be dissolved or dispersed in water during the grinding process. Soymilk for tofu making also could be made from soybean powder or soybean flakes (Moizuddin et al. 1999), which require less time to rehydrate. In addition, dry unsoaked soybeans or dehulled soybeans can be partially soaked at high temperature for a very short time and milled with hot water (80°C) to extract soymilk with a low beany flavor (Wilkens et al. 1967). However, such practice will reduce the yield of soymilk. In preparing consumer soymilk products by

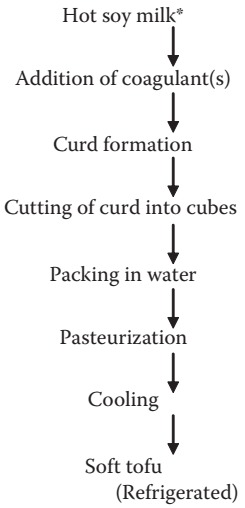

FIGURE 8.3 Schematic diagram for production of soft tofu. *13°Brix or higher soymilk solid for soft tofu production.

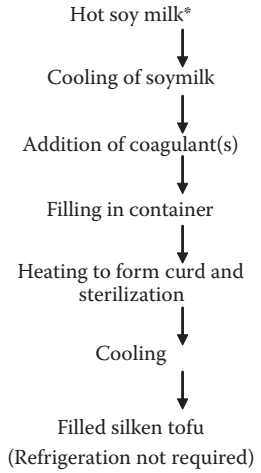

FIGURE 8.4 Schematic diagram for production of shelf-stable–filled silken tofu. *13°Brix or higher soymilk solid for filled silken (Kinogoshi) tofu production.

some manufacturers, dehulling soybeans before extraction also removes germs (hypocotyls) and is carried out to improve the flavor of soymilk and to extend shelf life.

8.2.1.2 Grinding

Grinding soaked soybeans breaks down the intact structure of the dry beans. The amount of water added in the grinding process should be carefully controlled to meet the requirements for the different types of tofu to be manufactured. For example, the water dosage for silken tofu, soft tofu, and regular tofu is 5, 7 to 8, and 10 times of raw soybean weight, respectively (Watanabe 1997). This will give soymilk with 9 to 13°Brix or higher (see Figures 8.2, 8.3, and 8.4). Grinding can be conducted using a stone mill or stainless steel grinder. Solids content in °Brix can be rapidly measured using a hand-held refractometer for quality control. °Brix is proportionally related to soluble solid content of the soymilk.

Water-soluble components such as sugars, amino acids, and water-soluble vitamins are dissolved in the water added during the grinding process. Proteins and lipids are dispersed in the slurry, and the fibers are broken down to smaller particulates. Proper grinding gives appropriate small particle sizes in the slurry and facilitates the extraction of solids and nutrients into the soymilk. It is understandable that the finer the slurry, the more components can be extracted, but it makes it harder for the separation of the soymilk and the residue (*okara*). This will also affect the yield of soymilk.

8.2.1.3 Grinding Temperature

Temperature during grinding affects not only the flavor of the soymilk but also the texture of the resulting tofu. Obata and Matsuura (1993) reported that tofu firmness decreases as the grinding water temperature increases between 0°C and 50°C. They found that this is related to the contents of sulfhydryl (–SH) groups in soymilk that is critical in the curd formation process. Lipoxygenases in soybean decrease the availability of –SH groups in soymilk and makes the tofu less firm. Hot grinding at a temperature higher than 80°C can produce bland soymilk because lipoxygenases are inactivated. However, hot grinding could not prevent the formation of all lipid derived volatiles; therefore, other processing strategies have been suggested to eliminate soy odors (Sun et al. 2010; Wilkens et al. 1967).

8.2.1.4 Water Added during Grinding

Tofu contains approximately 88% to 90% water. It has long been claimed that water from certain sources such as mountains and streams are better for making tofu. Water containing proper amount (approximately 100 mg/l) and balance of minerals, including calcium, magnesium, sodium, potassium, iron, and manganese, provides a harmonious and mellow taste (Watanabe 1997). The amount of water absorbed during soaking and the amount added during grinding constitute the water-to-bean ratio. For regular tofu, a ratio of 10:1 is considered the best with recovered soymilk having 6.0% to 6.3% solids and 3.0% protein (Watanabe et al. 1964). However, ratios of 5:1 to 7:1 are required for making soft or silken tofu (Saio 1979). For making filled tofu, soy isolate can be added to increase protein content, thereby increasing firmness of tofu because pressing is not carried out in filled tofu.

8.2.1.5 Heating and Separation

Heating the slurry or after separation of the soymilk and the residue serves several purposes:

- Inactivation of microorganisms
- Improvement of nutritional quality of soymilk by inactivating the trypsin inhibitor (TI)
- Denaturation of proteins to facilitate their curdling in the presence of coagulant(s)
- Improvement on yield of soymilk
- Reduction of beany flavor

Abundant microorganisms are present during the soaking process and introduced during grinding. Mild heating serves as a mechanism to reduce its microbial load, although it will not destroy all the microorganisms at this stage.

One of the major disadvantages of soybeans is the natural presence of TI that affects the digestion of proteins by trypsin. TI is heat resistant. Upon heating, the activity of TI can be reduced considerably. Chang and Hou (2004) summarized the effect of various combinations of temperature and time of heating to reduce the TI activity. Watanabe (1997) recommends that heating soymilk for 3 to 5 minutes at 100°C is adequate for tofu making. Heating temperature and time range of 90°C to 105°C for 4 to 20 minutes are used by tofu manufacturers. These heat treatments can reduce the TI activity considerably and at the same time facilitate the separation of soymilk and *okara* (residue). Ultrahigh temperature (UHT) processing has been recently used to heat soymilk in a very short time within 1 minute, depending on temperature (135°C–150°C; Kwok et al. 1993; Rouhana et al. 1996). The optimal residual TI

activity is in the range of 4% to 10%, but some believe up to 15% to 20% of residual activity is satisfactory. However, absolute TIs remained may be more important than percentage of the residues because soybean varieties vary greatly in the TI they contain. UHT heating could result in significant TI activities if not heated properly as that reported in some soymilk produced in Hong Kong (Guo et al. 1997a). Some commercial soymilk products produced in the United States also contain high TI activities (Yuan and Chang 2010b). The consumption of the alarmingly high levels of TI may be harmful to the pancreas. However, recent discoveries of the potential health benefit of the Bowman–Birk inhibitor show that a different strategy of heating may be necessary to retain the Bowman–Birk inhibitor in soy food products. Our research (Yuan et al. 2008) shows blanching of soaked soybean at 70°C to 85°C for 30 to 450 seconds could inactivate 25% to 50% of total TIs. However, the combination of blanching at 80°C for 2 minutes and UHT at 135°C for 10 seconds inactivates only 62% of TIs in soymilk. When UHT temperature increases to 150°C for 10 seconds, 27% of TIs still remain active. When heating conditions increase to 150°C and 50 seconds, the percentage of residual TI decreases to 11%. UHT processing of soymilk has been used to produce aseptically packaged tofu products (Alan et al. 1999).

Soy proteins in their nature state maintain their globular structure with the hydrophobic regions wrapped inside. Upon heating, soy proteins are denatured, subsequently unfolding the natural molecules with exposure of the hydrophobic groups to the outside. Thus, protein solubility decreases because of aggregation. The viscosity of soymilk is affected by heating procedure. A two-step heating method (70°C for 5–10 minutes followed by heating to 90°C for 5–10 minutes) resulted in a higher viscosity than that heated by one-step heating (90°C for 5–10 minutes; Liu et al. 2004; Liu and Chang 2007).

For a small quantity of slurry, the separation of soymilk and residue (*okara*) can be accomplished by filtering the slurry in a cotton cloth or nylon bag accompanied by manual pressing. For a large volume, it is accomplished by drum pressing, screw pressing, centrifugation, or shake filtration. The Chinese procedure in the preparation of milk is to separate the soymilk for the residue (*okara*) first followed by heating the filtered soymilk subsequently. However, the Japanese procedure is to heat the slurry first before separating soymilk and *okara*. It is claimed that the Japanese procedure can increase the soymilk yield and reduce the beany flavor. The *okara* contains 17% and 29% of the original protein and solids in the original soybeans, respectively. When the *okara* is rewashed and repressed, an additional 15% to 20% soymilk can be recovered (Shurtleff and Aoyagi 1990).

Soy products including tofu usually carry a beany flavor that is not familiar to the Western consumers and hence is a barrier for consumption. The presence of lipoxygenases in soybeans is the major cause of beany flavor in soy products including tofu. The iron-containing lipoxygenases catalyze the oxidation of polyunsaturated fatty acids such as linoleic acid, producing fatty acid hydroperoxides, which are then fragmented to produce hexanal, the major compound of beany flavor in soy products. When soaked soybeans are blanched at 80°C for 2 minutes, no hexanal can be detected after grinding (Yuan et al. 2008).

Soybeans are known to be the most abundant source of lipoxygenases, with four isozymes, L-1, L-2, L-3, and L-4 (Axelrod et al. 1981). L-3 and L-4 are very similar in behavior and composition, and they are sometimes considered as one isozyme, L-3. Heating is known to be capable of inactivating enzymes in the raw soymilk. L-1 is heat stable, with loss of activity of 50% at 69°C for 25 minutes (Christopher et al. 1970), whereas L-2 and L-3 are much less heat stable with loss of activity of 50% at the same temperature for 0.7 minutes (Christopher et al. 1970). L-1 has optimal activity at pH 9 and L-2 and L-3 at pH 7. L-2 and L-3 have more activity on fatty acids and triglycerides with increased activity at the presence of calcium ions. However, L-1 is more active on fatty acids but not activated by calcium ions. Although L-2 is the least abundant, it has the highest specific activity at the positions of 9 and 13 of linoleic acid and is mainly responsible for the beany flavor (Christopher and Axelrod 1971; Christopher et al. 1972; Takamura et al. 1991).

The beany flavor can be developed rapidly when the substrate is available and the temperature is appropriate. For soymilk, it is at the grinding step where enzyme, fatty acids, and triglycerides are liberated with excess of water present. Wilkens et al. (1967) found that when the grinding temperature is at 80°C, no volatiles are formed. Several techniques are developed to overcome this beany flavor by heating the soybeans mildly, adjusting the moisture or pH, or using aqueous alcohol to soak the soybeans or their combinations (Hajika et al. 1995; Trawatha et al. 1995). Soybean cultivars with the absence of lipoxygenases are also available (Wilson 1996). Soymilk made from lipoxygenase-free cultivars has the same

functional properties, less beany flavor, and less astringency as compared with soybeans made from regular soybeans, but the soymilk is rated more yellow and darker (Torres-Penaranda et al. 1998). Fatty acids also play an important role in generating beany flavor compounds in addition to lipoxygenases (Yuan and Chang 2007a). Hexanal can be produced by lipoxygenases-null cultivars by the autoxidation of unsaturated fatty acids, particularly the linoleic acids (Yuan and Chang 2007a,b).

Besides contributing to the beany flavor in soymilk, lipoxygenases have also been found to affect tofu texture (Obata and Matsuura 1993; Obata et al. 1996). During grinding of soaked soybean at 2°C to 50°C, lipoxygenases not only oxidize lipids to hydroperoxides but also subsequently oxidize the free –SH groups liberated from the denatured proteins to disulfide bonds and possibly cysteinic or cysteic acids. The oxidation of –SH groups affects their availability to participate in the interchange of free –SH groups with disulfide bonds during heating to form the protein networks, thereby decreasing the firmness of the tofu products. Firmer tofu products can be prepared by grinding soybeans under anaerobic conditions. Among the lipoxygenase isozymes, L-2 has the greatest SH-degrading capacity.

8.2.2 Coagulation of Soymilk

The coagulation of soymilk to form the curd is the most critical and difficult step in tofu manufacturing as there are many interdependent variables that can affect the outcome of coagulation. These are the following:

- Solid content and pH of soymilk
- Types and amount of coagulant(s) used
- Temperature
- Coagulation (mixing or stirring) method
- Cutting or breaking curd
- Pressing

8.2.2.1 Solids Content and pH of Soymilk

Soymilk for tofu manufacturing has solid concentration ranging between 9 and 13°Brix. For regular or firm (momen) tofu manufacturing, it is between 9 and 10°Brix. For making silken (Kinugoshi) and filled silken tofu, it is 13°Brix or higher, and for making soft tofu, it is 10 to 12°Brix. In making regular or firm tofu, although it requires a lower solid content in the soymilk, it takes longer time to coagulate the curd and thus more solids can be coagulated. In addition, it requires pressing to firm the tofu. For making soft tofu, a higher solid content requires shorter coagulation time as compared with the regular or firm tofu. For silken tofu, the soymilk is coagulated without pressing, and therefore there is no excretion of whey. Thus, the requirement for a higher solid/protein content in the soymilk is understandable. Soy protein isolate has been added to increase protein content in making filled tofu products. The pH of soymilk before coagulation usually is about neutral or slightly below as this is affected by the original pH of soaking and water added during grinding. This near-neutral pH in soymilk is therefore most suitable for lipoxygenases to work on the substrate to produce the beany flavor compounds as indicated earlier.

8.2.2.2 Types of Coagulants Available

Hot soymilk is usually coagulated to form curd by the addition of a salt or an acid coagulant. Tofu coagulants are classified into four basic types:

- Chloride-type or *nigari*-type fast-acting salt coagulant such as calcium chloride, magnesium chloride, and seawater
- Sulfate-type slow action salt coagulant such as calcium sulfate and magnesium sulfate
- Glucono-delta-lactone (GDL), also slow action, but works differently from salt coagulant
- Acid coagulants such as citrus juices, vinegar, and lactic acid

Each type of coagulant has its advantages and disadvantages. *Nigari* may be natural or refined. Regular *nigari* is composed of mainly magnesium chloride hexahydrate ($MgCl_2 \cdot 6H_2O$, 92.3%), and the residual components in seawater (magnesium sulfate, 3.8%; sodium chloride, 1.7%; and calcium sulfate, 1%) after salt (sodium chloride) are removed and dried. Refined *nigari* is mainly magnesium chloride (99.5%). Calcium chloride is not found in seawater, but it gives tofu an excellent flavor, almost identical to tofu made with regular or refined *nigari*. In addition, it is generally recognized as safe in the United States, available in food-grade and the cheapest chloride-type coagulant. Tofu made with *nigari*-type coagulants is considered the most delicious with subtle sweet flavor and aroma. However, compared with tofu made with calcium sulfate or GDL, *nigari*-type coagulants react very rapidly with soymilk and therefore require skills and attention. They must be added slowly, and the curd is destroyed while agitation is going on. This type of tofu contains less water as compared with the sulfate-type, and the yield is less with a coarser texture. *Nigari is* not suitable for making silken tofu as the reaction is too fast for the high solid content and high temperature of the soymilk, making it very difficult to form uniform blocks of tofu. However, for filled silken tofu, this problem can be overcome by cooling down the soymilk to 2°C to 4°C before coagulant addition followed by heating to slowly coagulate the proteins.

Calcium sulfate or gypsum is the most widely used sulfate-type coagulant for making tofu in the world. Gypsum is not very water soluble at room temperature (3.0 g/L), making it a determining factor on the coagulation reaction. It reacts slowly with the soymilk; consequently, it allows the formation of curds with higher water-holding capacity and soft and smooth texture. Thus, they give 15% to 20% higher bulk yield as compared with the *nigari* process. Calcium sulfate can be used in making regular, firm, soft, silken, and even packed (packaged-filled) tofu, whereas the last two types cannot be easily accomplished with *nigari*. Calcium sulfate is easy to use, even with unskillful tofu manufacturers, with fairly consistent yields and texture. Calcium sulfate-coagulated tofu has a mild or bland taste, and the taste is slightly inferior to that of *nigari*-type tofu. It should be noted that the coagulation of soymilk using salts followed by pressing will remove the water-soluble constituents not trapped in the final curd.

GDL was first used in Japan to make silken tofu in the 1960s (Shurtleff and Aoyagi 1990). It differs from the chloride- and sulfate-type coagulated tofu in that it uses an acid to form the curd. In a water solution, GDL is slowly hydrolyzed into gluconic acid by water in 2 to 3 hours with a pH drop from 3.5 to 2.5 for a 1% solution at room temperature. In manufacturing packed and silken tofu, GDL is dissolved in previously cooled soymilk to allow gradual formation of gluconic acid. Upon heating, the soymilk coagulates because of the action of gluconic acid with the formation of a homogenous solidified curd without losing any of the water in the container. GDL-coagulated tofu has a sour flavor, and its sourness increases with the increased concentrations used in making firm and extra firm GDL tofu. GDL is often used together with calcium sulfate to improve the flavor of the final tofu product. This type of tofu contains all the components of the soymilk used to make tofu in addition to the coagulant.

The acid-type coagulant such as vinegar, lactic acid, and lemon juice can be used as natural coagulants. However, the use of this group of coagulant produces low yield with slightly crumbly texture and a slightly tart flavor as compared with the *nigari*- and sulfate-type tofu.

8.2.2.3 Amount of Coagulant Used

The amount of coagulant required to reach the optimum coagulation varies with the solid content of soymilk. It is also a kind of proprietary to the tofu manufacturers. It greatly affects the yield, texture, taste, and aroma of the final product. In general, tofu makers can tell by observing the curd formed and whey produced during pressing whether the appropriate amount of coagulant has been added. Whey usually is transparent and amber or pale yellow. When proper coagulation is achieved, the curds have a smooth and cohesive texture in traditional tofu making. If coagulation is incomplete (too little coagulant), the whey is cloudy containing some uncoagulated soymilk. When too much coagulant is used, the texture will become coarse and crumbly. There are some published methods for determining appropriateness of coagulation. Whey transmittance is one of the methods in determining the optimum amount of coagulant added for maximum yield. Watanabe et al. (1964) reported that more concentrated soymilk requires 3% to 8% more coagulant to reach the same level of whey transmittance, and the dosage for calcium sulfate is 20% more than that for *nigari*. Chang and Hou (2004) reported that the coagulant

concentration required for reaching the optimum coagulation of silken tofu increases linearly with the soymilk solid content in the range of 6% to 11%, and the concentration for magnesium chloride is approximately 13% to 15% more than that for calcium chloride. Kao et al. (2003) reported that in making firm tofu by calcium sulfate, 0.4% resulted in the most uniform and homogeneous microstructure and retained the highest protein and water in the tofu gel. Whey conductance is claimed to be a better indication for proper coagulation with faster and more reproducible results than whey transmittance (Beddows and Wong 1987a). A rapid titration method based on the flow properties of the soymilk during titration with coagulant was developed in our laboratory (Liu and Chang 2003, 2004) for determining optimal coagulants of the *nigari* type (calcium chloride and magnesium chloride) for filled tofu making. This method allows the manufacturer to determine coagulants rapidly for reducing waste that may be caused by the improper use of *nigari* coagulants. The procedure can be modified for sulfate type of coagulants.

8.2.2.4 Coagulation Temperature

Coagulation temperature is another crucial factor in tofu making. The rate of soymilk coagulation and tofu quality are affected by the soymilk temperature. The yield and moisture content of tofu decrease as the temperature of coagulation increases, whereas the hardness and elasticity of the resultant tofu increase (Wang and Hesseltine 1982). When soymilk is at high temperature, proteins possess high active energy. This can lead to fast coagulation, resulting in the formation of curd with low water holding capacity; consequently, tofu has hard texture and low bulk yield. The hotter the soymilk at the time of coagulation, the less the amount of coagulant required. When tofu is coagulated at a high temperature, a small increase in the amount of coagulant may lead to a large decrease in yield. In the tofu industry, the temperature of coagulation varies from one factory to another, depending on the type of coagulant used. Generally, coagulation temperature ranges from 68°C to 95°C for those using *nigari*, whereas those using calcium sulfate prefer the range from 70°C to 80°C in Japan. Beddows and Wong (1987b) reported that the optimum coagulation temperature is 75°C to 80°C for silken tofu with gypsum as coagulant in a small bench scale. We found (Shih et al. 1997) that the optimum coagulation temperature is 85°C to 91°C for making soft tofu with $CaSO_4$ in a medium scale. The operational temperature of coagulation also varies from one region to another. In the United States, tofu makers prefer a relatively high temperature (85°C) for using *nigari* and calcium sulfate because less coagulant makes the curd form quickly; hence, tofu has firm and dense texture but no significant drop in yield.

8.2.2.5 Coagulation Method

The coagulant addition procedure, the stirring speed, and the follow-up stirring after the coagulant is added have very definite effect on tofu yield and quality. Generally, the controlling techniques of this coagulation rely on the experienced tofu maker's judgment.

Traditionally, calcium sulfate in a suspension is added to soymilk, which has been stirred vigorously but rhythmically by hand with a paddle, and the mixture continues to be mixed six to eight more times.

Nigari-type coagulant could be divided into three portions and added in three steps to coagulate soymilk slowly and get high yield and smooth texture. The first portion of *nigari* is poured from a height of several feet into the soymilk being swirled with a paddle. Coagulation starts from the bottom of the container and slowly works up while the uncoagulated soymilk constantly rises to the surface. The second portion is sprinkled over the soymilk surface. The content is covered and stands for approximately 5 minutes. Then, the last portion of the *nigari* is sprinkled over the surface. The curd is allowed to stand for 15 to 20 minutes to solidify completely (Shurtleff and Aoyagi 1990).

The optimum combination of stirring time and speed are critical in obtaining quality tofu with proper yield. This depends considerably on the experience of the tofu manufacturer and his desired yield and quality. Chang and Hou (2004) and Shih et al. (1997) determined the optimum combinations of soymilk solids, coagulant concentration, soymilk temperature for adding coagulant, and stirring time after adding coagulant for traditional soft tofu making by using a medium scale and a stirrer fixed at 285 rpm. Tofu yield is affected mainly by soymilk solid content and coagulant concentration. Tofu solids and

protein content are affected by soymilk solids, coagulant concentration, and stirring time. Solid content of soymilk is the most important factor affecting textural properties of tofu. The optimum combinations are soymilk 11.8 to 12.3°Brix, coagulant 0.27% to 0.32% of soymilk volume, stirring temperature 85°C to 91°C, and stirring time of 5 to 11.3 seconds (Shih et al. 1997).

8.2.2.6 Cutting of Curd

Depending on the type of tofu manufactured, the curd may or may not be cut or broken up before pressing to expel the whey. Cutting or breaking curd is required in the manufacturing of regular or firm and extra firm tofu, whereas filled silken and aseptically packaged tofu do not require cutting as the curd is formed inside the container.

8.2.3 Pressing of Curd

For tofu requiring a cutting step, a pressing step follows to expel the whey. The yield, the moisture contents, and the texture in these final products, therefore, depend on the pressure applied during this pressing step and its length. It is obvious that longer time and higher pressure are needed for firmer tofu. Pressing usually is conducted in molds of various sizes depending on the volume of tofu to be produced. For research purposes, we have used a mold for small-scale production with a dimension of 12.5L × 12.5W × 5.5H cm, a mold for medium-scale production with a dimension of 25L × 25W × 7H cm, and a mold for large-scale production with a dimension of 40L × 40W × 4.5H cm. For industrial production, the molds may be larger. Widely inconsistent conditions of pressures and duration of pressing have been used by different researchers (Chang and Hou 2004) for making various types of tofu. Because of differences in soymilk- and tofu-making methodology, it is difficult to compare reported data, and a standardized procedure is needed for determining the quality of soybeans in tofu making. Because of this reason, we (Yuan and Chang 2010a,b) have recently published a method for small-scale tofu making and textural analysis. As a general guideline, tofu manufacturers apply a light initial pressure of 2 to 4 g/cm square for approximately 5 to 10 minutes followed by a stronger pressure of approximately 5 to 15 g/cm square for 10 to 15 minutes to make soft tofu. For firm tofu, a pressure of 20 to 100 g/cm square is used for 20 to 30 minutes (Shurtleff and Aoyagi 1990). Generally, silken tofu is not pressed, but Beddows and Wong (1987b) reported an optimum pressure of 4 to 6 g/cm square for pressing silken tofu until dripping ceased. Pressures above or below this range will produce unacceptable silken tofu.

8.3 Effect of Soybeans and Components on Soymilk and Tofu Manufacturing and Quality

8.3.1 Soybean Raw Materials

Soybean cultivar is one of the major factors influencing the quality of tofu. It is well known that good tofu can only be prepared from good soybeans. In addition, location (environment of growth) and handling practice at harvest and storage practices after harvest together can also affect soybean chemical compositions, which in turn affect curd formation and sensory properties of tofu.

Over the years, substantial interest has been placed on the understanding of the quality of various soybean cultivars for tofu making. This is of practical importance in soybean trading because a good quality identity-preserved soybean cultivar developed especially for tofu making would commend a premium. Several researchers have reported the differences in the quality of various soybean cultivars for making tofu. Soybean cultivars with higher protein content have generally lower oil and total sugar content. The chemical composition of soybeans is closely related to that in soymilk and tofu (Wang et al. 1983; Lim et al. 1990). The higher protein content in soybeans, the higher protein content is retained in soymilk or in packed tofu. However, cultivars with higher protein contents may not produce tofu with harder texture because protein content alone is not adequate to explain the observations related to hardness.

Therefore, a thorough understanding of the composition in various cultivars and changes during storage is important to relate to tofu quality.

In choosing a laboratory-scale method for evaluating soybean quality for making tofu, it is important that the selected small-scale method has the ability to detect the differences of soybeans with different quality characteristics, and such a method could produce a similar trend of results in a large-scale tofu manufacture process. Because manufacturing processes of tofu vary from manufacturer to manufacturer, one evaluation method cannot be applied to all manufacturers and product types. However, for a simple purpose of comparison among different varieties/materials for tofu making, one method developed for a specific tofu product may be appropriate. Most methods reported for tofu making are small-scale methods and have not been described in detail, and they have not compared the reported small-scale method with a large-scale method. Our research results (Cai et al. 1997; Chang and Hou 2004) revealed that the small bench and the large-scale method correlated significantly ($P \geq 0.05$) in tofu yield, color, texture, and chemical composition (moisture, protein, lipid, ash, calcium, and magnesium). The quality and the yield of tofu were significantly affected by soybean cultivars and processing methods. Because tofu quality made by the small bench scale was well correlated to the production method, the bench-scale method may be used for determining the quality of soybeans for making tofu. Murphy et al. (1997) also suggested a production-scale method for determining the soybean quality for suitability of commercial processing. However, the small bench-scale method developed in our laboratory (Yuan and Chang 2010a,b) is appropriate for evaluating soybean quality because tofu quality made by the small bench scale is well correlated to a production method.

8.3.2 Storage Conditions

Most soybeans are produced by a few major countries and shipped to other nonproducing countries for further processing into various soy products including tofu (Chang et al. 2004; Hou and Chang 2005a). These beans may be stored up to 1 year or longer after harvest in a wide variety of environmental circumstances before they are processed. Prolonged postharvest storage of beans, generally called aging, will decrease the quality of edible soybeans and the viability. The mechanism of soybean aging is not completely understood. A commonly acceptable hypothesis is that lipid peroxidation plays an important role in the initial stage of seed aging process (Parrish and Leopold 1978; Stewart and Bewley 1980; Wilson and McDonald 1986). Hydroperoxides resulting from the lipid peroxidation of polyunsaturated fatty acids in the presence of oxygen not only destroy the lipid itself but also damage cell membranes and other cellular components. In addition, they can break down to form secondary volatile oxidation products, which may contribute to the off-flavor formation in soy products during storage of soybean (Clark and Synder 1991; Hou and Chang 1998). The magnitude of the quality of deterioration of seeds depends on storage conditions, including time, temperature, relative humidity (RH), and microbial contamination. Among these factors, RH/water activity is the most important. Low humidity may effectively preserve the original bean qualities even at a temperature as high as 30°C (Saio et al. 1980). However, at a temperature higher than ≥30°C, soybean quality can be changed greatly during storage even at medium range of RH 50% (Chang 2010; Kong et al. 2008; Kong and Chang 2009). In general, when the RH increased to 80% and the temperature is 30°C or higher, the soybean quality deteriorated quickly. Humidity has a higher effect on the soybean quality for soymilk or tofu making, whereas when humidity is low, temperature increase is important in soybean deterioration.

The reported changes of components in soybeans induced by storage include the following:

- Surface discoloration, Maillard reaction, loss in protein extractability (Kong and Chang 2009; Kong et al. 2008; Chang et al. 2004; Hou and Chang 2005a; Thomas et al. 1989; Narayan et al. 1988)
- Increase in the acidity or decrease in pH (Liu and Chang 2008; Hou and Chang 1998)
- Decrease in phospholipid content (Mounts and Nash 1990; List and Mounts 1993; Nakayama et al. 1981)

- Changes in isoflavone forms with a decrease in malonyl forms and increase in aglycone forms (Hou and Chang 2002)
- Decrease in the extractability of glycinin and β-conglycinin and changes in the subunit composition of glycinin and structures (Chang et al. 2004; Hou and Chang 2004; Hou and Chang 2005b; Murphy et al. 1997; Saio et al. 1982)

Adverse conditions of soybeans storage result in the decrease in the yield and quality of both soymilk and tofu with off-flavor and coarse tofu texture (Hou and Chang 1998, 2005; Genovese and Lajolo 1992). However, soybeans in conditions of 57% RH 20°C, cool 4°C, or in an uncontrolled ambient temperature condition in North Dakota could retain their soymilk and tofu qualities for up to 18 months (Hou and Chang 2005a,b). In general, whole soybeans are more resistant to deterioration during storage than soy meal or damaged beans, including split and seed coat cracking. Usually, the amount of broken or damaged beans tends to increase with prolonged storage, especially when moisture content is low (<13%; Genovese and Lajolo 1992).

Under the conditions of 30°C, 82% RH, reducing sugar content in soybeans was first reduced and later nonreducing oligosaccharides in the soybeans were hydrolyzed. A part of the reducing sugars formed by the hydrolysis of the oligosaccharides may participate in the nonenzymatic glycosylation and in the Maillard reactions with the amino residues in the soy proteins (Hou and Chang 2004 and 2005a,b; Locher and Bucheli 1998; Wettlaufer and Leopold 1991). Sugar content in soybeans and tofu has significance in color and sweetness of the products.

Phytic acid affects the coagulation of soymilk during tofu making by decreasing pH after calcium salt is added. Phytate in soybeans degrades gradually with storage time in the adverse environment. However, under the mild or cold conditions, the hydrolysis of phytate also could occur, but at a lesser degree (Hou and Chang 2003). The hydrolysis of phytate in soybeans during storage contributes not only to the decrease of soymilk pH but also to the loss in chelating ability with calcium ions and subsequently affects protein coagulation behavior to lead to softer textural quality (Hou and Chang 2003). The effect of phytate on soymilk and tofu making and quality is discussed in the following sections.

8.3.3 Proteins

On a dry basis, approximately 35% to 40% of soybeans is made up of soy proteins (mostly globulins) and approximately 90% of these proteins is extractable with water or dilute salt solutions. Soy proteins consist of discrete groups of polypeptides that have a wide range of molecular size. A typical ultracentrifuge pattern of water-extractable soy proteins has four major fractions designated as 2S, 7S, 11S, and 15S on the basis of their sedimentation rates. Each fraction is a complex mixture of proteins. The 7S and 11S proteins are the two major storage proteins in soybeans, which comprise approximately 70% of storage protein. The 2S fraction accounts for approximately 20% of the extractable proteins, which contain protease inhibitors (Kunitz and the Bowman–Birk TIs) and cytochrome *c* (Steiner and Frattali 1969).

The 7S fraction has been classified into three major components with different physicochemical properties named β-conglycinin, γ-conglycinin, and basic 7S globulin (Catsimpoolas 1969; Hirano et al. 1987). β-Conglycinin is the most prevalent of these three and accounts for approximately 30% to 35% of the total seed protein, which is used interchangeably with 7S protein because it is the major 7S protein. The 11S fraction, designated as glycinin, accounts for an additional third of the total seed protein and is generally simple protein. The 15S fraction accounts for approximately 10% of the total seed protein, which is an aggregate of 11S protein (Wolf and Nelsen 1996).

Tofu is made from heated soymilk that is a turbid solution containing approximately 5% protein and 3% lipid. Soy proteins are the dominant components in tofu dry matter (more than 50% of the total solids on dry basis), which provide the major network structure of tofu gel. Soy proteins form an emulsion gel by a combination of heating and the addition of a coagulant, which is either an acid or divalent salt or a combination of both. This tofu emulsion is permanent because heating is not able to separate lipids from the protein system. Besides protein and lipid, other components in soymilk such as phytate, isoflavones, saponins, and lipoxygenases also may play important roles in the coagulation of proteins during curd

formation. Because of the complexity of the soymilk–tofu system, the mechanisms of tofu formation are also complex and are not fully understood. Although reports on understanding the interactions between nonprotein constituents (e.g., phytate and lipid) and proteins on the coagulating reaction in tofu making are available (Saio et al. 1969; Kumagai et al. 1998), there is a need for a comprehensive approach to put all factors together in one picture to understand the gel formation in tofu making.

Recent studies have shown that various soybean cultivars have various ratios of 11S/7S proteins that may influence the textural quality of tofu (Cai and Chang 1999; Poysa and Woodrow 2002; Mujoo et al. 2003). We found that the β-conglycinin (7S) and glycinin (11S) contents in 13 varieties are 17.2% to 23.1% and 36.3% to 51.3% of total proteins, respectively, and the 11S/7S protein ratio varied from 1.64 to 2.51 among the varieties (Cai and Chang 1999). Furthermore, positive correlations existed between tofu firmness and the 11S/7S ratios in various (13–16) soybean cultivars (Chang and Hou 2004; Cai and Chang 1999; Zhang and Chang 1996). Processing methods also affect 7S- and 11S-protein content of tofu and their contribution to tofu hardness, yield, and sensory quality. Thus, processing methods affect the relationships between 11S/7S ratios and textural quality because different coagulation processes and pressing steps are used for preparing different types of tofu (Cai and Chang 1999). However, conflicting results on the relationships between 11S/7S ratio and tofu firmness have been reported by other researchers (Utsumi and Kinsella 1985; Murphy et al. 1997; Skurray et al. 1980; Taira 1990). The conflicting report may be partly due to different methods used for processing in different laboratories.

8.3.4 Lipids and Phospholipids

Approximately 60% of total lipids in raw soymilk are associated with the protein particles; however, only 3% are found in the protein particles of cooked soymilk (Ono et al. 1996). Liu and Chang (2010) found that oil-protein particle in raw soymilk was significantly affected by soymilk preparation. At specific conditions, 80% to 90% of the total lipids and 30% to 40% of the total proteins could be precipitated by low speed centrifugation. The supernatant could be used to prepare low fat or fat free soymilk. The precipitate is 11S rich fraction with high lipid content and might be used as food ingredient with special functionality. After being heated to 65°C, part of the lipids and almost all α and α′ subunits of β-conglycinin in the particulate fraction begin to liberate to soluble fraction. At temperature higher than 90°C, almost all neutral lipids in the protein particles of raw soymilk are liberated to a floating fraction, and one half of the phospholipids remains in the particles (Ono et al. 1996; Guo et al. 1997b). The coagulation of soymilk depends on the concentration of coagulant, the pH of soymilk, and the temperature, the last being an external factor that accelerates soymilk coagulation. In fact, the addition of coagulant causes not only the coagulation of protein and gelation but also the incorporation of lipids into the protein gel with the lipid droplets being located in the networks of the protein gel (Guo et al. 1999; Yamano et al. 1981; Saio et al. 1969).

Phospholipids (including lecithin) are polar compounds. More than 50% of these lipids extracted from hexane-defatted soy meal are phopsholipids. They are believed to play an important role in combining the particulate proteins with neutral lipids (Ono et al. 1996). 11S and 7S proteins isolated from defatted soy meal contain approximately 0.8% and 2.3% phospholipids, respectively. Phospholipids bind to the hydrophobic sites of β-conglycinin (Ohtsuru et al. 1979). 7S protein is more hydrophobic than 11S protein, and therefore phospholipids bind stronger to the 7S proteins. Removal of lipids, particularly the phospholipids, from the surface of 7S proteins by extraction with a chloroform–methanol solution makes 7S proteins vulnerable to form insoluble aggregates, thus decreasing the ability to complex with protein particles. Adding phospholipids to soymilk increases the formation of protein particles. Lecithin-supplemented gels exhibit a fine network structure.

8.3.5 Phytic Acid

Phytic acid (phytate) content comprises 1% to 2% of soybeans on a dry basis and accounts for approximately 70% to 80% of the phosphorus in seeds (Cheryan 1980). It is structurally integrated with the protein bodies as phytin, a mixed potassium, magnesium, and calcium salt of inositol (Erdman 1979). Xu

et al. (2010) found that thermal processing condition may slightly reduce phytic acid content in soymilk. Phytate has strong chelating ability with multivalent metallic ions, especially zinc, calcium, and iron. According to Graf (1983), calcium ion can bind to phytic acid over a wide pH range (pH 4.8–10.4), the degree and tightness of binding being affected by pH, temperature, ionic strength, and size and valence of the cation. The affinity of phytic acid for calcium increases sharply with pH; the higher the pH (alkaline), the higher affinity; the affinity in pH 10.4 is a thousandfold higher than in pH 4.8.

It is reported that phytate attaches to the glycinin at pH between 2.5 and 5.0, and the extent of binding increases with decreasing pH; above the pI (isoelectric point) of glycinin (pH 4.9), no binding is found (Okubo et al. 1976). A portion of phytate in soymilk is bound to particulate and soluble proteins (approximately 35% and 23%, respectively). The others are present in the free form (approximately 42%; Ono et al. 1993). Therefore, when calcium is added to soymilk, it binds to phosphate groups of phytate and binds to proteins as well.

The role of phytic acid in the coagulation step during tofu making has been related to a decrease in pH after calcium salt is added (Ono et al. 1993; Tezuka and Ono 1995). When calcium is added, the phytate-calcium salts form at approximately the neutral pH (6.6). Hydrogen ions that bound originally with phosphate groups in phytate are liberated. Therefore, the pH decrease on the addition of calcium may be mainly due to the formation of phytate-calcium salts. The decrease in pH of soymilk from approximately 6.6 to 5.8 after the addition of calcium chloride allows the use of a lower concentration of Ca salts for coagulation. When calcium is present, it binds simultaneously to proteins and phytate, and calcium binding to proteins can retard the decrease of pH because of less phytate-calcium formation. Tofu curd contains both types of calcium bound to protein or phytate.

Phytate is also very important in relation to the speed of coagulation during tofu making. It has been found that the higher content of phytic acid results in a slower coagulation of soymilk during tofu making and gives a higher tofu yield (Saio et al. 1969). Our study (Liu and Chang 2004) shows that there is a significant correlation between the level of phytic acid in soymilk and the amount of coagulant required to reach the optimum coagulation. Recently, this relationship was confirmed by actual tofu making with different types of coagulants (Ishiguro and Ono 2010; Ishiguro et al. 2006, 2008; Toda et al. 2006). It is well known that the amount of coagulant greatly affects tofu texture and yield. Therefore, the phytate content in soymilk can affect yield and the textural properties of tofu. To understand the role of phytate on yield and textural properties of tofu, we conducted a series of experiments using Proto soybean as the raw materials that were stored under a series of environmental conditions to induce the changes of phytate (Hou and Chang 2003). Significant positive relationships exist between soybean phytate and tofu yield ($r = 0.93$) and between soymilk phytate and tofu yield ($r = 0.95$). Negative correlations between soybean phytate and tofu hardness and brittleness are observed ($r = -0.92$ and -0.84, respectively). Negative correlation coefficients are found between soymilk phytate and tofu hardness and brittleness ($r = -0.94$ and -0.86, respectively). Although statistical correlation between phytate content existed, phytate does not have a direct effect on tofu yield because when phytate changes in soybeans during storage, other components such as protein solubility, protein molecular structures, and titratable acidity also are changed simultaneously (Hou and Chang 2004 and 2005a,b). To understand the separate effect of phytate, we used phytase to hydrolyze the phytate in soymilk to that of the stored beans. Reducing phytate actually increases tofu yield but reduces firmness. Therefore, phytate plays a minor role in affecting tofu quality as long as the coagulant used is optimal. Besides its effect on texture of tofu, phytate has been recently reported to be responsible for the astringency of soymilk (Al Mahfuz et al. 2004 a,b). The astringency in soymilk disappears when the soymilk is coagulated with calcium salt to tofu. Therefore, the calcium salt form of phytic acid has no astringent taste.

8.3.6 Isoflavones and Saponins

The distribution of isoflavones, a subclass of the more familiar flavonoids, is extremely limited in nature. Soybeans and soy foods are the major foods containing a significant amount of isoflavones. The main isoflavones found in soybeans are genistein, daidzein, and glycitein, each of which exists in four chemical forms, as an aglycone form (genistein, daidzein, and glycitein), a β-glucoside form

(genistin, daidzin, and glycitin), a malonylglucoside form (6″-*O*-malonylgenistin, 6″-*O*-malonyldaidzin, and 6″-*O*-malonylglycitin), and an acetylglucoside form (6″-*O*-acetylgenistin, 6″-*O*-acetyldaidzin, and 6″-*O*-acetylglycitin). Isoflavone glucoside form has lower bioavailability than its aglycone form. It was reported that 84.5% of isoflavone glucoside could be converted to aglycone form when 0.02% (w/v) β-glycosidase was added to soymilk and kept at 55°C for 30 minutes (Rye et al. 2010).

Isoflavone content varies among soybean varieties, which contain approximately 1 to 4 mg/g soybean. The isoflavone content of soybeans is markedly affected by crop year and growing condition (Wang and Murphy 1994; Hoeck et al. 2000). Hui et al. (2001) found large variation in isoflavone aglycones content of tofu not only between different brands but also between different batches of the same brand. Isoflavones are quite heat stable. Wang and Murphy (1994) reported that cooking did not influence the isoflavone retention during tofu making but alter the distribution of isoflavones by dramatically decreasing in malonylglucoside forms and increasing in acetylglucoside forms. Xu and Chang (2009) found that the thermal processing of soymilk caused significant increases in glucoside and acetylglucoside forms but caused significant decreases in malonylglucoside and aglycone forms. In addition, indirect UHT processing converted more isoflavones from malonylglucoside form into glucoside form than the direct UHT did. Minimal heat processing can convert substantial amounts of malonylglucoside to the β-glucosides. Total isoflavone content in soy products decreased most likely due to leaching of isoflavones into water during processing. Wang and Murphy (1996) reported that 44% of total isoflavones were lost during the processing of tofu. Coagulant type and concentration affect the recovery of isoflavones in tofu (Kao et al. 2004; Rekha and Vijayalakshmi 2010). The concentration of 0.3% calcium sulfate gives the highest tofu recovery as compared with that made by calcium chloride. Isoflavones have several potential health benefits (Chang and Hou 2004). Liu et al. (2005) reported that tofu has *in vitro* and *in vivo* antioxidant activity as high as that of 250 ppm isoflavone crude extracts. Recently, we found that soymilk phenolic extract could inhibit human tumor cell proliferation *in vitro*. Raw and processed soymilks show antiproliferative activities against human HL-60 leukemia cells, AGS gastric tumor cells, and DU 145 prostate cancer cells in a dose-dependent manner (Xu et al. 2010).

Saponins are widely distributed in plants and are glycosides composed of a sapogenin that makes up the aglycone and a sugar moiety. The sapogenin is a triterpeneoid alcohol. At least five sapogenins have been found in soybeans (Birk 1969). Xylose, arabinose, galactose, glucose, rhamnose, and glucuronic acid have been found in the glucoside portion of soy saponins. Saponins exist in two groups, A and B (Shiraiwa et al. 1991a,b; Kudou et al. 1992). The group A saponins consist of six different kinds of saponins (Aa, Ab, Ac, Ad, Ae, and Af), which are acetyl-soyasaponins. The group B saponins, on the other hand, consist of eight kinds of saponins (Ba, Bb, Bb′, Bc, Bd, Be, BdA, and BeA), which are not acetylated and are different from the group A. BdA is the major natural soybean saponin in soybean seeds (Kudou et al. 1992). Saponins are polar compounds because of the associated sugars (oligosaccharides), which are found in the soybean meal in amounts of approximately 0.5% of the dry weight (Fenwick and Oakenfull 1981). Xu et al. (2010) found that thermal processing condition may reduce total saponin content in soymilk.

It is known that isoflavones and saponins impart bitter and astringent aftertastes in the flavor of soy products. Glucoside forms of saponins and isoflavones are the major compounds that cause objectionable aftertaste in soybeans, and saponin A group contributes most strongly to the undesirable taste. The undesirable taste becomes weaker when saponins decompose from glucoside forms to aglycone forms, but isoflavone glucosides show a reverse tendency. The aglycones of isoflavones have stronger objectionable aftertaste than those of glucosides (Okubo et al. 1992; Matsuura et al. 1989).

Saponins, glycitin, and glycitin derivatives are present primarily in hypocotyl, and all of these substances can give objectionable aftertaste. In Japan, the removal of these compounds by processing methods has been reported to produce good-tasting tofu using the Namashibori technique (Watanabe 1997). In the method, the soybean is first cracked, and seed coat and hypocotyl are removed. The resulting materials are soaked in water for a short period, ground to make the slurry "go," and filtered. The raw soymilk is boiled, and the brown foams (which contain saponins) are scooped away to reduce aftertaste. However, in light of potential health benefits of these isoflavone and saponin compounds (Hasler 1998), a different strategy may be needed to preserve these compounds to produce soy foods with the maximum health benefits.

8.3.7 Lipoxygenases

Lipoxygenases and hydroperoxide lyase (Matoba et al. 1985) are involved in the oxidation of polyunsaturated fatty acids such as linoleic acid and linolenic acids to hydroperoxides and secondary breakdown products, some of which have a beany flavor. Heating can reduce the activity of lipoxygenases and hydroperoxide lyase (see Section 8.2.1.5, on the reduction of beany flavor, and Section 8.2.1.3).

8.4 Mechanism of Tofu Curd Formation

There are several reviews on the mechanism of tofu curd formation (Saio et al. 1969; Tezuka et al. 2000; Ono et al. 1996; Guo et al. 1997a,b, 1999; Ono et al. 1991, 1993; Ono 2000; Tezuka and Ono 1995; Ono et al. 2010). It could be summarized into three steps.

- The total of the large- and medium-sized particles constitutes more than 50% of the proteins in soymilk. Before heating the raw soymilk, most of the particles are still intact. Most lipids are present in the protein particles.
- When the soymilk is heated up to 90°C, the proteins denature and the lipid droplets are liberated to floating fraction. Approximately 75% of the large particles is degraded to supernatant proteins, but the amount of medium-sized particles increases because of the combination of β subunit of 7S and the basic polypeptide of 11S from the supernatant proteins that contain mainly 11S and 7S globulin.
- The addition of coagulant (calcium ion, magnesium ion, and/or GDL) to the heated soymilk is the key step. At low concentration of coagulant(s), protein particles combine with lipid droplets, and the gel network is first formed through the binding of calcium to protein particles to neutralize the negatively charged protein molecules and to cause protein aggregation due to a reduction in electrostatic repulsion. When approximately 50% of the proteins is coagulated with coagulant, almost all lipids in soymilk are trapped and become inseparable by the association with particulate proteins. With the availability of more coagulant(s), it leads to a decrease in pH. The soluble proteins aggregate at a higher concentration of coagulant and bind to the protein particles–oil droplet complex to form a stable tofu–curd emulsion network.

8.5 Food Quality Evaluation of Soybeans, Soymilk, and Tofu: Trading and Industry Perspective

8.5.1 Soybean Quality

As discussed earlier, soymilk and tofu quality characteristics are affected by raw material characteristics, processing, and storage. In trading, identity-preserved and organic food soybeans are preferred by the industry. Soybeans are alive, and each lot of the soybeans may have unique properties and can change over time. The seller and the purchasers should maintain a record of the history of the soybeans with respect to variety (cultivar), production environment (location, soil, water, temperature, and sunlight of cultivation), harvest and storage conditions (humidity, temperature, and time), and shipping conditions. Mechanical drying after harvest should be performed very carefully so that the proteins are not denatured.

The following are the factors that are commonly considered in trading soybeans for food uses.

8.5.1.1 Size and Hilum Color of Soybeans

Traditionally, large size is preferred for making tofu. However, our research shows that size is not a factor to affect tofu quality (Wang and Chang 1995). Size uniformity and % hard-to-soak beans (stone beans) are important. Clear hilum soybeans have been preferred by the industry. However, our research shows

that soymilk and tofu color is not related to hilum color. We have observed that a brown hilum color soybean produces soymilk with whiter color than some white hilum soybeans.

8.5.1.2 Chemical Composition as Related to Food Quality

Protein content and protein subunit patterns (11S and 7S subunit distributions) affect the yield, composition, and texture of soymilk and tofu. Lipoxygenases, lipid, and fatty acid content affect the flavor of soymilk and tofu. Sugar content affects the taste. Phytate, saponins, and isoflavones affect the bitterness and astringency of the soymilk products. However, the exact contributions of these compounds to food quality are not well established.

8.5.1.3 Storage Changes

Undesirable storage changes can be measured. Chemical changes include protein solubility, titratable acidity, or pH of soymilk. Rancidity or oxidized off-flavor may be resulted from improper storage. Color is a good indicator of the changes of soybeans during storage and can be measured using a surface colorimeter such as the Minolta colorimeter with the Hunter's Lab scale. The optimal coagulant concentration for making tofu decreases with storage (Liu and Chang 2003, 2008). Soymilk and tofu yield and texture need to be measured before trading. Soybeans should be kept in a low RH and cool temperature to preserve the quality for food making (Chang 2010; Hou and Chang 2005a).

8.5.2 Soymilk Quality

Indicators for soymilk include protein recovery, sugar content, color, beany aroma or off-flavor, and taste (sweetness, bitterness, astringency, aftertaste, and chalky taste). Instrumental analysis can be used to measure color. Protein recovery in soymilk should be measured. Additional composition of interest could also be measured. Aroma and taste should be measured using appropriate methods.

8.5.3 Tofu Quality

In addition to the yield of tofu such as tofu made from 100 g soybean, color, texture (firmness, cohesiveness, elasticity, and smoothness), and taste are important characteristics of tofu. Small-scale (Yuan and Chang 2010a,b; Chang and Hou 2004) or pilot-scale making equipment (Figures 8.5 and 8.6) are useful for processing tofu and soymilk for quality evaluation of soybean materials (Cai et al. 1997; Chang and Hou 2004). Chemical methods and sensory methods are available for measuring the above characteristics. Instrumental methods are commonly used for textural analyses (Yuan and Chang 2010a,b, Yuan and Chang 2007c). Hexanal, a major indicator of grassy, beany flavor compound commonly analyzed by gas chromatography, is strongly affected by raw materials and processing methods (Yuan and Chang 2007a,b). Because tofu researchers use different parameters for textural analysis, the interlaboratory

FIGURE 8.5 A pilot-plant small production scale tofu machine (Model PT-60 Ta-Ti-Hsing Co.) housed in Dr. Chang's laboratory at the North Dakota State University for traditional pressed tofu making.

FIGURE 8.6 A small pilot-scale UHT processor (Microthermics Co.) housed in Dr. Chang's laboratory at North Dakota State University for soymilk quality research.

results are difficult to compare. The Instron universal testing equipment (Bourne 1982) is the most reported method for textural profile analysis of tofu (Chang and Hou 2004; Yuan and Chang 2007c). Yuan and Chang (2007c) investigated the optimal Instron settings for measuring textural qualities of 13 different types of tofu and found that the instrumental analysis using the selected parameters (75% plunger penetration and cross-head speed of 60 to 100 mm/minutes for cylindrical sample size of 44 mm diameter and 1.5 cm height) correlated very well with the results obtained from the human sensory testing methods.

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REFERENCES

Al Mahfuz A, Tsukamoto C, Ono T, editors. 2004a. The decrease mechanism of undesirable astringent taste in soymilk during tofu curd formation. Proceedings of VII World Soybean Research Conference and IV International Soybean Processing and Utilization Conference and III Brazilian Soybean Congress; 2004 February 29–March 5; 1039–54. Foz do Iguassu, Brazil.

Al Mahfuz A, Tsukamoto C, Kudou S, Ono T. 2004b. Changes of astringent sensation of soy milk during tofu curd formation. *J Agric Food Chem* 52:7070–4.

Alan LJL, Ho LJ, Kong TY, inventors; Method for producing an aseptic packaged tofu product. U.S. patent 5,863,590.

Axelrod B, Cheesbrough TM, Laakso S. 1981. Lipoxygenase from soybeans. In: Lowenstein JM, editor. *Methods in enzymology*. Vol. 71. New York: Academic Press. p 441–51.

Beddows CG, Wong J. 1987a. Optimization of yield and properties of silken tofu from soybeans: I. The water:bean ratio. *Int J Food Sci Technol* 22:15–21.

Beddows CG, Wong J. 1987b. Optimization of yield and properties of silken tofu from soybeans: III. Coagulant concentration, mixing and filtration pressure. *Int J Food Sci Technol* 22:29–34.

Birk Y. 1969. Saponins. In: Liener IE, editor. Toxic constituents of plant foodstuffs. New York: Academic Press. p 169–210.

Bourne MC. 1982. Textural profile analysis. *Food textures and viscosity: Concept and measurement*. New York: Academic Press. p 114–7.

Cadwallader KR, Chang SKC, editors. 2010. Chemistry, texture and flavor of soy. In: ACS Symposium Series 1059. Washington, DC: American Chemical Society.

Cai TD, Chang KC. 1999. Processing effect on soybean storage proteins and their relationship with tofu quality. *J Agric Food Chem* 47:720–7.

Cai TD, Chang KC, Shih MC, Hou HJ, Ji M. 1997. Comparison of bench and production scale methods for making soymilk and tofu from 13 soybean varieties. *Food Res Int* 30:659–68.

Catsimpoolas N. 1969. A note on the proposal of an immunochemical system of reference and nomenclature for the major soybean globulins. *Cereal Chem* 46:369–72.

Chang KC. 2010. Storage-induced color and biological changes of soybeans as related to soymilk and tofu making. In: Cadwallader KR, Chang SKC, editors. *Chemistry, texture and flavor of soy*. ACS Symposium Series 1059. Washington, DC: American Chemical Society. p 113–30.

Chang KC, Hou HJ. 2004. Science and technology of tofu making. In: Hui YH, Ghazala S, Murrell KD, Nip W-K, editors. *Handbook of vegetable preservation and processing*. New York: Marcel Dekker. p 443–78.

Chang KC, Liu ZS, Hou HJ, Wilson LA, editors. 2004. Influence of Storage on the Characteristics of Soybean, Soymilk and Tofu.: Proceedings of VII World Soybean Research Conference and IV International Soybean Processing and Utilization Conference and III Brazilian Soybean Congress; 2004 February 29–March 5; 977–83. Foz do Iguassu, Brazil.

Chang SKC. 2006. Chemistry and technology of tofu making. In: Hui YH, editor. *Handbook of food science, technology and engineering*. Vol. IV. Food Technology and Food Processing; Chapter 171. Boca Raton, FL: CRC Press. p 171.1–171.24.

Chen S, editor. 1989. Preparation of fluid soymilk. Proceedings of the World Congress on Vegetable Protein Utilization in Human Foods and Animal Feedstuffs; 1988; Singapore. Champaign, IL: American Oil Chemists' Society. p 341–52.

Cheryan M. 1980. Phytic acid interactions in food systems. *CRC Crit Rev Food Sci Nutri* 20:297–335.

Christopher J, Axelrod B. 1971. On the different positional specificities of peroxidation of linoleate shown by two isozymes of soybean lipoxygenase. *Biochem Biophys Res Commun* 44:731–6.

Christopher JP, Pistorius EK, Axelrod B. 1970. Isolation of an isoenzyme of soybean lipoxygenase. *Biochim Biophys Acta* 198:12–9.

Christopher JP, Pistorius EK, Axelrod B. 1972. Isolation of a third isoenzyme of soybean lipoxygenase. *Biochim Biophys Acta* 284:54–62.

Clark DK, Snyder HE. 1991. Hydroperoxide formation in soybean seeds during storage. *J Amer Oil Chem Soc* 68:346–7.

Erdman JW Jr. 1979. Oilseed phytates: Nutritional implications. *J Amer Oil Chem Soc* 56:736–41.

Fenwick DE, Oakenfull D. 1981. Saponin content of soybeans and some commercial soybean products. *J Sci Food Agric* 32:273–8.

Genovese MI, Lajolo FM. 1992. Physicochemical properties of isolated soy proteins from normal, broken or damaged seeds. *J Food Sci* 57:1378–81.

Graf E. 1983. Calcium binding to phytic acid. *J Agric Food Chem* 31:851–5.

Guo QC, Liang HH, Qin WH. 1997a. The research on trypsin inhibitor activity for commercial soy beverage. *China Dairy Ind* 25(6):8–10.

Guo ST, Ono T, Mikami M. 1997b. Interaction between protein and lipid in soybean milk at elevated temperature. *J Agric Food Chem* 45:4601–5.

Guo ST, Ono T, Mikami M. 1999. Incorporation of soy milk lipid into protein coagulum by addition of calcium. *J Agric Food Chem* 47:901–5.

Hajika M, Igita K, Nakazawa Y. 1995. Induction of a soybean line lacking all seed lipoxygenase isozymes. *Jpn Agric Res Quart* 29:73–6.

Hasler CM. 1998. Functional foods: Their role in disease prevention and health promotion. *Food Technol* 52(11):63–70.

Hirano H, Kagawa H, Kamata Y, Tamauchi F. 1987. Structural homology among the major 7S globulin subunits of soybean seed storage proteins. *Phytochemistry* 26:41–5.

Hoeck JA, Fehr WR, Murphy PA, Welke GA. 2000. Influence of genotype and environment on isoflavone contents of soybean. *Crop Sci* 40:48–51.

Hou HJ, Chang KC. 1998. Yield and quality of soft tofu as affected by soybean physical damage and storage. *J Agric Food Chem* 46:4798–805.

Hou HJ, Chang KC. 2002. Interconversion of isoflavones in soybeans as affected by storage. *J Food Sci* 67:2083–9.

Hou HJ, Chang KC. 2003. Yield and textural properties of soft tofu as affected by changes of phytate content during soybean storage. *J Food Sci* 68:1185–91.

Hou HJ, Chang KC. 2004. Structural characteristics of purified glycinin from soybeans stored under various conditions. *J Agric Food Chem* 52:3792–800.

Hou HJ, Chang KC. 2005a. Storage affects color and chemical composition and tofu making quality. *J Food Process Preserv* 28:473–88.

Hou HJ, Chang KC. 2005b. Structural characteristics of purified β-conglycinin from soybeans stored under various conditions. *J Agric Food Chem* 52:7931–7.

Hui E, Henning SM, Park N, Heber D, Liang V, Go W. 2001. Genistein and daidzein/glycitein content in tofu. *J Food Compos Anal* 14:199–206.

Ishiguro T, Ono T. 2010. Soybean phytate content and its influence on tofu texture. In: Cadwallader KR, Chang SKC, editors. *Chemistry, texture and flavor of soy*. ACS Symposium Series 1059. Washington, DC: American Chemical Society. p 249–54.

Ishiguro T, Ono T, Nakasato K. 2008. The localization of phytate in tofu curd formation and effects of phytate on tofu texture. *J Food Sci* 73(2):C67–71.

Ishiguro T, Ono T, Wada T, Tsukamoto C, Kono Y. 2006. Changes in soybean phytate content as a result of field growing conditions and influence on tofu texture. *Biosci Biotechnol Biochem* 70(4):874–80.

Kao FJ, Su NW, Lee MH. 2003. Effect of calcium sulfate concentration in soymilk on the microstructure of firm tofu and the protein constitutions in tofu whey. *J Agric Food Chem* 51(21):6211–6.

Kao TH, Lu YF, Hsieh HC, Chen BH. 2004. Stability of isoflavone glucosides during processing of soymilk and tofu. *Food Res Int* 37(9):891–900.

Kong FB, Chang KC. 2009. Statistical and kinetic studies of the changes in soybean quality during storage as related to soymilk and tofu making. *J Food Sci* 74(2): S81–9.

Kong FB, Chang KC, Liu ZS, Wilson. 2008. Changes of soybean quality during storage as related to soymilk and tofu making. *J Food Sci* 73(3):S134–44.

Kudou S, Uchida T, Okubo K. 1992. Off-flavor of soybean and fermented foods. *J Brew Soc Jpn* 87:29–35.

Kumagai H, Shizawa Y, Sakurai H, Kumagai H. 1998. Influence of phytate removal and structural modification on the calcium-binding properties of soybean globulins. *Biosci Biotechnol Biochem* 62:341–6.

Kwok KC, Liang HH, Niranjan K. 2002. Optimizing conditions for thermal processes of soymilk. *J Agric Food Chem* 50:4834–8.

Kwok KC, Qin WH, Tsang JC. 1993. Heat inactivation of trypsin inhibitors in soymilk at ultra high temperatures. *J Food Sci* 58:859–62.

Lim BT, deMan JM, deMan L, Buzzell RI. 1990. Yield and quality of tofu as affected by soybean and soymilk characteristics. Calcium sulfate coagulant. *J Food Sci* 55:1088–92, 1111.

List GR, Mounts TL. 1993. Origin of the nonhydratable soybean phosphatides: Whole beans or extraction? *J Amer Oil Chem Soc* 70:639–41.

Liu JB, Chang KC, Wiesenborn DP. 2005. Antioxidant properties of soybean isoflavone extract and tofu *in vitro* and *in vivo*. *J Agric Food Chem* 53:2333–40.

Liu KS. 1997. Soybeans: Chemistry, technology, and utilization. New York: Chapman & Hall. p 137–97.

Liu ZS, Chang KC. 2003. Development of a titration method for predicting the optimal concentrations of coagulants for making filled tofu. *J Agric Food Chem*. 51:5214–21.

Liu ZS, Chang KC. 2004. Effect of soymilk characteristics and cooking conditions on optimal coagulant concentration for making filled tofu. *J Agric Food Chem* 52:3405–11.

Liu ZS, Chang KC. 2007. Soymilk viscosity as influenced by heating methods and soybean varieties. *J Food Process Preserv* 31:320–33.

Liu ZS, Chang KC. 2008. Optimal coagulant concentration, soymilk and tofu quality as affected by a short-term model storage of Proto soybeans. *J Food Process Preserv* 32:39–59.

Liu ZS, Chang SK. 2010. Chemical characteristics of low-fat soymilk prepared by low-speed centrifugal fractionation of the raw soymilk. *J Food Sci* 75(5):C420–7.

Liu ZS, Chang SKC, Li L, Tatsumi E. 2004. Effect of selective thermal denaturation of soybean proteins on soymilk viscosity and tofu‘s physical properties. *Food Res Int* 37:815–22.

Locher R, Bucheli P. 1998. Comparison of soluble sugar degradation in soybean seed under simulated tropical storage conditions. *Crop Sci* 38:1229–35.

Matoba T, Hidaka H, Kitmura K, Kaizuma H, Kito M. 1985. Contribution of hydroperoxide lyase activity to n-hexanal formation in soybean. *J Agric Food Chem* 33:856–8.

Matsuura M, Obata A, Fukushima D. 1989. Objectionable flavor of soy milk developed during the soaking of soybeans and its control. *J Food Sci* 54:602–5.

Moizuddin S, Harvey G. Fenton AM, Wilson, LA. 1999. Tofu production from soybeans or full-fat soyflakes using direct and indirect heating processes. *J Food Sci* 64:684–7.

Mounts TL, Nash AM. 1990. HPLC analysis of phospholipids in crude oil for evaluation of soybean deterioration. *J Amer Oil Chem Soc* 67:757–60.

Mujoo R, Trinh DT, Ng PKW. 2003. Characterization of storage protein in different soybean varieties and their relationship to tofu yield and texture. *Food Chem* 82(2):265–73.

Murphy PA, Chen HP, Hauck CC, Wilson LA. 1997. Soybean protein composition and tofu quality. *Food Technol* 51:86–8, 110.

Nakayama Y, Saio K, Kito M. 1981. Decomposition of phospholipid in soybeans during storage. *Cereal Chem* 58:260–4.

Narayan R, Chauhan GS, Verma NS. 1988. Changes in the quality of soybean during storage: Part I. Effect of storage on some physico-chemical properties of soybean. *Food Chem* 27:13–23.

Obata A, Matsuura M. 1993. Decrease in the gel strength of tofu caused by an enzyme reaction during soybean grinding and its control. *Biosci Biotechnol Biochem* 57:542–5.

Obata A, Matsuura M, Kitamura K. 1996. Degradation of sulfhydryl groups in soymilk by lipoxygenases during soybean grinding. *Biosci Biotechnol Biochem* 60:1229–32.

Ohtsuru M, Yamashita Y, Kanamoto R, Kito M. 1979. Association of phosphatidylcholine with soybean 7S globulin and its effect on the protein conformation. *Agric Biol Chem* 43:765–70.

Okubo K, Iijima M, Kobayashi Y, Yoshikoshi M, Uchida T, Kudou S. 1992. Components responsible for the undesirable taste of soybean seeds. *Biosci Biotechnol Biochem* 56:99–103.

Okubo K, Myers DV, Iacomucci GA. 1976. Binding of Phytic Acid to Glycinin. *Cereal Chem* 53:513–24.

Ono T, editor. The mechanisms of curd formation from soybean milk to make a stable lipid food. Proceedings of the Third International Soybean Processing and Utilization Conference; 2000; Ibaraki, Japan. 2000. p 51–52.

Ono T, Choi MR, Ikeda A, Odagiri S. 1991. Changes in the composition and size distribution of soymilk protein particles by heating. *Agric Biol Chem* 55:2291–7.

Ono T, Katho S, Mothizuki K. 1993. Influences of calcium and pH on protein solubility in soybean milk. *Biosci Biotechnol Biochem* 57:24–8.

Ono T, Onodera Y, Chen Y, K. Nakasto. 2010. Tofu structure is regulated by soymilk protein composition and coagulant concentration. In: Cadwallader KR, Chang SKC, editors. *Chemistry, texture and flavor of soy*. ACS Symposium Series 1059. Washington, DC: American Chemical Society. p 219–29.

Ono T, Takeda M, Guo ST. 1996. Interaction of protein particles with lipids in soybean milk. *Biosci Biotechnol Biochem* 60:1165–9.

Parrish DJ, Leopold AC. 1978. On the mechanism of aging in soybean seeds. *Plant Physiol* 61:365–8.

Poysa V, Woodrow L. 2002. Stability of soybean seed composition and its effect on soymilk and tofu yield and quality. *Food Res Int* 35(4):337–45.

Rekha C, Vijayalakshmi G. 2010. Influence of natural coagulants on isoflavones and antioxidant activity of tofu. *J Food Sci Technol* 47(4):387–93.

Rouhana A, Adler-Nissen J, Cogan U, Frokiaer H. 1996. Heat inactivation kinetics of trypsin inhibitors during high temperature-short time processing of soymilk. *J Food Sci* 61:265–9.

Rye YG, Won B, Park HR, Ghafoor K, Park J. 2010. Effects of the β-glycosidase reaction on bio-conversion of isoflavones and quality during tofu processing. *J Sci Food Agric* 90:843–9.

Saio K. 1979. Tofu—relationships between texture and fine structure. *Cereal Foods World* 24:342–54.

Saio K, Kobayakawa K, Kito M. 1982. Protein denaturation during model storage studies of soybeans and meals. *Cereal Chem* 59:408–12.

Saio K, Koyama E, Yamazaki S, Watanabe T. 1969. Protein–calcium–phytic acid relationships in soybean: Part III. Effect of phytic acid on coagulative reaction in tofu-making. *Agric Biol Chem* 33:36–42.

Saio K, Nikkuni I, Ando Y, Otsuru M, Terauchi Y, Kito M. 1980. Soybean quality changes during model storage studies. *Cereal Chem* 57:77–82.

Shih MC, Hou HJ, Chang KC. 1997. Process optimization of soft tofu. *J Food Sci* 62:833–7.

Shiraiwa M, Harada K, Okubo K. 1991a. Composition and structure of 'group a saponin' in soybean seed. *Agric Biol Chem* 55:315–22.

Shiraiwa M, Harada K, Okubo K. 1991b. Composition and structure of 'group b saponin' in soybean seed. *Agric Biol Chem* 55:911–7.

Shurtleff W, Aoyagi A. 1990. *Tofu and soymilk production*, Vol. 2. Lafayette, CA: Soya Food Center. p 115–31.

Skurray G, Cunich J, Carter D. 1980. The effect of different varieties of soybean and calcium ion concentration on the quality of tofu. *Food Chem* 6:89–95.

Steiner RF, Frattali V. 1969. Purification and properties of soybean protein inhibitors of proteolytic enzymes. *J Agric Food Chem* 17:513–8.

Stewart RRC, Bewley D. 1980. Lipid peroxidation associated with accelerated aging of soybean axes. *Plant Physiol* 65:245–8.

Sun C, Cadwallader KR, Kim H. 2010. Composition of key aroma components between soymilks prepared by cold and hot grinding methods. In: Cadwallader KR, Chang SKC, editors. *Chemistry, texture and flavor of soy*. ACS Symposium Series 1059. Washington, DC: American Chemical Society. p 361–72.

Taira H. 1990. Quality of soybeans for processed foods in Japan. *Jpn Agric Res Quart* 24:224–30.

Takamura H, Kitamura K, Kito M. 1991. Inhibition by lipoxygenase-3 of *n*-hexanal generation in soybeans. *FEBS Lett* 292:42–4.

Tezuka M, Ono T. 1995. Properties of soymilk prepared from soybeans of different varieties. *Nippon Shokuhin Kogyo Kagaku Kaishi* 42:556–61.

Tezuka, M, Taira H. Igarashi Y, Yagasaki K, Ono T. 2000. Properties of tofus and soymilks prepared from soybeans having different subunits of glycinin. *J Agric Food Chem* 48:1111–7.

Thomas R, deMan JM, deMan L. 1989. Soymilk and tofu properties as influenced by soybean storage conditions. *J Amer Oil Chem Soc* 66:777–82.

Toda K, Takahashi K, Ono T, Kitamura K, Nakamura Y. 2006. Variation in the phytic acid content of soybeans and its effect on consistency of tofu made from soybean varieties with high protein content. *J Sci Food Agric* 86:212–9.

Torres-Penaranda AV, Reitmeier CA, Wilson LA, Fehr WR, Narvel JM. 1998. Sensory characteristics of soymilk and tofu made from lipoxygenase-free and normal soybeans. *J Food Sci* 63:1084–7.

Trawatha SE, Tekrony DM, Hildebrand DF. 1995. Soybean lipoxygenase mutants and seed longevity. *Crop Sci* 35:862–8.

Utsumi S, Kinsella JE. 1985. Forces involved in soy protein gelation: Effect of various reagents on the formation, hardness and solubility of heat-induced gels made from 7S, 11S and soy isolate. *J Food Sci* 50:1278–82.

Wang CCR, Chang KC. 1995. Physicochemical properties and tofu quality of soybean cultivar proto. *J Agric Food Chem* 43:3029–34.

Wang HJ, Murphy PA. 1994. Isoflavone composition of american and japanese soybeans in Iowa: Effects of variety, crop year, and location. *J Agric Food Chem* 42:1674–7.

Wang HJ, Murphy PA. 1996. Mass balance study of isoflavones during soybean processing. *J Agric Food Chem* 44:2377–83.

Wang HL, Hesseltine CW. 1982. Coagulation conditions in tofu processing. *Process Biochem* 17:7–12.

Wang HL, Swain EW, Kwolek WF. 1983. Effect of soybean varieties on the yield and quality of tofu. *Cereal Chem* 60:245–8.

Watanabe T. 1997. Science of tofu. Kyoto, Japan: Food Journal Co. p 14–54.

Watanabe T, Fukamachi E, Nakayama O, Teremachi T, Abe K, Seruga S, Miyagana S. 1964. Research into the standardization of the tofu making process. National Food Institute Reports, Parts 1–3. Japan: National Food Institute.

Wettlaufer SH, Leopold AC. 1991. Relevance of amadori and maillard products to seed deterioration. *Plant Physiol* 97:165–9.

Wilkens WF, Mattick LR, Hand DB. 1967. Effect of processing method on oxidative off-flavors of soybean milk. *Food Technol* 21:1630–2.

Wilson DO Jr., McDonald MB. 1986. The lipid peroxidation model of seed aging. *Seed Sci Technol* 14:269–300.

Wilson LA. 1996. Comparison of lipoxygenase-null and lipoxygenase-containing soybeans for foods. In: Piazza GJ, editor. *Lipoxygenase and lipoxygenase pathway enzymes*. Champaign, IL: AOCS Press. p 209–25.

Wolf W, Nelsen TC. 1996. Partial purification and characterization of the 15S globulin of soybeans, a dimer of glycinin. *J Agric Food Chem* 44:785–91.

Xu B, Chang SK. 2009. Isoflavones, flavan-3-ols, phenolic acids, total phenolic profiles, and antioxidant capacities of soy milk as affected by ultrahigh-temperature and traditional processing methods. *J Agric Food* Chem 57(11):4706–17.

Xu B, Chang SK, Liu Z, Yuan S, Zou Y, Tan Y. 2010. Comparative studies on the chemical and cell-based antioxidant activities and antitumor cell proliferation properties of soy milk manufactured by conventional and commercial UHT methods. *J Agric Food Chem* 58(6):3558–66.

Yamano Y, Miki E, Fukui Y. 1981. Incorporation of lipid into soybean protein gel and the role of 11S and 7S protein. *Nippon Shyokuhin Kogyo Gakkaishi* 28:136–41.

Yuan SH, Chang KC. 2007a. Selected odor compounds in soymilk as affected by chemical composition and lipoxygenases in five soybean materials. *J Agric Food Chem* 55:426–31.

Yuan SH, Chang KC. 2007b. Selected odor compounds in cooked soymilk as affected by soybean materials and direct steam injection. *J Food Sci* 72: S481–6.

Yuan SH, Chang KC. 2007c. Texture profile of tofu as affected by instron parameters and sample preparation, and correlations of instron hardness and springiness with sensory scores. *J Food Sci* 72:S135–45.

Yuan SH, Chang KC. 2010a. Standardization of physical parameters for instron texture analysis for tofu quality evaluation. In: Cadwallader KR, Chang SKC, editors. *Chemistry, texture and flavor of soy*. ACS Symposium Series 1059. Washington, DC: American Chemical Society. p 231–48.

Yuan SH, Chang KC. 2010b. Trypsin inhibitor activity in laboratory-produced and commercial soymilk. In: Cadwallader KR, Chang SKC, editors. *Chemistry, texture and flavor of soy*. ACS Symposium Series 1059. Washington, DC: American Chemical Society. p 23–43.

Yuan S, Chang SK, Liu Z, Xu B. 2008. Elimination of trypsin inhibitor activity and beany flavor in soy milk by consecutive blanching and ultrahigh-temperature (UHT) processing. *J Agric Food Chem* 56(17):7957–63.

Zhang GH, Chang KC. 1996. Tofu quality as related to soybean and soymilk composition. Abstract of 1996 Annual Meeting of Institute of Food Technologists, New Orleans, LA.

Part III

Fruits and Fruit Products

9

Sensory Analysis of Fruit and Fermented Fruit Product Flavors

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CONTENTS

9.1 Introduction

Sensory evaluation is defined as a scientific method used to evoke, measure, analyze, and interpret those responses to foods and beverages as perceived through the five senses of sight, smell, touch, taste, and hearing (Stone and Sidel 1993). Integrating sensory science techniques assists in identifying specific properties of foods that may not be found instrumentally and also provides important and useful information to product developers, food scientists, and managers about the sensory characteristics of their products (Lawless and Heymann 1998).

Sensory evaluation has been used significantly in the identification and characterization of fruit and fermented fruit flavors. The main test methods used to assess these flavors have been descriptive analysis (DA), affective tests, quality rating methods, and difference tests. DA is conducted to determine how products differ in specific sensory characteristics and produces analytical data from highly trained panelists, which can be compared with instrumental measurements. Affective tests, which are also known as consumer tests, can be used to investigate how well products are accepted or preferred among a population of untrained regular users of the products (Lawless and Heymann 1998). Quality rating methods, like the Bux Baum method, use experts on the food form to judge various aspects of the product to determine the product's overall degree of quality (Meilgaard et al. 1999). The following chapter will further discuss these sensory methods in the context of the studies that have focused on fruit and fermented fruit

flavors. This chapter will discuss how these methods have been applied, first, to fruit flavors and, second, for fermented fruit flavors. For the fruit flavors section, the outline will follow (1) various DA methods, (2) integration of consumer and descriptive studies, and (3) the correlation of instrumental and sensory measurements. For the fermented fruit flavors section, the outline will follow (1) DA methods, (2) quality rating methods, (3) consumer and affective testing methods, and (4) difference testing methods.

9.2 Fruit Flavors

9.2.1 DA Methods Used to Evaluate Fruits

DA allows a sensory scientist to obtain a complete sensory characterization of the attributes of a single product or several products for comparison. It can assist in identifying fundamental ingredients or processing variables, and may help in determining which attributes are important in consumer acceptance. DA is beneficial for determining sensory differences between your product and the competitor's product, testing for shelf life, accessing the suitability of prototypes, and is also advantageous for quality assurance. In DA, panelists are trained to be consistent and reproducible, which is especially critical during term generation (descriptors) to describe the sensory attributes of the products (Lawless and Heymann 1998).

DA is one of the most widely used methods to evaluate fruit flavors. Several different fruits have been profiled using DA, and it is often helpful when evaluating the storage and shelf life properties of fruit. The methods of DA that have been used for identifying fruit flavors include quantitative DA (QDA), Spectrum method, free-choice profiling (FCP), and customized DA.

9.2.1.1 Quantitative DA

QDA has been described as a descriptive technique that does not use consensus rating, the panel leader is only a facilitator, not a participant, and line scales are generally unstructured (Murray et al. 2001). The data is viewed as relative values and may pertain to more than one sample or a standard product. Compared with the Spectrum method, less time is required for QDA training, which becomes advantageous especially when evaluating multiple fruits. Strawberries stored under modified atmosphere packaging have been examined through QDA. Judges established descriptive terms to characterize strawberries kept under several different storage conditions. Several fruits stored in air-treated or mixed gas (N_2 + O_2 + CO_2)–treated packages exhibited off-flavors that were most likely caused by anaerobic enzymatic reactions (Shamaila et al. 1992). The effects of storage temperature and time on orange juice concentrate were also examined using QDA. Seven orange juice flavor and aroma attributes were identified using an unstructured scale varying from weak to strong (Spoto et al. 1997).

9.2.1.2 The Spectrum Method

The Spectrum method has also been used as a DA technique in evaluating fruit flavors. The method is different from QDA in that panelists do not generate a panel-specific vocabulary to describe the attributes of a product; instead, a standardized lexicon (vocabulary) of terms is used (Lawless and Heymann 1998; Civille and Lyon 1996). Scales are standardized and reference points are extensively used for each attribute detected (Murray et al. 2001). The data obtained from the Spectrum method are considered absolute and can be compared with data acquired from different panels (Lawless and Heymann 1998). In an orange juice study, the Spectrum method was performed and data were compared with a customized DA on the same juices. The lexicons developed in both analyses were similar for describing the flavor of the orange juices (Lotong et al. 2002).

9.2.1.3 Free-Choice Profiling

FCP is another method used for DA, which allows each member of the sensory panel the ability to develop their own list of perceived qualities for a product rather than using a standard lexicon. Although

assessors are recruited and are minimally trained, they must be able to detect the differences among the products and quantify them similarly to other DA methods (Oreskovich et al. 1991). Passion fruit juice was evaluated under FCP using the participation of consumers who had never tried the juice before. This particular study showed the usefulness of using FCP to differentiate between variations of a novel fruit juice while having the ability to reveal consumers' perceptions of the product (Deliza et al. 2005). A study on black currant drinks from fruit concentrates was also conducted using FCP. However, this method did not yield useful data due to the inconsistency of individuals' descriptions, difficulties in evaluating the several samples presented in different sessions, and the confusion in the use of vocabulary sets (Boccorh et al. 2001). Fruits can be difficult to differentiate especially in a beverage form when the only difference is between fruits from fresh and frozen fruit concentrates. In this type of situation, more training and a smaller range of descriptors are recommended.

9.2.1.4 Customized DA

It is common for a DA technique to be a combination of various methods to fit the focus and objective of the project. This technique is labeled as customized DA and is a fusion of DA methods (Murray et al. 2001). In a shelf life study of minimally processed honeydew, kiwifruit, papaya, pineapple, and cantaloupe, trained panelists developed a list of adjectives to describe all the fruits and were able to add other descriptive comments to score sheets when tasting (O'Connor-Shaw et al. 1994). The sensory effects of storage on different fresh-cut cantaloupe varieties were examined using DA, because flavors tend to deteriorate at a quicker rate compared with appearance in fresh-cut fruit (Ayhan et al. 1998; Bett-Garber et al. 2003). Fruit juices have commonly been investigated using descriptive sensory studies; in particular, red raspberry genotypes (Harrison et al. 1998) and Spanish mandarin juices (Carbonell et al. 2007). Several descriptive studies have been performed on apples and all studies have generated their own unique lexicons to describe the fruit's attributes. A storage and shelf life test on five apple cultivars harvested at different maturity stages was performed using a general DA (Zerbini et al. 1999). The sensory characteristics of irradiated apple cider were quantified using DA (Wang et al. 2003), as well as apple juices made from a wide range of apple cultivars (Cliff et al. 2000). Thirty apple genotypes processed as apple pies were also evaluated with DA, which revealed that six of the apple selections had the potential for processed products (Sanford et al. 2001). Table 9.1 is a joint lexicon from several apple studies which show the wide array of terms that were generated when testing different apple genotypes. It is noticeable that even though the descriptors may vary, there is still a pattern in the terms generated. Vague descriptors such as "apple flavor" were commonly used, but most often, specific references were identified to support the terms.

9.2.2 Integrating Consumer Studies with DA to Assess Fruit Flavors

Consumer methods used to evaluate fruit flavors vary depending on several factors such as the type of fruit, harvest method, cut, and storage variability. Consumer sensory testing is typically performed toward the end of the product development cycle (Lawless and Heymann 1998); however, in the case of fruit flavors, it varies. A consumer test differs from DA in that it involves approximately 100 to 500 targets, untrained consumers who assess the product by giving their preference or acceptance. The primary objectives for conducting a consumer test are for product maintenance, product optimization, product development, and assessment of the potential market (Meilgaard et al. 1991). Consumer sensory studies have been conducted to determine the desired physical attributes of fruit because the physical appearance of the fruit is an important aspect of the quality and overall taste of the fruit. The tactile and visual cues of the fruit can dictate the likelihood of purchase. Richardson-Harman and others investigated the external properties of apples through maturity and ripening and how these characteristics affect consumer preference (Richardson-Harman 1998). Several other consumer methods have been studied for fruit flavors, most often relating consumer results through sensory testing to instrumental measurements. These methods will be discussed further in this chapter.

It is not uncommon for researchers to combine sensory DA with consumer testing when assessing fruits (Péneau et al. 2007; Carr et al. 2001). Several studies have been done on fruit flavors in which

TABLE 9.1

Selective Sensory Descriptors for Apples and Apple-Related Products in the Literature

	Definition	Reference
Aroma		
Green-grassy[a]	NA	NA
Green apple[a]	NA	NA
Fruity[a]	NA	NA
Cooked/caramelized[a]	NA	NA
Oxidized[a]	NA	NA
Honey/caramel[a]	NA	NA
Apple cider[e]	NA	Duché de Longueville/Antoinette/cidre de cru Normand[e]
Chemical flavor[e]	NA	NA
Cucumber[e]	NA	2,6-nonadienal[e]
Earthy[e]	NA	NA
Flowers[e]	NA	β-Ionone, linalool[e]
Fusel[e]	NA	NA
Lactony[e]	NA	δ-Decalactone[e]
Pear/candy[e]	NA	Amyl acetate, "peredrups" candy[e]
Tin/metallic[e]	NA	NA
Texture		
Hardness[f]	Overall impression of hardness for entire bite[f]	NA
Mealiness[f]	Extent to which sample was dry and grainy and broke down into granular pieces during chewing[f]	NA
Crunchiness[f]	Strength of sound produced during chewing that leads to the sensation of crunchiness[f]	NA
Degree of breakdown[f]	Overall estimate of size and number of particles in mouth[f]	NA
Firmness[c]	The force required to completely compress a 12-mm apple slice between molar teeth[c]	Cream cheese 12 mm cube[c]
Crispness[c]	The degree to which rupture is heard as the sample breaks during full compression[c]	Digestive biscuit 12 mm square[c]
Rubberiness[c]	After two chews, the degree to which the apple slice springs back to original shape[c]	Cheddar cheese 12 mm cube[c]
Juiciness[c,f]	The degree of moisture released after two chews[c]; amount of juice released into the mouth[f]	Canned pear 12 mm cube[c]
Graininess[c]	The size of particles present[c]	100:10 (w/w) applesauce/sugar[c]
Fibers/strings[c]	The presence of strings or fibers[c]	Pineapple tidbits[c]
Outer skin[c]	The degree to which an outer skin has formed on the individual apple slice[c]	Rehydrated golden raisins[c]
Flavor		
Tartness[c]	The taste stimulated by acid[c]	100:10 (w/w) applesauce/sugar[c]
Off-flavor[c]	The intensity of an old stored apple off-flavor (alcoholic, acetaldehyde)[c]	Apple juice from apples held anaerobic for 7 days[c]
Bitterness[c]	The taste stimulated by substances such as caffeine[c]	Unsweetened grapefruit juice[c]
Green-grassy[a]	NA	0.004% (w/v) hex-3-enol[a]
Cardboard-like flavor[b]	Aromatic associated with slightly oxidized fats/oils, reminiscent of wet cardboard packaging[b]	Wet cardboard[b]

(*continued*)

TABLE 9.1 (Continued)

Selective Sensory Descriptors for Apples and Apple-Related Products in the Literature

	Definition	Reference
Flavor		
Cooked apple flavor[a,b,d]	Heated apple with milder taste and soft texture[b]; characteristic of stewed apple[d]	5.2 g HFCS cooked in microwave for 100 seconds, cooled and dissolved in 200 mL juice[a]; microwave-heated Braeburn apple slice 3 sec[b] 400 g Granny Smith apples stewed with one cup of water and 1 tablespoon of sugar[d]
Fruity[a]	NA	15% (w/w) ascorbic acid–treated McIntosh juice + 0.03% (w/v) IFF pear and 0.03% (w/v) Universal melon essences[a]
Oxidized[a]	NA	NA
Yeasty[d]	Characteristic of fermented apples[d]	Appletise brand carbonated apple juice[d]
Lemon[d]	Characteristic of fresh lemons[d]	50% juice + 35 ml lemon juice + scored rind of two lemons steeped for 1 hour[d]
Stalky[d]	Characteristic of apple stalks/cores[d]	50% juice + 32.5 mL cis-3-hexen-1-ol[d]
Cereal[d]	Characteristic of cooked baby cereals[d]	Farex brand baby cereal made up with filtered water[d]
Metallic[d]	Characteristic of canned foods[d]	Fresh Up Old Fashioned brand apple juice[d]
Honey/caramel[a,d]	Characteristic of clover honey[d]	0.7% (w/v) pasteurized honey[a]
Apple flavor[a,b,c]	Fresh and mature apple flavor[b]; strength of apple flavor[c]	15% ascorbic acid–treated McIntosh juice[a]; Braeburn fresh apple slice[b]; 50:50 (w/w) apple juice/water[c]
Sweet[a,b,c,d]	Taste of sugar[b,c,d]	2.5% (w/w) HFCS[a]; 15% (w/w) sucrose solution[b]; 15% (w/w) sucrose solution[c]; 50% juice + 50% water[d]
Sour[a,b,d]	Taste stimulated by acids[b,d]	0.3 malic acid/250 mL juice[a]; 0.2% (w/w) citric acid[b]; 50% juice + 50% water[d]
Astringent[a,b,c,d]	Shrinking or puckering of mouth surfaces[b,c]; drying mouthfeel[d]	0.08 g tannin/250 mL juice[a]; 0.15% (w/w) alum[b]; 100:10 (w/w) applesauce/sugar[c]; Ocean Spray brand cranberry juice[d]
Viscosity[d]	Thickness of solution[d]	Filtered water, "Sunraysia" brand prune juice, "Golden Circle" brand mango nectar[d]
Sweet aftertaste[e]	NA	NA
Bitter aftertaste[e]	NA	NA
Dry/harsh after-feeling[e]	NA	NA

[a] *Source:* Cliff, M. A. et al., *J Food Qual*, 23(1), 73–86, 2000. With permission.
[b] *Source:* Wang, H. et al., *J Food Sci*, 68(4), 1498–503, 2003. With permission.
[c] Sanford, K. A., et al., *J Food Qual*, 24(4), 301–25, 2001. With permission.
[d] *Source:* Walker, S., J. Prescott, *J Sens Stud*, 15(3), 285–307, 2000. With permission.
[e] *Source:* King, B. M. et al., *Food Qual Pref*, 18(2), 286–95, 2007. With permission.
[f] *Source:* Ioannides, Y. et al., *Food Qual Pref*, 18(6), 825–33, 2007. With permission.

trained panelists developed a lexicon for a particular fruit and the products or prototypes are further examined through consumer methodology in an attempt to discover drivers of liking through acceptance or preference tests. A predicted model evaluated through DA and ingredient information may also be further optimized and tested with consumer methods. This type of application was performed on tropical mixed jams containing pineapple, papaya, and carambola fruits. Significant attributes were included in an optimization model and subjected to a consumer test which validated that the predicted equations and contour plots did in fact determine the optimum formulation (Abdullah and Cheng 2001). Pear leather

fruits have been characterized by first using DA, then by a consumer acceptance test using a nine-point hedonic scale (Huang and Hsieh 2005). The probability of consumer purchase was translated as an index of acceptability after conducting a modified Spectrum method on different varieties of mango fruit (Malundo et al. 2001). The regression tree model, a statistical comparison method, was applied to compare the relationship between sensory attributes and overall acceptability of fresh pineapple (Schulbach et al. 2007). A study performed on the U.K. apple market related the sensory properties of several types of peeled and unpeeled apples to the consumer acceptance of these apples (Daillant-Spinnler et al. 1996). A study on consumer's perceived freshness of strawberries was performed in which ratings of freshness by the expert panel in the DA portion of the study shown to be the most reliable approach of measuring freshness. Freshness is a multidimensional sensory attribute and can be affected by several characteristics (Péneau et al. 2007; Fillion and Kilcast 2000). By performing DA as well as consumer tests, a more accurate definition of freshness was obtained. Finally, in an orange juice study comparing food acceptability in younger and older consumers, partial least squares regression was used to relate the sensory characteristics of the juice to liking measures (Forde and Delahunty 2004).

9.2.3 Relationship of Taste and Aroma Evaluations to Instrumental Measurements

Although sensory methods alone can provide important information about fruits and fruit flavors, many researchers study fruit flavors with the intention of correlating sensory findings with instrumental data. Indeed, many facilities do not have the resources or training necessary to adequately conduct sensory analysis on fruit flavors, yet have some instruments available to run analytical tests on chemical and physical properties. Furthermore, the inherent cost associated with running a full sensory panel makes instrumental analyses an attractive alternative. Therefore, much research has been undertaken to relate descriptive sensory attributes of fruits and fruit flavors to their chemical or physical characteristics.

As sweet and sour tastes are both closely associated with fruits, measurements of sweetness- and sourness-eliciting compounds are some of the most common methods used, particularly soluble solids content (SSC), titratable acidity (TA) or pH, and individual sugar or acid assays. SSC has been reported to correlate significantly with sweet taste attributes in peaches and nectarines (Crisosto et al. 2006), plums and pluots (Crisosto et al. 2007), apples (Cliff et al. 1998; Harker et al. 2002b), raspberries (Shamaila et al. 1993), and kiwifruits (Marsh et al. 2004). Likewise, TA has been shown to correlate closely with sour taste in peaches and nectarines (Crisosto et al. 2006), apples (Cliff et al. 1998; Harker et al. 2002b), raspberries (Shamaila et al. 1993), and to a lesser degree, kiwifruits (Marsh et al. 2004).

Although SSC and TA (and particularly SSC to TA ratios) have proven to be effective measures of the sensory characteristics of fruit products, attempts to relate sweetness or sourness to specific sugars and acids have had more mixed success. Harker et al. (2002b) found that quantification of malate was somewhat correlated to acid taste and overall flavor in apples, but quinate and citrate were not. In the same study, assays of fructose, glucose, sorbitol, and sucrose all had poor correlation with sweet taste.

In kiwifruits, Marsh et al. (2004) found a relationship between malate content and sensory acidity, whereas also noting that the malate increase was coupled with other changes that made it difficult to attribute the acid taste increase to malate alone. Finally, Rega et al. (2004) found that TA gave results similar to measurement of citric acid by high performance liquid chromatography in orange juice, thereby indicating that measuring individual acids did not provide any more information than a simple titration.

Interestingly, the results also indicate that these taste compounds have confounding effects on other sensory attributes. Sweet and sour compound levels have been shown to affect the perception of overall fruit flavor and aroma (Crisosto et al. 2007; Harker et al. 2002b). This phenomenon, known as the halo effect, was also observed for perceptions of specific aromas and flavors in orange and apple beverages such as increased cooked, lactony, and candy notes with increased soluble solids and decreased green, metallic, and terpene notes with decreased acidity (King et al. 2007).

Aroma compounds obviously contribute significantly to sensory properties of fruit flavors as well, as each fruit or fruit flavor has a unique combination of volatiles that make up its aromatic "signature." These volatiles can be characterized and qualified to aid in understanding their role in the sensory experience of fruits. In cling peaches, Spencer et al. (1978) reported that a combination of pH, cis-3-hexenyl acetate, linalool, an unidentified monoterpene, and α-terpineol levels accounted for "overall peach

flavor" as assessed by a descriptive panel, whereas other specific attributes were likewise accounted for by specific chemical components. Rega et al. (2004) found "artificial flavor" in orange juice to be correlated with levels of ethyl acetate, 2-propanone, and octanal, whereas "vegetal" odor correlated with hexenal and "freshly squeezed" odor to relate to terpenic compounds. However, other studies have found poor correlation between sensory attributes and volatiles in a variety of fruits and fruit products (Harker et al. 2002b; Shamaila et al. 1993). Therefore, some sensory qualities in fruit flavors seem to be more readily elucidated by measurement of volatiles than in others, so each product should be evaluated on a case-by-case basis.

9.2.4 Measuring Texture of Fruit by Sensory and Instrumental Methods

Texture is considered an important part of the sensory experience of fruits and fruit products, so much work has been done to correlate sensory texture attributes with physical characteristics measured via instruments, albeit with mixed results. As shown in Figure 9.1, puncture tests using common instruments such as a penetrometer provided good prediction for sensory characteristics such as crispness, crunchiness, and hardness in apples (Harker et al. 2002a; Ioannides et al. 2007). Similarly, firmness tests were seen to correlate with sensory firmness in Gala apples (Cliff et al. 1998). Tests on canned peaches show a correlation between work done during back extrusion and sensory firmness as well as between work done on wire extrusion and sensory fibrousness (Apostolopoulos and Brennan 1994). *In vivo* tests have also shown some promise as the methods to predict sensory attributes in fruits. Electromyography of facial muscles during chewing of Red Delicious apples and the size and shape of the chewed particles were both found to relate to sensory hardness (Ioannides et al. 2007). However, measurements of chewing sounds of apples did not show good correlation with sensory attributes (Harker et al. 2002a). Some chemical properties of fruits also seem to correlate with sensory textural attributes. Again, in canned peaches, hardness and graininess were found to relate to TA, whereas graininess was also closely related to the amount of alcohol-insoluble fibers (Lin and Rao 1982). Conversely, textural attributes can help predict sensory properties due to the influence of physical characteristics on flavor release and cognitive perception of fruit products. Rega et al. (2004) found that although pulp in orange juice contains a high quantity of aroma compounds, it also contributes to viscosity and inferred that this would impede volatilization of flavor compounds. This theory was confirmed by the fact that orange juice with added pulp (and therefore higher viscosity) underwent a significant increase in aroma volatiles measured instrumentally, but not in flavor assessed by a sensory panel. However, in a study of added viscosifying agents

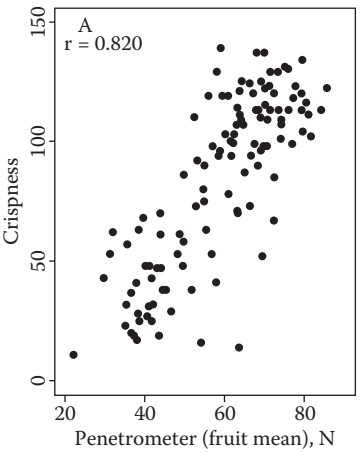


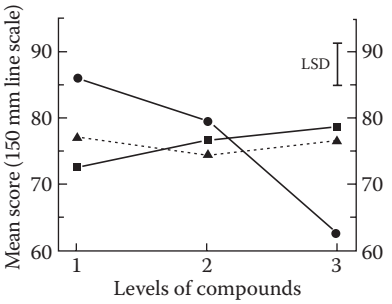
FIGURE 9.1 Relationship between penetrometer puncture force and scores for crispness of apples. Points represent the score given by an individual panelist for a single apple. (From Harker, F. R., et al., *Postharvest Biol Technol*, 24(3), 225–239, 2002a. With permission. Harker, F. R., et al., *Postharvest Biol Technol*, 24(3), 241–250, 2002b. With permission.)

in apple juice (Walker and Prescott 2000), the opposite tendency was found. Although sensory differences were perceived for sourness, cooked apple flavor, honey, and lemon aromas in juices with varying viscosities, no corresponding differences were found in the headspace gas chromatograms, suggesting that other mechanisms not accounted for by the instrumental analysis were responsible for the flavor differences. This reiterates the importance of using sensory methodology in tandem with instrumental measures to adequately characterize fruits and fruit flavors.

9.2.5 Relating Consumer Evaluations to Instrumental Measures

Instrumental measurements have been shown to correlate with hedonic ratings of fruits and fruit products as well. In general, high SSC leads to a high degree of liking, whereas high TA leads to poor liking scores. However, the ratio of soluble solids to acid (usually calculated as degrees Brix to percent acid ratio) is often used as a predictor of liking, especially at intermediate levels of sugar and acid because of the interaction between sweetness and sourness sensations. Studies have found this trend to hold when consumers were asked to evaluate kiwifruit (Crisosto and Crisosto 2001), table grapes (Crisosto and Crisosto 2002), cherries (Guyer et al. 1993; Crisosto et al. 2003), and grapefruit juice (Fellers et al. 1988). In fact, this ratio is a common measure of fruit quality and acceptability used by federal and state agencies like the U.S. Department of Agriculture's (USDA 2008) Agricultural Marketing Service (7 CFR 905.306). However, Fellers et al. (1986a,b) have seen that tartness and bitterness, as evaluated in orange and grapefruit juices by naïve consumers rather than trained panelists, can be easily confused for each other and can affect sweetness perception despite their independent variation. Therefore, correlations may be seen between tastants and seemingly unrelated attributes in the case of consumer testing due to lack of training to differentiate and identify sensations.

Although much of the literature focuses on sweet and sour perceptions, other attributes can also contribute to liking in fruits and fruit flavors. Given constant sugar and acid levels in a model kiwifruit-flavored solution, consumers were shown to be sensitive to changes in volatile flavor compounds and their effect on kiwifruit aroma intensity and preferred specific levels (Gilbert et al. 1996; Figure 9.2). Crisosto et al. (2003) found color to be a factor in purchase intent of cherries by consumers, with darker color leading to increased purchase intent. Fellers et al. (1986b) found that consumers rated grapefruit juices with high levels of limonin and naringin as too bitter when rated by the just-about-right scale. Heavily heat-treated orange juice was found to be high in octanoic acid and also received



	μg/30 mL		
	Level 1	Level 2	Level 3
Ethyl butanoate	0	172.8	518.4
E-2-hexenal	0	77.4	335.1
Hexanal	0	32.4	165.0

FIGURE 9.2 Mean intensities for overall liking of kiwifruit model solution with various levels of added compounds. Square, ethyl butanoate; circle, E-2-hexenal; triangle, hexanal. Solid lines represent significant effects. (From Gilbert, J. M., et al.: *J Sens Stud.* 1996, 11(3), 247–259. Copyright Wiley-VCH Verlag GmbH & Co. KGaA. Reproduced with permission.)

poorer liking scores (Moshonas and Shaw 1997). The contrary has been found as well, with poor correlations found between hedonic ratings and volatile concentrations in raspberries (Shamaila et al. 1993). There seems to be many factors at work in consumer perception of fruit flavors that have not yet been elucidated.

9.3 Fermented Fruit Flavors

9.3.1 DA and Attribute Rating Methods Used to Evaluate Fermented Fruits

Being able to identify and quantify attributes of flavor in fermented fruit products is essential to being able to understand how a product is perceived. DA has been used to show how both the raw materials as well as the variables within the fermentation process can affect the taste and aroma of fermented fruit products. In a sea buckthorn juice study, juices were evaluated with and without fermentation after inoculation with a strain of *Oenococcus oeni*. Malic acid fermentation took place at varying degrees in different samples, depending on their initial amount of malic acid. After being evaluated by a DA panel trained on attributes generated in previous work performed on sea buckthorn, a higher degree of malic acid fermentation was correlated with a significant decrease in sourness and astringency, which are negatively perceived sensory properties. Fermented samples also showed higher fruity and fermented flavors, due to esters being formed during the fermentation process (Tiitinen et al. 2007).

With fermented fruit products, such as wines, the flavors are often so complex that trained, experienced tasters of the products are used in place of a traditionally trained panelist. Such was the case in a study on Syrah wines, in which the amount of juice allowed to "run-off" before being fermented was varied to see the effects on various sensory characteristics. Instead of rating the characteristics on a scale, 36 experts ranked each sample in terms of intensity of aroma, flavor, color, and astringency. Color increased with more juice run-off, although other sensory differences were inconsistent (Gawel et al. 2001).

Industry professionals with experience with a specific product have also been trained to evaluate various flavor and aroma attributes on a line scale. In a different study on Syrah wines, which were made with different yeast preparations, professionals were trained with references to quantify-specific flavors in the wines. They found that the wines made with active dry yeast were higher in intensities most associated with the Syrah varietal, such as black pepper, meaty, and fruity flavors as well as mouthfeel and richness (Bohlscheid et al. 2007).

9.3.2 Quality Rating Methods Used to Evaluate Fermented Fruits

With wines and other fermented beverages, there is typically a wide range of products on the market in terms of quality. To judge the value of a specific product, panelists experienced with a certain product use quality rating systems that take into account multiple sensory aspects of the product. These methods typically only require a handful of experts on the product and are fairly straightforward to use. One such method is the Bux Baum method, in which each sample can receive up to 20 points (2 points for color, 2 points for clarity, 4 points for smell, and 12 points for taste; Meilgaard 1999). Other rating systems are similar, and can provide more or less priority to different aspects of the product, depending on the investigator's interest or unique characteristics of the product. The data generated from this type of study can show that one fermented fruit product is deemed a higher quality than another, but should not be confused with preference or acceptance testing data that are typically obtained with a much larger number of untrained consumers. It may also be harder to compare data between studies using quality rating test methods because using a small number of expert panelists introduces risk for variability and bias. On the other hand, this type of test requires significantly less resources and time than traditional preference and acceptance testing, so long as experts are available.

The Bux Baum method was used in a study on mango wines to understand the effect of pectinase treatment on the overall quality of the wine. Five expert panelists rated the samples, and it was found that the samples that underwent the pectinase treatment were rated higher in quality overall, especially in taste.

Analysis of the products showed a higher alcohol content, which is what the authors believed most likely contributed to the higher sensory quality scores (Reddy and Reddy 2009).

The Bux Baum method was also carried out on a study understanding the effect of indigenous yeasts on plum brandies. The brandies were made with different yeasts (three natural and two commercial) and distilled to an alcoholic concentration of approximately 65%. Samples made with distillery strains of *Saccharomyces cerevisiae* received the highest quality ratings. In this study, volatile compound analysis was also conducted on the samples, and thus informal correlations between the sensory results and the samples' volatile compound profile could be made. The sample receiving the highest quality ratings had the lowest level of analyzed volatile compounds and had a higher alcohol level. Samples rated lower contained higher levels of acetaldehydes, fusel alcohols, and esters, which were associated with sharp, acrid tastes. The lowest rated sample contained methylating sulfuric compounds, and those were associated with a burning sour taste and sauerkraut aroma (Satora and Tuszynski 2010).

A modified quality rating system was used in a study done on cider making, in which eight different *Saccharomyces* strains were used in the fermentation of the samples. Ten expert panelists used a quality rating system similar to the Bux Baum method, and scored the samples on a 100-point scale, based on the sensory categories of appearance, aroma, taste, and style. The samples were also analyzed with a gas chromatography–mass spectrometry to quantify the volatile compounds and statistical analysis was performed to correlate the two sets of data. The samples scoring lowest in the sensory evaluation were correlated to lower intensity of aroma compounds produced by the yeasts (Peng et al. 2008).

9.3.3 Consumer Affective Testing Methods Used to Evaluate Fermented Fruits

Affective testing, also known as consumer testing, can also be a valuable tool in evaluating fermented fruit products. These tests can be used to understand how untrained consumers like a product as a whole or how much they like individual aspects of the product. Some consumer studies are set up to understand how certain types of consumers will react to their product, and demographic criteria are set for who evaluates the product. This can be carried out if the product is going to be marketed toward a certain group, or if groups have certain built-in biases such as cultural familiarity or past experience with the food.

This was seen in a study performed on Tepache, a Mexican fruit beverage made with pineapple and other fruits, fermented with water and brown sugar. The samples evaluated underwent differences in the fermentation process, some being double-fermented and some triple-fermented. The sensory methods used included paired comparison testing, overall acceptance testing, and attribute acceptance testing of appearance, consistency, odor, flavor, and sweetness to sourness ratio. Panelists for this study were required to be regular consumers of the product with an income of less than five thousand dollars per year. Samples that were triple-fermented had more fermented flavor than double-fermented samples, but that did not directly correlate to liking (Moreno-Terrazas et al. 2001).

Unique fermented fruit products can be assessed by consumer testing to understand the viability of commercially developing such a product. One such study evaluated wine made with the South American fruit gabiroba, fermented with both *S. cerevisiae* as well as natural yeast strains indigenous to the region. A preference test was performed with 50 untrained panelists, which showed that wine fermented naturally with indigenous yeasts was preferred. Volatile compound analysis was also carried out on these samples, and the indigenously fermented sample had higher concentrations of compounds responsible for certain aromatics, the reason the author thought was responsible for it being preferred (Duarte et al. 2009).

When investigating several variables in fermented fruit products, response surface methods can be used to find the optimum level of each variable to create the most accepted product. In a study on banana puree fermented with *Lactobacillus acidophilus*, the variables investigated were fermentation temperature, total inoculum level, and peeled fruit to water ratio. The bacterial cell suspensions were immobilized in both sodium alginate solution as well as potassium carrageenan solution to stabilize the inoculation. A consumer test with 50 untrained panelists showed that the optimal sample for each preparation was well received, with the sodium alginate prepared solution being slightly preferred over the potassium carrageenan solution (Tsen et al. 2006).

9.3.4 Discrimination Methods Used to Evaluate Fermented Fruits

Discrimination testing is used to gauge whether or not trained or untrained human subjects are able to tell the difference between different samples. Discrimination testing can be done with various methods. General difference test methods, such as the triangle test, ask panelists to find a difference between products using any and all aspects of the products. Alternatively, a specific difference test can be done, in which the panelists are prompted to find the sample that is lesser or greater in the intensity of a specific attribute. Difference tests may be conducted by untrained, unbiased panelists to understand at what point a typical consumer will be able to tell the difference between two products, although selecting specific panelists (i.e., trained or expert panelists) can be done as well depending on the scope of the project.

In a study on Arbequina natural green olives, the olives were fermented with different starters—one with yeast (*Candida*), two with *Lactobacillus* strains, and a sample that was naturally fermented. To understand if the olives would be perceived differently, 12 trained judges performed triangle tests between each pair of the three samples and were also asked for their preferred sample in each pair. The trained judges were able to differentiate among all of the samples, but only showed significant preference in one pair (*Lactobacillus pentosus* 5E3A18–fermented olives were preferred over naturally fermented olives). The study showed the importance of using a starter culture in this type of olive, and the degree of difference it can have on the characteristics of the final product (Hurtado et al. 2010).

Difference testing was also paired with preference testing in fermented melon juice distillates. Three melon substrates were processed as juice, melon paste with skin, and melon paste without skin. The samples were fermented into two groups, one with adjusted pH and the other, unadjusted. The samples were then distilled twice to a final alcohol content of 58% to 69%. A triangle test was done with 18 panelists familiar with the products, and they did not find a difference between the unadjusted and adjusted pH varieties of juice distillates and paste without skin distillates, but they did find differences in the paste with skin distillates. For the unadjusted pH samples, the paste without skin was preferred over the paste with skins, and the juice was preferred over the paste with skins, but no preference was shown between juice and paste without skins. In the pH-adjusted distillates, the juice was significantly preferred over paste with skins, and paste without skins was preferred over paste with skins. Volatile compound analysis was also analyzed with a principal component analysis plot, which grouped distillates based on substrate pH. These results substantiated the findings from the difference tests (Hernandez-Gomez et al. 2005).

9.4 Conclusions

Based on the body of work reviewed here, it is apparent that many varied methods have been applied to evaluate the sensory properties of fruits, fresh and fermented. DA is an effective tool for characterizing complex products like fruits, which, like most agricultural products, are subject to natural variation. Although QDA, Spectrum, and other established DA methods can be, and have been, used effectively, many researchers choose to customize the methods and data analyses to fit their studies in a manner which they feel is appropriate for their topic and product of interest. Affective methods also use a mixture of recognized and made-to-order methodologies. These methods provide valuable information about consumer attitudes toward fruits, and when combined with descriptive methods, can elucidate drivers of liking. Quality rating methods can be a useful tool to understand what factors affect how experienced professionals perceive fruit products. Difference testing can also be used to understand what changes in formulation or processing truly change how a fruit or fermented fruit product is perceived. Finally, instrumental measurements can be coupled with sensory data to correlate physical or chemical characteristics of fruits with their sensory attributes, and in some cases, to allow instrumental measurements to replace sensory assessments. However, not all sensory traits can be linked to instrumentally measured properties, and thus, sensory evaluation will continue to be a necessary and indispensable tool in evaluating fruit and fermented fruit flavors and overall fruit quality.

The authors would like to stress that great care must be taken when designing a new sensory method or making modifications to existing practices because improper data collection can compromise the

validity of the study. Fruits and fermented fruit products are inherently and widely variable and difficult to generalize, but careful use of appropriate sensory tools can yield insightful results. Because individualized methods for fruit and fermented fruit flavor evaluation are as numerous as the fruits to be evaluated, careful review of sensory principles is recommended to ensure scientifically sound results.

REFERENCES

Abdullah A, Cheng TC. 2001. Optimization of reduced calorie tropical mixed fruits jam. *Food Qual Pref* 12(1):63–8.

Apostolopoulos C, Brennan JG. 1994. Interrelationships between sensory and mechanical characteristics of canned peaches. *J Texture Stud* 25(2):191–206.

Ayhan Z, Chism GW, Richter ER. 1998. The shelf-life of minimally processed fresh cut melons. *J Food Qual* 21(1):29–40.

Bett-Garber KL, Beaulieu JC, Ingram DA. 2003. Effect of storage on sensory properties of fresh-cut cantaloupe varieties. *J Food Qual* 26(4):323–35.

Boccorh RK, Paterson A, Piggott JR. 2001. Quantifying flavor character in blackcurrant drinks from fruit concentrates. *J Sens Stud* 16(6):619–41.

Bohlscheid JC, Specht G, Ortiz-Julien A, Maloney J, Bertheau B, Ross CF, Edwards CG. 2007. Application of a new yeast preparation for problem grape musts. *J Wine Res* 18(3):173–85.

Carbonell L, Izquierdo L, Carbonell I. 2007. Sensory analysis of Spanish mandarin juices. Selection of attributes and panel performance. *Food Qual Pref* 18(2):329–41.

Carr BT, Craig-Petsinger D, Hadlich S. 2001. A case study in relating sensory descriptive data to product concept fit and consumer vocabulary. *Food Qual Pref* 12(5–7):407–12.

Civille GV, Lyon BG. 1996. *Aroma and flavor lexicon for sensory evaluation*. West Conshohocken, PA: ASTM.

Cliff MA, Lau OL, King MC. 1998. Sensory characteristics of controlled atmosphere- and air-stored 'Gala' apples. *J Food Qual* 21(3):239–49.

Cliff MA, Wall K, Edwards BJ, King MC. 2000. Development of a vocabulary for profiling apple juices. *J Food Qual* 23(1):73–86.

Crisosto CH, Crisosto GM. 2001. Understanding consumer acceptance of early harvested 'Hayward' kiwifruit. *Postharv Biol Technol* 22(3):205–13.

Crisosto CH, Crisosto GM. 2002. Understanding American and Chinese consumer acceptance of 'Redglobe' table grapes. *Postharv Biol Technol* 24(2):155–62.

Crisosto CH, Crisosto GM, Echeverria G, Puy J. 2006. Segregation of peach and nectarine (*Prunus persica* (L.) Batsch) cultivars according to their organoleptic characteristics. *Postharv Biol Technol* 39(1):10–8.

Crisosto CH, Crisosto GM, Echeverria G, Puy J. 2007. Segregation of plum and pluot cultivars according to their organoleptic characteristics. *Postharv Biol Technol* 44(3):271–6.

Crisosto CH, Crisosto GM, Metheney P. 2003. Consumer acceptance of 'Brooks' and 'Bing' cherries is mainly dependent on fruit SSC and visual skin color. *Postharv Biol Technol* 28(1):159–67.

Daillant-Spinnler B, MacFie HJH, Beyts PK, Hedderley D. 1996. Relationships between perceived sensory properties and major preference directions of 12 varieties of apples from the Southern Hemisphere. *Food Qual Pref* 7(2):113–26.

Deliza R, MacFie H, Hedderley D. 2005. The consumer sensory perception of passion-fruit juice using free-choice profiling. *J Sens Stud* 20(1):17–27.

Duarte WF, Dias DR, Pereira GVDM, Gervasio IM, Schawn RF. 2009. Indigenous and inoculated yeast fermentation of gabrioba (*Campomanesia pubescens*) pulp for fruit wine production. *J Ind Microbiol Biotechnol* 36:557–69.

Fellers PJ, Carter RD, Jager G. 1988. Influence of the ratio of degrees Brix to percent acid on consumer acceptance of processed modified grapefruit juice. *J Food Sci* 53(2):513–5.

Fellers PJ, Jager G, Poole MJ. 1986a. Quality of retail Florida-packed frozen concentrated orange juice as determined by consumers and physical and chemical analyses. *J Food Sci* 51(5):1187–90.

Fellers PJ, Jager G, Poole MJ, Hill EC, Mittal P. 1986b. Quality of Florida-packed retail grapefruit juices as determined by consumer sensory panels and chemical and physical analyses. *J Food Sci* 51(2):417–20.

Fillion L, Kilcast D. 2000. Concept and measurement of freshness of fruits and vegetables. Leatherhead Food RA Research Reports No. 770.

Forde CG, Delahunty CM. 2004. Understanding the role cross-modal sensory interactions play in food acceptability in younger and older consumers. *Food Qual Pref* 15(7–8):715–27.

Gawel R, Iland PG, Leske PA, Dunn CG. 2001. Compositional and sensory differences in Syrah wines following juice run-off prior to fermentation. *J Wine Res* 12(1):5–18.

Gilbert JM, Young H, Ball RD, Murray SH. 1996. Volatile flavor compounds affecting consumer acceptability of kiwifruit. *J Sens Stud* 11(3):247–59.

Guyer DE, Sinha NK, Chang T, Cash JN. 1993. Physicochemical and sensory characteristics of selected Michigan sweet cherry (*Prunus avium* L.) cultivars. *J Food Qual* 16(5):355–70.

Harker FR, Maindonald J, Murray SH, Gunson FA, Hallett IC, Walker SB. 2002a. Sensory interpretation of instrumental measurements 1: Texture of apple fruit. *Postharv Biol Technol* 24(3):225–39.

Harker FR, Marsh KB, Young H, Murray SH, Gunson FA, Walker SB. 2002b. Sensory interpretation of instrumental measurements 2: Sweet and acid taste of apple fruit. *Postharv Biol Technol* 24(3):241–50.

Harrison RE, Muir DD, Hunter EA. 1998. Genotypic effects on sensory profiles of drinks made from juice of red raspberries (*Rubus idaeus* L.). *Food Res Int* 31(4):303–9.

Hernandez-Gomez LF, Ubeda-Iranzo J, Garcia-Romero E, Briones Perez A. 2005. Comparative production of different melon distillates: Chemical and sensory analyses. *Food Chem* 90:115–25.

Huang X, Hsieh F. 2005. Physical properties, sensory attributes, and consumer preference of pear fruit leather. *J Food Sci* 70(3):E177–86.

Hurtado A, Reguant C, Bordons A, Rozes N. 2010. Evaluation of a single and combined inoculation of a *Lactobacillus pentosus* starter for processing *cv. Arbequina* natural green olives. *Food Microbiol* 27:731–40.

Ioannides Y, Howarth MS, Raithatha C, Defernez M, Kemsley EK, Smith AC. 2007. Texture analysis of Red Delicious fruit: Towards multiple measurements on individual fruit. *Food Qual Pref* 18(6):825–33.

King BM, Duineveld CAA, Arents P, Meyners M, Schroff SI, Soekhai ST. 2007. Retronasal odor dependence on tastants in profiling studies of beverages. *Food Qual Pref* 18(2):286–95.

Lawless HT, Heymann H. 1998. *Sensory evaluation of food: Principles and practices*. New York: Chapman & Hall.

Lin RR, Rao VNM. 1982. Sensory, physical and chemical properties of canned peaches. *J Food Sci* 47(1):317–8.

Lotong V, Chambers DH, Dus C, Chambers EI, Civille GV. 2002. Matching results of two independent highly trained sensory panels using different descriptive analysis methods. *J Sens Stud* 17(5):429–44.

Malundo TMM, Shewfelt RL, Ware GO, Baldwin EA. 2001. An alternative method for relating consumer and descriptive data used to identify critical flavor properties of mango (*Mangifera indica* L.). *J Sens Stud* 16(2):199–214.

Marsh K, Attanayake S, Walker S, Gunson A, Boldingh H, MacRae E. 2004. Acidity and taste in kiwifruit. *Postharv Biol Technol* 32(2):159–68.

Meilgaard M, Civille GV, Carr BT. 1991. *Sensory evaluation techniques*. 2nd ed. Boca Raton, FL: CRC Press.

Meilgaard MC, Civille GV, Carr BT. 1999. *Sensory evaluation techniques*. 3rd ed. Boca Raton, FL: CRC Press. 387 p.

Moreno-Terrazas R, Reyes-Morales H, Huerta-Ochoa S, Guerrero-Legarreta I, Vernon-Carter EJ. 2001. Consumer awareness of the main sensory attributes of tepache, a traditional fermented fruit beverage. *Food Sci Technol Int* 7(5):411–5.

Moshonas MG, Shaw PE. 1997. Flavor and chemical comparison of pasteurized and fresh Valencia orange juices. *J Food Qual* 20(1):31–40.

Murray JM, Delahunty CM, Baxter IA. 2001. Descriptive sensory analysis: past, present and future. *Food Res Int* 34(6):461–71.

O'Connor-Shaw RE, Roberts R, Ford AL, Nottingham SM. 1994. Shelf life of minimally processed honeydew, kiwifruit, papaya, pineapple and cantaloupe. *J Food Sci* 59(6):1202–6.

Oreskovich DC, Klein BP, Sutherland JW. 1991. Procrustes analysis and its applications to free-choice and other sensory profiling. In: Lawless HT, Klein BP, editors. *Sensory science theory and applications in foods*. New York: Marcel Dekker. p 353–93.

Péneau S, Brockhoff PB, Escher F, Nuessli J. 2007. A comprehensive approach to evaluate the freshness of strawberries and carrots. *Postharvest Biol Technol* 45(1):20–9.

Peng B, Yue T, Yuan Y. 2008. A fuzzy comprehensive evaluation for selecting yeast for cider making. *Int J Food Sci Technol* 43:140–4.

Reddy LVA, Reddy OVS. 2009. Effect of enzymatic maceration on synthesis of higher alcohols during mango wine fermentation. *J Food Qual* 32:34–47.

Rega B, Fournier N, Nicklaus S, Guichard E. 2004. Role of pulp in flavor release and sensory perception in orange juice. *J Agric Food Chem* 52(13):4204–12.

Richardson-Harman N, Phelps T, McDermott S, Gunson A. 1998. Use of tactile and visual cues in consumer judgments of apple ripeness. *J Sens Stud* 13(2):121–32.

Sanford KA, McRae KB, Deslauriers MLC, Sarsfield P. 2001. Sensory descriptive analysis and correspondence analysis aids in the choosing of apple genotypes for processed products. *J Food Qual* 24(4):301–25.

Satora P, Tuszynski T. 2010. Influence of indigenous yeasts on the fermentation and volatile profile of plum brandies. *J Food Microbiol* 27:418–24.

Schulbach KF, Portier KM, Sims CA. 2007. Evaluation of overall acceptability of fresh pineapple using the regression tree approach. *J Food Qual* 30(6):993–1008.

Shamaila M, Powrie WD, Skura BJ. 1992. Sensory evaluation of strawberry fruit stored under modified atmosphere packaging (MAP) by quantitative descriptive analysis. *J Food Sci* 57(5):1168–84.

Shamaila M, Skura B, Daubeny H, Anderson A. 1993. Sensory, chemical and gas chromatographic evaluation of five raspberry cultivars. *Food Res Int* 26(6):443–9.

Spencer MD, Pangborn RM, Jennings WG. 1978. Gas chromatographic and sensory analysis of volatiles from cling peaches. *J Agric Food Chem* 26(3):725–32 ER.

Spoto MHF, Domarco RE, Walder JMM, Scarminio IS, Bruns RE. 1997. Sensory evaluation of orange juice concentrate as affected by irradiation and storage. *J Food Process Preserv* 21(3):179–91.

Stone H, Sidel JL. 1993. S*ensory evaluation practices*, 2nd ed. San Diego: Academic.

Tiitinen K, Vahvaselka M, Laakso S, Kallio H. 2007. Malolactic fermentation in four varities of sea buckthorn (*Hippophae rhamnoides* L.). *Eur Food Res Technol* 224:725–32.

Tsen JH, Lin YP, King AE. 2009. Response surface methodology optimization of immobilized *Lactobacillus acidophilus* banana puree fermentation. *Int J Food Sci Technol* 44:120–7.

U.S. Department of Agriculture (USDA). 2008. Oranges, grapefruit, tangerines and tangelos grown in florida: subpart-grade and size requirements. Title 7, Code of Federal Regulations, Part 905.306.

Walker S, Prescott J. 2000. The influence of solution viscosity and different viscosifying agents on apple juice flavor. *J Sens Stud* 15(3):285–307.

Wang H, Reitmeier CA, Glatz BA, Carriquiry AL. 2003. Mixed model analysis of sensory characteristics of irradiated apple cider. *J Food Sci* 68(4):1498–503.

Zerbini PE, Pianezzola A, Grassi M. 1999. Poststorage sensory profiles of fruit of five apple cultivars harvested at different maturity stages. *J Food Qual* 22(1):1–17.

10

Wine Fermentation and Production

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CONTENTS

10.1 Introduction

Wine production is a complex process, not only from the microbiological point of view, but also in terms of biochemistry, with the transformation of must into wine by the metabolic activity of yeasts, particularly, *Saccharomyces cerevisiae*. The key metabolic process that occurs is the alcoholic fermentation catabolic pathway, which involves the conversion of the hexoses present in the grape juice into ethanol and carbon dioxide. In addition, other compounds initiate significant changes in the organoleptic properties of wine. In wine, malolactic fermentation takes place after alcoholic fermentation. This second fermentation reduces the acidity and adjusts the sensory profile of the wine, which makes this an indispensable process in the production of many wines, especially red wines. There are also special wines available on the market—all of which have emerged from the winemaking process, such as sparkling wines and biologically aged wines. Both have undergone a long and unique production that is characterized by their development through two main phases; in the case of sparkling wines, there are two processes involving fermentation and an aging period with yeast, and in the case of the biologically aged wines, a biological process that involves yeast oxidative metabolism.

A full description of the processes that take place during the production of wine can be found in various texts (Moreno-Arribas and Polo 2009; Reynolds 2010); the aim of this chapter is to provide the reader with an updated overview of the biochemical and microbiological phenomena that occur during the production of wine, and the relationship with the sensory quality and health properties of this product. Also included is a detailed description of the use of commercial preparations of inactive dry yeast (IDY); its properties for promoting fermentation and technological processes, and improving the organoleptic quality of wines, make them very interesting. These additives are becoming more widely used in wine production.

10.2 Alcoholic Fermentation

The alcoholic fermentation of grape must is the transformation of the sugars (glucose and fructose) present in musts into ethanol and carbon dioxide by the action of yeast from the genus *Saccharomyces*, generally, *S. cerevisiae* and *S. bayanus*. In addition, several other reactions and transformations take place in the acidic, sweet, and unflavored must, eventually producing a highly flavored alcoholic beverage (Boulton et al. 1996; Ugliano et al. 2009). This process is probably the oldest form of the biotechnological application of a microorganism and has been used by humans for several thousand years (Samuel 1996).

10.2.1 Yeast Fermentation

Different species of yeast are found naturally in grape must, depending on the grape variety, climatic conditions, agricultural treatments, etc. These indigenous yeasts, in addition to their interaction with other microorganisms, such as fungi, bacteria, and viruses, can produce "spontaneous" alcoholic fermentation (Fleet 1993, 1998; Pretorius 2000). Obviously, all these microorganisms contribute to the change in the must, with the metabolites of some microorganisms being the substrates of others (Pizarro et al. 2007). With regard to the indigenous yeast, a number of different yeasts act in the initial stages of fermentation, but when the level of ethanol increases, *S. cerevisiae* predominates because of its high resistance to ethanol (Fleet 1993; Fleet and Heard 1993; Beltran et al. 2002; Zamora 2009). The organoleptic characteristics of the final wine depend on the types of yeast acting during alcoholic fermentation and, sometimes, the indigenous yeasts and other microorganisms cause sensorial defects in wines. For this reason, and to avoid such spoilage, sulfur dioxide is normally added to the must to avoid the development of indigenous yeast, and selected strains (normally *S. cerevisiae*) are inoculated so that alcoholic fermentation can take place. The yeast is also selected based on the variety of grape, the composition of the must, the conditions of fermentation, and the type of wine (Bisson 2004).

The environmental conditions of must are not ideal for the development of yeast: the low pH, high sugar concentration, and high sulfur dioxide levels mean that the yeast has to adapt to this unfavorable medium. The evolution of the yeast population during alcoholic fermentation consists of three phases: latency, exponential growth, and the stationary stage. After a latency period (or adaptation of yeast to the environment; initial value $\approx 10^4$ cells/mL in noninoculated medium and 10^6 cells/mL in inoculated must), an exponential growth process (3–6 days; population increases to 10^7–10^8 cells/mL) takes place. During this stage, one-third of the ethanol is formed and most of the nutrients present in the must are consumed. Finally, in the stationary phase (2–10 days), because of a deficiency of nutrients, yeast growth stops and the remaining two-thirds of the ethanol are formed. In addition, the viable yeast population decreases because of the lack of nutrients and the increased level of ethanol. During these fermentation stages, different stress situations affect the yeast: oxidative stress, ionic stress, increased temperatures, nutrient limitation and starvation, and osmotic and ethanol stress, the latter two being the most important (Attfield 1997; Bauer and Pretorius 2000).

The yeast used in winemaking is also important because of its strong influence on the characteristics and quality of the resulting wine. Several works have been published in which the qualitative and quantitative profiles of the metabolites of different strains (*Saccharomyces* and non-*Saccharomyces*) were studied (Fleet 1992, 2008; Lambrechts and Pretorius 2000; Romano et al. 2003; Swiegers et al. 2005).

These studies provide information about the positive (such as the formation of glycerol or ester formation) and negative (such as the production of acetic acid) aspects, depending on the strain. More recently, interest has also been focused on the study of the capacity to produce glycosidases or cysteine lyase to enhance the varietal characteristics of the grapes by hydrolysis of the aroma precursors (Fia et al. 2005; Maicas and Mateo 2005; Swiegers et al. 2005; Villena et al. 2007; Dubourdieu et al. 2006; Swiegers and Pretorius 2007; Fleet 2008). Moreover, at the end of fermentation, yeast autolysis frees the wine of components such as mannoproteins, fatty acids, peptides, amino acids, etc., which also influence the sensorial characteristics of wines, both directly through new volatile compounds and through the interaction of liberated compounds with volatile wine compounds (Chalier et al. 2007; Lubbers et al. 1994a,b; Rodríguez-Bencomo et al. 2010). Nowadays, the use of genomic techniques for improving strains is directed toward obtaining new yeasts to elaborate new wine types adapted to consumer requirements and to obtain strains that are more resistant to ethanol, antimicrobial compounds, and other stress effects (Pizarro et al. 2007; Fleet 2008).

10.2.2 Sugars and Nitrogen Catabolism

The catabolism of sugars starts with glycolysis, which is an intracellular transformation of glucose into pyruvate, also called the Embdem–Meyerhoff pathway (Kresge et al. 2005). In addition, yeast uses pentoses (ribose) to synthesize nucleotides and NADPH, substances used in cell multiplication, and other catabolism processes (Horecker 2002). After glycolysis, the pyruvate produced can be transformed into ethanal and carbon dioxide, and ethanal is reduced to ethanol (alcoholic fermentation). Another possibility is the transformation of pyruvate into acetyl-coenzyme A and carbon dioxide (respiration process). Although the respiration process is more energetically efficient than alcoholic fermentation, it needs oxygen and is inhibited at high concentrations of sugars. Under wine fermentation conditions, yeast can only ferment sugars and the respiration process is only possible at low sugar levels and in the presence of oxygen (Barnett et al. 2005; Zamora 2009). In addition to alcoholic fermentation, there is another important biochemical pathway. During the growth of the yeast population, to obtain components such as proteins, lipids, etc., most of the pyruvate is consumed and regenerated by glycopyruvic fermentation, which produces pyruvate and glycerol.

There is a wide variety of nitrogen compounds in grape must, such as amino acids, ammonia, peptides, and proteins. Among them, only ammonia and amino acids (except proline) are assimilated by *S. cerevisiae*, thus these components are called easily assimilable nitrogen. The different sources of nitrogen are taken up and degraded in a specific order depending on environmental, physiological, and specific strain factors (Salmon and Barre 1998). If the levels of easily assimilable nitrogen are low, the likelihood of fermentation problems increases. An excess may cause microbiological instability in the wine, producing unwanted compounds (Ribereau-Gayon et al. 2000; Bisson and Butzke 2000), in which case, the use of sulfur amino acids by yeast could lead to the formation of mercarptans and hydrogen sulfide, which could have a negative effect on wine quality (Jiranek et al. 1995). For this reason, the habitual supplementation of musts with ammonia salts should be carried out carefully, taking into account the initial nitrogen and sugar contents.

10.2.3 Volatile Compounds Generated during Alcoholic Fermentation

Alcoholic fermentation generates a great variety of volatile compounds that have an important effect at a sensorial level. These compounds belong to different chemical families, such as higher alcohols, fatty acids, esters, carbonyl compounds, sulfur compounds, etc. (Rapp and Versini 1991; Cordonnier 1979; Etiévant 1991; Câmara 2004). Quantitatively, fermentative volatile compounds are the most abundant compounds of wine aroma, and are responsible for the common-wine character of more neutral wines. However, in the case of wines with more floral notes (such as muscat wines), fermentative compounds are of less sensorial importance. Moreover, the compounds generated during alcoholic fermentation and their concentrations depend heavily on the yeast strain, fermentation conditions, grape variety, and must composition.

Higher alcohols (or fusel alcohols) are quantitatively the most important group of volatile compounds produced by yeast during alcoholic fermentation. This group of compounds includes alcohols that contain more than two carbon atoms, such as isobutanol, 2-methyl-1-butanol, 3-methyl-1-butanol, 2-phenylethanol, and tyrosol. The influence on wine quality of these compounds depends on their concentration. At levels lower than 300 mg/L, they give complexity to the aroma of wines; however, at high levels, they can lower the quality of wine aroma. In the case of 2-phenylethanol, this normally enhances wine aroma with rose-like notes (Rapp and Versini 1996; Swiegers et al. 2005).

The formation of these compounds is related to the amino acid metabolism by two different pathways: first, the catabolism of the corresponding amino acids (valine, leucine, isoleucine, and 2-phenylalanine), and second, the anabolism of amino acids such as threonine, valine, leucine, isoleucine, and glutamic acid through the catabolism of sugars (Etiévant 1991; Ugliano and Henschke 2009). Therefore, the content of higher alcohols increases when high levels of the corresponding amino acids are present (Schulthess and Ettlinger 1978). The production of higher alcohols during alcoholic fermentation is also influenced by different factors, such as the yeast strain, the composition and pH of the initial must, conditions of fermentation (temperature), the assimilable nitrogen content, grape variety, aeration, etc. (Soufleros et al. 1980). The production of higher alcohols during fermentation decreases with low temperatures and low pH, anaerobiosis, and high levels of ammonium ions (Etiévant 1991).

Volatile acids are mainly synthesized during fermentation by yeast and bacteria, and can have a positive or negative effect on aroma and flavor, depending on their concentration and the type and style of wine. During fermentation and aging, organic acids are involved in the reactions of ester formation and hydrolysis. In fact, there is a link between the acid and ethyl ester content, probably because they share some of their metabolism pathways (Etiévant 1991). With regard to the factors that affect the formation of volatile acids, low temperatures during fermentation increase their production. Moreover, the strain used during fermentation has a strong effect on their final content in wines (Shinohara 1986).

Acetic acid usually constitutes more than 90% of the total content of volatile acids (Swiegers et al. 2005; Henschke and Jiranek 1993). Acetic acid is also the most sensorially important volatile acid produced during alcoholic fermentation, and at high levels (>0.7 g/L), it produces sensorial defects with unpleasant vinegary notes, although this may depend on the type of wine (Dubois 1994). Other volatile acids, with a larger carbon chain (butyric, hexanoic, octanoic, and decanoic acids), provide sour, cheese-like, or rancid sensorial notes, the extent of their sensorial influence depending on the type of wine. In some wines, acids such as octanoic, hexanoic, decanoic, and some branched-chain acids could be present at levels slightly higher than their odor thresholds, contributing to the freshness and equilibrating the fruity notes (Etiévant 1991; Shinohara 1985).

Of all the chemical families, more compounds have been identified in wines from the ester group, and their origin is mainly fermentative. These compounds are responsible for the fruity aroma of wines, and most of the more common esters are in concentrations similar to or higher than their odor thresholds even in old wines (Table 10.1). The principal esters derived from alcoholic fermentation are the ethyl esters (butyrate, hexanoate, octanoate, decanoate, and dodecanoate) and the group of acetates (ethyl, isoamyl, phenylethyl, isobutyl acetates, etc.). Their combined impact, taking into account their joint synergic/additive and suppressive effects, produces the final aroma in the wine (Etiévant 1991). Quantitatively, the most important ester—and the one most studied—is ethyl acetate. It represents more than 80% of the total content of volatile esters (Pinsun 1996). Its contents vary between 50 and 100 mg/L and this may contribute to the complexity of the wine aroma. If its concentration is higher than 150 mg/L, the likely result is an unpleasant, vinegary, and bitter flavor.

The formation of esters during fermentation is influenced by several factors. The effect of the yeast strain on the production of esters is important (Ugliano and Henschke 2009). For example, some non-*Saccharomyces* yeast strains produce higher contents of ethyl acetate than *S. cerevisiae* yeast. In addition, the esterase activity of the yeast also affects the final ester content in the wine. Moreover, the fermentation conditions strongly affect ester production and the type of ester produced. Low temperatures favor the production of esters such as isoamyl, isobutyl, and hexyl acetates, whereas high temperatures tend to lead to the formation of esters of high molecular weight such as ethyl octanoate and decanoate and

TABLE 10.1

Esters Present in Wines and Their Sensory Nuances

Compound	Odor Threshold (mg/L)[a]	Odor Description[b]
Ethyl butyrate	0.020	Fruity
Ethyl isobutyrate	0.015	Strawberry
Ethyl hexanoate	0.014	Fruity
Ethyl octanoate	0.005	Fruity, fresh
Ethyl decanoate	0.200	Fruity, fatty acid
Ethyl cinnamate	0.001	Flowery, sweet
Ethyl acetate	12.27	Solvent
Isobutyl acetate	1.605	Solvent, flowery
Isoamyl acetate	0.030	Banana
2-Phenylethyl acetate	0.250	Roses

[a] *Source:* Ferreira, V. et al., *J Agric Food Chem*, 50(814), 4048–54, 2002. With permission.

[b] *Sources:* Ferreira, V. et al., *J Agric Food Chem*, 50(814), 4048–54, 2002. With permission. Culleré, L. et al., *J Agric Food Chem*, 52(6), 1653–60, 2004. With permission. Campo, E. et al., *Anal Chim Acta*, 563, 180–7, 2006. With permission.

phenylethyl acetate, and favor the hydrolysis process (Killian and Ough 1979). Aeration of must, low levels of sulfur dioxide, low fermentation temperatures, and clarified must help ester production, whereas turbid must tends to lower ester production. Also, the composition of nitrogens in must has a strong effect on the accumulation of esters. In general, the production of esters increases when the amino acid nitrogen increases, and the addition of ammonium salt also stimulates the production of esters (Etiévant 1991; Ugliano and Henschke 2009).

Carbonyl compounds such as aldehydes, ketones, and lactones are produced by yeast metabolism. Quantitatively, the most important of these is acetaldehyde, which is presented at levels from 10 to 75 mg/L (for dry wines) and is formed as an intermediate in ethanol production. It contributes "nutty" notes, although it is linked with oxidation to "off-flavors." However, this compound plays an important role in sherry-type wines and other oxidized wines. The production of this compound increases with high levels of sulfur dioxide in must. In addition, yeast resistant to sulfur dioxide generally tends to produce higher levels of acetaldehyde (Casalone et al. 1992). With regard to ketones, diacetyl (2,3-butanedione) is also produced during fermentation, contributing to wine aroma with a buttery note. Diacetyl is formed by decarboxylation of α-acetolactase and is regulated by valine and threonine availability (Dufour 1989). If the levels of assimilable nitrogen are low, valine is synthesized, and therefore α-acetolactate is also synthesized, which is then transformed into diacetyl by spontaneous oxidative decarboxylation. In addition, threonine represses the uptake of valine when there is enough assimilable nitrogen, and the formation of diacetyl decreases. Moreover, some γ-lactones are formed during fermentation, their precursor being glutamic acid (Wurz et al. 1988), and with γ-butyrolactone being quantitatively the most important lactone, although their sensorial importance is not clear (Ugliano and Henschke 2009).

During alcoholic fermentation, some volatile phenols, such as 4-vinylguaiacol and 4-vinylphenol, are synthesized from nonflavonoid hydroxycinnamic acids such as *p*-coumaric acid and ferulic acid by decarboxylation in a nonoxidative process (Chatonnet et al. 1993). High levels of some of these compounds, such as 4-ethylphenol, are seen as a defect and this is associated with wines contaminated by *Brettanomyces* yeast (Chatonnet et al. 1993).

Another important group of compounds is the sulfur compounds which, in most cases, have been linked to negative sensory effects. However, some of them, such as varietal thiols, play a key role in the characteristic aroma of some varieties of wine (Tominaga et al. 1996, 1998a,b; Dubourdieu et al. 2006). The formation of these sulfur compounds by yeast is produced by different pathways, such as the reduction of elemental sulfur, degradation of sulfur amino acids or sulfur-containing pesticides, or by

an enzymatic process on sulfur-containing aroma precursors from grapes (Mestres et al. 2000; Swiegers et al. 2005).

Hydrogen sulfide (H_2S) is the sulfur compound most commonly generated during yeast metabolism. Typically, it provides an aroma similar to rotten eggs and because of its odor threshold, this is common in wines that have just been fermented, but can be solved by the addition of copper salt or by aeration. The concentration of H2S produced is influenced by the composition of the must, especially at the nutritional level, conditions of fermentation, and the type of yeast. In musts with some amino acid deficiency, yeast produces them from inorganic sulfur sources that also produce H2S (Henschke and Jiranek 1991; Rauhut 1993; Spiropoulos et al. 2000). In addition, the yeast autolysis process can also produce H2S by the degradation of sulfur-containing amino acids.

Other sulfur-containing compounds such as ethanethiol, and some polysulfides, such as dimethyl sulfide, dimethyl disulfide, trimethyl disulfide, etc., present sensorial notes that can produce sensorial defects in wines. The formation of these compounds is also related to the metabolism of some amino acids and peptides by yeast or by the oxidation of mercaptans. The production of mercaptans (ethyl mercaptan and methyl mercaptan), which produce unpleasant sensorial notes of onion and rubber, is related to the metabolism of methionine (Swiegers et al. 2005). In addition, some compounds, such as methionol (with a cauliflower and cabbage odor), 4-methylthio-1-butanol (onion and garlic odor), and 2-mercapto-1-ethanol (poultry and farmyard odor), could result from the metabolism of methionine, homocysteine, and cysteine yeasts (Mestres et al. 2000).

With regard to varietal thiols, 4-methyl-4-mercapto-2-pentanone, 3-mercaptohexyl acetate, and 3-mercaptohexanol present very low odor thresholds and characterize the aroma of wines elaborated with some grape varieties such as sauvignon and colombard. These compounds are not present in grapes and are formed from the aroma precursors of cysteine during fermentation by yeast (Darriet et al. 1995).

10.3 Malolactic Fermentation

Winemaking is a complex microbial process involving yeasts and bacteria. Although they are both naturally present on grape skins (Renouf et al. 2005), they can also be found in barrels, tanks, and the equipment used during vinification. Yeasts are predominant in wine and are used to carry out alcoholic fermentation, whereas lactic acid bacteria are responsible for malolactic fermentation. During winemaking, malolactic fermentation reduces the acidity of wine (by conversion of L-malic acid into L-lactic acid), contributing positively to the microbial stability and organoleptic quality of wines.

Research on malolactic fermentation and wine lactic acid bacteria from the second half of the last century has greatly enriched our basic knowledge of this subject, so that now, the winemaker has the necessary tools to allow him or her to take various actions to accelerate or, where appropriate, avoid malolactic fermentation. However, in terms of the impact of malolactic fermentation on wine quality, there are still many unresolved issues. These mostly concern innovative aspects, such as the incidence of bacterial growth in the organoleptic and hygienic qualities of wine. This section summarizes the main contributions of literature about the positive contribution of malolactic fermentation toward wine quality, and describes the progress of the main changes produced by the metabolism of lactic acid bacteria during the production of wine.

10.3.1 Lactic Acid Bacteria

The involvement of lactic acid bacteria in winemaking, and their distribution and succession in musts, wines, and during fermentation, have been extensively studied (Lonvaud-Funel 1999; Versari et al. 1999; Moreno-Arribas and Lonvaud-Funel 2001; Liu et al. 2002; Matthews et al. 2006). Wine lactic acid bacteria have a complex ecology and, in the same way as many other fermented food products, they experience a steady growth rate during vinification. Because of the low pH of musts and wines, the low content of nutrients and the relatively high ethanol content (in the case of wine), only a limited number of bacterial species can grow and develop in grape must and wine (Table 10.2). Lactic acid bacteria from grape must

or wine belong to two families representing three genera. Lactobacillaceae are represented by the genus *Lactobacillus*, and Streptococcaceae are represented by *Oenococcus* and *Pediococcus*. *Oenococcus oeni* is the bacterial species which predominates at the end of alcoholic fermentation. This is the species best adapted to grow in difficult conditions, such as those imposed by the medium (low pH and high ethanol concentration; Davis et al. 1986); therefore, for most wines, it is the main species responsible for malolactic fermentation.

Over the last few decades, different molecular methods have been developed for the identification of lactic acid bacteria of enological interest with the aim of reducing the analysis time usually required to perform phenotypic assays. Moreover, molecular methods allow more reliable identification results because they are not influenced by physiological and environmental conditions. They can be used to identify lactic acid bacteria in grape must at different stages of winemaking, aging, and storage. Tools comprise culture-dependent and culture-independent methods. Methods can identify the species and the strain level. Bacterial strain can be differentiated, and in addition, strains carrying specific genes can be detected whatever the species. Molecular tools for identifying lactic acid in grapes and wine have recently been included in a draft resolution (Oeno-Micro-09-409) by the International Organization of Vine and Wine. The document outlines the main applications of molecular methods to identify lactic acid bacteria in wine and presents the main literature in this field.

TABLE 10.2

Description of the Lactic Acid Bacteria from Musts and Wines

Genus	Species	Winemaking Step Used for Bacteria Isolation	References
Oenococcus	*O. oeni*	Grape, must, alcoholic fermentation, malolactic fermentation, oak barrel aging	Dicks et al. (1995)
Leuconostoc	*L. mesenteroides*	Grape, must, wine	Martinez-Murcia et al. (1993)
Pediococcus	*P. damnosus*	Must, alcoholic fermentation, wine, spoiled wine (“ropiness”)	Garvie (1986); Davis et al. (1986); Edwards and Jensen (1992); Gindreau et al. (2001)
	P. parvulus	Must, alcoholic fermentation, wine	
	P. pentosaceus	Must, alcoholic fermentation, wine	
Lactobacillus	*L. plantarum*	Grape, must, wine, base wine for brandy manufacture	Fornachon (1957); Costello et al. (1983); Lafon-Lafourcade et al. (1983); Davis et al. (1986); Sieiro et al. (1990); Edwards et al. (2000); Du Plessis et al. (2004); Beneduce et al. (2004); Moreno-Arribas and Polo (2008)
	L. brevis	Must, alcoholic fermentation, wine	
	L. hilgardii	Wine, alcoholic fermentation	
	L. casei	Must, wine	
	L. paracasei	Must, wine	
	L. zeae	Biologically aged wine	
	L. vini	Wine	
	L. mali	Grape, must, wine	
	L. kunkeei	Grape, alcoholic fermentation, alcoholic fermentation in spoiled wines	
	L. lindneri	Grape	
	L. kefiri	Grape	
	L. vermiforme	Wine	
	L. trichodes	Spoiled wine	
	L. fermentum	Alcoholic fermentation	
	L. cellobiosus	Alcoholic fermentation	
	L. nageli	Alcoholic fermentation in spoiled wines	

Source: Pozo-Bayon, M. A. et al., *Afr J Biotechnol*, 8 (17), 3995–4001, 2009. With permission.

10.3.2 Malolactic Fermentation and Its Positive Organoleptic Contribution

In biochemical terms, malolactic fermentation involves the decarboxylation of one molecule of L-isomer of malic acid (dicarboxylic acid) to yield a molecule of L-malic acid (monocarboxylic acid) and one molecule of carbon dioxide. Malic acid is one of the most abundant organic acids in wine. Its concentration ranges between 2 and 10 g/L, depending on the area of production. In lactic acid bacteria, the enzyme responsible for this decarboxylation is the malolactic enzyme, and acts in the presence of Mn2+ and NAD+. This enzyme has been purified in different strains of lactic acid bacteria in wine (Lonvaud-Funel and Strasser de Saad 1982; Naouri et al. 1990).

The main effect of malolactic fermentation and the reason for its presence in winemaking is the biological deacidification of wine as a result of the decarboxylation of a dicarboxylic acid to obtain a monocarboxylic acid. The total acidity decreases, and the strong flavor of malic acid is replaced by the less powerful flavor of lactic acid. Therefore, malolactic fermentation is not recommended for those young wines and some white wines in which acidity is essential. As a result of bacterial activity during malolactic fermentation, other reactions that modify the aroma compounds and "flavor" of the wine take place. For example, during this process, some varietal aromas arising from fermentation will change or disappear (Costantini et al. 2009). As a result of citric acid metabolism, organoleptically important compounds such as diacetyl and other carbonyl compounds will be produced (Martineau and Henick-Kling 1995; Nielsen and Richelieu 1999). The increase in fruit flavors may be due to the formation of lactic acid bacteria esters in wine. In addition to fruity, buttery aromas, malolactic fermentation has been associated with characteristic aromas such as floral, roasted, vanilla, sweet, woody, smoked, bitter, honey, etc.

It has also been shown that malolactic fermentation can incur changes in the color of wine. There is a decrease in the intensity of color with a blue drop, mainly because of the possible adsorption of anthocyanins by bacterial cell walls, which also contribute to the increase in pH produced by the transformation of malic acid into lactic acid and decreased free sulfur dioxide levels. Lactic acid bacteria may modify the phenolic composition of wines. Hernández et al. (2006) showed that *trans*-caftaric and *trans*-coutaric acids are substrates of lactic acid bacteria that can exhibit cinnamoyl esterase activity during malolactic fermentation, increasing the concentration of hydroxycinnamic acids. An additional source of caffeic and *p*-coumaric acids may come from the hydrolysis of cinnamoyl-glucoside anthocyanins (Moreno-Arribas and Polo 2008), as well as from other hydroxycinnamic derivatives by bacterial enzymatic activity. Furthermore, according to Hernández et al. (2007), it seems that this activity could be strain-dependent and could also depend on the isomeric form of the above-mentioned esters because only the *trans*-isomers were involved in the reaction.

Different studies have focused on the biosynthesis of aroma compounds during malolactic fermentation and the concomitant organoleptic consequences (Maicas et al. 1999; Laurent et al. 1994). Pozo-Bayón et al. (2005) investigated the changes in volatile compounds before and after malolactic fermentation, carried out by four different starter cultures of the species *O. oeni* and *Lactobacillus plantarum*, and found significant metabolic differences between both species. Aroma/flavor attributes also seemed to vary according to the strain used for inducing malolactic fermentation.

Malolactic fermentation increases the fruity and buttery aromas but reduces vegetable or grassy aromas (Henick-Kling 1993). The formation and hydrolysis of esters during malolactic fermentation may also lead to an increase in the fruity aroma and this is probably because of the action of the lactic acid bacteria esterases responsible for the synthesis and degradation of these compounds. However, to date, there have been no studies that demonstrate these changes. The reduction in vegetable or grassy aromas could be because of the catabolism of aldehydes by lactic acid bacteria. *O. oeni* can catabolize acetaldehyde, converting it into ethanol and acetate (Osborne et al. 2000). In addition to fruity and buttery aromas, malolactic fermentation has also been associated with other characteristic aromas such as floral, roasted, vanilla, sweet, woody, smoked, bitter, honey, etc. (Henick-Kling 1993; Sauvageot and Vivier 1997).

Lactic acid bacteria may cause polysaccharides to be released in a wine (Dols-Lafargue et al. 2007). These compounds can increase the sensation of volume or body in wines, and can also be polymerized with the grape or wood tannins, reducing sensations of roughness or astringency, and producing more complex flavors.

It is also important to add that the microbiological stability of wine is enhanced by this stage of winemaking. After malolactic fermentation, the concentration of nutrients will be lower and this will prevent the growth of other bacteria. On the other hand, during malolactic fermentation, lactic acid bacteria synthesize antimicrobial compounds (Costantini et al. 2009).

10.3.3 Metabolic Traits Involved in Wine Spoilage

10.3.3.1 Spoilage of the Organoleptic Quality of Wines

In most cases, if you carry out a control of malolactic fermentation, lactic acid bacteria contribute positively to wine quality. However, some species or strains of lactic acid bacteria can produce changes that affect wine quality and acceptability, even to the extent of making it unfit for marketing. Among various reviews of these alterations are the works of Lonvaud-Funel (1999) and Costantini et al. (2009).

One of the most frequent alterations is the well-known "lactic disease" (piqûre lactique), which is characterized by a highly volatile acidity that impairs wine. This may occur during the winemaking process and during storage. When alcoholic fermentation is too slow or is not complete, conditions are very favorable for lactic acid bacteria growth. In this case, lactic acid bacteria can metabolize sugars, producing ethanol and carbon dioxide, which are also produced by yeasts, as well as acetic acid, resulting in an increase in the volatile acidity of wine. If this volatile acidity exceeds the limit of 1 g/L, the wine is unmarketable (Lonvaud-Funel 1999).

Some lactic acid bacteria convert glycerol to 3-hydroxypropionaldehyde by the enzyme glycerol dehydratase. This compound is the precursor to acrolein, a compound that is not problematic on its own, but which may lead to bitter tastes when it reacts with phenolic compounds such as tannins. Glycerol is one of the main compounds in wine, in concentrations of 5 to 8 g/L.

Wine with an increased viscosity and a slimy appearance is called "ropy." This aspect is due to the production of dextrane or glucane from *Leuconostoc* and *Pediococcus* (Fugelsang 1997; Lonvaud-Funel 1999). These extracellular polysaccharides are mainly produced by *Pediococcus damnosus*, and their production is linked to a plasmid of approximately 5500 bp; the ropy phenotype disappears when the plasmid is lost. These ropy strains are much more tolerant to ethanol than others. Concentrations of glucane of approximately 100 mg/L are high enough to give the wine this abnormal tolerance.

As a result of lactic acid bacteria metabolism, compounds can be produced that give wine an unpleasant aroma. The compounds related to this alteration and associated with so-called animal taints are volatile phenols. In red wines, the main volatile phenols detected are 4-vinylphenol, 4-vinylguayacol, 4-ethylphenol, and 4-ethylguayacol. Vinylphenols are generated from the decarboxylation of grape phenolic acids by the action of *S. cerevisiae* during alcoholic fermentation. In the case of ethylphenols, their origin has been under debate for many years, but it has been demonstrated that its biosynthesis is involved in some bacterial strains of *Pediococcus* and *Lactobacillus* (Cavin et al. 1993; Chatonnet et al. 1993) and yeasts of the genera *Brettanomyces/Dekkera* (Chatonnet et al. 1993).

Other compounds produced are aromatic heterocyclic bases responsible for a "mousy" character. Some lactic acid bacteria such as *L. plantarum*, *Lactobacillus brevis*, and *O. oeni* are associated with the presence of odors and flavors in wine such as the "smell of mouse urine" or "acetamide" (Grbin and Henschke 2000). This alteration of the wine is associated with the metabolism of the amino acids, ornithine and lysine, and the production of three volatile heterocyclic bases: 2-acetyltetrahydropyridine, 2-ethyltetrahydropiridina, and 2-acetyl-1 pyrroline. As in the production of volatile phenols, some strains of lactic acid bacteria as well as the yeast *Brettanomyces* may be responsible for the formation of these compounds (Grbin and Henschke 2000).

10.3.3.2 Spoilage of the Hygienic Quality of Wines

The study of the metabolic role of lactic acid bacteria in the formation of compounds which are undesirable because of their implications for health has been focused on two groups of compounds: ethyl carbamate and biogenic amines.

Ethyl carbamate (also referred to as urethane) is a naturally occurring component of fermented foods and beverages, including wine. Some heterofermentative lactic acid bacteria are capable of forming small amounts of citrulline from the degradation of the amino acid arginine. The excretion of citrulline is of toxicological concern because it is a precursor to the formation in wine of the potential carcinogenic ethyl carbamate (Zimmerli and Schlatter 1991). According to Mira de Orduña et al. (2001), the risk of citrulline formation by malolactic bacteria in wines with high residual arginine concentrations can be reduced by carrying out malolactic fermentation with pure oenococcal cultures and by establishing precisely complete malolactic conversion, which must be followed by the inhibition of bacterial activity.

Biogenic amines are low molecular weight organic bases with high biological activity which have shown undesirable effects in foods and beverages. Histamine is the most widely studied biogenic amine and can cause headaches, hypotension, and digestive problems, whereas tyramine is associated with migraines and hypertension (Bover-Cid and Holzapfel 1999; Moreno-Arribas et al. 2010). In wine, as in other fermented products, biogenic amines are mainly derived from the presence of lactic acid bacteria that produce enzymes capable of decarboxylating the corresponding precursor amino acids. The most common biogenic amines in wine are histamine, tyramine, putrescine, and cadaverine (Marcobal et al. 2006), produced by the decarboxylation of histidine, tyrosine, ornithine/arginine, and lysine, respectively. The production of biogenic amines in wine should be considered an important criterion in the selection of starter cultures and in the study of the characteristics of the autochthonous microbiota present in the wine's environment. Several articles have reported conflicting results but, in general, the presence of biogenic amines in wine is correlated with wine spoilage and, in particular, the action of different *Lactobacillus* strains (Straub et al. 1995; Moreno-Arribas et al. 2000, 2003; Costantini et al. 2006). More information about the chemical and biochemical features of the production of biogenic amines in wines is found in Ancín-Azpilicueta et al. (2008), Smit et al. (2008), and Moreno-Arribas et al. (2010).

10.4 Special Vinifications

10.4.1 Sparkling Wines

The elaboration of sparkling wines is based on a secondary fermentation of a still wine (base wine) and an aging period of wine with yeasts. The carbon dioxide that characterizes these wines is exclusively produced during this second fermentation. These wines also present a relatively high economical added value and, for some regions of the main producer countries (France, Italy, and Spain), form the basis of the economy. Recently, the main chemical and biochemical features involved in sparkling wine production have been reviewed (Pozo-Bayón et al. 2009a).

10.4.1.1 Production of Sparkling Wines

A sparkling wine is a wine obtained through a second fermentation of a still wine, called base wine. The characteristic carbon dioxide of these wines is exclusively produced during the second fermentation. Figure 10.1 shows an example of different sparkling wine types, classified according to their different production technologies into sparkling wines fermented in the bottle and those fermented in large *cuves* or *granvas*. The latter are produced in hermetically sealed tanks, where the wine has to spend at least 20 days before being transferred to the bottles in which they are sold. Some Italian wines, such as Lambrusco and Asti, are produced in this way. The sparkling wines fermented in the bottle can be filtered and transferred to another bottle (transfer method) or the entire process (fermentation and aging) can be produced in the same bottle that goes to market (classical or traditional method). The latter includes some high-quality sparkling wines such as Talento, Cava, and Champagne wines, from Italy, Spain, and France, respectively. Although they share the same production process, most of their differences are because of the variety of grape, the production areas, and the length of time the wine is kept with yeast during aging. Specific legislation in each country regulates the raw materials, some base wine physicochemical parameters, and winemaking technologies to guarantee the quality of these wines.

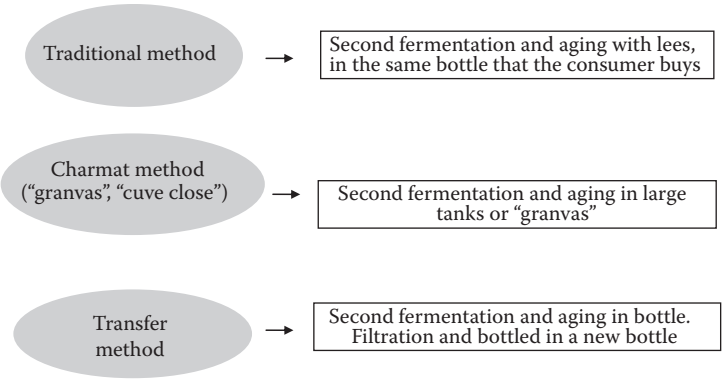

FIGURE 10.1 Main winemaking technologies for sparkling wine production.

The first step in the elaboration of sparkling wines using the traditional method is the filling of the bottles with the base wine, which can be a monovarietal white wine or a mixture of wines. The "tirage" liquor is also added to the bottle. This tirage liquor is a mixture that contains grape must (or partially fermented grape must), saccharose, and yeast (which will produce the second fermentation) in specific proportions to produce the carbon dioxide pressure desired. The yeast used in this process should be highly resistant to ethanol and carbon dioxide pressure, and high activity at low temperature, and have a high flocculation capacity (Bidan et al. 1986; Martinez-Rodríguez and Pueyo 2009). The characteristics of the base wine are also important: it should have lower residual sugars, moderate alcohol content, and low volatile acidity and should be free of yeast or bacteria. The sealed bottles are located in places specially designed for the fermentation and aging processes to take place.

During the aging process, the bottles are turned from time to time so that the sediments move to the neck of the bottle, and when all the lees are accumulated in the neck, this is frozen (by immersion in a cryogenic bath) and the cap is removed, producing gas pressure and leading to the expulsion of the frozen lees deposit. Usually, during this process, called disgorging, some wine is lost and this is replaced using a "liquor of expedition." The liquor of expedition could be the same sparkling wine, base wine, saccharose, or grape must (partially fermented or not) and will give the sweetness desired to the sparkling wine. Finally, the bottles are sealed with a cork and a wire cap is used to secure the cork.

10.4.1.2 Importance of Yeast Autolysis

During aging, yeast autolysis takes place. This process involves the hydrolysis of biopolymers under the action of hydrolytic enzymes. The final result is the release of cytoplasmic components such as peptides, amino acids, fatty acids and nucleotides, and cell wall compounds such as glucans and mannoproteins. All of these components are involved in the sensorial and functional properties of sparkling wines (Alexandre and Guilloux-Benatier 2006; Charpentier and Feuillat 1993; Feuillat and Charpentier 1982; Pozo-Bayon et al. 2009).

With regard to nitrogen compounds, in the first stages of the autolysis process, high molecular weight peptides, mainly of a hydrophobic nature, are released; however, after hydrolysis, these become lower molecular weight peptides or amino acids (Moreno-Arribas et al. 1996, 1998). These low molecular weight peptides present important properties related to tensioactivity and some bioactive effects (antioxidant, antimicrobial, antihypertensive; Aldaide-Hidalgo et al. 2007, 2008). However, during aging and after hydrolysis, these break down glucanes and release cell wall mannoproteins (Feuillat et al. 2003). This is related to important improvements in protein haze, tartaric stability, and foaming properties (Dupin et al. 2000; Nuñez et al. 2006). In addition, lipids and nucleic acids, which are present in lower concentrations, also have some sensorial implications. During aging, lipids are released, and because

of their tensioactive properties, they are related to foam stability (Pueyo et al. 1995, 2000; Gallart et al. 2002), although some lipids could have negative effects (Gallart et al. 2004). Nucleic acids are also released during autolysis (Aussenat et al. 2001; Charpentier et al. 2005) and some of them could act as enhancers of flavor and mouthfeel of other compounds.

While the wine is aging with the yeast, the degradation and synthesis of volatile compounds occur simultaneously (Pozo-Bayon et al. 2003). Some studies suggest that the concentrations of some esters (hexyl and isoamyl acetates and ethyl esters of butyric and octanoic acids and diethyl succinate) could be used to distinguish between cava wines of different ages (Pozo-Bayon et al. 2003). Also, compounds derived from the enzymatic degradation of carotenoids, such as some c13 norisoprenoids, have been suggested as markers of aging time (Bosch-Fuste et al. 2007; Riu-Aumatell et al. 2006; Loyaux et al. 1981).

Additionally, the interaction of volatile compounds with different components released by lees also has an effect at the sensorial level. Thus, mannoproteins released by yeast could bind to different volatile compounds, reducing their volatility, which could affect the final perceived aroma (Chalier et al. 2007; Lubbers et al. 1994a,b).

10.4.1.3 Foam Characteristics

The characteristic foam of sparkling wines and its properties is an important quality marker of this type of wine. On the basis that wine proteins are the compounds that have the most positive effect on the formation of adequate foam, the characteristics of the foam may be affected negatively by technological processes, such as clarifications or filtrations, which have a direct effect on the protein concentration, reducing their contents (Dambrouck et al. 2005; Vanrell et al. 2007). On the other hand, foamability decreases during aging, although its persistence increases (Andrés-Lacueva et al. 1996). In this context, the use of yeast strains with enhanced autolytic capacity can help achieve better foaming properties in shorter times (Nuñez et al. 2005). Finally, grape variety also affects the characteristics of foam. Thus, Chardonnay wines have the best foamability but the worst stability (Andrés-Lacueva et al. 1996, 1997).

10.4.2 Biologically Aged Wines

10.4.2.1 Production of Biologically Aged Wines

Biologically aged wines are also developed, traditionally, from selected wine grape varieties and the subsequent biological or foster parenting by "flor" yeast. This is truly a group of Spanish wines that are obtained in denominations—Jerez-Xères-Sherry, Manzanilla-Sanlucar de Barrameda, Montilla-Moriles, and Condado de Huelva (Andalucia, Southern Spain) are produced in this way. There are also some biologically aged wines produced in other countries, such as France (Jura Valley), Italy (Sardinia and Sicily), Hungary (Tokay), United States (California), Australia, and South Africa, using some different technological processes to those used in Spain.

The production of these wines has two distinct stages. The first is to obtain the wine of the current year, with the vinification of the grape must in fermentation control. So production is always based on new wines which have just finished fermenting. Then, the selected wines are headed, if necessary, with a premium wine to raise the alcohol content from 15°C to 15.5°C. The second stage, which is what differentiates these wines, is the biological aging process through a system of "criaderas and soleras." Breeding takes place in wooden vessels that are filled to five-sixths full, leaving the cap set for the aerobic process (with oxygen). The wine, at this stage, is known as "sobretablas."

In the special conditions of the aging cellars, which provide high humidity, low temperature, and very little ventilation and lighting in which these wines are kept, a "velum" or flor quickly and spontaneously develops on the surface of these wines, consisting of a pale creamy-looking yeast, similar to that made by alcoholic fermentation but which now lives in contact with the oxygen in the air, while protecting the wine from it. The metabolic activity of the yeasts, in particular, the very slow diffusion of oxygen through the timber of the vats and the transfer of volatiles from this wood to the wine, suggest a series of biological and physical mechanisms that give rise to the chemicals and organoleptic

characteristics of these wines that is so unique. The wine for the market is extracted from the hearth at a third of the total volume, and distributed in several "bags" a year. The gap left in each bag is replaced by wine from the immediate criadera and so on until reaching the last criadera, where it is replaced by the sobretablas. This system aims to always obtain a wine of uniform characteristics and qualities over time.

Fino wines are the best example of this group of wines. The yeasts that make up the velum have an oxidative metabolism, creating a reducing atmosphere in the wine that is responsible for its pale color and much of its aromatic complexity, characterized by nutty (hazelnut), balsamic, and spicy notes, closely linked to the presence of acetaldehyde. It is known that different yeast strains have different metabolic characteristics allowing targeted breeding stock based on the selected flor yeasts.

10.4.2.2 Importance of the Flor Velum

Biologically aged wines have a complex microbiota consisting of fungi, yeasts, and bacteria. Correction of acidity and high alcohol content allow the subsequent selection of only a few yeasts. Physiological and molecular characterization of the yeasts present in the velum of sherry wines has shown that most of them belong to different species of *S. cerevisiae*, mainly *beticus*, *cheresiensis*, *montuliensis*, and *rouxii* (Martínez et al. 1995, 1997; Benítez and Codón 2005). These flor yeasts differ physiologically from the typical fermentative yeasts (which are unable to grow aerobically in wine), and they have different metabolic and genetic characteristics (Esteve-Zarzoso et al. 2001, 2004; Budroni et al. 2005).

During biological aging, the concentration of ethanol decreases because of its consumption by flor yeast, which uses it as the main source of carbon and energy. Acetaldehyde is the main subproduct from ethanol metabolism, but other volatile compounds, such as acetic acid, butanediol, diacetyl, and acetoin, can also be formed. Additionally, ethanol can be metabolized via the tricarboxylic acid pathway (Suárez-Lepe and Iñigo-Leal 2004). Ethanol can also be lost by evaporation through the oak cask, and therefore, a decrease in the alcoholic level can be observed in these wines during aging (Charpentier et al. 2000). Flor yeast can use glycerol as a carbon source, therefore its concentration decreases during wine aging, which could be useful as an indicator of the age of the wine (Peinado and Mauricio 2009).

The biological aging of wines reduces the content of amino acids and other nitrogenous wine components (ammonium and urea). The main source of nitrogen for the yeast during the biological aging process is L-proline, although yeasts differ in the amount of assimilable nitrogen, they prefer consuming amino acids (Mauricio and Ortega 1997; Valero et al. 2003).

The content of organic acids decreases during biological aging. Before its incorporation into the biological aging system (criadera system), wine is stored after its fortification with ethanol (encabezado), and it is during this period that it undergoes malolactic fermentation (wine stored in this way is called sobretablas wine). Therefore, most of the malic acid is converted into lactic acid and an insignificant amount of the former occurs in biologically aged wines.

Fino wines submitted to biological aging present some volatile compounds characteristic of the metabolism of flor yeast, and therefore acquire a typical and distinct flavor. Some volatile compounds, such as acetaldehyde, constitute some of the most important compounds produced during biological aging. Besides their contribution to the ethereal and overripe apple notes, they are responsible for the pungent aroma of fino-sherry wines (Zea et al. 2007). On the other hand, flor yeasts increase the content of other aroma compounds such as higher alcohols, ethyl esters, lactones, and terpenes (Zea et al. 1995). Sotolon is an important lactone produced during the biological aging of fino wines, but it is also important in wines produced under oxidative aging conditions. This compound is produced from the aldolization reaction between α-ketobutyric acid (from the deamination of L-threonine by flor yeast) and acetaldehyde through a mechanism proposed by Pham et al. (1995). Because of its low perception threshold (10 μg/L), this compound has been described as an important odor impact compound, with nutty, curry, and cotton candy notes in both biologically aged sherry wines and those produced under oxidative aging conditions (Cutzach et al. 2000; Escudero and Etievant 1999; Kotseridis and Baumes 2000, among others).

10.5 Use of Enological Additives for Wine Quality Improvement: IDY Preparations

Nowadays, different enological IDY preparations based on thermally inactivated *S. cerevisiae* yeasts are commercially available. Depending on how these preparations are obtained, they can be classified as inactive yeast obtained by thermal inactivation and drying; yeast autolysates in which an incubation stage allows the enzymes to be released from the vacuoles, thus degrading part of the intracellular cell content; yeast hulls or walls (the insoluble component formed by yeast walls without cytoplasmic content); and yeast extracts (the soluble extract after the total degradation of the cytoplasmic content).

TABLE 10.3

Commercial Products Formulated with Inactive Yeast Preparations and Their Applications

Product Category[a]	Composition[b]	Action in Musts and Wines[c]
Alcoholic fermentation enhancers	Inert support elements, yeast walls, inert yeast, ammonium salts (phosphate, sulfate), thiamine, microcrystalline cellulose, silica gel	Promote synthesis of yeast membrane constituents Improve assimilation of nitrogen compounds Enhance yeast metabolism and promote yeast growth Protection and stimulation of yeast during rehydration Prevention of undesirable compound formation (H_2S) Better resistance of yeast to osmotic shock and to ethanol Increase in polyunsaturated fatty acids, vitamins, sterols of yeast: Help yeast survival at the end of fermentation Reduction of duration of alcoholic fermentation and restart stuck fermentations Lower risk of native flora development Aroma preservation and improvement of wine sensory profile Increase in colloidal wine content Adsorption of toxic compounds
Malolactic fermentation enhancers	Inactivated yeast rich in amino acids and polysaccharides, mineral cofactors, vitamins, cellulose diammonium phosphate	Complex nutrients for *O. oeni* Favors and allows faster malolactic fermentation Favors rapid growth of starter bacteria and increases their survival Detoxification Reduces the risk of malolactic fermentation by undesirable bacteria
Wine quality enhancers	Yeast cell walls, yeast autolysates rich in peptides such as glutathione, wall yeast mannoproteins, microcrystalline cellulose, inactive yeast, inactive yeast with antioxidant properties, biammonium, phosphate and sulfate salts, thiamine, silica gel, inactive yeast and pectolitic enzymes, yeast autolysate and β-glucanases	Increase sweetness and decrease bitterness of wines Favors the elimination of astringent polyphenols Tartaric stabilization Antioxidant properties (glutathione) Keep aroma freshness and color in white and rosé wines Quick release of mannoproteins to wine Avoid formation of off-flavors, acidity, etc. Promote fermentation and restart stuck fermentation More structured wines: increase volume and mouthfeel Faster release of polysaccharides and antioxidant peptides into the wine Remove toxic compounds from must and wines

Source: Pozo-Bayon, M. A. et al., *Food Res Int*, 42(7), 754–761, 2009. With permission.

[a] Commercial products.

[b] One or more of these components can be included in the composition.

[c] The applications are in agreement with the manufacturer's descriptions included in the products.

The composition of these IDY preparations varies according to the methods used to obtain it (Guilloux-Benatier and Chassagne 2003; Pozo-Bayon et al. 2009), although, in general, they consist of yeast autolysis (amino acids, peptides, proteins, polysaccharides [mannoproteins], nucleotides, and fatty acids), yeast walls, vitamins, and minerals. In general, all of these preparations have been shown to improve the organoleptic characteristics of elaborated wines or to encourage the fermentation process. In Table 10.3, some applications of commercial IDY during winemaking are summarized.

With regard to their effects on fermentation, some IDY preparations are designed as protectors of active yeast during their hydration to avoid membrane damage (Dulau et al. 2002). Moreover, IDYs have also been shown to be enhancers of alcoholic and malolactic fermentations. Because yeast and lactic bacteria need assimilable nitrogen sources (free amino acids and ammonia) for their development, yeast manufacturers have recommended the use of fermentation enhancers based on yeast components such as nutrients. The effects of these enhancers of alcoholic fermentation have been evaluated, concluding that their effects are due to the release of nitrogen compounds and to the detoxification effect that yeast walls can have on some toxic compounds (ethanol and medium chain fatty acids; Lafon-Lafourcade et al. 1984; Feuillat et al. 1996). This detoxification effect has also been observed in the case of malolactic fermentation (Lonvaud-Funel et al. 1985; Edwards and Beelman 1987). The stimulation of malolactic fermentation depends on the compounds provided by the IDY (Remize et al. 2006).

Probably the most interesting application of commercial IDY preparations is related to its use for improving the sensory quality of wines. In this context, the releasing of glutathione by some of these IDY preparations and its antioxidant capacity have focused on studies protecting the color of white wines and avoiding the oxidation of aroma compounds (Roland et al. 2010; Papadopoulou et al. 2008; Vaimakis et al. 1996; Roussis et al. 2007, 2008). Moreover, the polysaccharides and mannoproteins released by IDYs could interact with tannins and anthocyanins, preventing precipitation and thus leading to an increase in color stability (Doco et al. 2003; Escot et al. 2001; Feuillat 2001; Francois et al. 2007). This effect of the interaction with mannoproteins may be related to the decrease in astringency produced by proanthocyanidins (Vidal et al. 2004). On the other hand, mannoproteins could also interact with aroma compounds, decreasing their effective concentration and reducing their sensorial effect (Langourieux and Crouzet 1997; Chalier et al. 2007). This property could be used to remove off-flavors from wine (Chassagne et al. 2005; Pradelles et al. 2008).

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REFERENCES

Alcaide-Hidalgo JM, Martinez-Rodriguez AJ, Martin-Alvarez PJ, Pueyo E. 2008. Influence of the elaboration process on the peptide fraction with angiotensin I-converting enzyme inhibitor activity in sparkling wines and red wines aged on lees. *Food Chem* 111(4):965–9.

Alcaide-Hidalgo JM, Pueyo E, Polo MC, Martinez-Rodriguez AJ. 2007. Bioactive peptides released from *Saccharomyces cerevisiae* under accelerated autolysis in a wine model system. *J Food Sci* 72(7):M276–9.

Alexandre H, Guilloux-Benatier M. 2006. Yeast autolysis in sparkling wine—a review. *Aus J Grape Wine Res* 12(2):119–27.

Ancin-Azpilicueta C, Gonzalez-Marco A, Jimenez-Moreno N. 2008. Current knowledge about the presence of amines in wine. *Crit Rev Food Sci Nutr* 48(3):257–75.

Andrés-Lacueva C, Gallart M, Lopez-Tamames E, Lamuela-Raventos RM. 1996. Influence of variety and aging on foaming properties of sparkling wine (Cava): 1. *J Agric Food Chem* 44(12):3826–9.

Andrés-Lacueva C, Lamuela-Raventos RM, Buxaderas S, delaTorreBoronat MDC. 1997. Influence of variety and aging on foaming properties of cava (sparkling wine): 2. *J Agric Food Chem* 45(7):2520–5.

Attfield PV. 1997. Stress tolerance: the key to effective strains of industrial baker's yeast. *Nat Biotechnol* 15(13):1351–7.

Aussenac J, Chassagne D, Claparols C, Charpentier M, Duteurtre B, Feuillat M, Charpentier C. 2001. Purification method for the isolation of monophosphate nucleotides from champagne wine and their identification by mass spectrometry. *J Chromatogr A* 907(1–2):155–64.

Barnett JA, Entian KD. 2005. A history of research on yeasts—9: regulation of sugar metabolism. *Yeast* 22(11):835–94.

Bauer FF, Pretorius IS. 2000. Yeast stress response and fermentation efficiency: how to survive the making of wine—a review. *S Afr J Enol Vitic* 21:27–51.

Beltran G, Torija MJ, Novo M, Ferrer N, Poblet M, Guillamon JM, Rozes N, Mas A. 2002. Analysis of yeast populations during alcoholic fermentation: a six year follow-up study. *Syst Appl Microbiol* 25(2):287–93.

Beneduce L, Spano G, Vernile A, Tarantino D, Massa S. 2004. Molecular characterization of lactic acid populations associated with wine spoilage. *J Basic Microbiol* 44:10–6.

Benítez T, Codón AC. 2005. Levaduras. *Saccharomyces cerevisiae* III. Levaduras de vinos de crianza biológica. In: Carrascosa AV, Muñoz R, González R, editors. *Microbiología del vino*. Madrid, Spain: A.M.V. Ediciones. p. 78–113.

Bidan P, Feuillat M, Moulin JP. 1986. Les vins mousseux. Rapport de la France. 65eme Asemblee Generale de L'OIV. *Bull OIV* 59:563–626.

Bisson LF. 2004. Biotechnology of wine yeast. *Food Biotechnol* 18(1):63–96.

Bisson LF, Butzke CE. 2000. Diagnosis and rectification of stuck and sluggish fermentations. *Am J Enol Vitic* 51(2):168–77.

Bosch-Fuste J, Riu-Aumatell M, Guadayol JM, Calxach J, Lopez-Tamamaes E, Buxaderas S. 2007. Volatile profiles of sparkling wines obtained by three extraction methods and gas chromatography mass spectrometry (GC-MS) analysis. *Food Chem* 105(1):428–35.

Boulton RB, Singleton VL, Bisson LF, Kunkee RE. 1996. Yeast and biochemistry of ethanol fermentation. In: RB Boulton, editor. *Principles and practices of winemaking*. New York: Springer. p. 139–72.

Bover-Cid S, Holzapfel WH. 1999. Improved screening procedure for biogenic amine production by lactic acid bacteria. *Int J Food Microbiol* 53(1):33–41.

Budroni M, Zara S, Zara G, Pirino G, Mannazzu I. 2005. Peculiarities of flor strains adapted to Sardinian sherry-like wine ageing conditions. *Fems Yeast Res* 5(10):951–8.

Câmara JS. 2004. Caracterização Aromatiza de Castas Productoras De Vinho Madeira. [Doctoral thesis]. Universidade da Madeira.

Campo E, Ferreira V, Escudero A, Marqués JC, Cacho J. 2006. Quantitative gas chromatography-olfactometry and chemical quantitative study of the aroma of four Madeira wines. *Anal Chim Acta* 563:180–7.

Casalone E, Colella CM, Daly S, Gallori E, Moriani L, Polsinelli M. 1992. Mechanism of resistance to sulfite in *Saccharomyces cerevisiae*. *Curr Genet* 22:435–40.

Cavin JF, Andioc V, Etievant PX, Divies C. 1993. Ability of wine lactic-acid bacteria to metabolize phenol carboxylic-acids. *Am J Enol Vitic* 44(1):76–80.

Chalier P, Angot B, Delteil D, Doco T, Gunata Z. 2007. Interactions between aroma compounds and whole mannoprotein isolated from *Saccharomyces cerevisiae* strains. *Food Chem* 100(1):22–30.

Charpentier C, Etiévant P, Guichard E. 2000. Vinificación de los vinos de velo: vino Jaune, Jerez y otros. In: Flanzy C, editor. *Enología: fundamentos científicos y tecnológicos*. Madrid, Spain: A. Madrid Vicente, Ediciones, and Ediciones Mundi-Prensa. p. 531–39.

Charpentier C, Feuillat M. 1993. Yeast autolysis. In: Fleet GH, editor. *Wine microbiology and biotechnology*. Switzerland: Harwood Academic Publishers. p. 225–42.

Charpentier C, Aussenac J, Charpentier M, Prome JC, Duteurtre B, Feuillat M. 2005. Release of nucleotides and nucleosides during yeast autolysis: kinetics and potential impact on flavor. *J Agric Food Chem* 53(8):3000–7.

Chassagne D, Guilloux-Benatier M, Alexandre H, Voilley A. 2005. Sorption of wine volatile phenols by yeast lees. *Food Chem* 91(1):39–44.

Chatonnet P, Dubourdieu D, Boidron JN, Lavigne V. 1993. Synthesis of volatile phenols by saccharomyces-cerevisiae in wines. *J Sci Food Agric* 62(2):191–202.

Cordonnier CL. 1979. Les composants variétales et prefermentaires de l'arôme des vins. *Rev Fr Oenol* 16:79–90.

Costantini A, Cersosimo M, Del Prete V, Garcia-Moruno E. 2006. Production of biogenic amines by lactic acid bacteria: screening by PCR, thin-layer chromatography, and high-performance liquid chromatography of strains isolated from wine and must. *J Food Protect* 69(2):391–6.

Costantini A, Vaudano E, Del Prete V, Danei M, Garcia-Moruno E. 2009. Biogenic amine production by contaminating bacteria found in starter preparations used in winemaking. *J Agric Food Chem* 57(22):10664–9.

Costello PJ, Morrison GJ, Lee TH, Fleet GH. 1983. Numbers and species of lactic acid bacteria in wines during vinification. *Food Technol Aust* 35:14–8.

Culleré L, Escudero A, Cacho J, Ferreira V. 2004. Gas chromatography-olfactometry and chemical quantitative study of the aroma of six premium quality Spanish aged red wines. *J Agric Food Chem* 52(6):1653–60.

Cutzach I, Chatonnet P, Dubourdieu D. 2000. Influence of storage conditions on the formation of some volatile compounds in white fortified wines (vins doux naturels) during the aging process. *J Agric Food Chem* 48(6):2340–5.

Dambrouck T, Marchal R, Cilindre C, Parmentier M, Jeandet P. 2005. Determination of the grape invertase content (using PTA-ELISA) following various fining treatments versus changes in the total protein content of wine. Relationships with wine foamability. *J Agric Food Chem* 53(22):8782–9.

Davis CR, Wibowo DJ, Lee TH, Fleet GH. 1986. Growth and metabolism of lactic-acid bacteria during and after malolactic fermentation of wines at different pH. *Appl Environ Microbiol* 51(3):539–45.

Darriet P, Tominaga T, Lavigne V, Boidron J, Dubourdieu D. 1995. Identification of a powerful aromatic compound of *Vitis vinifera* L. var. sauvignon wines: 4-mercapto-4-methylpentan-2-one. *Flavor Fragr J* 10:385–92.

Dicks LMT, Dellaglio F, Collins MD. 1995. Proposal to reclassify *Leuconostoc oenos* as *Oenococcus oeni* [corrig.] gen.nov, comb. nov. *Int J Syst Bacteriol* 45:395–7.

Doco T, Vuchot P, Cheynier V, Moutounet M. 2003. Structural modification of wine arabinogalactans during aging on lees. *Am J Enol Vitic* 54(3):150–7.

Dols-Lafargue M, Gindreau E, Le Marrec C, Chambat G, Heyraud A, Lonvaud-Funel A. 2007. Changes in red wine soluble polysaccharide composition induced by malolactic fermentation. *J Agric Food Chem* 55(23):9592–9.

Dubois P. 1994. Les aromes des vins et leurs defauts. *Rev Fr Oenol* 145:27–40.

Dubourdieu D, Torninaga T, Masneuf I, Des Gachons CP, Murat ML. 2006. The role of yeasts in grape flavor development during fermentation: the example of sauvignon blanc. *Am J Enol Vitic* 57(1):81–8.

Dufour JP. 1989. Effect of wort amino acids on beer quality. *Louvain Brewing Lett* 2:11–9.

Dulau LA, Ortiz-Julien A, Trioli G. 2002. Method for dry yeast rehydration and rehydration medium. International patent PCT/FR02/01949.

Dupin IVS, McKinnon BM, Ryan C, Boulay M, Markides AJ, Jones GP, Williams PJ, Waters EJ. 2000. *Saccharomyces cerevisiae* mannoproteins that protect wine from protein haze: their release during fermentation and lees contact and a proposal for their mechanism of action. *J Agric Food Chem* 48(8):3098–105.

Du Plessis EM, Dicks LMT, Pretorius IS, Lambrechts MG, du Toit M. 2004. Identification of lactic acid bacteria isolated from South African brandy base wines. *Int J Food Microbiol* 91:19–29.

Edwards CG, Beelman RB. 1987. Inhibition of the malolactic bacterium, leuconostoc-oenos (psu-1), by decanoic acid and subsequent removal of the inhibition by yeast ghosts. *Am J Enol Vitic* 38(3):239–42.

Edwards CG, Jensen KA. 1992. Occurrence and characterization of lactic acid bacteria from Washington state wine: *Pediococcus* spp. *Am J Enol Vitic* 43:233–8.

Edwards CG, Collins MD, Lawson PA, Rodriguez AV. 2000. *Lactobacillus nagelii* sp. nov., an organism isolated from a partially fermented wine. *Int J Syst Evol Microbiol* 50:699–702.

Escot S, Feuillat M, Dulau L, Charpentier C. 2001. Release of polysaccharides by yeasts and the influence of released polysaccharides on colour stability and wine astringency. *Aus J Grape Wine Res* 7(3):153–9.

Escudero A, Etievant P. 1999. Effect of antioxidants on the flavor characteristics and the gas chromatography/olfactometry profiles of champagne extracts. *J Agric Food Chem* 47(8):3303–8.

Esteve-Zarzoso B, Peris-Toran MJ, Garcia-Maiquez E, Uruburu F, Querol A. 2001. Yeast population dynamics during the fermentation and biological aging of sherry wines. *Appl Environ Microbiol* 67(5):2056–61.

Esteve-Zarzoso B, Fernandez-Espinar MT, Querol A. 2004. Authentication and identification of *Saccharomyces cerevisiae* 'flor' yeast races involved in sherry ageing. *Antonie van Leeuwenhoek* 85(2):151–8.

Etiévant PX. 1991. Wine. In: Maarse H, editor. *Volatile compounds in foods and beverages*. New York: Marcel Dekker. p. 483–546.

Ferreira V, Ortin N, Escudero A, Lopez R, Cacho J. 2002. Chemical characterization of the aroma of Grenache rose wines: aroma extract dilution analysis, quantitative determination, and sensory reconstitution studies. *J Agric Food Chem* 50(814):4048–54.

Feuillat M. 2001. Nouveaux adjuvants oenologiques possibles d'origine levurienne. *Bull OIV* 849–50:753–71.

Feuillat M. 2003. Yeast macromolecules: origin, composition, and enological interest. *Am J Enol Vitic* 54(3):211–3.

Feuillat M, Charpentier C. 1982. Autolysis of yeasts in champagne. *Am J Enol Vitic* 33(1):6–13.

Feuillat M, Guerreau J. 1996. Les nouveaux activateurs de la fermentation alcoolique. *Bull OIV* 789–90:987–98.

Fia G, Giovani G, Rosi I. 2005. Study of β-glucosidase production by wine-related yeasts during alcoholic fermentation. A new rapid fluorimetric method to determine enzymatic activity. *J Appl Microbiol* 99(3):509–17.

Fleet GH. 1992. Spoilage yeasts. *Crit Rev Biotechnol* 12:1–44.

Fleet GH. 1993. The microorganisms of winemaking—isolation, enumeration and identification. In: Fleet GH, editor. *Wine microbiology and biotechnology*. Switzerland: Harwood Academic Publishers. p. 1–25.

Fleet HG. 1998. The microbiology of alcoholic beverages. In: Wood BJB, editor. *Microbiology of fermented foods*. Vol. 1. London: Blackie Academic and Professional. p. 217–72.

Fleet GH. 2008. Wine yeasts for the future. *FEMS Yeast Res* 8(7):979–95.

Fleet GH, Heard GM. 1993. Yeast-growth during fermentation. In: Fleet GH, editor. *Wine microbiology and biotechnology*. Switzerland: Harwood Academic Publishers. p. 27–54.

Fornachon JCM. 1957. The occurrence of malo-lactic fermentation in Australian wines. *Aust J Appl Sci* 8:120–9.

Francois JM, Alexandre H, Granes D, Feuillat M. 2007. Vers une meilleure connaissance des produits dérivés de levures. *Rev Enologues* 122:9–12.

Fugelsang KL. 1997. *Wine microbiology*. London: Chapman and Hall. p. 117–42.

Gallart M, Lopez-Tamames E, Suberbiola G, Buxaderas S. 2002. Influence of fatty acids on wine foaming. *J Agric Food Chem* 50(24):7042–5.

Gallart M, Tomas X, Suberbiola G, Lopez-Tamames E, Buxaderas S. 2004. Relationship between foam parameters obtained by the gas-sparging method and sensory evaluation of sparkling wines. *J Sci Food Agric* 84(2):127–33.

Garvie EI. 1986. Genus Pediococcus Claussen 1903, 68AL. In: Sneath PHA, Mair NS, Sharpe ME, Holt JG, editors. *Bergey's manual of systematic bacteriology*. Vol. 2. Baltimore: Williams and Wilkins. p. 1075–9.

Gindreau E, Wailling E, Lonvaud-Funel A. 2001. Direct polymerase chain reaction detection of ropy *Pediococcus damnosus* strains in wine. *J Appl Microbiol* 90:535–42

Grbin PR, Henschke PA. 2000. Mousy off-flavor production in grape juice and wine by *Dekkera* and *Brettanomyces* yeasts. *Aus J Grape Wine Res* 6:255–62.

Guilloux-Benatier M, Chassagne D. 2003. Comparison of components released by fermented or active dried yeasts after aging on lees in a model wine. *J Agric Food Chem* 51(3):746–51.

Henick-Kling T. 1993. Malolactic fermentation. In: Fleet GH, editor. *Wine microbiology and biotechnology*. Berlin: Springer–Verlag. p. 286–326.

Henschke PA, Jiranek V. 1991. Hydrogen sulfide formation during fermentation: effect of nitrogen composition in model grape must. Proceedings of the International Symposium on Nitrogen in Grapes and Wine. Davis, CA, USA: American Society for Enology and Viticulture, p. 172–84.

Henschke PA, Jiranek V. 1993. Yeast—metabolism of nitrogen compounds. In: Fleet GH, editor. *Wine microbiology and biotechnology*. Switzerland: Harwood Academic Publishers. p. 77–164.

Hernandez T, Estrella I, Carlavilla D, Martin-Alvarez PJ, Moreno-Arribas MV. 2006. Phenolic compounds in red wine subjected to industrial malolactic fermentation and ageing on lees. *Anal Chim Acta* 563(1–2):116–25.

Hernandez T, Estrella I, Perez-Gordo M, Alegria EG, Tenorio C, Ruiz-Larrrea F, Moreno-Arribas MV. 2007. Contribution of malolactic fermentation by *Oenococcus oeni* and *Lactobacillus plantarum* to the changes in the nonanthocyanin polyphenolic composition of red wine. *J Agric Food Chem* 55(13):5260–6.

Horecker BL. 2002. The pentose phosphate pathway. *J Biol Chem* 277(50):47965–71.

Jiranek V, Langridge P, Henschke PA. 1995. Regulation of hydrogen-sulfide liberation in wine-producing *Saccharomyces-cerevisiae* strains by assimilable nitrogen. *Appl Environ Microbiol* 61(2):461–7.

Killian E, Ough CS. 1979. Fermentation esters—formation and retention as affected by fermentation temperature. *Am J Enol Vitic* 30(4):301–5.

Kotseridis Y, Baumes R. 2000. Identification of impact odorants in Bordeaux red grape juice, in the commercial yeast used for its fermentation, and in the produced wine. *J Agric Food Chem* 48(2):400–6.

Kresge N, Simoni RD, Hill RL. 2005. JBC Centennial—1905–2005—100 years of biochemistry and molecular biology—Otto Fritz Meyerhof and the elucidation of the glycolytic pathway. *J Biol Chem* 280(4):124–6.

Lafon-Lafourcade S, Carre E, Ribereau-Gayon P. 1983. Occurrence of lactic acid bacteria during the different stages of vinification and conservation of wines. *Appl Environ Microbiol* 46:874–80.

Lafon-Lafourcade S, Geneix C, Ribéreau-Gayon, P. 1984. Ways of initiating the action of yeast ghosts during winemaking. *Conn Vigne Vin* 18:111–25.

Lambrechts MG, Pretorius S. 2000. Yeast and its importance to wine aroma—a review. *S Afr J Enol Vitic* 21:97–128.

Langourieux S, Crouzet JC. 1997. Study of interactions between aroma compounds and glycopeptides by a model system. *J Agric Food Chem* 45(5):1873–7.

Laurent MH, Henick-Kling T, Acree TE. 1994. Changes in the aroma and odor of Chardonnay wine due to malolactic fermentation. *Wein-Wiss* 49:3–10.

Liu SQ, Pritchard GG, Hardman MJ, Pilone GJ. 1994. Citrulline production and ethyl carbamate (urethane) precursor formation from arginine degradation by wine lactic-acid bacteria leuconostoc-oenos and lactobacillus-buchneri. *Am J Enol Vitic* 45(2):235–42.

Lonvaud-Funel A. 1999. Lactic acid bacteria in the quality improvement and depreciation of wine. *Antonie van Leeuwenhoe*k 76:317–31.

Lonvaud-Funel A, Strasser de Saad AM. 1982. Purification and properties of a malolactic enzyme from a strain of *Leuconostoc mesenteroides* isolated from grapes. *Appl Environ Microbiol* 43:357–61.

Lonvaud-Funel A, Desens C, Joyeux A. 1985. Stimulation de la fermentation malolactique par l'addition au vin d'enveloppes cellulaires de levure et différents adjuvants de nature polysaccharidique et azotée. *Conn Vigne Vin* 9:229–40.

Loyaux D, Roger S, Adda J. 1981. The evolution of champagne volatiles during aging. *J Sci Food Agric* 32(12):1254–8.

Lubbers S, Charpentier C, Feuillat M, Voilley A. 1994a. Influence of yeast walls on the behavior of aroma compounds in a model wine. *Am J Enol Vitic* 45(1):29–33.

Lubbers S, Voilley A, Feuillat M, Charpentier C. 1994b. Influence of mannoproteins from yeast on the aroma intensity of a model wine. *Lebensm Wiss Technol* 7(2):108–14.

Maicas S, Mateo JJ. 2005. Hydrolysis of terpenyl glycosides in grape juice and other fruit juices: a review. *Appl Microbiol Biotechnol* 67(3):322–35.

Maicas S, Gil JV, Pardo I, Ferrer S. 1999. Improvement of volatile composition of wines by controlled addition of malolactic bacteria. *Food Res Int* 32(7):491–6.

Marcobal A, Martin-Alvarez PJ, Polo MC, Munoz R, Moreno-Arribas MV. 2006. Formation of biogenic amines throughout the industrial manufacture of red wine. *J Food Protect* 69(2):397–404.

Martineau B, HenickKling T. 1995. Formation and degradation of diacetyl in wine during alcoholic fermentation with *Saccharomyces cerevisiae* strain EC11 and malolactic fermentation with *Leuconostoc oenos* strain MCW. *Am J Enol Vitic* 46(4):442–8.

Martínez-Rodríguez A, Pueyo E. 2009. Sparkling wines and yeast autolysis. In: Moreno-Arribas MV, Polo C, editors. *Wine chemistry and biochemistry*. New York: Springer. p. 61–80.

Martínez P, Codon AC, Perez L, Benitez T. 1995. Physiological and molecular characterization of flor yeasts—polymorphism of flor yeast populations. *Yeast* 11(14):1399–411.

Martínez P, Pérez Rodríguez L, Benítez T. 1997. Evolution of flor yeast population during the biological aging of fino sherry wine. *Am J Enol Vitic* 48:160–8.

Martínez-Murcia AJ, Harland NM, Collins MD. 1993. Phylogenetic analysis of some leuconostocs and related organism as determined from the large-subunit rRNA gene sequences: assessment of congruence of small- and large-subunit rRNA derived trees. *J Appl Microbiol* 12:48–55.

Matthews A, Grbin PR, Jiranek V. 2006. A survey of lactic acid bacteria for enzymes of interest to oenology. *Aus J Grape Wine Res* 12 (3):235–44.

Mauricio JC, Ortega JM. 1997. Nitrogen compounds in wine during its biological aging by two flor film yeasts: an approach to accelerated biological aging of dry sherry-type wines. *Biotechnol Bioeng* 53(2):159–67.

Mestres M, Busto O, Guasch J. 2000. Analysis of organic sulfur compounds in wine aroma. *J Chromatogr A* 881(1–2):569–81.

Mira de Orduna R, Patchett ML, Liu SQ, Pilone GJ. 2001. Growth and arginine metabolism of the wine lactic acid bacteria *Lactobacillus buchneri* and *Oenococcus oeni* at different pH values and arginine concentrations. *Appl Environ Microbiol* 67(4):1657–62.

Moreno-Arribas V, Lonvaud-Funel A. 2001. Purification and characterization of tyrosine decarboxylase of *Lactobacillus brevis* IOEB 9809 isolated from wine. *Fems Microbiol Lett* 195(1):103–7.

Moreno-Arribas MV, Polo C. 2008. Occurrence of lactic acid bacteria and biogenic amines in biologically aged wines. *Food Microbiol* 25:875–81.

Moreno-Arribas MV, Polo C, editors. 2009. *Wine chemistry and biochemistry*. New York: Springer.

Moreno-Arribas V, Pueyo E, Polo MC. 1996. Peptides in musts and wines. Changes during the manufacture of cavas (Sparkling wines). *J Agric Food Chem* 44(12):3783–8.

Moreno-Arribas V, Pueyo E, Polo MC, Martin-Alvarez PJ. 1998. Changes in the amino acid composition of the different nitrogenous fractions during the aging of wine with yeasts. *J Agric Food Chem* 46(10):4042–51.

Moreno-Arribas V, Torlois S, Joyeux A, Bertrand A, Lonvaud-Funel A. 2000. Isolation, properties and behaviour of tyramine-producing lactic acid bacteria from wine. *J Appl Microbiol* 88(4):584–93.

Moreno-Arribas MV, Polo MC, Jorganes F, Munoz R. Screening of biogenic amine production by lactic acid bacteria isolated from grape must and wine. *Int J Food Microbiol* 84(1):117–23.

Moreno-Arribas MV, Gomez-Cordoves C, Martin-Alvarez PJ. 2008. Evolution of red wine anthocyanins during malolactic fermentation, postfermentative treatments and ageing with. *Food Chem* 109(1): 149–58.

Moreno-Arribas MV, Smit AY, Du Toit M. 2010. Biogenic amines in the winemaking process. In: Reynols AG, editor. *Managing wine quality*. Cambridge, UK: Woodhead Publishing. p. 494–522.

Naouri P, Chagnaud P, Arnaud A, Galzy P. 1990. Purification and properties of a malolactic enzyme from *Leuconostoc oenos* ATCC-23278. *J Basic Microbiol* 30(8):577–85.

Nielsen JC, Richelieu M. 1999. Control of flavor development in wine during and after malolactic fermentation by *Oenococcus oeni*. *Appl Environ Microbiol* 65(2):740–5.

Nunez YP, Carrascosa AV, Gonzalez R, Polo MC, Martinez-Rodriguez AJ. 2005. Effect of accelerated autolysis of yeast on the composition and foaming properties of sparkling wines elaborated by a champenoise method. *J Agric Food Chem* 53(18):7232–7.

Nunez YP, Carrascosa AV, Gonzalez R, Polo MC, Martinez-Rodriguez A. 2006. Isolation and characterization of a thermally extracted yeast cell wall fraction potentially useful for improving the foaming properties of sparkling wines. *J Agric Food Chem* 54(20):7898–903.

Oeno-Micro-09-409. Herramientas moleculares para la identificación de bacterias lácticas en la uva y el vino. Paris, France: International Organization of Wine and Vine.

Osborne JP, Mira de Orduna R, Pilone GJ, Liu SQ. 2000. Acetaldehyde metabolism by wine lactic acid bacteria. *Fems Microbiol Lett* 191(1):51–5.

Papadopoulou D, Roussis IG. 2008. Inhibition of the decrease of volatile esters and terpenes during storage of a white wine and a model wine medium by glutathione and N-acetylcysteine. *Int J Food Sci Technol* 43(6):1053–7.

Peinado R, Mauricio J. 2009. Biologically aged wines. In: Moreno-Aribas MV, Polo C, editors. *Wine chemistry and biochemistry*. New York: Springer. p. 81–102.

Pizarro F, Vargas FA, Agosin E. 2007. A systems biology perspective of wine fermentations. *Yeast* 24(11):977–91.

Pham TT, Guichard E, Schlich P, Charpentier C. 1995. Optimal conditions for the formation of sotolon from ketobutyric acid in the french 'Vin Jaune.' *J Agric Food Chem* 43:2616–9.

Pozo-Bayon MA, Pueyo E, Martin-Alvarez PJ, Martinez-Rodriguez AJ, Polo MC. 2003. Influence of yeast strain, bentonite addition, and aging time on volatile compounds of sparkling wines. *Am J Enol Vitic* 54(4):273–8.

Pozo-Bayon MA, Alegria EG, Polo MC, Tenorio C, Martin-Alvarez PJ, De La Banda MTC, Ruiz-Larrea F, Moreno-Arribas MV. 2005. Wine volatile and amino acid composition after malolactic fermentation: effect of *Oenococcus oeni* and *Lactobacillus plantarum* starter cultures. *J Agric Food Chem* 53(22):8729–35.

Pozo-Bayon MA, Martinez-Rodriguez A, Pueyo E, Moreno-Arribas MV. 2009a. Chemical and biochemical features involved in sparkling wine production: from a traditional to an improved winemaking technology. *Trends Food Sci Technol* 20(6–7):289–99.

Pozo-Bayon MA, Andujar-Ortiz I, Moreno-Arribas MV. 2009b. Scientific evidences beyond the application of inactive dry yeast preparations in winemaking. *Food Res Int* 42(7):754–61.

Pradelles R, Alexandre H, Ortiz-Julien A, Chassagne D. 2008. Effects of yeast cell-wall characteristics on 4-ethylphenol sorption capacity in model wine. *J Agric Food Chem* 56(24):11854–61.

Pretorius IS. 2000. Tailoring wine yeast for the new millennium: novel approaches to the ancient art of winemaking. *Yeast* 16(8):675–729.

Pueyo E, MartinAlvarez PJ, Polo MC. 1995. Relationship between foam characteristics and chemical composition in wines and cavas (sparkling wines). *Am J Enol Vitic* 46(4):518–24.

Pueyo E, Martinez-Rodriguez A, Polo MC, Santa-Maria G, Bartolome B. 2000. Release of lipids during yeast autolysis in a model wine system. *J Agric Food Chem* 48(1):116–22.

Rapp A, Versini G. 1991. Influence of nitrogen compounds in grapes on aroma compounds of wines. Proceedings of the International Symposium on Nitrogen in Grapes and Wine. Davis, CA, USA: American Society for Enology and Viticulture. p. 156–64.

Rapp A, Versini G. 1996. Influence of nitrogen on compounds in grapes on aroma compounds in wines. *J Int Sci Vigne Vin* 51:193–203.

Rauhut D. 1993. Yeasts—Production of sulfur compounds. In: Fleet GH, editor. *Wine microbiology and biotechnology*. Chur, Switzerland: Harwood Academic Publishers. p. 183–223.

Remize F, Gaudin A, Kong Y, Guzzo J, Alexandre H, Krieger S, Guilloux-Benatier M. 2006. *Oenococcus oeni* preference for peptides: qualitative and quantitative analysis of nitrogen assimilation. *Arch Microbiol* 185(6):459–69.

Renouf V, Claisse O, Lonvaud-Funel A. 2005. Numeration, identification and understanding of microbial biofilm on grape berry surface. *Aus J Grape Wine Res* 11:316–27.

Reynolds A, editor. 2010. *Managing in wine quality*. Cambridge, UK: Woodhead Publishing.

Ribereau-Gayon P, Glories Y, Maujean A, Dubourdieu D. 2000. Chemical nature, origins and consequences of the main organoleptic defects. In: Ribereau-Gayon P, editor. *Handbook of enology*. Chichester, UK: Wiley, Vol. 2. p. 209–53.

Riu-Aumatell M, Bosch-Fuste J, Lopez-Tamames E, Buxaderas S. 2006. Development of volatile compounds of cava (Spanish sparkling wine) during long ageing time in contact with lees. *Food Chem* 95(2):237–42.

Rodriguez-Bencomo JJ, Ortega-Heras M, Perez-Magarino S. 2010. Effect of alternative techniques to ageing on lees and use of non-toasted oak chips in alcoholic fermentation on the aromatic composition of red wine. *Eur Food Res Technol* 230(3):485–96.

Roland A, Vialaret J, Razungles A, Rigou P, Schneider R. 2010. Evolution of S-cysteinylated and S-glutathionylated thiol precursors during oxidation of Melon B. and sauvignon blanc musts. *J Agric Food Chem* 58(7):4406–13.

Romano P, Fiore C, Paraggio M, Caruso M, Capece A. 2003. Function of yeast species and strains in wine flavor. *Int J Food Microbiol* 86(1–2):169–80.

Roussis IG, Lambropoulos I, Tzimas P. 2007. Protection of volatiles in a wine with low sulfur dioxide by caffeic acid or glutathione. *Am J Enol Vitic* 58(2):274–8.

Roussis IG, Sergianitis S. 2008. Protection of some aroma volatiles in a model wine medium by sulphur dioxide and mixtures of glutathione with caffeic acid or gallic acid. *Flavor Fragr J* 23(1):35–9.

Salmon JM, Barre P. 1998. Improvement of nitrogen assimilation and fermentation kinetics under enological conditions by derepression of alternative nitrogen-assimilatory pathways in an industrial *Saccharomyces cerevisiae* strain. *Appl Environ Microbiol* 64(10):3831–7.

Samuel D. 1996. Investigation of ancient Egyptian baking and brewing methods by correlative microscopy. *Science* 273(5274):488–90.

Sauvageot F, Vivier P. 1997. Effects of malolactic fermentation on sensory properties of four Burgundy wines. *Am J Enol Vitic* 48(2):187–92.

Schulthess D, Ettlinger L. 1978. Influence of concentration of branched-chain amino-acids on formation of fusel alcohols. *J Instit Brew* 84(4):240–3.

Shinohara T. 1985. Gas-chromatographic analysis of volatile fatty-acids in wines. *Agric Biol Chem* 49(7):2211–2.

Shinohara T. 1986. Factors affecting the formation of volatile fatty-acids during grape must fermentation. *Agric Biol Chem* 50(12):3197–9.

Sieiro C, Cansado J, Agrelo D, Velázquez JB, Villa TG. 1990. Isolation and enological characterization of malolactic bacteria from the vineyards of North-western Spain. *Appl Environ Microbiol* 56:2936–8.

Smit AY, du Toit WJ, du Toit M. 2008. Biogenic amines in wine: understanding the headache. *S Afr J Enol Vitic* 29(2):109–27.

Soufleros E, Bertrand A. 1980. Incidence de l'action conjuguée de la température de fermentation et de l'acidité du mileieu sur les sur les teneurs en substances volatiles formés par les levures. *Conn Vigne Vin* 14:97–109.

Spiropoulos A, Tanaka J, Flerianos I, Bisson LF. 2000. Characterization of hydrogen sulfide formation in commercial and natural wine isolates of *Saccharomyces*. *Am J Enol Vitic* 51(3):233–48.

Straub BW, Kicherer M, Schilcher SM, Hammes WP. 1995. The formation of biogenic-amines by fermentation organisms. *Z Lebensm Unters Forsch* 201(1):79–82.

Suárez-Lepe JA, Iñigo-Leal B. 2004. Microbiología enológica. *Fundamentos de vinificación*. Madrid: Ediciones Mundi-Prensa. p. 673–716.

Swiegers JH, Pretorius IS. 2007. Modulation of volatile sulfur compounds by wine yeast. *Appl Microbiol Biotechnol* 74(5):954–60.

Swiegers JH, Bartowsky EJ, Henschke PA, Pretorius IS. 2005. Yeast and bacterial modulation of wine aroma and flavor. *Aus J Grape Wine Res* 11(2):139–73.

Tominaga T, Darriet P, Dubourdieu D. 1996. Identification of 3-mercaptohexyl acetate in sauvignon wine, a powerful aromatic compound exhibiting box-tree odor. *Vitis* 35(4):207–10.

Tominaga T, des Gachons CP, Dubourdieu D. 1998a. A new type of flavor precursors in *Vitis vinifera* L cv sauvignon blanc: S-cysteine conjugates. *J Agric Food Chem* 46(12):5215–9.

Tominaga T, Furrer A, Henry R, Dubourdieu D. 1998b. Identification of new volatile thiols in the aroma of *Vitis vinifera* L. var. sauvignon blanc wines. *Flavor Fragr J* 13(3):159–62.

Ugliano M, Henschke PA. 2009. Yeast and wine flavor. In: Moreno-Arribas MV, Polo C, editors. *Wine chemistry and biochemistry*. New York: Springer. p. 313–92.

Vaimakis V, Roussis IG. 1996. Must oxygenation together with glutathione addition in the oxidation of white wine. *Food Chem* 57(3):419–22.

Valero E, Millan C, Ortega JM, Mauricio JC. 2003. Concentration of amino acids in wine after the end of fermentation by *Saccharomyces cerevisiae* strains. *J Sci Food Agric* 83(8):830–5.

Vanrell G, Canals R, Esteruelas M, Fort F, Canals JM, Zamora F. 2007. Influence of the use of bentonite as a riddling agent on foam quality and protein fraction of sparkling wines (Cava). *Food Chem* 104(1):148–55.

Versari A, Parpinello GP, Cattaneo M. 1999. *Leuconostoc oenos* and malolactic fermentation in wine: a review. *J Ind Microbiol Biotechnol* 23(6):447–55.

Vidal S, Francis L, Williams P, Kwiatkowski M, Gawel R, Cheynier W, Waters E. 2004. The mouth-feel properties of polysaccharides and anthocyanins in a wine-like medium. *Food Chem* 85(4):519–25.

Villena MA, Iranzo JFU, Perez AIB. 2007. β-Glucosidase activity in wine yeasts: application in enology. *Enzyme Microb Technol* 40(3):420–5.

Wurz REM, Kepner RE, Webb AD. 1988. The biosynthesis of certain gamma-lactones from glutamic-acid by film yeast activity on the surface of flor sherry. *Am J Enol Vitic* 39(3):234–8.

Zamora F. 2009. Biochemistry of alcohlic fermentation. In: Moreno-Arribas MV, Polo C, editors. *Wine chemistry and biochemistry*. New York: Springer. p. 3–26.

Zea L, Moreno J, Ortega JM, Mauricio JC, Medina M. 1995. Comparative study of the gamma-butyrolactone and pantolactone contents in cells and musts during vinification by three *Saccharomyces cerevisiae* races. *Biotechnol Lett* 17(12):1351–6.

Zea L, Moyano L, Moreno JA, Medina M. 2007. Aroma series as fingerprints for biological ageing in fino sherry-type wines. *J Sci Food Agric* 87(12):2319–26.

Zimmerli B, Schlatter J. 1991. Ethyl carbamate: analytical methodology, occurrence, formation, biological activity and risk assessment. *Mutat Res* 259:325–50.

11

Fermentation of Caper Products

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CONTENTS

11.1 Introduction

The vegetable caperbush, *Capparis* spp. (Capparidaceae), is a Mediterranean periannual shrub that grows wild in semiarid regions. It is a plant that has medicinal and aromatic properties and is mainly cultivated for its flower buds (or caper) and fruits (or caper berries). These products are very popular in Mediterranean countries for their flavor and digestive properties. The annual production is approximately 10,000 tons, and the main producer and exporter countries are Spain, Morocco, Turkey, and Italy. Capers buds and, to a lesser extent, caper berries have gained importance in the food industry and international trade, but manufacturing processes are expensive due to the lack of extensive farming of *Capparis* spp. (production often relies on wild-growing plants), the seasonal availability of the raw materials, and the absence of large enough processing industries. Although capers are fermented in brine, caper berries can be either pickled or fermented in brine or in water, usually on a small-scale production or at home. Fermentation of caper and caper berries relies on the spontaneous growth of naturally occurring lactic acid bacteria (LAB), and there is always a risk for stuck fermentations and economic losses.

11.2 Fermentation of Caper Berries

Traditional fermented caper berry fruits (or caper berries) are highly appreciated for their unique organoleptic properties, and often consumed as appetizers or used as ingredients in the preparation of traditional dishes. The main producers of fermented caper berries are Mediterranean countries, especially Greece, Italy, Turkey, Morocco, and Spain, and the final products are exported mainly to central European countries, the United States, and the United Kingdom as a delicatessen product.

Caper fruits are fermented in a traditional way, often in small enterprise or at home (Luna and Pérez 1985). During the months of June and July, the fruits are harvested and immersed directly in tap water. Usually, fermentation vessels of less than 25 L are used. The vessels are left on sunny terraces where temperatures may well reach 45°C during the day, and drop to approximately 20°C at night. The

fermentation lasts about 5 days, and the process is finished when capers are considered to reach the desired organoleptic properties. After fermentation, the capers are placed in brine and stored in this way until consumption. Some producers may also use brine for fermentation instead of plain water. According to Özcan (1999), the most suitable caper berries fermentation for LAB activity was 5% to 10% NaCl, and the best length of fermentation with respect to product color, flavor, acidity, pH, and LAB activity in brine was 20 to 25 days. In this case, the quality of fermented caper berries was maintained when stored in fresh brine with a concentration of 15% NaCl at equilibrium.

11.2.1 Microbiology of Caper Berry Fermentation

The production of fermented caper fruits is a spontaneous lactic acid fermentation that relies on microorganisms present in the raw material and processing environment (Alvarruiz et al. 1990; Özcan 1999, 2001; Özcan and Akgül 1999a,b). Pérez Pulido et al. (2005) studied the microbial communities during caper berry fermentation by using a combination of culture-dependent and culture-independent methods. Shortly after initiation of fermentation, the pH in the fermentation broths dropped rapidly to values close to 4.5, and the enterobacteria and yeasts initially present were displaced by a lactic acid microbiota. Identification of representative cultivable bacteria after plate count analysis revealed that lactobacilli were the predominant species in the fermentation. By using species-specific probes and 16S rDNA gene sequencing, the following species could be identified: *Lactobacillus plantarum*, *Lactobacillus paraplantarum*, *Lactobacillus pentosus*, *Lactobacillus brevis*, *Lactobacillus fermentum*, *Pediococcus pentosaceus*, *Pediococcus acidilactici*, and *Enterococcus faecium* (Pérez Pulido et al. 2005). Among them, *Lb. plantarum* was the most abundant species during the entire fermentation period, accounting for more than 49% of the isolates. Other lactobacilli (*Lb. brevis* and *Lb. pentosus*) were detected more intermittently due to their presence in much lower concentrations in the fermentation broths. One species (*Lb. fermentum*) was detected at the end of fermentation, probably due to its broader capacity to use residual sugars. In contrast, the pediococci were detected at the beginning of fermentation, and were rapidly displaced by the lactobacilli.

A culture-independent approach was also applied in parallel for studying caper berry fermentation, based on DNA extracted from the whole microbial community, amplification of the 16S rDNA V3 region followed by reamplification by nested polymerase chain reactor (PCR), separation of the amplicons by temporal temperature gradient electrophoresis (TTGE), sequencing of the separated DNA bands, and comparison of the corresponding DNA sequences with suitable databases (Pérez Pulido et al. 2005). The band profiles obtained by TTGE analysis corresponded mainly to LAB (*Lb. plantarum*, *Lb. brevis*, *Lb. fermentum*, and *Pediococcus* sp.). Among them, an intense band corresponding to *Lb. plantarum* was present in all samples from the beginning to the end of fermentation, in agreement with cultivation data. Enterococci (*Enterococcus* sp., *E. faecium*, and *E. casseliflavus*) were also detected by TTGE. Plating in selective media also confirmed that they were present during fermentation, although in quite low numbers, and it was difficult to assess their role as accidental contaminants or as true members of the fermentation community.

The combination of both culture-dependent and culture-independent analysis was found to be complementary, providing additional information than either approach singly. Results from other works also revealed significant differences in the microbial compositions of fermented foods depending on the use of culture-dependent or culture-independent methods (Ben Omar and Ampe 2000; Ercolini et al. 2001; Meroth et al. 2003; Miambi et al. 2003), suggesting that polyphasic studies should be used to better understand the microbial ecology of foods. The results of culture-dependent and molecular studies altogether reveal the microbial complexity of caper berry fermentation. This would be expected for a natural fermentation, which relies solely on the microbiota of the fruit's surface, manufacturing plant, and other natural sources. The microbiology of caper berry fermentation resembles other vegetable fermentations regarding the fast drop in pH observed (which is due to the low buffering capacity of vegetables) and the predominance of lactobacilli of *Lb. plantarum* species along with pediococci (Daeschel et al. 1987; Ruiz-Barba et al. 1994; Garrido Fernández et al. 1995; Leal-Sánchez et al. 2003). Although species of the *Leuconostoc* genus may be present in other vegetable fermentations, but not so much in caper fermentations because of the fast acidification and high fermentation temperature (Pederson et al. 1954; Montano et al. 1992).

11.2.2 Characterization of Strains Isolated from Caper Fermentation

A collection of strains isolated from caper berry fermentation was investigated concerning strain diversity and functional properties. Random amplification of polymorphic DNA-polymerase chain reactor (RAPD-PCR) fingerprints were investigated by cluster analysis, revealing a high diversity among the lactobacilli, with four different genomic groups (including several subgroups) that included different species: G1 (*Lb. plantarum* and *Lb. pentosus*), G2 (*Lb. fermentum*), G3 (*Lb. brevis*, *Lb. plantarum*, *Lb. paraplantarum*, and *Lb. pentosus*), and G4 (*Lb. brevis*). A high diversity was also found for *L. plantarum* fingerprints, which clustered in two main groups with two clearly defined subgroups each (Figure 11.1). Nevertheless, lactobacilli were far more homogeneous when analyzed by biochemical tests, and most strains of *Lb. plantarum* clustered in a single group (Pérez Pulido et al. 2007). The pediococci clustered as a highly homologous group represented by two highly related subgroups of *Ped. pentosaceus* and a single *Ped. acidilactici* isolate (Pérez Pulido et al. 2005). Nevertheless, strains of pediococci were found to differ in plasmid content (Pérez Pulido et al. 2006).

Fermented capers were also shown to be a valuable source of LAB strains with functional properties. Carbohydrate fermentation analysis revealed up to 26 different patterns for lactobacilli strains, which is an indication that caper fermentation is carried out by a metabolically diverse bacterial community (Pérez Pulido et al. 2007). Furthermore, the predominance of *Lb. plantarum* in this fermentation is important for the rapid production of lactic acid and fast acidification, ensuring early preservation conditions of the fermented product. The presence of heterofermentative bacteria, such as *Lb. brevis* and *Lb. fermentum*, is also expected to contribute to the development of aroma and specific flavors typical of this fermented food. This could be especially true for *Lb. fermentum*, which was isolated predominantly at the end of the fermentation. Analysis of enzymatic activities revealed a broad spectrum of activities among the lactobacilli, but very restricted activity among the pediococci (Figure 11.2). Enterococci also exhibited a large number of enzyme activities, but as mentioned previously, were present in low numbers in the fermentation. Among the lactobacilli, we found some strains with lipolytic activities, mainly heterofermentative species (*Lb. brevis* and *Lb. fermentum*), although activity was restricted to esters of short to medium fatty acids. Given the low fat content of capers, the contribution of esterase activities to the generation of aroma compounds should rely on limited hydrolysis of the fruit tissue fatty acids (Pérez Pulido et al. 2007). Galactosidase and glucosidase activities were also frequently found among isolates (Figure 11.2). These enzymes may play a role in the degradation of toxic plant glucosides or cyanogens, and could contribute to the elimination of the bitter taste of caper buds during fermentation. It has been reported that the flower buds of *Capparis spinosa* are rich in glucosinolates, the main component being glucocapperin (Matthäus and Özcan 2002). Other metabolic complementary tests revealed that many isolates degraded α-galactoside sugars, such as raffinose and stachyose, activities that may provide health benefits by contributing to the reduction of flatulence after consumption of foods containing α-galactoside sugars. Additional properties of interest were also detected in some of the isolates, such as bile salt hydrolase activity (Pérez Pulido et al. 2007). This property has been related to the capacity to

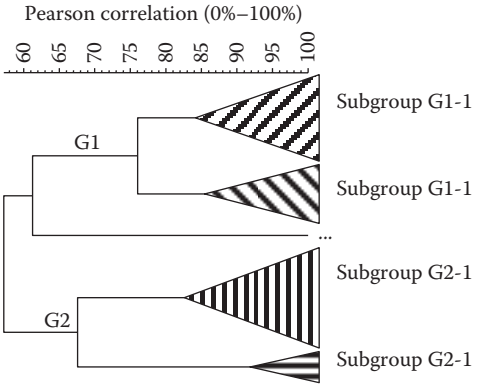

FIGURE 11.1 Diversity of *Lb. plantarum* strains from caper berry fermentations according to cluster analysis of RAPD-PCR fingerprints.

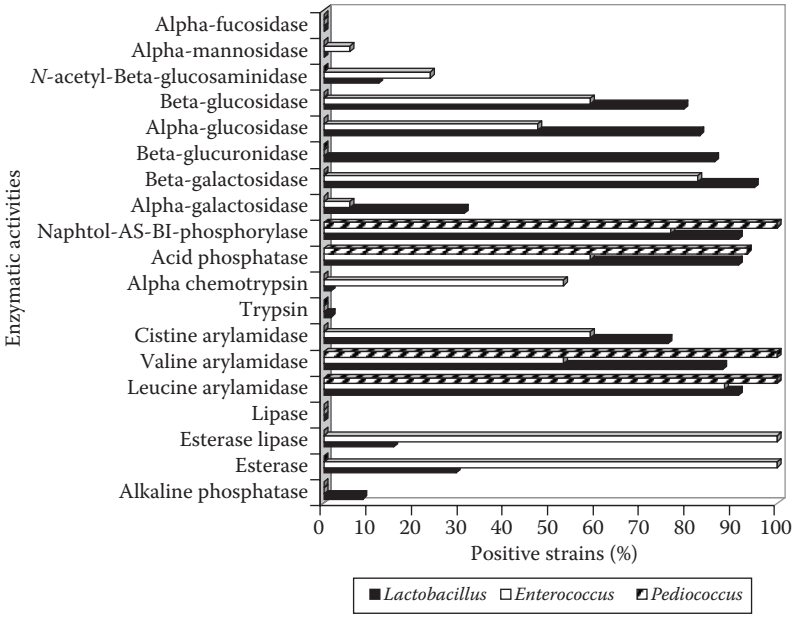

FIGURE 11.2 Enzymatic activities detected among bacterial isolates from caper berry fermentations.

lower blood serum cholesterol levels (De Smet et al. 1994; Lim et al. 2004), and preparations of encapsulated *Lb. plantarum* with bile salt hydrolase activity have been suggested as a probiotic (Jones et al. 2004). Therefore, this traditional fermentation process may be a valuable source of new strains with potential industrial applications.

11.3 Fermentation of Caper Buds

Capers are a dark olive green and about the size of a fresh kernel of corn and are used as an important seasoning in Mediterranean kitchens as a complement of salads, pasta, and other foods, and are known in nearly all the world. Fermented products are greatly appreciated for their flavor and digestive properties. Capers have a very important role in the food industry, with brined flower buds being a high-value commodity in the world market during recent years. The main caper producer and manufacturer-exporter countries are Spain, Morocco, Italy, and Turkey. Capers are exported to other countries such as the United States, United Kingdom, and Venezuela (Sánchez et al. 1992).

Caper buds are harvested from June to August, and the habitual treatment consists of submerging the freshly harvested capers in a solution of salt for approximately 40 days until fermentation takes place and the bitter taste resulting from the glycoside glucocapparin disappears (Arslan et al. 2008). The enzymatic reaction which removes this glycoside also leads to the formation of rutin, often seen as crystallized white spots on the surfaces of individual caper buds. After fermentation, the fruits are crushed, mixed with salt, spices, oil and vinegar, and kept at room temperature for 3 months. After storage, the sensory properties of the samples are determined.

In contrast to caper berry fermentation, the microbial communities and population dynamics of caper bud fermentation have not been studied in so much detail, but is probably very similar to that found in caper berry and other vegetable fermentations. Most probably, *Lb. plantarum* is also the predominant LAB in this fermentation, together with other LAB species.

Some research work has been published dealing with caper bud fermentation technology. Although, in principle, capers can be fermented with high salt brines, this has been objected to (Alvarruiz et al. 1990; Rodrigo et al. 1992). Organoleptic characteristics and preservation of the final product proved to

be the same over at least 27 months when capers had been pretreated with 10%, 15%, or 20% NaCl at equilibrium (Alvarruiz et al. 1990). In another work, Özcan and Akgül (1999a) studied caper fermentation using raw materials from different plant species (*Capparis ovata* and *C. spinosa*), bud sizes, harvest times, and in different brines (5%, 10%, 15%, and 20% NaCl). The most suitable salt concentration for LAB activity was 5%, and partly 10%, because high salt concentrations inhibited both the growth of undesirable microorganisms (coliforms, yeasts, and molds) and the activity of LAB. Lower NaCl brines (i.e., 5%) were more likely to permit the growth of coliform bacteria, yeasts, and molds, but the pH values were markedly decreased in these brine due to the increase of acidity of LAB, which inhibit or diminish the harmful microorganisms. LAB growth in 5% brine of *C. ovata* from 20 days was higher that of *C. spinosa*. Minor differences between LAB counts of *C. ovata* and *C. spinosa* brines at the same concentrations were determined at 30, 40, and 50 days of pickling. Acidity, LAB activity, sedimentation, and hardness were reduced by increasing bud size in *C. ovata*. As a pickling product, the small buds of *C. ovata* had the advantage in color and flavor. However, the formation of more sediment and partial softening were noticeable disadvantages. For both species, pickling time was determined to be 40 to 50 days with regard to end product flavor and odor, brine acidity and pH, and LAB activity (Özcan and Akgül 1999a).

Various storage experiments were carried out for fermented caper buds to determine the effect on sensory, chemical, and microbiological qualities from different plant species (Özcan and Akgül 1999b). The buds were stored for 180 days in 10% and 20% brines. Old and freshly made brines were tested. The results were that for product storage, fresh brines were beneficial for eliminating off-flavors; also, no sediment was observed. However, the color of the buds in both plant species (*C. ovata* and *C. spinosa*) changed during storage, in old and fresh brines, although the color of *C. spinosa* was more yellowish-green than desired. However, no important changes occurred in bud firmness during storage because old and fresh brines had similar effects on firmness. Acidity in old brines was usually higher than in fresh brines during storage. Microbiologically, total and coliform bacterial counts in old brines of *C. spinosa* were lower than in other samples. The growth of yeasts and molds was observed in different brines and times. The growth of LAB were observed only in 10% fresh brine, and it decreased from initial storage and at 30 days of *C. ovata* and *C. spinosa* growth, respectively. LAB were not found in old brines with the same salt concentration, probably because of the soluble inhibitory substances of buds. The fresh brine containing at least 10% salt is suggested for the storage of pickled capers. Alvarruiz et al. (1990) also reported that salt concentration should not be less than 10% for keeping firmness and color in caper storage. They indicated, otherwise, some off-flavor or softening defects. In other work (Özcan 2001), the storage of fermented capers was studied in two different species, with some preservatives added (acetic acid, citric acid, sugar, or tarragon extract), and with or without pasteurization at 80°C (Özcan 2001). They found that the best results were *C. ovata* buds fermented in 5% brine and stored in 2% brine plus 1% acetic acid, 6% brine plus 1% acetic acid, or 6% brine plus citric acid. Furthermore, buds of *C. ovata* kept their bright yellowish color during storage. Although, for all samples, firmness gradually decreased during storage, *C. spinosa* samples decreased in firmness more than *C. ovata*. Finally, the texture of the buds was significantly protected by pasteurization and by the addition of sugar and tarragon extract.

Recently, there has been an increased interest in processed caper buds, with an increasing demand throughout the world market. For that reason, some additional research has been published recently about the improvement of caper bud fermentation (Arslan et al. 2008; Douieb et al. 2010). In the first research, Arslan et al. (2008) studied lactic acid (1%), citric acid (1%), yogurt (2.5%), and a starter culture (*Lb. plantarum*) singly in brines (10%) for the fermentation of raw caper buds (*C. ovata* Desf. var. *canescens*). The buds were divided into two different sizes, small and big, according to their diameters before fermentation, and the effects of bud size on the fermentation were also investigated. Small buds could be recommended for their higher acidity levels and hardness scores. The addition of citric and lactic acid revealed very low pH values (2.14–2.60) and high acidity levels (1.2%–1.7%), which may help reduce the risk of spoilage during the first days of fermentation (Garrido Ferndez et al. 1997; Leal-Sánchez et al. 2003). The fermentation with starter culture inoculation or the addition of yogurt to the brine resulted in lower acidity values, similar to the control brine. During fermentation of caper buds, there were changes in microbial profiles but bud size was not significant for the microbial counts. Lactic bacteria were higher in the inoculated samples on day 1 of fermentation (ranging between 5.5 and 6.9 log cfu/mL), but on week

6 of fermentation, these samples had the lowest LAB counts (ranging between 3.0 and 4.5 log cfu/mL), whereas control and yogurt supplemented samples had the highest LAB numbers during the fermentation period. The total microbial flora in the different brine conditions had similar total bacterial populations, ranging between 4.6 and 6.9 log cfu/mL at the beginning of fermentation, whereas these numbers decreased to 3.0 and 4.4 log cfu/mL on week 6. The lowest total bacterial population was observed in the citric acid supplemented samples on week 6. The high number of molds and yeasts at the beginning of the fermentation period decreased through the end of fermentation, with the lowest values in lactic acid– and citric acid–containing samples. The coliform numbers were higher early on in week 1 of fermentation (between 1.1 and 2.0 log cfu/mL), but rapidly decreased later in the week and did not grow during fermentation. The differences between flavor and color scores of samples were not statistically significant for bud size, whereas these differences were significant for hardness scores (small buds had the highest scores). Regarding flavor, citric acid was preferred, and *Lb. plantarum*–inoculated and yogurt supplemented samples had the lowest flavor scores. Furthermore, the color of buds in *Lb. plantarum*–inoculated brines were grayish pale green, whereas the colors of the other buds were bright green. Hardness was the best in citric acid– and lactic acid–supplemented samples. The desired bright yellow color of brine was obtained in control, citric acid–, and lactic acid–supplemented samples, whereas yogurt-supplemented and *Lb. plantarum*–inoculated brines were more pale and turbid. Yogurt-supplemented brines revealed much more sediment at the bottom, which could be attributed to the highly dry material of this brine type. The best initial brine conditions deduced from the physicochemical, microbiological, and sensory results were citric acid and lactic acid-supplemented brine types, and the use of small buds for fermentation was also recommended. The reason for undesirable results in *Lb. plantarum*–inoculated samples may be attributed to the high glucosinolate, especially glucocapparin and glucocleomin, content of caper buds.

Recently, Douieb et al. (2010) studied the best values of factors, brines (10% and 20%), lactic acid (0.1% and 1%), citric acid (0.1% and 1%), and lactic fermentum (*Lb. plantarum*; 0.5% and 2.5%), and the best combinations between the factors susceptible to reach a decrease in pH to inhibit the growth of undesirable microorganisms and, consequently, to achieve a good fermentation. They found that brine, lactic acid, and citric acid have a significant effect on the decrease in pH (with the brine with the most important effect and citric acid with relatively little effect), on the contrary with the lactic ferment, which did not have any significant effect. The interactions with significant effects on the decrease in pH were brine and lactic acid, between brine and lactic ferment, between lactic acid and citric acid, and between lactic acid and lactic ferment. As to the effect of the interactions, it is the interaction between lactic acid and citric acid that had the most important effect, followed by the effect of the interaction between lactic acid and lactic ferment, and then, the effect of the interaction between brine and lactic acid. The interaction between brine and lactic ferment has the lowest effect. In conclusion, the best decrease of pH was produced when the factors are at the highest level of their variations.

11.4 Concluding Remarks

Caper berries and caper buds are traditional fermented foods with an increasing importance in the world market. These fermentations are often carried out by small to medium enterprises or at home, making innovation and technological improvements on fermentation processes more difficult. Knowing the influence of internal and external factors and deciphering the composition and dynamics of the microbial communities involved in these fermentations is of crucial importance for optimization of production processes at an industrial scale. Work carried out on the fermentation of caper buds suggests a complex microbial community of LAB responsible for the fermentation, in which *Lb. plantarum* is the main actor. The influence of technological factors on the fermentation of caper buds has been quite studied in detail, but the microbiological aspects of this fermentation have not been deciphered. The fact that caper buds are most frequently fermented in water whereas caper buds are fermented in brine precludes from extrapolation of results about one fermentation process to another. Therefore, the influence of technological factors on caper berry fermentation and the composition and dynamics of the microbial communities involved in the fermentation of caper buds still require further studies.

REFERENCES

Alvarruiz A, Rodrigo M, Miquel J, Girer V, Feria A, Vila R. 1990. Influence of brining and packing conditions on product quality of capers. *J Food Sci* 55:196–8.

Arslan D, Unver A, Özcan M. 2008. Determination of optimum fermentation quality of capers (*Capparis ovata* desf. var. *canescens*) in different brine conditions. *J Food Process Preserv* 32:219–30.

Ben Ornar N, Ampe F. 2000. Microbial community dynamics during production of the Mexican fermented maize dough pozol. *Appl Environ Microbiol* 66:3664–73.

Daeschel MA, Anderson RE, Fleming HP. 1987. Microbial ecology of fermenting plant materials. *FEMS Microbiol Rev* 46:357–67.

De Smet I, Van Hoorde L, De Saeyer N, Vande Woestyne M, Verstraete W. 1994. In vitro study of bile salt hydrolase (BSH) activity of BSH isogenic *Lactobacillus plantarum* 80 strains and estimation of cholesterol lowering through enhanced BSH activity. *Microb Ecol Health Dis* 7:315–29.

Douieb H, Benlemlih M, Errachidi F. 2010. Improvement of the lactic acid fermentation of capers (*Capparis spinosa* L) through an experimental factorial design. *Grasas Aceites* 61(4):398–403.

Ercolini D, Moschetti G, Blaiotta G, Coppola S. 2001. The potential of a polyphasic PCR-DGGE approach in evaluating microbial diversity of natural whey cultures for water buffalo mozzarella cheese production: bias of culture-dependent and culture-independent analyses. *Syst Appl Microbiol* 24:610–7.

Garrido Fernández A, Fernández Diez MJ, Adams MR. 1997. *Table olives: production and processing*. London: Chapman & Hall.

Garrido Fernández A, García García P, Brenes Balbuena M. 1995. Olive fermentations. In: Rehm HJ, Reed G, editors. *Enzymes, biomass, food and feed*. New York: VCH. p. 593–627.

Jones ML, Chen H, Ouyang W, Metz T, Prakash S. 2004. Microencapsulated genetically engineered *Lactobacillus plantarum* 80 (pCBH1) for bile acid deconjugation and its implication in lowering cholesterol. *J Biomed Biotechnol* 2004:61–9.

Leal-Sánchez MV, Ruiz-Barba JL, Sánchez AH, Rejano L, Jiménez-Díaz R, Garrido A. 2003. Fermentation profile and optimization of green olive fermentation using *Lactobacillus plantarum* LPCO10 as a starter culture. *Food Microbiol* 20:421–30.

Lim HJ, Kim SY, Lee WK. 2004. Isolation of cholesterol-lowering lactic acid bacteria from human intestine for probiotic use. *J Vet Sci* 5:391–5.

Luna F, Pérez M. 1985. La Tapanera o alcaparra. Cultivo y aprovechamiento. Ministerio de Agricultura, Pesca y Alimentación, Madrid, Spain.

Matthäus B, Özcan M. 2002. Glucosinolate composition of young shoots and flower buds of capers (*Capparis* species) growing wild in Turkey. *J Agric Food Chem* 50:7323–5.

Meroth CB, Walter J, Hertel C, Brandt MJ, Hammes WP. 2003. Monitoring the bacterial population dynamics in sourdough fermentation processes by using PCR-denaturing gradient gel electrophoresis. *Appl Environ Microbiol* 69:475–82.

Miambi E, Guyot JP, Ampe F. 2003. Identification, isolation and quantification of representative bacteria from fermented cassava dough using an integrated approach of culture-dependent and culture-independent methods. *Int J Food Microbiol* 82:111–20.

Montano A, de Castro A, Rejano L. 1992. Transformaciones bioquímicas durante la fermentación de productos vegetales. *Grasas Aceites* 43:352–60.

Özcan M. 1999. Pickling and storage of caperberries (*Capparis* spp.). *Z Lebensm Unters Forsch A* 208:379–82.

Özcan M. 2001. Pickling caper flower buds. *J Food Qual* 24:261–9.

Özcan M, Akgül A. 1999a. Pickling process of capers (*Capparis* spp.) flower buds. *Grasas Aceites* 50:94–9.

Özcan M, Akgül A. 1999b. Storage quality in different brines of pickled capers (*Capparis* spp.) flower buds. *Grasas Aceites* 50:269–74.

Pederson CS, Albury MN. 1954. The influence of salt and temperature on the microflora of sauerkraut fermentation? *Food Technol* 8:1–5.

Pérez Pulido R, Abriouel H, Ben Ornar N, Lucas López R, Martínez Cañamero M, Gálvez A. 2006. Plasmid profile patterns and properties of pediococci isolated from caper fermentations. *J Food Prot* 69: 1178–82.

Pérez Pulido R, Ben Ornar N, Abriouel H, Lucas López R, Martínez Cañamero M, Gálvez A. 2005. Microbiological study of lactic acid fermentation of caper berries by molecular and culture-dependent methods. *Appl Environ Microbiol* 71:7872–9.

Pérez Pulido R, Ben Ornar N, Abriouel H, Lucas López R, Martínez Cañamero M, Guyot JP, Gálvez A. 2007. Characterization of lactobacilli isolated from caper berry fermentations. *J Appl Microbiol* 102:583–90.

Rodrigo M, Lazaro M, Alvarruiz A, Giner V. 1992. Composition of capers (*Capparis spinosa*): influence of cultivar, size, and harvest date. *J Food Sci* 57:1152–4.

Ruiz-Barba JL, Cathcart DP, Warner PJ, Jiménez-Díaz R. 1994. Use of *Lactobacillus plantarum* LPCO10, a bacteriocin producer, as a starter culture in Spanish style green olive fermentations. *Appl Environ Microbiol* 60:2059–64.

Sanchez AH, Castro de A, Rejano L. 1992. Controlled fermentation of caperberries. *J Food Sci* 57:675–8.

12

Apple Cider Fermentation

Alessandro Nogueira and Gilvan Wosiacki

CONTENTS

12.1 History

The first use of apple juice as a raw material for beverage fermentation is lost in the mists of human history. However, there are some reports of the consumption of fermented beverages in the East Mediterrean approximately 2000 years ago (Jarvis et al. 1995, quoted by Laplace et al. 2001). Ancient papers contain words such as *shekar* (Hebrew), *Sikera* (Greek), and *Sicera* (Latin). Plinius, in the first century AD *(Histoire Naturelle*) and Hippocrates, in the fifth century AD, both refer to apple- and pear-based fermented beverages (Robin and De La Torre 1988; Ravier 1985).

During the first millennium AD, cider was considered to be a special beverage for kings, warriors, sailors, and pirates, but it had its golden era in Brittany and Normandy in France mid-thirteenth century when the cider press was hailed as one as the greatest discoveries in the history of alcoholic beverages. At that time, there were several cold winters that ravaged the vineyards of Brittany and

Normandy, and the production of beer was not allowed because such processing would consume too much cereal, necessary for the diet of humans. During the First World War (1914–1918), many places used for cider processing were affected because apple orchards were abandoned or destroyed to be substituted by sugar beet. People moved from the countryside to the cities and at this time cider was still more popular than wine in Western France. Later, in the 1960s, the decline in consumption of cider occurred because of the availability of beer, but at the beginning of the twenty-first century, it is still third in the ranking of alcoholic beverages, behind beer and wine in France (Drilleau 1991a,b; Ravier 1985).

12.2 Definitions and Characteristics

There are more than 25 cider-producing countries in the world. The most important of these in amount and quality are France, England, Switzerland, Belgium, and Spain in Europe, the United States and Canada, and Brazil, Argentina, Chile, and Paraguay in South America (Lea and Drilleau 2003; Nogueira and Wosiacki 2010).

In the United States and England, the word "cider" relates to apple juice consumption without any thermal treatment, and the words "hard cider" refer to the same product but in its fermented state. In France, cider is known as *cidre*, in Spain and Brazil *sidra*, in Argentina and Paraguay *cidra*, but all these words refer exclusively to the fermented product (Lea and Drilleau 2003). In Germany, a similar product can be found in the area of Trier, where it is known by the name of *viez,* and it is considered to be a technological derivation of *apfelwein* (Possmann 1992).

In France, Germany, Spain, and Brazil, the definitions of cider are restricted by law, and the profiles of quality are quite different. The minimum permitted amount of alcohol in Germany is 5%, and there is no maximum figure; in France, the minimum is 4% (v/v; see Table 12.1). In Brazil and England, the maximum permitted amount of alcohol can be up to 8% (v/v). French ciders are characterized by their mild taste, astringency (tannins), and fruity aroma, although in German-speaking countries (Germany, Switzerland, and Austria), the product may be even drier and more acidic. In Spain, especially in the area of Asturias, the preference is for a product that is spicy in flavor with a predominance of acetic acid. In Brazil, the product is mild, with low aromatics and low acid levels because it is produced mainly from table apples (Mangas et al. 1999; Lea 1995; Lea and Drilleau 2003; Nogueira and Wosiacki 2010).

In France, the beverage has the following legal definition: "No beverage can be transported with the intention of sale with the name of cider if such a beverage is not derived exclusively from the fermentation of fresh apple juice or of a mixture of fresh apple and pear juices extracted with or without water" (Lea and Drilleau 2003). In England, cider is defined as "A beverage obtained by partial or complete fermentation of apple juice or apple concentrate with or without addition, before processing, of sugars and potable water" (National Association of Cider Makers 1998).

12.3 Raw Materials

Apples destined for fermentation processing may belong to two different groups: fruit that is unqualified for commercial use and industrial fruit. In Brazilian processing, fruit that has previously been discharged in the preprocessing stage of selection without any physical or microbiological damage is exclusively used because it is accepted that these fruits are desirable for human consumption at the moment of harvesting or after cold storage for a period of time (Nogueira and Wosiacki 2010). In France alone, there are more than 300 apple varieties that have been studied or submitted to genetic improvement to obtain reasonable physical chemical and agronomic features that might positively enhance the quality of the resultant beverage (Boré and Flecking 1997). In the English, German, and Spanish methods of processing, mixtures of both categories may occur (Lea and Drilleau 2003).

TABLE 12.1

Specifications of the Standards of Identity and Quality of the Cider Apple in Some European Countries

Sugar addition (and concentrate)	
England	Allows ad libitum[a]
France	Not allowed, but apple juice concentrate can be added up to 50%[b]
Germany	Allows a maximum of 1.055 kg m^{-3} [b]
Alcoholic degree	
England	Minimum of 1.2% and maximum of 8.5%[a]
France	Minimum of 1.5% (maximum of 3% for sweet cider)[b]
Germany	Minimum of 5%[b]
Acid addition	
England	Allows *ad libitum*: malic, citric, tartaric, lactic[a]
France	Only allows citric acid, malic acid (maximum of 5 g L^{-1})[b]
Germany	Only allows lactic acid (maximum of 3 g L^{-1})[b]
Sweeteners	
England	Allows *ad libitum* sugar and sweeteners[a]
France	Only allowed in apple juice. Residuals in the cider are the following: demi-sec 28–42 g L^{-1}, sec < 28 g L^{-1}, and sweet > 35 g L^{-1} [b]
Germany	Only allows sugars (maximum of 10 g L^{-1})[b]
Colorant	
England	Allows all food colorants[a]
France	Allows cochineal and caramel[b]
Germany	Only allows small amounts of caramel[b]
Free sugar in dry extract	
England	Minimum of 13 g L^{-1} [a]
France	Minimum of 16 g L^{-1} [b]
Germany	Minimum of 18 g L^{-1} [b]

Source: Adapted from Nogueira, A., G. Wosiacki, Sidra, in: Filho, W. V., editor, Tecnologia de Bebidas Alcoólicas, Blucher, São Paulo, 2010.

[a] *Source:* National Association of Cider Makers, Code of practice for the production of cider and perry, National Association of Cider Makers, London, 1998. With permission.

[b] *Source:* Lea, A. G. H., Cidermaking, in: Lea, A. G. H., and J. R. Piggott, editors, Fermented beverage production, pp. 66–96, Blackie Academic, London, 1995. With permission.

12.4 Processing

In those countries where industrial varieties and mixtures are processed, the fraction of each variety that is supposed to be considered to maintain a composition profile is directly dependent on the fruit (acidity, sweetness, dryness, etc.) which is reflected in the sensorial quality of the beverage. The fruits should pass through a process of sanitization followed by selection in which trained people separate the bad fruits (spoiled fruits with phytopathological defects and also those with mechanical and phenological defects) and discard them to be processed in another industrial unit. This raw material is washed by showering or immersion and after that passes through a knife mill. In some countries, such as Brazil and Argentina, during the milling process, kalium bisulfite (70–150 mg L^{-1}) can be added to control the browning reaction and to postpone a natural fermentation because harvesting and processing occur in the summer with high daily temperatures varying from 25°C to 35°C (Nogueira and Wosiacki 2010). After this, extraction is carried out in a belt press, and the obtained apple must is modified by adding industrial pectinolytic preparation in amounts according to the instructions of the distributor. If SO_2 has not been added at this

stage, it is then added as follows: 75 mg L^{-1} (pH 3.0–3.3), 100 mg L^{-1} (pH 3.3–3.5), and 150 mg L^{-1} (pH 3.5–3.8) of sulfide anhydride (Lea and Drilleau 2003). After depectinization and flotation, within 6 to 10 hours, the must is racked and the pomace sediment is put through a second pressing, usually in a press filter.

In some industrial plants, after the extraction of the must enzyme, yeast and sulfite are added; however, the pectinolytic activity must be controlled because methanol may be released, which may exceed the permitted levels set by local legislation. In Brazil, after the depectinization stage, water is added through a soluble sugar solution as well as a fermentation enhancer, ammonium salts, vitamins, ergosterol, and unsaturated fatty acids and citric acid, aiming to increase the processing yield. The addition of water is allowed in Brazil (Ministério da Agricultura, Pecuária e Abastecimento 2009), but it can cause a reduced level of quality in the final product. After this, the must is allowed to naturally ferment because of the yeast present in the fruit, which is the custom in France where the processing temperature varies from 7°C to 11°C; however, in other producing countries, the must receives industrial dry active yeast at a ratio from 20 to 40 g hL^{-1}, which represents an initial population of approximately 2.0 to 4.0×10^6 colony-forming units (CFU) mL^{-1}. Alcoholic fermentation occurs in vessels equipped with gas-escaping devices (batoc) to avoid the entry of oxygen but allowing for excess carbon dioxide (CO_2) outlet. Fermentation can be continued until all the sugar is converted to ethanol and volatile carbonic dioxide or can be interrupted maintaining fruity residual sugars in the final product. If the level of fruit organic acids was high after the alcoholic fermentation, the product requires a resting period of around 20 to 35 days to support the transformation of malic acid to lactic acid and consequently to reduce the acid taste of the beverage. In some countries, after the alcoholic fermentation, mixtures of fermented must with or without sucrose are added to correct (variation of 40–100 g L^{-1}) the final sugar level. If necessary, the acidity is corrected with lactic or citric acid. The addition of preservatives normally conforms to kalium metabisulfite with 20 to 80 mg L^{-1} of free SO_2 and kalium sorbate at a concentration allowed by law (Wosiacki et al. 1997). The fermented beverage is then clarified by protein glue, generally with gelatin or casein, using bentonite as a coadjuvant. The next step is stabilization by a thermal treatment or preservative using the addition of CO_2 at a low temperature, bottling in glass bottles at low temperature as well as in high-pressure–resistant bottles with metal forcing stoppers, and finally, labelling.

Figure 12.1 shows some of the main processes in cider making all around the world. Brazilian cider is produced exclusively in large industrial quantities, whereas the French and the British versions of cider may be obtained from small, medium, and large producers. Christen and LéQuére (2002) classified small producers as those who buy the raw material or produce in small amounts and use only the apple juice to pass through natural gasification in the bottle without any thermal stage. Medium producers use pure juice—artificial gasification (in general) without any thermal procedure. Large or industrial producers use artificial gasification—pasteurization and diffusion of the pomace after pressing to add to the process.

12.4.1 Preparation of the Fermentation Substract

12.4.1.1 Raw Materials

The fruits that are considered as "industrial" or raw material in Brazil are those that have no commercial value because of their physiological and morphological defects, small size, poor color distribution, inadequate shape, scarring, and mechanical injuries; that is, fruit that is unsuitable for fresh consumption. Industrial apples differ from those that are rejected because the latter include apples infected by microorganisms, which should form only a small fraction of the disposal of production. The raw material is processed as it arrives in the industry, where there is no separation of varieties; thus, it constitutes a mixture of varieties of commercial fruit and its pollinators; also in most cases it is commercial (Paganini et al. 2004).

For comparative purposes, the areas of the production of apples in France include Brittany and Normandy, among others, where a more temperate climate is characteristic, with very clear definition of the seasons. The harvest of apples for making cider in those regions of France runs from October 15 to

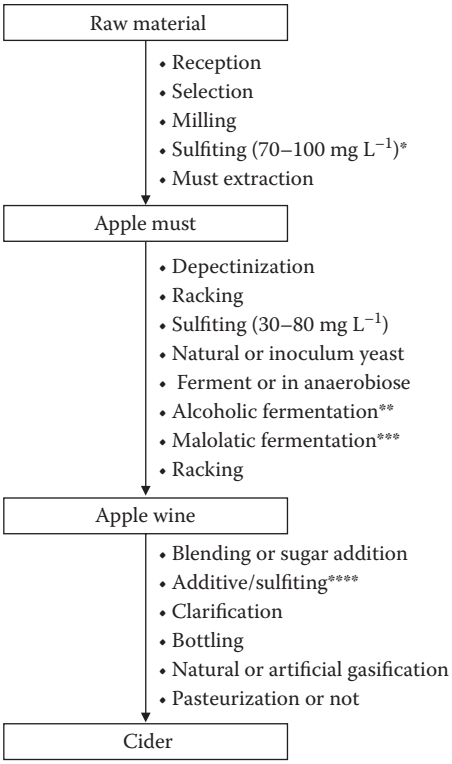

FIGURE 12.1 Different operations in cider processing worldwide. *Used in South America to block enzymatic browning and to prevent initial contaminations in apple must. **Can be complete or partial with residual sugars (30–60 g L^{-1}). ***Used in apple wine acid with inoculation or natural bacteria (20–35 days). ****Added in agreement with the legislation of each country.

December 15, that is, typically during the fall. Harvesting can be performed either manually or mechanically, using specialized machines, but is very rare for a cider to be produced from a single variety of apple, as is the product obtained from the Guillevic apple, the only such drink to be marketed (Lepage 2002).

In France, it is necessary to mix some varieties to obtain a proper balance of sugar, acidity, and tannins, significant parameters for obtaining a quality product, not easily found in a single variety at the levels required. Thus, the industries have control of the mixture of varieties in their orchards and maintain appropriate contracts with producers, although small and medium producers produce their own blends. Unlike other countries, France exclusively uses cider apples (*pommes à cidre*) as raw material for the preparation of the drink, and more than 300 varieties have been listed and characterized by Bore and Flecking (1997, 756).

12.4.1.2 Industrial Classification of Apples

These are fruits with different shapes or even with bad color patterns in the epicarp. These fruits represent a fraction (20%) both quantitatively and qualitatively of the total apple production. Even so, they maintain the same physical and chemical properties, especially those related to aroma compounds (Nicolas et al. 1994; Wosiacki et al. 1991).

In Europe, the relationship between sugar and acid as well as tannin and acid allows the classification of varieties into four taste groups: sweet, bitter sweet, bitter, and acid (Drilleau 1991a,b, 1993; Le Quere and Drilleau 1993). The sweet apple, with approximately 15 g/100 mL, and the bittersweet are the references for mixtures that aim to obtain high alcohol levels.

Older classifications clearly differentiate those apples that are more acidic from bitter ones (Tavernier and Jacquin 1946). Those classified as acidic, with total tritable acid of 70 to 90 meq L^{-1}, clearly favor the perception of the body of the cider and mediate the formation of what is known as *chapeau brun*; meanwhile, the sour apples point to a fresher product and a better taste. Sour fruit provides better protection against microbiological alterations such as *casse oxydasique* and *framboisé*.

In England, the raw material comprises a mixture of apples for cider and of table apples, and the fruit used by the cider-making industry is split into four classes: sharp, bittersharp, bittersweet, and sweet.

These varieties, grouped only for this situation, allow the producer a selection of the components for a mixture of different fruits, which permits an annual production of high quality balanced cider.

The sound fruits are stored for some days to reach the required maturity, and it is possible to check this by slightly pressing the fruit with the finger tips (Nogueira and Wosiacki 2010).

In France, three levels of ripeness are considered (Drilleau 1991a,b). Apples from the first season are fruits that ripen on the tree by the end of September and are difficult to store. Those from the second season are hard fruits that ripen from the beginning of October until the middle of November and may be stored for some time to reach the required level of ripeness. Third season apples have a firm texture and must be stored to finish the ripening process until December or January.

12.4.1.3 Chemical Components of Apples

The composition of the must made with European industrial apples is slightly different from that made with Brazilian table fruits (Table 12.2). Sugars constitute the highest carbohydrate fraction and are also the largest components of soluble solids of apples. The main types of sugars are fructose, glucose, and sucrose, evenly distributed and transformed into alcohol by the metabolism of yeast. The must shows an average total content of reducing sugars of 12.0 g/100 mL and 11.9 g/100 mL for European and Brazilian varieties, respectively (Table 12.2).

Fructose is the main simple sugar in apples, and its concentration can be two times or three times that of glucose, and in some special varieties, can represent up to 80% of the total sugar (Smock and Neubert 1950; Wosiacki et al. 2005).

Total starch levels (amylase and amylopectin) in ripened fruits do not exceed 1% (Table 12.2). In cider processing, fruits with higher levels of starch—that is, those which are not completely mature—will produce a lower alcohol level because yeast does not hydrolyze these polysaccharides. Starch may also be responsible for the turbidity of the final product (Smock and Neubert 1950; Beveridge 1997; Demiate et al. 2003).

Pectin, composed of polygalacturonic acid units linked in large chains, is a plant-like cement in the cellular wall, which may explain part of the turbidity and viscosity of pressed apple must. Such substances should be hydrolyzed and withdrawn from the must before fermentation because the process of filtration will be impaired because of blocked filters and also because of the possibility of methanol fixing by the yeast action over pectin.

Table 12.3 demonstrates that the total levels of nitrogen found in apples used in the European industries show a larger amplitude (27–574 mg L^{-1}) than Brazilian fruits (59–330 mg L^{-1}), which reflects the status of the nutrients that may affect the processing and final quality of the beverage (Downing 1989).

TABLE 12.2

English Apple Classification for Cider

Category	Acidity (g/100 mL)	Tannin (g/100 mL)
Sharp	>0.45	<0.20
Bittersharp	>0.45	>0.20
Bittersweet	<0.45	>0.20
Sweet	<0.45	<0.20

Source: Beech, F., *Prog Ind Microbiol*, 11, 133–213, 1972. With permission.

TABLE 12.3

Organic and Mineral Content in Apple Juice Produced in Europe and Brazil

	Europe	Brazil
Compounds	**Min–Max**	**Min–Max**
Fructose (g/100 mL)	5.2–13.0	3.2–11.5
Glucose (g/100 mL)	0.9–3.0	0.8–5.2
Sucrose (g/100 mL)	2.0–4.5	0.1–9.3
Total sugar (g/100 mL)	9.0–15.0	7.6–16.3
Sorbitol (g/100 mL)	0.2–1.0	–
Starch (%)	<1[a]	<1[a]
Pectin (g/100 mL)	0.1–1.0	0.37–0.70[b]
Nitrogen (mg L^{-1})	27–574	59–330
Amino acid (mg L^{-1})	25–2000[c]	1.3–7.7[d]
Thiamine (µg/100 mL)	2.8–13.2	–
pH	3.3–4.0	2.9–4.3
Malic acid (g/100 mL)	0.4–1.3	0.07–1.1
Ash (g/100 mL)	0.14–0.46	0.50–2.49
Potassium (mg L^{-1})	1200	861–2628
Calcium (mg L^{-1})	–	12.7–49.3
Phosphorus (mg L^{-1})	–	22.5–99.0
Magnesium (mg L^{-1})	–	28–45
Total phenols (mg L^{-1})	188–3000	101–833
Chlorogenic acid (mg L^{-1})	149–900	370–399
Phloredzin (mg L^{-1})	9.0–200	155–237
Epicatechin and procyanidol (mg L^{-1})	4.0–1700	6.0–1169

Sources: Lea, A. G. H., F. W. Beech, *J Sci Food Agric*, 29, 493–6, 1978. With permission. Beech, F., *Prog Ind Microbiol*, 11, 133–213, 1972. With permission. Lea, A. G. H., *Fermented beverage production*, Blackie Academic & Professional, London, 1995. With permission. Drilleau, J. F., *Pomme*, 20, 19–20, 1990a. With permission. Drilleau, J. F., *Pomme*, 21, 20–22, 1990b. With permission. Drilleau, J. F., *Pomme*, 23, 23–25, 1991a. With permission. Drilleau, J. F., *Technologie*, 23, 21–22, 1991b. With permission. Goverd, K. A., J. G. Carr, *J Sci Food Agric*, 25, 1185–90, 1974. With permission. Czelusniack, C. et al., *Braz J Food Technol*, 6, 25–31, 2003. With permission. Paganini, C. et al., Ciênc Agrotec, 28, 1336–43, 2004. With permission. Fan, X. et al., Hortscience, 30, 104–5, 1995. With permission. Nogueira, A. et al., *Ciênc Tecnol Alimentos*, 27, 259–64, 2007. With permission. Kovacs, E. et al., *Acta Hortic*, 219–24, 1999. With permisison. Canteri-Schemin, M. H., Obtenção de pectina alimenticia a partir de bagaço de maçã, Dissertation (M.A. in food technology, mestrado em tecnologia de alimentos), Universidade Federal do Paraná, Curitiba, 2003. Wosiacki, G. et al., *Acta Aliment*, 37, 9–22, 2008. With permission. Alberti, A. et al., *Braz Arch Biol Technol*, 54, 10–18, 2011. With permission. Zardo, D. B. M. et al., *Ciênc. Tecnol. Alimentos*, 29, 148–54, 2009. With permission.

Note: –, not determined.

[a] More than 2% for immature fruits.

[b] Percentage in juice.

[c] Asparagine and aspartic acid represent 90%.

[d] Expressed in formal number.

Thiamine quickly disappears from the must at the beginning of fermentation. The addition of thiamine is authorized by the laws of several EEC countries, with a maximum of 50 mg hL^{-1}, not to accelerate the fermentation but to reduce, by decarboxylation, the important ketone acids that are likely to participate in combinations with sulfur dioxide (Ribereau-Gayon et al. 1998).

Apple must shows mineral composition both qualitatively and quantitatively appropriate for the performance of yeast. Little is known about this precise function; however, it is known that some minerals at low concentrations are components of enzyme systems. Table 12.3 shows the main phenolic differences between European (French and English) industrial apples and the Brazilian variety.

12.4.1.4 Phenolic Compounds

In apples, phenolic substances can be found in the vacuoles (95%) and also in the epicarp and subcarp. These substances are found at higher levels in the epicarp than in internal fruit tissues. In different varieties, the skin to pulp ratio may be 3 to 10, respectively (Nicolas et al. 1994).

The composition of phenolic compounds in cider must is dependent on the variety of apple, the degree of ripeness, the cultural condition, and the pressing method (Lea and Arnold 1978). During processing, the phenolic substances may be altered by the enzymatic browning because of the action of the endogenous enzyme system (Sataque and Wosiacki 1987) and also by precipitation (Lea and Timberlake 1978; Cliff et al. 1991). The enzymatic oxidation may be stopped by the use of antioxidative agents such as sulfur dioxide and ascorbic acid, which may prevent the enzyme activity or even react with the enzyme oxidant intermediary. They may also act as reducing agents, driving the formed quinones back to their original polyphenolic substance form (Shahidi and Naczk 1995; Sayavedra-Soto and Montgomery 1986; Nicolas et al. 1994). In this way, larger losses of phenolic compounds occur because of oxidative reaction during and after the milling operation because of the incomplete extraction from the fruit tissues and in the clarification of the juice (Shahidi and Naczk 1995; Nogueira et al. 2004).

The tannin and the acidity levels of the must are older criteria used in the French (Tavernier and Jacquin 1946) and English classifications (Beech 1972) of apple varieties for cider making. These compounds are linked to the bitterness and astringency of cider contributing to the formation of the body of the beverage (Drilleau 1991a,b, 1993; Lea 1990) and are important markers in the final quality. The color of cider is essentially due to the phenolic compound linked to oxidative reactions. Some of them, such as the phenolic hydroxycinnamic acids, are precursors of volatile constituents that may contribute to the aroma of the cider. The apples used for cider making (industrial apples) may have up to 10 times more phenolic compounds than table fruit (Whiting and Coggins 1975; Van Buren 1970, quoted by Sanoner et al. 1999).

There is currently much interest in phenolic compounds because of their antioxidative activity, which can prevent the negative effects of oxidative stress on human health (Lee and Smith 2000; Mangas et al. 1999). Five phenolic compound classes may be found in apples: [1] hydrocinnamic acid derivatives (caffeic acid, 5-caffeoylquinic acid, 4-caffeoylquinic acid, 3-caffeoylquinic acid, 5-ρ-coumaroylquinic acid, ρ-coumaryc, 4-ρ-coumaroylquinic acid, ρ-coumarylglucose acid, cafeoilglucose, feruloilglucose, ferulic acid), [2] flavonols (quercetin-3-*O*-β-D-galactopyranoside, quercetin-3-*O*-β-D-glucopyranoside, quercetin-3-*O*-β-D-xylose, quercetin-3-*O*-α-L-rhamnopyranoside, quercetin-3-*O*-α-L-arabinofuranoside, quercetin-3-*O*-rutinoside), [3] anthocyanins (cyanidin-3-galactoside, cyanidin-3-glucoside, cyanidin-3-xyloside, cyanidin-3-arabinoside, cyanidin-3-arabinose, cyanidin-3-glucoside, and cyanidin-3-xyloside), [4] dihydrochalcones (phloretin-2′-*O*-glucoside (phlorizin), phloridzin, and phloretin xyloglucoside), and [5] flavan-3-ols monomers ((–)-epicatechin; (+)-catechin; procyanidin B1, B2, B5, C1; and high polymerization forms; Nicolas et al. 1994; Marks et al. 2007; Hamauzu et al. 2006).

These compounds can also be classified into two larger categories, phenol acids and flavonoids, the latter being clustered into three subgroups: flavan-3-ols, flavanols, and dihydrochalcones (Drilleau 1991a,b). *p*-coumaric and caffeic acid, most frequently found in industrial apple must and usually in apples, both appear in the esterified form with chinic acid to form *p*-coumaroilchinic and 5′-cafeoilchinic acid also known as chlorogenic acid, the substrate of polyphenol oxydase (PPO) from the initial phase of browning enzymatic reaction.

The monomers of catechin represent between 15% and 20% of the total of polyphenolic compounds in apple varieties for cider. The polyphenols of (–)epicatechin, also known as procianidols, are composed of important monomers in apple cider because they can make up 90% of the phenolic compound of some varieties (Sanoner et al. 1999). The phenolic compounds are characterized by the number of constituent units or degree of polymerization (GPn). Other flavonoids are divided into two other smaller classes, the flavonoids derived from quercetin and located in the epicarp and the dihydrochalcones like floridzine, the xyloglucoside of phloretin.

12.4.2 Must Extraction

The raw material is stored in silos or piled up. The precleaning stage removes soil and vegetable waste, selecting the fruits and rejecting the bad. Finally, the apples are washed in tap water. The process of

milling is carried out in a mill or grater. The milling equipment produces a thin pulp, which allows high yields within a short pressing time. The control of pulp thickness is quite important because the texture of apples may vary from one group to another. It should be thin and solid, avoiding the formation of an amorphous mass without any resistance that would result in losses in pressing yields (Nogueira et al. 2005; Nogueira and Wosiacki 2010).

Mashing is the stage before pressing. It can take up to 5 minutes (hot procedure) or several hours (cold procedure), depending on the technology used by the processor. At this processing stage, commercial enzymes with pectin and cellulolytic activities may be added to the mash to increase the yield, sometimes reaching between 80% and 85% based on the amount of raw material (Shahidi and Naczk 1995).

The traditional form of extraction using a hydraulic press consists of pouring small amounts of mash in 20 × 20 cm plastic or nylon sieves, piling them on top of each other, and the fluid is obtained by pressing. This is the traditional press method by which the juice is extracted from the higher layers of cellular material passing through the lower layers with a filtration occurring at this stage that supports the process.

A low initial pressure allows the extraction of the inner juice, creating a flow channel, and then a higher pressure is maintained until the juice stops flowing. Such old type pressure equipment is now almost obsolete and has been substituted by belt presses or, in high production units, by a larger horizontal press. The principle is still basically the same, extracting the juice from the broken raw material using the factors time × temperature to achieve a maximum yield. The mash consistency of the raw material is what determines the resistance. Depending on many factors, within approximately 30 minutes, the yield may reach between 65% and 70% (Michel 1987). Some alternative procedures on the basis of the use of coadjutants like cellulose and rice hull in the ratio of 1% to 2% may provide an increase of approximately 10% of the yield, but it then becomes impossible to use the pomace (Binnig and Possmann 1993).

The extraction process normally produces natural juice of approximately 600 to 700 L ton^{-1} and the pomace (pressing solid waste) that represents approximately 300 to 400 kg per apple ton. Pomace has a large amount of soluble sugar that may be extracted by the addition of water in the process known as diffusion, which is normally used when apple production is low. Industrial processing uses the addition of water in a continuous way in belt presses in countercurrent, which is only viable if the final product is submitted to a traditional concentration (concentrated juice) for further commercialization. Accordingly, there are three kinds of must resulting from this process: must derived from pure juice, must derived from diffusion, and must derived from a mixture of both (Drilleau 1985, 1996).

12.4.2.1 Apple Must Clarification

The stage known as prefermentation includes the period between pressing until the beginning of alcoholic fermentation (Drilleau 1990a,b). Arriving from the extraction press, the must contains hydrocolloidal pectic substances that cause fluid viscosity.

Industrial must clarification is performed by one of two available processes in the agro-industrial sector, by flotation, by pasting, or both, after a preliminary enzymatic depectinization. Such procedures include two main kinds of reaction, which cause the reduction of molecular size and the loss of the identity of polysaccharides as hydrocolloids: that of the hydrolysis and the other by beta-transelimination. The enzymatic hydrolysis of the methoxyl group only modifies the electrostatic negative charges that arise after the action of the pectinesterase.

Clarification through flotation is the method most used in cider processing and requires a period that may vary from 3 to 8 days depending on the temperature. The processing is based on enzymatic activity in the juice or must derived from raw material or added as an adjuvant. The pectinametilesterase promotes a change at the molecular level releasing methanol in the environment and increasing the number of electric charges, which keeps in balance as a function of pH. This structure is loaded with more negative charges and can bind more with calcium, leading to the formation of a gel when in the presence of excess calcium and sucrose but in the processing of juice can be removed more easily as pectinate calcium.

When the oxidative yeasts begin the fermentation process, the CO_2 produced causes a drop in the apparent density of this product, which rises to the surface in a shape of a brown mousse known as a

"brown hat." However, for this operation to be successful, the must cannot be sulfated nor can the outside temperature be beyond the range of 10°C to 15°C, which favors the start of natural fermentation in a slow way. If not, with the start of rapid fermentation, the great amount of bubbles released by the yeast cells would prevent the flotation process of the brown hat.

Pectinesterase and calcium are present in apples at low concentrations, which prevent clarification without the utilization of coadjutants, such as the addition of commercial enzyme preparations and of calcium chloride in soluble form (Le Quere and Drilleau 1993). The density of the must after flotation is close to that when flowing from the press, and the liquid must should be separated by siphoning, then the clean liquid is transported to the industrial fermentor (Michel 1987). However, clarifying by flotation is less efficient with the must obtained from acid apples because the low content of pectic substances and the high levels of malic acid interfere with the construction of the gel structure of the calcium pectinic salt.

In this way, the more effective depectinization is an interesting alternative for this kind of must. The process consists of breaking some glycosidic linkages in the main chain of the pectic substance by using a commercial pool containing, among others, polygalacturonases and transeliminases; the first breaks the glycosidic links by a hydrolysis reaction, and the second, by an electronic shift that favors the formation of a double linkage among the carbon C4 and C5 of the methoxylated galacturonic residue. This breakage in the main reaction promotes a drop in viscosity whereas the molecular chain is dramatically reduced, which withdraws them from the hydrocolloid system, allowing them to be sedimented from the solids in suspension, making what is known as *lees,* possible. Such a reaction occurs in a period between 10 and 24 hours, depending on various factors, among them, the enzymatic activity and the system temperature, and the clarification process finishes with the removal of hydrolyzed and oxidated compounds by pasting with gelatin and bentonite, the latter by filtration (Drilleau 1985; Le Quere 1991; Lepage 2002).

12.4.2.2 Sulfur Dioxide

Sulfur dioxide (SO_2), also known as sulfurous anhydride or gas, was used in the nineteenth century in wine processing, but it was only at the beginning of the twentieth century that this procedure was studied by academic researchers. Currently, SO_2 is being used under the structure of anhydride and expressed in milligrams per liter (ppm) or grams per hectoliter. Whether the SO_2 is gas or liquid solution of potassium metabisulfite, the effects are in the same order of magnitude.

The equilibrium between the different structures is identical, but it will depend on the pH and on the presence or absence of $-SO_2$ combining compounds (Ribereau-Gayon et al. 1998; Beech 1993).

Sulfur dioxide's properties include the following: (1) antiseptic—it prevents the development of microorganisms and is more effective on bacteria than yeast. In this way, it is used in the must after pressing to eliminate bacterial contaminations and to promote the development of yeast *Saccharomyces* and in the final product as a microbiological stabilization agent. (2) Antioxidant—it reacts with dissolved oxygen and prevents oxidation but the enzymes are faster. (3) Antioxidasic—it prevents the activity of oxidases present in the culture media, but such a process also occurs very slowly. Enzyme browning, in some cases, is necessary to attenuate cider color (Ribereau-Gayon et al. 1998; Lea 1995; Blouin 1993; Sayavedra and Montgomery 1986).

The treatment of must with a low concentration of sulfur dioxide (SO_2) before fermentation is the method used in the control of undesirable microorganisms and also to promote the growth of the yeast *Saccharomyces*, which shows a low resistance to SO_2. In Switzerland, the must is treated with 35 to 40 mg L^{-1} of SO_2 and inoculated 2 days later. In Brazil, the concentration of SO_2 varies from 30 to 50 mg L^{-1}, depending on the phytosanitary conditions of the fruit. In countries that use natural fermentation like France and Spain, such treatment is seldom used (Downing 1989; Nogueira 2003).

Sulfur dioxide, as a solution of potassium metabisulfite with approximately 50% free SO_2, may be added as follows: 75 mg L^{-1} between pH 3.0 and 3.3; 100 mg L^{-1} between pH 3.3 and 3.5; and 150 mg L^{-1} between pH 3.5 and 3.8 (Beech 1972; Burroughs and Sparks 1973). In musts with pH higher than 3.8, 200 mg L^{-1} of sulfite, the maximum limit in France and England, does not reach the desired level of efficiency (in Brazil, the limit is 350 mg L^{-1}). It is recommended that musts with high pH levels should be mixed with musts from acid fruits or receive DL-malic acid before sulfating. After 6 hours of resting, the dosage of free sulfur dioxide is performed for process control (Downing 1989; Lea 1995).

The SO_2 is used in the finished product in two situations: first, in a preservation dose when the product will not be placed on the shelf for 3 to 6 months (dry cider, 30–40 mg L^{-1}; sweet cider, 40–80 mg L^{-1}) and, second, in cider for immediate consumption (dry cider, 20–30 mg L^{-1}; sweet cider, 30–50 mg L^{-1}) where the product is for sale. Thus, the product is protected against oxidation, the development of contaminating microorganisms, and re-fermentation in sweet ciders (Nogueira and Wosiacki 2010).

SO_2 is essential in the manufacture of cider in Brazil, given the high temperatures at the time of processing, with a mean temperature of 25°C, which can cause contamination at different stages of processing. However, the excess sulfur dioxide masks the aroma of cider as well as producing a suffocating, irritating odor and the feeling of "burning" at the end of tasting. Another drawback is that SO_2 can be toxic and can cause symptoms of nausea, regurgitation, and gastric irritation when used above the dosage allowed by law for humans. For asthma sufferers, the effects of intoxication may be higher. The tolerable dose for humans established by the North American Food and Drug Administration is 0.7 mg kg^{-1} per day, which makes it acceptable for a daily dose of 42 to 56 mg depending on body weight of between 60 and 80 kg, respectively. It has still not been possible to find a product to substitute SO_2 in terms of its oenological properties and which does not display these drawbacks.

12.4.3 Fermentation

The fermentation of cider is the result of the action of a complex microbiota on a substrate that is also complex (Le Quere and Drilleau 1993). The alcoholic fermentation of French cider demonstrates different characteristics from other industrial fermentations in that it is slow (taking from 1 to 2 months), partial (retaining residual sugars because the level of alcohol does not exceed 5%), and mixed because it is led by a complex microbiota (Michel et al. 1990). These three fundamental differences are not present in the Brazilian method of processing.

Table 12.4 identifies the main microbial species found in the fermentation of cider, including 500 strains of yeast isolated from the must and the equipment in both the artisanal and the industrial production of cider in the French regions of Brittany and Normandy. The *Saccharomyces cerevisiae* strain is the most common (Le Quere and Drilleau 1993, 1998; Michel et al. 1988). Beech (1993) confirmed these results by analyzing the natural microbiota of apple musts in England, and he noted the presence of *S. cerevisiae* and species such as *Pichia*, *Torulopsis*, *Hansenula*, and *Kloeckera apiculata*, which is the imperfect form of *Hanseniaspora valbyensis* (Shehata et al., quoted by Michel et

TABLE 12.4

Major Microorganisms Found in Apple Wine Fermentation in France and Spain

Class of Microorganism	French Cider Processing[a]	Spanish Cider Processing[b]
Yeast	*Brettanomyces* sp. *H. valbyensis* *M. pulcherrima* *S. cerevisiae* var. *uvarum*	*M. pulcherrima* *Pichia guilliermondii* *H. valbyensis* *H. uvarum* *C. parapsilosis* *S. cerevisiae* *Saccharomyces bayanus* *Saccharomyces pastorianus* *Saccharomyces kudriavzevii* *Saccharomyces mikatae*
Lactic acid bacteria	*Lactobaclillus brevis* *Leuconostoc mesenteroides* *L. oenos*	ND
Acetic acid bacteria	*A. aceti* *G. oxydans*	ND

Note: ND, not determined.

[a] *Source:* Michel, A., et al., *Belg J Food Chem Biotechnol*, 45, 98–102, 1990. With permission.

[b] *Source:* Bedriñana et al., *Food Microbiol*, 27, 503–8, 2010. With permission.

al. 1988). In Spain, yeasts were isolated from apple juice and at different stages of fermentation (see Table 12.4). The species *Candida parapsilosis* is reported here for the first time in cider (Bedriñana et al. 2010).

12.4.3.1 Apple Wine Fermentation

The French cider fermentation process comprises two distinct phases: first oxidation and then fermentation.

12.4.3.1.1 Oxidation Phase

The oxidation phase, carried out by low action yeast fermentation, can last between 5 and 15 days after the preparation of the must and generally corresponds to the consumption of approximately 10 g L^{-1} of sugars and 30–40 mg L^{-1} of nitrogen (Le Quere and Drilleau 1998). The yeast species found at this stage, especially *Metschnikowia pulcherrima* and *H. valbyensis*, participate in the process of clarification by flotation through the release of CO_2 and dominate the medium of fermentation for a few days before being overtaken by the yeast strains, *S. cerevisiae* var. *uvarum* and sometimes *S. cerevisiae* (Michel et al. 1988; Nogueira 2003). The action time of the oxidative strains varies depending on the microorganism, its initial population, and the availability of oxygen during the growth phase. The fermentation of cider achieved only with yeast fermentation produces sensorial results such as a neutral aroma and/or less typically with the presence of the *Hanseniaspora* strains a fruity aroma and esters such as ethyl acetate and phenyl-ethyl. However, alcoholic fermentation is sometimes achieved solely under the influence of oxidative yeasts (peaked), resulting in a significant production of ethyl acetate, harmful to the aroma of the product (Drilleau 1996). The oxidative microorganisms and weakly fermentative yeasts can be completely inhibited by the addition of sulfurous anhydride, a common practice in many countries.

12.4.3.1.2 Fermentation Phase

At the start of fermentation, the oxidative yeasts are superseded by fermentative strains (Figure 12.2). Alcoholic fermentation, responsible for the transformation of sugars into ethanol and carbon dioxide, occurs in most cases under the action of the microorganism present in the fruit and processing material (Valles et al. 2007; Michel et al. 1988; Dierings 2008). However, there are disadvantages such as the variation in the quality of the beverage and the risk of contamination. The use of pure cultures of yeast, usually in the active dry form, is an interesting tool for the standardization of the product (Valles et al. 2008; Cabranes et al. 1997). The *Saccharomyces* yeasts grow during the oxidative stage with initial population of approximately 6.0×10^4 CFU mL^{-1}, stabilizing its population after an intake of from 10 to 15 g L^{-1} of sugars and presenting maximum population of approximately 1.0 to 4.0×10^7 CFU mL^{-1}, in which case the fermentation speed reaches its maximum value (Le Quere and Drilleau 1998; Drilleau 1996).

The slow pace of fermentation is intended to obtain a product with a better sensorial quality and brings practical benefits for the producers, facilitating the organization of the treatments during the process.

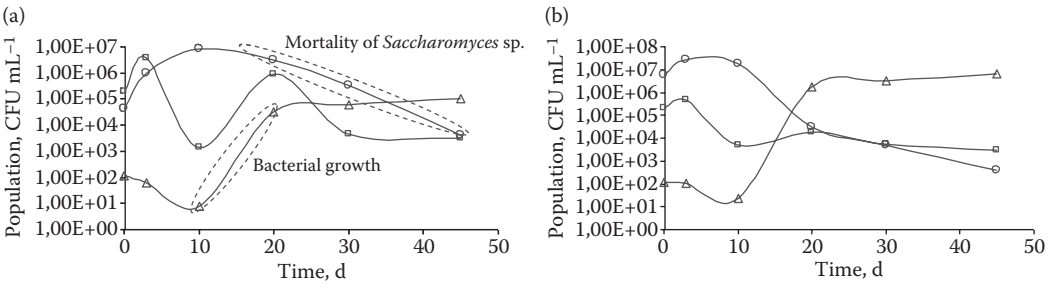

FIGURE 12.2 Development of the (○) fermentative yeast, (□) non-*Saccharomyces* yeast, and (△) lactic bacteria during fermentation, (a) natural and (b) with inoculum of *S. cerevisiae* Arôme plus (2.0×10^6 CFU mL^{-1}) in apple wine processing. The fermentations were led at 20°C.

To reduce the speed of fermentation, some technological strategies are used, such as low temperatures, clarification by flotation, transfers, filtrations, and centrifugations.

12.4.3.1.3 Sensory Quality

In the traditional French method, slow fermentation has always been regarded as necessary to obtain a quality cider. Among the products used to aid rapid fermentation, the presence of the aroma of yeast masks or substitutes the fruity aroma considered beneficial to quality. This undesirable aroma of yeast decreases slightly during ripening, but if it is expressed during fermentation, then it will remain in the final product (Le Quere 1991). Compounds such as diacetyl and acetoin show undesirable sensory effects typical of rancid products. These substances are produced in large quantities in rapid fermentations because of high temperatures and the presence of a large population of yeasts (Drilleau 1991a,b).

In Brazil, the situation is different because the fermentation is complete and rapid, that is, with total consumption of sugars. Thus, there is a high intensity of undesirable flavor at the end of fermentation because the oxidative microorganisms are affected by the action of SO_2. In addition, yeasts can use other substrates such as organic acids because the sulfation of the must affects or inhibits the growth of lactic bacteria, which impairs the body of the final product. The phenolic compounds decrease because of the associations with the cell wall of the yeast, the reactions during the oxidation, and the clarification operations, which result in a product with little color and astringency (Renard et al. 2001; Nogueira et al. 2004).

12.4.3.1.4 Technical Strategies to Slow the Pace of Fermentation

Fermentation should be stopped before the total consumption of sugars, that is, with residual sugar content compatible with the type of product desired. If the fermentation is fast, the company will have a very short amount of time to achieve "stabilization"; that is, stop the fermentation before all the sugars are consumed. Finally, slowing the speed of fermentation makes the organization of work easier, allowing treatment in times of increased availability of labor (Drilleau 1991a,b).

Low temperatures can reduce the activity of yeast cells. Temperatures lower than 5°C provide interesting results in relation to aromas but require costly installations with thermal insulation and adequate cooling capacity (Drilleau 1991a,b). The temperature range in the fermentation of the cider is between 7°C and 15°C, interfering in its speed; however, this is not the only factor (Lepage 2002).

The flotation method also affects fermentation kinetics as confirmed experimentally with a test without clarification (control), which lasts 20 days, compared with the same must clarified by the flotation method, which can reach 48 days (Lepage 2002). The elimination of yeast and bacteria in the *chapeau brun* promotes a nutritional depletion of the must in particular by reducing the concentration of nitrogen (Beech and Challinor 1951) because the first generations of these microorganisms used the nitrogen to grow, consuming approximately 30% to 60% of the initial nitrogen (Le Quere 1991; Drilleau 1990a,b). In addition to slowing down the speed of fermentation, this practice allows a clear fermentation, considered favorable to the formation of aroma constituents. When flotation is performed correctly, the fermentation stops naturally before all the sugars are consumed (Drilleau 1985).

Traditionally, producers carry out successive rackings to remove the precipitated biomass, called sludge, reducing the nutritional potential of the medium by the same principle of flotation. However, when the initial fermentation rate is high, this causes a convection current in the system that prevents sedimentation.

Currently, these two practices (flotation and racking) are rarely sufficient to slow the fermentation because the nitrogen concentration doubled or tripled in the last 50 years because of excessive nitrogen fertilization. Therefore, flotation and racking are often ineffective, and thus it is necessary to compensate for this change in the raw material by centrifugation or filtration. To systematize the removal of the biomass, a clarification is used (during the growth of the yeast) by filtration or centrifugation, performed after the consumption of 10 g L^{-1} of sugars. Until this moment, the maximum population is not achieved, and growth can restart; providing a slow fermentation rate from 2 to 6 g L^{-1} of sugar per week (Le Quere 1991).

Centrifugation is widely used to decrease the amount of biomass and consequent speed of fermentation. Its action is little influenced by the amount of suspended material and by the presence of residual pectic substances and can thus be used as a security operation, when flotation is not enough to achieve

the expected results. Centrifugation can reduce the yeast population from 7.5×10^7 to 3.8×10^4 CFU mL^{-1}, but there is the inconvenience that the bacteria are not separated in the centrifugation process by their size. The elimination of yeast can lead to a proliferation of the bacterial population because of a lack of competition or inhibition between them. To make matters worse, the difficulty of cleaning certain machinery and pipes increases the inoculum potential of bacteria, such as aeration produced by the operation. The bacterial population of 10 (before the trial) can move 1.8×10^4 CFU mL^{-1} (Le Quere 1991; Drilleau 1990a,b).

Filtrations on silica gel plates and on membranes, commonly used in finishing the product, are effective in reducing the biomass and thus slowing down the fermentation. However, to be carried out (in terms of cost), the filtration should be used with a must without pectic material and with few particles in suspension. Flotation is a vital preoperation. When filtration is performed after reducing density by five points, the normal fermentation period of 20 to 30 days (final density of 1015–1020 kg m^{-3}) extends to 2 to 3 months because of the elimination of 80 mg L^{-1} of total nitrogen. Another benefit of this operation is the reduction of bacteria; however, this reduction is lower than that observed in the amount of yeast. In contaminated cider, silica gel filtration decreased the yeast population from 3.2×10^4 to 20 CFU mL^{-1} and the acetic bacteria population from 1.4×10^5 to 2.0×10^4 CFU mL^{-1} (Le Quere 1991; Drilleau 1990a,b). In the 1980s, microfiltration and ultrafiltration membranes began to be used in cider processing. In this operation, the liquid travels tangentially to the membrane with pores that ensure the conduct of the filtrate. The microfiltration or ultrafiltration membranes contain pores, the size of tenths of microns, which eliminate, after filtration, most microorganisms, still requiring however an inoculation with dry active yeast (Drilleau 1985). There are precautions required regarding the efficiency of the operation because if the filtration is carried out too late, there is a risk that fermentation will be stopped completely, but if it is carried out too early, it may be ineffective (Drilleau 1991a,b; Nogueira et al. 2008).

12.4.3.1.5 Fermentative Metabolism of the Yeast Saccharomyces

The simple sugars (fructose and glucose) are degraded within the cytoplasm, and only sucrose is hydrolyzed by invertase bound to the cell wall on the outside of the yeast. Water, carbon dioxide, and oxygen molecules are the only molecules that are absorbed or excreted by simple diffusion because of the solubility in lipids of the biomembranes. All other molecules entering or leaving the cytoplasm require a process of either facilitated diffusion or active transport (Marc 1982). The hexoses penetrate the cell by facilitated diffusion, which corresponds to the passage of a compound through the membrane against a concentration gradient, accelerated by a transporter of protein nature (Lagunas 1993). Once in the cell, carbohydrates and nitrogenous substrates undergo various transformations. The simple sugars glucose and fructose are direct substrates of glycolysis (Embden–Meyerhof–Parnas pathway) being rapidly degraded (Figure 12.3). This phase requires the involvement of three enzymes for the phosphorylation of these sugars: the hexokinase PI and PII and glucokinase (Maitra 1970). The two hexokinases enzymes PI and PII are able to perform the phosphorylation of glucose and fructose, but with different yields (3:1 ratio in favor of glucose), whereas the glucokinase phosphorylates the glucose only when in high concentrations. These differences explain why the glucose is consumed faster than fructose in fermentation and also why there is a higher concentration of glucose to fructose at the end of the process (D'Amore et al. 1989; Marc 1982).

The phosphate sugars are subsequently transformed into pyruvate by the glycolytic pathway (Figure 12.3). The hexose phosphates (fructose-6-phosphate and glucose-6-phosphate) are important molecules used in other secondary metabolisms, especially in the synthesis of polysaccharides, molecules involved in cell wall synthesis in yeast. For each sugar molecule metabolized, two molecules of pyruvate are formed (Walker 1998). The pyruvic acid is an intermediate in glycolysis and the precursor of many compounds (Whiting and Coggins 1975, quoted by Drilleau 1996).

In aerobiosis, breathing permits the entire substrate oxidation of carbon through the Krebs cycle and oxidative phosphorylation (Alberts et al. 1997). In anaerobiosis, pyruvate is mainly oriented toward the production of ethanol by regenerating the cofactor NAD^+, consumed at the level of glyceraldehyde-3-phosphate. Pyruvate is decarboxylated into acetaldehyde (final receptor of electrons) by the enzyme pyruvate decarboxylase (cofactor, thiamine pyrophosphate) and then reduced to ethanol by alcohol dehydrogenase to form NAD^+ (Figure 12.3). The synthesis of a degree of ethanol (1% v/v) in alcoholic

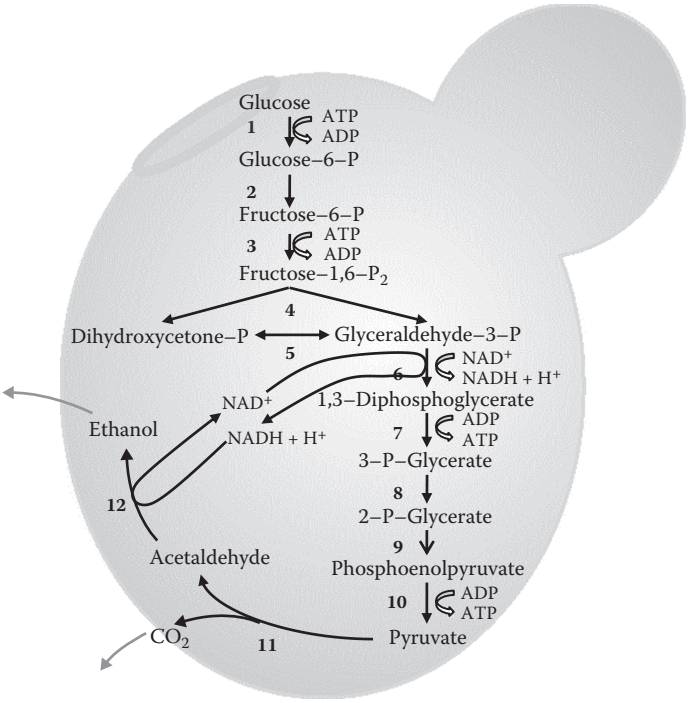

FIGURE 12.3 Simplified metabolic pathway of the *S. cerevisiae* during alcoholic fermentation. Glycolysis enzymes: (1) hexokinase; (2) phosphoglucose isomerase; (3) phosphofructokinase; (4) aldolase; (5) triosephosphate isomerase; (6) glyceraldeyde-3-phosphate dehydrogenase; (7) phosphoglycerate kinase; (8) phosphoglycerate mutase; (9) enolase; (10) pyruvate kinase; (11) pyruvate decarboxylase; and (12) alcohol dehydrogenase.

fermentation represents an average consumption of 17 g L^{-1} of reducing sugars (glucose and fructose). Ethanol, as a small hydrophilic molecule, crosses the plasma membrane by simple diffusion (Jones 1988; Walker 1998). From the standpoint of energy, glycolysis provides two molecules of ATP per molecule of degraded glucose, that is, 14.6 kcal/61.08 kJ biologically usable per mole of fermented glucose (Ribereau-Gayon et al. 1998).

The yeast *S. cerevisiae* synthesizes its proteins during fermentation through the incorporation of nitrogen compounds present in the must. This finding explains the fact that in winemaking conditions, yeast is able to synthesize the amino acids it needs from sources of available nitrogen. For this reason, *S. cerevisiae* has an extraordinary capacity for transamination between amino acids and ketone α (Jones et al. 1969).

The microorganisms use part of the Krebs cycle to synthesize the amino acids and lipids necessary for growth. The yeast is able to synthesize the vitamins, sterols, and nucleic acids necessary for their multiplication, but the inorganic elements essential for the functioning of enzymes are directly extracted from the must (Ribereau-Gayon et al. 1998).

12.4.3.2 Importance of Nitrogen Compounds

The nitrogen compounds in the must play an important anabolic role in the biosynthesis of proteins and in the enzymatic functions, influencing the growth and metabolism of yeasts, which constitutes 10% of its dry matter (Walker 1998; Henschke and Jiranek 1992).

Currently, French producers seek less radical or cheaper techniques to limit the number of interventions on the product and carry out fermentations with small amounts of yeast. This concern is common in factories that receive apples from new orchards with high nitrogen concentration, even after mixing with other musts that are low in nitrogen concentration (Drilleau 1993). However, not all producers, whether large, medium, or small, provide an analytical control of the nitrogen in the must before starting the production of cider, which may further undermine the process.

12.4.3.2.1 Nitrogen Compounds in Must

Apple must contain different amounts (44–329 mg L^{-1}) of nitrogen (total nitrogen by Kjeldahl): amine form, 15.2% to 61.2%; amide form, 5% to 30%; and maximum ammonia form, 1% (Burroughs 1957). Alberti et al. (2011) evaluated 50 musts from Brazilian apples and found similar values from 59 to 330 mg L^{-1}. The analysis of total nitrogen in 27 musts of apples harvested in France in 1993 showed that 25% had less than 75 mg L^{-1} (low levels), 51% had 75 to 155 mg L^{-1} (normal levels), and the remaining 24% had values higher than 150 mg L^{-1} (high concentration). For the same variety at harvest, the nitrogen content varied among the orchards, and even in the same orchard, a single variety varied in terms of nitrogen content according to the harvest (Drilleau 1996; Lea 1995).

The concentration of total nitrogen is constantly increasing because of the age of the orchards. It was observed that the younger the orchard, the greater the level of total nitrogen in the must, reaching as high as 2000 mg L^{-1}, and the older the trees, the lower the concentrations of these compounds. Because many of the old orchards are being relocated, as well as excessive nitrogen fertilization, this no longer allows the partial fermentation of must just by controlling the nitrogen concentration, as recommended by Jacquin and Tavernier (1951).

By analyzing the different forms of nitrogen in apples and musts, it was demonstrated that after pressing, a fractionation of nitrogen compounds occurs, some are retained in the bagasse et al. go to the must in amounts that vary according to the physiological state of the fruit, including the degree of ripening (Baron et al. 1982).

Nitrogen, in the form of free α-amine present in apple must, constitutes up to 50% of total nitrogen of the fruit, and this value tends to decrease after the fruit-ripening period. Among the amino acids, five of them (asparagine, glutamine, aspartic acid, glutamic acid, and serine) may represent 86% to 95% of the total amino acids of apple must and are the most easily assimilated by the yeast. When the fruit comes from new or old orchards with high nitrogen levels, the presence of these amino acids can be multiplied by a factor of 2 to 5 times (Baron et al. 1977).

12.4.3.2.2 Influence of Nitrogen in Fermentation

The growth of microorganisms occurs in the presence of nutritional factors that provide the different needs of the cell, such as carbohydrates, nitrogen, minerals, and vitamins. If one of these factors is in low concentration, not catering to the needs of the cell, it will be fully and rapidly consumed causing the growth of the microorganism to be interrupted. This is known as a limiting factor. Traditionally, nitrogen is considered as a limiting factor in the fermentation of cider. In the processing of cider, a low concentration of total nitrogen was considered a necessary condition for the conduct of slow fermentation and stabilization of density; that is, the presence of residual sugar in the final product without nutrients will not allow re-fermentation. It was observed that for this to happen, the ratio *N/A* of the must should be less than 0.4 (*N* is the total concentration of nitrogen in the must mg L^{-1}, and *A* is the concentration of total sugars in g L^{-1}; Jacquin and Tavernier 1951).

The same authors found, through synthetic means, that the total organic nitrogen of 50 mg L^{-1} was completely consumed during fermentation; after day 12, it was almost completely stabilized, and at the end of day 17, it showed a density of 1025 kg m^{-3}. The same musts with 100 and 150 mg L^{-1} completed fermentation, reaching a density of 1000 kg m^{-3} at the end of day 17 with residual nitrogen, showing that the fermentative power of yeast is directly proportional to the concentration of nitrogen. This explains the stabilization "in density" of musts with low concentrations of nitrogen. Moreover, the consumption of amino acids by the yeast makes the environment less favorable for the development of bacteria in the first two weeks of fermentation. During fermentation, the yeasts release glutamic acid and valine, essential for bacterial growth during the process of malolactic bioconversion.

12.4.3.3 Importance of Oxygen

Gas exchange between cider and the environment is a function of equilibrium of partial pressures of oxygen, via the atmospheric air. This is a gas that is slightly soluble in fluids, noting that its solubility at a certain temperature in pure water is comparable with that observed in various water–alcohol solutions

such as wine (Moutounet and Mazauric 2001; Vivas 1999). Moreover, the presence of colloid substances promotes the solubilization. Oxygen is not a gas that is found in high concentrations in wine, and unlike gaseous nitrogen or CO_2, oxygen in wine, once dissolved, is quickly consumed and used in redox mechanisms, until it completely disappears (Vivas 1999).

Figure 12.4 illustrates the magnitude of the presence of oxygen in the face of dissolution after different prefermentation unit operations, from extraction of the must until clarification. Similar results were obtained in the processing of wine by Vivas et al. (2003). However, it is worth mentioning that these values may vary depending on the technology used in processing.

12.4.3.3.1 Oxygen and the Must

In enology, oxygen influences the quality of wine by the biological action of yeast and alcoholic fermentation and the chemical and biochemical action on the must before or after fermentation. Regarding the latter point, it is observed that after the addition of oxygen, large quantities of oxygen are consumed spontaneously by the must through the oxidation of phenolic substances catalyzed by polyphenoloxidase. The oxidation reactions transform the native phenolic compounds of the apple must, most of which are either colorless or yellow or brown compounds, which is known as the browning reaction. For this to occur, three actors are required: polyphenols, polyphenoloxidases, and oxygen. In general, the reaction speeds can be influenced by the concentrations of these agents, by nature, by the proportions of native phenols, and also by the effect of acidity (pH) and temperature of the must.

The browning mechanism has two steps: an enzymatic reaction and a chemical oxidation reaction. The main property of hydroxycinnamic acids is their sensitivity to oxidation by an enzyme (Nicolas et al. 1994). In fruits, they are substrates for polyphenoloxidase, which functions as an oxidoreductase, which in the presence of oxygen catalyzes two types of reactions: first, the hydroxylation of cresols in ortho-diphenols through cresolase activity and, second, the oxidation reaction of ortho-diphenols to yellowish ortho-quinones through catecholase (Figure 12.5). In apples, chlorogenic acid and catechins are particularly sensitive to the action of polyphenoloxidase (Gunata et al. 1987; Aubert et al. 1992, quoted by Lepage 2002). However, *para-coumaroylquinic* acid is a competitive inhibitor of the reaction, and *procianidols* have an inhibitory effect on polyphenoloxidase (Janovitz-Klapp et al. 1990). The intensity of inhibition increases with the degree of polymerization (GPn) substrate. The laccases of essentially fungal origin are of a different class from the polyphenoloxidases (Mayer and Harel 1991). They are able to oxidize the *o*-diphenols and *p*-diphenols to *o*-quinones and *p*-quinones, respectively. They are not found in healthy apples, but when the cider is processed with raw material of an advanced degree of maturity, there may be a darkening upon exposure to the air, forming a reddish brown complex called *casse oxidasic*.

The ortho-quinones react very quickly in the environment by various mechanisms (e.g., additions and coupled reactions) using other phenolic compounds such as catechins and procianidols. The procianidols

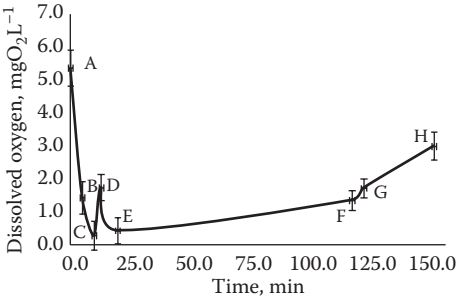

FIGURE 12.4 Dissolved oxygen during apple juice processing. (A) Pressing; (B) 5 minutes after pressing; (C) 10 minutes after pressing; (D) pectinase addition; (E) heating at 40°C for enzyme action; (F) end of depectinization process; (G) racking; (H) filtration. Brazilian cider processing conditions: commercially discarded apples (Gala and Fuji), temperature at 25°C, 975 m a.s.l., and pilot plant of the apple work group.

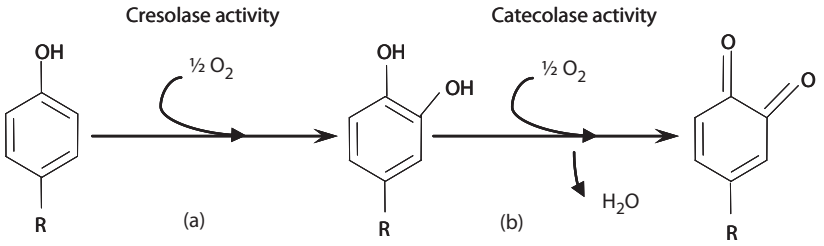

FIGURE 12.5 Reactions catalyzed by polyphenoloxidase. (a) Hydroxylation of monophenol in *o*-diphenol. (b) Dehydrogenization of *o*-diphenol in *o*-quinone.

of the apple are PPO inhibitors but can be oxidized by an oxide-reduction system, and these reactions lead to the formation of yellow and brown pigments called melanoidins (Lea 1984a,b).

12.4.3.3.2 Oxygen and Alcoholic Fermentation

Studies carried out in the wine and beer industries have shown the favorable effect of the addition of oxygen in the kinetics of alcoholic fermentation. It was shown that to develop and maintain a good viability (reproductive capacity) and a good vitality (ability to produce energy), yeasts require low oxygen concentrations (10 mg L^{-1}; Salmon et al. 1998). This is necessary for the synthesis of sterols and unsaturated fatty acid constituents of the plasma membrane essential for cell growth (Andreasen and Stier 1953, 1954). Nogueira et al. (2004) and Alberti (2011) found that the dissolved oxygen content in apple must for the complete fermentation of sugars, when nitrogen is not limiting, is between 2.0 and 3.0 mg L^{-1}.

The relationship between oxygen and fermentation depends on the mode of conduct from the start. The moment of the addition of oxygen and also the quantity added are important criteria to achieve the expected result. In the case of wine, it is undesirable to add oxygen before or at the inoculum of yeasts, when many enzymes such as polyphenoloxidase may still be active, thus increasing the risk of oxidation, and the addition of oxygen is less effective for yeasts. The optimum time recommended for the additions is during the first quarter of alcoholic fermentation. Only oxygen is added and perhaps delayed until the middle of fermentation to add the assimilated combined oxygen and nitrogen (Sablayrolles and Barre 1986; Sablayrolles et al. 1996). Different methods of adding oxygen to the process have been discussed. These steps, when performed with nitrogen, can help improve the kinetics of fermentation and decrease the risk of sluggish fermentations (Blateyron et al. 1998).

12.4.3.3.3 Oxygen and the Yeast Saccharomyces

S. cerevisiae belongs to the facultative anaerobic groups of yeast that are capable of using glucose both aerobically and anaerobically, with preference for the latter (Van Dijken et al. 1993). The concentration of sugars and oxygen in the fermentation system directs the metabolism of the yeast for the fermentation process or the respiration. The different pathways involved in oxygen consumption have been much more studied in conditions of respiratory metabolism than in alcoholic fermentation. The growth of yeast under anaerobic conditions normally requires oxygen in minimum rates to encourage the synthesis of sterols and unsaturated fatty acids, and these compounds, known as anaerobic growth factors, are essential for the integrity of the plasma membrane. Molecular oxygen is also necessary in the cyclization of squalene for the synthesis of fatty acids where $½O_2$ is necessary for the formation of each double bond in positions Δ^9, Δ^{10}, and Δ^{15} of carbons 16 and 18 of the respective fatty acids (Ratledge and Evans 1989). During beer fermentation, the maximum oxygen levels for the biosynthesis of fatty acids is 0.3 to 1.0 mg L^{-1} of dissolved oxygen (Kirsop 1977).

12.4.3.3.4 Metabolic Aerobic Pathway

Cellular respiration consists of a multistep energy recovery process necessary to maintain the yeast cell structure and functionality, including cellular growth, and is supported by the oxidative phosphorylation of ADP to ATP, representing the easiest mechanism to manage the stored chemical energy. In this

high-energy compound, produced by the regeneration of many cofactors in a stepwise way down to the final acceptor electron, the oxygen is driven to the metabolic transformation according to the physiological needs of the microbial fermenting cell. The metabolism of oxygen in yeast is mainly achieved using the Embden–Meyerhof–Parnas pathway located in the mitochondria where many enzyme systems drive the oxygen consumption in this classic set of reactions or to an alternative pathway (Ainsworth et al. 1980: Goffeau and Crosby 1978; Henry and Nyns 1975; Laties 1982).

12.4.3.4 Volatile Compounds

The volatile compounds of cider are largely qualitatively identical to those of all other fermented beverages. Hundreds of volatile compounds that contribute to the aroma of cider isolated from different yeasts have been identified and listed (Williams and Tucknott 1971, 1987; Williams 1980; Williams and May 1981, quoted by Lea and Drilleau 2003). Ciders have a characteristically high content of secondary alcohols, especially 2-phenyl-ethanol, formed over the status of nutrients in the must (Lea 1995).

However, during fermentation, a compound, which is considered to be exclusive to cider and is known for its "cidery" aroma, can be formed. This has a molecular weight of 172 Da and is present in low concentrations, but it contributes to the aroma of the product (Lea 1995).

Diacetyl is synthesized from pyruvate by *Leuconostoc* species during malolactic fermentation and positively contributes to the buttery aroma in wines and ciders. Even at this stage, aromas that can be described as spicy and phenolic can be formed, especially when fruits are classified as acid-bitter. These compounds are the ethyl–ethyl–phenol and catechol resulting from hydrolysis, decarboxylation, and reduction of *p-coumaroyl*quinic and chlorogenic acid, respectively (Beech and Carr 1977). Ciders of superior quality taste sweet and fruity and fragrant, whereas products with spicy, choking aromas and with acid, astringent flavors are not well accepted (Leguerinel et al. 1987). According to these authors, isobutanol increases fruity and fragrant aromas, and 2,3-butanediol has an inhibitory action on these aromas.

Massiot et al. (1994) analyzed the volatile clarified must and the fermented apple product and observed an increase of several compounds during the fermentation processes (3-methyl-1-ol, 2,3-butanediol, 2-methyl-1-propanol, 2-phenyl-ethanol, 2-methyl-butanoic acid, octanoic acid, and 3-hydroxy-2-butanone).

Considering the use of sequential fermentations, a strain of non-*Saccharomyces* yeast that has a high capacity to form aromatic compounds is *Hanseniaspora*, which has great ability to form acetates that are important aromatic contributors. As a result of these mixed fermentations, Xu et al. (2006) observed that the strains of *H. valbyensis* produce high levels of ethyl esters and the strains of *S. cerevisiae* produce high amounts of alcohol, and the result of these levels is a positive contribution to the final product. The major esters formed are ethyl acetate and phenyl ethyl acetate (considered to be the most influential on the aroma), and the alcohols are isoamyl alcohol and isobutyl. Within the *Hanseniaspora* strain, the species *Hanseniaspora uvarum* produces high levels of ethyl acetate, but unlike other yeasts, this one produces acetoin in multiculture trials. This species does not cause an increase in volatile acidity in mixed cultures, does not maintain residual sugar, and uses less nitrogen in both mixed and sequential fermentations, indicating the absence of competition between them (Williams 1980; Bilbao 1997; Rojas et al. 2003; Ciani et al. 2006).

12.4.3.5 Malolactic Fermentation

During alcoholic fermentation, the yeasts can leave the fermentation medium deficient in vitamins and amino acids, making it less favorable for the development of bacteria in the first weeks of fermentation (Drilleau 1996). However, during the decline, cellular phase nutrients are released, which act as growth factors for the development of lactic acid bacteria (Figure 12.2; King and Bellman 1986; Dierings 2008).

In the 1970s and 1980s, British researchers found acid-tolerant, lactic, and acetic bacteria in cider and proved that the genus *Leuconostoc* and *Lactobacillus* existed in fruit (Salih et al. 1988). Among these, *Lactobacillus plantarum* and *Bacillus homofermentativo* were the most common.

Malolactic fermentation is a microbiological process that allows the deacidification of cider through decarboxylation of L-malic acid (dicarboxylic acid) in L-lactic acid (monocarboxylic acid). It is desirable

for three factors: decreased acidity, increased contribution to the sensory characteristics, and microbiological stability (Xue et al. 2005; Liu 2002; Versari et al. 1999).

During fermentation, the homofermentative bacilli are no longer detected and the homofermentative strains of the genus *Leuconostoc* (*Leuconostoc oenos*) predominate in cider, being responsible for the malolactic bioconversion (TML), which is the transformation of L-malic acid of the raw material into acid L-lactic acid in the product (Drilleau 1991a,b). However, because these bacteria are heterofermentative and in an environment rich in sugars such as cider consumption, it can cause the appearance of what is conventionally called *lactic bite*, from the French expression *piqûre láctique*, characterized by the excessive production of acetic acid that compromises the quality of the final product (Drilleau 1991a,b). Some other compounds that could result in the same loss of quality are also produced, such as diacetyl, acetoin, ethyl lactate, and some volatile phenols (Drilleau 1991a,b).

Malolactic transformation is initiated at the end of the main fermentation and lasts approximately 5 weeks, when the population of lactic bacteria increases by 1000 or even 10,000 times (Salih et al. 1988). For this to take place, a bacterial population of approximately 1.0×10^6 to 1.0×10^7 CFU mL^{-1} is required (Drilleau 1990a,b). The decrease in acidity, required in ciders with high acidity levels, is due to the transformation of mono-di-hydroxy-4-carboxylic carbons (malic acid) to a monohydroxy monocarboxylic acid 3-carbon (lactate), leaving the product less aggressive to the palate. However, it is necessary to take control measures because low-acid products can reduce the characteristic texture of the cider (Rankine 1970; Drilleau 1991a,b; Van Vuuren and Dicks 1993; Laplace et al. 2001).

Oenococcus oeni is recognized as the lactic acid bacteria with the greatest ability to develop malolactic fermentation in the modern fruit wine industry (Xue et al. 2005; Liu 2002; Herrero et al. 1999; Louvaud-Funel 1995).

The start of malolactic conversion can be influenced by two factors: the rate of lactic acid bacteria inoculum and the amount of phenolic compounds in the environment. A rate exceeding 10^5 CFU mL^{-1} shortens the period of grace before the onset of biotransformation and prevents the formation of lactic bite in cider with a higher degradation speed of malic acid. A high concentration of phenolic compounds has an adverse effect at the beginning of the process and may be a consequence of the inhibited growth of lactic acid bacteria or their enzymatic functions. However, the rate of conversion of L-malic acid is little affected (Salih et al. 1987, 1988).

Secondary fermentation or maturation has some of the following characteristics: duration of 3 to 6 months, limited consumption of sugar not exceeding 2 to 4 g L^{-1} per month in an environment containing 30 to 50 g L^{-1} of sugars (yeast eliminated by filtration), low alcohol content of approximately 2% to 3% (v/v), relatively high pH (3.8–4.2), and presence of complex microbiota (Le Quere and Drilleau 1993, 1998).

Malolactic bacteria can be controlled by sulfur dioxide (SO_2), and the resistance of these microorganisms to antiseptic depends on the concentration of free sulfur dioxide, which can vary according to the pH of the must.

12.4.3.6 Natural Gasification

Natural gasification or *prise de mousse* is considered to be a slow process of accumulation of CO_2 released during secondary fermentation, which may occur directly in bottles or in closed pressure-resistant vats. When natural gasification occurs in the bottle, unlike sparkling wines made according to the *crémant* method (formerly *champenoise*), ciders remain with the yeast inside the bottle. This operation is not performed in Brazil, where gasification is essentially artificial.

To avoid a super-gasification resulting from excess growth of yeast in the bottle, it is necessary that gasification is slow and interrupted naturally after several months of storage (from 1 to 2 months at 10°C to 15°C). The optimum concentration of dissolved carbon dioxide should be between 4 and 6 g L^{-1}, corresponding to a pressure of 2 to 3 bars at 10°C. For this, the yeast will consume between 9 and 13 g L^{-1} of sugars; that is, the natural gasification should be started between five and seven points of the final desired density (Drilleau 1993; Le Quere 1992).

Two important factors vary the speed of *prise de mousse*—the temperature and the yeast population. Using temperature as a tool to speed up or slow down the production of carbon dioxide may be

interesting from the practical aspect; however, low temperatures may mask an instability in the product, which will appear later in the producer's cellar, in marketing, or in the consumer's cellar. Control of the yeast population, however, can ensure a regular *prise de mousse* and stability in the cider once the fermentation speed is proportional to the number of live yeast (Simon 1992). This control of the initial population of yeast is simple. After the elimination of yeast from the cider by a fine filtration, a population of yeast is added before bottling, and this addition may be made in the form of active dry yeast (4 g hL^{-1}) or by a small volume of unfiltered apple cider that has been previously analyzed. The advantage of this operation in relation to artificial carbonation is that oxygen is incorporated into the filtering operation (4–7 mg L^{-1}), and bottling (3 mg L^{-1}) will be consumed by yeast in one case and in the other the oxygen after heat treatment can cause chemical reactions of oxidation, altering the flavor and aroma of the final product (Le Quere 1992; Ribereau-Gayon 1998).

12.4.4 Treatments after Fermentation

Cider producers often mix fermentations to provide a balance and the desired residual sugar. Fermentation is stopped by a filtration designed to eliminate the yeast and bacteria, and the density at which the filtration takes place depends on the desired category of cider, which can be dry, semidry, or sweet. Various types of filtration can be used in the stabilization of cider, such as using plates with diatoms and membrane modules for tangential filtration.

12.4.4.1 Bottling

Bottling is performed when the cider has stabilized; that is, the density does not vary by more than two points a month (Michel 1987). The technique of natural *prise de mousse* in bottles does not require special equipment for filling the bottles; however, in artificial gasification, producers use machines called isobaric *tireuses* with or without adjustment of the level of carbon dioxide. In this system, the liquid at low temperature is maintained at higher pressure than the saturation to prevent loss of gas and foaming during the transfer. The first step is to place the bottle under pressure; the second step begins when the liquid starts to be loaded into the inside of the bottle when the pressure in the bottle is the same as in the reservoir. The cider is then closed, labeled, and ready for commercialization (Lepage 2002).

12.4.4.2 Artificial Gasification

Saturation or gasification is a unit operation used at the time of bottling that allows the creation of a free artificial effervescence that is not present in the fermentation process. The transference is usually provided by gravity (a closed dome with entrance on the top) to a storage tank located under the machine where the level is regulated by a float. A pump ensures the filling of the column containing *Raschig* rings. The saturation pressure of 3 bars allows a CO_2 concentration of 5 g L^{-1} in the final product; however, in this case, up to 8 mg L^{-1} of oxygen can be added with this operation if antioxidant preventative measures are not taken (Le Quere 1992). Then, the bottle is closed with different types of devices (cork toppers, plastic corks, mechanical stoppers, and capsules). The most common type used in Europe is the cork stopper and in Brazil the most common is plastic; both have a metal fixing that serves to fix the stopper and act as a safety measure because of internal pressure. Thus, the bottling and carbonation stages are important to provide a quality product.

12.5 Apple Wine Defects

12.5.1 Bacterial Contamination

The composition of must makes it prone to bacterial contamination, and the main problems are those known in the manufacture of wine as *fat* (*graisse* or *ripeness*), lactic bite, acetic bite, and acroleic bite. The only disease that is specific to cider is *framboise*. Acetic bite is due to the action of aerobic bacteria

such as *Gluconobacter oxydans* in the early stages fermentation and *Acetobacter aceti* at the end of maturation. This contamination occurs mainly in dry cider or in interrupted fermentation, but as is usual, the persistence of a small production of CO_2 prevents the dissolution of oxygen that could cause acetylation, making it a rare problem in cider consumption. However, this contamination is more common in cider for distillation, which does not have adequate protection against oxygen. Lactic bite is often a major problem, and it is mainly caused by the bacteria responsible for malolactic conversion. Fat (*graisse* or *ripeness*) is considered a disease in ciders when high pH develops. It develops in the bottle preventing its treatment. Its main feature is the increase in viscosity linked to the production of glucan chains by lactic bacteria (*Lactobacillus* and *Leuconostoc* spp.) from sugars, giving the appearance of oil, detectable by its brightness.

Although the aroma is not affected, the cider can be only treated with rigorous stirring to break the glucan chains and by adding 100 ppm of sulfite to inhibit new growth (Le Quere and Drilleau 1998; Lea 1995). Acroleic bite concerns ciders specifically intended for distillation; it is a degradation of glycerol formed by yeast fermentation by lactic acid bacteria such as *Lactobacillus brevis* or *Lactobacillus colinóides*. The degradation of glycerol forms a precursor to 3-hydroxypropionaldeyde, which can decompose in acrolein during distillation. In the presence of this precursor in cider, French legislation prohibits distillation (Le Quere and Drilleau 1998). *Framboise* (France) or "sickness" (England), which is specific to cider, is characterized by the production of high concentrations of acetaldehyde and the formation of a milky viscosity due to the combinations of ethanol with certain phenolic compounds.

Two organisms were isolated in cider with this contamination, *Acetobacter rancens*, responsible for the transformation of lactic acid to acetaldehyde, and *Zymomonas anaerobia* var. *pomaceae*, able to transform glucose and fructose in ethanol with an accumulation of acetaldehyde in cider with a pH level higher than 3.7. However, there still remain many questions, mainly because of the difficulty of reproducing the phenomenon in the laboratory. SO_2 is not effective because of the high concentrations of ethanol (from 1000 to 2000 mg L^{-1}) produced in the environment; that is, there are no measures to avoid this problem. Good hygiene and rigorous working procedures reduce the risk, but if it is present in the locale of production, there is always a good chance that the problem may resurface (Le Quere and Drilleau 1998; Beech and Carr 1977; Lea 1995).

12.5.2 Sediments in Apple Wine

The yeast *Saccharomycodes ludwigii* is found in Europe, and especially England. It is present in apple peel and is sulfite resistant. It has especially large cells (25 mm in diameter), which precipitate in the bottle and are called *protein deposit* (Lea 1995). Protein deposits in cider are rare because of a low concentration of proteins in apples (100 mg L^{-1}). Normally, this deposit appears after a clarification treatment with excess gelatin.

Phenolic compounds are also responsible for deposits, through associations with proteins and polysaccharides, especially after the cooling of the product. These compounds are very important for cider but are also responsible for a series of undesirable aromas, such as the presence of naphthalene in stored ciders. The action of microorganisms (found in fruits with high degree of maturity) promotes a series of unwanted aromas from phenols (stable, sulfidic, wood, etc.; Shahidi and Naczk 1995; Lea 1995).

REFERENCES

Ainsworth PJ, Ball AJS, Tustanoff ER. 1980. Cyanide-resistant respiration in yeast; isolation of a cyanide-insensitive NAD(P)H oxidoreductase. *Arch Biochem Biophys* 202:172–86.

Alberti A. 2011. Efeito do nitrogênio e do oxigênio na elaboração de fermentados varietais de maçã. Dissertation (M.A. in Science and Food Technology), Universidade Estadual de Ponta Grossa, Ponta Grossa.

Alberti A, Wosiacki G, Nogueira A. 2011. Apple wine processing with nitrogen contents. *Braz Arch Biol Technol* 54:10–8.

Alberts B, Bray D, Lewis J, Raff M, Roberts K, Watson JD. 1997. *Biologia Molecular da Celula*. 3rd ed. Porto Alegre: Artes Médicas Sul.

Andreasen AA, Stier TJ. 1953. Anaerobic nutrition of *Saccharomyces cerevisiae*: I. Ergosterol requirement for growth in a defined medium. *J Cell Comp Physiol* 41:23–6.

Andreasen AA, Stier, TJ. 1954. Anaerobic nutrition of *Saccharomyces cerevisiae*: II. Unsaturated fatty acid requirement for growth in a defined medium. *J Cell Comp Physiol* 43:217–81.

Baron A, Bohuon G, Drilleau JF. 1977. Remarques sur l'indice formol des concentrés de jus de pomme. *Ann Falsif Expert Chim* 70:19–26.

Baron A, Goas M, Drilleau JF. 1982. Évolution au cours de la maturation, des fractions azotées et des acides aminés dans la pomme et dans le moût correspondant. *Sci Aliment* 2:15–23.

Bedriñana RP, Simón AQ, Valles BS. 2010. Genetic and phenotypic diversity of autochthonous cider yeasts in a cellar from Asturias. *Food Microbiol* 27:503–8.

Beech F. 1972. English cidermaking: technology, microbiology and biochemistry. *Prog Ind Microbiol* 11:133–213.

Beech FW. 1993. Yeasts in cider-making. In Rose AH, Harrison JS, editors. *The yeasts, yeast technology*. 2nd ed. London: Academic Press. p 169–213.

Beech FW, Carr JG. 1977. Cider and perry. In *Economic Microbiology*, edited by Rose HA, 139–313. London: Academic Press.

Beech FW, Challinor SW. 1951. Maceration and Defecation in Cider-Making: I. Changes Occurring in the Pectin and Nitrogen Contents of Apple Juices, 143–61. Annual Report of the Agricultural and Horticultural Research Station, Long Ashton, UK.

Beveridge T. 1997. Haze and cloud in apple juices. *Critical Reviews in Food Science and Nutrition* 37:75–91.

Bilbao A, Irastorza A, Duenas M, Fernandez K. 1997. The effect of temperature on the growth of strains of *Kloeckera apiculata* and *Saccharomyces cerevisiae* in apple juice fermentation. *Lett Appl Microbiol* 24:37–9.

Binnig R, Possmann P. 1993. Apple juice. In: Nagy S, Chen CS, Shaw PE, editors. Fruit juice. Processing technology. Auburndale: Agscience. p 271–317.

Blateyron L, Aguera E, Dubois C, Gerland C. 1998. Control of oxygen additions during alcoholic fermentations. *Vitic Enol Sci* 53:131–5.

Boré JM, Flecking J. 1997. Pommiers à cidre. Variétés de France, vol. 1. Paris: INRA.

Burroughs LF. 1957. The amino-acids of apple juices and ciders. *J Sci Food Agric* 8:122–31.

Burroughs LF, Sparks AH. 1973. Sulphite-binding power of wines and ciders: I. Equilibrium constants for the dissociation of carbonyl bisulphite compounds. *J Sci Food Agric* 24:187–98.

Blouin J. 1993. Le SO_2. Qu'en savons-nous en 1993? *Rev Oenologues* 67:13–7.

Cabranes C, Mangas JJ, Blanco D. 1997. Selection and biochemical characterisation of *Saccharomyces cerevisiae* and *Kloeckera apiculata* strains isolated from Spanish cider. *J Inst Brew* 103:165–70.

Canteri-Schemin MH. 2003. Obtenção de pectina alimenticia a partir de bagaço de maçã. [Dissertation]. M.A. in Food Technology, Mestrado em Tecnologia de Alimentos, Universidade Federal do Paraná, Curitiba.

Christen P, Léquére JM. 2002. La fabrication du cidre. *Process* 92:1186.

Ciani M, Beco L, Comitini F. 2006. Fermentation behaviour and metabolic interactions of multistarter wine yeast fermentations. *Int J Food Microbiol* 108:239–45.

Cliff M, Dever MC, Gayton R. 1991. Juice extraction process and apple cultivar influences on juice properties. *J Food Sci* 56:1614–27.

Czelusniack C, Oliveira MCS, Nogueira A, Silva NCC, Wosiacki G. 2003. Qualidade de maçãs comerciais produzidas no Brasil. Aspectos fisico-quimicos. *Braz J Food Technol* 6:25–31.

D'amore T, Russell I, Stewart GG. 1989. Sugar utilization by yeast during fermentation. *J Ind Microbiol* 4:315–24.

Demiate IM, Lara PSB, Nogueira A, Wosiacki G. 2003. Propriedades físicas, químicas e funcionais de amido de maçã. *Semina: Ciências Agrárias* 24:289–98.

Dierings LR. 2008. Abordagem microbiológica da fementação oxidativa alcoólica e malolática no processamento da sidra. [Dissertation]. M.A. in Science and Food Technology, Mestrado em Ciência e Tecnologia de Alimentos, Universidade Estadual de Ponta Grossa, Ponta Grossa.

Downing DL. 1989. Apple cider. In: Downing DL, editor. *Processed apple products*. New York: Van Nostrand Reinhold. p 169–87.

Drilleau JF. 1985. French fruit processing for cider. *Flüssiges Obst* 8:429–33.

Drilleau JF. 1990a. Comportement du cidre. Effets de quelques traitements préfermentaires. *Pomme* 20:19–20.

Drilleau JF. 1990b. La fermentation en cidrerie. Quelques généralités et principes d'élaboration. *Pomme* 21:20–2.

Drilleau JF. 1991a. Consolider les connaissances et maîtriser la qualité du produit fini. *Pomme* 23:23–5.

Drilleau JF. 1991b. Produits cidricoles. Quelques mots sur les composés phénoliques (tanins). *Technologie* 23:21–2.

Drilleau JF. 1993. Réunion annuelle Certec: azote et fermentation; composés phénoliques et oxydation. *Pomme* 33:24–5.

Drilleau JF. 1996. La cidrerie. In: Bourgeois CM, Larpent JP, editors. *Microbiologie alimentaire, aliments fermentés et fermentations alimentaires, Tome* 2. Paris: Apria. p 138–61.

Fan X, Mattheis JP, Patterson ME, Fellman JK. 1995. Changes in amylose and total starch content in Fuji apples during maturation. *Hortscience* 30:104–5.

Goffeau A, Crosby BA. 1978. New type of cyanide-insensitive respiration in the yeasts *Schizosaccharomyces pombe* and *Saccharomyces cerevisiae*. In: Bacile M, Horecker BL, Stoppani AOM, editors. *Biochemistry and genetics of yeasts: pure and applied aspects*. New York: Academic Press. p 81–6.

Goverd KA, Carr JG. 1974. The content of some B-group vitamins in single-variety apple juices and commercial ciders. *J Sci Food Agric* 25:1185–90.

Hamauzu Y, Inno T, Kume C, Irie M, Hiramatsu K. 2006. Antioxidant and antiulcerative properties of phenolics from Chinese quince, quince, and apple fruits. *J Agric Food Chem* 54:765–72.

Henry MF, Nyns EDJ. 1975. Cyanide-insensitive respiration. An alternative mitochondrial pathway. *Subcell Biochem* 4:1–65.

Henschke PA, Jiranek V. 1992. Yeasts—metabolism of nitrogen compounds. In: Fleet GH, editor. *Wine microbiology and biotechnology*. Switzerland: Harwood Academic Publishers. p 77–164.

Herrero M, De La Roza C, Garcia LA, Dãaz M. 1999. Simultaneous and sequential fermentations with yeast and lactic acid bacteria in apple juice. *J Ind Microbiol Biotechnol* 22:48–51.

Jacquin F, Tavernier J. 1951. Sur l'importance relative de l'azote et du phosphore dans la fabrication des cidres doux. *C R Acad Agric Fr*, 1–5.

Janovitz-klapp AH, Richard FC, Goupy PM, Nicolas JJ. 1990. Kinetic studies on apple polyphenol oxidase. *J Agric Food Chem* 38, 1437–41.

Jones M, Pragnell MJ, Pierce JS. 1969. Absorption of amino acids from wort by yeasts. *J Inst Brew* 70: 307–15.

Jones RP. 1988. Intracellular ethanol—accumulation and exit from yeast and other cells. *FEMS Microbiol Rev* 54:239–58.

King SW, Beelman RB. 1986. Metabolic interactions between *Saccharomyces cerevisiae* and *Leuconostoc oenos* in a model grape juice/wine system. *Am J Enol Vitic* 37:53–60.

Kirsop BH. 1977. Oxygen and sterol synthesis during beer fermentations. Proceedings of the EUCHEM Conference on Metabolic Reactions in the Yeast Cell in Anaerobic and Aerobic Conditions, 41–42. Helsinki, Finland: Alko Offset.

Kovacs E, Sass P, Al-Ariki K. 1999. Cell-wall analysis of different apple cultivars. *Acta Hortic* 219–24.

Lagunas R. 1993. Sugar transport in *Saccharomyces cerevisiae*. *FEMS Microbiol Rev* 104:229–42.

Laplace JM, Jacquet A, Travers I, Simon JP, Auffray Y. 2001. Incidence of land and physicochemical composition of apples on the qualitative and quantitative development of microbial flora during cider fermentations. *J Inst Brew* 107:227–33.

Laties L. 1982. The cyanide-resistant, alternative path in higher plant respiration. *Annu Rev Plant Biol* 35:519–55.

Lea AGH. 1984a. Oxidation of apple phenols. Bulletin de Liaison—Groupe Polyphenols. p 462–4.

Lea AGH. 1984b. Colorants et tanins dans les pommes a cidre anglais. *Flüssiges Obst* 51:356–61.

Lea AGH. 1990. Bitterness and astringency: the procyanidins of fermented apple ciders. In: Rousseff RL, editor. *Bitterness in foods and beverages*. Amsterdam: Elsevier. p 123–43.

Lea AGH. 1995. Cidermaking. In: Lea AGH, Piggott JR, editors. *Fermented beverage production*. London: Blackie Academic & Professional. p 66–96.

Lea AGH, Arnold GM. 1978. The phenolics of ciders: bitterness and astringency. *J Sci Food Agric* 29:478–83.

Lea AGH, Drilleau JF. 2003. Cidermaking. In: Lea AGH, Piggott JR, editors. *Fermented beverage production*. 2nd ed. New York: Kluwer Academic/Plenum Publishers. p 59–87.

Lea AGH, Timberlake CF. 1978. The phenolics of ciders: effect of processing conditions. *J Sci Food Agric* 29:484–92.

Lee CY, Smith NL. 2000. Apples: an important source of antioxidants in the American diet. *N Y Fruit Q* 8:15–7.

Leguerinel JJ, Cleret CM, Bourgeois P, Mafart I. 1987. Essai d'évaluation des caractéristiques organoleptiques des cidres par analyses instrumentales. *Sci Aliment* 7:223–39.

Lepage A. 2002. Cidre devariété Guillevic: vers la maîtrise d'uncidre de qualité supérieure. Monographie. Scienses et Techniques des Industries Agro-Alimentaire. Conservatoire National des Arts et Metiers, Paris. 158 p.

Le Quere JM. 1991. Fermentation lente du cidre. Pour une élaboration de qualité. *Pomme* 22:17–9.

Le Quere JM. 1992. Effervescence naturelle et gazéification. *Pomme* 25:15–7.

Le Quere JM, Drilleau JF. 1993. Microorganismes et typicité cidre. *Pomme* 31:16–9.

Le Quere JM, Drilleau JF. 1998. Microbiologie et technologie du cidre. *Rev Oenologues* 88:17–20.

Liu SQ. 2002. A review: malolactic fermentation in wine—beyond deacidification. *J Appl Microbiol* 92:589–601.

Lonvaud-Funel A. 1995. Microbiology of the malolactic fermentation: molecular aspects. *FEMS Microbiol Lett* 126:209–14.

Maitra PK. 1970. A glucokinase from *Saccharomyces cerevisiae. J Biol Chem* 245:2423–31.

Mangas JJ, Rodríguez R, Suárez B, Picinelli A, Dapena E. 1999. Study of the phenolic profile of cider apple cultivars at maturity by multivariate techniques. *J Agric Food Chem* 47:4046–52.

Marc I. 1982. La levure en fermentation: etude bibliographique. *Bios* 13:45.

Marks SC, Mullen W, Crozier A. 2007. Flavonoid and hydroxycinnamate profiles of English apple ciders. *J Agric Food Chem* 55:8723–30.

Massiot P, Le Quere JM, Drilleau JF. 1994. Biochemical characteristics of apple juices and fermented products from musts obtained enzymatically. *Fruit Process* 4:108–13.

Mayer AM, Harel E. 1991. Polyphenol oxidase and their significance in fruits and vegetables. In: Fox PF, editor. *Food enzymology*. London: Elsevier.

Michel A. 1987. Production du cidre a la ferme. *Pomme* 17:1.

Michel A, Bizeau C, Drilleau JF. 1988. Flore levurienne présente dans les cidreries de l'ouest de la France. *Sci Aliment* 8:359–68.

Michel A, Bizeau C, Drilleau JF. 1990. Relations métaboliques entre levures impliquées dans la fermentation du cidre. *Belg J Food Chem Biotechnol* 45:98–102.

Ministério da Agricultura, Pecuária e Abastecimento. Decreto no. 6.871, June 4, 2009. Regulamenta a Lei no. 8.918, de 14 de julho de 1994, que dispõe sobre a padronização, a classificação, o registro, a inspeção, a produção e a fiscalização de bebidas. Diário Oficial da República Federativa do Brasil, Brasília, DF, June 4, 2009.

Moutounet M, Mazauric JP. 2001. L'oxygène dissous dans les vins. *Rev Française Œnologie* 186:12–5.

National Association of Cider Makers. 1998. Code of practice for the production of cider and perry. London: National Association of Cider Makers.

Nicolas JJ, Richard-Forget FC, Goupy PM, Amiot MJP, Aubert SY. 1994. Enzymatic browning reactions in apple and apple products. *Crit Rev Food Sci Nutr* 34:109–57.

Nogueira A. 2003. Tecnologia de processamento sidricola. Efeitos do oxigênio e do nitrogênio na fermentação lenta da sidra. [PhD thesis in Biotechnical and Agroindustrial Processes]. Doutorado em Processos Biotecnológicos Agroindustriais—Setor de Engenharia Química, Universidade Federal do Paraná, Curitiba. 210 p.

Nogueira A, Le Quere JM, Gestin P, Michel A, Wosiacki G, Drilleau JF. 2008. Slow fermentation in French cider processing due to partial biomass reduction. *J Inst Brew*114:102–10.

Nogueira A, Mongruel CE, Oliveira MC, Passos M, Wosiacki G. 2005. Avaliação da trituração e de tratamentos enzimáticos na obtenção de suco de maçã por centrifugação. Publicatio UEPG—Ciências Exatas e da Terra, Agrárias e Engenharias 11:7–12.

Nogueira A, Prestes RA, Simões DRS, Wosiacki G. 2004. Análise dos indicadores físico-químicos de qualidade da sidra brasileira. *Semina: Ciências Agrárias* 24:289–98.

Nogueira A, Teixeira, SH, Demiate IM, Wosiacki G. 2007. Influencia do processamento no teor de minerais em sucos de maçãs. *Ciênc Tec Alimento* 27:259–64.

Nogueira A, Wosiacki G. 2010. Sidra. In: Filho WV, editor. Tecnologia de Bebidas Alcoólicas, Vol. 1. São Paulo: Blucher.

Paganini C, Nogueira A, Denardi F, Wosiacki G. 2004. Aptidão industrial de seis cultivares de maçã (dados da safra 2001/2002). *Ciênc Agrotecnol* 28:1336–43.

Possmann P. 1992. A comparison of cider and apfelwein. *Flüssiges Obst* 59:486–92.

Rankine BC. 1970. La fermentation malo-lactique et son importance dans les vins rouges de table australiens. *Bull OIV* 4:383–97.

Ratledge C, Evans CT. 1989. Lipids and their metabolism. In: Rose AH, Harrison JS, editors. The yeasts, metabolism and physiology of yeasts. 2nd ed. New York: Academic Press. p 367–455.
Ravier M. 1985. Le cidre hier et aujourd'hui, Vol. 1. Paris: La Nouvelle Librairie. 64 p.
Renard CMGC, Baron A, Guyot S, Drilleau JF. 2001. Interactions between Apple Cell Walls and Native Apple Polyphenols: quantification and Some Consequences. *Int J Biol Macromol* 29:115–25.
Ribereau-Gayon PDD, Donèche B, Lonvaud A. 1998. Traité d'oenologie: microbiologie du vin vinifications. Paris: Dunod. 617 p.
Robin P, De La Torre M. 1988. Le cidre, la pomme, le calvados. Paris: Editions du Papyrus. 192 p.
Rojas V, Gil JV, Piñaga F, Manzanares P. 2003. Acetate ester formation in wine by mixed cultures in laboratory fermentations. *Int J Food Microbiol* 86:181–88.
Sablayrolles JM, Barre P. 1986. Evaluation des besoins en oxygene de fermentations alcooliques en conditions oenologiques simulées. *Sci Aliment* 6:373–84.
Sablayrolles JM, Barre P, Salmon JM. 1996. Carences nutritionnelles des moûts. Efficacité des ajouts combinés d'oxygène et d'azote ammoniacal. *Rev Française Œnologie* 159:25–32.
Salih AG, Drilleau JF, Divies C, Lenzi P. 1987. Facteurs contribuant au contrôle de la transformation malolactique dans les cidres. *Sci Aliment* 7:205–221.
Salih AG, Le Quere J-M, Drilleau J-F, Fernandez JM. 1988. A survey of microbiological aspects of cidermaking. *J Inst Brew* 94:5–8.
Salmon JM, Fornairon C, Barre P. 1998. Determination of oxygen utilization pathways in an industrial strain of *Saccharomyces cerevisiae* during enological fermentation. *J Ferment Bioeng* 86:154–63.
Sanoner P, Guyot S, Marnet N, Molle D, Drilleau JF. 1999. Polyphenols profiles of French cider apple varieties (*Malus domestica* sp.). *J Agric Food Chem* 47:4847–53.
Sataque EY, Wosiacki G. 1987. Caracterização da polifenoloxidase de maçã (variedade Gala). *Arq Biol Tecnol* 30:287–99.
Sayavedra-Soto LA, Montgomery MW. 1986. Inhibition of polyphenoloxidase by sulfite. *J Food Sci* 51:1531–6.
Shahidi F, Naczk M. 1995. Food phenolics—sources, chemistry, effect, applications. Pennsylvania: Technomic. 321 p.
Simon D. 1982. Dissolution d'oxygene au soutirage. *Bios* 13:33.
Smock RM, Neubert AM. 1950. *Apples and apples products*. New York: Interscience Publishers. 486 p.
Tavernier J, Jacquin P. 1946. Influence de l'elimination partielle des matières azotées des moûts des pommes sur la fermentation des cidres. *C R Acad Sci* 222:1–10.
Valles BS, Bedriñana RP, Queipo AL, Alonso JJM. 2008. Screening of cider yeasts for sparkling cider production (champenoise method). *Food Microbiol* 25:690–7.
Valles BS, Bedriñana RP, Tascón NF, Simón AQ, Madrera RR. 2007. Yeast species associated with the spontaneous fermentation of cider. *Food Microbiol* 24:25–31.
Van Dijken J, Weusthuis R, Pronk J. 1993. Kinetics of growth and sugar consumption in yeasts. *Antonie Leeuwenhoek* 63:343–52.
Van Vuuren HJ, Dicks LMT. 1993. *Leuconostoc oenos*: a review. *Am J Enol Vitic* 44:99–112.
Versari A, Parpinello GP, Cattaneo M. 1999. *Leuconostoc oenos* and malolactic fermentation in wine: a review. *J Ind Microbiol Biotechnol* 23:447–55.
Vivas N. 1999. Acquisitions récentes sur l'oxydoréduction des vins rouges los de leur élevage. *Rev Oenologues* 90:15–20.
Vivas N, Viva N, Debèda N, Vivas de Gaujelac NV, Nonier MF. 2003. Mise en evidence du passage de l'oxygène au travers des douelles constituents lês barriques par l'utilisation d'un dispositif original de mesure de la porosité du bois. Premiere resultats. *Sci Aliments* 23:655–78.
Walker GM. 1998. *Yeast physiology and biotechnology*. Scotland: John Wiley & Sons. 350 p.
Whiting JC, Coggins RA. 1975. Estimation of the monomeric phenolics of ciders. *J Sci Food Agric* 26:1833–8.
Williams A. 1980. Flavor research and the cider industry. *J Inst Brew* 5:455–70.
Williams A, May H. 1978. Observations on the use of porous polymers for collecting volatiles from synthetic mixtures reminiscent of fermented ciders. *J Sci Food Agric* 29:1041–54.
Williams A, May HV. 1981. Examination of an extract of cider volatiles using both electron impact and chemical ionization gas chromatography–mass spectrometry. *J Inst Brew* 87:372–5.
Williams A, Tucknott OG. 1971. Volatile constituents of fermented cider: I. Draught dry cider blend. *J Sci Food Agric* 22:264–9.

Williams A, Tucknott OG. 1987. Examples of cryogenic matrix isolation GC/IR in the analysis of flavor extracts. In: *Flavor Science and Technology*. Chichester: Wiley. p 259–70.

Wosiacki G, Cherubin RA, Santos DS. 1997. Cider processing in Brazil. *Fruit Process* 7:242–9.

Wosiacki G, Kamikoga ATM, Neves JR. 1991. Características do suco clarificado de maçãs. *Alimentos Tecnol* 8:76–9.

Wosiacki G, Nogueira A, Silva NCC, Denardi F. 2005. The apple and its fructose content cultivar Sansa—a case study. *Publicatio UEPG—Ciências Exatas e da Terra, Agrárias e Engenharias* 11:27–39.

Wosiacki G, Nogueira A, Silva NCC, Denardi F, Vieira RG. 2008. Quality profile of samples of 139 apples—Cultivars harvested in Brazil from 1982 to 2006. *Acta Alimentaria* 37:9–22.

Xu Y, Zhao GA, Wang LP. 2006. Controlled formation of volatile components in cider making using a combination of *Saccharomyces cerevisiae* and *Hanseniaspora valbyensis* yeast species. *J Ind Microbiol Biotechnol* 33:192–6.

Xue W, Zhou L, Li X, Shi X. 2005. Study on isolation and detection of thermo-acidophilic bacteria in apple juice concentrate. Journal of Xi'an Jiaotong University (Medical Sciences) 26:199–200.

Zardo DBM, Dantas AP, Vanz R, Wosiacki G, Nogueira A. 2009. Intensidade de pigmentação vermelha em maçãs e sua relação com os teores de compostos fenólicos e capacidade antioxidativa. *Ciênc Tec Alimento* 29:148–54.

13

Fermentation and Cashew Apple Juice

Ana Lúcia Fernandes Pereira and Sueli Rodrigues

CONTENTS

13.1 Introduction

13.1.1 Cashew Fruit

The cashew (*Anacardium occidentale* L.) is native to tropical America. Originating from Brazil, it has become naturalized in many tropical countries such as Vietnam, India, Nigeria, Tanzania, Ivory Coast, Mozambique, and Benin. Cashew is formed by the developed peduncle (apple) attached to the nut (actual fruit composed of shell + kernel). The peduncle, which is also called the pseudo-fruit, false fruit, cashew apple, or simply cashew, represents the edible portion, *in natura* and also as juices, pulp, and preserves (Filgueiras et al. 1999). Apart from the usual presence of sugars and organic acids, one important characteristic of cashew apple is its high vitamin C content, four times higher than that of sweet orange (Rufino et al. 2010; Akinwale 2000).

Cashew apple is the peduncle of the cashew fruit, which is rich in reducing sugars (fructose and glucose), vitamins, minerals, and some amino acids. Although cashew apples can be consumed as juice, ice cream, and other foodstuffs, cashew tree cultivation is an agricultural activity directed at the production of cashew nuts. The nuts represent only 10% of the total fruit weight, and large amounts of cashew apples are lost in the field after nut removal. The expected yield for the cashew tree under rainy conditions is approximately 1 t/ha of raw cashew nut and 10 t/ha of cashew apple. Under irrigated conditions, it may reach 3.8 t/ha of raw cashew nut and 30 t/ha of cashew apple (Rabelo et al. 2009). The average yield of juice extraction is approximately 85% (v/w), thus the juice productivity can reach 25.5 m^3/ha. The amount of sugar in cashew apples depends on the cultivar. Naragaraja et al. (2007) reported sugar contents from 50 to 200 g/L. Thus, sugar productivities may reach up to 5 t/ha. According to government data (National Agriculture Research Agency–EMBRAPA/CNPAT, Fortaleza, Ceará, Brazil), the Brazilian Northeast presents an annual production of approximately two million tons of cashew apples, and 85% to 90% of this production is lost or underutilized. As such, cashew apple is considered an agricultural residue and its nutritive juice can be a cheap raw material. Because of its availability and low

TABLE 13.1

Brazilian Cashew Apple Composition

Component	Range of Mean Values
Reducing sugars (g/L)	80.0–82.4
Vitamin C (mg/100 g)	153–261
Titratable acidity (% p/p)	0.35–0.49
Soluble solids (Brix)	9.74–10.76
Tannins (%)	0.27–0.76
pH	3.90–4.34
Moisture (%)	85.98–87.20
Ash (%)	0.32–0.33
Calcium (mg/100 g)	13.65–16.75
Iron (mg/100 g)	0.28–0.34
Phosphate (mg/100 g)	25.85–32.55
Protein (% p/p)	0.64–0.92

cost, cashew apple has been studied as a substrate for fermentative and enzymatic process for several applications (Silveira et al. 2010; Fontes et al. 2009; Honorato and Rodrigues 2009; Rabelo et al. 2009; Chagas et al. 2007; Rocha et al. 2007; Honorato et al. 2007). Table 13.1 presents the physicochemical composition of cashew apples cultivated in Brazil.

Variations in the mineral, phenol, tannin, vitamin C, and sugar composition of Ghana's cashew apple juice were investigated by Lowor and Agyent-Badu (2009). The mean proximate composition was as follows: phenolics (0.27% p/v), condensed tannins (0.27% p/v), vitamin C (231.4 mg/100 mL), and sugars (12.05 g/L). The mineral composition (mg/100 mL) showed potassium (76.0) to be the highest, followed by calcium (43.0), magnesium (10.92), phosphorous (0.79), and sodium (0.41). Zinc, copper, and iron concentrations were much lower and ranged from 0.05 to 0.08 mg/100 mL. The physicochemical properties of the juice were as follows: pH (4.31), color (light yellow for juice from yellow apples and yellow with traces of red pigments for juice from red apples). The phenol and tannin contents of the juice showed significant variations among samples from different cultivation areas. No significant differences in the quantitative composition of calcium, iron, zinc, and phosphorus could be attributed to the cultivation zone or color from which the juice was extracted. Actually, physicochemical, proximal composition, and the vitamin C contents of the cashew apple may change due to the variety and cultivation area as well as from harvest to harvest. However, cashew apple juice is always a good source of vitamin C, reducing sugars (glucose and fructose), and minerals.

13.1.2 Cashew Apple Juice Production

After the nut removal, cashew apples are washed with chlorinated water (10–50 ppm) and rinsed with tap water. The juice is usually obtained by pressing the cashew peduncles, through a mechanical process, to extract the juice in an expeller. The juice may be processed for human consumption as fruit juice or can be used as substrate for fermentation or enzyme synthesis. The juice content of the cashew apple is approximately 85%, and good extraction yields are usually obtained. The integral juice is also very perishable and needs thermal treatment as well as the addition of chemical products, usually sodium metabisulfite, to preserve the characteristic color. Cashew apple juice contains tannins, giving an astringent taste and a dry mouthfeel, making it less attractive in comparison to other fruit juices. Tannins are removed by clarification techniques. Gelatin and membrane processes are usually used for juice clarification. The microbial profile of *in natura* cashew apple juice is comprised of mesophilic bacteria, yeasts, and filamentous fungi. However, the natural flora of cashew apple juice is responsible for juice spoilage. Even when stored under refrigeration (4°C), the juice spoils relatively quickly. Better microbial stability is found when the juice is stored frozen (Lavinas et al. 2006). Isolates of the genera *Leuconostoc, Lactobacillus, Pediocoecus, Aspergillus, Rhizopus,* and some yeast strains were obtained from the fresh

pomace (Aderiye and Mbadiwe 1993). Adesioye (1991) reported the isolation of *Saccharomyces cerevisiae* sp. from cashew apple juice.

As a matter of fact, the native flora of cashew apple juice may have plenty of variation because of the region they are cultivated in and the processing methods used.

13.1.3 Fermented Foods

Fermentation is a traditional way of preserving foods. Since ancient times, even without knowing that microorganisms were responsible for the food transformation, fermented foods have been used for human feed and nutrition. In general, fermentation stabilizes food by making it so acid (low pH) or alcoholic that undesirable microorganisms find it difficult to grow. Fermented products are often further protected from oxidation and infection by microorganisms using air-tight containers or heat. The word fermentation typically refers to the fermentation of sugar to alcohol using yeast in microaerobic or anaerobic conditions. However, nowadays, the term fermentation is widely applied even for processes that are not anaerobic, such as vinegar production. Several foodstuffs are now produced using microorganisms such as bakery products, yogurts, fermented milks, wines, beer, sausages, fish, fermented soy, fermented beans, and sauerkraut among many others. Because of the large availability of cashew apples as a cashew nut by-product, cashew apple juice has been studied as a substrate for biotechnological process. In this chapter, some applications of cashew apple juice in fermentative and enzymatic processes are presented.

13.2 Background of Fermented Cashew Apple Juice

13.2.1 Alcoholic Fermentation

Alcoholic fermentation was the first process studied applying cashew apple juice as a substrate due to its high content of reducing sugars, which are a readily fermentable sugar. However, there are only few studies on fermented beverages produced from cashew apples.

Cashew apple wine was produced by Mohanty et al. (2006) using fully ripened and undamaged cashew apples (var. *Vengurla-6*). The apples were processed just after the harvest. Cashew apples were cleaned by washing in tap water and by immersing for 3 days in 5% salt solution to reduce tannin content, after which they were steamed for 15 minutes at a pressure of 15 lb. The apples were then crushed in a mixer and grinder (TTK Prestige, Ltd., Bangalore, India) and the juice was extracted by using a juice squeezer. The juice (must) was filtered through a cotton cheesecloth at 12°Brix and was treated with sodium metabisulfite (100 mg/mL) to inhibit the growth of undesirable microorganisms such as acetic acid bacteria, wild yeasts, and molds. Then, cane sugar and tartaric acid were added into the juice (amelioration) to attain 17°Brix and pH 3.6, respectively. The ameliorated must was inoculated with 2% (v/v) starter culture (prepared with grape juice) of *S. cerevisiae* var. *bayanus*, and fermentation was carried out at room temperature (32 ± 2°C) for 6 days. Racking of wine was carried out when total soluble sugars reached 2 to 3°Brix. Two or three more rackings were done at 15-day intervals to remove any sediment deposited in the wine. After racking, the wine was clarified with the addition of 0.04% bentonite and analyzed. Sodium metabisulfite (100 mg/mL) was added as a preservative before bottling. A general flowchart for cashew wine production is shown in Figure 13.1. Despite the high level of tannin content, cashew apples could be processed into beverage owing to its fleshy pulp, soft peel, lack of seeds, high sugar concentration, and strong exotic flavor.

Because of fermentation, the composition of cashew apple juice changes. The titratable acidity increased from 0.24 ± 0.02 g (tartaric acid/100 mL) in must to 1.21 ± 0.06 g (tartaric acid/100 mL) in the finished wine. The increase in titratable acidity was concomitant with a decrease in pH from 4.63 ± 0.07 in must to 2.92 ± 0.06 in wine. As expected, the reducing sugar (g/100 mL) content decreased from an initial value of 6.44 ± 0.24 in must to 0.9 ± 0.02 in wine. However, the phenolic content remained unchanged in wine samples, which was expected. The cashew wine presented a light yellow color and alcohol content of 7.0%. The wine was slightly acidic (1.21 g tartaric acid/100 mL), which, together with comparatively high tannin content (1.9 ± 0.22 mg/100 mL), imparted the characteristic cashew

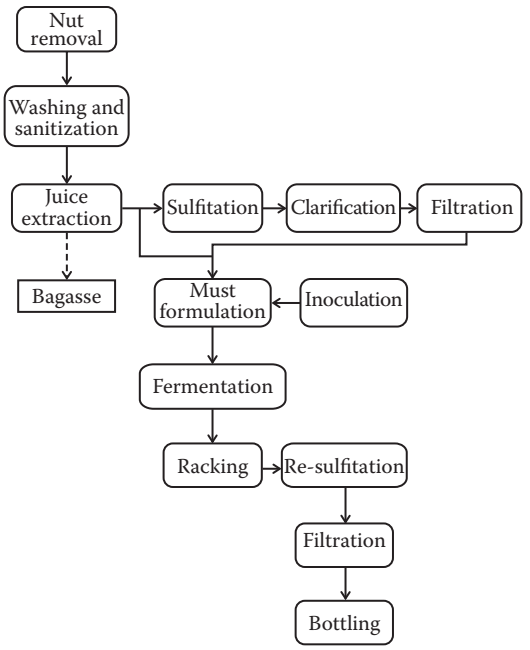

FIGURE 13.1 Basic flow chart for cashew apple wine production.

apple flavor and astringency. Lactic acid concentration in the wine was very low (2.5 mg/100 mL). The standard grape wine presented less acidity (titratable acidity, 0.53 g tartaric acid/100 mL and lactic acid, 0.21 mg/100 mL), and consequently, higher pH but with higher ethyl alcohol content (10% v/v). The other parameters, such as soluble solids, reducing sugar, and phenol concentrations were somewhat similar in both cashew apple and grape wine samples. As expected, the tannin content in grape wine was very low compared with cashew apple wine.

Sensory attributes were evaluated by trained panelists who ranged in age from 20 to 35 years old and were familiar with wine consumption. The cashew wine produced along with a selected commercial brand of grape wine was presented to the trained panel of sensory analysts. Sensory evaluation showed that the panelists rated cashew wine as somewhat inferior (except color/appearance) to commercial grape wine, but the attributes taste, aftertaste, and color/appearance were scored as "like much." However, the panelists rated aroma and flavor scores as "like moderately" to "like much" probably because of the high tannin content in cashew wine, which imparted an astringent flavor. Nevertheless, the cashew wine was acceptable to all the trained panelists.

Abreu (1997) produced a sparkling wine from cashew apple with an alcoholic content of 7.72% (v/v), pH of 3.6, total acidity of 0.597 g citric acid/100 mL, soluble solid content of 11.53°Brix, and total tannin concentration of 1.92 mg/100 mL. However, the sensory evaluation of the wine was not tested. Similarly, Osho and Odunfa (1999) fermented cashew apple juice using a commercially available wine yeast (*S. cerevisiae*) and three other wine yeasts. The wine produced using *S. cerevisiae* NCYC 125 was found acceptable by the consumer panelists on the basis of color, aroma, bouquet, and taste. However, in both studies, cashew wine was not compared with any standard/grape wine. Cashew wine, prepared by fermentation of cashew apple juice using *S. cerevisiae*, would be another good prospect for the alcoholic beverage industry. However, more research should be conducted to find methods for the reduction of tannin concentration in cashew apple must and, consequently, in wine to minimize the astringent flavor.

In another study, clarified fresh cashew juice was inoculated with *Saccharomyces bayanus* and fermented at 18°C and 30°C (Garruti et al. 2006). Sulfur dioxide was added at 0, 50, 100, and 200 mg/kg. The headspace volatiles were identified by gas chromatography-mass spectrometry and sniffed using the Osme technique. The analysis of the eight cashew wine treatments showed the presence of 41 volatile

compounds. The aromagram of samples containing sulfur dioxide at 100 mg/kg showed 25 odoriferous compounds. Fermentation temperature affected the volatile profile of cashew apple wine. Low fermentation temperature increased the concentration of esters with sweet, fruity, and cashew-like aromas, but also increased the level of compounds that presented unpleasant aromas (isopentyl acetate, styrene, and hexanoic acid) and decreased the concentration of isobutanol, which has been described as smelly. Must sulfitation, in general, produced an increase in the ester concentration, except ethyl lactate. Styrene was not affected by sulfitation, but hexanoic acid increased significantly.

The refrigerated fermentation provided better wines, increasing the levels of compounds with cashew, fruity, and sweet notes and decreasing undesirable compounds perceived as "fermented," "plastic," or "smelly." A low level of sulfitation was required to obtain a fruity cashew wine whereas too much sulfite favored the production of isobutanol and hexanoic acid, which could impair the sensory quality of the product.

The principal component analysis carried out with the eleven volatiles which produced a greater impact on the cashew wine aroma and flavor showed that wines fermented at 18°C and supplemented with sulfur dioxide presented a greater concentration of volatiles, whereas samples made at 30°C were characterized by a higher intensity of isobutanol. Wines with no sulfitation had a smaller concentration of all odor-active compounds identified. Sensory descriptors determined by qualitative descriptive analysis showed that wines fermented at 30°C were characterized by "fermented aroma," "acid taste," and "alcoholic flavor," whereas the samples fermented at 18°C were associated with "fruit flavor."

13.2.2 Other Processes Using Cashew Apple Juice as Substrate

The use of agricultural excesses as a substrate in industrial fermentations became an interesting alternative to reduce production costs and to reduce negative environmental impact caused by the disposal of these products. Mannitol is a sugar alcohol applied as a sweetener. Heterofermentative lactic acid bacteria, such as *Leuconostoc mesenteroides* sp. are able to produce mannitol from fructose. Because the carbohydrates of cashew apple juice are glucose and fructose, Fontes et al. (2009) studied the use of cashew apple juice as a substrate for mannitol production. The best results were obtained by applying only cashew apple juice as a substrate, containing 50 g/L of total reducing sugar (28 g/L of fructose), yielding 18 g/L hour^{-1} of mannitol with 67% of fructose conversion into mannitol and productivity of 1.8 g/L hour^{-1}.

The use of cashew apple juice as a low-cost substrate for *Lactobacillus casei* B-442 cultivation and lactic acid production was studied by Silveira et al. (2010). Lactic acid is applied in the food industry as a preservative and *L. casei* is a probiotic bacteria. Because of the low protein content of cashew apple juice, ammonium sulfate was used as the only exogenous nitrogen source, replacing the use of yeast extract, which is a more expensive nitrogen source for industrial applications. The effect of cashew apple juice reducing sugars, ammonium sulfate concentration, the fermentation pH, and temperature on biomass formation, lactic acid production, and productivity were evaluated. The highest productivity (2.36 g/L hour^{-1}) was obtained by applying 50 g/L of reducing sugar from the cashew apple juice supplemented with 6 g/L of ammonium sulfate. The process yield was approximately 95% when fermentation was carried out at 37°C with pH controlled at 6.5 using sodium hydroxide (120 g/L). The biomass produced can be used as a supplement in the food industry due to its probiotic effects.

Cashew apple juice was also evaluated for dextransucrase production. Dextransucrase is an enzyme produced by *L. mesenteroides* sp. that converts sucrose into dextran, and in the presence of acceptors such as maltose, glucose, fructose, and other simple sugars, produces prebiotic oligosaccharides. The effect of yeast extract and the addition of phosphate was evaluated using factorial planning tools. The addition of both phosphate and yeast extracts were significant factors for biomass growth, but had no significant effect on maximum enzyme activity. The enzyme activities found in cashew apple juice assays were at least 3.5 times higher than the activity found in the synthetic medium. Assays with pH control (pH 6.5) were also carried out. The pH-controlled fermentation enhanced biomass growth, but decreased the enzyme activity (Chagas et al. 2007).

Honorato and Rodrigues (2010) studied the stability and partially purified dextransucrase in clarified cashew apple juice at room temperature. The stability of crude dextransucrase produced in a fermentation

medium using cashew apple juice as substrate was evaluated. The crude enzyme was stable at 30°C for 30 hours at pH ranging from 4.5 to 5.5. The partially purified enzyme, produced in a culture medium containing sucrose as substrate, was stable in nonfermented cashew apple juice at pH 5.0 for 96 hours at 30°C. The high stability of the partial enzyme in cashew apple juice at room temperature allows its industrial use without the need for enzyme purification and, consequently, reducing processing costs.

Biosurfactants are a group of compounds that can significantly reduce the surface tension of liquids at a very low concentration. They are widely used in the field for emulsification, foaming, dispersion, and solubilization. They have very high surface activity and emulsification activity. They are biodegradable and nontoxic and can be used in extreme temperatures and under acid or alkaline conditions. Surfactin is a widely used biosurfactant. Surfactin is used in the food industry and, because of its excellent emulsifying property, can play an important role in the formation of a certain concentration, texture, and dispersal phase of raw food materials. It can also be used in the production of bread and meat to improve the rheological properties and emulsify part of the cracked fat. *Bacillus subtilis* LAMI008, a strain isolated from the tank of the Chlorination at the Wastewater Treatment Plant on Campus do Pici in Federal University of Ceará, Brazil, has been screened for surfactin production in mineral medium containing clarified cashew apple juice (MM-CAJC). Results were compared with the ones obtained using mineral medium with glucose as a carbon source. The influence on growth and surfactin production of culture medium supplementation with yeast extract was also studied. The substrate concentration analysis indicated that *B. subtilis* LAMI008 was able to degrade all carbon sources studied and produce biosurfactant. The highest reduction in surface tension was achieved with the fermentation of MM-CAJC, supplemented with yeast extract, which decreased from 58.95 ± 0.10 to 38.10 ± 0.81 dyne/cm. The biosurfactant produced was capable of emulsifying kerosene, achieving an emulsification index of 65%. Surfactin concentration of 3.5 mg/L was obtained when MM-CAJC, supplemented with yeast extract, was used, thus indicating that it is feasible to produce surfactin from clarified cashew apple juice, a renewable and low-cost carbon source (Rocha et al. 2009).

Ethanol was another product obtained using cashew apple juice. Using a commercial strain of *S. cerevisiae*, growth kinetics and ethanol productivity were obtained for batch fermentation with different initial sugar (glucose + fructose) concentrations. Maximal ethanol, cell, and glycerol concentrations were obtained when an initial sugar concentration of 103.1 g/L was used. Cell yield ($Y_{X/S}$) was calculated as 0.24 (g microorganism)/(g glucose + fructose) using cashew apple juice medium with 41.3 g/L of initial sugar concentration. Glucose was exhausted first, followed by fructose. Furthermore, the initial concentration of sugars did not influence ethanol selectivity. These results indicate that cashew apple juice is a suitable substrate for yeast growth and ethanol production (Pinheiro et al. 2009). The results obtained in this study indicated that the sugar profile consumption depends on the microbial strain because, in mannitol production using cashew apple juice as a substrate, glucose and fructose were consumed simultaneously (Fontes et al. 2009).

13.3 Functional Cashew Apple Juice

13.3.1 Functional Foods

Consumers are increasingly interested in the health benefits of foods and have begun to look beyond the basic nutritional benefits of food to the disease prevention and health-enhancing compounds contained in many foods. This, combined with a more widespread understanding of how diet affects disease, health care costs, and aging populations, has created a market for functional foods and natural health products. Thus, functional foods are defined as processed foods containing ingredients that aid specific bodily functions in addition to being nutritious.

The large intestine contains up to 500 different types of bacteria, which contribute to a number of biological functions in the body. To maintain a well-balanced flora in the large intestine, the number of beneficial bacteria must be higher than the pathogenic bacteria. Therefore, it is essential to promote adequate levels of "good bacteria." This balance can be obtained by direct microbial intake, which is done by consuming fermented milks containing lactobacillus or bifidobacteria (good bacteria). Probiotics

represent probably the archetypal functional food, and are defined as live microbial supplements, which beneficially affect the host by improving its intestinal microbial balance (Kalliomaki et al. 2001; Brown and Valiere 2004).

Another method is through the consumption of foods containing prebiotic oligosaccharides. A prebiotic is a nondigestible food ingredient that affects the host beneficially by selectively stimulating the growth or activity of bacteria in the colon. Prebiotic oligosaccharides are not digested in the stomach and are able to reach the large intestine where they are metabolized by the beneficial intestinal microbiota (*Bifidobacteria* and *Lactobaccillus*). In vitro tests have found that inulin and oligofructose are excellent and selective growth media and energy substrates for Bifidus bacteria. These bacteria have been shown to inhibit the development of a number of harmful strains. The results of these studies have been confirmed in numerous clinical human studies. Other oligosaccharides, such as gluco-oligosaccharides, malto-oligosaccharides, and galacto-oligosaccharides, were also studied and presented good results. However, the commercial foods containing prebiotics and probiotics are limited to milk-based beverages.

13.3.2 Prebiotic Cashew Apple Juice

Dextransucrase is a bacterial extracellular glucosyltransferase produced by *Leuconostoc* strains that promote dextran synthesis. Fructose is a natural side product released when the enzyme polymerizes glucose from sucrose into dextran. The same enzyme is also responsible for the synthesis of prebiotic oligosaccharides through an acceptor reaction (Rodrigues et al. 2003, 2006; Rabelo et al. 2006; Monchois et al. 1999; Monsan and Paul 1995). The acceptor reaction was first described by Koepsell et al. (1953). In the presence of sucrose, the introduction of other carbohydrates (acceptors) shifts the enzyme pathway from dextran synthesis toward the production of oligosaccharides (Tsuchiya et al. 1952; Pereira et al. 1998; Heincke et al. 1999; Rodrigues et al. 2006; Rabelo et al. 2006). This shifted pathway has been called acceptor reaction (Figure 13.2). Although several *L. mesenteroides* strains are able to produce glucosyltransferases, the acceptor reaction has been widely studied with dextransucrase from *L. mesenteroides* NRRL B-512-F (Monsan and Paul 1995; Rodrigues et al. 2005, 2006; Rabelo et al. 2006). According to the acceptor reaction, homologous series of acceptor products are formed during the enzymatic reaction. These acceptor products are oligosaccharides with a degree of polymerization between 2 and 10, which are considered prebiotic carbohydrates (Chung and Day 2002, 2004). Interest in the synthesis of oligosaccharides has increased because these carbohydrates present functional and prebiotic activities for animals and humans. A prebiotic is a nondigestible food ingredient that beneficially affects the host by selectively stimulating the growth and activity of one, or a limited number of, bacteria in the colon, improving the host's health (Kolida et al. 2002). Prebiotic carbohydrates act by increasing the growth activity of bifidobacteria in the human intestine, with the advantage of being selectively used by bifidobacteria and not by pathogenic microorganisms such as *Salmonella* and *Escherichia coli*. Bifidobacteria are known as useful microorganisms that are able to maintain human health and prevent the increase of pathogenic microorganisms.

Prebiotic oligosaccharide synthesis in cashew apple juice was studied because of the presence of simple sugars (glucose and fructose) that act as acceptors. Vergara et al. (2010) studied the production and prebiotic effect of cashew apple juice fermented with *L. mesenteroides* B-512F and *Leuconostoc citreum* B-742. The strains were cultivated in clarified cashew apple juice to produce prebiotic oligosaccharides. Yeast extract (20 g/L), K_2HPO_4 (g/L), and sucrose (50 g/L) were added to the juice to promote microbial growth and dextransucrase production. Initial pH was adjusted to 6.5. Fermentations were carried out at

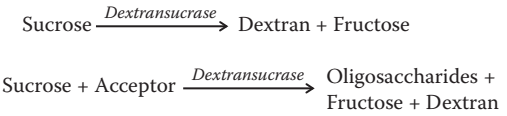

FIGURE 13.2 Enzyme reaction for oligosaccharide synthesis in cashew apple juice.

30°C and at 150 rpm for 24 hours. The prebiotic effect of the fermented cashew apple juice, containing oligosaccharides, was evaluated through *Lactobacillus johnsonii* B-2178 growth. *L. johnsonii* was incubated for 48 hours using fermented cashew apple juice as substrate. Lactobacillus growth was compared with microbial growth in nonfermented juice and in De Man, Rogosa and Sharp (MRS). *L. johnsonii* growth in the fermented cashew apple juice was threefold higher than the amount observed in the nonfermented juice.

Rabelo et al. (2009) used the crude enzyme of *L. citreum* B-742 produced in cashew apple juice. After fermentation, cells were removed by centrifugation. The crude enzyme in cashew apple juice was mixed with fresh cashew apple juice containing sucrose, and the synthesis was carried out for 24 hours. Results showed that cashew apple juice is a good source of reducing sugars and can be used as a substrate for the production of dextransucrase by *L. citreum* B-742 for the synthesis of oligosaccharides using the crude enzyme. Optimal oligosaccharide yield (~80%) was obtained for sucrose concentrations lower than 60 g/L and reducing sugar concentrations higher than 100 g/L.

Oligosaccharides with a degree of polymerization of up to 6 were obtained using dextransucrase from two different strains: *L. mesenteroides* NRRL B-512F, the industrial strain, and *L. citreum* NRRL B-742. As dextransucrase was produced directly into cashew apple juice, the costs of enzyme recovery and purification were avoided.

13.3.3 Probiotic Cashew Apple Juice

Technological advances have made it possible to alter some of the structural characteristics of fruit and vegetable matrices by modifying food components in a controlled way (Betoret et al. 2003). This could make them ideal substrates for the culture of probiotics because they already contain beneficial nutrients such as minerals, vitamins, dietary fibers, and antioxidants (Yoon et al. 2004), while lacking the dairy allergens that might prevent consumption by certain segments of the population (Luckow and Delahunty 2004). There is a genuine interest in the development of fruit juice–based functional beverages with probiotics because they have taste profiles that are appealing to all age groups and because they are perceived as healthy and refreshing foods (Tuorila and Cardello 2002; Yoon et al. 2004; Sheehan et al. 2007).

Comparing dairy and nondairy probiotic products, Zhou et al. (2009) reported viable cell counts of 8.59 ± 0.04 log CFU/mL after fermentation, when *L. casei* was inoculated in mare milk, whereas Sheehan et al. (2007) reported viable cell counts of 8.20 ± 0.01 log CFU/mL of *L. casei* in pineapple juice. Fruits and vegetables have been suggested as ideal media for probiotic growth because they inherently contain essential nutrients, they are good-looking, and have good taste (Luckow and Delahunty 2004; Sheehan et al. 2007). However, the survival of probiotics in fruit-based matrix is more complex in dairy products because the bacteria usually need protection from the acidic conditions in these media (Shah 2007).

Pereira et al. (2011) studied *L. casei* NRRL B-442 cultivation in cashew apple juice. Cashew apples from EMBRAPA's Experimental Station in Pacajús (Brazil) were used and clarified juice was used. *L. casei* was inoculated into the cashew apple juice and fermentation was carried out at the optimal conditions. The product was stored under refrigeration (4°C) as usually done for ready-to-drink probiotic beverages (fermented milk). The product stability was evaluated along 42 days.

Color component L^* (lightness) describes the brightness of the color. It separates color into bright and dark colors. During fermentation, there was an increase in cashew apple juice turbidity because of the biomass increase of *L. casei* in the juice, which resulted in a reduction of lightness compared to the nonfermented juice. The color component b^* (yellowness) of cashew apple juice, inoculated with *L. casei*, increased with fermentation because of carotenoid isomerization. This probably happened because of the reduction in pH during the fermentation period as result of lactic acid production. The slight decrease of a^* values during this process indicated that, in cashew apple juice fermentation, the green color component increased. The total color change ΔE^* also increased during fermentation. However, the color change observed after juice fermentation did not change the characteristics of the juice or its acceptability. In fact, because of the increase in yellowness, the color of the juice was enhanced, making it more attractive compared with the nonfermented juice (Pereira et al. 2011).

Viable cell counts were the main concern during the shelf life of probiotic cashew apple juice. The minimal value desirable is usually 6 log CFU/mL, which corresponds to an ingestion of 8 log UFC (10^8 CFU) per serving portion of 100 mL. Fermented cashew apple juice presented a slight increase of viable cells counts of *L. casei* at the beginning of storage, but from day 35, there was a slight reduction of viable cell counts. However, at the end of the storage period, the viable cell counts of *L. casei* in fermented cashew apple juice was higher than 8.00 log CFU/mL, which is considered a great value for fermented products containing probiotics and assures an ingestion of 10^{10} CFU per serving portion of 100 mL. No protection agents were necessary for probiotic cashew apple juice.

Few works have been published on probiotic juices. Ross and Fitzgerald (2007) reported viable cell counts of 8.20 ± 0.01 log CFU/mL of *L. casei* in pineapple juice. Yoon et al. (2004) found that *Lactobacillus plantarum, L. casei,* and *Lactobacillus delbrueckii* grew rapidly on sterilized cabbage juice without nutrient supplementation, reaching nearly 8.00 log CFU/mL at 48 hours of fermentation at 30°C. It is important to emphasize that for probiotic cashew apple juice preparation, no heat treatment was necessary and there was no evidence of contamination. Thus, the *L. casei* overlapped and controlled other microbial growth and juice spoilage factors, avoiding the added costs of heat treatment as well as its adverse effects, such as nutritional losses and sensory changes.

Fermented cashew apple juice was evaluated by 10 untrained panelists after the addition of sugar (8% p/v) and the juice was considerably acceptable. Enzyme browning, which is characteristic of cashew apple juice and is usually avoided by using chemical products such as sodium metabisulfite, was avoided by applying fermentation technology only. Aside from taste, color is an important factor in food acceptance and the maintenance of the characteristic color without preservatives is a technological advantage.

Complementary studies on the effect of the fermentation process on sensory acceptance with a broader range of panelists, aroma, and on the juice's nutritional values such as carbohydrate (sugar) content, carotenoids, antioxidant activity, and vitamin content, especially B vitamins, are under development by our group and will be published elsewhere. For now, we can conclude that cashew apple juice fermented with *L. casei* is a good, healthy alternative for functional foods containing probiotics.

REFERENCES

Abreu FAP. 1997. Aspectos tecnológicos da gaseificação do vinho de caju (*Anacardium occidentale* L.). [Master's Thesis]. Universidade Federal do Ceará, Fortaleza, Brazil. 95 p.

Aderiye BI, Mbadiwe UV. 1993. Alcohol production in submerged cashew pomace. *Plant Foods Hum Nutr* 43:273–8.

Adesioye HO. 1991. Production of wine from cocoa (*Theobroma cacao* L. *Kuntze*) juice using *Saccharomyces* sp. Isolated from palm wine and cashew juice. Paper presented at the 15th Annual Conference of NIFST, Otta, Nigeria.

Akinwale TO. 2000. Cashew apple juice: its use in fortifying the nutritional quality of some tropical fruits. *Eur Food Res Technol* 211:205–7.

Betoret N, Puente L, Díaz MJ, Pagán MJ, García MJ, Gras ML. 2003. Development of probiotic-enriched dried fruits by vacuum impregnation. *J Food Eng* 56:273–7.

Brown AC, Valiere A. 2004. Probiotics and medical nutrition therapy. *Nutr Clin Care* 7:56–68.

Chagas CMA, Honorato TL, Pinto GAS, Maia GA, Rodrigues S. 2007. Dextransucrase production using cashew apple juice as substrate: effect of phosphate and yeast extract addition. *Bioprocess Biosyst Eng* 30:207–15.

Chung CH, Day DF. 2002. Glucooligosaccharides from *Leuconostoc mesenteroides* B-742 (ATCC 13146): a potential prebiotic. *J Ind Microbiol Biotechnol* 29:196–9.

Chung CH, Day DF. 2004. Efficacy of *Leuconostoc mesenteroides* (ATCC 13146) isomaltooligosaccharides as a poultry prebiotic. *Poult Sci* 83:1302–6.

Filgueiras HAC, Alves RE, Mosca JL, Menezes JB. 1999. Cashew apple for fresh consumption: research on harvest and postharvest technology in Brazil. *Acta Hortic* 485:155–60.

Fontes CPML, Honorato TL, Rabelo MC, Rodrigues S. 2009. Kinetic study of mannitol production using cashew apple juice as substrate. *Bioprocess Biosyst Eng* 32:493–9.

Garruti DS, Abreu FAP, Franco MRB, Silva MAP. 2006. The influence of fermentation temperature and sulfur dioxide on the volatile composition and flavor profile of cashew wine. In: Flavor science: Vol. 43. *Recent advances and trends (developments in food science)*. New York: Elsevier. p. 109–12.

Heincke K, Demuth B, Jördening HJ, Buchholz K. 1999. Kinetics of the dextransucrase acceptor with maltose-experimental results and modeling. *Enzyme Microb Technol* 24:523–34.

Honorato TL, Rabelo MC, Gonçalves LRB, Rodrigues S. 2007. Fermentation of cashew apple juice to produce high added value products. *World J Microbiol Biotechnol* 23:1409–15.

Honorato TL, Rodrigues S. 2010. Dextransucrase stability in cashew apple juice. *Food Bioprocess Technol* 3:105–10.

Kalliomaki M, Salminen S, Arvilommi H, Kero P, Koskinen P, Isolauri E. 2001. Probiotics in primary prevention of atopic disease: a randomized placebo controlled trial. *Lancet* 357:1076–9.

Koepsell HJ, Tsuchiya HM, Hellman NN, Kazenko A, Hoffman CA, Sharpe ES, Jackson RW. 1953. Enzymatic synthesis of dextran: acceptor specificity and chain initiation. *J Biol Chem* 200:793–801.

Kolida S, Thuhy K, Gibson GR. 2002. Prebiotic effect of inulin and oligofructose. *Br J Nutr* 87:S193–7.

Lavinas FC, Almeida NC, Miguel MAL, Lopes MLM, Valente-Mesquista VL. 2006. Estudo da estabilidade química e microbiológica do suco de caju *in natura* armazenado em diferentes condições de estocagem. *Ciênc Tecnol Aliment* 26:875–83.

Lowor TS, Agyent-Badu CK. 2009. Mineral and proximate composition of cashew apple juice from Northern, Savannah, Forest and Coastal regions in Ghana. *Am J Food Technol* 4:154–61.

Luckow T, Delahunty C. 2004. Which juice is healthier? A consumer study of probiotic non-dairy juice drinks. *Food Qual Prefer* 15:751–9.

Mohanty S, Ray P, Swain MR. 2006. Fermentation of cashew apple (*Anacardium occidentale* L.) apple into wine. *J Food Process Preserv* 30:314–22.

Monchois V, Willemot RM, Monsan P. 1999. Glucansucrases: mechanism of action and structure function-relationships. *FEMS Microbiol Rev* 23:131–51.

Monsan P, Paul F. 1995. Enzymatic synthesis of oligosaccharides. *FEMS Microbiol Rev* 16:187–92.

Naragaraja KV, Bhuvaneshwari S, Swamy KRM. 2007. Biochemical characterization of cashew (*Anacardium occidentale* L.) apple juice and pomace in India. PGR Newsletter—FAO Biodivers 149, 9–13.

Osho A, Odunfa AS. 1999. Fermentation on cashew apple juice using the wine yeast strain NCYC 125 and three other isolated yeast strains. *Adv Food Sci* 21:23–9.

Pereira ALF, Maciel TC, Rodrigues S. 2011. Probiotic beverage from cashew apple juice fermented with *Lactobacillus casei*. *Food Res Int* 44:1276–83.

Pereira AM, Costa FAA, Rodrigues MI, Maugeri F. 1998. In vitro synthesis of oligosaccharides by acceptor reaction of dextransucrase from *Leuconostoc mesenteroides*. *Biotechnol Lett* 20:379–401.

Pinheiro ADT, Rocha MVP, Macedo GR, Gonçalves LRB. 2009. Evaluation of cashew apple juice for the production of fuel ethanol. *Appl Biochem Biotechnol* 155:366–78.

Rabelo MC, Fontes CPML, Rodrigues S. 2009. Enzyme synthesis of oligosaccharides using cashew apple juice as substrate. *Bioresource Technol* 100:5574–80.

Rabelo MCC, Honorato TL, Gonçalves LRB, Pinto GAS, Rodrigues S. 2006. Enzymatic synthesis of prebiotic oligosaccharides. *Appl Biochem Biotechnol* 133:31–40.

Rocha MVP, Barreto RVG, Melo VMM, Gonçalves LRB. 2009. Evaluation of cashew apple juice for surfactin production by *Bacillus substilis* LAMI008. *Appl Biochem Biotechnol* 155:63–75.

Rocha MVP, Souza MCM, Benedicto SCL, Bezerra MS, Macedo GR, Pinto GAS, Gonçalves LRB. 2007. Production of biosurfactant by *Pseudomonas aeruginosa* grown on cashew apple juice. *Appl Biochem Biotechnol* 137–40:185–9.

Rodrigues S, Lona LMF, Franco TT. 2003. Effect of phosphate on production of *Leuconostoc mesenteroides* NRRL B512F. *Bioprocess Biosyst Eng* 23:57–62.

Rodrigues S, Lona LMF, Franco TT. 2005. The effect of maltose on dextran yield and molecular weight distribution. *Bioprocess Biosyst Eng* 28:9–14.

Rodrigues S, Lona LMF, Franco TT. 2006. Optimizing panose production by modeling and simulation using factorial design and surface response analysis. *J Food Eng* 75:433–40.

Rufino MSM, Pérez-Jiménez J, Tabemero M, Alves RE, Brito ES, Saura-Calixto F. 2010. Acerola and cashew apple as sources of antioxidants and dietary fibre. *Int J Food Sci Technol* 45:2227–33.

Shah NP. 2007. Functional cultures and health benefits. *Int Dairy J* 17:1262–77.

Sheehan VM, Ross P, Fitzgerald GF. 2007. Assessing the acid tolerance and the technological robustness of probiotic cultures for fortification in fruit juices. *Innov Food Sci Emerg Technol* 8:279–84.

Silveira MS, Fontes CPML, Guilherme AA, Fernandes FAN, Rodrigues S. 2010. Cashew apple juice as substrate for lactic acid production. *Food Bioprocess Technol* (in press). doi:10.1007/s11947-010-0382-9.

Tsuchiya HM, Koepsell HJ, Corman J, Bryant G, Bogard MO, Feger VH, Jackson RW. 1952. The effect of certain cultural factors on production of dextransucrase by *Leuconostoc mesenteroides*. *J Bacteriol* 64:521–7.

Tuorila H, Cardello AV. 2002. Consumer responses to an off/flavor in juice in the presence of specific health claims. *Food Qual Prefer* 13:561–9.

Vergara CMAC, Honorato TL, Maia GA, Rodrigues S. 2010. Prebiotic effect of fermented cashew apple (*Anacardium occidentale* L.) juice. *LWT—Food Science and Technology* 43:141–5.

Yoon KY, Woodams EE, Hang YD. 2004. Probiotication of tomato juice by lactic acid bacteria. *J Microbiol* 42:315–8.

Zhou Q, Wang JC, Guo Z, Yan L, Zhang Q, Chen W, Liu X-M, Zhang HP. 2009. Fermentation characteristics and transit tolerance of *Lactobacillus casei* in reconstituted mare milk during storage. *Int J Dairy Technol* 62:249–54.

14

Production and Characterization of Wine from Mango (Mangifera indica L.) Fruit Juice

Lebaka Veeranjaneya Reddy and Obulam Vijaya Sarathi Reddy

CONTENTS

14.1 Introduction

Winemaking is one of the most ancient of man's technologies and is now one of the most commercially prosperous biotechnological processes. Historians believe that wine was being made in Caucasus and Mesopotamia as early as 6000 BC. Records for wine have been found in Egypt and Phoenicia, dating as far back as 5000 BC. By 2000 BC, it was being produced in Greece and Crete. Colonization by Romans spread winemaking all around the Mediterranean. By 500 BC, it was spread to Sicily, Italy, France, Spain, Portugal, and Northern Africa. The cultivation of wine also spread in to Balkan states, and the Romans took it into Germany and other parts of Northern Europe, eventually reaching as far as Britain (Robinson 1994).

The production and consumption of fermented beverages like wine is an ancient practice. However, the production and consumption of fruit-based distilled alcoholic beverages is a later development. Different aspects of fruit-based alcoholic beverages other than grapes have been investigated (Barnett 1980). The Rig-Veda amply testifies that wine is perhaps the oldest fermented product known to man. However, the actual birthplace of wine is still unknown, although it had been prepared somewhere in 350 BC (Joshi and Devender 2005). European explorers in the sixteenth century introduced wine into the new world (Amerine et al. 1980). The early spreading and world distribution of the vine and winemaking technology is shown in Figure 14.1.

Wine has been made in India for as long as 5000 years. It was the early European travelers to the courts of the Mughal emperors Akbar, Jehangir, and Shah Jehan in the sixteenth and seventeenth centuries who reported tasting wines from the royal vineyards. Advances in the second half of the twentieth century have clearly shown that the production of quality wines is not quite as simple a process as Pasteur, the founder of modern enology, suggested over a century ago. The most important transformation that takes place in the juice during vinification is the alcoholic fermentation of the sugars, especially hexoses (glucose and fructose), which mainly result in the production of ethanol and carbon dioxide (CO_2), and the generation of a large number of by-products. In most of the world's wine regions, at least until around the middle of the 1980s, wine had been made by fermentation with the indigenous yeasts present on the grapes when harvested, or introduced from the equipment and cellar during the vinification process. Recently, to ensure that must fermentation is rapid and complete and can produce wines of reproducible character and quality, the practice of adding selected yeasts to slightly sulfited musts

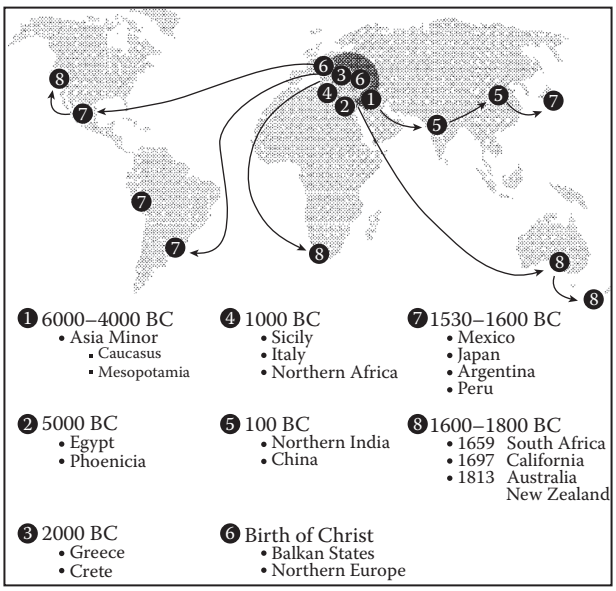

FIGURE 14.1 The early spreading and world distribution of the vine and winemaking technology.

has become widespread. Presently, most commercial wine yeasts are strains of *Saccharomyces cerevisiae*, including those described by enologists as *Saccharomyces bayanus*, which has been reidentified in most cases as *S. cerevisiae*. In the initial phases of fermentation, different species of indigenous yeasts, called *wild yeasts*, present in the grape, make an important contribution. The predominant species belong to the genera *Brettanomyces*, *Candida*, *Cryptococcus*, *Debaryomyces*, *Dekkera*, *Hanseniaspora*, *Hansenula*, *Kloechera*, *Kluyveromyces*, *Pichia*, *Rhodotorula*, *Torulaspora*, *Schizosaccharomyces*, and *Zygosaccharomyces*. As fermentation progresses, these so-called "non-*Saccharomyces*" species die off, leaving the more ethanol-tolerant *S. cerevisiae* to predominate and complete the fermentation. This species has long been known as the *wine yeast*. Considerable progress has been made over the last decade in understanding the biochemistry and interactions of yeast, lactic acid bacteria, and other microorganisms during the winemaking process. This knowledge has been of great benefit to winemakers in their quest to produce high-quality wines.

14.1.1 Wine Production from Fruits Other than Grapes

In European usage, wine and brandy refer to exclusively fermented by-products of grapes—member of the genus *Vitis*, notably cultivars of vinifera. In England, especially in the New World, wines and brandies may refer to the fermented by-products of any fleshy fruit or flower. The quality and the quantity of grapes depend on geographical, geological, and climatic conditions in the vineyards and the grape variety and methods of cultivation (Joshi and Pandey 1999).

In general, grapes are the main raw materials that have been used for wine production for the past few decades. However, many research groups have investigated the suitability of fruits other than grapes (Table 14.1). Compared with the quantity of grape wine produced and consumed in the world, the amount

TABLE 14.1

Different Types of Wine Produced from Different Fruits

Fruits	Investigators
Apple	Sandhu and Joshi (1995)
Apricot	Joshi et al. (1990)
Banana	Kotecha et al. (1994)
Ber	Gautam and Chundawt (1998)
Cashew	Mandal (1997)
Custard apple	Kotecha et al. (1995)
Dates	Ali and Dirar (1984)
Jamun	Shukla et al. (1991)
Grape fruit and kinnow	Joshi and Thakur (1994)
Kiwi fruit	Heatherbell et al. (1980), Soufleros et al. (2001)
Litchi	Vyas et al. (1989)
Mandarin	Selli et al. (2004)
Mango	Kulkarni et al. (1980), Onkarayya and Singh (1984), Reddy and Reddy (2005)
Marula	Fundira et al. (2002)
Muskmelon	Teotia et al. (1991)
Orange	Selli et al. (2002, 2003)
Palm sap and coconut	Nathanael (1955)
Peach	Joshi and Shah (1998)
Pear	Atrri et al. (1994)
Plum	Vyas and Joshi (1982), Joshi and Sharma (1995)
Pomegranate	Adsule et al. (1992)
Sapota and guava	Bardiya et al. (1974)
Strawberry	Pilando et al. (1985)

of wine produced from fruits other than grape is insignificant (Amerine et al. 1980; Sandhu and Joshi 1995). However, in some countries, wines made from other fruits like apple (Spain, France, Belgium, Switzerland, and England), plum (Germany), and cashew apple (India) have a very high demand (Joshi and Pandey 1999).

Wines are made from a complete or a partial alcoholic fermentation of grape or any other fruit and contain certain ethyl alcohols as the intoxicating agent, essential elements, vitamins, sugars, acids, and phenolics. Wines from fruits are preferable to distilled liquors for stimulatory and healthful properties (Gasteineau et al. 1979). These beverages also serve as an important adjunct to the human diet by increasing the satisfaction and by contributing to the relaxation necessary for the proper digestion and absorption of food. Joshi and John (2002) have reported the antimicrobial effect of apple wine on pathogenic bacteria.

14.1.2 Fruit Wines Production and Postharvest Losses

Fruits are highly perishable commodities and have to be either consumed immediately or preserved in one form or another. In developed countries, a considerable quantity of fruit is used, but in developing countries, the lack of proper utilization results in considerable postharvest losses, estimated to be 30% to 40%. The increased production can be soaked up profitably if fruit wines are produced (Sandhu and Joshi 1995; Joshi and Parmar 2004). Setting up fruit wineries, aside from industrializing the fruit-growing belts, could result in the economic upliftment of the people, generating employment opportunities and providing better returns of their produce to the orchardists (Amerine et al. 1980; Fowles 1989; Joshi et al. 2004). The availability of technology is the single most important factor determining the production through cost, and the type of the product is also a significant consideration in popularizing the product. Hence, the production of fruit wines in those countries where fruits other than grapes are grown would certainly be advantageous.

14.1.3 Mango

It is a matter of astonishment to many that mango (*Mangifera indica* L.), one of the most celebrated of tropical fruits, is a member of the family Anacardiaceae, which is notorious for including several highly poisonous plants. The mango fruit is one of the most highly prized desert fruits of the tropics. It has a rich, luscious, aromatic flavor and a delicious taste in which sweetness and acidity delightfully blended. Mango production has experienced continuous growth in the last decades of the twentieth century (Baisya 2004).

The world's total annual mango fruit production was estimated at 22 million metric tons. The global production of mangoes is concentrated mainly in Asia and more precisely in India, which produced 12 million metric tons per annum. Mangoes are cultivated in 85 countries. The total world production in 2004 was 26,147,900 metric tons (Food and Agriculture Organization of the United Nations 2007). Asia and the oriental countries produced approximately 80% of the world's total production. Major mango-producing countries are India, Mexico, China, and Pakistan.

Mango is the choicest fruit of the country. It has been cultivated in India for the last 4000 years. Mango is called the king of fruits and the pride fruit of India. The crop is significantly important in the fruit economy of India and is the third largest industry in the country. In India, mango is grown in 2 million acres and occupies 39% of the total fruit production (Figure 14.2). It is the most cultivated area occupied by crops in India, with 60% of the total area under fruits. More than 25 types of mango cultivars, which are widely cultivated all over the world, are available in India (Anonymous 1962; Table 14.2).

Andhra Pradesh, being blessed with varied soil types and agroclimatic conditions, is suitably located for the cultivation of large varieties of fruit crops and is one of the largest fruit-producing states in India. The major fruit crops grown in Andhra Pradesh are mango, sweet orange, banana, grape, pomegranate, coconut, and cashew. Mango, occupying an area of 3.7 lakh hectares with an annual production of 32 lakh metric tons, has placed the state in first position with a share of 20% of India's production together with the highest productivity (Baisya 2004).

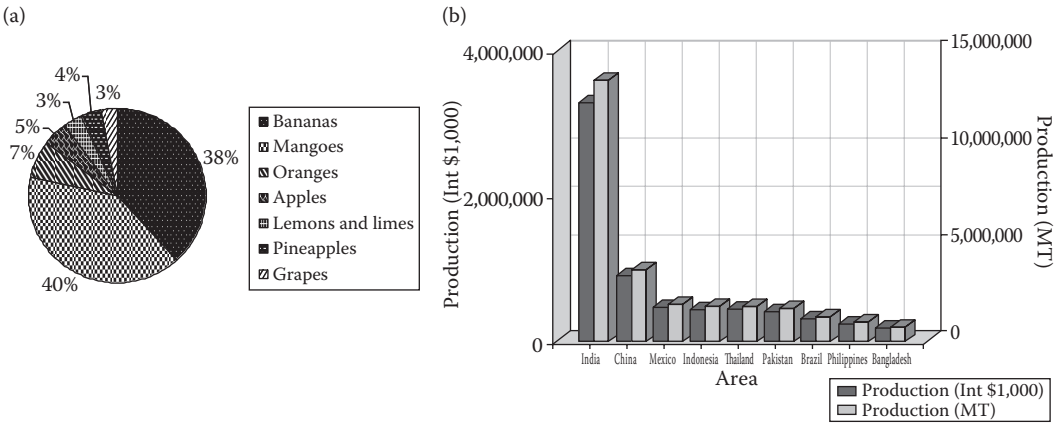

FIGURE 14.2 Production of different fruits in India (2004). (From Food and Agriculture Organization of the United Nations, *FAOSTAT statistic database agriculture*, Rome, Italy, http://faostat.fao.org/faostat/collection, 2007.)

Mango contains a high concentration of sugar (16%–18% w/v) and acids with organoleptic properties and also contains antioxidants like carotene (i.e., vitamin A, 4800 IU). Sucrose, glucose, and fructose are the principal sugars in ripened mango, with small amounts of cellulose, hemicellulose, and pectin. The green tender fruits are rich in starch, and during ripening, the starch that is present is hydrolyzed to reducing sugars (Anonymous 1962). The unripened fruit contains citric acid, malic acid, oxalic acid, succinic acid, and other organic acids, whereas the ripened fruit mostly contains malic acid (Giri et al. 1953). Mango juice along with aromatics is recommended as a restorative tonic; it contains good concentrations of vitamins A and C, which are useful in heat apoplexy. Mangoes with higher initial concentrations of β-carotene are helpful as cancer-preventing agents. Fruits like mango are highly perishable commodities. In developing countries like India, 20% to 30% of fruits produced undergo spoilage because of the lack of proper utilization and undeveloped postharvesting technology (Sandhu and Joshi 1995).

TABLE 14.2

Varieties of Mangoes Produced in India from Different States

State	Varieties
Andhra Pradesh	Banganapalli, Suvarnarekha, Neelum, and Totapuri
Bihar	Bombay Green, Chausa, Dashehari, Fazli, Gulabkhas, Kishen Bhog, Himsagar, Zardalu, and Langra
Gujarat	Kesar, Alphonso, Rajapuri, Jamadar, Totapuri, Neelum, Dashehari, and Langra
Haryana	Chausa, Dashehari, Langra, and Fazli
Himachal Pradesh	Chausa, Dashehari, and Langra
Karnataka	Alphonso, Totapuri, Banganapalli, Pairi, Neelum, and Mulgoa
Madhya Pradesh	Alphonso, Bombay Green, Dashehari, Fazli, Langra, and Neelum
Maharashtra	Alphonso, Kesar, and Pairi
Punjab	Chausa, Dashehari, and Malda
Rajasthan	Bombay Green, Chausa, Dashehari, and Langra
Tamil Nadu	Alphonso, Totapuri, Banganapalli, and Neelum
Uttar Pradesh	Bombay Green, Chausa, Dashehari, and Langra
West Bengal	Fazli, Gulabkhas, Himsagar, Kishenbhog, Langra, and Bombay Green

14.2 Wine Production from Mango Pulp

The ever-increasing fruit production needs improvement in preservation technologies. In India, efforts have been made to use the surplus (after export) and the small and unattractive mangoes for various purposes; one of the alternatives is wine production. Fruits are used to produce a variety of alcoholic beverages, including different types of fruit wines, and their distillates are known as brandy. The Rig-Veda has also mentioned the medicinal power of wines (Vyas and Chakravorthy 1971). These beverages have been a part of the food of man ever since his settlement in Tigris Euphrates basins and were used as therapeutic agents. Although fruit wines are produced and consumed worldwide, hard liquors still constitute a big chunk of the alcoholic beverages consumed in India. The research on wine production carried out in India reveals that an impressive progress has been made in the development of technologies for the preparation of wines of different types from various fruits. Successful marketing of grapes and apple wines in India is an indicator of potential Indian market waiting for fruit wines.

Mangoes are a highly perishable tropical fruit, with a shelf life of 2 to 4 weeks at 10°C to 15°C (Yahia 1998), limiting their availability in fresh markets. The shelf stability and the physical and chemical qualities of mangoes can be threatened by required insect quarantine treatments, chilling temperatures, and fungal rot, as these conditions may induce abiotic or biotic stress to the fruit (Cisneros-Zevallos 2003). Postharvest physiologists have extensively studied postharvest treatments to identify optimal conditions for the retention of fruit quality, enabling these fruit to be shipped to distant markets. An alternative and profitable method of using mangoes for winemaking could become widely accepted. Investigators have carried out much research on mango composition and on cultivation aspects. Anonymous (1963) first reported the wine production from mango pulp, and then Czyhrinciwk (1966) reported the technology involved in mango wine production. However, Kulkarni et al. (1980), Onkarayya and Singh (1984), and Onkarayya (1985, 1986) screened some varieties of mango for winemaking and found that mango wine had similar characteristics to that of grape wine. These authors have not given details on the vinification techniques and the chemical composition of the wine produced from mango. Their work was inadequate, particularly in the area of mango wine production and its composition. Varakumar et al. (2010) have carried out extensive investigations on mango wine production using 13 locally abundantly available mango cultivars.

14.2.1 Screening of Mango Varieties

The suitability of mango cultivars for wine production is generally screened on the basis of juice quality, quantity, and other properties. The screening of some commercial mango varieties for winemaking has been reported by Kulkarni et al. (1980) and Onkarayya and Singh (1984). According to their reports, the composition of wine changes with the mango variety used in the fermentation. Mango juice is prepared by washing the mangoes with 1% HgCl and peeled and pulped manually. To obtain good yields, pulp was treated with Trizyme P50 (800 U/mL), a pectinase enzyme. Among the 10 varieties they used, 6 varieties showed promising juice yields (450–550 mL/kg mango). The juice yield and the physical and chemical compositions are presented in Table 14.3. In mango, three types of sugars are present: glucose, fructose, and sucrose. The total soluble solids of mango juice are between 14.2% and 20.5%. The Banginapalli (20.5%) variety had high total soluble solids. The sugar content of mango juice ranged from 15% to 18% (w/v), whereas the titrable acidity as tartaric acid ranged from 0.310% to 0.462% (w/v). The pH of the juices is between 3.8 and 4.5 (Reddy and Reddy 2005).

Carbohydrate metabolism plays an important role during mango fruit development, particularly in the changes in sugar content (Lakshminarayana et al. 1983). Fructose and glucose come from sucrose hydrolysis, glucose also being produced by starch hydrolysis. These hexoses are used in fuel respiration and for fruit growth. Generally, fructose represents approximately 20% to 30% of total sugars during mango fruit development and may be considered as a storage sugar. Moreover, fructose sweetness is more pronounced than the other sugars, and it may thus account for a significant part of the sweet taste of mango. The pH increases during the maturation and ripening of the fruit. According to this, at low pH values, such as during the early stages of development, pH increases result in a decrease in malic acid content, whereas at higher pH values in the final stages of fruit development in mango flesh (pH from

TABLE 14.3

Physicochemical Characteristics of Mango Pulp

Mango Variety	Juice Yield (mL/kg)	Reducing Sugars (% w/v)	Titrable Acidity (%)	pH	TSS (%)
Alphonso	570 ± 10	16.3 ± 1.32	0.33	4.1 ± 0.53	16.0 ± 1.2
Raspuri	600 ± 13	15.5 ± 2.21	0.43	3.9 ± 0.86	14.2 ± 1.8
Banginapalli	550 ± 17	18.5 ± 1.24	0.32	4.0 ± 0.6	20.5 ± 0.79
Totapuri	500 ± 22	16.0 ± 1.0	0.31	4.2 ± 1.0	16.5 ± 1.2
A. Banesha	500 ± 15	18.0 ± 0.8	0.32	4.5 ± 0.45	20.1 ± 1.42
Neelam	480 ± 20	15.5 ± 1.7	0.42	4.3 ± 0.8	15.5 ± 1.5
Mulgoa	468 ± 8	14.3 ± 1.4	0.42	4.3 ± 0.5	15.0 ± 1.24
Suvarnarekha	470 ± 12	15.0 ± 0.55	0.40	3.9 ± 1.3	14.4 ± 0.58
Rumani	475 ± 14	14.5 ± 1.0	0.39	4.2 ± 0.72	14.6 ±1.43
Jahangir	460 ± 10	15.6 ± 1.62	0.46	4.6 ± 0.56	14.2 ± 1.3

Note: TSS, total soluble solids.

3 to 4.5 during fruit growth), pH results increase in malic acid content. Citric acid is synthesized when fruit growth becomes slower and when energy demand is low (Selvaraj and Kumar 1994). At maturation, however, malic acid concentration is linked to total cation concentrations of ripe fruit.

14.2.2 Fermentation

Batch fermentation is preferable by inoculating the actively growing yeast into must contained in fermenters or conical flasks fitted with a rubber cork fixed with a bent glass tube for CO_2 release under stationary conditions for a period of 20 days.

14.2.3 Influence of Mango Variety on the Composition of Wine Produced

Differences in the composition between mango varieties have been previously reported. The distinctiveness or the intensity of mango aroma varies between varieties of *M. indica* and within a variety, depending on the climate, the ripeness of the fruits, the crop size, and other factors. Of interest to the winemaker are these major fruit components: sugar, organic acids, aroma and flavor compounds, phenolic compounds/tannins, certain amino acids, and certain metallic compounds such as potassium. Fully ripe mangoes contain almost equal quantities of glucose and fructose, which are simple sugars, and when fermented with yeast, form ethanol and CO_2. Ripe mango fruits contain 70% to 80% of water by weight. Reddy (2005) reported the composition of 10 mango wines produced from different cultivars. He suggested that the concentration of ethanol, organic acids, tannins, and aromatic volatile compounds produced differed with mango variety. The concentration of higher alcohols ranged from 131 to 343 mg/L (Table 14.4). The highest is in the wine produced from the Banginapalli variety, and the lowest is in the wine produced from the Neelam variety. Higher alcohols may influence certain sensory characteristics, although they constitute a relatively lesser quantity of the total substances.

Esters have a crucial importance in wine quality as they provide pleasant aroma sensations. The formation of esters in wine depends on various factors such as type of fruit, yeast strain, and fermentation conditions like temperature, pH, and incubation time. Although the yeast used in fermentation was the same, the ester concentration was different in each type of mango fruit. In the mango wine produced from different varieties, the concentration of esters varied from 15 to 35 mg/L (Table 14.4).

14.2.4 Screening of Yeast Strains for Mango Wine Production

The inoculation of must with selected yeasts minimizes the influence of wild yeast on wine quality. However, little is known about the contribution of wild yeasts to the synthesis of volatile compounds during inoculated fermentations. In mango wine fermentation, different investigators used different yeast strains.

TABLE 14.4

Physicochemical Characteristics of Mango Wine Produced at 22°C ± 2°C and pH 5

Mango Variety	Ethanol (% w/v)	Total Acidity (% v/v)	Volatile Acidity (% v/v)	pH	Residual Sugars (g/L)	Higher Alcohols (mg/L)	Total Esters (mg/L)	Tannins (% w/v)	Color Optical Density at 590 nm
Alphonso	7.5	0.650	0.100	3.8	2.1	300	25	0.011	0.22
Raspuri	7	0.735	0.210	3.8	2.4	200	29	0.072	0.18
Banginapalli	8.5	0.600	0.181	3.7	2.0	343	35	0.012	0.23
Totapuri	7	0.622	0.121	4.0	2.0	230	20	0.012	0.17
A. Banesha	8	0.610	0.110	4.0	2.0	320	30	0.013	0.25
Neelam	6.5	0.826	0.234	3.6	2.5	131	15	0.014	0.21
Mulgoa	6.3	0.621	0.109	3.9	3.0	152	18	0.065	0.28
Suvarnarekha	6.8	0.630	0.153	4.1	2.3	175	22	0.025	0.19
Rumani	6.9	0.618	0.125	4.0	2.1	212	15	0.027	0.24
Jahangir	7.1	0.646	0.138	3.8	2.0	256	21	0.042	0.16

Onkarayya and Singh (1984) used the *S. cerevisiae* strain, and Reddy (2005) screened the three types of yeast strains isolated from different sources. To evaluate the fermentation performance, the yeast strains are subjected to different temperature and pH during fermentation. From the three yeast strains, *S. cerevisiae* CFTRI 101 shows promising results at all conditions followed by the yeast strain isolated from palm wine and the yeast strain purified from commercial baker's yeast (PWY1). The baker's yeast could not ferment or use the complete sugar from the mango must and has a slow fermentation rate. The palm wine yeast strain's fermentation rate is comparable with that of CFTRI 101, but it cannot tolerate high concentrations of ethanol, which is the most desirable character for wine fermentation. The characteristic feature of lower ethanol tolerance might be the reason for the low ethanol production from the remaining yeasts.

14.2.5 Influence of Yeast Strain on the Composition of Mango Wine

The compounds formed during alcoholic fermentation have a decisive influence on the volatile composition of wine. The major volatile products of yeast metabolism, ethanol, glycerol, and CO_2 make a relatively small yet fundamental contribution to wine flavor. The main groups of compounds that form the *fermentation bouquet* are organic acids, higher alcohols, and esters and, to a lesser extent, aldehydes (Lambrechts and Pretorius 2000). The CFTRI 101 yeast strain completely use the sugar in mango must and convert it into ethanol (residue 1–2 g/L). This strain showed vigorous fermentation capacity during the fermentation of mango must. The strain is well adopted to the conditions of mango must and started ethanol production within 4 hours of inoculation and completed the fermentation within 70 to 76 hours, and it showed good ethanol tolerance capacity up to 10% (w/v). The higher alcohol concentration varies in wines produced by three yeast strains. Strain 101 produce higher concentrations of alcohols compared with the other two strains. The concentration with CFTRI 101 ranged between 150 and 300 mg/L, followed by baker's yeast (123–200 mg/L) and palm wine isolate (100–185 mg/L). Esters are mainly used to synthesize all mango must samples after the consumption of the first 20% to 30% of sugars by the yeast. The greatest synthesis takes place during the fermentation at 50% to 75% of the sugars. High amounts of esters are present in the samples with CFTRI 101 yeast strain, which has a greater capacity for generating these volatile compounds compared with the other two.

14.3 Optimization of Mango Wine Fermentation Conditions

14.3.1 Enzyme Treatment

Pectolytic enzymes have been used in fruit juice processing for several purposes: extraction (Rombouts and Pilnik 1978), clarification (Pilnik 1983), liquefaction and maceration (Sreenath et al. 1987, 1995),

and cloud stabilization (Askar et al. 1990). The maceration action of enzymes consists of a controlled action of the pectolytic enzymes (Voragen and Pilnik 1981) on the highly esterified pectins constituting cell walls rather than the pectins in the middle lamella. The use of lyases or polygalacturonases with low pectinesterase activity enabled the recovery of intact cell suspensions. Conversely, during liquefaction, the degradation of the middle lamella is followed by cell wall lysis under the action of pectolytic enzymes, cellulases, and hemicellulases. In particular, the mango pulp contains high levels of pectin and needs pectinase treatment for the clarification of juice. A decrease of 50% or more of the relative viscosity of mango pulp resulted from different times of maceration with pectolyase and with three of the four commercial enzyme preparations (Sakho et al. 1998). Preliminary studies were carried out to optimize the conditions for maximum extraction of juice, using different levels of pectinase enzyme and different incubation periods at ±28°C. On the basis of these studies, 0.6% pectinase and 8 hours of incubation time were selected for obtaining the juice from the pulp. The juice was separated by centrifugation, and the clear juice was used for the preparation of wine by slight modification of the method described. Pectinase treatment increased the juice yield and the fermentability of mango pulp (Table 14.5). In addition, pectinase treatment increases the fermentation performance as well as the chemical component production during wine fermentation. Pectinase treatment also enhances the juice extraction and decreases the viscosity of juice, which could make the wine production from mango economical. The total alcohol content was higher in pectinase-treated wines in comparison with control wines (Table 14.5). Finally, we suggest that pectinase treatment is essential to produce more quality wines from fruits like mango.

14.3.2 Fermentation Conditions

Kumar et al. (2009) optimized the fermentation conditions, temperature, pH, and inoculum size using the response surface methodology. Reddy and Reddy (2010) investigated the effect of fermentation conditions such as temperature, pH, sulfur dioxide (SO_2), and aeration on mango wine composition and yeast growth.

14.3.2.1 Temperature

Temperature has a positive influence on fermentation—to some extent, temperature increases the yeast growth, the speed of enzyme action (which approximately doubles with every 10°C increase in temperature), and the cell sensitivity to the toxic effect of alcohol, which increases with temperature because of increased membrane fluidity. This may partially explain the rapid decline in yeast viability

TABLE 14.5

Effect of Pectinase Enzyme Treatment on Mango Juice Recovery and Wine Composition

	Banginapalli		Totapuri	
Character of Juice or Wine	**Untreated**	**Treated**	**Untreated**	**Treated**
Juice yield (mL/kg)	458 ± 12	550 ± 10, $p < .0001$	416 ± 8.0	500 ± 22, $p < .0001$
TSS (% w/v)	15 ± 0.60	20.5 ± 0.79, $p < .001$	12 ± 0.80	16.5 ± 1.2, $p < .001$
Reducing sugars (% w/v)	15.8 ± 1.5	18.5 ± 1.6, $p < .0130$	15 ± 1.0	16 ± 1.3, $p < .1662$
Acidity (% w/v)	0.43 ± 0.06	0.56 ± 0.08, $p = .0097$	0.31 ± 0.03	0.44 ± 0.05, $p < .0003$
pH	4.0 ± 0.53	3.7 ± 0.3, $p = .2553$	4.2 ± 0.5	4.0 ± 0.45, $p < .4831$
Ethanol (% w/v)	6.3 ± 1.1	8.5 ± 1.4, $p < .0127$	5.1 ± 0.9	7.0 ± 1.2, $p < .0112$
Higher alcohols (mg/L)	265 ± 8.2	340 ± 10.5, $p < .0001$	279 ± 6.8	358 ± 12.7, $p < .0001$
Total esters (mg/L)	20 ± 1.3	32 ± 1.5, $p < .0001$	16 ± 1.2	25 ± 0.8, $p < .0001$
Residual sugars (g/L)	10 ± 1.6	2.5 ± 1, $p < .0001$	15 ± 1.0	3 ± 1.3, $p < .0001$
Acidity (% w/v)	0.45 ± 0.04	0.66 ± 0.08, $p < .0002$	0.36 ± 0.03	0.54 ± 0.05, $p < .0001$
pH	4.4 ± 0.50	4.7 ± 0.41, $p = .2823$	4.5 ± 0.5	4.2 ± 0.5, $p = .3232$

Note: Values are presented as mean + SD.

at temperatures higher than 20°C during wine fermentation (Torija et al. 2002). At higher temperatures, more glycerol is produced. The enhanced production of glycerol at warm temperatures counters the bitterness of tannins and generates a smoother mouthfeel. Fermentation at low temperatures (<15°C) leads to more aromatic and paler wines (Walker 1998). Reddy and Reddy (2010) reported that at the start of fermentation, the time to reach maximal cell population and decline of yeast population at 15°C and 20°C were slow compared with fermentations at 25°C, 30°C, and 35°C. It was observed that cell viability decreased as temperatures increased. They also observed that fermentations at 30°C were completed in a short time and all the available sugars were consumed. Further increases in temperature (at 35°C) delayed the fermentation and left residual sugars. In contrast, low temperature fermentations, which started more slowly, consumed all the sugars faster because of the high biomass maintained throughout the process. High yeast mortality might have caused a slower fermentation rate in the final stages of the fermentation at 35°C.

Reddy and Reddy (2010) performed the analyses of the mango wine volatiles and suggested that the yeast used 99% of the available sugars (residual sugars, 1–2 g/L). The sum of all the secondary metabolism products increased as fermentation temperature increased from 15°C to 35°C. Glycerol concentrations increased as temperatures increased (5.3 g/L at 15°C and 8.4 g/L at 35°C; Table 14.6). The concentrations of acetaldehyde and esters decreased as the temperature increased. The concentration of total esters ranged from 18 to 40 mg/L, and ethyl acetate levels ranged from 12 to 35 mg/L, the lowest values observed in experiments with high temperature (35°C). The acceptable range of acetaldehyde and ethyl acetate is 13 to 40 g/L and 50 to 75 mg/L, respectively. The concentration of higher alcohols increased with temperature. The total concentrations of higher alcohols were 256 mg/L at 15°C and 380 mg/L at 30°C; in contrast, the concentration decreased to 350 mg/L at 35°C as compared with 30°C (Table 14.6). In general, the threshold value of higher alcohols was 200 to 400 g/L. The highest total ester concentration was produced at 15°C (40 mg/L). This ester concentration drastically decreased as temperature increased (18 mg/L at 35°C). The changes in yeast growth and metabolism between 30°C and 35°C are very high and dramatic. Temperature affects not only the fermentation kinetics (rate and length of fermentation) but also the yeast metabolism, which determine the chemical composition and in turn the quality of wine. The alcohol concentration decreases as the temperature increases, which is due to the increase in the concentration of products from other metabolic pathways such as glycerol, acetic acid, and so forth. There are no reports in the literature on all the secondary products because of their low concentrations. Glycerol is the main secondary product after ethanol in wine fermentations using *S. cerevisiae*, and it ranges between 9 and 10 g/L. Several authors observed the increased glycerol levels as compared with the controls at high temperature (Torija et al. 2002; Caridi 2003; Kourkoutas et al. 2003a,b; Reddy and Reddy 2005). On the basis of the published work, it can be said that the fermentation temperature has much influence on the wine quality and yeast growth compared with all tested variables. Increase in temperature increased the volatile composition and also the yeast biomass.

TABLE 14.6

Effect of Temperature on Volatile Compounds Production (mg/L)

Metabolite (mg/L)	15°C	20°C	25°C	30°C	35°C
Acetaldehyde	19 ± 0.83	15 ± 0.51	17.1 ± 1.22	23.0 ± 1.15	23.0 ± 1.15
Ethyl acetate	35 ± 0.72	33 ± 1.28	27 ± 0.46	20 ± 0.78	9 ± 0.63
1-Propanol	40 ± 0.83	44.2 ± 2.45	49.5 ± 2.33	50 ± 1.53	42.5 ± 3.6
Isobutanol	73 ± 4.5	89 ± 1.31	105 ± 5.48	112 ± 1.98	90 ± 1.88
Isoamyl and acetyl alcohol	95.5 ± 8.3	125 ± 5.78	147 ± 3.63	200 ± 5.68	152 ± 7.49
Phenyl ethanol	18.5 ± 0.74	29 ± 1.23	24 ± 1.20	25.5 ± 0.58	20 ± 1.83
Glycerol	5.3 ± 0.43	6 ± 0.38	7.2 ± 0.61	7.5 ± 0.56	8.4 ± 0.67
Acetic acid	0.130 ± 0.06	0.158 ± 0.04	0.334 ± 0.05	0.450 ± 0.03	0.525 ± 0.05
MethaSnol	300 ± 8.23	284 ± 6.124	250 ± 9.23	254 ± 5.42	268 ± 6.86

Note: Values are presented as mean ± SD.

14.3.2.2 pH

Optimum pH value is necessary for yeast growth and ethanol production. Most of the yeasts grow very well between pH 4.5 and 6.5, and nearly all species are not able to grow in more acidic or alkaline media. Low or high pH values are known to cause chemical stress on yeast cell. The enzyme aldehyde dehydrogenase activity is increased at high pH values, and acetic acid is produced. This oxidation generates a molecule of NADH, which requires reoxidation to maintain the redox balance of the cell (Walker 1998). It has also been confirmed that the optimum pH is very important to produce good quality wines.

14.3.2.3 Sulfur Dioxide

SO_2 plays two important roles. First, it is an antimicrobial agent and as such is used to help curtail the growth of undesirable fault-producing yeasts and bacteria. Second, it acts as an antioxidant, safeguarding the wine's fruit integrity and protecting it against browning. Despite its chemical simplicity, SO_2 can take on a few different forms in a wine. One form is called *molecular* SO_2. When in this form, it is approximately 500 times more effective in killing wine microbes compared with any of the other forms that it can take. Luckily for us, the desirable yeasts that undertake wine fermentation are more resistant to SO_2 than most of the spoilage yeasts. Hence, having some SO_2 around helps give the desirable bugs a leg up in their competitive dog-eat-dog world in which they coexist (Sneyd et al. 1993).

SO_2 is used as a preservative agent in wine because it has several functions, such as preventing the enzymatic oxidation of the must and inhibiting the growth of undesirable microorganisms in wine preservation. At present, it is difficult to make quality wines without the addition of SO_2 because sulfur is an essential component for yeast growth. Aeration is not required during the ethanol production phase, but its presence has many advantages during crushing and yeast proliferation in the production of essential sterols (ergosterol and lanosterol) and unsaturated fatty acids, such as linoleic and linolenic acids. Oxygen (O_2) also favors the accumulation of urea, associated with the production of ethyl carbamate (Bafrncova et al. 1999). The addition of SO_2 does not cause any delay on the onset of the alcoholic fermentation except in higher concentrations (200 mg/L). At normal dose (100 mg/L, which is generally used in commercial wine fermentations), slight stimulatory effect was observed in the initial stages of fermentation (first 2–3 days). Alcoholic fermentation, in both cases, is completed in 7 days. Apart from slight enhancement in ethanol production, SO_2 also causes some effects on volatile compound synthesis during fermentation. It causes a slight increase in acetaldehyde concentration (Table 14.7), and the effect is dose-dependent (at 100 ppm, it produced 30 mg/L as compared with 25 mg/L without SO_2). Methanol and ethyl acetate production affects fermentation severely. However, the concentration of 1-propanol is not affected by the addition of SO_2. 1-Propanol may be formed from the carbon skeleton corresponding to the amino acid threonine by transformation (data not presented). SO_2 has little effect on wine quality. However, high concentrations can decrease the growth of yeasts. It has been suggested that SO_2 can stimulate fermentation by *Saccharomyces* in wine by inhibiting the polyphenol oxidase. This is an enzyme that competes directly for available dissolved O_2, therefore making O_2 not readily available for *Saccharomyces*. Another reason is that SO_2 can neutralize the free carbonyl compounds that are known to cause the inhibition of yeast fermentation in the free state (Bisson 1999). On the basis of the results obtained, it could be confirmed that the addition of SO_2 induces acetaldehyde formation using yeast in mango wine fermentation. Similar results have been observed in the production of grape wine and cider (Herraiz et al. 1989; Herrero et al. 2003).

14.3.2.4 Oxygen

O_2 makes up approximately 20% of the air we breathe and is found everywhere in the winery. In general, winemakers are mostly aware of the detrimental effects exposure to O_2 have on wines: at best, a dulling of the fruit with a loss of once-present vitality, with volatile acidity and sherry-like aldehydic flaws developing in a worst-case scenario. In fact, it is because of these potentially negative reactions that most winery decisions (e.g., processing fruit, racking, bottling, etc.) usually seek to carefully limit or even

TABLE 14.7

Composition of Volatiles from Three Different Mango Varieties (Banginapalli, Alphonso, and Totapuri) Fermented at 25°C ± 2°C and pH 4.5 for 10 Days

Serial Number	Retention Time	Name of the Compound	Banginapalli (mg/L)	Alphonso (mg/L)	Totapuri (mg/L)
Alcohol					
1	1.271	Ethanol	8.5	7.5	7
2	1.350	Ethyl ether	Solvent	Solvent	Solvent
3	1.492	1-Propanol	54.11	42.32	47.13
4	1.729	Isobutyl alcohol	102.40	115.14	98.87
5	2.581	Isoamyl alcohol	125.2	108.40	140.44
6	2.850	Pentan-2-one	1.43	1.15	1.51
7	4.823	2-Furan methanol	0.123	ND	0.216
8	6.535	Hexane-1-ol	1.42	1.02	ND
9	12.900	Phenethyl alcohol	22.15	24.15	20.48
10	19.414	Cyclohexane methanol	1.13	ND	1.34
11	42.58	*n*-Pentanedecanol	0.610	ND	TR
Ester					
12	1.665	Ethyl acetate	35.15	30.42	27.48
13	6.876	Ethyl hexanoate	0.942	0.671	0.552
14	15.92	Ethyl octanoate	1.150	1.06	1.451
15	20.124	Ethyl decanoate	2.34	1.86	1.43
16	33.62	β-Phenylethyl butanoate	ND	0.62	0.92
17	19.67	Dimethyl styrene	1.11	1.34	1.09
Acid					
18	1.950	Acetic acid	0.201	0.163	0.155
19	3.292	Propanoic acid	0.145	0.217	0.184
20	3.829	Butanoic acid	0.932	0.745	0.874
21	12.655	2-Furoic acid	0.910	0.548	0.745
22	15.482	Benzoic acid	1.08	1.21	1.43
23	15.750	Phenyl formic acid	0.643	0.912	0.434
24	16.723	Octanoic acid	0.735	0.427	ND
25	37.99	Decanoic acid	1.18	0.963	TR
Ketone					
26	2.850	Pentan-2-one	1.43	1.15	1.51
27	6.245	Furanone	1.12	1.51	1.22
28	11.489	Hydroxy-dimethyl-furanone	0.238	0.452	0.331
29	25.967	Phenol 2,6-bis-4-methoxy-one	0.451	0.432	0.312
Unknown					
30	15.165	Unknown	0.183	0.412	0.243
31					
32	23.377	Benzene methane-4-hydroxy	0.531	0.256	0.231
33	35.68	Unknown	0.441	0.131	ND
34	38.86	Unknown	0.12	TR	TR
35	46.34	Unknown	TR	TR	ND

Note: ND, not detect; TR, trace levels.

eliminate the exposure of wines to O_2 in the first place. However, there is one time when O_2 exposure on the macro level is actually quite beneficial to any wines' development, and that is during the fermentation itself. When correctly applied, O_2 interacts with both the yeast and the wine/must in such a way that the yeast's health is improved, fermentations encounter fewer problems, and the resultant wine quality is often more approachable with fresher aromas and tastes than it had previously.

Reddy and Reddy (2010) reported that aeration can improve the cell growth and the initial fermentation rate. Fermentation is completed faster (5 days) with aeration, which took longer time in the absence of O_2. The viability of yeast cells is increased (8×10^6 cells/mL) with aeration when compared with the absence of O_2 (5×10^6 cells/mL). In their experiments, final ethanol concentration decreased in case of shaking fermentation experiments. Aeration increased glycerol concentration (4%–6% of total ethanol). The ester concentration was high in aerated samples. It was observed that the high degree of aeration results in an increase in biomass production and less ethanol. The cause for the reduction in final ethanol may be the evaporation of the product with oxidation to other products at shaking conditions. In aerated samples, the first 50% of the ethanol formed very rapidly. The aeration also affected the formation of volatile compounds, and our results are in accordance with previous reports (Grosz and Stehanopoulos 1990; Alfenore et al. 2004). O_2 appears to be involved in the synthesis of oleic acid and ergosterol, which stimulate yeast growth under anaerobic conditions. The presence of dissolved O_2 increases both the ethanol and the yeast biomass. Finally, it can be concluded that the optimization of fermentation conditions is very crucial in obtaining best quality wine from mango juice.

14.4 Characterization of Mango Wine

Mango wine has been characterized in terms of ethanol and glycerol concentration, higher alcohols, carotenoids, polyphenols, and volatiles present (Reddy et al. 2009; Varakumar et al. 2010).

14.4.1 Analysis of Mango Wine Volatiles by Gas Chromatograph-Flame Ionization Detector (GC-FID) and Gas Chromatography–Mass Spectroscopy

14.4.1.1 Ethanol and Glycerol

Ethanol and glycerol are the principal metabolites produced during wine fermentation. From the fermentation of mango juice, ethanol is produced in highest concentration than other metabolites. In general, the concentration of ethanol contributes to the whole characteristic quality and flavor of the produced wine. The percentage of ethanol produced in mango wines is between 7% and 8.5% (w/v), comparable with moderate grape wines. Glycerol concentration in mango wines is between 5.7 and 6.9 g/L. Glycerol production mainly depends on the yeast strain used, the fruit variety, and the fermentation conditions such as temperature and aeration. An increase in temperature results in higher glycerol production (Gardner et al. 1993; Remize et al. 1999). It is reported that the optimum temperature for maximum glycerol production by commercial wine yeast strains of *S. cerevisiae* varies between 22°C and 32°C (Scanes et al. 1998). Another parameter, which highly influences the quality of wine, is acidity. The main organic acid present in mango musts and produced wine was malic acid, and the other acids were less than 1 g/L.

14.4.1.2 Higher Alcohols and Other Flavor Compounds

Mango fruit is known to have good aroma and flavor. The flavor of mango fruit is due to the volatile components that are present. The flavor of wine depends on many varietal or fermentative compounds, which are present in highly variable amounts and are mainly alcohols, esters, terpenes, sulfur compounds, acids, and lactones (Rapp 1988; Nobel 1994). Volatile aroma compounds are present in fruit juices, and many are synthesized by wine yeast during wine fermentation. As far as consumers are concerned, the aroma and the flavor of wine are among the main characteristics that determine its quality and value (Mauricio et al. 1997; Swiegers et al. 2005; Molina et al. 2007). Aroma profile is

important in wine, as it contributes to the quality of the final product. It is due to the combined effects of several volatile compounds, mainly alcohols, aldehydes, esters, acids, and monoterpenes, and other minor components already present in the grapes that are being formed during the fermentation and maturation process (Verzera et al. 2008). The aroma complexity dramatically increases during alcoholic fermentation because of the synthesis of important volatile compounds by the wine yeast and the release of some varietal aroma precursors (Mauricio et al. 1997; Swiegers et al. 2005). The nature and the amount of the synthesized volatile compounds depend on multiple factors, such as the nitrogen content of the must, the temperature of fermentation, and the yeast strain (Lambrechts and Pretorius 2000; Swiegers et al. 2006). The volatile compounds synthesized by wine yeasts include higher alcohols (fusel, marzipan, and floral aromas), medium and long-chain volatile acids (fatty, cheesy, and sweaty aromas), acetate esters and ethyl esters (fruity and floral aromas), and aldehydes (buttery, fruity, and nutty aromas), among others (Stashenko et al. 1992). The volatile fatty acids also contribute to the aroma of wines. Fatty acids are essential constituents of the plasma membrane and precursors of more complex molecules, such as phospholipids (Lambrechts and Pretorius 2000). Isoamyl alcohol is the major higher alcohol found in wines (>50%), and its concentration has been reported in the range of 90 to 292 mg/L (Useeglio-Tomasset 1975; Boulton et al. 1996).

Reddy et al. (2009) screened many varieties for wine production, and they found that the three varieties of mango cultivars, Banginapalli, Alphonso, and Totapuri, are well suitable. Hence, the same varieties have been selected for gas chromatography–mass spectroscopy (GC-MS) analysis. The composition of three wines is as shown in Table 14.7. The three major compounds (alcohols, esters, and organic acids) are present in different concentrations. The isoamyl alcohol (140 mg/L) was higher in quantity as compared with all the compounds detected by GC-MS, followed by isobutyl alcohol (115.4 mg/L), *n*-propanol (54.11 mg/L), ethyl acetate (35.15 mg/L), and phenyl ethanol (24.15 mg/L). Ethyl hexanoate, ethyl decanoate, and ethyl octanoate are produced in large amounts in Banginapalli variety. The volatile acids present in the mango wine are acetic acid, propanoic acid, and benzoic acid. Recently, Pino and Queris (2010) reported the presence of 102 volatile compounds using advanced headspace solid-phase microextraction–GC-MS. According to them, mango wine accounted for approximately 9 mg/L of volatile compounds, which included 40 esters, 15 alcohols, 12 terpenes, 8 acids, 6 aldehydes and ketones, 4 lactones, 2 phenols, 2 furans, and 13 miscellaneous compounds. Isopentanol and 2-phenylethanol were the major constituents. A tentative study to estimate the contribution of the identified compounds to the aroma of the wine, on the basis of their odor activity values, indicated that the compounds potentially most important to mango wine included ethyl butanoate and decanal.

14.4.2 Polyphenols and Carotenoid Composition

Mangoes are considered one of the most preferred fruits in the world because of their attractive color, delicious taste, and excellent nutritional properties. Abundant in antioxidants, mangoes are among the many fruits consumed for their potential health benefits, including anticancer and antiviral activities and reduced risk of cardiac disease associated with the antioxidant activities of polyphenolic and carotenoid compounds (Shieber et al. 2000). β-Carotene is the most abundant carotenoid in several cultivars. The nutritional value of mango as a source of vitamin C and provitamin A should also be emphasized. Studies carried out using rats fed with a diet supplemented with 10% mango confirmed the reduction in cytotoxic effects induced by dimethylhydrazine through the optimization of enzymatic oxidative mechanisms (Anilakumar et al. 2003). Despite mango's importance and wide acceptance by the consumer, there have been few studies on the content of antioxidant components in mango varieties. The studies have focused on the nutritional value as source of vitamin C and β-carotene, but information on the total phenolic content is scarce. Mango cultivars differ in their antioxidant component content because of genotypic variation and preharvest factors, including climatic conditions, agricultural practices, and ripening stage (Lee and Kader 2000).

Phenolic compounds are considered as the basic components of wines, and more than 200 compounds have been identified. Two primary classes of phenolics that occur in grapes and wine are flavonoids and nonflavonoids. The most common flavonoids in white and red wines are flavonols, catechins

(flavan-3-ols), and anthocyanins, the latter being found only in red wine. Recent awareness of the role of antioxidants plays in the promotion of health because of their ability to act as chemoprotective agents (Teissedre and Waterhouse 2000). Wine polyphenols contribute to wine color and to other sensorial characteristics of wines such as bitterness and astringency (Perez-Magarino and Gonzalez-Sanjose 2004). The concentration of total polyphenols varies with the type of mango fruit used in wine fermentation. The Totapuri mango variety contains the highest concentration of polyphenols (1050 mg/L), followed by Alphonso, Banginapalli, and Sindhura (725, 610, and 490 mg/L, respectively; Figure 14.3). Eleven different phenolic compounds were identified in a detailed analysis of Alphonso phenolic compounds using liquid chromatograph-mass spectroscopy (Table 14.8).

Wine polyphenols are widely known to have a protective action on the organism against cardiovascular and degenerative diseases (Hertog et al. 1993; Soleas et al. 1997). Some of these beneficial effects are directly related to their ability for scavenging free radicals. This ability depends on their chemical structure because polyphenols can donate a hydrogen atom from their hydroxyl group and stabilize the phenoxy radical formed by the delocalization of the unpaired electron within the aromatic structure. The antiradical activity ranged from 27.57% to 36.70% ascorbic acid equivalents, the antioxidant activity ranged from 73.90% to 85.95% gallic acid equivalents, and the reducing power varied from 0.71 to 2.906 m/L ascorbic acid equivalents. The free radical quenching of the Totapuri wine is as shown in Figure 14.4. The quenching of DPPH is dose dependent (Reddy 2005; Varakumar et al. 2010).

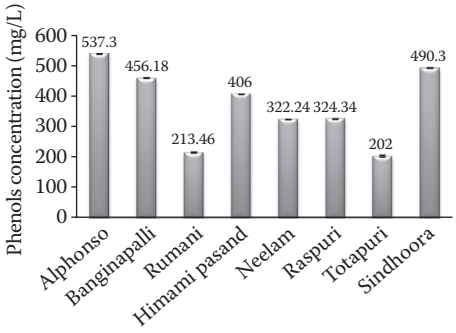

FIGURE 14.3 Total phenol content in wines produced from different varieties of mango fruits.

TABLE 14.8

LC-MS Determination of Phenolic Compounds in Alphonso Mango Wine

Serial Number	Compound Name	Retention Time (Minutes)	Molecular Weight	% (w/v)
1	*P*-Hydroxy benzoic acid	9.93	137	50.2
2	Caffeic acid	14.98	175	3.4
3	Vanillic acid	16.52	169	6.4
4	Ferulic acid	21.12	193	7.9
5	Sulfonic acid	22.17	187	11.21
6	Syringic acid	30.01	197	1.5
7	Protocatechuic acid	32.22	155	0.8
8	Leutolin	34.46	281	3.4
9	Sinapic acid	41.41	223	1.2
10	Quinaldinic acid	46.38	173	0.34
11	Phenylphosphoramidic acid	47.94	325	0.99

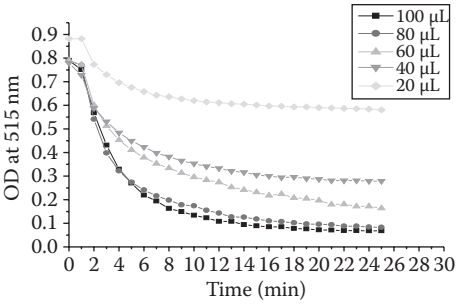

FIGURE 14.4 Kinetics of DPPH scavenging activity by Totapuri wine sample.

Carotenoids are responsible for the bright yellow color of mango and are important lipophilic radical scavengers found in many fruits and vegetables. Mango carotenoids are synthesized in mango fruit, and their concentrations increase toward ripening (Vazquez-Caicedo et al. 2005). Godoy and Rodriguez-Amaya (1989) reported that β-carotene is the dominant carotenoid in mango, comprising 48% to 84% of the total carotenoid content, and the presence of 16 other carotenoids including neo-β-carotene, mono-epoxy-β-carotene, auroxanthin, and zeaxanthin was confirmed in the ripe mangoes (Kiaui and Bauernfeind 1981). In white grapes and in mangoes, the carotenoid levels significantly increased toward maturity in both the peel and the pulp. Medlicott et al. (1986) had shown the breakdown of chlorophyll and, similarly, the increase in peel carotenoids in ripe fruit of mango var. Tommy Atkins. Mango fruit carotenoids in pulp and peel tend to increase with ripening.

The total amount of carotenoids in mango wines are in the range of 578 to 4330 µg/100 g, and the highest amount of total carotenoids from mango wine was in Alphonso (4330 µg/100 g), followed by Sindhura (4101 µg/100 g), Banginapalli (2943 µg/100 g), Rumani (2857 µg/100 g), Totapuri (690 µg/100 g), Raspuri (634 µg/100 g), and Neelam (578 µg/100 g; Varakumar et al. 2010). Among the carotenoids analyzed in puree, β-carotene was the major carotenoid ranging from 65.4 to 94.1, followed by lutein (4.5–29.4), violaxanthin (0.6–3.8), and neoxanthin (0.7%–3.1%). The relative highest total carotenoid levels in puree were found in Sindhura (5810), followed by Alphonso (5720), Rumani (3970), Banginapalli (3955), Totapuri (1920), Raspuri (1080), and Neelam (980 µg/100 g), which is a poor source of carotenoids with respect to the mango purees studied (Table 14.9). In this mango wine, xanthophylls (oxygenated carotenoids) were degraded more than β-carotene (hydrocarbon carotenoid). Among xanthophylls, lutein was degraded more ranging from 78.7% to 93.9%, followed by neoxanthin (26.8%–83.3%) and violaxanthin (50%–74.3%; Varakumar et al. 2010). This is due to the combined effect of acidic conditions of wine with temperature responsible for the significant degradation of xanthophylls compared with β-carotene (17.9%–60.7%), which might be related to the presence of hydroxyl groups of xanthophylls. The degradation of β-carotene during carrot juice fermentation was reported by Kun et al. (2008) by using the *Bifidobacterium bifidum* B3.2 strain, and similarly the losses of β-carotene and zeaxanthin in spontaneous (noninoculated) fermentation were reported by Li et al. (2007) during the preparation of the fermented porridge from maize.

14.4.3 Effect of Storage Bottle Color and Temperature on Wine Color

The browning of bottled wines during storage has been reported by several researchers (Fabios et al. 2000; Selli et al. 2002; Benítez et al. 2003). Alaka et al. (2003) reported the effect of bottle color and storage conditions on mango juice chemical attributes. According to them, glass bottles will give greater protection against the degradation of the chemical attributes of the mango juices. Packaging Ogbomoso mango juices in glass bottles and storing them at 6°C will give good protection against the quality degradation of mango juices. Reddy (2005) examined the change in the color of the mango wine at 8°C, 16°C, and 25°C stored in white, green, and brown storage on the basis of the browning index. Wine stored in darker brown bottles at low temperature showed low browning indices when compared

TABLE 14.9

Total Carotenoid Content Present in Different Cultivars of Mango Puree and Wine (μg/100 g)

	Neoxanthin		Violaxanthin		Lutein		β-Carotene		Total Carotenoids	
Variety	**Puree**	**Wine**	**Puree**	**Wine**	**Puree**	**Wine**	**Puree**	**Wine**	**Puree**	**Wine**
Alphonso	41 ± 2.42	12 ± 1.4***	35 ± 2.1	9 ± 0.9***	258 ± 15	55 ± 7.8***	5385 ± 142	4251 ± 138**	5720 ± 240	4330 ± 176*
Banginapalli	56 ± 3.02	12 ± 1.3***	42 ± 1.54	13 ± 0.97***	326 ± 24	20 ± 5.5***	3531 ± 92	2898 ± 101*	3955 ± 151	2943 ± 141*
Neelam	14 ± 3.42	4 ± 0.95*	37 ± 1.21	12 ± 1.08***	288 ± 13	19 ± 6.4***	641 ± 159	543 ± 53	980 ± 152	578 ± 101
Raspuri	12 ± 0.9	2 ± 0.55***	16 ± 2.06	8 ± 0.51*	212 ± 19	42 ± 3.5***	840 ± 114	617 ± 59	1080 ± 132	634 ± 113
Rumani	123 ± 1.7	90 ± 1.84***	30 ± 1.14	12 ± 0.67***	270 ± 15	28 ± 4.6***	3550 ± 121	2753 ± 91**	3970 ± 151	2857 ± 104**
Sindhura	102 ± 3.6	31 ± 0.84***	62 ± 1.43	21 ± 0.51***	452 ± 21	37 ± 4.1***	5192 ± 109	4012 ± 163**	5810 ± 216	4101 ± 163**
Totapuri	52 ± 2.94	25 ± 0.51***	15 ± 1.01	7 ± 0.49**	213 ± 12	13 ± 5.4***	1642 ± 97	645 ± 74***	1920 ± 147	690 ± 125**

Note: Values are presented as mean ± SD.

*,**, and *** Significant when compared with the carotenoids in puree to wine at $p = 0.0001$, 0.001 and 0.01 respectively.

TABLE 14.10

Sensorial Evaluation of 8 Mango Wines Produced from 8 Different Mango Varieties

Serial Number	Wine Variety	Flavor	Taste	Texture	Appearance	Mouthfeel	Overall Acceptability
1	Alphonso	8.90	8.26	8.59^{b}	8.23^{ac}	8.85^{cd}	8.62^{cd}
2	Raspuri	7.22^{bc}	7.68^{ac}	8.14^{ac}	7.97^{bc}	8.0^{ac}	7.94^{ac}
3	Banginapal	8.56^{ac}	8.35^{bd}	8.45^{bd}	8.76^{cd}	8.5^{cd}	8.3^{bd}
4	Totapuri	7.84^{ac}	7.45^{ac}	7.98^{ac}	8.1^{ac}	7.6^{ac}	7.4^{ac}
5	Sindhoora	6.9^{ab}	6.4^{b}	7.2^{ab}	8.45^{bd}	7.3^{bc}	7.2^{ad}
6	Neelam	6.5^{b}	6.65^{ab}	6.9^{b}	6.3^{a}	6.7^{b}	6.6^{b}
7	Rumani	7.1^{bc}	6.8^{bc}	7.3^{bc}	7.5^{ab}	6.6^{a}	7.0^{ab}
8	Himami pasand	6.1^{c}	6.0^{a}	6.3^{a}	6.7^{b}	6.9^{ab}	6.2^{a}

Note: Values are presented as mean ± SD; values not sharing a common superscript letter are significant at $p < .05$ Duncan's multiple range test.

with wine stored in white bottles. The contents of ascorbic acid and sugars together with storage temperatures, sunlight, and color of bottles affect the browning in orange juice and orange wines (6 ± 12). Sunlight can cause undesired changes in wines. Therefore, in winemaking, the use of bottles that will prevent the transmission of shortwave lights such as ultraviolet, violet, and blue is recommended because shortwave lights have high energy and can initiate some chemical reactions (Boulton et al. 1996). In the brewery industry, brown bottles are commonly used to prevent the transmission of lights with a wavelength between 350 and 550 nm and the formation of undesired aroma compounds (Moll 1999).

14.4.4 Sensorial Evaluation of Mango Wine

Mango wine quality is tested on the basis of the main characteristics: visual, aroma, taste, and harmony. The sensorial results are as shown in Table 14.10. From Table 14.10, it can be concluded that all the different mangoes produced different types of wines, including the three yeast strains that produced three wines with different characters. Banginapalli wines with yeast strain CFTRI 101 got the highest score, followed by Alphonso and Totapuri.

14.5 Cost Economics

Approximately 500 mL of juice can be obtained from 1 kg of mangoes. To produce 1 L of wine, it requires approximately 1250 mL juice, as there would be fermentation and evaporation losses. This means that 2.5 kg of mangoes is required, costing approximately Rs 50.00 as the present raw material cost. In addition, approximately 40% of the raw material cost would be the processing cost. Hence, 1 L of wine would cost approximately Rs 70.00. However, scale-up studies need to be carried out for an actual assessment of the cost of the production of mango wine.

14.6 Immobilization Studies

Cell immobilization in alcoholic fermentation is a rapidly expanding research area because of its beneficial technical and economic advantages compared with the conventional free cell system (Stewart and Russell 1986). For applications in winemaking, various supports have been proposed for potential use in main or secondary fermentation. The most commonly used polysaccharides for cell immobilization in winemaking are alginates, cellulose, carrageenan, agar, pectic acid, and chitosan. Sodium, calcium, and barium salts of alginates have been extensively used for cell entrapment, but calcium alginate gels are considered more suitable for alcoholic fermentation (Colagrande et al. 1994). To satisfy the

demand for natural products combined with consumer acceptance, some researchers have proposed the use of fruit pieces as cell immobilization carriers for wine or beer production (Bekatorou et al. 2002) and reported products with fine taste and aroma and a distinct fruity character. Apple (Kourkoutas et al. 2001) and quince (Kourkoutas et al. 2003a,b) pieces are considered cheap, abundant supports of food-grade purity for immobilization and led to a product with improved sensory characteristics. Other authors (Tsakiris et al. 2004) proposed the use of grape products, such as residual grape skins, as a support for immobilization in winemaking. They are cheap, readily available, and natural, and their use needs no pretreatment. This biocatalyst showed a good stability and produced wines with special flavor and improved quality.

A novel yeast biocatalyst was prepared by using watermelon pieces as immobilizing supports for yeast, the *S. cerevisiae* 101 strain for use in wine production. Immobilization was confirmed by electron microscopy (Figure 14.5; Reddy 2005; Reddy et al. 2008). The fermentation rate and other parameters compared with free yeast cells at different temperatures. In all cases, fermentation time was short (22 hours at 30°C and 80 hours at 15°C) and produced high ethanol productivities (4 g/L/hour). The volatile compounds, methanol, ethyl acetate, 1-propanol, isobutanol, and amyl alcohols, that formed during fermentation were analyzed with the help of GC-FID. The concentrations of ethyl acetate and methanol were approximately 100 mg/L in all cases. The cell metabolism of immobilized yeast was not significantly affected by immobilization. The immobilization of yeast on watermelon pieces reportedly increased the fermentation rate, vitality, and viability of yeast cells. Preliminary sensory tests suggest the fruity aroma, the fine taste, and the overall improved quality of the produced wines.

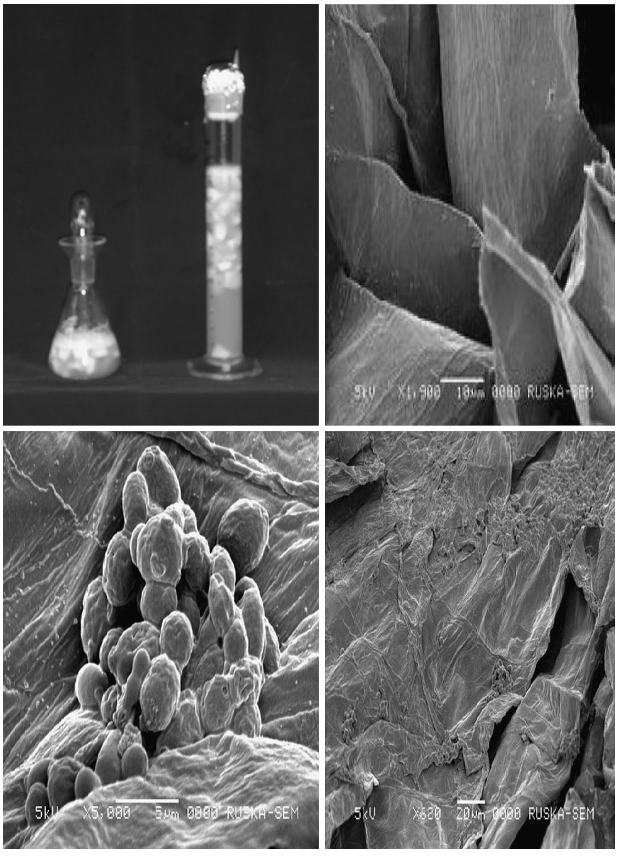

FIGURE 14.5 Immobilization of yeast cells on watermelon pieces in mango wine fermentation.

14.7 Other Fermented Products from Mango Juice

14.7.1 Mango Vermouth

Vermouth is officially classified as an "aromatized fortified wine." This is prepared by the fortification and infusion of the proprietary recipe of different plants, barks, seeds, and fruit peels collectively known as *botanicals*. This type of wine is quite popular in Europe, the United States, Russia, and Poland. The word vermouth, derived from the German word *wermut*, means man with courage or spirit. The alcohol content in vermouth varies from 15% to 17%.

The aromatic wine produced from mango juice is known as mango vermouth (Ossa et al. 1987). The base wine was made from a Banginapalli cultivar raised to 22°Brix, with the addition of 100 ppm SO_2, 0.5% pectinol enzyme, and carrying fermentation at 22°C using the Montrachet strain 522 of *S. cerevisiae*. The composition of mango vermouth, with respect to pH, ethanol, total acidity, total phenols, and aldehyde, is comparable with vermouth prepared from grapes. The herbs and spices used in mango vermouth are black pepper, coriander, cumin, Bishop's weed, clove, large cardamom, saffron, fenugreek, nutmeg, cinnamon, poppy seeds, ginger, lichen, and flame of forest. The sensory evaluation tests suggested very good quality compared with grape vermouth.

14.8 Conclusions and Future Perspectives

Wines are made from a complete or a partial alcoholic fermentation of grape or any other fruit and contain ethyl alcohol as the intoxicating agent, essential elements, vitamins, sugars, acids, and phenolics. Wines from fruits are preferable to distilled liquors for stimulatory and healthful properties. These beverages also serve as an important adjunct to the human diet by increasing the satisfaction and by contributing to the relaxation necessary for the proper digestion and absorption of food.

Fruits produced from the tropics are highly perishable commodities and have to be either consumed immediately or preserved in one form or another. In developed countries, a considerable quantity of fruit is utilized, but in developing countries, the lack of proper utilization results in considerable postharvest losses, estimated to be 30% to 40%. The increased production can be soaked up profitably if fruit wines are produced. Setting up fruit wineries, aside from industrializing the fruit-growing belts, could result in the economic upliftment of the people, generating employment opportunities, and providing better returns of their produce to the orchardists. The availability of technology is the single most important factor determining the production through cost, and the type of the product is also a significant consideration in popularizing the product. Hence, the production of fruit wines in those countries where fruits other than grape are grown would certainly be advantageous. It is also essential to carry out and stimulate research and development on the production and evaluation of wines made from fruits other than grapes.

REFERENCES

Adsule RN, Kotech PM, Kadam SS. 1992. Preparation of wine from pomegranate. *Beverage Food World* 19:13–6.

Alaka O, Aina JO, Falade KO. 2003. Effect of storage conditions on the chemical attributes of Ogbomoso mango juice. *Eur Food Res Technol* 218:79–82.

Alfenore S, Cameleyre X, Benbadis L, Bideaux C, Uribelarrea JL, Goma G, Molina-Jouve C, Guillouet E. 2004. Aeration strategy: a need for very high ethanol performance in *Saccharomyces cerevisiae* fed batch process. *Appl Microbiol Biotechnol* 63:1–12.

Amerine MA, Berg HW, Kunkee RE, Qugh CS, Singleton VL, Webb AD. 1980. *The technology of wine making*. 4th ed. Westport, CT: AVI.

Anilakumar KR, Khanum F, Krishna KRS, Santhanam K. 2003. Reduction of dimethylhydrazine-induced cytotoxicity by mango fruit bar: changes in antioxidant enzymes in rats. *Plant Foods Hum Nutr* 58:1–11.

Anonymous. 1962. *Wealth of India—raw materials 6 (L–M)*. New Delhi, India: Publication and Information Directorate, CSIR.

Anonymous. 1963. Mango wine. *Int Bottr Pack* 37:157–9.
Askar A, Gierschner KH, Siliha H, El Zoghbi M. 1990. Polysaccharides and cloud stability of tropical nectars. In *Proceedings XXth Symposium of the International Federation of Fruit Juice Producers*. Küdig Druck AG, Zug (eds)., parries. pp. 207–223.
Attri BL, Lal BB, Joshi VK. 1994. Technology for the preparation of the pear vermouth. *Indian Food Packer* 46:39–44.
Bafrncova P, Smogrovicova D, Salvikova I, Patkova J, Domeny Z. 1999. Improvement of very gravity ethanol fermentation by media supplementation using *S. cerevisiae*. *Biotechnol Lett* 21:337–41.
Baisya RK. 2004. Post harvest management of fruits and vegetables—a technology management perspective. *Indian Food Packer* 58:78–82.
Bardiya MC, Kundu BS, Tauro BS. 1974. Studies on fruit wines: guava wine. Haryana. *J Hort Sci* 3:140–6.
Barnett JA. 1980. A history of research on yeasts: work by chemists and biologists 1739–850. *Yeast* 14:1439–51.
Bekatorou A, Sarellas A, Ternan NG, Mallouchos A, Komaitis M, Koutinas AA, Kanellaki M. 2002. Low-temperature brewing using yeast immobilized on dried figs. *J Agric Food Chem* 50(25):7249–57.
Benítez P, Castro R, García Barroso C. 2003. Changes in the polyphenolic and volatile contents of 'fino' sherry wine exposed to ultraviolet and visible radiation during storage. *J Agric Food Chem* 51(22):6482–7.
Bisson LF. 1999. Stuck and sluggish fermentations. *Am J Enol Vitic* 50:107–19.
Boulton RB, Singleton VL, Bisson LF, Kunkee RE. 1996. *Principles and practices of winemaking*. New York: Aspen.
Caridi A. 2003. Protective agents used to reverse the metabolic changes induced in wine yeasts by concomitant osmotic and thermal stress. *Lett Appl Microbiol* 35:98–101.
Cisneros-Zevallos. 2003. The use of controlled post harvest abiotic stresses as a tool for enhancing the nutraceutical content and adding value of fresh fruits and vegetables. *Journal of Food Science* 68:1560–1565.
Colagrande O, Silva A, Fumi MD. 1994. Recent applications of biotechnology in wine production. *Biotechnol Progress* 10:2–18.
Czyhrinciwk N. 1966. The technology of passion fruit and mango wines. *Am J Enol Vitic* 17:27–30.
Fabios M, Lopez-Toledano A, Mayen M, Merida J, Medina M. 2000. Phenolic compounds and browning in sherry wines subjected to oxidative and biological aging. *J Agric Food Chem* 48(6):2155–9.
Food and Agriculture Organization of the United Nations. 2007. FAOSTAT Statistic database agriculture, Rome, Italy. http://faostat.fao.org/faostat/collection.
Fowles G. 1989. The complete home wine maker. *New Scientist* 9:38–42.
Fundira M, Blom M, Pretorius IS, Van Rensburg P. 2002. Selection of yeast starter culture strains for the production of marula fruit wines and distillates. *J Agric Food Chem* 50:1535–42.
Gardner N, Rodrigue N, Champagne CP. 1993. Combined effects of sulfites, temperature and agitation time on production of glycerol in grape juice by *Saccharomyces cerevisiae*. *Appl Environ Microbiol* 59:2022–8.
Gasteineau FC, Darby JW, Tuner TB. 1979. Fermented food beverages. In: *Nutrition*. New York: Academic Press.
Giri KV, KrishnaMurthy DV, Narashimha Rao PL. 1953. Separation of organic acids. *J Indian Inst Sci* 35A: 77–98.
Godoy HT, Rodriguez-Amaya DB. 1989. Carotenoid composition of commercial mangoes from Brazil. *Lebensm Wiss Technol* 22:100–3.
Grosz R, Stehanopoulos G. 1990. Physiology, biochemical and mathematical studies of Micro-aerobic continuous ethanol fermentation by *Saccharomyces cerevisiae*: I. Hysteresis, oscillations, and maximum specific ethanol productivities in chemostat culture. *Biotechnol Bioeng* 38:1006–19.
Heatherbell DA, Struebi P, Eschenbruch R, Withy LM. 1980. A new fruit wine from kiwifruit: a wine of unusual composition and Riesling Sylvaner character. *Am J Enol Vitic* 31:114–21.
Herraiz T, Martin-Alvarez PJ, Reglero G, Herraiz M, Cabezudo MD. 1989. Differences between wines fermented with and without sulphur dioxide using various selected yeasts. *J Sci Food Agric* 49:249–58.
Herrero M, Garcia LA, Diaz M. 2003. The effect of SO_2 on the production of ethanol, acetaldehyde, organic acids and flavor volatiles during industrial cider fermentation. *J Agric Food Chem* 52:3455–9.
Hertog MGL, Feskens EJM, Hollman PCH, Katan MB, Kromtout D. 1993. Dietary antioxidant flavonoids and risk of coronary heart disease: the Zutphen elderly study. *Lancet* 342:1007–1013.
Joshi VK, Bhushan S, Devender A. 2004. Fruit based alcoholic beverages. In: Ashok Pandey P, editor. *Concise encyclopedia of bioresource technology*. New York: Haworth Inc. p. 335–50.
Joshi VK, Bhutani VP, Sharma RC. 1990. Effect of dilution and addition of nitrogen source in chemical, mineral and sensory qualities of wild apricot wine. *Am J Enol Vitic* 41:229–31.

Joshi VK, Devender A. 2005. Panorama of research and development of wines in India. *J Sci Ind Res* 64:9–18.
Joshi VK, John S. 2002. Antimicrobial activity of apple wine against some pathogenic and microbes of public health significance. *Alimentaria* 2:67–72.
Joshi VK, Pandey A. 1999. Biotechnology: Vol. II. *Food fermentation (microbiology, biochemistry and technology)*. New Delhi: Educational Publishers and Distributors.
Joshi VK, Parmar M. 2004. Present status, scope and future strategies of fruit wines production in India. *Indian Food Industry* 24:48–52.
Joshi VK, Shah PK. 1998. Effect of wood treatment on chemical and sensory qualities of peach wine during aging. *Acta Alimenteria* 27:307–18.
Joshi VK, Sharma SK. 1995. Comparative fermentation behavior and physico-chemical sensory characteristics of plum wine as affected by the type of preservatives. *Chem Microbial Technol (Lebensmittel)* 17:65–9.
Joshi VK, Thakur NS, Thakur KS. 1994. Effect of different yeast strains on the physico-chemical and sensory qualities of plum wine. *Ibid Abst* 148.
Kiaui H, Bauernfeind JC. 1981. Carotenoid as food colours. In: Bauernfeind JC, editor. *Carotenoids as colorants and vitamin a precursors*. New York: Academic Press.
Kotecha PM, Adsule RN, Kadam SS. 1994. Preparation of wine from over ripe banana fruit. *Beverage Food World* 21:28–9.
Kotecha PM, Adsule RN, Kadam SS. 1995. Processing of custard apple: preparation of ready-to-serve beverage and wine. *Indian Food Packer* 49:5–7.
Kourkoutas Y, Douma M, Koutinas AA, Kanellaki M, Banat IM, Marchant R. 2003a. Room and low temperature continuous wine making using yeast immobilized on quince pieces. *Process Biochem* 39(2):143–8.
Kourkoutas Y, Komaitis M, Koutinas AA, Kaliafas A, Kanellaki M, Marchant R, Banat IM. 2003b. Wine production using yeast immobilized on quince biocatalyst at temperatures between 30°C and 0°C. *Food Chem* 82:353–60.
Kourkoutas Y, Komaitis M, Koutinas AA, Kanellaki M. 2001. Wine production using yeast immobilized on apple pieces at low and room temperatures. *J Agric Food Chem* 49:1417–25.
Kulkarni JH, Singh H, Chada KL. 1980. Preliminary screening of mango varieties for wine making. *J Food Sci Technol* 17:218–21.
Kumar YS, Prakasam RS, Reddy OVS. 2009. Optimization of fermentation conditions for mango (*Mangifera indica* L.) wine production by employing response surface methodology. *Int J Food Sci Technol* 44:2320–7.
Kun S, Rezessy-Szabo JM, Nguyen QD, Hoschke A. 2008. Changes of microbial population and some components in carrot juice during fermentation with selected *Bifidobacterium* strains. *Process Biochem* 43:816–21.
Lakshminarayana G, Chandrasekhara-Rao, Ramaligaswamy PA. 1983. Varietals variations in content characteristics and composition of mango seed and fat. *J Am Oil Chem Soc* 60:880–9.
Lambrechts MG, Pretorius IS. 2000. Yeast and its importance to wine aroma—a review. *South Afr J Enol Vitic* 21:97–129.
Lee SK, Kader A. 2000. A preharvest and postharvest factors influencing vitamin C content of horticultural crops. *Postharvest Biol Technol* 20:207–20.
Li S, Tayie, FAK, Young, MF, Rocheford T, White WS. 2007. Retention of provitamin A carotenoids in high b-carotene maize (*Zea mays*) during traditional African household processing. *J Agric Food Chem* 55:10744–50.
Mandal RC. 1997. *Cashew production and technology*. Udaipur, India: Agro Botanical Publisher.
Mauricio JC, Moreno J, Zea L, Ortega JM, Medina M. 1997. The effects of grape must fermentation conditions on volatile alcohols and esters. *J Sci Food Agric* 75:155–60.
Medlicott AP, Bhogal M, Reynolds SB. 1986. Changes in peel pigmentation during ripening of mango fruit (*Mangifera indica* var. Tommy Atkins). *Ann Appl Biol* 109:651–6.
Molina AM, Swiegers JH, Varela C, Pretorius IS, Agosin E. 2007. Influence of wine fermentation temperature on the synthesis of yeast-derived volatile aroma compounds. *Appl Microbiol Biotechnol* 77:675–87.
Moll M. 1991. *Beers and coolers*: Berlin: Sprenger-Verlag.
Nathanael WRN. 1955. Toddy yields from coconut palms in Ceylon. *Ceylon Coconut Quart* 6:8–16.
Nobel AC. 1994. Wine flavor. In: Piggott JR, Patterson A, editors. *Understanding natural flavors*. Glasgow: Blakie Academic and Professional. p. 226–42.
Nordstrom, K, Carlsson BO. 1965. Yeast growth and formation of fusel alcohols. *J Inst Brew* 71:171–4.

Onkarayya H. 1985. Mango vermouth—a new alcoholic beverage. *Indian Food Packer* 39:85–8.
Onkarayya, H. 1986. A rapid modernization process to improve mango dessert wines. *J Food Sci Technol* 23:175–6.
Onkarayya H, Singh H. 1984. Screening of mango varieties for dessert and Mandeira-style wine. *Am J Enol Vitic* 35:63–5.
Ossa de M, Caro I, Bonat M, Perez I, Domecqe B. 1987. Dry extract in sherry and its evolution in the aging process. *Am J Enol Vitic* 38:293–7.
Perez-Magarino S, Gonzalez-Sanjose ML. 2004. Evolution of flavonols, anthocyanins, and their derivatives during the aging of red wines elaborated from grapes harvested at different stages of ripening. *J Agric Food Chem* 50:4076–9.
Pilando L, Wrolstad RE, Hetherbell DA. 1985. Influence of fruit composition, maturity and mold contamination of the colour and stability of strawberry wine. *J Food Sci* 50:112–9.
Pilnik W. 1983. Enzymes in beverage industry (fruit juices, nectar, wine, spirits and beer). In: Dupuy P, editor. *Utilisation des enzymes en technologie alimentaire*. Paris: Tec. & Doc Lavoisier. p. 425–36.
Pino JA, Queris O. 2011. Analysis of volatile compounds of mango wine. *Food Chem* 125(4):1141–6.
Rapp A. 1998. Volatile flavor of wine: correlation between instrumental analysis and sensory analysis. *Nahrung* 42:351–63.
Reddy LVA. 2005. Production and characterization of wine like product from mango fruits (*Mangifera indica* L.). [Thesis]. Sri Venkateswara University, Tirupati, India.
Reddy LVA, Reddy OVS. 2005. Production and characterization of wine from mango fruits (*Mangifera indica* L.). *World J Microbiol Biotechnol* 21:1345–50.
Reddy LVA, Reddy OVS. 2007. Production of ethanol from mango (*Mangifera india*) juice fermentation. *Res J Microbiol* 21:441–5.
Reddy LVA, Reddy OVS. 2009a. Effect of enzymatic maceration on synthesis of higher alcohols during mango wine fermentation. *J Food Qual* 32:34–47.
Reddy LVA, Reddy OVS. 2009b. Production, optimization and characterization of wine from mango (*Mangifera indica* L.). *Nature Product Radiance* 8(4):426–35.
Reddy LVA, Reddy OVS. 2011. Effect of fermentation conditions on yeast growth and volatile composition of wine produced from mango (*Mangiferia indica* L.) fruit juice. *Food Bioprod Process* 89(4):487–91.
Reddy LVA, Reddy YHK, Reddy LPA, Reddy OVS. 2008. Wine production by novel yeast biocatalyst prepared by immobilization on watermelon (*Citrullus vulgaris*) rind pieces and characterization of volatile compounds. *Process Biochem* 43:748–52.
Reddy LVA, Sudheer Kumar Y, Reddy OVS. 2009. Evaluation of volatile flavor compounds from wine produced from mango fruits (*Mangifera indica* L.) by gas chromatography and mass spectrometry (GC-MS). *Indian J Microbiol* 50(2):183–91.
Remize F, Roustan JL, Sablayrolles JM, Barre P, Dequin S. 1999. Glycerol overproduction by engineered *Saccharomyces cerevisiae* wine yeast strains leading to substantial changes in by-product formation and a stimulation of fermentation rate in stationary phase. *Appl Environ Microbiol* 65:143–9.
Robinson K. 1994. *The Oxford companion to wine*. Oxford: Oxford University Press.
Rombouts FM, Pilnik W. 1978. Enzymes in fruits and vegetables juice technology. *Process Biochem* 13:9–13.
Sakho M, Chassagne D, Jaus A, Chiarazzo E, Crouzet J. 1998. Enzymatic maceration: effects on volatile components of mango pulp. *J Food Sci* 63:975–8.
Sandhu DK, Joshi VK. 1995. Technology, quality and scope of fruit wines especially apple beverages. *Indian Food Ind* 11:24–8.
Scanes KT, Hohmann S, Prior BA. 1998. Glycerol production by the yeast *Saccharomyces cerevisiae* and its relevance to wine: a review. *South Afr J Enol Vitic* 19:17–22.
Selli S, Canbas A, Unal U. 2002. Effect of bottle colour and storage conditions on browning of orange wine. *Food* 46:64–7.
Selli S, Kurkcuoglu M, Kafkas E, Cabaroglu T, Demirci B, Baser KHC, Canbas A. 2004. Volatile flavour components of mandarin wine obtained from clementines (Citrus reticula Blanco) extracted by solid-phase micro extraction. *Flavour Fragr J* 19:413–6.
Selvaraj Y, Kumar R. 1994. Enzymatic regulation in ripening mango fruit. *Indian J Hortic* 51:316–23.
Shieber A, UlrichW, Carle R. 2000. Characterization of polyphenols in mango puree concentrate by HPLC with diode array and mass spectrometric detection. *Innov Food Sci Emerg Technol* 1:161–6.

Shukla KG, Joshi MC, Sarswati Y, Bisht NS. 1991. Jambal wine standardization of methodology and screening of cultivars. *J Food Sci Technol* 28:142–4.

Sneyd TN, Leske PJ, Dunsford PA. 1993. In: Stockley C, Johnstone R, Leske P, Lee T, editors. *Proceedings of the 8th Wine Industry Technical Conference*. Adelaide, South Australia: Winetitles. p. 161–6.

Soleas GJ, Diamandis EP, Goldberg DM. 1997. Wine as a biological fluid: history, production, and role in disease prevention. *J Clin Lab Anal* 11:287–313.

Soufleros EH, Pissa I, Petridis D, Lygerakis M, Mermelas K, Boukouvalas M, Tsimitakis E. 2001. Instrumental analysis of volatile and other compounds of Greek kiwi wine; sensory evaluation and optimization of its composition. *Food Chem* 75:487–500.

Sreenath HK, Nanjundaswamy AM, Sreekantiah KR. 1987. Effect of various cellulases and pectinases on viscosity reduction of mango pulp. *J Food Sci* 52:230–1.

Sreenath HK, Sudarshana Krishana KR, Santhanam K. 1995. Enzymatic liquefaction of some varieties of mango pulp. *Lebensm Wiss Technol* 28:196–200.

Stashenko H, Macku C, Shibamato T. 1992. Monitoring volatile chemicals formed from must during yeast fermentation. *J Agric Food Chem* 40:2257–9.

Stewart GG, Russel I. 1986. One hundred years of yeast research and development in the brewing industry. *J Inst Brew* 92:537–58.

Swiegers JH, Bartowksy EJ, Henschke PA, Pretorius IS. 2005. Yeast and bacterial modulation of wine aroma and flavor. *Aust J Grape Wine Res* 11:127–38.

Swiegers JH, Francis IL, Herderich MJ, Pretorius IS. 2006. Meeting consumer expectations through management in vineyard and winery: the choice of yeast for fermentation offers great potential to adjust the aroma of Sauvignon Blanc wine. *Aust N Z Wine Ind J* 21:34–42.

Teissedre P, Waterhouse A. 2003. Inhibition of oxidation of human low-density lipoproteins by phenolic substances in different essential oil varieties. *J Agric Food Chem* 48:3801–5.

Teotia MS, Manan JK, Berry SK, Seghal RC. 1991. Beverage development from fermented (*S. cerevisiae*) Muskmelon (*C. melo*) juice. *Indian Food Packer* 45:49–51.

Torija MJ, Roes N, Pblet M, Guillamon MJ, Mas A. 2002. Effects of fermentation temperature on the strain population of *Saccharomyces cerevisiae*. *Int J Food Microbiol* 80:47–53.

Tsakiris A, Bekatorou A, Psarianos C, Koutinas AA, Marchant R, Banat IM. 2004. Immobilization of yeast on dried raisin berries for use in dry white wine-making. *Food Chem* 87:11–5.

Useeglio-Tomasset L. 1975. Volatiles of wine dependant on yeast metabolism. *Proceedings of the 4th International Oenological Symposium*, Valencia, Spain, p. 346–70.

Varakumar S, Kumar YS, Reddy OVS. 2011. Carotenoid composition of mango (*mangifera indica* L.) wine and its antioxidant activity. *J Food Biochem* 35:1538–47.

Vazquez-Caicedo AL, Sruamsiri P, Carle R, Neidhart S. 2005. Accumulation of all-*trans*-β-carotene and its 9-*cis* and 13-*cis* stereoisomers during postharvest ripening of nine Thai mango cultivars. *J Agric Food Chem* 53:4827–35.

Verzera A, Ziino M, Scacco A, Lanza CM, Mazzaglia A, Romeo V, Condurso C. 2008. Volatile compound and sensory analysis for the characterization of an Italian white wine from "Inzolia" grapes. *Food Anal Methods* 1(2):144–51.

Voragen AGJ, Pilnik W. 1981. Spezifische enzymeinwirkung bei der verflüssigung von apfelzellwandpräparaten. *Flüss Obst* 48:261–4.

Vyas SR, Chakravorthy SC. 1971. *Wines from Indian grapes*. Hisar: Haryana Agricultural University. p. 1–69.

Vyas KK, Joshi VK. 1982. Plum wine making: standardization of methodology. *Indian Food Packer* 36:80–6.

Vyas KK, Sharma RC, Joshi VK. 1989. Application of osmotic technique in plum wine fermentation. Effect on physico-chemical and sensory qualities. *J Food Sci Technol* 26:126–8.

Walker GM. 1998. *Yeast physiology and biotechnology*. New York: Wiley.

Yahia EM. 1998. Modified and controlled atmosphere for tropical fruits. *Hortic Rev* 22:123–83.

15

Stone Fruit: Wine and Brandy

Vinod Kumar Joshi, Rakesh Sharma, and Ghan Shyam Abrol

CONTENTS

15.1 Introduction

Stone fruit, or in botany, a drupe, is a fruit in which an outer fleshy part (exocarp or skin and mesocarp or flesh) surrounds a shell (pit or stone) of hardened endocarp with a seed inside. These fruits develop from a single carpel, and mostly from flowers with superior ovaries. The definitive characteristic of a drupe is that the hard, lignified stone (or pit) is derived from the ovary wall of the flower. Apricots, plums, peaches, and cherries are the major stone fruits that are grown worldwide (Westwood 1978). These stone fruits are climacteric in nature, that is, the mature fruit is harvested and ripened off-the-plant. These fruits are highly perishable commodities and have to be either consumed immediately or preserved in one form or another. In developed countries, a considerable quantity is used to prepare processed products. However, in developing countries, lack of proper use results in considerable postharvest losses, estimated to be 30% to 40% (Joshi et al. 2000). The conversion of such fruits into wine of acceptable qualities, especially in developing countries, could save these precious resources to a greater extent.

Wines have been a part of man's diet ever since his settlement in the Tigris Euphrates basins, where wine was used as a therapeutic agent. The *Rig-Vedas* also mention the medicinal power of wine. Wines are known to have been prepared by Assyrians since 3500 BC. The production and consumption of fermented beverages such as wine is an ancient practice (Vyas and Chakravorty 1971; Amerine et al. 1980; Joshi et al. 2011). Moderate consumption of low levels of alcohol have been associated with decreased mortality, not only from all coronary heart diseases but also from other diseases (Delin and Lee 1991). These beverages also serve as an important adjunct to the human diet by increasing the satisfaction, contributing to the relaxation necessary for proper digestion and absorption of food, and have a glucose tolerance factor (a chromium-containing compound that stimulates insulin production and is used for curing diabetes). Resveratrol (an anticancer agent) is also found in wine (Gasteineau et al. 1979; Joshi and Devi 2009). Although wine is produced largely by the fermentation of grape juice, it has also been created extensively from fruits such as apples, cherries, currants, peaches, plums, strawberries, etc. Plum wines are also quite popular in many countries, particularly in Germany and the Pacific Coastal states (Amerine et al. 1980). Stone fruits, including plums, have many characteristics in common, such as their pulpy nature, appealing color, high sugar and acid contents, etc., and can be used for the preparation of wine and brandy. Thus, the production of wines in countries where stone fruits are grown would be advantageous. A brief review of the technology of wine production, and the composition of wine and brandies from stone fruits, has been made in this chapter.

15.2 Production of Stone Fruit Wines—General Aspects

15.2.1 Production

Stone fruits are grown the world over and occupy an area of 5.07 million hectares, with 35.24 million tons in production. In India, an area of approximately 43,000 hectares is covered by plantations of

apricots, peaches and nectarines, plums, and cherries with an annual production of approximately 0.25 million tons (Food and Agriculture Organization 2008). The increased production of these fruits can be soaked up profitably if fruit wines are produced, thus generating employment opportunities and providing better returns to the orchardists (Amerine et al. 1980; Sandhu and Joshi 1995).

15.2.2 Composition of Fruit

Compared with grapes, almost all of the stone fruits are low in sugar (the most essential component for alcoholic fermentation) and have thus to be ameliorated to the sugar level needed for producing a table wine (Table 15.1). Even when the sugar level is satisfactory, the high acidity demands dilution, consequently, requiring the addition of more sugar to the must (Amerine et al. 1980; Bhutani and Joshi 1995; Joshi and Sharma 1994). However, stone fruits are a very good source of anthocyanins, polyphenols, minerals (such as potassium, sodium, calcium, magnesium, and iron), and vitamins.

TABLE 15.1

Chemical Composition of Some Stone Fruits

Parameters	Peach	Plum	Apricot	Cherry
Total sugar (% fresh weight)	8–9	6–8	6–7	9–13
Major sugars (% total)				
Fructose	10	20	10	55
Glucose	10	55	20	40
Sucrose	80	25	70	5
Acid (% tartaric acid)	0.5–0.8	1.4–1.7	1.1–1.3	0.4–0.6
Major acids (% total)				
Citric	25	–	25	10
Malic	75	95	5	90
pH	3.27	1.4–1.7	1.1–1.3	3.75
Minerals (mg/100 g)				
Potassium	453	120–190	296	–
Sodium	2.0	0.0–3.0	1	–
Calcium	15	6.0–8.0	–	–
Magnesium	21	4.0–7.0	–	–
Iron	2.4	0.1–0.4	0.54	0.4
Zinc	–	0.1	–	–
Vitamins (mg/100 g)				
Vitamin C	1–27	4.0–11.0	10	13–56
Thiamine	0.02	0.02–0.05	–	–
Riboflavin	0.04	0.04–0.05	–	–
Niacin	0.5	0.2–0.9	–	–
Carotene (%)	0.75–0.79	0.26–0.78	–	–
Anthocyanine (mg/100 g)	20.3–178	926	–	–
Phenolic compounds (mg/100 g)	3–14	46.3–57.0	–	–

Sources: Fowles, G., New Scientist, 38, 1989. With permission. Bhutani, V. P., and V. K. Joshi, *Handbook of Fruit Science and Technology, Cultivation, Storage and Processing*, Marcel Dekker, New York, pp. 243–96, 1995. With permission. Wills, R. B. H. et al., *J Sci Food Agric*, 34, 1383–8, 1983. With permission.

15.2.3 Problems in Wine Production

The technique for the production of wine from stone fruits is essentially the same and involves basic alcoholic fermentation of the juice or the pulp, but modifications are made with respect to the physicochemical characteristics depending on the type of wine needed and fruit used. The major difference in the production of these wines, however, arises from the difficulty of extracting the sugar from the pulp of some of the fruits (Amerine et al. 1980; Joshi 1997). The juices extracted from most stone fruits lack the requisite sugar content or have poor fermentability. The higher acidity in some of the fruits makes it all the more difficult to prepare a palatable wine. The production of quality wine is affected by several factors, namely; fruit variety, stage of harvest and maturity of fruit, total sugar and acid contents, and total phenols, pigments, nitrogen compounds, etc., in the fruit or in the must. Additives like nitrogenous compounds, pectin esterase, sulfur dioxide, or other preservatives, fruits yielding pulp or juice, yeast strains, and other vinification practices, and postfermentation operations (especially the length of maturation and method of preservation used) influence the quality of wine. Fruits, sweetening agents such as fruit concentrate, sugar, acid, nitrogen source, clarifying enzyme, filtering aids, etc., are the raw materials required to make wine. Furthermore, spices and herbs, or their extracts, constitute essential raw materials for the preparation of fortified wines like vermouth. Mostly, the pectin-splitting pectin esterase is used in the clarification of wines. The pectin esterase enzyme has also been used for the extraction of juices from stone fruits such as plum and peach (Joshi et al. 1991a), which might later on be used for the production of wines. Several nitrogen sources can be used but diammonium hydrogen phosphate has many advantages (less shifting in pH of the must and lower cost); thus, it is the most commonly used nitrogen source.

15.3 General Method of Wine Preparation

In the preparation of table, fortified, and sparkling wines, many steps are common. Different unit operations involved in the general preparation of wine from stone fruits are outlined here.

15.3.1 Preparation of Yeast Starter Culture

A good strain of wine yeast from *Saccharomyces cerevisiae* is a prerequisite and needs to be procured for making quality wine. Starter culture in the form of slants, tablets, or compressed yeast can be used for this purpose. Before addition to the must for fermentation, the yeast culture is activated in the juice/pulp intended for wine making. The container with the juice is plugged with cotton and kept at a warm place (25°C–28°C) and the culture is ready after 24 to 48 hours. The amount of active culture is added at a rate of 2% to 5% (v/v) to the must. The yeast from the tablet is activated in sterilized water at an optimum temperature for yeast growth. However, compressed yeast can be used directly in wine fermentation.

15.3.2 Preparation of Must

The "must" is prepared depending on the fruits used and type of wine to be made. Either the juice is extracted or the fruit is made into pulp. Fruits such as plum or apricot, which are highly acidic, can also reduce the fermentability of the juice which can be corrected by dilution or by adding calculated amounts of potassium or calcium carbonate. The carbonate is added to the heated mixture at 66°C to 71°C to hasten the reaction to make calcium citrate less soluble. Ion exchange treatment may also be used for this purpose. Usually, pectinol is added to the must for clarification of the wine. Its addition has been found to enhance the quality of wine. SO_2 is usually added to the must at a concentration of 100 to 150 ppm as potassium metabisulfite. In plum wine fermentation, the addition of sodium benzoate gave better quality wine than potassium metabisulfite (Joshi and Sharma 1995; Joshi et al. 1999a). The addition of ammonium sulfate along with thiamine and biotin increased the rate of fermentation.

15.3.3 Fermentation

The must is allowed to ferment at a temperature range of 20°C to 25°C after inoculation with yeast culture. Temperature higher than 26°C are avoided because it causes loss of volatile components and alcohol. The container in which fermentation is carried out should be equipped with an air bag. The sugar content or Brix is measured periodically to monitor the progress of fermentation. Normally, fermentation is allowed to proceed until all of the sugar content is consumed completely (usually a reading of approximately 8°Brix). When the fermentation is complete, bubbling due to the production of carbon dioxide stops.

15.3.4 Siphoning/Racking

After completion of fermentation, the yeast and other materials settle at the bottom of the container with the clear liquid separating out, which is siphoned/racked, or in the case of pulpy must, it is filtered through a cheese/muslin cloth followed by siphoning. Two or three rackings are usually done every 15 to 20 days. During the inter-racking period, no headspace is kept in the bottle or container, that is, the bottles are closed tightly to prevent the acetification of wine.

15.3.5 Maturation

The newly made wine is harsh in taste and has a yeasty flavor. The process of maturation makes the wine mellow in taste and fruity in flavor aside from undergoing clarification. This period may take 6 months and extend up to 2 to 3 years. The process of maturation is complex and the formation of esters takes place, thus, improving the flavor of such beverages (Amerine et al. 1980). The use of *Quercus* wooden barrels for maturation of table wines is an age-old practice (Quinn and Singeleton 1985); however, maturation of wines from stone fruits with different wood chips has produced similar characteristics as that of barrel-aged table wines (Joshi et al. 1994; Joshi and Shah 1998).

15.3.6 Clarification

If the wine after racking and maturation is not clear, it is clarified using filtering aids such as bentonite, celite, or by tannin/galatin treatment in a filter press. These treatments usually make the wine crystal clear.

15.3.7 Blending

The wine from fruits such as apricot or plum are acidic and may need some amount of sweetening before final bottling. For apricots and plum wine, the extent of sweetening to a total soluble solids (TSS) of 12°Brix was found to be optimum (Joshi et al. 1999a). The wine is refiltered, if needed.

15.3.8 Pasteurization

Wines, being low alcoholic beverages, are pasteurized at 62°C for 15 to 20 minutes after keeping some headspace in the bottle and crown corking. Heating precipitates tannins and other such materials which are heat-sensitive in addition to preserving the wines. However, the pasteurized wines, once opened, have to be kept at a low temperature to prevent spoilage. Alternatively, table wines can be preserved by the addition of preservatives such as sulfur dioxide, sodium benzoate, sorbic acid, etc. (Amerine et al. 1980). Wines with higher alcohol contents, such as fortified wines, need no preservation as the alcohol itself acts as a preservative (more than 15% alcohol). Carbon dioxide in conjunction with ethyl alcohol and low levels of SO_2 prevents the spoilage of carbonated wines.

15.4 Table Wine

15.4.1 Plum Wine

15.4.1.1 Plum Fruit Cultivars

Among the stone fruits, plum (*Prunus salicina* Linn.) is an important fruit grown throughout the temperate regions of the world (Bhutani and Joshi 1995). With an attractive color and high fermentability, plum has potential for the preparation of alcoholic beverages including wine (Vyas and Joshi 1982; Joshi et al. 1991a). Among the cultivars evaluated, Santa Rosa, Methley, Green Gage, and Santa Rosa plum made the best quality wine (Joshi and Bhutani 1990).

15.4.1.2 Methods of Preparation

The plum fruit is acidic in nature and thus, makes unpalatable wine. To prepare wine, basically all the methods revolve around either dilution of the pulp, use of microorganisms to degrade the acids, or osmotic treatment of the fruits to leach out the acids (Amerine et al. 1980; Vyas and Joshi 1982; Vyas et al. 1989). According to one method of preparing plum wine, for every pound of plum, one liter of water is added followed by the addition of starter culture (Amerine et al. 1980). The mixture is allowed to ferment

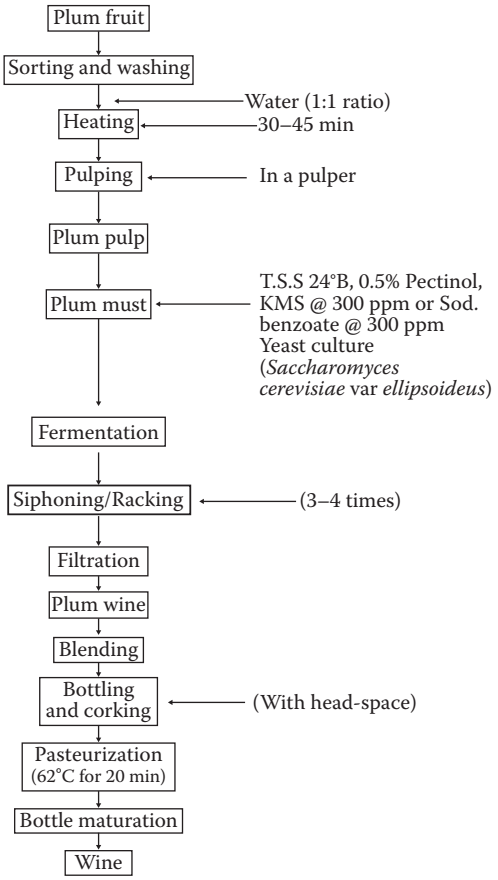

FIGURE 15.1 Schematic flowchart for making plum wine.

for 8 to 10 days before pressing. Because the fruits cannot be practically pressed before fermentation, the addition of pectolytic enzyme before fermentation facilitates processing by increasing the yield of juice and hastening wine clarification. Additional sugar may be added to the partially fermented juice, depending on the type of wine required, that is, table or dessert. Another method for preparation of wine stated that the plum fruits should be fully ripe, diluted with water in a 1:1 ratio (whole fruit basis), with the addition of 0.3% pectinol, 150 ppm SO_2, and enough sugar to increase the TSS of the must to 24°Brix (Vyas and Joshi 1982). A generalized flow diagram of plum wine making is shown in Figure 15.1. The composition of the plum wine produced is given in Table 15.2.

15.4.1.3 Effect of Skin Retention and Use of Honey on Wine Quality

Plum wine can be prepared from plum fruits either with skin or without skin of the fruit, but the wines have considerable differences in physicochemical characteristics. A comparison of the characteristics of plum wine is made in Table 15.3. Use of honey instead of sugar in plum wine fermentation has also been made (Joshi et al. 1990a).

15.4.1.4 Effect of Initial Sugars on Wine Quality

In the preparation of wine, initial sugar concentration (ISC) is one of the most important quality parameters because it is known to influence the quality and type of wine. In plum (cv. Black Beauty), an increase in ISC decreased the rate of fermentation, increased ethanol, and sugar concentration. Differences in

TABLE 15.2

Chemical Composition of Plum Wine

Characteristics	Range
Ethanol (% v/v)	8.8–11.0
Total soluble solids (°Brix)	8.0–12.0 (sweet)
Titratable acidity (% malic acid)	0.62–0.88
Volatile acidity (% acetic acid)	0.028–0.040
Esters (mg/L)	104–109
Color (tintometer color units)	
Red	6–10
Yellow	10
Total coloring matter and tannins (mg/100 mL)	119

Source: Vyas, K. K., and V. K. Joshi, *Indian Food Packer*, 36(6), 80, 1982. With permission.

TABLE 15.3

Comparison of Some Characteristics of Plum Wine Fermentation with and without Skin

Characteristics	Fermentation With Skin	Fermentation Without Skin
Acidity (% MA)	1.43	1.12
pH	3.68	3.75
Alcohol (% w/v)	8.71	8.65
Sugar use (%)	16.2	16.0
Color value (OD 540 nm)	0.80	0.21
Volatile acidity (% AA)	0.043	0.041
Total coloring matter (mg/100 mL)	208	116

Source: Vyas, K. K., and Joshi, V. K., *Indian Food Packer*, 36(6), 80, 1982. With permission.

TABLE 15.4

Effect of ISC (oB) on Physicochemical Characteristics of Wines from Black Beauty Cultivar of Plum

Characteristics	ISC-20	ISC-24	ISC-30
Total soluble solids (oB)	7.9	8.8	12.2
Titratable acidity (% MA)	0.94	0.95	0.88
Fermentation rate (°Brix/24 hours)	2.13	1.45	1.48
Ethanol (% v/v)	10.8	12.8	14.4
Tannins (mg/L)	565	432	550
Total aldehyde (mg/L)	92	75	72
pH (before blending)	2.71	2.89	2.89
Total sugar (%)	0.248	0.22	2.80
Methanol (µL/L)	277	310	480
Volatile acidity (% AA)	0.050	0.040	0.060
Color values (tintometer color units)			
Yellow	7.0	7.4	20.8
Red	4.8	13.8	15.8
Blue	0.1	0.1	0.1

Source: Joshi, V. K. et al., *J Northeast Foods*, 8(1,2), 1–7, 2009. With permission.

other parameters were also obtained (Table 15.4). The sensory quality of wine was the best at ISC-30 (as a table wine), but ISC-24 was considered suitable. However, depending on the type of wine and ethanol contents needed, the required ISC can be chosen.

15.4.1.5 Effect of Osmotic Treatment of Fruit on Wine Quality

To produce quality wine, aside from acids, a proper balance of tannin and good fruity flavor are prerequisites. Application of osmotic techniques after blanching the fruit in water increased TSS and decreased acids, although a little loss of anthocyanin and mineral content also occurred. The sensory quality of wine prepared from water-blanched and osmotically treated fruits were the best (Vyas et al. 1989).

15.4.1.6 Use of Preservatives

The type of preservative used reportedly affects the quality of wine produced (Joshi and Sharma 1995). The use of sodium benzoate instead of potassium metabisulfite produces wine with better color and sensory qualities without affecting the physicochemical characteristics. Potassium metabisulfite is commonly used in most of the wine fermentations, but it reduces the color of wine. Higher ethanol, propanol, and amyl alcohol contents have been reported in potassium metabisulfite–treated wine compared with sodium benzoate–treated wine. Thus, the use of sodium benzoate does not interfere with normal alcoholic fermentation of plum into wine (Figure 15.2). With respect to sensory qualities, sodium benzoate–treated wine was found to be superior to potassium metabisulfite–treated wine (Joshi and Sharma 1995).

The mineral composition of the wines was reportedly affected by the addition of different preservatives (Joshi and Bhutani 1990; Figure 15.3).

15.4.1.7 Microbiology of Fermentation

The qualitative and quantitative composition of the yeast microbiota in fermenting must depends mainly on the type of fruit, origin, production procedure, type of the produced beverage, temperature, pH, SO_2, and ethanol concentration (Satora and Tuszynski 2005). However, the spontaneous fermentation of plums has also been carried out to determine the fermentation behavior and quality of the product

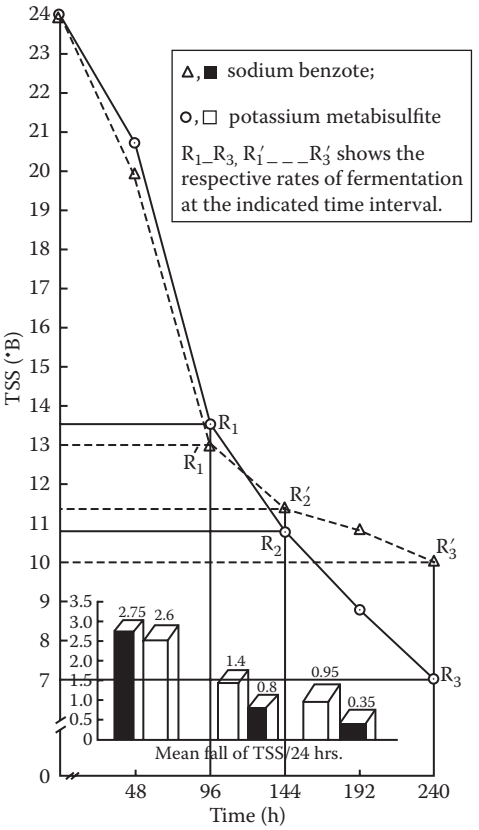

FIGURE 15.2 Comparison of rate of fermentation of plum must treated with potassium metabisulfite and sodium benzoate.

obtained. In general, wine yeast *S. cerevisiae* var. *ellipsoideus* is used to produce plum wine. In such a fermentation, the early stages of alcoholic fermentation are dominated by non-*Saccharomyces* species especially *Kloeckera apiculata*/*Hanseniaspora uvarum* and *Candida pulcherrima*. These yeast strains were identified using killer sensitivity analysis. The activity of these non-*Saccharomyces* species however decreased after two days, at which point *Saccharomyces* species took over the fermentation process. This pattern is almost similar to that of spontaneous fermentation of grapes (Fleet and Heard 1994). A comparison of the physicochemical characteristics of fresh and fermented plum must using different sources of fermentation/spontaneous fermentation is given in Table 15.5.

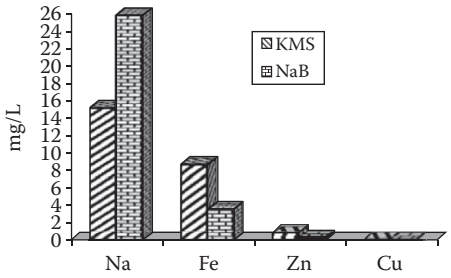

FIGURE 15.3 Effect of preservatives on mineral contents of plum wine.

TABLE 15.5

Physicochemical Composition of Fresh and Fermented Plum Musts

Plum Must	Extract	Total Sugars	Reducing Sugars	Titratable Acidity[a]	pH	Ethanol (% vol.)	Fermentation Rate (%)
	(g/L)						
Fresh	175.0 (±1.0)	132.6 (±2.3)	78.7 (±2.5)	7.84 (±0.18)	3.87 (±0.07)	–	–
Fermented with							
Distillery *S. cerevisiae*	45.0 (±0.0)	4.8 (±0.1)[a]	1.2 (±0.4)[a]	5.40 (±0.15)[a]	3.77 (±0.05)[a]	6.5 (±0.1)[a]	76.9
Wine *S. cerevisiae*	43.0 (±4.2)	3.8 (±0.7)[b]	3.4 (±0.5)[b]	6.73 (±0.10)[b]	3.78 (±0.04)[a]	6.5 (±0.1)[a]	76.2
Indigenous *S. cerevisiae*	40.0 (±14.1)	4.6 (±0.1)[a]	3.9 (±0.2)[b]	6.89 (±0.18)[b]	3.78 (±0.03)[a]	6.5 (±0.1)[a]	76.8
K. apiculata	45.0 (±2.5)	2.4 (±0.3)[c]	1.2 (±0.2)[a]	5.61 (±0.19)[a]	3.85 (±0.04)[b]	6.3 (±0.1)[b]	72.8
Aureobasidim sp.	48.0 (±2.0)	4.8 (±0.3)[a]	2.4 (±0.2)[c]	6.11 (±0.24)[c]	3.75 (±0.03)[a]	6.3 (±0.1)[b]	74.1
Spontaneous fermentation	43.0 (±3.0)	1.2 (±0.2)[d]	0.0	6.03 (±0.23)[c]	3.90 (±0.01)[b]	8.4 (±0.0)[c]	96.9

Source: Satora, P., and T. Tuszynski, *Food Technol Biotechnol*, 43(3), 277–282, 2005. With permission.

Note: Values not showing the same superscript letter within the horizontal line are different according to the Duncan test.

[a] Titratable acidity expressed as grams per liter of malic acid.

15.4.1.8 Effect of Wine Yeast Strains

In inoculated studies or commercial production, the use of wine yeast *S. cerevisiae* var. *ellipsoideus* is an established practice. How the different wine yeast strains affect the physicochemical characteristics of plum wine was also studied (Joshi et al. 2009). The rate of fermentation of all the yeasts showed that the "Tablet" and "W" yeast strains gave the highest reduction in TSS, whereas UCD 505/522 gave the lowest rates. All the yeast strains recorded rates of fermentation higher than 1.6 and strains UCD 595, W, and Tablet had the same rate of fermentation. The yeasts did not influence the acid with the wines and was desirable (Joshi et al. 2009). It was concluded that plum wines prepared by using strain UCD 595, W, and Tablet have better sensory qualities compared with that produced from UCD 522 and UCD 505 (Table 15.6).

15.4.1.9 Effect of Dilution of Pulp

Besides dilution of pulp with water, the other alternative available is the use of yeast *Schizosaccharomyces pombe* as a deacidifying yeast in plum must. Deacidification behavior of *Schiz. pombe* was studied in plum must (Vyas and Joshi 1988) of varying sugar contents (Table 15.7). Clearly, the deacidification activity of the yeast decreased with the increase in TSS of the musts.

The effect of varying levels of pH, ethanol, SO_2, and N_2 source on the deacidification activity of *Sc.* pombe during plum must fermentation was examined (Joshi et al. 1991a). The deacidification activity of *Sc. pombe* with different nitrogen sources indicated that the highest amount of acid was degraded in must containing ammonium sulfate (Table 15.8). Higher amounts of acids were metabolized in musts of pH values between 3.0 and 4.5 as compared with the control (2.8). The use of yeast, no doubt, holds promise as a deacidification method; however, more in-depth studies would be needed to use the yeast in plum wine fermentation.

15.4.1.10 Low Alcoholic Plum Beverages

Attempts to prepare plum wine with no or low alcohol contents were made, with variable success. Plum wine was prepared with four different treatments, namely, by conventional methods (using *S. cerevisiae*), by using *S. cerevisiae* but ameliorated with honey, by conventional methods but followed by distillation

TABLE 15.6

Effect of Different Wine Yeast Strains on the Sensory Characteristics of Fermented Plum Wine

	Yeast Strains				
Attributes[a]	**UCD 522**	**UCD 595**	**UCD 505**	**W Yeast**	**Tablet Yeast**
Appearance (2)	1.6	1.9	1.7	1.7	1.8
Color (4)	1.8	1.6	1.8	1.5	1.6
Aroma (2)	2.5	3.4	3.0	3.4	3.4
Volatile acidity (2)	1.0	1.7	1.7	1.7	1.7
Total acidity (2)	1.0	1.6	1.3	1.6	1.6
Sweetness (1)	0.4	0.6	0.5	0.6	0.6
Body (1)	0.5	0.7	0.6	0.7	0.7
Flavor (2)	1.0	1.7	0.8	1.5	1.6
Bitterness (1)	0.4	0.7	0.5	0.7	0.5
Astringency (1)	0.3	0.8	0.4	0.8	0.8
Overall impression (2)	1.3	1.7	1.3	1.7	1.7
Total	11.8	16.4	13.6	15.9	16.0

Source: Joshi, V. K. et al., *J Northeast Foods*, 8(1,2), 1–7, 2009. With permission.

[a] Maximum scores used for each attribute are shown in parentheses.

for removal of alcohol, and by deacidification with *Schiz. pombe* followed by fermentation with *S. cerevisiae*. These products were preserved using different preservation methods (Amandeep et al. 2009), that is, pasteurization (at 80°C for 2 minutes or 70°C for 10 minutes or at 60°C for 15 minutes) and chemical preservatives (sodium benzoate, sulfur dioxide at 100 ppm). There was no microbial/yeast count (CFU/mL) in the treatments that were pasteurized at 70°C or 80°C (Figures 15.4a and 15.4b). The microbial counts remained the same, except in the treatment, where alcohol was removed. Pasteurization at 60°C for 15 minutes inhibited the increase of microbial load.

15.4.1.11 Maturation

For maturation, it is an age-old practice to use wooden casks for storage of alcoholic beverages produced from grape. The addition of wood chips of *Albizia* and *Quercus* produced wines of the highest sensory qualities (including plum wine; Joshi et al. 1994). In general, treatment of wines with wood chips increased the methanol content; wood chips from *Populus, Celtis*, and *Salix* resulted in higher methanol levels compared with *Albizia* and *Quercus* (Table 15.9). Wood chips of *Quercus* and *Albizia* were found to be most suitable for maturation of plum wine.

TABLE 15.7

Characteristics of Plum Musts Fermented with *Schiz. pombe*

	Initial				Final			
Musts	**TSS (°Brix)**	**Titratable Acidity (% MA)**	**pH**	**Alcohol (% v/v)**	**TSS (°Brix)**	**Titratable Acidity (% MA)**	**Volatile Acidity (% AA)**	**pH**
P_1	6.8	0.92	3.27	2.7	2.8	0.11	0.04	4.42
P_2	12.0	0.92	3.28	5.5	4.0	0.22	0.09	4.02
P_3	15.0	0.91	3.28	8.2	5.0	0.30	0.09	3.93
P_4	20.0	0.91	3.28	11.6	6.0	0.32	0.13	4.15
P_5	25.0	0.92	3.27	12.6	8.6	0.34	0.20	4.16

Source: Vyas, K. K., and V. K. Joshi, *J Food Sci Technol*, 25, 306, 1988. With permission.

Note: MA, malic acid; AA, acetic acid.

TABLE 15.8

Final Characteristics of Plum Musts Fermented with *Schiz. pombe*

Plum Musts	Titratable Acidity (% MA)	Deacidification Activity (%)	TSS (°Brix)	Total Soluble Solids Used (%)	pH
Variable SO_2					
50	0.12	90.00	5.5	45	3.92
100	0.12	90.00	4.8	52	4.02
150	0.07	94.76	4.8	52	4.20
200	0.08	92.50	4.9	51	4.44
250	0.08	92.50	5.0	50	4.44
Control	0.11	90.18	4.2	58	4.04
Variable nitrogen source					
Yeast extract	0.20	90.19	3.5	65	4.16
Ammonium nitrate	0.44	78.43	3.0	70	3.69
Ammonium sulfate	0.12	94.11	4.0	60	4.15
Tryptone	0.19	90.68	3.0	70	4.18
Peptone	0.16	92.15	3.2	68	4.28
DAHP	0.28	89.21	4.2	58	4.35
Control	0.23	88.23	4.0	60	4.11
Variable pH					
2.5	0.23	90.00	4.2	58	3.72
3.0	0.06	97.20	4.3	57	5.87
3.5	0.07	95.20	4.4	56	6.18
4.0	0.07	95.23	5.0	50	6.38
4.5	0.07	95.23	5.2	48	7.10
Control (2.8)	0.09	92.00	4.1	59	4.63

Source: Joshi, V. K. et al., *J Appl Bacteriol*, 70, 385, 1991a. With permission.

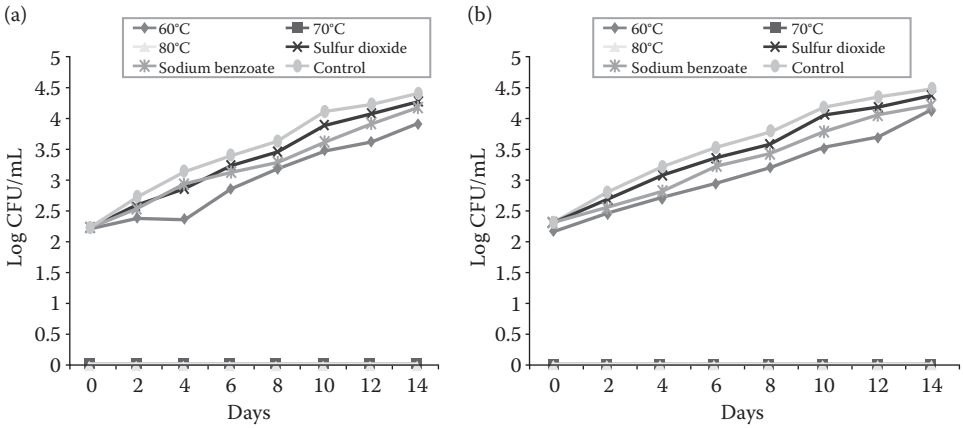

FIGURE 15.4 (a) Effect of different preservation methods on total plate count in 20% blended plum wine (T1). (b) Effect of different preservation methods on total plate count in 30% blended plum wine (T1).

TABLE 15.9

Effect of Wood Chips on Physicochemical and Sensory Characteristics of Plum Wine

			Maximum Score							
Treatment	**Tannins (mg/100 g)**	**Methanol (µL/L)**	**Appearance 2**	**Color 2**	**Aroma 4**	**Volatile Acidity 2**	**Total Acidity 2**	**Sweetness 1**	**Body 1**	**Flavor 2**
Control	139.00	240.00	1.53	1.51	3.07	1.42	1.32	0.68	0.72	1.20
Alnus nitida	96.00	236.00	1.53	1.49	2.99	1.49	1.41	0.67	0.64	1.35
Populus ciliate	107.00	338.00	1.56	1.49	3.08	1.50	1.52	0.58	0.64	1.45
Celtis australis	122.00	327.00	1.55	1.54	3.08	1.54	1.54	0.61	0.58	1.57
Toona ciliate	148.00	181.00	1.57	1.60	2.99	1.42	1.30	0.61	0.58	1.10
Salix tetrasperma	100.00	229.00	1.50	1.49	2.83	1.25	1.32	0.68	0.61	1.32
Bombax cieba	122.00	180.00	1.59	1.48	2.99	1.36	1.36	0.68	0.72	1.39
Albizia chinensis	111.00	249.00	1.64	1.61	3.09	1.62	1.64	0.74	0.64	1.58
Quercus leucotrichohora	139.00	198.00	1.74	1.68	3.41	1.61	1.70	0.73	0.78	1.68
CD (p = .05)	20.15	11.29	0.10	0.10	0.10	0.10	0.10	0.10	0.10	0.10

Source: Joshi, V. K. et al., *J Tree Sci*, 27–36, 1994. With permission.

15.4.2 Peach Wine

15.4.2.1 Fruit and Cultivars

The peach fruit has a lower acid content compared with plum or apricot; however, because it is pulpy, it has to be diluted with water to prepare the must for fermentation (Joshi and Bhutani 1995). However, production of wine from dried peaches has not been successful (Amerine et al. 1980). Among the cultivars evaluated, Redhaven, Sunhaven, Flavourcrest, Rich-a-haven, J.H. Hale, and July Elberta were rated suitable for conversion into wine of acceptable sensory quality (Joshi et al. 2005).

15.4.2.2 Method of Preparation

The method used for making peach wine consists of dilution of the pulp at a ratio of 1:1, increasing the initial TSS to 24°Brix, and adding pectinol and diammonium hydrogen phosphate (DAPH) at the rate of 0.5% and 0.1%, respectively. Then, 100 ppm of SO_2 is added to the must to control the activity of undesirable microflora. Differences in fermentation behavior of different peach cultivars were also observed. Among the cultivars evaluated, Redhaven, Sunhaven, Flavourcrest, Rich-a-haven, J.H. Hale and July Elberta were rated suitable for conversion into wine of acceptable sensory quality (Joshi et al. 2005). Some of the physicochemical characteristics of wines produced from different cultivars of peach are given in Table 15.10. In another study, the peach pulp was fermented at 25°C for 2 weeks using *S. cerevisiae* KCCM 12224, aged at 15°C for 14 weeks, and it is seen that the fermentation increased to yeast count 2.8×10^6 CFU/mL after 2 weeks, but decreased to 7.0×10^3 CFU/mL after 14 weeks (Chung et al. 2003). Benomyl fungicide is demonstrated to be fungistatic to three strains of *S. cerevisiae* at concentrations ranging from 10 to 200 μg/mL in an agar medium prepared from peach pulp. However, heating peach juice containing 30 μg/mL of fungicide for 10 minutes at 80°C significantly reduced the growth-retarding effects on the three strains tested (Beuchat 1973).

15.4.2.3 Maturation

Wooden barrels or wood chips have been used to mature peach wine. The effect of three different woodchips (*Quercus*, *Bombax,* and *Albezia)* treatment on the chemical composition and sensory qualities of peach wine (eight cultivars) has revealed significant changes compared with the control (Joshi and Shah 1998). Wine aged with *Quercus* wood has increased total phenols, aldehydes, and more total ester contents than the control (Tables 15.11 and 15.12). Furthermore, wines treated with wood chips of *Quercus* gave the wine with the best sensory qualities.

15.4.3 Apricot Wine

15.4.3.1 Fruit and Cultivar

Apricot is a delicious fruit grown in many parts of hilly, temperate countries including India. The fruit, due to its rich flavor, holds promise for conversion into wine. In India, wild apricot fruit grown at higher altitudes are being used locally to make liquor, which is completely lacking in nutrients. Furthermore, preparation of apricot wine from New Castle variety and wild apricot (*chulli*) has been reported (Joshi et al. 1990b; Joshi and Sharma 1994). The physicochemical characteristics of wine produced from apricot cv. New Castle are given in Table 15.13.

15.4.3.2 Method of Preparation

A method for the preparation of wine from wild apricot has been developed which consists of diluting the pulp at a ratio of 1:2, the addition of DAHP at 0.1% and 0.5% pectinol, TSS of 24°Brix, and fermentation with *S. cerevisiae*. Furthermore, with the increase in the dilution level, the rate of fermentation, alcohol content, and pH of the wines increased, whereas a decrease in titratable acidity and volatile acidity, total phenols, TSS, color values, and mineral contents took place. The addition of DAHP at a rate of 0.1%

TABLE 15.10

Physicochemical Characteristics of Wines from Different Peach Cultivars

Cultivars	Ethanol % (v/v)	Higher Alcohols (mg/L)	Volatile Acidity (% AA)	Total Esters (mg/L)	Total Aldehyde (mg/L)	Total Phenols (mg/L)	Titratable Acidity (% MA)	pH	Total Sugars (mg/L)	Color Intensity
Rich-a-haven	10.73 (3.27)	113.6	0.023 (0.723)	98.23	48.23	241.8	0.66 (0.81)	3.60	1.33 (1.15)	0.53
J.H. Hale	10.67 (3.26)	126.9	0.029 (0.727)	90.92	43.30	259.0	0.71 (0.84)	3.80	1.24 (1.12)	0.33
Redhaven	11.10 (3.33)	121.4	0.023 (0.723)	98.40	45.53	301.7	0.72 (0.85)	3.71	1.26 (1.12)	3.86
Flavourcrest	11.33 (3.36)	134.4	0.029 (0.722)	94.33	49.32	306.1	0.80 (0.89)	3.61	1.19 (1.89)	0.33
Sunhaven	11.67 (3.41)	131.6	0.022 (0.725)	101.50	53.97	278.6	0.63 (0.71)	3.71	1.22 (1.05)	0.36
Kateroo	11.83 (3.24)	154.3	0.029 (0.727)	92.37	56.83	230.4	0.61 (0.73)	3.84	1.22 (1.10)	0.23
July Elberta	10.87 (3.14)	151.5	0.020 (0.721)	95.50	63.10	203.3	0.70 (0.83)	0.74	1.36 (1.17)	0.48

Source: Joshi, V. K. et al., *J Food Sci Technol*, 42(1), 83–89, 2005. With permission.

Note: Values within parentheses are the transformed values.
AA, acetic acid; MA, malic acid; blue color units, 0 in all the wines; TCU, tintimeter color unit.

TABLE 15.11

Effect of Different Wood Chips on Total Phenols, Total Esters, Aldehyde and Higher Alcohols of Peach Wine of Different Cultivars

	Total Phenols (mg/L)				Total Esters (mg/L)				Aldehyde (mg/L)				Higher Alcohols (mg/L)			
Cultivars	W_0	W_1	W_2	W_3	W_0	W_1	W_2	W_3	W_0	W_1	W_2	W_3	W_0	W_1	W_2	W_3
Rich-a-haven	253.8	264.6	256.0	251.3	115.1	122.2	121.7	120.6	53.26	52.26	51.54	49.11	125.4	123.1	129.9	127.5
J.H. Hale	270.5	279.4	270.2	271.0	109.3	118.1	117.0	114.9	48.81	47.96	51.43	51.41	148.6	147.2	149.0	140.8
Redhaven	315.9	320.4	315.9	313.5	115.2	122.1	119.5	119.3	55.10	56.56	48.91	46.99	144.6	139.1	150.8	164.1
Flavourcrest	317.8	337.5	322.0	321.2	114.9	120.8	119.2	118.2	60.17	56.70	58.87	51.41	126.9	134.3	133.3	129.0
Sunhaven	290.5	307.0	295.0	291.2	120.3	127.3	124.2	125.1	56.91	60.81	55.38	57.91	138.1	130.1	142.5	156.8
Stark Early Giant	223.2	241.2	227.2	226.2	116.7	121.9	157.4	122.1	68.83	66.80	68.58	68.40	154.6	158.2	161.1	167.4
Kateroo	288.2	309.3	298.9	293.4	109.0	114.3	116.6	113.8	54.04	53.66	50.30	50.56	159.8	197.9	185.6	185.0
July Elberta	214.4	230.6	221.0	218.8	108.0	113.3	111.7	111.8	73.31	73.54	74.31	75.64	215.6	250.8	245.7	256.5

Source: Joshi, V. K., and P. K. Shah, *Acta Aliment Budapest*, 27(4), 307–318, 1998. With permission.
Note: W_0, Control; W_1, Quercus chips; W_2, Albizia chips; W_3, Bombax chips.

TABLE 15.12

Changes in Total Phenols, Total Esters, Aldehyde and Higher Alcohols of Peach Wine during Maturation

	Total Phenols (mg/L)				Total Esters (mg/L)				Higher Alcohols (mg/L)				Aldehyde (mg/L)			
Cultivars	**M_0**	**M_1**	**M_2**	**M_3**	**M_0**	**M_1**	**M_2**	**M_3**	**M_0**	**M_1**	**M_2**	**M_3**	**M_0**	**M_1**	**M_2**	**M_3**
Rich-a-haven	241.8	244.2	256.0	269.1	98.23	100.0	125.6	134.1	113.6	133.2	124.4	121.8	48.23	47.69	51.89	55.43
J.H. Hale	259.0	259.0	273.3	286.1	90.27	92.2	120.1	132.1	126.9	137.6	155.3	146.2	43.30	42.33	50.32	57.02
Redhaven	301.7	302.7	319.2	333.4	98.40	99.9	121.8	135.3	121.4	157.4	140.1	143.4	45.53	45.62	52.75	57.30
Flavourcrest	306.1	306.6	324.9	342.4	94.30	95.6	123.6	135.6	154.4	127.9	138.4	126.3	49.32	48.44	57.82	64.09
Sunhaven	278.6	278.63	299.9	309.9	101.50	100.9	131.8	140.0	131.6	145.3	146.1	141.0	53.97	51.51	56.94	64.83
Stark Early Giant	206.0	209.0	230.6	247.9	97.80	98.6	130.5	139.4	144.2	164.5	161.8	154.6	65.73	64.77	67.80	71.88
Kateroo	230.4	275.8	297.4	319.1	97.37	93.2	116.2	130.3	154.3	199.1	186.1	176.1	56.83	50.47	50.78	55.16
July Elberta	203.5	203.0	223.5	237.1	92.50	96.3	112.9	124.3	215.6	233.1	252.2	233.6	63.10	72.82	74.51	75.27

Source: Joshi, V. K., and P. K. Shah, *Acta Aliment. Budapest*, 27(4), 307–318, 1998. With permission.
Note: M_0, without maturation; M_1, 3 months; M_2, 6 months; M_3, 12 months.

TABLE 15.13

Physicochemical Composition of Apricot Wine

Characteristics	Apricot (New Castle) Mean ± SD*
TSS (°Brix)	8.20 ± 0.07
Titratable acidity (% MA)	0.76 ± 0.02
pH	3.15 ± 0.02
Ethanol (% v/v)	10.64 ± 0.09
Reducing sugars (%)	0.34 ± 0.01
Total sugars (%)	1.11 ± 0.02
Volatile acidity (% AA)	0.025 ± 0.002
Total phenols (mg/L)	253.60 ± 0.8
Total esters	120.6 ± 0.6
Color (units)	
Red	0.70 ± 0.05
Yellow	4.30 ± 0.08
Blue	0.60 ± 0.05

Source: Joshi, V. K. et al., *Am J Enol Vitic*, 41(3), 229, 1990b. With permission.

enhanced the rate of fermentation. The wine from 1:2 diluted pulps was rated as the best (Joshi et al. 1990b). In another method, honey was used to ameliorate the wild apricot must instead of sugar. A comparison of physicochemical characteristics of various wines produced is given in Table 15.14. Alcohol and total phenol contents in honey wine were found to be higher than that of sugar wine (Ghan Shyam 2009). Depending on the initial sugar contents, ethanol was produced. However, out of three different ISCs, that is, 22, 24, and 26°Brix, wild apricot wine of 22°Brix and treated with *Quercus* wood chips was rated the best.

For preparation of apricot wine from New Castle variety, the extraction of pulp either by hot method (Method I) or addition of enzyme and water to the fruits (Method II) was studied (Joshi and Sharma 1994). The comparison of rate of fermentation, alcohol content, physicochemical characteristics, and mineral contents of these wines is given in Table 15.15. The wine prepared from the later method had higher titratable acidity, K, Na, Fe content and lower phenolic contents. The effect of initial sugar levels on the quality of apricot wine was also reported by Joshi and Sharma (1994). It was found that dilution of pulp in the ratio of 1:1 with water, addition of DAHP at 0.1%, and raising the TSS to 30°Brix instead of 24°Brix made the wine of superior quality. The higher sensory quality of wine with 30°Brix could be attributed to balanced acid/sugar/alcohol content besides production of lower amount of volatile acidity due to higher initial sugar concentration of the must (30°Brix).

TABLE 15.14

Effect of Different Initial TSS Levels Using Sugar and Honey on Chemical Characteristics of Wild Apricot Wine

	TSS (°Brix)		Ethanol (%v/v)		Higher Alcohols (mg/L)		Total Phenols (mg/L)	
Treatment	**Sugar**	**Honey**	**Sugar**	**Honey**	**Sugar**	**Honey**	**Sugar**	**Honey**
Initial TSS 22°Brix	8.33	8.27	10.23	9.18	113.0	121.0	245.0	238.0
Initial TSS 24°Brix	8.57	8.37	11.70	9.86	131.5	136.5	264.0	255.0
Initial TSS 26°Brix	8.77	8.60	12.17	10.38	153.0	158.0	279.0	270.0

Source: Ghan Shyam, A., Preparation and evaluation of wild apricot mead and vermouth [MSc thesis], Dr. Y. S. Parmar, University of Horticulture and Forestry, Nauni, Solan, India, 2009.

TABLE 15.15

Comparison of Rate of Fermentation, Physicochemical Characteristics, and Mineral Contents of Apricot Wine Produced by Different Methods of Must Preparation

	Wines from Musts of Different Methods (Mean ± SD)	
Parameters	**Method (I)**	**Method (II)**
Rate of fermentation (°Brix/24 hours)	2.71 ± 0.08	2.75 ± 0.04
Ethanol (% v/v)	10.17 ± 0.3	10.22 ± 0.3
Titratable acidity (% malic acid)	0.72 ± 0.03	0.82 ± 0.02
pH	3.70 ± 0.05	3.65 ± 0.03
Total phenols (mg/L)	182 ± 3.0	112 ± 8.0
Volatile acidity (% acetic acid)	0.037 ± 0.001	0.039 ± 0.001
Minerals (mg/L)		
Potassium	1463 ± 124	2048 ± 120
Magnesium	18.7 ± 0.2	20.77 ± 0.9
Calcium	70.0 ± 5.8	78.0 ± 1.4
Sodium	11.13 ± 1.6	23.81 ± 4.5
Ferrous	1.69 ± 0.06	7.83 ± 0.04
Zinc	1.94 ± 0.02	1.39 ± 0.01
Copper	1.07 ± 0.01	1.08 ± 0.01
Manganese	0.81 ± 0.01	0.99 ± 0.01

Source: Joshi, V. K., and S. K. Sharma, *Res India*, 39(4), 25. 1994. With permission.

15.4.3.3 Maturation

In maturation of apricot wine cv. New Castle, the wood chips of *Quercus, Albizia, Bombax* and *Toona* were found to increase the tannin contents appreciably, whereas the *Populus, Celtis* and *Salix* woodchips increased the methanol levels in the treated wine (Joshi et al. 1994). There was significant improvement in sensory qualities of all the wines except those treated with woods of *Toona, Populus* and *Alnus*. Out of different woodchips tried, *Albezia* and *Quercus* produced wine of highest sensory qualities (Table 15.16). The addition of different wood chips, namely, *Quercus, Bombax,* and *Acacia* on ethanol, phenols and sensory quality characteristics of wild apricot wine was also reported (Ghan Shyam 2009). It was found that wood chips-treated wines showed more decrease in ethanol content than control wine after maturation for six months and lowest ethanol was recorded in wine treated with *Quercus* wood chips (Figure 15.5).

15.4.4 Cherry Wines

15.4.4.1 Fruit and Cultivar

Like other stone fruits, cherry fruits can also be used for preparations of wine, especially the sour type of cultivars that are more preferred (Schanderl and Koch 1957; Benk et al. 1976). However, the cherry fruit has been found more suitable for preparation of dessert wine than a table wine. The alcohol content of such wines may range from 12% to 17%. A blend of current and table variety of cherries can also be used for wine making.

15.4.4.2 Method of Preparation

To prepare a dessert wine of 16% alcohol, each liter of juice is ameliorated with 430 g of sugar. The addition of potassium metabisulfite before fermentation is advisable. The clarity of the wine is improved considerably if pectolytic enzyme is used. However, the addition of urea to the cherry must did not

TABLE 15.16

Effect of Wood Chips on Physicochemical and Sensory Characteristics of Apricot Wine

			Maximum Score							
			Appearance	Color	Aroma	Volatile Acidity	Total Acidity	Sweetness	Body	Flavor
Treatment	Tannins (mg/100 g)	Methanol (µL/L)	2	2	4	2	2	1	1	2
Control	57.00	182.00	1.57	1.45	3.08	1.57	1.34	0.69	0.57	1.00
Alnus nitida	63.00	167.00	1.55	1.62	3.14	1.59	1.32	0.77	0.61	1.06
Populus ciliate	74.00	238.00	1.56	1.56	3.03	1.53	1.28	0.71	0.62	1.38
Celtis australis	104.00	232.00	1.57	1.61	3.019	1.55	1.47	0.60	0.68	1.44
Toona ciliate	118.00	188.00	1.53	1.51	3.03	1.55	1.18	0.52	0.63	1.22
Salix tetrasperma	139.00	220.00	1.54	1.55	3.04	1.54	1.10	0.59	0.53	1.18
Bombax cieba	126.00	192.00	1.60	1.56	3.33	1.60	1.42	0.66	0.66	1.52
Albizia chinensis	122.00	198.00	1.64	1.48	3.14	1.61	1.33	0.65	0.56	1.50
Quercus leucotrichohora	83.00	209.00	1.67	1.67	3.57	1.68	1.60	0.67	0.71	1.69
CD (p = .05)	16.42	14.07	0.15	0.15	0.15	0.15	0.15	0.15	0.15	0.15

Source: Joshi, V. K. et al., *J Tree Sci*, 27–36, 1994. With permission.

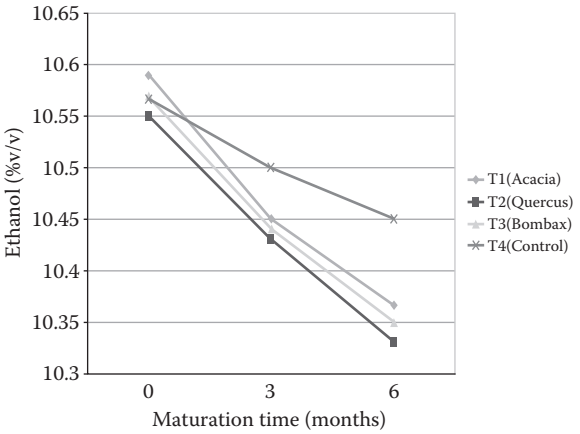

FIGURE 15.5 Effect of different wood chips on ethanol level during maturation of wild apricot wine.

improve fermentability (Yang and Weiganrd 1940). To enhance the flavor, approximately 10% of the pits may be broken down while crushing the cherries. However, production of hydrogen cyanide from the hydrolysis of amygdalin, present in the pit of cherries, has been detected (Baumann and Gierschner 1974; Misselhorn and Adam 1976; Stadel Mann 1976). The cherry wine does not require a long aging period. To make the wine sweet, sugar can be added before bottling. The bottled wine may be preserved either by germ-proof filtration or pasteurization. Yoo et al. (2010) investigated the protective activities of juice and wine produced from tart and sweet cherries. It was found that both the juice and wine making processes reduced the total phenolics to approximately 30% to 40% and 42% to 60%, respectively, and total anthocyanin capacity to approximately 60% to 77% in juice and 85% to 95% in wine. However, the radical-scavenging activity, SOD, and CAT enzyme levels were higher in cherry wine than in cherry juice (Table 15.17).

TABLE 15.17

Contents of Total Phenolics and Total Anthocyanins, Total Antioxidant Capacity, and DPPH Radical-Scavenging Activity of Cherry Fruits and Their Products

Cultivar		Total Phenolics*	Total Anthocyanins†	Total Antioxidants‡	DPPH-Radical-Scavenging Activity (%)§
Danube	Fruit	188 ± 8.1^{b}	70.1 ± 5.4^{b}	338 ± 14.0^{bc}	60.711.0^{c}
	Juice	56.7 ± 3.1^{e}	7.9 ± 2.8^{e}	202 ± 5.3^{e}	24.9 ± 0.7^{e}
	Wine	79.4 ± 4.4^{d}	29.6 ± 5.9^{d}	289 ± 2.9^{d}	53.0 ± 2.9^{d}
Balaton	Fruit	221 ± 2.0^{a}	93.5 ± 2.2^{a}	404 ± 8.1^{a}	75.2 ± 4.1^{a}
	Juice	86.8 ± 7.4^{d}	50.1 ± 4.5^{c}	311 ± 6.5^{c}	54.2 ± 5.6^{d}
	Wine	149 ± 1.5^{c}	63.4 ± 4.3^{c}	387 ± 4.7^{b}	69.0 ± 0.3^{b}

Source: Yoo, K. M. et al., *Food Chem*, 123, 734–40, 2010.

Note: All mean values are from triplicate determinations. Values in the same column with different superscript letters differ significantly ($p < .01$) by Duncan's multiple-range test.

* Total phenolics are expressed in milligrams of gallic acid equivalents (GAE) per 100 g of fresh cherries or milligrams of GAE per liter of liquor.

† Total anthocyanins are expressed in milligrams of cyanidine 3-glucoside equivalents (CGE) per l00 g of fresh cherries or milligrams of CGE per liter of liquor.

‡ Total antioxidant activity is expressed as milligrams of vitamin C equivalent antioxidant capacity (VCEAC) per l00 g of fresh cherries or mg of VCEAC per liter of liquor.

§ DPPH-radical-scavenging activities of each extract arc l00 µg/mL or 100 µL of liquor.

15.5 Vermouth

The term "vermouth" is derived from the German word for wormwood (*wermut*). It is supposedly derived from *wer* (man) and *mut* (courage, spirit, manhood; Pilone 1954). Officially, it is classified as an "aromatized fortified wine," referring to its derivation from a white base wine fortified and infused with a proprietary set of different plant parts: bark, seeds, and fruit peels. Vermouths are particularly popular in Europe and in the United States (Amerine et al. 1980; Panesar et al. 2009). When vermouth was introduced into Bavaria in the first half of the seventeenth century by the Piedmont producer Alessio, *Vinum absinthianum* was probably translated literally as *Wermutwein*. When it reached France, it was changed to vermouth. Largely, vermouth has been defined as a fortified wine (15%–21% alcohol content) flavored with a mixture of herbs and spices. Vermouth could be the sweet or Italian type and the dry or French type. In the Italian type, alcohol content varies from 15% to 17% with 12% to 15% sugar, whereas the French type has 18% alcohol with 4% reducing sugar. For the preparation of vermouth, a part of the base wine is initially distilled into brandy according to standard procedures (Amerine et al. 1980). Then, the first one-tenth of the expected distillate called "head" is not taken. Similarly, the last one-tenth of the distillate is also not taken. Only the middle portion of the distillate is collected and further double distillation is carried out to increase the strength of the brandy. Spice extracts are prepared by taking wine and brandy at a 1:1 ratio. Spices are immersed and gently heated for 10 minutes for 10 days continuously (Joshi 1997). The extract is kept at a low temperature for 2 days. The supernatant is separated by filtration and used in the preparation of vermouth.

15.5.1 Plum Vermouth

Besides the use of plum for making wine, attempt to prepare plum vermouth of commercial acceptability has also been made (Joshi et al. 1991b). Basically, the method to prepare plum vermouth is the same as that practiced for grape. Base wine is prepared as indicated by the method used to make wine. It is fortified and blended with spices and herb extracts. On an industrial scale, the brandy is prepared by fractional distillation. The herbs, spices, and parts used are shown in Table 15.18. It was found that increasing the alcohol concentration of plum vermouth increased the aldehydes, esters, total phenols, and TSS but the acidity and vitamin C decreased, whereas the addition of herbs/spice extracts increased the

TABLE 15.18

Spices and Herbs Used in the Preparation of Plum Vermouth

Common Name	Botanical Name	Parts Used	Quantity (g/L)
Black pepper	*Piper nigrum* L.	Fruit	0.75
Coriander	*Coriander sativum* L.	Seeds	0.70
Cumin	*Cuminum cyaninum* L.	Seeds	0.50
Clove	*Syzygium aromaticum* L.	Fruit	0.25
Large cardamom	*Amomum subulatum* Roxb.	Seeds	0.50
Saffron	*Crocus sativus* L.	Flower	0.01
Nutmeg	*Myristica fragrans*	Seed	0.25
Cinnamon	*Cinnamomum zeylanicum* Beryn	Bark	0.25
Poppy seed	*Papaver somniferum* L.	Seed	1.00
Ginger	*Zingiber offocinale* Rosc.	Dried root	1.00
Woodfordia	*Woodfordia floribunda*	Flower	0.25
Asparagus	*Asparagus* sp.	Leaves	0.10
Withania	*Withania somnifera*	Roots	0.20
Adhatoda	*Adhatoda* sp.	Leaves	0.25
Rosemary	*Rosmarinus offocinalis*	Flowering plant	0.10

Source: Joshi, V. K. et al., *J Food Sci Technol*, 28, 138–140, 1991b. With permission.

TABLE 15.19

Physicochemical Characteristics of Dry and Sweet Plum Vermouth

	Type of Vermouth	
Physicochemical Characteristics	**Dry**	**Sweet**
Total sugar (%)	ND	4.8
Titratable acidity (% malic acid)	0.81	0.79
Ethanol (% v/v)	15.0	14.5
Volatile acidity (% acetic acid)	0.03	0.04
pH	3.38	3.34
Vitamin C (mg/100 mL)	3.5	3.2
Total phenols (mg/L)	417	390
Aldehyde (mg/L)	411	112
Esters (mg/L)	204	219

Source: Joshi, V. K. et al., *J Food Sci Technol*, 28, 138–140, 1991b. With permission.
Note: ND, not detected.

total phenols, aldehyde, and ester content of vermouth. A comparison of sweet and dry plum vermouth has been made in Table 15.19. The sensory evaluation of the products showed the sweet products to be superior. The sweet vermouth with 15% alcohol content, spice extracts (10%), and sweeteners (6%) was considered to be the best product.

15.5.2 Apricot Vermouth

Wild apricot fruit has successfully been made into vermouth of acceptable physicochemical characteristics. In wild apricot vermouth, must of 24°Brix was prepared by diluting the pulp in a 1:2 ratio with water, 200 ppm of SO_2, 0.1% DAHP, and 0.5% pectinase enzyme were added. A 24-hour-old active culture was prepared and used for fermentation (Joshi et al. 1991a,b). Attempts were made to prepare vermouth from wild apricot with respect to alcohol content (Table 15.20), sugar level, and quantity of spices (Ghan Shyam 2009). Out of three different alcohol levels used, the highest TSS, ethanol, higher alcohols, and total esters were found in vermouth with 19% alcohol level, whereas total sugars, titratable acidity, and total phenols were recorded in wild apricot vermouth with 15% alcohol level (Figure 15.6).

TABLE 15.20

Effect of Alcohol Levels on Chemical Composition of Wild Apricot Vermouth

		Alcohol Level (%)		
Characteristics	**Base Wine (Mean ± SD)**	**15**	**17**	**19**
TSS (°Brix)	8.20 ± 0.07	17.09	17.23	17.45
Reducing sugars (%)	0.34 ± 0.01	5.55	5.46	5.38
Total sugars (%)	1.11 ± 0.02	10.41	10.16	9.96
Titratable acidity (% MA)	0.76 ± 0.02	0.85	0.78	0.77
Ethanol (% v/v)	10.64 ± 0.09	15.09	17.10	19.04
Total esters (mg/L)	135.40 ± 0.55	260.8	262.7	267.4
Total phenols (mg/L)	253.60 ± 0.8	454.4	451.6	446.6

Source: Ghan Shyam, A., Preparation and evaluation of wild apricot mead and vermouth [MSc thesis], Dr. Y. S. Parmar, University of Horticulture and Forestry, Nauni, Solan, India, 2009.

FIGURE 15.6 Wild apricot vermouth.

15.6 Sparkling Wine

A wine is called sparkling if it is surcharged with carbon dioxide (not less than 5 g/L at 20°C; Lee and Baldwin 1988). Sparkling wine such as champagne from grape is produced by secondary fermentation in closed containers such as bottles or tanks to retain the carbon dioxide produced. There are four types of sparkling wines that could be made (Amerine et al. 1980) as described below:

1. Sparkling wines with an excess of carbon dioxide produced by fermentation of residual sugar from the primary fermentation (Australian, German, Loire and Italian and the Muscato, Ambila of California).
2. Sparkling wines with an excess of carbon dioxide from a malolactic fermentation (Vinho verela wines of northern Portugal).
3. Sparkling wines containing an excess of carbon dioxide from sugar added after the fermentation process. Most of the sparkling wines in the world are of this type.
4. Sparkling wines in which an excess of carbon dioxide is added and includes the carbonated wines.

The different steps involved in the production of sparkling wine are preparation of a base wine, sugaring, yeasting, bottling, proper blending, secondary fermentation in bottles or tank maturation, finishing, and disgorging (Amerine et al. 1980; Joshi 1997). Maturation is a very important step in the production of sparkling wine and may extend up to 3 years. Bottle-aging results in the acquiring of physicochemical and sensory characteristics usually associated with such wines. In the production of sparkling wine, preparation of base wine is the first and foremost step. The quality of sparkling wine depends largely on the characteristics of the base wine, which in turn are dictated by a number of factors such as cultivar, yeast culture, preservatives, temperatures, nitrogen source, etc.

15.6.1 Sparkling Plum Wine

Plum fruits have been evaluated for the preparation of sparkling wine. The base wines from plums must were prepared with two preservatives, such as potassium metabisulfite and sodium benzoate. It was concluded that both types of wine were suitable for conversion into sparkling wine, although sodium benzoate treated–wine was considered better (Joshi et al. 1995). In preparation for sparkling wine from plum, the effects of different concentrations of sugar (1%, 1.5%, and 2%) and diammonium hydrogen phosphate (0.1%–0.3%), two strains of *Saccharomyces*, namely, UCD 595 and UCD 522 in two base wines, were reported. Increasing ethanol content up to 6% in plum base wine and decreasing the temperature of incubation from 24°C to 8°C reduced the viable count of both the yeast strains, although it remained at more than 80%. The UCD 595 strain was found to give better acclimatization to ethanol and low temperature compared with UCD 505. A sugar concentration of 1.5% and DAHP of 0.2% in 200 mL juice bottles at 15 ± 2°C were found to be optimum for bottle fermentation (Sharma and Joshi 1996). Furthermore,

TABLE 15.21

Comparison of Physicochemical Characteristics of Two Base Wines after Secondary Fermentation

	Base Wines		
Parameters	**Potassium Metabisulfite**	**Sodium Benzoate**	**CD ($p < 0.05$)**
Total soluble solids (°Brix)	9.31	10.94	0.04
Titratable acidity (% MA)	1.03	1.03	NS
CO_2 pressure (lbs/sq. in.)	20.92	19.04	0.66
pH	3.26	3.38	0.03
Ethanol (% v/v)	11.70	11.33	0.04

Source: Sharma, S. K., and V. K. Joshi, *Ind J Exp Biol*, 34(3), 235, 1996. With permission.
Note: Means irrespective of sugar and DAHP levels.

a comparison of the physicochemical characteristics of two base wines after secondary fermentation, irrespective of the sugar and DAHP levels, is given in Table 15.21.

A comparison of the physicochemical characteristics of sparkling plum wine prepared by bottle and tank fermentation was also made (Table 15.22). Bottle-fermented sparkling wine was rated better in both physicochemical and sensory quality (Joshi et al. 1995). Effect of different cultivars, yeast type on physicochemical and sensory quality of plum based sparkling wine production has been reported by Joshi and Bhardwaj (2011).

For maintaining foam stability in beer and wines, proteins, particularly polysaccharide glycoproteins (hydrophobic) having molecular weights of more than 5000 Daltons, act as surface-active agents, contributing to the bubble size, whereas low molecular weight proteins reduce these characteristics. The combined effect of cultivars, the addition of yeast extracts, type and form of yeast culture on the foam

TABLE 15.22

Comparison of Physicochemical Characteristics of Sparkling Plum Wine

Characteristics	**Bottle-Fermented Wine**	**Tank-Fermented Wine**
Aldehydes (mg/L)	27.50	25.45
Esters (mg/L)	121.67	113.33
Total phenols (mg/L)	303.67	293.67
Total anthocyanins (mg/100 mL)	111.50	110.33
Volatile acidity as acetic acid (g/100 mL)	0.020	0.023
Titratable acidity (% MA)	0.94	0.98
Crude proteins (%)	0.47	0.45
Total sugar (%)	2.16	2.52
Ethanol (% v/v)	11.97	11.57
Pressure (lbs/sq. in.)	52.50	8.75
Methanol (µL/L)	312.13	298.49
Amyl alcohol (µL/L)	301.75	327.10
Minerals (ppm)		
Iron	6.70	6.57
Zinc	2.16	2.13
Potassium	1417.33	1404.33
Sodium	29.28	28.52
Manganese	0.51	0.48
Copper	0.12	0.14

Sources: Joshi, V. K., D. K. Sandhu, and N. S. Thakur, *Biotechnology: Food Fermentation (Microbiology, Biochemistry and Technology*), Educational Publisher & Distributors, Ernakulum, New Delhi, Vol. II, p. 647, 1999a. With permission; Joshi, V. K., S. K. Sharma, R. K. Goyal, and N. S. Thakur, *Braz Arch Biol Technol,* 42(3), 315, 1999b. With permission.

TABLE 15.23

Effect of Cultivar, Addition of Yeast Extract, and Treatment (Yeast Type and Culture Form) on Foam Expansion (Expanded Volume/Original Volume) of Sparkling Plum Wine

	Control					+0.5% Yeast					
Cultivars	**Free Cell Liquid Culture, *S. cerevisiae***	**Immobilized Cell, *S. cerevisiae***	**Free Cell Culture, *Sc. pombe***	**Immobilized Cell, *Sc. pombe***	**Mean**	**Free Cell Liquid Culture, *S. cerevisiae***	**Immobilized Cell, *S. cerevisiae***	**Free Cell Culture, *Sc. pombe***	**Immobilized Cell, *Sc. pombe***	**Mean**	**Grand Mean**
Green Gage	1.50	1.42	1.33	1.10	1.34	3.33	1.20	1.65	1.50	1.92	1.63
Methley	1.00	1.10	3.00	1.05	1.54	1.10	1.05	1.58	1.20	1.23	1.38
Kanto-5	1.10	1.54	1.42	1.60	1.41	2.10	1.00	2.58	1.84	1.88	1.65
Santa Rosa	1.20	1.20	1.60	1.20	1.30	2.40	1.05	2.53	1.05	1.76	1.53
Frontier	1.10	1.10	1.60	1.42	1.30	1.54	1.05	1.40	1.05	1.26	1.28
Meriposa	2.50	1.84	1.33	1.20	1.72	1.54	1.42	2.38	1.60	1.73	1.73

Source: Bhardwaj, J. C., and V. K. Joshi, *Nat Prod Rad*, 8(4), 452–464, 2009.

TABLE 15.24

Effect of Cultivar, Addition of Yeast Extract, and Treatment (Yeast Type and Culture Form) on Foam Stability (as Seconds) of Sparkling Plum Wine

	Control					+0.5% Yeast					
Cultivars	**Free cell Liquid Culture, *S. cerevisiae***	**Immobilized Cell, *S. cerevisiae***	**Free Cell Culture, *Sc. pombe***	**Immobilized Cell, *Sc. pombe***	**Mean**	**Free cell Liquid Culture, *S. cerevisiae***	**Immobilized Cell, *S. cerevisiae***	**Free Cell Culture, *Sc. pombe***	**Immobilized Cell, *Sc. pombe***	**Mean**	**Grand Mean**
Green Gage	20.0	15.0	15.5	16.0	16.6	25.0	30.0	30.0	25.0	28.1	22.4
Methley	16.0	15.0	12.5	15.0	14.6	20.0	15.5	28.0	20.0	20.9	17.7
Kanto-5	12.5	12.5	20.0	15.0	15.0	20.0	19.0	25.0	20.0	18.5	16.7
Santa Rosa	12.5	12.5	20.0	13.5	14.6	20.0	15.0	20.0	15.0	20.7	17.7
Frontier	16.0	12.5	20.0	15.5	16.0	20.0	15.0	20.0	25.0	20.0	18.0
Meriposa	17.0	12.5	12.5	15.5	14.4	20.0	25.0	20.0	15.0	20.0	17.2

Source: Bhardwaj, J. C., and V. K. Joshi, *Nat Prod Rad*, 8(4), 452–464, 2009.

characteristics, and physicochemical and sensory quality characteristics of sparkling plum wine were determined (Bhardwaj and Joshi 2009). It was found that the addition of 0.5% yeast extract (*S. cerevisiae* and *Sc. pombe*) to the base wine at the beginning of secondary fermentation improved both foam stability and foam expansion. The values of foam expansion of the sparkling wine prepared from six plum cultivars (Table 15.23) showed that all three factors affected the foam expansion of the wines significantly. The addition of yeast extract significantly increased the mean expansion values of the sparkling wines, whereas immobilized yeast culture significantly decreased this parameter. Foam life time (L_F) of the sparkling wines, according to the factor studied (Table 15.24), showed that the addition of yeast extract in the base wine significantly enhanced L_F in the sparkling wine. However, the type of yeast did not influence the L_F values of sparkling wine significantly. The significant effect of cultivar on the L_F values can be attributed to the different surface active agents present in different wines. The studies showed the potential of plum fruits for sparkling wine production by the "Methode Champagnose," although more work is needed to commercialize this method.

15.7 Brandy

The word "brandy" captures all the wine distillates. Grapes are the fruits which are most frequently used to prepare brandy. These wine distillates have been called *aqua vini, eaux-de-vie, weinbrand, Cognac, Branntwein, aquardiente, aquavit*, and in English-speaking countries, brandy. It is made by distilling wine, and then, aging in oak barrels. The complete process of brandy production includes fermentation of juice or mash, distillation of wine, followed by aging in oak wood barrels.

15.7.1 Plum Brandy

The generic name of plum brandy is *Silvovitz*, and is a national drink of middle European countries such as Romania, Hungary, and Yugoslavia (Anonymous 2005). Plum brandies produced in the Alsace region of France are mostly named after the variety of fruit used. The varieties include quetsch (from Alsatian plums and are light-green and prune-like) and mirabelle (made from yellow plums), which are similar to those used for the preparation of dried prunes. However, plum brandy is usually colorless.

Although for brandy production, plums must have a high sugar content, yet the type of sugar is also important. Some plums contain sorbitol (0%–36% of total sugar) which is not a fermentable sugar. If the sorbitol content is high, the sucrose content is low and *vice versa*. Cultivars with high sorbitol content include German Prune, Italian Prune, Chrudimes, Tulver Gras, and Buhler Fruhzwetsche. The cultivars with little or no sorbitol are Stanley, Ersinger, Bluefire, and Grand Prize. The content of sorbitol is also influenced by climatic conditions, that is, in warm and sunny weather, the yield is low and the sorbitol content is high, which may therefore, cause bad fermentation (Hartmann 1982).

The method of making plum brandy is similar to that of grape brandy produced in large quantities. Imported Yugoslavian Silvovitz is often aged in oak, mulberry, or acacia cooperage (Litchev 1961). Most of the samples are light yellow and have little evidence of wood-aged character.

The aroma components of an experimental brandy made from California French prunes include aliphatic fatty acids, free acids, free fatty acids, aromatic acid, esters, alcohols, ethyl acetate lactone, and unsaturated aliphatic aldehydes (Crowell and Guymon 1973). Among the volatiles were the carboxylic acid, alcohol, and aldehydes found in plum brandy. It is reported that 50% of all plums grown in Yugoslavia belong to a Pozegaca cultivar and another 49.5% are local cultivars which are used for brandy production (Silvovitz; Paunovic and Bulatovic 1978). The quantitative and qualitative composition of plum brandies obtained using different fermentation sources is given in Table 15.25. Sensory evaluation of these brandies conducted by positive rating (Table 15.26) showed that the samples obtained using the distillery strain of *S. cerevisiae* received the highest score (18.2) and were characterized by a well-harmonized taste and aroma (Satora and Tuszynski 2010).

TABLE 15.25

Chemical Composition of Analyzed Plum Spirit Samples

Sample	Ethanol (% vol.)	Acetaldehyde	2,3-Butanediol	Acetion	Ethyl Acetate	Total Esters	Methanol	Propanol	Isobutanol	Butanol	Amyl Alcohols	Acetic Acid
		(mg/L 100° EtOH)										
Distillery *S. cerevisiae*	69.6 (±0.6)[a]	124 (±2)[a]	33.7 (±1.8)[a]	0.0 (±0.0)[a]	462 (±11)[a]	626 (±6)[a]	9634 (±295)[ab]	1484 (±81)[ab]	838 (±46)[a]	0.8 (±0.1)[a]	2080 (±115)[a]	169 (±3)[a]
Wine *S. cerevisiae*	69.5 (±4.2)[ab]	225 (±5)[bc]	51.0 (±3.3)[b]	7.8 (±1.5)[a]	277 (±10)[a]	394 (±8)[a]	7600 (±135)[c]	1563 (±180)[a]	1449 (±83)[b]	0.7 (±0.3)[a]	1938 (±108)[a]	379 (±19)[b]
Indigenous *S. cerevisiae*	71.0 (±0.6)[a]	246 (±38)[b]	39.1 (±6.5)[a]	9.2 (±1.4)[ab]	1183 (±25)[b]	1380 (±30)[b]	8936 (±367)[a]	1327 (±54)[b]	794 (±17)[a]	0.4 (±0.0)[b]	1266 (±5)[b]	248 (±11)[c]
Aureobasidium sp.	69.5 (±0.0)[ab]	169 (±4)[d]	37.3 (±4.0)[a]	10.3 (±2.8)[ab]	858 (±9)[c]	922 (±10)[c]	9632 (±675)[ab]	894 (±111)[c]	777 (±98)[a]	0.6 (±0.1)[ab]	1241 (±117)[b]	137 (±2)[d]
K. apiculata	66.3 (±0.6)[b]	182 (±4)[de]	14.7 (±2.4)[c]	6.5 (±2.1)[a]	1655 (±33)[d]	1829 (±17)[d]	9744 (±80)[b]	802 (±24)[c]	562 (±23)[c]	0.4 (±0.1)[b]	923 (±13)[c]	230 (±6)[e]
Spontaneous fermentation	74.3 (±1.4)[c]	212 (±18)[ce]	32.6 (±1.1)[a]	20.0 (±1.4)[b]	1925 (±107)[e]	2470 (±256)[e]	7550 (±378)[c]	617 (±78)[d]	338 (±7)[d]	0.6 (±0.1)[ab]	716 (±49)[d]	170 (±7)[a]

Source: Satora, P., and T. Tuszynski, *Food Microbiol,* 27, 418–424, 2010. With permission.

Note: Values not sharing the same superscript letter within the horizontal line are different according to the Duncan test ($p < .05$).

TABLE 15.26

Sensory Analysis of Tested Plum Spirits after 6 Months of Maturation—Buxbaum Model of Positive Ranking

	Assessment Characteristics				
Sample	**Color (Maximum 2 Points)**	**Clearness (Maximum 2 Points)**	**Odor (Maximum 4 Points)**	**Taste (Maximum 12 Points)**	**Total (Maximum 20 Points)**
Distillery *S. cerevisiae*	2.0 (±0.0)	2.0 (±0.0)	3.4 (±0.2)[a]	10.8 (±0.4)[a]	18.2 (±0.3)[a]
Wine *S. cerevisiae*	2.0 (±0.0)	2.0 (±0.0)	3.4 (±0.3)[a]	9.6 (±0.4)[b]	17.0 (±0.4)[bc]
Indigenous *S. cerevisiae*	2.0 (±0.0)	2.0 (±0.0)	3.0 (±0.1)[b]	9.6 (±0.2)[b]	16.6 (±0.3)[b]
Aureobasidium sp.	2.0 (±0.0)	2.0 (±0.0)	2.4 (±0.2)[c]	7.8 (±0.4)[c]	14.2 (±0.2)[d]
K. apiculata	2.0 (±0.0)	2.0 (±0.0)	3.4 (±0.1)[a]	10.2 (±0.2)[ab]	17.6 (±0.3)[ac]
Spontaneous fermentation	2.0 (±0.0)	2.0 (±0.0)	3.0 (±0.2)[b]	10.2 (±0.4)[ab]	17.2 (±0.5)[bc]

Source: Satora, P., and T. Tuszynski, *Food Microbiol*, 27, 418–424, 2010. With permission.

15.7.2 Peach Brandy

The brandy produced from peach fruit is called peach brandy and is popular in some countries. There have been some attempts to make brandies from fruits, including peaches, in California (Amerine et al. 1980). Various workers (Piepet et al. 1977; Stanciulescu et al. 1975) have described methods for preparing fruit brandy. A method for preparing peach brandy has been previously described (Amerine et al. 1980; Piepet et al. 1977). It can be made in a similar way as grape brandy, except for the method of wine preparation. Stone fruits like peaches are very pulpy, and for fermentation, these have to be diluted. This results in the reduction of sugar content to a very low level. The production of brandy can soak up any surplus of peach fruit, especially the cullage, but the use of poor quality waste fruit usually has little chance of resulting in quality brandy. Peach fruits from the July Elberta cultivar have the potential to produce commercially acceptable brandy. However, dried peach has not been found suitable for wine making (Amerine et al. 1980) nor, for that purpose, would it make a good brandy. The process of distillation is similar as that for any brandy. A blend of tree-ripened Sunny slope peaches is carefully distilled to preserve the delicate aroma. The resulting product is rich in aroma and fruit flavor. A simple chromatography method to estimate ethylcarbomate in spirits including peach has also been documented (Andrey 1980).

The peach fruit, because it is pulpy, has to be diluted before fermentation, which results in reduced sugar content, and hence, the need for amelioration (Joshi and Bhutani 1995). Brandy was produced by using peach pulp from July Elberta variety ameliorated with sugar, jaggary, and molasses as fermentable sugar sources in peach must (Shah and Joshi 1999). Sugar-based peach must gave the highest ethanol content among jaggary and molasses (Table 15.27).

The addition of different wood chips, namely, *Quercus, Albizia*, and *Bombax*, to the brandy increased the esters, tannins, higher alcohols, ethanol, and furfural contents compared with untreated brandy. Peach brandy should be matured with *Quercus* wood chips for improvement in all the quality characteristics (Shah and Joshi 1999). Composition and properties of peach brandies are affected by peach variety.

15.7.3 Apricot Brandy

Apricots fruits are used for making renowned apricot brandy, especially in Hungary (Nyujto and Guranyi 1981). Brands include the French Abricotine and the Hungarian Barack Palinka. In 10 authentic laboratory brandies of stone fruit (sour and sweet cherry, damson plum, and apricot), 0 to 6.4 mg of benzaldehyde per 100 mL of absolute alcohol and traces to 8.6 mg of hydrocyanic acid have been revealed. In 12 commercial samples, benzaldehyde ranged from traces to 13.4 mg/100 mg and hydrocyanic acid

TABLE 15.27

Biochemical Characteristics of Peach Brandy from Must with Different Sources of Sugar

	Peach Brandy		
Component	**Sugar**	**Molasses**	**Jaggery**
Total esters (mg/100 mL)	21.68	31.63	27.60
Fusel oil (mg/L)	48.71	36.33	41.43
Furfural (mg/L)	15.12	9.45	12.90
Aldehyde (mg/L)	24.59	19.19	21.43
Titratable acidity (mg/L)	58.47	92.22	60.76

Source: Shah, P. K., and V. K. Joshi, *J Sci Ind Res*, 58(6), 995, 1999. With permission.

from traces to about 10 mg. Brandy can also be prepared from wild apricot (Joshi et al. 1989). The pulp is diluted in a 1:1 ratio with water fortified with 0.1% DAHP and fermented to completion with TSS of 25°Brix. The wine is then distilled to make brandy. The brandy made from 1:1 diluted pulp was superior to that from 1:2 dilution. The brandy so produced had an intense apricot flavor and high sensory acceptability. The product was treated with slightly roasted oak wood chips during maturation to impart color and flavor. Comparison of the methanol content of brandy made experimentally with that of a locally prepared brandy showed a considerable reduction in the quantity of this alcohol (Joshi and Sadhu 2000).

15.7.4 Cherry Brandy

The fruit-growing regions of the upper Rhine River are the prime *eau de vie* production area of Europe. The Black Forest Region of Bavaria in Germany and the Alsace in France are known for their cherry brandies named *Kir* in France and *Kirschwasser* in Germany. Dutch sour cherries have been grown almost exclusively for processing. However, large amounts of inexpensive, deep-frozen cherries are being imported from countries outside the European Economic Community, particularly, Yugoslavia. Assessment of Dutch fruits used for various purposes for attributes like flavor and appearance by a panel have concluded that among all, brandy wine scored the highest taste assessment (Dieren and Joosse 1987). The suitability of different cultivars of cherry for processing (as whole fruit) brandy or alcohol-free juice has been reported (Aeppli et al. 1982).

15.8 Conclusions

The role of wine from grapes and its constituents in preventing coronary heart disease is well established. However, there is no information on stone fruit wines, which could be a potential area for future research. The use of enzymes in juice extraction, flavor improvements, continuous fermentation using bioreactor technology, and their possible applications in wine production from stone fruits needs serious consideration. We can foresee a future in which biological deacidification with malolactic bacteria or deacidifying yeast like *Schiz. pombe* is used. Different types of wines such as vermouth, sparkling wine, and sherry-type wines have been prepared from grapes, but can such a variety of wines be prepared from stone fruits? Despite the efforts made and described here, there are a large number of gaps in the research that could be elaborated in the future. It could certainly be a fruitful area of research especially in those countries and regions where stone fruit cultivation is practiced. Brandy is an alcoholic product and its consumption has been associated with medicinal properties, which enhances its importance among alcoholic beverages, and it is also relished for its elegant aroma and taste. These characteristics are based on a number of compounds. The role of these compounds in the development of brandy flavor is entirely related to the type/variety of fruit, distillation, activity of processes of interaction, and chemical conversion. The complexity of these processes requires a thorough understanding. There is a need to identify the components of brandy responsible

for medicinal value. Conclusively, the production of wine and brandy from stone fruits is certainly an exciting field for future research and development.

REFERENCES

Aeppli A, Gremminger U, Nyfeler A, Zbinden W. 1982. Cherry Cultivars, p. 95.

Amandeep G, Joshi VK, Rana N. 2009. Evaluation of preservation methods of low alcoholic plum wine. *Nat Prod Rad* 8(4):392–405.

Amerine MA, Berg HW, Kunkee RE, Qugh CS, Singleton VL, Webb AD. 1980. *The technology of wine making*. 4th ed. Westport, CT: AVI.

Andrey D. 1980. A sample gas chromatography method of ethylcarbamate in spirits. *Z Lebensmitt Untersuch Forschung* 185(1):21.

Anonymous. 2005. *All-natural accelerated aging of distilled spirits*. U.S. Patent 6869630.

Baumann G, Gierschner K. 1974. Studies on the technology of juice manufacture from sour cherries in relation to the storage of the product. *Fluess Obst* 41(4):123.

Benk E, Borgmann R, Cutka I. 1976. Quality control of sour cherry juices and beverages. *Fluess Obst* 43(1):17, 18, 23.

Beuchat LR. 1973. Inhibitory effects of benomyl on *Saccharomyces cerevisiae* during peach fermentation. *Am J Enol Vitic* 24(3):110–15.

Bhardwaj JC, Joshi VK. 2009. Effect of cultivar, addition of yeast type, extract and form of yeast culture on foaming characteristics, secondary fermentation and quality of sparkling plum wine. *Nat Prod Rad* 8(4):452–64.

Bhutani VP, Joshi VK. 1995. Plums. In: Salunkhe DK, Kadam SS, editors. *Handbook of fruit science and technology, cultivation, storage and processing*. New York: Marcel Dekker. p. 243–96.

Bhutani VP, Joshi VK, Chopra SK. 1989. Mineral composition of experimental fruit wines. *J Food Sci Technol* 26:332.

Chung JH, Mok CY, Park YS. 2003. Changes of physicochemical properties during fermentation of peach wine and quality improvement by ultrafiltration. *J Korean Soc Food Sci Nutr* 32(4):506–12.

Crowell EA, Guymon JF. 1973. Aroma constituents of plum brandy. *Am J Enol Vitic* 24(4):159–65.

Delin CR, Lee TH. 1991. The J-shaped curve revisited: Wine and cardiovascular health update. *Aust N Z Wine Ind J* 6(1):15(FSTA 24(5): 5H77, 1992).

Dieren JPA-Van, Joosse ML. 1987. Are the sour cherries written off? *Fruitteelt* 77(24):14.

Fleet GH, Heard GM. 1994. Yeast—growth during fermentation. In: *Wine microbiology and biotechnology*. Chur: Hartwood Academic Publishers. p. 27–54.

Food and Agriculture Organization. 2008. *FAO Production Statistics*. Available from: http://faostat.org/default/567.

Fowles G. 1989. The complete home wine maker. *New Scientist* 38.

Gasteineau FC, Darby JW, Turner TB. 1979. *Fermented food beverages in nutrition*. New York: Academic Press.

Ghan Shyam A. 2009. *Preparation and evaluation of wild apricot mead and vermouth*. [MSc thesis]. Dr. Y.S. Parmar, University of Horticulture and Forestry, Nauni, Solan, India.

Hartmann W. 1982. The importance of sugar content and patterns of plum for brandy production. *Abstracts XXIst International Horticultural Congress*, Vol. 1, Abstract No. 1159 A.

Joshi VK. 1997. *Fruit wines*. Dr. Y.S. Parmar, University of Horticulture and Forestry, Nauni, Solan (HP). 155 p.

Joshi VK, Attri BL, Gupta JK, Chopra SK. 1990a. Comparative fermentation behaviour, physicochemical characteristics of fruit honey-wines. *Indian J Hortic* 47(1):49.

Joshi VK, Attri BL, Mahajan BVC. 1991b. Studies on preparation and evaluation of vermouth from plum. *J Food Sci Technol* 28, 138–40.

Joshi VK, Bhardwaj JC. 2011. Effect of different cultivars yeast (free and immobilized cultures) of *S. cerevisiae* and *Schiz. pome* on physico-chemical and sensory quality of plum based wine for sparkling wine production. *Int J Food Ferm Tech* 1(2):69–81.

Joshi VK, Bhutani VP. 1990. Evaluation of plum cultivars for wine preparation. In: XXIII International Horticultural Congress, held at Italy. *Abstract* 3336.

Joshi VK, Bhutani VP. 1995. Peach. In: Salunkhe DK, Kadam SS, editors. *Hand book of fruit science and technology*. New York: Marcel Dekker. 230 p.

Joshi VK, Bhutani VP, Sharma RC. 1990b. Effect of dilution and addition of nitrogen source on chemical, mineral and sensory qualities of wild apricot wine. *Am J Enol Vitic* 41(3):229.

Joshi VK, Devi PM. 2009. Resveratrol: importance, role, content in wine and factors influencing its production. *Proceedings of the National Academy of Sciences*, India, 79, Part III, p. 76–9.

Joshi VK, Mahajan BVC, Sharma KR. 1994. Treatment of fruit wines with wood chips—effect on physico-chemical and sensory qualities. *J Tree Sci* 13(1):27–36.

Joshi VK, Sandhu DK. 2000. Quality evaluation of naturally fermented alcoholic beverages, microbiological examination of source of fermentation and ethanolic productivity of the isolates. *Acta Alimentaria* 29(4):323.

Joshi VK, Sandhu DK, Thakur NS. 1999a. Fruit based alcoholic beverages. In: Joshi VK, Pandey A, editors. *Biotechnology: food fermentation (microbiology, biochemistry and technology)*. Vol. II. Ernakulum, New Delhi: Educational Publisher & Distributors. p. 647.

Joshi VK, Shah PK. 1998. Effect of wood treatment on chemical and sensory quality of peach wine during aging. *Acta Alimentaria Budapest* 27(4):307–18.

Joshi VK, Shah PK, Kumar K. 2005. Evaluation of peach cultivars for wine preparation. *J Food Sci Technol* 42(1):83–9.

Joshi VK, Sharma PC, Attri BL. 1991a. A note on deacidification activity of *Schizosaccharomyces pombe* in plum musts of variable composition. *J Appl Bacteriol* 70:385.

Joshi VK, Sharma PC, Chauhan SK. 1989. Preparation of brandy from wild apricot fruit. *National Seminar on Recent Advances in Postharvest Management of Temperate Fruits, Vegetable and Ornamental Plants*, held on September 29 and 30, 1989. p. 47.

Joshi VK, Sharma S, Devi MP, Bhardwaj JC. 2009. Effect of initial sugar concentration on the physico-chemical and sensory qualities of plum wine. *J Northeast Foods* 8 (1,2):1–7.

Joshi VK, Sharma S, Shashi B, Attri D. 2004. Fruit based alcoholic beverages. In: Pandey A, editor. *Concise encyclopedia of bioresource technology*. New York: Haworth, Inc. 335 p.

Joshi VK, Sharma SK. 1994. Effect of method of must preparation and initial sugar levels on the quality of apricot wine. *Res Industry* 39(4):25.

Joshi VK, Sharma SK. 1995. Comparative fermentation behaviour, physico-chemical and sensory characteristics of plum wine as effected by the type of preservatives. *Chem Mikrobiol Technol Lebensm* 17(3/4):65.

Joshi VK, Sharma SK, Goyal RK, Thakur NS. 1999b. Sparkling plum wine: effect of method of carbonation and the type of base wine on physico-chemical and sensory qualities. *Braz Arch Biol Technol* 42(3):315.

Joshi VK, Sharma SK, Thakur NS. 1995. Technology and quality of sparkling wine with special reference to plum—an overview. *Indian Food Packer* 49:49.

Joshi VK, Thakur NS, Bhat A, Garg C. 2011. Wine and brandy: a perspective. In: *Handbook of enology—principles, practices and recent innovations*. New Delhi: Asiatech Publishers. p. 1–45.

Lee TH, Baldwin GE. 1988. Developments in the production and consumption of sparkling wines in Australia. *Food Technol Aust* 40(4):138.

Litchev V. 1961. Aldehyde composition of Bulgarian plum brandy. *Lozarstvo i Vinarstvo* 7:42–6.

Misselhorn K, Adam R. 1976. On the cyanides contents in stone-fruit products (Transl). *Braunt Wein Wirt and Chaft* 116(4):49.

Nyujto F, Guranyi D. 1981. *Apricots*. Kajszibarack. 465 p.

Panesar PS, Kumar N, Marwaha SS, Joshi VK. 2009. Vermouth production technology—an overview. *Nat Prod Rad* 8(4):334–44.

Paunovic R, Bulatovic S. 1978. The effect of peach variety on the composition and properties of peach brandy. *Zbornik-Radova-Pol.ioprivrednog-Fakulteta-Beograd* 586:15.

Piepet HJ, Bruchmann EE, Koeb E. 1977. *Technologie der obstrannerie*. Eugen Ulmar, Stuttgart.

Pilone FJ. 1954. Production of vermouth. *Am J Enol Vitic* 19:69.

Quinn KM, Singleton VL. 1985. Isolation and identification of elligitannin from white oak wood and an estimate of their roles in wines. *Am J Enol Vitic* 36:148–55.

Satora P, Tuszynski T. 2005. Biodiversity of yeasts during plum *Wegierka zwykla* spontaneous fermentation. *Food Technol Biotechnol* 43(3), 277–82.

Satora P, Tuszynski T. 2010. Influence of indigenous yeasts on the fermentation and volatile profile of plum brandies. *Food Microbiol* 27:418–24.

Schanderl H, Koch J. 1957. *Die Fruchtwein be reitung*. Stuttgart: Eugen Ulmer.

Shah PK, Joshi VK. 1999. Influence of different sugar sources and addition of wood chips on physicochemical and sensory quality of peach brandy. *J Sci Ind Res* 58(6):995.

Sharma SK, Joshi VK. 1996. Optimization of some parameters of secondary fermentation for production of sparkling wine. *Indian J Exp Biol* 34(3):235.

Stadel Mann W. 1976. Content of hydrocyanic acid in stone fruit juices. *Fluss Obst* 43(2):45.

Stanciulescu G, Rusnac D, Bortes G. 1975. *Technologia Distilator Alcoolial din Fruete si Vin*. Bucharest: Cares.

Vyas KK, Joshi VK. 1982. Plum wine making: standardization of a methodology. *Indian Food Packer* 36(6):80.

Vyas KK, Joshi VK. 1988. Deacidification activity of *Schizosaccharomyces pombe* in plum musts. *J Food Sci Technol* 25:306.

Vyas KK, Sharma RC, Joshi VK. 1989. Application of osmotic technique in plum wine fermentation—effect on physico-chemical and sensory qualities. *J Food Sci Technol* 26:126.

Vyas SR, Chakravorty SR. 1971. *Wine making at home*. Hissar, Haryana, India: Haryana Agricultural University Bulletin.

Westwood MN. 1978. *Temperate zone pomology*. San Francisco: W.H. Freeman. p. 233–5.

Wills RBH, Scriven FM, Greenfield H. 1983. Nutrient composition of stone fruit (*Prunus* spp) cultivars: apricot, cherry, nectarine, peach and plum. *J Sci Food Agric* 34:1383–8.

Yang HY, Weiganrd EH. 1940. Production of fruit wines in the Pacific North West. *Fruit Prod* J 29:8.

Yoo KM, Al-Farsi M, Lee H, Yoon H, Lee CY. 2010. Antiproliferative effects of cherry juice and wine in Chinese hamster lung fibroblast cells and their phenolic constituents and antioxidant activities. *Food Chem* 123:734–40.

16

Fermentation of Olive Fruit

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CONTENTS

16.1 Introduction

The olive fruit is a drupe. It has a bitter component (the glucoside oleuropein), a low sugar concentration (2.6%–6.0%), and a high oil content (10%–30%). Such characteristics prevent olives from being consumed directly from the tree and promote diverse processes to make them edible, which can slightly differ between areas of production (Garrido Fernández et al. 1997). The *Trade Standard Applying to Table Olives* (International Olive Oil Council [IOOC] 2004) defined this food as "the product obtained from suitable olive cultivars, processed to remove their natural bitterness, and preserved (by natural fermentation, heat treatment, or preservatives) with or without brine until consumption."

The oldest written reference on the preparation of table olives is by Columela in his manuscript *De Re Rustica*. In this way, the production of table olives originally started at a small, domestic scale millennia

ago. Currently, the sector is formed not only by small-scale domestic operators but also by large, international industries.

With the exception of certain presentations, table olives are fermented products. Thus, information on their physicochemical and microbiological characteristics is essential for understanding and improving their processing, storage, and safety. In the last few decades, various books have been published on the subject (Fernández Díez et al. 1985; Garrido Fernández et al. 1997; Kailis and Harris 2007). The present chapter is an overview based on the most recent contributions to this field.

16.2 Trends in Table Olive Production and Consumption

Table olives are a traditional fermented vegetable of Mediterranean countries. The production of this food was a craft process until the middle of the twentieth century, when mass fermentation and storage were introduced together with pitting and pitting/stuffing automation. The progressive mechanization in the industries has led to a rapid expansion of the sector.

FIGURE 16.1 Changes in table olive production, consumption, and exportation in the international market during the last seasons according to the IOOC (2009).

TABLE 16.1

Main (>10,000 tons) Table Olive Production, Consumption, and Exportation Countries in the 2007–2008 Season

Country	Production	Consumption	Exportation
Argelia	91	86	0
Argentina	100	13	91
EC	721	577	248
Iran	40	41	0
Jordan	30	19	11
Lebanon	23	20	11
Morocco	90	36	66
Syria	100	94	23
Tunisia	18	18	0
Turkey	200	190	20
Egypt	432	350	110
United States	109	241	4
Chile	14	16	3
Peru	112	60	18

Source: IOOC, *Key figures on table olives*. November 2010, Madrid, Spain.
Note: Data are expressed as tons × 1000.

Nowadays, table olives are one of the major fermented vegetables, with an overall production and consumption of more than 2,100,000 tons (Figure 16.1). Most of the table olives are produced in the European Community (EC; Table 16.1) with Spain (553,000 tons), Greece (95,000 tons), and Italy (56,000 tons) as the main contributors. Approximately 25% of the EC table olives are destined for exportation to third countries. There are also important productions in Egypt (432,000 tons), Turkey (200,000 tons), and Peru, the United States, Syria, Argentina, Algeria, and Morocco (these latter countries with approximately 100,000 tons each). The EC, Egypt, Argentina, and Morocco are strongly committed to the international market, but only a few producers require importations to cover their internal demand (United States, 240,000 tons; Italy, 122,000 tons).

16.3 Main Cultivars Devoted to Table Olive Production

The wild olive tree (*Olea europaea* L.) originated in Asia Minor. The Phoenicians started disseminating the olive throughout the Greek isles and Greek mainland. Later, the olive spread throughout the Mediterranean countries reaching Tripoli, Tunis, Sicily, and southern Italy. The Romans continued the expansion of the olive tree to the countries bordering the Mediterranean Basin. Olive cultivation was introduced in Spain by the Phoenicians (1050 BC). During the fifteenth and sixteenth centuries, the Spanish extended olive planting to the Americas, where there is an increasing production nowadays. The olive tree has also recently expanded to the Far East (Japan), South Africa, and Australia (IOOC 1996).

Olive cultivars devoted to the production of table olives (Figure 16.2) must meet several requirements (IOOC 2004). The varieties that can only be used are those whose volume, shape, flesh-to-stone ratio, fine flesh, taste, firmness, and ease of detachment from the stone make them particularly suitable for processing.

In Spain, the most well-known olive cultivar is Manzanilla, which has a moderate size (200–300 fruits per kilogram). It is an apple-shaped fruit with a fine skin, an excellent taste, and a flesh-to-pit ratio of 6:1. The Cacereña variety is very similar to Manzanilla, but with a slightly lower size. However, it is prone to softening and gaseous spoilage. Gordal is also very popular in Spain because of its big size (100–120 fruits per kilogram) and its flesh-to-pit ratio (8:1). It has a fine epicarp with typical white spots and a mesocarp with a good texture. Its stone is right and regular, making this cultivar also adequate for mechanization. Hojiblanca is an olive cultivar with a double use (oil extraction or table olives). Its flesh-to-pit ratio is 6:1 and its size, very variable, is approximately 230 fruits per kilogram. Other Spanish cultivars are Aloreña, Verdial, Picual, Lechin, Arberquina, and Morona. Manzanilla and Gordal are usually devoted to the production of green Spanish-style table olives, although Gordal is also prepared as natural seasoned turning color olives. Hojiblanca may be used as both green and ripe olives. On the contrary, Aloreña is exclusively used as seasoned olives and possesses the only Spanish table olive Protected Denomination of Origin.

FIGURE 16.2 Manzanilla olive cultivar in Seville (Spain) devoted to the production of table olives.

In Greece, the most abundant olive cultivar is Coservolea, with a flesh-to-pit ratio of 8:1 and a size of 180 to 200 fruits per kilogram. Their fruits are round to oval-shaped, similar to Hojiblanca. It is used for green and natural black olives. Kalamon is the second most important Greek cultivar. Its size ranges from 180 to 360 fruits per kilogram. Its fruits are curved, showing a prominent tip at the end. Kalamon is used exclusively in specialties from natural black olives. Other Greek cultivars are Chalkidiki, Throbolea, Megaritici, and Kothreici.

The most appreciated cultivar in Turkey is Domat. Its size is 180 to 190 fruits per kilogram. Gemlik is an appropriate cultivar to prepare natural black olives. Its flesh-to-pit ratio is 6:1 and its size is 270 to 290 fruits per kilogram. Memeçik, the most abundant double olive cultivar in Turkey, is processed as green and natural black olives. Other Turkish cultivars are Memeli, Edremit, and Sofralik.

In the United States, olive cultivars were imported from different countries. The variety most abundant is Manzanillo, with a flesh-to-pit ratio of approximately 8:1 and an average size of approximately 210 fruits per kilogram. Mission was introduced from Mexico, and it has a flesh-to-pit ratio of 7:1 and an average size of approximately 250 fruits per kilogram. Other cultivars in the United States are Sevillano, Ascolano, and Barouni.

16.4 Commercial Presentations of Table Olives

According to the IOOC (2004), table olives can be classified as a function of the type of olive fruit and the method of preparation used. Types are mainly related to the color of raw material used in the process. There are three main types: (i) green olives (normal-sized fruits with their skin still completely green), (ii) turning color olives (fruits with a yellow to pink surface color; Figure 16.3), and (iii) natural black olives (fruits picked with a dark color not only on the surface but also on the interior of the flesh).

In contrast, preparations allude to the system used for debittering, the way they are kept, and the preservation procedure. The IOOC Trade Standard includes (i) treated olives (when fruits are debittered using lye treatments and subjected to fermentation in brine), (ii) natural olives (when fruits are brined directly and debittering occurs only by dilution or natural hydrolysis; they are also maintained in brine for a total or partial fermentation), (iii) dehydrated or shriveled olives (when fruits, regardless of lye treatment or not, are subjected to dehydration by salt, heat, or any other technological process), and (iv) olives darkened by oxidation (when fruits are darkened by oxidation and preserved by sterilization).

Trade preparations may be offered in several presentations and forms, such as (i) whole olives, which includes cracked and split olives; (ii) stoned (pitted) olives, which includes halved, quartered, divided, sliced, chopped (or minced), and broken fruits; (iii) stuffed olives, with different products (pimento paste, anchovies, etc.); (iv) salad olives, a mixture of olives with pieces of stuffing material or capers (or both); (v) olives with capers; (vi) olive paste; and (vii) other styles.

FIGURE 16.3 Hojiblanca turning color olives ready to be pickled.

16.5 Description of the Major Table Olive Elaborations

16.5.1 Green Spanish-Style Olives

Green Spanish-style olives are obtained from green fruits, which are treated with alkali, washed, and put in brine where they undergo lactic acid fermentation.

16.5.1.1 Debittering Process

The lye treatment consists of a NaOH solution (2%–3.5% w/v), which penetrates up to two-thirds of the olive flesh. The main transformation during the debittering process is the chemical hydrolysis of the glucoside oleuropein, which is transformed into elenolic acid and hydroxytyrosol (Brenes et al. 1995). According to Marsilio et al. (1996), lye also dissolves the epicuticular waxy coating and solubilizes the intercellular cement in the middle lamella, causing softening because of the hydrolysis of insoluble protopectins in the cell walls. This favors the solubilization of olive components (sugars, minerals, organic acids, etc.) from olive flesh into brine. Alkali treatment time should be approximately 7 to 8 hours for most cultivars and more than 10 hours for sensible ones (Gordal). El-Makhzangy and Abdel-Rhman (1999) reported that concentrations of NaOH between 1% and 2% were the most suitable for the Àzizi variety (Egypt). Chammem et al. (2005) also found a concentration of 2% NaOH as adequate for "Meski" (Tunisian). The addition of calcium and sodium salts during this treatment, in combination with low temperatures, may also improve texture and prevent sloughing in Manzanilla cultivars (Rejano Navarro et al. 2008). After lye treatment, olives are washed to remove the excess alkali. Traditionally, two or three washings were done. However, they have now been reduced to a single wash, with a duration ranging from 6 to 24 hours (Garrido Fernández et al. 1997).

16.5.1.2 Fermentation

16.5.1.2.1 Initial Brine Conditions

The washed olives are then brined in a 10% to 12% NaCl solution, which is rapidly enriched with the nutrients leached from olives. This brine may also be supplemented with organic acids to lower the pH at levels appropriate to control the growth of spoilage microorganisms and to facilitate the growth of lactobacilli (Garrido Fernández et al. 1997). Benkeblia (1997) reported that the initial pH levels of brine and temperature were determinants for the relative abundance of *Lactobacillus plantarum* and *Lactobacillus brevis* in Sigoise (Algeria) fermentations. Durán Quintana et al. (1999) found that an initial pH level of 5.0 and 3.0% NaCl led to the highest acidification in fermentations carried out at 12°C. Asehraou et al. (2002) recommend adjusting the initial pH level to 4.0 with lactic acid and the addition of sodium benzoate (0.05% w/v) to a 5% NaCl brine to control bloater damage. Low levels of NaCl (≤4.0%) and initial pH level correction (4.5–6.5) were also favorable for establishing a proper green olive fermentation (Vega Leal-Sánchez et al. 2003). Chammem et al. (2005), using Meski (Tunisia) olives, observed that 9% NaCl was preferable for achieving a good fermentation. The addition of glucose (0.5%–1.0%) also led to a more rapid acidification and a decrease in the pH level (Chorianopoulos et al. 2005). Finally, the reuse of ozonated alkaline solutions as fermentation brines has been studied as an alternative to reduce the amount of effluents generated by this industry (Segovia Bravo et al. 2007a) without any negative effect on the overall quality.

16.5.1.2.2 Microbial Growth

Spanish-style table olive fermentations are usually spontaneous. The fermentative process is divided into four phases. The first lasts from brining until the initial growth of lactic acid bacteria (LAB) (~72 hours). The most relevant species found are Gram-negative bacteria of the genera *Enterobacter*, *Enterococcus*, *Escherichia*, and *Citrobacter* (Fernández Díez et al. 1985; Garrido Fernández et al. 1997; Campaniello et al. 2005). It is recommendable to reduce the length of this phase as much as possible to prevent spoilage. The second phase begins when the pH level of brine decreases up to approximately 6.0; the

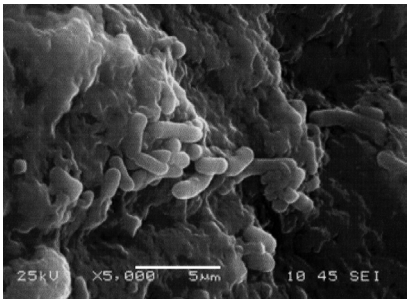

FIGURE 16.4 Polymicrobial communities on the surface of fermented Spanish-style green olives.

Gram-negative counts decrease and, in parallel, there is an increase of the lactic cocci populations, mainly *Leuconostoc* and *Pediococcus* spp. (González Cancho and Durán Quintana 1981; Campaniello et al. 2005). Its length is approximately 7 days. The third and more important phase is characterized by an abundant growth of *Lactobacillus* spp. (Figure 16.4), mainly *L. plantarum* and *Lactobacillus pentosus*, although other lactobacilli (*Lactobacillus casei*, *Lactobacillus curvatus*, *Lactobacillus fermentum*, *Lactobacillus cellobiosus*, *Lactobacillus coryniformis*, *Lactobacillus rhamnosus*, and *Lactobacillus brevis*) have also been reported (Garrido Fernández et al. 1997; De Castro et al. 2002; Kulisic et al. 2005; Campaniello et al. 2005; De Bellis et al. 2010). Its length is approximately 25 days. The fourth phase begins at the end of the lactic acid fermentation and lasts until olive packing. This phase takes place in the fermentation containers and later in the plastic drums used for keeping the olives during the conditioning operations. Only *Propionibacterium* species have been identified in this phase (De Castro and Rejano Navarro 1990). These species can consume the lactic acid produced by LAB, which originate as the pH level increases with the consequent risk of spoilage. This is prevented by increasing the NaCl concentration to more than 8% when the temperature rises (Garrido Fernández et al. 1997). Yeasts are also present during fermentation and contribute to the sensory attributes of the final product, but several species have been recognized as spoilage organisms in olive fermentations (Arroyo-López et al. 2008). The main species isolated from this elaboration are *Candida krusei*, *Candida boidinii*, *Pichia anomala*, *Pichia membranifaciens*, *Rhodotorula glutinis*, *Saccharomyces cerevisiae*, and *Candida tropicalis* (Arroyo-López et al. 2008). Proper lactic fermentation usually implies an adequate yeast population. The control of yeasts and bloater damages was achieved using *L. plantarum* as starter and 0.05% sodium sorbate (Asehraou et al. 2002). The new bulk fermentation systems (Figure 16.5) have somewhat altered the classical microbial evolution, decreasing the presence of Gram-negative bacteria, cocci, and yeasts and favoring the predominance of lactobacilli.

16.5.1.2.3 Physicochemical Changes in Brine

The initial amino acid content in green olive brines is approximately 1000 mg/L (80% as free amino acids), but reductions of 40%, 23%, and 36% were observed at the end of fermentation when brines

FIGURE 16.5 Bulk fermentation system used in large industries. Each fermenter has a capacity of approximately 10 tons.

were inoculated with *Enterobacter cloacae*, *Leuconostoc mesenteroides*, and *L. pentosus*, respectively (Montaño et al. 2000). The main sugars in solution are glucose (~1.0%), fructose (~0.4%), mannitol (~0.1%), and sucrose (~0.1%) (Sánchez et al. 2000). Throughout fermentation, the main process is the transformation of sugars into lactic acid by LAB, which decreases the pH level to values that make the product safe (<4.5) preventing *Clostridium botulinum* growth. A typical Spanish green olive brine is composed of the following: lactic acid (~1.3%), acetic acid (~0.3%), ethanol (~0.1%), methanol (~0.1%), formic acid (~0.05%), succinic acid (~0.05%), and propanol, propionic acid, 2-butanol, or acetaldehyde in lower proportions (Montaño et al. 2003). Panagou and Tassou (2006) studied the composition of Conservaolea brines. In addition to the previously mentioned compounds, they also reported 4-methyl-1-pentanol (~5 mg/L), ethyl acetate (~400 mg/L), isobutyl acetate (~300 mg/L), isobutyric acid (170–1200 mg/L), and isovaleric acid (18–40 mg/L). Iraqi et al. (2005), using gas chromatography–olfactometry/aroma extraction dilution analysis, also found the volatile compounds (*Z*)-3-hexenal, (*E*,*E*)-2,4-decadienal, and (*Z*,*Z*)-2,4-decadienal, which were related to green and coriander/paraffin oil odors, whereas guaiacol gave a bad olive, phenolic note. Moreover, methional and several terpenes were detected in the fruits as well. Biogenic amines have been found in green olives. Putrescine was detected first, but cadaverine, histamine, tyramine, and tryptamine were quantified after 12 months of storage with a high presence in "Zapatera" olives (García García et al. 2000). Storage at 20°C and 25°C produced a gradual increase in cadaverine and tyramine, which was markedly lower in unwashed olives with a practical stabilization of the product (García García et al. 2004).

16.5.1.2.4 Physicochemical Changes in Fruits

Through fermentation, the sugars in olive flesh gradually decrease. With respect to color, the evolution of chlorophylls and carotenoids led to the formation of an attractive yellow surface color (Garrido Fernández et al. 1997). However, in the case of Gordal, a green staining spoilage may appear. Its origin has been related to the degradation of proteins and phospholipids, forming pigment–lipoprotein complexes. The local accumulation of Cu-metallochlorophyll complexes becomes a macroscopically visible stain (Gallardo Guerrero et al. 2003a). Apparently, lipoxygenase is involved in the oxidative processes associated with this alteration (Gallardo Guerrero et al. 2003b). The interaction between oxidized chlorophylls and endogenous Cu was modulated by lye treatment and pH level, producing staining only in those fruits with prior oxidized chlorophylls (Gallardo Guerrero et al. 2007). The texture of fruits is also affected. The most important changes during processing were the decrease in the molecular weight of xyloglucans and the cellulose fraction which, in quantitative terms, was the largest decrease of the total cell wall polysaccharides (Sánchez-Romero et al. 1998b). An increase in the neutral branched arabinans in the water-soluble fraction, an increase in homogalacturonans, rhamnogalacturonans, and uronic acid, and a solubilization of arabinans in the respective solutions of analysis were also noticed (Sánchez-Romero et al. 1998a).

16.5.1.2.5 Application of Starter Cultures

The use of starter cultures, mainly LAB species, have been widely studied in Spanish-style olive fermentations. Many LAB species produce bacteriocins, which are proteins with a wide spectrum of inhibition against pathogens and spoilage microorganisms (Peres et al. 2008). Four strains of *L. plantarum* were found appropriate for achieving Spanish-style fermentations at 12°C (Durán Quintana et al. 1999). Sánchez et al. (2001) used *L. pentosus* CECT 5138 to initiate fermentation at alkaline pH (>9), showing a rapid growth and acidification that reduced the *Enterobacteriaceae* population and thereby the risk of spoilage. The use of a starter culture of *L. plantarum* led to a higher death rate of *Escherichia coli* O157:H7 compared with natural fermentations (Spyropoulou et al. 2001). The use of *Enterococcus casseliflavus* cc45 and *L. pentosus* 5138A sequentially applied with one day delay just after brining produced a quicker acidification (De Castro et al. 2002). The use of *L. plantarum* 1159 inoculation was useful, together with other conditions, to prevent bloater damage in the presence of high counts of *P. anomala* S18 (Asehraou et al. 2002). *L. plantarum* LPCO10, at a level of approximately 7 $\log_{10}$ CFU/mL in brine, improved the traditional fermentation when inoculated 1 to 4 days after brining at ≤4% NaCl and pH 4.5 to 6.5 (Vega Leal-Sánchez et al. 2003). Segovia Bravo et al. (2007b) observed an improvement in the growth of *L. pentosus* when it was inoculated together with *S. cerevisiae* in ozonated alkaline

solutions reused as fermentation brines. Recently, *Lactobacillus paracasei* IMPC2.1, obtained from the human intestine, has been tested as a probiotic starter in olive fermentations, allowing the control of the fermentation and a final probiotic product (De Bellis et al. 2010). However, starter cultures are still scarcely used at the industrial scale in this sector.

16.5.1.3 Conditioning (Grading, Pitting, and Stuffing)

Defective and browning spotted olives are removed after fermentation. Algorithms for the classification of olives by machine vision have been developed for this purpose (Diaz et al. 2000), and nowadays, their application at an industrial scale is common. Healthy olives are graded by size, according to their transversal diameters (IOOC 2004). Recently, a new machine, which takes into consideration the extremely variable dimensional characteristics of olives, was developed to improve grading by the size of oval-shaped and elongated drupes (Zimbalatti and Giametta 2002).

Most of the olives are commercialized as pitted or stuffed with diverse products. During pitting, some olives may break whereas others are pitted without damage. It was noticed that firsts were poorer in homogalacturonans, xylans, and xyloglucans compared with healthy olives. These results are in agreement with the role attributed to these compounds in maintaining the cell wall structure (Jiménez et al. 2000). Usually, fruits in the green olive maturation are properly pitted, whereas turning color olives are too disorganized to give good results. In addition, olives subjected to very strong lye treatment (high concentration and full flesh penetration) were affected by major losses. A system based on nuclear magnetic resonance for the detection of pits in olives under motion on a conveyor belt was developed to substitute the current method of separation by density. The projections were used to distinguish between pitted and nonpitted olives (Zion et al. 1997). The stuffing operation is normally achieved simultaneously to pitting. In addition to natural products, many diverse pastes of vegetal and animal origin products are used for this purpose.

16.5.1.4 Packing

The shelf life of green olives is affected by diverse factors. Nonpasteurized olives with low acidity and salt levels usually suffer a significant increase in pH level during storage, indicative of microbiological instability. On the contrary, pasteurized fruits are less firm and had worse colors than nonpasteurized olives, but they have a higher stability. During storage, firmness decreases and color fades with time, according to a first-order kinetic, particularly in pasteurized olives kept in the dark (Sánchez et al. 1997). Diverse preservatives (sorbic acid, benzoic acids or their salts, etc.) are recognized for their use in table olive packing to improve microbiological stability.

16.5.2 Ripe Olives

Ripe olives are obtained from green or turning color fruits, which were previously stored in brine and darkened by oxidation in an alkaline medium.

16.5.2.1 Storage

Ripe olives can be darkened directly or stored for further treatment. Storage can be achieved in cold rooms for a limited period of time. Agar et al. (1999) showed that the best conditions were 0°C to 5°C in air or in 2kPaO_2 for up to 4 weeks. However, olives can be also stored at room temperature in diverse holding solutions for longer periods of time. There are several storage systems, which were recently studied by De Castro et al. (2007).

In brine, traditionally, olives were kept in a 5% to 7% NaCl solution, which was progressively raised up to 8% to 10% in summer. Sometimes, brines were also acidified with acetic acid or supplemented with calcium chloride to prevent olive softening. Vaughn (1982) mentioned that the initial stages of this type of storage were dominated by Gram-negative bacteria, but after 4 to 5 days, LAB began to appear and the other bacteria began to decline. Among the most relevant LAB species found were *L. mesenteroides*, *Pediococcus cerevisiae*, and *L. plantarum*. Yeasts were also present throughout this period. This storage in brine can be

combined with the application of an aerobic process developed by the Instituto de la Grasa (CSIC) (Garrido Fernández et al. 1997). The presence of air favors the growth of yeasts. Fernández González et al. (1993) and Durán Quintana et al. (1994) isolated from Hojiblanca cultivar the species *P. membranifaciens*, *Pichia fermentans*, and *Hansenula polymorpha*, whereas in Cacereña cultivar, *Pichia minuta*, *P. fermentans*, *P. membranifaciens*, and *Rhodotorula* spp. were found. In these fermentations, the presence of LAB was reduced, showing its maximum between the 30th and the 60th day. It has been proven that the presence of LAB in aerobic storage is conditioned by the cultivar. Some of these cultivars (Manzanilla, Hojiblanca, and Cacereña) hardly support bacterial growth, whereas Gordal is prone to its development.

In acidic solutions, this storage is achieved by a combination of acid, food preservatives, temperature, and anaerobiosis. The procedure was initially developed by Vaughn et al. (1969), who found that the best conditions for storage were 0.67% lactic acid, 1.0% acetic acid, and 0.03% of both sodium benzoate and potassium sorbate. With this system, the Gram-negative bacteria and yeasts were markedly reduced. The accumulation of fermentable material and the lack of other microorganisms provided a very rich medium in which LAB grew when spring arrived. The presence of LAB increased the lactic acid, which contributed to the decrease in the pH level and the reinforcement of the product's stability. However, an important drawback of this process is the slow, but constant, degradation of the preservatives as well as their accumulation into the olive oil (approximately 30%–50% of the original preservative contents go into the oil, whereas the other 30% to 40% is in the flesh), which increases the microbial growth and the risk of alterations. The presence of added calcium in the storage solution might make it difficult to eliminate these preservatives from the flesh (Brenes et al. 2004). It is also possible to achieve storage without preservatives in anaerobic conditions, but with higher acetic acid concentrations and calcium chloride. In this case, the growth of yeasts is favored. The two most important yeast species found in acidified holding solutions (from both Manzanilla and Hojiblanca cultivars) were *S. cerevisiae* and *Pichia galeiformis*, which were present throughout the entire storage process, whereas *P. membranifaciens* was found only at the early stages and *C. boidinii* during the last period of storage. Only the last one showed lipolytic activity (Rodríguez Gómez et al. 2010). The storage of ripe olives produced changes in the olive oil, increasing acidity, peroxide value, polar compounds, and erythrodiol but decreasing triacylglycerols, polyunsaturated fatty acids, and Δ^5-avenasterol (López López et al. 2010).

16.5.2.2 Darkening Process

This phase consists of a series of one to three alkaline treatments, which progressively penetrate into the olive flesh up to the pit, with washing periods in between to remove the excess alkali. Then, a solution of ferrous gluconate or lactate is added for 10 to 18 hours to fix the black color. A shorter process was suggested by El-Makhzangy et al. (2008), which consisted of only one lye treatment with NaOH (1% w/w), followed by three washings until the pH level reached approximately 7.5 units. Then, acetic acid was added to lower the pH level to approximately 5 units during the color fixation (0.4% ferrous solution). The addition of certain amino acids (L-tryptophan, L-histidine, L-cysteine, and L-cystine) to solutions containing caffeic acid and hydroxytyrosol, which is mainly responsible for the color formation during darkening (Brenes et al. 1995; Marsilio et al. 2001), may improve the color fixation (Romero et al. 1998a). Manganese salts also improved the black color homogeneity at an industrial scale (Romero et al. 1998b,c). The reuse of ripe olive storage solutions may improve surface color and olive firmness (Brenes et al. 1998). The best conditions for color fixation were obtained at a low pH level (4.0), using tap water for preparing the ferrous solution and no aeration during the first 3 hours of immersion (García García et al. 2001). Jepson et al. (1998) suggested heating the ferrous solution at high temperatures. Recently, a new procedure based on the dry oxidation of polyphenols in an overpressure of oxygen at 40°C has been developed (García García et al. 2008).

16.5.2.3 Conditioning and Packing

Fixed black oxidized olives are then passed into a light brine (3%–5% NaCl), which can be slightly acidified with acetic acid to a final pH level of approximately 5.5 units. Because of the high pH level obtained in the equilibrium, these olives must be sterilized (Figure 16.6; Garrido Fernández et al. 1997).

FIGURE 16.6 Retorts used for the sterilization of olives at industrial scale.

Most of these fruits are commercialized as pitted or sliced. An approach to the shelf life estimation of this product was reported by García García et al. (2008) using an accelerated shelf life test (ASLT). Changes in pH level, color, and texture followed a first-order kinetic degradation. Degradation rates increased rapidly with temperature. Thus, an overexposure to this factor may completely ruin the product in a short period.

16.5.2.4 Changes during Ripe Olive Processing

Ripe olive processing has important effects on fruit texture. The lye treatment produces degradation of the cell walls because of the generalized loss of cellulose, pectic, and hemicellulosic polysaccharides, caused by the breakage of ester and hydrogen bonds. The thermal treatment also produces degradation of cellulose and increases the solubilization of polysaccharides (Mafra et al. 2007). Processing produces changes in the composition of ripe olive oil (acidity, fatty acids, polar compounds, diacylglycerols, and monoacylglycerols; López López et al. 2009). Ripe olives are particularly rich in iron and manganese (if added). A flame absorption spectrometric method for the analysis of both elements was validated and can be used by the regulatory agencies and industries (García García et al. 2002).

16.5.3 Directly Brined Olives

Product obtained from green, turning color, or black fruits, not treated with lye and directly brined, where they undergo fermentation.

16.5.3.1 Brining

Initial salt concentration in brines ranges from 5% to 10% NaCl, according to the sensitivity of the olive cultivar, but its content is periodically increased as salt is absorbed by fruits. Initial pH level correction with lactic, acetic, or HCl to prevent excessive growth of undesirable microorganisms is also fairly common. The leaching of nutrients from the olive flesh into the solution is slow because of the strong barrier of the untreated skin, but microbial growth begins just after brining.

16.5.3.2 Fermentation

16.5.3.2.1 Green-Turning Color Olives

These are characterized by a strong texture, a high concentration of polyphenols, and a slow diffusion of nutrients. Piga and Agabbio (2003) studied the effect of controlling pH level (4.0), salt content (2% and 4%), and temperature (~25°C) on Tonga de Cagliari olives. The diffusion rate of sugars and polyphenols into the brine was higher in 2% than that in 4% NaCl, but fruits fermented in 4% were preferred by panelists and showed a lower incidence of irreversible shriveling. The main groups of microorganisms involved in the fermentation of this type of olives are, like in other table olive elaborations, *Enterobacteriaceae*, yeasts, and LAB. Mourat et al. (2004) identified on the basis of morphological and biochemical criteria *Lactococcus lactics*, *L. plantarum*, and *Enterococcus* spp. from Algerian, naturally

green olives. In naturally fermented Sicilian green olives, *L. casei* resulted in being the dominant species, but *L. plantarum* and *Enterococcus faecium* were also reported (Randazzo et al. 2004). The microbial characterization of green Bella di Cerignola olives (Campaniello et al. 2005) showed the presence of different species of yeasts (*Candida* spp., *Rhodotorula mucilaginosa*, and *Cryptococcus laurentii*) and LAB (*L. plantarum*, *L. pentosus*, and *L. mesenteroides*). Aponte et al. (2010) worked with four Sicilian table olive cultivars brined in 8% NaCl and acidified with lactic acid. Only yeasts (*Candida parapsilopsis*, *Pichia guilliermondii*, and *Pichia kluyveri*) were found on this occasion. Hurtado et al. (2008) studied the microbial population dynamics during processing of Arbequina cultivar in industrial fermenters. They found that *Enterobacteriaceae* (mainly *Proteus vulgaris* and *Providencia rettgeri*) reached high population levels in the first days after brining (between 5 and 7 $\log_{10}$ CFU/mL), but they disappeared after 5 days. Yeasts (mainly *Candida diddensiae*, *R. glutinis*, *P. kluyveri*, *Kluyveromyces lactis*, and *C. boidinii*) were found from the very beginning of the process and reached its maximum populations after approximately 2 months. LAB species (*L. pentosus*, *Lactobacillus paraplantarum*, and *L. plantarum*) were detected from the 15th day and reached its maximum levels between the 40th and 60th days. However, in a further study (Hurtado et al. 2009), using 5% and 10% NaCl and different maturation degrees, the only LAB found was *L. plantarum*, whereas the main yeasts were *P. anomala*, *Candida sorbosa*, and *C. boidinii*. In conclusion, apparently, the biodiversity of LAB in directly brined green table olives is reduced, whereas that of yeasts is wider.

Starter cultures can also be used to improve natural green olive fermentations. Panagou et al. (2003) noticed a decrease in the survival of *Enterobacteriaceae* and a quicker acidification when a commercial strain of *L. pentosus* was used in Conservolea fermentations. The use of a *L. plantarum* strain able to hydrolyze oleuropein led to a slowly debittering but to a higher sensory quality (Idrissi et al. 2004). A selection of *L. plantarum* strains, isolated from Algerian table olive fermentations to be used as starter (Mokhbi et al. 2009), showed high to moderate acidifying activity but no exopolysaccharide production. *L. pentosus*, alone or in coculture with *C. diddensiae* and *L. plantarum*, was used in Arbequina green olives (Hurtado et al. 2010). The incorporation of *C. diddensiae* was favorable to reduce the survival period of *Enterobacteriaceae* but failed to colonize the brine until the end of the process. The LAB counts reached 6 $\log_{10}$ CFU/mL only when *L. pentosus* was added.

16.5.3.2.2 Natural Black Olives

They must be picked when completely mature. The initial salt concentration in brine is diverse (between 6% and 10%), but its content progressively decreases and must be periodically supplemented to reach 8% to 10% NaCl. Traditionally, the initial pH level is corrected to 4.0 with acetic acid. There are two fermentation systems: (i) the traditional anaerobic and (ii) the recently developed aerobic process.

In the anaerobic process, sugars are quickly consumed as they pass into the solution. Kotzekidou (1997) identified the yeast population associated with Greek olive cultivars from several factories. The predominant species were *Torulaspora delbrueckii*, *Debaryomyces hansenii*, and *C. laurentii*. In Spanish cultivars, the main yeast species found were *S. cerevisiae* and *P. anomala*. As in the case of Greek cultivars, the presence of LAB was not systematic and included only *Pediococcus* spp. (in the case of low initial NaCl) and *L. plantarum* (Garrido Fernández et al. 1997). Balatsouras (1966) considered the natural black olives as a lactic process because microorganisms were initially represented by *Streptococcus*, followed by *Leuconostoc* and *Lactobacillus*. Turantas et al. (1999) used potassium sorbate and sodium benzoate to control the yeast counts and to increase the LAB population, but preservatives accumulated into the olive flesh. Tassou et al. (2002) showed that the use of 6% NaCl at 25°C conducted to a high titratable acidity and low pH level. The LAB species found in this case were identified as *L. mesenteroides*, *L. brevis*, *L. plantarum*, and *L. pentosus*. Nychas et al. (2002) observed the initial growth in brine of *Enterobacteriaceae* and *Pseudomonas* spp., which decreased as the LAB and yeast populations increased. In French natural black olives, Coton et al. (2006) found sequential apparition of yeasts, with *P. anomala*, *C. boidinii*, and *Debaryomyces etchellsii* being the predominant species. Nisiotou et al. (2010) have identified in Conservolea Greek olive cultivar up to 17 species of yeasts, which coexisted with the LAB throughout the process. *P. membranifaciens* and *P. anomala* were the prevalent species. The use of starter culture for natural black olives has also been checked. *L. pentosus* 1MO was reported to shorten the debittering process time of black table olives from Itrana and Leccino cv. at controlled

temperature to only 8 hours (Servilli et al. 2006). Kumral et al. (2009) noticed that the inoculation with *Leuconostoc cremoris* at 10–12°C led to the highest titratable acidity, lowest pH level, and least yeast growth, without the presence of *Pseudomonas* or *Enterobacter.* Psani and Kotzekidou (2006) studied diverse technological characteristics of *D. hansenii* and *T. delbrueckii* for their use as starter adjuncts in Greek-style black olive fermentations. Some mycogenic strains showed activity against *Listeria monocytogenes*, *Bacillus cereus*, and *Salmonella typhimurium*. Mixed cultures of *L. plantarum* ATCC8014 and *D. hansenii* 2114 were used as starters in mature olive juice (Tsapatsaris and Kotzekidou 2004). The inoculation of the yeast previously to the *Lactobacillus* improved the performance of the bacteria.

The aerobic process was initially developed at Instituto de la Grasa (Sevilla) to prevent the formation of gas pockets in directly brined color turning and naturally black olives (García García et al. 1985), although its use has been also extended to ripe olive storage (De Castro et al. 2007). Basically, it consists of the injection of air into the brine through a column device with a sufficient number of holes around the surface of its bottom to permit brine entrance (Figure 16.7). The brine is forced into the column by the ascending movement of the air bubbles and ascends up to the top where it is mixed with the rest of the fermenter's brine (Garrido Fernández et al. 1985). An image of its implementation is shown in Figure 16.8. Usually, the air flux is approximately 0.2 L of air per hour per liter capacity of the container. Normally, the pH level of the initial brine is corrected with acetic acid, and the NaCl may range from 2% to 8%. The presence of aerobic conditions prevents the growth of anaerobic *Clostridium* and *Propionibacterium* but may favor the presence of *Enterobacteriaceae*. Because LAB are oxygen tolerant, the system does not interfere with lactic acid fermentation. The main species of microorganisms found in these conditions were *Citrobacter freundii* and *Proteus mirabilis* (among *Enterobacteriaceae*). In the case of low NaCl concentrations, the genera *Pediococcus* and *Leuconostoc* persist up to the 20th to 25th day, followed by *Lactobacillus*, which may end the fermentation, except where the level of salt increases by more than 8% to 10%. Yeasts are the predominant microorganisms, and their populations increase with the air flux, with *P. membranifaciens*, *Hansenula mrakii*, and *C. boidinii* being the most representative species identified (Durán Quintana et al. 1986). The product obtained from Farga olives (Argentina) under aerobic conditions and in the presence of $CaCl_2$ had better texture, more attractive color, and significantly lower gas pocket spoilage than fruits processed following the anaerobic system (Álvarez et al. 2003).

16.5.3.3 Changes during Fermentation

After fermentation, olives lose most of their initial bitter taste and are ready for commercialization. The debittering process was studied in detail by Servilli et al. (2008), who supposed that the hydrolysis of the glucosides (oleuropein, demethyloleuropein, and verbascoside) reduces the polyphenol molecular weights and makes their solubilization easy, which is also favored by the degradation of the outer cuticle of the skin. Romero et al. (2002) carried out a study of the phenolic compounds in Spanish black olives, identifying hydroxytyrosol-4-β-D-glucoside as the main phenol, together with hydroxytyrosol, salidroside, tyrosol, verbascoside, luteolin-7-glucoside, rutin, demethyloleuropein, and oleuropein. In general, natural olives are rich in phenols. Blekas et al. (2002) observed that hydroxytyrosol, tyrosol, and luteolin were abundant in Greek-style natural and in Kalamata olives. Galega and Negrinha de Freixo (Portugal) showed good antioxidant capacity and antimicrobial activity because of polyphenols (Pereira et al. 2006). Directly brined olives are also rich in triterpenic acids, which reach approximately 2000 mg/kg of flesh. Among anthocyanins, cyanidine-3-*O*-glucoside and cyanidine-3-*O*-rutinoside were found. These compounds suffer polymerization as the fermentation time progress, which is related to the presence of oxygen in the brine (Romero et al. 2004).

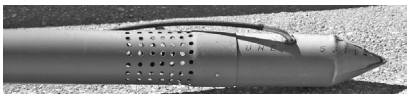

FIGURE 16.7 Air column device used for the injection of air into the brine in the aerobic storage/fermentation system. Holes around the surface of its bottom allow brine entrance.

FIGURE 16.8 Details of the aerobic fermentation system applied to an underground 10-ton fiberglass fermenter.

16.5.3.4 Conditioning and Packing

Usually, directly brined olives are commercialized as whole or cracked and are sold in just brine or as seasoned olives, with the addition of vinegar, garlic, red or green pepper, aromatic herbs, and so forth. They are relatively unstable and need pasteurization or the addition of preservatives for their complete stabilization. Panagou (2004) studied different packing treatments (acidified brines and modified atmospheres) for olives in brine stored at room temperature (~20°C). The microbial flora of LAB and yeasts continued during shelf life but no *Enterobacteriaceae* or *Pseudomonas* were detected. Best results were noticed using brines supplemented with 0.3% lactic and 0.3% citric acids. In Spanish commercial samples, the LAB population in directly brined olives was between 4.5 and 7.3 $\log_{10}$ CFU/mL, whereas the yeast counts were markedly lower (between 2.2 and 5.5 $\log_{10}$ CFU/mL). Packed natural green and black olives from the Portuguese market showed generally acceptable safety, with the exception of four samples that had *S. aureus* (Pereira et al. 2008). Finally, Romeo et al. (2009) reported that reusing the fermentation brines in pasteurized natural green olives was favorable for keeping the composition of the product (polyphenols, pigments, and other compounds that characterize aroma and nutritive value), even if subjected to slight browning due to thermal treatment.

16.5.4 Dry-Salted Olives

Panagou (2006) described the elaboration of dry-salted olives from Thassos cultivar, which was achieved by mixing coarse salt (40%) and freshly harvested olives (60%). The initial microflora comprised LAB (~4 $\log_{10}$ CFU/g), yeasts (~5.7 $\log_{10}$ CFU/g), *Enterobacteriaceae* (~3.7 $\log_{10}$ CFU/g), and *Pseudomonas* (~4 $\log_{10}$ CFU/g), but at the end of fermentation, only yeasts were isolated. The prevailing yeast species was *Candida famata* (Panagou et al. 2002). This preparation has recently been identified as a nutrition-rich source of oleuropein by Zoidou et al. (2010). Dry olives can also be obtained by oven drying. Scanning electron microscopy revealed that heat treatment was highly damaging, affecting the intracellular pectin substances and producing cell separation (Marsilio et al. 2000).

16.5.5 Specialties

Some commercial presentations are not specifically defined in the IOOC (2004) standards. They may be included under the "specialties" group. Seasoned, not heat treated, olives are fairly popular. They are usually obtained from green or turning color directly brined olives and destined for local markets. Yeast species isolated in high frequencies from the elaboration of seasoned green table olives are *P. anomala*, *Kluyveromyces marxianus*, and *S. cerevisiae* (Hernández et al. 2007). These olives, however, have a limited shelf life and may soften rapidly. Among the factors affecting the changes in texture of seasoned olives are the enzymes (cellulase and polygalacturonase) coming from the seasoning products (garlic, lemon, etc.) as well as those of endogenous origin (Fernández Bolaños et al. 2002).

One specialty is the "Aloreña de Malaga" Protected Denomination of Origin, which is characterized by its crispy flesh. They are seasoned with garlic, pepper, fennel, thyme, orange, and so forth. Stabilization is usually achieved by preservatives because thermal treatment produces a brownish color. Arroyo-López et al. (2007) showed that the previous storage phase followed the normal sequence of microorganisms in brined olives: *Enterobacteriaceae*, yeasts, and LAB. Among the species of yeasts

isolated were *S. cerevisiae*, *C. tropicalis*, *C. diddensiae*, *Issatchenkia occidentalis*, and *Geotrichum candidum* (Arroyo-López et al. 2006). However, the only LAB species isolated from these olives was *L. pentosus*. A lactic process can also be induced for the fermentation of this cultivar by using low salt and acetic acid (García García et al. 1992). Arroyo-López et al. (2007) used different types of antioxidants to preserve the characteristic Aloreña green color during storage, with good results for ascorbic acid and sodium metabisulfite. Partial substitutions of the traditional NaCl by KCl and $CaCl_2$ salts have been proposed recently by Bautista Gallego et al. (2010) in this type of elaboration.

16.6 Nutritional Characteristics

Table olives are mainly used as an appetizer or ingredient of some dishes. It was already mentioned that these products are a good source of antioxidants (hydroxytyrosol, oleuropein, methyloleuropein, etc.) and triterpenic acids, all of them with unquestionable biological value. From the nutritional labeling perspective, olives are mainly composed of moisture, fat, ashes (mineral), fiber, and proteins. However, they are low in carbohydrates. Several publications have recently revised the nutritional composition of table olives. The fat content ranges from approximately 10 (in Gordal) to approximately 31 g/100 g of flesh (in cultivars destined for oil). Most of this is monounsaturated fat (~5% to ~19%) because of its abundance of oleic acid. It is followed by saturated fat (~1.5% to ~6%), with palmitic acid as the most outstanding component of this fraction, and polyunsaturated fat (~0.5% to ~3.9%), with linolenic acid being the major contributor. Table olives also have small proportions of trans fat (~0.08% to ~0.44%), mainly elaidic acid (López et al. 2006). In general, table olives are a good source of monounsaturated fatty acids with a reasonable level of polyunsaturated fat. Fiber is also an important constituent, which reaches from approximately 2% to approximately 5% of flesh. Some stuffing material (hazelnut, almond, etc.) can produce an increment in fiber concentration. Thus, in general, table olives are a good source of this nutrient. The levels of lactic acid are approximately 0.5% in flesh, but the proportion of sugars is negligible after fermentation (López López et al. 2007). Average protein levels are between approximately 0.9% and approximately 1.2%, its proportion being higher in the presence of stuffing materials of animal origin (anchovies, almonds, etc.). Protein proportion is relatively low but has all the essential amino acids with lysine as the limiting amino acid, followed by cysteine and methionine (López et al. 2007). Table olives are rich in sodium (~0.5% to ~1.8%), but they are also a good source of other minerals such as calcium (~0.3% to ~0.8%), potassium (~0.05% to ~0.12%), magnesium (~0.05% to ~0.2%), and phosphorous (~0.05% to ~0.14%), containing important proportions of microelements (copper, ~0.17 to ~1.10 mg/100 g; zinc, ~0.15 to ~0.36 mg/100 g; and manganese, ~0.02 to ~0.15 mg/kg). Finally, table olives are a good source of phytosterols (~20 to ~52 mg/100 g), with β-sistorol being the most abundant (López López et al. 2008), vitamin E (~1.3 to ~5.23 mg/100 g; López López et al. 2005a), and vitamin A (~197 to ~1387 μ/100 g), which is particularly abundant in those presentations including red pepper as stuffing material (López López et al. 2005b).

REFERENCES

Agar T, Hess-Pierce B, Sourour M, Kader AA. 1999. Identification of optimum preprocessing storage conditions to maintain quality of black ripe "Manzanillo" olives. *Postharvest Biol Technol* 15:53–64.

Álvarez DME, Sánchez A, Lamarque AL. 2003. Natural black olives. Comparative study of three fermentative processes using "Farga" cultivar. *Olivae* 97:47–51.

Aponte M, Ventorino V, Blaiotta G, Volpe G, Farina V, Avellone G, Lanza CM, Moschetti G. 2010. Study of green Sicilian table olive fermentations through microbial chemicals and sensory analysis. *Food Microbiol* 27:162–70.

Arroyo-López FN, Durán Quintana MC, Romero C, Rodríguez Gómez F, Garrido Fernández A. 2007. Effect of storage on the sugars, polyphenols, color, and microbiological changes in cracked Manzanilla-Aloreña olives. *J Agric Food Chem* 55:7434–44.

Arroyo-López FN, Durán Quintana MC, Ruiz Barba JL, Querol A, Garrido Fernández A. 2006. Use of molecular methods for the identification of yeast associated with table olives. *Food Microbiol* 23:791–6.

Arroyo-López FN, Querol A, Bautista Gallego J, Garrido Fernández A. 2008. Role of yeasts in table olive production. *Int J Food Microbiol* 128:189–96.

Asehraou A, Peres C, Faid M, Brito D. 2002. Reducing bloater spoilage incident in fermented green olives during storage. *Grasas Aceites* 53:330–4.

Balatsouras GD. 1966. The chemical composition of the brine of stored Greek black olives. *Grasas Aceites* 17:83–8.

Bautista Gallego J, Arroyo-López FN, Durán Quintana MC, Garrido Fernández A. 2010. Fermentation profiles of Manzanilla-Aloreña cracked green table olives in different chloride salt mixtures. *Food Microbiol* 27:403–12.

Benkeblia N. 1997. Efecto del pH inicial en el desarrollo de *Lactobacillus plantarum* and *Lactobacillus brevis* durante la fermentación láctica de aceitunas verdes en salmuera. *Olivae* 65:52–5.

Blekas G, Vassilakis C, Harizanis C, Tsimidou M, Boskou DG. 2002. Biophenols in table olives. *J Agric Food Chem* 50:3688–92.

Brenes M, García P, Romero C, Garrido A. 1998. Ripe olive storage liquids reuse during the oxidation process. *J Food Sci* 63:117–21.

Brenes M, Rejano L, García P, Sánchez AH, Garrido A. 1995. Biochemical changes in phenolic compounds during Spanish-style green olive processing. *J Agric Food Chem* 46:2702–6.

Brenes M, Romero C, García P, Garrido A. 2004. Absorption of sorbic and benzoic acids in the flesh of table olives. *Eur Food Res Technol* 219:75–9.

Campaniello D, Bevilacqua A, D'Amato D, Corbo MR, Altieri C, Sinigaglia M. 2005. Microbial characterization of table olives processed according to Spanish and natural styles. *Food Technol Biotechnol* 43:289–94.

Chammem N, Kachouri M, Mejri M, Peres C, Boudabous A, Hamdi M. 2005. Combined effect of alkali pretreatment and sodium chloride addition on the table olive fermentation process. *Bioresour Technol* 96:1311–16.

Chorianopoulos NG, Boziaris IS, Stamatiou A, Nychas GJE. 2005. Microbial association and acidity development of unheated and pasteurized green-table olives fermented using glucose or sucrose supplements at various levels. *Food Microbiol* 22:117–24.

Columela LJM. 54 AC. De Re Rustica. Vol II. Spain: Nestlé, A.E.P.A. Santander.

Coton E, Coton M, Levert D, Casaregola S, Sohier D. 2006. Yeast ecology in French cider and black olive natural fermentations. *Int J Food Microbiol* 108:130–5.

De Bellis P, Valerio F, Sisto A, Lonigro SL, Lavermicocca P. 2010. Probiotic table olives: microbial populations adhering on olive surface in fermentation sets inoculated with the probiotic strain *Lactobacillus paracasei* IMPC2.1 in an industrial plant. *Int J Food Microbiol* 140:3–13.

De Castro A, García P, Romero C, Brenes M, Garrido A. 2007. Industrial implementation of black ripe olive storage under acid conditions. *J Food Eng* 80:1206–12.

De Castro A, Montaño A, Casado FJ, Sánchez AH, Rejano L. 2002. Utilization of *Enterococcus casseliflavus* and *Lactobacillus pentosus* as starter culture for Spanish-style green olive fermentation. *Food Microbiol* 19:637–44.

De Castro A, Rejano Navarro L. 1990. Incidencia de la fermentación propiónica en la conservación de aceitunas verdes de mesa. Seminario Últimos avances en la Tecnología de Preparación de Encurtidos. Aplicación a Pepinillos y otros cultivos de interés en Andalucía. Seville, Spain.

Diaz R, Faus G, Blasco M, Blasco J, Moltó E. 2000. The application of a fast algorithm for the classification of olives by machine vision. *Food Res Int* 33:305–9.

Durán Quintana MC, García García P, Brenes Balbuena M, Garrido Fernández A. 1994. Induced lactic acid fermentation during the preservation stage of ripe olives from Hojiblanca cultivar. *J Appl Bacteriol* 76:377–82.

Durán Quintana MC, García García P, Garrido Fernández A. 1986. Fermentación en medio aeróbico de aceitunas maduras en salmuera con inyección alternante de aire. *Grasas Aceites* 37:242–6.

Durán Quintana MC, García García P, Garrido Fernández A. 1999. Establishment of conditions for green table olive fermentation at low temperature. *Int J Food Microbiol* 51:133–43.

El-Makhzangy A, Abdel-Rhman A. 1999. Physico-chemical properties of Ázazi' green pickled olives as affected by alkaline process. *Nahrung* 43:320–4.

El-Makhzangy A, Ramadan-Hassenien MF, Suleiman ARM. 2008. Darkening of brined olives by rapid alkaline oxidation. *J Food Process Preserv* 32:586–99.

Fernández Bolaños J, Rodríguez R, Saldaña C, Heredia A, Guillén R, Jiménez A. 2002. Factors affecting the changes in texture of dressed ("aliñadas") olives. *Eur Food Res Technol* 214:237–41.

Fernández Díez MJ, Castro Ramos R, Garrido Fernandez A, González Cancho F, González Pellissó F, Nosti Vega M, Heredia Moreno A, Mínguez Mosquera MI, Rejano Navarro L, Durán Quintana MC, Sánchez Roldán F, García García P, Castro-Gómez Millán A. 1985. Biotecnología de la Aceituna de Mesa. Consejo Superior de Investigaciones Científicas, Instituto de la Grasa, Seville, Spain.

Fernández González MJ, García García P, Garrido Fernández A, Durán Quintana MC. 1993. Microflora of the aerobic preservation of brined green olives from Hojiblanca cultivar. *J Appl Bacteriol* 75:226–33.

Gallardo Guerrero L, Gandul Rojas B, Mínguez Mosquera MI. 2007. Physicochemical conditions modulating the pigment profile in fresh fruits (*Olea eoropaea* Var. *Gordal*) and favouring interaction between oxidized chlorophylls and endogenous Cu. *J Agric Food Chem* 55:1823–31.

Gallardo Guerrero L, Jarén Galán M, Hornero-Méndez D, Mínguez Mosquera MI. 2003b. Evidence of the involvement of lipoxygenase in the oxidative processes associated with the appearance of green staining alteration in the Gordal olive. *J Sci Food Agric* 83:1487–92.

Gallardo Guerrero L, Milicua JC, Salvador AM, Jarén Galán M, Mínguez Mosquera MI. 2003a. Pigment–licoprotein complexes in table olives (cv. Gordal) with green staining alterations. *J Agric Food Chem* 51:1724–7.

García García P, Brenes M, Romero Barranco C, Garrido Fernández A. 2001. Colour fixation in ripe olives: effects of type of iron salt and other processing factors. *J Sci Food Agric* 81:1364–70.

García García P, Brenes Balbuena M, Hornero Méndez D, García Borrego A. 2000. Content in biogenic amines in table olives. *J Food Prot* 63:111–6.

García García P, Durán Quintana MC, Brenes Balbuena M, Garrido Fernández A. 1992. Lactic acid fermentation during the storage of "Aloreña" cultivar untreated green table olives. *J Appl Bacteriol* 73:324–30.

García García P, Durán Quintana MC, Garrido Fernández A. 1985. Fermentación aeróbica de aceitunas maduras en salmuera. *Grasas Aceites* 36:14–20.

García García P, López López A, Garrido Fernández A. 2008. Study of the shelf life of ripe olives using an accelerated test approach. *J Food Eng* 84:569–75.

García García P, Romero Barranco C, Brenes M, Garrido Fernández A. 2002. Validation of a method for the analysis of iron and manganese in table olives by flame atomic absorption spectrometry. *J Agric Food Chem* 50:3654–9.

García García P, Romero Barranco C, Durán Quintana MC, Garrido Fernández A. 2004. Biogenic amine formation and "Zapatera" spoilage of fermented green olives: effect of storage temperature and debittering process. *J Food Prot* 67:117–23.

Garrido Fernández A, Fernández-Díez MJ, Adams RM. 1997. Table olives production and processing. London: Chapman & Hall.

Garrido Fernández A, García García P, Sánchez Roldán F. 1985. Nuevo proceso aeróbico de aceitunas negras al natural. Optimización de la columna de aireación. *Alimentación Equipos Tecnología* 4:73–81.

González Cancho F, Durán Quintana MC. 1981. Bacterias cocaceas del ácido láctico en el aderezo de aceitunas verdes. *Grasas Aceites* 32:373–9.

Hernández A, Martín A, Aranda E, Perez-Nevado F, Córdoba MG. 2007. Identification and characterization of yeasts isolated from the elaboration of seasoned Green table olives. *Food Microbiol* 24:346–51.

Hurtado A, Reguant C, Bordons A, Ròzes N. 2009. Influence of fruit ripeness and salt concentration on the microbial processing of Arbequina table olives. *Food Microbiol* 26:827–33.

Hurtado A, Reguant C, Bordons A, Rozès N. 2010. Evaluation of a single and combined inoculation of a *Lactobacillus pentosus* starter for processing cv. Arbequina natural green olives. *Food Microbiol.* doi:10.016/j.fm.2010.03.006. In press.

Hurtado A, Reguant C, Esteve-Zarzoso B, Bordons A, Ròzes N. 2008. Microbial population dynamics during the processing of Arbequina table olives. *Food Res Int* 41:738–44.

Idrissi IJ, Rahmani M, Souizi AA. 2004. Ensayo de endulzado biológico de las aceitunas de mesa a escala industrial. *Olivae* 101:34–7.

International Olive Oil Council. 2004. Trade standard applying to table olives. COI/NC No. 1. December 2004. Madrid, Spain.

International Olive Oil Council. 2010. Key figures on table olives. November 2010. Madrid, Spain.

Iraqi R, Vermeulen C, Benzekri A, Bouseta A, Collin S. 2005. Screening for key odorants in Moroccan green olives by gas chromatography-olfactometry/aroma extract dilution analysis. *J Agric Food Chem* 53:1179–84.

Jepson D, Moore R, Samimi M, Kashefi C, Bodine M. 1998. Olive processing method. U.S. Patent No. 5,837,304.

Jiménez A, Rodríguez R, Felizón B, Fernández-Caro I, Guillén R, Fernández-Bolaños J, Heredia A. 2000. Cell wall polysaccharides implied in green olive behaviour during the pitting process. *Eur Food Res Technol* 211:181–4.

Kailis S, Harris D. 2007. *Producing table olives*. Collingwood, Australia: Landlink Press.

Kotzekidou P. 1997. Identification of yeasts from black olives in rapid system microtitre plates. *Food Microbiol* 14:609–16.

Kulisic T, Berkovic K, Pavic S, Sustra A. 2005. Preliminary study of physicochemical and microbiological features of brines in the process of preserving the green olives of oblica cultivar. *Acta Alimentaria* 34:499–505.

Kumral A, Basoglu F, Sahin I. 2009. Effect of the use of different lactic starters on the microbiological and physicochemical characteristics of naturally black table olives of Gemlik cultivar. *J Food Process Preserv* 33:651–64.

López A, Garrido A, Montaño A. 2007. Proteins and aminoacids in table olives: relationship to processing and commercial presentations. *Ital J Food Sci* 19:217–28.

López A, Montaño A, García P, Garrido A. 2006. Fatty acid profile of table olives and its multivariate characterization using unsupervised (PCA) and supervised techniques (DA) chemometrics. *J Agric Food Chem* 54:6747–53.

López López A, Jiménez Araujo A, García García P, Garrido Fernández A. 2007. Multivariate analysis for the evaluation of fiber, sugars, and organic acids in commercial presentations of table olives. *J Agric Food Chem* 55:10803–11.

López López A, Montaño A, Garrido Fernández A. 2005a. Evaluation of vitamin E by HPLC in a variety of olive-based foodstuffs. *J Am Oil Chem Soc* 82:129–33.

López López A, Montaño A, Garrido Fernández A. 2005b. Provitamin A carotenoids in table olives according to processing styles, cultivars, and commercial presentations. *Eur Food Res Technol* 221:406–11.

López López A, Montaño A, Ruiz Méndez MV, Garrido Fernández A. 2008. Sterol, fatty alcohols and triterpenic alcohols in commercial table olives. *J Am Oil Chem Soc* 85:253–62.

López López A, Rodríguez Gómez F, Cortés Delgado A, García García P, Garrido Fernández A. 2010. Effect of the previous storage of ripe olives on the oil composition of fruits. *J Am Oil Chem Soc* 87:705–14.

López López A, Rodríguez Gómez F, Cortés Delgado A, Montaño A, Garrido Fernández A. 2009. Influence of the ripe table olive processing on oil characteristics and composition as determined by chemometrics. *J Agric Food Chem* 57:8973–81.

Mafra I, Barros SA, Coimbra MA. 2007. The combined effects of black oxidising table olive process and ripening on the cell wall polysaccharides of olive pulp. *Carbohydr Polym* 68:647–57.

Marsilio V, Campestre C, Lanza B. 2001. Phenolic compounds change during California-style ripe olive processing. *Food Chem* 74:55–60.

Marsilio V, Lanza B, Campestre C, de Angelis M. 2000. Oven-dried table olives: textural properties as related to pectic composition. *J Sci Food Agric* 80:1271–6.

Marsilio V, Lanza B, de Angelis M. 1996. Olive cell wall components: physical and biochemical changes during processing. *J Sci Food Agric* 70:35–43.

Mokhbi A, Kaid-Harche M, Lamri K, Rezki M, Kacem M. 2009. Selection of *Lactobacillus plantarum* strains for their use as starter cultures in Algerian olive fermentations. *Grasas Aceites* 60:82–8.

Montaño A, Sánchez AH, Casado FJ, de Castro A, Rejano L. 2003. Chemical profile of industrially fermented green olives of different varieties. *Food Chem* 82:297–302.

Montaño A, Sánchez AH, de Castro A. 2000. Changes in the amino acid composition of green olive brine due to fermentation by pure culture of bacteria. *J Food Sci* 65:1022–7.

Mourat K, Haklima ZK, Nour-Eddine K. 2004. Isolation of lactic acid bacteria for its possible use in the fermentation of green Algerian olives. *Grasas Aceites* 55:385–93.

Nisiotou AA, Choranopoulos N, Nychas G.JE, Panagou EZ. 2010. Yeast heterogeneity during spontaneous fermentation of black Conservolea olives in different brine solutions. *J Appl Microbiol* 108:396–405.

Nychas GJE, Panagou EZ, Parker ML, Waldron KW, Tassou CC. 2002. Microbial colonization of naturally black olives during fermentation and associated biochemical activities. *Lett Appl Microbiol* 34:173–7.

Panagou EZ. 2004. Effect of different packing treatments on the microbiological and physicochemical characteristics of untreated green olives of the Conservolea cultivar. *J Sci Food Agric* 84:757–64.

Panagou EZ. 2006. Greek dry-salted olives: monitoring the dry-salting process and the subsequent physicochemical and microbiological profile during storage under different packing conditions at 4° and 20°C. *LWT Food Sci Technol* 39:322–9.

Panagou EZ, Tassou CC. 2006. Changes in volatile compounds and related biochemical profile during controlled fermentation of cv. Conservolea green olives. *Food Microbiol* 23:738–46.

Panagou EZ, Tassou CC, Katsabouxakis KZ. 2002. Microbiological, physicochemical and organoleptic changes in dry-salted olives of Thassos variety stored under different modified atmospheres at 4 and 20°C. *Int J Food Sci Technol* 37:635–41.

Panagou EZ, Tassou CC, Katsabouxakis C. 2003. Induced lactic acid fermentation of untreated green olives of the Conservolea cultivar by *Lactobacillus pentosus*. *J Sci Food Agric* 83:667–74.

Pereira JA, Pereira APG, Ferreira ICFR, Valentão A, Andrade PB, Seabra R, Estevinho ML, Bento A. 2006. Table olives from Portugal: phenolic compounds, antioxidant potential and antimicrobial activity. *J Agric Food Chem* 54:8425–31.

Pereira AP, Pereira JA, Bento A, Estevinho ML. 2008. Microbiological characterization of tables olives commercialized in Portugal with respect to safety aspects. *Food Chem Toxicol* 46:2895–902.

Peres C, Serrano C, Brito D, Damaso A, Delgado A. 2008. Bacteriocin diversity among LAB from table olives. *Acta Hortic* 791:685–91.

Piga A, Agabbio M. 2003. Quality improvement of naturally green table olives by controlling some processing parameters. *Ital J Food Sci* 15:259–68.

Psani M, Kotzekidou P. 2006. Technological characteristics of yeasts strains and their potential as starter adjuncts in Greek-style black olive fermentation. *World J Microbiol Biotecnol* 22:1329–39.

Randazzo CL, Restuccia C, Romano AD, Caggia C. 2004. *Lactobacillus casei*, dominant species in naturally fermented Sicilian green olives. *Int J Food Microbiol* 90:9–14.

Rejano Navarro L, Higinio Sánchez AH, Vega Macías V. 2008. Nuevas tendencias en el tratamiento alcalino "cocido" de las aceitunas verdes aderezadas al estilo español o sevillano. *Grasas Aceites* 59: 197–204.

Rodríguez Gómez F, Arroyo López FN, López López A, Bautista Gallego J, Garrido Fernández A. 2010. Lipolytic activity of the yeast species associated with the fermentation/storage phase of ripe olive processing. *Food Microbiol* 27:604–12.

Romeo FV, De Luca S, Piscopo A, Perri E, Poiana M. 2009. Effect of post-fermentation processing on the stabilization of naturally fermented green olives (cv. *Nocellara etnea*). *Food Chem* 116:873–8.

Romero C, Brenes M, García P, Garrido A. 1998a. Effect of amino acids on the chemical oxidation of olive *o*-diphenols in model systems. *Food Chem* 63:319–24.

Romero C, Brenes M, García P, García A, Garrido A. 2004. Polyphenol changes during fermentation of naturally black olives. *J Agric Food Chem* 52:1973–9.

Romero C, García P, Brenes M, Garrido A. 1998b. Colour improvement in ripe olive processing by manganese cations: industrial performance. *J Food Eng* 48, 75–81.

Romero C, García P, Brenes M, Garrido A. 1998c. Use of manganese in ripe olive processing. *Z Lebensm Unters Forsch A* 206:297–302.

Romero C, García P, Brenes M, García A, Garrido A. 2002. Phenolic compounds in natural black Spanish olive varieties. *Eur Food Res Technol* 215:489–96.

Sánchez AH, de Castro A, Rejano L, Montaño A. 2000. Comparative study on chemical changes in olive juice and brine during green olive fermentation. *J Agric Food Chem* 48:5975–80.

Sánchez AH, Montaño A, Rejano L. 1997. Effect of preservation treatment, light, and storage time on quality parameters of Spanish-style green olives. *J Agric Food Chem* 45:3881–6.

Sánchez AH, Rejano L, Montaño A, de Castro A. 2001. Utilization of a high pH starter cultures of lactobacilli for Spanish-style green olive fermentation. *Int J Food Microbiol* 67:115–22.

Sánchez-Romero C, Guillén R, Heredia A, Jiménez A, Fernández-Bolaños J. 1998a. Degradation of pectic polysaccharides in pickled green olives. *J Food Prot* 61:78–86.

Sánchez-Romero C, Guillén R, Heredia A, Jiménez A, Fernández-Bolaños J. 1998b. Degradation of hemicellulosic and cellulosic polysaccharides in pickled green olives. *J Food Prot* 61:87–93.

Segovia Bravo KA, Arroyo López FN, García García P, Durán Quintana MC, Garrido Fernández A. 2007a. Reuse of ozonated alkaline solutions as fermentation brines in Spanish green table olive. *J Food Sci* 72:M126–M133.

Segovia Bravo KA, Arroyo López FN, García García P, Durán Quintana MC, Garrido Fernández A. 2007b. Treatment of green table olive solutions with ozone: effect on their polyphenol content and on the *Lactobacillus plantarum* and *Saccharomyces cerevisiae* growth. *Int J Food Microbiol* 114:60–8.

Servilli M, Minnocci A, Veneziani G, Tatichii A, Urbani S, Esposito S, Sebastiani L, Valmorri S, Corsetti A. 2008. Compositional and tissue modifications induced by the natural fermentation process in table olives. *J Agric Food Chem* 56:6389–96.

Servilli M, Settani L, Veneziani G, Esposto S, Massitti O, Tatichi A, Urbani S, Montedoro GF, Corsetti A. 2006. The use of *Lactobacillus pentosus* 1MO to shorten the debittering process time of black table olives (Cv. Itrana and Leccino): a pilot-scale application. *J Agric Food Chem* 54:3869–75.

Spyropoulou KE, Chorianopoulos NG, Skandamis PN, Nychas GJE. 2001. Survival of *Escherichia coli* O157-H7 during the fermentation of Spanish-style green olives (Conservolea variety) supplemented with different carbon sources. *Int J Food Microbiol* 66:3–11.

Tassou CC, Panagou EZ, Katsaboxakis KZ. 2002. Microbiological and physicochemical changes of naturally black olives fermented at different temperatures and NaCl levels in brine. *Food Microbiol* 19:605–15.

Tsapatsaris S, Kotzekidou P. 2004. Application of a central composite design and response surface methodology to the fermentation of olive juice by *Lactobacillus plantarum* and *Debaryomyces hansenii*. *Int J Food Microbiol* 95:157–68.

Turantas F, Göksungur Y, Dinçer AH, Ünlütürk A, Güvenç U, Zorlu N. 1999. Effect of potassium sorbate and sodium benzoate on microbial population and fermentation of black olives. *J Sci Food Agric* 79:1197–202.

Vaughn RH. 1982. The fermentation of olives. In: Reed G, editor. *Industrial microbiology*. 4th ed. Westport, CT: AVI Publishing Co. p. 207–39.

Vaughn RH, Martin MH, Stevenson KE, Johnson MG, Crampton VM. 1969. Salt-free storage of olives and other produce for future processing. *Food Technol* 23:316–20.

Vega Leal-Sánchez M, Ruiz Barba JL, Sánchez AH, Rejano L, Jiménez Díaz R, Garrido A. 2003. Fermentation profile and optimization of green olive fermentation using *Lactobacillus plantarum* LPCO10 as a starter culture. *Food Microbiol* 20:421–30.

Zimbalatti G, Giametta F. 2002. Selection and calibration of eating olives: technical and economic aspects. *Acta Hortic* 586:679–82.

Zion B, Kim SM, McCarthy MJ, Chen P. 1997. Detection of pits in olives under motion by nuclear magnetic resonance. *J Sci Food Agric* 75:496–502.

Zoidou E, Mellidou E, Gikas E, Tsarbopoulos A, Magiatis P, Skaltsounis AL. 2010. Identification of Throuba Thassos, a traditional Green table olive variety, as a nutritional reach source of oleuropein. *J Agric Food Chem* 58:46–50.

17

Noni Fruits

Scot Nelson

CONTENTS

17.1 Introduction

Morinda species comprise a useful and widely distributed genus of tropical trees, shrubs, and vines. Most of the approximately 80 species originated from Borneo, New Guinea, Northern Australia, and New Caledonia. At least 20 species, including *Morinda citrifolia* L. (Rubiacea), have significant modern economic and historical values in the Pacific Islands and in Asia. The buoyant-seeded species such as *M. citrifolia* became essential components of many tropical coastal and forest ecosystems, where they served important functions as medicines, foods, dyes, or wood for several ancient, indigenous societies (Nelson and Elevitch 2006).

Botanists recognize three distinct varieties of *M. citrifolia* (McClatchey 2003):

- *M. citrifolia* var. *citrifolia* (the commercial noni)
- *M. citrifolia* var. *bracteata* (has conspicuous bracts subtending the fruits)
- *M. citrifolia* cv. "Potteri" (has green and white colored leaf variegation)

M. citrifolia var. *citrifolia* is the most important variety in the species, having the widest geographic distribution and most significant economic, cultural, and medicinal values. Noni is a small, evergreen tree or shrub reaching a height of 3 to 10 m and a mature stem diameter of 15 cm or more. The global population of noni varies significantly for plant form, fruit and leaf sizes and shapes, palatability, ripe fruit odor, color, and seed numbers. *M. citrifolia* var. *citrifolia* is morphologically diverse but has no clear subpopulations bearing unique characteristics (McClatchey 2003). Noni has undergone no plant breeding or significant selection for crop improvement by humans. However, the large-fruited type found in Hawaii produces much more juice and has higher sugar and fruit yields than the more bitter tasting, small-fruited, narrow-leafed types, which predominate in some Pacific regions such as Samoa (Newton 2003), the Federated States of Micronesia, and the Northern Marianas islands (Nelson, unpublished).

Today, naturalized and cultivated noni plants grow throughout the global tropics and subtropics. Noni is naturalized principally as a coastal plant but can grow inland at elevations up to 800 m (Nelson 2006). Noni plants tolerate high salinity, often growing in brackish tide pools or on ocean

beaches. Noni tolerates and often thrives in drought-prone areas and in marginal soils. It is often one of the first dicotyledonous species in the succession of plants that colonize newly deposited lava fields in Hawaii (Nelson 2006). The noni plant produces syncarps throughout the year, from which widely used products such as fresh and fermented juice or powders are manufactured and marketed in the United States as dietary supplements or as novel foods in the European Union (Potterat and Hamburger 2007).

17.2 Traditional Uses of Noni

The history of the geographic dissemination and the use of noni throughout the Pacific Islands began approximately 3600 years ago as Polynesian voyagers sequentially colonized new islands. Noni was carried by these explorers to the Hawaiian Islands as a "canoe plant" around AD 400, where inhabitants used the fruits as a famine food and in medicines (Nelson and Elevitch 2006).

Noni was an important traditional component of folk medicines throughout the Pacific Islands for more than 2000 years, treating a broad range of indications (Dixon et al. 1999; McClatchey 2002; Nelson 2006). Many of the ancient uses of the plant persist today in Pacific Island cultures. All parts of the plant had important social uses and medicinal applications. Noni fruits or fruit juices were widely used in common practices by ancient cultures in the Pacific Ocean. More frequently and over a broader geographic area, Pacific islanders applied noni plant organs and/or their extracts topically to the human body to relieve symptoms of various diseases, conditions, or maladies (Table 17.1). Although in modern times people refer to the "traditional" method of noni juice extraction (e.g., drip extracted and fermented in a closed container), such a tradition does not derive directly from a common practice in ancient cultures but is more accurately characterized as a modern phenomenon.

TABLE 17.1

Some Traditional Uses of Noni (*M. citrifolia* var. *citrifolia*) as a Medicinal Plant among Pacific Islands Societies

Plant Part	Preparation	Use
Leaf	Fresh	Topical burns, headaches, fever, ghost medicine
	Tea	Malaria, fever, pain
	Poultice	Tuberculosis, sprains, deep bruising, rheumatism, sciatica, fever, stings from stonefish, bone fractures, dislocations
	Extract	Hypertension, bleeding caused by a bone puncture, stomach ache, fractures, diabetes, loss of appetite, urinary tract ailments, abdominal swelling, hernias, vitamin A deficiency
	Vapor of broken leaves	Sties
Fruit	Unripe	Sores or scabs around the mouth, ghost medicine
	Ripe	Sore throat gargle (mashed), peeling or cracking of toes and feet (crushed), body or intestinal worms, cuts, wounds, abscesses, mouth and gum infections, toothaches, appetite and brain stimulant
	Poultice	Boils, carbuncles, tuberculosis, sprains, deep bruising, rheumatism
	Oil	Stomach ulcers
	Extract	Hypertension
Stem	Decoction of bark	Jaundice
	Wood or bark extract	Hypertension
Seed	Oil	Scalp insecticide
Flowers		Sties
Roots	Juice	Badly infected cut
All parts		Mild laxative

Sources: Nelson, S. C., and C. R. Elevitch, *Noni: The Complete Guide for Consumers and Growers,* Permanent Agriculture Resources, Holualoa, Hawaii, 2006. With permission. McClatchey, W., *Integr Cancer Ther,* 1, 110–120, 2002. With permission."

17.3 Modern Uses of Noni and Compositional Analysis

The modern consumption of fermented noni juice as a medicine developed in the 1990s because of modern advertising claims, marketing programs, and scientific research, which piqued interest in properties and potential of noni as a healing agent. During the late 1990s, manufactured noni juice (both fresh and fermented), fruit powders, and a variety of other products derived from noni fruits (foods and cosmetics) became popular among consumers in North America, Europe, and Asia. Noni juice marketing began in the United States as a dietary supplement on July 1, 1996, as well as in Canada, Japan, Australia, Mexico, Norway, and Hong Kong (McClatchey 2002). Noni juice gained approval by the European Commission as a novel food in Europe in 2003 (European Commission 2003).

Noni juice and juice products are readily available internationally in health food stores, grocery markets, via Internet retailers, and through pyramid or multilevel marketing enterprises as freshly squeezed or fermented products or as beverages derived from noni fruit juice concentrates. Noni juice products are marketed either as 100% pure noni juice or as juice or beverage mixtures consisting of noni juice and other fruit juices or flavors. Some noni juice products also contain added preservatives, sugar, and colors, and some are marketed as organically grown.

The initial popularity and marketing success of noni was based on various unsubstantiated claims made about noni products and their effects on human health. For example, noni was marketed as an immune system stimulant, an anticancer agent, a menstrual cycle regulator, or a blood cleanser (Yang et al. 2007b). Some medical doctors in Hawaii prescribe noni for use by patients in cancer therapies and for other indications (Nelson, unpublished).

Noni is popularly credited with the ability to treat an impressive array of human health problems. A recent review of the ethnobotanical literature for traditional medicines, the modern science-based literature, and some popular press articles indicates that at least 36 indications are purportedly treatable by noni, including arthrosis, ascaris, asthma, cancer, colds, colic, constipation, cramps, diabetes, diarrhea, dysentery, dysuria, fevers, fungus, gallstone, gout, headaches, heart problems, hepatosis, hypertension, infections, insomnia, leukorrhea, mycosis, nervousness, neuralgia, pain, rheumatism, sapremia, sores, sore throats, stomach aches, stones, tumors, ulcers, and wounds (Nelson and Elevitch 2006). In reality, much more scientific research and clinical studies are needed to fully substantiate these health claims.

Biochemical research indicates that the noni plant contains several interesting compounds, which can probably provide benefits to human health. The physiologically useful and biologically active compounds isolated from noni fruits and leaves include iridoids, alkaloids, complex polysaccharides, glycosides, scopoletin, sterols, lignans, anthraquinones, trisaccharide fatty acid esters, triterpenoids, and vitamins and minerals (Table 17.2).

Laboratory experiments, both *in vivo* and *in vitro*, confirm that noni fruit juice, extracts, or its isolated biological compounds can scavenge free radicals, provide anticarcinoma and antimutagenic activities, inhibit low-density lipoprotein oxidation, serve as an anticlastogenic, purify the blood, regulate cell function and cholesterol, stimulate the immune system, act as a neuroprotective in Alzheimer's dementia, facilitate insulin secretion, provide antigenotoxic effects, provide antifungal effect, protect the liver, inhibit elastase and tyrosinase, provide 1,1-diphenyl-2-picrylhydrazyl radical scavenging activity, inhibit xanthine oxidase, and provide analgesic and anti-inflammatory activities (Basar et al. 2010; Furusawa et al. 2003; Harada et al. 2010; Hirazumi and Furusawa 1999; Hornick et al. 2003; Jainkittivong and Langlais 2009; Kamiya et al. 2004; Muralidharan et al. 2010; Palu et al. 2009; Ratanavalachai et al. 2008; Saludes et al. 2002; Taşkin et al. 2009; Wang et al. 2002, 2008; Yamaguchi et al. 2002; Zin et al. 2006).

During the past 10 years, basic research has accelerated in this area as a natural precursor to the clinical trials with humans that will be needed to establish scientifically that noni can be useful within human health programs. Today, recommendations are relatively conservative for the intake of noni juice (30 mL/day), in part because science is not fully aware of the applications for or consequences of ingesting the material. Noni juice is generally recognized as safe. However, Noni does not appear on the U.S. Food and Drug Administration's generally recognized as safe list because it has never been considered unsafe. The Food and Drug Administration's Center for Food Safety and Applied Nutrition

TABLE 17.2

Some Important Chemical Constituents of Noni (*M. citrifolia* var. *citrifolia*) Juice and Their Potential Health Applications

Chemical Group or Molecule	Activities, Diseases, or Applications
Anthraquinones • Damnacanthal • Morenone 1 and 2 • Several others	Antibacterial, antiviral, type I collagen synthesis, cholesterol reduction, triglyceride reduction, antitumor, analgesic, sedative
Glycosides and glucosides (e.g., flavonol glycosides, iridoid glycosides, lipid glycosides) • Asperuloside • Citrifolinin A and B • Rutin	Anticancer, antitumor
Lignans/neolignans • Americanin A • Morindolin, others	Antioxidant, arteriosclerosis
Polysaccharides	Immunomodulatory, anticancer, antitumor
Sterols • β-Sitosterol	Steroid hormone production
Scopoletin	Hypertensive, antibacterial, antifungal, anti-inflammatory, analgesic, histamine inhibiting, arthritis, allergies, sleep disorders, migraine headaches, depression, Alzheimer's disease
Triterpenoids • Ursolic acid	Antitumor (skin cancer), hepatoprotective, anti-inflammatory (oral and topical), antiulcer, antimicrobial, antihyperlipidemic, antiviral

Sources: Nelson, S. C., and C. R. Elevitch, *Noni: The Complete Guide for Consumers and Growers*, Permanent Agriculture Resources, Holualoa, Hawaii, 2006. With permission. Potterat, P., and M. Hamburger, *Planta Med*, 73, 191–199, 2007. With permission.

focuses on products where a decision is needed and focuses primarily on food additives, food colors, and similarly synthetic products rather than on naturally growing fruits and vegetables. In addition, the U.S. War Department's Technical Manual, *Emergency Food Plants and Poisonous Plants of the Islands of the Pacific* (Merrill 1943), listed *M. citrifolia* as an edible survival plant for soldiers in the Pacific Islands.

Compositional analyses for fermented noni juices from the Pacific Islands are presented (Table 17.3). The data indicate variability for nutritional components between juices. It is not clear whether these differences are nutritionally significant, derived from differences in noni plant types (i.e., as natural variation within the *M. citrifolia* var. *citrifolia*), the result of the type of fermentation (bacterial species or microbes involved, fermentation environment, or equipment), or the extent or duration of the fermentation (note the pH difference between juices). Research is needed to determine the effects of these factors on the results of compositional analyses.

17.4 Manufacture of Noni Juice Products

Table 17.4 and Figure 17.1 show the actual standard operating procedures and a flow chart for noni juice processing by selected manufacturers of noni juice in the Pacific Islands. The noni juice from some processors in the Pacific Islands is certified by the Hazard Analysis and Critical Control Points. Most processors apply hygienic rules during juice processing, starting by cleaning the noni fruit and discarding all bruised or rotting fruit, steam cleaning vessels and utensils, pasteurizing before bottling, and checking each pasteurized batch for bacteria, yeast and mold. If there is contamination, then the batch has to be reprocessed. Records are kept throughout the process for review if there are problems. The key points

TABLE 17.3

Compositional Profile (per 100 mL) of 100% Pure Noni (*M. citrifolia* var. *citrifolia*) Juice from Selected Manufacturers in the Pacific Islands

Aspect	Hawaiian	Pacific Island Noni Association
Dry matter	7.6 g	
Water	94.8 g	96.7 g
Fat	<0.1 g	
Total protein	0.5 g	0.5 g
Ash	0.4 g	0.8 g
Total dietary fiber	0.6 g	0.1 g
Sucrose	1.3 g	<0.1 g
Glucose	1.5 g	1.7 g
Fructose	1.5 g	1.6 g
Carbohydrates	6.0 g	4.2 g
Calories (energy)	27 (113 kJ)	19.6 (82 kJ)
Sodium (Na)	9 mg	49 mg
Potassium (K)	150 mg	98 mg
Calcium (Ca)	6 mg	3.9 mg
Magnesium (Mg)	11 mg	20 mg
Iron (Fe)	0.4 mg	0.6 mg
Phosphorous (P)	10 mg	2.0 mg
Chloride (Cl)/Salt (as NaCl)	62 mg	0.2 g
Molybdenum		<0.1 mg/L
Vitamin B_1	0.0006 mg	
Vitamin B_2	0.035 mg	
Vitamin B_6	<0.05 mg	
Vitamin B_{12}	70 µg	
Pantothenic acid	0.169 mg	
Niacin	0.194 mg	
Biotin	4.07 µg	
Folic acid	11.4 µg	
Ascorbic acid (vitamin C)	53.2 mg	20 mg
Vitamin E (total)	0.05 mg	
Beta-carotene	<0.0005 mg	
Total carotene	0.0035 mg	<0.1 mg/L
Acidity	pH 3.43	pH 3.83
Amino acids (mg)		Aspartic acid (12), threonine (6), serine (9), glutamic acid (9), asparagine (0), proline (3), glycine (5), alanine (21), cysteine (2), valine (6), methionine (3), isoleucine (3), leucine (9), tyrosine (7), phenylalanine (7), GABA (2), histidine (9), lysine (7), arginine (11), tryptophan (0)
Alcohols		0.3 (ethanol, % v/v), 0.2 (methanol, % v/v)
Specific gravity		1.025

Sources: Nelson, S. C., and C. R. Elevitch, *Noni: The Complete Guide for Consumers and Growers*, Permanent Agriculture Resources, Holualoa, Hawaii; 2006. With permission. CAMedica Ltd., *Request for an Opinion on the Equivalence of Noni Juice (Juice from the Fruit of Morinda citrifolia L.)*, Brook House, Tarrington, Herefordshire, UK, 2004. With permission.

TABLE 17.4

Standard Operating and Manufacturing Procedures for Manufacture of Fermented Noni Juice

Harvesting	Ripe and mature fruits are used. Mature fruits are identified in terms of color—greenish-yellow to white. Ripe fruit is acceptable, but overripe fruit is not; that is, the fruit should be firm and able to be handled without it falling apart. Green, bruised, and damaged fruit must be rejected because they are susceptible to the development of molds producing mycotoxins such as patulins. Green fruit produces a lower Brix measurement—approximately 4% or less. Fruit that has fallen to the ground must not be collected to avoid mold problems. Fruit is collected in sterile or clean containers (see sanitizing program).
Sorting	At the factory collection point, further sorting of fruit is conducted. The following type of fruit must be rejected: green, bruised, and damaged fruit. Also excluded are any foreign matter such as stones, sticks, stem, leaves, bark, and plant root material.
Washing	The fruit is washed thoroughly with clean, running water (not recycled water) and left to air-dry.
Processing	Transfer fruit to clean containers for processing (see step 2, sanitizing program). These containers must be food-grade plastic or stainless steel. Fruit can be whole or pulped (hammer mill machine is commonly used for pulping the fruit). Seal the containers. The container must be sealed to prevent contamination. It is important to ensure the seal is tamperproof. Leave to mature for a minimum of 10 days to a maximum of 60 days.
Juice extraction	Various types of presses can be used to extract the juice. The matured fruit is transferred to fruit pressing equipment. The resultant juice is transferred from the press to storage containers of food-grade plastic or stainless steel and sealed.
Filtration	Bulk juice is filtered to exclude pulp, seed, and foreign particles.
Pasteurizing	The juice is pasteurized at 87.5°C for a minimum of 3 seconds.
Labeling	The minimum information required is botanical name, common name, batch number, manufacture date, and manufacturer's details. For retail labels, ensure compliance with the regulations in the country of destination.
Sanitizing program	(1) Fruit is washed with clean running water. Visual inspection is conducted to ensure that all foreign particles are removed from the fruit. (2) Maturing containers—clean with fresh running water, scrub with water and food-grade approved detergent solution, rinse with clean running water to remove all traces of detergent, spray with food-grade approved sanitizer, and leave to air-dry. The drums are visually inspected for cleanliness. (3) Fruit press—clean with high-pressure fresh running water to remove all traces of fruit particles at the end of each day and apply sanitizer. At the start of each day, rinse off the sanitizer and thoroughly wash the machine. (4) Accessory equipment (hammer mill, pumps, etc.): at completion of the work cycle, thoroughly rinse the equipment with fresh running water to remove all traces of fruit particles, using detergent if necessary, rinse with water to remove traces of detergent, and then spray with sanitizing solution, leave to air-dry, and rinse with water before reusing. The accessory equipment is visually inspected for cleanliness. (5) Approved plastic or stainless steel drums: containers for the pressed juice are treated in the same way as the maturing containers. (6) Bottles: only new bottles are used; visual inspection for cleanliness occurs before filling. If any contamination is found, then appropriate cleaning process is implemented. On receipt of empty bottles into factory, bottles are stored in a clean environment. (7) Bottling of juice: all equipment used in bottling of juice is thoroughly cleaned, sanitized, and dried before and after use. After filling, drums and bottles should be inspected for any signs of spillage and, if necessary, sanitized.
Personal hygiene	Footwear, gloves, and hairnets are to be supplied to all workers. Factory workers must be trained to thoroughly wash their hands any time they return to the production area.
Sorting tables/mesh	Cleaned with fresh running water before and after use.
Factory	Good housekeeping implemented at all times.
Staff training	Staff training is carried out in all the previously mentioned operations. Staff training in food handling and food safety is carried out on an ongoing basis.
Records	A written recording system is used for all of the factory operations.

(*continued*)

TABLE 17.4 (Continued)

Standard Operating and Manufacturing Procedures for Manufacture of Fermented Noni Juice

Compositional standards	Appearance: reddish-brown to dark brown nonviscous liquid with minimal sediment. Odor: sharp, pungent. Taste: strong piquant, slightly bitter. Pasteurization: 87.5°C for a minimum of 3 seconds. Brix: 7%–14%, pH 3.55–4. Acidity: 0.095%–0.135%. Ethanol: 0.3% v/v. Microbiology: aerobic total viable count (colony-forming units/g or mL), 10^2 (100) per mL/g. Enterobacteriaceae: 10^2 (100) per mL/g. *Escherichia coli*: 0 per mL/g. *Salmonella*: 0 per mL/g. Yeast: 10^2 (100) per mL/g. Mold: 10 per mL/g. Other substances that may be called on for testing: pesticide residues, levels and methods as specified in BP 2000 (or latest); patulins (mycotoxins derived from mold), 0.25 µg/L; anthraquinones, 2 mg/100 mL; isoflavones, 1 mg/100 mL.

Source: Nelson, S. C., and C. R. Elevitch, *Noni Processing, Marketing, and Field Training Workshop for Yap*, Permanent Agriculture Resources, Holualoa, Hawaii, 2007. With permission.

Note: Standard operating and manufacturing procedures for the production of fermented (aged) noni juice vary geographically and for the intended markets. Some Pacific Island processors use the described procedures for the production of 100% pure noni juice that is processed using the traditional method of maturing (fermenting) the fruit and juice. These procedures and their derived products were approved by the U.K. Food Standard Agency for noni juice sold in the European Union as a novel food.

in the juice processing (e.g., sanitizing program, pasteurization) are related to food and consumer safety issues.

Today, the fermentation of noni juice is not controlled by processors for the microbes involved in the fermentation. These organisms remain unidentified normally and occur at random. Also, key variables in the environment that are important to the fermentation of noni juice (e.g., temperature, light) are not managed with precision or consistency by processors of noni juice. Therefore, the production of fermented noni juice is not sophisticated in relation to similar fermented products (e.g., vinegar, wine, beer, yogurt, etc.) in which fermentation is strictly managed.

Farmers harvest noni fruits from trees by hand at the "hard white" stage of fruit phenology. Such fruits have no photosynthetic tissues, which, if used for juice production, produce a more darkly colored, astringent, and bitter-tasting juice product. Harvested fruits are washed with fresh water and allowed to soften for several days, whereupon the juice begins to drip or exude from them gradually for a period of one to several weeks.

Freshly squeezed noni juice products are lighter in color (amber) than fermented products (dark brown), are sweeter, and have a pH level of approximately 5.5. The freshly squeezed products derived from ripe, soft fruits are first frozen and then thawed before pressing and filtering. Freezing the fruits breaks the cell walls to allow a higher juice yield compared with fruits left unfrozen and then squeezed. These freshly squeezed products must be pasteurized and refrigerated after opening to prevent the fermentation of the juice by microbes in the environment.

Fermented noni juice products are generally sour (having a pH level of approximately 3.5 or less). The fermented products are derived from ripe fruits, which soften and undergo aging in closed vessels for 2 to 3 months and fermentation by acidifying bacteria. During the aging process, the juice emerges from the fruit pulp naturally over time and is fermented by naturally occurring microbes (bacteria, yeasts, or fungi) that are ambient in the crop cultivation or fruit processing environments. These microbes become associated with the fruits as airborne particles, by contact with equipment or tables, and by contact with human hands or animals such as insects.

Some manufacturers produce "juice" products from noni fruit puree. However, to be accurate, these are not really fruit juices but rather beverages derived from macerated fruit pulp. Other companies produce noni juice beverages derived from noni concentrates that are manufactured from the raw juice by vacuum boiling and evaporative procedures.

Manufacturing protocols for noni juice products vary globally. For products sold in Europe, prescribed safe handling and sanitary practices during manufacture, such as for food hygiene, processing facilities, equipment, packaging, presentation, customer service, labeling, and advertising, must be followed. Many juice products produced in the Pacific Islands undergo no such strict handling practices. Labeling of products varies globally.

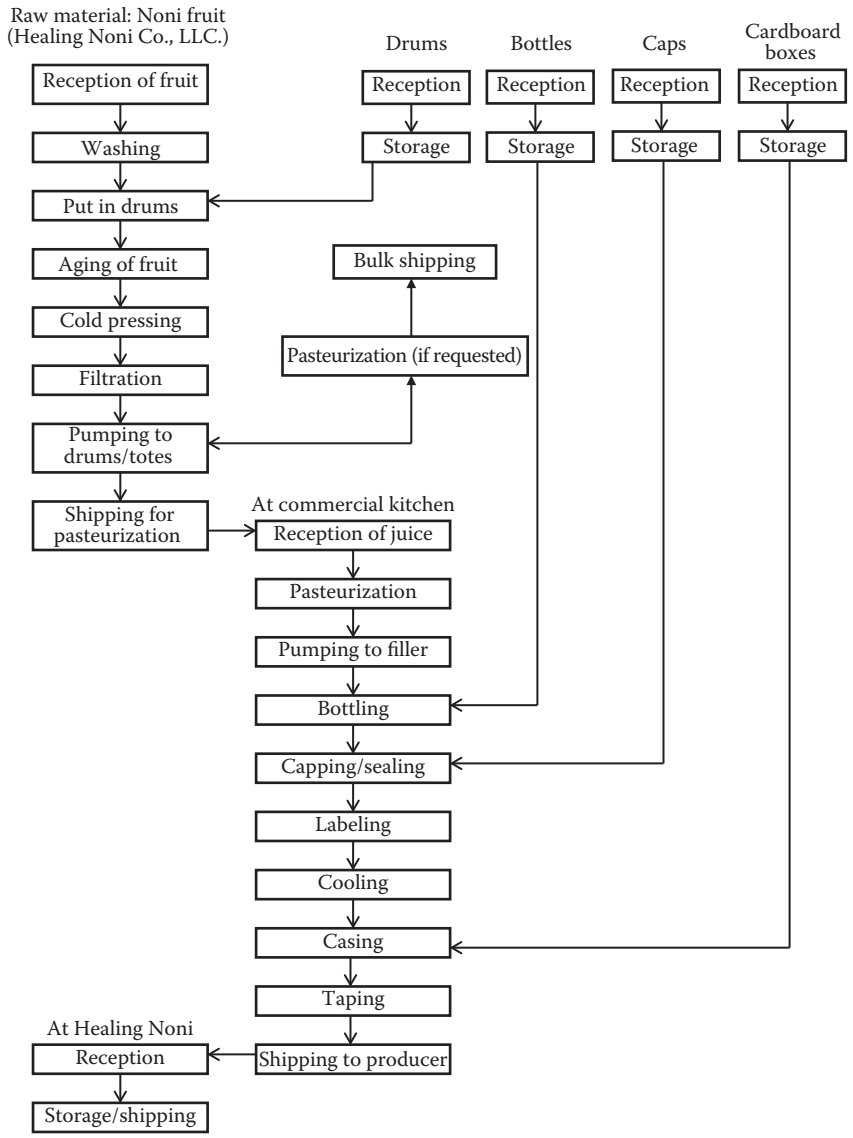

FIGURE 17.1 A flow chart for noni juice processing in Hawaii.

17.5 Quality Evaluation of Fermented Noni Juice

A common question raised by consumers is which is healthier to consume, fresh or fermented noni juice? This question could be answered in part by an analysis of biologically active compounds in the respective products. For example, the consumption of fresh frozen noni juice rather than fermented noni juice was recommended by Yang et al. (2007b) because of the superior antioxidant properties of freshly squeezed noni juice products. In their experiments, the fermentation of noni juice for 3 months caused a 90% loss of free radical scavenging activity.

What effects does fermentation actually have on the qualities and chemistry of noni juice? First, manufacturers of fermented noni juice do not pitch any fermenting microbes into the fermentation vessels. As a result, the process of fermentation is competitive, random, and uncontrolled. This results in

significant variability among juice batches and among geographic regions for color and organoleptic quality parameters such as flavors and microbial content. The between- and among-batch variability and the lack of quality controls for fermented juice could affect both the chemical content and the medicinal attributes of noni juice.

Various acidifying bacterial species and fungal species (molds such as *Rhizopus*, yeasts) can ferment or contaminate noni juice (Nelson, unpublished). However, little research and few scientific publications identify or specify the microbial species normally associated with or responsible for the fermentation of noni juice. For industrial applications, bacterial fermentation provides alcohol-free products wherein the natural sugars in noni juice are converted to organic acids such as acetic acid or lactic acid. Fermentation by bacteria can be confirmed by checking the pH of fermented juice. The pH level of fully bacteria-fermented noni juice decreases from approximately 5.5 (fresh juice) to approximately 3.5 (fully aged or fermented juice; Nelson and Elevitch 2007), whereas the pH level of noni juice fermented by yeasts does not change (Nelson, unpublished). Where fungi such as yeasts contaminate noni fermentation vessels, alcohol can be produced, which is undesirable for many markets (e.g., Japan). Also, some fungi capable of contaminating noni juice are potentially toxic, allergenic, or pose human health hazards. Therefore, fungi (and air, which bears fungal spores) should be excluded from noni juice fermentation vessels in favor of the acidifying bacterial species.

The question of the effect of industrial fermentation on noni juice should focus on fermentation by bacterial species and the effects on the compositional properties and organoleptic attributes of noni juice. During fermentation, temperature, oxygen, and microorganisms can cause undesirable chemical reactions, which may reduce the quality of fermented products. However, little is known about changes that occur in the microbiological, physiochemical, and functional properties of noni juice during fermentation by bacteria.

Chan-Blanco et al. (2007) found that initially populations of molds, yeasts, and mesophyllic bacteria increased exponentially in noni juice undergoing fermentation. After 2 weeks, microbial growth changed abruptly. The growth of yeasts, molds, and mesophyllic bacteria stabilized, whereas the growth of lactic acid bacteria decreased abruptly and became undetectable after 5 weeks. All phenolic compounds, including scopoletin and rutin, varied significantly immediately after harvest but remained more or less steady during aging. Vitamin C and total phenols content remained constant. Their results indicated that the "traditional" processing treatment of fermentation did not appear to affect the main composition of noni fruits because significant changes occurred mostly during ripening and imperceptibly during aging. The microbiological activity did not significantly affect the content of phenolic compounds and vitamin C or the oxygen radical absorbance capacity. However, further work is needed to determine the effects of various bacterial species on medicinally important chemistry in the noni fruit juice.

There is inconsistency among studies for the ability to recover important biologically active chemistry from noni fruits. For example, Chan-Blanco et al. (2007) could not detect aucubin and alizarin, whereas Wang et al. (2009) and McKoy et al. (2002) detected aucubin and alizarin, respectively. This disparity suggests a possible variation among noni genotypes for medically active chemical composition. We know that noni is an extremely variable species with a wide range of plant morphologies across the Pacific Islands (McClatchey 2003). The disparity could also suggest differences among researchers in extraction or chemical detection methods and the need for more research to clarify questions about these apparent discrepancies.

Chan-Blanco et al. (2007) did not note any significant reduction in pH in their experiments but noted the presence of molds and yeasts in the juice. This indicates that fermentation was done primarily by the molds and yeasts rather than by bacteria normally associated with noni fruits. Chan-Blanco et al. (2007) also noted an increase in ethanol production in their experiments, another indication that fermentation was accomplished primarily by molds and/or yeasts. This conclusion is supported by their observation that lactic acid bacterial populations crashed after several weeks of fermentation. The air in their plastic containers also probably favored the molds and yeasts.

Aging (ripening and fermentation) of noni fruits has a significant effect on the concentration of various biologically active chemistries. For example, the concentration of scopoletin is low in unripe fruits but increases during ripening (Chan-Blanco et al. 2007). Conversely, unripe noni fruits with physiological maturity presented the highest concentration of rutin, which then decreased during ripening but

remained almost constant during aging. These data suggest that noni processing can be adjusted to enhance the content of specific compounds found in the fruits. For example, for the production of products high in rutin, one could extract juice from unripe noni fruits having near-physiological maturity.

Yang et al. (2007a) examined phenolic compounds content and free radical and superoxide anion radical scavenging activities in two noni juices derived from "traditional" fermentation processes that differed in juice maturity. Juice from ripe fruits was lighter in color, contained higher total quantities of phenolic compounds, condensed tannins, flavonoids, and scopoletin, and thereby exhibited superior scavenging activity for free radicals, superoxide anion radicals, and hydrogen peroxide and higher angiotensin converting enzyme–inhibitory activity. Traditional fermentation also yielded more juice. However, prolonged fermentation time reduced the reductive phytochemical content and some related bioactive compounds.

The processing and storage of noni products can degrade bioactive properties (Yang et al. 2007a,b). Yang et al. (2010) examined the total phenolics, ascorbic acid, and antioxidant capacity of noni juice and powder during storage at 24°C. After 2 weeks of storage, illuminated noni juice lost 32% of total phenolics, 89% of ascorbic acid, and 46% to 65% of antioxidant capacity (~8%, 22%, and 9%–15% more than unilluminated noni juice). However, all juice lost 97% of ascorbic acid by 4 weeks. Protection from light reduced the degradation of the antioxidant characteristics of noni juice for only 2 weeks. These and other results (Yang et al. 2007b) indicate that the most effective means by which to retain the antioxidant characteristics of noni juice is to restrict light and to control temperature during juice storage.

17.6 Probiotic Potential of Fermented Noni Juice

Most probiotic bacteria used in fermented products are members of the genera *Lactobacillus* and *Bifidobacterium* (Daly and Davis 1998). People normally consume probiotics as part of fermented foods having active, live probiotic cultures such as yogurt or dietary supplements. Such bacteria when administered in adequate amounts confer health benefits on humans, including stimulation of immune systems, reduction of serum cholesterol levels, improved lactose digestion, and management and prophylaxis of certain gastrointestinal infections (Gilliand 1990; Lin et al. 1998; Salimnen et al. 1998; Mattila-Sandholm et al. 1999). Recently, lactic acid bacteria such as *Lactobacillus acidophilius*, *Lactobacillus plantarum*, *Lactobacillus casei*, *Bifodobacterium longum*, and *Bifidobacterium lactis* have been used in fruits to produce probiotic beverages derived from fruits (Gardner et al. 2001; Tien et al. 2005; Yoon et al. 2006; Kun et al. 2008).

Wang et al. (2009) examined the potential for the probiotic fermentation of noni juice by examining the growth of lactic acid bacteria (*L. casei* and *L. plantarum*) and bifidobacteria (*B. longum*) and the effects of fermentation on pH, acidity, sugar content, cell survival, and antioxidant properties of the juice. They found that each bacterial strain tested grew well in noni juice at 30°C, reaching nearly 109 colony-forming units/mL after 48 hours of fermentation (Wang et al. 2009). *L. casei* produced less lactic acid than *B. longum* and *L. plantarum*. *B. longum* and *L. plantarum* survived well in the acidified, fermented juice under cold storage for 4 weeks at 4ºC. Noni juice fermented with *B. longum* had a high antioxidant capacity that did not differ from that of noni juice fermented by lactic acid bacteria (Wang et al. 2009). This work established that *B. longum* and *L. plantarum* are potentially excellent probiotic organisms for the industrial fermentation of noni juice. This suggests that new noni-based health beverage products could be developed for the probiotics market using these bacterial species without external nutrient supplement.

17.7 Future Research and Conclusion

The fermented noni juice industry is still very young, having been in existence for only 15 years or so. There is great opportunity and need for fermentation research and product development for the best products to be manufactured. Research on the effects of fermentation on the major bioactive chemical components is desperately needed. In the area of product development, the best bacterial species for noni

juice fermentation should be identified and fermentation processes be improved to allow their controlled use. An example of such improved products lies in probiotics. Optimum environmental parameters for the fermentation of noni juice also need to be identified, such as fermentation temperature.

REFERENCES

Basar S, Uhlenhut K, Högger P, Schöne F, Westendorf J. 2010. Analgesic and antiinflammatory activity of *Morinda citrifolia* L. (noni) fruit. *Phytother Res* 24:38–42.

Chan-Blanco Y, Vaillant F, Pérez AM, Belleville M, Zúniga C, Brat P. 2007. The ripening and aging of noni fruits (*Morinda citrifolia* L.): microbiological flora and antioxidant compounds. *J Sci Food Agric* 87:1710–1716.

Daly C, Davis R. 1998. The biotechnology of lactic acid bacteria with emphasis on applications in food safety and human health. *Agric Food Sci Finland* 7:219–250.

Dixon AR, McMillen H, Etkin NL. 1999. Ferment this: the transformation of noni, a traditional Polynesian medicine (*Morinda citrifolia*, Rubiaceae). *Econo Bot* 53:51–68.

European Commission. 2003. Commission Decision of 5 June 2003 authorizing the placing on the market of "noni juice" (juice of the fruit of *Morinda citrifolia* L.) as a novel food ingredient under Regulation (EC) Nr. 258/97 of the European Parliament and of the Council. *Official Journal of the European Union L* 144/12; 12.6.2003.

Furusawa E, Hirazumi A, Story S, Jenson J. 2003. Antitumor potential of a polysaccharide-rich substance from the fruit juice of *Morinda citrifolia* (noni) on sarcoma 180 ascites tumor in mice. *Phytother Res* 17:1158–1164.

Gardner NJ, Savard T, Obermeier P, Caldwell G, Champagne CP. 2001. Selection and characterization of mixed starter cultures for lactic acid fermentation of carrot, cabbage, beet and onion vegetable mixtures. *Int J Food Micribiol* 64:261–275.

Gilliand SE. 1990. Health and nutritional benefits from lactic acid bacteria. *FEMS Microbiol Rev* 87: 175–188.

Harada S, Fujita-Hamabe W, Kamiya K, Satake T, Tokuyama S. 2010. Involvement of glycemic control in the inhibiting effect of *Morinda citrifolia* on cerebral ischemia-induced neuronal damage. *Yakugaku Zasshi* 130:707–712.

Hirazumi A, Furusawa E. 1999. An immunomodulatory polysaccharide-rich substance from the fruit juice of *Morinda citrifolia* (noni) with antitumor activity. *Phytother Res* 13:380.

Hornick CA, Meyers A, Sadowska-Krowika H, Anthony CT, Woltering EA. 2003. Inhibition of angionic initiation and disruption of newly established human vascular networks by juice from *Morinda citrifolia* (noni). *Angiogenesis* 6:143–149.

Jainkittivong A, Langlais RP. 2009. Antifungal effect of *Morinda citrifolia* fruit extract against *Candida albicans*. *Oral Surg Oral Med Pathol Oral Radiol Endod* 108:394–398.

Kamiya K, Tanaka Y, Endang H, Umar M, Satake T. 2004. Chemical constituents of *Morinda citrifolia* fruits inhibit copper induced low-density lipoprotein oxidation. *J Agric Food Chem* 52:5843–5848.

Kun S, Rezessy-Szabó JM, Nguyen QD, Hoschke Á. 2008. Changes of microbial population and some components in carrot juice during fermentation with selected Bifidobacterium strains. *Process Biochem* 43:816–821.

Lin MY, Yen CL, Chen CH. 1998. Management of lactose indigestion by consuming milk containing lactobacilli. *Digest Dis Sci* 43:133–137.

Mattila-Sandholm T, Matto J, Saarela M. 1999. Lactic acid bacteria with health claims—interactions and interference with gastrointestinal flora. *Int Dairy J* 9:25–35.

McClatchey W. 2002. From Polynesian healers to health food stores: changing perspectives of *Morinda citrifolia* (Rubiaceae). *Integr Cancer Ther* 1:110–120.

McClatchey W. 2003. Diversity of uses and growth forms in the *Morinda citrifolia* complex. In: Nelson SC, editor. *Proceedings of the 2002 Hawaii Noni Conference*. Honolulu, HI: University of Hawaii at Manoa, College of Tropical Agriculture and Human Resources. p. 5–10.

McKoy MLG, Thomas EA, Simon OR. 2002. Preliminary investigation of the anti-inflammatory properties of an aqueous extract from *Morinda citrifolia* (Noni). *Proc West Pharmacol Soc* 45:76–78.

Merrill DE. 1943. *Emergency food plants and poisonous plants of the Islands of the Pacific*. War Department Technical Manual TM 10-420. Washington, DC: United States Government Printing Office.

Muralidharan P, Ravi Kumar V, Balamurugan G. 2010. Protective effect of *Morinda citrifolia* fruits on β-amyloid (25–35) induced cognitive dysfunction in mice: an experimental and biochemical study. *Phytother Res* 24:252–258.

Nelson SC. 2006. *Morinda citrifolia* (noni). Species profiles for Pacific Island agroforestry. Available from: http://www.traditionaltree.org.

Nelson SC, Elevitch CR. 2006. *Noni: the complete guide for consumers and growers*. Holualoa, HI: Permanent Agriculture Resources.

Nelson SC, Elevitch CR. 2007. *Noni Processing, marketing, and field training workshop for yap*. Holualoa, HI: Permanent Agriculture Resources.

Newton K. 2003. Production of noni juice and powder in Samoa. In: Nelson SC, editor. *Proceedings of the 2002 Hawaii Noni Conference*. Honolulu, HI: University of Hawaii at Manoa, College of Tropical Agriculture and Human Resources. p. 29–32.

Palu A, Deng S, West B, Jensen J. 2009. Xanthine oxidase inhibiting effects of noni (*Morinda citrifolia*) fruit juice. *Phytother Res* 23:1790–1791.

Potterat P, Hamburger M. 2007. *Morinda citrifolia* (noni) fruit—phytochemistry, pharmacology, safety. *Planta Med* 73:191–199.

Ratanavalachai T, Thitiorul S, Nandhasri P. 2008. *In vitro* genotoxic and antigenotoxic studies of Thai Noni fruit juice by chromosomal aberration and sister chromatid exchange assays in human lymphocytes. *Songklanakarin J Sci Technol* 30:583–589.

Salimnen S, Ouwenhand AC, Isolauri E. 1998. Clinical applications of probiotic bacteria. *Int Dairy J* 8:563–572.

Saludes JP, Garson MJ, Franzblau SG, Aguinaldo AM. 2002. Antitubercular constituents from the hexane fraction of *Morinda citrifolia* Linn. (Rubiaceae). *Phytother Res* 16:683–685.

Taşkin Eİ, Akgün-Dar K, Kapucu A, Osanç E, Doğruman H, Eraltan H, Ulukaya E. 2009. Apoptosis-inducing effects of *Morinda citrifolia* L. and doxorubicin on the Ehrlich ascites tumor in Balb-c mice. *Cell Biochem Funct* 27:542–546.

Tien YY, Ng CC, Chang CC, Tseng WS, Kotwal S, Shyu YT. 2005. Studies on the lactic-fermentation of sugar apple (*Annona squamosa* L.) puree. *J Food Drug Anal* 13:377–381.

Wang CY, Ng CC, Su H, Tzeng WS, Shyu YT. 2009. Probiotic potential of noni juice fermented with lactic acid bacteria and bifidobacteria. *Int J Food Sci Nutr* 60(S6):98–106.

Wang MY, Nowicki D, Anderson G, Jensen J, West B. 2008. Liver protective effects of *Morinda citrifolia* (noni). *Plant Foods Hum Nutr* 63:59–63.

Wang MY, West B, Jensen CJ, Nowicki D, Su C, Palu AK. 2002. *Morinda citrifolia* (Noni): a literature review and recent advances in Noni research. *Acta Pharmacol Sin* 23:1127–1141.

Yamaguchi S, Ohnishi J, Sogawa M, Maru I, Ohta Y, Tsukada Y. 2002. Inhibition of angiotensin I converting enzyme by noni (*Morinda citrifolia*) juice. *J Jpn Soc Food Sci Technol* 49:624–627.

Yang J, Gadi R, Paulino T, Thomson T. 2010. Total phenolics, ascorbic acid, and antioxidant capacity of noni (*Morinda citrifolia* L.) juice and powder as affected by illumination during storage. *Food Chem* 122:627–632.

Yang J, Paulino R, Janke-Stedronsky S, Abawi F. 2007b. Free-radical scavenging activity and total phenols of noni (*Morinda citrifolia* L.) juice and powder in processing and storage. *Food Chem* 102:302–308.

Yang SC, Chen TI, Li KY, Tsai TC. 2007a. Change in phenolic compound content, reductive capacity and ACE inhibitory activity in noni juice during traditional fermentation. *J Food Drug Anal* 15:290–298.

Yoon YK, Woodams EE, Hang YD. 2006. Production of probiotic cabbage juice by lactic acid bacteria. *Bioresour Technol* 97:1427–1430.

Zin ZM, Hamid AA, Osman A, Saari N. 2006. Antioxidative activities of chromatograph fractions obtained from root, fruit and leaf of Mengkudu (*Morinda citrifolia* L.). *Food Chem* 94:169–178.

18

*Wine Production in the United States: Regulatory Requirements**

Y. H. Hui

CONTENTS

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18.1 Introduction

For any country, when a private company produces wine for public consumption, the government has a tight control over the business for three important reasons, among others: tax, economic integrity, and safety. In the United States, two federal agencies prescribe regulations for the commercial production of wine:

1. Alcohol and Tobacco Tax and Trade Bureau (TTB; www.ttb.gov)
2. Food and Drug Administration (www.fda.gov/)

18.2 Laws and Regulations

In this country, TTB has issued the following regulations governing the commercial production of wine:

- 27 CFR 1: Basic permit requirements under the Federal Alcohol Administration Act, nonindustrial use of distilled spirits and wine, bulk sales, and bottling of distilled spirits
- 27 CFR 4: Labeling and advertising of wine
- 27 CFR 9: American viticultural area
- 27 CFR 12: Foreign nongeneric names of geographic significance used in the designation of wines
- 27 CFR 13: Labeling proceedings
- 27 CFR 16: Alcoholic beverage health warning statement
- 27 CFR 24: Wine
- 27 CFR 27: Importation of distilled spirits, wine, and beer
- 27 CFR 28: Exportation of alcohol
- Public Law 107-188, the Public Health Security and Bioterrorism Preparedness and Response Act of 2002

For reference, 27 CFR 4 refers to Volume 27, U.S. Code of Federal Regulations, Part 4. The abbreviations used in this chapter also include the U.S. Codes, for example, 26 U.S.C. 5384 means Volume 26, U.S. Codes, Part 5384.

This chapter discusses the major focus of the regulations. If a company starts a wine production and distribution business, the original legal documents should be consulted for details. We start with some definitions.

18.3 Meaning of Terms

The following provides the meaning of some selected legal terms related to the production of wine (for complete details, refer to 27 CFR 24).

Agricultural wine. Wine made from suitable agricultural products other than the juice of grapes, berries, or other fruits.

Allied products. Commercial fruit products and by-products (including volatile fruit-flavor concentrate) not taxable as wine.

Amelioration. The addition to juice or natural wine before, during, or after fermentation, of either water or pure dry sugar, or a combination of water and sugar to adjust the acid level.

Artificially carbonated wine. Effervescent wine artificially charged with carbon dioxide and containing more than 0.392 g of carbon dioxide per 100 mL.

Brix. The quantity of dissolved solids expressed as grams of sucrose in 100 g of solution at 60°F (20°C) (percent by weight of sugar).

Chaptalization (Brix adjustment). The addition of sugar or concentrated juice of the same kind of fruit to juice before or during fermentation to develop alcohol by fermentation.

Concentrate plant. An establishment qualified for the production of volatile fruit-flavor concentrate.

Distilled spirits plant. An establishment qualified for producing, warehousing, or processing of distilled spirits (including denatured spirits) or manufacturing of articles.

Distilling material. Any fermented or other alcoholic substance capable of or intended for use in the original distillation or other original processing of spirits.

Effervescent wine. A wine containing more than 0.392 g of carbon dioxide per 100 mL.

Export or exportation. A severance of goods from the mass of things belonging to the United States with the intention of uniting them to the mass of things belonging to some foreign country and will include shipments to any possession of the United States. Shipments to the Commonwealth of Puerto Rico and to the territories of the Virgin Islands, American Samoa, and Guam will also be treated as exportations.

Fold. The ratio of the volume of the fruit must or juice to the volume of the volatile fruit-flavor concentrate produced from the fruit must or juice; for example, 1 gallon of volatile fruit-flavor concentrate of 100-fold would be the product from 100 gallons of fruit must or juice.

Foreign wine. Wine produced outside the United States.

Formula wine. Special natural wine, agricultural wine, and other than standard wine (except for distilling material and vinegar stock) produced on bonded wine premises under an approved formula.

Fruit wine. Wine made from the juice of sound, ripe fruit (other than grapes). Fruit wine also includes wine made from berries or wine made from a combination of grapes and other fruit (including berries).

Grape wine. Wine made from the juice of sound, ripe grapes.

Hard cider. Still wine derived primarily from apples or apple concentrate and water (apple juice, or the equivalent amount of concentrate reconstituted to the original Brix of the juice before concentration, must represent more than 50% of the volume of the finished product) containing no other fruit product or any artificial product, which imparts a fruit flavor other than apple; containing at least one half of 1% and less than 7% alcohol by volume; having the taste, aroma, and characteristics generally attributed to hard cider; and sold or offered for sale as hard cider.

Heavy bodied blending wine. Wine made from fruit without added sugar, with or without added wine spirits, and conforming to the definition of natural wine in all respects except as to maximum total solids content.

High-proof concentrate. A volatile fruit-flavor concentrate (essence) that has an alcohol content of more than 24% by volume and is unfit for beverage use (nonpotable) because of its natural constituents, that is, without the addition of other substances.

Invert sugar syrup. A substantially colorless solution of invert sugar, which has been prepared by recognized methods of inversion from pure dry sugar and contains not less than 60% sugar by weight (60°Brix).

Juice. The unfermented juice (concentrated or unconcentrated) of grapes, other fruit (including berries), and authorized agricultural products exclusive of pulp, skins, or seeds.

Kind. The class and type of wine.

Lees. The settlings of wine.

Liquid sugar. A substantially colorless refined sugar and water solution containing not less than the equivalent of 60% pure dry sugar by weight (60ºBrix).

Lot. Wine of the same type. When used with reference to a "lot of wine bottled," lot means the same type of wine bottled or packed on the same date into containers.

Must. Unfermented juice or any mixture of juice, pulp, skins, and seeds prepared from grapes or other fruit (including berries).

Natural wine. The product of the juice or must of sound, ripe grapes or other sound and ripe fruit (including berries) made with any cellar treatment authorized by subparts F and L of this part and containing not more than 21% by weight (21°Brix dealcoholized wine) of total solids.

Nonbeverage wine. Wine or wine products made from wine, rendered unfit for beverage use in accordance with §24.215.

Proof. The ethyl alcohol content of a liquid at 60°F, stated as twice the percentage of ethyl alcohol by volume.

Proof gallon. A U.S. gallon of liquid at 60°F, which contains 50% by volume of ethyl alcohol having a specific gravity of 0.7939 at 60°F referred to water at 60°F as unity, or the alcoholic equivalent thereof.

Pure dry sugar. Refined sugar 95% or more by weight dry, having a dextrose equivalent of not less than 95% on a dry basis, and produced from cane, beets, or fruit, or from grain or other sources of starch.

Reconditioning. The conduct of operations, after original bottling or packing, to restore wine to a merchantable condition. The term includes relabeling or recasing operations.

Same kind of fruit. In the case of grapes, all of the species and varieties of grapes. In the case of fruits other than grapes, this term includes all of the several species and varieties of any given kind, except that this will not preclude a more precise identification of the composition of the product for the purpose of its designation.

Sparkling wine or champagne. An effervescent wine containing more than 0.392 g of carbon dioxide per 100 mL of wine resulting solely from the secondary fermentation of the wine within a closed container.

Special natural wine. A product produced from a base of natural wine (including heavy bodied blending wine) to which natural flavorings are added and made pursuant to an approved formula in accordance with subpart H of this part.

Specially sweetened natural wine. A product made with a base of natural wine and having a total solids content in excess of 17% by weight (17°Brix dealcoholized wine) and an alcohol content of not more than 14% by volume.

Spirits. That substance known as ethyl alcohol, ethanol, or spirits of wine in any form (including all dilutions or mixtures thereof, from whatever source or by whatever process produced), but not denatured spirits unless specifically stated.

Standard wine. Natural wine, specially sweetened natural wine, special natural wine, and standard agricultural wine, produced in accordance with requirements.

Still wine. Wine containing not more than 0.392 g of carbon dioxide per 100 mL.

Sugar. Pure dry sugar, liquid sugar, and invert sugar syrup.

Sweetening. The addition of juice, concentrated juice, or sugar to wine after the completion of fermentation and before tax payment.

Total solids. The degrees Brix of unfermented juice or dealcoholized wine.

U.S. wine. Wine produced on bonded wine premises in the United States.

Unmerchantable wine. Wine that has been taxpaid, removed from bonded wine premises, and subsequently returned to bonded wine premises for the purpose of reconditioning, reformulation, or destruction.

Vinegar. A wine or wine product not for beverage use produced in accordance with the provisions of this part and having not less than 4.0 g (4.0%) of volatile acidity (calculated as acetic acid and exclusive of sulfur dioxide) per 100 mL of wine.

Volatile fruit-flavor concentrate. Any concentrate produced by any process, which includes evaporations from any fruit mash or juice.

Wine. When used without qualification, the term includes every kind (class and type) of product produced on bonded wine premises from grapes, other fruit (including berries), or other

suitable agricultural products and containing not more than 24% of alcohol by volume. The term includes all imitation, other than standard, or artificial wine and compounds sold as wine. A wine product containing less than one half of 1% alcohol by volume is not taxable as wine when removed from the bonded wine premises.

Wine spirits. Brandy or wine spirits authorized under 26 U.S.C. 5373 for use in wine product.

18.4 Standards of Identity

The TTB has provided the Standards of Identity for Wine in 27 CFR 4.21. The sections and paragraphs mentioned in this chapter refer to 27 CFR 4.21. The standards of identity for the several classes and types of wine are defined in the following sections.

18.4.1 Class 1: Grape Wine

1. *Grape wine* is wine produced by the normal alcoholic fermentation of the juice of sound, ripe grapes (including restored or unrestored pure condensed grape must), with or without the addition, after fermentation, of pure condensed grape must and with or without added grape brandy or alcohol, but without other addition or abstraction except as may occur in cellar treatment, provided that the product may be ameliorated before, during, or after fermentation by either of the following methods:
   (i) By adding, separately or in combination, dry sugar or such an amount of sugar and water solution as will not increase the volume of the resulting product more than 35%, but in no event should any product so ameliorated have an alcoholic content derived by fermentation, of more than 13% by volume, or a natural acid content, if water has been added, of less than 5 parts per thousand, or a total solids content of more than 22 g per 100 cm^3.
   (ii) By adding, separately or in combination, not more than 20% by weight of dry sugar or not more than 10% by weight of water.
   (iii) In the case of domestic wine, in accordance with the U.S. Codes.
   (iv) The maximum volatile acidity, calculated as acetic acid and exclusive of sulfur dioxide, is 0.14 g per 100 mL (20°C) for natural red wine and 0.12 g per 100 mL (20°C) for other grape wine, provided that the maximum volatile acidity for wine produced from unameliorated juice of 28°Brix or more is 0.17 g per 100 mL for red wine and 0.15 g per 100 mL for white wine. Grape wine deriving its characteristic color or lack of color from the presence or absence of the red coloring matter of the skins, juice, or pulp of grapes may be designated as "red wine," "pink (or rose) wine," "amber wine," or "white wine" as the case may be. Any grape wine containing no added grape brandy or alcohol may be further designated as "natural."
2. *Table wine* is grape wine having an alcoholic content not in excess of 14% by volume. Such wine may also be designated as "light wine," "red table wine," "light white wine," "sweet table wine," and so forth, as the case may be.
3. *Dessert wine* is grape wine having an alcoholic content in excess of 14% but not in excess of 24% by volume. Dessert wine having the taste, aroma, and characteristics generally attributed to sherry and an alcoholic content, derived in part from added grape brandy or alcohol, of not less than 17% by volume, may be designated as "sherry." Dessert wines having the taste, aroma, and characteristics generally attributed to angelica, madeira, muscatel, and port and an alcoholic content, derived in part from added grape brandy or alcohol, of not less than 18% by volume may be designated as "angelica," "madeira," "muscatel," or "port," respectively. Dessert wines having the taste, aroma, and characteristics generally attributed to any of the previously mentioned products and an alcoholic content, derived in part from added grape brandy or alcohol, in excess of 14% by volume but, in the case of sherry, less than 17% or, in other cases, less than 18% by volume, may be designated as "light sherry," "light angelica," "light madeira," "light muscatel," or "light port," respectively.

18.4.2 Class 2: Sparkling Grape Wine

1. *Sparkling grape wine* (including "sparkling wine," "sparkling red wine," and "sparkling white wine") is grape wine made effervescent with carbon dioxide resulting solely from the fermentation of the wine within a closed container, tank, or bottle.
2. *Champagne* is a type of sparkling light wine that derives its effervescence solely from the secondary fermentation of the wine within glass containers of not greater than 1 gallon capacity and possesses the taste, aroma, and other characteristics attributed to champagne as made in the champagne district of France.
3. (i) A sparkling light wine having the taste, aroma, and characteristics generally attributed to champagne but not otherwise conforming to the standard for "champagne" may, in addition to but not in lieu of the class designation "sparkling wine," be further designated as
   (A) "Champagne style"
   (B) "Champagne type"
   (C) "American (or New York State, Napa Valley, etc.) champagne," along with one of the following terms: "bulk process," "fermented outside the bottle," "secondary fermentation outside the bottle," "secondary fermentation before bottling," "not fermented in the bottle," or "not bottle fermented." The term "charmat method" or "charmat process" may be used as additional information.

   (ii) Labels should be so designed that all the words in such further designation are readily legible under ordinary conditions and are on a contrasting background. In the case of paragraph (3)(i)(C) of this section, the TTB will consider whether the label as a whole provides the consumer with adequate information about the method of production and origin of the wine. The TTB will evaluate each label for legibility and clarity on the basis of such factors as type size and style for all components of the further designation and the optional term "charmat method" or "charmat process," as well as the contrast between the lettering and its background and the placement of information on the label.

   (iii) Notwithstanding the provisions of paragraphs (3)(i)(A), (B), and (C) of this section, the appropriate TTB officer may authorize the use of a term on sparkling wine labels as an alternative to those terms authorized in paragraph (3)(i) of this section, but not in lieu of the required class designation "sparkling wine," upon a finding that such term adequately informs the consumer about the method of production of the sparkling wine.
4. *Crackling wine*, *petillant wine*, or *frizzante wine* (including cremant, perlant, reciotto, and other similar wine) is sparkling light wine normally less effervescent than champagne or other similar sparkling wine but containing sufficient carbon dioxide in solution to produce, upon pouring under normal conditions, after the disappearance of air bubbles, a slow and steady effervescence evidenced by the formation of gas bubbles flowing through the wine. Crackling wine, which derives its effervescence from secondary fermentation in containers greater than 1-gallon capacity, should be designated "crackling wine—bulk process," and the words "bulk process" should appear in lettering of substantially the same size as the words "crackling wine."

18.4.3 Class 3: Carbonated Grape Wine

Carbonated grape wine (including carbonated wine, carbonated red wine, and carbonated white wine) is grape wine made effervescent with carbon dioxide other than that resulting solely from the secondary fermentation of the wine within a closed container, tank, or bottle.

18.4.4 Class 4: Citrus Wine

1. (i) *Citrus wine or citrus fruit wine* is wine produced by the normal alcoholic fermentation of the juice of sound, ripe citrus fruit (including restored or unrestored pure condensed

citrus must), with or without the addition, after fermentation, of pure condensed citrus must and with or without added citrus brandy or alcohol, but without any other addition or abstraction except as may occur in cellar treatment, provided that a domestic product may be ameliorated or sweetened in accordance with the provisions of 26 U.S.C. 5384 and any product other than domestic may be ameliorated before, during, or after fermentation by adding, separately or in combination, dry sugar or such an amount of sugar and water solution as will not increase the volume of the resulting product more than 35%, or in the case of products produced from citrus fruit having a normal acidity of 20 parts or more per thousand, not more than 60%, but in no event should any product so ameliorated have an alcoholic content, derived by fermentation, of more than 14% by volume, or a natural acid content, if water has been added, of less than 5 parts per thousand, or a total solids content or more than 22 g per 100 cm^3.

(ii) The maximum volatile acidity, calculated as acetic acid and exclusive of sulfur dioxide, should not be more than 0.14 g for natural citrus wine and more than 0.12 g for other citrus wine, per 100 mL (20°C).

(iii) Any citrus wine containing no added brandy or alcohol may be further designated as "natural."

2. *Citrus table wine* or *citrus fruit table wine* is citrus wine having an alcoholic content not in excess of 14% by volume. Such wine may also be designated "light citrus wine," "light citrus fruit wine," "light sweet citrus fruit wine," and so forth, as the case may be.
3. *Citrus dessert wine* or *citrus fruit dessert wine* is citrus wine having an alcoholic content in excess of 14% but not in excess of 24% by volume.
4. Citrus wine derived wholly (except for sugar, water, or added alcohol) from one kind of citrus fruit should be designated by the word "wine" qualified by the name of such citrus fruit, for example, "orange wine" and "grapefruit wine." Citrus wine not derived wholly from one kind of citrus fruit should be designated as "citrus wine" or "citrus fruit wine" qualified by a truthful and adequate statement of composition appearing in direct conjunction therewith. Citrus wine rendered effervescent by carbon dioxide resulting solely from the secondary fermentation of the wine within a closed container, tank, or bottle should be further designated as "sparkling," and citrus wine rendered effervescent by carbon dioxide otherwise derived should be further designated as "carbonated."

18.4.5 Class 5: Fruit Wine

1. (i) *Fruit wine* is wine (other than grape wine or citrus wine) produced by the normal alcoholic fermentation of the juice of sound, ripe fruit (including restored or unrestored pure condensed fruit must), with or without the addition, after fermentation, of pure condensed fruit must and with or without added fruit brandy or alcohol, but without other addition or abstraction except as may occur in cellar treatment, provided that a domestic product may be ameliorated or sweetened in accordance with the provisions of 26 U.S.C. 5384 and any product other than domestic may be ameliorated before, during, or after fermentation by adding, separately or in combination, dry sugar or such an amount of dry sugar and water solution as will increase the volume of the resulting product, in the case of wines produced from any fruit or berry other than grapes, having a normal acidity of 20 parts or more per thousand, not more than 60%, and in the case of other fruit wines, not more than 35%, but in no event should any product so ameliorated have an alcoholic content, derived by fermentation, of more than 14% by volume, or a natural acid content, if water has been added, of less than 5 parts per thousand, or a total solids content of more than 22 g per 100 cm^3.

(ii) The maximum volatile acidity, calculated as acetic acid and exclusive of sulfur dioxide, should not be more than 0.14 g for natural fruit wine and more than 0.12 g for other fruit wine, per 100 mL (20°C).

(iii) Any fruit wine containing no added brandy or alcohol may be further designated as "natural."

2. *Berry wine* is fruit wine produced from berries.
3. *Fruit table wine* or *berry table wine* is fruit or berry wine having an alcoholic content not in excess of 14% by volume. Such wine may also be designated "light fruit wine" or "light berry wine."
4. *Fruit dessert wine* or *berry dessert wine* is fruit or berry wine having an alcoholic content in excess of 14% but not in excess of 24% by volume.
5. Fruit wine derived wholly (except for sugar, water, or added alcohol) from one kind of fruit should be designated by the word "wine" qualified by the name of such fruit, for example, "peach wine" and "blackberry wine." Fruit wine not derived wholly from one kind of fruit should be designated as "fruit wine" or "berry wine," as the case may be, qualified by a truthful and adequate statement of composition appearing in direct conjunction therewith. Fruit wines that are derived wholly (except for sugar, water, or added alcohol) from apples or pears may be designated "cider" and "perry," respectively, and should be so designated if lacking in vinous taste, aroma, and characteristics. Fruit wine rendered effervescent by carbon dioxide resulting solely from the secondary fermentation of the wine within a closed container, tank, or bottle should be further designated as "sparkling," and fruit wine rendered effervescent by carbon dioxide otherwise derived should be further designated as "carbonated."

18.4.6 Class 6: Wine from Other Agricultural Products

1. (i) Wine of this class is wine (other than grape wine, citrus wine, or fruit wine) made by the normal alcoholic fermentation of sound fermentable agricultural products, either fresh or dried, or of the restored or unrestored pure condensed must thereof, with the addition before or during fermentation of a volume of water not greater than the minimum necessary to correct natural moisture deficiencies in such products, with or without the addition, after fermentation, of pure condensed must, and with or without added alcohol or such other spirits as will not alter the character of the product, but without other addition or abstraction except as may occur in cellar treatment, provided that a domestic product may be ameliorated or sweetened in accordance with part 24 of this chapter, and any product other than domestic may be ameliorated before, during, or after fermentation by adding, separately or in combination, dry sugar or such an amount of sugar and water solution as will not increase the volume of the resulting product more than 35%, but in no event should any product so ameliorated have an alcoholic content, derived by fermentation of more than 14% by volume, or a natural acid content, if water has been added, of less than 5 parts per thousand, or a total solids content of more than 22 g per 100 cm^3.
   (ii) The maximum volatile acidity, calculated as acetic acid and exclusive of sulfur dioxide, should not be more than 0.14 g for natural wine of this class and more than 0.12 g for other wine of this class, per 100 mL (20°C).
   (iii) Wine of this class containing no added alcohol or other spirits may be further designated as "natural."
2. *Table wine* of this class is wine having an alcoholic content not in excess of 14% by volume. Such wine may also be designated as "light."
3. *Dessert wine* of this class is wine having an alcoholic content in excess of 14% but not in excess of 24% by volume.
4. *Raisin wine* is wine of this class made from dried grapes.
5. *Sake* is wine of this class produced from rice in accordance with the commonly accepted method of manufacture of such product.
6. Wine of this class derived wholly (except for sugar, water, or added alcohol) from one kind of agricultural product should except in the case of "sake," be designated by the word "wine" qualified by the name of such agricultural product, for example, "honey wine," "raisin wine," and "dried blackberry wine." Wine of this class not derived wholly from one kind of agricultural

product should be designated as "wine" qualified by a truthful and adequate statement of composition appearing in direct conjunction therewith. Wine of this class rendered effervescent by carbon dioxide resulting solely from the secondary fermentation of wine within a closed container, tank, or bottle should be further designated as "sparkling," and wine of this class rendered effervescent by carbon dioxide otherwise derived should be further designated as "carbonated."

18.4.7 Class 7: Aperitif Wine

1. *Aperitif wine* is wine having an alcoholic content of not less than 15% by volume, compounded from grape wine containing added brandy or alcohol, flavored with herbs and other natural aromatic flavoring materials, with or without the addition of caramel for coloring purposes, and possessing the taste, aroma, and characteristics generally attributed to aperitif wine and should be so designated unless designated as "vermouth" under paragraph 2 of this section.
2. *Vermouth* is a type of aperitif wine compounded from grape wine, having the taste, aroma, and characteristics generally attributed to vermouth and should be so designated.

18.4.8 Class 8: Imitation and Substandard or Other than Standard Wine

1. "Imitation wine" should bear as a part of its designation the word "imitation" and should include
   (i) Any wine containing synthetic materials.
   (ii) Any wine made from a mixture of water with residue remaining after thorough pressing of grapes, fruit, or other agricultural products.
   (iii) Any class or type of wine the taste, aroma, color, or other characteristics of which have been acquired in whole or in part, by treatment with methods or materials of any kind (except as permitted in §4.22(c)(6)), if the taste, aroma, color, or other characteristics of normal wines of such class or type are acquired without such treatment.
   (iv) Any wine made from must concentrated at any time to more than 80° (Balling).
2. "Substandard wine" or "other than standard wine" should bear as a part of its designation the words "substandard" or "other than standard" and should include
   (i) Any wine having a volatile acidity in excess of the maximum prescribed therefore in §§4.20 to 4.25.
   (ii) Any wine for which no maximum volatile acidity is prescribed in §§4.20 to 4.25, inclusive, having a volatile acidity, calculated as acetic acid and exclusive of sulfur dioxide in excess of 0.14 g per 100 mL (20°C).
   (iii) Any wine for which a standard of identity is prescribed in this §§4.20 to 4.25, inclusive, which, through disease, decomposition, or otherwise, fails to have the composition, color, and clean vinous taste and aroma of normal wines conforming to such standard.
   (iv) Any "grape wine," "citrus wine," "fruit wine," or "wine from other agricultural products" to which has been added sugar and water solution in an amount which is in excess of the limitations prescribed in the standards of identity for these products, unless, in the case of "citrus wine," "fruit wine," and "wine from other agricultural products" the normal acidity of the material from which such wine is produced is 20 parts or more per thousand and the volume of the resulting product has not been increased more than 60% by such addition.

18.4.9 Class 9: Retsina Wine

Retsina wine is grape table wine fermented or flavored with resin. Table 18.1 summarizes the classes and types of wine.

TABLE 18.1

Wine Classes and Types

(a) Class 1; grape wine

(1) *Grape wine* is wine produced by the normal alcoholic fermentation of the juice of sound, ripe grapes (including restored or unrestored pure condensed grape must), with or without the addition, after fermentation, of pure condensed grape must, and with or without added grape brandy or alcohol, but without other addition or abstraction except as may occur in cellar treatment, provided that the product may be ameliorated before, during or after fermentation by either of the following methods:

(i) By adding, separately or in combination, dry sugar, or such an amount of sugar and water solution as will not increase the volume of the resulting product more than 35%; but in no event shall any product so ameliorated have an alcoholic content derived by fermentation, of more than 13% by volume, or a natural acid content, if water has been added, of less than 5 parts per thousand, or a total solids content of more than 22 g per 100 cm^3.

(ii) By adding, separately or in combination, not more than 20% by weight of dry sugar, or not more than 10% by weight of water.

(iii) In the case of domestic wine, in accordance with 26 U.S.C. 5383.

(iv) The maximum volatile acidity, calculated as acetic acid and exclusive of sulfur dioxide is 0.14 g per 100 mL (20°C) for natural red wine and 0.12 g per 100 mL (20°C) for other grape wine, provided that the maximum volatile acidity for wine produced from unameliorated juice of 28 or more degrees Brix is 0.17 g per 100 mL for red wine and 0.15 g per 100 mL for white wine. Grape wine deriving its characteristic color or lack of color from the presence or absence of the red coloring matter of the skins, juice, or pulp of grapes may be designated as "red wine," "pink (or rose) wine," "amber wine," or "white wine," as the case may be. Any grape wine containing no added grape brandy or alcohol may be further designated as "natural."

(2) *Table wine* is grape wine having an alcoholic content not in excess of 14% by volume. Such wine may also be designated as "light wine," "red table wine," "light white wine," "sweet table wine," etc., as the case may be.

(3) *Dessert wine* is grape wine having an alcoholic content in excess of 14% but not in excess of 24% by volume. Dessert wine having the taste, aroma, and characteristics generally attributed to sherry and an alcoholic content, derived in part from added grape brandy or alcohol, of not less than 17% by volume, may be designated as "sherry." Dessert wines having the taste, aroma, and characteristics generally attributed to angelica, madeira, muscatel, and port and an alcoholic content, derived in part from added grape brandy or alcohol, of not less than 18% by volume, may be designated as "angelica," "madeira," "muscatel," or "port," respectively. Dessert wines having the taste, aroma, and characteristics generally attributed to any of the above products and an alcoholic content, derived in part from added grape brandy or alcohol, in excess of 14% by volume but, in the case of sherry, less than 17%, or, in other cases, less than 18% by volume, may be designated as "light sherry," "light angelica," "light madeira," "light muscatel" or "light port," respectively.

(*continued*)

TABLE 18.1 (Continued)

Wine Classes and Types

(b) Class 2; sparkling grape wine	
(1) *Sparkling grape wine* (including "sparkling wine," "sparkling red wine," and "sparkling white wine") is grape wine made effervescent with carbon dioxide resulting solely from the fermentation of the wine within a closed container, tank, or bottle.	
(2) *Champagne* is a type of sparkling light wine which derives its effervescence solely from the secondary fermentation of the wine within glass containers of not greater than one gallon capacity, and which possesses the taste, aroma, and other characteristics attributed to champagne as made in the champagne district of France.	
(3)	(i) A sparkling light wine having the taste, aroma, and characteristics generally attributed to champagne but not otherwise conforming to the standard for "champagne" may, in addition to but not in lieu of the class designation "sparkling wine," be further designated as: (A) "Champagne style;" or (B) "Champagne type;" or (C) "American (or New York State, Napa Valley, etc.) champagne," along with one of the following terms: "Bulk process," "fermented outside the bottle," "secondary fermentation outside the bottle," "secondary fermentation before bottling," "not fermented in the bottle," or "not bottle fermented." The term "charmat method" or "charmat process" may be used as additional information. (ii) Labels shall be so designed that all the words in such further designation are readily legible under ordinary conditions and are on a contrasting background. In the case of paragraph (b)(3)(i)(C) of this section, TTB will consider whether the label as a whole provides the consumer with adequate information about the method of production and origin of the wine. TTB will evaluate each label for legibility and clarity, based on such factors as type size and style for all components of the further designation and the optional term "charmat method" or "charmat process," as well as the contrast between the lettering and its background, and the placement of information on the label. (iii) Notwithstanding the provisions of paragraphs (b)(3)(i)(A), (B), and (C) of this section, the appropriate TTB officer may authorize the use of a term on sparkling wine labels, as an alternative to those terms authorized in paragraph (b)(3)(i) of this section, but not in lieu of the required class designation "sparkling wine," upon a finding that such term adequately informs the consumer about the method of production of the sparkling wine.

(*continued*)

TABLE 18.1 (Continued)

Wine Classes and Types

(4) *Crackling wine, petillant wine, frizzante wine* (including cremant, perlant, reciotto, and other similar wine) is sparkling light wine normally less effervescent than champagne or other similar sparkling wine, but containing sufficient carbon dioxide in solution to produce, upon pouring under normal conditions, after the disappearance of air bubbles, a slow and steady effervescence evidenced by the formation of gas bubbles flowing through the wine. Crackling wine which derives its effervescence from secondary fermentation in containers greater than 1-gallon capacity shall be designated "crackling wine—bulk process," and the words "bulk process" shall appear in lettering of substantially the same size as the words "crackling wine."

(c) Class 3; carbonated grape wine. "Carbonated grape wine" (including "carbonated wine," "carbonated red wine," and "carbonated white wine") is grape wine made effervescent with carbon dioxide other than that resulting solely from the secondary fermentation of the wine within a closed container, tank, or bottle

(d) Class 4; citrus wine

(1)

(i) *Citrus wine* or *citrus fruit wine* is wine produced by the normal alcoholic fermentation of the juice of sound, ripe citrus fruit (including restored or unrestored pure condensed citrus must), with or without the addition, after fermentation, of pure condensed citrus must, and with or without added citrus brandy or alcohol, but without any other addition or abstraction except as may occur in cellar treatment, provided that a domestic product may be ameliorated or sweetened in accordance with the provisions of 26 U.S.C. 5384 and any product other than domestic may be ameliorated before, during, or after fermentation by adding, separately or in combination, dry sugar, or such an amount of sugar and water solution as will not increase the volume of the resulting product more than 35%, or in the case of products produced from citrus fruit having a normal acidity of 20 parts or more per thousand, not more than 60%, but in no event shall any product so ameliorated have an alcoholic content, derived by fermentation, of more than 14% by volume, or a natural acid content, if water has been added, of less than 5 parts per thousand, or a total solids content or more than 22 g per 100 cm^3.

(ii) The maximum volatile acidity, calculated as acetic acid and exclusive of sulfur dioxide, shall not be, for natural citrus wine, more than 0.14 g, and for other citrus wine, more than 0.12 g per 100 mL (20°C).

(iii) Any citrus wine containing no added brandy or alcohol may be further designated as "natural."

(2) *Citrus table wine* or *citrus fruit table wine* is citrus wine having an alcoholic content not in excess of 14% by volume. Such wine may also be designated "light citrus wine," "light citrus fruit wine," "light sweet citrus fruit wine," etc., as the case may be.

(*continued*)

TABLE 18.1 (Continued)

Wine Classes and Types	
(3) *Citrus dessert wine* or *citrus fruit dessert wine* is citrus wine having an alcoholic content in excess of 14% but not in excess of 24% by volume.	
(4) Citrus wine derived wholly (except for sugar, water, or added alcohol) from one kind of citrus fruit, shall be designated by the word "wine" qualified by the name of such citrus fruit, e.g., "orange wine," "grapefruit wine." Citrus wine not derived wholly from one kind of citrus fruit shall be designated as "citrus wine" or "citrus fruit wine" qualified by a truthful and adequate statement of composition appearing in direct conjunction therewith. Citrus wine rendered effervescent by carbon dioxide resulting solely from the secondary fermentation of the wine within a closed container, tank, or bottle shall be further designated as "sparkling"; and citrus wine rendered effervescent by carbon dioxide otherwise derived shall be further designated as "carbonated."	

18.5 Labeling Samples

The TTB has distributed the information is this section to provide examples of approvable wine labels along with additional comments to further explain some aspects of the labeling rules. Again, details are provided in the regulations (27 CFR 4).

To maximize the usefulness of this guide, all of the pages and comments should be reviewed. Every label is unique, and certain information on a label may trigger other labeling requirements. The TTB regulations are quite detailed about the production of a wine and the information appearing on the label. Samples provided will prove useful but are not all-encompassing.

18.5.1 Generic Table Wine Label

This label contains all of the mandatory information for a typical domestic grape wine that contains at least 7% alcohol by volume but no more than 14% alcohol by volume (see Figure 18.1).

In this example, the name of the bottling winery also serves as the brand name for the label. "Table wine" is the class and type designation. Also, when the words "table wine" are present on the brand label, the alcohol content need not be specifically shown on the label as long as the alcohol content of the product is between 7% and 14% by volume.

This label contains all of the mandatory information for a typical domestic grape wine that contains more than 14% but not more than 24% alcohol by volume.

18.5.2 Generic Dessert Wine Label

"Dessert wine" is how the TTB refers to wine of this tax class. It is an acceptable class and type designation for grape wine with an alcohol content in excess of 14%, so it could be stated on the label in place of "Red Wine" as the class and type designation. Unlike "table wine," it does not substitute as an alcohol

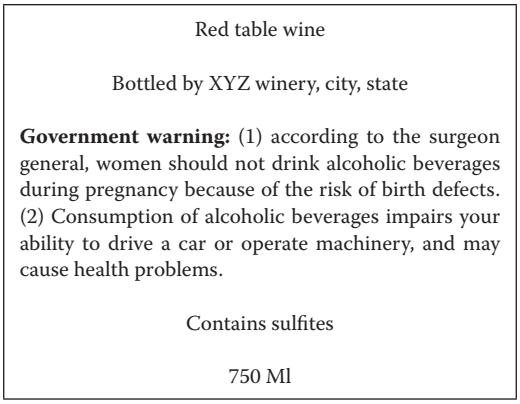


FIGURE 18.1 Generic table wine label.

content statement. A specific alcohol content statement is required for any wine with an alcohol content of more than 14% alcohol by volume. In this example, the name of the bottling winery also serves as the brand name for the label (see Figure 18.2).

18.5.3 Standard One-Piece Wine Label

This wine is designated as "American merlot." This means that at least 75% of the wine is derived from merlot grapes grown in the United States. Because a varietal designation ("merlot") is used on this label, an appellation of origin ("American") is required. In this case, a vintage date would not be permitted on the label. Vintage-dated wines require an appellation of origin that is smaller than a country (see Figure 18.3).

18.5.4 Standard Two-Piece Wine Label

Please note that a fanciful name is optional and may be shown on the label, but it does not replace the need for a class and type designation (in this case, "rose wine"). Also keep in mind that "rose" must be followed by "wine" in order for it to serve as the class and type designation. In addition, although a

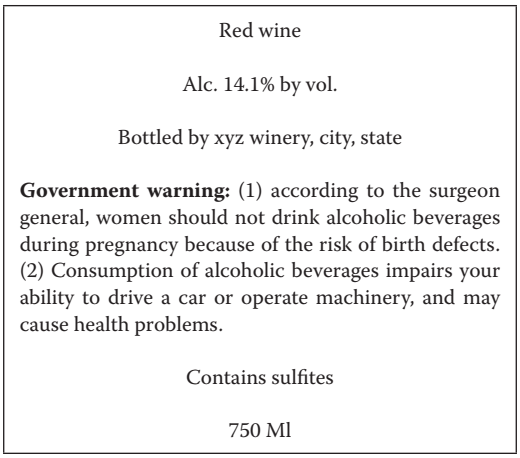


FIGURE 18.2 Generic dessert wine label.

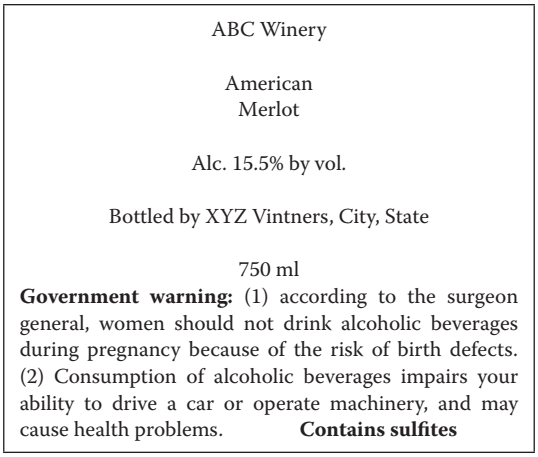


FIGURE 18.3 Standard one-piece wine label.

varietal designation is not present, an appellation of origin smaller than a country must be shown on the brand label because the wine is vintage-dated (see Figure 18.4).

18.5.5 Imported Wine Label with Additional Strip Label

The "Downunder Winery" label is the label usually applied by the foreign winery. To qualify for TTB approval, the U.S. importer may have the foreign winery affix additional labels to the bottle to bring it into compliance with the requirements of the regulations. The "strip" label must appear on the same face of the container as the label with the brand name. When designing a strip label such as this or a two-piece brand label, it is important to remember that the *appellation of origin* (in this case, "Victoria") and the *class and type designation* (in this case, "Red Wine") *must appear on the same label* (see Figure 18.5).

18.5.6 Domestic Three-Piece Wine Label

When designing two-piece brand labels for a wine with a varietal designation or a vintage date, it is important to remember that the *appellation of origin* (in this case, "California") and the *class and type designation* (in this case, the varietals and their percentages) must appear on the same label. The brand name is "ABC Winery." Note that when multiple varietals are shown on a brand label, the percentages of each grape type must be shown and must total 100% (see Figure 18.6).

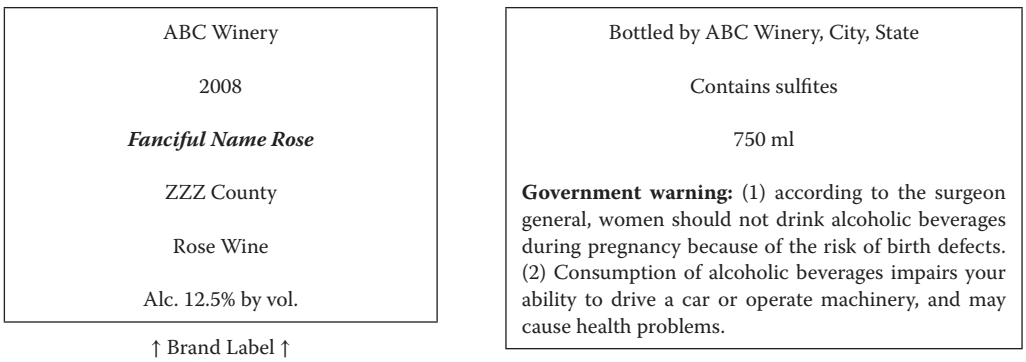


FIGURE 18.4 Standard two-piece wine label.

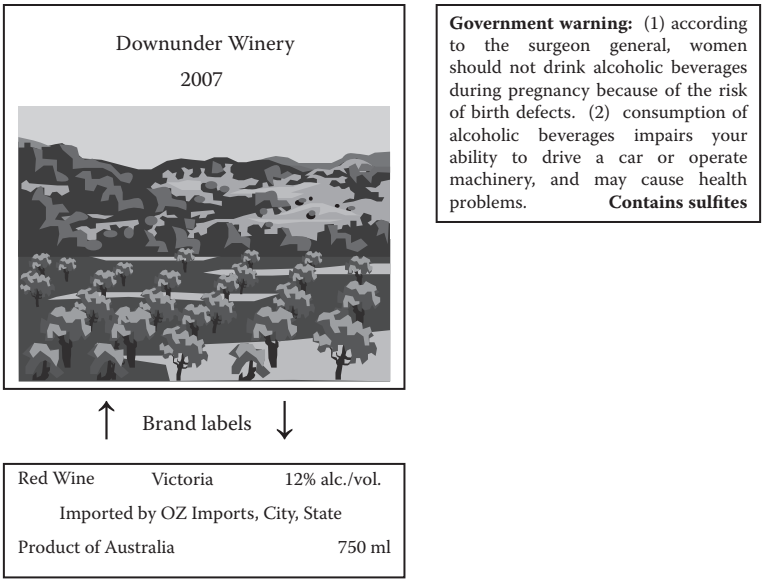


FIGURE 18.5 Imported wine label with additional strip label.

18.5.7 Bottler or Importer-Designated Brand Label

This label is acceptable because it contains the mandatory brand-label information, and the bottler or importer has designated it as their "brand" label. However, because this label is to be considered the brand label, it has to meet all of the brand-label requirements, and brand-label requirements are more stringent than back-label requirements (Figure 18.7).

For instance, brand-label varietal references take precedence as the class and type designation. When using a varietal or two or more grape varietals as the type designation, the varietals (and their percentages) must be in at least 2-mm print and appear separate and apart or more conspicuous than the surrounding text. If the mandatory information appears surrounded by other text, it must be in bold print or at least twice the size of the surrounding text.

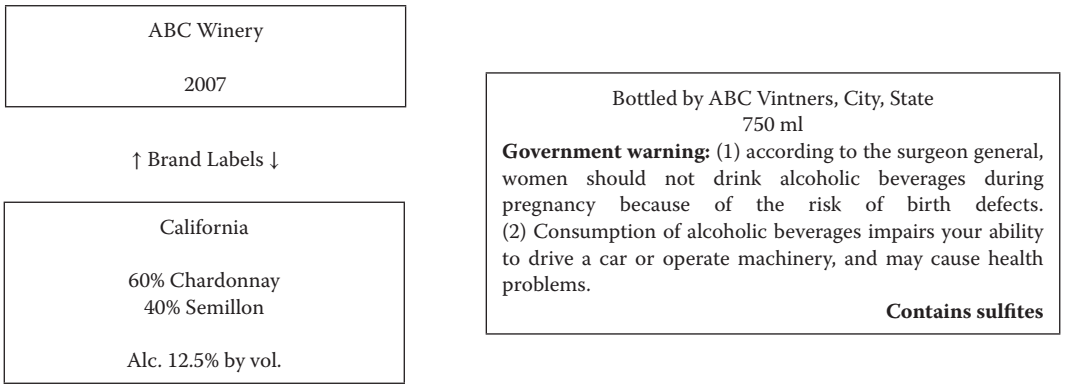
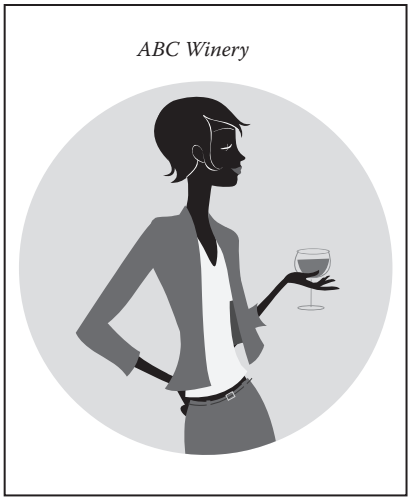


FIGURE 18.6 Domestic three-piece wine label.



ABC Winery
American Red Wine

This red wine is a blend of 50% **MERLOT** and 50% **CABERNET SAUVIGNON**. We find it quite yummy.

ALC. 13% by vol.

Bottled by XYZ Cellars, City, State

Contains sulfites

750 ml

Goverment warning: (1) According to the surgeon general, women should not drink alcholic beverages during pregnancy because of the risk of birth defects. (2) Consumption of alcoholic beverages impairs your ability to drive a car or operate machinery, and may cause health problems.

↑ Brand label ↑

FIGURE 18.7 Bottler or importer-designated brand label.

18.5.8 "Nonstandard Wine" Label

These labels contain all of the mandatory information for wines, which must have a formula (domestic) or a preimport product evaluation (imported) approved before the submission of the label. The formula or preimport evaluation, when approved, will include a suggested statement of composition (e.g., "grape wine with natural flavors added"), which is the mandatory class and type designation and must appear on the brand label (see Figure 18.8).

Please note that a fanciful name is optional and may be shown on the label, but it does not replace the need for the statement of composition. Also keep in mind that vintage dates and grape varietals are not permitted on these wines.

ABC Winery

(Optional Fanciful Name)

Red Wine with natural flavors

Alc. 12.5% by vol.

↑ Brand Label ↑

Bottled by ABC Vintners, City, State

Contains sulfites

750 ml

Government warning: (1) according to the surgeon general, women should not drink alcoholic beverages during pregnancy because of the risk of birth defects. (2) Consumption of alcoholic beverages impairs your ability to drive a car or operate machinery, and may cause health problems.

FIGURE 18.8 "Nonstandard wine" label.

18.6 Appellation of Origins

18.6.1 Introduction

According to the TTB, this appellation of origin page is intended to serve as a "warehouse" of all U.S. and foreign appellations of origin. The information provided here is as comprehensive as possible based on the information available to us. First to be posted were U.S. appellations of origin, approved U.S. viticultural areas identified in the 50 states, and U.S. counties. We are now adding, country by country, foreign appellations of origin as the respective foreign government verifies its information. We update the listing of U.S. viticultural areas whenever a new U.S. viticultural area is added to our regulations. Listings of foreign appellations of origin will be updated when we are officially notified of an addition or amendment by an authorized representative of the foreign government. The information provided here covers the following.

18.6.2 Definition

Under U.S. regulations, an appellation of origin is

- A country
- A U.S. state or the foreign equivalent
- For U.S. wine, a listing of up to three states (multistate appellation)
- A U.S. county or the foreign equivalent
- For U.S. wine, a listing of up to three counties (multicounty appellation)
- A U.S. or foreign government recognized delimited grape-growing area (called a *viticultural area* under U.S. regulations)

18.6.3 Application

- Appellations of origin are only defined for use on wine
- All appellations of origin apply to grape wine
- U.S. or foreign viticultural area appellations of origin apply only to grape wine
- Country, state (or foreign equivalent), multistate (U.S. wine only), county (or foreign equivalent), or multicounty (U.S. wine only) appellations of origin apply to non–grape wine only if permissible under the laws and regulations of the labeled appellation of origin

18.6.4 Requirements for Use

Whether mandatory or optional, an appellation of origin may be used on a wine label only if

- For U.S. wine:
  - With an American (country) state or county appellation of origin:
    1. Not less than 75% of the volume of the wine is derived from grapes (or other agricultural commodity) grown in the labeled appellation of origin
    2. The wine is fully finished (except for cellar treatment and/or blending, which does not alter the class and type of the wine) in the labeled appellation of origin, except that in the case of a state appellation of origin, the wine is fully finished (except for cellar treatment and/or blending, which does not alter the class and type of the wine) in the labeled state or an adjacent state
    3. The wine conforms to the laws and regulations of the labeled appellation of origin governing the composition, the method of production, and the designation of wine produced in the labeled appellation area

- With a multistate appellation of origin composed of not more than three states:
  1. The states are contiguous

  Note: Contiguous means that (a) both or all three states touch at a common border or (b) in the case of three states, all three states are in an unbroken line
  2. 100% of the wine is derived from grapes (or other agricultural commodity) grown in the labeled states
  3. The wine is fully finished (except for cellar treatment and/or blending, which does not alter the class and type of the wine) in one of the labeled states
  4. The percentage of wine derived from grapes (or other agricultural commodity) grown in each of the labeled states is shown on the label
  5. The wine conforms to the laws and regulations governing the composition, the method of production, and the designation of wine in all the states listed in the appellation
- With a multicounty appellation of origin composed of not more than three counties:
  1. All of the counties are in the same state
  2. One hundred percent of the wine is derived from grapes (or other agricultural commodity) grown in the labeled counties
  3. The percentage of wine derived from grapes (or other agricultural commodity) grown in each county is shown on the label
- With a viticultural area appellation of origin:
  1. The labeled area is an American viticultural area approved under U.S. regulations
  2. Not less than 85% of the volume of the wine is derived from grapes grown in the labeled viticultural area
  3. The wine is fully finished (except for cellar treatment and/or blending, which does not alter the class and type of the wine) in the state or one of the states where the viticultural area is located

- For imported wine:
  - With a country, foreign equivalent of a state or foreign equivalent of a county appellation of origin:
    1. Not less than 75% of the volume of the wine is derived from grapes (or other agricultural commodity) grown in the labeled appellation of origin
    2. The wine conforms to the requirements of the foreign laws and regulations governing the composition, the method of production, and the designation of wine available within the country of origin
  - With a viticultural area appellation of origin:
    1. The labeled area is recognized by the government of the country of origin as a delimited grape-growing or viticultural area
    2. Not less than 85% of the volume of the wine is derived from grapes grown in the labeled viticultural area
    3. The wine conforms to the requirements of the foreign laws and regulations governing the composition, method of production, and designation of wine available within the country of origin

18.6.5 When an Appellation of Origin Is Required

- Generally, appellations of origin are only required for grape wine
- An appellation of origin is required on grape wine when the wine is labeled with
  - A grape varietal designation

- One of the following designations and the wine is not from the origin indicated*:
  Angelica (United States)
  Burgundy (France)
  Claret (France)
  Chablis (France)
  Champagne (France)
  Chianti (Italy)
  Haut Sauterne (France)
  Hock (Germany)
  Malaga (Spain)
  Marsala (Italy)
  Madeira (Portugal)
  Moselle (France)
  Port (Portugal)
  Rhine Wine (Germany)
  Sauterne (France)
  Sherry (Spain)
  Tokay (Hungary)
- A vintage date
- The phrase "estate bottled"

18.6.6 Type of Appellation of Origin Required

Generally, with two exceptions, any one of the types of appellations (i.e., a country, state, etc.) may be used when an appellation is required. The two exceptions are

- Wine labeled with a vintage date:
  The appellation of origin must be a state (or foreign equivalent), a multistate (U.S. wine only), a county (or foreign equivalent), a multicounty (U.S. wine only), or a viticultural area
- Wine labeled as "estate bottled":
  The appellation of origin must be a viticultural area

18.6.7 Type Size Requirements

When an appellation of origin is required, the type size requirements are as follows:

- For containers of 187 mL or less:
  The appellation of origin must be at least 1 mm in size and substantially as conspicuous as the product designation

* There has been a change relative to the use of all of the mentioned names except Angelica. On March 10, 2006, the U.S. and the European Union (EU) signed an Agreement on Trade in Wine in which the United States committed to seek to change the legal status of the names (with the exception of Angelica, which is a U.S. wine and therefore not included in the agreement) to restrict their use solely to wine originating in the applicable EU member state, except as provided for under a "grandfather" provision. The grandfather provision excepts certain non-EU wines labeled with one of the names (other than Angelica), provided the applicable label was approved on a certificate of label approval or certificate of exemption issued before March 10, 2006. The legislative proposal that effected the change in legal status of the names was included in the Tax Relief and Health Care Act of 2006 that was enacted on December 20, 2006.

- For containers of more than 187 mL:
  The appellation of origin must be at least 2 mm in size and substantially as conspicuous as the product designation

18.6.8 Placement Requirements

When an appellation of origin is required, it must appear on the same label and in the same view as the product designation.

Part IV

Vegetables and Vegetable Products

19

Vegetable Fermentation with Economical Significance in South America: Use of Ensilage as a Probiotic Vehicle

Mario Eduardo Arena, Ana Lidia Apás, and Silvia Nelina González

CONTENTS

19.1 Vegetable Fermentation

There are different methods to conserve foods, and one of them is increasing the acidity. This can be achieved artificially with the addition of weak acids or naturally by fermentation, obtaining free additive products. The preservation of food by fermentation is an ancient and widely practiced technology. These processes were crafted in nature and, obviously, the role of bacteria was not appreciated. Nowadays, it is accepted that the fermentation processes can be developed spontaneously by the action of native microflora or by lactic acid bacteria (LAB) inoculation. Fermentation increases not only the product's shelf life and microbiological food safety, but also improves the digestibility and nutritional value of the food (Caplice and Fittzgerald 1999).

The demand for fermented products has experienced an important increase in recent years ever since consumers recognized that fermentation plays an important and beneficial role in human nutrition (Spyropoulou et al. 2001). In fact, nearly 25% of the European diet and 60% of the diet in many developing countries consists of fermented foods (Stiles 1996).

19.1.1 Advantages of Fermenting Vegetables

Heating could destroy vitamins, provoke Maillard reactions, and reduce the availability of free amino acids. Lactic fermentation does not require or only requires very little energy in the form of heat, allowing

the preservation of fresh or minimally processed vegetables. It also does not destroy amino acids or vitamins, unlike any form of thermal conservation. Moreover, fermentation increases the digestibility of plants and, if it is made with starter strains with beneficial properties, might have a probiotic effect. For example, it has been demonstrated that fermentation with selected strains contributes to the maintenance of folate concentrations that are usually destroyed by heating methods (Jägerstad et al. 2004).

19.1.2 Use of Starters

Fermentation can be carried out by natural flora or by inoculated starters like LAB. In many cases, the fermentation is led by indigenous flora, which varies based on the substrate, temperature, and conditions of storage. Thus, a product with variable sensorial properties is usually obtained.

Spontaneous fermentations typically result from the competitive activities of a variety of autochthonous and contaminating microorganisms. Those best adapted to the conditions during the fermentation process will eventually dominate. Initiation of a spontaneous process takes a relatively long time, with a high risk for failure. Failure of fermentation processes can result in spoilage or the survival of pathogens, thereby creating unexpected health risks in food products. Thus, from both a hygiene and safety point of view, the use of starter cultures is recommended (Rodriguez et al. 2009). The use of starter cultures would be an appropriate approach for the control and optimization of the fermentation process to minimize variations on organoleptic quality and microbiological stability.

The selected starters of LAB ferment many vegetables, and this process can contribute to the flavor, texture, and aroma characteristics of the food. Additionally, it can result in hygienic conservation and commercial stability.

In many works, starter cultures are mixed and include bacteria with different metabolisms (homofermentative and heterofermentative). The selection of mixed starter cultures is based principally on the competitiveness between the starter and natural flora.

To obtain the desirable properties of fermented vegetables, it is preferable that LAB are adapted to the intrinsic characteristics of the raw materials (Di Cagno et al. 2008, 2009a,b). Food processing that includes the use of selected autochthonous starters may favor the storage of vegetables at room temperature for extended shelf life.

19.1.3 Presence of LAB in Vegetables

LAB are responsible for fermentation of many vegetables. The most important commercial vegetable fermentation in Europe is for olives, cucumbers (pickles), and cabbages; in these vegetables, LAB represents 1.5% of the total bacterial population (Buckenhüskes 1997).

Some species of LAB are commonly found in various types of fermented vegetables. *Lactobacillus plantarum* and *Leuconostoc mesenteroides* were commonly identified within the microbiota of cucumber, tomato, pumpkin, persimmon, and in eggplant when they ferment spontaneously (Buckenhüskes 1997; Seseña and Palop 2007). Occasionally, *Weissella* spp. have been identified in vegetables such as tomato and chard (Björkroth and Holzapfel 2006; Di Cagno et al. 2008); *Lactobacillus curvatus/ Lactobacillus sakei* were found in peppers (Choi et al. 2003; Lee et al. 2004). *L. plantarum* strains have been used to increase health-promoting and sensory properties of tomato juices (Di Cagno et al. 2009a).

Gardner et al. (2001) used pure and mixed starters of three LAB: *L. plantarum* NK 312, *Pediococcus acidilactici* AFERM 772, and *Le. mesenteroides* BLAC to lead the fermentation of a juice from a mixture of vegetables (onion, carrot, and beet), and selected the most suitable starter—the mix composed of the three LAB.

L. curvatus and *L. sakei* dominated the microbiota of kimchi and sauerkraut during late fermentation (Vogel et al. 1993; Choi et al. 2003; Lee et al. 2004). *L. plantarum* was isolated from orange juice and apple (Arena et al. 1996, 2004b), whereas *L. plantarum*, *L. brevis*, *L. pentosaceus*, and *Leuconostoc* spp. have been found to occur mainly in the spontaneous fermentation of cucumbers (Tamminen et al. 2004).

L. plantarum is one of the most ubiquitous microorganisms in vegetables because of its flexible and wide metabolic capacity to rapidly adapt to changing environmental conditions, including acid stress (Corsetti and Gobbetti 2002; Klaenhammer et al. 2005). One of the main advantages of LAB is the

ability to inhibit the development of spoilage microorganisms that could affect consumer health or deteriorate the food.

19.1.4 Antimicrobial Activity

Three mechanisms may explain the antimicrobial efficiency of LAB: organic acid production (with pH diminution), competition for nutrients, and production of antagonistic compounds.

19.1.4.1 Organic Acids

It is well known that LAB can produce lactic acid (homofermentative bacteria) or lactic acid over acetic acid (heterofermentative) from carbohydrates. Lactic acid, the most widely occurring hydroxylcarboxylic acid, has traditionally been used as a food preservative.

Acidification inhibits the growth of microorganisms not adapted to low pH. Therefore, the capability for rapid acidification is one of the most desirable properties of a starter culture.

Organic acids occurring in foods are additives or end products of the carbohydrate metabolism of LAB (Arena et al. 1996). The direct antimicrobial effects of organic acids, including lactic and acetic acids, were observed against Enterobacteriaceae (Apás et al. 2008).

19.1.4.2 Microbial Interference

General microbial interference is an effective nonspecific control mechanism common to all populations and environments including foods (Caplice and Fittzgerald 1999).

19.1.4.3 Against Yeasts

Not all microorganisms are inhibited by low pH produced by the formation of acids by LAB. Some yeasts have a great capacity to develop in acidic environments.

In fermented vegetables, spoilage yeasts appear after fermentation by LAB, but when a lactic starter is used, the growth of yeasts is usually inhibited. Results suggest that prefermentation of vegetables with LAB does not prevent the subsequent growth of yeast because residual sugars are found in the fermented products. Furthermore, acidification with lactic acid alone to pH 3.74 is insufficient to prevent the growth of spoilage yeast in fermented vegetables, and the presence of other organic acids is desirable in this aim. The production of acetic acid by *Le. mesenteroides* during fermentation would thus seem to be a critical aspect of the preservation of fermented vegetables.

The prevention of yeast spoilage would be improved by reducing the contamination levels of yeast, and by preventing the growth of yeast in the initial stages of fermentation when the pH of the vegetable mixture is high. The inoculation of a selective lactic starter to rapidly lower the pH seems recommendable.

19.1.4.4 Inhibition of Mycotoxin-Producing Fungi

Food and feed spoilage molds cause great economic losses worldwide. It is estimated that between 5% and 10% of the world's food production is wasted because of fungal deterioration (Pitt and Hocking 1997).

Some strains of *Lactococcus* species such as *Lc. lactis, Lc. lactis* subsp. *diacetylactis*, and *Lc. lactis* subsp. *cremoris* were reported to control mycotoxinogenic mold growth (Luchese and Harrigan 1990; Batish et al. 1989; Florianowicz 2001).

Two LAB, *Lactobacillus fermentum* and *Lactobacillus rhamnosus*, which are widely used in the fermentation and preservation of food, were inhibitory against *Aspergillus nomius* assayed on their fungal inhibitory properties (Muñoz et al. 2010).

Therefore, the reduction of mold growth during the production and storage of food and feed is of great importance. *Aspergillus* frequently produces mycotoxins in grains and consequently represents a direct threat to crops. For many years now, it has been clear that the most effective means to prevent

the contamination of food with mycotoxins is to avoid the growth of mycotoxigenic fungi (Battcock and Azam-Ali 1998).

19.2 South American Vegetable Fermentations

In northwest Argentina, two kinds of cultures are being fermented to increase their shelf lives. One is pepper, which is used for human consumption, and the other is a residual of sugar cane industries, which is used for animal feed. Both are of great importance for economic activities and for environmental control. Both are fermented using native strains of LAB.

19.2.1 Pepper Fermentation

Pepper is a solanaceous agricultural crop belonging to the *Capsicum annum* L. species. Health-promoting, nutritional, and sensory attributes make pepper one of the most consumed vegetables worldwide that contain a large spectrum of antioxidant compounds (polyphenols, vitamin C, flavonoids, and carotenoids) with free radical scavenging properties, are essential antioxidants that may protect against the propagation of the oxidative chain (Namiki 1990; Deepa et al. 2007). When consumed in the daily diet, these compounds may prevent several human diseases, including several forms of cancer, arteriosclerosis, and cardiovascular diseases (Harris 1996; Bramley 2000).

Pepper represents an important culture in the northwest of Argentina. Its main production takes place in the provinces of Salta and Catamarca; represented per 1300 to 1400 hectares cultivated. Production varies between 1200 and 2500 kg/ha according to the type of irrigation. Pepper reproduces by seeds. Its culture takes place in the period that passes from summer to autumn, does not resist low temperatures or frosts, and because of pickling, they are available in the market throughout the year, but its price increases during the winter.

Peppers are consumed mature or immature, as crude fruits, or in Spanish stews, and in preserves or pickles. There is little information available on the fermentation of peppers.

A protocol for the manufacture of fermented peppers includes a thermal blanching at 85°C for 2 minutes. Overall, blanching is used to decrease the microbial population and, especially, to inactivate deleterious enzymes (e.g., peroxidase, polyphenol oxidase, and pectin methylesterase) that may cause undesirable nutritional and sensory changes (Bahçeci et al. 2004; Castro et al. 2008). In addition, blanching of peppers in the presence of water promotes chemical or enzymatic reactions leading to positive variations of the aroma profile (Jiang and Kubota 2001; Plessi et al. 2002).

However, in our experience, treatment with fluent steam is better to avoid altering the organoleptic properties of the pepper (Arena et al. 2004a). The results indicated that all heat treatments affect the color and consistency of pepper. A smaller alteration in the product's sensory properties occurred when it was put under fluent steam and when it was put at 3/4 atmosphere in an autoclave and extinguished. After treatment, the samples were tested to evaluate the modification of media pH for seven days. There was a decrease of two pH units in the control media with or without inoculation. The pH values decreased by 0.3 units after seven days in the samples treated with fluent steam. The treatment that involved heating in an autoclave at 3/4 atmosphere and extinguishing was effective for inhibiting the pH modifications for seven days and produced smaller modifications that were the same for treatment at 3 minutes. It is possible to infer that microbial growth was produced in the natural control medium and in the medium treated with fluent steam. From these results, the treatment of peppers in solution at 3/4 atm in an autoclave and then switched off was the technique selected for fermentation.

Considering the technological importance to the industry of the development of controlled vegetable fermentation, we used culture initiators for the fermentation of peppers cultivated in the northwest of the country to obtain a product of stable quality in a controlled process and in a relatively short time compared with natural fermentation procedures.

The advantage of using a starter culture is that the pH decreases more quickly and it confers microbiological stability to the product. A spontaneous fermentation generally needs four to six days before the pH stabilizes, but a high-level inoculation (10^6–10^7 colony forming unit [CFU]/g) of a mixed culture can

be controlled and accelerates the process to prevent spoilage and to obtain products of consistently high and desired quality (Aukrust et al. 1994).

The starters were inoculated at concentrations 100 times greater than the native flora, according to methods proposed by Gardner et al. (2001) and consistent with Seseña et al. (2001), which uses LAB starters to lead the fermentation of eggplants in the order of 10^7 CFU/mL.

The maximum values of viable cells are obtained between the second and fifth days of fermentation, and, as of this time, the number of viable cells begins to diminish or remains stable (Arena et al. 2004a). This occurs in a diverse number of vegetable fermentations, such as in cucumbers and in col for the elaboration of chucrut (Frazir and Westhoff 1993).

The percentage of survival is lower at 30°C compared with 22°C. A smaller percentage of survival is also seen in fermentations controlled and inoculated with the *Le. mesenteroides* strain; this can be a consequence of the acid production that is exerted over the other bacteria. The results for carrots, onions, and coles agree with those reported by Gardner et al. (2001).

The microorganisms that we used in these fermentations consume as much glucose as fructose. From these results, it is possible to suggest that fermentation is controlled at 30°C because the fast pH diminution confers greater microbiological stability to the product (Perera et al. 2004).

The fact that the combination of one heterofermentative *Le. mesenteroides* and one homofermentative *Pediococcus pentosaceus* was selected as the best starter by the people who tested pepper's sensorial properties is in agreement with the findings reported previously in other vegetables.

Fermentation can be carried out with different concentrations of sugar and sodium chloride. In the fermentation of peppers, the acid production depends on the initial glucose concentration. It has been previously reported that with 20 g/L of glucose in the fermentation medium, pH diminution takes place more quickly and the maximum growth is greater compared with 2 g/L of glucose in the medium; nevertheless, the fermentation of peppers with the smaller concentration of glucose is excellent, contributing to the possibility of reducing the costs for the manufacturing industry (Arena et al. 2004a). The lactic acid production is between nine and ten times greater when the glucose concentration is increased from 2 to 20 g/L.

Spyropoulou et al. (2001) reported that in the fermentation of olives, the lactic acid production is five times greater when the initial glucose concentration is increased from 1 to 10 g/L. In the production of fermented cabbage (chucrut), the relation between lactic acid and acetic acid is optimal at a range between 3.5 and 5 (Gardner et al. 2001; Adams 1995).

For the pepper fermentation starter, as selected by the tasting panel (*Le. mesenteroides*/*P. pentosaceus*), the lactic acid and acetic acid relation is between 2.8 and 3.1. In the mixed culture, the acetic acid in the heterofermentative microorganism contributes toward reaching the balanced acidity in the taste examination (Arena et al. 2004a).

The fermentations in the juice of peppers have patterns similar to the fermentations of the fruit in solution with 20 g/L of glucose. In the juice, the pH decreases at a greater speed when it is incubated to 30°C. Overall, the dominant species identified in peppers were frequently found in other vegetable matrices.

The dominant presence of health-promoting compounds and the sensory features of pepper fruits may encourage food processing that aims at preserving the functional compounds and agreeable sensory characteristics of pepper for extended shelf life, possibly at room temperature (Navarro et al. 2006; Castro et al. 2008). Besides the definite economic advantages, storage at room temperature may also allow processed peppers to overcome their sensitivity to chilling injuries (Mercier et al. 2001; Vicente et al. 2005). An important fact to keep in mind, and which will be discussed later, is the good survival rate of some LAB.

19.2.2 Sugar Cane Fermentation

In northern Argentina, sugarcane is a major agricultural product; however, its harvest produces an abundant amount of residues. Sugarcane blunting is the upper part of the cane that usually contains lower sugar levels than the rest of the cane, and is discarded to increase the sugar yield of a given amount of cane. With no other use for the discards, the residues are commonly placed in earthen excavations (i.e., sanitary landfills). Because of their high humidity levels, they decompose, generating abundant effluents.

Blunting may have lower sugar content than the rest of the cane; however, it contains sufficient sugar to be a carbohydrate source for the growth of pathogenic or detrimental organisms when discarded. Therefore, using rather than discarding this residue could reduce the pollution.

Sugar production from sugarcane generates residual products, many of which are, currently, waste products. Because of its sugar content, its discards can be fermented, which will reduce the levels of sugar and the pH. Consequently, the fermentation product will be microbiologically stable. Silage produced in this way can definitely be used as a nutrient for cattle, goats, and sheep.

The effects of silage inoculation on animal performance, food intake, and live weight gain have been reported; however, the causes are unclear (Weinberg et al. 2003). The benefits could be associated with silage fermentation rather than from the effects of LAB in the rumen (Khuntia and Chaudhary 2002).

Apás et al. (2008) assessed product improvement by pH diminution, lactic acid production, and decrements in the presence of potentially human pathogenic (Enterobacteriaceae, fungus, or *Clostridia*) or detrimental microorganisms (fungi and yeast; Fegan and Desmarchelier 2002; Bueno et al. 2006). Using this approach, they attempted to develop a product that could be used as a dietary supplement in mammal feeding. Such products are particularly necessary for the enrichment of the diet of fodder-consuming animals during the winter. Organic remainders from seasonal vegetative harvests are being studied as alternative dietary products to be used instead of conventional ingredients in the diets of animals. A disadvantage in their use as a fixed ingredient in diets is their seasonal availability and their high moisture content. The amount of dry matter (DM) is an important parameter in the ensilage process. If silage has less than 30 g/kg of DM, the fermentation process is considered inadequate (McDonald et al. 1991). DM values below that level result in increased amounts of gas and liquid effluents, nutrient loss, as well as bacterial and fungal contamination. Humid storage in a silo allows *Clostridia* growth, stimulates butyric acid production, and consequently, diminishes silage quality (McDonald et al. 1991).

Apás et al. (2008) demonstrated that a residue from sugarcane harvests, blunting, could be used as a ruminant animal food supplement with suitable DM, carbohydrate, and fiber contents. Moreover, a ruminant probiotic survives on this product, increasing blunting's effectiveness. Our results indicate that sugarcane blunting has a significant amount of sugar content, even after being fermented for 60 days. When ruminant diets are supplemented, there is often a concomitant reduction in pasturing time because the animals' energy demand is reduced (Adams 1985). Furthermore, the presence of glucose in the food enhances the survival of the inoculants LAB in the rumen fluid (Yusof et al. 2000). These factors indicate the advantages of fermented sugarcane blunting silage.

From the microbiological point of view, the maximum number of viable LAB cells was obtained between the second and fifth days of fermentation. Afterward, the number of viable cells diminished or remained stable. Despite these increases in total cell numbers, the LAB inoculations produced a decrement in Enterobacteriaceae, fungi, and yeast levels. The effect of LAB fermentation on *Escherichia coli* O157:H7 in barley forage has produced a similar decrease in *E. coli* levels, resulting in total elimination between 15 and 42 days of fermentation (Bach et al. 2002). In addition, total inhibition of Enterobacteriaceae was obtained with ensilages from other vegetative sources (Pagan Riestra 2006). A reduction in Enterobacteria is considered advantageous from a medical perspective because those bacteria are frequently associated with infections after intestinal surgery (Nichols and Smith 1994). It has been reported that some strains of *Lactobacillus* may protect the host from infection (Adlerberth et al. 1996).

LAB, under certain growth conditions, seems to inhibit the development of fungus (Bueno et al. 2006). Similar effects have been observed after inoculating vegetable juices with a LAB mixture to enhance the fermentation growth of yeasts (Gardner et al. 2001). The inhibition of spoilage yeasts in fermented salads has been associated with a combination of lactic acid and carbon dioxide formation, along with a reduction in the concentration of residual oxygen (Bonestroo et al. 1993).

However, it has been reported that inhibition of yeasts can be a result of their competition for nutrients with LAB and not to the production of lactic acid (Bayrock and Ingledew 2004). In the presence of the two LAB isolated from sugarcane, the inhibition of yeast growth was higher than that observed in the presence of the goat probiotic bacteria alone. These results could be due to acetic acid being more inhibitory with yeasts, molds, and bacteria compared with lactic acid (Blom and Mortvedt 1991). Decreases in fungi cell numbers in ensilages of vegetative products after 65 days of incubation of 2.87 log CFU/g in grenadines and 3.15 log CFU/g in citrus products have been reported (Pagan Riestra 2006).

Lactobacillus species are found in the gut of humans and other animals, but their numbers may vary with the animal species, the age of the host, or the location within the gut. Although LAB may enhance the digestion of silage, not all microorganisms improve silage after fermentation. For example, direct-fed microbial cultures of *Saccharomyces cerevisiae* did not improve fermentation or digestion in sheep fed sugarcane tops (Arcos-García et al. 2000).

The postfermentation characteristics of sugarcane blunting were deemed beneficial, as suggested by the low pH values, high lactic acid levels, low Enterobacteriaceae and fungus abundance, and high LAB contents relative to the uninoculated, control blunting.

The lactic acid production was higher than that observed in the fermentation of some fruits (Pagan Riestra 2006). In addition, the fermentation of this residue can serve as a medium for introducing probiotic bacteria to goats, suggesting its use as a useful ruminant feed supplement. LAB-enhanced silage production of vegetative residuals from sugarcane processing plants can produce potentially beneficial dietary supplements for ruminants.

Given the good results obtained by fermentation using the strains *Lactobacillus* sp. FB965 and *Lactobacillus* sp. FB974 isolated from sugarcane and were selected for their acidification capacity (Draksler et al. 2004), it was proposed that fermentation with probiotic strains isolated from one animal to consume the silage. We tested different strains of *L. plantarum*, *Bifidobacterium*, *Lac. fermentum*, and *Enterococcus faecalis*. The best fermentation showed a decrease in pH, acceptability for livestock consumption, and overall survival of probiotic strains and was obtained for two strains of *L. plantarum* (Apás et al. 2008).

These results suggested that LAB-enhanced silage production of vegetative residuals from sugarcane processing plants can produce potentially beneficial dietary supplements for ruminants.

19.3 Perspectives

The concept that LAB are beneficial to health is generally well accepted. However, it is known that the beneficial properties to the consumer are strain-dependent. On the other hand, when one thinks of probiotics, what usually only comes to mind are dairy products.

Why do we not think of a fermented vegetable product as a probiotic supplement? Why not use probiotic strains with proven properties to carry out the fermentation of a plant?

We demonstrated that probiotic strains, combined with lactic acid strains with good fermentative capabilities, can ferment sugar cane silage and survive at least 60 days (Apás et al. 2011). When these strains were administrated to goats, the fecal mutagenicity diminished 60% and the putrescine levels, cancer markers in the gut, decreased nearly 10 times, whereas the fecal microflora were enhanced (Apás et al. 2010).

On the other hand, the survival of LAB in peppers at 22°C was greater than at 30°C. At 30°C, there was a greater percentage of survival in the uncontrolled fermentations. At 30 days of incubation, a lower level of microbial survival was observed in selected control peppers heating inoculated with *Le. mesenteroides* in pure culture or combined with one or two of the other LAB. In the mix of fermentation that involves different combinations between heterofermentative and homofermentative LAB, survival at 30 days was log 5.40 CFU/g sample (Arena et al. 2004b); indicating the possibility of considering the medium to preserve the probiotic bacteria.

This is the time to add value to the fermentation of vegetables, and design it as a functional food. It is well established that lactic acid has beneficial properties in the gastrointestinal tract of humans; this is the main acid formed in the fermentation of vegetables. This clearly demonstrates that early removal of Enterobacteriaceae eliminates the risk of the formation of mutagens from promutagens in food.

These ideas will have to be new prospects for silage; it is certainly time to add these foods to probiotic strains, which requires new scientific studies and a new field of research for this potential application.

REFERENCES

Adams DC. 1985. Effect of time of supplementation on performance, forage intake, and grazing behavior of yearling beef steers grazing Russian wild ryegrass in the fall. *J Anim Sci* 61:1037–42.

Adams MR, Mosa MO. 1995. Microbiología de los Alimentos. Capítulo 9: Alimentos Fermentados y Alimentos Microbianos, Zaragoza, Spain: Editorial Acribia.

Adlerberth I, Ahrne S, Johansson M, Molin G, Hanson L, Wold A. 1996. A mannose-specific adherence mechanism in *Lactobacillus plantarum* conferring binding to the human colonic cell line HT-29. *Appl Environ Microbiol* 62:2244–51.

Apás AL, Arena ME, Draksler D, González SN. 2008. Utilization of sugarcane industrial residues as animal food and probiotic medium. *J Biosci Bioeng* 106:363–6.

Apás AL, Dupraz J, Ross R, González SN, Arena ME. 2010. Probiotic administration effect on fecal mutagenicity and microflora in the goat's gut. *J Biosci Bioeng* 110:537–40.

Apás AL, Arena ME. González SN. 2011. Goats fed with probiotic supplements obtained from fermentation of sugar cane residue. *Biocell* 35:A119.

Arcos-García JL, Castrejón FA, Mendoza GD, Pérez-Gavilán EP. 2000. Effect of two commercial yeast cultures with *Saccharomyces cerevisiae* on ruminal fermentation and digestion in sheep fed sugarcane tops. *Livest Prod Sci* 63:153–7.

Arena ME, Perera MF, Manca de Nadra MC. 2004a. Fermentación láctica de *Capsicum annum*. Simposio Internacional de Biotecnología. Aplicaciones en Alimentos, Salud y Medio Ambiente. II Simposio Argentino-Italiano de Bacterias Lácticas, p. 34.

Arena ME, Saguir FM, Manca de Nadra MC. 1996. Inhibition of growth of *Lactobacillus plantarum* isolated from citrus fruits in the presence of organics acids. *Microbiol Aliments Nutr* 14:219–26.

Arena ME, Sánchez LA, Manca de Nadra MC. 2004b. Estudio de la microflora natural de manzanas. Simposio Internacional de Biotecnología. Aplicaciones en Alimentos, Salud y Medio Ambiente. II Simposio Argentino-Italiano de Bacterias Lácticas. p. 35.

Aukrust T, Blom H, Sandtorv B, Slinde E. 1994. Interaction between starter culture and raw material in lactic acid fermentation of sliced carrot. *LWT Food Sci Technol* 27:337–41.

Bach SJ, Mc Alllister, TA, Baah J, Yanke LJ, Veira DM, Gannon VPJ, Holley RA. 2002. Persistence of *Escherichia coli* O157:H7 in barley silage: effect of bacterial inoculant. *J Appl Microbiol* 93:288–94.

Bahçeci SK, Serpen A, Gökmen V, Acar J. 2004. Study of lipoxygenase and peroxidase as indicator enzymes in green beans: change of enzyme activity, ascorbic acid and chlorophylls during frozen storage. *J Food Eng* 66:187–92.

Batish VK, Grover S, Lal R. 1989. Screening lactic starter cultures for antifungal activity. *Cultured Dairy Prod J* 24:21–5.

Battcock M, Azam-Ali S. 1998. Fermented fruits and vegetables—a global perspective. *FAO Agricultural Services Bulletin No. 134*. Rome: Food and Agriculture Organization of the United Nations.

Bayrock DP, Ingledew WM. 2004. Inhibition of yeast by lactic acid bacteria in continuous culture: nutrient depletion and/or acid toxicity? *J Ind Microbiol Biotechnol* 31:362–8.

Björkroth J, Holzapfel W. 2006. Genere *Leuconostoc, Oenococcus* and *Weissella*. *Prokariotes* 4:267–319.

Blom H, Mortvedt C. 1991. Anti-microbial substances produced by food-associated microorganisms. *Biochem Soc Trans* 19:694–8.

Bonestroo MH, Wit JC, Kusters BJ, Rombouts FM. 1993. Inhibition of the growth of yeasts in fermented salads. *Int J Food Microbiol* 17:311–20.

Bramley PM. 2000. Is lycopene beneficial to human health? *Phytochemistry* 54:233–6.

Buckenhüskes HJ. 1997. Fermented vegetables. In: Doyle MP, Beuchat LR, Montville TJ, editors. *Food microbiology: fundamentals and frontiers*. Washington, DC: ASM Press. p. 595–609.

Bueno DJ, Silva JO, Oliver G, González SN. 2006. *Lactobacillus casei* CRL 431 and *Lactobacillus rhamnosus* CRL 1224 as biological control for *Aspergillus flavus* strains. *J Food Prot* 69:2544–8.

Caplice E, Fittzgerald GF. 1999. Food fermentations: role of microorganisms in food production and preservation. *Int J Food Microbiol* 50:131–49.

Castro SM, Saraiva JA, Lopes-da-Silva JA, Delgadillo I, Van Loey A, Smout C, Hendrickx M. 2008. Effect of thermal blanching and high pressure treatments on sweet green and red bell peppers fruits (*Capsicum annum* L.). *Food Chem* 107:1436–49.

Choi IK, Jung SH, Kim BJ, Park SY, Kim J, Han HU. 2003. Novel *Leuconostoc citreum* starter culture system for the fermentation of kimchi, a fermented cabbage product. *Antonie van Leeuwenhoek* 84:247–53.

Corsetti A, Gobbetti M. 2002. *Lactobacillus plantarum*. In: Roginski H, Fuquay W, Fox PF, editors. *Encyclopedia of dairy sciences*. Missouri, USA: Academic Press, Ltd. p. 1501–7.

Deepa N, Kaur C, Binoy G, Balraj S, Kapoor HC. 2007. Antioxidant constituents in some sweet pepper (*Capsicum annum* L.) genotypes during maturity. *Lebensm Wiss Technol* 40:121–9.

Di Cagno R, Surico RF, Minervini G, De Angelis M, Rizzello CG, Gobbetti M. 2009b. Use of autochthonous starters to ferment red and yellow peppers (*Capsicum annum* L.) to be stored at room temperature. *Int J Food Microbiol* 130:108–16.

Di Cagno R, Surico RF, Paradiso A, De Angelis M, Salmon JC, Buchin S, De Gara L, Gobbetti M. 2009a. Effect of autochtonous lactic acid bacteria starters on health promoting and sensory properties of tomato juices. *Int J Food Microbiol* 128:473–83.

Di Cagno R, Surico RF, Siracusa S, De Angelis M, Paradiso A, Minervini F, De Gara L, Gobbetti M. 2008. Selection of autochthonous mixed starter for lactic acid fermentation of carrots, French beans or marrows. *Int J Food Microbiol* 127:220–8.

Draksler D, González SN, Oliver G. 2004. Preliminary assays for the development of a probiotic for goats. *Reprod Nutr Dev* 44:397–405.

Fegan N, Desmarchelier P. 2002. Comparison between human and animal isolates of Shiga toxin-producing *Escherichia coli* O157 from Australia. *Epidemiol Infect* 128:357–62.

Florianowicz T. 2001. Antifungal activity of some microorganisms against *Penicillium expansum*. *Eur Food Res Technol* 212:282–6.

Frazier W, Westhoff D. 1993. Microbiología de los alimentos. Acribia, S.A. Zaragoza, Spain.

Gardner NJ, Savard T, Obermeier P, Caldwell G, Champagne CP. 2001. Selection and characterization of mixed starter cultures for lactic acid fermentation of carrot, cabbage, beet and onion vegetable mixtures. *Int J Food Microbiol* 64:261–75.

Harris JR. 1996. Ascorbic acid: biochemistry and biomedical cell biology. In: Harris JR, editor. *Subcellular biochemistry*. New York: Plenum Press. p. 25.

Jägerstad M, Jastrebova J, Svensson U. 2004. Folates in fermented vegetables—a pilot study. *Lebensm Wiss Technol* 37:603–11.

Jiang J, Kubota K. 2001. Formation by mechanical stimulus of the flavor compounds in young leaves of Japanese pepper (*Xanthoxylum piperitum* DC.). *J Agric Food Chem* 49:1353–7.

Khuntia A, Chaudhary LC. 2002. Performance of male crossbred calves as influence by substitution of grain by wheat bran and the addition of lactic acid bacteria to diet. *Asian-Australian J Anim Sci* 50:217–26.

Klaenhammer TR, Barrangou R, Buck BL, Azcarate-Peril MA, Altermann E. 2005. Genomic features of lactic acid bacteria effecting bioprocessing and health. *FEMS Microbiol Rev* 29:393–409.

Lee J, Jang J, Kim B, Kim J, Jeong G, Han H. 2004. Identification of *Lactobacillus sakei* and *Lactobacillus curvatus* by multiplex PCR-based restriction enzyme analysis. *J Microbiol Methods* 59:1–6.

Luchese RH, Harrigan WF. 1990. Growth of and aflatoxin production by *Aspergilus parasiticus* when in the presence of either *Lactococcus lactis* or lactic acid and at different initial pH values. *J Appl Bacteriol* 69:512–9.

McDonald P, Henderson AR, Heron SJE. 1991. *The biochemistry of silage*. 22nd ed. Marlow, UK: Chalcombe Publications.

Mercier J, Baka M, Reddy B, Corcuff R, Arul J. 2001. Short-wave ultraviolet irradiation for control of decay caused by *Botrytis cinerea* in bell pepper: induced resistance and germinicidal effects. *J Am Soc Hortic Sci* 126:128–33.

Muñoz R, Arena ME, Silva J, González SN. 2010. Inhibition of mycotoxin-producing *Aspergillus nomius* vsc 23 by lactic acid bacteria and *Saccharomyces cerevisiae. Brazilian J Microbiol* 41:1019–26.

Namiki M. 1990. Antioxidants/antimutagenes in food. *CRC Crit Rev Food Sci Nutr* 29:273–300.

Navarro JM, Flores P, Garrido C, Martinez V. 2006. Changes in the contents of antioxidant compounds in pepper fruits at different ripening stages, as affected by salinity. *Food Chem* 96:66–73.

Nichols RL, Smith JW. 1994. Anaerobes from a surgical perspective. *Clin Infect Dis* 18:S280–6.

Pagan Riestra S. 2006. Caracterización del proceso fermentativo de ensilajes de residuos orgánicos de plantas procesadoras de piña (*Ananas comosus*) y china (*Citrus sinensis*) y su evaluación en dietas para ovinos. [Thesis magister]. Puerto Rico University.

Perera MF, Arena ME, Manca de Nadra MC. 2004. Proceso de fermentación de pimientos. V Jornadas Científicas y Encuentro de Jóvenes Investigadores Universidad Nacional de Tucumán.

Pitt JI, Hocking AD. 1997. *Fungi and food spoilage*. 2nd ed. London: Blackie.

Plessi M, Bertelli D, Miglietta F. 2002. Effect of Microwaves on Volatile Compounds in White and Black Pepper. *LWT - Food Sci Technol* 35:260–264.

Seseña S, Palop MLl. 2007. An ecological study of lactic acid bacteria from Almagro eggplant fermentation brines. *J Appl Microbiol* 103:1553–61.

Seseña S, Sanchez Hurtado I, González Vinas MA, Palop L. 2001. Contribution of starter culture to the sensory characteristics of fermented Almagro eggplants. *Int J Food Microbiol* 67:197–205.

Spyropoulou KE, Chorianopoulos NG, Skandamis, PN, Nycha GJ. 2001. Survival de *Escherichia coli* O157:H7 during the fermentation of Spanish style green olives (conserveola variety) supplemented with different carbon sources. *Int J Food Microbiol* 66:3–11.

Stiles ME. 1996. Biopreservation by lactic acid bacteria. *Antonie van Leeuwenhoek* 70:331–45.

Tamminen M, Joutsjoki T, Sjöblom M, Joutsen M, Palva A, Ryhänen E-L. 2004. Screening of lactic acid bacteria from fermented vegetables by carbohydrate profiling and PCR-ELISA. *Lett Appl Microbiol* 39:439–44.

Vicente AR, Pineda C, Lemoine L, Civello PM, Martinez GA, Chaves AR. 2005. UVC treatments reduce decay, retain quality and alleviate chilling injury in pepper. *Postharvest Biol Technol* 35:69–78.

Vogel RF, Lohmann M, Nguyen M, Weller AN, Hammes WP. 1993. Molecular characterization of *Lactobacillus curvatus* and *Lactobacillus sake* isolated from sauerkraut and their application in sausage fermentations. *J Bacteriol* 74:295–300.

Weinberg ZG, Muck RE, Weimer PJ. 2003. The survival of silage inoculants lactic acid bacteria in rumen fluid. *J Appl Microbiol* 94:1066–71.

Yusof S, Shian LS, Osman A. 2000. Changes in quality of sugar-cane juice upon delayed extraction and storage. *Food Chem* 68:395–401.

20

Fermented Red Beet Juice

Zsolt Zalán, Anna Halász, and Ágnes Baráth

CONTENTS

20.1 Introduction

Red beet (*Beta vulgaris*, also known as beetroot, table beet, garden beet, or blood turnip) is a popular vegetable all over the world. This vegetable plant is a considerable source of vitamins C and B (B1, B2, and B6), minerals (such as calcium, iron, potassium, magnesium, phosphorus), and moreover, it contains a relatively high level of folic acid (Wang and Goldman 1997; Váli et al. 2007; Sárdi et al. 2009). However, the most important bioactive agents of the red beet are the water-soluble plant pigments, the betalains. These nitrogen-containing pigments, which are synthesized from the amino acid tyrosine, are composed of two main groups, the red betacyanins and the yellow betaxanthins. The red beet betalains contain two major soluble pigments, the betanin (red) and the vulgaxanthine I (yellow; Azeredo 2009). Betanin is the main coloring component present in the food color additive, E-162. Although the tops of the red beet plant can be cooked or served fresh as greens, the root is the most valuable part of the plant, which may be eaten fresh or pickled for salads or cooked whole, then sliced or diced, and moreover, it can be consumed as a juice or in fermented form.

The fermentation of vegetables is an ancient practice that has been applied by Asian and Mediterranean people for the past 4000 years (Hulse 2004). The original purpose for the fermentation of raw materials was to preserve it, and this was accomplished with naturally occurring fermentations because the ancient people were not aware of the role of the microorganisms in this process. The knowledge and the methodologies of the production of ancient fermented foods were handed down from generation to generation. Nevertheless, this preservation process, and the fermented vegetables themselves, have spread all over the world and after millennia they could be found among our meals; for example, sauerkraut and olives from the Western World, gari from West-Africa, kimchi from Korea, gundruk from Nepal, or sunki from Japan. The lactic acid bacteria (LAB) not only preserves the raw material, but also makes some plant material more digestible, thus improving the nutritional properties of the food and moreover developing the flavor and aroma, creating a higher value product.

Fermentation depends considerably on the biological activity of microorganisms. The raw material (vegetables) provides the substrate for the LAB, which excrete a range of microbial metabolites; therefore, both

the type of substrate and the genera of bacteria and their enzyme activity considerably influence the quality of the end product. LAB could produce energy for their activity or for propagation through lactofermentation. The uptaken or intracellularly hydrolyzed carbohydrates can be fermented by lactobacillus strains through two major pathways: glycolysis (Embden–Meyerhof pathway) is used by the homofermentative LAB, and the 6-phosphogluconate/phosphoketolase pathway is used by heterofermentative LAB. During the fermentation of carbohydrate, when the sugars are converted to cellular energy, LAB excrete lactate as a metabolic by-product. Generally, lactic acid is the predominant end product (always by the obligate homofermentative strains), but under various conditions, the amount of other by-products (acetic and other acids, alcohol, and carbon dioxide) could be increased by the facultative homofermentative strains, whereas the obligate heterofermentative strains always produce significant amounts of by-product, other than lactic acid. The acids produced play an important role in the preservation of the product as well as enhancing the shelf life and microbiological safety of the fermented food. Aside from organic acids (lactic, acetic, formic, caproic, propionic, butyric, and valeric acids), LAB can produce several other antimicrobial components, such as hydrogen peroxide (Rodrígues et al. 1997; Ito et al. 2003), carbon dioxide, alcohol, diacetyl (Ammor et al. 2006), and proteinaceous, ribosomally synthesized antimicrobial compounds, so-called bacteriocins (Cleveland et al. 2001; Plocková et al. 2001), which also take part in the preservation as well as forming altogether synergistically the microbiological safety of the product. Beyond preservation, acids have remarkable effects on the organoleptical properties of the product; however, in the formation of flavor, several other metabolites play a role. Depending on the enzymes present in the bacterial strains, different flavors can develop because of the contribution of many enzymes, which lead to various flavor compounds and, in such a way, different products from the point of view of aroma. In LAB, pyruvate is a starting molecule for the formation of short-chain flavor compounds such as acetaldehyde, acetate, acetoin, diacetyl, and ethanol. Lactobacilli can also metabolize citrate to produce acetoin, acetolactate, and diacetyl (Marilley and Casey 2004). The amino acid–converting enzymes of LAB can also play an essential role in the flavor development of the product. Amino acids in the vegetable substrate may contribute to the production of such flavor and aroma compounds such as aldehydes, acids, alcohols, esters, and sulfur compounds (Ayad et al. 2001). Beyond preservation and aroma formation, LAB could also detoxify the substrate. Vegetables provide one of the main sources for nitrate and nitrite intake in human nutrition. The red beet, leafy greens, kohlrabi, and radish are considered as high–nitrate accumulation plants (Nagy-Gasztonyi et al. 2006). During fermentation, LAB can reduce or, in some cases, completely remove nitrites from the substrate (Heród-Leszczyńska and Miedzobrodzka 1992; Walkowiak-Tomczak and Zielińska 2006). Many microorganisms, such as Enterobacteriaceae and certain *Lactobacilli*, *Pediococci*, and *Enterococci*, are particularly active in the formation of biogenic amines. The amount and type of amine formed in a food depends on the nature of the product and the microorganisms present. The lactofermented food products contain considerable amounts of putrescine, cadaverine, histamine, and tyramine (Santos 1996). Amine production of bacteria depends on the amino acid decarboxylase activity of that certain strain. Some biogenic amines are indispensable components of living cells; however, the consumption of foods that contain high concentrations of biogenic amines may have a toxicological effect. As this short introduction has shown, the activity and metabolite production of LAB strongly influence the quality of the product and have a great effect on the shelf life, taste, digestibility, and safety of fermented food.

Spontaneous fermentation could result in a unique product with appropriate properties; however, it is unrepeatable, the quality of the product is changing and the safety of the product is questionable because of the lack or absence of control in the process. Constant and appropriate quality is absolutely necessary for the application of a selected starter culture with good fermentation properties and low amine production.

20.2 Starter Culture Selection

For the lactofermentation of red beet, the most applicable LAB are the strains from the *Lactobacillus* genus because the members of this genus can be found in many different habitats, as well as on the plants and plant materials themselves, and they are already used as starter cultures for different fermented foods (cheese, meat, sourdough, etc.). Several lactobacilli strains are also regarded as probiotics and are applied in probiotic food (Stiles and Holzapfel 1997; Holzapfel and Schillinger 2002).

20.2.1 Biogenic Amine Production

Aside from the several positive effects of LAB in food preservation, their ability to decarboxylize amino acids and produce biogenic amines is also very important in food production. These biogenic amines may be formed and degraded during the normal metabolism of animals, plants, and microorganisms. In food, they may result either from endogenous amino acid decarboxylase activity in food materials or from the activity of decarboxylase-positive bacteria. Depending on the spoilage association (e.g., Enterobacteriaceae), microbial deterioration of food may be accompanied by an increased production of amino acid decarboxylases. Experimental data indicates a correlation between bacterial numbers and the biogenic amine content of particular foods. This has been intensely studied for fish, meat, and some vegetables. Several authors have published data about fermented foods such as sauerkraut, cheese, sausage, beer, and wine (Stratton et al. 1991; Halász et al. 1994, 1999a,b; Leitão et al. 2000; Kalač et al. 2002; Suzzi and Gardini 2003). Concentrations of biogenic amines show extreme variations in traditional "spontaneously" fermented food and beverages. Quality criteria, with respect to the presence of histamine and other biogenic amines in foods and food products, are necessary from the toxicological point of view and also from a technological aspect. There is evidence of the involvement of histamine and tyramine as causative agents in food poisoning. Because histamine has one of the highest biological activities of all amines, its production is of particular interest. From the point of view of good manufacturing practices, levels of 50 to 100, 100 to 800, and 30 mg/kg for histamine, tyramine, and β-phenylethylamine, respectively, or a total of 100 to 200 mg/kg, are regarded as acceptable. Some countries have regulated the maximum amounts of specific products allowable. In Switzerland, 10 mg of histamine per liter of wine is the permissible limit; in the United States, 50 mg/100 g fish indicates a danger to health. The European Union established legislative limits only for histamine in fish (Maintz and Novak 2007). Amine production of bacteria depends on the amino acid decarboxylase activity of that certain strain. Halász et al. (1994) measured histidine activity levels of 3.7×10^{-2} mol/min for *Lactobacillus brevis* var. *lindneri*, and in *Lactobacillus plantarum*, it was 7.6×10^{-2} mol/min. The highest activities were detected in the stationary growth phase. This result is in agreement with the findings of Künsch et al. (1990), who found that a significant accumulation of histamine and tyramine only occurred in the final period of sauerkraut fermentation. However, as redox potential also influences histidine decarboxylase activity, conditions resulting in reduced redox potential stimulate amine formation, and histidine decarboxylase activity seems to be inactivated or destroyed in the presence of oxygen. Amine synthesis is also influenced by stress conditions. Cold-shocked *Lb. plantarum* increased amine synthesis in comparison with untreated cells (Amal Ahmed 1996). Because some people suffer adverse reactions after consuming amines, the production of fermented foods with predictably low levels of specific amines needs to be addressed by the food industry. It is possible to select starter culture strains with low amine production, no histamine synthesis, and reduced tyramine secretion.

The authentic LAB must be investigated for their biogenic amine production. Aside from histamine and tyramine, the total amine synthesis also has to be considered. Only low amine producers should be considered for further selection, and should include the ability to grow on red beet as the only substrate, acid production, speed of pH reduction, and last but not least, hydrogen peroxide synthesis. Halász et al. (1994, 1999a,b) found that the amino acid decarboxylase enzyme activity of bacteria used as starter cultures varied widely, and some of them even produce histamine (Table 20.1). Despite this, the biogenic amine contents of spontaneously fermented samples were compared with a potential starter culture–fermented red beet; in the spontaneously fermented sample, the amount of total biogenic amine was five to seven times greater and histamine level contents were also detected (Table 20.2). This demonstrates that the application of a well-chosen starter culture is preferable to spontaneous fermentation.

20.2.2 Growth and Acidification

One of the most important properties of a starter culture is fast growth on the raw material to quickly reach an appropriately high cell count. The cells of starter cultures with fast growth, on the one hand, compete with the other coexisting microbes for substrates and a niche and, on the other hand, remove fermentable carbohydrates from the raw material; in this way, preventing the growth of other microbes. At the same time, during its growth, the starter culture produces several metabolic end products from

TABLE 20.1

Biogenic Amine Production of Selected Strains (μg/mL)

	Biogenic Amines, Produced in MRS							
Strain	**PUT**	**HIST**	**CAD**	**SPED**	**AGM**	**SPER**	**TYRM**	**Σ**
Lb. fermentum DT 41	0.50	TR	TR	1.45	0.10	0.60	1.85	4.50
Lb. acidophilus N2	TR	TR	TR	2.83	ND	ND	3.42	6.25
Lb. plantarum 2142	TR	ND	TR	0.45	ND	0.38	0.54	1.37
Lb. casei-casei 2750	0.22	ND	0.19	0.14	TR	0.25	0.56	1.36
Lb. casei-casei 2752	TR	ND	TR	0.26	ND	0.20	0.98	1.44
Lb. curvatus 2770	0.70	ND	TR	0.33	0.38	0.67	6.20	8.28
Lb. curvatus 2775	0.26	ND	TR	0.30	0.3	0.78	6.43	8.2
Lb. plantarum 2739	0.52	0.20	0.86	1.40	1.78	0.90	12.74	18.40

Source: Halász, A. and Z. Zalán, Akadémiai Kiado, 71–85, 2009. With permission.

which the greatest amount of organic acids are produced. These acids, during the fast-growing phase, cause quick acidification and therefore the decrease in pH is another good sign of the process of fermentation. The low pH and the undissociated organic acids also have an inhibitory effect on many organisms. According to our results, the *Lactobacillus* strains grow well on red beet juice. From the initial 10^6 CFU/mL level, the viable cell count reached 1.6 to 4.3 × 10^8 CFU/mL during 32 hours at 30°C (Baráth et al. 2004). Other authors reported that the cell count of lactobacilli could even reach 10^9 CFU/mL after 48 hours of fermentation at 30°C (Yoon et al. 2005), 9.48 log CFU/mL on sliced red beet at 20°C after 4 days (Walkowiak-Tomczak and Zielińska 2006), and 10.7 log CFU/mL in juice after 8 hours of incubation at 37°C if the initial cell count is high enough (8.71 log CFU/mL, according to Buruleanu et al. 2009); however, in this case, a rapid cell count decrease could then be observed. It can be established that the type of substrate (chopped red beet or juice) and the *Lactobacillus* strains used only slightly influence the reachable cell count, rather the parameters of the incubation significantly influence the final cell number. In contradistinction to this, the pH changes not only according to the strain but also the variety of the red beet (Table 20.3). The pH decrease depends considerably on the acid production (the enzyme activity of the cells). The different qualities and quantities of the acids produced could cause different decreases in the pH of the environment, even at the same cell count. Thus, in the metabolic pathways, the fermentation profile of the strain plays an important role in acidification. The decrease in pH was faster in every case in which starter cultures were applied compared with the spontaneously fermented samples. The pH of the fermented juice decreased from the initial values of 5.5 to lower than 4 at 24 hours of fermentation. Yoon et al. (2005) reported the same results when the pH decreased to less than 4.5 from the initial 6.3 after 48 hours. Because rapid acidification is essential for the microbial stability of the product, for the selection of the starter culture, this property of the strain is very important. Aside from the low pH, the remaining undissociated weak acids also have an antimicrobial effect, an effect which could be increased by the production of hydrogen peroxide.

TABLE 20.2

Biogenic Amine Content in the Spontaneously and with a Starter Culture Fermented Product (μg amine/mL brine)

	Biogenic Amines							
Strains	**PUT**	**HIST**	**CAD**	**SPD**	**AGM**	**SPER**	**TYRM**	**Σ**
Lb. plantarum	0.89	ND	ND	5.10	4.49	6.10	ND	16.58
Lb. brevis var. *lindneri*	7.22	ND	0.49	6.14	4.40	6.11	ND	24.36
Spontaneous fermentation	6.42	3.22	29.38	7.01	14.17	5.17	60.04	125.51

Source: Halász, A. and Z. Zalán, Akadémiai Kiado, 71–85, 2009. With permission.

TABLE 20.3

pH Drop in Fermented Red Beet after 24 Hours

	LAB Strains			
Beet Variety	***Lb. plantarum* 2142**	***Lb. curvatus* 2770**	***Lb. casei-casei* 2745**	**Control**
Bonel	3.85	3.64	3.82	5.45
Bolivar	3.90	3.15	3.87	5.55
Pablo	3.70	3.62	3.72	5.32

Source: Halász, A. and Z. Zalán, Akadémiai Kiado, 71–85, 2009. With permission.

20.2.3 Hydrogen Peroxide Production

Lactobacilli are able to generate hydrogen peroxide in the presence of oxygen by flavoprotein oxidase, nicotinamide adenine dinucleotide (NADH) oxidase, and pyruvate oxidase, which then accumulates because of the lack of catalase in lactobacilli (Murphy and Condon 1984). Hydrogen peroxide is a strong oxidizing agent and thus is a well-known antibacterial component (Ouwehand 1998; Ito et al. 2003). However, it only possesses short-lived action because hydrogen peroxide reacts with the organic material of the environment. This oxidizing effect can cause bleaching of colored components of red beet and, in this way, causes undesired loss of the color of the product aside from the degradation of the antioxidant components. Betalains, the biologically active pigments of red beet, are also sensitive to the oxidative effect of hydrogen peroxide (Wasserman et al. 1984). Because of this, the starter strains for red beet juice fermentation should not form hydrogen peroxide or only at low concentrations. The difference in the production of hydrogen peroxide between the spontaneous and starter culture fermentations is significant (Table 20.4).

20.2.4 Antimicrobial Activity

The original purpose of fermentation was the preservation of the raw material. The growth and antimicrobial activity of the starter culture plays the greatest role in this preservation effect. The best starter cultures have a wide antimicrobial activity against spoilage bacteria, yeasts, and molds. This activity is very important because the surface of the raw material contains a wide range of microbes. According to our investigations, 2.3×10^7 to 7.5×10^8 CFU live cells, 0.9 to 9.3×10^2 CFU coliforms, and 1.7×10^5 to 3.4×10^6 CFU molds and yeasts, and also Enterobacteriaceae can be found on the surface of different varieties of red beet. Walkowiak-Tomczak and Zielińska (2006) found 2.07 log CFU/g of mesophilic bacteria on fresh red beet root and, in some cases, 1.52 log CFU/g of molds and yeasts. The number of microbes on the surface of red beets is influenced by several external parameters such as the cropland, the weather, and we have even obtained slight differences between varieties of red beet. Nevertheless, these microbes can contaminate the prepared red beet raw material. Klewicka et al. (2004) reported mesophilic bacteria at a concentration of 10^3 CFU/mL, as well as yeasts and molds in small quantities in fresh beet juice. LAB are able to inhibit the growth of many pathogens and food spoilage microorganisms, such as *Escherichia coli*, *Listeria monocytogenes*, *Salmonella enteritidis*, and molds (Harris et al. 1989; Schillinger et al. 1991; Corsetti et al. 1998; Park et al. 2005). According to our investigations, the potential starter cultures show various inhibitory activities against food-borne microorganisms (Table 20.5). However, some strains, which have a relatively broad inhibitory activity against bacteria and molds can also be found (Hudáček et al. 2007).

TABLE 20.4

Hydrogen Peroxide Production of LAB on Red Beet

	Incubation Time (hours)			
	2	**24**	**48**	**72**
Spontaneous	2.07	1.93	1.79	1.63
Lb. plantarum 2142	0.34	0.34	0.33	0.33
Lb. curvatus 2770	0.58	0.51	0.60	0.57

Source: Halász, A. and Z. Zalán, Akadémiai Kiado, 71–85, 2009. With permission.

TABLE 20.5

Antimicrobial Spectrum of Some Potential Starter *Lactobacillus* Strains

		Antimicrobial Activity							
Indicator Microorganism	**Sign of Strain**	***Lb. fermentum* DT41**	***Lb. acidophilus* N2**	***Lb. plantarum* 2739**	***Lb. plantarum* 2142**	***Lb. casei-casei* 2750**	***Lb. casei-casei* 2752**	***Lb. curvatus* 2770**	***Lb. curvatus* 2775**
Listeria innocua	DSM 2257	–	–	+	–	–	+	–	–
Staphylococcus aureus	ATTC 14438	–	–	–	–	–	–	–	–
Enterobacter		–	–	–	–	–	–	–	–
Enterobacter	10	–	–	–	–	+	+	+	+
Bacillus		+	±	–	–	–	–	–	–
E. coli	2	–	–	–	+	+	+	–	+
Pseudomonas		±	–	–	+	+	+	+	+
Pseudomonas	7	+	+	+	–	+	–	–	+
Klebsiella oxtocoa	4	–	–	–	–	+	+	–	+
Alcaligenes	8	–	–	–	–	+	–	–	–
Enterococcus		–	–	–	+	–	+	+	+
Micrococcus	5	+	+	+	+	+	+	+	+
Asp. flavus	31	–	–	–	+	–	–	–	–
Asp. parasiticus	1039	–	–	–	–	–	–	–	+

Note: –, no inhibitory effect; ±, weak inhibitory effect; +, strong inhibitory effect.

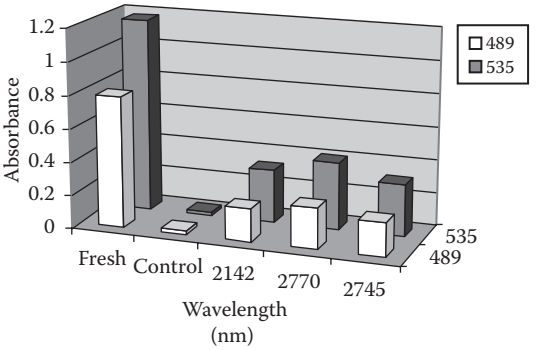


FIGURE 20.1 Effect of lactofermentation on pigment components of red beet (Pablo variety). (With kind permission from Springer Science+Business Media: Eur Food Res Technol, 218, 2004, 184–7, Baráth, Á., A. Halász, E. Németh, Zs. Zalán, Fig. 1.)

20.2.5 Changes in Betalains

Because the betalains are the most important bioactive component of the red beet, the investigation of the effect of starter culture on this component is also very important. The changes in the absorbances of the red and yellow pigments unambiguously show the effects on the betalains. Investigating the absorbances at 535 and 489 nm, a great decrease could be observed in the fermented samples in comparison with the initial values (Figure 20.1). The decrease was the greatest in the spontaneously fermented (control) sample. Among the different *Lactobacillus* strains, no significant difference could be detected, which is in agreement with their similar hydrogen peroxide production. Betalains are also partly decolorized because of the low pH, and furthermore, the red beet has several endogenous enzymes such as polyphenoloxidases and beetroot cell wall–associated peroxidase enzymes, which may cause the degradation of betalain and loss of color (Lashley and Wiley 1979; Azeredo 2009). These peroxidases make contact with their substrates after slicing and, as the cell wall destruction is more intensive during maceration, the decolorization is greater under optimal circumstances for peroxidase.

20.2.6 Viability

Because several lactobacilli strains are regarded as probiotics and their beneficial effects are connected with their live forms, the living cells of the starter *Lactobacillus* strains and their viability after fermentation (during storage until consumption) is also important. The strains show various viabilities, the cell counts of several strains decreased to the initial level during storage at 4°C for 4 weeks, but some strains remained at 10^8 CFU/mL after 4 weeks (Baráth et al. 2004; Yoon et al. 2005).

20.3 The Process of Fermentation

The first step in fermentation is the preparation of the raw material for inoculation with the starter culture. As previously mentioned, the surface of the harvested red beet root contains a wide range of numerous microorganisms, and thus the elimination of soil and microbes from the surface of the roots by washing is the first important step. This is followed by the peeling of the root and a repeat washing. The cleaned red beet root, after chopping, can be used directly in this form or a juice could be made from it. Heat treatment (blanching or pasteurization) can be considered before the inoculation. On the one hand, with this step, the microbial safety of the product can be increased and the chopped form will be softer, but on the other hand, the heat treatment can cause change in the color, the red-violet can turn to brown and the activity of the bioactive molecules could vanish. However, good manufacturing practice—what is necessary during the whole process—can produce a microbiologically safe raw material without heat

treatment. The juice can be produced both mechanically by centrifuge and enzymatically by liquefaction. For this enzymatic procedure, the red beet root should be mixed with the same amount of water and, after heat treatment at 80°C for 2 minutes, cooled down to 40°C. A macerating-type pectolytic enzyme mixture should be added to this mixture and treated at 40°C for 2 hours. Then, the reaction should be stopped by heating at 80°C and the mixture homogenized and the pH adjusted. For the fermentation of red beet slices, these should be filled up with brine before the inoculation. After the preparation of red beets, the next step is inoculation with the starter culture. The population of the starter culture should be at the exponential growth phase, thus culturing for one night is optimal. The amount of inoculum is usually 1% to 2% of the volume of the raw material. The initial cell concentration in the product should be at least 10^6 cell/mL juice/brine to guarantee fast growth and rapid acidification to keep down the other potential microbes present. The optimal incubation temperature is 30°C to 37°C. The optimal incubation time depends on the temperature and the initial cell concentration, but at 30°C, the 10^6 cell/mL initial cell concentration and pH drop reaches the maximum after 32 to 48 hours.

20.4 Health Benefits of Fermented Products

Both the red beet and the potential probiotic starter culture have in themselves beneficial health effects on human consumers. Hippocrates, Galen, Avicenna, and Paracelsus have previously used red beet in the treatment of several gastrointestinal diseases, fevers, and anemia, as well as in wound healing (Sárdi et al. 2009). Red beet has traditionally been used in folk medicine because it has been considered to have blood-forming and antitumor properties. The most important bioactive components of the red beet are the betalains. Similar to other natural plant colorants, betalains have a wide range of desirable biological activities. Betalains are radical scavengers and show antioxidant activity, whereby they prevent active oxygen-induced and free radical–mediated oxidation of biological molecules (Pavlov et al. 2002). Together with the antioxidant activity, the betalains show anti-inflammatory, hepatoprotective, and anticancer properties (Georgiev et al. 2010); therefore, their presence in the human diet may reduce the risk of cancer, cardiovascular disease, and other diseases associated with aging (Pavlov et al. 2005). Kapadia et al. (1996) found that beetroot contains one of the most useful, natural, cancer-preventive agents. In the red beet, the main betalains are betanin (red) and vulgaxanthine I (yellow). According to previous investigations, there is a very close relation between the content of red pigments and the antioxidant capacity of red beet, which shows that the red betanin is primarily responsible for the antioxidant activity, whereas a less strict relationship was found between the yellow pigments and the antioxidant capacity (Czapski et al. 2009). The stability of these pigments is low; nevertheless, the betalains remain stable in the gastrointestinal tract without any significant loss in their antioxidative properties (Georgiev et al. 2010); however, particularly during heating and storage, they can degrade and lose their activity. Therefore, lactic acid fermentation can be an alternative method to preserve (even partially) the active components. The red beet can accumulate a significant amount of nitrates which, after consumption, can easily be reduced to nitrites in the human organism and can be hazardous to human health. Nitrite can react with secondary amines and result in nitroso-amino compounds. This reaction may occur either during food processing, in technological processes, or in the digestive tract (Halász and Bartáth 1998). At the same time, recent findings show that consumption of increased nitrate from red beet juice improves brain function and reduces blood pressure and the occurrence of cardiovascular diseases (Ahluwalia 2010; Presley et al. 2010). Furthermore, it should be mentioned that the red beet—as the vegetable generally—is the source of several important minerals, vitamins (see the Introduction), and fibers.

Among the lactobacilli, there are several probiotic strains. After a circumspect selection, the probiotic strain can be used as starter culture; however, the starter culture can also show probiotic properties. The established benefits of probiotic strains are that they can improve the health of the consumers by their modulation of gut microflora composition (blocking of adhesion of pathogens and production of antimicrobial agents), antidiarrheal capabilities, enhancing the immune system function, and several other potential benefits which have been assigned to probiotics, such as preventing the occurrence of inflammatory bowel disease, reducing the risk of colorectal cancer, and hypocholesterolemic effects (Tuohy et al. 2003; Rastall et al. 2005; Parvez et al. 2006).

The health benefits of fermented foods have been well known for centuries. As early as AD 76, the Roman historian Plinio proposed the consumption of fermented milks for treating gastrointestinal infections (Stanton et al. 2005); however, the scientific establishment of the beneficial effects of fermented foods began with Metchnikoff in the early 1900s. The health benefits of fermented red beet derive from the above-mentioned beneficial effects of red beet and LAB. These good properties exert their effect not only additively, but the LAB also protect the beneficial components of red beet and moreover can increase their effect. It was observed that the lactofermented red beet juice showed stronger antioxidant properties compared with the fresh juice (Walkowiak-Tomczak and Zielińska 2006). Klewicka et al. (2009) found that the intake of fermented beetroot juice containing live *Lactobacillus* cells positively modulated the cecal microflora and its metabolic activity in rats. If we use proven probiotic strains as starter culture or give probiotic strains in addition to the fermented red beet, the product has an increased health-promoting effect. Because most of the probiotic foods are dairy products, the fermented, probiotic red beet represents a perfect alternative to dairy products for consumers who do not want to eat milk-based food or are lactose intolerant or allergic to milk proteins.

20.5 Concluding Remarks

Lactofermented red beet combines the beneficial effects of the mineral-, vitamin-, and fiber-rich, high antioxidant source red beet with the health-promoting effects of LAB and results in a well-digestible, preservative-free, minimally or nontreated functional food with several health benefits. For such a product, a carefully selected starter culture is required, screened for biogenic amine and hydrogen peroxide production, growth, and antimicrobial properties. Mainly, red beet juice (e.g., Beet Juice by Biotta (Tägerwilen, Switzerland) and Bio Red Beet Juice by Dr. Steinberger (Unkel, Germany)) and red beet powder made from juice (e.g., ^{AIM}RediBeets (Tucson, AZ), Whole beet plant juice tablets by Sonne's (Kansas City, MO), Beet Juice POWder by Eclectic Institute (Sandy, OR), and Beet Juice Powder by Pines International, Inc. (Lawrence, KS)) are present in the market, and to a smaller degree, the pickled form of red beet can be found. Nevertheless, the fermented form of red beet is not widespread, although knowledge of the health benefits of lactofermented red beets should be increased.

REFERENCES

Ahluwalia A. 2010. Dietary nitrate intake in the form of beetroot juice shown to be an effective strategy to combat the prevalence of CVD. *Nutr Res Newslett*, 2010.

Amal Ahmed HM. 1996. Effect of heat shock treatment on heat resistance of microorganisms and biogenic amine synthesis. [DPhil dissertation]. Budapest: Hungarian Academy of Sciences. 93 p.

Ammor S, Tauveron G, Dufour E, Chevallier I. 2006. Antibacterial activity of lactic acid bacteria against spoilage and pathogenic bacteria isolated from the same meat small-scale facility 1—screening and characterization of the antibacterial compounds. *Food Control* 17:454–61.

Ayad EHE, Verheul A, Engels WJM, Wouters JTM, Smit G. 2001. Enhanced flavour formation by combination of selected lactococci from industrial and artisanal origin with focus on completion of a metabolic pathway. *J Appl Microbiol* 90:59–67.

Azeredo HMC. 2009. Betalains: properties, sources, applications, and stability—a review. *Int J Food Sci Technol* 44:2365–76.

Baráth Á, Halász A, Németh E, Zalán Zs. 2004. Selection of LAB strains for fermented red beet juice production. *Eur Food Res Technol* 218:184–7.

Buruleanu LC, Nicolescu CL, Gorghiu G, Bratu MG, Avram D, Manea I. 2009. Lactic acid fermentation of carrot and red beet juices by probiotic bacteria. *Bull UASVM Agr* 66:252–8.

Cleveland J, Montville TJ, Nes IF, Chikindas ML. 2001. Bacteriocins: safe, natural antimicrobials for food preservation. *Int J Food Microbiol* 71:1–20.

Corsetti A, Gobetti M, Rossi J, Daminiani P. 1998. Antimould activity of sourdough lactic acid bacteria: identification of mixture of organic acids by *Lactobacillus sanfrancisco* CB1. *Appl Microbiol Biotechnol* 50:253–6.

Czapski J, Mikołajczyk K, Kaczmarek M. 2009. Relationship between antioxidant capacity of red beet juice and contents of its betalain pigments. *Pol J Food Nutr Sci* 59:119–22.

Georgiev VG, Weber J, Kneschke EM, Denev PN, Bley T, Pavlov AI. 2010. Antioxidant activity and phenolic content of betalain extracts from intact plants and hairy root cultures of the red beetroot *Beta vulgaris* cv. Detroit Dark Red. *Plant Food Hum Nutr* 65:105–11.

Halász A, Baráth Á, Holzapfel WH. 1999a. The biogenic amine content of beer; the effect of barley, malting and brewing on amine concentration. *Z Lebensm Unters Forsch A* 208:418–23.

Halász A, Baráth Á, Holzapfel WH. 1999b. The influence of starter culture selection on sauerkraut fermentation. *Z Lebensm Unters Forsch A* 208:434–8.

Halász A, Baráth Á, Simon-Sarkadi L, Holzapfel WH. 1994. Biogenic-amines and their production by microorganisms in food. *Trends Food Sci Technol* 5:42–9.

Halász A, Bartáth Á. 1998. Biogenically active amines in food. II. Cost 917. Brussels, Belgium: European Communities Publications. p. 1–7.

Harris LJ, Daeschel MA, Stiles ME, Klaenhammer TR. 1989. Antimicrobial activity of lactic acid bacteria against *Listeria monocytogenes*. *J Food Prot* 52:384–7.

Heród-Leszczyńska T, Miedzobrodzka A. 1992. Effect of the fermentation process on levels of nitrates and nitrites in selected vegetables. *Rocz Panstw Zakl Hig* 43:253–8.

Holzapfel WH, Schillinger U. 2002. Introduction to pre- and probiotics. *Food Res Int* 35:109–16.

Hudáček J, Zalán ZS, Chumchalová J, Halász A. 2007. Antifungálny účinok laktobacilov na plesne rodu *Fusarium* a *Aspergillus* (antifungal effect of lactobacilli on *Fusarium* and *Aspergillus* molds). *Chem Listy* 101:730–7.

Hulse JH. 2004. Biotechnologies: past history, present state and future prospects. *Trends Food Sci Technol* 15:3–18.

Ito A, Sato Y, Kudo S, Sato S, Nakajima H, Toba T. 2003. The screening of hydrogen peroxide-producing lactic acid bacteria and their application to inactivating psychrotrophic food-borne pathogens. *Curr Microbiol* 47:231–6.

Kalač P, Šavel J, Křížek M, Pelikánová T, Prokopová M. 2002. Biogenic amine formation in bottled beer. *Food Chem* 79:431–4.

Kapadia GJ, Tokuda H, Konoshima T, Nishino H. 1996. Chemoprevention of lung and skin cancer by *Beta vulgaris* (beet) root extract. *Cancer Lett* 100:211–4.

Klewicka E, Motyl I, Libudzisz Z. 2004. Fermentation of beet juice by bacteria of genus *Lactobacillus* sp. *Eur Food Res Technol* 218:178–83.

Klewicka E, Zdujczyk Z, Jumkiewicz J. 2009. Effect of lactobacillus fermented beetroot juice on composition and activity of cecal microfora of rats. *Eur Food Res Technol* 229:153–7.

Künsch U, Schärer H, Temperli A. 1990. Study on the formation of biogenic amines during sauerkraut fermentation. *Food Biotechnol* 4:240–3.

Lashley D, Wiley RC. 1979. A betacyanine decolorizing enzyme found in red beet tissue. *J Food Sci* 44:1568–9.

Leitão MC, Teixeira HC, Barreto Crespo MT, San Romão MV. 2000. Biogenic amines occurrence in wine. Amino acid decarboxylase and proteolytic activities expression by *Oenococcus oeni*. *J Agric Food Chem* 48:2780–4.

Maintz L, Novak N. 2007. Histamine and histamine intolerance. *Am J Clin Nutr* 85:1185–96.

Marilley L, Casey MG. 2004. Flavours of cheese products: metabolic pathways, analytical tools and identification of producing strains. *Int J Food Microbiol* 90:39–159.

Murphy MG, Condon S. 1984. Correlation of oxygen utilization and hydrogen peroxide accumulation with oxygen induced enzymes in *Lactobacillus plantarum* cultures. *Arch Microbiol* 138:44–8.

Nagy-Gasztonyi M, Kardos-Neumann Á, Takács-Hájos M. 2006. Characterization of red beet and carrot with special emphasis on nitrate accumulation. *Acta Alimentaria* 35:131–8.

Ouwehand AC. 1998. Antimicrobial components from lactic acid bacteria. In: Salminen S, von Wright A, editors. *Lactic acid bacteria: microbiology and functional aspects*. New York: Marcel Dekker. p. 139–59.

Park JH, Seok SH, Cho SA, Baek MW, Lee HY, Kim DJ, Chung MJ, Kim SD, Hong UP, Park JH. 2005. Antimicrobial effect of lactic acid producing bacteria culture condensate mixture (LCCM) against *Salmonella enteritidis*. *Int J Food Microbiol* 101:111–7.

Parvez S, Malik KA, Ah Kang S, Kim HY. 2006. Probiotics and their fermented food products are beneficial for health. *J Appl Microbiol* 100:1171–85.

Pavlov A, Kovatcheva P, Georgiev V, Koleva I, Ilieva M. 2002. Biosynthesis and radical scavenging activity of betalains during the cultivation of red beet (*Beta vulgaris*) hairy root cultures. *Z Naturforsch* 57:640–4.

Pavlov A, Kovatcheva P, Tuneva D, Ilieva M, Bley T. 2005. Radical scavenging activity and stability of betalains from *Beta vulgaris* hairy root culture in simulated conditions of human gastrointestinal tract. *Plant Food Hum Nutr* 60:43–7.

Plocková M, Stiles J, Chumchalová J, Halfarová R. 2001. Control of mould growth by *Lactobacillus rhamnosus* VT1 and *Lactobacillus reuteri* CCM 3625 on milk agar plates. *Czech J Food Sci* 19:46–50.

Presley TD, Morgan AR, Bechtold E, Clodfelter W, Dove RW, Jennings JM, Kraft RA, King SB, Laurienti PJ, Rejeski WJ, Burdette JH, Kim-Shapiro DB, Miller GD. 2010. Acute effect of a high nitrate diet on brain perfusion in older adults. *Nitric Oxide* 24(1):34–42.

Rastall RA, Gibson GR, Gill HS, Guarner F, Klaenhammer TR, Pot B, Reid G, Rowland IR, Sanders ME. 2005. Modulation of the microbial ecology of the human colon by probiotics, prebiotics and synbiotics to enhance human health: an overview of enabling science and potential applications. *FEMS Microbiol Ecol* 52:145–52.

Rodrígues E, Tomillo J, Nuñez M, Medina M. 1997. Combined effect of bacteriocin-producing lactic acid bacteria and lactoperoxidase system activation on *Listeria monocytogenes* in refrigerated milk. *J Appl Environ Microbiol* 83:389–95.

Santos SMH. 1996. Biogenic amines: their importance in foods. *Int J Food Microbiol* 29:213–31.

Sárdi É, Stefanovits-Bányai É, Kocsis I, Takács-Hájos M, Fébel H, Blázovics A. 2009. Effect of bioactive compounds of table beet cultivars on alimentary induced fatty livers of rats. *Acta Alimentaria* 38:267–80.

Schillinger U, Kaya M, Lücke FK. 1991. Behaviour of *Listeria monocytogenes* in meat and its control by a bacteriocin-producing strain of *Lactobacillus sake. J Appl Microbiol* 70:473–8.

Stanton C, Ross RP, Fitzgerald GF, Van Sinderen D. 2005. Fermented functional foods based on probiotics and their biogenic metabolites. *Curr Opin Biotech* 16:198–203.

Stiles ME, Holzapfel WH. 1997. Lactic acid bacteria of foods and their current taxonomy. *Int J Food Microbiol* 36:1–29.

Stratton JE, Hutkins RW, Taylor SL. 1991. Biogenic amines in cheese and other fermented foods: a review. *J Food Prot* 54:460–70.

Suzzi G, Gardini F. 2003. Biogenic amines in dry fermented sausages: a review. *Int J Food Microbiol* 88:41–54.

Tuohy KM, Probert HM, Smejkal CW, Gibson GR. 2003. Using probiotics and prebiotics to improve gut health. *Drug Discov Today* 8:692–700.

Váli L, Stefanovits-Bányai É, Szentmihályi K, Fébel H, Sárdi É, Lugasi A, Kocsis I, Blázovics A. 2007. Liver-protecting effects of table beet (*Beta vulgaris* var. *rubra*) during ischemia—reperfusion. *Nutrition* 23:172–8.

Walkowiak-Tomczak D, Zielińska A. 2006. Effect of fermentation conditions on red-beet leaven quality. *Pol J Food Nutr Sci* 15/56:437–44.

Wang M, Goldman IL. 1997. Accumulation and distribution of free folic acid content in red beet (*Beta vulgaris* L.). *Plant Food Hum Nutr* 50:1–8.

Wasserman BP, Eiberger LL, Guilfoy MP. 1984. Effect of hydrogen peroxide and phenolic compounds on horseradish peroxidase-catalyzed decolorization of betalain pigments. *J Food Sci* 49:536–8.

Yoon KY, Woodams EE, Hang YD. 2005. Fermentation of beet juice by beneficial lactic acid bacteria. *Lebensm Wiss Technol* 38:73–5.

21

Health Benefits of Fermented Vegetable Juices

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CONTENTS

21.1 Nutritional Quality of Vegetables

In recent years, increasing attention has been paid to the role of diet in human health. Fruits and vegetables are important components of a healthy diet, but intakes in most countries are well below the recommended five servings a day (400 g).

Vegetables contain most of the essential components of human nutrition. The nutrient composition of vegetables is very complex and difficult to assess. However, among amino acids, fatty acids, and vitamins, there is recognition now that many compounds in vegetables, such as dietary fiber, flavonoids, sterols, phenolic acid, glucosinolates, and so forth, are associated with lower disease risk. These compounds might exert protective effects against many diseases such as cancer, coronary heart disease, diabetes, high blood pressure, cataracts, degenerative diseases, and obesity (Liu et al. 2000; Hung et al. 2004). Oxidative stress seems to be the critical factor in many diseases because reactive oxygen species (ROS) have the ability to damage macromolecules like DNA, proteins, and lipids. Therefore, it is necessary to supply adequate amounts of ROS scavengers. Fruits and vegetables are considered to be the major contributors of ROS-scavenging antioxidants.

21.1.1 Chemical Components in Vegetables and Their Health Effects

21.1.1.1 Primary Metabolites

The primary metabolites of vegetables are classified as carbohydrates, fats, nucleic acids, and proteins. Of these primary metabolites, carbohydrates, fats, and proteins are considered nutrients. Primary metabolites are involved in growth and development, respiration and photosynthesis, and hormone and protein synthesis. However, the focus of this section is on the secondary metabolites of vegetables.

21.1.1.2 Secondary Metabolites

Vegetables are a valuable source of a wide range of secondary metabolites, which determine the color of vegetables, protect plants against herbivores and microorganisms, and act as signal molecules under stress conditions (Seiger 1998). It is estimated that there are more than 200,000 chemical structures that are synthesized in plants. In recent years, some secondary metabolites have been used as protective dietary constituents. Vegetable phytochemicals are summarized in Table 21.1.

21.1.1.2.1 Carotenoids

More than 600 different carotenoids have been detected in plants, microorganisms, and animals. Approximately 20 of them can be identified in human blood serum after the intake of fruits and vegetables. Carotene occurs in many forms, designated as α-carotene and β-carotene followed by γ-, δ-, and ε-carotene. β-carotene is comprised of two retinyl groups and is broken down and used in the body as both retinoic acids and retinals, which are active forms of vitamin A. Vegetables, including carrots, sweet potatoes, and pumpkin, are rich sources of carotenoids.

Lycopene is a red color pigment and in contrast with most other carotenoids that are widely distributed among a great variety of vegetables, tomatoes and tomato products are the main sources of lycopene in the diet. Lycopene is a linear carotenoid with 11 double conjugated bonds. It is the precursor of all carotenoids because they are formed by the cyclization of its structure followed by the hydroxylation of specific carbons. Because of its high number of conjugated dienes, it is the most potent singlet oxygen quencher among the natural carotenoids. In humans, differently from β-carotene, lycopene is not transformed into vitamin A. Lycopene's role has also been associated with the decreased incidence of prostate and lung cancer. Xanthophylls are yellow carotenoid pigments involved in photosynthesis and are found in the leaves of most plants. Zeaxanthin and lutein are the xanthophylls that are absorbed and

TABLE 21.1

Vegetable Phytochemicals with Antioxidant Properties

Phytochemicals	Dietary Source
Carotenoids	Carrots, tomatos, celery, brocolli
Betalains	Beetroot
Glucosynolates	Family Brassicaceae
Phenolic acids	
Hydroxybenzoic acids	Carrots, spinach, tomatoes, eggplants
Flavonoids	
Anthocyanins	Red cabbage, purple potato, eggplants, radishes, red onions
Flavonols	Beans, lettuce, onions, olive, pepper, tomatoes
Flavones	Celery, parsley, spinach, rutin
Isoflavones	Soybeans
Others phenolics	
Capsaisinoids	Pepper
Coumarins	Carrot, celery, parsley, cabbage
Secoiridoids	Olives

bioavailable. They are found in the macula of the eye, and they decrease the risk of developing macular degeneration and cataracts.

21.1.1.2.2 Betalains

Betalains are a class of red and yellow indole-derived pigments—betacyanins and betaxanthins. These pigments are synthesized from tyrosine and constitute a class of secondary metabolites found in species of the order Caryophyalles, with the exception of the families Caryophyllaceae and Molluginacae, the species of which accumulate anthocyanins. An example of a betacyanin-producing plant is the beetroot, in which the main betacyanin, betanin, is found in concentration in the store root of 0.5 g betanin/kg. Several studies showed that beetroot is a good source of natural antioxidants. Vinson et al. (1998) ranked beets among the ten most potent vegetables. It was found that betanin from beetroots inhibit lipid peroxidation in membranes and provide protection against oxidative stress–related disorders by acting as antioxidants *in vivo.* Besides these hydrophilic pigments, there is the presence of further health-promoting constituents of beetroots such as phenolics (phenolic acids, phenolic acid esters, and flavonoids) and folic acid. Therefore, consumers may benefit from the consumption of beetroot or beetroot juice.

21.1.1.2.3 Glucosoinolates

Glucosoinolates are a unique large group of thioglucosides naturally occurring in plants of the order *Brassicales*. These are known to efficiently reduce the risk of cancer, to be involved in the inhibition of enzymes modifying steroid hormone metabolism, and in protection against oxidative damage.

21.1.1.2.4 Phenolics

Phenolics are also secondary metabolites synthesized by plants. The chemical structure of phenols possesses one or more aromatic rings, with one or more hydroxyl groups. Phenolic substances are a category of phytonutrients that exert strong antioxidant properties. They can be classified into simple phenols, phenolic acids, coumarins, flavonoids, stilbenes, hydrolyzable and condensed tannins, and lignans and lignin. Phenols in food may contribute to the bitterness, astringency, color, flavor, odor, and oxidative stability of food. The ability of some of the phenolic substances to work as potent antioxidant components is of great importance.

Several studies have reported that there is relationship between phenolic content and antioxidant activity. Vinson et al. (1998) found that beets had the highest total phenolic content, followed by red onion, broccoli, and kidney beans. Environmental factors, such as climatic growth conditions, growth, ripening stage, temperature, and duration of storage may influence antioxidant activity.

A diet rich in vegetables may provide protection against cardiovascular disease, several common cancers, and other chronic diseases. The scientific base supporting the health benefits derived from eating vegetables is rapidly growing. Adequate supplementation with vegetables, as well as fruit, might play an important role in the control of acute and chronic diseases via immunomodulation. A human study showed that a diet low in carotenoids reduces T lymphocyte and the addition of vegetable juice, such as tomato juices, improves these functions.

Phenolic compounds are potent blocking agents of *N*-nitroso components, the class of versatile carcinogens in rodents. Human exposure to endogenously formed *N*-nitroso components relates to an increased risk of cancer. Shenoy and Choughuley (1989) reported that onion and garlic juice were effective in reducing the chemical formation of nitrosamines and assumed that cysteine and several other sulfhydryl compounds might also act as nitrite scavengers. Garlic and onions contain large quantities of allyl sulfur compounds. Chung et al. (2002) recently reported that the intake of whole strawberries, garlic juice, and kale juice showed inhibiting effects on the formation of *N*-nitroso compounds in human volunteers.

Earlier epidemiological studies have suggested a positive role of vegetables in the interruption of oxidative stress and, therefore, in disease prevention. This effect was initially associated with the presence of fiber, unsaturated fatty acids, minerals, and vitamins. The health-protective mechanisms of fiber intake involve other molecules bound to polysaccharides.

21.1.1.3 Oligoelements, Vitamins, and Fatty Acids

Numerous studies have been developed regarding oligoelements and vitamins as a factor able to prevent oxidative damage. Oligoelements are metals that have a role in the synthesis and structural stabilization of proteins and nucleic acids. Magnesium, copper, zinc, selenium, and manganese are the most important of them. Among these, attention is targeted to selenium, the consumption of which is inversely correlated with the risk of developing cancer. Hydrogen selenide, methylselenol, and selenomethionine are able to regulate gene expression, protect DNA from damage, and stimulate the repair of cell cycles. Oligoelements enter into the mechanism of preventive protection against free radicals, performed by antioxidant enzymes.

21.1.1.3.1 Vitamin C

The chemical structure of vitamin C is similar to that of the hexose sugars, with an ene-diol group involving carbons 2 and 3 of this group that makes vitamin C a strong reducing agent, which is oxidizable to dehydroascorbic acid. Most vegetables synthesize ascorbic acid (vitamin C). Because primates are unable to synthesize vitamin C, this vitamin must be introduced with fresh vegetables and fruits. Vitamin C is involved in several metabolic processes and also improves the immune response and favors the elimination of xenobiotics and radicals. Vegetables rich in ascorbic acid include spinach, onions, spring cabbage, broccoli, sweet peppers, and beans.

21.1.1.3.2 Vitamin B Complex

The vitamin B complex of vegetables includes the water-soluble vitamins thiamine (B1), riboflavin (B2), nicotinic acid (B3), panthothenic acid (B5), pyridoxine (B6), biotin (B7), and folic acid (B7; Bender 2003). Vitamin B, in humans, is involved in tissue respiration and carbohydrate, fatty acid, and amino acid metabolism. Green vegetables such as spinach, Brassiceae vegetables, asparagus, turnip, and lettuce are good sources of vitamin B.

21.1.1.3.3 Vitamin E

Vitamin E is known under the term tocopherols. They are homologous, derived from the 6-hydroxychromane structure. Tocopherols come largely from green leafy vegetables, seeds, and their oils. Vitamin E is a lipid-soluble antioxidant, which can react synergistically with selenium to prevent the oxidation of fatty acids, membrane phospholipids, and proteins.

21.1.1.3.4 Fatty Acids

Fatty acids are major components of fats. They supply the human body with energy and structural material for cell membranes and organ padding. Fatty acids are involved in the absorption of certain vitamins, blood clotting, and the immune response (Nettleton 1995; Shahidi and Miraliakbari 2005). Two unsaturated fatty acids that cannot be produced in the body are linoleic acid and α-linolenic acid. Linoleic acid can be found in most vegetable oils. α-Linolenic acid is found in green vegetables such as Chinese cabbage, Brussels sprouts, and parsley. The consumption of monounsaturated fatty acids (oleic, palmitoleic) has been shown to reduce cholesterol and has a beneficial effect on some of the risk factors for cardiovascular disease and type 2 diabetes (Simopoulos 1999; Connor 2000).

21.1.2 Antioxidant Capacity of Fresh, Stored, and Processed Vegetables

Collection and transport of vegetables from the field to the storage facility or marketplace does not significantly decrease the nutritional and organoleptic properties or the antioxidant capacity. However, lengthy storage at storage temperatures in the range 5°C to 8°C, humidity levels of 80%, can modify the content of vitamins and polyphenol structures. Therefore, some significant fluctuations of phenols, and consequently, of antioxidant capacity are observed.

The various factors that occur during the processing of vegetables affect nutrient quality and degrade phenolic constituents, single marker compounds like vitamin C and other antioxidants.

Minimally processed products are increasingly consumed nowadays. These are the "ready-to-eat" fruits and vegetables which are washed, cleaned, cut, and then finally submitted to modified atmosphere packaging. Organoleptic characteristics and phenol concentrations strictly depend on the type of storage and modified atmosphere packaging. These parameters also seem to be relevant in determining the bioavailability of phenolics in humans (Watson and Preedy 2010).

Thermal processing techniques such as blanching have raised concerns regarding the importance of functional components, yet thermal processing is an important method of preserving foods and the maintenance of functional characteristics including texture, flavor, and color. Thermal treatment of vegetables has been known to affect antioxidant activity. The phenolic content in fresh vegetables is 10% to 20% higher than in thermally treated vegetables. Blanching of Brassiceae vegetables reduces the functional properties of the glucosinolates by inactivation of the myrosinase. On the contrary, temperature increases the release of lycopene from tomato peel, and the total antioxidant capacity of the final product increases as well (Yoon et al. 2004).

Processed vegetables show a wide range of phytochemical losses. The technology in the food industry should reduce the loss of antioxidants and micronutrients to the minimum level by using mild processes in processing and preservation of vegetables.

21.2 Probiotic Fermented Vegetable Juices

The lactic acid fermentation of vegetable products, applied as a preservation method for the production of finished and half-finished products, is considered as an important technology. Because of their positive effects on health, most fermented foods today are included in a special group known as functional or therapeutic foods. The presence of functional foods in the daily diet could greatly improve the quality of human life, helping in the reduction of the risk of degenerative chronic diseases.

Today, on the world market, different varieties of functional foods are present, from which most are dairy-based fermented products. Analysis shows that consumption of functional dairy-based products in Western Europe, the United States, and Japan increased by 12% since 2005, and that functional foods are especially popular in Japan, where more than 53 different types of products are present on the market. Problems with severe lactose intolerance and allergy to milk proteins in some persons, as well as the unfavorable cholesterol content of fermented dairy products, has encouraged producers to develop a group of nondairy functional products, which include fermented vegetable juices. In such a way, the list of potential functional food products are constantly expanding at a very fast rate. Consumption of nondairy functional foods are of great importance especially for vegetarians whose diet is mostly based on fruits, vegetables, legumes, and cereal products. Unlike dairy-based products, fruits and vegetables do not contain any allergens, so they can be used by many different groups of peoples.

21.2.1 Microflora of the Human Gut

The study of the intestinal microflora of humans is of great importance for probiotic research and development. The gastrointestinal tract is, after the respiratory tract, the second largest body surface area, with more than 400 m^2 which, during human life, participates in four main functions: ingestion, digestion, absorption, and defecation. The large intestine of the mammalian gut is the main site of microbial colonization and it always contains more than 10^{12} microorganisms/g of fecal material, which is more than the number of somatic cells present in the human body.

Analysis of the human gut microflora showed that it is a complex and dynamic community which contains more than 800 different bacterial species (aerobic and anaerobic), yeast, and fungi, among which only 30 to 40 species form almost 99% of the intestinal flora. Their distribution along the gut progressively increases from the stomach down to the feces, which is made up of 50% bacteria. The least amount of microorganisms are present in the stomach where only few bacteria (mainly acid-tolerant species of *Lactobacillus*) can survive acid conditions because of the gastric juices. A similar situation is

found in the duodenum, in which the scarcity of bacteria is a consequence of the composition of luminal medium (acid, bile, and pancreatic secretions) which kills most ingested microorganisms. Unlike the stomach, in the colon much higher numbers of different groups of bacteria are present (up to 10^{12} cells per gram of feces) because of better growth conditions such as higher concentration of nutrient, low-redox potential, and slow transit. Among them, anaerobic species of bacteria have a very important role for the host because they perform metabolic functions including fermentation in the colon, provide short-chain fatty acids, produce vitamins, aid in the development of the immune system, and assist to the trophic action of the epithelium controlling epithelial cell proliferation and differentiation (Iannitti and Palmieri 2010).

In the mother's uterus, the human fetus is sterile, and during delivery, microbes start to colonize the intestinal tract of newborn babies. In the first few weeks after birth, the presence of different populations of microorganisms in a baby's gut greatly depends not only on the type of birth (vaginal or caesarean delivery), but also on the exposure to numerous bacteria present in the environment (e.g., skin, mouth, and mother's milk, nursing stuff, other infants, air, and equipment). This initial acquisition of intestinal mirobiota shows low diversity and plays a key role in the development of immune processes which provides protection against pathogens. Its composition is relatively unstable and has a tendency to change during the initial period of life.

Among the microorganisms, lactic acid bacteria (LAB; *Bifidobacterium* and *Lactobacillus*) play significant roles in maintaining a healthy gut ecosystem. In general, in the first months of life, especially in breast-fed infants, the dominant bacteria are members of the genus *Bifidobacterium* (up to 90% of the total fecal bacteria) because of the bifidogenic effect of breast milk, whereas in formula-fed infants, weaning children, and adults bacteria are more diverse. Also, it is established that although the species of *Bifidobacterium* are among the predominant anaerobic bacteria in the intestinal microbiota from early infancy until old age, their numbers decrease during aging and their species/strain composition changes from infancy to adult years and old age. The distribution of microorganisms in the adult human gut is shown in Table 21.2.

In a healthy individual, all microorganisms mentioned in Table 21.2 live in the intestinal tract in a natural balance called symbiosis, the composition of which has a strong influence on the nutritional and health status of the host by improving its metabolic and immune function. A detailed analysis of healthy microflora of the intestinal tract by Round et al. showed that it can be divided into three separate groups: (1) symbionts, which include species with very well known health-promoting functions; (2) commensals, which are permanent residents and do not show either benefits or detrimental effects to the host; and (3) patobionta, which are also permanent residents of the gut but unlike commensals, they have the potential to induce pathology.

In the presence of some endogenous and environmental factors (diet, antibiotic intake, xenobiotics, etc.), the equilibrium present in the gut can be changed and cause dysbiosis, which leads to metabolic or immunologic feedback of the host such as halitosis, adrenal stress, diarrhea, candida, leaky gut syndrome, obesity, and even colon and breast cancer. In all these cases of dysbiosis, it is noted that changes in the composition of microflora present in the gut are manifested either as a reduction in the number of symbionts and as an increase in the number of pathobionts. Much clinical evidence proved that everyday consumption of food with probiotic microorganisms could help in maintaining or improving the microbial balance in gut. That is especially important today when the modern way of life (busy lifestyle, irregular and improper nutrition, and stress) is a main reason for changes in bacterial diversity of the human gut.

21.2.2 Development of the Probiotic Concept

Interest in probiotics dates to the beginning of the 20th century (1905), when Nobel Laureate Ilya Metchnikoff noticed that Bulgarian peasants had an average life span of 87 years, and that four out of every thousand lived to more than 100 years of age. At that time, it was very rare and he attributed this longevity to their diet rich in fermented milk. As a result of these observations, Metchnikoff established an autointoxication theory in which he pointed out that, during the life time, the human body is being slowly poisoned by toxins produced by enteric pathogens. This constant presence of toxins in the human

TABLE 21.2

Microflora Present in Gut and the Function of Some Parts of the Gastrointestinal Tract

Part of the Gastrointestinal Tract	Number of Microorganisms	pH	Different Groups of Microorganisms Present in the Gut	Function
Stomach	$<10^2$	1–2	*Streprococcus*, *Lactobacillus*, *Bifidobacterium*, *Bacteroides*, *Enterobacteriaceae*, Yeasts	Digestion and acid secretion
Small intestine				
Duodenum	$10^{1–3}$	6–7	*Streptococcus*, *Lactobacillus*, *Veillonella*, *Bacteroides*, *Bifidobacterium*, Yeasts *Enterobacteriaceae*	Digestion and absorption of carbohydrates, proteins and fats
Ileum	$10^{7–9}$	6–7	*Streptococcus*, *Lactobacillus*, *Bifidobacterium*, *Bacteroides*, *Clostridium*, Yeasts *Enterobacteriacea*e	Absorption of bile acids and vitamin B12
Large intestine				
Colon	$10^{10–12}$	5–7	*Bacteroides*, *Eubacterium*, *Ruminococcus*, *Coprococcus*, *Peptostreptococcus*, *Bifidobacterium*, *Clostridium Streptococcus*, *Lactobacillus*, *Enterobacteriaceae*	Absorption of water, electrolytes, and short chain fatty acids

body steadily weakened its resistance which could be successfully prevented by the consumption of not only sour milk but also other fermented food which contains lactic acid–producing bacteria.

At the same time, Grigoroff isolated, from a starter culture for the production of Bulgarian fermented milk, a LAB which he called *Lactobacillus bulgaricus.* Stimulated by Metchnikoff's theory, Grigoroff made the assumption that after consumption of this popular fermented milk from Bulgaria, the *Lb. bulgaricus* present could successfully establish itself in the intestinal tract and prevent the multiplication and even decrease the number of putrefactive bacteria. Although subsequent studies conducted by Herter and Kendall have shown that *Lb. bulgaricus* failed to establish itself in the gut, this species of LAB may lead to other substantial changes in the gut microflora and indirectly affect the general health of the organism (Ouwehand et al. 1999).

By definition, probiotics are nonpathogenic live microorganisms or microbial mixtures which, when ingested in proper amounts, exert a positive influence on the health or physiology of the host by improving its intestinal microflora balance. Most probiotics isolated thus far belong to two genera: *Bifidobacterium* and *Lactobacillus*, but health-promoting effects are also observed in some species which belong to the genera *Lactococcus*, *Enterococcus*, *Saccharomyces*, and *Propionibacterium*. In Table 21.3, common probiotic microorganisms used for the production of different functional food and pharmaceutical products (capsules, pills, and tablets) are presented.

Most vegetables are consumed in the human diet as fresh, minimally processed, pasteurized, or cooked in boiling water, or by microwaving. Such vegetable-based products have a very short shelf life, and their durability can be greatly extended by lactic acid fermentation. The tradition of lactic acid fermentation of vegetables dates back to ancient times. Since then, the technology of the production of these products

TABLE 21.3

Some Probiotic Microorganisms Used in Commercial Applications

***Lactobacillus* Species**	***Bifidobacterium* Species**	**Other**
Lb. acidophilus	*Bf. adolescentis*	*Bacillus cereus*
Lb. amylovorus	*Bf. animalis*	*Clostridium botyricus*
Lb. brevis	*Bf. breve*	*Enterococcus faecalis*
Lb. casei	*Bf. bifidum*	*Enterococcus faecium*
Lb. rhamnosus	*Bf. infantis*	*E. coli*
Lb. crispatus	*Bf. lactis*	*Lac. lactis* ssp. *cremoris*
Lb. delbrueckii ssp. *bulgaricus*	*Bf. longum*	*Lac. lactis* ssp. *lactis*
Lb. fermentum		*Leuconostoc mesenteroides* ssp. *dextranicum*
Lb. gassei		
Lb. helveticus		*Pediococcus acidilactici*
Lb. johnsonii		*Propionibacterium freudenreichii*
Lb. lactis		*Saccharomyces boulardii*
Lb. paracasei		*S. salivarius* ssp. *thermophilus*
Lb. plantarum		
Lb. reuteri		

has developed to improve not only their shelf life properties but also their safety, nutritional, and sensory characteristics. Lactic acid fermentation of vegetable juice can be performed spontaneously by autochthonous LAB present as an integral part of the microflora on the surface of vegetables, or by starter cultures that are designed to be added into raw vegetable juices which previously could be heat-treated (Liepe and Junker 1984). Among many species of LAB, *Lactobacillus plantarum*, *Lactobacillus brevis*, and *Lactobacillus xylosus* are the most frequently used starter cultures for the fermentation of vegetables juices (Schobinger 1987). In many studies, it has been observed that commercial starter cultures do not always fully correspond to autochthonous species of LAB which are better adapted to the intrinsic characteristics of raw vegetable materials used for fermentation and also do not require additional supplementation of vegetable juices with nutrients or pH adjustment during fermentation. Therefore, the application of such autochthonous species of LAB produces fermented vegetable juices with desirable properties (Di Cagno et al. 2009).

Recently, in several studies, different vegetable juices such as tomato, carrots, beetroot, cabbage, celery, and many others, were pointed out as ideal substrates for the growth of probiotic microorganisms, especially LAB (Karovičová et al. 1999; Kohajdová and Karovičová 2005; Klewicka et al. 2004; Rakin et al. 2003, 2004a,b, 2007; Yoon et al. 2005, 2006; Demir et al. 2006; Moraru et al. 2007; Nazzaro et al. 2008; Burleanu et al. 2009; Koh et al. 2010). The presence of minerals, vitamins, dietary fibers, and antioxidants in vegetable juices can fully satisfy the nutritional needs of fastidious microorganisms such as LAB. With LAB, particularly good fermentability is achieved in juices prepared from Chinese cabbage, cabbage, pH-adjusted tomato (pH 7.2), carrots, and spinach, which contain more fermentable saccharides than other types of vegetables. Such probiotic fermented vegetable juices do not only have pleasant sensory properties (flavor and taste), but also could promote the health and well-being of humans. During fermentation, probiotic microorganisms further improve the nutritional value of vegetable juices, enhancing their sensory properties, and enabling at the same time their preservation. For this reason, fermented probiotic vegetable juices are very popular in many countries and their consumption increases every day.

21.2.3 Criteria for the Selection of Probiotic Microorganisms

A probiotic is said to have good features if it has several desirable properties such as: (1) being nonpathogenic, noninvasive, noncarcinogenic, and safe; (2) being resistant to acid, bile, and pancreatic secretions present in the stomach and upper part of the gastrointestinal tract; (3) having the ability to adhere to human epithelial cells and to colonize human intestine; (4) being able to produce antimicrobial

substances (acids, hydrogen peroxide, and bacteriocins), which prevent the growth of pathogenic microorganisms, during growth in the gut; (5) being able to reduce or exclude pathogenic adherence; (6) having good growth characteristics and beneficial effects on human health; and (7) being able to coaggregate to form a normal balanced flora. Two species of LAB listed in Table 21.3, *Streptococcus salivarius* ssp. *thermophilus* and *Lactobacillus delbrueckii* ssp. *bulgaricus*, which are traditionally used for the production of yogurt, do not survive in the host's intestinal tract, and because of these characteristics many scientists do not categorize them as probiotics and consider them as a yogurt starter cultures. In addition to the above-mentioned features, the probiotic must maintain its viability during processing, storage, and product application and show resistance to the physicochemical processing of the food. Also, from an economic point of view, the probiotics used for the production of functional food and pharmaceutical products should also be of low cost, which provides that probiotic-containing products are accessible for everyone (Prado et al. 2008; Randaheera et al. 2010).

21.2.4 Origin of Strains, Importance of Their Correct Identification, and Safety

When probiotics became initially available, many authors pointed out the importance of the origin of strains for specific commercial applications. They believed that for achieving a successful outcome in lactobacilli therapy, it was necessary that strains used for production must be normal inhabitants of the intestinal tract of humans. In this way, a compatibility with the human gut microflora is achieved and the chances of bacterial survival during passage through the gastrointestinal tract are maximized. Although most of the species mentioned in Table 21.3 are important and normal constituents of human or animal gastrointestinal tract, some recent studies indicate that certain strains isolated from other environments, especially fermented substrates such as dairy and nondairy-based products, also have probiotic characteristics. These results led the experts at the Food and Agriculture Organization/World Health Organization (Vasiljevic and Shah 2008) to come to the conclusion that in the selection of probiotics, much more attention should be paid to the specificity of probiotic action rather than to their origin. This is also supported by the fact that despite numerous studies conducted today, very little is known about the true origin of the human intestinal microflora, and that many strains present in the intestinal tract of humans were once a part of the surrounding areas where humans lived.

In addition to origin, it is very important that every probiotic strain should be correctly identified before it is approved for human consumption. Thanks to developments in modern molecular techniques, it is now possible to precisely identify any species of probiotics and to put the real name on the product label. In this way, consumers could always be informed of which species and strains are used for production and what their link is to a specific health effect. For scientists, this enables accurate surveillance and epidemiological studies.

Several centuries-long applications of different species of LAB (lactobacilli, bifidobacteria, and lactococci) in the production of traditional fermented products confirmed no pathogenic or virulent properties in these groups of bacteria, and for these reasons, they obtained "generally recognized as safe" status. However, the application of strains which are not of human origin in the food fermentation industry prompted the question of whether they are really safe for human use because they do not have a complete history of safe use. Also, there is a constant concern regarding the safety of probiotics other than *Lactobacillus* and *Bifidobacterium* because some genera, such as *Enterococcus*, contain many pathogenic species which could be multidrug resistant. For these reasons, it is necessary that before any "new" strain becomes an integral part of a probiotic product or commercially available to consumers, to carry out a profound research. It should encompass not only their technological properties but also their safety, functionality, and health benefits. Therefore, today's experts at the Food and Agriculture Organization/World Health Organization underlined the need for all strains, regardless of origin, to be tested in experiments *in vitro* and *in vivo* (in humans). Only strains which demonstrate good probiotic characteristics in both experimental conditions should be considered as probiotics and could be used in the production of functional food.

Also, for safety reasons, probiotic strains need to be tested for their natural resistance against antibiotics because resistant genes could be transferred to other endogenous bacteria present in the colon. In this way, it is possible to prevent the spread of antibiotic resistance especially in cases in which

antibiotic-resistant genes are located on plasmids. At this moment, the information that we have regarding the antibiotic resistance of probiotic strains are rather contradictory. Although results at first indicated that certain strains of *Bifidobacterium* and *Lactobacillus* could show a strain-dependent resistance against the tested antibiotics, recently completed studies on 50 strains of *Bifidobacterium* showed that these strains were not resistant to antibiotics and that they could be used without risk as a supplement in the human diet (Moubareck et al. 2005; Espinoza and Navarro 2010).

21.2.5 Survival within the Gastrointestinal Tract

After being ingested, the major problem of probiotic microorganisms is to arrive at a sufficient amount in the large intestine, where they will adhere to colonic mucosa and colonize the colon. During passage through the stomach and upper intestine, probiotic microorganisms are faced with the adverse conditions present in these areas and the microbial strains must be able to both tolerate the acidic and protease-rich conditions in the stomach and survive and grow in the presence of bile acids. Up to now, everything we know about the resistance of probiotics have been from experiments conducted *in vitro* with artificial (simulated) gastric juice, bovine or pig bile, and various types of animal pancreatic extracts. The experimental data showed that various species and probiotic strains show different levels of tolerance to gastrointestinal conditions.

Acidity in the stomach is very low and ranges between 1 and 2 pH because of gastric juice secretions during digestion, which constitutes a primary defense mechanism against most ingested microorganisms. In such acid conditions, most probiotic organisms show a moderate tolerance to acid pH during the first 90 minutes of incubation; after that, they steadily lose their viability and after 2 hours of incubation, the rate of decrease varies considerably between different species and even among strains from the same species. Among probiotics, the most acid-sensitive are from the *Bifidobacterium* species (*Bifidobacterium longum*, *Bifidobacterium lactis*), the growth of which is significantly delayed at pH lower than 5.0. It has been noted that acid tolerance of species of *Bifodobacterium* depend on cultivation condition, strain and species, and that among all species of *Bifidobacterium*, *Bifidobacterium animalis* ssp. *lactis* has the highest acid tolerance and is thus preferably used in "bifidus" products. Unlike *Bifidobacterium* species, *Lactobacillus acidophilus* and *Lactobacillus salivarius* are acid-tolerant strains, and more than 10^4 CFU/mL could survive after 2 hours of incubation at pH 2.0 (Ding and Shah 2009). For *Lb. acidophilus*, it has been confirmed that it has a high cytoplasmic buffering capacity (pH 3.72–7.74), which allows this species to resist changes in cytoplasmic pH and gain stability under acidic conditions. Also, Lorca and de Valdez (2001) reported that *Lb. acidophilus* can also mediate acid tolerance by membrane ATPases. The same mechanism has also been noted with *Bf. animalis and Bf. lactis* (Matsumoto et al. 2004).

Although many studies *in vitro* showed that most bacteria are sensitive to gastric juices, *in vivo* studies conducted in humans indicated that high rates of bacteria can be isolated from feces, especially in circumstances when bacteria are consumed together with food matrix which protects bacterial cells from the harmful effects of gastric juice, increasing the pH in the stomach to 3.0 or higher. Also, Del Piano et al. (2006) performed *in vitro* experiments on seven probiotic strains and reported better survival from *Lb. plantarum* after exposure for 1 hour in human gastric juice withdrawn from an empty stomach of healthy individuals than in simulated gastric juice (15% and 45%, versus less than 20% bacteria survival, respectively).

Probiotic microorganisms which survive acid conditions present in the stomach enter the small intestine (duodenum and ileum) where pH is not very low (pH 6–7) but there are bile acids and pancreatic juices present which may have adverse effects on probiotic cells. Bile acids are synthesized in the liver from cholesterol and are secreted (500–700 mL/day) from the gall bladder into the duodenum in conjugated form, which then undergo extensive chemical modifications in the colon (deconjugation, dehydroxylation, dehydrogenation, and deglucuronidation) because of microbial activity. Both conjugated and deconjugated bile acids exhibit antibacterial activity, however, the deconjugated forms are more inhibitory. Gram-positive bacteria are found to be more sensitive than Gram-negative bacteria. Experimental data obtained for probiotic microorganisms showed that many strains are particularly sensitive to deconjugated bile salts, although resistant to the presence of conjugated bile salts. Also, many

experiments conducted *in vitro* showed that most probiotics are susceptible to 0.3% of bovine and porcine bile, but they are resistant to the same percentage of human bile, which explains why they survive in the human gastrointestinal tract. In addition, it has been noticed that bile acid resistance and relative ability to grow in the presence of bile acids greatly differ among strains of each species of probiotic. It was noted that *Lb. reuteri* species hydrolyzed bile salt and thus achieved resistance, also allowing the participation of cholesterol. Similar effects are noted in experiments with *Lb. acidophilus*, which also allowed the participation of cholesterol after deconjugation of bile salts. In *Lb. acidophilus* NCFM, two genes which encode bile salt hydrolysis are identified. One of them, *bsh*A is very similar to bile salt hydrolases found in *Lb. johnsonii*, *Bf. longum*, and *Listeria monocytogenes.*

Pancreatic juice is one of the most aggressive fluids in human and animal bodies. Its essential components are enzymes which are released as zymogens and subsequently activated in the small intestine. Unlike gastric juice and bile salts, human pancreatic juice is not only very perishable and instable but is also difficult to find or obtain in adequate quantities for testing the resistance of probiotic microorganisms. For these reasons, there is insufficient experimental data about the resistance of probiotics in the presence of human pancreatic juice. However, on the basis of existing results, it can be concluded that pancreatic juice can inhibit the growth of multiresistant bacteria strains and some probiotic bacteria.

21.2.6 Adhesion to Intestinal Cells

The ability of probiotic microorganisms to adhere to intestinal surfaces is another feature that is desirable because it later allows the colonization of the human gastrointestinal tract. Owing to adhesion, the retention time of probiotics in the intestine is lengthened, which is particularly important in the small intestine where the intestinal material has a short residence time. Also, adhered strains have better chances to show metabolic and immunomodulatory effects compared with nonadhered strains. The adhesion may also provide means of competitive exclusion of pathogenic bacteria from the intestinal epithelium. It has been noticed that *Lb. rhamnosus* GG effectively shortened the duration of rotavirus diarrhea in infants because of the ability to adhere and colonize the small intestine. A similar behavior was observed in the highly adhesive strain, *Bf. lactis* Bb12, which was effective in preventing and treating acute diarrhea in infants.

Today, it is known that mechanisms of adherence to epithelial surface include both receptor-specific binding as a charge and hydrophobic interaction between the probiotic and epithelial cell. Also, some LAB possesses the ability to coaggregate with cells of the same strain or with cells from other species, and this mechanism can enable adhesion, for example, to the mucus. LAB can be bound to extracellular matrix molecules such as collagens, fibronectin, and vitronectin, which may be shed from the epithelium to the mucus layers, as well as to mucosal components. In addition, it has been noticed that some strains of LAB, such as *Lb. acidophilus*, *Lb. gasseri*, *Lb. johnsonii*, *Lb. crispatus*, and so forth, form an S-layer during their growth, which covers the cell surface and may contain substances that help in adhesion to the intestinal surface or convey cell surface hydrophobicity. Also, these S-layers may protect the cell of probiotics from host defense mechanisms.

Although many research efforts have been devoted to probiotic adhesion studies, the role of adhesion in successful probiotic function remains speculative, mostly because some probiotic strains are poorly adherent *in vitro* or *in vivo*, but still show positive effects in the host. Also, *in vivo* studies have shown that most probiotic strains colonize the human gut only temporarily. In addition, some other studies have shown that strong adhesion ability may increase the risk of infection in the host. For these reasons, it is now widely accepted that administration of a daily dose of probiotics is the best way to maintain probiotic activity in the gut on a safe basis.

21.2.7 Production of Antimicrobial Compounds during the Growth of Probiotics

During the growth phase, some probiotic strains can produce a wide range of antimicrobial compounds (organic acids, carbon dioxide, hydrogen peroxide, bacteriocins, various low molecular mass peptides, antifungal peptides/proteins, fatty acids, diacetyl, bacteriocins, phenyllactic acid, and OH-phenyllactic

acid), the major task of which is to prevent the growth of harmful microorganisms during fermentation and to help in the preservation of food products by effective competitive exclusion of pathogenic microorganisms in the intestine, and additionally, to make possible probiotic effects for the host. To achieve this activity in the host, probiotic strains must be alive and metabolically active during their passage through the intestine, which gives the probability that some of these antimicrobial compounds will be really produced on a target location. In some circumstances, the activity of these antimicrobial compounds can be further enhanced by the acid conditions present in the stomach (Ganzle et al. 1999).

Lactic and acetic acids are main organic acids produced by probiotic strains, which lower pH in the gastrointestinal tract and show bactericidal or bacteriostatic effects toward Gram-negative pathogenic bacteria such as *Salmonella*, *Escherichia coli*, and *Shigella*. Effects toward Gram-negative pathogenic bacteria can be further improved if relevant concentrations of lactic acid in the microenvironment can be combined with a detergent such as bile salts (Begley et al. 2005). In addition, the analysis of cell-free culture supernatant of probiotic strains showed the presence of heat-stable low molecular weight antibacterial substances other than lactic acid, which caused inactivation of a wide range of Gram-negative bacteria and participated in inhibition of adhesion and invasion of Caco-2 cell by *Salmonella enterica* ser. *typhimurium* (Vasiljevic and Shah 2008).

Another characteristic of some probiotic strains is that they can produce various antimicrobial peptides or proteins which are known as bacteriocins. Bacteriocins are synthesized on ribosomes and could be efficient against other bacteria, which belong to the same species (narrow spectrum) or are members of other genera (broad spectrum) including genera such as *Salmonella*, *E. coli*, or *Shigella*. One very well known bacteriocin is nisin (E234) produced by *Lactococcus lactis* ssp. *lactis* which has been, for decades, applied as a food additive for the preservation of different food products or are produced *in situ* by the starter culture. After nisin, many different bacteriocins were isolated and characterized from LAB. They have different antimicrobial spectra, modes of action, molecular weights, genetic origins, and biochemical properties. They are divided into four groups: (1) antibiotics (to which nisin belongs); (2) small, heat-stable peptides; (3) large, heat-labile proteins; and (4) protein complexes. The production of this group of bacteriocins is stimulated by the presence of pathogens and the producers have immunity to their own bacteriocin.

For bacteriocin present in food or produced in the small intestine, it is important to show their activity in the presence of bile, which are amphoteric molecules and could interact with hydrophobic part of bacteriocin such as curvacin A. For this bacteriocin, Ganzle et al. (1999) showed in a dynamic model of the gastrointestinal tract that its activity against *Listeria innocula* could be increased in the presence of bile. The stimulatory effect on bacteriocin activity was also evident if the bile was diluted tenfold below the bile concentration effective in the absence of bacteriocin.

21.2.8 Antioxidative Activity of Probiotic Strains

Although oxidation is of great importance for many living organisms because it allows the production of energy necessary for the development of biological processes, oxidative stress can cause serious damage on biological molecules. During its lifetime, the human body continuously produces free radicals and other ROS. This occurs especially during the passage of nutrients through the gastrointestinal tract. Many of these free radicals and ROS attack biological molecules present in the cells, causing further formation of new free radicals, leading to a free radical chain reaction which can result in serious damage to living organisms. Thus, they can play an important pathological role in cancer, emphysema, cirrhosis, atherosclerosis, arthritis, and could be the main reason for human aging which is closely related to oxidation. Humans and other organisms have evolved antioxidant and repair systems to protect themselves from oxidative damage. These systems are not effective enough to totally prevent damage, especially when the natural production of host antioxidants decreases. In such circumstances, one way to solve this problem is to take additional antioxidant supplements or to include foods which contain antioxidants in the daily diet. Both methods can help the human body reduce oxidative damage.

Recent studies have shown that some species of LAB, such as intestinal strain of *Bf. longum* ATCC15708 and, to a lesser extent, *Lb. acidophilus* ATCC4356, can possess antioxidative effects and thus can inhibit linoleic acid peroxidation (28%–48%) and scavenge the free radicals α,α-diphenyl-β-picrylhydrazyl

(21%–52%). These two strains demonstrated excellent antioxidative activity both as intact cells (10^9) as well as intracellular cell-free extracts. These strains were able to protect plasma lipid from oxidation to different degrees. Their cell-free extracts demonstrated higher inhibition than intact cells, probably because the cells could not enter the blood (Lin and Chang 2000). On the other hand, whether the intracellular cell-free extracts will really show the antioxidative effect in the human body will mainly depend on how much of the intracellular extract will be absorbed from the small intestine into the blood. This is of great importance because *Bifidobacterium* spp. and *Lb. acidophilus* make up a significant part of the natural microflora of the human intestinal tract and today they are also included in the composition of fermented products such as fermented vegetable juices.

Kruszewska et al. (2002) pointed out that antioxidant activity is strain dependent. For example, although the *Lactobacillus paracasei* F19 strain had slightly less antioxidant activity than species *Pediococcus pentosaceus* 16:1 and *Lb. plantarum* 2592, another strain of *Lb. paracasei* did not exert antioxidative activity. Research carried out afterward by Annuk et al. (2003) showed that high antioxidant activity have obligately homofermentative lactobacilli whereas this characteristic is highly strain-dependent among facultatively and obligately heterofermentative lactobacilli. In addition, Ljungh and Wadström (2005) showed that *Ped. pentosaceus* 16:1 and *Lb. plantarum* 2592, after 18 hours of growth, produced an amount of antioxidants in which activity corresponded to 100 μg of vitamin C.

Lb. plantarum belongs to a group of industrially important species of LAB. It is involved in many vegetable fermentations (Buckenhüskes 1997); it is also a frequent inhabitant of the human intestinal tract (Johansson et al. 1993). The strain *Lb. plantarum* 299v has been previously used as a probiotic microorganism (Rodgers 2008). For this species, Fernández et al. (2010) showed, in experiments carried out with 18 strains isolated from different environments, that they could produce carotenoid compounds.

The antioxidant properties of carotenoids are very well known and in Gram-positive bacteria, such as *Lb. plantarum* for instance, these pigments play an important role in protecting cells from oxidative stress by scavenging free radicals with their conjugated double bonds (Clauditz et al. 2006). *In vivo*, carotenoids are commonly located in membranes and their functional effects depend on their structures. In the membrane, carotenoids exert two functions: (1) as scavengers for singlet oxygen, and (2) for the stabilization of the membrane. Genetic analyses through PCR revealed that all of the *Lb. plantarum* strains tested by Fernández et al. (2010) contained the genes (*crtN* and *crtM*) for carotenoid production, arranged as an operon. The same genes are described in the annotated complete genome sequence of *Lb. plantarum* WCFS1, a strain isolated from the human pharynx (Kleerebezem et al. 2003).

21.3 Important Characteristics of Fermented Vegetable Products

21.3.1 Viability of Probiotic Strains in Fermented Vegetable Products

To show healthy effects after consumption, probiotic microorganisms must be not only alive but also present in high numbers in the product at the time of purchase. Analysis of commercial probiotic foods conducted in the past decade showed that most of these products contained variable and, in some cases, very low concentrations of probiotics. This especially refers to probiotic foods containing species from the genera *Bifidobacterium*. For these reasons, consumers require that every type of probiotic food produced, including fermented vegetable juice, should be labeled with the correct information not only about the species of probiotic bacteria used for fermentation, but also about the minimum daily quantities necessary to achieve confirmed specific health benefits to the consumer.

Moreover, there have been no agreements about the recommended levels until now, whereas the suggested levels have a range from 10^6 CFU/mL (Kurman and Rasic 1991) to more than 10^7 and 10^9 CFU/mL (Lourens-Hattingh and Viljeon 2001). Such large differences in the data for dose required to obtain a therapeutic effect are mainly caused by a possible decline in the concentration of probiotic organisms during processing and storage of the probiotic product, as well as by the fact that the fermented products could lower the activity during passage through the upper and lower parts of the gastrointestinal tract. Also, recommended daily dose may vary from species to species, and even among strains within the same species. Because of all the above-mentioned reasons, the Fermented Milks and Lactic Acid

Bacteria Beverages Association from Japan has developed a standard in which at least 10^7 viable bifidobacteria per gram of a product is required to be present in human probiotic food to provide a therapeutic effect (Ishibashi and Shimamura 1993).

However, to achieve and maintain such a large number of live probiotic cells in food, it is important to avoid many different stress conditions during the manufacturing process and subsequent storage of the probiotic products. In addition to environmental factors such as water activity, redox potential (presence of oxygen), incubation, and storage temperature, the final acidity of the product, and the viability and activity of the probiotic is also linked with genus, species, and strain biotype of the probiotic, interactions between microbial species used as a starter culture for the production of fermented vegetable juices, production of hydrogen peroxide due to bacterial metabolism, availability of nutrients, growth promoters and inhibitors, concentration of sugar, composition and quality of other active ingredients present in the fermented vegetable juices, buffering capacity of the medium, inoculation level, and fermentation time (Del Piano et al. 2006; Vasiljevic and Shah 2008). From all these reasons, the conditions under which probiotic strains and finished probiotic fermented vegetables juices are produced and stored should be precisely defined and controlled. This will prevent variability among batches and will allow the optimization of viability/efficacy of probiotic strains in the final products.

Sometimes, in the production of certain types of probiotic fermented foods, it is recommended that starter cultures formed from several probiotic strains with different beneficial properties be used. In such cases, it is important to show that the probiotic strains incorporated in the starter culture do not inhibit each others' *in vivo* conditions. In addition, it should be taken into account that probiotic strains such as *Lb. acidophilus* and *Bifidobacterium* should not be combined with *Lb. delbruckii* ssp. *bulgaricus*, because these species produce hydrogen peroxide and considerable amounts of acid during fermentation in the presence of oxygen, which may affect the survival of probiotic strains. On the other hand, the presence of *S. salivarius* ssp. *thermophilus* in a starter culture, together with probiotic strains, may stimulate their growth because of the consumption of oxygen (Vasiljevic and Shah 2008).

Oxygen can be dissolved in the fermentation substrate and final products or can enter through packaging materials during storage. Its presence during fermentation and later in probiotic-containing products (positive redox potential) can have a detrimental effect on the viability of microaerophilic and anaerobic probiotic strains such as *Lb. acidophilus* and *Bifidobacterium* spp. Their characteristic is that they do not have electron-transport chains and incompletely reduce oxygen to hydrogen peroxide. Furthermore, they do not have catalase to convert hydrogen peroxide into water and its intracellular accumulation over time becomes toxic to the cell and leads to death. Studies have shown that species of *Bifidobacterium* are generally more susceptible to the deleterious presence of oxygen than *Lb. acidophilus*, which must be taken into account in their application.

As mentioned previously, many researchers have shown that raw vegetable juices without nutrient supplementation and pH adjustment (tomato, cabbage, beetroot, carrot, or celery) are ideal substrates for the growth of probiotic strains. In Table 21.4, the total number of viable cells reached in certain types of fermented vegetable juice at the end of the fermentation process and their stability during cold storage at 4°C are presented. As can be seen from the table, the total number of viable cells in most fermented vegetable juices is in the range of 10^8 to 10^9 CFU/mL at the end of the fermentation process, which indicates that the consumption of fermented vegetable juices immediately after the fermentation process could have a positive effect on human health.

It is evident from Table 21.4 that the total number of viable cells in fermented cabbage and beetroot juice at the end of fermentation largely depends on the strain and species of LAB used for fermentation. Therefore, although Yoon et al. (2005) achieved approximately 10^9 CFU/mL of fermented cabbage juice with strains of *Lb. plantarum* C3, *Lb. casei* A4, and *Lb. delbruckii* D7, Karovičová and Kohajdová (2003) obtained little more than 10^6 CFU/mL with some other strains from the same species in fermented cabbage juice. Similarly, this can be observed in beetroot juice fermented with different strains of *Lb. acidophilus*, *Lb plantarum*, *Lb. delbruckii*, and *Lb. casei* (Klewicka et al. 2004; Rakin et al. 2004a,b).

The reason for this variation in cell number could be a deficiency of some essential growth nutrients necessary for the growth of some strains from the same species in cabbage or beetroot juices, or similarly, in fresh cabbage juice, the presence of some antibacterial substance which could inhibit the growth of some LAB. Analysis of the growth-inhibitory substances from fresh cabbage juice showed that they

TABLE 21.4

Cell Viability in Fermented Vegetable Juices after Fermentation and Its Stability during Cold Storage at 4°C

Vegetable Juice	Strain Used for Fermentation	Cell Viability after Fermentation CFU/mL	Stability after 4 Weeks of Cold Storage at 4°C	Reference
Tomato	*Lb. acidophilus* LA39	$1.7 \pm 0.4 \times 10^9$	$1.4 \pm 0.4 \times 10^9$	Yoon et al. 2004
	Lb. plantarum C3	$2.0 \pm 0.5 \times 10^9$	$3.4 \pm 1.0 \times 10^6$	
	Lb. casei A4	$9.0 \pm 1.9 \times 10^8$	$1.7 \pm 0.5 \times 10^8$	
	Lb. delbrueckii D7	$7.7 \pm 1.0 \times 10^9$	$8.1 \pm 3.5 \times 10^8$	
	Lb. acidophilus BCRC 10695 (free cells)	1.3×10^9	2.7×10^8	Tsen et al. 2008
	Lb. acidophilus BCRC 10695 (immobilized in κ-carrageenan)	5.6×10^{10}	2.4×10^9	
Cabbage	*Lb. plantarum* C3	$1.75 \pm \times 0.705\ 10^9$	$4.1 \pm \times 0.16\ 10^7$	Yoon et al. 2006
	Lb. casei A4	$1.1 \pm \times 0.01\ 10^9$	ND	
	Lb. debrueckii D7	$1.1 \pm \times 0.15\ 10^9$	$4.5 \pm \times 3.32\ 10^5$	
	Lb. casei 589	1.2×10^6	Not tested	Karovičová and Kohajdová 2005
	Lb. lactis 447	1.5×10^6	Not tested	
	Lb. helveticus CCM 3826	2.3×10^6	Not tested	
	Lb. plantarum BIL	4.8×10^6	Not tested	
	Lb. pentosus	1.7×10^6	Not tested	
	Lb. acidophilus CCM 2913	3.5×10^5	Not tested	
	Lb. plantarum 190	1.2×10^6	Not tested	
	Lb. plantarum 195	3.2×10^5	Not tested	
	Lb. delbrueckii 237	1.9×10^6	Not tested	
	Lb. delbrueckii 238	2.5×10^6	Not tested	
	Lb. plantarum 181	3.0×10^5	Not tested	
	Lb. plantarum 187	1.6×10^6	Not tested	
	Lb. plantarum 192	1.9×10^6	Not tested	
	Lb. plantarum 976	4.0×10^5	Not tested	
	Lb. plantarum CCM 551	2.6×10^5	Not tested	
Carrot	*Lb. delbrueckii* ssp. *bulgaricus* DSM 20081 and ATCC 11842	5.1×10^9	2.9×10^9	Nazzaro et al. 2008
	Lb. delbrueckii ssp. *bulgaricus* DSM 20081 and ATCC 11842 + inulin	5.0×10^9	3.0×10^9	
	Lb. delbrueckii ssp. *bulgaricus* DSM 20081 and ATCC 11842+ fructooligosaccharides	5.2×10^9	3.3×10^9	
	Lb. rhamnosus DSM 20711	5.0×10^9	2.8×10^9	
	Lb. rhamnosus DSM 20711 + inulin	5.12×10^9	3.1×10^9	
	Lb. rhamnosus dsm 20711 + fructooligosaccharides	4.8×10^9	1.6×10^9	
	Bf. lactis Bb-12	1.65×10^8	Not tested	Kun et al. 2008
	Bifidobacterium bifidum B7.1	9.12×10^8	Not tested	
	Bifidobacterium bifidum B3.2	3.46×10^8	Not tested	
	Lb. plantarum RSKK 1062	4.32×10^9	Not tested	Demir et al. 2006
Beetroot	*Lb. acidophilus* LA39	$2.78 \pm 0.002 \times 10^9$	$1.61 \pm 0.182 \times 10^5$	Yoon et al. 2005
	Lb. plantarum C3	$9.2 \pm 5.0 \times 10^8$	$7.7 \pm 1.19 \times 10^7$	
	Lb. casei A4	$1.67 \pm 0.77 \times 10^9$	$7.2 \pm 3.53 \times 10^7$	
	Lb. delbrueckii D7	$1.53 \pm 0.23 \times 10^9$	$9.0 \pm 3.61 \times 10^6$	

(*continued*)

TABLE 21.4 (Continued)

Cell Viability in Fermented Vegetable Juices after Fermentation and Its Stability during Cold Storage at 4°C

Vegetable Juice	Strain Used for Fermentation	Cell Viability after Fermentation CFU/mL	Stability after 4 Weeks of Cold Storage at 4°C	Reference
Beetroot	*Lb. acidophilus* Ch-5	2.51×10^4	Not tested	Klewicka et al. 2004
	Lb. plantarum ŁOCK 0858	2.51×10^3	Not tested	
	Lb. delbrueckii subsp. *delbrueckii* ŁOCK 0854	3.16×10^4	Not tested	
	Lb. plantarum 2142	7.0×10^8	10^6	Barath et al. 2004
	Lb. curvatus 2770	7.0×10^8	10^6–10^7	
	Lb. casei pseudoplantarum 2745	7.0×10^8	10^6–10^7	
	Bifidobacterium BB12 (cloudy beetroot juice)	1.0×10^8	Not tested	Moraru et al. 2007
	Lb. plantarum A112	3.1×10^8	Not tested	Rakin et al. 2004a,b
	Lb. acidophilus NCDO1748	3.98×10^6	Not tested	
	Lb. acidophilus BGSJ15-3	3.98×10^8	Not tested	
Celery	*Bifidobacterium* BB12 (cloudy celery juice)	1.0×10^8	Not tested	Moraru et al. 2007
Cabbage-carrot	*Lb. plantarum* 178	1.1×10^4	Not tested	Karovičová et al. 1999
	Lb. plantarum 186	2.4×10^4	Not tested	
	Lb. plantarum 189	2.5×10^4	Not tested	
	Lb. casei 589	9.7×10^5	Not tested	
	Lb. lactis 447	1.3×10^6	Not tested	
	Lb. helveticus CCM 3826	1.4×10^6	Not tested	
	Lb. plantarum BIL	2.5×10^6	Not tested	
	Lb. pentosus	9.0×10^5	Not tested	
	Lb. acidophilus CCM 2913	1.8×10^6	Not tested	
	Lb. plantarum 190	6.3×10^5	Not tested	
	Lb. delbrueckii 237	1.0×10^5	Not tested	
	Lb. plantarum 181	1.6×10^5	Not tested	
	Lb. plantarum 187	8.3×10^5	Not tested	
	Lb. plantarum 192	1.0×10^6	Not tested	
	Lb. plantarum 976	2.1×10^5	Not tested	
	Lb. plantarum CCM 551	1.4×10^5	Not tested	
Beetroot juice and brewer's yeast autolysate in a 1:1 ratio	*Lb. acidophilus* NCDO1748	$7.0 \times 10^8 \pm 1.2 \times 10^8$	Not tested	Rakin et al. 2007
	Lb. plantarum A112	3.16×10^7	Not tested	Rakin et al. 2004a,b
	Lb. acidophilus BGSJ15-3	1.12×10^8	Not tested	
Carrot juice and brewer's yeast autolysate in a 1:1 ratio	*Lb. acidophilus* NCDO1748	$9.2 \times 10^7 \pm 0.6 \times 10^7$	Not tested	Rakin et al. 2005
Mixture of beetroot and carrot juice and brewer's yeast autolysate (1:1)	*Lb. plantarum* A112	3.1×10^7	Not tested	Rakin et al. 2003, 2004a,b
	Lb. acidophilus NCDO 1748	7.9×10^6	Not tested	
Mixture of beetroot and carrot juice and brewer's yeast autolysate (2:1)	*Lb. plantarum* A112	1.25×10^7	Not tested	
	Lb. acidophilus NCDO 1748	5.01×10^6	Not tested	

are carbohydrates in nature and of a low molecular weight. It is found that the inhibition of fresh juice could be eliminated if the cabbage is heated (steamed for 10 minutes) before juice extraction (Dickerman and Liberman 1952; Kyung and Fleming 1994a,b). In fermented beetroot juice, the total number of viable cells of *Lb. acidophilus* NCDO1748 can be increased in the final product if the beetroot juice is supplemented with brewer's yeast autolysate before fermentation (Rakin et al. 2005, 2007). The same authors also noticed that different contents of brewer's yeast autolysate in beetroot or carrot juice individually, or in mixtures, positively influenced the course of lactic acid fermentation and total number of cells in the final product. The main reason for this is the negligible amounts of vitamin B group members in beetroot and carrot juice, and the presence of brewer's yeast autolysate contributes to an increase in the content of these vitamins in the mixture and allows better growth and activity of especially fastidious bacteria such as LAB.

Such a large number of viable cells in fermented vegetable juices must be retained in products during cold storage. Research conducted thus far have shown that temperatures ranging from 4°C to 6°C were the most favorable for storage of different fermented products, including fermented vegetable juices because it facilitated the survivability of lactic fermentation bacteria and maintained appropriate sensory attributes. However, as can be seen from Table 21.3, only few research groups have thus far studied the stability of viable cell numbers in fermented vegetable juices during cold storage. Their studies confirmed earlier research on fermented dairy products which stated that the final pH of a product is a crucial factor for the survival of probiotic organisms. Many probiotic organisms do not thrive well if the pH of the substrate is lower than 4.4, which is what occurs in the case of fermented vegetable juices in which pH in the final product reaches 3.11 to 4.11. Also, it has been noticed that after fermentation, the pH of some fermented vegetable juices continue to decline during cold storage, in a process known as postacidification. Postacidification in fermented vegetable juices could lead to a further decrease in viable cell numbers. The rate of reduction depends on the probiotic strain and type of fermented vegetable juice.

Yoon et al. (2004, 2005, 2006) studied the stability of some probiotic strains (*Lb. acidophilus* LA39, *Lb. plantarum* C3, *Lb. casei* A4, and *Lb. delbrueckii* D7) in different fermented vegetable juices (tomato, cabbage, and beetroot) during 4 weeks of cold storage at 4°C. As can be seen from Table 21.3, although the total number of viable cells in all fermented vegetable juices reached approximately 10^9 CFU/mL after fermentation, the fastest decline was observed in fermented cabbage juices. In this fermented vegetable juice, the viable cell number of *Lb. plantarum* C3 and *Lb. delbrueckii* D7 were 4.1×10^7 and 4.5×10^5, respectively, after 4 weeks of storage at 4°C, whereas *Lb. casei* A4 lost cell viability completely after only 2 weeks of cold storage, indicating that it was unable to survive the low pH and high acidity conditions present in the fermented cabbage juice. Slightly better results were achieved in fermented beetroot juice after 4 weeks of storage at 4°C, in which *Lb. acidophilus* LA39 was considerably less stable (1.61×10^5) in comparison with other strains *Lb. plantarum* C3, *Lb. casei* A4, *Lb. delbrueckii* D7 (7.7×10^7, 7.2×10^7, and 9.0×10^6, respectively). The best stability was observed in fermented tomato juice where the number of viable cells obtained after 4 weeks of cold storage at 4°C were 1.4×10^9, 3.4×10^6, 1.7×10^8, and 8.1×10^8 for *Lb. acidophilus* LA39, *Lb. plantarum* C3, *Lb. casei* A4, and *Lb. delbrueckii* D7, respectively. Based on these results, it can be concluded that except for fermented cabbage juice with *Lb. casei* A4 and fermented beetroot juice with *Lb. acidophilus* LA39, all other fermented vegetable juices contained more than 10^6 CFU/mL of viable cells after 4 weeks of cold storage at 4°C. This viable number is a prerequisite for fermented products to achieve maximum health benefits at the time of consumption.

21.3.2 Current Studies of Health-Promoting Properties of Some Fermented Vegetable Juices

The consumption of lactic acid–fermented food should be increased in the population (Kohajdová and Karovičová 2005). Another important factor is the supply of lactic acid–fermented vegetable juices with new quality characteristics, without application of preserving agents. Fermented vegetable juices are abundant in sugars, vitamins, specific substances, and various metabolites which are beneficial to our health.

Fermented beetroot juice can modify the metabolic activity of animals and thereby reduce the risk of diseases associated with food intolerance. Consumption of fermented food which contain live cells of LAB can positively modulate intestinal microflora and its metabolic activity, particularly enzymes which participate in the process of carcinogenesis.

Regular intake of fermented beetroot juice can contribute to the stabilization of the intestinal microbial ecosystem. Klewicka et al. (2004) studied the effects of beetroot juice fermented by *Lb. casei* 0920 and *Lb. brewis* 0944 on the state of intestinal ecosystems of experimental animals (rats). Supplementation of the diet of experimental rats with fermented beetroot juice (groups of animals were fed for 4 weeks) considerably reduced the number of Enterobacteriaceae family bacteria. The decrease in their number grew with the volume of ingested juice and approached 0.8 log units for 1.5 mL of juice to –2.1 log units for 6.0 mL of juice.

A study by Klewicka (2010) provides evidence that the beetroot juice fermented by *Lb. paracasei* 0916 strain maintains its antimutagenic activity. The juice (a dose of 10 μL) decreased the number of mutations of *Salmonella typhimurium* TA98 and *S. typhimurium* TA 100 by 61% and 65%, respectively. The beetroot juice fermented by *Lb. brevis* 0944 retained its ability to reduce mutations of *S. typhimurium* TA98 and *S. typhimurium* TA 100 by 56% and 55%, respectively. The LAB used in this study were characterized by relatively heterofermentative metabolisms (*Lb. paracasei*) or strictly heterofermentative metabolisms (*Lb. brevis*). These bacteria form a mixture of organic acids rich in lactic and acetic acids. Lankaputhra and Shah (1998) found that acetic acid showed higher antimutagenic activity than lactic acid.

In search of nontoxic cancer-chemopreventive agents, the antitumor promoting effects of several fermented vegetable juices or extracts as well as the natural colorants used in food and pharmaceutical products need to be evaluated and reported. The specific mechanism of how fermented foods prevent carcinogenesis is still unclear, but it may be attributed in part to the metabolisms of certain substances. More research has to be carried out to ascertain the factors or combination of factors responsible for the positive consequences of fermented diets. Ming et al. (2007) investigated the antitumor activity of vegetables and fruits fermented liquids. Their results indicate that vegetables and fruits fermented liquids may inhibit proliferation and promote apoptosis of hematomas. Apoptosis is an important mechanism in the cytocidal effect of antitumor drugs.

Beetroot juice has also been studied for promoting health. *In vitro* tests of beetroot extract for antitumor promoting effect on Epstein–Barr virus showed a high order of activity. The activity was higher than that of extracts of peppers, red onion skin, and cranberry. Kapaida et al. (1996) evaluated *in vivo* antitumor promoting activity against mice skin and lung assays. The results of these bioassays indicated that orally administered beetroot extract to mice inhibited TPA-induced promotion of lung tumors in this species. The results of pulmonary tumor inhibitory effect indicated that compared with control, although a crude extract was tested, there was a 60% reduction of lung tumors. These findings indicate that beetroot is a useful cancer-preventive vegetable.

21.3.3 Future Perspectives in the Production of Fermented Vegetable Juices

Fermented vegetable juices would be the next food category where healthy bacteria will make their mark. Probiotic bacteria must be live to exert their health benefits. The stability of probiotic strains during fermentation and cold storage in vegetable juices could be further improved with the development of microencapsulation technologies or by supplementation of fermented vegetable juices with an appropriate quantity of prebiotic.

Microencapsulation is one of the newest and highly efficient methods, which is very intensively used today in several different fields, including food technology, because it prevents interfacial inactivation, stimulates production and excretion of secondary metabolites, and allows continuous use and protection against disadvantageous and unfavorable environments. During microencapsulation, probiotic cells remain closed within encapsulating membrane, which protects cells and enables them to survive various stress conditions present in the external environment, during the fermentation and cold storage. In addition, inside the microcapsule, probiotic cells better survive gastroduodenal transit and, in high concentrations, enter the jejunum and the ileum where they could attach to epithelial cells and temporarily or permanently inhabit the environment.

Several studies have reported that different materials such as gelatin, vegetable gum, alginate, starch, mixture of xanthane-gelan, carrageenan, cellulose acetate phthalate, and chitosan, could be used as encapsulating materials for food application (Mortazavian et al. 2007). The research conducted thus far has shown that microcapsules prepared from gelatin and vegetable gum could provide good protection, especially when acid-sensitive probiotic organisms are used. In addition, because of the process requirements and overall cost of food products, a great potential for future application showed alginate microcapsules (Del Piano et al. 2006; Vasiljevic and Shah 2008). However, until now, only one research study used κ-carrageenan as an encapsulating material for immobilization of *Lb. acidophilus* BCRC 10695 and fermentation of tomato juice (Tsen et al. 2008). Results obtained in this study showed that after 80 hours of fermentation of tomato juice, final viable cell numbers in gel beads (average diameterm approximately 3.0 mm) were more than 1010 CFU/mL, ten times more than in sample fermented with free cells (Table 21.4). Also, they noticed that after 10 weeks of cold storage at 4°C, the viable cell numbers in a sample with immobilized cells were significantly higher in comparison with the sample fermented with free cells (10^6 and 10^4 CFU/mL, respectively). Therefore, these results confirmed that immobilized cells could better withstand the unfavorable low pH in tomato juice (pH 4.2) during fermentation and cold storage. Furthermore, fermented tomato juice with immobilized cells of *Lb. acidophilus* BCRC 10695 had better sensory quality than the juices produced with free cells which open the possibility for further research in this field.

Incorporating both prebiotics and alginate in coating materials may better protect probiotics in the food systems and gastrointestinal tract due to synbiosis. Nazzaro et al. (2008) studied microencapsulated *Lb. acidophilus* DSM 20079 within an alginate-xantan-inulin matrix and evaluated its ability to ferment carrot juice and withstand gastrointestinal stresses after fermentation and 8 weeks of storage at 4°C. Their results indicated that the encapsulated cells showed higher fermentation activity than free cells. Immobilized cells were more resistant to both the low pH and the pancreatic juice, and concentration-immobilized cells remained constant. A similar trend was noticed after 8 weeks at 4°C. Burleanu et al. (2009) studied the addition of inulin to the process of lactic acid fermentation of beetroot juice using *Lb. acidophilus* and multiple cultures of *Bifidobacterium*. They also concluded that in samples with inulin, the lactic fermentation process began faster than in those without inulin. Also, the addition of sugars such as fructooligosaccharide promoted the fermentation process and improved the tastes of fermented juice (Koh et al. 2010).

Summarizing the overall findings, we can conclude that the fermented vegetable juices represent a basis for functional food products with high added value and may benefit consumers searching for an alternative beverage to replace fermented dairy products. Because of this, we hypothesize that fermented vegetable juices would become an increasingly important category in the future.

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REFERENCES

Annuk H, Shchepetova J, Kullisaar T, Songisepp E, Zilmer M, Mikelsaar M. 2003. Characterization of intestinal lactobacilli as putative probiotic candidates. *J Appl Microbiol* 94:403–12.

Barath A, Halas A, Nemeth E, Zalan Z. 2004. Selection of LAB strains for fermented red beet juice production. *Eur Food Res Technol* 218:184–7.

Begley M, Gahan CGM, Hill C. 2005. The interaction between bacteria and bile. *FEMS Microbiol Rev* 29:625–51.

Bender DA. 2003. *Nutritional Biochemistry of the Vitamins*. 2nd ed. Cambridge, UK: Cambridge University Press. 512 p.

Buckenhüskes HJ. 1997. Fermented vegetables. In: Doyle MP, Beuchat LR, Montville, IM, editors. *Food microbiology. Fundamentals and frontiers*. Washington, DC: A.S.M. Press. p. 595–609.

Burleanu L, Manea I, Bratu MG, Avram D, Nicolescu CL. 2009. Effects of prebiotics on the quality of lactic acid fermented vegetable juices. *Ovidius Univ Ann Chem* 20:102–7.

Chung MJ, Sung HL, Sung NJ. 2002. Inhibitory effect of whole strawberries, garlic juice or kale juice on endogenous formation of *N*-nitrosodimethylamine in humans. *Can Lett* 182:1–10.

Clauditz A, Resch A, Wieland KP, Peschel A, Gotz F. 2006. Staphyloxanthin plays a role in the fitness of *Staphylococcus aureus* and its ability to cope with oxidative stress. *Infect Immun* 74:4950–3.

Connor WE. 2000. Importance of n-3 fatty acids in health and disease. *Am J Clin Nutr* 71:171S–5S.

Del Piano M, Morelli L, Strozzi GP, Allesina S, Barba M, Deidda F, Lorenzini P, Ballarė M, Montino F, Orsello M, Sartori M, Garello E, Carmagnola S, Pagliarulo M, Capurso L. 2006. Probiotics: from research to consumer. *Dig Liver Dis* 38:248–55.

Demir N, Bahceci KS, Acar J. 2006. The effects of different initial *Lactobacillus plantarum* concentrations on some properties of fermented carrot juice. *J Food Proc Preserv* 30:352–63.

Di Cagno R, Surico RF, Paradiso A, De Angelis M, Salmon J C, Buchin S, De Gara L, Gobbetti M. 2009. Effect of autochthonous lactic acid bacteria starters on health-promoting and sensory properties of tomato juices. *Int J Food Microbiol* 128:473–83.

Dickerman JM, Liberman S. 1952. Studies on the chemical nuture of an antibiotic present in water extract of cabbage. *Food Res* 17:438–41.

Ding WK, Shah NP. 2009. Effect of various encapsulating materials on the stability of probiotic bacteria. *J Food Sci* 74:100–7.

Espinoza YR, Navarro YG. 2010. Non-dairy probiotic products. *Food Microbiol* 27:1–11.

Fernández JG, Barragán AM, Guerrero BC, Méndez DH, Barba JLR. 2010. Carotenoid production in *Lactobacillus plantarum. Int J Food Microbiol* 140:34–9.

Ganzle M, Hertel C, van der Vossen J, Hammes W. 1999. Effect of bacteriocin producing lactobacilli on the survival of *Escherichia coli* and *Listeria* in a dynamic model of the stomach and the small intestine. *Int J Food Microbiol* 48:21–35.

Hung HC, Joshipura KJ, Jiang R, Hu FB, Hunter D, Smith-Warner SA, Colditz GA, Rosner B, Spiegelman D, Wilett WC. 2004. Fruit and vegetable intake and risk of major chronic disease. *J Natl Cancer Inst* 96:1577–84.

Iannitti T, Palmieri B. 2010. Therapeutical use of probiotic formulations in clinical practice. *Clin Nutr* 29:701–25.

Ishibashi N, Shimamura S. 1993. Bifidobacteria: research and development in Japan. *Food Tech* 46:126–35.

Johansson ML, Molin G, Jeppsson B, Nobaek S, Ahrné S, Bengmark S. 1993. Administration of different *Lactobacillus* strains in fermented oatmeal soup: *in vivo* colonization of human intestinal mucosa and effect on the indigenous flora. *Appl Environ Microbiol* 59:15–20.

Kapaida GJ, Tokuda H, Konoshima T, Nishino H. 1996. Chemoprevention of lung and skin cancer by *Beta vulgaris* (beet) root extract. *Cancer Lett* 100:211–4.

Karovičová J, Drdák M, Greif G, Hybenová E. 1999. The choice of strains of *Lactobacillus* species for the lactic acid fermentation of vegetable juices. *Eur Food Res Technol* 210:53–6.

Karovičová J, Kohajdová Z. 2003. Lactic acid fermented vegetable juices. *Hortic Sci* 30:152–8.

Karovičová J, Kohajdová Z. 2005. Lactic acid–fermented vegetable juices—palatable and wholesome foods. *Chem Pap* 59:143–8.

Kleerebezem M, Boekhorst J, van Kranenburg R, Molenaar D, Kuipers OP, Leer R, Tarchini R, Peters SA, Sandbrink HM, Fiers MWEJ, Stiekema W, Lankhorst RMK, Bron PA, Hoffer SM, Groot MNN, Kerkhoven R, de Vries M, Ursing B, de Vos WM, Siezen RJ. 2003. Complete genome sequence of *Lactobacillus plantarum* WCFS1. *Proc Natl Acad Sci USA* 100:1990–5.

Klewicka E. 2010. Fermented beetroot juice as a factor limiting chemical mutations induced by MNNG in *Salmonella typhimurium* TA98 and TA100 Strains. *Food Technol Biotechnol* 48:229–33.

Klewicka E, Motyl I, Libudzisz Z. 2004. Fermentation of beet juice by bacteria of genus *Lactobacillus* sp. *Eur Food Res Technol* 8:178–83.

Koh JH, Kim Y, Oh JH. 2010. Chemical characterization of tomato juice fermented with *Bifidobacteria. J Food Sci* 75:428–32.

Kohajdová Z, Karovičová J. 2005. Sensory and chemical evaluation of lactic acid–fermented cabbage-onion juices. *Chem Pap* 59:55–61.

Kruszewska D, Lan J, Lorca G, Yanagisawa N, Marklinder I, Ljungh Å. 2002. Selection of lactic acid bacteria as probiotic strains by *in vitro* tests. *Microb Ecol Health Dis* 29:37–49.

Kun S, Rezessy-Szabo J, Nguyen Q, Hoschke A. 2008. Changes of microbial population and some components in carrot juice during fermentation with selected *Bifidobacterium* strains. *Proc Biochem* 43:816–21.

Kurman JA, Rasic JL. 1991. The health potential of products containing bifidobacteria. In: Robinson RK, editor. *Therapeutic properties of fermented milks*. London: Elsevier Applied Food Sciences. p. 117–58.

Kyung KH, Fleming HP. 1994a. Antibacterial activity of cabbage juice against lactic acid bacteria. *J Food Sci* 59:125–9.

Kyung KH, Fleming HP. 1994b. *S*-metyl-L-cysteine sulfoxide as the precursor of methanethiolsulfinate, the principal antibacterial compound in cabbage. *J Food Sci* 59:350–5.

Lankaputhra WEV, Shah NP. 1998. Antimutagenic properties of probiotic bacteria and of organic acids. *Mutat Res* 397:169–82.

Liepe HU, Junker M. 1984. Gemüsesaefte. *Flüssiges Obst* 50:304–7.

Lin MY, Chang FJ. 2000. Antioxidative effect of intestinal bacteria *Bifidobacterium longum* ATCC 15708 and *Lactobacillus acidophilus* ATCC 4356. *Dig Dis Sci* 45:1617–22.

Liu S, Manson JE, Lee IM, Cole SR, Hennekens CH, Wilett WC, Buring JE. 2000. Fruit and vegetable intake and risk of cardiovascular disease:the women health study. *Am J Clin Nutr* 72:922–8.

Ljungh A, Wadström T. 2005. Lactic acid bacteria as probiotics. *Curr Issues Intestinal Microbiol* 7:73–90.

Lorca GL, de Valdez F. 2001. A low-pH-inducible stationary-phase acid tolerance response in *Lactobacillus acidophilus* CRL 639. *Curr Microbiol* 42:21–5.

Lourens-Hattingh A, Viljeon CB. 2001. Yogurt as probiotic carrier food. *Int Dairy* J 11:1–17.

Matsumoto M, Ohishi H, Benno Y. 2004. H+ ATPase activity in *Bifidobacterium* with special reference to acid tolerance. *International J Food Microb* 93:109–13.

Ming L, Toshima Y, Xiao Wu, Zhang X, Cai Y. 2007. Inhibitory effects of vegetable and fruit fermented liquid on tumor growth in Hepatoma-22 inoculation model. *Asia Pac J Clin Nutr* 16:443–6.

Moraru D, Bleoanca I, Segal R. 2007. Probiotic vegetable juices. The Annals of the University Dunareade Jos of Galati—Food Technology. Gelati, Romania: Fascicle IV.

Mortazavian A, Razavi SH, Ehsani MR, Sohrabvanci S. 2007. Principles and methods of microencapsulation of probiotic microorganisms. *Iranian J Biotechnol* 5:1–18.

Moubareck C, Gavini F, Vaugien L, Butel MJ, Doucet-Populaire F. 2005. Antimicrobial susceptibility of bifidobacteria. *J Antimicrob Chemother* 55:38–44.

Nazzaro F, Fratianni F, Sada A, Orlando P. 2008. Synbiotic potential of carrot juice supplemented with *Lactobacillus* spp. and inulin or fructooligosaccharides. *J Sci Food Agric* 88:2271–6.

Nettleton JA. 1995. *Omega*-3 *fatty acids and health*. New York: Chapman Hall. 384 p.

Ouwehand AC, Kirjavainen PV, Shortt C, Salminen S. 1999. Probiotics: mechanisms and established effects. *Int Dairy J* 9:43–52.

Prado FC, Parada JL, Pandey A, Soccol CR. 2008. Trends in non-dairy probiotic beverages. *Food Res Int* 41:111–23.

Rakin M, Baras J, Vukašinović M. 2004a. The influence of brewer's yeast autolysate and lactic acid bacteria on the production of a functional food additive based on beetroot juice fermentation. *Food Technol Biotechnol* 42(2):109–13.

Rakin M, Baras J, Vukašinović M. 2005. Lactic acid fermentation in vegetable juices supplemented with different content of brewer's yeast autolysate. *Acta Period Technol* 36:71–80.

Rakin M, Baras J, Vukašinović M, Maksimović M. 2004b. The examination of parameters for lactic acid fermentation and nutritive value of fermented juice of beetroot, carrot and brewer's yeast autolysate. *J Serb Chem Soc* 69:625–34.

Rakin M, Baras J, Vukašinović M, Mijuca D. 2003. Optimization of lactic-acid fermentation of beetroot juice and brewer's yeast autolysate. *Roumanian Biotechnol Lett* 8(5–6):1421–30.

Rakin M, Vukasinovic M, Siler-Marinkovic S, Maksimovic M. 2007. Contribution of lactic acid fermentation to improved nutritive quality vegetable juices enriched with brewer's yeast autolysate. *Food Chem* 100:599–602.

Randaheera RDCS, Baines SK, Adams MC. 2010. Importance of food in probiotic efficacy. *Food Res Int* 43:1–7.

Rodgers S. 2008. Novel applications of live bacteria in food services: probiotics and protective cultures. *Trends Food Sci Technol* 19:188–97.

Schobinger U. 1987. *Frucht-und Gemuesesaefte: 2. Auflage*. Stuttgart: Verlag Eugen Ulmer. 637 p.

Seiger DS. 1998. *Plant secondary metabolilism*. Dordrecht, The Netherlands: Kluwer Academic Publishers. 592 p.

Shahidi F, Miraliakbari H. 2005. Omega-3 fatty acids in health and disease: part 2. Health effects of omega 3 fatty acids in autoimmune diseases, mental health and gene expression. *J Med Food* 8:133–48.

Shenoy NR, Choughuley ASU. 1989. Effect of certain plant phenolics on nitrosamine formation. *J Agric Food Chem* 37:721–5.

Simopoulos AP. 1999. Essential fatty acids in health and chronic disease. *Am J Clin Nutr* 70:560S–9S.

Tsen JH, Lin YP, Huamg HY, King VA. 2008. Studies on the fermentation of tomato juice by using κ-carrageenan immobilized *Lactobacillus acidophilus*. *J Food Proc Preserv* 32:178–89.

Vasiljevic T, Shah NP. 2008. Probiotics—from Metchnikoff to bioactives. *Int Dairy J* 18:714–28.

Vinson JA, Hao Y, Su C, Zubic L. 1998. Phenol antioxidant quantity and quality in foods: vegetables. *J Agric Food Chem* 46:3630–4.

Watson RR, Preedy VR. 2010. *Bioactive foods and extracts: cancer treatment and prevention*. Boca Raton, FL: CRC Press Taylor & Francis Group. 643 p.

Yoon KY, Woodams EE, Hang YD. 2004. Probiotication of tomato juice by lactic acid bacteria. *J Microbiol* 42:315–8.

Yoon KY, Woodams EE, Hang YD. 2005. Fermentation of beet juice by benefical lactic acid bacteria. *Lebensm Wiss Technol* 38:73–5.

Yoon KY, Woodams EE, Hang YD. 2006. Production of probiotic cabbage juice by lactic acid bacteria. *Biores Technol* 97:1427–30.

22

Almagro Eggplant: From Homemade Tradition to Small-Scale Industry

Susana Seseña and María Llanos Palop

CONTENTS

22.1 Introduction

Pickling and fermentation are preservation methods that extend fruit and vegetable shelf life using a simple and inexpensive technology. Vegetable production is usually seasonal, and these technologies are a traditional means of preserving harvested perishable and palatable products.

Brine fermentation involves complex microbial, chemical, and physical reactions and gives the final products unique flavor characteristics. Almost all vegetables provide sufficient nutrients for the growth of fermentation-related microorganisms, and the growth of certain fermentative microorganisms during fermentation results in sensory changes that are desired by the consumers.

"Almagro eggplant" (*Berenjena de Almagro*) is a pickle traditionally made in the province of Ciudad Real in the Castilla-La Mancha region of Spain. It is produced in a few villages around Almagro (Almagro is a village; Figure 22.1) over an area of approximately 108,616 hectares. For its production, an autochthonous variety of eggplant, *Solanum melongena* L. var. *esculentum depressum* or Almagro, is used. There are various preparations available on the market, such as seasoned, stuffed, stuffed with pepper paste, and sliced.

This pickle is commonly consumed as an appetizer, and its traditionally made seasoning of vinegar, vegetable oil, table salt, cumin, garlic, paprika, and water has been used since ancient times. At the turn of the century, locals bought the eggplants from the growers at harvest time and bottled them in glazed jars. The composition of the seasoning varied depending on the expected time of consumption. There were many homes where eggplants were seasoned and, although the formula was widely known, there were specialists who were able to get the seasoning just right and were very much in demand. In time, production went from a family tradition to small-scale industry.

Since 1994, its production has come under the "Berenjena de Almagro" Protected Geographical Indication (PGI; Reglamento de la Denominación Específica de la Berenjena de Almagro y de su Consejo

FIGURE 22.1 Map of the eggplant production area.

Regulador 1994), making it one of the first Spanish vegetables to obtain this recognition. Information provided by the Berenjena de Almagro PGI Regulatory Board indicate that, in 2009, 2000 tons of eggplants were accepted and marketed under the PGI, which gives one an idea of the economic importance of this product to this region of Spain.

22.2 Almagro Eggplant

Eggplant (*S. melongena*) cultivation began, according to various studies, in subtropical, tropical, and Asian regions such as India, China, and Burma. It was through the Silk Road, and the Arabs, true specialists in vegetable dishes and preserves, that it finally arrived in the west. However, it was in the Islamic world where the eggplant became important.

The variety used to make this pickle (*S. melongena* L. var. *esculentum depressum* or Almagro) depends on the farmers themselves, who select and sow the seeds, and it is closer to the wild variety than other eggplants consumed by humans.

The harvest normally begins in July and ends in October when the temperatures go down, although this period can vary because of climatic conditions. The harvesting is done by hand and takes place every three or four days when the fruit reaches optimum ripeness.

22.2.1 Characteristics of the Fruit

The fruit of the variety used in the production of "Almagro eggplants" is a fleshy berry, greenish when covered by the calyx, but becoming purple or dark when uncovered. It tends to be round, pear-shaped, or egg-shaped and measures from 7 to 10 cm in length (Figure 22.2). These characteristics make it a product that is very well suited to industrial production.

Typically, fruits have prickles on their bracts and stems, which must be removed at the selection stage during the production process. The flesh is whitish in color, and when the fruit is ripe, the seeds are visible inside (Figure 22.3).

22.2.2 Almagro Eggplant Production

The first written reference describing in certain detail the stages of the Almagro eggplant production process was reported by Morales Mocino (1985), and includes a diagram of the production process as shown in Figure 22.4.

Years later, when the Berenjena de Almagro Regulating Board was created in July 1994 with the aim of controlling and ensuring the quality of the products accepted under the PGI, regulations were made public describing aspects relating to the characteristics of the raw material and finished product, as well as the production process that would be mandatory for any product to be admitted under the PGI.

FIGURE 22.2 Fruit used in Almagro eggplant production.

With respect to the production process, the regulations state:

1. All the eggplants to be protected under the geographical indication must be carefully selected before they undergo the production process, removing those that are damaged or altered.
2. Almagro eggplant production will be comprised of the following stages:
   - Blanching in boiling water for 5 to 20 minutes, or in any event, sufficient time to inhibit undesired microbial activity while ensuring that the fruit preserves its texture, without softening.

FIGURE 22.3 Interior appearance of the fruit.

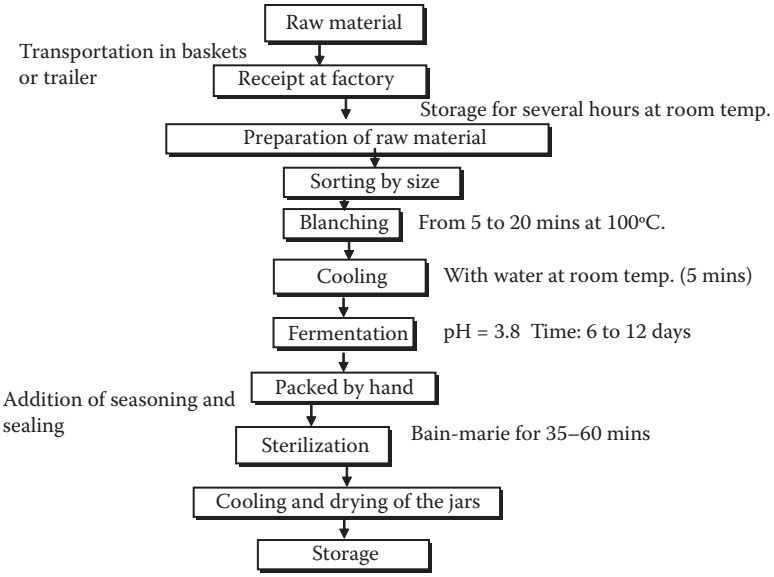

FIGURE 22.4 Diagram of the traditional production process of "Almagro eggplants."

- Fermentation: after blanching, the eggplants are placed in suitable containers and the brine is added. The fermentation time is from 4 to 20 days.
- Brine: the composition of the brine is vinegar, oil, salt, cumin, garlic, paprika, and water. Additives permitted by the corresponding quality standards may be used.
- Packing: packing is carried out using the permitted mechanical methods and in the necessary hygienic and preservation conditions. Containers of metal, glass, or other materials permitted under the legislation in force can be used.

The manufacture of Almagro eggplant, although simple in essence, is a laborious process that combines traditional methods with new production technologies.

The following stages can be defined:

1. Preliminary operations. In these operations, the fruit is prepared for subsequent processing. These include peeling, sorting, and grading. These operations are performed to discard products that are unsuitable for processing—damaged, unripe, or overripe fruit and fruits that fail to meet suitable size, color, or texture parameters.
   1.1. Peeling involves cutting off the stem and bracts so that they do not protrude from the fruit and as many prickles as possible are removed. These operations are carried out simultaneously and by hand.
   1.2. Next, the eggplants are measured and sorted according to size by using mechanical sorters.
   1.3. Grading homogenizes the product, improving heat processing efficiency. Trained staff normally carry out the quality control procedures.
2. Blanching. Also known as "cooking" of the eggplants. This heat process is applied to inhibit enzymatic activity or decrease microbial populations. The eggplants are placed in large stainless steel cages (Figure 22.5) that are submerged in boiling water for the appropriate time depending on the size of the fruit to be blanched.
3. Cooling. After blanching, the cages are placed in tubs of water at room temperature until cooled.

FIGURE 22.5 Cage used for blanching.

4. Fermentation. The blanched eggplants are placed in barrels, frequently of plastic, with a capacity of approximately 250 liters (Figure 22.6). Before they are filled, a certain volume of brine is added to the barrels to prevent damage to the fruit.

   Once the barrels are filled, the brine, prepared according to a "secret" recipe passed down from generation to generation, is added. The composition of brine, although varying greatly, always contains as essential ingredients vinegar, salt, and water, to which spices and condiments are added.

FIGURE 22.6 Eggplants fermenting.

The eggplants are left to ferment spontaneously and at room temperature for a period ranging from 4 to 20 days. In the traditional production process, fermentation takes place at room temperature, so the product is exposed to variations in temperature between day and night typical of the summer months, when most of the manufacturing takes place. The physical-chemical and microbiological changes that occur at the fermentation stage are described in detail later on.

After fermentation, the fruit that is going to be used for preparations such as stuffed, filled, or sliced eggplants will be processed accordingly. In the case of stuffed eggplants, for instance, the fruit is slit open and a piece of chili pepper or red pepper is held in place with a fennel stalk. To prepare the "sliced" eggplants, the stalks and bracts are removed from the fruit and sliced to facilitate consumption.

5. Packaging. The aim of this operation is to maintain the same hygiene and quality conditions from processing to consumption.

   During this stage, the eggplants are placed in containers of tin or glass and, although in this operation, the use of mechanical procedures is permitted, it is often carried out by hand, especially when small containers are used.

   After placing the fruit in containers, hot seasoning is added, the composition of which varies depending on the product that has been prepared, although it is normally made up essentially of salt, vinegar, and spices. The filled containers then go through a tunnel for both the eggplants and the brine to reach a minimum temperature of 65°C, so that, once the containers are sealed, a vacuum occurs inside (Figure 22.7). The containers of the products accepted under the PGI must have the corresponding labels (Figure 22.8), indicating this fact and the quality standards that they meet.

FIGURE 22.7 Preparations of Almagro eggplants.

FIGURE 22.8 Labeling of the PGI.

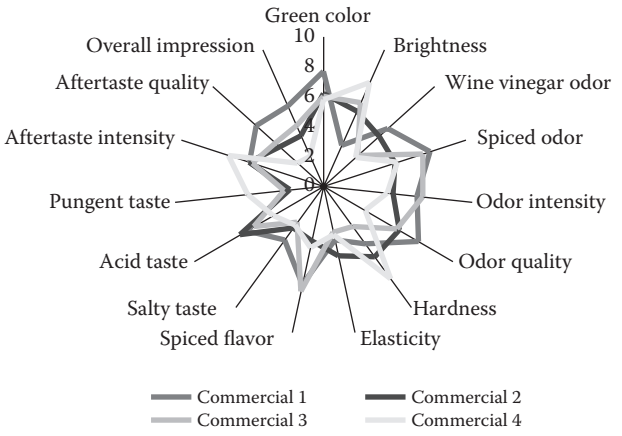

FIGURE 22.9 Sensory profiles for eggplants elaborated in different factories.

22.2.3 Sensory Characteristics of Almagro Eggplant

The organoleptic properties of the Almagro eggplant are primarily determined by the production method used. The texture essentially depends on the moment at which the fruit is collected and the softening effect of the cooking and fermentation stages. The flavor is also highly variable, depending on the composition of the seasoning. The fennel stalk at the end of the fruit in some preparations also adds a very unique flavor.

The Berenjena de Almagro PGI Regulating Board has compiled an official tasting sheet to be used to evaluate the sensory quality of the product. It is used to evaluate various attributes relating to the appearance, smell, and flavor of the eggplants, as well as the presence of defects.

In the studies carried out by our research group, sensory evaluations were conducted on eggplants produced at various factories for comparison, and spider web diagrams, like the one shown in Figure 22.9, were obtained.

Consumers can obtain numerous health benefits from Almagro eggplants, as a result of their nutritional properties. For instance, their high fiber content improves gastrointestinal transit. Their nutritional properties also include high vitamin A content, antioxidant substances such as ascorbic acid and polyphenols, riboflavin and thiamine, and minerals such as calcium, phosphorous, iron, sodium, and potassium, among others.

The polyphenolic compounds include hydroxycinnamic acid derivatives, such as chlorogenic acid, which constitutes 70% to 95% of the total polyphenols of the eggplant flesh. This chlorogenic acid is a powerful antioxidant and it has been shown to delay aging and reduce cholesterol levels in the blood. It also displays antitumor activity (Prohens et al. 2008). Consequently, genetic improvement programs are currently being conducted to develop varieties of eggplant with nutraceutical properties, resulting from its higher content of polyphenols, without its appearance being affected by browning during production.

22.3 Chemical Characterization of Commercial Traditionally Manufactured Almagro Eggplants

Although the PGI's regulations describe some of the chemical characteristics of the fermented eggplants, there is not an awful lot of information in this regard. Some years ago, our laboratory performed a study for chemical characterization and a large number of Almagro eggplants obtained from shopping centers were analyzed.

This study (Ballesteros et al. 1998) determined some physical properties (size, color, density, weight, etc.), mechanical properties (texture), chemical composition (ash, dry extract, reducing sugars, etc.), and defects (presence of piercings, bruises, lesions, etc.) of these eggplants.

The results of the study revealed that there was a great deal of heterogeneity of size in commercial containers, unlike with other similar products like cucumbers, which is a clear disservice to consumers. Likewise, it also revealed that there was considerable dispersion of data, resulting not just from the

different sizes of the fruits, but also owing to the differences in the technological treatments used in the process; blanching in particular.

The results of the chemical analysis showed that the values for the ash and protein nitrogen content were not significantly different, with average values of 2.42 and 1.48 g/100 g, respectively. In contrast, the values obtained for the dry extract were significantly different and the largest eggplants displayed lower values for dry extract as a result of the higher quantity of water present in their interstices. Significant differences were also found in the D-lactic acid content, a compound produced during the fermentation stage, which was greater in the larger fruits owing to their more advanced ripeness and the higher reducing sugar content (glucose and fructose) of the fresh fruits.

The residual glucose values in the fermented eggplants, although low, were in some cases significant, indicating that fermentation had been incomplete. Collective fermentation of fruits with different physical and chemical properties makes the fermentation occur in a different way and this could be the reason for why the fermented products also display differences in parameters, such as lactic acid, which are directly related to the evolution of the fermentation process.

Analysis with consumers revealed that the smaller eggplants were greatly preferred; their most highly valued attributes being aroma, flavor, juiciness, appearance (absence of defects), and taste sensation.

22.4 Microbiology of Almagro Eggplant Fermentation

A detailed study of this process (Ballesteros et al. 1998), carried out in several of the manufacturing companies accepted under the Berenjena de Almagro PGI, revealed that there is a diffusion of the sugars present in the fruits into the brine during the first few days of fermentation (Figure 22.10), and simultaneously, although with a delay of two days and as a consequence of fermentation, lactic acid appears in the brine (Figure 22.11).

This causes a slight drop in the pH of brine (Figure 22.12), although the buffering nature of the brine's ingredients prevents sharp declines, which could halt the fermentation process.

When a microbiological analysis of the brine was carried out (Figure 22.13), it was observed that, although the initial lactic bacteria counts were low (10^3 cells/mL), after a short time (6–10 hours), they underwent a sharp increase, reaching values approaching 10^8 cells/mL after 48 hours. These values remained constant

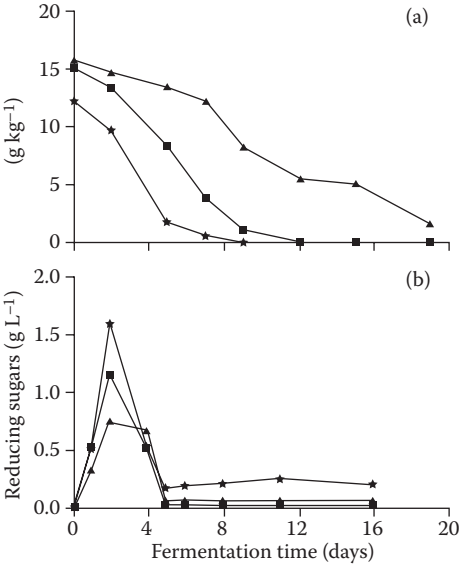

FIGURE 22.10 Changes in reducing sugar quantities during fermentation in fruits (a) and brine (b). ★ = F1: <34 g; ■ = F2: 34–44 g; ▲ = F3: >44 g.

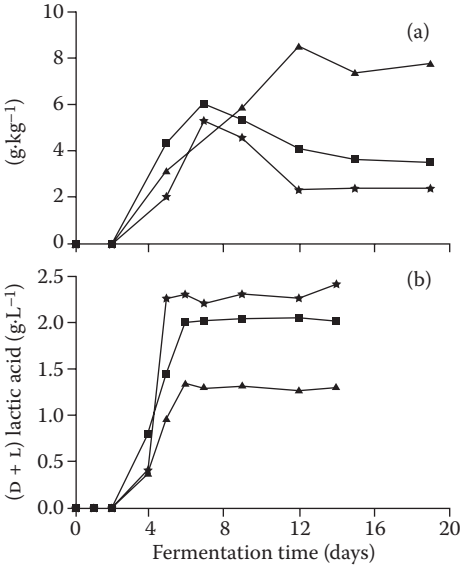

FIGURE 22.11 Changes in lactic acid quantity during fermentation in fruits (a) and brine (b). ★ = F1: <34 g; ■ = F2: 34–44 g; ▲ = F3: >44 g.

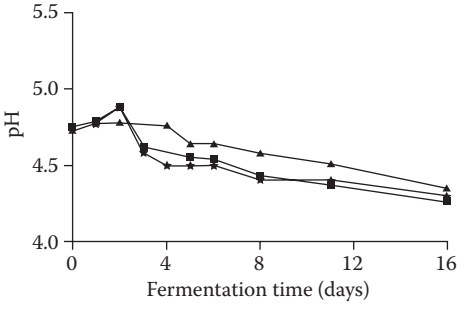

FIGURE 22.12 Changes in brine pH. ★ = F1: <34 g; ■ = F2: 34–44 g; ▲ = F3: >44 g.

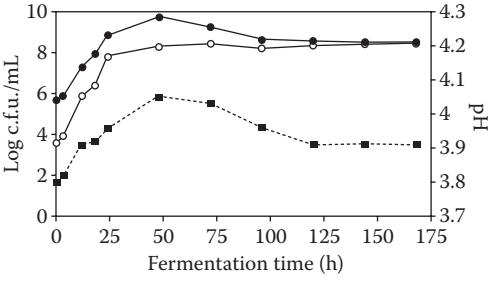

FIGURE 22.13 Changes in microflora (–○–, lactic acid bacteria; –•–, aerobic mesophilic bacteria) and pH values (–■–) during the spontaneous fermentation of Almagro eggplants.

until the end of the process. The total bacterial counts in these first 48 hours were higher than the lactic bacteria counts, although their evolution was parallel; leveling off after four days of fermentation.

To analyze the microbiota participating in fermentation, a daily sampling was carried out for seven days at an important manufacturing company for two consecutive years to obtain year-to-year variability (Seseña et al. 2004). The results of the polyphasic study of the profiles of the isolates from the

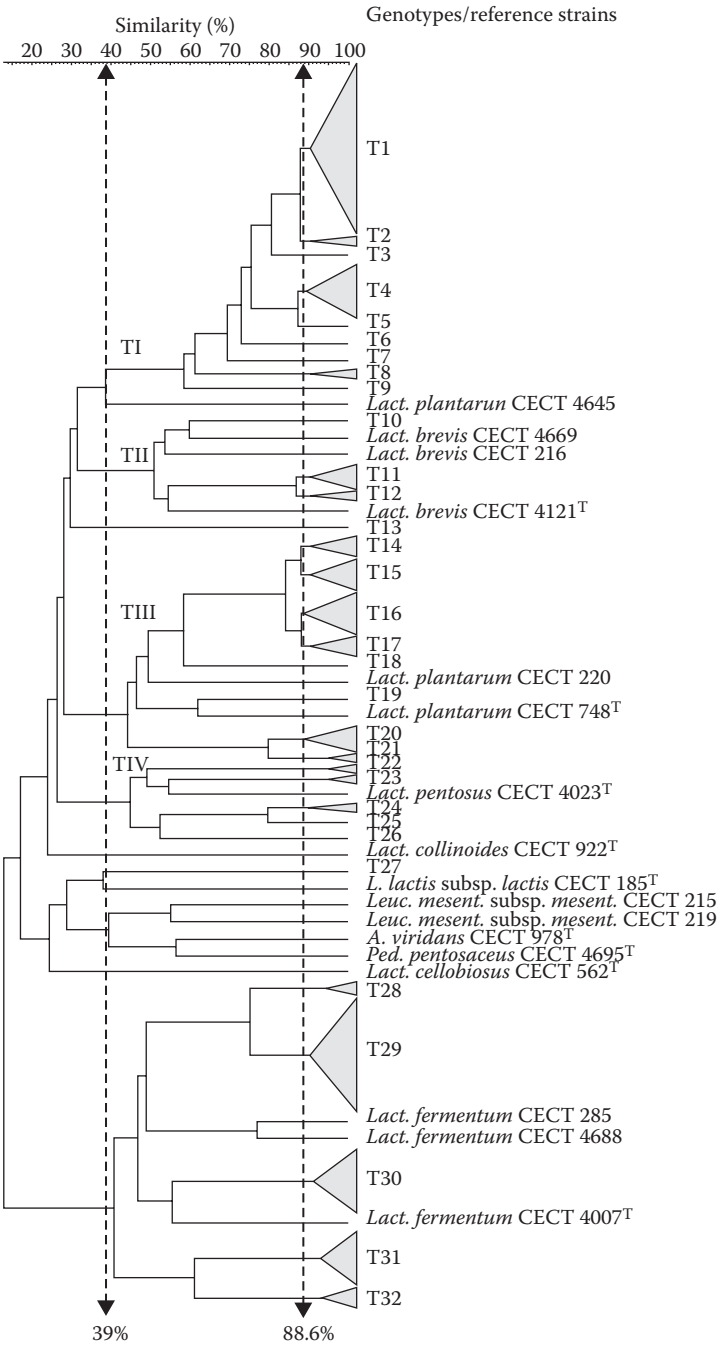

FIGURE 22.14 Consensus dendrogram obtained by combining RAPD, PFGE, and SDS-PAGE.

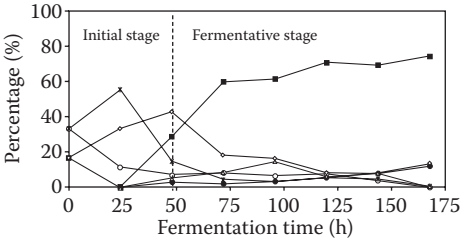

FIGURE 22.15 Evolution of species of *Lactobacillus* during spontaneous fermentation of Almagro eggplants: (–■–) *L. plantarum* biotype 1; (–•–) *L. pentosus*; (–○–) *L. brevis* biotype 3; (–◇–) *L. brevis* biotype 2; (–×–) *L. fermentum*; (–△–) *Lactobacillus* spp.

counting plates of the samples taken in the first year obtained from different molecular approaches RAPD-PCR (Randomly Amplified Polymorphic DNA-Polymerase Chain Reaction), PFGE (Pulsed-Field Gel Electrophoresis), SDS-PAGE (Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis) led to the dendrogram in Figure 22.14 (Sanchez et al. 2004). The dendrogram is composed of 32 genotypes grouped into five main clusters (TI–TV), which were assigned to the species *Lactobacillus (L.) plantarum*, *Lactobacillus brevis*, *L. plantarum*, *Lactobacillus pentosus*, and *Lactobacillus fermentum*, respectively.

The predominant species was *L. plantarum*, which comprised 54.7% of the isolates, followed by the species *L. fermentum* (36.13%), *L. pentosus* and *L. brevis* (both 4.7%), and finally, 1.29% of the isolates were assigned to *Lactobacillus* spp. Among the isolates from the second year, the predominant species was *L. fermentum/Lactobacillus cellobiosus* with a participation of 88.5%, followed by *L. plantarum* (8.7%), and *L. pentosus* (2.7%). Other major differences were the absence of *L. brevis* isolates and the presence of *L. cellobiosus* isolates.

From these results, it can be concluded that, unlike other fermented vegetables, such as olives, cucumbers, and sauerkraut, in which species of the genera *Lactobacillus*, *Pediococcus*, and *Leuconostoc* all commonly take part (Daeschel et al. 1987; Montaño et al. 1992), there is comparatively little diversity in the microbial genera involved in the spontaneous fermentation of Almagro eggplants.

As for the evolution of these species in the process (Figure 22.15), it is possible to argue that, with the exception of *L. pentosus*, all of the species were present in the brine from the beginning, their presence varying over the course of the process.

During fermentation, there seems to be two distinguishable stages: an initial stage lasting for the first 45 hours, in which the obligately heterofermentative *L. fermentum* and *L. brevis* predominate, their numbers increasing considerably in these initial hours before declining in the next stage; and a second stage, called the primary or fermentative phase, from day 2 until the end of the process. In this last stage, the sugars present in the brine are rapidly consumed and lactic acid is consequently formed. Although all the species continue to be present, there is a clear predominance of *L. plantarum*, a facultatively heterofermentative species, the population of which increases continuously from the beginning of the second stage and, after about two days, becomes predominant until the end of fermentation.

22.5 Future Research

New research projects are currently being undertaken on the microbiota responsible for the fermentation of the Almagro eggplant, in which culture-independent methods such as DGGE (Denaturing Gradient Gel Electrophoresis) and TGGE (Temporal Gradient Gel Electrophoresis) are being used. Numerous authors have reported that the results obtained using these two methodologies are complementary to those from culture-dependent methods and they do not always coincide. Research is also being conducted for the botanical analysis of the species to ensure the purity and continuity of the seed.

ACKNOWLEDGMENTS

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REFERENCES

Ballesteros C, Palop LI, Romero C. 1998. Changes in the chemical composition in eggplant fruits of different sizes during fermentation. *Sci Aliments* 18:617–26.

Daeschel MA, Anderson RE, Fleming HP. 1987. Microbial ecology of fermenting plant materials. *FEMS Microbiol Rev* 168:1–37.

Montaño A, de Castro A, Rejano L. 1992. Transformaciones bioquimicas durante la fermentación de productos vegetales. *Grasas Aceites* 43(6):352–60.

Morales Mocino A, 1985. El cultivo y principios de comercialización de la berenjena de Almagro. Proyecto Fin de carrera. Ciudad Real: EUITA.

Prohens J, Muñoz-Falcón JE, Rodríguez-Burruezo A, Nuez F. 2008. Strategies for the breeding of eggplants with improved nutritional quality. *Acta Hortic* 767:285–292.

Reglamento de la Denominación Específica de la Berenjena de Almagro y de su Consejo Regulador. 1994. Orden de la Consejería de Agricultura y Medio Ambiente del 15 de Junio de 1994 (DOCM no. 36, 22 de julio de 1994).

Sanchez I, Seseña S, Palop LI. 2004. Polyphasic study of the genetic diversity of lactobacilli associated with Almagro eggplants spontaneous fermentation, based on combined numerical analysis of randomly amplified polymorphic DNA and pulsed-field gel electrophoresis patterns. *J Appl Microbiol* 97:446–58.

Seseña S, Sánchez I, Palop Ll. 2004. Genetic diversity (RAPD-PCR) of lactobacilli isolated from "Almagro" eggplant fermentations from two seasons. *FEMS Microbiol Lett* 239:159–65.

23

*Olives in Commerce in the United States**

Y. H. Hui

CONTENTS

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23.1 Introduction

In the United States, for processed food products, there are two basic or mandatory approaches if manufacturers want to sell their products nationally. The science, technology, and engineering of processed food products are important if they want to manufacture quality products that the consumers prefer. To sell them throughout this country, the manufacturer must comply with federal requirements that are designed to eliminate economic frauds and to protect the public from injuries when consuming the products. This chapter covers olives from the perspectives of the latter approach including the importation of olives.

The information has been derived from the websites of:

1. U.S. Department of Agriculture (USDA, www.USDA.gov)
2. U.S. Food and Drug Administration (FDA, www.FDA.gov)

At each website, type in the word "olives," and all legal information discussed in the chapter will become available.

23.2 Economy and Crops

The Risk Management Agency of the USDA (www.USDA.gov) has provided the following short assessment of fruit olives.

23.2.1 Olive Tree

The olive is a long-lived, subtropical evergreen tree; some specimens have been reported to live for 1000 years. The tree's shallow root system penetrates only 3 or 4 feet, even in deep soils. The above-ground portion of the tree is recognizable by its dense assembly of limbs, short internodes, and compact foliage. Unless pruned to open light channels, the dense foliage excludes light from the center of the tree.

The flower bud inflorescence (flower bud cluster) is borne in the axil of each leaf. Buds form on the current season's growth and begin visible growth the next season. Buds may, however, remain dormant for more than a year and then begin growth, forming inflorescence a season later than expected. Each inflorescence contains between 15 and 30 flowers, depending on the cultivar and the growing conditions in that year. If all leaf axils produce an inflorescence, each twig has the potential to produce hundreds of flowers.

Full bloom in California occurs during May. Blooming generally occurs 1 to 2 weeks earlier in the southern part of the state than in the northern part. The fruit matures to the processing stage in September or October. The fruit's oil percentage, however, continues to rise as it approaches physiological maturity. Harvesting for oil may continue as late as February.

23.2.2 Olives

The olive is a drupe, botanically similar to the almond, apricot, cherry, nectarine, peach, and plum. The flesh of the olive fruit may be pickled for table use or it may be pressed or processed to recover its oil. The oil is prized for cooking and table use. Fresh olives are extremely bitter and are not considered palatable.

California's olive industry produces primarily black-ripe pickled olives, used as appetizers and condiments or in salads and sandwiches. Olives are also used to add color and seasoning to prepared dishes, such as pizza. Oil production is a salvage operation in California, for cull olives and those that are too small for canning.

California accounts for all commercial olive productions in the United States. Olive yields and production are highly variable from year to year. In the "on" year, trees produce abundant flowers and set more fruit than can grow to marketable sizes. In addition, the large crop of fruit draws down carbohydrate

reserves, causing subnormal shoot growth. A large crop also delays fruit maturity and increases the chance of losses due to early fall frosts. Because olive trees develop fruit buds on the previous year's shoots, repressed shoot growth diminishes the crop potential for the next year, the "off" year.

The alternate-bearing pattern in olives, however, occurs less dependably than among certain other tree crops, such as pecans and pistachios. Sometimes, olive trees produce large (small) crops for several years before yielding a small (large) crop. Olives are a minor item in the American diet. Per capita use of canned (pickled) olives averages between 1 and 1.5 lb. annually. Consumption of pickled cucumbers, in contrast, averages approximately 4.5 to 6 lb. per person per year. Among the olives consumed in the United States, 65% to 70% are produced in California. The remainder are imported, largely from Spain, Greece, Mexico, and Portugal.

The highest-quality olive production occurs in areas having mild winters and long, hot, dry summers. Being a subtropical tree, small limbs and shoots can be damaged by temperatures lower than 22°F. Temperatures lower than 15°F can kill large limbs and whole trees. Ideal temperatures range between 35°F and 65°F. This temperature range supplies the chilling hours required for good flower development without damaging vegetative growth.

Because olive trees do not "come true" from seed, trees for commercial production are vegetatively propagated. Historically, olives have been grown from cuttings as own-rooted plants. This method continues to be the preferred way of propagating olives. Sometimes, seedlings are grown and are then budded or grafted to a desired variety. It may take up to two years longer to produce a mature bearing tree from seedlings than from rooted cuttings.

The ideal crop load varies with cultivar and with tree age and vigor. As a rule, six fruit per foot of shoot appears to result in good fruit size while ensuring moderate shoot growth for next year's crop. Fruit thinning is the most effective method of controlling crop size. Thinning involves removing fledgling fruit, which allows the remaining crop to grow larger and shoot growth to proceed normally. Because of the high cost of hand thinning, chemical thinning is used in commercial crops.

The bulk of California's olives (90%–95%) are processed as canned ripe olives. Other uses include pressing for olive oil and producing Spanish-style green olives and other pickled products, such as Sicilian, Greek, and other styles of olives. All California olives sold for canning as black-ripe or green-ripe olives are subject to the regulations of the Federal Marketing Order. The Order provides for mandatory inspection of olives and canned olive products and for the industry-wide support of research, advertising, and public relations activities. Each lot of fruit designated for canned ripe olives must be size-graded by California state inspectors (incoming inspection). These inspectors classify the olives as "canning," "limited," "undersize," or "culls." This classification serves as the basis for paying growers for their fruit.

A cold, wet spring is the most frequently occurring natural peril in producing olives. Prolonged, abnormally low temperatures during April and May retard bud development and diminish the proportion of fruit-bearing, pistil-containing blossoms. A deficiency of pistil-containing flowers reduces fruit set and subsequent production. Other production perils include early fall freezes and excess moisture. Most disease and insect problems can be controlled through management practices.

23.3 Types of Pickled Olives

Pickled olives are prepared in several different styles that vary according to color and flavor. The style of preparation depends on the maturity of the fruit when harvested and the method of processing.

23.3.1 California-Style Black-Ripe Olives

California-style olives are prepared from fully developed (but not ripe) fruit, which is green to straw yellow in color when picked. The fruit is treated with dilute solutions of lye (sodium hydroxide) to remove its bitter flavor. A combination of the lye treatment and exposure to air, or aeration in water, produces the dark color. The olives are washed to remove the lye and placed in a mild salt solution for several days for curing. Olives intended for pitting are put through an automatic pitter after curing. The cured olives

are packed into cans or jars and heat-treated to destroy microorganisms that could cause spoilage. Pitted olives are canned in the same manner as unpitted olives. The black-ripe style reportedly accounts for most of California's pickled olive output.

23.3.2 California-Style Green-Ripe Olives

Canned green-ripe olives can be produced from Manzanillo, Mission, and Sevillano fruit, which are pink or straw yellow in color when harvested. As with the black-ripe olives, green-ripe olives are successively treated with lye solutions to remove bitterness. To avoid darkening between lye treatments, however, green-ripe olives are never exposed to air. After washing to remove the lye, green-ripe olives are canned in the same manner as black-ripe olives. Green-ripe olives constitute a very small portion of California's production.

23.3.3 Spanish-Style Green Olives

Spanish-style olives, like the ripe olives discussed previously, are processed from fully developed fruit, which is green to straw yellow in color when harvested. Similar to the production of California-style olives, the process for Spanish-style green olives consists of a lye solution treatment to remove bitterness. The lye treatment for Spanish-style olives, however, may be stopped before all of the bitterness is removed, contributing to the Spanish-style flavor. After the lye treatment, the olives are placed in a salt solution and fermented for two to twelve months. The pickled olives are then ready for sale or packaging. Spanish-style olives are always green when marketed. Most are pitted, or pitted and stuffed with pimento strips or other ingredients, such as onions or anchovies. Nearly all Spanish-style olives for retail sale are packed in glass bottles to which a brine solution has been added.

23.3.4 Greek-Style, Naturally Ripe Olives

Greek-style olives are made from ripened fruit that has turned purple or black in color before harvesting. The olives are fermented in salt brine for several months. When fermentation is completed, the olives are sorted and packed in fresh brine for consumer use. They may also be packed in vinegar brine for use as appetizers.

The color of Greek-style olives ranges from black (the most characteristic) to pale pink. As no lye treatment is used in processing, Greek-style olives exhibit a somewhat bitter flavor. A specialty product in California, Greek-style olives account for a minor portion of California's production.

23.3.5 Sicilian-Style Olives

Sicilian-style olives are prepared from fully developed olives that still retain their green color at harvest. They are prepared in a similar manner to Greek-style olives, and have the same characteristic bitter flavor. Sicilian-style olives are green in color when marketed and may be packed in glass bottles or plastic buckets. As with the Greek-style, Sicilian-style olives account for only a minor part of California's production.

23.4 Standards of Grades

To facilitate commerce, the USDA has established U.S. Standards for Grades of Canned Ripe Olives. Although the standard is a voluntary one, most manufacturers use it as a guide in commerce in this country. The next few sections discuss grades for domestic olives. For complete details, consult the USDA website.

23.4.1 Product Description

Canned ripe olives are prepared from properly matured olives that have first been properly treated to remove the characteristic bitterness, packed in a solution of sodium chloride, with or without spices, and sufficiently processed by heat in hermetically sealed containers. Canned olives that are not oxidized in

processing and that possess a tan to light bronze color indicative of preparation from olives of advanced maturity and commonly referred to as "tree ripened" or "home cured" are not covered here.

23.4.2 Types of Canned Ripe Olives

Canned ripe olives are processed as two distinct types. "Canned ripe olives" refers to olives of either "ripe type" or "green ripe type." Ripe-type olives are those that have been treated and oxidized in processing to produce a typical dark brown to black color. Green ripe–type olives are those that have not been oxidized in processing, which range in color from yellow-green, green-yellow, or other greenish casts, and may be mottled.

23.4.3 Styles and Designations of Canned Ripe Olives

- "Whole" olives are those that have not been pitted
- "Pitted" olives are those from which pits have been removed
- "Halved" olives are pitted olives in which each olive is cut lengthwise into two approximately equal parts
- "Segmented" olives are pitted olives in which each olive is cut lengthwise into three or more approximately equal parts
- "Sliced" olives consist of parallel slices of fairly uniform thickness prepared from pitted olives
- "Chopped" olives are random-size cut pieces or cut bits prepared from pitted olives
- "Broken pitted" olives consist substantially of large pieces that may have been broken in pitting but have not been sliced or cut

The "average count" for canned whole ripe olives is determined from all containers in the sample and is calculated on the basis of the drained weight of the olives. The diameters of canned whole and pitted ripe olives are determined by measuring the smallest diameters at the largest circumferences at right angles to the longitudinal axes of the olives. The longitudinal axis is a line running from the stem to the apex of the olive.

23.4.4 Size Determination and Minimum Drained Weights

The size of canned whole or pitted olives should conform to the applicable count per pound range indicated in Table 23.1 in the case of whole olives, or conform closely to the applicable illustration in Table 23.1 in the case of pitted olives. When the count per pound of whole olives falls between two count ranges, the size designation should be the next smaller size (Table 23.1).

A lot of canned ripe olives is considered to have met the minimum drained weights if the following criteria are met:

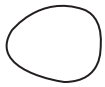
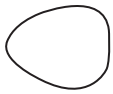
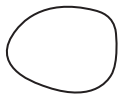
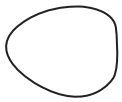
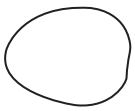
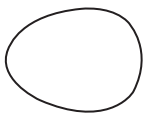
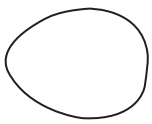
1. The average of the drained weights from all the sample units in the sample is equal to or greater than the acceptance value for drained weights for the size
2. There should be no unreasonable shortage in any individual container

23.4.5 Grades of Canned Ripe Olives

U.S. Grade A is the quality of canned ripe olives of whole, pitted, halved, segmented, sliced, and chopped styles that has a good flavor, that has a good color, that is practically free from defects, that has a good character, and that for those factors which are rated in accordance with the scoring system outlined in this subpart, the total score is not less than 90 points, provided that such canned ripe olives may have a reasonably good color if the total score is not less than 90 points; and further, provided that in the styles of whole and pitted olives, the variation in diameters does not exceed 4 mm, and of the 90%, by count,

TABLE 23.1

Size: Canned Whole and Pitted Ripe Olives

Designation	Count per Pound	Illustration	Approximate Diameter Range Illustrated (mm)
Small	128–140		16–17
Medium	106–121		17–19
Large	91–105		19–20
Extra large	65–88		20–22
Jumbo	51–60		22–24
Colossal	41–50		24–26
Super colossal	≤40		≥26

of the most uniform in size, the diameter of the largest does not exceed the diameter of the smallest by more than 3 mm.

U.S. Grade B is the quality of canned ripe olives of whole, pitted, halved, segmented, sliced, and chopped styles that has a good flavor, that has a reasonably good color, that is reasonably free from defects, that has a reasonably good character, and that for those factors which are rated in accordance with the scoring system outlined in this subpart, the total score is not less than 80 points, provided that for the styles of whole and pitted olives, the variation in diameters does not exceed 8 mm, and of the 80%, by count, of the most uniform in size, the diameter of the largest does not exceed the diameter of the smallest by more than 4 mm.

U.S. Grade C is the quality of canned ripe olives of whole, pitted, halved, segmented, sliced, chopped, and broken pitted styles that has a reasonably good flavor, that has a fairly good color, that is fairly free from defects, that has a fairly good character, and that for those factors which are rated in accordance with the scoring system outlined in this subpart, the total score is not less than 70 points, provided that for the styles of whole and pitted olives, of the 60%, by count, of the most uniform in size, the diameter of the largest does not exceed the diameter of the smallest by more than 4 mm.

Substandard is the quality of canned ripe olives of any style that fail to meet the applicable requirements for U.S. Grade C.

23.4.6 Determining the Grade of a Sample Unit

In addition to considering other requirements outlined in the standards, the following quality factors are evaluated. The factors not rated by score points include the following:

23.4.6.1 Flavor

"Good flavor" in ripe type means a distinctive flavor characteristic of ripe-type olives (including that of properly spiced olives), which have been properly prepared and processed and which are free from objectionable flavors of any kind.

Good flavor in green ripe type means a distinctive mellow flavor characteristic of green ripe–type olives, which have been properly prepared and processed and which are free from objectionable flavors of any kind.

"Reasonably good flavor" in either ripe type or green ripe type (including that of properly spiced olives) means that the flavor may be slightly lacking in distinctly characteristic flavor for the respective type but the olives are free from objectionable flavors of any kind.

23.4.6.2 Color

The evaluation of color should be determined within five minutes after the olives are removed from the container and is based on the uniformity of the exterior color or general appearance as to color of the olives within the container. The evaluation of color in "halved" style olives is based on the uncut surfaces.

The color of ripe-type olives is determined by comparison with USDA coloring instruments. Composite USDA Color Standards for Canned Ripe Olives are available and are comparable to the colors produced by the spinning discs.

Normal color for green ripe–type olives is yellow-green, green-yellow, or other greenish casts, any of which may have a mottled appearance that is typical of green ripe–type olives. Off-color means dark brown, dark purple, or black olives.

Grade A. Canned ripe olives that have a good color may be given a score of 27 to 30 points. "Good color" has the following meanings with respect to the applicable type and style:

RIPE TYPE

Whole, pitted, halved. The olives or units have a practically uniform black or dark brown color. Not less than 90%, by count, of the olives or units have a color equal to or darker than the appropriate USDA Composite Color Standard.

Segmented, sliced, and chopped. The general color impression of the olive as a mass is normal and typical of these styles prepared from olives with good color.

Green ripe type. The general color appearance of the olives should be normal. Not less than 90%, by count, should be practically uniform in such normal color for the type, and no off-color olives may be present.

Grade B. If the canned ripe olives have a reasonably good color, a score of 24 to 26 points may be given. "Reasonably good color" has the following meanings with respect to the applicable type and style:

RIPE TYPE

Whole, pitted, halved. The olives or units have a reasonably uniform black, dark brown or reddish-brown color. Not less than 80%, by count, of the olives or units have a color equal to or darker than the appropriate USDA Composite Color Standard.

Segmented, sliced, and chopped. The general color impression of the olives as a mass is normal and typical of these styles prepared from olives with reasonably good color.

Green ripe type. The general color appearance of the olives should be normal. Not less than 80%, by count, should be reasonably uniform in such normal color for the type, and no off-color olives may be present.

Grade C. If the ripe olives have a fairly good color, a score of 21 to 23 points may be given. Canned ripe olives that fall into this classification should not be graded above U.S. Grade C regardless of the total score for the product (this is a limiting rule). "Fairly good color" has the following meanings with respect to the applicable type and style:

RIPE TYPE

Whole, pitted, halved. The olives or units have a fairly uniform black, dark brown, or reddish-brown color. Not less than 60%, by count, of the olives or units have a color equal to or darker than the appropriate USDA Composite Color Standard.

Segmented, sliced, and chopped. The general color impression of the olives as a mass is normal and typical of these styles prepared from olives of fairly good color.

Broken pitted. The general color impression of the olives as a mass is normal and may be variable, but is typical of this style prepared from olives of good, reasonably good, or fairly good color.

GREEN RIPE TYPE

The general color impression of the olives should be normal but may vary markedly for the type. No more than 10%, by count, of off-color olives may be present.

Substandard. Canned ripe olives that are abnormal in color for any reason may be given a score of 0 to 20 points and should not be graded above substandard, regardless of the total score for the product (this is a limiting rule).

23.4.6.3 Defects

General. The factor of absence of defects refers to the degree of freedom from harmless extraneous vegetable material (HEVM), stems, and portions thereof, blemishes, wrinkles, mutilated olives, and from any other defects that affect the appearance or edibility of the product.

DEFINITION OF DEFECTS

Blemishes mean dark-colored surface marks in either ripe-type or green ripe–type olives, which may or may not penetrate into the flesh. Olives or pieces of olives affected by blemishes are classified as follows:

Minor blemishes mean surface discolorations on olives or pieces of olives, which individually or collectively materially affect the appearance of the unit.

Major blemishes mean surface discolorations or black flesh (oxidized) on olives or pieces of olives, which may or may not be associated with a soft texture below the skin and which individually or collectively seriously affect the appearance or edibility of the unit.

Severe blemishes mean dark brown, dark purple, or black surface areas on olives or pieces of olives of the green ripe type, or any other blemishes, whether or not specifically defined, which severely affect the appearance or edibility of the unit.

Blowout refers to a soft-pitted olive in which the pit has been pushed out instead of cut out, leaving an irregular ring of flesh that materially affects its appearance.

Broken piece in halved, segmented, and sliced style olives means any piece of olive flesh that appears to be less than three-fourths of a full unit. Also included are poorly cut units and end slices less than one-half the average size slice.

Cross-pitted refers to olives pitted along an axis other than the stem–flower axis. A defect is a unit where the angle of these two axes exceeds 45 degrees.

Harmless extraneous vegetable material (or HEVM), harmless extraneous material, and extraneous vegetable material are synonymous terms and mean any vegetable substance that is harmless.

Mechanically damaged means a unit in whole, pitted, and halved styles that is punctured, cut, or damaged by means other than pitting so that its appearance is materially affected.

Misshapen refers to an olive that does not have a normal shape for a given variety.

Mutilated refers to an olive in whole or pitted styles that is so pitter-torn or damaged by other means that the entire pit cavity is exposed or the appearance of the olive is seriously affected.

Obvious split pit means a pit in an olive that can be determined visually as split.

Pitter damage means a loss of skin and flesh from a pitted olive caused by the pitter on the cut end exceeding the area of a circle 3 mm in diameter but is not mutilated.

Plunger damage means a loss of skin and flesh from a pitted olive equal to or exceeding the area of a circle 5 mm in diameter.

Stem means a stem that measures 3 mm or more from the shoulder of the olive. Stems are classified as follows:

Minor stem is a stem that measures more than 3 mm but not more than 4 mm from the shoulder of the olive.

Major stem is a stem that measures more than 4 mm from the shoulder of the olive.

Detached stem, when it measures 4 mm or more, is a defect that should be scored as a minor stem for whole pitted, halved, and broken pitted-style olives and a major stem for segmented, sliced, and chopped-style olives.

Wrinkles are grooves 0.5 mm or more in width. Classification of wrinkles should be determined immediately after removing surface moisture and any increase in wrinkles because of dehydration after removing from the container should not be considered. Olives or pieces of olives affected by wrinkles are classified as follows:

Minor wrinkles are wrinkles that collectively do not more than materially affect the appearance of the unit.

Major wrinkles are wrinkles that collectively more than materially affect the appearance of the unit.

Grade A. Canned ripe olives of whole, pitted, halved, segmented, sliced, and chopped styles that are practically free from defects may be given a score of 36 to 40 points. "Practically free from defects" means that any defects present, but not specifically limited in Table 23.5, may not more than slightly affect the appearance or edibility of the olives; and in addition, specified defects may be present in all other styles except "broken pitted" not to exceed the allowances for grade A provided in Table 23.5.

Grade B. If canned ripe olives of whole, pitted, halved, segmented, sliced, and chopped styles are reasonably free from defects, a score of 32 to 35 points may be given. Canned ripe olives that fall into this classification should not be graded above U.S. Grade B regardless of the total score for the product (this is a limiting rule). "Reasonably free from defects" means that any defects present but not specifically limited may not more than materially affect the appearance or edibility of the olives; and in addition, specified defects may be present in all other styles except "broken pitted" not to exceed the allowances for grade B provided in USDA regulations.

Grade C. If canned ripe olives of whole, pitted, halved, segmented, sliced, chopped, and broken pitted styles are fairly free from defects, a score of 28 to 31 points may be given. Canned ripe olives that fall into this classification should not be graded above U.S. Grade C, regardless of the total score for the product (this is a limiting rule). "Fairly free from defects" means that any defects present but not specifically limited by the FDA may more than materially affect the appearance and edibility of the olives; and in addition, specified defects may be present in all other styles not to exceed the allowances for grade C provided by the USDA.

Substandard. Canned ripe olives that fail to meet the requirements of paragraph (e) of this section may be given a score of 0 to 27 points and should not be graded above substandard, regardless of the total score for the product (this is a limiting rule).

23.4.6.4 Character

The factor of character refers to the firmness, tenderness, and texture characteristics for the variety and type.

Grade A. Canned ripe olives of whole, pitted, halved, segmented, sliced, and chopped styles that have a good character may be given a score of 27 to 30 points. "Good character" means that, for the type, the olives have a fleshy texture characteristic for the variety and size; that not less than 95%, by count, of whole, pitted, and halved olives and by weight of other style olives are practically uniform in texture and are tender but not soft. The remaining 5% may be soft but not excessively soft.

Grade B. If canned ripe olives of whole, pitted, halved, segmented, sliced, and chopped styles have a reasonably good character, a score of 24 to 26 points may be given. Canned ripe olives that fall into this classification should not be graded above U.S. Grade B regardless of the total score for the product (this is a limiting rule). "Reasonably good character" means that, for the type, the olives generally have a fleshy texture characteristic for the variety and size; that not less than 90%, by count, of whole, pitted, and halved olives, and by weight of other style olives are practically uniform in texture and are tender but not soft. The 10% may be soft but not more than 1/2, or 5%, may be excessively soft.

Grade C. If canned ripe olives of whole, pitted, halved, segmented, sliced, chopped, and broken pitted styles have a fairly good character, a score of 21 to 23 points may be given. Canned ripe olives that fall into this classification should not be graded above U.S. Grade C regardless of the total score for the product (this is a limiting rule). "Fairly good character" means that the olives generally have a fleshy texture characteristic for the variety and size; that not less than 80%, by count, of whole, pitted, and halved olives and by weight of other style olives are practically uniform in texture and are tender but not soft. The remaining 20% may be soft but not more than 1/2, or 10%, may be excessively soft.

Substandard. Canned ripe olives that fail to meet the requirements of paragraph (d) of this section may be given a score of 0 to 20 points and should not be graded above substandard, regardless of the total score for the product (this is a limiting rule).

23.5 FDA Compliance Requirements

Canning olives must comply with requirements issued by the FDA and this chapter is not the proper forum to discuss the safety of canning. However, there are two important FDA requirements that should be mentioned.

One is the use of color additives in canning olives. Ferrous gluconate and ferrous lactate may be safely used in amounts consistent with good manufacturing practices for the coloring of ripe olives.

The other important consideration is adulteration. The FDA will take legal actions for olives with adulterations (pits, rot, or insect infestation) as indicated.

23.5.1 Pits

1. Pitted whole. Examine a minimum of 500 olives from each code or from the lot if no codes are present. The average is 1.3% or more by count of olives with whole pits or pit fragments 2 mm or longer measured in the longest dimension.
2. Pitted salad olives, including broken pitted, halved, quartered, sliced, and chipped or minced. Examine a minimum of 1500 g from each code or from the lot if no codes are present. The average is 1.3 or more fragments per 300 g, including whole pits and fragments 2 mm or longer measured in the longest dimension.

23.5.2 Insect Infestation

1. Salt-cured olives. Examine a minimum of six subs from each code or from the lot if no codes are present. The average is 10% or more by count of olives with 10 or more scale insects each.
2. Imported black. Actionable if 10% or more by count show damage by Dacusa (olive fruit fly).
3. Imported green. Actionable if 7% or more by count show damage by Dacusa (olive fruit fly).
4. Salad type, including broken, pitted, halved, quartered, sliced, and chopped or minced. Actionable if 9% or more by weight of olives show damage by Dacusa (olive fruit fly).

"Damage by Dacusa" means the presence of any form or part thereof of the olive fruit fly or maggot tunneling affecting an area of 1/4 in. or greater average diameter.

As for rot, moldy olives are identified. As an example, for salt-cured olives, examine a minimum of six subs from each code or from the lot if no codes are present. The average is 25% or more by count of moldy olives.

The following describes the indication of violations:

PITS

1. Olives are adulterated if they contain pit fragments that have been substituted wholly or in part for pitted olives
2. Olives are misbranded when the label statement "pitted olives" is false and misleading as applied to a product containing olives with pit fragments

INSECT INFESTATION

Olives are adulterated when they consist wholly or in part of a filthy substance caused by the presence of insects or insect-damaged olives.

MOLD

Olives are adulterated when they consist wholly or in part of a decomposed substance caused by the presence therein of moldy, decomposed olives.

23.6 Olive Importation

The United States has stringent requirements about the importation of canned olives and the USDA works with the U.S. Customs to make sure that importers comply with these requirements.

The U.S. regulations provide the following definitions:

1. *Canned ripe olives* means olives in hermetically sealed containers and heat sterilized under pressure, of the two distinct types, "ripe" and "green ripe" as defined in the current U.S. Standards for Grades of Canned Ripe Olives. The term does not include Spanish-style green olives.
2. *Spanish-style green olives* means olives packed in brine and that have been fermented and cured, otherwise known as green olives.
3. *Variety group 1* means the following varieties and any mutations, sports, or other derivations of such varieties: Aghizi Shami, Amellau Ascolano, Ascolano dura, Azapa, Balady, Barouni, Carydolia, Cucco, Gigante di Cerignola, Gordale, Grosane, Jahlut, Polymorpha, Prunara, Ropades, Sevillano, St. Agostino, Tafahi, and Touffahi.
4. *Variety group 2* means the following varieties and any mutations, sports, or other derivations of such varieties: Manzanillo, Mission, Nevadillo, Obliza, and Redding Picholine.

"Limited use" means the use of processed olives in the production of packaged olives of the halved, segmented (wedged), sliced, or chopped styles, as defined under USDA grade standards.

The terms used in this section have the same meaning as those given to the respective terms in the current U.S. Standards for Grades of Canned Ripe Olives discussed previously in this chapter including the terms *size*, *character*, *defects*, and *ripe type*. However, *broken pitted olives* consist of large pieces that may have been broken in pitting but have not been sliced or cut.

The importation into the United States of any canned ripe olives is prohibited unless such olives are inspected and meet the following applicable requirements. Olives imported in bulk form and used in the production of any canned ripe olives are subject to other applicable USDA requirements.

23.6.1 Minimum Quality Requirements

Canned ripe olives should meet the following quality requirements, except that no requirements should be applicable with respect to color and blemishes for canned green-ripe olives (see Tables 23.2–23.5).

1. Canned whole and pitted olives of the ripe type should meet the minimum quality requirements prescribed in Table 23.2
2. Canned sliced, segmented (wedged), and halved olives of the ripe type should meet the minimum quality requirements prescribed in Table 23.3
3. Canned chopped olives of the ripe type should meet the minimum quality requirements prescribed in Table 23.4 of this section and should be practically free from identifiable units of pit caps, end slices, and slices ("practically free from identifiable units" means that not more than 10%, by weight, of the unit of chopped style olives may be identifiable pit caps, end slices, or slices)
4. Canned broken pitted olives of the ripe type should meet the minimum quality requirements prescribed in Table 23.5, provided that broken pitted olives consist of large pieces that may have been broken in pitting but have not been sliced or cut

TABLE 23.2

Whole and Pitted Style (Defects by Count per 50 Olives)

Flavor	Reasonably good; no off-flavor
Flavor (green ripe type)	Free from objectionable flavors of any kind
Salometer	Acceptable range in degrees: 3.0–14.0
Color	Reasonably uniform with not less than 60% having a color equal or darker than the USDA Composite Color Standard for ripe type
Character	Not more than 5 soft units or 2 excessively soft units
Uniformity of size	60%, by visual inspection, of the most uniform in size. The diameter of the largest does not exceed the smallest by more than 4 mm
Defects	
Pitter damage (pitted style only)	15
Major blemishes	5
Major wrinkles	5
Pits and pit fragments (pitted style only)	Not more than 1.3% average by count
Major stems	Not more than 3
HEVM	Not more than 1 unit per sample
Mutilated	Not more than 3
Mechanical damage	Not more than 5
Split pits or misshapen	Not more than 5

TABLE 23.3

Sliced, Segmented (Wedged), and Halved Styles (Defects by Count per 255 g)

Flavor	Reasonably good; no off-flavor
Salometer	Acceptable range in degrees: 3.0–14.0
Color	Reasonably uniform with no units lighter than the USDA Composite Color Standard for ripe type
Character	Not more than 13 g excessively soft
Defects	
Pits and pit fragments	Average of not more than 1 by count per 300 g
Major stems	Not more than 3
Broken pieces and end caps	Not more than 125 g by weight

TABLE 23.4

Chopped Style (Defects by Count per 255 g)

Flavor	Reasonably good; no off-flavor
Salometer	Acceptable range in degrees: 3.0–14.0
Color	Reasonably uniform with no units lighter than the USDA Composite Color Standard for Ripe Type
Defects	
Pits and pit fragments	Average of not more than 1 by count per 300 g
Major stems	Not more than 2 units per sample
HEVM	Not more than 2 units per sample

TABLE 23.5

Broken Pitted Style (Defects by Count per 255 g)

Flavor	Reasonably good; no off-flavor
Salometer	Acceptable range in degrees: 3.0–14.0
Color	Reasonably uniform with no units lighter than the USDA Composite Color Standard for Ripe Type
Character	Not more than 13 g excessively soft
Defects	
Pits and pit fragments	Average of not more than 1 by count per 300 g
Major stems	Not more than 3
HEVM	Not more than 2 units per sample

5. A lot of canned ripe olives is considered to meet the requirements if it complies with specifications in Tables 23.2 through 23.5. Further:
   a. The number of sample units that do not meet the requirements should be less than an accepted number prescribed by regulations.
   b. There is no "off-flavor" in any sample unit.

23.6.2 Weight Considerations

1. Canned whole ripe olives of variety group 1, except the Ascolano, Barouni, and St. Agostino varieties, should be of such a size that not more than 25%, by count, of the olives may weigh less than 1/75 lb. (6.0 g) each, except that not more than 10%, by count, of the olives may weigh less than 1/82 lb. (5.5 g) each
2. Canned whole ripe variety group 1 olives of the Ascolano, Barouni, and St. Agostino varieties should be of such a size that not more than 25%, by count, of the olives may weigh less than 1/105 lb. (4.3 g) each, except that not more than 10%, by count, of the olives may weigh less than 1/116 lb. (3.9 g) each
3. Canned whole ripe olives of variety group 2, except the Obliza variety, should be of such a size that not more than 35%, by count, of the olives may weigh less than 1/140 lb. (3.2 g) each, except that not more than 7%, by count, of the olives may weigh less than 1/160 lb. (2.8 g) each
4. Canned whole ripe variety group 2 olives of the Obliza variety should be of such a size that not more than 35%, by count, of the olives may weigh less than 1/127 lb. (3.5 g) each, except that not more than 7%, by count, of the olives may weigh less than 1/135 lb. (3.3 g) each
5. Canned whole ripe olives not identifiable as to variety or variety group should be of such a size that not more than 35%, by count, of the olives may weigh less than 1/140 lb. (3.2 g) each, except that not more than 7%, by count, of the olives may weigh less than 1/160 lb. (2.8 g) each
6. Canned pitted ripe olives of variety group 1, except the Ascolano, Barouni, and St. Agostino varieties, should be at least "extra large" as defined in §52.3754 of the U.S. Standards for Grades of Canned Ripe Olives
7. Canned pitted ripe variety group 1 olives of the Ascolano, Barouni, and St. Agostino varieties should be at least "large" as defined in §52.3754 of the U.S. Standards for Grades of Canned Ripe Olives
8. Canned pitted ripe olives of variety group 2, except the Obliza variety, should be at least "small" as defined in §52.3754 of the U.S. Standards for Grades of Canned Ripe Olives
9. Canned pitted ripe variety group 2 olives of the Obliza variety should be at least "medium" as defined in §52.3754 of the U.S. Standards for Grades of Canned Ripe Olives
10. Canned pitted ripe olives not identifiable as to variety or variety group should be at least "small" as defined previously (U.S. Standards for Grades of Canned Ripe Olives)

Imported bulk olives that do not meet the applicable minimum size requirements may be imported for limited use, but any such olives so used should not be smaller than the following applicable minimum size:

a. Whole ripe olives of variety group 1, except Ascolano, Barouni, or St. Agostino varieties, of a size that not more than 35% of the olives, by count, may be smaller than 1/105 lb. (4.3 g) each
b. Whole ripe olives of variety group 1 of the Ascolano, Barouni, or St. Agostino varieties, of a size that not more than 35% of the olives, by count, may be smaller than 1/180 lb. (2.5 g) each
c. Whole ripe olives of variety group 2, except the Obliza variety, of a size that not more than 35% of the olives, by count, may be smaller than 1/205 lb. (2.2 g) each
d. Whole ripe olives of variety group 2 of the Obliza variety of a size that not more than 35% of the olives, by count, may be smaller than 1/180 lb. (2.5 g) each
e. Whole ripe olives not identifiable as to variety or variety group of a size that not more than 35% of olives, by count, may be smaller than 1/205 lb. (2.2 g) each

23.7 USDA Inspection

23.7.1 General

According to the USDA, the following information applies: All canned ripe olives are required to be inspected and certified by the USDA before importation, including a release from custody of the U.S. Customs and Border Protection. Also, bulk olives for processing into canned ripe olives must be inspected and certified before canning.

Olives failing to meet the previously mentioned import requirements may be (1) exported, (2) disposed of under Federal or Federal-State Inspection Service supervision with the importer bearing the costs of certifying the disposal of such olives, (3) diverted to an exempt outlet approved by the USDA, or (4) reinspected with failed portion disposed of under the procedures of number (2) or (3) above.

USDA inspection and certification services will be available upon application. The cost of inspection and certification should be borne by the applicant. Inspection applications should be accompanied by either (1) an "On Board" bill of lading designating the lots to be entered as canned ripe olives, (2) a list of such lots by variety and their identifying marks, or (3) a list identifying lots by variety of imported bulk olives.

23.7.2 Definitions

The USDA has provided the following additional definitions:

1. Canned ripe olives means olives in hermetically sealed containers and heat-sterilized under pressure, of the two distinct types "ripe" and "green-ripe."
2. "Ripe" olives are those that have been treated and oxidized in processing to produce a typical dark brown to black color.
3. "Green-ripe" olives are those that have not been oxidized in processing, which range in color from yellow-green or other greenish casts and may be mottled.
4. Spanish-style green olives are packed in brine and are fermented and cured. They are otherwise known as "green olives."
5. "Limited use" means the use of processed olives in the production of packaged olives of the halved, segmented (wedged), sliced, or chopped styles.

Imported bulk olives, when used in the production of canned ripe olives, must be inspected and certified as prescribed. Imported bulk olives that do not meet the applicable minimum size requirements specified previously may be imported for processing into limited use styles, but must meet certain requirements.

23.7.3 Size and Quality Requirements

Importing any canned ripe olives, or bulk olives for processing into canned olives, into the United States is prohibited unless such olives are inspected and meet the following minimum size and quality requirements:

Variety 1 (Aghizi Shami, Amellau, Azapa, Balady, Carydolia, Cucco, Gigante di Cerignola, Gordale, Grosane, Jahlut, Polymorpha, Prunara, Ropades, Sevillano, Tafahi, and Touffahi). Imported by size/weight (by count per pound and grams). Canned, whole ripe: not more than 25% may weigh less than (1/75 lb.) 6 g each, not more than 10% may weigh less than (1/82 lb.) 5.5 g each. Canned, pitted ripe: not more than 25% may measure less than 21 mm in diameter. Whole ripe (limited use requirements): not more than 35% may be smaller than (1/105 lb.) 4.3 g each.

Ascolano, Barouni, and St. Agostino. Imported by size/weight (by count per pound and grams). Canned, whole ripe: not more than 25% may weigh less than (1/105 lb.) 4.3 g each, not more than 10% may weigh less than (1/116 lb.) 3.9 g each. Canned, pitted ripe: not more than 25% may measure less than 19 mm in diameter. Whole ripe (limited use requirements): not more than 35% may be smaller than (1/180 lb.) 2.5 g each.

Variety 2 (Manzanillo, Mission, Nevadillo, and Redding Picholine). Imported by size/weight (by count per pound and grams). Canned, whole ripe: not more than 35% may weigh less than (1/140 lb.) 3.2 g each, not more than 7% may weigh less than (1/160 lb.) 2.8 g each. Canned, pitted ripe: not more than 35% may measure less than 16 mm in diameter. Whole ripe (limited use requirement): not more than 35% may be smaller than (1/205 lb.) 2.2 g each.

Obliza. Imported by size/weight (by count per pounds and grams). Canned, whole ripe: not more than 35% may weigh less than (1/127 lb.) 3.5 g each, not more than 7% may weigh less than (1/135 lb.) 3.3 g each. Canned, pitted ripe: not more than 35% may measure less than 17 mm in diameter. Whole ripe (limited use requirements): not more than 35% may be smaller than (1/180 lb.) 2.5 g each.

Unidentifiable varieties. Imported by size/weight (by count per pound and grams). Canned, whole ripe: not more than 35% may weigh less than (1/140 lb.) 3.2 g each, not more than 7% may weigh less than (1/160 lb.) 2.8 g each. Canned, pitted ripe: not more than 35% may measure less than 16 mm in diameter. Whole ripe (limited use requirements): not more than 35% may be smaller than (1/205 lb.) 2.2 g each.

Canned ripe olives should meet the minimum quality requirements previously specified, which apply as appropriate to canned whole, pitted, sliced, segmented, halved, chopped, and broken pitted olives. No requirements should be applicable with respect to color and blemishes for canned green-ripe olives.

24

*Pickle Manufacturing in the United States: Quality Assurance and Establishment Inspection**

Y. H. Hui

CONTENTS

24.1 Introduction

This chapter is not about the science and technology of pickles. Instead, information presented deals with regulatory requirements for pickles in commerce in the United States:

1. Safety. This ensures that the product is wholesome and does not harm the consumers.
2. Economic integrity. This ensures that the consumers are buying what the products are claimed to be, a genuine container of pickles in terms of quality and quantity.

* The information in this chapter has been modified from *Food Safety Manual*, published by Science Technology System of West Sacramento, CA. Copyrighted 2012©. Used with permission.

The information has been derived from the websites of

1. U.S. Department of Agriculture (USDA, www.USDA.gov)
2. U.S. Food and Drug Administration (FDA, www.FDA.gov)

24.2 Quality Assurance

24.2.1 Introduction

Pickled cucumbers are acid fermented in the presence of salt. Cucumbers for pickling are harvested while still immature. The fermentation of cucumbers varies according to the salt concentration used: 8%–15% and 3%–5%. Usually, the salt solution is poured into the cucumbers in tanks and then fermentation is allowed to proceed. Fermentation of sugar yields lactic acid, carbon dioxide, some volatile acids, ethanol, and other flavoring and aromatic chemicals.

Once the cucumbers have been brined and the tank closed, there is a rapid development of microorganisms in the brine. The normal mixed flora in the cucumbers serves as a starter culture. The rate and depth of fermentation depend on two major factors:

- Concentration of salt in the brine
- Temperature control

In general, with a lower salt concentration, more varieties of microorganisms will be initially available, resulting in faster acid production and a lower pH. The lactic acid bacteria chiefly responsible for production of high-salt pickles are *Pediococcus cerevisae*, *Lactobacillus plantarum*, and *Lactobacillus brevis.*

In controlled fermentations, the cucumbers are washed and soaked in a chlorinated brine. The brine is acidified with acetic acid, buffered with sodium acetate or sodium hydroxide, and inoculated with *L. plantarum* and *P. cerevisae*.

This section is not designed to explain how pickles are manufactured in the United States. Rather, it is designed to show you the critical factors you should look for in assuring the quality of your pickles. The information has been modified from a document issued by the USDA: *United States Standards for Grades of Pickles*. Consult the original document for complete details.

This chapter has 11 tables of reference data, numbering 1 to 11, and they do not appear in the same order as presented in the text. This presentation parallels that used by the USDA.

24.2.2 Product Description

Pickles means the product prepared entirely or predominantly from cucumbers (*Cucumis sativus* L). Clean, sound ingredients are used that may or may not have been previously subjected to fermentation and curing in a salt brine. The product is prepared and preserved through natural or controlled fermentation or by direct addition of vinegar to an equilibrated pH of 4.6 or lower. The equilibrated pH value must be maintained for the storage life of the product. The product may be further preserved by pasteurization with heat or refrigeration and may contain other vegetables, nutritive sweeteners, seasonings, flavorings, spices, and other permissible ingredients defined by the FDA. The product is packed in commercially suitable containers to ensure preservation.

24.2.3 Styles of Pickles

1. Whole style means the pickles are whole and are relatively uniform in diameter as indicated in a latter discussion (Table 24.2).
2. Whole, mixed sizes style means the pickles are whole pickles of mixed sizes.

3. Sliced lengthwise style means the pickles are cut longitudinally into halves, quarters, or other triangular shapes (spears, strips, or fingers), or otherwise into units with parallel surfaces with or without ends removed.
4. Sliced crosswise, cross-cut, or waffle cut style means the pickles are cut into slices transversely to the longitudinal axis. The cut surfaces may have flat-parallel or corrugated-parallel surfaces.
5. Cut style means the pickles are cut into chunks or pieces that are of various sizes and shapes.
6. Relish style means finely cut or finely chopped pickles containing no less than 60% of cucumber ingredient and may contain other vegetable ingredients (cauliflower, onions, pepper, tomatoes, cabbage, olives, mustard, or any other suitable vegetable).

24.2.4 Types of Pack

24.2.4.1 Cured Type

The pickles are cured by natural or controlled fermentation in a salt brine solution and may contain the dill herb or extracts thereof. The pickle ingredient may be partially desalted. The pickles may be further processed or preserved by the addition of vinegar and may contain other ingredients (spices, flavorings, firming, and preserving agents) that constitute the characteristics of the particular type of pickle. The pickles are preserved by acidification to maintain an equilibrated pH of 4.6 or lower. The characteristics of the various types of cured pickles are as follows:

1. Dill pickles (natural or genuine) are cucumbers that are cured in a brine solution with dill herb and other flavoring agents.
2. Dill pickles (processed) are brine-cured pickles that have undergone a freshening process and are packed in a vinegar solution with dill flavoring and other flavoring agents.
3. Sour pickles are cured pickles that are packed in a vinegar solution with or without spices.
4. Sweet pickles and mild sweet pickles are cured pickles that are packed in a vinegar solution with suitable nutritive sweetening ingredient(s).
5. Sour mixed pickles are cured pickles that are packed in a vinegar solution. The pickles may be of any style or combination of styles other than relish and may contain other vegetable ingredients as outlined in Table 24.1 or any other suitable vegetable.
6. Sweet mixed pickles and mild sweet mixed pickles are cured pickles that are packed in a vinegar solution with suitable nutritive sweetening ingredient(s). The pickles may be of any style or combination of styles other than relish and may contain other vegetable ingredients as outlined in Table 24.1 or any other suitable vegetable.
7. Sour mustard pickles or sour chow chow pickles are cured pickles of the same styles and ingredients as sour mixed pickles except the pickles are packed in a prepared mustard sauce of proper consistency with or without spices and flavorings.
8. Sweet mustard pickles or sweet chow chow pickles are cured pickles of the same styles and ingredients as sweet mixed pickles except the pickles are packed in a sweetened, prepared mustard sauce of proper consistency with or without spices and flavorings.
9. Sour pickle relish consists of finely cut or chopped cured pickles that are packed in a vinegar solution. Sour pickle relish may contain other chopped or finely cut vegetable ingredients as listed in Table 24.1 and may contain a stabilizer such as a starch or gum.
10. Sweet pickle relish and mild sweet pickle relish are finely cut or chopped cured pickles that are packed in a vinegar solution with a suitable nutritive sweetening ingredient(s). Sweet pickle relish and mild sweet pickle relish may contain other chopped or finely cut vegetable ingredients as listed in Table 24.1 and may contain a stabilizer such as a starch or gum.
11. Hamburger relish consists of relish style pickles and other chopped or finely cut vegetable ingredients as listed in Table 24.1 with tomato product added.

TABLE 24.1

Proportions of Pickle Ingredients in Certain Types and Styles

	Cured, Fresh-Pack, and Refrigerated Types	
Pickle Ingredients and Styles	**Sour Mixed, Sweet Mixed, Mild Sweet Mixed, Sour Mustard or Sour Chow Chow, Sweet Mustard or Sweet Chow Chow**	**Sour Pickle Relish, Sweet Pickle Relish, Dill Relish, Hamburger Relish, Mustard Relish**
Cucumbers, any style other than relish	Percent by weight of drained weight of product 60%–80%	
Cucumbers, chopped, or finely cut		60%–100%
Cauliflower pieces	10%–30%	
Cauliflower, chopped or finely cut		30% maximum (optional)
Onions, whole (maximum diameter of 1–0.25 in.), sliced, or cut	5%–12%	
Onions, chopped or finely cut		12% maximum (optional)
Green tomatoes, whole or pieces	10% maximum (optional)	
Green tomatoes, chopped or finely cut		10% maximum (optional)
Red, green, or yellow peppers or pimientos, cut, finely cut, or pieces	Optional	Optional
Celery	Optional	Optional
Cabbage	Optional	Optional
Olives	Optional	Optional
Tomato paste	Optional	Required in hamburger relish
Mustard or prepared mustard	Required in chow chow and mustard pickles	Required in mustard relish, optional in hamburger relish

12. Mustard relish consists of sweet pickle relish with mustard and other chopped or finely cut vegetable ingredients as listed in Table 24.1.
13. Dill relish consists of relish style pickles containing dill flavoring and other chopped or finely cut vegetable ingredients as listed in Table 24.1.

24.2.4.2 Fresh-Pack Type

The pickles are prepared from uncured, unfermented cucumbers and are packed in a vinegar solution with other ingredients to produce the characteristics of the particular type of pack. The pickles are preserved by acidification to maintain an equilibrated pH of 4.6 or lower. In addition, the pickles are sufficiently processed by heat to ensure preservation of the product in hermetically sealed containers. The distinguishing characteristics of the various types of fresh-pack pickles are as follows:

1. Fresh-pack dill pickles are pickles that are packed in a vinegar solution with dill flavoring.
2. Fresh-pack sweetened dill pickles are pickles that are packed in a vinegar solution with suitable nutritive sweetening ingredient(s) and dill flavoring.
3. Fresh-pack sweetened dill relish consists of finely cut or chopped pickles packed in a vinegar solution with suitable nutritive sweetening ingredient(s) and dill flavoring. The relish may contain other finely cut or chopped vegetable ingredients as listed in Table 24.1.
4. Fresh-pack sweet pickles and fresh-pack mild sweet pickles are pickles that are packed in a vinegar solution with nutritive sweetening ingredient(s).
5. Fresh-pack sweet pickle relish and fresh-pack mild sweet pickle relish consists of finely cut or chopped pickles that are packed in a vinegar solution with suitable nutritive sweetening ingredient(s). The relish may contain other finely cut or chopped vegetable ingredients as listed in Table 24.1.

6. Fresh-pack hamburger relish consists of relish style pickles and other chopped or finely cut vegetable ingredients as listed in Table 24.1 with tomato product added.
7. Fresh-pack mustard relish consists of sweet pickle relish with mustard and other chopped or finely cut vegetable ingredients as listed in Table 24.1.
8. Fresh-pack dill relish consists of relish style pickles containing dill flavoring and other chopped or finely cut vegetable ingredients as listed in Table 24.1.
9. Fresh-pack dietetic pickles are pickles that are packed with or without the addition of sweetening ingredient(s), salt (NaCl), or other suitable ingredient(s) as declared and permitted under FDA regulations.

24.2.4.3 Refrigerated Type

The pickles are prepared from fresh cucumbers and are packed in a vinegar solution with other ingredients to produce the fresh crisp characteristic of refrigerated type. The pickles are preserved by acidification to maintain an equilibrated pH of 4.6 or lower. They are stored, distributed, and displayed under refrigeration and may or may not contain one or more chemical preservatives. The various types of refrigerated pickles are the same as the types listed for fresh-pack type in earlier discussion with respect to ingredients except that they conform to the requirements for refrigerated type.

24.2.5 Sizes of Whole Pickles

Sizes of whole pickles are based on the diameter and the relationship of diameter to the count per gallon. Size designations, applicable counts, and diameters are outlined in Table 24.2. The diameter of a whole cucumber is the shortest diameter at the greatest circumference measured at right angles to the longitudinal axis of the cucumber.

24.2.6 Definitions of Terms

For an interpretation of this standard, some definitions of terms are given:

Analytical definitions refer to analytical laboratory requirements.

1. *Acid* means total acidity calculated as acetic acid in accordance with the official methods of analysis of the Association of Official Analytical Chemists.
2. *Brix value* (Brix) means the percent sugar, by weight, corrected to 20°C (68°F), as determined with a sugar scale Brix hydrometer or other instrument that gives equivalent results.

TABLE 24.2

Sizes of Processed Whole Pickles Approximate Counts in Glass and Metal

Word Designation	Diameter	Glass			Metal	
		1 qt.	0.5 gal	1 gal	No. 10	No. 12 (1 gal)
Midget	19 mm (0.75 in.) or less	67 or more	135 or more	270 or more	202 or more	270 or more
Small gherkin	Up to 2.4 cm (0.94 in.)	33–66	67–134	135–269	101–201	135–269
Large gherkin	Up to 2.7 cm (1.06 in.)	16–32	32–66	65–134	48–100	65–134
Small	Over 2.7 cm (1.06 in.) but not over 3.5 cm (1.38 in.)	10–15	20–31	40–64	30–47	40–64
Medium	Over 3.5 cm (1.38 in.) but not over 3.8 cm (1.50 in.)	6–9	13–19	26–39	19–29	26–39
Large	Over 3.8 cm (1.50 in.) but not over 4.4 cm (1.73 in.)	4–5	9–13	18–25	13–18	18–25
Extra large	Over 4.4 cm (1.73 in.)	2–3	6–8	12–17	9–12	12–17

3. *Degrees Baumé* means the density of the packing medium determined with a Baumé hydrometer (modulus 145) corrected to 20°C (68°F).
4. *Equalization* means the natural (osmotic) or simulated blending between the soluble solids of the pickle ingredient and the packing medium.

   Natural equalization means equalization brought about after a period of time has elapsed after processing as follows. Sweetened pickles are considered to be equalized 15 days or more after processing. If the pickles have been sweetened in a tank before packing, the pickles will be considered equalized 15 days after the sweetening process began. Sour and dill pickles are considered to be equalized 10 days or more after processing.

   Simulated equalization means a method of simulating equalization by comminuting the finished product in a mechanical blender, filtering the suspended material from the comminuted mixture, and making the required tests on the filtrate.
5. *Total chlorides or salt* means the salt content expressed as grams of sodium chloride (NaCl) per 100 mL packing medium, except that total chlorides in mustard pickles and chow is determined and expressed in grams NaCl per 100 g of product.

Blemished means any unit that is affected by discoloration, pathological injury, insect injury, or similar causes to the extent that the appearance or edibility of the product is adversely affected:

1. *Slightly* refers to those blemishes that detract only slightly from the appearance of the unit;
2. *Seriously* refers to those blemishes that strongly detract from the appearance or edibility of the unit.

Color

1. *Good color* in cured type means the typical skin color of the pickles ranges from a translucent light green to dark green and is practically free from bleached areas. Not more than 10%, by weight, of the pickles may vary markedly from such typical color. In mixed pickles, chow chow pickles, and pickle relish, all of the ingredients possess a practically uniform color typical for the respective ingredient. The pickles and other vegetable ingredients should be free of off-colors.
2. *Good color* in fresh-pack and refrigerated types means the typical skin color of the pickles ranges from an opaque yellow-green to green. Not more than 15%, by weight, of the pickles may vary markedly from such typical color. In pickle relish, all of the ingredients possess a good uniform color typical for the respective ingredient. The pickles and other vegetable ingredients should be free of off-colors.
3. *Reasonably good color in cured type* means the typical skin color of the pickles ranges from light green to dark green and is reasonably free from bleached areas. Not more than 25%, by weight, of the pickles may vary markedly from such typical color. In mixed pickles, chow chow pickles, and pickle relish, all of the ingredients possess a reasonably uniform color typical for the respective ingredient. The pickles and other vegetable ingredients should be free of off-colors.
4. *Reasonably good color in fresh-pack and refrigerated types* means the typical skin color of the pickles ranges from light yellow-green to green. Not more than 30%, by weight, of the pickles may vary markedly from such typical color. In pickle relish, all of the ingredients possess a good, fairly uniform color typical for the respective ingredient. The pickles and other vegetable ingredients should be free of off-colors.
5. *Poor color in all types of pickles* means the pickles fail to meet the requirements for good or reasonably good color for the respective type.

Crooked pickles mean whole pickles that are curved at an angle greater than 60°, as illustrated by Figure 24.1. See also the definition of *misshapen*.

Curved pickles mean whole pickles that are curved at an angle of 35° to 60° when measured, as illustrated by Figure 24.2.

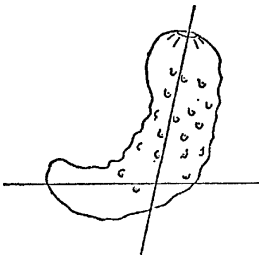


FIGURE 24.1 Crooked pickles.

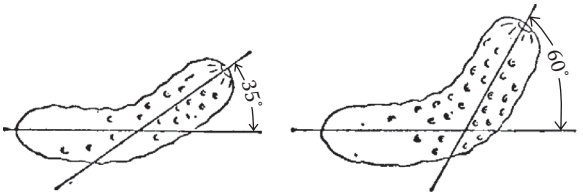


FIGURE 24.2 Curved pickles.

Diameter in whole style means the shortest diameter measured transversely to the longitudinal axis at the greatest circumference of the pickle. *Diameter* in cross-cut style is the shortest diameter of the largest cut surface.

Defect means an imperfection such as curved, misshapen, mechanically damaged, discolored, and other imperfection that affects the appearance or edibility of the product.

End cut means a pickle unit intended for cross-cut (sliced crosswise) style that has only one cut surface.

Extraneous vegetable material (EVM) means any harmless vegetable material, other than stems, that is not normally part of the pickle ingredient. EVM such as leaves or other vegetable material not associated with proper pickle preparation or packaging is considered a defect if it affects the appearance or edibility of the product.

Slightly means practically free of EVM and does not more than slightly affect the appearance or edibility.

Materially means reasonably free of EVM and does not more than materially affect the appearance or edibility.

Flavor and odor

1. *Good flavor and odor* means characteristic flavor and odor (e.g., characteristic dill flavor or the like) typical of properly processed pickles, for the type, that is free from objectionable flavor and odor of any kind.
2. *Reasonably good flavor and odor* means flavor and odor that may be lacking in characteristic flavor for the type but is free from objectionable flavor and odor.
3. *Poor flavor and odor* means flavor and odor that fails to meet the requirements for good or reasonably good flavor and odor.

Length in sliced lengthwise style means the longest straight measurement at the approximate longitudinal axis.

Mechanical damage refers to crushed or broken units that affect the appearance of the units. In relish, mechanical damage refers to units that are poorly cut and have a ragged or torn appearance.

Misshapen pickles mean whole pickles that are crooked or otherwise deformed (such as nubbins). Also see the definition for crooked pickles.

Nubbin is a misshapen pickle that is not cylindrical in form, is short and stubby, or is not well developed.

Texture means the firmness, crispness, and condition of the pickles and any other vegetable ingredient(s) and freedom from large seeds, detached seeds, and tough skins that may be present. The following terms also relate to texture:

1. *Hollow centers in whole style* means the pickles, when cut transversely to the longitudinal axis, are missing a third or more of the seed cavity.
2. *Soft, shriveled, and slippery units* refers to pickles that are wrinkled, not crisp, slick, flabby, or lack firmness.
3. *Good texture* means the pickle units have been properly processed and possess a texture that is firm and crisp.
4. *Reasonably good texture* means the pickle units have been properly processed but lack some of the firmness and crispness that is characteristic for the style and type of pack.
5. *Poor texture* means the pickle units do not meet the requirements for good or reasonably good texture.

Uniformity of size (relish style only)

1. *Practically uniform in size* means the size of the units may vary moderately in size but not to the extent that the appearance or the eating quality is seriously affected.
2. *Poor uniformity of size* means the units fail the requirements for practically uniform.

Unit means one whole, half, slice, or piece of pickle as applicable for the style.

Units missing a third or more of the seed cavity in cross-cut style means pickles that have lost a substantial portion of the seed cavity such as a cross-cut unit missing a third or more of the seed cavity portion.

24.2.7 Recommended Fill of Container

The recommended fill of container is not a factor of quality for the purposes of these grades. Each container of pickles should be filled with pickle ingredient, as full as practicable, without impairment of quality. The product and packing medium should occupy not less than 90% of the total capacity of the container.

24.2.8 Quantity of Pickle Ingredient

The recommended minimum quantity of pickle ingredient is designated as the percentage of the declared volume of product in the container for all items except pickle relish. Minimum quantity of pickle relish is designated as a relationship of the drained weight of the pickle ingredient to the declared volume of the container. The minimum quantities recommended in Tables 24.3 and 24.4 are not factors of quality for the purposes of these grades.

TABLE 24.3

Recommended Pickle Ingredients All Styles except Relish

Type of Pack	Minimum Fill (Volume)
Cured	55%
Fresh-Pack	57%
Refrigerated	57%

TABLE 24.4

Recommended Drained Weight-to-Container Volume: Relish

Type of Pack	Minimum Fill (Weight/Volume)
Cured	
Sweet	92%
Other than sweet	88%
Fresh-pack	
Sweet	85%
Other than sweet	80%

The percent volume of pickle ingredient is determined for all styles, except relish, by one of the following methods in accordance with the procedures prescribed by the USDA:

1. Direct displacement (overflow-can method)
2. Displacement in a graduated cylinder
3. Measurement of pickle liquid
4. Any other method that gives equivalent results and is approved by the USDA

24.2.8.1 Drained Weight/Volume

The percent weight/volume (w/v) of relish shown in Table 24.4 is determined as follows:

The drained weight of pickle relish of all types is determined by emptying the contents of the container upon a U.S. Standard No. 8 circular sieve of proper diameter containing 8 meshes to the inch (0.0937 in. ± 3%, square openings) so as to distribute the product evenly, inclining the sieve slightly to facilitate drainage, and allowing to drain for 2 minutes. The drained weight is the weight of the sieve and the pickles less the weight of the dry sieve. A sieve 8 in. in diameter is used for 1 quart and smaller size containers and a sieve 12 in. in diameter is used for containers larger than 1 quart in size.

24.2.9 Sample Unit Size

For all styles of pickles and types of pack, the sample unit used in analyzing the quality factors is the entire contents of the container unless otherwise specified in 7 CFR Part.

24.2.10 Grades

1. U.S. Grade A is the quality of pickles that meets the applicable requirements of Tables 24.5 to 24.11 and scores not less than 90 points
2. U.S. Grade B is the quality of pickles that meets the applicable requirements of Tables 24.6 to 24.11 and scores not less than 80 points
3. Substandard is the quality of pickles that fails the requirements of U.S. Grade B

24.2.11 Factors of Quality

The grade of pickles is based on the following quality factors:

1. Analytical requirements in Table 24.5
2. Flavor and odor
3. Color
4. Uniformity of size

TABLE 24.5

Analytical Requirements Cured Type Pickles: All Styles

	Total Acidity Expressed as Acetic Acid g/100 mL, Unless Otherwise Indicated	Total Chlorides Expressed as NaCl grams/100 mL, Unless Otherwise Indicated	Degrees Brix	Degrees Baumé
	Maximum	Maximum	Minimum	Minimum
Cured type all styles				
Dills (natural, genuine, or processed)	1.1	5.0		
Sour, sour mixed, dill pickle relish, sour relish	2.7	5.0		
Sweet whole, sweet mixed, and sweet relish	2.7	3.0	27.0	15.0
Mild sweet, mild sweet mixed, mild sweet relish			20.0	12.0
Sour mustard or sour chow chow	2.7[a]	3.0[a]		
Sweet mustard or sweet chow chow	2.7[a]	3.0[a]	28.0	15.5
Fresh-pack and refrigerated types, all styles				
Dills and sweetened dills	1.1	4.25		
Sweetened dill relish	1.1	4.25		
Sweet and mild sweet relish	1.65	2.75		
Sweet and mild sweet pickles	1.65	2.75		
Dietetic				

Note: All pickle products must have an equilibrated pH of 4.6 or lower.

[a] Expressed as grams/100 g.

5. Defects
6. Texture

24.2.12 Requirements for Grades

See Tables 24.5 to 24.11.

24.3 Establishment Inspection

The USDA has issued guidelines for the inspection of a pickles processing plant. Some of the information is provided in this chapter. The quality control officer in such a plant should use the information to implement its in-plant inspection procedure.

The information is presented in the teacher/student format for ease of reference.

1. Direct special attention to the following areas when inspecting these types of food establishments. If the establishment is producing acidified fresh-pack pickles, determine if the establishment is complying with the requirements of 21 CFR 114, Acidified Foods.
2. Salt stations and salt stock tanks
3. Insects that breed in decomposed pickles or other decaying organic matter such as the lesser or little house fly, the latrine fly, the house fly, the rat-tailed maggot, and drosophila are of major sanitary significance. Examine 25% of the tanks for insect filth.

TABLE 24.6

Quality Requirements Whole-Style Pickles

	Grade A		Grade B	
	Maximum (by Count)	**Score**	**Maximum (by Count)**	**Score**
Flavor and odor	Good		Reasonably good[a]	
Color	Good	18–20	Reasonably good[a]	16–17
Uniformity of size[b]		18–20		16–17
Diameter variation				
Midget and gherkin [over 8 mm (0.31 in.)]	10%		20%	
Small and medium [over 10 mm (0.39 in.)]	10%		20%	
Large and extra large [over 12 mm (0.47 in.)]	10%		20%	
Defects	Practically free	27–30	Reasonably free[a]	24–26
Blemished (slightly and seriously)	15%		25%	
Blemished (seriously)	5%		10%	
Curved pickles	10%		20%	
Misshapen	5%		15%	
Mechanical damage	10%		15%	
Attached stems [over 2.5 cm (0.98 in.)]	10%		20%	
EVM	Practically free		Reasonably free[a]	
Texture	Good	27–30	Reasonably good[a]	24–26
Large seeds, detached seeds, tough skins	Practically free			
Soft, shriveled, and slippery units	5%		10%	
Hollow centers	15%		25%	
Total score (minimum)	90 points		80 points	

[a] Cannot be graded above U.S. Grade B, regardless of the total score.
[b] Pickles that are substandard for uniformity of size cannot be graded above U.S. Grade B, regardless of the total score.

TABLE 24.7

Quality Requirements Whole-Style Pickles, Mixed Sizes

	Grade A		Grade B	
	Maximum (by Count)	**Score**	**Maximum (by Count)**	**Score**
Flavor and odor	Good		Reasonably good[a]	
Color	Good	18–20	Reasonably good[a]	16–17
Defects	Practically free	27–30	Reasonably free[a]	24–26
Blemished (slightly and seriously)	15%		25%	
Blemished (seriously)	5%		10%	
Curved pickles	10%		20%	
Misshapen	5%		15%	
Mechanical damage	10%		15%	
Attached stems [over 2.5 cm (0.98 in.)]	10%		20%	
EVM	Practically free		Reasonably free[a]	
Texture	Good	27–30	Reasonably good[a]	24–26
Large seeds, detached seeds, tough skins	Practically free		Reasonably free	
Soft, shriveled, and slippery units	5%		10%	
Hollow centers	15%		25%	
Total score (minimum)[b]	90 points		80 points	

[a] Cannot be graded above U.S. Grade B, regardless of the total score.
[b] Total score is adjusted by dividing the total score by .80 to allow for the absence of the quality factor of uniformity of size in whole mixed sizes style.

TABLE 24.8

Quality Requirements Sliced Lengthwise Style Pickles

	Grade A		Grade B	
	Maximum (by Count)	**Score**	**Maximum (by Count)**	**Score**
Flavor and odor	Good		Reasonably good[a]	
Color	Good	18–20	Reasonably good[a]	16–17
Uniformity of size[b]		18–20		16–17
Length variation [over 2.6 cm (1.02 in.)]	10%		20%	
Defects	Practically free	27–30	Reasonably free[a]	24–26
Blemished (slightly and seriously)	15%		25%	
Blemished (seriously)	5%		10%	
Mechanical damage	10%		15%	
Attached Stems [over 2.5 cm (0.98 in.)]	10%		20%	
EVM	Practically free		Reasonably free[a]	
Texture	Good	27–30	Reasonably good[a]	24–26
Large seeds, detached seeds, tough skins	Practically free		Reasonably free[a]	
Soft, shriveled, and slippery units	5%		10%	
Total score (minimum)	90 points		80 points	

[a] Cannot be graded above U.S. Grade B, regardless of the total score.
[b] Pickles that are substandard for uniformity of size cannot be graded above U.S. Grade B, regardless of the total score.

TABLE 24.9

Quality Requirements Sliced Crosswise or Cross-Cut Style Pickles

	Grade A		Grade B	
	Maximum (by Count)	**Score**	**Maximum (by Count)**	**Score**
Flavor and odor	Good		Reasonably good[a]	
Color	Good	18–20	Reasonably good[a]	16–17
Uniformity of size[b]		18–20		16–17
Diameter [over 5.4 cm (2.13 in.)]	10%		20%	
Defects	Practically free	27–30	Reasonably free[a]	24–26
Blemished (slightly and seriously)	15%		25%	
Blemished (seriously)	5%		10%	
Mechanical damage	15%		25%	
Broken pieces and end cuts	10%		15%	
Thickness over 10 mm (0.38 in.)	10%		15%	
Attached stems [over 2.5 cm (0.98 in.)]	10%		15%	
Units missing one third of the seed cavity	10%		15%	
EVM	Practically free		Reasonably free[a]	
Texture	Good	27–30	Reasonably good[a]	24–26
Large objectionable seeds, detached seeds, and tough skins	Practically free		Reasonably free[a]	
Soft, shriveled, and slippery units	5%		10%	
Total score (minimum)	90 points		80 points	

[a] Cannot be graded above U.S. Grade B, regardless of the total score.
[b] Pickles that are substandard for uniformity of size cannot be graded above U.S. Grade B, regardless of the total score.

TABLE 24.10

Quality Requirements Cut Style Pickles

	Grade A		Grade B	
	Maximum (by Count)	**Score**	**Maximum (by Count)**	**Score**
Flavor and odor	Good		Reasonably good[a]	
Color	Good	18–20	Reasonably good[a]	16–17
Uniformity of size[b]		18–20		16–17
Small pieces 5 g or less	5%		10%	
Defects	Practically free	27–30	Reasonably free[a]	24–26
Blemished (slightly and seriously)	15%		25%	
Blemished (seriously)	5%		10%	
Mechanical damage	10%		15%	
Attached stems over 2.5 cm (0.98 in.)	10%		15%	
EVM	Practically free		Reasonably free[a]	
Texture	Good	27–30	Reasonably good[a]	24–26
Large objectionable seeds, detached seeds, and tough skins	Practically free		Reasonably free[a]	
Soft, shriveled, and slippery units	5%		10%	
Total score (minimum)	90 points		80 points	

[a] Cannot be graded above U.S. Grade B, regardless of the total score.

[b] Pickles that are substandard for uniformity of size cannot be graded above U.S. Grade B regardless of the total score.

4. "Mill run" salt may be used, but workers should not walk in the salt.
5. Tanks should be skimmed daily for debris and insects, and the skimmings should be properly disposed.
6. Newly salted stock ferments–scum growth should be removed regularly and disposed so that insects are not attracted.

TABLE 24.11

Quality Requirements Relish

	Grade A		Grade B	
	Maximum (by Weight)	**Score**	**Maximum (by Weight)**	**Score**
Flavor and odor	Good		Reasonably good[a]	
Color	Good	18–20	Reasonably good[a]	16–17
Uniformity of size		18–20		16–17
Overall appearance	Good		Reasonably good[a]	
Defects	Practically free	27–30	Reasonably free[a]	24–26
Blemished (slightly and seriously)	15%		25%	
Blemished (seriously)	5%		10%	
Poorly cut	10%		15%	
Loose stems over 3.0 mm (0.12 in.)	10%		15%	
EVM	Practically free		Reasonably free[a]	
Texture	Good	27–30	Reasonably good[a]	24–26
Large objectionable seeds, detached seeds, and tough skins	Practically free		Reasonably free[a]	
Soft, shriveled, and slippery units	5%		10%	
Total score (minimum)	90 points		80 points	

[a] Cannot be graded above U.S. Grade B, regardless of the total score.

Examine raw materials used in relish as follows.

1. Obtain the usual composition of relish in percent by weight of cucumbers as well as other ingredients to help appraise the filth load found in the sample.
2. Salt stock used for relish may consist of poor quality pickles, i.e., deformed, bloated, or blemished. However, in the absence of filth, grit, or partly/wholly rotted pickles, there is no objection to their use. Mushy pickles are caused by certain pectin splitting enzymes during fermentation. Soft pickles may be invaded by bacteria and fungi, but it is frequently difficult to determine if any mold or bacteria are present by field examination.
3. Examination of cucumber salt stock for relish—when whole pickles or large pieces are used, examine a representative sample of 100 units going to chopper.
4. Segregate and list objectionable pickles as follows:

Class	Number	Percent
1: With rot spots over 0.5 in.	_____	_____
2: Insect infested or damaged	_____	_____
3: Mushy or very soft	_____	_____

5. For class 1 pickles, make a further determination of the surface area of the rot spots by size: up to 1 in., from 1 in. to half of the pickle, and over half of the pickle. Take close-up color photographs of objectionable pickles. Collect exhibits of pickles showing typical rot and insect damage.
6. Laboratory examination of mushy pickles for mold is necessary to establish if they are objectionable. If over 5% of the units are mushy, cut a thin cross section from each pickle. Place the slices in a quart jar with water and add 20 mL formaldehyde for later examination.
7. When small pieces of salt stock cucumbers, cauliflower, and peppers are used, rot determination by count is impractical. If rotten pieces are observed, collect a separate quart of each pickled vegetable. Preserve the samples with 20 mL formaldehyde. At the same time, collect a sample totaling half a gallon of finished relish.

24.3.1 Peppers

1. Check for insect larvae (maggots or larvae of pepper weevil) in fresh and salt stock peppers and figure the percentage of infestation on a representative sample. Examine any fresh-pack peppers in which infested stock was used.
2. If peppers with rot are found, evaluate in the same fashion as for cucumbers.
3. Examine vinegar storage tanks for drosophila infestation and for vinegar eels.
4. Insect filth in sweet stock pickles—insects, particularly drosophila, are attracted to the sweetening tanks, and may be found in the finished sweet pickle products.
5. Sweet brine is frequently circulated within a tank and from one tank to another dispersing insects in the circulating brine. It is sometimes difficult to estimate the number of insects and parts in such circulating brine. Close examination of the inside tank walls may reveal drosophila above the brine level. These are the best indices of infestation in a tank.

When insects are found in a sweetening tank,

1. Determine whether sweet brine in the tank is an intermediate or finishing brine and if it is circulated within the tank or between sweetening tanks.
2. If the finishing brine is used as a packing medium, determine whether it is filtered before use, and evaluate the filtration step.

3. If sweet stock is held in infested tanks, determine anticipated date of packing.
4. Evaluate tank covers used.
5. List quantitatively the extent of insect infestation by the collection of representative samples of filth from a definite area, e.g., square feet of the walls of the tank on the sweet stock and in a specified amount of brine from different areas of the tank if the infestation is widespread. If infestation seems to be isolated, collect specimens showing the types of insects.

Pay attention to other points of interest.

1. Grit in pickles—excessive grit is frequently found in fresh-pack pickles and in midget sweet pickles. Salt stock may occasionally contain excessive grit. If dirty cucumbers are packed, collect in-line and finished product samples.
2. Use of color and preservatives—green artificial color is sometimes used in relish without label declaration. Ascertain if the color is permitted for use and declared on the label.
3. Sorbic acid may be used in salt stock to prevent yeast growth and in finished pickle products as a preservative. Where sorbic acid is present in the finished product, determine if it is declared on the label.
4. Examination of warehouse stocks—examine for evidence of spoilage, particularly in fresh-pack pickles, which may have been inadequately pasteurized.
5. If heavy insect infestation is found, examine 24 jars of the pickle product (other than relish) most likely to contain insects by inverting jars under strong light. Collect jars containing insects as a factory sample.

24.3.2 Sample Collection

24.3.2.1 Bulk Salt Stock for Filth

If in barrels, collect a minimum of 12 half-gallon jars of salt with their brine; 2 from each of 6 previously unopened barrels to make 6 duplicate subs. Collect 1 sub from the top and the other sub from the bottom, if possible. If in tank cars, collect a minimum of 12 half-gallon jars of salt stock and brine. If live flies are observed inside tank during sampling, note and estimate their number.

24.3.2.2 Finished Pickle Product—All Types

Filth and grit

Quarts and smaller jars	Minimum to collect
Up to 100 cases in lot	24 jars
More than 100 cases	48 jars
Gallon jars	Minimum to collect
Up to 100 cases	12 jars
More than 100 cases	24 jars

Undeclared color and chemical additives

Collect 6 quarts or 12 pints for examination.

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*Canned Sauerkraut: Manufacturing, Inspection, and Grade Standards**

Y. H. Hui

CONTENTS

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25.1 Introduction

The term *sauerkraut* literally means *sour* (*sauer*) *cabbage* (*kraut*). It is a traditional German fermented vegetable product that has spread to other cultures, and it is used on its own or in food preparations. Its sequential growth of lactic acid bacteria has long been recognized. Each lactic acid bacterium dominates the fermentation until its end product becomes inhibitory for its own development and creates another environment suitable for another lactic acid bacterium to take over. The fermentation continues until most of the available fermentable sugars are exhausted. The production of sauerkraut is not risk-free and sanitary precautions must be taken to avoid spoilage. Table 25.1 presents the basic steps in sauerkraut processing.

The remaining information in this chapter has been derived from the websites of the U.S. Department of Agriculture (USDA, www.usda.gov).

25.1.1 Canned Sauerkraut and Crops

Sauerkraut, including both canned and bulk, is an important processed product. Cabbage harvested for sauerkraut is an important cash crop for growers in areas where kraut is produced. A substantial percent of the total U.S. cabbage, crop was utilized in the manufacture of sauerkraut.

It is estimated that 70%–80% of the sauerkraut produced has been packed in cans. The remainder is generally marketed in bulk in barrels or packed in plastic bags or in glass. Sauerkraut packed in glass is usually preserved by the addition of not to exceed one tenth of 1% benzoate of soda. Sauerkraut packed in plastic bags may be preserved either by refrigeration or by the addition of benzoate of soda.

In this country, the principal producing areas are New York, Wisconsin, and Ohio, which are the principal kraut-packing states, accounting for about 75% of the total annual production. Moderate quantities are produced in Indiana and Michigan in the Midwest and in Washington, Oregon, Colorado, and Utah in the West.

All but a very small proportion of the sauerkraut pack is made in northern areas. This may be attributed in part to the heavier yields of fall cabbage obtained in those areas and in part to the greater solidity and whiteness of heads. The greater proportion of the cabbage grown for the fresh market and used in the manufacture of sauerkraut is of varieties of the domestic type.

Nearly all early cabbage grown in the South for the fresh market and the greater part of the late crop cabbage grown in the North is of the domestic type. This type includes many varieties, with heads ranging from medium to large in size and from round and pointed to distinctly flattened in shape. Among the leading kraut varieties are All Head (All Head Early), Marion Market, and Copenhagen Market for early season processing; All Seasons, Flat Dutch, and Glory (Glory of Enkhuizen) are for later packing.

TABLE 25.1

Basic Steps in Sauerkraut Processing

Selection and trimming of white head cabbage
Coring and shredding of head cabbage to 1/8 in. thick
Salting with 2.25% to 2.50 % salt by weight with thorough mixing
Storage of salted cabbage in vats with plastic cover weighted with water to exclude air in the cabbage
Fermentation at 7°C to 23°C for 2–3 months or longer to achieve an acidity of 2.0% (lactic)
Heating of kraut to 73.9°C before filling the cans or jars followed by exhausting, sealing, and cooling
Storage and distribution

Sources: Brady, P.L., *Making brined pickles and sauerkraut*, Cooperative Extension Service, University of Arkansas, Little Rock, AR, 1994; Desrosier, N.W., *Elements of food technology*, Westport, CT: AVI Publishing, 1977. With permission. Fleming, H.P., McFeeters, R.F., Ethchells, J.L., Bell, T.A., Pickled Vegetables, in Speck, M.L., editor, *Compendium of Methods for the Microbiological Examination of Foods*, APHA Technical Committee on Microbiological Methods of Foods, pp. 663–681, 1984; Hang, D.Y., Western Fermented Vegetables: Sauerkraut. In: Hui, Y.H., Ghazala, S., Murrell, K.D., Graham, D.M., Nip, W.K., editors, *Handbook of Vegetable Preservation and Processing*, Marcel Dekker, New York, pp. 223–230, 2003.

In some seasons when there is a shortage of domestic types, a considerable tonnage of Danish-type cabbage may be used in the manufacture of kraut. The Danish type is characterized by medium sized, very compact heads, general spherical or somewhat top-shaped. The Danish type is used for storage to be sold later as fresh cabbage.

Red and Savoy cabbages are grown for the fresh market and are generally not used for sauerkraut. Red cabbage may be used in the production of a canned pickled cabbage.

25.2 Manufacture of Sauerkraut

Sauerkraut is produced by the action of lactic acid bacteria, which transforms the sugar in the cabbage into lactic acid. These bacteria are essentially anaerobic; that is, they grow best in a medium from which air is excluded. This condition is created by the addition of salt, which draws enough juice from the cabbage to help create the anaerobic conditions under which the desired bacteria will thrive. The steps of manufacturing sauerkraut are described below:

1. Head cabbages selection and trimming
2. Head cabbage coring and shredding (~1/8 in. thick)
3. Salting (2.25%–2.50% by weight)
4. Thorough mixing
5. Storage of salted cabbage in vats, excluding air in containing by various mechanical means such as using weights
6. Fermentation: 7°C to 23°C, 2–3+; final acidity, 2.0% lactic acid
7. Heating: end temperature ~73.9°C
8. Filling cans or jars
9. Exhausting, sealing, and cooling
10. Storage and distribution

25.2.1 Delivery and Storage of Cabbage

Cabbage may be used immediately on delivery or stored in bins at the plant until used. These storage bins should be enclosed and protected so that the cabbage can be stored into the winter, if necessary, without damage by freezing. The cabbage may be allowed to wilt slightly before shredding. The shreds of cabbage are not so easily broken and longer shreds may be obtained. The length of the shreds will depend principally on the size of the heads of cabbage used. Small heads will provide only short shreds with sometimes a "choppy" appearance. Large heads will provide long shreds when the cutter is properly adjusted.

25.2.2 Coring and Trimming

Heads of cabbage are cored before shredding. The heads are placed under a rapidly revolving augur with small horizontal blades. The blades cut the core into very fine pieces that are not objectionable in the finished product.

The heads are trimmed before coring and shredding or chopping. Trimming consists of removing portions of stems and heavy green outer leaves and cutting out blemished or discolored areas.

25.2.3 Cutting, Shredding, or Chopping

The heads of cabbage are cut into shreds by curved knives set into a rapidly revolving disc about 3 ft in diameter. The blades are usually set to cut shreds 1/32 in. in thickness. The cutter blades must be very sharp and properly set to give a clean, even cut. Chopped kraut is prepared by means of a mill that cuts the cabbage into small pieces of varying degrees of fineness.

25.2.4 Filling the Tanks

Kraut tanks, which may be constructed of cypress wood, are usually set on a firm concrete foundation. The top of the tanks, which may be 8 to 10 ft deep, project 3 to 3.5 ft above the flooring built around the tanks. The tanks are usually placed in groups of 10 or 12 or more, depending on the size of the plant and the number of shredding and packing lines to be in operation during the season. The tanks are usually of a size to hold from 20 tons to as much as 100 tons of chopped or shredded cabbage. Each tank is provided with an opening in the bottom to drain off the excess juice after the tank is opened for canning and to allow for cleaning after the tank has been emptied. A small opening may be provided in the side of the tank for sampling juice for analysis to determine the progress of fermentation.

The shredded cabbage from the cutter is usually conveyed by conveyor belt into a two-wheeled dump truck designed to carry 100 lb of cabbage. The shredded or chopped cabbage is weighed into the truck in 100-lb lots, which is then pushed to the side of the tank and dumped.

The cabbage is spread evenly by workmen in the tank using hay forks. A weighted quantity of salt is then uniformly scattered over the cabbage. From 2 to 3 lb of salt may be used for each 100 lb of cabbage. The usual amount is 2–1/2 lb of salt per 100 lb of cabbage.

When the tank is full, heavy planks or wood sections cut to fit the inside of the tank may be placed on the cabbage and weighted down or may be held in place by a screw press. A new and improved method is to weight the cabbage down with water held in a plastic cover giving a depth of about 2 ft of water.

25.2.5 Fermentation

A number of different kinds of bacteria, yeasts, and mold spores are present in or on the cabbage as it comes from the field, ready to develop when conditions are favorable. The addition of salt to the shredded or chopped cabbage inhibits the growth of many of the undesirable organisms. The juice drawn from the shredded cabbage by the salt helps to create a condition favoring the growth of lactic acid bacteria.

An initial growth of yeast takes place, causing a frothy appearance and the evolution of carbon dioxide gas. Yeasts are aerobic (oxygen-loving) organisms, and their growth helps to exhaust the air present throughout the shredded cabbage, which favors the development of the lactic acid bacteria. A small amount of alcohol is formed by the activity of the yeast. The alcohol, usually less than 0.5%, is oxidized to acetic acid, which also contributes to the flavor of the kraut.

Fermentation to the minimum acidity of 1.5% may be completed in a period as short as 10 days, or it may take several weeks, depending on the temperature. A temperature of about 85°F is most favorable on the growth of lactic acid bacteria. The rate of fermentation is determined principally by the temperature of the cabbage in the tank. Fermentation tanks should be in a heated room in northern production areas where low temperatures prevail during the curing period. Some kraut manufacturers warm the shredded cabbage before it goes into the tank in cold weather or when customers' demands emphasize the need for as rapid fermentation as possible. It may be heated by passing it on an endless belt through a covered chamber where it is exposed for a few minutes to live steam. Kraut that has been cured rapidly and then canned promptly will usually be lighter in color than when it is slowly fermented and packed after holding in tank for long periods.

Fully fermented sauerkraut should have a lactic acid content of 1.6% to 2.0% and a residual sugar content of about 1.5%. The kraut may be held in the tanks in acceptable condition for a period of several months. Kraut tends to darken when left in the tank and may acquire off-flavors due to growth of contaminating mold yeast and bacteria.

25.3 Canning Sauerkraut

The canning of sauerkraut must comply with U.S. Food and Drug Administration (FDA) requirements for

- Acidified canned food
- Low-acid canned food

The acidity of the kraut should be determined by means of laboratory tests to ensure a properly cured product before the tank is opened. The tank should not be disturbed until the kraut is to be removed. Once opened, the tank should be packed out without delay. If not completely emptied within a day or two, enough juice should be left to cover the kraut and the cover replaced; otherwise, the exposed kraut will dry and discolor.

The excess juice is drained off by means of a tap in the bottom of the tank. In most cases, this juice is discarded. A limited quantity is canned, however, and sold as sauerkraut juice. Some canners may use a portion of this juice to add to the kraut when canned. However, a hot salt (2% to 3% NaCl) brine is generally added to fill the cans.

The sauerkraut is usually heated to about 120°F before handfilling. The cans are then exhausted in a steam exhaust box for 4 to 8 or 10 min depending on the size of the cans. When a mechanical filler is used, the kraut may be filled at a temperature of 180°F–185°F. The cans are then immediately closed and no further processing is necessary. After filling, the cans are passed under a flow of hot brine containing 2% to 3% salt. Care should be taken not to overfill the cans, since this may result in the development of springers or flippers.

Exhausting is required to ensure a good vacuum on the canned product. An exhaust of 4 to 8 or 10 min in a steam exhaust box is desirable, depending on the size of the can. Canned kraut is processed in an open kettle at boiling temperature. The period of processing will depend on the size of the can and the closing temperature. The closing temperature of 140°F in canning sauerkraut, the pH, temperature, and can sizes must comply with FDA regulations.

After processing, the cans may be water cooled to 100°F to 110°F. At this temperature, the cooking is stopped and enough heat is left to dry the cans. Plain cans are ordinarily used for sauerkraut. The acid brine acts slowly on the tinplate and may in time cause the production of enough hydrogen gas to produce flippers or springers, especially if the initial vacuum is low. Enamel-lined cans are sometimes used to reduce loss from hydrogen springers and to give the product a better appearance.

25.3.1 FDA Requirements

All requirements of the FDA must be complied with canning sauerkraut. The product must be packed under sanitary conditions, must not be adulterated or contaminated with decay, insects, or filth, and must be truthfully labeled.

No standard of identity or quality has been issued by the FDA for canned kraut. However, under a previous definition adopted as a guide for enforcement officials, the product was defined as follows:

> Sauerkraut: The product, of characteristic flavor, obtained by the full fermentation, chiefly lactic, of properly prepared and shredded cabbage in the presence of not less than 2% nor more than 3% of salt. It contains upon completion of fermentation not less than 1.5% of acid, expressed as lactic acid. Sauerkraut which has been rebrined in the process of canning or repacking contains not less than 1% of acid expressed as lactic acid.

It is implied that the product is clean and sound as required under the law.

Also, in the absence of a standard of identity, the term *sauerkraut* is considered the common or usual name for a product obtained by the lactic acid fermentation of cabbage in the presence of salt. Products that have not been fermented but owe their acidity to added vinegar, acetic acid, or other acidifiers are not entitled to the name sauerkraut. Ingredients of sauerkraut must be listed on the label by their common or usual name in descending order of predominance.

Regarding adulteration by thrips, FDA will recommend legal action if the following is confirmed:

The sauerkraut exceeds an average of 50 thrips per 100 g.

In an advisory issued by the FDA on the fill of container for canned kraut, the following minimum drained weights have been specified as conforming to good commercial practice.

Size of Container (Can)	Drained Weight (oz)
No. 2	16
No. 2-1/2	23
No. 10	80

The drained weight recommendations for these and other sizes of containers as provided in the grade standards for canned kraut conform to FDA recommendations. However, with modern packaging practices, options are available and they require consultation with the FDA and USDA.

In addition to the name of the product, *sauerkraut*, the style should be shown on the label as shredded or chopped. The net contents and the name of the packer or distributor must also be shown on the label.

25.4 Inspection of the Product

The USDA sampling requirements should be followed. The condition of the containers in the lot, such as freedom from swells, springers, leakers, and rusty containers should be ascertained at time of sampling in accordance with applicable USDA instructions. The number of cases in the lot and warehouse location should be recorded as a means of identification in the event that supervision of loading is requested. Whenever it appears that there may be some question as to the grade of the lot or the quality is likely to be irregular or a wide variation in drained weight or other deviation is anticipated, the appropriate USDA sampling plan should be selected.

The U.S. Standards for Grades of Canned Sauerkraut define and describe the requirements for the different grades and outline the procedure for inspecting the product. Such standards are discussed later in this chapter. The inspection process must use these standards as a frame of reference.

25.4.1 Inspection during Packing Operations

25.4.1.1 Sanitation, Raw Product, and Trimming

A continual check should be made of the condition of the equipment and building, with particular attention to adequate lighting, cleanliness of the working areas, accessibility for efficient cleaning, and proper ventilation. It is the duty of the inspector to see that a high standard of sanitation is maintained. Insanitary conditions can be prevented by careful daily cleaning of all pieces of equipment and keeping floors free from an accumulation of trash and waste material. Floors should be flushed and drains kept open to carry off wash water and waste material.

The quality and condition of the raw product should be observed for color or maturity, condition, and freedom from damage by worms or insects.

The heavy outer green leaves should be removed by the trimming operation. When heads are damaged by infestation all of the area of infestation should be carefully removed, as well as any other damage that may affect the quality of the kraut.

25.4.1.2 Coring, Cutting, or Shredding

When shredded style kraut is being cut, the heads are cored following the trimming operation. The core is not removed by the coring operation but is cut into very fine pieces. The coring machine consists of a single blade, augur-like knife that revolves at high speed. The head is placed in position under the augur and then raised by a foot lever to engage the cutting blades. The head is raised to force the cutting blades through the entire length of the core. Coring should completely disintegrate the core. Check to determine efficiency of the coring operation.

When chopped kraut is made, the coring operation may be dispensed with. The heads of cabbage, after trimming, may go directly to the chopper or disintegrator. Check operation of chopper for efficiency. Pieces should be of fairly uniform size without an excess of very fine or coarse pieces.

The cutter should be operated at the speed recommended by the manufacturer. The knives should be uniformly set to slice the cabbage to give approximately 1/16-in.-thick shreds. Knives should be very sharp to give a clean cut shred as long as can be obtained from average size head.

25.4.2 Heating, Steaming, Weighing, and Salting

When kraut is made during cool weather, the shreds or pieces of chopped cabbage may be heated by steaming at atmospheric pressure sufficient to raise the temperature of the cabbage to about 80°F in the tank. Heating accelerates fermentation, enabling the processor to pack out the kraut in from 10 days to 2 weeks, if necessary, to meet delivery schedules. Steaming should be carefully checked to prevent overheating and destruction of lactic acid bacteria, which are essential for normal fermentation.

The shredded or chopped kraut should be weighed into a dump cart or other container for conveying and emptying into the kraut tank. The cabbage should be evenly distributed in the tank. From 2 to 3 lb of canner's grade salt is uniformly added over the cabbage as it is being distributed.

25.4.3 Covering Tank after Filling

When wooden tank covers or planks are used, they may be weighted down with building blocks treated with an acid-resisting, nonabsorbent coating. The cover may also be held in place by a screw press that can be adjusted as required to hold the kraut under the juice that collects during fermentation. The cabbage should be covered with cheese cloth before the wooden tank covers are put in place. The cheese cloth keeps shreds and pieces of cabbage from working loose and floating in the juice that accumulates over the kraut. Off-flavors may develop due to fermentation of this floating material by aerobic bacteria and scum-forming yeasts.

The use of plastic covers is rapidly replacing other methods of weighing down the kraut after the tank is filled. The covers are designed to hold the amount of water required to press the kraut below the surface of the juice. The use of these covers has resulted in improved sanitary conditions and has practically eliminated the loss of off-flavored and discolored kraut that frequently develops at the top of the tank when other methods are used.

25.4.4 Curing Progress, Tanking Opening, and Packing

Samples of juice for acidity determinations should be taken from near the center of the tank at intervals of 3 to 6 days, depending on the rate of fermentation. Samples should be taken by means of a 4- or 5-ft length of 0.25-in. stainless steel tubing. The salt content as well as the acidity should be determined by the applicable method outlined in the standards. The kraut may be canned when the acidity reaches 1.5% or higher, calculated as lactic acid.

Before removing the wooden tank cover, the juice over the top of the cover should be siphoned off and the cover scrubbed to remove any decomposed material. The scrub water is also siphoned off. The weights or screw press can then be removed to release the cover. The cover is taken off and the tank is then open for packing. When plastic covers are used, the water in the cover can be siphoned off and the cover folded up and removed, opening the tank for packing. The bottom plug is then removed from the tank to drain off all or part of the excess of kraut juice that is not needed for canning.

25.4.5 Filling

In a typical hand-packing operation, the kraut taken from the tank is heated to 110°F–125°F in a tank of kraut juice or kraut juice and brine. When a mechanical kraut filler is used, the temperature of filling is increased to 1700°F–1850°F. No further processing is necessary when kraut is filled at the higher temperature.

The filling operation should be carefully checked. Containers should be checked for ingoing weights at regular intervals to avoid drained weights below the recommended minimum for the size. The coding of containers should be checked after filling and closing. Errors in coding may be avoided by careful checking at this point.

25.4.6 Brining and Exhausting

When the product is hand packed, salt brine (2%–3% NaCl) at near-boiling temperature is added to completely fill the container. The containers then pass through a steam exhaust box to expel air and raise the temperature to 1110°F–150°F at the center of the can. Containers then pass through closing machines and are then placed in retort crates for open-kettle processing in water at boiling temperature.

The salt brine should be checked for temperature and percentage of salt content. The brine should be added at a temperature near the boiling point to ensure a good vacuum after processing and cooling to atmospheric temperature.

The time and temperature of the exhaust should be carefully checked. The resulting vacuum on containers after processing and cooling and the efficiency of the sterilizing process are directly related to the time and temperature of the exhaust.

25.4.7 Cooking and Cooling

Sufficient heat treatment to preserve the product in hermetically sealed containers without overcooking is the objective of the cooking process. Warehouse stacks should be observed daily for any evidence of spoilage, which may be due to understerilization or leaky containers. Overcooking, which may unfavorably affect the color of the product, should be avoided.

Overcooking may result from failure to cool the containers after processing. When containers are water cooled, excessive moisture should be removed from the cans after immersion in the cooling tank. Cooling to 100°F is recommended before the cans are cased. At this temperature, no further cooking occurs and sufficient heat is retained to dry off the containers, thereby avoiding the rusting of metal containers and the metal closures of glass containers.

25.5 U.S. Standards for Grades of Bulk Sauerkraut

25.5.1 Product Description

Bulk or barreled sauerkraut, hereinafter referred to as sauerkraut or bulk sauerkraut, is the product of characteristic acid flavor, obtained by the full fermentation, chiefly lactic, of properly prepared and shredded cabbage in the presence of not less than 2% nor more than 3% of salt. It contains, upon completion of the fermentation, not less than 1.5% of acid, expressed as lactic acid.

25.5.2 Grades of Bulk Sauerkraut

U.S. Grade A (first quality) bulk sauerkraut is of a color approximating or is lighter than olive buff (according to Ridgway's Color Standards and Nomenclature), with the shreds uniformly cut to approximately 1/32 in. in thickness. The product is practically free from defects and blemishes; is of fine, crisp texture; possesses a normal, well-developed, sauerkraut flavor; and scores not less than 85 points when scored according to the scoring system outlined herein.

U.S. Grade C (second quality) bulk sauerkraut may have a variable straw color, but not darker than dark olive buff (according to Ridgway's Color Standards and Nomenclature); the shreds may lack uniformity of thickness. The product is reasonably free from defects and blemishes; is of reasonably fine, crisp texture; possesses a normal sauerkraut flavor; and scores not less than 70 points and need not score more than 84 points when scored according to the scoring system outlined herein.

Substandard bulk sauerkraut is sauerkraut that fails to meet the requirements of the foregoing grades or when any one of the grading factors falls in the D classification.

25.5.3 Ascertaining the Grade

The grade of bulk sauerkraut may be ascertained by considering, in addition to the requirements in the definition, the following factors: color, cut, absence of defects, texture, and flavor. The relative

importance of each element has been expressed numerically on a scale of 100. The maximum number of credits that may be given for each factor is

Factors	Points
Color	15
Cut	15
Absence of defects	10
Texture	15
Flavor	45
Total score	100

25.5.4 Ascertaining the Rating of Each Factor

The essential variations within each factor are so described that the value may be ascertained for each factor and expressed numerically. The numerical ranges within each factor are inclusive. For instance, the range 13 to 15 means 13, 14, and 15.

25.5.4.1 Color

A classification: Sauerkraut that possesses a color approximating or is lighter than olive buff (according to Ridgway's Color Standards and Nomenclature) may be given a credit of 13 to 15 points.

C classification: If the sauerkraut possesses a color ranging from olive buff to (but not darker than) dark olive buff, or is slightly greenish or yellowish, or possesses a tint of light brown, a credit of 10 to 12 points may be allowed. Sauerkraut that falls in this classification should not be graded above U.S. Grade C (second quality), regardless of the total score for the product.

SStd classification: If the sauerkraut possesses a decidedly dark or pink-tinted color, a credit within the range of 0 to 9 points may be allowed.

25.5.4.2 Cut

A classification: If the shreds of sauerkraut are uniformly cut to approximately 1/32 in. in thickness and are of a reasonable length, a credit of 13 to 15 points may be allowed.

C classification: If the cut is very irregular, the shreds being choppy and poorly cut, a credit of 10 to 12 points may be allowed. Sauerkraut that falls in this classification may not be graded above U.S. Grade C (second quality), regardless of the total score for the product.

SStd classification: Sauerkraut that is very uneven in cut, containing decidedly thick or short shreds, may be given a credit of not more than nine points.

25.5.4.3 Absence of Defects

Absence of defects refers to presence or absence of defects such as large pieces of leaves, dead leaves, large pieces of core, spotted shreds, or other defects.

A: Sauerkraut that is practically free from the defects mentioned may be given a credit of 9 to 10 points.

C: If the product is reasonably free from the defects mentioned, a credit of 6 to 8 points may be allowed. Sauerkraut that falls in this classification should not be graded above U.S. Grade C (second quality), regardless of the total score for the product.

SStd: If pieces of dead leaves, large pieces of core, spotted shreds, or any similar blemishes are prominently present to the extent of injuring the appearance of the product, a credit within the range of 0 to 5 points may be allowed.

25.5.4.4 Texture

Texture refers to the condition of the product and the tendency of the shreds to be firm, fresh, crisp, and easy to cut, as opposed to soft or mushy.

A: Sauerkraut that is firm and crisp may be given a credit of 13 to 15 points.

C: If the product is somewhat tough, or is slightly soft, a credit of 10 to 12 points may be allowed. Sauerkraut that falls in this classification should not be graded above U.S. Grade C (second quality), regardless of the total score for the product.

SStd: If the sauerkraut is tough or, on the contrary, is soft and mushy, a credit within the range of 0 to 9 points may be allowed.

25.5.4.5 Flavor

A: Sauerkraut that has a highly acid (expressed as lactic), palatable, clean, characteristic sauerkraut flavor may be given a credit of 40 to 45 points.

C: If the product possesses a good sauerkraut flavor that may be suggestive of improper bacterial action, but not markedly so, it may be given a credit of 34 to 39 points. Sauerkraut that falls in this classification should not be graded above U.S. Grade C (second quality), regardless of the total score for the product.

SStd: If the flavor is poor and unpalatable from any cause (acetic, butyric, yeasty, moldy, very salty, bitter stale, or rancid), a credit within the range of 0 to 33 points may be allowed.

TABLE 25.2

Score Sheet for Bulk Sauerkraut

Identification marks on packages	. . . . .	. . . . .	. . . . .
Net weight as indicated on package	. . . . .	. . . . .	. . . . .
Percent acid	. . . . .	. . . . .	. . . . .
Factors		**Score Points**	
		A	13–15
Color	15	C	10–12[a]
		SStd	0–9[b]
		A	13–15
Cut	15	C	10–12[a]
		SStd	0–9[b]
		A	9–10
Absence of defects	10	C	6–8[a]
		SStd	0–5[b]
		A	13–15
Texture	15	C	10–12[a]
		SStd	0–9[b]
		A	40–45
Flavor	45	C	34–39[a]
		SStd	0–33[b]
. . . . .			
Total score	100		
. . . . .	. . . . .	. . . . .	. . . . .
Grade			

[a] Sauerkraut that falls in this classification may not be graded above U.S. Grade C (second quality).

[b] Sauerkraut that falls in this classification may not be graded above substandard.

25.5.5 Ascertaining the Grade

The grade of a lot of bulk sauerkraut covered by these standards is determined by USDA procedures. Use Table 25.2 as a score sheet for bulk sauerkraut.

REFERENCES

Brady PL. 1994. *Making Brined Pickles and Sauerkraut*. Little Rock, AR: Cooperative Extension Service, University of Arkansas.

Desrosier NW. 1977. *Elements of food technology*. Westport, CT: AVI Publishing.

Duncan A. 1987. Perfecting the pickle. *Oregon's Agric Prog* 33(2/3):6–9.

Fleming HP, McFeeters RF, Ethchells JL, Bell TA. 1984. Pickled vegetables. In: ML Speck, editor. *Compendium of methods for the microbiological examination of foods*. APHA Technical Committee on Microbiological Methods of Foods. Washington, DC, p. 663–681.

Hang DY. 2003. Western fermented vegetables: sauerkraut. In: YH Hui, S Ghazala, KD Murrell, DM Graham, WK Nip, editors. *Handbook of vegetable preservation and processing*. New York: Marcel Dekker. p. 223–230.

26

Mexican Jalapeño Pepper: Properties, Manufacture, and Flavor

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26.1 Fermentation and Pickling of Jalapeño Pepper

Preservation of vegetables by brining and fermentation is an extremely ancient culinary art that can be traced back thousands of years. The organoleptic properties and stability of fermented vegetables make them a popular food commodity in many countries. Fermentation and pickling are the preservation methods most commonly used to extend the shelf life of the jalapeño pepper, since they are relatively simple and accessible technologies. The processed pepper is well accepted by the consumer, as can be confirmed by the increasing demand for pickled jalapeño for nachos and other similar products.

In Mexico, peppers are added to traditional dishes to increase their flavor and color, and they may be fermented and pickled or acidified only (Guerra et al. 2001). Sometimes, they are mixed with other vegetables such as kale, carrots, and onions.

26.2 Fermentation Process

From the biochemical point of view, fermentation is defined as the group of reactions that result in usable energy for the cell (Nout et al. 1999). This process depends on the biological activity of microorganisms for the production of a range of metabolites, which improve the sensory and nutritional characteristics of foodstuffs, as well as suppresses the growth and survival of undesirable microflora. Fermented

vegetables by themselves offer a series of advantages before the pickling process (such as the presence of lactic cultures that favor a healthy microbial balance in the gut), the fibers of the vegetables maintain their structures since they are not altered by the sterilization processes, and the fermentation itself requires no energy. Several traditional fermentation methods are found in different countries (Ross et al. 2002; Zamora 2009).

Lactic acid fermentation is favored in tropical climates as an alternative to improve the quality and safety of foods at a low cost, due to the restrictions that still exist in some tropical countries for the production, preservation, and distribution of foods, which are related to the lack of the technological infrastructure necessary to carry out other preservation methods such as freezing, refrigeration, or canning (Nout et al. 1999).

Lactic acid bacteria (LAB) are the main microorganism responsible for the natural fermentation of vegetables, including jalapeños. They are defined as a group of facultative anaerobic Gram-positive bacteria and they typically lack a respiratory chain, fermenting various sugars to produce the energy required for cellular maintenance and growth. LAB can produce a wide variety of antagonistic primary and secondary metabolites and even antibiotics and bacteriocins such as reuterocyclin and nisin. The indigenous LAB flora varies as a function of the quality of the raw material, temperature, and harvesting conditions. Species belonging to the Lactobacillaceae family and to the *Lactobacillus* genus are the most common bacteria in natural vegetable lactic fermentation, the most abundant being *Lb. plantarum* (Ross et al. 2002; Rivera-Espinoza 2010; Liu et al. 2006). Other bacteria commonly identified in the fermentation process belong to the Streptococcaceae family and include the genera *Leuconostoc*, *Streptococcus*, *Pediococcus*, and *Lactococcus* (Granito et al. 2006; Breidt et al. 2007).

The fermentation process of jalapeño is based on the fermentation of the sugars naturally present in the vegetable and requires the addition of salt, which must remain constant throughout the process since LAB are facultative anaerobic, halophilic bacteria and therefore require a minimum concentration of NaCl. The product is not only acidified by lactic acid, since other compounds are also produced, such as acetic acid, ethanol, CO_2, esters, and aldehydes that confer special characteristics of texture, flavor, and color to the final product (Colquichagua 1998). These compounds are characteristic of LAB heterofermentative fermentation, which is produced via the pentose phosphoketolase pathway (Liu et al. 2010).

LAB have GRAS (generally recognized as safe) status; however, the good safety record of fermented vegetables depends on a number of factors that result from the selection and growth of lactic acid bacteria. These factors include rapid acid production for lowering pH and, thus, inhibiting the growth of undesirable bacteria during the initiation stage of fermentation and conversion of fermentable sugars to acids, in order to prevent the possible later growth of pathogenic microorganisms (which could produce disease in consumers) and secondary yeast fermentations (which could deteriorate the product). The rapid decrease of acidity minimizes the influence of spoilage bacteria. In slowly acidified environments, lactic acid fermentation may be suppressed by butyric bacteria and a level of acidity lower than pH 3.6 is undesirable from the sensory point of view, because it causes degradation of carotenoids and therefore loss of color in the final product (Karovikova 2005).

Washing the peppers before fermentation is a crucial step to avoid contamination by fungi or other microorganisms. It is carried out in a rotating washer, which has a cylinder with perforations and is semisubmerged in water and connected to a perforated transportation band. Showers of water under pressure wash the vegetables inside the cylinder. Then the peppers are classified by size, either manually or automatically. This is an important step since the process may be altered when peppers are not uniform in size due to the formation of void spaces during fermentation or packing (Infoagro 2011).

The fermentation of the jalapeño pepper is carried out by the immersion of the vegetable in pickles with concentrations of sodium chloride between 18% and 20%, and it is important that the peppers are completely covered by the brine so as to generate an anaerobic environment. During this process the peppers transfer cellular fluids to the brine, so the brine tends to be diluted. For this reason, it is necessary to add 1% of salt everyday during the first week, and three times a week during the remainder of the immersion to have the pickle concentration at between 18% and 20% (Galicia-Cabrera 2004; Hui 2004).

The fermentation takes place for 4 to 6 weeks. Industrially, it is carried out in closed tanks of a capacity between 120 and 14,000 L, with a valve that allows the elimination of the gas formed during the

process, although in some tropical places the tanks are open and placed outdoors. After the period of fermentation, the peppers that were originally luminous green change their color to olive green. This process normally intensifies the pepper characteristics of flavor and aroma and avoids the proliferation of pathogenic microorganisms obtaining a stable product (Adams and Nout 2001; Enachescu 1995).

During fermentation, a white film may form in the inside of the tanks, which is constituted by oxidative yeast and molds that consume the lactic acid produced by the LAB and must be eliminated periodically. The tanks may also be treated with UV light in order to eliminate microorganisms growing on the surface (Breidt et al. 2007).

When the fermented peppers are not going to be immediately packed, they may be stored in a brine of 20% salt concentration and 1% acidity expressed as lactic acid. To achieve this last parameter, it is often necessary to add commercial lactic acid. In this manner the development of unwanted yeast may be prevented (Infoagro 2011).

Some authors recommended the use of a lactic acid bacteria starter culture to improve the quality of the product and shorten the processing time, such as *Lactobacillus plantarum* (Lázaro 1998; Karovikova 2005; Hui 2004).

As soon as the fermentation process is finished, the acid concentration in the product increases from 0.8% to 1.5% (expressed as lactic acid); then the peppers are washed to eliminate the salt excess, classified in accordance to size, placed in glass recipients or plastic bags, mixed with other vegetables (usually carrots and onions), and finally covered with vinegar. The fermented peppers are highly perishable if the brine contains less than 3% of acetic acid, in which case a pasteurization process is necessary. Finally, the product is labeled, packed, and stored (Galicia-Cabrera 2004).

Among the advantages offered by the finished fermented product is a long shelf life due to the production of metabolites, combined with good manufacturing practices that hinder the development of other microorganisms (Zamora et al. 2009). LAB produce several antimicrobials, including organic acids such as lactic, formic, phenyl acetic, and caproic acids, carbon dioxide, oxygen peroxide, diacetyl, and ethanol. Bacteriocins produced by *Lactobacillus casei* have shown an inhibitory effect on *E. coli* isolated from African products, as well as reuterin and reutericyclin produced by *L. reuteri*, among others (Granito et al. 2006; Nout et al. 1999; Soomro et al. 2002; Gálvez et al. 2007). Due to this bactericidal effect, LAB proliferate during the first stages of fermentation, however, some pathogens, viruses, and parasites may survive lactic fermentation in the absence of a thermal treatment and good hygienic practices (Nout et al. 1999).

The kinetics obtained in our laboratory for a spontaneous fermentation of jalapeño peppers is shown as follows. Changes in free phenolic compounds, capsaicinoids, antioxidant activity, and color were monitored.

26.2.1 Methods

26.2.1.1 Experimental Design

A central composite design 2^2 with five replications of the central point was chosen to optimize the fermentation process and to establish the influence of the most relevant factors, as well as the possible interactions among them, using a reduced number of trials (Blanes et al. 2006).

In the case of the spontaneous fermentation of jalapeño pepper, it is possible to vary the temperature and salt concentration to set the optimal conditions to obtain a quality product. Table 26.1 shows the parameters observed during the fermentation.

An optimization trial was carried out based on the data from Table 26.1 using the Design-Expert® Version 7.0.3 software (Stat-Ease, Minneapolis, MN).

26.2.1.2 Capsaicinoid Extraction

The extraction of capsaicinoids from jalapeño peppers during fermentation was made according to the methodology proposed by Cázares et al. (2005). One gram of lyophilized sample was extracted with 10 mL of acetonitrile for 5 hours in a water bath at 60°C.

TABLE 26.1

Central Composite Design: Fermentation Parameters

Experiment	NaCl (%)	*T* (°C)	pH	Acidity (% Lactic Acid)
1	5.75	30	3.8	0.72
2	1.5	35	6.3	0.09
3	5.75	40	3.5	0.27
4	10	35	4.3	0.36
5	2.75	31.50	4.8	0.36
6	8.76	31.50	5	0.18
7	2.75	38.5	4.6	0.45
8	8.76	38.5	6.1	0.18
9	5.75	35	6.5	0.09
10	5.75	35	5.6	0.18
11	5.75	35	4.1	0.63
12	5.75	35	4.4	0.45
13	5.75	35	4.9	0.36

26.2.1.3 Free Phenolics

Two grams of sample was added to 8 mL of 80% ethanol and moderately agitated for 10 minutes. Afterward, the samples were centrifuged at 3700 rpm at a temperature of –4°C for 10 minutes. The supernatant was then recovered for analysis (Kafui 2002).

26.2.1.4 Free Phenolics Quantification

Quantification of phenolics was carried out according to the methodology of Kafui (2002). 100 µL of the extract were added with 750 µL of Folin reagent, kept for 5 minutes, then neutralized with 60% sodium bicarbonate, and kept in the dark for 90 minutes. Afterward, the absorbance is read at 750 nm and interpolated in a ferulic acid curve made previously.

26.2.1.5 Antioxidant Activity

The antioxidant activity was determined using the ABTS radical technique (Charurin et al. 2002). A 2.45 mM solution of ABTS (2,2′-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) is added with 0.66 g of sodium persulfate and kept in the dark for 12–16 hours. Five hundred microliters of this solution is then transferred into a beaker, and the volume is taken up to 50 mL with 80% alcohol, adjusting the absorbance to 0.7 ± 0.2 at a wavelength of 734 nm. Nine hundred ninety microliters from this adjusted solution is mixed with 10 µL of the sample in order to determine the difference in absorbance from reaction time 0 to 5 minutes.

A difference in absorbance is obtained, which magnitude is calculated with the following formula:

$$\Delta A_{\text{sample}} = \frac{(A_{t=0(\text{sample})} - A_{t=5(\text{sample})})}{A_{t=0(\text{sample})} - \dfrac{(A_{t=0(\text{solvent})} - A_{t=5(\text{solvent})})}{A_{t=5(\text{solvent})}}}$$

The percentage of inhibition is interpolated into a previously made curve to obtain the millimolars of Trolox equivalent to the antioxidant activity of the sample.

$$\text{mM of Trolox} = \frac{\%\ \text{of inhibition} - 0.9905}{44.035}$$

26.2.1.6 High-Performance Liquid Chromatography Assay

The analysis of capsaicinoids was carried out with reverse phase HLPC, using a C18 column in isocratic regime 50:50 with 100% acetonitrile and 10% acetonitrile and a flow of 1 mL/min at 280 nm. The injection volume was of 10 μL.

The capsaicin standard curve was made with solutions of 0.1 to 0.05 mg/mL of methanol. The dihydrocapsaicin standard curve was carried out with 0.22 to 0.055 mg/mL of methanol, with five intermediate points, respectively.

26.2.1.7 Color Analysis

Changes in color were studied during the fermentation process of jalapeño pepper using a Chroma meter CR 400 Konica Minolta Version 1.100 (Japan) colorimeter, with a D65 luminant. The CIELAB space coordinates were used, where the *L** axis corresponds to lightness and starts in 0 (black) and ends in 100 (white). The other axes are *a** and *b**, which represent variations between red-green and yellow-blue, respectively (Figure 26.1).

26.2.2 Results

The optimal conditions for the fermentation of jalapeño pepper were obtained at a salt concentration of 8.42% and a temperature of 31.5°C, which yield a theoretical pH of 4.27.

Under these optimal conditions, the fermentation follows the predicted behavior regarding the production of lactic acid and pH reduction (Figure 26.2). Figure 26.2 shows that salt transfer by osmosis into the inside of the pepper increases as the fermentation goes on, with the resulting decrease in pH,

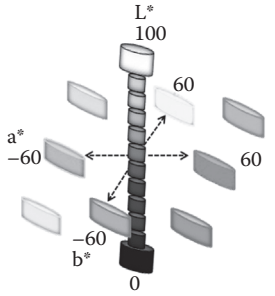

FIGURE 26.1 CIELAB color coordinates. (Adapted from Manresa, A., and I. Vicente, El color en la industria de los alimentos, Ciudad de la Habana: Editorial Universitaria, 2007.)

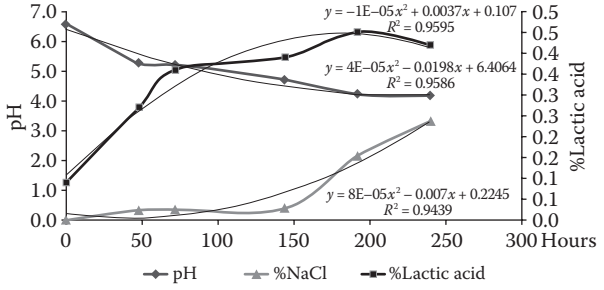

FIGURE 26.2 Changes observed during fermentation of jalapeño peppers (*Capsicum annuum* L.) on pH, lactic acid, and NaCl.

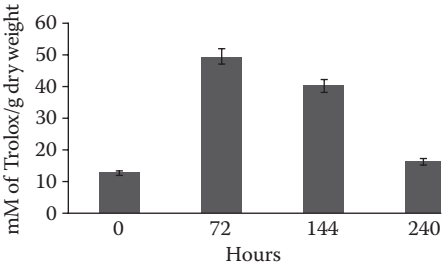

FIGURE 26.3 Antioxidant activity of macerated jalapeño peppers during fermentation.

starting with an initial pH of 6.6. This value is similar to the starting pH value of pickles (4.5), which are fermented products widely commercialized and studied by the scientific community (Breidt et al. 2007).

In the case of pickles, the concentration of salt has been reported between 2% and 4% (Breidt et al. 2007). After 2 weeks of fermentation, jalapeño peppers reached a salt concentration of 3.6%.

The products inside the brine show a transfer of fluids, minerals, and nutrients by osmosis until reaching the equilibrium, resulting in products with very peculiar flavors, great stability, and high nutritional qualities.

Figure 26.3 shows changes in antioxidant activity of the macerated peppers. A fourold increase in antioxidant activity is observed after 72 hours of fermentation, which is due to the availability of the compounds released during the fermentation process, among other factors (Fereidoon et al. 1995). However, after 144 hours of fermentation, a slight decrease in the antioxidant activity is observed, which continues until reaching values close to the initial.

The quantification of free phenolics (Figure 26.4) shows that after 72 and 240 hours, there is a higher concentration than the initial. These changes in concentration may be due to the changes in microbial populations, which gradually modify the compounds along fermentation and lead to the appearance or disappearance of some of them, as well as to the release of those that were attached to the cell membranes and walls. Furthermore, it has been reported that some phenolic compounds are more active than others, which depends on their particular chemical structure and their concentration on the extract along the fermentation process.

The highest antioxidant activity due to free phenolics was found along the fermentation process (Figure 26.5). An increase was observed after 72 hours and at the end of fermentation, suggesting that there is a period of accumulation of such compounds. However, after 144 hours the activity decreases because LAB use phenolics as a substrate or otherwise break them, since certain type of bacteria are able to grow in the presence of phenolics such as *p*-coumaric, ferulic, and caffeic acids, as reported by Acero et al. (2005). However, the metabolic process carried out by lactic bacteria in order to use or break down these compounds is unknown.

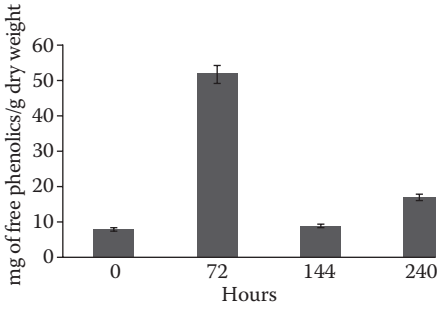

FIGURE 26.4 Concentration of free phenolics of jalapeño pepper (*Capsicum annuum* L.) during fermentation.

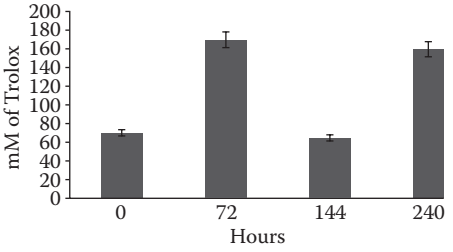

FIGURE 26.5 Antioxidant activity of free phenolics from jalapeño peppers (*Capsicum annuum* L.) during fermentation.

Figure 26.6 shows the changes in concentration of capsaicin and dihydrocapsaicin during fermentation. The generation of new compounds and disappearance of others, with the corresponding effect on antioxidant activity, is observed. There is a clear decrease in the capsaicinoid concentration, showing the capability of the microbial flora to bioconvert them.

Along the fermentation process, the antioxidant activity given by capsaicinoids is lower than the total antioxidant activity, and gradually decreases until reaching a minimum at 144 hours, which is kept constant up to 240 hours. This may be due to the activity of LAB on these compounds, either using or transforming them.

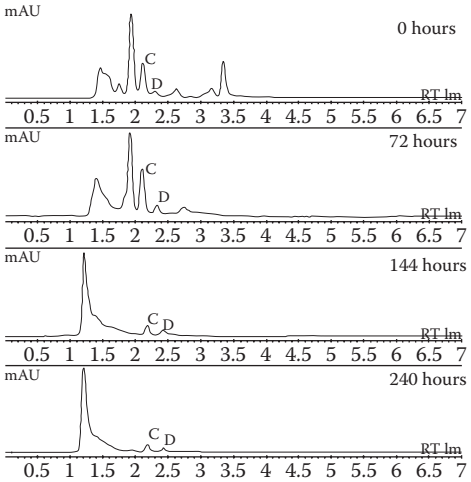

FIGURE 26.6 Changes in concentration of capsaicinoids during germentation of jalapeño pepper. C, capsaicin; D, dihydrocapsaicin.

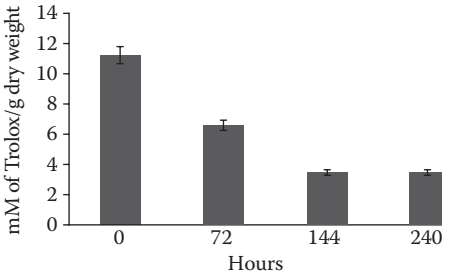

FIGURE 26.7 Antioxidant activity of capsaicinoids along fermentation of jalapeño pepper (*Capsicum annuum* L.).

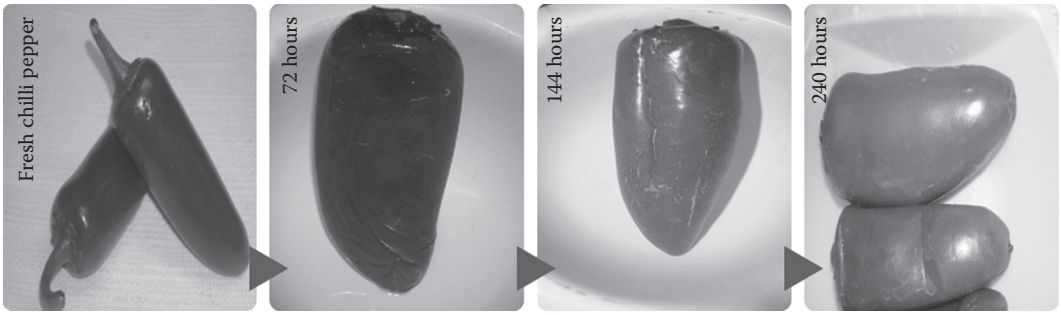

FIGURE 26.8 Changes in color of jalapeño peppers (*Capsicum annuum* L.) during fermentation.

The data from Table 26.2 shows the color of each sample in the chromatic coordinates a^* and b^*, together with the lightness index at the center of the axes, in order to determine variations in the chromatic space (Manresa and Vicente 2007).

The color of jalapeño pepper is defined with a negative a^* value, which places it in the green region. However, due to fermentation, it tends to lose the green and yellow colors and gain lightness, which explains the change in the perception of the color with the naked eye. This change is better appreciated with the Euclidian difference (ΔE), defined as the distance between two points in a tridimensional space (Manresa and Vicente 2007) based on the CIELAB color system and calculated with the following formula:

$$\Delta E = \sqrt{(\Delta L^*)^2 + (\Delta a^*)^2 + (\Delta b^*)^2}$$

In this case, the result indicates the difference in the color perceived by the eye of the analyst and the real difference according to the measurement performed by the instrument, according to an established scale of perception (Table 26.3).

Based on the data in Table 26.3, the fermented samples were compared with the nonfermented samples to determine color differences.

TABLE 26.2

Color Changes in Jalapeño Peppers (*Capsicum annuum* L.) during Fermentation

Parameter	Fresh Pepper	72 Hours	144 Hours	240 Hours
L^*	30.64 ± 0.3	31.0025 ± 0.3	33.125 ± 0.4	32.5225 ± 0.2
a^*	–8.03 ± 0.05	–5.9275 ± 0.4	–3.3325 ± 0.3	–0.3225 ± 0.04
b^*	8.94 ± 0.03	6.07 ± 0.5	8.09 ± 0.4	8.5825 ± 0.2

TABLE 26.3

Perception of Color in the CIELAB Scale

Sensory Perceived Difference	ΔE Value (Instrumental)
Traces	0–0.5
Slight	0.5–1.5
Noticeable	1.5–3.0
Appreciable	3.0–6.0
Remarkable	6.0–12.0
Extraordinary	>12

TABLE 26.4

Perception of the Color Difference of Jalapeño Peppers (*Capsicum annuum* L.) during Fermentation

Relation of Samples	Δ*E*	Sensory Perceived Difference
Nonfermented–fermented 72 hours	3.58	Appreciable
Nonfermented–fermented 144 hours	5.38	Appreciable
Nonfermented–fermented 240 hours	7.94	Remarkable

26.3 Pickling

In Mexico, jalapeños are commonly commercialized like canned pickled products (not fermented), mixed with carrots and onions, in vinegar and in different presentations. The peppers are prepared without seeds, in halves cut along, or in rings and they may or may not be submitted to thermal treatment. According to Pederson and Luh (1988), pickled products are produced by lactic or acetic acid (vinegar) being incorporated into the raw material to modify its sensory properties and to extend its shelf life.

At industrial scale, jalapeño peppers are pickled or cold-packed by dipping fresh vegetables in a hypertonic solution of acetic acid and sodium chloride in big plastic tanks, which causes dehydration and incorporation of the pickling solutes into the vegetable tissue (Valdez-Fragoso et al. 2007). Raw materials are previously washed, reduced in size, and blanched in the case of fresh vegetables or they may also be fermented. Numerous types of pickled products may be prepared by the addition of sugars, spices, essences, and aromas, always using vinegar since this ingredient is fundamental for pickling (Breidt et al. 2007). Figure 49.2 shows the flowchart (Galicia-Cabrera 2004).

The result of the dehydration/impregnation phenomena is the reduction of water activity to levels lower than 0.950 and the reduction of pH (3.3–4.5), which protects the product against microbial growth and confer stability and safeness. The final acidity and salinity levels impart the expected flavor of the pickled product (Valdez-Fragoso et al. 2009; Fleming et al. 1993).

The preserving levels of acetic and NaCl in the industrial pickling of whole jalapeño pepper at atmospheric pressure are of 1.5%–2.0% and 6%–7%, respectively and more than 6 weeks are required to attain such levels. During the process, the pepper is partially dehydrated and the solutes penetrate into the vegetable tissue to provide the sensory properties characteristic of the product. This dehydration-impregnation process is controlled by diffusion phenomena and occurs at relatively low mass transfer rates. Among the variables that affect the process are the concentration of the osmotic solution, type of solute, processing time, temperature, pH, agitation, ratio of hypertonic solution to product, size and geometry of the solid, and nature of the raw material (Daeschel et al. 1990; Valdez-Fragoso et al. 2007).

An optimal jalapeño pickling process should minimize water loss, maximize the pickling solutes impregnation, attain equilibrium water activity, and increase the process yield. According to Valdez-Fragoso et al. (2007), the use of vacuum pulses is a useful technique. They achieved a maximum ratio of solids gain to water loss by using a 12% NaCl brine, 4.6 brine to pepper ratio (w/w), and 22 days of processing, combined with an initial vacuum pulse of 666 mbar for 5 minutes. Derossi et al. (2010) carried out a similar study on pepper slices and found that vacuum level was the most important variable influencing total mass and pH. The vacuum process led an increase in the diffusion rate of hydrogen ions into the vegetable tissue, due to the increase in contact area between the acid solution and the cells.

Pickled jalapeños are commonly canned. In this case, Gu et al. (1999) recommend rotary heating as opposed to static heating, since it has been observed that in the second process the top of the container pasteurizes much faster and cools much slower than the middle and bottom, which is consistent with convection heating. The cold spot was located 4.5 cm above the bottom on the longitudinal axis of the can. Static heating results in soft peppers at the top and firm ones at the bottom. In contrast, peppers processed with rotary heating at 19 rpm had uniform heat penetration and uniform cooling. They came up to pasteurization temperature in 6 minutes as opposed to 10 minutes in the static mode, which results

in substantial energy savings for commercial processors. The agitated process also resulted in a more uniform texture as a result of more uniform penetration throughout the product.

Softening of the pepper during pickling may result from nonenzymatic cell degradation, since pectolytic enzymes such as polygalacturonase have an optimal pH between 3.5 and 5.6. Acetic acid by itself may also cause degradation of pectic substances. However, these effects may be opposed by the presence of calcium ions (Ca^{2+}), which have been used to firm many acidified foods including cucumbers and jalapeños. Calcium treated jalapeño rings were firmer than the non-calcium–treated ones due to the ability of these ions to bind with demethylated polyuronides to form calcium pectates, which prevent pectic depolymerization and solubilization allowing to keep the cell wall structure and giving a firmer product (Gu 1999; Valdez-Fragoso et al. 2009).

The flavor of the pickled jalapeño is the result of the contribution of multiple compounds such as capsaicinoids, volatile compounds (described previously), sodium chloride, acetic acid, carotenoids and derivatives, sucrose, glucose, and fructose that are the principal sugars used in case of fermentation, organic acids such as citric, malic, and ascorbic acid in addition to the phenolic acids, such as ferulic, cinnamic, and chlorogenic acids, and all the volatile compounds mentioned above.

26.3.1 Biochemical Changes during Fermentation

Sucrose, glucose, and fructose are the main sugar components of jalapeño pepper, with fructose and glucose being the fermentable sugars (Breidt et al. 2007). During the first 48–72 hours of fermentation water, sugars, proteins, minerals, and other substances present in the fruits diffuse to the brine by osmosis. As a consequence, the product loses weight and may shrink, but salt begins to penetrate the tissue and produces a gain of water, which in turn causes a weight gain and a recovery of the original shape. The change of texture during fermentation is the most important physical characteristic of this process, and it determines the qualitative differences among pickled and fresh products.

TABLE 26.5

Interaction of Capsaicin and Sensory Perception of Foods

Parameter	Effect of Capsaicin	Reference
Sweetness	Reduces	Lawless et al. 1985; Simons et al. 2002
Flavor intensity	No effect, unclear results	Prescott 1999; Mcbride and Finlay 1990; Prescott and Francis 1999
Olfactory sensations (lemon and celery)	Reduces	Lawless et al. 1985
Perceived oiliness of carbohydrate-based foods	Increases	Yoshioka et al. 1998
Flavor in solid foods	Suppresses (concentration dependent effect)	Reinbach 2007a
Intensity of warmed-over flavor	Reduces	Emrick et al. 2005
Temperature	Burning sensation increases with temperature	Reinbach 2007b
Texture	No effect	Reinbach 2007b
Bitterness	Increases	Prescott et al. 1993; Prescott 1999
Overall flavor of foods	Enhances	Prescott et al. 1993; Mcbride and Finlay 1990; Prescott and Francias 1999
Fruit flavors	No effect	Lawless et al. 1985; Prescott and Stevenson 1995
Meat flavor	Suppresses (concentration dependent effect)	Reinbach 2007a
Food with increased fat levels	Increases burning sensation	Emrick et al. 2005

Source: Adapted from Reinbach, H. C., Hot spices—Effects on appetite, energy intake and sensory properties of a meal, DPhil thesis, University of Copenhagen, Faculty of Life Sciences, Department of Food Science, Denmark, 2008.

The main biochemical change is the transformation of sugars contained in the fresh fruits to lactic acid due to the microbial action. Even though the main product of fermentation is lactic acid, as mentioned above, other compounds such as alcohol and esters are also formed, which confer aroma and taste to pickled jalapeños (Infoagro 2011).

Fresh jalapeño peppers are high in carotenoids and therefore a good source of provitamin A and antioxidants. However, these compounds suffer changes along processing. If a thermal treatment is carried out to inactivate enzymes before fermentation with starting cultures, carotenoids may accumulate due to a loss of water and soluble solids. Furthermore, thermal treatment induces the isomerization of different carotenoids, generating subproducts such as 13-*cis*-β-carotene (Guerra et al. 2001).

The kinetics of a fermentation obtained in our laboratory is presented as follows, together with the change on some metabolites such as capsaicinoids and phenols.

According to Reibach (2008), hot spices such as chili peppers interact with tastes and flavors. At low concentrations, irritants such as capsaicin in chili, allicin in garlic, diallyl sulfide in onion, and isothiocyanates in mustard enhance tastes and odors and produce pungency at higher concentrations. Both the persistence and the intensity of the oral burn from chili are affected by the concentration of capsaicin, type of capsacinoids in the product, serving temperature, type of food, and individual sensitivity and experiences. Table 26.5 presents a summary of the current research on interaction of capsaicin and some sensory attributes of foods.

This research indicates that there is indeed an interaction among irritants, tastes, and flavors, which have some impact on the overall flavor sensation of foods.

ACKNOWLEDGMENTS

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REFERENCES

Adams M, Nout MJR. 2001. *Fermentation and Food and Safety.* Maryland: Aspen Publishers.

Blanes P, Garro O, Giménez M, Hunzicker G. 2006. Aplicación de un diseño central compuesto para la determinación de especies orgánicas e inorgánicas de arsénico en agua por HPLC-HG-AAS. Comunicaciones Científicas y Tecnológicas. Universidad Nacional del Nordeste. Res. E051.

Breidt F, McFeeters R, Díaz I. 2007. Fermented vegetables. In: Doyle MP, Beuchat LR, editors. *Food Microbiology Fundamentals and Frontiers*, 3rd ed. Washington, D.C.: ASM Press. Chapter 36. p 783–93.

Charurin P, Ames JM, del Castillo MD. 2002. Antioxidant activity of coffee model systems. *J Agric Food Chem* 50(13):3751–6.

Colquichagua D. 1998. *Encurtidos*. Lima, Peru: Intermediate Technology Development Group.

Daeschel MA, Fleming HP, Pharr DM. 1990. Acidification of brined cherry peppers. *J Food Sci* 55(1):186–92.

Derossi A, De Pilli T, Severini C. 2010. Reduction in the pH of vegetables by vacuum impregnation: A study on pepper. *J Food Eng* 99:9–15.

Emrick ME, Penfield MP, Bacon CD, et al. 2005. Heat intensity and warmed-over flavor in precooked chicken patties formulated at 3 fat levels and 3 pepper levels. *J Food Sci* 70:S600–4.

Enachescu DM. 1995. Fruit and vegetable processing. Rome: FAO Agricultural Services Bulletin No. 119.

Fleming HP, Thompson RL, McFeeters RF. 1993. Firmness retention in pickled peppers as affected by calcium chloride, acetic acid, and pasteurization. *J Food Sci* 58(2):325–30.

Galicia-Cabrera RM. 2004. Jalapeno pepper preservation by fermentation or pickling. In: Hui YH, Ghazala S, Graham DM, Murrell KD, Nip WK, editors. *Handbook of Vegetable Preservation and Processing.* New York: Marcel Dekker. p 179–88.

Granito M, Alvarez G. 2006. Lactic acid fermentation of black beans (*Phaseolus vulgaris*): microbiological an chemical characterization. *J Sci Food Agric* 86:1164–71.

Gálvez A, Abriouel H, Lucas R, Ben N. 2007. Bacteriocin-based strategies for food biopreservation. *Int J Food Microbiol* 120:51–70.

Gu YS, Howard LR, Wagner AB. 1999. Firmness and cell wall characteristics of pasteurized jalapeño pepper rings as affected by calcium chloride and rotary processing. *J Food Sci* 64(3).

Guerra M, Jaramillo M, Dorantes L, Hernández H. 2001. Carotenoid retention in canned pickled jalapeño peppers and carrots as affected by sodium chloride, acetic acid, and pasteurization. *J Food Sci* 66(4):620–6.

Hui H, Ghazala S, Grahm D, Murrell K, Nip W. 2004. *Handbook of Vegetable Preservation and Processing.* New York: Marcel Dekker.

Infoagro. 2010. Fabricación de encurtidos primera parte. Available from http://www.infoagro.com/conservas/fabricacion_encurtidos.htm.

Kafui K. 2002. Antioxidant activity of grains. *J Agric Food Chem* 50(21):6182–7.

Karovikova J. Kohaidova Z. 2005. Lactic-acid fermentable juices—palatable and wholesome foods (review). *Chem Pap* 59(2):143–8.

Lawless H, Rozin P, Shenker J. 1985. Effects of oral capsaicin on gustatory, olfactory and irritant sensations and flavor identification in humans who regularly or rarely consume chili pepper. *Chem Sens* 10:579–89.

Lázaro López-Francos M-J. 1998. Análisis del proceso de fermentación dirigida. Aislamiento y selección de bacterias acido lácticas para su utilización como iniciadores. [DPhil thesis]. Spain: Universitat de Valencia. Available from: Full Text, ProQuest. Web. 31 January 2011.

Liu et al. 2006. Metabolic engineering of a Lactobacillus plantarum double ldh knockout strain for enhanced ethanol production. *J Ind Microbiol Biotechnol* 33:1–7.

Manresa A, Vicente I. 2007. El color en la industria de los alimentos. Ciudad de la Habana: Editorial Universitaria.

Mcbride RL, Finlay DC. 1990. Perceptual integration of tertiary taste mixtures. *Percept Psychophysics* 48: 326–30.

Nout M, Sarkar P. 1999. Lactic acid food fermentation in tropical climates. *Antonie van Leeuwenhoek* 76: 395–401.

Prescott J. 1999. Introduction to the trigeminal sense: The role of pungency in food flavours. In: Bell GA, Watson AJ, editors. *Tastes & Aromas: The Chemical Sense in Science and Industry*. Sydney: University of New South Wales Press.

Prescott J, Allen S, Stephens L. 1993. Interactions between oral chemical irritation, taste and temperature. *Chem Sens* 18:389–404.

Prescott J, Francis J. 1997. The effects of capsaicin on flavor intensity in foods: A time intensity study. *Chem Sens* 22:772.

Prescott J, Stevenson RJ. 1995. Effects of oral chemical irritation on tastes and flavors in frequent and infrequent users of chili. *Physiol Behav* 58:1117–27.

Reinbach HC. 2008. Hot spices—Effects on appetite, energy intake and sensory properties of a meal. [DPhil thesis]. Denmark: University of Copenhagen, Faculty of Life Sciences, Department of Food Science.

Reinbach HC, Meinert L, Ballabio D, et al. 2007a. Interactions between oral burn, meat flavor and texture in chili spiced pork patties evaluated by time-intensity. *Food Qual Pref* 18:909–19.

Reinbach HC, Meinert L, Ballabio D, Aaslyng MD, Bredie WLP, Olsen K, et al. 2007b. Interactions between oral burn, meat flavor and texture in chili spiced pork patties evaluated by time-intensity. *Food Qual Pref* 18:909–19.

Rivera-Espinoza Y, Gallardo-Navarro Y. 2010. Non-dairy probiotic products (review). *Food Microbiol* 27:1–11.

Ross RP, Morgan S, Hill C. 2002. Preservation and fermentation: Past, present and future. *Int J Food Microbiol* 79:3–16.

Shahidi F, Naczk M. 1995. *Food Phenolics Sources Chemistry Effects Applications*. Lancaster. Basel: Technomic Publishing. p 75–99.

Simons CT, O'Mahony M, Carstens E. 2002. Taste suppression following lingual capsaicin pre-treatment in humans. *Chem Sens* 27:353–65.

Soomro AH, Masud T, Anwaar K. 2002. Role of lactic bacteria (LAB) in food preservation and human health. A review. *Pakistan J Nutri* 1(1):20–2.

Valdez-Fragoso A, Martínez-Monteagudo SI, Salais-Fierro F, Welti-Chanes J, Mujica-Paz H. 2007. Vacuum pulse-assisted pickling whole jalapeño pepper optimization. *J Food Eng* 79:1261–68.

Valdez-Fragoso A, Sáenz-Hernández CM,Welti-Chanes J, Mújica-Paz H. 2009. Cherry pepper pickling: Mass transport and firmness parameters and stability indicators. *J Food Eng* 95:648–55.

Yoshioka M, St-Pierre S, Suzuki M, et al. 1998. Effects of red pepper added to high-fat and high-carbohydrate meals on energy metabolism and substrate utilization in Japanese women. *Br J Nutri* 80:503–10.

Zamora-Hernández E, Flores-Rodríguez L, González-González L, García-Martínez I. 2009. Estudios preliminares de la fermentación de jugo de chile jalapeño (*Capsicum annuum L.*) empleando *Lactobacillus plantarum.* Ciencia y Tecnología. *Investigación Universitaria Multidisciplinaria* 8(8):105–12.

Part V

Cereals and Cereal Products

27

Fermented Bread

Weibiao Zhou and Nantawan Therdthai

CONTENTS

27.1 Background

Bread is among the earliest "processed" foods made and consumed by mankind, and today, it is still a staple food for many parts of the world. The latest archeological discoveries showed that the first bread eaten by human ancestors was most likely a quick bread, which was made by mixing flour with water and baking the dough on hot stones. Fermented bread was first made by ancient Egyptians more than 5000 years ago (i.e., ~3000 BC). Specially designed bread-baking ovens were used as early as 2500 BC, where bread was baked on the inner surface of the ovens (Zhou and Therdthai 2006).

There is a large variety of bread, differentiated by either their ingredients or methods of baking. Fermented bread makes up the majority of bread sold in today's market, particularly in the Western countries. Most of the fermented bread belongs to the category of leavened bread in which the leavening agent is usually bakers' yeast, i.e., *Saccharomyces cerevisiae*, for most of time. Such bread may also be called yeast bread and requires one or two fermentation (or called proving) steps before baking. The fermentation increases the volume of dough considerably. Examples of yeast bread include the most common white bread, different styles of brown bread, whole grain (or called wholemeal) bread, and many types of gourmet bread (mixed grain, fruits, etc.).

Meanwhile, sourdough bread has been a part of European diets for about 5000 years. In the past, sourdough was almost always used as a leavening agent for bread production (Corsetti and Settanni 2007). Fermented bread with sourdough exists all over the world. Some variation can be observed, due to varied flours, microorganisms, ingredients, and fermentation technology. Sourdough fermentation was traditionally applied to wheat and rye baked products (Ganzle et al. 2008). In the 19th century, industrial bakeries appeared, and bakers' yeast was used as a common leavening agent, instead of sourdough. Sourdough is limited to only being used in artisan and rye bread (Corsetti and Settanni 2007). Straight dough process has been used in industrial production, as it results in less variation and is less laborious. However, sourdough bread has drawn a strong interest recently, due to its health benefits and unique flavor (Ganzle et al. 2008). Consequently, many researches have been conducted aiming to understand the scientific issues behind the sourdough fermentation to integrate it into the modern industrial bread production.

This chapter describes dough fermentative microorganisms, fermentation mechanism, and fermented bread-baking technology.

27.2 Microorganisms and Fermentation in Bread

27.2.1 Microorganisms for Bread Fermentation

27.2.1.1 Bakers' Yeast

Commercial yeast for bread making was introduced in the late 19th century. Today, bakers' yeast is most likely *Saccharomyces cerevisiae*. It is produced in aseptic systems in which the suitable strain is selected and propagated under sterile conditions. Yeast cells are grown in specific media for cultivation. Then, several transfers are carried out from an initial fermentation container to larger containers, until there is a sufficient amount of yeast mass for inoculums. The media in the inoculums include sugars, minerals, vitamins, and salts. Batch fermentation of pure culture is conducted. During major propagation steps, nutrients are gradually fed, following the so-called Zulauf process. Intensive aeration should be applied to enhance the multiplication of yeast cells. Therefore, nutrient feeding, pH regulating, aeration rate, and temperature monitoring should be carefully scheduled and controlled. The fully fermented mash, which is concentrated to 50% solid, is called "yeast cream." When the growth media is removed from the yeast cream by washing, the concentrate of the residue is called "cream yeast," which contains around 82% moisture. It can be stored at 2°C–4.5°C for 3–4 weeks. Among other forms of bakers' yeast are dry yeasts including active dry yeast, instant active dry yeast, and protected active dry yeast. Normally, dry yeast is prepared using a tunnel or conveyor belt dryer. Due to its high activity, the instant active dry yeast requires better equipment such as fluidized-bed dryer. Consequently, the instant active dry yeast has a high porosity and a rapid rehydration property. There is no requirement of preprocessing for rehydration before dough mixing. The protected active dry yeast is dried to 5%–6% moisture content. Some antioxidant agents may be added to reduce the effect of oxygen, which eliminate the need of special packaging. It is usually used for the development of complete mixes (Kulp 2003).

27.2.1.2 Lactic Acid Bacteria

Traditional sourdough contains a diversity of flora to yield unique flavor and texture of the final product, i.e., bread. Heterofermentative species of *Lactobacillus* are typical flora in sourdough ecosystems. In contrast, *Enterococcus*, *Lactococcus*, *Leuconostoc*, *Pediococcus*, *Streptococcus*, and *Weissella* are less

frequently encountered. The dominance of some microorganisms over others is dependent on fermentation temperature, acidity, type of substrate, and interaction among the microorganisms. Antagonistic interaction can be a significant factor involving competitiveness of microorganisms (De Vuyst et al. 2009). Dominant lactic acid bacteria in sourdoughs from various flours are listed in Table 27.1.

In German rye sourdough, *Lactobacillus reuteri* was dominant and stable due to the production of antibiotic reutericyclin (De Vuyst et al. 2009). In French wheat sourdough, *Lactobacillus plantarum* and *Pediococcus pentosaceus* were the main flora (76% of total isolation) (Robert et al. 2009). In durum wheat sourdough from Apulia (southern Italy), 88% of the isolates were identified as *Lactobacillus plantarum*, *Lactobacillus paracasei*, and *Lactobacillus casei* (Ricciardi et al. 2005). Isolates (81.3%) from the sourdough from Sardinia (southern Italy) comprised *Lactobacillus pentosus*, *Lactobacillus plantarum*, *Lactobacillus brevis*, *Lactobacillus sanfranciscensis*, and *Weissella confusa*.

27.2.1.3 Yeasts Found in Sourdough

The most frequently found yeasts in sourdough fermentation are *Saccharomyces cerevisiae*, *Kazachstania exigua*, and *Candida humilis*. Stability of sourdough fermentation could be dependent on interactions between yeasts and lactic acid bacteria. *Saccharomyces cerevisiae* is always present in the fermentation because it is bakers' yeast and can contaminate sourdough in a bakery (De Vuyst et al. 2009). However, Cabellero et al. (1995) reported superior proofing activity of *Kluyveromyces marxianus* in the presence of lactose and whey protein in dough, compared with *Saccharomyces cerevisiae*. *Kazachstania exigua* and *Candida humilis* are characterized as maltose-negative and acid-tolerant. They should work well with maltose-positive lactic acid bacteria such as *Lactobacillus sanfranciscensis*. *Lactobacillus sanfranciscensis* can hydrolyze maltose by an intracellular maltose phosphorylase. As a result, unphosphorylated glucose plus glucose-1-phosphate is produced, without adenosine triphosphate (ATP) utilization. To avoid an intracellular accumulation, the unphosphorylated glucose is excreted outside the cells and used by the maltose-negative yeasts. Otherwise, the excreted unphosphorylated glucose can induce glucose repression in maltose-positive yeasts (De Vuyst et al. 2009).

With the presence of lactic acid bacteria during dough fermentation, the maximum growth rate of yeast is reduced, but with no effect on the final cell counts. The consequent ethanol production is decreased. However, yeast fermentation favors the production of mannitol and acetic acid by the lactic acid bacteria (Paramithiotis et al. 2006). Stability of the population dynamics of lactic acid bacteria can be observed within a few days. This implies efficiency of carbohydrate and peptide or amino acid utilization, efficient

TABLE 27.1

Lactic Acid Bacteria Found in Sourdoughs

Flour Base	Nation	Lactic Acid Bacteria	References
Rye	Germany	*Lactobacillus reuteri*	De Vuyst et al. (2009)
Wheat	France	*Lactobacillus plantarum, Lactobacillus curvatus, Lactobacillus paracasei, Lactobacillus sanfranciscensis, Lactobacillus pentosus, Lactobacillus paraplantarum, Lactobacillus sakei, Lactobacillus brevis, Pediococcus pentosaceus, Leuconostoc mesenteroides, Leuconostoc citreum, Weissella cibaria, Weissella confusa, Lactococcus lactis,* and *Enterococcus hurae*	Robert et al. (2009)
Durum wheat flour	Italy (Apulia)	*Lactobacillus plantarum, Lactobacillus paracasei, Lactobacillus casei, Lactobacillus brevis,* and *Leuconostoc mesenteroides*	Ricciardi et al. (2005)
	Italy (Sardinia)	*Lactobacillus pentosus, Lactobacillus plantarum, Lactobacillus brevis, Lactobacillus sanfranciscensis, Weissella confusa, Lactobacillus casei, Lactobacillus zeae, Lactobacillus sakei, Lactobacillus alimentarius, Lactobacillus farciminis, Pediococcus pentosaceus,* and *Leuconostoc citreum*	Catzeddu et al. (2006)
Amaranth flour	–	*Pediococcus pentosaceus, Lactobacillus sakei,* and *Lactobacillus plantarum*	Sterr et al. (2009)

regulation of redox balance, and tolerance toward acidification. Stability of the final population can be determined by the rate and intensity of acidification (De Vuyst et al. 2009).

27.2.2 Selection of Microbial Growth Phase in Sourdough for Fermented Bread

In general, microbial growth can be divided into six phases including lag, acceleration, exponential growth, transition, stationary, and death phases. The lag phase is the period when microorganisms adjust to a new environment before growth. When a dried form of microorganisms is used, the lag phase can be extended due to rehydration required. After the microorganisms are adjusted to the new environment, they start to multiply, until the highest multiplication rate is reached. This period is called the acceleration phase. In the exponential growth phase, the organisms are doubling their numbers by binary fission. The doubling time for the microorganisms of sourdough can be in the hour range. After substantially growing, nutrients are exhausted and waste is accumulated and can be toxic. Therefore, their growth rate is reduced in the transition phase. During the stationary phase, the number of microorganisms is static due to an equal number of growth and death. When the number of new growth is nearly zero and the existing microorganisms keep dying, the life cycle enters the death phase.

For sourdough making, microorganisms in their transition and stationary phases should be selected, due to their high number. These phases can ensure a high degree of acidification and flavor development. Obviously, death phase is not recommended. Exponential growth phase may be used for replacing bakers' yeast due to the fast production of carbon dioxide gas during this phase. However, environmental parameters including temperature, time, and water content have to be tightly controlled. For example, after a 4-hour incubation at 25°C, a decrease in pH was much less than an incubation of the same duration at 37°C (Gaggiano et al. 2007).

27.2.3 Mechanisms during Fermentation in Bread

Metabolic trails include lactic acid fermentation, proteolysis, and synthesis of volatile and antimold compounds (Robert et al. 2009). The actual mechanisms depend on the types and quality of flour, fermentation condition, and microorganisms and their nutritional and trophic relationships (De Vuyst et al. 2009).

27.2.3.1 Lactic Acid Fermentation

In homolactic fermentation, lactic acid bacteria produce only lactic acid through glycolysis, starting from glucose. As shown in Figure 27.1, glucose is converted into pyruvate through 10 reaction steps, resulting in 2 ATP.

In heterolactic fermentation, lactic acid bacteria produce carbon dioxide gas, acetic acid, ethanol through the 6-phosphogluconate/phosphoketolase (6-PG/PK) pathway (Figure 27.2). The key enzymes involved are glucose-6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase. The pathway yields 1 ATP and 2 ATP when glucose and pentose are used as substrate, respectively. The ratio of acetic acid to ethanol depends on the redox potential of the fermentation system.

Besides entering the pathway as glucose-6-phosphate or fructose-6-phosphate, hexose can also be decarboxylated to a pentose. Pentose is then phosphorylated and converted to ribulose-5-phosphate or xylulose-5-phosphate, using epimerases or isomerases. After that, it enters the pathway through the lower half of 6-PG/PK pathway. Pentose phosphate is then cleaved to 2 compounds: glyceraldehyde-3-phosphate (GAP) and acetyl phosphate. GAP is then converted to lactic acid with 2 ATP produced by phosphorylation of the substrate. Acetyl phosphate is reduced to ethanol without producing ATP.

Lactic acid bacteria involved in sourdough fermentation can be classified into three groups including obligately homofermentative (such as *L. amylovorus*, *L. acidophilus*, *L. farciminis*, *L. mindensis, L. crispatus*, *L. johnsonii*, and *L. amylolyticus*), obligately heterofermentative (such as *L. acidifarinae*, *L. brevis*, *L. fermentum*, *L. reuteri, L. rossiae*, *L. frumenti*, and *L. zymae*), and facultatively heterofermentive (such as *L. plantarum*, *L. pentosus*, *L. casei*, *L. paralimentarius*, and *L. alimentarius*). Facultatively heterofermentive lactic acid bacteria are used for hexose fermentation through glycolysis because they contain a constitutive fructose-1,6-diphosphate aldolase, which is a key enzyme for glycolysis. To

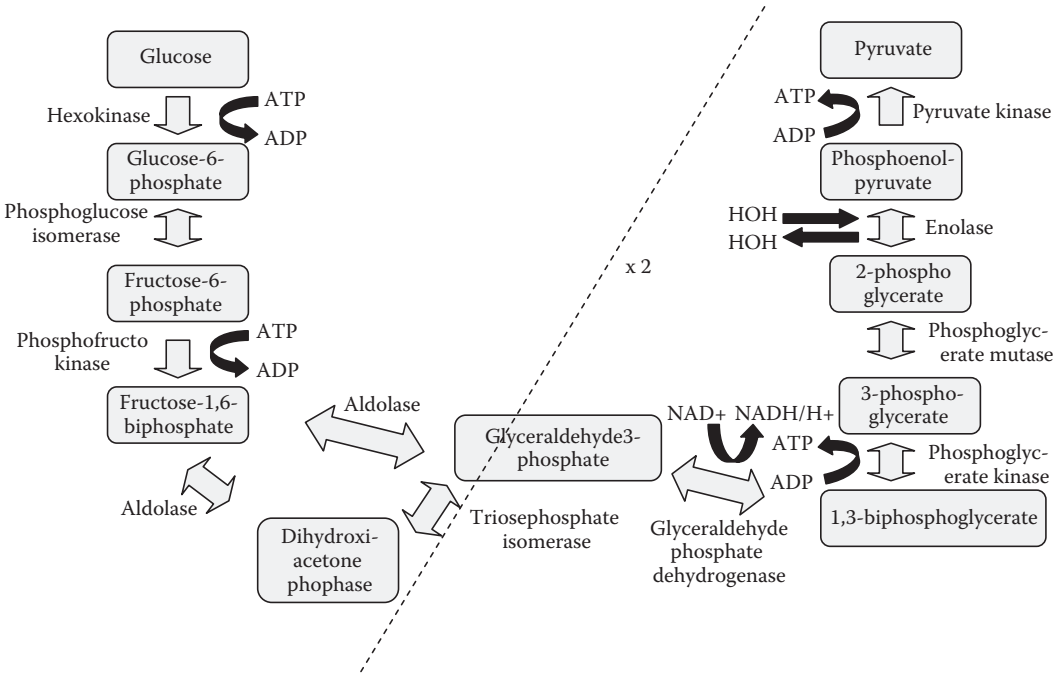

FIGURE 27.1 Conversion of glucose to pyruvate via glycolysis. (Modified from Kulp, K., Chapter 5: Baker's yeast and sourdough technologies in the production of U.S. bread products, in: Kulp K., Lorenz K., editors, *Handbook of dough fermentations*, pp. 97–143, Marcel Dekker, New York, 2003.)

ferment pentose, the obligately heterofermentive lactic acid bacteria are used, as it contains a constitutive phosphoketolase, which is a key enzyme in the 6-PG/PK pathway. However, the available pentose sugar induces phosphoketolase for facultatively heterofermentive lactic acid bacteria. Therefore, facultatively heterofermentive lactic acid bacteria ferment pentose by the same way as the obligately heterofermentive lactic acid bacteria. However, the obligately homofermentive lactic acid bacteria do not ferment pentose. With pentose fermentation, lactic acid and acetic acid are equally produced without formation of carbon dioxide gas. Because there are no dehydrogenation steps, acetyl phosphate is used by acetate kinase. As a result, acetate and ATP are produced (Corsetti and Settanni 2007).

27.2.3.2 Proteolysis in Sourdough during Fermentation

In wheat grains, the major storage protein is gluten, and consists of glutenins and gliadins. Gliadins are monomeric alcohol-soluble proteins responsible for the viscous property of dough. Glutenins, which are soluble in dilute acid, are classified into two types: high-molecular-weight (HMW) glutenins and low-molecular-weight (LMW) glutenins. The HMW ones are responsible for dough strength and elasticity, while the LMW ones are responsible for dough viscous properties. When proteins are degraded, viscoelastic properties of dough could be modified. In addition, the subunits of glutenins can form together and become glutenin macropolymers. The amount of the glutenin macropolymers affects the bread loaf volume. Proteolysis in dough involves proteolytic enzymes (protease) including the proteinase group (catalyzing protein degradation to yield small fraction of peptides) and peptidase group (hydrolyzing and breaking down specific peptide bonds to yield amino acids). Commonly, proteolytic enzymes in rye and wheat dough are aspartic proteinase and carboxypeptidase. Both enzymes are active in the acidic environment that results from lactic acid bacteria fermentation. However, the activity of lactic acid bacteria on proteolysis is likely attributed to peptide hydrolysis, not the degradation of HMW proteins (Ganzle et al. 2008).

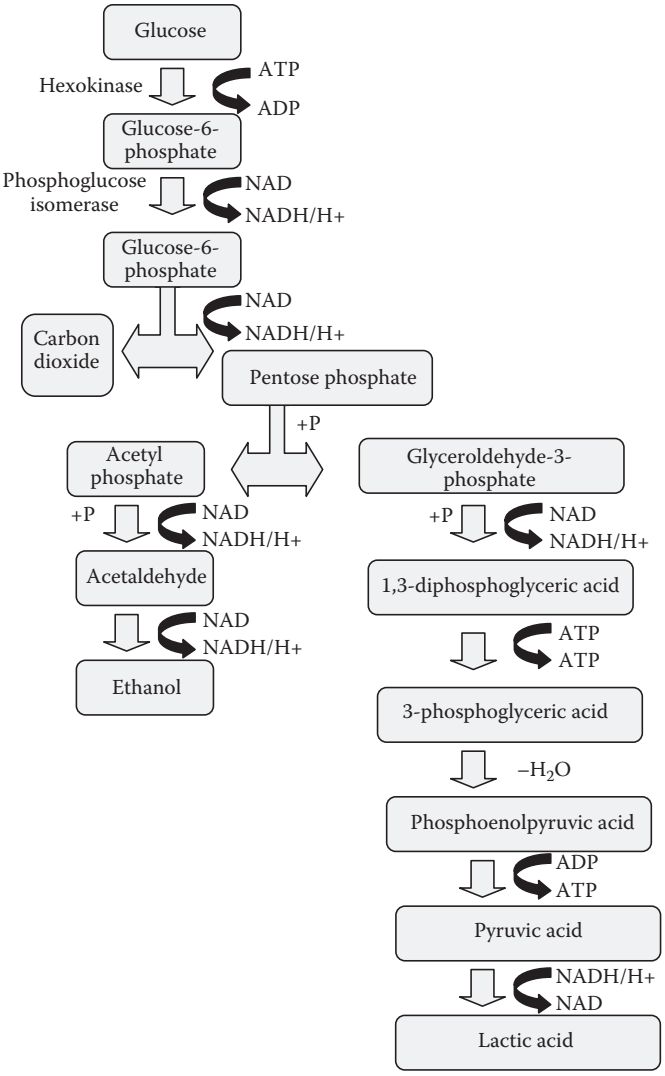

FIGURE 27.2 6-Phosphogluconate/phosphoketolase pathway. (Modified from Kulp, K., Chapter 5: Baker's yeast and sourdough technologies in the production of U.S. bread products, in: Kulp K., Lorenz K., editors, *Handbook of dough fermentations*, pp. 97–143, Marcel Dekker, New York, 2003.)

The primary proteolysis that changes proteins to peptides is mainly the responsibility of endogenous cereal proteases. Later, the proteolysis that provides liberation of amino acids is enhanced by acidic conditions. The lactic acid bacteria fermentation can bring the acidity to an optimum pH (3.5–4.0) for the activity of aspartic proteinase. *Lactobacillus pontis* was reported as a lactic acid bacterium good for proteolysis. Not only does it provide acidification, but it also reduces the disulfide bonds in gluten proteins and increases the solubility of proteins and the accessibility of enzymes. As yeasts required amino acids for their growth, the amino acids were increased after the yeast growth was terminated (Ganzle et al. 2008).

Amino acids produced by proteolysis can be a precursor of or converted to flavor molecules during baking. However, the extent of proteolysis has to be limited due to its effect on dough viscoelastic properties. It has to produce enough precursors of flavor molecules but not too much gluten degradation. Actually, proteolysis in wheat sourdough is limited by enzymatic reaction and by the solubility of glutens. In some products such as soda crackers, a large extent of gluten degradation might be required. To

increase proteolysis, a combination of chemical acidification, protease addition, and reducing agent addition is recommended. The reducing agent can reduce intermolecular and intramolecular disulfide bonds. As a result, gluten proteins can be soluble and easily accessed by proteolytic enzymes. Degradation of gluten proteins is also of current interest as a processing tool to produce gluten-free products (Ganzle et al. 2008).

27.2.3.3 Synthesis of Volatile Compounds

Flavor of bread can be developed during both fermentation and baking. Normally, fermentation is responsible for crumb flavor, while baking is responsible for crust flavor. Major compounds strongly influencing bread flavor include organic acids, alcohols, esters, and carbonyls. The flavor developed during fermentation depends on types of microorganisms, material substrates, and fermentation condition. Homofermentative lactic acid bacteria produce lactic acid through glycolysis. Heterofermentative lactic acid bacteria produce lactic acid, carbon dioxide gas, acetic acid, and/or ethanol. Fermentation quotient (i.e., the ratio of lactic acid and acetic acid) can be an important factor affecting aromatic profiles. Flavor compounds such as ethyl acetate and hexyl acetate from the fermentation of heterofermentative lactic acid bacteria seem to be higher than those from homofermentative lactic acid bacteria. In contrast, a higher amount of flavor compounds in aldehyde groups is obtained from homofermentative lactic acid bacteria. Moreover, fermentation by a combination of yeast and lactic acid bacteria produces more aromatic compounds than that by lactic acid bacteria or yeast alone. Actually, to obtain enough volatile compounds, fermentation by lactic acid bacteria has to be reasonably long (12–24 hours). However, in the presence of yeast fermentation, the flavor generation process can be completed within a few hours. As described above, proteins can be degraded through proteolysis. The obtained amino acids can be converted to flavor compounds. The key reactions for amino acid degradation to produce aldehydes and alcohols are those via the Ehrlich pathway (Corsetti and Settanni 2007).

27.2.3.4 Synthesis of Antifungal Compounds

In bakery goods, fungal growth is one of the frequent causes of spoilage. Spoiling fungi are commonly from the genera of *Aspergillus*, *Cladosporium*, *Endomyces*, *Fusarium*, *Monilia*, *Mucor*, *Penicillium*, and *Rhizopus*. Sourdough addition into bread is a natural method to delay the fungal spoilage. Antifungal activity in sourdough bread is attributed to lactic acid bacteria. Its fungistatic effect is mainly from the production of acetic acid rather than lactic acid. Therefore, heterofermentative lactic acid bacteria are required. *Lactobacillus sanfranciscensis* can produce a mixture of acid varieties including acetic, caproic, formic, propionic, butyric, and *n*-valeric acids. Thus, it is claimed to have the widest range of antifungal activity among the heterofermentative lactic acid bacteria. *Lactobacillus plantarum*, one of the most widely used strains in sourdough, produces phenyllactic and 4-hydroxy-phenyl-lactic acids whose fungicidal activity can remain in bread after baking. Phenyllactic acid was found to delay the growth of *Aspergillus niger* and *Penicillium roqueforti* for up to 7 days (Corsetti and Settanni 2007).

27.2.4 Role of Fermentation in Bread Quality

27.2.4.1 Development of Flavor

As described earlier, volatile compounds in bread are mainly developed from microbial activities during fermentation and from Maillard reactions during baking. From fermentation, volatile compounds extracted from bread include ethyl acetate, ethanol, 2,3-butanedione, 1-propanal, 2-methyl-1-propanol, 2-methyl-1-butanol, 3-methyl-1-butanol, 3-hydroxy-2-butanone, acetic acid, propionic acid, benzaldehyde, isobutyric acid, butyric acid, isovaleric acid, hexanoic acid, and phenylethyl alcohol (Poinot et al. 2008).

Volatile compounds obtained from fermentation of wheat germ by *Lactobacillus plantarum* LB1, *Lactobacillus rossiae* LB5 were mainly alkanes and alkens (Rizzello et al. 2010). Although the concentration of volatile compounds is very low, they play an important role in sensorial quality especially

freshness. This is due to their low threshold values. The developed volatile compounds could be reduced dramatically during storage. Using sourdough could better maintain flavor of bread, compared with only using yeast for leavening. The sourdough bread with the addition of kefir (which has a naturally mixed flora including lactic acid bacteria, yeasts, and others) presented a complex profile of volatile compounds and contained a higher concentration of total headspace volatiles than the one without kefir (Plessas et al. 2011). When only yeast fermentation is used, addition of some sugars could improve the intensity of flavor compounds. Torner et al. (1992) observed a large amount of flavor compounds from yeast fermentation in dough with an addition of either glucose or sucrose but not maltose.

During baking, Maillard reactions are enhanced and yield volatile compounds including 2-methylpropanal, 2-methylfuran, 2-butanone, 2-methylbutanal, 3-methylbutanal, 2,3-pentanedione, 1 methylpyrrole, 2-pentylfuran, pyrazine, dihydro-2-methyl-3(2*H*)-furanone, 2-methylpyrazine, 2-ethylpyrazine, 2,5-dimethylpyrazine, 2,3-dimethylpyrazine, 2-ethyl-6-methylpyrazine, 2-ethyl-5-methylpyrazine, 2-ethyl-3-methylpyrazine, 3-furfural, pyrrole, 2-acetylfuran, 5-methyl-2-furfural, furfuryl alcohols, maltol, etc. The degree of baking and baking process conditions can affect the quantity of those volatile compounds. Generally, those compounds are responsible for giving roasty, malty, and sweet notes. Some studies also found that a decrease in yeast content could affect the quantity of those compounds, due to the fact that yeast contributes to the formation of Maillard reaction precursors (Poinot et al. 2008).

In addition to fermentation and Maillard reactions, lipid oxidation produces volatile compounds in bread such as hexanal, 1-hexanol, 1-pentanol, 1-octen-3-ol, benzyldehyde, and hexanoic acid. The activity of lipoxygenase (which is responsible for converting fatty acids into aldehydes by using oxygen) can be affected by yeast activity. When yeast content is reduced, there is an increase in the amount of oxygen available for lipid oxidation (Poinot et al. 2008).

27.2.4.2 Development of Structure and Texture

During fermentation, pores can be formed in the dough and is described by the Boltzmann sigmoidal function (Equation 27.1). An analysis of the number of pores and the volume of dough can be conducted using the magnetic resonance microscopy technique. With yeast fermentation, the number of pores in dough is dramatically increased. However, adding some edible seeds could reduce the number of pores and dough volume (Bajd and Sersa 2011).

$$YF(t) = \frac{A}{1 + \exp(-(t - t_0)/\Delta)} + B \tag{27.1}$$

where A is the amplitude, B is the offset, t_0 is time lag, and Δ is transition interval.

In addition to yeast, lactic acid fermentation also impacts on viscoelastic properties of dough and thereby bread, due to protein degradation and development of exopolysaccharides. In gluten-free bread, hydrocolloids have been added to improve texture of the bread. Recently, exopolysaccharides obtained from lactic acid fermentation have been proposed as an alternative ingredient. Lactic acid fermentation generally yields exopolysaccharides including homopolysaccharides (containing one type of monosaccharide, either glucose or fructose) and heteropolysaccharides (containing a combination of monosaccharides such as glucose, galactose, and rhamnose) (Bounaix et al. 2010). To synthesize homopolysaccharides by extracellular glucans and fructosyltransferase (glycosyltransferase), sucrose could be added as a glycosyl donor (Corsetti and Settanni 2007). Then not only homopolysaccharides were formed, but acid formation was also observed. An increase in acids could be beneficial to bread due to their antifungal property. Nonetheless, it may negatively affect the taste and volume of the bread. Heteropolysaccharides were intracellularly synthesized from sugar nucleotides by glycosyltransferase. Without a sucrose addition, acid formation was reduced. Therefore, lactic acid bacteria that formed heteropolysaccharides without sugar addition and reduced acetate formation became suitable for sourdough. Fermentation of wheat and sorghum flours with *Lactobacillus buchneri* FUA3154 could produce high molecular mass heteropolysaccharides with less than 10 mM acetate production. In the sorghum sourdough, the heteropolysaccharides produced by the fermentation caused the lowest resistance to deformation and elasticity of the

sourdough. The low resistance to deformation possibly increased the specific volume of the bread and decreased the hardness of its crumb. However, in wheat sourdough, the heteropolysaccharides produced by the fermentation did not show any significant effect on the viscoelastic properties of the dough and the bread. Therefore, the effect of heteropolysaccharides on the viscoelastic properties of sourdough bread may depend on the strength of gluten network (Galle et al. 2011).

27.2.4.3 Shelf Life Extension

Bread is normally spoiled by changes in physicochemical properties and thereby in its flavor and texture, which is called staling. Mold spoilage could also occur. By using heterofermentative lactic acid bacteria, the acidity of bread can be increased. This helps to inhibit or slow down mold spoilage. Better moisture retention was also observed in sourdough bread; as a result, starch retrogradation was slowed down and softness of bread crumb was maintained. Plessas et al. (2011) monitored changes of volatile compounds in sourdough bread during a 5-day storage. The total concentration of volatile compounds was used as an indicator for bread freshness. Normally, it was reduced dramatically during the first 3 days of storage. However, in sourdough bread containing kefir, it was decreased at a slower rate than that in regular sourdough bread. As a result, freshness of the bread was maintained for a longer period, compared with bread without kefir.

27.2.4.4 Nutritional Improvement

Sourdough fermentation mainly involves lactic acid bacteria and yeast. Lactic acid bacteria fermentation yields lactic acid and acetic acid that can decrease the pH value of the dough to below 5, while yeast fermentation yields carbon dioxide gas and ethanol. The obtained substances change the condition during fermentation and subsequently enzyme activity. Changes in pH can selectively enhance the performance of certain enzymes such as proteases, amylases, hemicellulases, and phytases. Activity of enzymes and microbial metabolites affect the nutritional quality of bread including proteins, starches, lipids, dietary fibers, vitamins, minerals, sterols, and phenolics via different mechanisms (Poutanen et al. 2009).

Proteins: During fermentation, proteolysis causes degradation of proteins and peptides and yields amino acids. In traditional rye sourdough, rye proteins (especially secalins) can be hydrolyzed by endogenous rye proteases, particularly aspartic proteases. As a result, amino acids and bioactive peptides are generated and act as precursors of flavor (Poutanen et al. 2009). Fermentation of wheat germ by *Lactobacillus plantarum* LB1 and *Lactobacillus rossiae* LB5 could increase the concentration of free amino acids by 50%, particularly Leu, Lys, Phe, Val, His, Ala, and Met. In vitro protein digestibility was also significantly increased ($p < 0.05$) (Rizzello et al. 2010). Proteolysis by lactic acid bacteria has been recommended as an alternate process to produce bakery goods for celiac persons (Poutanen et al. 2009).

Starches and dietary fibers: Common bakery goods contain a large amount of rapidly digestible carbohydrates that can generate glucose quickly and produce a high glycemic response. The type of starch used in dough is a crucial factor for glucose response. Amylose-rich starch has a higher resistance to amylolysis than amylopectin-rich starch. In addition, amylolysis can be increased quickly during baking, due to starch gelatinization. The higher the degree of starch gelatinization, the more rapidly it can be digested. Therefore, the glycemic response of bread is increased by starch gelatinization. However, acidification from sourdough fermentation can reduce pH, which retards starch digestibility; as a result, its glycemic index is reduced (Poutanen et al. 2009). Furthermore, soluble carbohydrates (i.e., glucose, fructose, sucrose, maltose, and raffinose) were decreased, particularly raffinose (Rizzello et al. 2010). The main effect on starch digestibility is from lactic acid bacteria fermentation, not yeast. Lactic acid resulting from the lactic acid bacteria fermentation reduces the pH (4.1–4.5) and slows down digestion rate. Acetic acid and propionic acid prolong gastric emptying rate. In addition to acidification from fermentation, tissue integrity of grain kernels, product porosity, and structure of starch influence the glycemic index of bakery products. Rye bread, which has a more rigid and less porous structure, has a lower glycemic index than wheat flour bread (Poutanen et al. 2009).

Some of nonstarch polysaccarides are dietary fibers, such as arabinoxylan. During fermentation, solubilization of dietary fibers can be observed. This is due to the contribution of endogenous enzymes, particularly xylanases (Poutanen et al. 2009).

Vitamins: Cereals are known as a good source of vitamins such as folate, thiamine, and vitamin E. The baking process can reduce the amount of folate. However, some folate can be obtained from yeast. Actually, 45%–70% of the folate in bread is originally from yeast (Patring et al. 2009). Folate content in dough with yeast fermentation can be increased threefold in the best case. The increasing trend of folate has been confirmed in both wheat and rye dough. Compared to yeast fermentation, lactic acid bacteria fermentation has a minimal impact on folate content. For thiamine, its content is increased during yeast fermentation especially with a long fermentation time but decreased during baking. The long yeast fermentation also increases riboflavin by 30%. However, in sourdough fermentation, there is no synergistic effect from yeast and lactic acid bacteria on the contents of B group vitamins. Some losses of vitamin E are observed during sourdough fermentation, possibly due to its sensitivity to oxygen in the air (Poutanen et al. 2009).

Minerals: With the presence of phytate (myo-inositol hexaphosphate), bioavailability of minerals may be limited. In grain, phytic acid, concentrated on aleurone layers, has a strong chelating capacity. When forming with dietary cations, it impairs mineral absorption in human. Phytases can dephosphorylate phytate and form free inorganic phosphate and inositol phosphate esters. Both substances have less influence on mineral solubility and bioavailability. The phytase in yeast has an optimum activity at pH 3.5. Wheat phytase has an optimum activity at pH 5.0 (Poutanen et al. 2009). Flour phytase itself is insufficient to significantly reduce the amount of phytate. However, together with sourdough fermentation, reduction of phytate is improved (Corsetti and Settanni 2007). By lowering the pH to 5.5 through sourdough fermentation, the amount of phytate could be decreased by 70% using the phytase in the flour. Degradation of phytate by enzymatic activity can be varied, depending on the amount of phytase present, flour particle size, acidity, temperature, time, and water content (Poutanen et al. 2009).

27.2.4.5 Development of Prebiotic Effect

Wheat and rye flours contain fructo-oligosaccharides of inulin that can be prebiotic (Corsetti and Settanni 2007). Moreover, exopolysaccharides obtained from lactic acid bacteria fermentation (such as glucans, fructans, gluco-, and fructo-oligosaccharides) are suggested as a gut health promoter (Poutanen et al. 2009). *Lactobacillus sanfranciscensis* is suggested to possess health-promoting activity. Fructans produced from *Lactobacillus sanfranciscensis* can act as a prebiotic, due to its ability to stimulate bifidobacterial growth. The biogenic substance produced from certain lactic acid bacteria is recognized as safe, resistant to high acidity, and resistant to high temperature during baking. Therefore, fructans are not degraded during fermentation and are still available after baking (Corsetti and Settanni 2007).

27.3 Technology in Fermented Bread Manufacture

Fermented bread can be manufactured using either natural fermentation or starter fermentation. Generally, indigenous flora in natural sourdough fermentation contains a mixture of yeasts, mainly *Saccharomyces cerevisiae* and lactic acid bacteria, mainly the genera of *Lactobacillus, Leuconostoc*, *Pediococcus*, and *Weissella* (Robert et al. 2009). However, with natural fermentation, it is not easy to control the consistency of fermented bread characteristics, depending on available microorganisms in the system.

In industrial baking, fermentation with a starter culture is preferred, due to it being less laborious, having less or rare variation of microbial composition and having more standardized quality of baked goods (Minervini et al. 2010). A starter culture has high viable microorganism counts, which may be added to a substrate to produce desired characteristics. It enables a strict control of a fermentation process; as a result, the outcome is predictable. The first pure starter culture is *Lactococcus lactis* applied to industrial milk fermentation (Holzapfel 1997). Currently, a wide range of starter cultures has been developed for being used in industrial processes. Commercially available starter cultures of lactic acid bacteria for bread making are *Lactobacillus plantarum*, *Lactobacillus casei*, *Lactobacillus brevis*, *Lactobacillus fructivorans*, and *Lactobacillus fermentum*. Different mixtures of those starter strains yield variations of sourdough acidity, pH, and flavor. To make gluten-free sourdough from amaranth flour, *Lactobacillus plantarum* RTa12 and *Pediococcus pentosaceus* RTa11 were recommended as starters, due to the resulted fast acidification and stable fermentation under various temperatures (Sterr et al. 2009). Besides the type

of microorganisms, available nutrients are also important to the results of fermentation. Supplementing flour with some enzymes may generate fermentable substrates. For example, adding α-amylase can help break down starch to maltose for yeast to grow in the dough. Sometimes, α-amylase is used together with proteases, which can hydrolyze gluten proteins. The enzymes can be from various sources and have different optimum temperatures for their activity (Sahlstrom and Brathen 1996).

27.3.1 Straight Dough and No-Time Dough Processes

Typically, straight dough and no-time dough processes are used for bread making in countries that mainly produce English-type bread. In the straight dough process, dough is prepared by incorporating all ingredients in a single stage. Then bulk fermentation is conducted for around 3–4 hours. In the no-time dough process, an even shorter fermentation of 15–40 minutes is applied. The processes are not time-consuming and are economical. However, the quality of bread may be reduced. Some enriching agents such as malt and lard could be added to improve its quality. Moreover, in the straight dough process, additional operation known as knocking-back (or "cutting-back" or "punching down") can be integrated into the original process. The knocking-back is an operation for partially degassing dough, in order to control dough volume and possibly redistribute yeast and substrates. Therefore, the rate of gas production during fermentation can be intensified. Normally, the knocking-back process should be conducted twice during the 3- to 4-hour fermentation (at 1 and 2.5 hours). Together with the knock-back operation, delayed salt method and delayed fat method can be used to improve dough quality. In the delayed salt method, all ingredients except salt are mixed together. After fermentation is carried out for two thirds of the total fermentation time, the knock-back is implemented and salt is added. Because salt inhibits yeast fermentation and slows down gluten development, the absence of salt at the beginning allows a rapid dough development. Similarly, in the delayed fat method, fat is added at the time of the knock-back operation. The obtained dough can be more tolerant to mechanical abuses. Therefore, it is suitable for automated production processes (Fowler and Priestley 1980).

In the straight dough/no-time dough processes, some reducing agents such as L-cysteine are used for dough development, which relies less on fermentation. To make white bread, straight dough contains 64% water, 2.5% compressed bakers' yeast, and other ingredients. Fermentation time at 30°C–35°C with 85% relative humidity is 2 hours. To use the no-time dough process, yeast should be increased to 3.5%. Additional ingredients including 40 ppm L-cysteine, 60 ppm ascorbic acid, 0.25% monocalcium phosphate, and 0.75% monoglyceride and diglyceride should be mixed with the main ingredients. This saves around 2 hours of fermentation time in the bread-making process (Kulp 2003).

27.3.2 Sponge and Dough Process

The sponge and dough process is widely used in bread making in the United States. The process is divided into two stages. In the first stage, flour (around 50%–80% of total), yeast and other ingredients are mixed with water, to obtain homogeneity of all ingredients. Fairly stiff dough called "sponge" was eventually obtained from this stage. Fermentation is then taken to proceed for about 3–5 hours. During the fermentation, a web-like structure is developed in the sponge due to gas retention. Dough growth is continued, until the volume is 4–5 times greater than the beginning. After 60%–70% of total fermentation time, dough may be collapsed. In the second stage, the sponge is mixed with the rest of ingredients using high-speed mixing. Dough is further fermented for another 15–30 minutes to complete the expansion (Fowler and Priestley 1980). Kulp (2003) reported a procedure for the sponge dough process, in which 70% flour was first mixed with yeast and other ingredients for 4 minutes and fermented at 30°C for 3–5 hours. In the second stage, dough was mixed for 11–13 minutes and rested for 15–20 minutes.

In the UK system, only one third of total flour is mixed to make sponge. Fermentation time is much longer (about 12 hours). A modification to the UK system is to add a greater quantity of yeast. Fermentation during the sponge stage is then accelerated. The modification is called "flying sponges." Half-sponge is another modification, and this is done by adjusting the amount of flour to make dough stiffer. Although the sponge dough process is time-consuming, the process is preferred particularly when hard flour is used. In contrast, the straight dough process is suitable to weak flour (Fowler and Priestley 1980).

27.3.3 Sourdough Fermentation

As described earlier, sourdough is a mixture of flour (most likely wheat flour or rye flour) and water, and the mixture is fermented with a combination of lactic acid bacteria and yeast. Flour extraction rate in the range of 0.54%–1.68% ash content affected the acidification. A decrease in the flour extraction rate may cause a reduced generation of acids. Acidification was slightly affected by fermentation temperature (25°C–35°C) (Martinez-Anaya et al. 1994). Basically, there are four types of sourdough fermentation, classified according to their preparation procedure and the metabolic activities of the main lactic acid bacteria.

27.3.3.1 Type 0 Sourdough

Type 0 sourdough may be described as the most traditional method to make fermented bread. It is made of a mixture of flour and water that is left alone for some time, until the mixture gets sour and starts to have bubbles. The microorganisms responsible for the type 0 sourdough are mostly homofermentative lactic acid bacteria.

27.3.3.2 Type I Sourdough

Type I sourdough is characterized by low incubation temperature and continuous back-slopping using the mother sponge taken from the previous fermentation. Done this way, the metabolic activity of microorganisms is kept at a high rate. In industrial bakeries, the frequency of back-slopping may be reduced. It can be weekly or monthly. In this case, starters should be used (Minervini et al. 2010). Microorganisms in this type are mainly *Lactobacillus brevis*, *Lactobacillus plantarum*, *Lactobacillus paralimentarius*, *Lactobacillus rossiae*, and *Lactobacillus sanfranciscensis* (De Vuyst et al. 2009).

27.3.3.3 Type II Sourdough

Type II sourdough is characterized by a high incubation temperature (higher than 30°C), long fermentation time (up to 5 days), and high water content. It is widely used as an acidifying agent and an aroma carrier (Minervini et al. 2010). Microorganisms in this type should be tolerant to heat and acid, such as *Lactobacillus amylovorus*, *Lactobacillus fermentum*, *Lactobacillus pontis*, and *Lactobacillus reuteri*. *Lactobacillus reuteri* is dominant and competitive in type II sourdough, due to its carbohydrate metabolism and antimicrobial potential. *Lactobacillus amylovorus* is also competitive in type II sourdough, due to its production of bacteriocin (De Vuyst et al. 2009). Unlike type I sourdough, type II requires addition of *Saccharomyces cerevisiae* as a leavening agent (Corsetti and Settanni 2007).

27.3.3.4 Type III Sourdough

Type III sourdough is used for production of dried sourdough as flavoring agents (Minervini et al. 2010). Therefore, it requires lactic acid bacteria that are resistant to a drying process. In addition, it requires addition of *Saccharomyces cerevisiae* as a leavening agent (Corsetti and Settanni 2007). To make bread, dried sourdough is mixed with other dry ingredients before dough making. Therefore, dried sourdough can be used as a starter in premixes, which ensured uniformity and consistency of bread. After it is mixed with flour (recommended ratio of dried sourdough to flour is 1:9) and water, the resulted dough has to be maintained at a suitable temperature (approximately 40°C) for revitalization of the selective microorganisms. This period is called reactivation phase. Fermentation condition during the reactivation phase may also influence the reactivation of the selective microorganisms. After being revitalized, selective microorganisms become active and start producing sourdough characteristics. Then the temperature should be reduced to 30°C, the optimal fermentation temperature of lactic acid bacteria. However, some other microorganisms can also be revitalized from spontaneous fermentation. They can spoil the selective microorganisms. Some problems of using dried sourdough are noted, e.g., the number of revitalized microorganisms may be too small or the reactivation time in dough may be too long. The number of

microorganisms that can be revitalized depends on the drying technology and conditions used in preparing the sourdough. During drying, the microorganisms in an active state are transformed to a latent state so that the microorganisms can resume proliferation and metabolic activity later. Hot air drying and freeze drying with optimum drying conditions can yield the microorganisms with a reasonable ability to revitalize. Nonetheless, an increase in storage time of dried sourdough may decrease the number of revitalized microorganisms and thereby affect the sourdough bread (Meuser et al. 1995).

27.3.4 Cell Immobilization

Cell immobilization is a biotechnology that increases fermentation productivity through the alteration in microbial physiology and metabolic activity. The immobilized cell can develop a high flavor intensity and become more tolerant to stress. Applications of immobilized cells are widely reported in alcohol fermentation for alcoholic beverages and lactic acid fermentation for liquid products. In comparison, there are very few reports on their applications in bread and solid-state fermentation. For obtaining a biocatalyst activity for alcoholic and lactic acid fermentation, a starch-gluten-milk matrix provides a good support to cell immobilization of yeast and lactic acid bacteria. Orange peel is also a good support to bakers' yeast immobilization. Using bakers' yeast immobilized in a starch-gluten-milk matrix (containing lactic acid bacteria) and kefir (containing natural coculture of yeast and lactic acid bacteria) immobilized on orange peel, the shelf life of bread (normally 3–4 days) was extended to 7–8 and 5–6 days, respectively, due to the delayed staling (Merchant and Banat 2007). Aside from that, pH was reduced and the rate moisture loss slowed down, compared with traditional bakers' yeast. Applying immobilized cells in sourdough bread shows a better improvement than that in straight dough bread. Bread aromatic volatile compounds, especially esters, are increased. Comparing between the two cell immobilization techniques in bread, the immobilized yeast with the starch-gluten-milk matrix, as a biocatalyst, produced bread with longer shelf-life and better quality. Regarding cost-effectiveness, the kefir immobilized on the orange peel was better (Merchant and Banat 2007). In addition to the starch-gluten-milk matrix and orange peel, the brew's spent grain has also been applied as a support to yeast and kefir. The brew's spent grain was recommended due to its high yeast loading capacity, high regeneration ability, low cost, simple preparation, and inert performance in fermentation environments (Plessas et al. 2007).

27.4 Conclusion

Fermented bread is part of the traditional diets for human. Among the fermented bread varieties, the popularity of sourdough bread was reduced in the past when bread made of bakers' yeast was first introduced to the market. However, more recently, its popularity has been regained due to its health benefits and unique flavor.

The main microorganisms in bread fermentation include yeast and lactic acid bacteria. Variations in the number of cell count and microbial growth phase and interactions between yeast and lactic acid bacteria all affect dough fermentation and formation. Mechanisms during the fermentation include lactic acid fermentation (which is often heterofermentation yielding lactic acid, alcohol, and acetic acid), proteolysis (which yields amino acids for yeast to grow and precursors of flavor compounds), and synthesis of volatile compounds and antifungal compounds (of which acetic acid is the major one). Roles of fermentation in bread manufacture are to develop flavor, structure, and texture. Due to improved moisture retention and development of antifungal compounds in fermented bread, the shelf life of bread can be extended. Moreover, biochemical reactions occurred during the fermentation can improve the nutritional value of bread and develop a prebiotic effect that is good for the human digestion system.

Various manufacture processes exist for fermented bread. For bread fermented with only bakers' yeast, the straight dough process and sponge and dough processes are the two major techniques. Variations in preparation methods lead to four varieties of sourdough, i.e., types 0–III. To improve the efficiency of fermentation, cell immobilization has recently been proposed for bread production. The technology might enable a better and easier control of fermentation processes and thereby provide better consistency of the quality of fermented bread in industrial scale productions.

REFERENCES

Bajd F, Serša I. 2011. Continuous monitoring of dough fermentation and bread baking by magnetic resonance microscopy. *Magn Reson Imag* 29(3):434–42.

Bounaix M-S, Gabriel V, Robert H, Morel S, Remaud-Siméon M, Gabriel B, Fontagné- Faucher C. 2010. Characterization of glucan-producing Leuconostoc strains isolated from sourdough. *Int J Food Microbiol* 144(1):1–9.

Caballero R, Olguín P, Cruz-Guerrero A, Gallardo F, García-Garibay M, Gómez-Ruiz L. 1995. Evaluation of *Kluyveromyces marxianus* as baker's yeast. *Food Res Int* 28(1):37–41.

Catzeddu P, Mura E, Parente E, Sanna M, Farris GA. 2006. Molecular characterization of lactic acid bacteria from sourdough breads produced in Sardinia (Italy) and multivariate statistical analyses of results. *Syst Appl Microbiol* 29(2):138–44.

Corsetti A, Settanni L. 2007. Lactobacilli in sourdough fermentation. *Food Res Int* 40(5):539–58.

De Vuyst L, Vrancken G, Ravyts F, Rimaux T, Weckx S. 2009. Biodiversity, ecological determinants, and metabolic exploitation of sourdough microbiota. *Food Microbiol* 26(7):666–75.

Fowler AA, Priestley RJ. 1980. The evolution of panary fermentation and dough development–review. *Food Chem* 5:283–301.

Gaggiano M, Cagno RD, De Angelis M, Arnault P, Tossut P, Fox PF, Gobbetti M. 2007. Defined multi-species semi-liquid ready-to-use sourdough starter. *Food Microbiol* 24(1):15–24.

Galle S, Schwab C, Arendt EK, Gänzle MG. 2011. Structural and rheological characterisation of heteropolysaccharides produced by lactic acid bacteria in wheat and sorghum sourdough. *Food Microbiol* 28(3):547–53.

Gänzle MG, Loponen J, Gobbetti M. 2008. Proteolysis in sourdough fermentations: mechanisms and potential for improved bread quality. *Trends Food Sci Technol* 19(10):513–21.

Holzapfel W. 1997. Use of starter cultures in fermentation on a household scale. *Food Control* 8(5–6):241–58.

Kulp K. 2003. Chapter 5: Baker's yeast and sourdough technologies in the production of U.S. bread products. In: Kulp K, Lorenz K, editors. *Handbook of Dough Fermentations*. New York: Marcel Dekker, Inc. p 97–143.

Martínez-Anaya MA, de Barber CB, Collar Esteve C. 1994. Effect of processing conditions on acidification properties of wheat sour doughs. *Int J Food Microbiol* 22(4):249–55.

Meuser F, Barber B, Fischer G. 1995. Determination of the microbial activity of dried sourdoughs by revitalization of their lactic acid bacteria and yeasts. *Food Control* 6(3):147–54.

Minervini F, De Angelis M, Cagno RD, Pinto D, Siragusa S, Rizzello CG, Gobbetti M. Robustness of *Lactobacillus plantarum* starters during daily propagation of wheat flour sourdough type I. *Food Microbiol* 27(7):897–908.

Paramithiotis S, Gioulatos S, Tsakalidou E, Kalantzopoulos G. 2006. Interactions between *Saccharomyces cerevisiae* and lactic acid bacteria in sourdough. *Process Biochem* 41(12):2429–33.

Patring J, Wandel M, Jägerstad M, Frølich W. 2009. Folate content of Norwegian and Swedish flours and bread analysed by use of liquid chromatography–mass spectrometry. *J Food Compos Anal* 22(7–8):649–56.

Plessas S, Alexopoulos A, Bekatorou A, Mantzourani I, Koutinas AA, Bezirtzoglou, E. 2011. Examination of freshness degradation of sourdough bread made with kefir through monitoring the aroma volatile composition during storage. *Food Chem* 124(2):627–33.

Plessas S, Bekatorou A, Kanellaki M, Koutinas AA, Marchant R, Banat IM. 2007. Use of immobilized cell biocatalysts in baking. *Process Biochemistry* 42(8):1244–9.

Plessas S, Trantallidi M, Bekatorou A, Kanellaki M, Nigam P, Koutinas AA. 2007. Immobilization of kefir and *Lactobacillus casei* on brewery spent grains for use in sourdough wheat bread making. *Food Chem* 105(1):187–94.

Poinot P, Arvisenet G, Grua-Priol J, Colas D, Fillonneau C, Bail AL, Prost C. 2008. Influence of formulation and process on the aromatic profile and physical characteristics of bread. *J Cereal Sci* 48(3):686–97.

Poutanen K, Flander L, Katina K. 2009. Sourdough and cereal fermentation in a nutritional perspective. *Food Microbiology* 26(7):693–9.

Ricciardi A, Parente E, Piraino P, Paraggio M, Romano P. 2005. Phenotypic characterization of lactic acid bacteria from sourdoughs for Altamura bread produced in Apulia (Southern Italy). *Int J Food Microbiol* 98(1):63–72.

Rizzello CG, Nionelli L, Coda R, De Angelis M, Gobbetti M. 2010. Effect of sourdough fermentation on stabilisation, and chemical and nutritional characteristics of wheat germ. *Food Chem* 119(3):1079–89.

Robert H, Gabriel V, Fontagné-Faucher C. 2009. Biodiversity of lactic acid bacteria in French wheat sourdough as determined by molecular characterization using species-specific PCR. *Int J Food Microbiol* 135(1):53–9.

Sahlström S, Bråthen E. 1997. Effects of enzyme preparations for baking, mixing time and resting time on bread quality and bread staling. *Food Chem* 58(1–2):75–80.

Sterr Y, Weiss A, Schmidt H. 2009. Evaluation of lactic acid bacteria for sourdough fermentation of amaranth. *Int J Food Microbiol* 136(1):75–82.

Torner MJ, Martínez-Anaya MA, Antuña B, de Barber CB. 1992. Headspace flavour compounds produced by yeasts and lactobacilli during fermentation of preferments and bread doughs. *Int J Food Microbiol* 15(1–2):145–52.

Zhou W, Therdthai N. 2006. Chapter 17: Bread manufacture. In: Hui YH, editor. *Bakery Products Science and Technology*. Oxford: Blackwell. p 301–17.

28

Sourdough Bread

Åse Solvej Hansen

CONTENTS

28.1 Introduction

Sourdough is used as an essential ingredient in the production of wheat and rye bread and mixtures thereof to achieve aromatic bread with long shelf life, and it is essential for production of traditional artisan bread. Sourdough has been used as leavening of bread dough during several hundreds of years, and sourdough bread was made in Egypt as early as 3000 BC where scenes of making leavened bread are depicted on the walls of the pyramids (Wood 1996).

The sourdough was and is still a piece of dough from the previous baking that was kept until the next baking, where it was mixed with flour, salt, and water to make the bread dough. The intervals between baking could be from 1 day in bakeries to 1 month in home baking. If the time between baking was long, salt could be added to the surface of the sourdough to avoid wrong fermentation. While this piece of dough is kept, lactic acid fermentation takes place due to the multiplication and metabolic activity of lactic acid bacteria (LAB) originally present in the flour. During this fermentation, selection and multiplication of yeasts from the flour also occur. The natural content of LAB and yeasts from the sourdough was responsible for the leavening capacity of the bread dough primarily due to their production of carbon dioxide. Yeast from beer or wine production could also be added to the dough to increase the leavening capacity until production of commercial baker's yeast began during the 19th century (Pederson 1979). The sourdough still holds a place of honor in many households throughout the world, and small portions are passed on to daughters at marriage (Pederson 1979).

28.1.1 Rye Sourdough

Sourdough is essential in rye bread making, and the tradition of rye sourdough fermentation corresponds to the rye-growing areas in the North, Central, and Eastern European countries including the Baltic States, where rye bread constitutes a considerable amount of the bread consumption. Rye sourdoughs have been characterized from Finland (Salovaara and Katunpaa 1984), Sweden (Spicher and Lonner 1985), Denmark (Knudsen S. 1924; Rosenquist and Hansen 2000), Germany (Böcker et al. 1995; Spicher and Stephan 1999), Poland (Wlodarczyk M. 1986), Czech Republic (Kratochvil and Holas 1983), Russia (Kazanskaya et al. 1983), Austria (Foramitti 1982), and Portugal (Rocha and Malcata 1999).

The tradition of production of rye bread without the addition of bakers' yeast has continued even in large-scale bakeries until today, and the leavening capacity of the sourdough is still very important in rye bread production. In the sixties and seventies, the time between baking and consumption of bread increased due to changes in society, and in some bakeries, preservative compounds such as vinegar, propionic acid, or sorbic acid were added to the dough for the prevention of molds. However, the natural content of yeasts from the sourdough is also inhibited by those preservatives, resulting in decreased leavening capacity, and it was necessary to add bakers' yeast to increase the bread volume. Stringent hygiene in bakeries in combination with addition of sourdough makes it today possible to produce bread with long shelf life without added preservatives.

Bread made from mixed wheat and rye is very common in many European countries, and sourdough should be used to enhance the sensory properties of the bread and prolong the microbial shelf life if more than 20% of the flour is from rye (Röcken 1996).

28.1.2 Wheat Sourdough

The tradition of making wheat bread with the addition of sourdough is widely used in the Mediterranean area as in Greece (De Vuyst et al. 2002; Simopoulos 2001), Italy (Corsetti et al. 2001; Ottogalli et al. 1996), France (Robert et al. 2009), Spain (Barber and Baguena 1988), Egypt (Abd-el-Malek et al. 1973), and Morocco (Faid et al. 1993). The sourdough tradition is also known from The Netherlands (Nout and Creemers 1987), Belgium (Scheirlinck et al. 2007c), Iran (Azar et al. 1977), South Korea (Aslam et al. 2006), Japan (Cai et al. 1999), and the San Francisco Bay area in the United States (Kline et al. 1970; Sugihara et al. 1971).

TABLE 28.1

Advantages of Using Sourdough in Bread-Making

Leavening of dough
Improved dough properties
— inhibition of α-amylase
Increased flavor and taste of bread
Improved nutritional value of sourdough bread
— higher bioavailability of minerals
— lower glycemic index
Extended shelf life of sourdough bread
— longer mold-free period
— prevention of rope in bread
— anti-staling effect

The cereal intake in the traditional diet of Greece is mostly in the form of sourdough bread rather than pasta (Simopoulos 2001). In Italy, sourdough is used in more than 30% of bakery products, which include more than 200 different types of sourdough bread. In some regions of southern Italy, most of the bread including sourdough bread is made from durum wheat instead of common bread wheat (Corsetti et al. 2001). The Italian sweet baked products as pandoro, colomba, and panettone is also leavened with sourdough (Foschino et al. 1995a; Ottogalli et al. 1996). In Morocco, commercial bakeries supply only part of the population with bread; most people eat home-made bread made with traditional sourdough, which has been carefully kept in every family. Addition of bakers' yeast is used mainly in towns and villages where refrigeration can be employed (Faid et al. 1993).

28.1.3 Why Is Sourdough Used?

The advantages of using sourdough for bread production include the possibility of leavening bread dough with little or no bakers' yeast added, improved dough properties, and the achievement of a better and more aromatic bread flavor and texture compared to bread leavened only by bakers' yeast (Table 28.1). Sourdough flavor is developed during a long fermentation process that requires 12–24 hours, while fermentation by bakers' yeast has to be finished within 1–2 hours. The addition of sourdough can also extend the shelf life of bread by several days by increasing the mold-free period of bread and retarding the development of rope. The nutritional value of sourdough bread made from high extraction flour is enhanced compared to bread made without sourdough because of a higher content of free minerals, which are separated from phytic acid during the long fermentation processes.

Interest in using sourdough in bread production has increased considerably in many European countries during recent decades. Today, a large part of the consumers prefer healthy bread with aromatic taste, good texture, and long shelf life without the addition of artificial preservatives. The demand for organic food is also on the rise, and a larger part of the bread made from organically grown cereals is made with sourdough due to its higher quality and better image. More consumers are also interested in food with a history, and sourdough bread is related to traditional and original food.

28.2 Characterization of Sourdough

28.2.1 Definition of Sourdough

Sourdough is a mixture of flour and water in which LAB have caused a lactic acid fermentation to occur. It is in general accepted that the LAB should still be able to produce acids when flour and water are added (metabolically active). The sourdough also has a natural content of sourdough yeasts, which are important for the leavening capacity of the dough. However, no official definition of sourdough exists, but it should include all different types of sourdough products with "living" LAB and excludes artificial

sourdough products. According to Lönner and Preve (1988), a sourdough should contain more than 5×10^8 metabolically active LAB/g, and have a pH value below 4.5.

28.2.2 Types of Sourdoughs

Sourdoughs can be started as follows:

During spontaneous fermentation
By adding a piece of mature sourdough (mother sponge)
By adding a defined starter culture

Most sourdoughs used in both wheat and rye bread baking are still initiated by adding a piece of mature or ripe sourdough, also called mother sponge. However, the use of defined starter cultures with specific fermentation patterns increases rapidly in industrial bread production as these cultures become commercially available (Böcker et al. 1995).

28.2.2.1 Spontaneous Fermentation

When dough made from flour and water is left for 1 to 2 days at ambient temperature, a spontaneous fermentation will take place due to the naturally occurring microorganisms in the flour. The dough will become acidified due to lactic acid fermentation. During the fermentation there is a successive flavoring of the Gram-positive LAB from the flour at the expense of the Gram-negative bacteria, which dominate the microflora of the flour (Lönner and Preve 1988; Spicher and Stephan 1999). The microflora of some spontaneously fermented rye sourdoughs were dominated by homofermentative *Lactobacillus* spp. and *Pediococcus* spp. (Lönner and Preve 1988). However, spontaneous sourdoughs do not always succeed and may result in products with off-flavor.

28.2.2.2 Mature Sourdough

Sourdoughs used by artisan bakers and in bakeries have traditionally been based on spontaneous fermentation, during which the sourdough has been kept metabolically active and probably microbially stable for decades by the daily addition of flour and water, the so-called "freshening" of the dough based on "back-slopping" (Figure 28.1). The fermented sourdough is used for bread production, but part of it is used to start a new sourdough. The terminology for sourdough and starter in different countries is listed in Table 28.2. A sourdough has a higher number of LAB than yeasts contrary to a leaven, biga, or levain, which is dominated by natural yeasts.

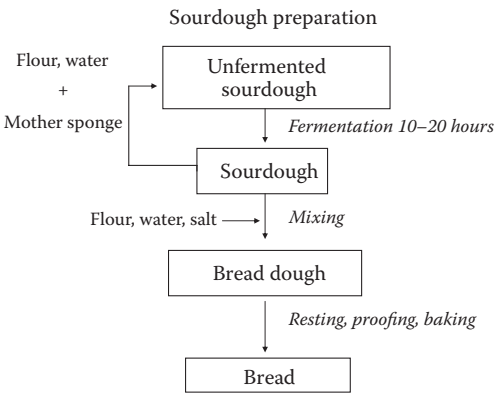

FIGURE 28.1 Schedule for production of sourdough bread.

TABLE 28.2

Terminology for Sourdough in Different Countries

	English	German	French	Spanish	Italian
Sourdough for bread production	Sourdough Leaven	Sauerteig	Levain natural	Masa madre (masa agria)	Lievito naturale (impasto acido)
Sourdough used as starter for a new sourdough	Mother sponge Starter	Anstellgut Reinzucht-sauer®	Le chef	Pie	Madre Capolievieto

Source: Ottogalli, G. et al., *Adv Food Sci*, 18, 131–44, 1996. With permission.

In commercial rye bread baking, the bakeries can use their own adapted sourdough, or if they have quality problems due to unstable process control, they can add a commercial sourdough as a starter. Some bakeries in Germany and Denmark regularly add commercial sourdoughs composed of a well-adapted microflora derived from natural sourdough fermentation. Examples of commercial sourdoughs are the Sanfrancisco sour for wheat bread production (Sugihara et al. 1970) and the Böcker-Reinzucht-Sauer® for rye bread production. Some products sold as sourdough have no living microorganisms, and these products will not contribute to a natural acidification and development of flavor compounds in the dough.

28.2.2.3 Starter Cultures of Pure Strains of LAB

Starter cultures for sourdough fermentation can be pure cultures of freeze-dried LAB, or a mixture of LAB and sourdough yeast. They should be mixed with flour and water and kept for several hours for multiplication and fermentation of the microflora before this fermented dough can then be used as a sourdough. The microorganisms have been selected due to their ability to acidify dough in a short time and result in acceptable bread flavor when used in bread baking. Cultures containing *Lactobacillus sanfranciscensis*, *Lb. plantarum*, *Lb. brevis*, and *Lb. fructivorans* or *Lb. brevis*, *Lb. pontis*, and *Saccharomyces cerevisiae* are commercially available (Hammes et al. 1996). Use of defined starter cultures with specific properties gives rise to new interesting opportunities for controlling and regulating sourdough fermentation. The term "starter culture" is sometimes used in the literature for a mature sourdough that has to be mixed with flour and water to ferment, or for commercial sourdoughs.

28.2.3 Sourdough Parameters

A sourdough can be characterized by the chemical parameters—pH, content of total titratable acids (TTA), content of lactic and acetic acid—and the microbial parameters such as number and species of LAB and yeast (Table 28.3). The final pH of a mature sourdough is 3.5–3.8 in most rye and wheat

TABLE 28. 3

Characteristics of Sourdoughs

Flour/water	1:0.6 firm 1:2 fluid
Fermentation time	8–24 hours
Fermentation temperature	25°C–35°C
LAB (number)	10^8–10^{10} cells/g
Amount of mother sponge	10%–20% of flour (long fermentation time) 25%–35% of flour (short fermentation time)
Sourdough yeasts (number)	10^5–10^7 cells/g
pH	3.5–3.8
Total titratable acids (TTA)	
—Whole meal flour	15–26
—Straight grade flour	8–11

sourdoughs (Brümmer and Lorenz 1991; Hansen and Hansen 1994b; Lund et al. 1989). Sourdough pH values show less variation and differences than TTA values. The TTA values in sourdoughs are dependent on the fermentation temperature, extraction rate of the flour, and the water content. In wheat sourdoughs, TTA has been found to vary between 8 and 11 in sourdoughs made from low extraction flour and 16 to 22 in wholemeal sourdoughs (Brümmer and Lorenz 1991; Hansen and Hansen 1994a). Rye sourdoughs are often made from flours with higher extraction rate than wheat flour and TTA values vary between 15 and 26 (Lund et al. 1989; Spicher and Stephan 1999).

The content of lactic and acetic acid in sourdoughs is very important for the taste and flavor of sourdough bread (Hansen et al. 1989a; Hansen and Hansen 1996). The fermentation quotient (FQ), the molar ratio between lactic and acetic acid is used as a measure in German studies of sourdough for the balance in production of those acids in rye bread. The FQ should be around 4 in sourdoughs to result in a harmonic taste of bread (Spicher and Stephan 1999). The content of lactic acid was 1.2%–1.7% and acetic acid 0.3%–0.4% in bakery rye sourdoughs made from whole meal flour (Rosenquist and Hansen 2000), and this is in accordance with the recommended FQ.

28.3 Microbiology of Sourdough

The microflora of the sourdoughs includes adapted LAB and yeasts that have optimal conditions for growth and fermentation similar to the conditions for the sourdough (temperature, water content, pH) and which probably produce antimicrobial compounds (Messens and De Vuyst 2002). The microflora in bakery sourdoughs remains remarkably stable despite the use of nonaseptic fermentation conditions (Böcker et al. 1990; Rosenquist and Hansen 2000; Strohmar and Diekmann 1992; Sugihara et al. 1970).

Early systematic studies of the microflora responsible for the sourdough fermentation were made on sourdoughs from Germany by Hollinger in 1902, from Denmark by Knudsen in 1924 (Knudsen S. 1924), and from Russia by Sseliber in 1939 (cited by Spicher and Stephan 1999). Spicher and Stephan (1999) have carried out many investigations concerning the identification of the microflora from different types of sourdoughs, both adapted sourdoughs from bakeries and in commercial starter cultures for sourdough. In several of the sourdoughs investigated, only LAB or sourdough yeasts are identified.

Recent investigations on sourdough fermentation have mainly dealt with interactions between sourdough microorganisms, identification of new species by new identification methods, inhibitory substances of sourdough LAB, and induced specific enzymatic activities.

LAB are mainly responsible for the acidification of the sourdough, whereas the sourdough yeasts are very important for the production of flavor compounds and for a harmonic bread flavor in combination with the acids. The levels of LAB in sourdoughs are 10^8–10^9 cfu/g and yeasts are 10^6–10^7 cfu/g, respectively. The LAB:yeast ratio in sourdoughs is generally 100:1 (Spicher and Stephan 1999).

28.3.1 Lactic Acid Bacteria

28.3.1.1 Identification

LAB are a group of Gram-positive bacteria, which are catalase-negative, nonmotile, nonsporeforming rods or cocci that produce lactic acid as the major end product during the fermentation of carbohydrates. They are strictly fermentative, aerotolerant or microaerophile, acidophilic, salt-tolerant, and have complex nutritional requirements for carbohydrates, amino acids, peptides, fatty acids, salts, nucleic acids derivatives, and vitamins (Ganzle et al. 1998; Hammes and Vogel 1995).

The LAB have traditionally been classified taxonomically into different genera based on colony and cell morphology, sugar fermentation, growth at different temperatures, configuration of lactic acid produced, ability to grow at high salt concentration, acid tolerance, or cell wall analyses (Kandler and Weiss 1986). Genera of LAB identified from sourdoughs are *Lactobacillus*, *Leuconostoc*, *Pediococcus, Lactococcus*, and *Weissella*, and the majority of the sourdough LAB belongs to the genus *Lactobacillus*.

Lactobacillus have been divided into three groups according to their carbohydrate fermentation patterns (Kandler and Weiss 1986):

Obligately homofermentative LAB

Hexoses are almost completely fermented to lactic acid (>85%) by the Embden-Meyerhof-Parnas (EMP) pathway.

Fructose is also fermented, but neither gluconate nor pentoses are fermented.

Facultatively heterofermentative LAB

Hexoses are almost completely fermented to lactic acid by the EMP pathway. Pentoses are fermented to lactic acid and acetic acid by an inducible phosphoketolase.

Obligately heterofermentative LAB

Hexoses are fermented to lactic acid, acetic acid (ethanol), and CO_2.

Pentoses are fermented to lactic and acetic acid. In general, both pathways involve phosphoketolase.

In the presence of oxygen or other oxidants, increased amounts of acetate may be produced by the heterofermentative LAB at the expense of lactate or ethanol. Various compounds such as citrate, malate, tartrate, quinolate, and nitrate may also be metabolized and used as energy sources or electron acceptors (Ganzle et al. 1998; Hammes and Vogel 1995).

The traditional techniques used for classification of LAB are not adequate as some organisms grow poorly on laboratory media and may escape isolation and can therefore not be identified by standard procedures (Vogel et al. 1996). Newer techniques for rapid classification and identification of LAB include hybridization- and PCR-based techniques as well as recognizing specific sequences in the ribosomal genes. The application of 16S rRNA sequence analysis and DNA-DNA hybridization experiments has led to identification of many new species.

Newer species identified with the gene identification techniques are *Lb. pontis* (Vogel et al. 1994), *Lb. panis* (Wiese et al. 1996), *Lb. frumenti* (Muller et al. 2000), and *Lb. mindensis* (Ehrmann et al. 2003) isolated from rye sourdoughs, and *Lb. paralimentarius* (Cai et al. 1999), *Lb. hammesii* (Valcheva et al. 2005), *Lb rossiae* formerly *Lb rossii* (Corsetti et al. 2005), *Lb. acidifarinae*, and *Lb. zymae* (Vancanneyt et al. 2005), *Lb. nantensis* (Valcheva et al. 2006), *Lb. sigilinis* (Aslam et al. 2006), and *Lb. namurensis* (Scheirlinck et al. 2007a) were isolated from wheat sourdough. *Lb spicheri* (Meroth et al. 2004) has been isolated from wheat and rice sourdoughs. Around 50 different species of LAB have been isolated from sourdoughs and characterized (Corsetti and Settanni 2007). *Lactobacillus* isolated from sourdoughs divided into the three groups due to fermentation pattern are shown in Table 28.4.

28.3.1.2 Occurrence

Heterofermentative LAB play a major role in sourdough fermentation compared to other fermented food systems. *Lb. sanfranciscensis* (former names *Lb. brevis* var. *lindneri* and *Lb. sanfrancisco*) is by far the most dominant LAB in both rye and wheat sourdoughs (Tables 28.5 and 28.6). *Lb. brevis* and *Lb. plantarum* also occur frequently in both types of sourdoughs. Some strains initially classified as *Lb. brevis* were renamed *Lb. pontis* (Vogel et al. 1994). Several other lactobacilli have been identified from rye sourdoughs and (Table 28.5), e.g., the homofermentative *Lb. acidophilus*, *Lb. alimentarius*, *Lb. amylovorus*, *Lb. casei*, *Lb. delbrueckii*, *Lb. farciminis*, *Lb. leichmanii*, *Lb. mindensis*, and *Lb. rhamnosus*, and the heterofermentative *Lb. büchnerii*, *Lb. cellobiosus*, *Lb. curvatus*, *Lb. fructivorans*, *Lb. fermentum*, *Lb. frumenti*, *Lb. panis*, *Lb. pontis*, and *Lb. viridescens.*

Wheat sourdoughs have in general been investigated later than the rye sourdoughs and several new heterofermentative *Lactobacillus* species have been identified in wheat sourdoughs within the last decades (Table 28.6). However, the homofermentative *Pediococcus* and *Weissella* and the heterofermentative *Leuconostoc* have also been isolated from wheat sourdoughs.

The variation in the composition of the microflora depends on the fermentation conditions, such as flour type, extraction rate, water content, fermentation temperature, fermentation time, and how

TABLE 28.4

Lactobacillus Species Isolated from Sourdoughs

Characteristics	Obligately Homofermentative	Facultatively Heterofermentative	Obligately Heterofermentative
Growth at 15°C	–	+	+/–
Growth at 45°C	+	–	+/–
Pentose fermentation	–	+	+
CO_2 from glucose	–	–	+
CO_2 from gluconate	–	+[a]	+[a]
FDP[b] aldolase present	+	+	–
Phosphoketolase present	–	+[c]	+
Lactobacillus	*Lb. acidophilus* *Lb. amylovorus* *Lb. delbrueckii* spp. *bulgaricus* *Lb. delbrueckii* spp. *delbrueckii* *Lb. farciminis* *Lb. helviticus* *Lb. leichmanni* *Lb. mindensis*	*Lb. alimentarius* *Lb. casei* *Lb. curvatus* *Lb. paralimentarius* *Lb. plantarum* *Lb. rhamnosus*	*Lb. acidifarinae* *Lb. brevis* *Lb. buchneri* *Lb. fermentum* *Lb. fructivorans* *Lb. frumenti* *Lb. hammesii* *Lb. hilgardii* *Lb. nantensis* *Lb. panis* *Lb. pontis* *Lb. reuteri* *Lb. rossiae* *Lb. sanfranciscensis*[d] *Lb. siliginis* *Lb. spicheri* *Lb. viridescens* *Lb. zymae*

[a] When fermented.
[b] FDP, fructose-1,6-diphosphate.
[c] Inducible by pentoses.
[d] Former names of some of the bacteria: *Lb. brevis* spp. *lindneri* and *Lb. sanfrancisco.*

the sourdough is refreshed. Most sourdoughs are fermented about 30°C, but *Lb. delbrueckii* has been isolated from rye sourdoughs with a fermentation temperature above 50°C (Kazanskaya et al. 1983; Spicher and Lonner 1985). Hammes et al. (1996) found that the most predominant LAB in firm sourdoughs with fermentation temperature between 23°C and 30°C are *Lb. sanfranciscensis* and *Lb. pontis*.

Some industrial sourdoughs are characterized by high water content to fluid conditions (suitable for pumping), elevated fermentation temperature (>30°C), and shorter fermentation time (15–20 hours). Fluid sourdoughs can be produced in large volumes—often by continuous fermentation systems, and they can be cooled for storing in silos up to 1 week. The microflora in this type of sourdough is dominated by *Lb. panis* (Wiese et al. 1996), *Lb. reuteri*, *Lb. sanfranciscensis*, and *Lb. pontis* (Hammes et al. 1996). The development of sourdough yeast is poor in fluid sourdoughs, and consequently it is necessary to add bakers' yeast to the bread dough.

Sourdoughs kept at ambient temperature will continue acidification. LAB are sensitive to low pH in longer time and the LAB will thus die off. Therefore, dried sourdough preparations are preferred for commercial sourdough samples. However, LAB are rather sensitive to preservation by drying, and LAB present in commercial sourdoughs must survive drying. *Lb. plantarum*, *Lb. brevis*, *Lb. sanfranciscensis*, and *Pediococcus pentosaceus* have been identified from dried commercial sourdough preparations (Hammes et al. 1996).

TABLE 28.5

LAB and Yeasts Isolated from Rye Sourdoughs

	LAB			
Country	**Homofermentative**	**Heterofermentative**	**Yeasts**	**Reference**
Russia (1939)	*Streptobacterium plantarum (Lactobacillus plantarum)*	*Lactobacillus. brevis* *Lb. fermenti*	Nonidentified species	Spicher and Stephan 1999
Czech Republic (1955)	*Streptobacterium plantarum*	Nonidentified species	*Saccharomyces cerevisiae*	Spicher and Stephan 1999
Germany (1959)	*Lb. delbrueckii* *Lb. plantarum* *Lb. leichmanii*	*Lb. brevis* *Lb. fermenti*		Spicher and Stephan 1999
Germany (1978)	*Lb. alimentarius* *Lb. plantarum* *Lb. acidophilus* *Lb. casei* *Lb. farciminis*	*Lb. brevis* *Lb. brevis* subsp. *lindneri* *Lb. plantarum* *Lb. fermentum* *Lb. fructivorans*	*Candida krusei* *S. cerevisiae* *Pichia saitoi* *Torupolsis holmii*	Spicher and Stephan 1999
Austria	*Lb. alimentarius* *Lb. casei* *Lb. rhamnosus*	*Lb. brevis* subsp. *lindneri* *Lb. büchnerii* *Lb. fermentum* *Lb. fructivorans*	*S. cerevisiae* *C. krusei*	Foramitti 1982
Russia	*Lb. plantarum* (firm) *Lb. plantarum* (liquid) *Lb. leichmanii* *Lb. casei* var. *casei* *Lb. delbruckii* (48°C–52°C)	*Lb. brevis* *Lb. fermenti* *Lb. brevis* *Lb. büchnerii*	*S. minor/S. exiguus* *S. exiguus*	Kazanskaya et al. 1983
Finland	*Lb. acidophilus* *Lb. plantarum*	*Lb. büchnerii* *Lb. cellobiosus* *Lb. viridescens*	*Torulopsis holmii* *S. cerevisiae* *T. unisporus* *T. stellata* *Endomycopsis fibuliger* *Hansenula anomala*	Salovaara and Katunpaa 1984 Salovaara and Savolainen 1984
Sweden	*Lb. acidophilus* *Lb. plantarum* *Lb. farciminis* *Lb. casei rhamnosus* *Lb. delbruckii* (53°C)	*Lb. brevis* *Lb. brevis* subsp. *lindneri* *Lb. fermentum* *Lb. viridescens*		Spicher and Lönner 1985
Poland			*S. cerevisiae* *S. exiguus* *T. candida* *C. krusei*	Wlodarczyk M. 1986
Germany		*Lb. brevis* *Lb. sanfrancisco*		Böcker et al. 1990
		Lb. curvatus *Lb. sanfrancisco*		Okada et al. 1992
Germany		Nonidentified species closely related to *Lb. fermentum* and *Lb. reuteri*	*S. cerevisiae*	Strohmar and Diekmann 1992

(*continued*)

TABLE 28.5 (Continued)

LAB and Yeasts Isolated from Rye Sourdoughs

Country	LAB		Yeasts	Reference
	Homofermentative	Heterofermentative		
Germany		*Lb. brevis* *Lb. sanfranciscensis* *Lb. fermentum* *Lb. fructivorans* *Lb. pontis* sp. nov		Vogel et al. 1994
Portugal (rye and maize)			*S. cerevisiae* *Torulaspora delbrueckii* *Issatchenkia orientalis* *Pichia anomala* *P. membranaefaciens*	Almeida and Pais 1996
Germany		*Lb. panis* sp. nov.		Wiese et al. 1996
Finland			*C. milleri* *S. cerevisiae* *S. exiguus*	Mantynen et al. 1999
Germany		*Lb. frumenti* sp. nov.		Muller et al. 2000
Denmark	*Lb. acidophilus* *Lb. amylovorus* (liquid)	*Lb. panis*	*S. cerevisiae*	Rosenquist and Hansen 2000
Germany	*Lb. mindensis* sp. nov.			Ehrmann et al. 2003

28.3.2 Yeasts

Several species of yeasts have been isolated from bakery and commercial sourdoughs. However, the taxonomy of yeasts has been gradually changed since the 1970s, and various synonyms have been used (Table 28.7). The traditional systematization and identification of yeasts have been based on biochemical tests as well as morphological and physiological criteria (Kreger van Rij 1984), but new molecular characteristics have defined and changed the taxonomy of yeasts (Mantynen et al. 1999).

S. cerevisiae is the frequently dominant yeast species in both rye and wheat sourdoughs (Tables 28.5 and 28.6). Other yeast species often isolated from sourdoughs are *Kazanchastania exigua (S. exiguous)*, *Candida milleri*, *C. holmii*, and *Issatchenkia orientalis (C. krusei*). The yeast species *Pichia saitoi*, *P. norvegensis*, and *Hansenula anomala*, and some other *Saccharomyces* spp., have occasionally been isolated from sourdoughs.

The yeasts present in sourdoughs are generally acid-tolerant. Strains of bakers' yeast *S. cerevisiae* have poor tolerance of acetic acid in sourdoughs (Suihko and Maekinen 1984), whereas strains of *S. cerevisiae* isolated from sourdoughs can grow on MYGP broth acidified with acetic acid to pH 3.5 (Hansen and Hansen 1994b).

Candida spp. are members of *Deuteromycetes* (fungi imperfect) because they have lost their ability to undergo sexual development. *C. milleri* is physiologically similar to *C. holmii* but different according to DNA identification. Some strains identified as *Torulopsis holmii* in the literature before 1978 have subsequently been assigned to *C. milleri* (Mantynen et al. 1999). *C. humilis* does not assimilate sucrose and raffinose and can be differentiated from *C. milleri* by an assimilation test (Yarrow 1998).

C. milleri (*T. holmii*) and *S. cerevisiae* were the dominating yeasts in bakery rye sourdoughs from Finland, whereas *S. cerevisiae* dominated in rye sourdoughs used for home baking (Salovaara and Savolainen 1984). Wlodarczyk found that *S. cerevisiae* accounted for 99% of all yeasts found in starters from three industrial rye bread bakeries in Poland, whereas the sourdoughs from the smaller bakeries contained a wider range of yeast strains (Wlodarczyk M. 1986).

TABLE 28.6

LAB and Yeasts Isolated from Wheat Sourdoughs

Country	Sourdough Product	LAB		Yeasts	Reference
		Homofermentative	Heterofermentative		
United States	San Francisco bread		*Lb. sanfrancisco*	*Saccharomyces exiguus*	Kline et al. 1970
				S. inusitas	Sugihara et al. 1970
Egypt	Balady bread				Abd-el-Malek et al. 1973
Iran	Sangak bread	*Lb. plantarum*	*Lb. brevis* *Leuconostoc mesenteroides*	*Torulopsis collucolasa* *T. candida*	Azar et al. 1977
Sweden	Wheat bread	*Lb. plantarum*	*Lb. brevis*		Spicher and Lonner 1985
Germany	Wheat bread	*Lb. plantarum* *Lb. farciminis* *Lb. casei*	*Lb. brevis*		Spicher and Stephan 1999
The Netherlands	Wheat bread		*Lb. brevis* var. *lindneri* *(Lb. sanfranciscensis)*	*S. exiguus*	Nout and Creemers 1987
			Lb. brevis var. *lindneri*	*S. exiguus*	
Switzerland	Swiss panettone/cake		*Lb. brevis* var. *lindneri*		Spicher and Stephan 1999
	Swiss bread		*Lb. brevis* var. *lindneri*		Spicher and Stephan 1999
Italy	Panettone, brioches, wheat bread, crackers	*Lb. plantarum* *Pediococcus*	*Lb. sanfranciscensis* *Lb. fermentum* *Lc. mesenteroides*	*S. cerevisiae* *C. stellata* *C. milleri*	Galli et al. 1988
Spain	Wheat bread	*Lb. plantarum* *Leuconostoc mesenteroides*	*Lb. brevis* *Lb. cellobiosus*	*S. cerevisiae* *C. boidinii* *C. guilliermondii* *Rhodutorula glutinis* *Pichia polymorpha* *Tricocporon margaritiferum*	Barber and Baguena 1988
Morocco	Wheat bread	*Lb. plantarum* *Lb. delbrueckii* *Lactococcus casei*	*Lb. brevis*	*C. milleri* *S. cerevisiae*	Boraam et al. 1993
Italy	Pannettone, bread	*Lb. plantarum* *Lb. farciminis*	*Lb. brevis* var. *lindneri* *(Lb. sanfranciscensis)*	*S. cerevisiae* *S. exiguus* *C. krusei*	Gobbetti et al. 1994a
Italy	Wheat bread			*S. cerevisiae* *S. exiguus* *C. krusei* *Pichia norvegensis* *Hansenula anomala*	Rossi 1996

(*continued*)

TABLE 28.6 (Continued)

LAB and Yeasts Isolated from Wheat Sourdoughs

Country	Sourdough Product	LAB		Yeasts	Reference
		Homofermentative	Heterofermentative		
Portugal	Maize bread	*Lb. delbrueckii* *Lb. curvatus* *Lb. plantarum* *Lb. lactis* spp. *Lactis*	*Lb. brevis*	*S. cerevisiae* *Torulaspora delbrueckii*	Rocha and Malcata 1999
Japan	Wheat bread	*Lb. paralimentarius* sp. nov.			Cai et al. 1999
Italy	Cakes: panettone, colomba, brioche	*Lb. delbrueckii*	*Lb. sanfranciscensis* *Lb. brevis* *Lb. sanfranciscensis*	*C. holmii* *S. cerevisiae*	Foschino et al. 1999
	Bread—durum wheat and bread wheat	*Lb. alimentarius* *Lb. plantarum*	*Lb. sanfranciscensis* *Lb. brevis* *Lec. citreum* *Lb. fermentum*	*S. cerevisiae*	Corsetti et al. 2001
Belgium	Wheat bread	*Lb. paralimentarius* *Weissella cibaria*	*Lb. sanfranciscensis* *Lb. brevis*		De Vuyst et al. 2002
Germany	Rice sourdough for gluten free bread	*Lb. plantarum* *Lb. paracasei*	*Lb. fermentum* *Lb. pontis* *Lb. spicheri* sp. nov.	*S. cerevisiae* *C. krusei*	Meroth et al. 2004
France	Wheat bread	*Lb. plantarum* *Lb. paralimentarius*	*Lb. sanfranciscensis* *Lb. spicheri* *Lb. hammesii* sp. nov.		Valcheva et al. 2005
Belgium	Wheat bread		*Lb. spicheri* *Lb. bevis* *Lb. acidifarinae* sp. nov. *Lb. zymae* sp. nov.		Vancanneyt et al. 2005
Italy	Wheat bread		*Lb rossiae* sp. nov. (former *Lb rossi*)		Corsetti et al. 2005
France	Wheat bread		*Lb. nantensus* sp. nov.		Acheva et al. 2006
South Korea	Wheat bread		*Lb. siliginis* sp. nov.		Aslam et al. 2006
Belgium	Wheat bread		*Lb. namurensis* sp. nov. *Lb.crustosum* sp. nov.		Scheirlinck et al. 2007b Scheirlinck et al. 2007a

Traditional Portuguese sourdoughs prepared from maize and rye were dominated by *S. cerevisiae* and *Torulaspora delbrueckii* (Almeida and Pais 1996), and *S. cerevisiae* and *C. pelliculosa* (Rocha and Malcata 1999), respectively. *S. cerevisiae* was also the dominating yeast in wheat sourdoughs from Italy, followed by *K. exigua*, *C. krusei*, *P. norvegensis*, and *Hansenula anomala* (Rossi 1996). Addition of bakers' yeast is widely used in some Italian wheat sourdoughs (Corsetti et al. 2001), whereas many sourdoughs are also prepared without yeasts (Foschino et al. 1999).

28.3.3 Microbial Interactions

The high stability of sourdoughs used for a longer period might be caused by production of inhibitory substances (Messens and De Vuyst 2002), but also microbial interaction between the LAB and the sourdough yeasts are of importance. Several sourdough LAB produce inhibitory substances against spoiling

TABLE 28.7

Yeasts Isolated from Sourdoughs and Their Synonyms

Perfect Fungi	Imperfect Fungi	Synonyms/Former Names
Saccharomyces cerevisiae		
Kazanchastania exigua	*Candida holmii*	*Torula holmii*
		Saccharomyces rosei
		Saccharomyces exiguus
	Candida milleri	*Torulopsis holmii*
	Candia humilis	
Saccharomyces delbrueckii		*Torulaspora delbrueckii*
Saccharomyces uvarum		*Saccharomyces inusitatus*
Issatchenkia orientalis	*Candida krusei*	*Saccharomyces krusei*
		Endomyces krusei
Pichia anomala	*Candida peliculosa*	*Hansenula anomala*
Picia membrafaciens	*Candida valida*	
Picia norvegensis		
Picia polymorpha		
Picia satoi		
Endomycopsis fibuligera		*Saccharomyces fibuliger*

Source: Barnett, et al. 2000. Kurtzmann, C. P., and J. W. Fell, editors, *The yeasts: a taxonomic study*, Elsevier, Amsterdam, 1998. With permission.

microorganisms. These compounds are organic acids, in particular acetic acid, carbon dioxide, ethanol, hydrogen peroxide, and diacetyl (De Vuyst and Vandamme 1994). The inhibition, however, can also be caused by bacteriocins that are low molecule-mass peptides, or proteins, with a bactericidal or bacteriostatic mode of action, in particular against closely related species (Messens and De Vuyst 2002).

Microbial interaction was early demonstrated for the Sanfrancisco sourdough. The sourdough yeast *T. holmii (C. milleri*) does not assimilate maltose (Sugihara et al. 1970), whereas *Lb. sanfranciscensis* hydrolyzes maltose and excretes one of the glucose molecules to be used for the sourdough yeast (Stolz et al. 1993). The glucose uptake of the yeast cell can induce an outflow of amino acids, and this liberation of amino acids has made growth of *Lb. sanfranciscensis* possible even in a medium initially deficient in essential amino acids (Gobbetti 1998).

A real risk of bacteriophage contamination of sourdoughs exists because bacteriophages with activity against *Lb. fermentum* have been isolated from an Italian sourdough (Foschino et al. 1995b).

28.4 Technological Aspects

28.4.1 Production of Sourdough

Sourdough can be made with variations in flour type as wheat or rye, flour extraction rate, flour/water ratio, fermentation temperature and time, and amount of starter. Sourdough can be prepared in one to three steps. Flour, water, and mother sponge are mixed in the one-stage process, and the mature sourdough is used after fermentation. This is the basic way to make a sourdough and is widely used. In a multiple-step sourdough, the fermented dough is refreshed with flour and water two to three times before the sourdough is used for bread making. It has traditionally been used in rye bread production in many German bakeries (Spicher and Stephan 1999). Industrialization in bakeries has also included sourdough production, where the time consuming multiple-stage processes have changed to the work-saving one-stage process.

Traditional rye sourdoughs have often been based on firm sourdoughs, but in automated large scale bakeries, firm sourdoughs are difficult to handle, and they are replaced by pumpable semifluid to fluid sourdoughs, which are suitable for automated fermentation systems.

28.4.2 Flour Type

The flour in the sourdough is the substrate for the fermenting microorganisms. Wheat and rye flour are mostly used for sourdough making, but maize and rice flour can also be used (Meroth et al. 2004; Rocha and Malcata 1999; Sanni et al. 1998; Valcheva et al. 2005). The amount of fermentable carbohydrates in the flour varies with the type of cereal, but in particular with the activity of endogenous enzymes in the flour. The activities of amylases, xylanases, and peptidases are important for liberation of the fermentable low molecular weight carbohydrates and amino acids. In the dough stage, the α-amylase cannot degrade intact starch granules, but some granules are damaged during the milling process and may be partly degraded in the dough.

Starch is generally not degraded by LAB, and the content of fermentable monosaccharides and disaccharides is less than 3% in wheat and 1% in rye (Delcour and Hoseney 2010). However, *Lactobacillus amylovorus* is an LAB with amylolytic activity isolated from African fermented cereal products made from maize such as ogi, mawé, and kunu-zakki (Sanni et al. 2002). Rye has a high content of arabinoxylans (9%–12%) compared to wheat and they can be degraded to the pentoses xylose and arabinose by the corresponding enzymes during the bread-making processes (Hansen et al. 2002).

The content of maltose has been shown to increase during sourdough fermentation from 1.5 to 2.4%, and the content of fructose from 0.05 to 0.45% in a sourdough fermented with *Leuconostoc mesenteroides* (Lefebvre et al. 2002). The content of glucose was unchanged at the level of 0.17% as a result of a balance between bacterial consumption and hydrolysis by the enzymatic activity.

28.4.3 Extraction Rate

The extraction rate of the flour is one of the most important factors for determining the character of sourdough. Flour with a high extraction rate (80%–100%) as whole meal flour has a much higher content of nutrients such as B vitamins and minerals compared to flour with low extraction rate as wheat flour (65%–75%). Also the buffering capacity of the flour is high in whole meal flour compared to wheat flour primarily due to a high content of phytic acid from the aleurone layer of the cereals. This results in longer fermentation time and higher production of acids in the high extraction flour (Table 28.3), and the high content of nutrients stimulate the growth and biochemical activity of the microflora in the sourdough followed by a higher production of flavor compounds The content of lactic acid varied from 0.8% in wheat sourdoughs made from low extraction flour to 1.4% in whole meal flour, and the content of acetic acid varied in the sourdoughs from 0.09% to –0.14% (Hansen and Hansen 1994a).

28.4.4 Water Content

The water content in the sourdough determines the firmness of sourdoughs, and it can be expressed as the dough yield (DY), which is the amount of sourdough in kilogram per 100 kg flour. DY varies from 150 in firm sourdoughs to 300 in fluid sourdoughs. The development in TTA is lower in fluid rye sourdoughs (high DY) compared to firm sourdoughs (low DY), but if the acidity is measured per gram dry matter, it will be lower in firm sourdoughs. This indicates that the nutrients are better used by the LAB in fluid sourdoughs compared to firm sourdoughs. The production of lactic acid is not influenced by the DY, whereas the production of acetic acid is generally lower in fluid sourdoughs (Lund et al. 1989; Spicher and Stephan 1999). The water content in sourdoughs influences the acidification of the dough more than the temperature (Brümmer and Lorenz 1991).

The content of LAB was not influenced by the firmness of rye sourdoughs, whereas the yeast propagation was low in the firm sourdoughs with levels below 10^3 CFU/g in six of the seven sourdoughs (Lund et al. 1989).

28.4.5 Temperature

The fermentation temperature for most sourdoughs is between 25°C and 30°C. The temperature increases 6°C to 8°C during fermentation on an industrial scale, if the temperature is not thermostatically

regulated. The temperature of the sourdough greatly influences the microbial propagation and production of acids, as the optimal temperature for growth and acidification varies for the different species of LAB (Spicher and Stephan 1999).

The optimum temperature for growth of the LAB is close to the optimal temperature for acid production, and most LAB have temperature optima between 30°C and 35°C. In general, the final pH is reached more quickly at higher temperatures (30°C–35°C) compared to lower temperatures (20°C–25°C) (Hansen et al. 1989b; Spicher and Stephan 1999).

Some species, mostly heterofermentative, can grow below 15°C, such as *Lb. farciminis*, *Lb. plantarum*, *Lb. rhamnosus*, *Lb. brevis*, *Lb. fructivorans* and *Lb. sanfranciscensis*. The highest temperature for growth is between 45°C and 55°C, and most species that can tolerate high temperatures are homofermentative, such as *Lb. acidophilus*, *Lb. amylovorus*, and *Lb. delbrueckii*. However, also the heterofermentative species of *Lb. pontis*, *Lb. rhamnosus*, *Lb. fermentum*, and *Lb. reuteri* can grow above 45°C (Hammes and Vogel 1995).

The optimum temperature for growth of sourdough yeasts seems to be lower than for the LAB. The optimum temperature for growth of *C. milleri* was determined to be 27°C (Ganzle et al. 1998). The minimum temperatures for growth of LAB and yeast are important when sourdoughs are stored by cooling, as the sourdough should not develop during the storage. The minimum temperature for growth of most sourdough yeasts has been found to be 8°C (Mantynen et al. 1999).

The content of acids produced in sourdough increases with increased fermentation temperature due to higher production of lactic acid, whereas the production of acetic acid is only negligibly influenced by the temperature. This confirms the general rule that the relative content of acetic acid is higher in cold sourdoughs compared to warmer sourdoughs (Spicher and Stephan 1999).

28.4.6 Amount of Mother Sponge

The amount of mother sponge to be mixed with flour and water for a new sourdough should be so high that the content of LAB in the sourdough is able quickly to decrease the pH to inhibit the growth of the Gram-negative bacteria in the flour. The amount of mother sponge influences the pH-lowering capacity in a sourdough, as low pH is reached more quickly when the amount of added mother sponge is high (Brümmer and Lorenz 1991). However, higher levels of acids are produced when a lower amount of mother sponge is added, as the fermentation time is longer before the pH drops to the critical pH level (Rosenquist and Hansen 2000). The recommended amount of mother sponge is generally 10%–20% for long time fermented rye and wheat sourdoughs (Brümmer and Lorenz 1991; Spicher and Stephan 1999) (Table 28.3). The Sanfrancisco sourdough is rebuilt every 8 h or at least two to three times a day, 7 days a week. The amount of mother sponge used in preparing a new sourdough is 25%–40% of the sourdough (Kline et al. 1970). This high amount of mother sponge makes the sourdough very stable, and this sourdough has been continued for more than a century.

28.5 Dough Properties and Bread Quality

28.5.1 Dough Properties and Bread Texture

28.5.1.1 Wheat Dough and Bread

Addition of sourdoughs in wheat bread making influences the gluten proteins and the viscoelastic behavior of dough due to the drop in pH caused by the organic acids produced. The dough stability is decreased after mixing with sourdough (Collar et al. 1994; Wehrle and Arendt 1998). Proteolytic breakdown of proteins is enhanced at low pH during fermentation of wheat dough, and the effects are attributed both to changes in pH and to proteolytic activity from the cereal enzymes (Ganzle et al. 2008).

Despite the decreased stability in doughs with added sourdough, increased bread volume is reported for bread containing up to 20% sourdough (Collar et al. 1994; Hansen and Hansen 1996). The crumb structure of bread containing up to 20% sourdough has been comparable to standard bread without

sourdough, whereas inferior crumb structure was observed in bread containing 40% sourdough (Crowley et al. 2002).

28.5.1.2 Rye Dough and Bread

The main component of rye and wheat is starch, and its content has a crucial influence on the bread texture. It becomes sticky and pasty if the starch is degraded during the bread making due to too high activity of amylases. This problem is greater in rye bread making than for wheat bread, as the activity of the sprout-induced enzyme α-amylase is highest in rye (Seibel and Weipert 2001), but also because rye is used as flour with high extraction and the enzyme activity is highest in the germ and aleuron layer. Too high activity of amylases is seen in rainy summers in the rye-growing area. Furthermore, the period from harvest to possible sprouting is extremely short for rye; it can even sprout in the fields (Seibel and Weipert 2001). One of the main functions of sourdough in rye bread making is inactivation of the α-amylase activity, and a general rule in bakeries is to add a larger amount of sourdough when the activity of enzymes in the flour is high. Bread with a rye content of more than 20% normally requires the addition of sourdough to prevent degradation of starch (Crowley et al. 2002; Stolz and Boecker 1996). Rye starch begins to swell as low as 52°C, and subsequently, the α-amylase can degrade the starch until it will be heat-inactivated at 80°C (Seibel and Weipert 2001). Rye α-amylase has a pH optimum at pH 5.5 (Gabor et al. 1991) and the activity is totally inactivated in sourdough at pH below 4.

The activity of α-amylase is not only reduced considerably in the sourdough, but also in the rye dough with added sourdough. The activity of α-amylase in the bread dough after resting (pH 4.5), with 20% sourdough added, was about half the activity in the flour (Hansen et al. 2002).

Arabinoxylans play a key role in the viscosity of rye dough due to their high water-binding capacity. The viscosity of sourdoughs decreases during the sourdough fermentation due to the activity of the pentosan-degrading enzymes at the beginning of the fermentation. However, those enzymes are inactivated in the fermented sourdough (Hansen et al. 2002). Rye proteins are different from wheat proteins, as they do not form gluten structure. During sourdough fermentation, the proteins are partly degraded due to proteolytic activity caused by enzymes naturally present in the flour (Kratochvil and Holas 1983; Tuukkanen et al. 2005).

28.5.2 Flavor and Taste

The flavor of bread crumb depends mainly upon the flour type and the enzymatic reactions taking place due to yeast and sourdough fermentations, whereas the flavor of bread crust is more influenced by the thermal reactions during the baking process. Including sourdough in the bread recipe is recommended to achieve a more aromatic bread flavor, and sourdough bread has higher content of volatile compounds (Hansen and Schieberle 2005) and higher scores in sensory tests (Hansen and Hansen 1996; Varapha et al. 2000). Several flavor compounds in sourdough bread can be related to the content of the compounds in the corresponding sourdoughs (Hansen and Schieberle 2005). However, the content of flavor compounds in the bread is lower than in the sourdoughs because only 15%–25% of the flour has been fermented as sourdough, and some of the volatile compounds as, e.g., esters, evaporate during the baking process.

The content of volatile compounds produced during sourdough fermentation depends on the flour type (wheat, rye, maize, and rice), the extraction rate of the flour, the fermentation temperature, the water content in the sourdough, and the microorganisms in the sourdough. Generally, the LAB in the sourdough are mostly responsible for the acidification of the sourdough, and the sourdough yeasts for the production of flavor compounds. Lactic acid and acetic acid are the most important acids formed during sourdough fermentation. Lactic acid is a nonvolatile acid with an acid taste but without any smell, whereas acetic acid is a volatile acid with prickling smell together with the acid taste known from vinegar. The higher production of acetic acid in sourdoughs fermented with heterofermentative cultures can be increased with the addition of fructose as a hydrogen acceptor (Gobbetti et al. 1995a; Röcken et al. 1992).

The extraction rate of the flour and the water content in the sourdough mostly influences the acidification of the sourdough. Higher extraction rate of the flour results in higher production of lactic and

acetic acid (Gobbetti et al. 1995; Hansen and Hansen 1994a); however, sourdoughs fermented with heterofermentative cultures have much higher content of ethyl acetate.

Factors that favor the propagation of yeasts as higher water content in the sourdoughs and increased fermentation temperature will also result in increased production of the yeast fermentation products such as ethanol, isoalcohols, esters, and diacetyl (Gobbetti et al. 1995; Hansen et al. 1989b; Lund et al. 1989). The content of diacetyl and some other carbonyls are also higher in sourdoughs fermented with homofermentative LAB compared to heterofermentative cultures (Damiani et al. 1996; Hansen and Hansen 1994b).

Precursors, for the formation of important flavor compounds during the baking process as free amino acids and phenolic acids, can also increase during the sourdough fermentation (Gobbetti et al. 1994b; Hansen et al. 2002; Thiele et al. 2002). Free amino acids can affect bread flavor through the formation of products from nonenzymatic Maillard browning reactions, Strecker degradation, or by heat degradation. Ferulic acid is the most abundant phenolic acid in rye and wheat (Hansen et al. 2002), and it can be degraded to 4-vinylguajacol or vanillin during thermal reactions (Belitz et al. 2009), compounds that have also been identified in sourdough bread (Hansen et al. 1989a; Kirchhoff and Schieberle 2001).

Sensory evaluation of rye bread crumb shows that the most intense and breadlike flavor is associated with 2-propanone, 3-methyl-butanal, benzylalcohol, and 2-phenylethanol (Hansen et al. 1989a). However, vanillin, 2,3-butandione, 3-hydroxy-4,5-dimethyl-furanone, and methylbutanoic acids also contribute to the overall crumb flavor (Kirchhoff and Schieberle 2001). The perceived taste of salt is enhanced in sourdough rye bread compared to wheat bread, so less salt can be added in sourdough rye bread (Salovaara et al. 1982).

Sensory evaluation of wheat bread crumb showed that bread made with sourdough fermented with the heterofermentative *Lb. sanfranciscensis* had a pleasantly mild, sour odor and taste. Bread fermented with the homofermentative *Lb. plantarum* had an unpleasant metallic sour taste, but when the sourdough was also supplemented with the sourdough yeast *S. cerevisiae*, the bread acquired a more aromatic bread flavor. That bread had a higher content of methyl-butanol, methyl-propanoic acids, and 2-phenylethanol, which may, in part, cause the more aromatic flavor (Hansen and Hansen 1996). Mixed cultures with both LAB and yeast are recommended for an aromatic and pleasant sourdough bread flavor (Barber et al. 1989; Damiani et al. 1996; Hansen and Hansen 1996). A lexicon for description of the flavor of wheat sourdough bread has been developed (Lotong et al. 2000).

28.5.3 Longer Shelf Life

During storage of bread, several different physical and microbiological changes occur, lowering the quality of bread. The bread crumb becomes hard, the bread crust changes from crispy to leathery, and the characteristic and favorable bread flavor disappears. All these changes are characterized as the staling process. Within few days, the bread might be spoiled due to contamination and growth of molds on the surface or development of rope in the bread crumb caused by *Bacillus* spp. Addition of sourdough in the bread recipe can be used to retard the staling process of the bread, prevent the bread against ropiness and prolong the mold-free period. Sourdough addition is the most promising procedure to preserve bread from spoilage, since it is in agreement with the consumer demand for natural and additive-free food products.

28.5.3.1 Antimold Activity of Sourdough Bread

Mold is the most frequent cause of bread spoilage. Addition of sourdough in the bread recipe increases the mold-free period for rye bread (Lönner and Preve 1988; Spicher and Stephan 1999) and wheat bread (Barber et al. 1992; Salovaara and Valjakka 1987). The length of mold-free period was prolonged from 4 days in wheat bread to 6 to 8 days in sourdough bread. No correlation was found between pH and bread shelf life.

The mold-free period was prolonged 1 to 3 days in slices of sourdough rye bread inoculated with *Aspergillus glaucus* when the sourdough was fermented with heterofermentative LAB compared to

homofermentative LAB, or bread without addition of sourdough (Lönner and Preve 1988). The antimicrobial effect of the heterofermentative LAB was supposed to be the result of their production of acetic acid.

Two hundred thirty-two strains of sourdough LAB belonging to nine different species were screened for production of antimold substances against *Aspergillus niger*, *Fusarium graminearum*, *Penicillium expansum*, and *Monilia sitophila* using agar well-diffusion assay (Corsetti et al. 1998). The anti-mold activity varied very much among the strains and was mainly detected within heterofermentative LAB. *Lb. sanfrancisensis* had the largest spectrum of antimold activity. Not only the acetic acid had inhibitory effect, but the LAB produced also formic, propionic, butyric, *n*-valeric, and caproic acid, and a mixture thereof was responsible for the antimold effect.

28.5.3.2 Prevention of Rope Spoilage

Ropiness is spoilage of wheat bread noticed as an unpleasant odor similar to that of overripe melons, followed by the occurrence of a discolored sticky bread crumb and sticky threads that can be pulled from the crumb. This bread spoilage is caused by heat-resistant strains of *Bacillus* and occurs particularly in summer when the climate favors growth of the bacteria. It is mainly caused by *Bacillus subtilis*, formerly referred to as *B. mesentericus*, because the heat-resistant spores can survive the baking process, sporulate, and multiply in the baked bread. The rope symptoms can be recognized when the level of *Bacillus* in bread crumb is 10^8 bacillus/g (Rosenkvist and Hansen 1995). Its incidence has increased during the past decade, presumably because most bread is now produced without preservatives and often with the addition of raw materials such as oat products, wheat bran, and sunflower seed with a high contamination level of *Bacillus* spores (Rosenkvist and Hansen 1995). Even a low level of the heat-resistant spores (10^1–10^2 bacillus/g) in raw materials resulted in a level of 10^7 bacillus/g bread in 2 days.

One potential way to prevent development of rope is to include sourdough in the bread recipe. Addition of 10% sourdough inhibited the natural *Bacillus* contaminants in wheat dough, but it was insufficient to inhibit the bacillus strains inoculated at a level of 10^6 spores/g (Rosenquist and Hansen 1998). Addition of 15% sourdough was more efficient as the strains of rope-producing *Bacillus* were effectively inhibited by sourdough fermented by strains of *Lb. sanfranciscensis*, *Lb. brevis*, *Lb. maltaromicus*, or by three different strains of *Lb. plantarum*. In this investigation, *B. subtilis* tended to be inhibited if the TTA value in the sourdough was more than 10 and when the pH of the bread crumb was below 4.8. Röcken demonstrated that sourdough effectively decreased the heat resistance (D_{97} value) of a rope-producing strain of *Bacillus* (Röcken 1996). He found that the heat resistance was reduced from 143 minutes without the addition of sourdough to 5.9 and 6.9 minutes with the addition of 10% and 20% sourdough, respectively.

28.5.3.3 Bread Firmness and Staling Rate

Bread becomes firmer during storage, and retrogradation of starch toward a more crystalline form is considered to be the primary cause of this bread staling. Several sourdoughs have been investigated for their potential effect on delaying the development of bread firmness and staling rate of wheat bread, and delayed staling rate has been observed in sourdough bread (Corsetti et al. 2000). The rate of starch retrogradation was not influenced if the acidification was rather low, whereas a standard sourdough (*Lb. sanfranciscensis* 57, *Lb. plantarum* 13, *S. cerevisiae* 141) was able to retard the staling rate. The staling rate was mostly influenced if the starter culture had amylolytic activity (*Lb. amylovorus* or a genetic modified strain *Lb. sanfranciscensis CB1 Amy*).

28.6 Nutritional Value

The addition of sourdough to the bread recipe has a positive influence on the nutritive value of the bread, as the minerals become bioavailable (Lopez et al. 2001; Nielsen et al. 2007), and blood glucose and insulin responses are lowered after eating sourdough bread compared to wheat bread (Liljeberg and Bjorck 1996).

28.6.1 Reduced Phytate Content by Sourdough

Whole-meal cereals are good sources of minerals such as K, P, Mg, Fe, and Zn; but without treatment the bioavailability is poor for minerals that are stored as phytate, an insoluble complex with phytic acid (myoinositol hexa-phosphoric acid). The content of phytate is 6 mg/g rye grain (Greiner et al. 1998), 3–4 mg/g in flour of soft wheat, and 9 mg/g in hard wheat flour (Garcia-Estepa et al. 1999). Phytate accounts for more than 70% of the total phosphorus in cereals, and it can be degraded during the bread-making process due to the activity of endogenous phytase and thus liberate the bound minerals when the ester-bound phosphoric acids are hydrolyzed. The optimum pH of rye phytase is found to be at pH 6.0 (Greiner et al. 1998), so the positive influence of sourdough fermentation on the degradation of phytate is the long process where the cereal phytase can degrade the phytate even if the enzyme activity is only about half the activity at optimum pH (Nielsen et al. 2007).

Sourdough fermentation has been shown to be more efficient than yeast fermentation in reducing the phytate content in whole bread (minus 62% and 38%, respectively) due to the long fermentation time leading to increased solubility of Mg and P (Lopez et al. 2001). Recently, external phytase activity has been measured in selected strains of LAB and yeasts isolated from bakery sourdoughs (unpublished results).

28.6.2 Reduced Glycemic Response with Sourdough Bread

Conventional wheat bread products are rapidly digested and absorbed, thus giving rise to high blood glucose and insulin responses. Eating wholemeal sourdough bread resulted in both lowered blood glucose and insulin response compared with eating wholemeal bread made without sourdough (Liljeberg et al. 1995). This nutritional positive effect was possibly due to a reduced gastric emptying rate caused by the lactic acid produced during the sourdough fermentation.

REFERENCES

Abd-el-Malek Y, El-Leithy MA, Awad YN. 1973. Microbiological studies on Egyptian "balady" bread-making. I. Microbial content and chemical properties of the flour. *Chemie, Microbiologie, Technologie der Lebensmittel* 2:60–2.

Almeida MJ, Pais CS. 1996. Characterization of the yeast population from traditional corn and rye bread doughs. *Lett Appl Microbiol* 23:154–8.

Aslam Z, Im WT, Ten LN, Lee MJ, Kim KH, Lee ST. 2006. *Lactobacillus siliginis* sp nov., isolated from wheat sourdough in South Korea. *Int J Syst Evol Microbiol* 56:2209–13.

Azar M, Ter Sarkissian N, Ghavifek H, Ferguson T, Ghassemi H. 1977. Microbiological aspects of Sangak bread. *J Food Sci Technol* 14:251–4.

Barber B, Ortola C, Barber S, Fernandez F. 1992. Storage of packaged white bread.3. Effects of sour dough and addition of acids on bread characteristics. *Zeitschrift fur Lebensmittel-Untersuchung Und-Forschung* 194:442–9.

Barber S, Baguena R. 1988. Microflora of the sour dough of wheat-flour bread.5. Isolation, identification and evaluation of functional properties of sour doughs microorganisms. *Revista de Agroquimica y Tecnologia de Alimentos* 28:67–78.

Barber S, Torner MJ, Martinezanaya MA, Debarber CB. 1989. Microflora of the sour dough of wheat-flour bread.9. biochemical characteristics and baking performance of wheat doughs elaborated with mixtures of pure microorganisms. *Zeitschrift fur Lebensmittel-Untersuchung Und-Forschung* 189:6–11.

Barnett JA, Payne RW, Yarrow D. 2000. Yeast: Characteristics and identification. 3rd ed. Cambridge: Cambridge University Press. p. 15–22.

Belitz H-D, Grosch W, Schieberle P. 2009. *Food Chemistry*. 4th ed. Berlin: Springer.

Böcker G, Stolz P, Hammes WP. 1995. New results on the eco system sour dough and on the physiology of sour dough specific of *Lactobacillus sanfrancisco* and *Lactobacillus pontis. Getreide, Mehl und Brot* 49:370–4.

Böcker G, Vogel RF, Hammes WP. 1990. *Lactobacillus sanfrancisco* als stabiles element in einem reinzucht-sauerteig-präparat. *Getreide, Mehl und Brot* 44:269–74.

Boraam F, Faid M, Larpent JP, Breton A. 1993. Lactic-acid bacteria and yeast associated with traditional Moroccan sour-dough bread fermentation. *Sciences des Aliments* 13:501–9.

Brümmer JM, Lorenz K. 1991. European developments in wheat sourdoughs. *Cereal Foods World* 36:310–4.

Cai YM, Okada H, Mori H, Benno Y, Nakase T. 1999. *Lactobacillus paralimentarius* sp nov., isolated from sourdough. *Int J Syst Bacteriol* 49:1451–5.

Collar EC, Benedito dB, Martinez-Anaya MA. 1994. Microbial sour doughs influence acidification properties and breadmaking potential of wheat dough. *J Food Sci* 59:629–33.

Corsetti A, Gobbetti M, De Marco B, Balestrieri F, Paoletti F, Russi L, et al. 2000. Combined effect of sourdough lactic acid bacteria and additives on bread firmness and staling. *J Agric Food Chem* 48:3044–51.

Corsetti A, Gobbetti M, Rossi J, Damiani P. 1998. Antimould activity of sourdough lactic acid bacteria: identification of a mixture of organic acids produced by *Lactobacillus sanfrancisco* cb1. *Appl Microbiol Biotechnol* 50:253–6.

Corsetti A, Lavermicocca P, Morea M, Baruzzi F, Tosti N, Gobbetti M. 2001. Phenotypic and molecular identification and clustering of lactic acid bacteria and yeasts from wheat (species *Triticum durum* and *Triticum aestivum*) sourdoughs of Southern Italy. *Int J Food Microbiol* 64:95–104.

Corsetti A, Settanni L, van Sinderen D, Felis GE, Dellaglio F, Gobbetti M. 2005. *Lactobacillus rossii* sp nov., isolated from wheat sourdough. *Int J Syst Evol Microbiol* 55:35–40.

Corsetti A, Settanni L. 2007. Lactobacilli in sourdough fermentation. *Food Res Int* 40:539–58.

Crowley P, Schober TJ, Clarke CI, Arendt EK. 2002. The effect of storage time on textural and crumb grain characteristics of sourdough wheat bread. *Eur Food Res Technol* 214:489–96.

Damiani P, Gobbetti M, Cossignani L, Corsetti A, Simonetti MS, Rossi J. 1996. The sourdough microflora. Characterization of hetero- and homofermentative lactic acid bacteria, yeasts and their interactions on the basis of the volatile compounds produced. *Food Sci Technol-Lebensmittel-Wissenschaft Technol* 29:63–70.

De Vuyst L, Schrijvers V, Paramithiotis S, Hoste B, Vancanneyt M, Swings J, et al. 2002. The biodiversity of lactic acid bacteria in Greek traditional wheat sourdoughs is reflected in both composition and metabolite formation. *Appl Environ Microbiol* 68:6059–69.

De Vuyst L, Vandamme EJ. 1994. Antimicrobial potential of lactic acid bacteria. In: De Vuyst L, Vandamme EJ, editors. *Bacteriocins of Lactic Acid Bacteria: Microbiology, Genetics and Applications*. London: Blackie Academic and Professional. p 91–142.

Delcour JA, Hoseney RC. 2010. *Principles of Cereal Science and Technology*. 3rd. ed. St. Paul, MN: AACC.

Ehrmann MA, Muller MRA, Vogel RF. 2003. Molecular analysis of sourdough reveals *Lactobacillus mindensis* sp nov. *Int J Syst Evol Microbiol* 53:7–13.

Faid M, Boraam F, Achbab A, Larpent JP. 1993. Yeast-lactic acid bacteria interactions in Moroccan sour-dough bread fermentation. *Food Sci Technol-Lebensmittel-Wissenschaft Technol* 26:443–6.

Foramitti A. 1982. [Continuous mechanized production of sourdough.] Kontinuierliche und mechanisierte herstellung von sauerteig. *Getreide, Mehl und Brot* 36:47–50.

Foschino R, Galli A, Perrone F, Ottogalli G. 1995a. Bakery products obtained with sour dough: investigation and characterization of the typical microflora. *Tecnica Molitoria* 46:485–502.

Foschino R, Perrone F, Galli A. 1995b. Characterization of two virulent *Lactobacillus fermentum* bacteriophages isolated from sour dough. *J Appl Bacteriol* 79:677–83.

Foschino R, Terraneo R, Mora D, Galli A. 1999. Microbial characterization of sourdoughs for sweet baked products. *Italian J Food Sci* 11:19–28.

Gabor R, Taufel A, Behnke U, Heckel J. 1991. Studies on the germination specific alpha-amylase and its inhibitor of rye (*Secale cereale*).1. Isolation and characterization of the enzyme. *Zeitschrift fur Lebensmittel-Untersuchung Und-Forschung* 192:230–3.

Galli A, Franzetti L, Fortina MG. 1988. Isolation and identification of sour dough microflora. *Microbiol Aliments Nutri* 6:345–51.

Ganzle MG, Ehmann M, Hammes WP. 1998. Modeling of growth of *Lactobacillus sanfranciscensis* and *Candida milleri* in response to process parameters of sourdough fermentation. *Appl Environ Microbiol* 64:2616–23.

Ganzle MG, Loponen J, Gobbetti M. 2008. Proteolysis in sourdough fermentations: mechanisms and potential for improved bread quality. *Trends Food Sci Technol* 19:513–21.

Garcia-Estepa RM, Guerra-Hernandez E, Garcia-Villanova B. 1999. Phytic acid content in milled cereal products and breads. *Food Res Int* 32:217–21.

Gobbetti M. 1998. The sourdough microflora: interactions of lactic acid bacteria and yeasts. *Trends Food Sci Technol* 9:267–74.

Gobbetti M, Corsetti A, Rossi J, Rosa FL, de Vincenzi S. 1994a. Identification and clustering of lactic acid bacteria and yeasts from wheat sourdoughs of Central Italy. *Italian J Food Sci* 6:85–94.

Gobbetti M, Simonetti MS, Corsetti A, Santinelli F, Rossi J, Damiani P. 1995. Volatile compound and organic acid productions by mixed wheat sour dough starters: influence of fermentation parameters and dynamics during baking. *Food Microbiol* 12:497–507.

Gobbetti M, Simonetti MS, Rossi J, Cossignani L, Corsetti A, Damiani P. 1994b. Free d-amino-acid and l-amino-acid evolution during sourdough fermentation and baking. *J Food Sci* 59:881–4.

Greiner R, Konietzny U, Jany KD. 1998. Purification and properties of a phytase from rye. *J Food Biochem* 22:143–61.

Hammes WP, Stolz P, Gaenzle M. 1996. Metabolism of lactobacilli in traditional sourdoughs. *Adv Food Sci* 18:176–84.

Hammes WP, Vogel RF. 1995. The genus *Lactobacillus*. In Wood BJB, Holzapfel WH, editors. *The Genera of Lactic Acid Bacteria*. London: Blackie Academic & Professional. p 19–54.

Hansen A, Hansen B. 1994a. Influence of wheat flour type on the production of flavour compounds in wheat sourdoughs. *J Cereal Sci* 19:185–90.

Hansen A, Hansen B. 1996. Flavour of sourdough wheat bread crumb. *Zeitschrift fur Lebensmittel-Untersuchung Und-Forschung* 202:244–9.

Hansen A, Lund B, Lewis MJ. 1989a. Flavor of sourdough rye bread crumb. *Lebensmittel-Wissenschaft & Technologie* 22:141–4.

Hansen A, Lund B, Lewis MJ. 1989b. Flavour production and acidification of sourdoughs in relation to starter culture and fermentation temperature. *Lebensmittel Wissenschaft und Technologie* 22:145–9.

Hansen A, Schieberle P. 2005. Generation of aroma compounds during sourdough fermentation: applied and fundamental aspects. *Trends Food Sci Technol* 16:85–94.

Hansen B, Hansen A. 1994b. Volatile compounds in wheat sourdoughs produced by lactic-acid bacteria and sourdough yeasts. *Zeitschrift fur Lebensmittel-Untersuchung Und-Forschung* 198:202–9.

Hansen HB, Andreasen MF, Nielsen MM, Larsen LM, Knudsen KEB, Meyer AS, et al. 2002. Changes in dietary fibre, phenolic acids and activity of endogenous enzymes during rye bread-making. *Eur Food Res Technol* 214:33–42.

Kandler O, Weiss N. 1986. Genus *Lactobacillus*. In: Sneath PHA, Mair NS, Sharpe ME, editors. Bergey's Manual of Systematic Bacteriology. Baltimore: The Williams and Wilkins Company. p 1209–34.

Kazanskaya LN, Afanasyeva OV, Patt VA. 1983. Microflora of rye sours and some specific features of its accumulation in bread baking plants of the USSR. *Dev Food Sci* 5B:759–63.

Kirchhoff E, Schieberle P. 2001. Determination of key aroma compounds in the crumb of a three-stage sourdough rye bread by stable isotope dilution assays and sensory studies. *J Agric Food Chem* 49:4304–11.

Kline L, Sugihara TF, McCready LB. 1970. Nature of the sanfrancisco sour dough french bread process. I. Mechanics of the process. *Bakers' Dig* 44:48–50.

Knudsen S. 1924. Milchsäurenbakteries des Sauerteiges und ihre Bedeutung für die Sauerteiggärung. In: *Den Kongelige Veterinær-og Landbohøjskole, Årsskrift 1924*. Copenhagen: Kandrup and Wunsch. p 133–86.

Kratochvil J, Holas J. 1983. Study of proteolytic processes in rye sour. In: Holas J, Kratochvil J, editors. *Progress in Cereal Chemistry and Technology*. Oxford: Elsevier. p 791–8.

Kreger van Rij NJW. 1984. *The Yeasts, a Taxonomy Study*. 3rd ed. Amsterdam, The Netherlands: Elsevier Science Publisher.

Kurtzman CP, Fell JW. 1998. The Yeasts, A Taxonomic Study. Fourth edition. Amsterdam, Elsevier. p 1–7.

Lefebvre D, Gabriel V, Vayssier Y, Fontagne-Faucher C. 2002. Simultaneous HPLC determination of sugars, organic acids and ethanol in sourdough process. *Lebensmittel-Wissenschaft Und-Technologie-Food Science and Technology* 35:407–14.

Liljeberg HGM, Bjorck IME. 1996. Delayed gastric emptying rate as a potential mechanism for lowered glycemia after eating sourdough bread: studies in humans and rats using test products with added organic acids or an organic salt. *Am J Clin Nutr* 64:886–93.

Liljeberg HGM, Lonner CH, Bjorck IME. 1995. Sourdough fermentation or addition of organic-acids or corresponding salts to bread improves nutritional properties of starch in healthy humans. *J Nutr* 125:1503–11.

Lönner C, Preve AK. 1988. Acidification properties of lactic acid bacteria in rye sour doughs. *Food Microbiol* 5:43–58.

Lopez HW, Krespine V, Guy C, Messager A, Demigne C, Remesy C. 2001. Prolonged fermentation of whole wheat sourdough reduces phytate level and increases soluble magnesium. *J Agric Food Chem* 49:2657–62.

Lotong V, Chambers E, Chambers DH. 2000. Determination of the sensory attributes of wheat sourdough bread. *J Sens Stud* 15:309–26.

Lund B, Hansen A, Lewis MJ. 1989. The influence of dough yield on acidification and production of volatiles in sourdoughs. *Lebensmittel Wissenschaft und Technologie* 22:150–3.

Mantynen VH, Korhola M, Gudmundsson H, Turakainen H, Alfredsson GA, Salovaara H, et al. 1999. A polyphasic study on the taxonomic position of industrial sour dough yeasts. *Syst Appl Microbiol* 22:87–96.

Meroth CB, Hammes WP, Hertel C. 2004. Characterisation of the microbiota of rice sourdoughs and description of *Lactobacilius spicheri* sp nov. *Syst Appl Microbiol* 27:151–9.

Messens W, De Vuyst L. 2002. Inhibitory substances produced by lactobacilli isolated from sourdoughs—A review. *Int J Food Microbiol* 72:31–43.

Muller MRA, Ehrmann MA, Vogel RF. 2000. *Lactobacillus frumenti* sp nov., a new lactic acid bacterium isolated from rye-bran fermentations with a long fermentation period. *Int J Syst Evol Microbiol* 50:2127–33.

Nielsen MM, Daniels DGH, Thomsen AD, Rasmussen SK, Hansen Å. 2007. Phytase activity and degradation of phytic acid during rye bread making. *Eur Food Res Technol* 225:173-81.

Nout MJR, Creemers MT. 1987. Microbiological properties of some wheatmeal sourdough starters. *Chemie Mikrobiologie Technologie der Lebensmittel* 10:162–7.

Okada S, Ishikawa M, Yoshida I, Uchimura T, Ohara N, Kozaki M. 1992. Identification and characteristics of lactic-acid bacteria isolated from sour dough sponges. *Biosci Biotechnol Biochem* 56:572–5.

Ottogalli G, Galli A, Foschino R. 1996. Italian bakery products obtained with sour dough: characterization of the typical microflora. *Adv Food Sci* 18:131–44.

Pederson CS. 1979. Some cereal foods. In: Pederson CS, editor. *Microbiology of Food Fermentations*. 2nd ed. Westport, Connecticut: AVI Publishing Co. p 235–70.

Robert H, Gabriel V, Fontagne-Faucher C. 2009. Biodiversity of lactic acid bacteria in French wheat sourdough as determined by molecular characterization using species-specific PCR. *Int J Food Microbiol* 135:53–9.

Rocha JM, Malcata FX. 1999. On the microbiological profile of traditional Portuguese sourdough. *J Food Prot* 62:1416–29.

Röcken W. 1996. Applied aspects of sourdough fermentation. *Adv Food Sci* 18:212–6.

Röcken W, Rick M, Reinkemeier M. 1992. Controlled production of acetic-acid in wheat sour doughs. *Zeitschrift fur Lebensmittel-Untersuchung Und-Forschung* 195:259–63.

Rosenkvist H, Hansen A. 1995. Contamination profiles and characterization of *Bacillus* species in wheat bread and raw-materials for bread production. *Int J Food Microbiol* 26:353–63.

Rosenquist H, Hansen A. 1998. The antimicrobial effect of organic acids, sour dough and nisin against *Bacillus subtilis* and *B. licheniformis* isolated from wheat bread. *J Appl Microbiol* 85:621–31.

Rosenquist H, Hansen A. 2000. The microbial stability of two bakery sourdoughs made from conventionally and organically grown rye. *Food Microbiol* 17:241–50.

Rossi J. 1996. The yeasts in sourdough. *Adv Food Sci* 18:201–11.

Salovaara H, Hellemann U, Kurkula R. 1982. Effect of salt on bread flavour. *Lebensmittel-Wissenschaft Technologie* 15:270–4.

Salovaara H, Katunpaa H. 1984. An approach to the classification of lactobacilli isolated from Finnish sour rye dough ferments. *Acta Aliment Pol* 10:231–9.

Salovaara H, Savolainen J. 1984. Yeast type isolated from Finnish sour rye dough starters. *Acta Aliment Pol* 10:241–5.

Salovaara H, Valjakka T. 1987. The effect of fermentation temperature, flour type, and starter on the properties of sour wheat bread. *International J Food Sci Technol* 22:591–7.

Sanni AI, Morlon-Guyot J, Guyot JP. 2002. New efficient amylase-producing strains of *Lactobacillus plantarum* and *L. fermentum* isolated from different nigerian traditional fermented foods. *Int J Food Microbiol* 72:53–62.

Sanni AI, Onilude AA, Fatungase MO. 1998. Production of sour maize bread using starter-cultures. *World J Microbiol Biotechnol* 14:101–6.

Scheirlinck I, Van der Meulen R, Van Schoor A, Cleenwerck I, Huys G, Vandamme P, et al. 2007a. *Lactobacillus namurensis* sp nov., isolated from a traditional Belgian sourdough. *Int J Syst Evol Microbiol* 57:223–7.

Scheirlinck I, Van der Meulen R, Van Schoor A, Huys G, Vandamme P, De Vuyst L, et al. 2007b. *Lactobacillus crustorum* sp nov., isolated from two traditional Belgian wheat sourdoughs. *Int J Syst Evol Microbiol* 57:1461–7.

Scheirlinck I, Van der Meulen R, Van Schoor A, Vancanneyt M, De Vuyst L, Vandamme P, et al. 2007c. Influence of geographical origin and flour type on diversity of lactic acid bacteria in traditional Belgian sourdoughs. *Appl Environ Microbiol* 73:6262–9.

Seibel W, Weipert D. 2001. Bread making and other food uses around the world. In: Bushuk W, editor. *Rye Production, Chemistry and Technology*. 2nd ed. St. Paul, USA: American Association of Cereal Chemists. p 147–211.

Simopoulos AP. 2001. The Mediterranean diets: what is so special about the diet of Greece? The scientific evidence. *J Nutr* 131:3065S–73S.

Spicher G, Lonner C. 1985. The microflora of sourdough.21. The *Lactobacillus* species of sourdough from Swedish bakeries. *Zeitschrift fur Lebensmittel-Untersuchung Und-Forschung* 181:9–13.

Spicher G, Stephan H. 1999. *Handbuch sauerteig, biologie, biochemie, technologie.* 5th ed. Hamburg: BEHR´S Verlag. 403 p.

Stolz P, Bocker G, Vogel RF, Hammes WP. 1993. Utilization of maltose and glucose by lactobacilli isolated from sourdough. *FEMS Microbiol Lett* 109:237–42.

Stolz P, Boecker G. 1996. Technology, properties and applications of sourdough products. *Adv Food Sci* 18:234–6.

Strohmar W, Diekmann H. 1992. The microflora of a sourdough developed during extended souring phases. *Zeitschrift fur Lebensmittel-Untersuchung Und-Forschung* 194:536–40.

Sugihara TF, Kline L, McCready LB. 1970. Nature of the sanfrancisco sour dough french bread process. I: Microbiological aspects. *Bakers' Dig* 44:51–3.

Sugihara TF, Kline L, Miller MW. 1971. Microorganisms of sanfrancisco sour dough bread process.1. Yeasts responsible for leavening action. *Appl Microbiol* 21:456–8.

Suihko ML, Maekinen V. 1984. Tolerance of acetate, propionate, and sorbate by *Saccharomyces cerevisiae* and *Torulopsis holmii. Food Microbiol* 1:105–10.

Thiele C, Ganzle MG, Vogel RF. 2002. Contribution of sourdough lactobacilli, yeast, and cereal enzymes to the generation of amino acids in dough relevant for bread flavor. *Cereal Chem* 79:45–51.

Tuukkanen K, Loponen J, Mikola M, Sontag-Strohm T, Salovaara H. 2005. Degradation of secalins during rye sourdough fermentation. *Cereal Chem* 82:677–82.

Valcheva R, Ferchichi MF, Korakli M, Ivanova I, Ganzle MG, Vogel RF, et al. 2006. *Lactobacillus nantensis* sp nov., isolated from French wheat sourdough. *Int J Syst Evol Microbiol* 56:587–91.

Valcheva R, Korakli M, Onno B, Prevost H, Ivanova I, Ehrmann MA, et al. 2005. *Lactobacillus hammesii* sp nov., isolated from French sourdough. *Int J Syst Evol Microbiol* 55:763–7.

Vancanneyt M, Neysens P, De Wachter M, Engelbeen K, Snauwaert C, Cleenwerck I, et al. 2005. *Lactobacillus acidifarinae* sp nov. and *Lactobacillus zymae* sp nov., from wheat sourdoughs. *Int J Syst Evol Microbiol* 55:615–20.

Varapha L, Chambers-E-IV, Chambers DH. 2000. Determination of the sensory attributes of wheat sourdough bread. *J Sens Stud* 15:309–26.

Vogel RF, Bocker G, Stolz P, Ehrmann M, Fanta D, Ludwig W, et al. 1994. Identification of lactobacilli from sourdough and description of *Lactobacillus pontis* sp nov. *Int J Syst Bacteriol* 44:223–9.

Vogel RF, Mueller M, Stolz P, Ehrmann M. 1996. Ecology in sourdoughs produced by traditional and modern technologies. *Adv Food Sci* 18:152–9.

Wehrle K, Arendt EK. 1998. Rheological changes in wheat sourdough during controlled and spontaneous fermentation. *Cereal Chem* 75:882–6.

Wiese BG, Strohmar W, Rainey FA, Diekmann H. 1996. *Lactobacillus panis* sp nov., from sourdough with a long fermentation period. *Int J Syst Bacteriol* 46:449–53.

Wlodarczyk M. 1986. Characteristic of yeasts from spontaneously fermenting bread starters. *Acta Aliment Pol* 12:103–8.

Wood ED. 1996. Back to the first sourdough. In: Wood ED, editor. *World Sourdough from Antiquity*. Berkeley, California: Ten Speed Press. p 7–17.

Yarrow D. 1998. Methods for isolation, maintenance and identification of yeasts. In: Kurtzmann CP, Fell JW, editors. *The Yeasts: A Taxonomic Study*. Amsterdam: Elsevier. p 77–100.

29

Liquid Sourdough Fermentation

Paola Carnevali, Fabio Minervini, and Aldo Corsetti

CONTENTS

29.1 Introduction

Industrial wheat bread production started at the middle of the 20th century, after the introduction of bakers' yeast as a fast and ready-to-use leavening agent and therefore ideal substitute for traditional sourdough or brewing yeast. Over decades, many different bread-making processes have been developed, which have the common aim of converting wheat flour and other ingredients into a light aerated and palatable food. The most important quality characteristics for wheat breads are high volume, soft and elastic crumb structure, good shelf life, and microbiological safety of the product (Cauvain 2003). Unfortunately, wheat bread has a relatively short shelf life because of microbial spoilage and physical and chemical changes in the starch-protein matrix of the bread crumb, which start immediately after baking and lead to the staling process (Selomulyo and others 2007).

Currently, a number of additives are available for the baking industry: emulsifiers, hydrocolloids, enzymes, organic acids, and their salts. Such additives allow to ensure and possibly maintain good textural and sensory properties and prolong the shelf life. However, most of these substances are E-numbered additives, which do not tally with current consumer trends preferring high-quality products baked without chemical additives (Katina 2005). Few companies have proposed a range of additive-free products, but very often these have a limited shelf life. Sourdough has been, is, and probably will be more and more the unique traditional starter to obtain otherwise irreproducible quality of leavened baked goods (Gobbetti and Gänzle 2007). Indeed, the use of sourdough improves sensory, nutritional, and storage properties (Gobbetti et al. 2005) and it has remarkable effects on bread volume and crumb structure as well (Arendt et al. 2007). Unique sourdoughs with a selection of an immense biodiversity of lactic acid bacteria (LAB) and yeasts are manufactured and used at both artisan and industrial levels (Gobbetti and Gänzle, 2007). For instance, in Italy, sourdough is used for manufacturing several typical and/or traditional baked goods, such as the Apulian durum wheat bread "Pane di Altamura" PDO and a renowned Christmas cake named *Panettone*. An increasing number of studies is focusing on innovative properties and/or application of sourdough-based baked goods: antimicrobial activity for controlling spoilage microorganisms (for a review, see Settanni and Corsetti 2008); improvement of mineral bioavailability, decreased glycaemic index, and improved quality of whole-grain or fiber-rich products (for a review, see Poutanen et al. 2009); improved quality of gluten-free products (for a review, see Moroni et al. 2009); content in exopolysaccharides (EPS) benefiting texture and shelf life (Tieking and Gänzle, 2005). Therefore, since the last decades of the 20th century, the interest of the bakery industry focused on the applications of sourdough to a wide range of baked goods.

After an overview on the types of sourdough, this chapter will focus on the so-called liquid sourdough (alias type II sourdough) and its applications. Indeed, a clear preference of the bakery industry for this type of sourdough may be observed due to its easy management and great flexibility. Thus, liquid sourdough may be regarded by bakery industries as the best way to combine tradition and innovation in baked goods endowed with superior quality.

29.2 Types of Sourdough

Sourdough technology is diffused throughout the world to produce leavened baked products with characteristic features such as palatability, high nutritional values, and extended shelf life (Corsetti and Settanni 2007). Depending on the technology applied, three types of sourdough can be considered, as defined by Böcker et al. (1995).

Type I sourdoughs are generally firm doughs (dough yield [DY] of about 160) whose activity relies on a mixed population of LAB (mainly represented by obligately heterofermentative lactobacilli such as *Lactobacillus sanfranciscensis*, *Lactobacillus pontis*, *Lactobacillus brevis*, *Lactobacillus fermentum*, and *Lactobacillus fructivorans*) and yeasts (*Candida milleri*, *Candida holmii*, *Saccharomyces cerevisiae*, and *Saccharomyces exiguus*, recently renamed as *Kazachstania exigua*), which are kept metabolically active due to daily refreshments (or rebuildings, replenishments, back-slopping). In a traditional three-stage protocol, three refreshments per day are performed; by combining DY values, time, and temperature of fermentation, a correct balance between LAB and yeast is maintained. At the end of the last step of fermentation, sourdough is used as a natural starter culture containing many microbial strains (Hammes 1991). Type II sourdoughs are generally liquid doughs (DY of about 200) dominated by pH-resistant lactobacilli such as *Lactobacillus panis*, *Lactobacillus reuteri*, *Lactobacillus johnsonii*, *L. sanfranciscensis*, and *L. pontis* (Böcker et al. 1995; Wiese et al. 1996). They are obtained by a unique step of fermentation of 15–20 hours in bioreactors or tanks at a controlled temperature higher than 30°C, after which they can be stored for many days at low temperature (Decock and Cappelle 2005). When needed, a portion of the mature sourdough can be added to a dough to acidify and enrich it with flavor compounds characteristics of sourdough-leavened baked products. The leavening of the dough to be baked relies on the addition of commercial bakers' yeast, because the long fermentation time and high DY and temperature of fermentation inhibit spontaneous yeasts. With such a type of sourdough as the subject of the present chapter, it will be further discussed in the next paragraph. Type II sourdough also represent the basis for type III sourdoughs (Decock and Cappelle 2005). Those are actually dried preparations deriving from liquid sourdoughs and, due to the protocol applied to their manufacture (spray drying or drum drying (see Decock and Cappelle 2005), they are dominated by drying-resistant LAB such as *Pediococcus pentosaceus*, *Lactobacillus plantarum*, and *L. brevis* (Böcker et al. 1995). Type III sourdoughs are essentially used at the industrial level as flavoring agents, and their characteristics and flavoring activity are determined by various time/temperature combinations applied during drying, in their turn influencing Maillard reaction and degree of caramelization or toasting (Decock and Cappelle 2005). As type II sourdough, they require the addition of bakers' yeast (*Saccharomyces cerevisiae*) as a leavening agent (Corsetti and Settanni, 2007).

29.3 Technology of Liquid Fermentation

Currently, bakery industries require shortening of the leavening time, ready-to-use starters, an easy management of the biological leavening agent, and a high quality and standardization of the leavened baked goods. Although the use of bakers' yeast (*Saccharomyces cerevisiae*) leads to a very rapid dough fermentation and represents a microbial starter that is very easy to manage, it gives baked goods flavor, texture, and extension of shelf life inferior to those obtained through the use of sourdough (Carnevali et al. 2007). In this context, liquid sourdough, in combination with bakers' yeast, may represent a valid technological option for fulfilling demands of modern bakery industries, because it has a number of advantages compared to traditional sourdough: easier management and reproducibility of the fermentation; easier

control of fundamental fermentation parameters, such as DY, temperature, and pH; higher suitability to steer microbial metabolism toward the production of desirable compounds, for instance, through addition and uniform distribution of specific nutrients; and higher versatility, i.e., it may be used as a natural starter in the protocol of production of a number of different baked goods without substantial formulation variations (Carnevali et al. 2007).

Although it has been previously shown that parameters such as sugar concentrations and yeast cell density did not cause any significant changes in the rheology properties of liquid sourdough (Mastilovic and Popov 2001a,b), our industrial application demonstrated that the type and amount of flour and the temperature of fermentation significantly influenced the rheology properties of experimental liquid sourdoughs produced at a pilot plant (Carnevali et al. 2007). Besides studying the influence of those parameters on the rheology of liquid sourdough, Barilla's R&D in cooperation with the Universities of Bari and of Bologna, carried out several research projects on liquid sourdoughs aiming to optimize fermentation parameters such as flour composition, initial cell density of the starter, temperature, aeration, time; select an association between autochthonous LAB and yeasts capable of adapting to liquid sourdough technology and of improving overall quality of the baked goods derived from; and scout differences and implications coming from the use of fresh or frozen or freeze-dried microbial cells used to inoculate liquid sourdoughs. A common experimental scheme was followed (Figure 29.1) and all fermentations were carried out in a bioreactor (Figure 29.2) under controlled pH. Concentrations of primary (e.g., organic acids, ethanol) and secondary (e.g., free amino acids, esters, aldehydes, alcohols) metabolites were determined in liquid sourdoughs and different analytical fingerprints were obtained for each association subjected to selection (Carnevali et al. 2007). Among technological parameters, flour composition, initial cell density, and time of fermentation appeared to be the most important for optimizing the liquid fermentation. Only a few strains of LAB seemed to be suitable for liquid fermentation, alone or in association with yeast, and one strain, *L. sanfranciscensis* Lb1, belonging to the Barilla's collection, was used to produce a liquid sourdough to be tested under different bakery technologies. The best sensorial impact derived from the use of this selected sourdough was perceived in the resulting bread. Results from biochemical characterization of the liquid sourdough started with *L. sanfranciscensis* Lb1 and from bread were elaborated through a principal component analysis. Bread crust and crumb were separately analyzed, and a bread produced with bakers' yeast was used as control. In the loading plot shown in Figure 29.3, components 1 and 2 represent the map of metabolites at the end of sourdough fermentation and after baking, respectively. Distribution of samples (Lb1-liquid sourdough, crust of Lb1-bread, crumb of Lb1-bread and bakers' yeast bread) is shown in Figure 29.4. Although the majority of metabolites influencing the

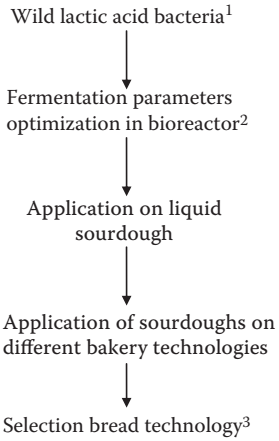

FIGURE 29.1 Scheme of the study carried out for wild strains selection. [1]Wild-type strains were isolated from traditional sourdough. [2]Setup of the fermentation conditions was made to favor the metabolic properties of specific strains. [3]Liquid sourdough technology was applied to different bakery processes to define the best procedure to obtain a bread with a particular flavor and sensory profile. (Reprinted from *Food Microbiology*, 24, Carnevali P. et al., Liquid sourdough: Industrial Application Perspectives, pp. 2–4, Copyright (2007), with permission from *Elsevier*.)

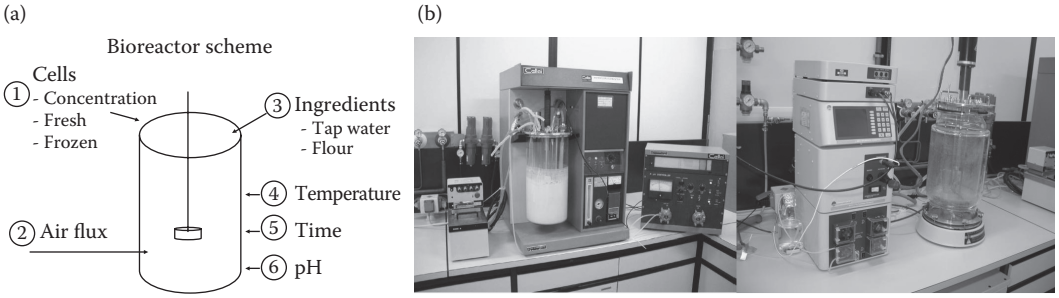

FIGURE 29.2 Bioreactor scheme and picture. The bioreactor was used to obtain the different experimental liquid sourdoughs. Various parameters one can set during liquid fermentation are reported.

flavor were produced upon baking, many aroma precursors were formed during sourdough fermentation. Use of liquid sourdough started with Lb1 mainly influenced the distribution of aroma-related metabolites in bread crust, whereas such metabolites were quite similarly distributed in crumb of bread made from liquid sourdough and in control bread (Carnevali et al. 2007). Performances of liquid sourdough started with Lb1 were reproducible at an industrial plant. Metabolite profiles were confirmed (data not shown) and a panel tested was carried out on bread made with Lb1 liquid sourdough and on bread made with bakers' yeast. Figure 29.5 shows the sensory profiles of two breads. Bread made with Lb1 received higher scores for acidity, typical note, taste intensity, and salty. Interestingly, comparable results were obtained when freeze-dried cells of Lb1 were used as starter for liquid sourdough production (data not shown) (Carnevali et al. 2007). Compared to the use of fresh cells, the use of freeze-dried starter culture would allow to avoid the preculture steps necessary to get a cell number suitable to start high volumes of sourdough, which may require 2–3 days.

Bakery industries interested in liquid sourdough usually demand fast acidification and maintenance of baking and sensorial properties (Messens et al. 2002). Moreover, it would be desirable to reduce the number of sourdough propagations (refreshments or back-slopping steps), in order to avoid risk of contamination and overcoming of selected starter by spontaneous lactobacilli. Therefore, Gaggiano et al. (2007) performed a study aiming to produce a liquid sourdough that could be stored for several weeks without losing its performance. Preliminarily 56 strains of sourdough LAB were screened based on the

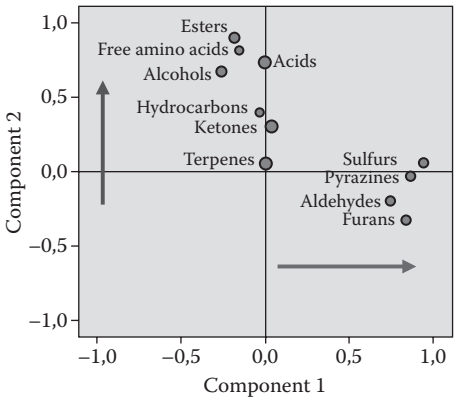

FIGURE 29.3 The loading plot of the results for the analytical fingerprint: volatile molecules and free amino acids were used as markers. Components 1 and 2 represent the map of the markers, volatile components, and free amino acids at the end of liquid fermentation and after baking in bread started with Lb1, respectively. (Reprinted from *Food Microbiology*, 24, Carnevali P. et al., Liquid sourdough: Industrial Application Perspectives, pp. 2–4, Copyright (2007), with permission from *Elsevier*.)

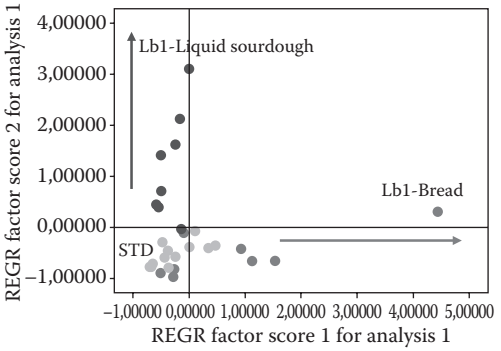

FIGURE 29.4 The score plot of the distribution of the different samples analyzed in the map of analytical markers regarding the volatile components. ● C, crust; ● M, bread crumb; ● LS, liquid sourdough Lb1; STD, bakers' yeast bread. (Reprinted from *Food Microbiology*, 24, Carnevali P. et al., Liquid sourdough: Industrial Application Perspectives, pp. 2–4, Copyright (2007), with permission from *Elsevier*.)

acidification kinetics during sourdough fermentation. *Lactobacillus casei* DPPMA27, *Weissella confusa* DPPMA20, and *L. fructivorans* DPPMA8 were selected and used to produce a liquid sourdough. Fermentation was carried out in a 2-L batch fermentation bioreactor (12 hours at 37°C), under controlled pH (5.6), by cultivating cells in a medium made of water (60%), flour, fructose, maltose, yeast extract, dipotassium phosphate, sodium acetate, manganese sulfate, and magnesium sulfate. Fructose and maltose either increase the concentration of fermentable carbohydrates of wheat flour or may be used by obligately heterofermentative strains in a cofermentation process that permits the synthesis of extramoles of ATP through the acetate kinase pathway (Gobbetti et al. 2005). Yeast extract and salts, as usual ingredients of culture media for LAB (e.g., MRS medium), were also found as indispensable growth factors to get the highest biomass yield. The liquid sourdough contained a high cell number (2×10^{10} CFU/g) of the above-mentioned LAB, and it was stored for 30 days under different conditions: at 4°C without additives or with 5% (w/w) NaCl or 5% (w/w) glycerol; at 15°C without any additives or with a buffering agent (sodium citrate, 2.5%); or directly frozen at –20°C. The sourdough showed no appreciable changes in pH during storage. The only exception was found during storage at 15°C with no sodium citrate added, which showed a decrease of the pH from 5.6 to below 5.0. During storage, no contaminant molds were found, and cell viability decreased by one log cycle just after 21 days, although it remained in the order of 10^9 CFU/g at the end of storage. The liquid sourdough was used for dough fermentation (DY = 160), and the microbial composition, acidifying activity, and fermentation end products were constant during 21 days of storage, independently from the storage condition (Gaggiano et al. 2007).

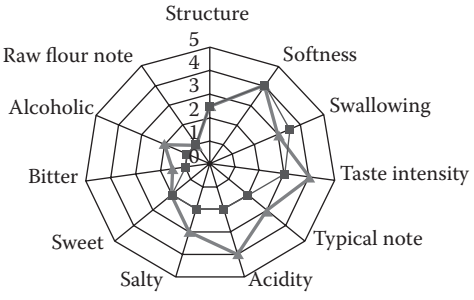

FIGURE 29.5 Results of sensory evaluation on the bread made with Lb1 liquid sourdough (▲) and on the bread made with bakers' yeast (■). (Reprinted from *Food Microbiology*, 24, Carnevali P. et al., Liquid sourdough: Industrial Application Perspectives, pp. 2–4, Copyright (2007), with permission from *Elsevier*.)

29.4 Perspectives and Potential of Liquid Sourdough for Industrial Application

As stated previously, liquid sourdough represents a concrete opportunity for bakery industries to get high-quality and attractive products. Some potential applications of this technology to industrial production are improvement of texture and nutritional quality through synthesis of EPS; improved quality of whole grain or fiber-rich baked goods; and control of spoilage microorganisms through synthesis of antimicrobial compounds.

LAB may synthesize extracellular polysaccharides, named EPS, associated with the cell surface in the form of capsules or secreted into the extracellular environment in the form of slime. In their natural environment, EPS protect the microbial cell against desiccation, phagocytosis, phage attack, and antibiotics and favor adhesion to solid surfaces and the formation of biofilms. Based on their composition, EPS are classified in homopolysaccharides and heteropolysaccharides. Homopolysaccharides consist of three or more units of the same monosaccharide, whereas heteropolysaccharides consist of several different monosaccharides (Sutherland 1998). Homopolysaccharides are either glucan (e.g., dextran, reuteran, mutan) or fructan (e.g., levan, inulin) and are usually synthesized by extracellular enzymes (glycosyltransferase) using sucrose as the glycosyl donor. Fructans may be synthesized by some sourdough strains of *L. sanfranciscensis* (levan) and of *L. reuteri* (inulin or levan). *L. reuteri* strains may produce dextran or reuteran (Korakli et al. 2006; Tieking and Gänzle 2005). Other sourdough-related LAB are also capable to synthesize homopolysaccharides (Bounaix et al. 2009; Di Cagno et al. 2006; Katina et al. 2009; Tieking and Gänzle 2005). EPS have positive repercussions on texture, mouth feel, taste perception, and stability of fermented foods (Tieking and Gänzle 2005). In addition, certain EPS are thought to have beneficial effects on human health such as cholesterol lowering ability (Pigeon et al. 2002), immunomodulating and antitumoral activities (Chabot et al. 2001; Kitazawa et al. 1998), prevention of gut colonization by enteropathogenic microorganisms (Kitov et al. 2000; Shoaf et al. 2006) and prebiotic effects (Dal Bello et al. 2001; Korakli et al. 2002). Bakery industries show remarkable interest on EPS as substitutes for additives from plant (starch, pectin, and cellulose) and seaweed (alginate and carageenans) sources for improving some characteristics (especially texture) of baked goods. According to previous researches on industrial application of EPS, they may be synthesized *in situ*, i.e., during sourdough fermentation, at a very variable concentration, ranging from 2.5 to 16 g/kg of dough. Although some authors report that levan synthesized at a concentration of ca. 0.5 g/kg of dough during fermentation of a liquid sourdough could be sufficient to influence positively the texture of baked goods (Tieking et al. 2003), the effect of EPS depends on many variables, such as structure and molecular weight, other than their concentration (Lacaze et al. 2007). Among EPS, dextran is the most studied homopolysaccharide for bakery applications. *Leuconostoc mesenteroides* LMGP-16878, previously isolated from a sourdough used for manufacturing *Panettone* cake, was capable of synthesizing dextran during fermentation of a liquid sourdough with added sucrose (ca. 10%). This sourdough improved the volume (up to 12%), moist feel in the mouth, and softness of rye breads and rye mixed breads due to its dextran content, which acts as a hydrocolloid (Lacaze et al. 2007). *W. confusa* VTT E-90392 was used as a starter for fermentation of a liquid sourdough containing sucrose due to its capacity to synthesize dextran. The resulting sourdough contained dextran at a concentration of 11–16 g/kg of dough, was characterized by high viscosity, and when applied to bread-making (addition level of ca. 40%), the final bread was characterized by improved volume (up to 10%) and crumb softness (25%–40%) during 6 days of storage (Katina et al. 2009). Besides benefiting technological and sensorial quality, some EPS are not digested in the small intestine and stimulate colonic carbohydrate fermentation to short-chain fatty acids, which have beneficial effects (Jann et al. 2006) and increase the cell count of beneficial members of the intestinal microbiota, thus acting as prebiotic compounds (for a review, see Candela et al. 2010). The levans produced by *L. sanfranciscensis* have been shown to possess prebiotic properties (Korakli et al. 2002). Gluco-oligosaccharides (Seo et al. 2007) and fructo-oligosaccharides formed during sourdough fermentation are potentially prebiotic (Tieking and Gänzle 2005). *L. reuteri* LTH5448 and *Weissella cibaria* 10M synthesize potentially prebiotic EPS in sorghum sourdoughs (Schwab et al. 2008), but fructo-oligosaccharides are not suitable for baking applications, as they are hydrolyzed by yeast invertase at the dough stage (Gänzle et al. 2009).

Consumption of foods rich in whole grains and cereal fiber has been shown to reduce the risk of chronic diseases such as diabetes, cardiovascular disease, and certain cancers (Jacobs et al. 2004; Larsson et al. 2005; Murtaugh et al. 2003). Whole grain or fiber-rich baked goods could give a valid contribution to reach the recommended dietary fiber intake, but they often are characterized with a lower sensorial quality with respect to baked goods made from more refined ingredients (Poutanen et al. 2009). During fermentation of rye-based liquid sourdoughs, degradation and/or solubilization of dietary fiber occurs (Boskov et al. 2002; Katina et al. 2007), with endogenous enzymes (e.g., xylanases) playing a pivotal role. During rye sourdough fermentation, endogenous rye proteases hydrolyze proteins, thus generating amino acids and small peptides, which act as flavor precursors (Tuukkanen et al. 2005). Fermentation of both wheat (Hassan et al. 2008; Salmenkallio-Marttila et al. 2001) and rye (Katina et al. 2007) brans could be an efficient pretreatment of bran both to improve sensorial quality of bran-containing bread and to degrade antinutritional factors, such as phytic acid (Hassan et al. 2008; Lioger et al. 2007). Bread containing bran prefermented by LAB and yeasts showed higher loaf volume and crumb softness during storage (Katina et al. 2006; Salmenkallio-Marttila et al. 2001).

Overall, compared to traditional sourdough, liquid sourdough has an intrinsic inferior stability, meaning it is more susceptible to change in the microbiota population during time. Sometimes, changes are confined to LAB and strains harbored in flour, which take the place of starter microorganisms, but in other cases, liquid sourdough may become dominated by deleterious microorganisms, resulting in the failure of the fermentation process or even in a rapid spoilage of baked goods (observations by the authors, data not published). The capacity of some LAB to synthesize antimicrobial compounds, such as organic acids and bacteriocins, may be exploited for increasing stability of liquid sourdough and, possibly, for guaranteeing a relatively long shelf life of baked goods, without using propionate or ethanol as additives. It has been shown that the stable persistence of *L. reuteri* in a German sourdough, prepared for producing a commercially available baking aid, is due to the production of reutericyclin, a natural low-molecular-weight antibiotic (Gänzle and Vogel 2003). Also, it has been shown that the bacteriocin producer *Lactobacillus amylovorus* DCE 471 is a competitive starter culture for liquid sourdough fermentation (Leroy et al. 2007) and that the in situ bacteriocinogenic activity of *Lactococcus lactis* subsp. *lactis* M30, isolated from nonmalted barley and producing a lacticin 3147-like bacteriocin, had a strong influence on the microbial consortium of sourdough LAB and showed an aptitude to support the dominance of selected, bacteriocin-insensitive strains, during sourdough fermentation (Corsetti and Settanni 2007; Settanni et al. 2005).

In conclusion, the potential of using liquid sourdough fermentation may be turned into reality only through application of basic knowledge to real issues emerging day by day in bakery industries strongly oriented to scientific research. Currently, the most attractive themes in which a lively and exponentially growing scientific community is engaged are LAB and yeast biodiversity (De Vuyst et al. 2009), microbial metabolism (for reviews, see Gänzle et al. 2007, 2009), influence of ecological parameters on microbial metabolism and quality of the final product (for a review, see Serrazanetti et al. 2009), and sourdough-leavened baked goods as functional food or carrier for bioactive compounds (for a review, see Gobbetti et al. 2010).

REFERENCES

Arendt EK, Ryan LAM, Dal Bello F. 2007. Impact of sourdough on the texture of bread. *Food Microbiol* 24:165–74.

Böcker G, Stolz P, Hammes WP. 1995. Neue Erkenntnisse zum Ökosysyem Sauerteig und zur Physiologie des sauerteig-typischen Stämme *Lactobacillus sanfrancisco* und *Lactobacillus pontis*. *Getreide Mehl und Brot* 49:370–4.

Boskov Hansen H, Andersen MF, Nielsen LM, Bach Knudsen K-E, Meyer AS, Christensen LP, Hansen A. 2002. Changes in dietary fibre, phenolic acids and activity of endogenous enzymes during rye bread making. *Eur Food Res Technol* 214:33–42.

Bounaix M-S, Gabriel V, Morel S, Robert H, Rabier P, Remaud-Siméon M, Gabriel B, Fontagné-Faucher C. 2009. Biodiversity of exopolysaccharides produced from sucrose by sourdough lactic acid bacteria. *J Agric Food Chem* 57:10889–97.

Candela M, Maccaferri S,Turroni S, Carnevali P, Brigidi P. 2010. Functional intestinal microbiome, new frontiers in prebiotic design. *Int J Food Microbiol* 140:93–101.

Carnevali P, Ciati R, Leporati A, Paese M. 2007. Liquid sourdough fermentation: Industrial application perspectives. *Food Microbiol* 24:150–4.

Cauvain S. 2003. Bread making: an overview In: Cauvain S, editor. *Bread Making, Improving Quality*. Cambridge: Woodhead Publishing Limited. p 8–20.

Chabot S, Yu HL, De Léséleuc L, Cloutier D, van Calsteren MR, Lessard M, Roy D, Lacroix M, Oth D. 2001. Exopolysaccharide from *Lactobacillus rhamnosus* RW-9595 stimulate TNF, IL-6 and IL-12 in human and mouse cultured inmunocompetent cells, and IFN-g in mouse splenocytes. *Lait* 81:683–97.

Corsetti A, Settanni L. 2007. Lactobacilli in sourdough fermentation: A review. *Food Res Int* 40:539–58.

Dal Bello FD, Walter J, Hertel C, Hammes WP. 2001. *In vitro* study of prebiotics properties of levan-type exopolysaccharides from lactobacilli and non-digestible carbohydrates using denaturing gradient gel electrophoresis. *Syst Appl Microbiol* 24:232–7.

De Vuyst L, Vrancken G, Ravyts F, Rimaux T, Weckx S. 2009. Biodiversity, ecological determinants, and metabolic exploitation of sourdough microbiota. *Food Microbiol* 26:666–75.

Decock P, Cappelle C. 2005. Bread technology and sourdough technology. *Trends Food Sci Technol* 16:113–20.

Di Cagno R, De Angelis M, Limitone A, Minervini F, Carnevali P, Corsetti A, Gänzle MG, Ciati R, Gobbetti M. 2006. Glucans and fructans production by sourdough *Weissella cibaria* and *Lactobacillus plantarum*. *J Agric Food Chem* 54:9873–81.

Gaggiano M, Di Cagno R, De Angelis M, Arnault P, Tossut P, Fox PF, Gobbetti M. 2007. Defined multi-species semi-liquid ready-to-use sourdough starter. *Food Microbiol* 24:15–24.

Gänzle MG, Vermeulen N, Vogel RF. 2007. Carbohydrate, peptide and lipid metabolism of lactic acid bacteria in sourdough. *Food Microbiol* 24:128–38.

Gänzle MG, Vogel RF. 2003. Contribution of reutericyclin production to the stable persistence of *Lactobacillus reuterii* in an industrial sourdough fermentation. *Int J Food Microbiol* 80:31–45.

Gänzle MG, Zhang C, Monang BS, Lee V, Schwab C. 2009. Novel metabolites from cereal associated Lactobacilli—novel functionalities for cereal products? *Food Microbiol* 26:712–9.

Gibson GR. 2008. Prebiotics as gut microflora management tools. *J Clin Gastroenterol* 42:S75–9.

Gobbetti M, Corsetti A. 1996. Co-metabolism of citrate and maltose by *Lactobacillus brevis* subsp. *lindneri* CB1 citrate negative strains: Effect on growth, end-products and sourdough fermentation. *Zeitschrift fur Lebensmittel-Untersuchung und-Forschung* 203:82–7.

Gobbetti M, De Angelis M, Corsetti A, Di Cagno R. 2005. Biochemistry and physiology of sourdough lactic acid bacteria. *Trends Food Sci. Technol* 16:57–69.

Gobbetti M, Di Cagno R, De Angelis M. 2010. Functional microorganisms for functional food quality. *Crit Rev Food Sci Nutr* 50:716–27.

Gobbetti M, Gänzle MG. 2007. Editorial. Third International Symposium on Sourdough. *Food Microbiol* 24:113.

Gobbetti M, Lavermicocca P, Minervini F, De Angelis M, Corsetti A. 2000. Arabinose fermentation by *Lactobacillus plantarum* in sourdough with added pentosans and a-L-arabinofunarosidase: A tool to increase the production of acetic acid. *J Appl Microbiol* 88:317–24.

Gobbetti M, Simonetti S, Corsetti A, Santinelli F, Rossi J, Damiani P. 1995. Volatile compound and organic acid productions by mixed wheat sour dough starters: influence of fermentation parameters and dynamics during baking. *Food Microbiol* 12:497–507.

Goesaert H, Brijs K, Veraverbeke WS, Courtin CM, Gebruers K, Delcour JA. 2005. Wheat flour constituents: how they impact bread quality, and how to impact their functionality. *Trends Food Sci Technol* 16:12–30.

Hammes WP. 1991. Fermentation of non-dairy food. *Food Biotechnol* 5:293–303.

Hammes WP, Brandt MJ, Francis KL, Rosenheim J, Seitter MFH, Vogelmann SA. 2005. Microbial ecology of cereal fermentations. *Trends Food Sci Technol* 16:4–11.

Hassan E, Awad A, Alkereem A, Mustafa A. 2008. Effect of fermentation and particle size of wheat bran on the antinutritional factors and bread quality. *Pak J Nutr* 7:521–26.

Jacobs Jr, Gallaher DD. 2004. Whole grain intake and cardiovascular disease: a review. *Curr Atherosclerosis Rep* 6(6):415–23.

Jann A, Arrigoni E, Rochat F, Schmid D, Bauche A, inventor; 2006. Methods for increasing the production of propionate in the gastrointestinal tract. U.S. Patent US7091194B1.

Katina K. 2005. Sourdough: a tool for the improved flavor, texture and shelf-life of wheat bread. [DPhil thesis]. University of Helsinki. VTT Publications 569.

Katina K, Laitila A, Juvonen R, Liukkonen K-H, Kariluoto S, Piironen V, Landberg R, Åman P, Poutanen K. 2007. Bran fermentation as a means to enhance technological properties and bioactivity of rye. *Food Microbiol* 24:175–86.

Katina K, Maina NH, Juvonen R, Flander L, Johansson L, Virkki L, Tenkanen M, Laitila A. 2009. In situ production and analysis of *Weissella confusa* dextran in wheat sourdough. *Food Microbiol* 26:734–43.

Katina K, Salmenkallio-Marttila M, Partanen R, Forssell P, Autio K. 2006. Effects of sourdough and enzymes on staling of high-fibre wheat bread. *LWT–Food Sci Technol* 39:479–91.

Kieronczyk A, Skeie S, Olsen K, Langsrud T. 2001. Metabolism of amino acids by resting cells of non-starter lactobacilli in relation to flavour development in cheese. *Int Dairy J* 11:217–24.

Kitazawa H, Harata T, Uemura J, Saito T, Kaneko T, Itoh T. 1998. Phosphate group requirement for mitogenic activation of lymphocytes by an extracellular phosphopolysaccharide from *Lactobacillus delbrueckii* spp. *bulgaricus*. *Int J Food Microbiol* 40:169–75.

Kitov PI, Sadowska JM, Mulvey G, Armstrong GD, Ling H, Pannu NS, Read RJ, Bundle DR. 2000. Shiga-like toxins are neutralized by tailored multivalent carbohydrate ligands. *Nature* 403:669–72.

Korakli M, Gänzle MG, Vogel RF. 2002. Metabolism by bifidobacteria and lactic acid bacteria of polysaccharides from wheat and rye, and exopolysaccharides produced by *Lactobacillus sanfranciscensis*. *J Appl Microbiol* 92:958–65.

Korakli M, Vogel RF. 2006. Structure/function relationship of homo-polysaccharide producing glycansucrases and therapeutic potential of their synthesised glycans. *Appl Microbiol Biotech* 71:790–803.

Lacaze G, Wick M, Cappelle S. 2007. Emerging fermentation technologies: development of novel sourdoughs. *Food Microbiol* 24:155–60.

Larsson SC, Giovannucci E, Bergkvist L, Wolk A. 2005. Whole grain consumption and risk of colorectal cancer: a population based cohort of 60,000 women. *Br J Cancer* 92:1803–7.

Leroy F, De Winter T, Moreno MRF, De Vuyst L. 2007. The bacteriocin producer *Lactobacillus amylovorus* DCE 471 is a competitive starter culture for type II sourdough fermentations. *J Sci Food Agric* 87:1726–36.

Lioger D, Leenhardt F, Demigne C, Remesy C. 2007. Sourdough fermentation of wheat fractions rich in fibres before their use in processed food. *J Sci Food Agric* 87:1368–73.

Mastilovic J, Popov S, editors. Investigation of rheological properties of liquid sour dough. Proceedings of 3rd Croatian Congress of Cereal Technologists; 2001 November 14–17; Opatija, Croatia. 2001.

Mastilovic J, Popov S, Psodorov D, editors. Optimization of the composition and process parameters for white wheat liquid sour dough production. Proceedings of 3rd Croatian Congress of Cereal Technologists; 2001 November 14–17; Opatija, Croatia. 2001.

Messens W, Neysens P, Vansieleghem W, Vanderhoeven J, De Vuyst L. 2002. Modeling growth and bacteriocin production by *Lactobacillus amylovorus* DCE 471 in response to temperature and pH values used for sourdough fermentations. *Appl Environ Microbiol* 68:1431–5.

Moroni AV, Dal Bello F, Arendt EK. 2009. Sourdough in gluten-free bread making: an ancient technology to solve a novel issue? *Food Microbiol* 26:676–84.

Murtaugh MA, Jacobs Jr, Steffen DR, Jacob B, Marquart L. 2003. Epidemiological support for the protection of whole grains against diabetes. *Proc Nutr Soc* 62:143–9.

Pigeon RM, Cuesta EP, Gilliland SE. 2002. Binding of free bile acids by cells of yogurt starter culture bacteria. *J Dairy Sci* 85:2705–10.

Poutanen K, Flander L, Katina K. 2009. Sourdough and cereal fermentation in a nutritional perspective. *Food Microbiol* 26:693–9.

Salmenkallio-Marttila M, Katina K, Autio K. 2001. Effect of bran fermentation on quality and microstructure of high-fibre wheat bread. *Cereal Chem* 78:429–35.

Schwab C, Mastrangelo M, Corsetti A, Gänzle MG. 2008. Formation of oligosaccharides and polysaccharides by *Lactobacillus reuteri* LTH5448 and *Weissella cibaria* 10M in sorghum sourdoughs. *Cereal Chem* 85:679–84.

Selomulyo VO, Zhou W. 2007. Frozen bread dough: Effects of freezing storage and dough improvers. *J Cereal Sci* 45:1–17.

Seo EC, Nam S-H, Kang H-K, Cho J-Y, Lee H-S, Ryu H-W, Kim D. 2007. Synthesis of thermo- and acid-stable novel oligosaccharides by using dextransucrase with high concentration of sucrose. *Enz Microb Technol* 40:1117–23.

Serrazanetti DI, Guerzoni ME, Corsetti A, Vogel RF. 2009. Metabolic impact and potential exploitation of the stress reactions in lactobacilli. *Food Microbiol* 26:700–11.

Settanni L, Corsetti A. 2008. Application of bacteriocin in vegetable food biopreservation. *Int J Food Microbiol* 121:123–38.

Settanni L, Massitti O, Van Sinderen D, Corsetti A. 2005. *In situ* activity of a bacteriocin-producing *Lactococcus lactis* strain. Influence on the interactions between lactic acid bacteria during sourdough fermentation. *J Appl Microbiol* 99:670–81.

Shoaf K, Mulvey GL, Armstrong GD, Hutkins RW. 2006. Prebiotic galactooligosaccharides reduce adherence of enteropathogenic *Escherichia coli* to tissue culture cells. *Infect Immun* 74:6920–28.

Sutherland IW. 1998. Novel and established applications of microbial polysaccharides. *Trends Biotechnol* 16:41–6.

Tieking M, Gänzle MG. 2005. Exopolysaccharides from cereal-associated lactobacilli. *Trends Food Sci Technol* 16:79–84.

Tieking M, Korakli M, Ehrmann MA, Gänzle MG, Vogel RF. 2003. *In situ* production of exopolysaccharides during sourdough fermentation by cereal and intestinal isolates of lactic acid bacteria. *Appl Environ Microbiol* 69:945–52.

Tieking M, Kühnl W, Gänzle MG. 2005. Evidence for formation of heterooligosaccharides by *Lactobacillus sanfranciscensis* during growth in wheat sourdough. *J Agric Food Chem* 53:2456–61.

Tomomatsu H. 1994. Health effects of oligosaccharides. *Food Technol* 48:61–5.

Tuukkanen K, Loponen J, Mikola M, Sontag-Strohm T, Salovaara H. 2005. Degradation of secalins during rye sourdough fermentation. *Cereal Chem* 82:677–82.

Wick M, Vanhoutte JJ, Adhemard A, Turini G. 2001. Automatic method for evaluating the activity of sourdough strains based on gas pressure measurements. *Appl Microb Biotechnol* 55:362–8.

Wiese BG, Strohmar W, Rainey FA, Diekmann H. 1996. *Lactobacillus panis* sp. Nov., from sourdough with a long fermentation period. *Int J Syst Bacteriol* 46:449–53.

30

Chinese Fermented Rice Noodles

Yong Yang

CONTENTS

30.1 Introduction

China is the largest rice-producing and rice-consuming country in the world (Hettiarachchy 2000; Yao 2004). Rice noodles are an important part of the diet in south China and are mainly manufactured and consumed in the following provinces: Sichuan, Yunnan, Guizhou, Guangxi, Guangdong, Hunan, Hubei, Jiangxi, and Fujian (Zhen 2003). However, fermented rice noodles are mainly produced and consumed in Guangxi, Guizhou, Hunan, and Yunnan. The most famous delicate types among all Chinese fermented rice noodles are Guilin Mifen (in Guangxi) and Guoqiao Mixian (in Yunnan) (Zhen 2003).

Chinese fermented rice noodles and instant fresh rice noodles are delicious, nutritional, and convenient and can be eaten in various ways such as with soup or salad, cold or hot, as a staple or as snack food, and they are being accepted and enjoyed by more and more northern Chinese and foreigners alike.

With the rapid development of the noodle industry, some Chinese companies have succeeded in developing fermented rice noodles from a simple, manually made diet to commercial products with long shelf life. However, due to limited production capacity, commercial Chinese fermented rice noodles are not enough to fulfill the home and abroad markets.

The manufacturers of fermented rice noodles have realized the bright future of this product and already set up some funds to support the development of the products. In this chapter, a concise review

on the processing technology, soup base making, and development trends of the Chinese fermented rice noodle is presented.

30.2 Processing Technology

The actual processing technology of fermented rice noodles may be different from factory to factory and from province to province but the basic principles involved are practically the same. The first automatic manufacturing line has been set up in the Hunan province, which is the largest rice-producing province in China and also famous for Changde Mifen, a typical processing technology of Hunan fermented rice noodle is introduced.

30.2.1 Fermented Rice Noodle Processing

The flowchart of fermented rice noodle processing is given in Figure 30.1 (Toh 1997; Sun 2001; Xu 2005).

30.2.2 Raw Materials

Long grain types of milled rice (*Oryza india*) are usually used to manufacture fermented rice noodles. Unlike wheat flour, there is no gluten in the rice flour to form the net texture in fermented rice noodles. Starch, especially amylase, which has high gelatinization character, contributes to the consistent structure in fermented rice noodles. The *O. india* rice has more amylose (23%–26%) than *Oryza sinica* (<16%–20%) (Hettiarachchy 2000), thus it is the main raw material for fermented rice noodle production.

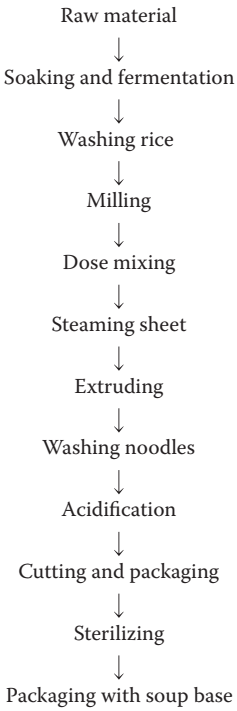

FIGURE 30.1 Flowchart of fermented rice noodle processing.

30.2.3 Fermentation

Fermentation of rice noodles occurs naturally, as the fermentation process often takes place spontaneously during the rice soaking stage (Blandinob 2003). The natural starters from previous batch are added into a new batch to initiate and accelerate the rice fermentation process. The whole rice grains are soaked in warm water (30ºC–40ºC), with a 1.5:1 ratio of rice to water. One to five percent of mixed starters (V/M), mainly lactic acid bacteria, is added into rice and water by back-slopping. Fermentation time in summer is 1–2 days, whereas it takes 2–3 days during the winter, depending on room temperature.

30.2.4 Milling

After the fermentation stage, wash the rice grains with flowing water and add more water to mill them into rice paste (particle size 120–150 μm). Since the rice grains become soft and easy to be milled into flour without heavy starch damage after 1–3 days of soaking and fermentation (Chiang 2002), fermented rice noodles are more elastic in the texture, with smoother mouth feel, and appears whiter than the non-fermented rice noodle, in which the rice is processed by dry milling.

30.2.5 Dose Mixing

The lack of gluten in rice flour makes it difficult to form a noodle structure as compared with wheaten noodles. In addition, the high (up to 65%) water content of fermented rice noodles makes it a challenge to prolong its shelf life. So other accessories and additives are used to deal with the above problems in fermented rice noodle production. The common raw materials and additives are listed in Table 30.1 (Fu 2000; Zhen 2003).

All components are first measured and then mixed directly into rice paste or prepared into a solution and then added into the mixed paste.

30.2.6 Steaming

To steam, spread out the well-mixed paste, 1–3 mm thick, on a steaming pan at 0.2–0.3 MPa for 50–120 seconds. After steaming, the degree of curing is over 90%. Temperature, water content, and steaming time are the critical factors in this process (Fu 1994). Too high a temperature creates bubbles and shrinkage on the surface of the noodle sheet, while too low a temperature makes it white and less lustrous. Water content in rice paste is 50%–55%.

TABLE 30.1

Ingredients of Chinese Fermented Rice Noodles

Component	Content (%)	Function
Rice	80–90	Gelatinization
Denatured starch	10–15	Improve strengthen and elastic characters of fermented rice noodle gel
Magic taro refined flour	3–5	Antiaging agent
Propylene glycol	2–3	Antiseptic agent
Soy bean oil	0.5–2	Glazing and greasing agents
Sodium chloride	0.5–1	Increase the water retention capacity
Glycine	0.2–0.5	Inhibit heat-resisting bacillus bacteria
Evaporated glycerin monostearate	0.3–0.5	Emulsifying agent
Complex phosphate	0.1–0.4	Chelating agent

30.2.7 Extrusion

The extrusion worm and cylinder are the main working parts in extruding. The pore size on the die is 1.5–2 mm in diameter. The rice noodle strands are molded and further cured during this process.

30.2.8 Washing of Noodles

Wash the noodles from the extruder die immediately with cold water to fix the gel structure of noodles and flush away the starch grains on the surface.

30.2.9 Acidification

Soak the noodles in an acid solution tank; lactic acid and citric acid are often used as acidulants (pH 3.8–4.0) for 20–60 seconds. The low pH of the product can extend the shelf-life associated with following the sterilizing process.

30.2.10 Cutting and Packaging

Long rice noodles are cut into serving size (25–40 cm) and then packed into clear polythene or other heat-enduring material package in fixed quantity.

30.2.11 Sterilization

Sterilization is done at 90°C–95°C for 30–40 minutes, and this depends on the thickness of the noodles and quantity of each package (150–300 g/bag). After sterilization, the noodle bags are cooled and sent to storage (He 2005).

30.2.12 Packaging with Soup Base

The soup base is placed in the bag along with the bag of noodles in a big package or bowl and is then heat-sealed.

30.3 Soup Base Making

Chinese fermented rice noodles are made in different places and have different flavors. In fact, soup base is the essential factor to determine the flavor style of the fermented rice noodles. Guilin Mifen (Guilin rice noodle) is the most famous rice noodle in China, and Lu Fen is the most popular one among Guilin Mifen. The ingredients and cooking methods of the soup base of Lu Fen are introduced here (Sha 2001).

30.3.1 Lu Shui (Soup)

Solid materials for preparation of Lu Shui (soup) are given in Table 30.2 (Sha 2003).

First, cut fresh ginger into thin slices and add, along with gauze bag (where dry spices and Chinese herbs are wrapped), into the water. Heat the water until boiling point and then switch into stewing temperature for about 1 hour. Then fish out the ginger and gauze bag; the Lu Shui will be ready for cooking meat.

30.3.2 Lu Cai (Meat Cooked with Lu Shui)

Beef and pork harslet (liver, tough, and bowel) are the favorable raw materials for Lu Cai cooking. Clean the meat and put into the pot with Lu Shui, add some salt, and boil. When cooked, fish out the meat,

TABLE 30.2

Solid Materials in Lu Shui

Solid Materials	Content (g)
Aniseed	10
Areca nut	6
Cassia	5
Cassia twig	5
Clove	1
Dried tangerine peel	5
Fennel	5
Fresh ginger	5
Fructus momordicae	3
Licorice	5
Pepper	3
Resurrection lily	7
Tsaoko	5
Zanthoxylum bungeanum	3

cool it down, and cut into slices for later use. If the meat was fished out, the Lu Shui would be ready for consumption as flavored fermented rice noodle.

30.3.3 Fermented Rice Noodle Serving

Put rice noodles into a strainer and then soak in boiling water to heat the noodles. Then load the hot noodles into a bowl and put some Lu Cai on the surface of the rice noodles. Spread garlic, onion, hot pepper, and sweet violet on the Lu Cai. Lastly, pour the hot Lu Shui into the bowl. The very delicious fermented rice noodles can then be eaten with hot soup, which is the most popular way to eat the noodles. However, there are two other ways to cook and eat the noodles: cold or stir-fried. In the hot summer, the fermented rice noodles can be dressed with salad instead of hot Lu Shui soup and consumed cold. Some people prefer this to hot greasy rice noodles cooked by stir-frying.

The Chinese fermented rice noodle manufacturers have adopted automatic production line in traditional fermented rice noodle production. The soup base bag has been developed after the sample of instant noodles. Except for the noodle bag in the package of a commercial product, there are some accessory bags: one meat bag (Lu Cai bag), one soup bag (Lu Shui, vinegar, and sauce bag), one dehydrated vegetable bag (garlic, onion, hot pepper, sweet violet), and one seasoning bag (salt, monosodium glutamate, seafood powder).

30.4 Looking Forward

Nowadays, consumption of a nutritionally unbalanced diet (more meat and dairy products) has led to numerous diseases resulting from poor nutrition. The growth of pasta consumption and production in the United States over the past 10–15 years coincided with the recognition by health-conscious consumers that pasta is a nutritious food, containing complex carbohydrates and being low in fat. It was also recognized that pasta is a low-cost, versatile, easy-to-prepare convenience food, giving it an added appeal to busy people (Seibel 1996).

Noodle is a kind of pasta mainly consumed in Asian countries. Three wheaten noodles (dry noodle, frying instant noodle, and long-life noodle) are mainly manufactured and consumed in China (Huang 1996). With noodles' improvement and development in terms of food safety, nutrition, convenience, and better flavor (Jiang 2003), the industry of long-life noodles are developing more rapidly than the dry noodle. Not only is the former more convenient for eating (i.e., it is ready for consumption after submersion

in hot water for just 2–3 minutes), it is also healthier, as it does away with the high fat content and toxic substances that come into being through the frying process of the latter type of noodles (Gotoh 2006). A new type of Chinese instant noodles was also developed with the application of lactic acid fermentation (Sawatari 2005).

Although rice noodles have already being consumed in the south of China for centuries, commercial products are not as popular as wheaten noodles because of the lack of mechanical processing technology. Currently, with the development of rice noodle processing technology, more and more companies are putting up large-scale rice noodle productions in China. Since the rice-consuming population in China is equal to the wheat-consuming population, it is estimated that the Chinese market of rice noodles will be much greater, at least up to 50% of the whole noodle (both wheat and rice noodle) market, in the future (Zhen 2002). There is no allergenic protein in rice, which is another attractive factor for the population, and persons with hypersensitivities prefer rice noodles to wheaten noodles (Hettiarachchy 2000).

Fermented rice noodles can be eaten directly without boiling and can also be served in various flavors and different ways depending on the consumers' preference, thus it is definitely one ideal type of rice noodle for future development. However, as the industry of fermented rice noodle is just starting, much deeper and more comprehensive research concerning fermented rice noodle processing technology (production process, product quality, and safety), flavoring art, and consumption patterns (associated with local diet habits and cultures) are needed.

REFERENCES

Blandinob A, Al-Aseeria ME, Pandiellaa SS, Canterob D, Webba C. 2003. Cereal-based fermented foods and beverages. *Food Res Int* 36:527–43.

Chiang PY, Yeh AI. 2002. Effect of soaking on wet-milling of rice. *J Cereal Sci* 35:85–94.

Fu XR. 1994. Improvement of steaming equipment in rice vermicelli production. *Food Mach* 41(3):32.

Fu XR. 2000. The common accessories and additives in the rice noodles. *Food Technol Cereals Oilseeds* 8(2):3–4.

Gotoh N. 2006. Novel approach on the risk assessment of oxidized fats and oils for perspectives of food safety and quality. I. Oxidized fats and oils induces neurotoxicity relating pica behavior and hypoactivity. *Food Chem Toxicol* 44:493–8.

He XY, Sun QJ. 2005. Research on sterilization and fermentation of fresh rice noodle. *Cereal Food Ind* 12(5):23–6.

Hettiarachchy NS, Ju ZY, Siebenmorgen T, Sharp RN. 2000. Rice: production, processing, and utilization. In: Kulp K, Ponte JG, editors. Handbook of Cereal Science and Technology. New York: Marcel Dekker. p 203–22.

Huang S. 1996. China—the world's largest consumer of paste products. In: Kruger JE, Matsuo RB, Dick JW, editors. Pasta and Noodle Technology. St. Paul: AACC. p 301–31.

Jiang ZH, Wu Y. 2003. Development and the status on the food of the noodles. *J Grain Oil (supplement)*:16–9.

Sawatari Y, Sugiyama H, Suzuki Y, Hanaoka A, Saito K, Yamauchi H, Okada S, Yokota A. 2005. Development of fermented instant Chinese noodle using *Lactobacillus plantarum*. *Food Microbiol* 22:539–46.

Seibel W. 1996. Future trends in pasta products. In: Kruger JE, Matsuo RB, Dick JW, editors. Pasta and Noodle Technology. St. Paul: AACC. p 331–49.

Sha XC. 2001. How to make Guilin Mifen (Guilin rice noodle). *Sichuan Cuisine* 3:36.

Sun QJ, inventor. 2001. Fresh instant noodle manufacturing method, Chinese Patent number 00113407.8.

Toh TS. 1997. Instant rice noodles. European patent application EP 0738473A2. *Trend Food Sci Technol* 8:249.

Xu W, He XY. 2005. Hunan rice noodle manufacturing methods. *Technol Econ Cereals Oilseeds* 2:48–9.

Yao HY. 2004. *Rice Profound Manufacture*. Beijing: Chemistry Industry Press. p 1–9.

Zhen HY, He XY, Lin LZ. 2003. The developing trend of instant rice noodle condiment. *J China Condiment* 1:9–13.

Zhen HY, Lin LZ. 2002. Market trend of instant rice noodle. *J Food Sci Technol* 11:22–4.

Zhen JX. 2003. *Modern New Style Food Development of Cereals*. Beijing: Science and Technology Document Press. p 120–35.

31

Boza: A Traditional Cereal-Based, Fermented Turkish Beverage

Sirma Yegin and Marcelo Fernández-Lahore

CONTENTS

31.1 Introduction to Fermented Cereal Beverages

Cereal grains are considered to be one of the most important sources of dietary proteins, carbohydrates, vitamins, minerals, and fiber for people all over the world. However, the nutritional quality of cereals and the sensorial properties of their products are sometimes of inferior quality in comparison with milk and milk-derived products. The reasons behind this are the lower protein content, deficiency of certain essential amino acids (e.g., lysine), low starch availability, presence of determined antinutrients (phytic acid, tannins, and polyphenols), and the coarse nature of the grains (Chavan and Kadam 1989).

A number of methods have been employed with the aim of ameliorating the nutritional qualities of cereals. These include genetic improvement and amino acid supplementation with the protein concentrates or other protein rich sources such as grain legumes or defatted oil seed meals of cereals. Additionally, several processing technologies, which include cooking, sprouting, milling, and fermentation, have been put into practice to improve the nutritional properties of cereals (Mattila-Sandholm 1998). Fermentation may be the most simple and economical way of improving the nutritional value, sensory properties, and functional qualities of cereals.

Several changes occur during fermentation of cereals. Natural fermentation of cereals increases the biological availability of essential amino acids such as lysine (Hamad and Fields 1979). Starch and fiber tend to decrease during fermentation of cereals (El-Tinay et al. 1979). Changes in the vitamin content of cereals with fermentation vary according to the fermentation process, and the raw material used

in the fermentation. Vitamins of the group B generally show an increase upon fermentation (Chavan et al. 1989). For example, during the fermentation of maize or kaffir corn in the preparation of kaffir beer, thiamine levels were virtually unchanged, but riboflavin and niacin contents were almost doubled (Steinkraus 1994). Fermentation has also a significant effect in the reduction of antinutrients. Cereals are rich in phytic acid, which reduces the bioavailability of minerals and the solubility, functionality, and digestibility of proteins and carbohydrates (Reddy et al. 1989). Fermentation of cereals provides optimum pH conditions for enzymatic degradation of phytate (Blandino et al. 2003). The phytate content reduced via the action of phytases that catalyze conversion of phytate to inorganic orthophosphate and a series of myo-inositols and lower phosphoric esters of phytate (Reddy and Pierson 1994). Tannin-protein complexes can cause inactivation of digestive enzymes and reduce protein digestibility by interaction of protein substrate with ionizable iron (Salunkhe et al. 1990). Fermentation of cereals also reduces the tannin content. As is evident, by the aid of a fermentation process, cereal grains can be transformed into value-added products.

Cereal-based fermented beverages are made from cereals such as maize, wheat, rice, oats, or a combination of any of these cereals and are mainly used as a milk or soy beverage substitute. Cereal-based beverages are mostly preferred by people who are allergic or intolerant to dairy and/or soy foods or by those who choose not to consume dairy products either for health or ethical reasons. There are several types of cereal-based fermented beverages produced around the world, which can be classified based on the raw materials used or type of fermentation involved in the manufacturing process. Some of these beverages are obiolor, ogi, pito, and skete in Nigeria (Adegoke et al. 1995); sorbia in Saudi Arabia (Gassem 2002); borde in Ethiopia (Abegaz et al. 2004); bushera in Uganda (Muianja et al. 2003); pozol in Mexico (Wacher et al. 2000); bouza in Egypt (Marcos et al. 1973); and boza in Turkey, Bulgaria, and other Balkan countries (Hancioglu and Karapinar 1997; Goetcheva et al. 2001).

31.2 Boza

Boza is a traditional Turkish beverage made by yeast and lactic acid fermentation of millet, cooked maize, wheat, or rice semolina or flour. Boza can also be produced by the combination of any of the mentioned cereals. It is a highly viscous colloid suspension, pale yellow in color, and can be classified as sweet or sour boza depending on acid content. The typical boza is the one known by its sourish taste. The taste of boza depends on the ingredients used and the method of preparation. The refreshing effect of lactic acid enables boza to be consumed during summer time; however, the high temperatures during that season result in rapid growth of indigenous microflora and, consequently, dramatic changes in sensorial attributes (Akpinar-Beyizit et al. 2010). Thus, boza is usually consumed in winter. It is served with roasted chick peas *leblebi* and cinnamon in Turkey. The popularity of the drink is due both to its pleasant taste and flavor and its nutritional properties. In Turkey, boza is produced both on the artisanal and industrial scale.

31.2.1 History of Boza

As a generic term, boza originated from the Persian word *buze*, meaning millet. The origin of boza dates back to the ancient populations that lived in the Anatolia and Mesopotamia regions. The preparation formula was taken by the Ottomans and spread over the countries they conquered. The Greek historian and philosopher, Xenophan, recorded that boza was made in eastern Anatolia in 401 BC and stored in clay jars that were buried beneath the ground. With the immigration of Turks, the production of this delicious beverage was introduced over a very large geographical area. Thus, it also became a characteristic beverage of Caucasus and Balkan countries such as Bulgaria, Albania, Macedonia, Romania, Serbia, and Montenegro. Although it is produced by many Balkan countries, there are quiet large differences between the products obtained mainly depending on the cereals used in the recipe. Usually the cereals, which are easily available in each region, are preferred for the production of boza locally. Similar cereal-based fermented beverages are produced in different parts of the world but are sometimes described in the literature under

similar names. *Bouza* (*buza*) is a fermented alcoholic beverage in Egypt (Baervald 1988; Sanni 1993) and busaa is also described as an acidic alcoholic beverage in Kenya (Nout 1980). These products are quiet similar in name but a main difference between the Turkish boza and the mentioned fermented beverages is their alcohol content. Turkish boza is accepted as a nonalcoholic beverage like Bulgarian boza (Goetcheva et al. 2001) and the boza produced in other Balkan countries. However, boza is believed to be one of the oldest predecessors of beer, although there are major differences between boza and beer.

31.2.2 Technological Process for Boza Production

Boza production consists of six steps: (1) preparation of raw materials, (2) boiling, (3) cooling and sieving, (4) sugar addition, (5) fermentation, (6) packaging and storage. Figure 31.1 depicts the steps for boza production.

31.2.2.1 Preparation of Raw Materials

Boza can be produced from various cereals (millet, wheat, maize, rice, and others). The best quality and taste is obtained when millet is used. The selected cereal or combination of two or more cereals are cleaned by an air-sieve separator to remove alien seeds, weeds, dusts, and other foreign materials. Then the grains are milled so as to obtain semolina or flour and sifted to remove remaining hull and bran.

31.2.2.2 Boiling

One volume of the mixture of cereal grains is mixed with 4 to 6 volumes of water under continuous stirring (Arici and Daglioglu 2002). It is then boiled in an open or steam-jacketed boiler after water addition. Stirring is necessary during boiling in order to prevent aggregation and to obtain a homogeneous mash (Birer 1987). Stainless steel boilers are used for the boiling process, and their capacity is varied

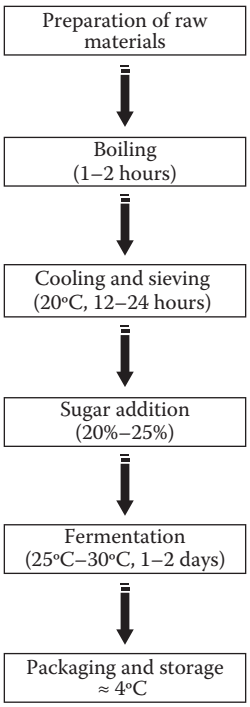

FIGURE 31.1 Steps for boza production.

depending on daily production. During the boiling process, the mixture absorbs water, and therefore hot water is added several times until the end of the process. The boiling process is stopped when a homogeneous mash is obtained, which takes approximately 1–2 hours, depending on the boiling temperature and the raw material used (Arici and Daglioglu 2002).

31.2.2.3 Cooling and Sieving

The mash obtained after boiling should be allowed to cool to around 20°C. Cooling time can be between 12 and 24 hours. Traditionally, cooling is carried out in marble vessels to accelerate the process. The cooled mash is then diluted with water under continuous stirring. It is further sieved for the elimination of undesirable particles.

31.2.2.4 Sugar Addition

The sieved boza is sweetened with saccharose powder to 20%–25%. Some of the sugar added is used as a substrate by lactic acid bacteria (LAB) and yeast. Therefore, the amount of sugar added has to be carefully adjusted.

31.2.2.5 Fermentation

Boza (2%–3% w/v) from a previous batch can be used as a starter culture. At the beginning of the production season, sourdough and yoghurt can also be used as starter culture if the boza is not available. The amount of starter culture depends on the season and fermentation temperature. Fermentation temperature may vary between 25°C and 30°C. Fermentation usually takes 1–2 days, depending on the fermentation temperature. Two percent inoculated mixture is ready for use, after a 24-hour incubation period at 25°C.

During boza production, two types of fermentation occur: lactic acid fermentation and alcohol fermentation. These fermentations take place at the same time (Copur and Tamer 2003). The fermentation of boza is normally carried out by natural fermentation involving a mixed culture of LAB and yeasts. Interactions between microorganisms are not controlled during traditional processing, which leads to variations in quality and stability of boza. LAB are always predominant. As a result of lactic acid production, the pH of the product decreased and the characteristic acidic sourish boza taste is obtained. Lactic acid fermentation contributes toward the safety, nutritional value, and acceptability of a wide range of cereal-based fermented beverages. Alcohol fermentation by yeasts is also important for the characteristic odor and mouthfeel. The alcohol fermentation produces CO_2 gas that results in an increase in volume. This is important in order to decide the level of substrates that should be loaded into each vessel.

Several physical and biochemical changes occur during boza fermentation. Hancioglu and Karapinar (1997) monitored the changes in numbers of LAB and yeast during the fermentation of Turkish boza for a 24-hour period. During this period, the population of LAB and yeast rose from 7.6×10^6 and 2.25×10^5/mL to 4.6×10^8 and 8.1×10^6/mL, respectively. Similarly, Gotcheva et al. (2001) monitored the changes during fermentation of different Bulgarian boza formulations during 48 hours. Although the LAB and yeast count vary from one sample to the other, the average value obtained was 10.5×10^7/mL, of which 70% were LAB. The microbial load of Bulgarian boza was approximately 10 times less than that observed for Turkish boza (Hancioglu and Karapinar 1997). This could be due to differences in boza preparation and variations in the microflora types.

31.2.2.6 Packaging and Storage

After fermentation (1–2 days), boza is cooled to 4°C and bottled in 1-kg plastic or glass containers. It should be consumed within 3–5 days for the best organoleptic characteristics since the fermentation still continues after packaging. The shelf life of boza is limited, at maximum 1 to 2 weeks, depending on the acidity of product; a strict temperature control is necessary to prolong the shelf life. Gotcheva et al. (2001) analyzed Bulgarian boza samples after fermentation during 10 days to study product stability.

It was concluded that boza could be kept in good condition for consumption for 11 days at 4°C, which shows that this could be considered a good storage temperature for the product.

31.3 Microflora of Boza

Hancioglu and Karapinar (1997) performed a pioneering study on identification of microflora of Turkish boza. Seventy-seven isolates of LAB and 70 yeast isolates were identified. The LAB isolated during fermentation included *Leuconostoc paramesenteroides* (25.6% of the total LAB), *Lactobacillus sanfrancisco* (21.9%), *Leuconostoc mesenteroides* subsp. *mesenteroides* (18.6%), *Lactobacillus coryniformis* (9.1%), *L. confuses* (7.8%), *Leuconostoc mesenteroides* subsp. *dextranicum* (7.3%), *Lactobacillus fermentum* (6.5%), and *Leuconostoc oenos* (3.7%) during fermentation of boza, and the yeasts isolated comprised *Saccharomyces uvarum* (83% of the total yeast) and *S. cerevisiae* (17.0%).

Gotcheva et al. (2000) investigated the microflora of Bulgarian boza. A total of 458 LAB and 194 yeast strains were isolated from three samples. The quantification of microorganisms show that the number of LAB varies from 6.0 to 8.8 × 10^7 CFU/cm^3, while yeast counts fall between 2.6 and 3.9 × 10^7 CFU/cm^3. The number of LAB was always higher than the number of yeasts and the ratio of LAB/yeasts was found to be more or less constant (2.4). The LAB isolated were identified as *Lactobacillus plantarum* (24% of the total LAB), *Lb. acidophilus* (23%), *Lb. fermentum* (19%), *Lb. coprophilus* (11%), *Leuconostoc raffinolactis* (9%), *Ln. mesenteroides* (9%), and *Lb. brevis* (5%). The yeasts isolated comprise *Saccharomyces cerevisiae* (47% of the total yeasts), *Candida tropicalis* (19%), *C. glabrata* (14%), *Geotrichum penicillatum* (12%), and *G. candidum* (9%).

Botes et al. (2007) isolated LAB and yeasts from three different preparations of boza and the isolates identified to species level by PCR with species-specific primers and primers designed to amplify the D1/D2 domain of 26S rDNA. Carbohydrate fermentation reactions and PCR with species-specific primers classified the isolates as *Lactobacillus paracasei* subsp. *paracasei*, *Lactobacillus pentosus*, *Lactobacillus plantarum*, *Lactobacillus brevis*, *Lactobacillus rhamnosus*, and *Lactobacillus fermentum*. No filamentous fungi were isolated. Results obtained from sequencing of the D1/D2 rDNA region identified the yeasts as *Candida diversa*, *Candida inconspicua*, *Candida pararugosa*, *Issatchenkia orientalis*, *Pichia fermentans*, *Pichia guilliermondii*, *Pichia norvegensis*, *Rhodotorula mucilaginosa*, and *Torulaspora delbrueckii*. *Saccharomyces cerevisiae*, commonly associated with fermented beverages, was not detected in any of the boza samples, despite enrichment.

Observation of different results on the identification of microflora of boza is possible since the production of boza is carried out by local microflora. The production of product-specific cultures is important to avoid variations in the quality and stability of product. Zorba et al. (2003) performed a study to select the appropriate starter culture combinations using sensory evaluation in order to produce a boza with characteristic properties under controlled conditions without quality variations. Different combinations (yeast/LAB, yeast/2 LAB, yeast/3 LAB ratios) with the previously selected isolates from sensory assessment of boza prepared with single-culture fermentations were used for boza production. It was concluded that boza inoculated with *S. cerevisiae* + *L. mesenteroides* subsp. *mesenteroides* (yeast/LAB) had the best quality and was more acceptable than any other sample.

Species of LAB and yeast have been also found in the fermentation of Kenyan busaa and kaffir beer and Nigerian ogi, pito, skete, and busa (Adegoke and Babalola 1988; Beuchat 1995; Sanni 1993).

31.4 Chemical Composition of Boza

Chemical composition of boza varies depending on ingredients used for the production and processing conditions. According to the national regulations in Turkey (Anonymous 1992), total dry matter and total sugar (saccharose) content should be minimum 20% and 10%, respectively. Total titratable acidity (lactic acid) should be 0.2%–0.5% in sweet boza and 0.5%–1.0% in sour boza. Volatile acidity (acetic acid) allowed up to 0.1% and 0.2% in sweet boza and sour boza, respectively. Table 31.1 shows the average composition of Turkish boza.

TABLE 31.1

Composition of Boza

	Range
Dry matter (%)	17.26–22.32
Total sugar (%)	16.11–22.59
Ash (%)	0.02–0.17
Total acidity (%)	0.15–0.50
Volatile acidity (%)	0.006–0.032
pH	3.2–3.8
Ethanol (%)	0.03–0.39
Thiamine (mg/100 g DM)	0.19–0.25
Riboflavin (mg/100 g DM)	0.18–0.21
Pyridoxine (mg/100 g DM)	0.32–0.36
Nicotinamide (mg/100 g DM)	0.51–0.60
Energy (kcal/100 g)	240–250

Sources: Adapted from Kose, E., and Yucel, U., *J Food Technol* 1(4), 191–193. Arici, M., and O. Daglioglu, *Food Rev Int*, 18, 39–48, 2002. With permission.

Note: DM, dry matter.

The raw materials being used for boza production have a major effect on the chemical composition of boza. The raw materials affect the amount and quantity of carbohydrates available as primary fermentation substrates, nitrogen sources, and growth factors for microbial activity (Akpinar-Bayizit et al. 2010). Operations such as size reduction, heating, and cooling also affect the final product properties (Nout and Motarjemi 1997). The compositional differences depending on the raw materials used for different boza recipes were investigated by Akpinar-Beyizit et al. (2010). Rice, maize, millet, and wheat flours were individually used as raw materials. Four different boza samples were evaluated in terms of titratable acidity, pH, alcohol, and organic acid profile. The total titratable acidity in terms of lactic acid was found to be lowest in millet boza (0.32%) and highest in wheat boza (0.61%), due to the probable high fermentable carbohydrate content of wheat compared to other raw materials. The final pH varied between 3.43 and 3.86 depending on the raw material. The final alcohol content was found lower in wheat boza. The organic acid composition of each boza samples was also determined. Oxalic, lactic, pyruvic, and acetic acid were found in all type of boza samples while citric, orotic, and malic acid were found in some of the boza samples. Production of organic acid during the metabolism of fermentable sugar are attributed mainly presence of homofermentative and heterofermentative LAB. This indicates that the composition of raw materials together with mircoflora has an important effect on the organic acid composition of boza. Gotcheva et al. (2001) also performed a study to show the effect of raw materials on the final product quality of boza. Boza was prepared both from whole wheat grains and wheat flour. When whole grains were used, the culture dry weight during this period decreased from 97 to 65 g/L, due mainly to starch conversation. Starch hydrolysis led to important viscosity changes (from 1137 to 500 cP), and the glucose concentration increased from 0.48 to 7.94 g/L for the same reason. The free amino nitrogen content decreased and the pH dropped after 24 hours from 5.1 to 3.4. The ethanol concentration measured at the end of fermentation was 0.5% v/v. When the whole-wheat flour's (average particle size 12.3 μm) used, dry matter content decreased from 142 to 126 g/L, free amino nitrogen content decreased from 32.3 to 18.2 mg/L and glucose concentration increased from 0.19 to 6.76 g/L during 24 hours. The ethanol concentration measured at the end of fermentation was similar to that obtained from whole wheat grain (i.e., 0.5% v/v). Significantly higher viscosity values were obtained due to the bran particles present in the flour. It has been concluded that the use of wheat flour resulted in a product with higher viscosity and dry matter content, when compared to that produced from whole-wheat grains.

Fermentation time is another important factor affecting the pH and the acidity of the boza. Higher fermentation time results in lower pH and higher acidity in terms of lactic acid. Hancioglu and Karapinar (1997) reported that pH of the boza samples dropped from 6.13 to 3.48, total titratable acidity of the samples increased from 0.02 to 0.27 mmol/g and the alcohol content increased from 0.02% to 0.79%. Similarly, Hayta et al. (2001) revealed that the pH of Turkish boza gradually decreases from 5.8 to 3.5 during 24-hour fermentation. Fermentation time also has effect on the production of alcohol by yeast. Higher fermentation times yields higher alcohol content, but the average alcohol content of traditional Turkish boza is between 0.03 and 0.39% w/v (Kose and Yucel 2003). According to the related national regulations, beverage contains ethyl alcohol less than 5 g/L as accepted nonalcoholic beverages (Anonymous 1998).

Fermentation temperature is also an important factor influencing the chemical composition of the final product. Gotcheva et al. (2001) studied the effect of different temperatures (4°C, 25°C, and 30°C) on the fermentation of boza. The initial glucose content (1.73 g/L) increased to 17.4, 13.8, and 2.6 g/L after 24 hours of fermentation period at 30°C, 25°C, and 4°C, respectively. Starch hydrolysis was most intensive at the highest temperature due to the faster microflora development. The initial free amino nitrogen content was 20.3 mg/L, and this value decreased to 7.3, 7.9, and 18.6 mg/L at the end of fermentation at 30°C, 25°C, and 4°C, respectively. It was mentioned that the highest microbial activity was observed at 30°C. The initial pH of 5.0 dropped to 3.4 either at 25°C or 30°C. Very small changes were observed for pH when the fermentation was carried out at 4°C. The biggest viscosity drop was observed at 30°C (from 820 to 630 cP).

31.5 Rheological Properties of Boza

The rheological behavior of a food matrix is an important physical property that has a direct association with product overall quality and processing characteristics, as well as on consumer acceptability. Boza is a highly viscous fermented beverage and, therefore, the description of its rheological properties becomes relevant. Genc et al. (2002) performed a study to link rheological behavior of boza with its performance during sensory analysis. The product showed a non-Newtonian behavior as indicated by a reduction in apparent viscosity values with an increase in shear rate. Such behavior was further described by a power law function indicating its pseudo-plastic nature. The calculated flow behavior index (n) showed an inverse relationship with "appearance" and "mouthfeel" scores while the fluid consistency (K) coefficient depicted a direct correlation with the mentioned sensory scores. It was concluded that rheological parameters of boza could be utilized as a predictor of consumer acceptance.

31.6 Antinutrients and Toxic Compounds in Boza

The concern in consuming innocuous foods and beverages free of pathogenic microorganisms or their toxins has emerged in the developing countries. Moreover, the products must not be contaminated with any foreign substances or, at least, they must fulfill the maximum limits of the residues allowed after exhausting studies demonstrating the risks (Hilliam 2000).

Fermentation of cereals leads to a general improvement in the shelf life, texture, taste, and aroma of the final product. However, the microorganisms responsible for the fermentation may produce some undesirable metabolite such as biogenic amines and mycotoxins. This becomes the case especially for the fermented products, which are produced with natural microflora.

Biogenic amines are basic nitrogenous organic compounds having a recognized activity and may occur naturally in foods and beverages. They are generated either as a result of endogenous amino acid decarboxylase activity in raw food material or by the growth of decarboxylase-positive microorganisms under conditions favorable to enzyme activity. Fermented foods and beverages are more susceptible to formation of biogenic amines, since several microorganisms are involved in the fermentation process (Shalaby 1996). Although they are needed for many critical functions in

humans and animals, high concentrations of these amines can have toxicological effects (Ten Brink et al. 1990). Since boza is a cereal-based fermented beverage containing protein, which might be used by decarboxylase-positive microorganisms, the formation of various biogenic amines might be expected. The microflora of boza includes decarboxylase-positive microorganisms. *Lactobacillus sanfrancisco*, *Leuconostoc oenos* (Silla Santos 1996) and *Leuconostoc mesenteroides* (Shalaby 1996) were mentioned before as biogenic amine-producing bacteria which were part of the microflora of boza. Consequently, Yegin and Uren (2008) investigated the biogenic amine content of boza. Of the 11 biogenic amines under study, putrescine, spermidine, and tyramine were detected in all boza samples. Tyramine was the prevailing biogenic amine. Tyramine concentrations of boza samples were between 13 and 65 mg/kg. Total biogenic amine contents of boza samples were between 25 and 69 mg/kg. The concentrations of biogenic amines detected in Turkish boza were below the maximum permissible limits. Maximum tyramine concentration was 65 mg/kg and this value was below the 100–800 mg/kg that was proposed by Ten Brink et al. (1990) and Nout (1994) as an upper limit for tyramine. Maximum total biogenic amine concentration was determined as 69 mg/kg, which was less than the 100–200 mg/kg legal limit given by Nout (1994). The presence of 10–25 mg of tyramine in one or two usual servings produces a severe adverse effect in those using monoamine oxidase inhibitor drugs (McCabe-Sellers et al. 2006). Consumption of boza is less than 0.5 kg per meal, and this consumption level appears to be safe for unmedicated individuals, but patients being treated with drugs containing monoamine oxidase inhibitors must limit their consumption of boza.

About 20% of the food and beverages, mainly of plant origin, are significantly contaminated with mycotoxins. Cereal and cereal-derived product because of resistance to technological processes are considered a major source of mycotoxins, mainly ochratoxin (OTA). Uysal et al. (2009) performed a study to determine the OTA levels in boza. OTA has been detected in only one sample as 3.58 μg/kg, and it was mentioned that this amount was above the limits of European Commission Regulations. However, fungi producing OTA were not identified in boza. Thus, their presence could be the result of contamination during processing or they could be coming from the cereals used in the boza recipe.

31.7 Conceptions of Functional Foods

A food can be regarded as functional if it is satisfactorily demonstrated to affect beneficially one or more target functions in the body beyond adequate nutritional effects, in a way which is relevant to either the state of well-being and health or the reduction of the risk of a disease (Roberfroid 2002). A functional food can be a natural one, with added components, or whose component has been eliminated by means of technological or biotechnological procedures (Prado et al. 2008).

Boza is accepted as a natural functional beverage because of having probiotic LAB. Todorov et al. (2008) evaluated the probiotic properties of strains isolated from Bulgarian boza. Strains of *Lactobacillus plantarum*, *L. rhamnosus*, *L. pentosus*, and *L. paracasei* were studied for antimicrobial activity, tolerance to gastric juice, bile salt hydrolase activity, and the ability to adhere to HT-29 and Caco-2 cell lines. All the strains survived conditions simulating the gastrointestinal tract and produced bacteriocins active against a number of pathogens. Adherence to HT-29 and Caco-2 cells was within the range reported for *Lactobacillus rhamnosus* GG, a well known probiotic. Of all the strains isolated from boza, strain *L. paracasei* ST284BZ had the highest auto-aggregation properties and revealed the best antiviral and antibacterial activity. Based on these characteristics, strain ST284BZ has been mentioned as the best probiotic. However, *L. plantarum* ST194BZ revealed the highest activity against *Mycobacterium tuberculosis* and thus has been proposed to be used to control tuberculosis. Tuncer and Ozden (2010) characterized the bacteriocin produced by *Lactococcus lactis* subsp. *lactis* YBD11 isolated from Turkish boza. It has been proved that the mentioned strain inhibited the growth of *Lactobacillus plantarum*, *Lactobacillus sake*, *Lactococcus lactis* subsp. *lactis*, *Lactococcus lactis* subsp. *cremoris*, *Micrococcus luteus*, *Listeria innocua*, *Enterococcus feacalis*, *Staphylococcus aureus*, *Staphylococcus carnosus*, *Pediococcus pentosaceus*, and *Bacillus cereus* but nisin producer strain *Lactococcus lactis* SIK83 and

Gram-negative bacteria were not inhibited. Different enzyme, pH, and heat treatments showed that the bacteriocin produced by *L. lactis* subsp. *lactis* YBD11 exhibited a similar behavior with control sample nisin. Tricine–SDS-PAGE of partially purified bacteriocin gave the same molecular weight of 3.5 kDa as that of nisin. These results indicated that bacteriocin produced by *L. lactis* subsp. *lactis* YBD11 was a nisin-like bacteriocin.

31.8 Future Remarks

Although boza is one of the famous cereal-based beverages in Turkey and other Balkan countries, there are several gaps related to the processing of that product. Since the fermentation is carried out by indigenous microflora, it is difficult to obtain a standardized product. Moreover, the shelf life of boza is also limited to 1–2 weeks. Thus, there is a need to develop product-specific starter cultures to obtain standardized quality. Development of an appropriate preservation method(s) to prolong the desired shelf life of the boza is also important. On the other hand, some modifications can be done in boza formulations such as enrichment with some functional ingredients and flavorings to develop new formulations with enhanced health benefits. Light boza and gluten-free boza can also be formulated by altering the raw materials used in the recipe according to the demand of consumers having health problems such as obesity and gluten intolerance.

REFERENCES

Abegaz K, Langsrud T, Beyene F, Narvhus JA. 2004. The effects of technological modifications on the fermentation of bode, an Ethiopian traditional fermented cereal beverage. *J Food Technol Afr* 9:3–12.

Adegoke GO, Babalola AK. 1988. Characteristics of microorganisms of importance in the fermentation of fufu and ogi—two Nigerian foods. *J Appl Bacteriol* 65:449–53.

Adegoke GO, Nwaigwe RN, Oguntimein GB. 1995. Microbiological and biochemical changes during the production of sekete ± a fermented beverage made from maize. *J Food Sci Tech* 32:516–8.

Akpinar-Bayizit A, Yilmaz-Ersan L, Ozcan T. 2010. Determination of boza's organic acid composition as it is affected by raw material and fermentation. *Int J Food Prob* 13:648–56.

Anonymous. 1992. Boza TS. 9778. Ankara: Institute of Turkish Standards.

Anonymous. 1998. Regulations of non-alcoholic beverages. No: 98/24, Republic of Turkey, Ministry of Agriculture and Rural Affairs, General Directorate of Protection and Control Vision.

Arici M, Daglioglu O. 2002. Boza: A lactic acid fermented cereal beverage as a traditional Turkish food. *Food Rev Int* 18:39–48.

Baervald G. 1988. The origin of bread based beverages. *Ge-treideMhel Brot* 42:335–8.

Beuchat, LR. 1995. Indigenous fermented foods. In: Reed G, Nagodawithana TW, editors. *Bio-Technology; Enzymes, Biomass, Food and Feed*. Weinheim, Germany: VCH. p 505–59.

Birer S. 1987. Boza Yapimi ve Ozeilikleri. *Gida* 12:341–3.

Blandino A, Al-Asseri ME, Pandiella SS, Cantero D, Webb C. 2003. Cereal-based fermented foods and beverages. *Food Res Int* 36:527–43.

Botes A, Todorov SD, Von Mollendorff JW, Botha A, Dicks LMT. 2007. Identification of lactic acid-bacteria and yeast from boza. *Process Biochem* 42:267–70.

Chavan JK, Kadam SS. 1989. Critical reviews in food science and nutrition. *Food Sci* 28:348–400.

Chavan UD, Chavan JK, Kadam SS. 1988. Effect of fermentation on soluble proteins and *in vitro* protein digestibility of sorghum, green gram and sorghum-green gram blends. *J Food Sci* 53:1574.

Copur U, Tamer CE. 2003. Boza ve yeni yaklasimlar. *Dunya Gida* 8(4):61–62 (in Turkish).

El-Tinay AH, Abdel-Gadir AM, El-Hidai, M. 1979. Sorghum fermented kisra bread. I. Nutritive value of kisra. *J Sci Food Agric* 30:859–63.

Gassem, MAA. 2002. A microbiological study of Sobia: a fermented beverage in the Western province of Saudi Arabia. *World J Microb Biot* 18:173–7.

Genc M, Zorba M, Ova G. 2002. Determination of rheological properties of boza by using physical and sensory analysis. *J Food Eng* 52:95–8.

Gotcheva V, Pandiella SS, Angelov A, Roshkova ZG, Webb C. 2000. Microflora identification of the Bulgarian cereal-based fermented beverage boza. *Process Biochem* 36:127–30.

Gotcheva V, Pandiella SS, Angelov A, Roshkova ZG, Webb C. 2001. Monitoring the fermentation of the traditional Bulgarian beverage boza. *Int J Food Sci Tech* 36:129–34.

Hamad AM, Fields ML.1979. Evaluation of protein quality and available lysine of germinated and ungerminated cereals. *J Food Sci* 44:456–9.

Hancioglu O, Karapinar M. 1997. Microflora of Boza, a traditional fermented Turkish beverage. *Int J Food Microbiol* 35:271–4.

Hayta M, Alpaslan M, Kose E. 2001. The effect of fermentation on viscosity and protein solubility of boza, a traditional cereal-based Turkish beverage. *Eur Food Res Technol* 213:335–7.

Hilliam M. 2000. Functional food. How big is the market? *The World of Food Ingredients* 12:50–3.

Kose E, Yucel U. 2003. Chemical composition of Boza. *J Food Technol* 1(4):191–3.

Mattila-Sandholm T. 1998. VTT on lactic acid bacteria. *VTT Symp* 156:1–10.

McCabe-Sellers BJ, Staggs CG, Bogle ML. 2006. Tyramine in foods and monoamine oxidase inhibitor drugs: A crossroad where medicine, nutrition, pharmacy, and food industry converge. *J Food Compos Anal* 19:58–65.

Morcos SR, Hegazi SM, El-Damhoughy ST. 1973. Fermented foods of common use in Egypt II. The chemical composition of bouza and its ingredients. *J Sci Food Agr* 24:1157–61.

Muianja CMBK, Narvhus JA, Treimo J, Langsrud T. 2003. Isolation, characterization and identification of lactic acid bacteria from bushera: A Ugandan traditional fermented beverage. *Int J Food Microbiol* 80:201–10.

Nout MJR. 1980. Microbiological aspects of the traditional manufacture of Busaa, a Kenyan opaque maize beer. *Chem Mikrobiol Technol Lebensm* 6:137–42.

Nout MJR. 1994. Fermented foods and food safety. *Food Res Int* 27(3):291–8.

Nout MJR, Motarjemi Y. 1997. Assessment of fermentation as a household technology for improving food safety: A joint FAO/WHO workshop. *Food Control* 8:221–6.

Prado FC, Parada JL, Pandey A, Soccol CR. 2008. Trends in non-dairy probiotic beverages. *Food Res Int* 41:111–23.

Reddy NR, Pierson MD. 1994. Reduction in antinutritional and toxic components in plant foods by fermentation. *Food Res Int* 27:281–90.

Reddy NR, Pierson MD, Sathe SK, Salunkhe DK. 1989. Interactions of phytate with proteins and minerals. In: Reddy NR, editor. *Phytates in Cereals and Legumes*. Boca Raton, Florida: CRC Press. p 57.

Roberfroid M. 2002. Functional food concept and its application to prebiotics. *Dig Liver Dis* 34:105–10.

Salunkhe DK, Chavan JK, Kadam SS. 1990. Occurrence, nature, and composition. In: Salunkhe DK, Chavan JK, Kadam SS, editors. *Dietary Tannins: Consequences and Remedies*. Boca Raton, Florida: CRC Press. p 29–68.

Sanni AI. 1993. The need for process optimization of African fermented foods and beverages. *Int J Food Microbiol* 18:85–95.

Shalaby AR. 1996. Significance of biogenic amines to food safety and human health. *Food Res Int* 29(7):675–90.

Silla Santos MH. 1996. Biogenic amines: Their importance in foods. *Int J Food Microbiol* 29:213–31.

Steinkraus KH. 1994. Nutritional significance of fermented foods. *Food Res Int* 27:259–67.

Ten Brink B, Damink C, Joosten HMLJ, Huis In't Veld JHJ. 1990. Occurrence and formation of biologically active amines in foods. *Int J Food Microbiol* 11:73–84.

Todorov SD, Botes M, Guigas C, Schillinger U, Wiid I, Wachsman MB, Holzapfel WH. 2008. Boza, a natural source of probiotic lactic acid bacteria. *J Appl Microbiol* 104:465–77.

Tuncer Y, Ozden B. 2010. Partial biochemical characterization of nisin-like bacteriocin produced by *Lactococcus lactis* subsp. *lactis* YBD11 isolated from Boza, a traditional fermented Turkish beverage. *Rom Biotechnol Lett* 15(1):4940–8.

Uysal UD, Oncu EM, Berikten D, Yılmaz N, Tuncel NB, Kıvanc M, Tuncel M. 2009. Time and temperature dependent microbiological and mycotoxin (ochratoxin-A) levels in boza. *Int J Food Microbiol* 130:43–8.

Wacher C, Canas A, Barzana E, Lappe P, Ulloa M, Owens JD. 2000. Microbiology of Indian and Mestizo pozol fermentations. *Food Microbiol* 17:251–6.

Yegin S, Uren A. 2008. Biogenic amine content of boza: A traditional cereal-based, fermented Turkish beverage. *Food Chem* 111:983–7.

Zorba M, Hancioglu O, Genc M, Karapinar M, Ova G. 2003. The use of starter cultures in the fermentation of boza, a traditional Turkish beverage. *Process Biochem* 38:1405–11.

32

Chinese Steamed Buns

Suwimon Keeratipibul and Naphatrapi Luangsakul

CONTENTS

32.1 Introduction

Originating from China, Chinese steamed buns or Chinese steamed bread (CSB) is a traditional wheat-type product in Chinese cuisine. They can be with or without filling. Their unique characteristics, which are distinctly different from baked bread, are their white color and round shape with a very thin and soft skin, and they are made by steaming instead of baking. Various names are used to call this product depending on location. In Shanghai, steamed bread, either with or without filling, is called *mantou,* whereas in northern China, a steamed bun with filling is called *baozi*, where *bao* means *wrapping.* CSB can be eaten at any meal in Chinese culture, and is often eaten for breakfast. Besides, steamed bread produced in northern China and southern China is distinctly different by their formulas. The northern-style CSB is produced from a starter mother dough, while the southern-style one is made with yeast (Peng et al. 2007). Nowadays, steamed bread is well-known and popularly consumed in Asia.

In Thailand, CSB were brought into the country by emigrating Chinese people. Presently, they are widely known and consumed not only by the emigrated Chinese but also by the Thais. CSB in Thailand has a round shape, with a typical diameter of 5–10 cm (Figure 32.1). Steamed buns in the Thai culture are eaten at anytime of day as a main dish, appetizer, or snack. Exceptionally, steamed buns are popular breakfast items in some provinces in the southern part of Thailand, such as Trang and Phuket. Generally, CSB are freshly made as small in-house–scale production and are kept warm in the steamer and served warm by street vendors, small retail shops, and convenience stores (Figure 32.2). They are also served in bamboo baskets in Chinese restaurants as a *dim sum* dish (Figure 32.3). Due to increased demand,

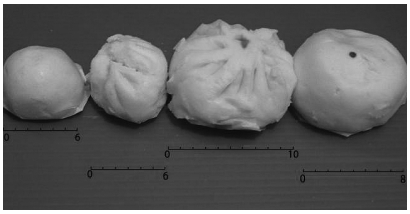

FIGURE 32.1 CSB in Thailand with a typical diameter of 5–10 cm.

FIGURE 32.2 CSB served warm at the sale point: (left) sold by street vendor and (right) sold in a small retail shop.

FIGURE 32.3 CSB served in a bamboo basket in Chinese restaurant.

production of CSBs in Thailand has been expanded to large-scale manufacturing where machinery is used (Figure 32.4) and the products are distributed in frozen form to supermarkets within Thailand as well as exported to nearby countries such as Japan, Hong Kong, and Singapore. Moreover, the steamed bun business in Thailand has further developed into a franchised system, such as Pui and Den brands (Figure 32.5). Nowadays, in response to popularity and increasing demand, a huge variety of CSB have been newly developed. These include colorful steamed buns (by mixing with healthy plants and cereals), healthy steamed buns with black sesame filling, and fancy steamed buns (Figure 32.6).

CSB are classified into two types according to the formula. One is produced from a starter "mother dough" and another is made with bakers' yeast. Both can be with or without filling. CSB without filling are called *mantou*, while the ones with filling are called *salapao* or *salabao.* Two major types of filling are available: savory (meat type) and sweet. The main ingredient of the savory type is either minced pork or chicken mixed with spices and vegetables and stuffed with mushroom and a piece of boiled egg or egg yolk. Barbecued pork or Chinese honey roast pork can also be savory filling (Figure 32.7). In addition, fried green vegetable filling is also available for vegetarians. The sweet filling can be red bean paste, black bean paste, taro paste, custard cream, or various flavors of custard (Figure 32.8).

FIGURE 32.4 CSB produced in large-scale manufacturer using machinery.

FIGURE 32.5 CSB sold in a franchised system: (left) Pui brand and (right) Den brand.

FIGURE 32.6 Newly developed CSB in Thailand: (left) healthy steamed buns with black sesame filling, (middle) colorful steamed buns, and (right) fancy steamed buns.

FIGURE 32.7 CSB filled with savory fillings: (left) barbequed pork and (right) spiced minced pork with egg yolk.

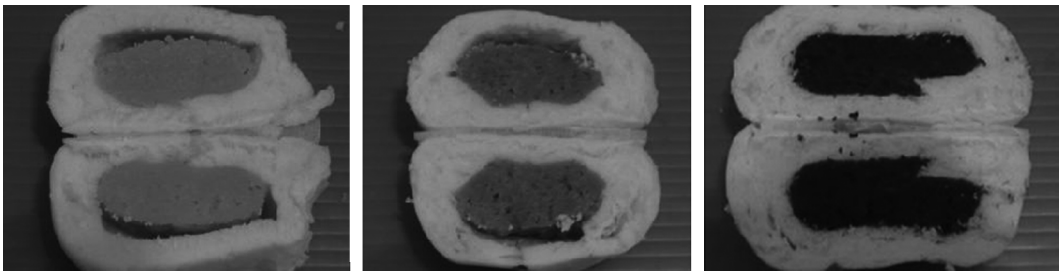

FIGURE 32.8 CSB filled with various types of sweet fillings: (left) custard cream, (middle) taro paste, and (right) black bean paste.

32.2 History of Chinese Steamed Buns

Steamed buns have a history of thousands of years. Chinese archaeologists were able to prove that steamed buns without filling had been available since 770–221 years BC, as the Chinese people already learned how to use a mill and thus they knew how to make food from wheat flour. Regarding the steamed bun's names, the words *Lu Bing* (fried dough), *Hu Bing* (fried dough), *Tang Bing* (noodles), *Long Bing* (steamed unfermented dough), *Huan Bing* (fried dough circles), were all previously called *mantou* (http://en.radio86.com/chinese-food/how-make-baozi-steamed-buns).

Another legend can be traced back to the Jin Dynasty when some history books contained records on "steamed cake," which referred to the steamed bun of the modern times. Legend has it that Zhu Geliang in the Three Kingdoms Period replaced a real human head with a human head–shaped object made of flour and beef or mutton in the memorial ceremony of Lishui River. This offering was called "steamed-bun head." From then on, steamed buns were used as oblation by people in the Jin Dynasty. Such steamed buns were of large size and usually stuffed with meat. In the Tang and Song dynasties, steamed buns were smaller as a kind of pastry, with or without stuffing. It was in the Qing Dynasty that the name of steamed buns was differentiated.

32.3 Types of Chinese Steamed Buns

CSBs can be broadly classified into two types. The first requires mixed culture as natural starter dough for fermentation, while the second needs bakers' yeast for fermentation. CSB made from starter dough have a more cake-like, dense, firm, and cohesive texture and are sticky to the teeth when chewed, while those made from yeast have a more bread-like, soft, elastic, and open texture and glossy skin. In Thailand, CSBs made from starter dough have bursting characteristics on the top showing their snowy white appearance (Figure 32.9). CSB made from yeast with stuffing are normally made in pleated style (Figure 32.10), while those without stuffing are made in a smooth, symmetry shape (Figure 32.11).

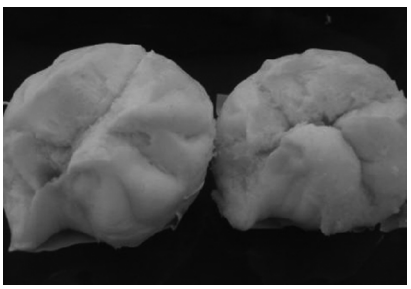

FIGURE 32.9 CSB with bursting characteristics on the top, produced from starter dough.

FIGURE 32.10 CSB in pleated style, produced from bakers' yeast.

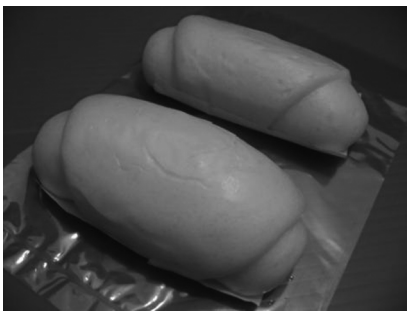

FIGURE 32.11 CSB without filling.

32.4 Microorganisms in Chinese Steamed Bun Starter Dough

CSB starter dough is similar to sourdough for Western baked bread. Sourdough is an intermediate product from natural fermentation mainly containing lactic acid bacteria (LAB) and yeasts, the amounts of which may vary depending on raw material, processing method, and area relating to geography and climate. CSB starter dough is also an intermediate product for CSB, which also contains LAB and yeasts (Luangsakul et al. 2009). The differences of taste, texture, and form of CSB are affected by the contents of LAB and yeasts in the starter dough. Until now, there have been only two pieces of scientific research that reported about microorganisms in CSB starter dough. Li et al. (2007) identified twenty LAB strains, which were isolated from CSB traditional starter dough in China by phenotypic characteristics. The identified LAB were *Lactobacillus vaccinostercus* (30% of total strains), *L. helveticus* (25% of total strains), *L. maltaromicus* (15% of total strains), *L. amylophilus* (15% of total strains), *L. delbrueckii* subsp. *bulgaricus* (5% of total strains), *L. delbrueckii* subsp. *delbrueckii* (5% of total strains), and *L. fructosus* (5% of total strains).

Another researcher group, Luangsakul et al. (2009), isolated both LAB and yeasts from the CSB starter doughs (CSB-SD) from four different commercial sources in Thailand. Thirty-one LAB and eight yeast strains were isolated. Total counts of LAB were from 1.8×10^4 to 10^8 CFU/g sample, while yeasts were from 10 to 2.3×10^2 CFU/g sample. The density of yeasts in the starter doughs of CSB was very low when compared to yeasts in sourdoughs for Western breads. All LAB isolates were identified as *Lactobacillus*. Based on the phenotypic characteristics, they were divided into two major groups, i.e., groups A and B, where the group B could be subdivided into four groups. Genotypic analysis, which was based upon partial 16S rRNA gene sequences, showed that isolates of group A, which all contained *meso*-diaminopimelic acid in their cell wall and produced DL-lactic acid, were closely related to *L. plantarum* (61.3% of total strains), while the strains of group B that produced L-lactic acid were closely

related to *L. casei* (38.7% of total strains). For yeasts, based on the D1/D2 domain sequences of 26S rRNA, eight yeast isolates were identified as *Candida tropicalis* (12.5% of total strains), *Pichia stipitis* (12.5% of total strains), *C. parapsilosis* (12.5% of total strains), *Issatchenkia orientalis* (12.5% of total strains), and *Saccharomyces cerevisiae* (50% of total strains).

From the reports presented above, more various species of LAB were found in the starter dough from China, while only two species of LAB were found in the one from Thailand. These results confirmed that the variations of LAB species depended on area, i.e., China and Thailand. Although yeasts were isolated from CSB starter dough from Thailand, the yeast density was considerably low when compared with the sourdoughs for Western breads. Therefore, in CSB formula, it is required that chemical leavening agents are regularly added during mixing of the activated dough with other ingredients. The yeasts in these starter doughs for CSB may not play any significant leavening role but may influence the sensory qualities. Thus, the association of LAB and yeasts or their community needs to be clarified in this regard and further study is needed.

The LAB and yeasts, which are naturally present in sourdough for Western bread, provide several benefits to baked bread. Gocmen et al. (2007) and Thiele et al. (2002) reported that the application of sourdough to wheat breads produced several effects, including leavening, acidification, improvement of the dough properties, bread flavor and texture, the delaying of firmness, nd staling, increasing the resistance to microbial spoilage and improving the nutrient availability of cereals. LAB and yeast species found in CSB starter dough are akin to those reported in sourdough for Western bread. Thus, Keeratipibul et al. (2010) applied CSB starter dough in making baked white bread. It was found that the 30% and 50% (w/w) substituted breads obtained a greater risen volume, finer crumb grain, retained more softness after 5-day storage, and showed a slightly increased mold stability than the one without substitution, somewhat similar to the Western sourdough. This study contributed a new alternative way of improving bread quality using an Eastern natural intermediate product to produce bread without chemical additives that can respond to current consumer demands.

32.5 Processing Methods of CSB

As mentioned earlier, there are two types of CSB. One is produced from starter dough and from bakers' yeasts. The processing methods for both types are described hereafter.

32.5.1 Starter Dough Chinese Steamed Buns

The production of CSB from starter dough requires several steps. First, starter dough (Figure 32.12) is prepared. This preparation step lasts several days and varies region by region, where its formula and method are kept secret and transferred only within family from generation to generation. After starter dough is prepared, a part of it is used to mix with wheat flour, water, sugar, fat, and chemical leavening agents, while the remainder is normally kept to seed the next production batch. Then the mixed dough is shaped as a bun, stuffed, and finally steamed.

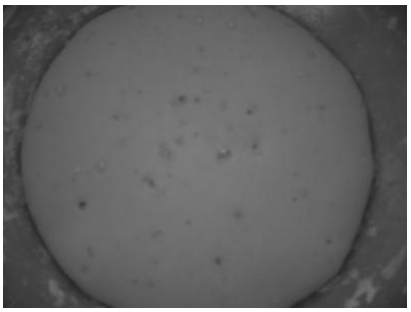

FIGURE 32.12 Starter dough.

Regarding the preparation of starter dough in Thailand, the interviews conducted with Thai manufacturers (both small and large scales) revealed that three methods are used, i.e., using wheat flour and water, using Thai dry traditional starter (Loog-Pang), and using vinegar. Each method is detailed below.

32.5.1.1 Method of Using Wheat Flour and Water

Soft wheat flour is mixed with water with a ratio of 1:1 and incubated at room temperature (average room temperature in Thailand is 30°C) for 12–24 hours. Then this first fermented starter dough is mixed with soft wheat flour and water with a ratio of 1:5:2.5 and incubated at room temperature for 12–24 hours. The third fermentation is continued by mixing the second fermented starter dough with soft wheat flour and water with a ratio of 1:4.7:2.3 and then incubated at room temperature for 12–24 hours. This results in the third starter dough, which is used to mix with other ingredients to make CSB. A part of this third starter dough is kept refrigerated to seed the next production batch.

32.5.1.2 Method of Using Thai Dry Traditional Starter (Loog-Pang)

Loog-Pang (Figure 32.13) is first mixed with soft wheat flour and water with a ratio of 1:20:10 and incubated at room temperature for 15–24 hours. The second fermentation is conducted by mixing the first starter dough with soft wheat flour and water by the ratio of 1:2:1; the mixture is incubated at room temperature for 15–24 hours. The second starter dough was further fermented under the same method and ratio of ingredients as in the second fermentation. The fourth fermentation is made by mixing the third starter dough with soft wheat and water by the ratio of 1:5:2.5 and letting it incubate at room temperature for 24 hours. The fourth starter dough is then used to mix with other ingredients to make CSB; similarly, a part of it is kept to seed in the next production batch.

32.5.1.3 Method of Using Vinegar

Soft wheat flour is mixed with vinegar, water, and oil by the ratio of 20:5:1:2 and incubated at room temperature for 15–24 hours. The second fermentation is done by mixing the first starter dough with soft wheat flour and water by the ratio of 4:4:1 and the mixture is incubated at room temperature for 15–24 hours. The second starter dough is then used to mix with other ingredients to make CSB.

Remark: The above-mentioned ingredient ratios and incubation times may vary according to manufacturer.

32.5.2 Yeast Chinese Steamed Buns

There are two methods to prepare yeast CSB: one mixing step and two mixing steps. Both methods are described herein. The ingredient ratios in the formulas below may be adjusted as needed.

FIGURE 32.13 Loog-Pang.

32.5.2.1 One Mixing Step

Ingredients: Soft wheat flour 600 g, baking powder 10 g, sugar 80 g, active dry yeast 10 g, shortening 20 g, and warm water 300 g.

Method: The yeast is activated by adding yeast into dissolved sugar solution. When the gas is produced and foam is formed, a mixture of wheat flour and baking powder is added and mixed. Then shortening is added and stirred until a stiff dough is formed. The dough is covered with a moistened cotton sheet and left to stand for 2 hours. After that, the dough is divided into 25–30 g each and rolled into a ball. Each ball is flattened into a 3-inch disc by hand or using a small wooden roller (Figure 32.14). For sweet-type filling, it is necessary to shape the disc in a way that the center is thicker than the edges, while for savory-type filling, the disc is shaped to give even thickness. Then the filling is added (Figure 32.15), and the edge is folded together in a sort of pleated style (Figures 32.16 and 32.17). Each bun is then placed on a

FIGURE 32.14 Flattening CSB dough using wooden roller.

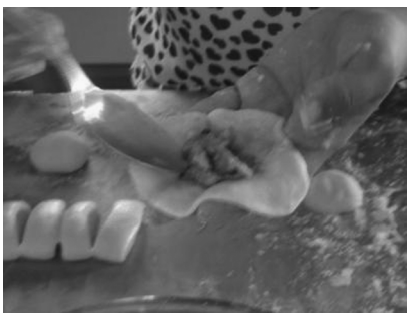

FIGURE 32.15 Placing the filling into CSB dough.

FIGURE 32.16 Folding the edges of the dough together in a sort of pleated style.

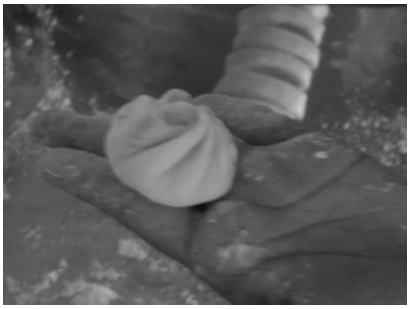

FIGURE 32.17 The finished pleated-style CSB dough.

FIGURE 32.18 CSB with sweet filling in a household-style steamer.

square of white paper. For savory-type filling, the pleated surface is usually on top of the bun, whereas for the sweet type, the pleated surface is at the bottom. Thus, the sweet type bun will have a smooth and round top. Then, both types of buns are proofed at 30°C around 1 hour. Before consuming, the buns are steamed for 15–20 minutes in a steamer (Figure 32.18).

32.5.2.2 Two Mixing Steps

Ingredients: Sponge uses soft wheat flour 325 g, yeast 8.5 g, granulated white sugar 10 g, and water 210 g. Dough uses soft wheat flour 175 g, baking powder 15 g, granulated white sugar 110 g, salt 3 g, shortening 35 g, and water 25 g.

Method: All dry ingredients of sponge are mixed together with water and fermented at 30°C until double volume is reached, which takes around 1 to 1.5 hours. The obtained fermented sponge is punched to release gas. Then this sponge and all other ingredients of dough are mixed together to obtain the final dough. Then the dough is divided and shaped in the same way as described in the one step mixing.

32.6 Factors Affecting Chinese Steamed Bun Quality

In recent years, researchers have studied the effects of several factors, such as protein, starch, and some food additives on CSB quality. The main ingredient in making CSB is wheat, which is also the main ingredient used in baked breadmaking; consequently, the scientific study of factors affecting CSB were conducted akin to those in baked wheat bread. However, the study has just started since the 1980s, thus there are not as numerous studies as those conducted about baked bread. Some literature reviews regarding the factors affecting CSB quality are summarized hereafter.

32.6.1 Wheat and Flour Property

There have been a number of research works regarding the properties of wheat and flour that affect the quality of CSB. These include protein content, dough strength, ash content, amylose content, starch damage, α-amylase activity, and paste viscosity. These properties are associated with CSB qualities on volume, spread ratio, skin brightness, smoothness, shape, firmness, cohesiveness, elastic, crumb cell structure and color, stickiness, and overall acceptance. The difference of wheat and flour properties depends upon the wheat variety, growing location, climate condition, and milling technique. Researchers have investigated wheat and flour properties from a number of wheat varieties that were grown in different countries and environmental conditions and correlated them with CSB qualities. These studies were useful for improvements of wheat variety in order to obtain products of desirable qualities. Scientific studies about the correlation of wheat and flour properties and CSB qualities are described below.

Huang et al. (1996) studied the relationship of Australian and Chinese wheat flour properties and the quality of northern-style CSB (produced from starter dough). It was found that both protein quantity and quality were very important factors affecting steamed bun volume. However, most dough strength parameters (Farinograph dough development time, stability, mixing tolerance), which were related to protein quality, played very important roles on the other qualities of steamed buns, which were the skin and crumb color, structure, eating quality, and total score. Flour peak viscosity was the parameter that showed the best correlation with specific volume, crumb structure, and total score. Flour of medium to strong strength (dough development time greater than 3–6 minutes, maximum resistance greater than 250 BU, extensibility greater than 19.5 cm), and flour peak viscosity over 280 RVU was recommended for the production of good quality steamed buns. Hard wheat flour (higher protein content) was found to be superior to soft wheat flour (lower protein content). The flour with lower ash and color grade was preferred for CSB with higher quality scores.

Crosbie et al. (1998) indicated wheat suitable for CSB making in his reviews on wheat quality requirements of Asian foods that flour of 10.0%–12.0% protein with medium to strong dough properties and moderately high to high flour paste viscosity was preferred for northern-style steamed buns (produced from starter dough). For southern-style steamed buns (produced from yeast), flour of medium protein content (9.5%–11.0%) and medium strength was preferred. In addition, other desirable properties were found in the flour with low α-amylase activity, white color, and a low level of damaged starch.

Koike et al. (1998) investigated and compared the quality of CSB made from white wheat grown in Colorado and from commercial all-purpose flour (medium protein content). Protein content of all tested white wheat and commercial all-purpose flour ranged from 9.0 to 11.6% (low to medium protein content). Mixograph peak times (mixing time) and height ranged from 2.0 to 3.25 minutes and 4.8 to 6.0 cm for the Colorado white wheat flours. The commercial flour had a lower mixing time and the curve had a lower peak height. Peak viscosity of wheat grown in Colorado ranged from 215 to 277 RVU, while peak viscosity of the commercial flour was lower (148 RVU). The white wheat grown in Colorado was found to be suitable for making steamed buns, except for one cultivar, which gave the lowest scores for smoothness, structure, elasticity, and stickiness on CSB. However, the commercial all-purpose flour made relatively good quality steamed buns even though its protein quality (shown by lower mixograph peak time and lower peak height) was different.

Zhu et al. (2001) studied the effects of selected Chinese and Australian wheat with different flour protein content and composition of high-molecular-weight glutenin subunits on northern-style CSB quality. The study revealed that wheat samples from different countries had different protein quantity and quality (dough strength), which resulted in different CSB quality. Flour protein content could be negatively or positively correlated with CSB quality depending on the protein content levels and the differences in dough strength. The difference in size distribution of glutenin polymers might correlate with dough properties, which referred to the different protein quality between the Australian and the Chinese wheat. Composition of high-molecular-weight (HMW) glutenin subunits 5 + 10, which could result in a greater size distribution of their glutenin polymers, might be associated with better CSB quality than the HMW glutenin subunits 2 + 12. Some Australian wheat cultivars appeared to be too strong for making good quality CSB, whereas Chinese wheat cultivars appeared to lack dough strength to gain better quality products. Some Chinese cultivars caused dough stickiness, which was a factor deteriorating CSB quality.

This difference might be related to the difference in the size distribution of glutenin polymers between Australian and Chinese wheat cultivars. This study suggested that, in order to improve the steamed bun quality, it was necessary to improve dough strength of Chinese wheat cultivars. In this study, it was also found that higher protein content could significantly improve dough properties.

He et al. (2003) investigated the association between Chinese and Australian wheat quality traits and the quality of northern-style CSB using manual and mechanized processing. They found that protein content, gluten strength, and extensibility were positively associated with loaf volume and steamed bread elasticity. The relationship among gluten strength, extensibility, appearance, and stickiness were highly dependent on processing methods. Medium protein content and medium-to-strong gluten strength with good extensibility were preferred for the mechanized methods, while the weak-to-medium gluten type was preferred for the manual methods. High flour whiteness and RVA peak viscosity was found desirable for CSB quality regardless of processing method.

Fu et al. (2005) made CSB from top-grade Canada Western Red Spring (hard wheat) and Canada Western Hard White Spring wheat flours. The results showed that both types of hard wheat were suited for steamed bun of lean (no sugar and shortening added) and rich formulations (sugar and shortening added). They could produce high quality steamed buns with round and symmetrical shape, smooth and bright white skin, as well as uniform and fine crumb structure.

Peng et al. (2007) studied the quality of steamed buns made from waxy wheat flour blends and found that waxy wheat flour was not suitable for producing steamed buns. Adding waxy wheat flour decreased the appearance, color, texture, elasticity, stickiness, and the total scores of CSB. However, it was capable of improving the qualities of frozen steamed buns when used at lower proportions. Normal flour blends with 10%–15% waxy wheat flour were the best proportions to decrease the firmness of frozen CSB without decreasing its eating qualities.

Recently, there has been an application of debranning wheat before milling. Debranning is a technology that sequentially and controllably removes outer layers from wheat kernels by abrasion and friction (Dexter and Wood 1996). Lijuan et al. (2007) studied the effects of applying debranning techniques on wheat flour and steamed bun quality, comparing to the conventional milling method. It was found that debranning lowered the gluten index, maximum resistance, and starch damage, while increased the pericarp content, ash content, L* value, falling number, and particle size distribution in wheat flour. In addition, the effects of debranning on pasting properties of flour were different among wheat varieties. Debranning had positive effects on the quality scores, volume, volume/weight, height, and structure of steamed buns. It was also suggested that debranning had different effects on soft, medium, and hard wheat and cultivars. Lin et al. (2010) investigated the effects of different debranning degrees on the qualities of various flours and northern-style CSB. The results showed that debranning (2.5, 5.0, 7.0% degree of debranning) increased the total flour yield and decreased starch damage contents in all cultivars. Debranning at any studied degree showed a negative effect on the qualities of steamed buns in some wheat cultivars (AHBM and JM20); however, the qualities of steamed buns were improved for some wheat cultivars (GY503, JN17, and XN979) by suitable degrees of debranning (5.0%). It was indicated in this study that both kernel hardness and the debranning degree affected the flour properties and the qualities of steamed buns.

It can be concluded that protein quantity and quality (dough strength) affect CSB quality. Wheat from different countries has different protein quantity and quality, which subsequently result in different quality of CSB.

32.6.2 Processing Methods

To obtain CSB of good quality, not only good quality of wheat flour is required, but suitable formulations and optimum processing conditions, depending on type of CSB, should also be taken into consideration. Water absorption, mixing time, and the amounts of shortening and sugar added could greatly affect the final product quality (Fu et al. 2005). Additionally, there have been studies on optimization of CSB processing methods as follows.

Huang et al. (1998) optimized the laboratory processing procedures for southern-style CSB using a response surface methodology. The results indicated that proofing time (10–30 minutes) had a significant

positive effect on total score of steamed buns made from strong flour, whereas fermentation time (60–180 minutes) had a positive effect on total score of CSB made from medium flour. There was a strong interaction between fermentation time and proofing time, which positively affected the total score of steamed buns made from medium flour. Sheeting had positive effects on the total score and the exterior smoothness score for both types of flour.

Fu et al. (2005) studied the effects of mixing time and water absorption content of wheat flour on the quality of CSB. It was found that CSB of lean formulation (no sugar or shortening added) collapsed when mixing time was reduced from 10 to 6 minutes, while CSB of rich formulation (sugar and shortening added) did not collapse when mixing time was reduced from 7 to 4 minutes. Besides, the lean formulation CSB collapsed when water absorption increased from 40 to 43%, while there was no effect on the rich formulation CSB when water absorption increased from 42 to 45%. It was concluded that in steamed bun production, CSB of rich formulation showed higher processing tolerance than the lean formulation under increased water absorption and reduced mixing time. Therefore, the formulation and processing conditions should be addressed as well.

Kim et al. (2009) evaluated the effects of amount of sourdough, fermentation time, and amount of yeast on the quality of CSB produced using all-purpose and whole-wheat flours. The results showed that the amount of yeast significantly influenced the pH of sourdough steamed buns produced with whole-wheat flour, but did not affect steamed buns produced with all-purpose flour. The amount of sourdough in steamed buns and fermentation time affected the total titratable acidity values of the steamed buns even though different types of wheat flour were used. It was also found that as the fermentation time increased, the crumb texture becomes softer in both steamed buns. An amount of 10%–25% sourdough produced the softest crumb texture for both steamed buns. One percent of yeast also gave the softest bun texture when produced with whole-wheat flour. In addition, this study also found that ethanol and 3-methyl-1-butanol were the two most abundant volatile compounds detected in the steamed buns, with and without sourdough.

It can be seen that proofing time, fermentation time, amount of sourdough (starter dough), and amount of yeast added affect CSB quality.

32.6.3 Food Additives

Some additives such as endoxylanases (used to improve dough handling properties, oven spring, and loaf volume in breadmaking), diacetyl tartaric acid ester of monoglycerides (DATEM), and thermophilic xylanases were reported as food additives used to improve dough and steamed bun properties. Their different effects on dough and steamed bun properties were studied.

Su et al. (2005) investigated the effects of two types of endoxylanase, i.e., solid-phase enzyme (S-XYL) and liquid-phase enzyme (L-XYL), on dough properties and CSB quality. Endoxylanases decreased the degree of polymerization of arabinxylans (AX), which is contained in wheat at 1.5%–2.5%, and liberated oligomers, xylobiose, and xylose (Kent 1983). It was found in this study that both enzymes significantly decreased the water absorption, dough development and stability time, and mixing tolerance index (MTI) of dough in a farinograph test. Adding both enzymes decreased the dough hardness. As a consequence, adhesiveness of dough decreased as well. Additionally, they shortened the fermentation time. The effects of S-XYL and L-XYL addition on CSB quality were to increase specific volume and spread ratio and decrease crumb softness and whiteness, but there were no significant differences in elasticity. In all cases, the effect of L-XYL addition was more obvious than that of S-XYL.

DATEM, another food additive used in CSB making, is reported to be an effective bread emulsifier. Its best functions are to increase the specific volume and to improve the structure of bread (Stampfli et al. 1996). The effects of DATEM on bread vary with flour protein content or gluten strength (Farvili et al. 1997).

Ying et al. (2009) studied the effects of the DATEM on the qualities of CSB made from different flour types. It was reported that the effects of DATEM on gluten strength and dough stability varied with different flour types. In case of weak flour, DATEM increased gluten strength and dough stability. On the contrary, DATEM weakened gluten strength in strong flour. However, the overall qualities of CSB made from either weak or strong wheat flours were improved by the use of this additive. The results of the

effects of DATEM on medium strong flour were not consistent. The researchers speculated that the lipid content of medium strong flour might be too low for DATEM to bind with; therefore, the dough strength was not increased.

Jiang et al. (2010) studied the effect of xylanase on the quality of CSB using thermophilic xylanases produced from *Chaetomium* sp nov. It was found that various levels of thermophilic xylanase (0.5–5 ppm) improved the specific volume increased the whiteness and decreased the spread ratio and crumb firmness of CSB.

From these studies, food additives can be used to improve CSB quality. However, the type of wheat flour and the amount of food additive should be carefully considered.

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REFERENCES

Anonymous. 2010. [Online]. Available from: (http://en.radio86.com/chinese-food/how-make-baozi-steamed-buns).

Crosbie GB, Huang S, Barclay IR. 1998. Wheat quality requirements of Asian foods. *Euphytica* 100:155–6.

Dexter JE, Wood PJ. 1996. Recent application of debranning of wheat before milling. *Trends Food Sci Technol* 7:35–40.

Farvili N, Walker CE, Qarooni J. 1997. The effects of protein content of flour and emulsifiers on tanoor bread quality. *J Cereal Sci* 26:137–43.

Fu BX, Assefaw EG, Carson GR, editors. Evaluation of flour quality for steamed bread processing—a model using Canadian wheat. Proceeding 55th Australian Cereal Chemistry Conference and Pacific Rim Symposium—The 12th Convention Royal Australian Chemical Institute (RACI) Convention; 2005 July 3–7; Sydney, Australia. 2005.

Gocmen D, Gurbuz O, Kumral AY, Dagdelen AF, Sahin I. 2007. The effects of wheat sourdough on glutenin patterns, dough rheology and bread properties. *Eur Food Res and Technol* 225:821–30.

He ZH, Liu AH, Javier Peña R, Rajaram S. 2003. Suitability of Chinese wheat cultivars for production of northern style Chinese steamed bread. *Euphytica* 131:155–63.

Huang S, Quail K, Moss R. 1998. The optimization of a laboratory processing procedure for southern-style Chinese steamed bread. *Int J Food Sci Technol* 33:345–57.

Huang S, Yun S, Quil K, Moss R. 1996. Establishment of flour quality guidelines for Northern style Chinese steamed bread. *J Cereal Sci* 24:179–85.

Jiang Z, Cong Q, Yan Q, Kumar N, Du X. 2010. Characterisation of a thermostable xylanase from *Chaetomium* sp. and its application in Chinese steamed bread. *Food Chem* 120:457–62.

Keeratipibul S, Luangsakul N, Otsuka S, Sakai S, Hatano Y, Tanasupawat S. 2010. Application of the Chinese steamed bun starter dough (CSB-SD) in breadmaking. *J Food Sci* 75:596–604.

Kent NL. 1983. *Technology of Cereals*. 3rd ed. New York: Pergamon Press.

Kim Y, Huang W, Zhu H, Rayas-Duarte P. 2009. Spontaneous sourdough processing of Chinese Northern-style steamed breads and their volatile compounds. *Food Chem* 114:685–92.

Koike N, Lorenz K, Bechtel PJ. 1998. Steamed breads made from Colorado grown white wheat. *J Food Process Preserv* 22:41–52.

Li H, Cao Y, Liu X, Fu L. 2007. Differentiation of *Lactobacillus* spp. isolated from Chinese sourdoughs by AFLP and classical methods. *Ann Microbiol* 57:687–90.

Lijuan S, Guiying Z, Guoan Z, Zaigui L. 2007. Effects of different milling methods on flour quality and performance in steamed breadmaking. *J Cereal Sci* 45:18–23.

Lin Q, Liu L, Bi Y, Li Z. 2010. Effects of different debranning degrees on the qualities of wheat flour and Chinese steamed bread. *Food Biop Technol*. doi:10.1007/s11947-010-0335-3.

Luangsakul N, Keeratipibul S, Jindamorakot S, Tanasupawat S. 2009. Lactic acid bacteria and yeasts isolated from the starter doughs for Chinese steamed buns in Thailand. *LWT—Food Sci Technol* 42:1404–12.

Peng Q, Shun-He C, Chum-Xi MA. 2007. Effect of waxy wheat flour blends on the quality of Chinese steamed bread. *Agric Sci China* 6:1275–82.

Stampfli L, Nersted B, Molteberg EL. 1996. Effects of emulsifiers on farinograph and extensograph measurements. *Food Chem* 57:523–30.

Su DM, Ding CH, Li LT, Su DH, Zheng XY. 2005. Effect of endoxylanases on dough properties and making performance of Chinese steamed bread. *Eur Food Res Technol* 220:540–5.

Thiele C, Ganzle MG, Vogel RF. 2002. Contribution of sourdough lactobacilli, yeast and cereal enzymes to the generation of amino acids in dough relevant for bread flavour. *Cereal Chem* 79:45–51.

Ying B, Jun L, Yunzi F, Zhuo C, Zaigui L. 2009. Applicability of DATEM for Chinese steamed bread made from flours of different gluten qualities. *J Sci Food Agric* 89:227–31.

Zhu J, Huang S, Khan K, O'Brien L. 2001. Relationship of protein quantity, quality and dough properties with Chinese steamed bread quality. *J Cereal Sci* 33:205–12.

33

*Whiskey Manufacture**

Y. H. Hui

CONTENTS

33.1 Introduction

Whiskey (Gaelic, *uisge beatha* = water of life) is classified as a spirit made from cereal starch, the manufacture of which involves hydrolytic breakdown of the starch into fermentable sugars, followed by fermentation, distillation, and maturation. Whiskies themselves may be classified on the basis of the cereal used (wheat, barley, corn, rye), on the degree of blending, and on the country of origin; almost all whiskey is produced in Scotland, Ireland, Canada, and the United States. Malt (scotch) whiskey (from barley) is made in the Scottish highlands and the West Coast, and grain whiskey (from cereals other than barley) is manufactured in the Scottish lowlands and Ireland. Rye (rye-based) and Bourbon (corn-based) whiskies originate in the North American continent. In the raw cereals, the essential component for whiskey production is the starch content, which is approximately 70% on a dry weight basis (see the following references for discussions throughout the chapter: Barnard 2008; Bryce et al. 2009; Herstein 2007; Lee et al. 2003; Nettleton 2009; Russell et al. 2003).

Figure 33.1 shows the steps in processing and manufacturing whiskey. Table 33.1 shows the processing steps and application principles.

33.2 Malting and Kilning (Malt Whiskey)

The barley is cleaned and then steeped in cold water for 2 to 3 days. The wet grain is allowed to germinate at 24°C over a period of 8 to 12 days. This procedure results in an increase in the activities of the enzymes α-amylase and β-amylase. These enzymes catalyze the hydrolytic breakdown of starch and

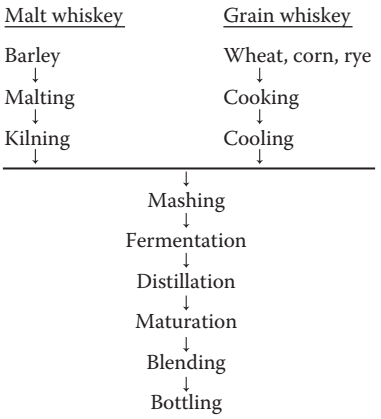


FIGURE 33.1 Flow chart for the manufacture of whiskey.

are specific toward the α-1,4-glucosidic linkages of the amylose and amylopectin components of starch. They differ in their modes of action: α-amylase carries out internal random hydrolysis, while β-amylase removes two glucose residues at a time from the nonreducing end of the polysaccharide chain. The principal end product of the joint action of α- and β-amylases upon starch is the fermentable sugar maltose. Some dextrin is also produced since neither of these amylases hydrolyzes the α-1,6-glucosidic linkage branch points of amylopectin (Lee et al. 2003).

Malting also encourages the development of β-glucanase and protease activities. These enzymes act upon components of the starchy endosperm (84% of the total barley grain), respectively, the endosperm cell wall structure, and the storage proteins (12% of the raw cereal on a dry weight basis), to enable germination to take place.

The sprouted grain is subjected to kilning, in which the grain is heated gradually to approximately 52°C to stop germination but not to destroy the enzymes. During kilning, the moisture level of the germinated barley (malt) is reduced to approximately 5%. In the manufacture of scotch, the malt is dried in the presence of smoke from a peat fire, which in turn contributes phenolic flavor compounds (phenol, isomeric cresols, xylenols, and guaiacol) to the malt. The dry malt may be stored until required.

TABLE 33.1

Processing Steps and Application Principles for Whiskey Processing

	Stages of Processing	Application Principles
A	Raw material	Starch content
B	Pretreatment before mashing	Amylases, β-glucanase, proteases, starch gelatinization
C	Mashing	Hydrolysis of starch, solubilization of sugars and amino acids
D	Fermentation	Ethanol formation
E	Distillation	Recovery of ethanol
F	Maturation	Flavor development
G	Blending	Mixtures of malt and grain whiskies
H	Bottling	Dilution of matured spirit

33.3 Mashing

The dried malt is milled to form the *grist*, which is then mixed with water at approximately 68°C in mash tuns for 0.5–1.5 hours. This process of mashing is characterized chemically by the continuing hydrolysis of starch by amylase activity into fermentable sugars, chiefly maltose, with smaller amounts of sucrose, glucose, and fructose. The other principal purpose of mashing is to bring into solution not only the sugars but also amino acids, resulting from protease activity, which serve as a nitrogen source for yeast growth during fermentation (Barnard 2008).

At the end of the mashing period, the resulting liquor (wort) is recovered by filtration, while the remaining residue (draff) is used as cattle feed.

33.4 Grain Whiskey

In the production of grain whiskey, an initial cooking stage at 120°C for 1.5 hours is required to gelatinize the starch of cereals other than barley. The resulting mash is cooled to 60°C–65°C before the addition of 10%–15% of freshly malted barley, which provides the amylase activity for the hydrolysis of the gelatinized starch (Russell et al. 2003).

33.5 Fermentation

The wort is cooled to 20°C–25°C and then inoculated with yeast (*Saccharomyces cerevisiae*). Fermentation, in vessels of capacity ranging from 9,000 to 45,000 L, requires 2–3 days and is principally characterized by the growth of the yeast and the fermentation (through the Embden-Meyerhof-Parnas, or glycolytic, metabolic pathway) of the wort sugars to ethanol (Figure 33.2).

The pH of the fermenting medium may decrease from 5.0–5.5 to 4.2–4.5 due to the production of organic acids, especially acetic, succinic, and pyruvic acids. Many other compounds, such as higher alcohols, carboxylic acids, esters, aldehydes, and ketones, are formed at this time: these compounds are sufficiently volatile to carry over into the distillate and therefore to make a major contribution to the final flavor of the whiskey. Glycerol, also formed during fermentation, is nonvolatile (boiling point 290°C)

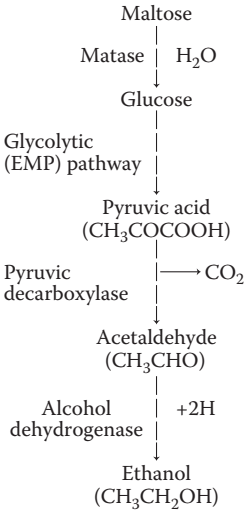


FIGURE 33.2 Fermentation of maltose to ethanol and carbon dioxide.

and is therefore a nondistillable organic material that might otherwise have contributed to the whiskey flavor. The yeast, in addition, has the ability to convert sulfur-containing amino acids into volatile sulfur-containing flavor compounds such as dimethyl disulfide (CH_3-S-S-CH_3) (Herstein 2007).

In whiskey, an important component in terms of contribution to flavor is a small optimal amount of the fusel oil fraction (*n*-amyl alcohol and isoamyl alcohol), which originates during fermentation as a result of the deamination of leucine and isoleucine.

These amino acids in turn arise from the hydrolysis of grain proteins and autolyzed yeast protein. The product of this fermentation is a liquid (the *wash*) that contains about 10% ethanol. The carbon dioxide produced during fermentation is recovered and used, for instance, in the manufacture of soft drinks.

33.6 Distillation

33.6.1 Malt Whiskey

The production of malt whiskey involves batch distillation of the fermented liquid in two copper pot stills, namely, the wash still and the spirit still (Nettleton 2009).

All the contents of the fermentation vat are transferred to a large wash still and boiled for 5–6 hours to produce a distillate (the low wines) that contains 20%–25% ethanol. The low wines are distilled in the spirit still to produce the following three fractions:

1. foreshots
2. whiskey (potable spirit containing 63%–71% ethanol)
3. feints (approximately 25% ethanol)

The careful selection of the cut points during this distillation procedure greatly influences the flavor of the final product. The first cut from foreshots to spirit governs the level of aldehydes and short-chain esters, while the second cut (spirit to feints) determines the concentrations of higher alcohols and acids. The foreshots and feints are returned to the process, being redistilled in the spirit still with the next batch of low wines. The conditions of distillation also enable further reactions to occur with respect to whiskey flavor development:

1. Continuing ester formation
2. Maillard reaction and the accompanying Strecker degradation to produce aldehydes (especially acetaldehyde, 2-furaldehyde, 5-hydroxymethyl-2-furaldehyde)
3. Continuing formation of volatile sulfur-containing compounds from corresponding nonvolatile precursors
4. The conversion of β-hydroxypropionaldehyde to acrolein:

$$HOCH_2CH_2CHO \rightarrow CH_2{=}CHCHO$$

$$-H_2O$$

5. The oxidation of unsaturated fatty acids (derived from barley malt) to carbonyl compounds

33.6.2 Grain Whiskey

Grain whiskey is normally produced by continuous distillation in a special still. Usually, the incoming material is the cold wash of 10% ethanol content, and the principal product from this process is raw spirit containing 95% ethanol (Nettleton 2009).

The distillation of aqueous ethanolic mixtures is governed by specific physical properties of water and ethanol, most notably that an aqueous ethanolic mixture cannot be completely separated into ethanol and

water. These are liquids that are completely miscible with each other, and therefore, of all the possible ethanol–water mixture compositions, there is one particular mixture (96% ethanol and 4% water) that has a maximum vapor pressure and that therefore boils at a lower temperature than any other mixture of these two liquids. This mixture is an azeotropic (constant boiling) mixture, which is able to distill without changing its composition, that is, the distillate has the same composition as the original liquid. In this case, it is a minimum boiling mixture (a particular kind of azeotropic mixture) since the boiling point is lower than for any other mixture of ethanol and water.

In the still, the wash is steam-distilled, and the resulting vapor is richer in ethanol than the original wash. However, in the rectifier column, the principal distillate fraction recovered is the azeotropic ethanol–water mixture (raw spirit) containing 95%–96% ethanol. A high level of ethanol is required in the maturation of whiskey, but in any event, the matured spirit is diluted with water before bottling.

33.7 Maturation

The whiskey obtained directly from the distillation step is colorless and has a harsh taste, and therefore, a long period of maturation is essential to develop the final flavor, which is a major determinant of whiskey quality. The raw spirit is stored in air-permeable oak barrels (casks) for up to 12 or 15 years; sometimes even longer: a minimum maturation period of 3 years is mandatory for scotch and Canadian whiskies. It is this maturation step that distinguishes whiskey from rawer spirits such as gin and vodka. Maturation, the progress of which is governed by the factors of temperature, time, and humidity, is characterized by gradual changes in the flavor and color of the maturing spirit, together with a concurrent decline in both volume and alcoholic content. There is also at this time a corresponding decline in the level of volatile sulfur-containing flavor compounds, which were generated during the fermentation and distillation stages. The flavor changes occur essentially as a result of reaction among the chemical components both of the barrel wood and of the raw distillate and continuing interaction between these two sets of chemical components (Barnard 2008; Bryce et al. 2009; Herstein 2007).

Barrels used in whiskey maturation are made either from American white oak (*Quercus alba*) or from oaks of European origin (*Q. petraea* and *Q. robur*). Not only is the original wood important, but a key step in barrel manufacture is the heat charring (over 200°C) of the inside of the barrel, a procedure that brings about the degradation of the major structural components of the wood. In addition, the whiskey industry uses recharred (regenerated) barrels that had previously been used for the maturation of other alcoholic beverages, especially sherry.

In chemical terms, the maturation of whiskey is far from being fully understood. However, the degradation of the structural components of oak heartwood by heat charring may be outlined as follows (see Figure 33.3):

1. Lignin to methoxy (for example, vanillin) and dimethoxy phenols and cresols
2. Hemicellulose to hexoses and pentoses, which lose molecules of water to become furfural and hydroxymethyl furfural
3. Cellulose to D-glucose
4. Maillard reaction with its accompanying Strecker degradation: eventual production of pyrazines, pyrroles, and pyridines, together with the direct heat pyrolysis of sugars

In addition to the structural compounds, oak heartwood contains extractives, especially phenolic compounds, fatty acids, and lactones, which become extracted directly into the raw spirit during maturation.

All of these low-molecular weight compounds are highly soluble in aqueous ethanol and such extraction, into the spirit, of these compounds originating from the wood of the barrel is an important part of maturation.

The species of oak used in barrel construction also influences the final whiskey flavor. The use of wood from *Q. alba* results in increased levels of vanillin and of lactones, especially the *cis* and *trans* isomers of 4-hydroxy-3-methyloctanoic acid lactone (also known as 3-methyl-4-hydroxy caprylic acid

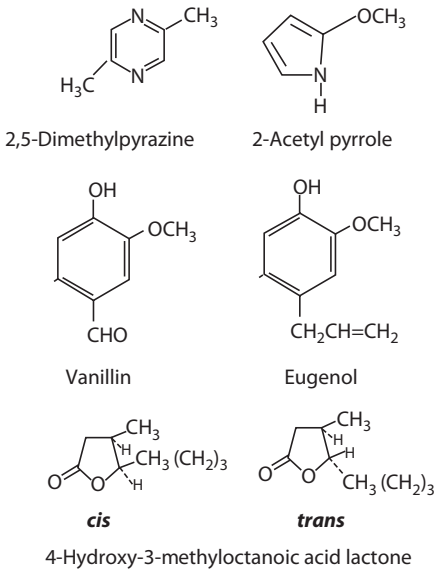


FIGURE 33.3 Structure of several compounds that impart unique flavors to whiskey during the aging and maturing process.

γ-lactone, β-methyl-γ-octalactone, whiskey lactone, or oak lactone; see Figure 33.3), in the maturing whiskey, where they impact a woody aroma, while the concentration of tannins is increased with the use of wood from European oaks. In this context, a characteristic component of oak-matured spirits is eugenol, which imparts a clove-like flavor.

The use of old sherry casks for whiskey maturation increases in its own right the level of tannins and of sugars such as glucose, arabinose, and xylose in the maturing spirit.

During maturation, the very many chemical reactions, involving both the components of the distillate and the wood-derived compounds, may be variously described as oxido-reductions, esterifications, Maillard reactions, polymerizations, and polycondensations. The first group of reactions are the most important, especially since the oak barrels are permeable to oxygen, and may be exemplified by the oxidation of some of the ethanol to acetaldehyde and acetic acid with the subsequent formation of ethyl acetate.

Maturation over a period of several years results also in the development of the amber color, which for the purpose of consumer acceptability may be standardized with added caramel.

33.8 Blending

Malt whiskey by itself has a heavy flavor and is therefore frequently blended with grain whiskey to increase its general acceptability. The most frequent composition of blended whiskey is 60%–70% grain whiskey and 30%–40% malt whiskey (Bryce et al. 2009).

33.9 Whiskey Flavor Components

The ethanol content excepted, the most important property, and hence quality criterion, of whiskey is the flavor, which is governed by the presence and relative concentrations of a large number of organic compounds, which together are less than 1% of the total whiskey composition. The flavor profiles are determined by gas chromatography (GC) for the volatile components and by high-performance liquid chromatography for the relatively nonvolatile components. Since each flavor component occurs at the

nanogram level or lower, final identification may have to be confirmed by mass spectrometry or by infrared spectroscopy. GC analysis has been able to identify 300–350 different volatile compounds, including 30+ alcohols and 20+ esters, from scotch whiskey, and such objective information may complement the more traditional but subjective organoleptic testing. The number of flavor components tends to be fewer in grain whiskey than in malt whiskey. The alcohols, especially of course ethanol, are very important components of whiskey (Lee et al. 2003; Nettleton 2009; Russell et al. 2003).

In whiskey, carboxylic acids are also present: acetic acid accounts for 50%–90% of the total volatile acid fraction. Other acids present are short-chain saturated volatile fatty acids [octanoic (caprylic) acid 8:0, decanoic (capric) acid 10:0, dodecanoic (lauric) acid 12:0] and lactic and succinic acids.

The conditions of fermentation, distillation, and maturation favor the interaction of alcohols and organic acids to produce esters, which are volatile and possess distinct aromas. The ethyl esters of the preceding acids are particularly well represented, and the most important ester is ethyl acetate (bp 77.1°C) formed from ethanol and acetic acid:

$$C_2H_5OH + CH_3COOH \rightarrow CH_3COOC_2H_5 + H_2O$$

Acetaldehyde is the principal carbonyl compound in whiskey, but other such compounds are also present. Acetals such as 1,1-diethoxyethane (diethyl acetal) also occur in whiskey and arise, during distillation and maturation, from the interaction of acetaldehyde and ethanol in acid conditions.

The conditions of whiskey manufacture greatly encourage the formation of many compounds from the materials used in production and the subsequent interaction of these compounds. Increasing knowledge of the chemistry of whiskey manufacture may result in closer correlation, in terms of quality control, between objective chemical analysis, for example, GC profiles and subjective sensory evaluation.

33.10 Bottling

The matured spirit is diluted with water to 40%–43% by volume of ethanol (80°–86° U.S. proof) before being bottled. Therefore, in terms of quantity, the major component of whiskey is actually water (approximately 60% by volume). Once bottled, the whiskey does not undergo subsequent change because oxygen no longer has access to the matured spirit. This represents the termination of maturation, but on the other hand there is no decline in whiskey quality due to age (Herstein 2007).

REFERENCES

Barnard A. 2008. *The Whisky Distilleries of the United Kingdom*. Edinburgh, Scotland: Birlinn.

Bryce JH, Piggott JR, Stewart GG. 2009. *Distilled Spirits: Production, Technology and Innovation*. Nottingham, UK: Nottingham University Press.

Herstein KM. 2007. *Chemistry and Technology—Wines and Liquors*. Dayton, OH: Herstein Press.

Lea AGH, Piggot JR. 2003. *Fermented Beverage Production*. New York: Springer.

Nettleton JA. 2009. *The Manufacture of Whisky and Plain Spirit*. Larbert, UK: Distilled Discourse.

Russell I, Bamforth C, Stewart G. 2003. *Whisky: Technology, Production and Marketing*. Burlington, MA: Academic Press.

34

Beer Production in the United States: Regulatory Requirements

Y. H. Hui

CONTENTS

34.1 Introduction

For any country, when a private company produces beer for public consumption, the government exercises a tight control over the business for three important reasons: tax, economic integrity, and safety. In the United States, two federal agencies prescribe regulations for the commercial production of beer:

1. Bureau of Alcohol, Tobacco, and Firearms Tax and Trade Bureau (TTB, www.ttb.gov)
2. Food and Drug Administration (FDA, www.fda.gov/)

This chapter describes some of these requirements. Let us first provide a brief discussion on the process of making beer. Only an outline is provided. To produce beer commercially, refer to standard references in beer making.

34.2 The Process of Making Beer: Brewing

Beer is produced by steeping cereal grains in water followed by fermentation with yeast. The process is called brewing. The basic ingredients of brewing are

- Water
- A starch source, e.g., malted barley, capable of being fermented to form alcohol.
- A brewer's yeast for fermentation.
- A supplementary or an adjunct starch source.
- Flavorings such as hops adjuncts, e.g., maize (corn), rice, or sugar. However, in some countries, the starch sources may include millet, sorghum, cassava root, potato, and agave. Grain mill refers to the quantity of each starch source used in a beer production recipe.

There are several steps in the brewing process and the major steps are as follows:

1. Barley (wheat, rice, other cereals) in vat with water
2. Malt
3. Milling
4. Mashing
5. Launtering
6. Wort: boiling, settling, clarification, cooling
7. Fermentation
8. Racking
9. Conditioning
10. Filtration
11. Carbonation
12. Bottling

One starts out with a formula. Using the formula as a guide, the brewing process is as follow. We will use barley as the grain.

Barley and malt: Although most beers are made from barley, others are used as well, e.g., wheat, rice, other cereals. The barley is put in a vat with water. The grain is soaked for 40 hours, referred to as steeping. Then, the grain is spread on the floor for about 5 days so they will germinate. The resulting produce is known as green malt. Germination makes enzymes, especially α-amylase and β-amylase. Enzymes convert grains to sugar. This green malt is dried in a kiln at high temperature (roasting). We now have the product malt, which will be subjected to milling.

Milling: The malt or processed barley is subject to cracking and milling. The malt is mixed with water, which extracts the sugar from the malt, which goes to the next stage of mashing.

Mashing: The milled grains are dropped into hot water in a large vessel known as a mash tun. Measured amounts of unmalted cereals are added; grain and water mixed to create a cereal mash. Thus, mashing converts released starches during malting into sugars that can be fermented.

Launtering: Lautering is the separation of the wort (the liquid containing the sugar extracted during mashing) from the grains. A mash tun is equipped with a filter, a launter tun, or simply a false bottom is used. The separation process usually contains two steps:

1. Sugar-rich water is drained from cereal mash to form a liquid known as wort.
2. The spent grains are then washed with hot water to recover any extracts not drained off. This is known as sparing.

Wort: boiling, cooling, settling, and clarification: The wort is moved into a large tank known as a "cooper" or "kettle." It is then subjected to an intensive boiling. The flower of the *hop* vine is used as a flavoring and preservative agent in most beer. Hops are added with herbs, sugars, and other flavoring compounds. At the end of the boil, the wort is transferred to vessel called "whirlpool." At this stage, the flavor, color, and aroma are determined. The end result is the formation of a cone in the center of the tank. This cone contains coagulated proteins, vegetable matter from hops, and other dense solids. After the whirlpool, the liquid wort is cooled and then moved to a fermentation tank. At this stage, yeast is added. The yeast ferments the sugars and other carbohydrate into alcohol, carbon dioxide, and other components. The beer at this stage requires refinement.

Racking and conditioning: Brewer racks the beer in a tank. This ages the beer to achieve the smooth flavor. Now, we have fresh (or "green") beer. After 1–3 weeks, the beer is then moved to a conditioning tank. The beer is conditioned for 1 week to several months. Further refinement is the next stage.

Filtration, carbonation, and bottling: The beer is filtered to stabilize flavor and to develop polish and color. It is then moved to a holding tank for carbonation. The beer is ready for bottling and distribution.

In the United States, the commerce of beer production is governed by many requirements. Some selected information is provided in the next few sections.

TABLE 34.1

Laws and Regulations in the United States

Law	Implementing Regulation	Regulation Title
Federal Alcohol Administration Act (FAA Act); 27 USC 201 et seq.	27 CFR 7	Labeling and Advertising of Malt Beverages
Alcoholic Beverage Labeling Act of 1988 (ABLA); 27 USC 213 et seq.	27 CFR 16	Alcoholic Beverage Health Warning Statement
Internal Revenue Code (IRC) 26 USC Chapter 51	27 CFR 25	Beer
	27 CFR 250	Liquors and Articles from Puerto Rico and the Virgin Islands
	27 CFR 251	Importation of Distilled Spirits, Wine, and Beer
	27 CFR 252	Exportation of Liquors

34.3 Laws and Regulations

Regulations for beer manufacture can be found in Table 34.1.

USC = United States Codes, e.g., 27 USC 201 means Volume 27 of the United States Code, Part 2011

CFR = United States Code of Federal Regulations, e.g., 27 CFR 7 means Volume 27 of the United States Code of Federal Regulations, Part 7

Considering the amount of regulations promulgated by the TTB, this chapter will highlight some major requirements. Refer to 27 CFR 25. Details for all requirements can be obtained from its website.

34.4 Materials for the Production of Beer

Beer must be brewed from malt or from substitutes for malt. Only rice, grain of any kind, bran, glucose, sugar, and molasses are substitutes for malt. In addition, you may also use the following materials as adjuncts in fermenting beer: honey, fruit, fruit juice, fruit concentrate, herbs, spices, and other food materials.

You may use flavors and other nonbeverage ingredients containing alcohol in producing beer. Flavors and other nonbeverage ingredients containing alcohol may contribute no more than 49% of the overall alcohol content of the finished beer. For example, a finished beer that contains 5.0% alcohol by volume must derive a minimum of 2.55% alcohol by volume from the fermentation of ingredients at the brewery and may derive not more than 2.45% alcohol by volume from the addition of flavors, and other nonbeverage ingredients containing alcohol. In the case of beer with an alcohol content of more than 6% by volume, no more than 1.5% of the volume of the beer may consist of.

34.5 Formulas for Fermented Products

Formulas are required by the TTB if a manufacturer is involved in any of the following:

1. Any fermented product that will be treated by any processing, filtration, or other method of manufacture that is not generally recognized as a traditional process in the production of a fermented beverage designated as “beer,” “ale,” “porter,” “stout,” “lager,” or “malt liquor”
2. Removal of any volume of water from beer, filtration of beer to substantially change the color, flavor, or character, separation of beer into different components, reverse osmosis, concentration of beer, and ion exchange treatments are examples of nontraditional processes for which you must file a formula

3. Pasteurization, filtration prior to bottling, filtration in lieu of pasteurization, centrifuging for clarity, lagering, carbonation, and blending are examples of traditional processes for which you do not need to file a formula
4. Any fermented product to which flavors or other nonbeverage ingredients (other than hop extract) containing alcohol will be added
5. Any fermented product to which coloring or natural or artificial flavors will be added
6. Any fermented product to which fruit, fruit juice, fruit concentrate, herbs, spices, honey, maple syrup, or other food materials will be added
7. Saké, including flavored saké and sparkling saké

TTB requires the following information in the beer formula:

1. For each formula, you must list each separate ingredient and the specific quantity used, or a range of quantities used. You may include optional ingredients in a formula if they do not impact the labeling or identity of the finished product.
2. For fermented products containing flavorings, you must list for each formula: The name of the flavor, the product number or TTB drawback number, and approval date of the flavor; the name and location (city and state) of the flavor manufacturer; the alcohol content of the flavor; and the point of production at which the flavor was added (that is, before, during, or after fermentation).
3. For formulas that include the use of flavors and other nonbeverage ingredients containing alcohol, you must explicitly indicate:
   (i) The volume and alcohol content of the beer base;
   (ii) The maximum volumes of the flavors and other nonbeverage ingredients containing alcohol to be used;
   (iii) The alcoholic strength of the flavors and other nonbeverage ingredients containing alcohol;
   (iv) The overall alcohol contribution to the finished product provided by the addition of any flavors or other nonbeverage ingredients containing alcohol. You are not required to list the alcohol contribution of individual flavors and other nonbeverage ingredients containing alcohol. You may state the total alcohol contribution from these ingredients to the finished product and
   (v) The final volume and alcohol content of the finished product.

In addition, other information is required:

1. For each formula, you must describe in detail each process used to produce a fermented beverage.
2. For each formula, you must state the alcohol content of the fermented product after fermentation and the alcohol content of the finished product.
3. You must refer in your formula to any approved formula number that covers the production of any beer base used in producing the formula product. If the beer base was produced by another brewery of the same ownership, you must also provide the name and address or name and registry number of that brewery.
4. The appropriate TTB officer may at any time require you to file additional information concerning a fermented product, ingredients, or processes, in order to determine whether a formula should be approved or disapproved or whether the approval of a formula should be continued.

You must file a new formula (with a new formula number) for approval by TTB, if you

1. Create an entirely new fermented product that requires a formula
2. Add new ingredients to an existing formulation
3. Delete ingredients from an existing formulation

4. Change the quantity of an ingredient used from the quantity or range of usage in an approved formula
5. Change an approved processing, filtration, or other special method of manufacture that requires the filing of a formula
6. Change the contribution of alcohol from flavors or ingredients that contain alcohol

34.6 Legal Definition for Malted Beverages

Malt beverage is legally defined as an alcohol or alcohol-free beverage made by the alcoholic fermentation of an infusion or decoction or combination of both, in potable brewing water, of

- malted barley comprising not less than 25% by weight of the total weight of fermentable ingredients
- hops (or their parts or products) in an amount equivalent to 7.5 pounds per 100 barrels (3100 gallons) of finished malt beverage

and with or without

- other malted cereals
- unmalted or prepared cereals
- other carbohydrates (or products prepared there from)
- carbon dioxide
- other wholesome products suitable for
- human food consumption

34.7 Legal Class and Types of Malted Beverages

Tables 34.2 and 34.3 describe the legal class and types of malted beverages.

34.8 Mandatory Label Information

TTB has required the following label:

Brand name

General features

– Usually, the most prominent piece of information on the label
– Name under which a malt beverage or line of malt beverages is marketed

Note: If the malt beverage is not sold under a brand name, the name of the bottler, packer, or importer (shown on the front of the container) becomes the brand name

Misleading brand name

A name that describes the age, origin, identity, or other characteristics of the malt beverage is prohibited unless the name, whether standing alone or in combination with other printed or graphic material

– Accurately describes the malt beverage and
– Conveys no erroneous impression about the malt beverage

or

is qualified with

The word “brand” or

A statement that dispels any erroneous impression created by the brand name

TABLE 34.2

Classes of Malt Beverages

Class	General Definition
Ale[a]	Malt beverage fermented at a comparatively high temperature containing 0.5% or more alcohol by volume possessing the characteristics generally attributed to and conforming to the trade understanding of "ale"
Beer[a]	Malt beverage containing 0.5% or more alcohol by volume possessing the characteristics generally attributed to and conforming to the trade understanding of "beer"
Cereal beverage[a]	Malt beverage containing less than 0.5% alcohol by volume
Lager/lager beer[a]	Malt beverage containing 0.5% or more alcohol by volume possessing the characteristics generally attributed to and conforming to the trade understanding of "lager"/"lager beer"
Malt beverage specialty[b]	Generally, any class and/or type of malt beverage that contains or is treated with (other than those recognized in standard brewing practices) flavoring and/or coloring materials and/or nonstandard blending or treating materials or processes
Malt liquor[a]	Malt beverage containing 0.5% or more alcohol by volume possessing the characteristics generally attributed to and conforming to the trade understanding of "malt liquor"
Near beer[a]	Malt beverage containing less than 0.5% alcohol by volume
Porter[a]	Malt beverage fermented at a comparatively high temperature containing 0.5% or more alcohol by volume possessing the characteristics generally attributed to and conforming to the trade understanding of "porter"
Stout[a]	Malt beverage fermented at a comparatively high temperature containing 0.5% or more alcohol by volume possessing the characteristics generally attributed to and conforming to the trade understanding of "stout"

[a] Sufficient as class and type designation.

[b] A distinctive or fanciful product name with a statement reflecting the composition and character of the product is sufficient as class and type designation, e.g., "honey ale, ale brewed with honey."

TABLE 34.3

Types of Malt Beverages

Type	General Description
Amber ale[a]	Malt beverage containing 0.5% or more alcohol by volume
Amber lager[a]	Malt beverage containing 0.5% or more alcohol by volume
Barley wine ale/barley wine style ale[a]	Malt beverage containing 0.5% or more alcohol by volume
Bavarian[b]	Malt beverage containing 0.5% or more alcohol by volume produced in Germany
Bitter ale[a]	Malt beverage containing 0.5% or more alcohol by volume
Black and tan[c]	Product containing 0.5% or more alcohol by volume composed of two classes of malt beverages
Bock beer[a]	Malt beverage containing 0.5% or more alcohol by volume
Brown ale[a]	Malt beverage containing 0.5% or more alcohol by volume
Cream ale[a]	Malt beverage containing 0.5% or more alcohol by volume
Cream stout[a]	Malt beverage containing 0.5% or more alcohol by volume
Dortmund/dortmunder[b]	Malt beverage containing 0.5% or more alcohol by volume produced in Germany
Draft beer/draught beer[a]	Unpasteurized[d] malt beverage containing 0.5% or more alcohol by volume
Dry beer[a]	Malt beverage containing 0.5% or more alcohol by volume
Export ale[a]	Malt beverage containing 0.5% or more alcohol by volume
Export beer[a]	Malt beverage containing 0.5% or more alcohol by volume
Golden ale[a]	Malt beverage containing 0.5% or more alcohol by volume
Golden lager[a]	Malt beverage containing 0.5% or more alcohol by volume
Half and half	Product containing 0.5% or more alcohol by volume composed of equal parts of two classes of malt beverages

(*continued*)

TABLE 34.3 (Continued)

Types of Malt Beverages

Type	General Description
Ice beer[a]	Malt beverage containing 0.5% or more alcohol by volume supercooled during the brewing process to form ice crystals
Imperial stout[a]	Malt beverage containing 0.5% or more alcohol by volume
India pale ale[a]	Malt beverage containing 0.5% or more alcohol by volume
Kulmbacher[b]	Malt beverage containing 0.5% or more alcohol by volume produced in Germany
Munich/munchner[b]	Malt beverage containing 0.5% or more alcohol by volume produced in Germany
Pale ale[a]	Malt beverage containing 0.5% or more alcohol by volume
Pils	Malt beverage containing 0.5% or more alcohol by volume
Pilsen/pilsener/pilsner	Malt beverage containing 0.5% or more alcohol by volume produced in the Czech Republic
Rye beer[a]	Malt beverage containing 0.5% or more alcohol by volume made from a fermentable base that consists of at least 5% by weight malted rye
Salvator[b]	Malt beverage containing 0.5% or more alcohol by volume produced in Germany
Stock ale[a]	Malt beverage containing 0.5% or more alcohol by volume
Vienna[b]	Malt beverage containing 0.5% or more alcohol by volume produced in Austria
Wein/Weiner[b]	Malt beverage containing 0.5% or more alcohol by volume produced in Germany
Wheat beer[a]	Malt beverage containing 0.5% or more alcohol by volume made from a fermentable base that consists of at least 25% by weight malted wheat
Wurtzburger[b]	Malt beverage containing 0.5% or more alcohol by volume produced in Germany

[a] Sufficient as class and type designation.

[b] Sufficient as class and type designation. For Bavarian, Dortmund/Dortmunder, Kulmbacher, Munich/Munchner, Salvator, Wein/Weiner, Wurtzburger produced outside of Germany or Vienna produced outside of Austria, the class and type designation "Bavarian," "Dortmund," etc., must include the word "type" or "American" or other adjective or statement, e.g., "brewed in the U.S.," indicating the true place of production.

[c] The class and type designation is the names of the two classes of malt beverages in conjunction with "black and tan," e.g., "black and tan, stout and ale." Note: The percentage of each class must also be shown if either class contributes less than 40% of the volume of the malt beverage, e.g., "black and tan, 70% stout and 30% ale."

The class and type designation is the names of the two classes of malt beverages in conjunction with "half and half," e.g., "half and half, porter and stout."

Not sufficient as class and type designation. "Malt beverage," "beer," "lager," or "lager beer" must appear as the class and type designation.

Sufficient as class and type designation for pilsen/pilsener/pilsner produced in the Czech Republic or the U.S. for pilsen/pilsener/pilsner produced outside of the Czech Republic or the United States, the class and type designation "pilsen" etc. must include the word "type" or "French" or other adjective or statement, e.g., "brewed in France," indicating the true place of production.

[d] Pasteurized beer may be described as "draft" (or "draught") only if the beer is packaged in containers of 1 gallon or larger and the contents of which are to be drawn off through a tap, spigot, faucet, or similar device. Other than this exception, pasteurized beer may not be described as "draft" (or "draught"). However, pasteurized beer may be described as "draft brewed," "draft beer flavor," etc., provided the fact that the beer is pasteurized is also noted on the label.

Type size

- Minimum 2 mm for containers larger than 0.5 pint
- Minimum 1 mm for containers of 0.5 pint or less

Legibility

- Must be readily legible
- Must appear on a contrasting background
- Must appear separate and apart from or be substantially more conspicuous than descriptive or explanatory information

Placement

Must appear on the front of the container

Class and type designation

Definition

The specific identity of the malt beverage

Type size

Minimum 2 mm for containers larger than 0.5 pint

Minimum 1 mm for containers of 0.5 pint or less

Legibility

- Must be readily legible
- Must appear on a contrasting background
- Must appear separate and apart from or be substantially more conspicuous than descriptive or explanatory information

Placement

Must appear on the FRONT of the container

Name and address

Required

For domestic malt beverages

The name and address of the producer/bottler or packer must appear on the label optionally preceded by an appropriate explanatory phrase such as "brewed and bottled/packed by," "brewed by," or "bottled/packed by"

Note:

- "Bottled by" is used for malt beverages bottled in containers of 1 gallon or less
- "Packed by" is used for malt beverages packed in containers of over 1 gallon

For imported malt beverages

The name and address of the importer must appear on the label preceded by an appropriate explanatory phrase such as "imported by," "sole agent," or "sole U.S. agent"

Definitions

Name

The company or corporate name or trade name identical to that shown on

Brewer's notice for domestic malt beverages

Basic permit for imported malt beverages

Address

For domestic malt beverages

City and state where malt beverage is bottled or packed

Note: The bottling/packing location may be contained within a listing of all the brewer's locations provided

1. The place of actual production/bottling or packing is given no less prominence than other locations listed
2. The actual location (address) where the malt beverage is produced/bottled or packed is indicated by printing, coding, or other markings on the label or container
3. The brewer files, prior to use, a notice explaining the coding system with the government

or

City and state of the bottler's/packer's principal place of business (see "principal place of business" section of this item)

Note: The address must be identical to that shown on the brewer's notice

For imported malt beverages

City and state of importer's principle place of business (see "principal place of business" section of this item)

Note: The principal place of business address must be identical to that shown on the basic permit

Principal place of business: use

For domestic malt beverages

The principal place of business address may be used on labels in lieu of the address of the premise where the malt beverage was produced/bottled or packed

For imported malt beverages

The principal place of business address must be used on the label

Conditions for use

The principal place of business address must be a location where production/bottling or packing operations occur

The actual location (address) where the malt beverage is produced/bottled or packed must be indicated by printing, coding, or other markings on the label or container

and

The brewer must file, prior to use, a notice explaining the coding system.

Note: These conditions for use apply only to domestic breweries; they do not apply to U.S. importers.

Type size

- Minimum 2 mm for containers larger than 0.5 pint
- Minimum 1 mm for containers of 0.5 pint or less

Legibility

- Must be readily legible
- Must appear on a contrasting background
- Must appear separate and apart from or be substantially more conspicuous than descriptive or explanatory information

Placement

For domestic malt beverages

Must appear on the FRONT of the container

or

May be burned or branded into the container

For imported malt beverages

May appear on the front, back, or side of the container

Net contents

General

- There are no standards of fill for malt beverages
- Malt beverages may be bottled or packed in any size container
- Net contents must be expressed in American measure but may also be expressed in metric measure

Form of statement

- If the net contents of the container are
- Less than 1 pint, the net contents must be shown in fluid ounces or fractions of a pint
- 1 pint, 1 quart, or 1 gallon, the net contents must be shown exactly that way
- More than 1 pint but less than 1 quart, the net contents must be shown in pints and fluid ounces or fractions of a quart

- More than 1 quart but less than 1 gallon, the net contents must be shown in quarts, pints, and fluid ounces or fractions of a gallon
- More than 1 gallon, the net contents must be shown in gallons and fractions thereof

Type size

- Minimum 2 mm for containers larger than 0.5 pint
- Minimum 1 mm for containers of 0.5 pint or less

Legibility

- Must be readily legible
- Must appear on a contrasting background
- Must appear separate and apart from or be substantially more conspicuous than descriptive or explanatory information

Placement

Must appear on the front of the container

or

May be blown, branded, or burned into the container

Alcohol content

General

A statement of alcohol content is optional unless

It is required by state law

or

It is prohibited by state law

Unless otherwise prescribed by state law, the optional statement of alcohol content must be expressed in percent by volume. See "Form of Statement" section of this item.

Form of statement

Alcohol content must be shown as

"ALCOHOL (ALC) _____% BY VOLUME (VOL)" or

"ALCOHOL (ALC) BY VOLUME (VOL) _____%" or

"_____% ALCOHOL (ALC) BY VOLUME (VOL)" or

"_____ % ALCOHOL (ALC)/VOLUME (VOL)"

Alcohol content must be expressed to the nearest 0.1% except that for malt beverages containing less than 0.5% alcohol by volume, the alcohol content may be expressed to the nearest 0.01%.

Table 34.4 describes miscellaneous definitions for alcohol content.

TABLE 34.4

Miscellaneous Terms to Express Alcoholic Contents

Term	May Be Used to Describe a Malt Beverage Containing
Low alcohol	Less than 2.5% alcohol by volume
Reduced alcohol	Less than 2.5% alcohol by volume
Nonalcoholic	Less than 0.5% alcohol by volume Note: The statement "contains less than 0.5% alc by vol" must appear with "nonalcoholic" on the label
Alcohol free	No alcohol (0.0% alc by vol) Note: The alcohol content statement "0.0% alc by vol" may not appear on the label unless the malt beverage is labeled "alcohol free"

Tolerances

For malt beverages containing 0.5% or more alcohol by volume

A tolerance of 0.3% above or below the alcohol content stated on the label is permissible except that regardless of this tolerance:

- A malt beverage labeled as containing 0.5% or more alcohol by volume may not contain less than 0.5% alcohol by volume
- A malt beverage labeled as "low alcohol" may not contain 2.5% or more alcohol by volume
- A malt beverage labeled as "reduced alcohol" may not contain 2.5% or more alcohol by volume

For malt beverages containing less than 0.5% alcohol by volume

The actual alcohol content may not exceed the labeled alcohol content

Type size

Unless otherwise required by state law:

- Minimum 2 mm for containers larger than 0.5 pint
- Minimum 1 mm for containers of 0.5 pint or less
- Maximum 3 mm for containers of 40 fl. oz. or less
- Maximum 4 mm for containers larger than 40 fl. oz.

Legibility

All portions of the alcohol content statement must be

- Of the same kind and size of lettering
- Of equally conspicuous coloring

Placement

May appear on the front, back, or side of the container

FD&C Yellow 5 disclosure

Required

The text "Contains FD&C Yellow 5" must appear on the label of any malt beverage containing FD&C Yellow 5

Type size

- Minimum 2 mm for containers larger than 0.5 pint
- Minimum 1 mm for containers of 0.5 pint or less

Legibility

- Must be readily legible
- Must appear on a contrasting background
- Must appear separate and apart from or be substantially more conspicuous than descriptive or explanatory information

Placement

May appear on the front, back, or side of the container

Saccharin disclosure

Required

The text "Use of this product may be hazardous to your health. This product contains saccharin which has been determined to cause cancer in laboratory animals." must appear on the label of any malt beverage containing saccharin.

Type size

- Minimum 2 mm for containers larger than 0.5 pint
- Minimum 1 mm for containers of 0.5 pint or less

Legibility
- Must be readily legible
- Must appear on a contrasting background
- Must appear separate and apart from all other label information

Placement

May appear on the front, back, or side of the container

Sulfite declaration

Required

The text "Contains sulfites" or "contains (a) sulfiting agent(s)" or identification of the specific sulfiting agent(s) must appear on the label of any malt beverage containing 10 or more parts per million (ppm) sulfur dioxide.

Type size
- Minimum 2 mm for containers larger than 0.5 pint
- Minimum 1 mm for containers of 0.5 pint or less

Legibility
- Must be readily legible
- Must appear on a contrasting background
- Must appear separate and apart from or be substantially more conspicuous than descriptive or explanatory information

Placement

May appear on the front, back, or side of the container

Aspartame disclosure

Required

The text "Phenylketonurics: contains phenylalanine," must appear on the label of any malt beverage containing aspartame.

Type size

The entire statement must appear in capital letters
- Minimum 2 mm for containers larger than 0.5 pint
- Minimum 1 mm for containers of 0.5 pint or less

Legibility
- Must be readily legible
- Must appear on a contrasting background
- Must appear separate and apart from all other label information

Placement

May appear on the front, back, or side of the container

Health warning statement

Required

The statement below must appear on all alcohol beverages for sale or distribution in the United States containing not less than 0.5% alcohol by volume, intended for human consumption and bottled on or after November 18, 1989:

GOVERNMENT WARNING: (1) According to the Surgeon General, women should not drink alcoholic beverages during pregnancy because of the risk of birth defects. (2) Consumption of alcoholic beverages impairs your ability to drive a car or operate machinery, and may cause health problems.

Format
- The words "GOVERNMENT WARNING" must appear in capital letters and in bold type.

TABLE 34.5

Maximum Number of Characteristics per Inch

Minimum Type: Size Requirement	Maximum Characters per Inch
1 mm	40
2 mm	25
3 mm	12

- The remainder of the statement may not appear in bold type.
- The statement must appear as a continuous paragraph.

Type size

- Minimum 3 mm for containers larger than 3 L (101 fl. oz.)
- Minimum 2 mm for containers larger than 237 mL (8 fl. oz.) to 3 L (101 fl. oz.)
- Minimum 1 mm for containers of 237 mL (8 fl. oz.) or less

Legibility

- Must be readily legible under ordinary conditions and appear on a contrasting background
- Must appear separate and apart from all other label information
- May not exceed maximum number of characters per inch as shown in Table 34.5

Placement

May appear on the front, back, or side of the container

Country of origin

Application

Required under U.S. Customs Service regulations for any imported malt beverage

Format

"Product/produce of _____"

(Fill in blank with name of country in which malt beverage was produced/brewed)

"Produced/brewed in" or "Produced/brewed and bottled or packed in _____"

(Fill in blank with name of country in which malt beverage was produced/brewed or produced/brewed and bottled or packed)

"Produced/brewed by" or "Produced/brewed and bottled or packed by _____"

(Fill in blank with name of producer/brewer or producer/brewer and bottler or packer and address [country or city and country] in which malt beverage was produced/brewed or produced/brewed and bottled or packed)

"_____ ale"

(Fill in blank with name of country in which ale was produced/brewed), i.e., name of country with class and/or class and type designation

Type size

There are no specific type size requirements

Legibility

There are no specific legibility requirements

Placement

May appear on the front, back, or side of the container

35

*Distilled Spirits Production in the United States: Regulatory Requirements**

Y. H. Hui

CONTENTS

35.1 Introduction

In any country, when a private company produces distilled spirits for public consumption, the government maintains a tight control over the business for three important reasons, among others: tax, economic integrity, and safety. In the United States, two federal agencies prescribe regulations for the commercial production of distilled spirits:

1. Bureau of Alcohol, Tobacco and Firearms Tax and Trade Bureau (TTB, www.ttb.gov)
2. Food and Drug Administration (FDA, www.fda.gov/)

Distilled spirits are obtained from the fermentation of cereals and grains. Whisky is a distilled spirit. Refer to Chapter 33 of this book for some details on the production of malt and grain whisky. This chapter focuses on the regulatory requirements in the production and distribution of distilled spirits in commerce in this country.

* The information in this chapter has been modified from *Food Safety Manual* published by Science Technology Systems (STS) of West Sacramento, CA. Copyrighted 2012. Used with permission.

35.2 Laws and Regulations

Table 35.1 describes the laws and regulations prescribed by TTB.

35.3 Standards of Identity

This section identifies the labeled distilled spirits as to its specific class and/or class and type, based on defined classes and types (27 CFR 5.22).

35.3.1 Definitions

Two definitions are given:

1. Class. The broad category "distilled spirits" is divided, under standards of identity, into a number of general but defined classes, e.g., "neutral spirits or alcohol," "whisky."
2. Type. Under most of the general classes are specific, defined types of distilled spirits, e.g., "vodka" is a specific type of "neutral spirits or alcohol"; "straight bourbon whisky" is a specific type of "whisky."

35.3.2 Class and Type Designation

Most type names are sufficient as class and type designations. Some class names are sufficient as class and type designations.

Table 35.2 categorizes and very broadly defines all classes and most types of distilled spirits. Those classes and types that are sufficient as class and type designations are notated.

35.4 Coloring/Flavoring/Blending Materials

Coloring/flavoring/blending materials may be used in or added to any class and/or type of distilled spirits. However, the use or addition of these materials may change the class and/or type of the distilled spirits.

EXAMPLE: FD&C Yellow #5 is added to straight bourbon whisky. The resulting product is no longer "straight bourbon whisky." The product is now a distilled spirits specialty and must be labeled with a statement of composition such as "straight bourbon whisky with FD&C Yellow #5 added."

The use or addition of coloring/flavoring/blending materials will not cause a change in the class and/or type if the materials used or added can be considered "harmless."

TABLE 35.1

Governing Laws and Regulations for Distilled Spirits

Law	Implementing Regulation	Regulation Title
Federal Alcohol Administration Act (FAA Act) 27 USC 201 et seq.	27 CFR Part 5	Labeling and Advertising of Distilled Spirits
Alcoholic Beverage Labeling Act of 1988 (ABLA) 27 USC 213 et seq.	27 CFR Part 16	Alcoholic Beverage Health Warning Statement
Internal Revenue Code (IRC) 26 USC Chapter 51	27 CFR Part 19	Distilled Spirits Plants
	27 CFR Part 250	Liquors and Articles from Puerto Rico and the Virgin Islands
	27 CFR Part 251	Importation of Distilled Spirits, Wine, and Beer
	27 CFR Part 252	Exportation of Liquors

TABLE 35.2

Distilled Spirits: Class and Type Designation

Class	General Class Definition	Type	General Type Definition
Neutral spirits or alcohol[1]	Spirits distilled from any material at or above 95% alcohol by volume (190 proof), and if bottled, bottled at not less than 40% alcohol by volume (80 proof)	Vodka[1]	Neutral spirits distilled or treated after distillation with charcoal or other materials so as to be without distinctive character, aroma, taste, or color
		Grain spirits[1]	Neutral spirits distilled from a fermented mash of grain and stored in oak containers
Whiskey[2]	Spirits distilled from a fermented mash of grain at less than 95% alcohol by volume (190 proof) having the taste, aroma, and characteristics generally attributed to whiskey and bottled at not less than 40% alcohol by volume (80 proof)	Bourbon whiskey[1]	Whiskey produced in the United States at not exceeding 80% alcohol by volume (160 proof) from a fermented mash of not less than 51% corn and stored at not more than 62.5% alcohol by volume (125 proof) in charred new oak containers
		Rye whiskey[3]	Whiskey produced at not exceeding 80% alcohol by volume (160 proof) from a fermented mash of not less than 51% rye and stored at not more than 62.5% alcohol by volume (125 proof) in charred new oak containers
		Wheat whiskey[3]	Whiskey produced at not exceeding 80% alcohol by volume (160 proof) from a fermented mash of not less than 51% wheat and stored at not more than 62.5% alcohol by volume (125 proof) in charred new oak containers
		Malt whiskey[3]	Whiskey produced at not exceeding 80% alcohol by volume (160 proof) from a fermented mash of not less than 51% malted barley and stored at not more than 62.5% alcohol by volume (125 proof) in charred new oak containers
		Rye malt whiskey[3]	Whiskey produced at not exceeding 80% alcohol by volume (160 proof) from a fermented mash of not less than 51% malted rye and stored at not more than 62.5% alcohol by volume (125 proof) in charred new oak containers
		Corn whiskey[3]	Whiskey produced at not exceeding 80% alcohol by volume (160 proof) from a fermented mash of not less than 80% corn, and if stored in oak containers, must be stored at not more than 62.5% alcohol by volume (125 proof) in used or uncharred new oak containers and not subjected in any manner to treatment with charred wood
		Straight bourbon whiskey[1]	• Bourbon whiskey stored in charred new oak containers for 2 years or longer • "Straight bourbon whiskey" may include mixtures of two or more straight bourbon whiskies provided all of the whiskies are produced in the same state

(*continued*)

TABLE 35.2 (Continued)

Distilled Spirits: Class and Type Designation

Class	General Class Definition	Type	General Type Definition
		Straight rye whiskey[3]	• Rye whiskey stored in charred new oak containers for 2 years or more • "Straight rye whiskey" may include mixtures of two or more straight rye whiskies provided all of the whiskies are produced in the same state
		Straight wheat whiskey[3]	• Wheat whiskey stored in charred new oak containers for 2 years or more • "Straight wheat whiskey" may include mixtures of two or more straight wheat whiskies provided all of the whiskies are produced in the same state
		Straight malt whiskey[3]	• Malt whiskey stored in charred new oak containers for 2 years or more • "Straight malt whiskey" may include mixtures of two or more straight malt whiskies provided all of the whiskies are produced in the same state
		Straight rye malt whiskey[3]	• Rye malt whiskey stored in charred new oak containers for 2 years or more • "Straight rye malt whiskey" may include mixtures of two or more straight rye malt whiskies provided all of the whiskies are produced in the same state
		Straight corn whiskey[3]	• Corn whiskey stored in used or uncharred new oak containers for 2 years or more • "Straight corn whiskey" may include mixtures of two or more straight corn whiskies provided all of the whiskies are produced in the same state
		Straight whiskey[3]	• Whiskey produced from a fermented mash of less than 51% of any one type of grain and stored in charred new oak containers for 2 years or more • "Straight whiskey" may include mixtures of two or more straight whiskies provided all of the whiskies are produced in the same state
		Whiskey distilled from bourbon mash[1]	Whiskey produced in the United States at not exceeding 80% alcohol by volume (160 proof) from a fermented mash of not less than 51% corn and stored in used oak containers
		Whiskey distilled from rye mash[1]	Whiskey produced in the United States at not exceeding 80% alcohol by volume (160 proof) from a fermented mash of not less than 51% rye and stored in used oak containers

(*continued*)

TABLE 35.2 (Continued)

Distilled Spirits: Class and Type Designation

Class	General Class Definition	Type	General Type Definition
		Whiskey distilled from wheat mash[1]	Whiskey produced in the United States at not exceeding 80% alcohol by volume (160 proof) from a fermented mash of not less than 51% wheat and stored in used oak containers
		Whiskey distilled from malt mash[1]	Whiskey produced in the United States at not exceeding 80% alcohol by volume (160 proof) from a fermented mash of not less than 51% malted barley and stored in used oak containers
		Whiskey distilled from rye malt mash[1]	Whiskey produced in the United States at not exceeding 80% alcohol by volume (160 proof) from a fermented mash of not less than 51% malted rye and stored in used oak containers
		Light whiskey[1]	Whiskey produced in the United States at more than 80% alcohol by volume (160 proof) [but less than 95% alcohol by volume (190 proof)] and stored in used or uncharred new oak containers
		Blended light whiskey or light whiskey—a blend[1]	Light whiskey blended with less than 20% straight whiskey on a proof gallon basis
		Blended whiskey or whiskey—a blend[3]	Whiskey produced by blending not less than 20% on a proof gallon basis (excluding alcohol derived from added harmless coloring, flavoring, or blending materials*) straight whiskey or a blend of straight whiskies and, separately or in combination, whiskey of any type or neutral spirits
		Blended bourbon whiskey or bourbon whiskey—a blend[1]	Blended whiskey produced in the United States containing not less than 51% on a proof gallon basis (excluding alcohol derived from added harmless coloring, flavoring, or blending materials*) straight bourbon whiskey
		Blended rye whiskey or rye whiskey—a blend[3]	Blended whiskey containing not less than 51% on a proof gallon basis (excluding alcohol derived from added harmless coloring, flavoring, or blending materials*) straight rye whiskey
		Blended wheat whiskey or wheat whiskey—a blend[3]	Blended whiskey containing not less than 51% on a proof gallon basis (excluding alcohol derived from added harmless coloring, flavoring, or blending materials*) straight wheat whiskey
		Blended malt whiskey or malt whiskey—a blend[3]	Blended whiskey containing not less than 51% on a proof gallon basis (excluding alcohol derived from added harmless coloring, flavoring, or blending materials*) straight malt whiskey

(*continued*)

TABLE 35.2 (Continued)

Distilled Spirits: Class and Type Designation

Class	General Class Definition	Type	General Type Definition
		Blended rye malt whiskey or rye malt whiskey—a blend[3]	Blended whiskey containing not less than 51% on a proof gallon basis (excluding alcohol derived from added harmless coloring, flavoring, or blending materials*) straight rye malt whiskey
		Blended corn whiskey or corn whiskey—a blend[3]	Blended whiskey containing not less than 51% on a proof gallon basis (excluding alcohol derived from added harmless coloring, flavoring, or blending materials*) straight corn whiskey
		A blend of straight whiskies or blended straight whiskies[3]	• Mixture of straight whiskies produced in different states • Mixture of straight whiskies produced in the same state to which harmless coloring, flavoring, or blending materials* have been added
		A blend of straight bourbon whiskies or blended straight bourbon whiskies[1]	A blend of straight whiskies produced in the United States consisting entirely of straight bourbon whiskies
		A blend of straight rye whiskies or blended straight rye whiskies[3]	A blend of straight whiskies consisting entirely of straight rye whiskies
		A blend of straight wheat whiskies or blended straight wheat whiskies[3]	A blend of straight whiskies consisting entirely of straight wheat whiskies
		A blend of straight malt whiskies or blended straight malt whiskies[3]	A blend of straight whiskies consisting entirely of straight malt whiskies
		A blend of straight rye malt whiskies or blended straight rye malt whiskies[3]	A blend of straight whiskies consisting entirely of straight rye malt whiskies
		A blend of straight corn whiskies or blended straight corn whiskies[3]	A blend of straight whiskies consisting entirely of straight corn whiskies
		Spirit whiskey[3]	Whiskey produced by blending neutral spirits and not less than 5% on a proof gallon basis whiskey, straight whiskey, or combination of whiskey and straight whiskey provided the straight whiskey is used at less than 20% on a proof gallon basis
		Scotch whiskey[1]	Unblended whiskey manufactured in Scotland in compliance with the laws of the United Kingdom
		Blended scotch whiskey or scotch whiskey—a blend[1]	A blend of whiskies manufactured in Scotland in compliance with the laws of the United Kingdom

(*continued*)

TABLE 35.2 (Continued)

Distilled Spirits: Class and Type Designation

Class	General Class Definition	Type	General Type Definition
		Irish whiskey[1]	Unblended whiskey manufactured in the republic of Ireland or in Northern Ireland in compliance with their laws
		Blended Irish whiskey or Irish whiskey—a blend[1]	A blend of whiskies manufactured in the republic of Ireland or in Northern Ireland in compliance with their laws
		Canadian whiskey[1]	Unblended whiskey manufactured in Canada in compliance with its laws
		Blended canadian whiskey or Canadian whiskey—a blend[1]	A blend of whiskies manufactured in Canada in compliance with its laws
Gin[1]	Spirits with a main characteristic flavor derived from juniper berries produced by distillation or mixing of spirits with juniper berries and other aromatics or extracts derived from these materials and bottled at not less than 40% alcohol by volume (80 proof)	Distilled gin	Gin produced by original distillation from mash with or over juniper berries and other aromatics or their extracts, essences, or flavors
		Redistilled gin	Gin produced by redistillation of distilled spirits with or over juniper berries and other aromatics or their extracts, essences, or flavors
		Compounded gin	Gin produced by mixing neutral spirits with juniper berries and other aromatics or their extracts, essences, or flavors
Brandy	Spirits distilled from the fermented juice, mash, or wine of fruit or from its residue at less than 95% alcohol by volume (190 proof) having the taste, aroma, and characteristics generally attributed to brandy and bottled at not less than 40% alcohol by volume (80 proof)	Fruit brandy	• Brandy distilled solely from the fermented juice or mash of whole, sound, ripe fruit, or from standard fruit wine, with or without the addition of not more than 20% by weight of the pomace of such juice or wine or 30% by volume of the lees of such wine or both. Such brandy may include up to 30% on a proof gallon basis of lees brandy • "Brandy" is grape brandy. Other types of fruit brandy must be further identified, e.g., "peach brandy" • Grape brandy must be stored in oak containers for a minimum of 2 years
		Pisco[1]	Peruvian grape brandy stored in other than oak containers
		Applejack or apple brandy[1]	Type of fruit brandy made from apples
		Kirschwasser[1]	Type of fruit brandy made from cherries
		Slivovitz[1]	Type of fruit brandy made from plums
		Immature brandy[1]	Grape brandy stored in oak containers for less than 2 years
		Cognac[1]	Grape brandy distilled in the Cognac region of France in compliance with the laws and regulations of the French government

(*continued*)

TABLE 35.2 (Continued)

Distilled Spirits: Class and Type Designation

Class	General Class Definition	Type	General Type Definition
		Armagnac[1]	Grape brandy distilled in the Armagnac region of France in compliance with the laws and regulations of the French government
		Calvados[1]	Apple brandy distilled in the Calvados region of France in compliance with the laws and regulations of the French government
		Dried fruit brandy	• Fruit brandy distilled from sound, dried fruit or from the standard wine of such fruit • For brandies of this type (except raisin brandy, see below), the word "brandy" must be qualified with the name of the fruit from which made preceded by the word "dried," e.g., "dried apricot brandy"
		Raisin brandy[1]	Type of dried fruit brandy made from raisins
		Lees brandy[1]	• Brandy distilled from the lees or sediment from fermentation of fruit wine • "Lees brandy" is grape lees brandy. Other types of lees brandy must be further identified, e.g., "cherry lees brandy"
		Pomace brandy or marc brandy[1]	• Brandy distilled from the skin and pulp of sound, ripe fruit after the withdrawal of the juice or wine • "Pomace brandy" or "marc brandy" is grape pomace or marc brandy. Other types of pomace or marc brandy must be further identified, e.g., "apple pomace brandy," "pear marc brandy"
		Grappa or grappa brandy[1]	Type of pomace brandy made from grapes
		Residue brandy[1]	• Brandy distilled wholly or in part from the fermented residue of fruit or wine • "Residue brandy" is grape residue brandy. Other types of residue brandy must be further identified, e.g., "orange residue brandy"
		Neutral brandy[1]	• Any type of brandy, e.g., "fruit brandy," "residue brandy," etc., distilled at more than 85% alcohol by volume (170 proof) but less than 95% alcohol by volume (190 proof) • "Neutral brandy" is grape neutral brandy. Other types of neutral brandy must be further identified, e.g., "neutral plum brandy," "neutral citrus residue brandy"

(*continued*)

TABLE 35.2 (Continued)

Distilled Spirits: Class and Type Designation

Class	General Class Definition	Type	General Type Definition
		Substandard brandy[1]	• Brandy: • (a) distilled from the fermented juice, mash, or wine having a volatile acidity calculated, exclusive of water added to facilitate distillation, as acetic acid and exclusive of sulfur dioxide, in excess of 0.2 g per 100 mL (20°C.) • (b) distilled from unsound, moldy, diseased, or decomposed juice, mash, wine, lees, pomace, or residue or which possesses any taste, aroma, or characteristic associated with products distilled from such material • "Substandard brandy" is grape substandard brandy. Other types of substandard brandy must be further identified, e.g., "substandard fig brandy"
Blended applejack or applejack—a blend[1]	Mixture of at least 20% on a proof gallon basis apple brandy that has been stored in oak containers for not less than 2 years and not more than 80% on a proof gallon basis neutral spirits, bottled at not less than 40% alcohol by volume (80 proof)	No type under this class	
Rum[1]	Spirits distilled from the fermented juice of sugar cane, sugar cane syrup, sugar cane molasses, or other sugar cane by-products at less than 95% alcohol by volume (190 proof) having the taste, aroma, and characteristics generally attributed to rum, and bottled at not less than 40% alcohol by volume (80 proof)	No type under this class	
Tequila[1]	Spirits distilled in Mexico in compliance with the laws and regulations of the Mexican government from a fermented mash derived principally from the *Agave tequilana* Weber ("blue" variety), with or without additional fermentable substances having the taste, aroma, and characteristics generally attributed to tequila and bottled at not less than 40% alcohol by volume (80 proof)	No type under this class	

(*continued*)

TABLE 35.2 (Continued)

Distilled Spirits: Class and Type Designation

Class	General Class Definition	Type	General Type Definition
Mescal/ mezcal[1]	Spirits distilled in Mexico in compliance with the laws and regulations of the Mexican government from a fermented mash derived from the Mezcal plant, having the taste, aroma, and characteristics generally attributed to mescal/mezcal and bottled at not less than 40% alcohol by volume (80 proof)	No type under this class	
Liqueur/ cordial[1]	Flavored spirits product containing not less than 2.5% by weight sugar, dextrose, levulose, or a combination thereof made by mixing or redistilling any class or type of spirits with or over fruits, flowers, plants, or pure juices therefrom or other natural flavoring materials or with extracts derived from infusions, percolation, or maceration of such materials	Sloe gin[1]	Liqueur/cordial deriving its main flavor characteristic from sloe berries
		Rye liqueur/rye cordial[1]	Liqueur/cordial with the predominant characteristic flavor of rye whiskey made with not less than 51% on a proof gallon basis rye whiskey, straight rye whiskey, or whiskey distilled from rye mash bottled at not less than 30% alcohol by volume (60 proof). Wine may be used but if used may not exceed 2.5% by volume of the finished product
		Bourbon liqueur/ bourbon cordial[1]	Liqueur/cordial produced in the United States with the predominant characteristic flavor of bourbon whiskey made with not less than 51% on a proof gallon basis bourbon whiskey, straight bourbon whiskey, or whiskey distilled from bourbon mash bottled at not less than 30% alcohol by volume (60 proof). Wine may be used but if used may not exceed 2.5% by volume of the finished product
		Rock and rye[1]	Liqueur/cordial with the predominant characteristic flavor of rye whiskey made with not less than 51% on a proof gallon basis rye whiskey, straight rye whiskey, or whiskey distilled from rye mash, rock candy, or sugar syrup and with or without the addition of fruit, fruit juices, or other natural flavoring materials, bottled at not less than 24% alcohol by volume (48 proof). Wine may be used but if used may not exceed 2.5% by volume of the finished product

(*continued*)

TABLE 35.2 (Continued)

Distilled Spirits: Class and Type Designation

Class	General Class Definition	Type	General Type Definition
		Rock and bourbon[1]	Liqueur/cordial produced in the United States with the predominant characteristic flavor of bourbon whiskey made with not less than 51% on a proof gallon basis bourbon whiskey, straight bourbon whiskey, or whiskey distilled from bourbon mash, rock candy, or sugar syrup and with or without the addition of fruit, fruit juices, or other natural flavoring materials, bottled at not less than 24% alcohol by volume (48 proof). Wine may be used but if used may not exceed 2.5% by volume of the finished product
		Rock and brandy[1]	Liqueur/cordial with the predominant characteristic flavor of brandy made with grape brandy as the exclusive distilled spirits base, rock candy, or sugar syrup and with or without the addition of fruit, fruit juices, or other natural flavoring materials, bottled at not less than 24% alcohol by volume (48 proof). Wine may be used but if used may not exceed 2.5% by volume of the finished product
		Rock and rum[1]	Liqueur/cordial with the predominant characteristic flavor of rum made with rum as the exclusive distilled spirits base, rock candy, or sugar syrup and with or without the addition of fruit, fruit juices, or other natural flavoring materials, bottled at not less than 24% alcohol by volume (48 proof). Wine may be used but if used may not exceed 2.5% by volume of the finished product
		Rum liqueur/rum cordial[1]	Liqueur/cordial with the predominant characteristic flavor of rum made with rum as the exclusive distilled spirits base, bottled at not less than 30% alcohol by volume (60 proof). Wine may be used but if used may not exceed 2.5% by volume of the finished product
		Gin liqueur/gin cordial[1]	Liqueur/cordial with the predominant characteristic flavor of gin made with gin as the exclusive distilled spirits base, bottled at not less than 30% alcohol by volume (60 proof). Wine may be used but if used may not exceed 2.5% by volume of the finished product

(*continued*)

TABLE 35.2 (Continued)

Distilled Spirits: Class and Type Designation

Class	General Class Definition	Type	General Type Definition
		Brandy liqueur/ brandy cordial[1]	• Liqueur/cordial with the predominant characteristic flavor of brandy made with brandy as the exclusive distilled spirits base, bottled at not less than 30% alcohol by volume (60 proof). Wine may be used but if used may not exceed 2.5% by volume of the finished product • "Brandy liqueur" or "brandy cordial" is grape brandy liqueur/cordial. Other types of brandy liqueurs/cordials must be specifically identified, e.g., "cherry brandy liqueur," "peach brandy cordial"
		Arak/arack/raki	Anise-flavored liqueur/cordial
		Amaretto[1]	Almond-flavored liqueur/cordial
		Kummel[1]	Caraway-flavored liqueur/cordial
		Ouzo[1]	Anise-flavored liqueur/cordial
		Anise/anisette[1]	Anise-flavored liqueur/cordial
		Sambuca[1]	Italian anise–flavored liqueur/cordial
		Peppermint schnapps[1]	Peppermint-flavored liqueur/cordial
		Triple sec[1]	Orange-flavored liqueur/cordial
		Curacao[1]	Orange-flavored liqueur/cordial
		Crème de _____[11]	Liqueur/cordial with the predominant flavor as indicated in the name, e.g., "crème de menthe"—mint-flavored liqueur/cordial
		Goldwasser[12]	German herb–flavored liqueur/cordial containing gold flakes
Flavored brandy[1]	• Brandy flavored with natural flavoring materials, with or without the addition of sugar, bottled at not less than 30% alcohol by volume (60 proof) • The name of the predominant flavor shall appear as part of the class and type designation, e.g., "apricot-flavored brandy" • Wine may be added up to 15% by volume of the finished product provided at least 12.5% of the wine is derived from the base commodity that corresponds to the labeled flavor of the product. If not, or if the wine addition exceeds 15% by volume of the finished product the classes and/or types and percentages (by volume) of the wine must be stated as part of the class and type designation	No type under this class	

(*continued*)

TABLE 35.2 (Continued)

Distilled Spirits: Class and Type Designation

Class	General Class Definition	Type	General Type Definition
Flavored gin[1]	• Gin flavored with natural flavoring materials, with or without the addition of sugar, bottled at not less than 30% alcohol by volume (60 proof) • The name of the predominant flavor shall appear as part of the class and type designation, e.g., "lime-flavored gin" • Wine may be added but if the addition exceeds 2.5% by volume of the finished product, the classes and/or types and percentages (by volume) of wine must be stated as part of the class and type designation	No type under this class	
Flavored rum[1]	• Rum flavored with natural flavoring materials, with or without the addition of sugar, bottled at not less than 30% alcohol by volume (60 proof) • The name of the predominant flavor shall appear as part of the class and type designation, e.g., "butterscotch-flavored rum" • Wine may be added but if the addition exceeds 2.5% by volume of the finished product, the classes and/or types, and percentages (by volume) of wine must be stated as part of the class and type designation	No type under this class	
Flavored vodka[1]	• Vodka flavored with natural flavoring materials, with or without the addition of sugar, bottled at not less than 30% alcohol by volume (60 proof) • The name of the predominant flavor shall appear as part of the class and type designation, e.g., "orange-flavored vodka" • Wine may be added but if the addition exceeds 2.5% by volume of the finished product, the classes and/or types and percentages (by volume) of wine must be stated as part of the class and type designation	No type under this class	

(*continued*)

TABLE 35.2 (Continued)

Distilled Spirits: Class and Type Designation

Class	General Class Definition	Type	General Type Definition
Flavored whiskey[1]	• Whiskey flavored with natural flavoring materials, with or without the addition of sugar, bottled at not less than 30% alcohol by volume (60 proof) • The name of the predominant flavor shall appear as part of the class and type designation, e.g., "cherry-flavored whiskey" • Wine may be added but if the addition exceeds 2.5% by volume of the finished product, the classes and/or types and percentages (by volume) of wine must be stated as part of the class and type designation	No type under this class	
Imitation distilled spirits	• Any class and/or type of distilled spirits treated with flavor(s) and/or color(s) to simulate a different class and/or type of distilled spirits • Any class and/or type of distilled spirits other than a distilled spirits specialty (see below) containing an artificial flavor**		
	• Any class and/or type of distilled spirits (except cordials, liqueurs, and specialties marketed under labels that do not indicate or imply that a particular class and/or type of distilled spirits was used in their manufacture) to which has been added any whiskey essence, brandy essence, rum essence, or similar essence or extract that simulates or enhances or is used in the particular product to simulate or enhance the characteristics of any class and/or type of distilled spirits • Any type of whiskey to which beading oil has been added • Any rum to which neutral spirits or distilled spirits other than rum have been added • Any brandy made from distilling material to which sugar (other than the kind and amount expressly authorized in the production of standard wine) has been added • Any brandy, except blended applejack, to which neutral spirits or distilled spirits other than brandy have been added	Specific class or type to which distilled spirits would otherwise belong, e.g., "imitation rum"[1]	

(*continued*)

TABLE 35.2 (Continued)

Distilled Spirits: Class and Type Designation

Class	General Class Definition	Type	General Type Definition
Recognized cocktails	Mixed drink that has gained trade and consumer recognition, containing one or more class(es) and/or type(s) of distilled spirits with flavoring and/or coloring materials	Apricot sour[13]	Apricot-flavored brandy or apricot liqueur/cordial and lemon juice or oil or natural lemon flavor**
		Black Russian[13]	Vodka and coffee-flavored brandy or coffee liqueur/cordial
		Bloody Mary[13]	Vodka and tomato juice or natural tomato flavor**
		Alexander[13], e.g., "Brandy Alexander"	Specific distilled spirits indicated in the cocktail name, e.g., brandy in the case of "Brandy Alexander," crème de cacao, and cream
		Daiquiri[13]	Rum and lime juice or oil or natural lime flavor**
		_____ daiquiri[13], e.g., "pineapple daiquiri"	Daiquiri with added flavor as indicated in the cocktail name, e.g., daiquiri with added pineapple juice or natural pineapple flavor** in the case of "pineapple daiquiri"
		Egg nog[13]	Brandy, rum or whiskey, milk or milk products of at least 6% butter fat, at least 1% egg yolk solids, sweetener, and natural flavor**
		Gimlet[13]	Gin and lime juice, oil, or natural lime flavor**
		Vodka gimlet[13]	Vodka and lime juice, oil, or natural lime flavor**
		Grasshopper[13]	Crème de menthe, crème de cacao, and cream
		Mai tai[13]	Rum and citrus juices, oils, or natural citrus flavors**
		Manhattan[13]	Whiskey (any type) and vermouth
		_____ Manhattan[13], e.g., "scotch manhattan"	Specific distilled spirits indicated in the cocktail name, e.g., blended scotch whiskey in the case of "scotch Manhattan" and vermouth
		Margarita[13]	Tequila, triple sec, and lime or lemon juice or oil or natural lime or lemon flavor**
		_____ margarita[13], e.g., "strawberry margarita"	Margarita with added flavor as indicated in the cocktail name, e.g., margarita with added strawberry juice or natural strawberry flavor** in the case of "strawberry margarita"
		Martini[13]	Gin and vermouth
		Vodka martini[13]	Vodka and vermouth
		Mint julep[13]	Straight bourbon whiskey and natural mint flavor**

(*continued*)

TABLE 35.2 (Continued)

Distilled Spirits: Class and Type Designation

Class	General Class Definition	Type	General Type Definition
		Mint julep[13], e.g., "vodka mint julep"	Specific distilled spirits indicated in the cocktail name, e.g., vodka in the case of "vodka mint julep," and natural mint flavor**
		Old fashioned[13]	Straight bourbon whiskey and bitters
		Pink squirrel[13]	Crème de almond, crème de cacao, and cream
		Screwdriver[13]	Vodka and orange juice or oil or natural orange flavor**
		Sloe gin fizz[13]	Sloe gin liqueur/cordial, lemon juice or oil or natural lemon flavor** and club soda
		Tom collins[13]	Gin, lemon juice or oil or natural lemon flavor**, and club soda
		_____ collins[13], e.g., "vodka collins"	Specific distilled spirits indicated in the cocktail name, e.g., vodka in the case of "vodka collins," lemon juice or oil or natural lemon flavor**, and club soda
		Wallbanger[13]	Vodka, orange juice or oil or natural orange flavor**, and galliano or similar type liqueur/cordial
		Whiskey sour[13]	Whiskey and lemon juice or oil or natural lemon flavor**
		_____ sour[13], e.g., "pisco sour"	Specific distilled spirits indicated in the cocktail name, e.g., pisco in the case of "pisco sour" and lemon juice or oil or natural lemon flavor**
		White Russian[13]	Vodka, coffee-flavored brandy, or coffee liqueur/cordial, and cream
Advocaat[1]	Egg-flavored distilled spirits product	No type under this class	
Aquavit[1]	Caraway-flavored distilled spirits product	No type under this class	
Bitters[1]	Distilled spirits product distinguished by its bitterness produced by blending extracts of plants, seeds, herbs, barks and/or roots with any class and/or type of distilled spirits	No type under this class	
Distilled spirits specialty	• Distilled spirits not defined under any other class • Generally, any class and/or type of distilled spirits that contain or are treated with flavoring and/or coloring materials and/or nonstandard blending or treating materials or processes	• No defined type(s) under this class • Product definition is unique to composition and production of the distilled spirits product[1]	

(*continued*)

*See Chapter 7 (Coloring/Flavoring/Blending Materials).
**See Flavor Materials section of Chapter 7 (Coloring/Flavoring/Blending Materials).
[1]Sufficient as class and type designation.
[2]Sufficient as class and type designation only for whiskies made by blending two or more specific types of whiskies, e.g., a blend of rye whiskey and corn whiskey should be designated "whiskey" or treating with harmless coloring, flavoring, or blending materials* a specific type of whiskey not customarily so treated, e.g., bourbon whiskey treated with caramel should be designated "whiskey."
[3]Sufficient as class and type designation for whiskey of this type produced in the United States.
■For whiskey of this type produced outside the United States, the class and type designation must include the words "American type" or "produced/distilled/blended [as appropriate] in _____" (fill in blank with country of origin).
■For whiskey of this type produced in the United States by blending domestic and imported whiskies, the percentage and origin of each foreign whiskey (e.g., "25% spirit whiskey produced in Japan") must be shown on the front of the container.
"Gin" is sufficient as class and type designation. "Distilled" may optionally appear as part of the class and type designation, i.e., "distilled gin."
"Gin" is sufficient as class and type designation. "Compounded" may appear but is not required as part of the class and type designation. Compounded gin may not be referred to or described as "distilled."
Sufficient as class and type designation only for grape brandy.
Brandy made by blending two or more specific types of brandies must be identified as "Brandy" followed by the percentage of (determined on a proof gallon basis) and name of each specific type of brandy, e.g., "brandy, 25% dried pear brandy, 75% lees brandy"
Percentages are not required if the brandy is derived from
• two specific types of brandies and neither contributes less than 40% of the alcohol on a proof gallon basis
• three specific types of brandies and each contributes at least 30% of the alcohol on a proof gallon basis
• four specific types of brandies and each contributes at least 20% of the alcohol on a proof gallon basis
Fruit brandy derived from two or more fruits must be identified as "fruit brandy" followed by the percentage of (determined on a proof gallon basis) and name of each fruit from which the brandy was made, e.g., "fruit brandy, 30% apple brandy, 70% cherry brandy."
Percentages are not required if the fruit brandy is derived from
• two fruits and neither contributes less than 40% of the alcohol on a proof gallon basis
• three fruits and each contributes at least 30% of the alcohol on a proof gallon basis
• four fruits and each contributes at least 20% of the alcohol on a proof gallon basis
See footnote 7; these provisions also apply to dried fruit brandy.
Sufficient as class and type designation for arak/arack/raki liqueur/cordial.
Arak/arack/raki containing less than 2.5% by weight sugar, dextrose, levulose, or combination thereof is a distilled spirits specialty and the class and type designation must include a truthful and adequate statement of composition (see Distilled Spirits Specialty under Class column of this chart).
[10]Sufficient as class and type designation. For Sambuca produced outside of Italy, the class and type designation "Sambuca" must include the word "type" or "American" or other adjective indicating the true place of production
[11]Sufficient as class and type designation. Predominant flavor becomes part of the class and type designation, e.g., "crème de almond," "crème de cocoa," "crème de peach."
[12]Sufficient as class and type designation. For Goldwasser produced outside of Germany, the class and type designation "Goldwasser" must include the word "type" or "American" or other adjective indicating the true place of production
[13]The class and type designation is the name of the cocktail with a declaration of the distilled spirits component(s) of the product, e.g., "screwdriver made with vodka."
[14]A distinctive or fanciful product name with a statement reflecting the composition and character of the product is sufficient as class and type designation, e.g., "spiced rum, rum with spice flavor."

35.4.1 Coloring Materials

35.4.1.1 Legal Status

The legal status is considered as follows:

1. Coloring materials must be approved by the U.S. FDA
2. Approved coloring materials are categorized as "certified" or "noncertified"

35.4.1.2 Approved Coloring Materials

All coloring materials approved for use in distilled spirits are listed below:

Certified colors:

FD&C Blue #1
FD&C Blue #2
FD&C Green #3
FD&C Red #3
FD&C Red #40
FD&C Yellow #5
FD&C Yellow #6

NOTE: The lake of each certified color, except for FD&C Red #3, listed above is an approved coloring material.

Noncertified colors:

Annatto extract
Beet extract
Beta carotene
Caramel
Carmine (cochineal extract)
Elderberry extract
Grapeskin extract (enocianina)
Paprika
Saffron
Titanium dioxide
Turmeric

35.4.2 Flavoring Materials

Flavoring materials include

Essential oils
Oleoresins
Spices
Herbs
Fruit juices/concentrates
Commercially prepared flavors (including essences, extracts, blenders, infusions, etc.)

35.4.2.1 Legal Status

All flavoring materials used in alcohol beverages must be

1. Approved by FDA under a specific regulation or
2. Affirmed as GRAS (generally recognized as safe) by FDA or
3. Self-affirmed as GRAS with no FDA objection

35.4.2.2 Flavor Categorization

Categories
For labeling purposes, all flavoring materials are categorized as

1. All natural or
2. Natural and artificial containing not more than 0.1% artificial topnote (i.e., an artificial material added to enhance the flavor, essence, extract, blender, etc., or replace a flavor note lost in processing) or
3. Natural and artificial containing greater than 0.1% artificial topnote or
4. All artificial

Nomenclature

1. Flavors categorized as "all natural" or "natural and artificial containing not more than 0.1% artificial topnote" are "natural" for labeling purposes
2. Flavors categorized as "natural and artificial containing greater than 0.1% artificial topnote" or "artificial" are categorized as "artificial" for labeling purposes

35.4.3 Blending Materials

Blending materials include wine and sugar.

35.4.4 Harmless Coloring/Flavoring/Blending Materials

35.4.4.1 Definition

Harmless coloring/flavoring/blending materials are coloring materials, flavoring materials, or blending materials that

- Are an essential component of the particular class and/or type of distilled spirits

EXAMPLE: By definition, a liqueur/cordial must contain flavoring material. Therefore, flavoring material is an essential component of this class/type of distilled spirits

- Are not an essential component but are, through established trade practice, customarily used in the particular class and/or type of distilled spirits provided that the total addition of coloring/flavoring/blending materials does not exceed 2.5% by volume of the finished product

EXAMPLE: Traditionally, to ensure consistency in color and smoothness, caramel color and blending sherry are added to blended whisky. Consequently, provided the total addition of caramel and blending sherry does not exceed 2.5% by volume of the blended whisky, these coloring and blending materials are considered "harmless."

NOTE: In the category of "nonessential but customarily used coloring/flavoring/blending materials," caramel is the only coloring material permitted.

The information in Table 35.3 specifies

1. Whether harmless coloring/flavoring/blending materials may be used in or added to each listed class/type
2. If harmless coloring/flavoring/blending materials may be used or added:
   a. Whether the use or addition of harmless coloring/flavoring/blending materials is limited to or may exceed 2.5% by volume of the finished product
   b. What category or type of flavoring materials may be used or added
   c. Whether the use or addition of harmless coloring/flavoring/blending materials must be disclosed on the label
   d. Whether there are any additional or specific limitations or restrictions that apply

A number of abbreviated references (both alpha and numeric) are used in the Table 35.3. A glossary of these abbreviations follows:

HCFBM = harmless coloring/flavoring/blending materials

NTE 2.5% = not to exceed 2.5% (i.e., harmless coloring/flavoring/blending materials may not exceed 2.5% by volume of the finished product)

ME 2.5% = may exceed 2.5% (i.e., harmless coloring/flavoring/blending materials may exceed 2.5% by volume of the finished product)

CFA = category of flavor allowed

The category of flavor is indicated by number:

1 = all natural

2 = natural and artificial containing not more than 0.1% artificial topnote

3 = natural and artificial containing greater than 0.1% artificial topnote

4 = all artificial

CM = coloring materials (if this column is marked, the use of coloring materials must be disclosed on the label)

FM = flavoring materials (if this column is marked, the use of flavoring materials must be disclosed on the label)

BM = blending materials (if this column is marked, the use of blending materials must be disclosed on the label)

35.4.4.2 What a Distilled Spirits Label Tells You

If you have ever taken a look behind a bar, you have seen rows upon rows of different bottles in a variety of heights, sizes, colors, and flavors. Usually the majority of these bottles are distilled spirits. Distilled spirits are generally produced by first combining various ingredients and fermenting them. The resulting fermented "mash," which has a low alcohol content, is then heated in a still until the alcohol turns into a vapor, which is captured and then turned back into liquid alcohol. This process is called "distillation" and is generally what distinguishes these products from wine and malt beverages. TTB regulations require that many types of distilled spirits, such as vodka, gin, rum, and tequila, must be bottled at no less than 40% alcohol by volume (80 proof). Other products, such as cordials, liqueurs, and specialties, may be bottled at a lower alcohol content. A careful review of the label will help you to understand the product in the bottle, and TTB designed this guide to help consumers make an informed choice when purchasing a distilled product. TTB regulations can be quite detailed in regard to the production of distilled spirits and the information appearing on the bottle; not every possibility is presented here, but this guide will give you a good grasp of the fundamentals. For more detailed information, please see the regulations listed on the back of this pamphlet. Table 35.4 describes this sample label for distilled spirits.

TABLE 35.3

Use of Harmless Coloring/Flavoring/Blending Materials

	HCFBM Allowed				Label Disclosure Required				
					Yes				
Class/Type	Yes NTE 2.5%	Yes ME 2.5%	No	CFA	CM	FM	BM	No	Qualification
Alexander		×		1, 2	×				
Advocaat		×		1, 2	×				
Amaretto		×		1, 2	×				
Anisette		×		1, 2	×				
Applejack, a blend	×			1, 2				×	
Apricot sour		×		1, 2	×				
Aquavit		×		1, 2	×				
Arak		×		1, 2	×				
Armagnac	×			1, 2				×	
Bitters		×		1, 2	×				
Black Russian		×		1, 2	×				
Bloody Mary		×		1, 2	×				
Bourbon liqueur/ cordial		*		1, 2	×				*Wine, if used, may not exceed 2.5% by volume of the finished product
Brandy (dried fruit)	×			1, 2				×	
Brandy (fruit)	×			1, 2				×	
Brandy (grape)	×			1, 2				×	
Brandy (immature)	×			1, 2				×	
Brandy (lees)	×			1, 2				×	
Brandy (neutral)	×			1, 2				×	
Brandy (pomace/marc)	×			1, 2				×	
Brandy (raisin)	×			1, 2				×	
Brandy (residue)	×			1, 2				×	
Brandy (substandard)	×			1, 2				×	
Brandy liqueur/ cordial		*		1, 2	×				*Wine, if used, may not exceed 2.5% by volume of the finished product
Calvados	×			1, 2				×	
Canadian whiskey			×						
Canadian whiskey, a blend			×						
Cognac	*			1				×	*HCFBM is limited to sugar, caramel, and oak chip infusion
Crème de		×		1, 2	×				
Curacao		×		1, 2	×				
Daquiri		×		1, 2	×				

(*continued*)

TABLE 35.3 (Continued)

Use of Harmless Coloring/Flavoring/Blending Materials

	HCFBM Allowed				Label Disclosure Required				
					Yes				
Class/Type	**Yes NTE 2.5%**	**Yes ME 2.5%**	**No**	**CFA**	**CM**	**FM**	**BM**	**No**	**Qualification**
Distilled spirits specialty		×		1, 2, 3, 4	×	×	*		*The use or addition of sugar does not require label disclosure
Egg nog		×		1, 2	×				
Flavored brandy		×		1, 2	×		*		*Wine may be added up to 15% by volume of the finished product provided at least 12.5% of the wine is derived from the base commodity that corresponds to the labeled flavor of the product. If not, or if the wine addition exceeds 15% by volume of the finished product, the percentages and classes/types of wine must also be disclosed on the label (as part of the class/type designation)
Flavored gin		×		1, 2	×		*		*If wine is used and exceeds 2.5% by volume of the finished product, the percentages and classes/types of wine must also be disclosed on the label (as part of the class/type designation)
Flavored rum		×		1, 2	×		*		*If wine is used and exceeds 2.5% by volume of the finished product, the percentages and classes/types of wine must also be disclosed on the label (as part of the class/type designation)

(*continued*)

TABLE 35.3 (Continued)

Use of Harmless Coloring/Flavoring/Blending Materials

	HCFBM Allowed				Label Disclosure Required				
					Yes				
Class/Type	**Yes NTE 2.5%**	**Yes ME 2.5%**	**No**	**CFA**	**CM**	**FM**	**BM**	**No**	**Qualification**
Flavored vodka		×		1, 2	×		*		*If wine is used and exceeds 2.5% by volume of the finished product, the percentages and classes/types of wine must also be disclosed on the label (as part of the class/type designation)
Flavored whiskey		×		1, 2	×		*		*If wine is used and exceeds 2.5% by volume of the finished product, the percentages and classes/types of wine must also be disclosed on the label (as part of the class/type designation)
Gimlet		×		1, 2	×				
Gin (compounded)		*		1, 2				×	*HCFBM is juniper berries and (optionally) other aromatics or their extracts, essences, or flavors blended with neutral spirits
Gin (distilled)		*		1, 2				×	*HCFBM is juniper berries and (optionally) other aromatics or their extracts, essences, or flavors added to mash prior to distillation
Gin (redistilled)		*		1, 2				×	*HCFBM is juniper berries and (optionally) other aromatics or their extracts, essences, or flavors added to distilled spirits prior to redistillation
Gin liqueur/cordial		*		1, 2	×				*Wine, if used, may not exceed 2.5% by volume of the finished product
Goldwasser		×		1, 2	×				
Grain spirits			×						

(*continued*)

TABLE 35.3 (Continued)

Use of Harmless Coloring/Flavoring/Blending Materials

	HCFBM Allowed				Label Disclosure Required				
					Yes				
Class/Type	**Yes NTE 2.5%**	**Yes ME 2.5%**	**No**	**CFA**	**CM**	**FM**	**BM**	**No**	**Qualification**
Grappa	×			1, 2				×	
Grasshopper		×		1, 2	×				
Imitation distilled spirits		×		1, 2, 3, 4	×				
Irish whiskey			×						
Irish whiskey, a blend			×						
Kirschwasser	×			1, 2				×	
Kummel		×		1, 2	×				
Liqueur/cordial		×		1, 2	×				
Mai tai		×		1, 2	×				
Manhattan		×		1, 2	×				
Margarita		×		1, 2	×				
Martini		×		1, 2	×				
Mescal			×						
Mint julep		×		1, 2	×				
Neutral spirits or alcohol			×						
Old fashioned		×		1, 2	×				
Ouzo		×		1, 2	×				
Peppermint schnapps		×		1, 2	×				
Pink squirrel		×		1, 2	×				
Pisco	×			1, 2				×	
Rock and bourbon		*		1, 2	×				*Wine, if used, may not exceed 2.5% by volume of the finished product
Rock and brandy		*		1, 2	×				*Wine, if used, may not exceed 2.5% by volume of the finished product
Rock and rum		*		1, 2	×				*Wine, if used, may not exceed 2.5% by volume of the finished product
Rock and rye		*		1, 2	×				*Wine, if used, may not exceed 2.5% by volume of the finished product
Rum	×			1, 2				×	
Rum liqueur/ cordial		*		1, 2	×				*Wine, if used, may not exceed 2.5% by volume of the finished product

(*continued*)

TABLE 35.3 (Continued)

Use of Harmless Coloring/Flavoring/Blending Materials

	HCFBM Allowed				Label Disclosure Required				
					Yes				
Class/Type	**Yes NTE 2.5%**	**Yes ME 2.5%**	**No**	**CFA**	**CM**	**FM**	**BM**	**No**	**Qualification**
Rye liqueur/ cordial		*		1, 2	×				*Wine, if used, may not exceed 2.5% by volume of the finished product
Sambuca		×		1, 2	×				
Scotch whiskey			×						
Scotch whiskey, a blend			×						
Screwdriver		×		1, 2	×				
Slivovitz	×			1, 2				×	
Sloe gin		×		1, 2	×				
Sloe gin fizz		×		1, 2	×				
Tequila			×						
Tom collins		×		1, 2	×				
Triple sec		×		1, 2	×				
Vodka	*			1				×	*HCFBM limited to citric acid (maximum of 0.1% by volume of the finished product) and sugar (maximum of 0.2% by volume of the finished product) added directly or by addition of a citric acid/sugar blender
Wallbanger		×		1, 2	×				
Whiskies (a blend of straight bourbon)	×			1, 2				×	
Whiskies (a blend of straight corn)	×			1, 2				×	
Whiskies (a blend of straight malt)	×			1, 2				×	
Whiskies (a blend of straight rye malt)	×			1, 2				×	
Whiskies (a blend of straight rye)	×			1, 2				×	
Whiskies (a blend of straight wheat)	×			1, 2				×	

(*continued*)

TABLE 35.3 (Continued)

Use of Harmless Coloring/Flavoring/Blending Materials

	HCFBM Allowed				Label Disclosure Required				
					Yes				
Class/Type	**Yes NTE 2.5%**	**Yes ME 2.5%**	**No**	**CFA**	**CM**	**FM**	**BM**	**No**	**Qualification**
Whiskies (a blend of straight)	×			1, 2				×	
Whiskey	×			1, 2				×	
Whiskey (blended bourbon)	×			1, 2				×	
Whiskey (blended corn)	×			1, 2				×	
Whiskey (blended light)	×			1, 2				×	
Whiskey (blended malt)	×			1, 2				×	
Whiskey (blended rye malt)	×			1, 2				×	
Whiskey (blended rye)	×			1, 2				×	
Whiskey (blended wheat)	×			1, 2				×	
Whiskey (blended)	×			1, 2				×	
Whiskey (bourbon)			×						
Whiskey (corn)	×			1, 2				×	
Whiskey (distilled from bourbon mash)	×			1, 2				×	
Whiskey (distilled from malt mash)	×			1, 2				×	
Whiskey (distilled from rye malt mash)	×			1, 2				×	
Whiskey (distilled from rye mash)	×			1, 2				×	
Whiskey (distilled from wheat mash)	×			1, 2				×	
Whiskey (light)	×			1, 2				×	
Whiskey (malt)	×			1, 2				×	
Whiskey (rye malt)	×			1, 2				×	
Whiskey (rye)	×			1, 2				×	
Whiskey (spirit)	×			1, 2				×	
Whiskey (straight bourbon)			×						
Whiskey (straight corn)			×						

(*continued*)

TABLE 35.3 (Continued)

Use of Harmless Coloring/Flavoring/Blending Materials

	HCFBM Allowed				Label Disclosure Required				
					Yes				
Class/Type	**Yes NTE 2.5%**	**Yes ME 2.5%**	**No**	**CFA**	**CM**	**FM**	**BM**	**No**	**Qualification**
Whiskey (straight malt)			×						
Whiskey (straight rye malt)			×						
Whiskey (straight rye)			×						
Whiskey (straight wheat)			×						
Whiskey (straight)			×						
Whiskey (wheat)	×			1, 2				×	
Whiskey sour		×		1, 2	×				
White Russian		×		1, 2	×				

TABLE 35.4

BRAND NAME

The brand name is used to identify and market a distilled spirits product. A brand name may not mislead the consumer about the age, identity, origin, or other characteristics of the distilled spirit.

NAME AND ADDRESS

The name and address of the bottler or importer must appear on the container. However, the address of the bottler's principal place of business may be used instead of the actual location where the bottling took place. It is also permissible for a bottler/importer to use a duly authorized trade name in place of its usual operating name.

ALCOHOL CONTENT

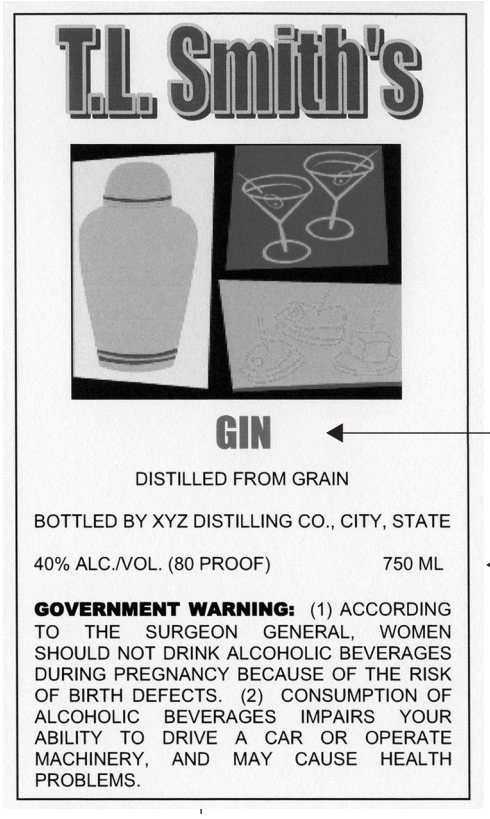
A statement of alcohol content expressed in percent by volume must appear on the brand label. An additional alcohol content statement expressed in degrees of proof may be shown in addition to the required alcohol by volume statement.

HEALTH WARNING STATEMENT

By law, this statement is required on all alcohol beverages containing 0.5% or more alcohol by volume.

COUNTRY OF ORIGIN

Pursuant to regulations issued by TTB, as well as requirements of U.S. Customs and Border Protection, a Country of Origin statement is required on containers of imported distilled spirits. Acceptable statements include "product of (*insert name of country*)" or "produced in (*insert name of country*)."



NET CONTENTS

The net contents of a distilled spirit container must be stated in metric units of measure. Distilled spirits must be bottled in sizes of 1.75 L, 1 L, 750 mL, 375 mL, 200 mL, 100 mL, or 50 mL. A can must be filled to 355 mL, 200 mL, 100 mL, or 50 mL.

CLASS/TYPE DESIGNATION

The brand label of a distilled spirit must contain a designation that accurately identifies the product in the bottle. The regulations are quite specific as to the ingredients and processes used to produce a product of a given class or type. For instance:

Gin must derive its main characteristic flavor from juniper berries. In addition, gin labels must also show the commodity from which the product was distilled (e.g., "Distilled from grain").

Vodka is defined as neutral spirits (alcohol produced from any material at or above 190 proof) so distilled, or so treated after distillation, as to be without distinctive character, aroma, taste, or color. Like gin, vodka labels must also show the commodity from which the product was distilled.

Rum must be made from the fermented juice of sugar cane, sugar cane syrup, sugar cane molasses, or other sugar cane by-products.

Tequila must be derived principally from the *Agave tequilana* Weber plant ("blue" variety). Tequila is a distinctive product of Mexico, manufactured in Mexico in compliance with the laws of Mexico.

Cordials and liqueurs are produced from spirits in combination with fruits, flowers, plants, juices, or natural flavors and with the addition of at least 2.5% by weight of certain sugars.

A distilled spirits product may not fit into any of the classes or types of spirits found in the regulations, usually because of the addition of flavoring materials or because it was made with a nonstandard blending or treating material. When this is the case, the product must be labeled with a truthful and adequate statement of composition such as "Rum with natural flavors." These products will also bear a mandatory fanciful name, such as "Spiced Rum."

Part VI

Specialty Products

36

Traditional Balsamic Vinegar: A Microbiological Overview

Lisa Solieri, Maria Gullo, and Paolo Giudici

CONTENTS

36.1 Introduction

Vinegar is an acidifying and flavoring agent of special importance for the safety and enrichment of human food, which results from microbial oxidation of ethanol contained in several substrates, such as wine, beer, fermented fruit, distilled ethanol, whey, and many others (Solieri and Giudici 2009). Vinegar was known and used by many ancient cultures: Egyptians, Sumerians, and Babylonians had experience and technical knowledge for making it from barley and any kind of fruit. Vinegar was very popular both in ancient Greece and Rome, where it was used in food preparations and as a remedy against a great number of diseases. In Asia, the first records about (malted) rice vinegar date back to the Chinese Zhou Dynasty (1027–221 BC) (Mazza and Murooka 2009).

Traditional balsamic vinegars of Modena and Reggio Emilia (here collectively referred to as TBV) are Italian aged condiments produced by spontaneous alcoholic and acetic fermentations. The raw material is cooked grape must with a soluble solids content ranging from 20 to 60°Brix (Bx). The acetified must is slowly aged for a minimum of 12 years in wood barrels by sequential refilling procedure. The first mention of a local, particular, and appreciated vinegar is reported in the poem *Acta Comitissae Mathildis*, written in the 12th century by the monk Donizo and later known as *Vita Mathildis*. This vinegar was first mentioned as "balsamic" at the end of 18th century, and only in the 19th century, a number of

references clearly spoke of "balsamic vinegar" and reported several recipes on how to produce it from the grape must (Benedetti 2004). Nowadays, TBV production is regulated by protected designation of origin (PDO) guidelines (Council Regulation EC No 813/2000) that specify raw material characteristics, production procedures, the production area, and the final product features. Control over TBV production and distribution is performed by the constitution of local associations of producers (*Consortia*). There are now four active *Consortia* in Modena and Reggio Emilia, which survey TBV quality according to specific Official Production Regulations (Disciplinare di Produzione 2000a,b).

Over time, the name "balsamic vinegar" has acquired an important trade value and is now being used to generally designate sweet and sour condiments with high viscosity and dark/brown color. Balsamic condiments could be grouped in four different categories: generic balsamic vinegars, balsamic vinegar with appellation of origin (PDO and Protected Geographical Indication or PGI), balsamic sauces, and dressing (Giudici et al. 2009). In particular "Balsamic Vinegar of Modena" (BVM) is an industrial class of vinegar legally recognized since 1965 and recently granted European PGI status (Commission Regulation EC No 583/2009). Despite their similar appellation, TBV and BVM are very distinct products due to different manufacturing technologies and starting materials. BVM is produced by aging a blend of cooked must and wine vinegar with addition of caramel, for a short time (minimum 2 months). Due to this different technology, BVM cannot duplicate the complexity flavor and the higher viscosity of TBV and it meets consumer's expectations to buy cheaper vinegar compared to TBV (Giudici et al. 2009a).

This chapter aims to describe the main technological and microbiological steps of TBV processing, underlying the role of microbial transformations in determining its quality.

36.2 Characteristics of TBV

TBV is a highly dense, aged vinegar, having analytical, and sensorial parameters stated as follows: (i) acidity (expressed as g of acetic acid per 100 g of product) not less than 4.5% (wt/wt) for TBV of Modena and 5% (wt/wt) for TBV of Reggio Emilia; (ii) density at 20°C not less than 1.240 g/L for the TBV of Modena and 1.20 g/L for the TBV of Reggio Emilia; (iii) dark brown color, nearly black, but full of "warm light"; (iii) good texture with a fluid- and syrup-like consistency; (iv) sharp and pleasantly acid fragrance; (v) full-body aroma with sweet and sour taste in perfect proportion (Disciplinare di produzione 2000a,b).

From a physicochemical point of view, several works attempt to characterize TBV samples. Dry residues and ashes range between 55.5% and 66.7% and between 0.675% and 0.859%, respectively (Masino et al. 2008). Viscosity was estimated between 2.3×10^{-3} and 3.5×10^{-2} Pa s, and it is related to Bx values, sugar content, and titratable acidity (Falcone et al. 2007). Other main physicochemical properties are summarized in Table 36.1 for 100 randomly selected TBV samples.

TABLE 36.1

Physical Properties of TBV

Physical Properties	Value Range	Description
pH	2.40–2.94	–
TA (%)	3.50–6.60	Bx, TA, and their ratio are used as a TBV quality criterion
Fixed acidity	1.59–3.40	
Volatile acidity	1.38–4.10	
Soluble solids (Bx)	61–82	Bx, TA, and their ratio are used as a TBV quality criterion
Soluble solids (Bx)–sugars	20–34	This parameter is related to TBV melanoidins content
Sugars (%)	39–48	
Density (g/L)	1.3036–1.3874	It measures the mass per unit volume and should be >1.24 for TBV of Modena and >1.20 for TBV of Reggio Emilia

Source: Data from Giudici, P., unpublished results. TA, titratable acidity expressed as g acetic acid/100 g of TBV; soluble solids were determined by refractometer; reducing sugars were determined by Feheling method.

Chemical composition of TBV is the result of complex microbial and chemicophysical transformations. The data available from the literature are not enough to give a definitive chemical picture, mainly because of the artisanal making practices that are slightly different from one producer to another one. Moreover, all the studies differ in sampling, sample extraction, and analytical procedures, thus the data are difficult to compare. Nevertheless, the chemical profile of the main nonvolatile and volatile compounds recovered until now in TBV is tentatively reported in Table 36.2.

TABLE 36.2

Chemical Composition of TBV

Components	Value Range	Origin and/or Description
Sugar (g/100 g)		
Reducing sugar	35.43–56.93	Glucose and fructose are the main hexose of grape juice.
Other sugars		Xylose, ribose, rhamnose galactose mannose arabinose, and sucrose were found in traces by Cocchi et al. (2006a)
Nonvolatile acids (g/100 g)		
Tartaric acid	0.24–0.76	Grape-derived acid, stable to microbial activity
Succinic acid	0.679–1.77	Formed via oxaloacetate and malate during the tricarboxylic acid (TCA) cycle by yeasts
Malic acid	0.84–1.88	Grape-derived acid, may be degraded by yeast and LAB; few yeasts may form it
Gluconic acid	0.33–3.34	Produced by AAB from D-glucose (via D-glucose dehydrogenase)
Lactic acid	0.039–0.071	Grape-derived acid; formed by LAB via malic acid degradation; small amount is produced by yeast glucose fermentation and during must cooking
Volatile compounds (mg/kg)		
Acetic acid	06.12–1.02	Produced by yeasts (via acetaldehyde dehydrogenase) and AAB (via ADH and ALDH)
Alcohols	23.7–70.2	Some aliphatic (propanol, isoamyl alcohol, isobutanol, and active amyl alcohol) and aromatic (2-phenylethyl alcohol and tyrosol) higher alcohols are produced by yeasts
Aldehydes	2.82–11.2	Formed from alcohol oxidation by yeast and AAB or wood release.
Acids	13.6–33.4	They include volatile acids different from acetic acid and mainly come from yeasts and AAB metabolism, such as phenylacetic acid and isovaleric acid
Acetates	3.38–12.3	Higher alcohol acetates, such as 2-phenylethyl acetate, are important contributors to vinegar aroma
Other esters	0.15–20.0	Ethyl esters of fatty acids, such as ethyl octanoate and ethyl decanoate, are important contributors to vinegar aroma
Enolic derivates	2.19–3.85	They arise from sugar thermal or acid-catalyzed degradation and from charred woods
Furanic and pyranic derivates	1689–3117	Formed from thermal sugar degradation
Ketones	0.59–1.25	Acetoin is produced by yeast and AAB metabolism (via acetolactate decarboxylase)
Lactones	7.67–12.80	Formed by cyclicization of hydroxyacids during fermentation or by sugar degradation
Phenols	127–320	Formed during wood aging (guaiacol, siringol, and eugenol) or by decarboxylation of cinnamic acids acted by LAB or yeasts species
Terpenes	2.01–30.5	Grape-derived compounds; formed by yeasts at low concentration

(*continued*)

TABLE 36.2 (Continued)

Chemical Composition of TBV

Biopolymers		
Melanoidins	–	0.2–2000 kDa biopolymers produced by Maillard reactions and showing antioxidant activity
Cellulose	–	Synthesized by cellulose-producing AAB from carbon compounds, such as hexoses, glycerol, dihydroxyacetone, pyruvate, and dicarboxylic acids
Polyphenols	1418.1–1500.6	They include phenolic acids, catechins, polymeric procyanidins, and flavonols with antioxidant activity

Sources: Giudici, P., R. N. Barbagallo, C. Altieri, and G. Masini, *Industrie delle Bevande*, 23:569–74, 1994; Falcone, P. M., and P. Giudici, *J Agric Food Chem*, 56:7057–66, 2008; Tagliazucchi, D., E. Verzelloni, and A. Conte, *Eur Food Res Technol*, 227:835–43, 2008; Chinnici, F., E. Durań Guerrero, F. Sonni, N. Natali, R. Natera Marin, and C. Riponi, *J Agric Food Chem*, 57:4784–92, 2009.

Note: Information on glucose, fructose, and tartaric, malic, and gluconic acids were from Giudici et al. (1994); melanoidins information were from Falcone and Giudici (2008); polyphenols were enzymatically calculated as sum of phenolic acid, catechin, polymeric procyanidin, and flavonol fractions by Tagliazucchi et al. (2008). Volatile compounds were characterized from five TBV "Extravecchio" samples using gas chromatography–mass spectrometry by Chinnici et al. (2009).

36.3 Manufacture of Traditional Balsamic Vinegar

Due to its ancient and homemade origin, different methods have been reported by historical references to obtain TBV (as quoted by Saccani and Ferrari Amorotti 1999). In recent decades, a standard protocol has been accepted and roughly described by official PDO guidelines. More precisely TBV processing consists of three steps: (1) preparation of the cooked grape must; (2) two-stage fermentation; and (3) aging. Finally, official sensory analysis is performed by the commission of expert tasters trained by the *Consortia*, who grades the TBV and certify its quality before marketing. A flowchart of TBV manufacturing is shown in Figure 36.1.

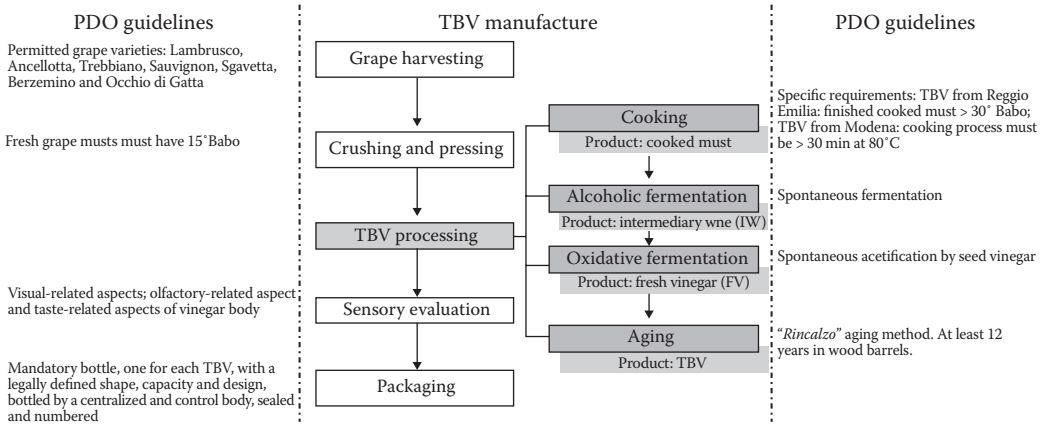


FIGURE 36.1 Overview of TBV-making process. TBV-specific steps are reported in gray.

36.3.1 Preparation of Cooked Grape Must

Grapes permitted in TBV production come from vineyards of the Emilia Romagna region and should have a minimum sugar content of 15°Babo. According to PDO rules, seven grape varieties are allowed, with white or red berries: Lambrusco, Ancellotta, Trebbiano, Sauvignon, Sgavetta, Berzemino, and Occhio di Gatta. Crushing and pressing of grape berry produce juice with a yield not higher than 70% w/w and pH around 3. The major grape constituents are sugars (glucose and fructose in the ratio 1:1) and organic acids, followed by anthocyanins, polyphenols, and tannins. The predominant nonvolatile organic acids are tartaric acid and malic acid, accounting for 90% of the titratable acidity of grape juice. Grape ripeness, timing of picking, and pressing conditions highly affect pH value and composition of grape juice, mainly the balance between sugars and organic acids. Fresh grape juice is boiled down to approximately 30% of the original volume to produce a concentrated must. Cooking is carried out in stainless steel pans operating at atmospheric pressure, heated by a direct flame burner for 12/24 hours at a temperature close to boiling point. The cooking is stopped when the soluble solids concentration reaches 30–40°Bx corresponding to density values of 1.13–1.18 kg/L, but in some cases, values of 60°Bx were detected (Solieri and Giudici 2008).

The heating causes a recorded decrease of 0.3 pH units and the formation of fouling materials, mainly foams and natural colloids formed by coagulated proteins, all needing to be removed mechanically. The heating also causes color change and water vaporization, with the subsequent lowering of the volume and increase of solute concentration and viscosity (Antonelli et al. 2004; Masino et al. 2005; Giudici et al. 2009a). Chemically, cooking profoundly changes grape must composition. Heat-induced deactivation of enzymes, like polyphenol oxidase, stops all enzymatic browning reactions that rapidly occur inside fresh grape musts (Falcone et al. 2010). In addition, the cooking was proved to be responsible for the nonenzymatic browning reactions giving a light brown color and caramel-like flavor to the musts (Piva et al. 2008; Falcone and Giudici 2008; Masino et al. 2008). These heat-induced sugar degradation reactions determine the formation of potential toxicant intermediary products such as methylglyoxal, furfuryl, 5-hydroxymethylfurfural (HMF), and other furanic derivates (Antonelli et al. 2004; Piva et al. 2008; Muratore et al. 2006; Masino et al. 2008). The last stage of sugar degradation includes the nonenzymatic formation of brown biopolymers, the so-called melanoidins, known for their antioxidant properties (Falcone and Giudici 2008; Tagliazucchi et al. 2008, 2010). Due to these polymerization reactions, phenolic compounds decrease simultaneously with the sugar degradation. Furthermore, malic acid and DL-lactic acid increase during thermal degradation of sugars, while acetic acid was found to rapidly decrease in the early stages of cooking, as a result of direct evaporation (Falcone et al. 2010).

36.3.2 Two-Stage Fermentation

TBV is achieved by the two-stage batch fermentation of cooked must: the fermentation of sugars to ethanol followed by the oxidation of ethanol to acetic acid. After cooking, the must is transferred into open wooden or stainless vessels, where it is contaminated by yeasts from the environment. An alternative is the accelerated batch fermentation (back-slopping) by addition of previously fermented cooked must to speed up the alcohol production and avoid mold contamination. According to PDO rules, neither starter culture nor dried yeast is used. The alcoholic fermentation is usually completed in a few weeks, during which yeasts increase rapidly from 10^2 to 10^6 CFU/g (Solieri et al. 2006). The product is an intermediary wine (IW) with an ethanol content around 7%–8% v/wt, reducing sugars about 19%–20% wt/v, and density around 1.079 kg/L (Solieri and Giudici 2008; Lemmetti and Giudici 2010a).

The second microbiological transformation is the bioconversion of ethanol to acetic acid by acetic acid bacteria (AAB). They may spontaneously grow forming a layer on the surface of IW, but they may be slowed down with ethanol values at 9%–10% v/v or completely inhibited at higher ethanol values. The oxidation can also be induced by "seed vinegar," the so-called mother of vinegar, that is an indigenous starter culture withdrawn from acetifying vinegar through back-slopping procedures (Gullo et al. 2006). Manual transfer of the bacterial film from one vessel in active oxidation to a new steady one has been suggested to enhance the success of this procedure (Giudici et al. 2006). As the occurrence of AAB is

limited to the surface of acidifying IW, the static acetification may need several weeks to obtain an acetic acid degree higher than 4.5%–5% wt/wt. The result of this two-stage fermentation is a sour and sugary fresh vinegar (FV) ready for aging.

36.3.3 Aging

The aging is a refilling (*rincalzo*)-based dynamic procedure that resembles the *Solera* method used for making sherry wine. The FV is aged in barrels set usually composed of at least five casks of different sizes and woods, mainly chestnut, acacia, cherry, oak, mulberry, ash, and, in the past, juniper. It is generally accepted that the type of wood plays an important role on the aging process, but no data are available on the topic. The casks are arranged in decreasing scalar volume, generally from 75 to 16 L. The smallest one contains the oldest vinegar and is conventionally numbered "1." Every year, a small quantity of the aged vinegar is withdrawn from the smallest barrel. This barrel is then filled from the content of the preceding barrel up to the first and largest cask, which receives the new FV. This refilling operation is aimed to reintegrate the vinegar lost either by evaporation or spilling, keeping constant the volume of vinegar in all the casks (Lemmetti and Giudici 2010b).

Chemical and physical changes occurred during aging and significantly contribute to define properties of the final product. The most important physical transformation is the solute concentration by water evaporation, decreasing pH values, and increasing viscosity and density (Falcone et al. 2007). As wooden cask works as a molecular separation device, high-steric dimension molecules are retained in the vinegar bulk, resulting in a general solutes increase (Siau 1984). Evaporation varies as a function of cask features (type of wood, thickness, size, and shape) and external conditions (no environmental conditions management is permitted by PDO guidelines). In general, low relative humidity of the cellar is the driving force for water transfer across the wood. By contrast, water activity decreases significantly during aging, and it progressively becomes the limiting driving factor for the evaporation. Moreover, solute precipitation may occur depending on the precise amount of insoluble matter and their degree of solubility (Lemmetti and Giudici 2010a).

In addition to physical changes, several heat-induced chemical reactions continue during aging driven by the low water activity and the sugary and acidic environment. Among them, there is the formation of melanoidins, HMF, and other furanic compounds (Muratore et al. 2006; Verzelloni et al. 2007; Falcone and Giudici 2008; Tagliazucchi et al. 2008). In particular, phenols and other compounds take part in polymerization reactions, resulting in accumulation of melanoidins with a relative molecular weight spreading between 0.2 and 2000 kDa (Falcone and Giudici 2008) and the following composition: carbohydrates (51% w/w), of which glucose (35% w/w) and fructose (10% w/w) are the main representatives, HMF (7.2% w/w), phenolic groups (4.6% w/w), and proteins (1.2% w/w) (Verzelloni et al. 2010). Finally, the polyphenol extraction from wood can occur during aging. This process has been supposed to contribute, together with solute concentration, to high concentration of polyphenols (Antonelli et al. 2004; Tagliazucchi et al. 2008).

36.3.4 Sensorial Analysis

The aging is strictly related to TBV quality and trade value. This value changes considerably with age and top prices are commanded for very old vinegars. From a legal point of view, TBV must be aged for at least 12 years, but no objective procedure has been set up for unambiguous TBV classification and authentication. Furthermore, the refilling procedure blends differently aged vinegars, resulting in a final product whose age is the average of each added aliquot. TBV age is objectively defined as the residence time that the product spends in the barrel set, that is the time it takes to completely replace in the cask (Lemmetti and Giudici 2010b). A mathematical model was elaborated to calculate the residence time accounting only for the volume of vinegar in the set of barrels and for the volume of the vinegar withdrawn and/or transferred from barrel to barrel (Giudici and Rinaldi 2007). Other attempts to identify descriptors of TBV age and quality include analytical (Chiavaro et al. 1988; Plessi et al. 1989; Giudici 1993; Giudici et al. 1994; Zeppa et al. 2002; Masino et al. 2005; Muratore et al. 2006), chemiometric (Consonni and Gatti 2004; Cocchi et al. 2002, 2006a,b; Durante et al. 2006; Consonni et al. 2008), and rheological measurements (Falcone et al. 2007, 2008).

Although these efforts, hitherto sensorial analysis is the only official procedure to evaluate age and quality of TBV. According to published PDOs, the sensorial profile of TBV is evaluated by hedonic judgment expressed in all cases through a numeric score by a panel that evaluates the following attributes: (1) visual-related aspects such as free flowing, color, and clearness; (2) olfactory-related aspects such as flavors (fragrance) and aroma in terms of their intensity, persistence, and pungency; and (3) taste-related aspects of vinegar body in terms of its intensity, harmony, and acidity. The score achieved is used to certify increasing levels of aging and price. According to product regulations, TBV from Modena should be labelled as "Affinato" ("fine") or "Extravecchio" ("fine old"); TBV from Reggio Emilia as "Orange," "Silver," and "Gold." No reference to the year of production or the presumed age of the product may be stated on packaging in both cases.

Recently, some criticisms were highlighted on effectiveness of such sensorial analysis, which is time-consuming and expensive. Implementation of the sensory procedure with near- and mid-infrared spectroscopy and instrumental determination of density, viscosity, and color were proposed to improve reliability of TBV classification (Giudici et al. 2009c; Versari et al. 2011).

36.4 Microbiology of Traditional Balsamic Vinegar

Yeasts and AAB are the biocatalysts of the microbial transformations in TBV. Yeasts are fungi with vegetative states that predominantly reproduce by budding or fission. The variety of yeasts is rather large: the current classification embraces over 700 species grouped in approximately 70 genera (Kurtzman and Fell 1998). The yeasts that are of interest in vinegar production are placed within the phylum *Ascomycota*, subphylum *Saccharomycotina*, which includes the class *Saccharomycetes*.

AAB are strictly aerobic bacteria belonging to the α-proteobacteria class. They occur mainly in sugary, acidic, and alcoholic habitats, such as foods, as indigenous microoganisms or intentionally added, contaminants as in fermented beverages, plants, fruits, and human pathogens (Sievers and Swings 2005; Greenberg et al. 2007). They are defined as "living oxidative catalysts" for their ability to perform very specific oxidation reactions and to channel the released electrons to molecular oxygen through processes called "oxidative fermentations" (Adachi et al. 2003; Deppenmeier and Ehrenreich 2009). The oldest known oxidative fermentation is vinegar production by which ethanol is oxidized into acetic acid.

Microbiological investigations on TBV can be grouped into two sequential periods of study. The first one is from 1970 to 1988 during which yeasts and AAB isolated from TBV were mainly physiologically characterized. The second one, from 1990 up to now, includes studies on microbiological aspects and analysis of the TBV composition related to microbial metabolism.

36.4.1 Yeasts

36.4.1.1 Diversity and Dynamics of Yeasts during TBV Processing

Although yeasts play a key role in the first stage of TBV fermentation, their ecology in cooked must remained unclear until the 1980s. Turtura and coworkers documented the occurrence of *Zygosaccharomyces bailii* and *Zygosaccharomyces rouxii* in fermenting cooked musts (Turtura, 1984, 1986; Turtura and Benfenati 1988), according to a previous study (Sacchetti 1970). Afterward, the strong fermenters and acetic acid–tolerant *Saccharomycodes ludwigii* was also identified in cooked must (Giudici 1990).

The information collected so far on the ecology of fermenting cooked must was obtained by traditional methods, based on plating analysis and biochemical and physiological identification of isolates using the taxonomic keys of Barnett et al. (1990) and Kurtzman and Fell (1998). The increasing availability of the sequences of the 26S rRNA gene and the intergenic regions flanking 5.8S rRNA gene allowed the development of different methods for the yeasts identification. Currently, polyphasic approaches combining PCR-based techniques and phenotypic assays have been optimized for the rapid and accurate detection and/or monitoring of yeasts in food (Schuller et al. 2004). Accordingly, Solieri et al. (2006) identified other species from TBV, including *Zygosaccharomyces mellis*, *Zygosaccharomyces pseudorouxii*,

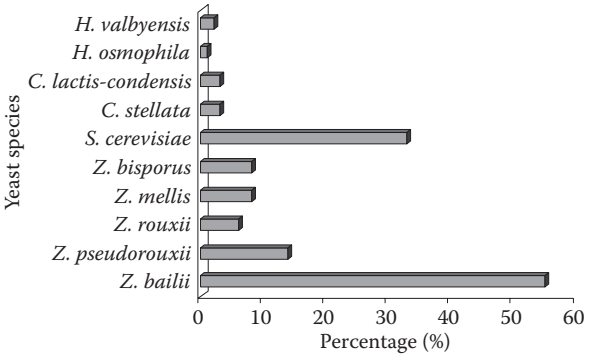


FIGURE 36.2 Percentage of yeast species isolated in 17 spontaneously fermenting cooked must samples. (Adapted from Solieri, L. et al., *J Appl Microbiol*, 101, 63–71, 2006.)

Zygosaccharomyces bisporus, *Hanseniaspora osmophila*, *Hanseniaspora valbyensis*, *Candida stellata*, *Candida lactis-condensi*, and *Saccharomyces cerevisiae* (Figure 36.2).

The cooked must fermentation not only involves the growth of different species but also the development of more than one strain within each species. Using molecular typing techniques, heterogeneous population of *S. cerevisiae* was found in fermenting cooked musts, in agreement to that found in some wine fermentation (Sabate et al. 1998; Pramateftaki et al. 2000; Cocolin et al. 2004; Ganga and Martinez 2004; Sipiczki et al. 2004; Santamaria et al. 2005). For non-*Saccharomyces* species, genetically distinct strains were rarely found in the same fermenting batch (Solieri et al. 2006).

From an ecological point of view, must cooking can be considered a heat sterilization during which temperatures between 55°C and 65°C are generally enough to kill yeasts living in grape juice within few minutes. After this treatment, the cooked must can be occasionally recontaminated by yeasts. Cooked grape must is relatively complete in nutrient content, but it can support the growth of a lower number of microbial species compared to fresh grape must (Solieri and Giudici 2008). Low pH values (in combination to high weak acid content) and high sugar amount exert strong selective pressure, such that only a few yeasts can proliferate. This selectivity is further strengthened when certain nutrients become depleted and anaerobic conditions with increasing ethanol values start to eliminate the most stress-sensitive yeasts. The *Zygosaccharomyces* yeasts are the most frequently species isolated from TBV due to their ability to withstand high sugar content (osmophilic behavior) and their acid resistance. *Z. bailii* is able to grow at a_w values from 0.61 to 0.75 and at pH values from 1.8 to 8.0 in high concentrations of glucose. While acetic acid concentrations of 80–150 mM totally inhibit the growth of *S. cerevisiae* at pH 4.5, the minimum inhibitory concentration of acetic acid ranges from 100 to 240 mM for *Z. bailii* (Fernandez et al. 1997).

The antimicrobial effects of acetic acid at low pH are generally attributed to intracellular acidification and anion accumulation, triggering ATP exhaustion and dissipation of the proton motive force. High acid resistance of *Z. bailii* may be explained by the fact that *Z. bailli* can consume acetate while growing on fermentable sugar (Sousa et al. 1996). Moreover, the low permeability of the plasma membrane to undissociated acid, the high buffering capacity of the cytosol, and/or the high amount of endogenous reserved energy can be also involved (Arneborg et al. 2000; Piper et al. 2001).

Temperature is another important parameter that affects yeast growth. Cooked must is stored at room temperature, generally in autumn, and low temperatures can inhibit mesophilic yeasts and determine sluggish and stuck fermentations. When temperature decreases below 20°C, growth rate of *S. cerevisae* is reduced and the contribution of non–*Saccharomyces cerevisiae* yeasts increases (Fleet and Heard 1993; Rainieri et al. 1998). Among osmotolerant yeasts, only *Z. lentus* has been described as suitable to grow at low temperature (Steels et al. 1999). For other *Zygosaccharomyces* yeasts, the minimum growth temperature increases in high sugary environments, in the range from 12°C to 13°C for *Z. bailii* and from 6.5°C to 10°C for the other species.

36.4.1.2 Yeast Metabolism and Quality of Cooked Grape Must

36.4.1.2.1 Sugars

Glucose and fructose, the main sugars present in cooked must, are metabolized by yeasts to pyruvate via the glycolytic pathway. Pyruvate is decarboxylated to acetaldehyde, which is then reduced to ethanol. The rate of fermentation and the amount of alcohol produced per unit of sugar are strain- and species-dependent. During yeast glycolysis, one molecule of glucose or fructose yields two molecules each of ethanol and carbon dioxide. However, the theoretical conversion of 180 g sugar into 92 g ethanol (51.1%) and 88 g carbon dioxide (48.9%) could only be expected in the absence of any yeast growth, production of other metabolites, and loss of ethanol as vapor. In a model fermentation, about 95% of the sugar is converted into ethanol and carbon dioxide, 1% into biomass and 4% into other products such as glycerol (Pronk et al. 1996).

S. cerevisiae is able to fast degrade carbohydrates to ethanol, with a minimization of by-products and biomass production and without completely oxidizing ethanol to CO_2, even in the presence of oxygen (Crabtree effect). This highly efficient fermentative metabolism and the high resistance to ethanol and anaerobic conditions determine the superiority of *S. cerevisiae* as a wine fermenter, with an ethanol production ranging from 6 to 23% v/v (Fleet and Heard 1993). Similarly *S'my. ludwigii* produces ethanol amounts ranging from 12 to 14% v/v in wine. *C. stellata* and apiculate yeasts like *H. uvarum* show very low fermentative vigor and rate, with an ethanol production of 5% ± 6% and 4% ± 6% v/v, respectively (Ciani and Maccarelli 1998). Compared to previously described yeasts, *Zygosaccharomyces* spp. showed an intermediate ethanol production, with values reached up to 10%, and a high ethanol tolerance (Romano and Suzzi 1993; Kalathenos et al. 1995).

In cooked must, high sugar concentration slightly affects ethanol production by yeasts. *S. cerevisiae* strains show a rapid decrease in ethanol production at increasing osmotic pressure conditions (Table 36.3). Compared to *S. cerevisiae*, species with a weaker fermentative vigor, such as *Z. bailii*, *Z. bisporus*, *Z. mellis*, *Z. pseudorouxii*, *Z. rouxii*, and *Candida* spp., are less affected in ethanol production by increasing sugar content due to their osmophilic behavior. In particular, *C. stellata* and *C. lactis-condensii* spp. strains produce the highest ethanol amount at the highest sugar concentration (Table 36.3).

TABLE 36.3

Ethanol Production, Glucose-to-Fructose Ratio, and Fermentation Rate of TBV-Associated Yeasts Species Evaluated in Cooked Musts

	Cooked Must A		Cooked Must B		Cooked Must C		
Species	**Ethanol (v/v)**	**FR**	**Ethanol (v/v)**	**FR**	**Ethanol (v/v)**	**FR**	**G/F**
Z. bailii (19)	7.0	0.6	7.2	0.7	5.9	0.4	9.24
Z. pseudorouxii (4)	6.3	0.3	6.5	0.2	6.3	0.3	5.36
Z. rouxii (1)	5.3	0.4	5.7	0.4	5.3	0.4	3.02
Z. mellis (3)	5.7	0.4	6.0	0.4	5.7	0.3	4.90
Z. bisporus (1)	5.8	0.5	6.1	0.4	4.6	0.3	3.05
C. stellata (2)	7.3	1.8	7.8	1.7	6.8	0.6	15.65
C. lactis-condensi (1)	7.4	1.8	8.2	1.9	7.3	0.8	122.10
H. osmophila (1)	5.0	1.4	5.3	1.5	4.4	0.7	0.75
H. valbyensis (1)	5.4	1.1	5.2	0.9	4.0	0.6	0.79
S. ludwigii (2)	9.7	2.1	8.1	1.4	4.4	0.7	–
S. cerevisiae (7)	8.6	2.0	8.2	1.9	5.9	1.0	0.44

Source: Data modified from Solieri, L. and P. Giudici, *Industrie delle Bevande*, 34:526–31, 2005.

Note: Ethanol production, glucose-to-fructose ratio (G/F), and fermentation rate (FR) of 11 TBV-associated yeasts species were evaluated in three cooked musts (A, B, and C) with increasing sugar content of 350, 400, and 550 g/L, respectively. The strain's number is in parentheses. Fermentation rate was evaluated as grams of free CO_2 for 100 mL cooked must after 72 hours. Ethanol amount (v/v) was evaluated at the end of fermentation. Residual sugars (g/L) were evaluated in cooked must C with 350 g/L as starting sugar. –, Not determined.

Furthermore, yeasts show different preferences for glucose and fructose, determining unbalanced glucose/fructose (G/F) ratio at end fermentation. This discrepancy between the glucose and fructose consumption considerably varies at strain and species level and is influenced by external conditions like nitrogen supplementation or alcohol addition. Generally, *S. cerevisiae* has a slightly higher preference for glucose than for fructose, resulting in a preponderance of fructose during the last phases of fermentation, which can be fermented by other yeasts under stress conditions (Fleet 1998; Bauer and Pretorius 2000; Berthels et al. 2004; Perez et al. 2005). Similarly, apiculate yeasts *H. valbyensis* and *H. osmophila* show a glucophilic behavior both in fresh and cooked grape musts (Ciani and Fatichenti 1999; Granchi et al. 2002; Solieri and Giudici 2005) (Table 36.3). By contrast, *H. guilliermondii*, *Candida*, and *Zygosaccharomyces* spp. consume faster fructose than glucose (Solieri and Giudici 2005; Moreira et al. 2008) (Table 36.3). The *Z. bailii* and *Z. rouxii* fructophilic phenotype has been correlated to fructose-specific transporters mediating the preferential fructose uptake via facilitated diffusion (Sousa-Dias et al. 1996; Pina et al. 2004; Leandro et al. 2010). From a technological point of view, unfermented glucose has been related to undesired TBV crystallization occurring either inside the wooden barrels or hermetic glasses and involving rearrangements of the α-D-glucose monohydrate molecules (Giudici et al. 2004). Significantly osmophilic yeasts are generally fructophilic with fructose consumed more than glucose (Solieri and Giudici 2005; Giudici and Falcone 2010).

36.4.1.2.2 Organic Acids

Yeast metabolism modifies significantly organic acids composition of fresh and cooked grape musts, with the exception of tartaric acid that is essentially stable to microbial activity. The metabolic impact on organic acid profile is well known in fresh grape must, but poorly in cooking must. Most yeasts can utilize significant concentrations of malic acid present in grape juice. *S. cerevisiae* typically degrade 3%–45% of malic acid during fermentation. Among TBV-associated species, *Z. bailii* has been reported to degrade malate when growing anaerobically on glucose but not on fructose (Baranowski and Radler 1984). Malate degradation activity was found also in *C. stellata* strains. By contrast, malate production is restricted to few species, such as *Saccharomyces uvarum* (Giudici et al. 1995).

Succinic acid is the main carboxylic acid produced during fermentation, where it typically accumulated to 2 g/L. *S. cerevisiae*, *Candida*, *Hanseniaspora*, and *Zygosaccharomyces* spp. produce succinic acid during must fermentation, but this production is highly strain-dependent (Swiegers et al. 2005 and references therein). The most likely pathway for its formation appears to involve the reductive branch (via oxaloacetate and malate) of the tricarboxylic acid (TCA) cycle during anaerobic fermentation (Roustan and Sablayrolles 2002; Camarasa et al. 2003).

Lactic acid is stable, and in wine, it might be present in amounts of up to 6 g/L after malolactic fermentation. Due to the inefficiency of the mitochondrial lacto-dehydrogenases under fermentation conditions, *S cerevisiae* strains produce only traces of lactic acid during alcoholic fermentation (Dequin and Barre 1994). No evidences of malolactic fermentation have yet been reported in cooked must.

36.4.1.2.3 Glycerol

Glycerol is a major product of alcoholic fermentation, after ethanol and CO_2 (Pronk et al. 1996; Scanes et al. 1998). Chemically, glycerol is a polyol with a colorless, odorless, and highly viscous character. It tastes slightly sweet, as well as having an oily and heavy mouth feel. Glycerol formation is initiated by reduction of the glycolytic intermediate dihydroxyacetone phosphate to glycerol-3-phosphate, catalyzed by NAD-dependent glycerol-3-phosphate dehydrogenase, with the concomitant oxidation of NADH to NAD^+. Subsequently, the glycerol-3-phosphate is hydrolyzed by glycerol-3-phosphatase to yield glycerol and inorganic phosphate. The shift in redox balance ($NADH/NAD^+$ ratio) caused by glycerol formation is corrected by using acetic acid as a redox sink to convert NAD^+ back to NADH. Therefore, increased glycerol production is usually linked to increased acetic acid production and inversely related to ethanol production (Pronk et al. 1996; Eglinton et al. 2002).

The amount of glycerol produced by *S. cerevisiae* is around one tenth of the amount of ethanol, and its concentrations in wine normally range between 4 and 9 g/L, with average values approximately of 7 g/L

(Scanes et al. 1998). The low ethanol producer *C. stellata* is characterized by a high glycerol production, around 10–14 g/L in wine and up to 16.3 g/L in synthetic medium, probably due to a low alcohol dehydrogenase activity (4-fold lower than that of *S. cerevisiae*) and a very high glycerol-3-phosphate dehydrogenase activity (40-fold higher than that of *S. cerevisiae*) (Ciani and Ferraro 1998; Soden et al. 2000; Ciani et al. 2000; Jolly et al. 2003; Magyar and Toth 2011).

Differently from *Candida stellata*, the strong fermenter *S'my. ludwigii* produces a high glycerol amount (around 9.91 g/L) without any inverse relationship between ethanol and glycerol production (Ciani and Maccarelli 1998). Finally, *Zygosaccharomyces* spp. has a good glycerol production, mainly in highly sugary cooked must. Like other osmophilic species, if exposed to osmotic stress, *Zygosaccharomyces* strains accumulate compatible solutes, such as glycerol, to balance the osmotic pressure across the cell membrane (Romano et al. 1997).

36.4.1.2.4 Volatile Acidity

Volatile acidity (VA) describes a group of volatile organic acids of short carbon chain length. The volatile acid content of wine is usually between 200 and 1000 mg/L (10%–15% of the total acid content), and of this, acetic acid usually constitutes about 90% of the volatile acids (Giudici et al. 1992; Henschke and Jiranek 1993). The rest of the volatile acids, principally propionic and hexanoic acids, is produced as the result of fatty acid metabolism by yeasts and AAB.

Acetic acid is of particular importance. It can be produced by yeasts as an intermediate of the pyruvate dehydrogenase (PDH) bypass, a pathway responsible for the conversion of pyruvate into acetyl-CoA through a series of reactions catalyzed by pyruvate decarboxylase (Pdc), acetaldehyde dehydrogenase (Ald), and acetyl-CoA synthase (as quoted by Vilela-Moura et al. 2011). The acetaldehyde-to-acetate conversion is catalyzed by different Ald isophorms, among them Ald6 (cytosolic isophorm), Ald5, and Ald4 (mitochondrial isoforms) are the main enzymes responsible for acetate formation during wine-like anaerobic growth on glucose (Saint-Prix et al. 2004).

Via PDH bypass, *S. cerevisiae* is able to produce a wide range of acetic acid, as little as 100 mg/L and up to 1.8 g/L (Giudici et al. 1992; Romano et al. 1992; Radler 1993; Ciani 1998). Non-*Saccharomyces* yeasts species isolated from fermenting cooked musts produce higher acetic acid concentrations. This trait is generally inversely related to ethanol production. The VA produced by *Hanseniaspora* spp. strains range from 0.75 to 2.25 g/L (Caridi and Ramondino 1999; Capece et al. 2005). Similarly, *C. stellata* produces a level of acetic acid around 1–2.5 g/L (Fleet and Heard 1993), while *S'my. ludwigii* higher than 0.75 g/L (Romano et al. 1999). *Zygosaccharomyces* species are generally known as acetic acid–producing yeasts (Romano and Suzzi 1993).

In addition to acetate formation, most yeasts are able to catabolize acetate by respiration according to a diauxic growth with consumption of acetic acid only after glucose exhaustion. By contrast, *Z. bailii* displays a biphasic growth in medium containing a mixture of glucose and acetic acid; the first phase is associated with simultaneous consumption of glucose and acetic acid, and the second with the utilization of the remaining acid (Sousa et al. 1998).

36.4.1.2.5 Higher Alcohols

Aliphatic (propanol, isoamyl alcohol, isobutanol, and active amyl alcohol) and aromatic (2-phenylethyl alcohol and tyrosol) higher alcohols are secondary by-products of alcoholic fermentation by yeast, obtained from α-ketoacids, which are formed via the catabolic or Ehrlich pathway or an anabolic pathway involving synthesis of branched-chain amino acids through their biosynthetic pathway from glucose (Hazelwood et al. 2008). Whereas *S. cerevisiae* is characterized by high production of isoamyl alcohol and 2,3-butanediol and low production of acetoin, *H. uvarum* and *C. stellata* have a high production of acetoin and ethyl acetate (higher in *C. stellata* than in *H. uvarum*) and a generally low production of other higher alcohols. The higher alcohol profile of other TBV-associated species is not well known. *Z. fermentati* shows a general low higher alcohols production in wine, with the exception of 2,3-butanediol. *S'my. ludwigii* can be considered a general high producer of acetoin, ethyl acetate, isoamyl alcohol, and isobutanol (Romano et al. 1992, 1999, 2003).

36.4.2 Acetic Acid Bacteria

36.4.2.1 Diversity and Dynamics of Acetic Acid Bacteria during TBV Processing

Among the 11 genera of AAB currently recognized in the family Acetobacteraceae, only species of the genera *Acetobacter* and *Gluconacetobacter* are frequently associated with vinegar production, as reported by several authors for wine and cereal vinegars (Joyeux et al. 1984; Kittelmann et al. 1989; Sievers et al. 1992; Schüller et al. 2000; Nanda et al. 2001; Azuma et al.; Vegas et al. 2010; Wu et al. 2010). However, there are a number of vinegars made from other raw materials and mainly produced at small scale, like TBV, for which there is not in-depth investigation. The first studies on AAB of TBV described *Acetobacter aceti* and *Acetobacter xylinum* (*Ga. xylinus*) as the main species (Sacchetti 1970; Turtura and Benfanti 1988). Using both culture-dependent and independent methods, recent studies recovered *Ga. europaeus*, *Ga. xylinus*, *A. pasteurianus*, *A. aceti*, and *A. malorum* with high frequencies in IW. In particular, strains of *Ga. europaeus* and *A. pasteurianus* are the most widespread. Other species such as *Gluconobacter oxydans* and *Ga. hansenii* have been detected in a lower number of samples (De Vero et al. 2006; Gullo and Giudici 2006; Gullo and Giudici 2008). These evidences are consistent with those dealing with other vinegars from which AAB strains mainly detected belong to *Ga. europaeus* and *A. pasteurianus* species (Hidalgo et al. 2010; Torija et al. 2010).

AAB population, in terms of occurrence, number, diversity, and dynamic of strains along the process is driven by intrinsic parameters of the IW and extrinsic parameters of the environment on which the process is performed. The high frequency of strains belonging to *Ga. europaeus* and *A. pasteurianus* could be the result of the selective pressure exerted by the IW environment. The intrinsic parameters influencing AAB population in IW are mainly: pH (3.0–3.4), ethanol (6.0%–8.0% v/v), residual sugars (130–250 g/L), and biological structures such as esopolysaccharides produced by AAB themselves. Whereas the main extrinsic parameters are temperature, relative humidity of environment, presence and concentration of oxygen, and presence and activities of other organisms with antagonistic behavior such as yeasts and molds.

36.4.2.2 Acetic Acid Bacteria Metabolism and Quality of Traditional Balsamic Vinegar

The most relevant phenotypic trait of AAB is the ability to carry incomplete oxidation of broad ranges of carbohydrates with aldehydic or ketonic functional groups whose products are secreted almost completely into the medium (Asai 1968). Carbon sources are utilized with different rates, e.g., *Acetobacter* and *Gluconacetobacter* species show diauxic growth on media containing both ethanol and glucose due to ethanol repression on glucose assimilation.

The incomplete oxidation is catalyzed by membrane-bound pyrroloquinoline quinone (PQQ)–dependent dehydrogenases, which are connected to the respiratory chain with generation of ATP by oxidative phosphorylation (Yakushi and Matsushita 2010). In general, organic acids are almost stoichiometrically accumulated in the medium and are not further utilized by the cells. However, in some conditions, alcohols and sugars are completely oxidized in the cytoplasm via membrane-bound PQQ-dependent dehydrogenases and the soluble cytoplasmatic dehydrogenases. In these cases, the substrates are utilized for both direct energy generation and carbon assimilation (assimilatory oxidation). At the first exponential phase, acetate is accumulated by incomplete oxidation of ethanol by the function of the periplasmatic membrane–bound PQQ–alcohol deydrogenase (ADH) and acetaldehyde dehydrogenase (ALDH). After ethanol consumption, the acetate is completely oxidized to H_2O and CO_2 (overoxidation) via acetyl CoA synthase and the cells have the second exponential phase (Matsushita et al. 2004). The mechanism of switch between incomplete oxidation and assimilatory oxidation as well as the control of carbon metabolism is the key aspect of TBV fermentation.

36.4.2.2.1 Acetic Acid

During acetic acid production by AAB, ethanol is dehydrogenated to acetaldehyde and then acetate by two successive oxidative reactions catalyzed by membrane-bound PQQ-ADH and ALDH, respectively. The fermentation proceeds according to the equation:

$$1C_2H_5OH + 1O_2 \rightarrow 1CH_3COOH + 1H_2O \ (\Delta G^\circ = -455 \text{ kJ/mol}).$$

Theoretically, 1 M ethanol solution (4.6 g/100 mL) gives rise to 6 g of acetic acid in a total volume of 102 mL, which, expressed in volume, corresponds to 5.8 mL ethanol/100 mL and 6 g/100 mL are 5.9 g of acetic acid/100 mL (Ebner and Follmann 1983). Empirical data collected during submerged fermentation show that the ethanol yield conversion is in the range of 95%–98% of the theoretical value (García-García et al. 2009).

The discrepancy between theoretical and observed yield is mainly due to loss by evaporation during the fermentation. In our knowledge, there are no data on yield in static fermentations of TBV; however, it is reasonable to assume that it is lower than in submerged fermentations because of the long exposition to air and the possible complete oxidation of ethanol by overoxidation.

As reported above, acetic acid is very toxic to the cells because it liberates protons (H^+) that contribute to dissipate the proton motive force. AAB have several mechanisms of acetic acid tolerance, such as overoxidation, efficiency of proton efflux, and integrity and lipid compositions of the cytoplasmatic membranes (Matsushita et al. 2005; Treck et al. 2007). Recently, it was found that the relationship between acetic acid resistance and exopolysaccharides formation during acetic acid fermentation by *A. pasteurianus* strains. In particular, strains producing exopolysaccharides show higher acetic acid tolerance than non-producing ones. The esopolysaccharides surrounding the cells contrast the passive diffusion of acetic acid into the cells acting as a biofilm-like barrier. This is one of the reasons why during long and uncontrolled acetification processes the risk of polysaccharides formation is unpredictable (Kanchanarach et al. 2010).

Acetic acid tolerance is a strain- and species-dependent trait, and considerable differences were observed among AAB isolated from TBV. Within *Gluconacetobacter* genus, *Ga. europaeus* is more resistant than other vinegar-related AAB, such as *Ga. intermedius*, *Ga. oboediens*, and *Ga. entanii* (Trcek et al. 2006). Moreover, *Ga. europaeus* strains exhibit the highest acetic acid tolerance during the long-term fermentation in static conditions. In contrast, *A. pasteurianus* strains show active fermentation up to 6% v/v of acetic acid during dynamic renewal of the substrate, but the vitality is strongly affected when ethanol is exhausted (Gullo et al. 2009).

36.4.2.2.2 Sugars

Among sugars metabolized by AAB, glucose and fructose have an important role in transforming IW to FV. Glucose can be oxidized to gluconic acid, which can be further oxidized to 2-ketogluconate and 2,5-diketogluconate by three membrane-bound PQQ-dependent dehydrogenases, which are linked to the respiratory chain. First, glucose is oxidized to gluconate by a membrane-bound, PQQ-dependent glucose dehydrogenase. In the second phase, gluconate is further oxidized to 2-ketogluconate and 2,5-diketogluconate mediated by gluconate dehydrogenase and 2-ketogluconate dehydrogenase, respectively (as quoted by Matsushita et al. 2004). The oxidation of glucose to gluconic acid was first noted by Brown and Snell (1954) and later confirmed by many other researchers. Now, AAB fermentation of glucose is also used for the industrial production of gluconic acid (as quoted by De Vero et al. 2010).

Fructose can be oxidized to 5-keto-D-fructose by the D-fructose dehydrogenase. Moreover, fructose can be formed from oxidation of intracellular sorbitol and can be channelled into the oxidative pentose phosphate pathway (Shibata et al. 2000).

In grape juice, strains belonging to *Acetobacter* and *Gluconobacter* species were reported to oxidize from 41% to 100% of glucose to gluconic acid, with amount higher than 100 g/L, while weak or no activity was observed on fructose (Giudici and Masini 1995). In TBV, gluconic acid ranges from 0.33 to 3.34 g/100 g, with highest values in most aged products, while oxidation of gluconic acid to ketogluconic acids is depressed by the low pH values. For these reasons, gluconic acid was suggested as a marker of TBV quality (Giudici et al. 1994; Giudici 1993). Furthermore, this acid has a pK_a in the range of 3.5–3.8, which is lower than of that acetic acid (4.75), and it could contribute to the low pH observed in TBV.

36.4.2.2.3 Glycerol and Other Polyols

Polyols (glycerol, D-mannitol, D-sorbitol) derive from grape and from metabolism of yeasts and AAB. As reported above, glycerol is the most abundant by-product of alcoholic fermentation and its concentration

is strongly affected by yeast strains and sugar concentration. The glycerol oxidation is well studied in *G. oxydans* where the enzyme glycerol/sorbitol dehydrogenase (sldAB) is considered the major polyol dehydrogenase exhibiting broad substrate specificity. It catalyzes the oxidation of D-sorbitol, gluconate, and glycerol, with the production of L-sorbose, 5-ketogluconate, and dihydroxyacetone (DHA), respectively (Matsushita et al. 2003; Prust et al. 2005). In *A. pasteurianus*, the metabolic pathway of glycerol was predicted by the results on *Ga. xylinus* where glycerol utilization is accompanied by the formation of DHA, cellulose, CO_2, and small amounts of acetate. In this species, DHA phosphate can be produced from glycerol by two pathways: one is via DHA catalyzed by glycerol dehydrogenase and the other one via glycerol 3-phosphate by glycerol kinase. The DHA phosphate may be converted to D-glyceraldehyde 3-phosphate by triosephosphate isomerase and thus enter into the glycolysis and gluconeogenesis pathway (Azuma et al. 2009).

In IW, glycerol is oxidized by AAB only when ethanol concentration decrease at a level close to zero (Giudici, unpublished results). Other polyols such as 2,3-butaneldiol and acetoin are also oxidized by AAB (Asai 1968). Being consistent secondary products of alcoholic fermentation, their oxidation could affect vinegar quality and composition.

36.5 Innovation Trends

Applications of selected starter cultures are widely diffused in several fermented food productions, such as wine, beer, bread, dairy products, fermented meat sausages, and various vegetables (Leroy and De Vuyst 2004), but they are rarely applied to vinegar production (Sokollek and Hammes 1997). In particular, TBV manufacture PDO rules allow only the use of "mother vinegar" to inoculate the cooked must, and any other specification are not reported on the microbiological composition of this seed vinegar. The microbiological fermentations of TBV are difficult to manage, especially acetic fermentation, since AAB are very fastidious microorganisms.

The main effects of AAB metabolism compromising the fermentation course occur at the startup of processes. Recently, a scaling-up procedures of selected AAB have been proposed to improve efficiency and reproducibility of acetic fermentation process in TBV manufacturing (Gullo and Giudici 2008; Gullo et al. 2009). Among tested strains, it was found the suitability of *A. pasteurianus* during the refilling stages and *Ga. europaeus* during long fermentation without refilling, suggesting the need for a double-culture fermentation to overcome fermentation slowness or breakdown. Effective refilling through the addition of aliquot of IW to the active selective starter culture is one of the most important operational procedures. For IWs, amounts of 1%–3% as upper and bottom limits for ethanol content and 3% as the bottom limit for acetic acid content were found advantageous (Gullo et al. 2009). Moreover, basics and specific traits of AAB strains for TBV starter cultures, considering the combined effect of their interaction, were defined (Table 36.4). For example, efficient oxidation of ethanol is strictly joined to a quick production of acetic acid that prevents the restarting of alcoholic fermentation, avoids spoilage by other organisms such as molds, and reduces the risk of massive esopolysaccharides production. The fast increase of acetic acid prevents undesired aroma but requires an adequate tolerance to this acid as well as to a wide temperature range. Moreover, the tolerance to acetic acid allows its accumulation without detrimental effects for cells and the maintenance into the medium at a concentration of 3%–4% is an alternative way to overcome overoxidation. The abilities to grow at high sugar concentration and produce gluconic acid contribute to the removal of D-glucose from the medium at the expense of cellulose production.

Among traits whose effects are not yet answered in IW, there is bacteriophage resistance. In industrial vinegar, fermentation breakdown is observed when bacteriophages are in concentrations 10^8–10^9 particles/mL. The problem is more serious in submerged acetification than in systems where a diversity of strains differing in phage susceptibility can be found, so that the fermentation can be slowed as a result of phage infection but does not stop completely. The use of pasteurized substrate, sterile filtered air, and the strict separation of submerged fermenters from seed vinegar are used as precautionary measures to avoid bacteriophage infections in the industrial field (Stamm et al. 1989; Sellmer et al. 1992).

The roles of yeast in TBV quality have not been given detailed consideration in the past, and possibly, this reflects their minor impact, compared to that of AAB. Nevertheless, such influences may be

TABLE 36.4

Basics and Specific Traits of AAB Strains for Use as TBV-Selective Starter Culture and the Combined Effect of Their Interaction

	Ethanol Oxidation	Fast Production of Acetic Acid	Tolerance to Acetic Acid	No Overoxidation of Acetate	Low pH Resistance	Bacteriophage Resistance	No Polysaccharides Production	No Undesired Aroma	Osmophily	Wide Temperature Growth Range
Ethanol Oxidation										
Fast Production of Acetic Acid	+									
Tolerance to Acetic Acid	+	+								
No Overoxidation of Acetate	+	/	/							
Low pH Resistance	–	–	–	/						
Bacteriophage Resistance	/	/	/	/	/					
No Polysaccharides Production	+	/	–	/	/	/				
No Undesired Aroma	+	+	/	+	/	/	+			
Osmophily	/	–	–	/	/	/	–	/		
Wide Temperature Growth Range	–	–	–	/	–	/	/	/	/	

Note: /, Unknown.

underestimated and could be of value in future developments. For instance, preferential consumption of glucose by yeasts could prevent glucose crystallization in the final product (Giudici et al. 2004). The example suggests how yeast's role in TBV extends far beyond the primary function to catalyze the rapid conversion of grape sugars into alcohol and carbon dioxide. Differently from wine production, ethanol concentration of 7%–8% v/v are enough to provide adequate substrate for subsequent acetic oxidation by AAB and ethanol amount greater than 8% v/v could inhibit AAB growth. Similarly, acetic acid production, which is a negative trait in oenology, is an amenable trait to promote subsequent AAB growth in TBV manufacture. According to these observations, effective management of alcoholic fermentation with starter cultures could be essential to ensure successful two-stage fermentation. Selected *Z. bailii* strains have been first proposed to produce a high-quality IW due to their ability to grow in high sugar and acidic environments. Recent advantages in yeast population knowledge have increased the range of yeasts suitable to test as a TBV-specific starter, providing an array of fitness and quality criteria for a starter culture development program (Solieri et al. 2006; Solieri and Giudici 2008).

36.6 Conclusions

TBV is the product of complex interactions among yeasts, AAB, and several technological practices and chemical processes that start in the vineyard and continue throughout the cooking of the must, the two-stage fermentation, until aging and packaging. Although physicochemical transformations of cooking and aging provide the foundations of TBV quality, yeasts and AAB act as a biocatalyst that modify aroma, flavor, mouth feel, and chemical complexity and impact on the subtlety and individuality of the final product. Consequently, it is important to identify and understand the interactions that occur between different microbial groups, species, and strains, as well as the ecological interactions between yeasts and cooked must and between AAB and IW, respectively. In the future, investigations on the biochemical, physiological, and molecular bases of these interactions will improve the reproducibility and quality of TBV.

REFERENCES

Adachi O, Moonmangmee D, Toyama H, Yamada M, Shinagawa E, Matsushita K. 2003. New developments in oxidative fermentation. *Appl Microbiol Biotechnol* 60:643–53.

Antonelli A, Chinnici F, Masino F. 2004. Heat-induced chemical modification of grape must as related to its concentration during the production of traditional balsamic vinegar: a preliminary approach. *Food Chem* 88:63–8.

Arneborg N, Jespersen L, Jakobsen M. 2000. Individual cells of *Saccharomyces cerevisiae* and *Zygosaccharomyces bailii* exhibit different short-term intracellular pH responses to acetic acid. *Arch Microbiol* 174:125–8.

Asai T. 1968. *Acetic Acid Bacteria Classification and Biochemical Activities*. Tokyo: University of Tokyo Press.

Azuma Y, Hosoyama A, Matsutani M, Furuya N, Horikawa H, Harada T, Hirakawa H, Kuhara S, Matsushita K, Fujita N, Shirai M. 2009. Whole-genome analyses reveal genetic instability of *Acetobacter pasteurianus*. *Nucleic Acids Res* 37:5768–83.

Baranowski K, Radler F. 1984. The glucose-dependent transport of L-malate in *Zygosaccharomyces bailii*. *Anton Leeuw* 50:329–40.

Barnett JA, Payne RW, Yarrow D. 1990. *Yeasts: Characteristics and Identification*. 2nd Ed. Cambridge: University Press.

Bauer FF, Pretorius IS. 2000. Yeast stress response and fermentation efficiency: how to survive the making of wine—A review. *S Afr J Enol Vitic* 21:27–51.

Benedetti B. 2004. *Fatti in casa l'aceto balsamico. Manuale illustrato per la formazione e conduzione di una acetaia*. Modena: Il Fiorino.

Berthels NJ, Cordero Otero RR, Bauer FF, Thevelein JM, Pretorius IS. 2004. Discrepancy in glucose and fructose utilisation during fermentation by *Saccharomyces cerevisiae* wine yeast strains. *FEMS Yeast Res* 4:683–9.

Brown GM, Snell EE. 1954. Pantothenic acid conjugates and growth of *Acetobacter suboxydans*. *J Bacteriol* 67:465–71.

Camarasa C, Grivet JP, Dequin S. 2003. Investigation by 13C-NMR and tricarboxylic acid (TCA) deletion mutant analysis of pathways for succinate formation in *Saccharomyces cerevisiae* during anaerobic fermentation. *Microbiol* 149(Pt 9):2669–78.

Capece A, Fiore C, Maraz A, Romano P. 2005. Molecular and technological approaches to evaluate strain biodiversity in *Hanseniaspora uvarum* of wine origin. *J Appl Microbiol* 98:136–44.

Caridi A, Ramondino D. 1999. Biodiversità fenotipica in ceppi di *Hanseniaspora* di origine enologica. *Enotecnico* 45:71–4.

Chiavaro E, Caligiani A, Palla G. 1988. Chiral indicators of ageing in balsamic vinegars of Modena. *Ital J Food Sci* 10:329–37.

Chinnici F, Durań Guerrero E, Sonni F, Natali N, Natera Marin R, Riponi C. 2009. Gas chromatography–mass spectrometry (GC-MS) characterization of volatile compounds in quality vinegars with protected European geographical indication. *J Agric Food Chem* 57:4784–92.

Ciani M. 1998. Wine vinegar production using base wines made with different yeast species. *J Sci Food Agric* 78:290–4.

Ciani M, Fatichenti F. 1999. Selective sugar consumption by apiculate yeasts. *Lett Appl Microbiol* 28:203–6.

Ciani M, Ferraro L. 1998. Combined use of immobilized *Candida stellata* cells and *Saccharomyces cerevisiae* to improve the quality of wines. *J Appl Microbiol* 85:247–54.

Ciani M, Ferraro L, Fatichenti F. 2000. Influence of glycerol production on the aerobic and anaerobic growth of the wine yeast *Candida stellata*. *Enzyme Microb Technol* 27:698–703.

Ciani M, Maccarelli F. 1998. Oenological properties of non-*Saccharomyces* yeasts associated with winemaking. *World J Microb Biot* 14:199–203.

Cocchi M, Bro R, Durante C, Manzini D, Saccani F, Sighinolfi S, Ulrici A. 2006b. Analysis of sensory data of aceto balsamico tradizionale di Modena (ABTM) of different ageing by application of PARAFAC models. *Food Qual Pref* 17:419–28.

Cocchi M, Durante C, Grandi M, Lambertini P, Manzini D, Marchetti A. 2006a. Simultaneous determination of sugars and organic acids in aged vinegars and chemometric data analysis. *Talanta* 69:1166–75.

Cocchi M, Lambertini P, Manzini D, Marchetti A, Ulrici A. 2002. Determination of carboxylic acids in vinegars and in aceto balsamico tradizionale di Modena by HPLC and GC methods. *J Agric Food Chem* 50:5255–61.

Cocolin L, Pepe V, Comitini F, Comi G, Ciani M. 2004. Enological and genetic traits of *Saccharomyces cerevisiae* isolated from former and modern wineries. *FEMS Yeast Res* 5:237–45.

Commission Regulation (EC) No 583/2009 of 3 July 2009 entering a name in the register of protected designations of origin and protected geographical indications [Aceto Balsamico di Modena (PGI)]. *Official Journal of the European Union*, L175, 4.7.2009, p 7.

Consonni R, Cagliani LR, Benevelli F, Spraul M, Humpfer E, Stocchero M. 2008. NMR and chemometric methods: A powerful combination for characterization of balsamic and traditional balsamic vinegar of modena. *Anal Chim Acta* 611:31–40.

Consonni R, Gatti A. 2004. ^{1}H NMR Studies on Italian Balsamic and Traditional Balsamic Vinegars. *J Agric Food Chem* 52:3446–50.

Council Regulation (EC) No 813/2000 of 17 April 2000 supplementing the Annex to Commission Regulation (EC) No 1107/96 on the registration of geographical indications and designations of origin under the procedure laid down in Article 17 of Regulation (EEC) No 2081/92. *Official Journal of European Communities*. 20 April 2000.

De Vero L, Gala E, Gullo M, Solieri L, Landi S, Giudici P. 2006. Application of denaturing gel electrophoresis (DGGE) analysis to evaluate acetic acid bacteria in traditional balsamic vinegar. *Food Microbiol* 23:809–13.

De Vero L, Gullo M, Giudici P. 2010. Acetic Acid Bacteria, Biotechnological Applications. In: Flickinger MC, editor. *Encyclopedia of Industrial Biotechnology: Bioprocess, Bioseparation and Cell Technology*. New York: John Wiley & Sons. p 9–25.

Deppenmeier U, Ehrenreich A. 2009. Physiology of acetic acid bacteria in light of the genome sequence of *Gluconobacter oxydans*. *Mol Microbiol Biotechnol* 16:69–80.

Dequin S, Barre P. 1994. Mixed lactic acid-alcoholic fermentation by *Saccharomyces cerevisiae* expressing the *Lactobacillus casei* L(+)-LDH. *BioTech* 12:173–77.

Disciplinare di produzione della denominazione di origine protetta «Aceto balsamico tradizionale di Modena». Provvedimento 15 Maggio 2000. G.U. Repubblica Italiana No.124, May 30, 2000a.

Disciplinare di produzione della denominazione di origine protetta «Aceto balsamico tradizionale di Reggio Emilia». Provvedimento 15 Maggio 2000. G.U. Repubblica Italiana No.124, May 30, 2000b.

Durante C, Cocchi M, Grandi M, Marchetti A, Bro R. 2006. Application of N-PLS to gas chromatographic and sensory data of traditional balsamic vinegars of Modena. *Chemometr Intell Lab* 83:54–65.

Ebner H, Follmann H. 1983. Acetic acid. In: Rehm HJ, Reed G, editors. *Biotechnology*. 1st ed. Vol. 3. Weinheim: Verlag Chemie. p 387–407.

Eglinton JM, Heinrich AJ, Pollnitz AP, Langridge P, Henschke PA, de Barros Lopes M. 2002. Decreasing acetic acid accumulation by a glycerol overproducing strain of *Saccharomyces cerevisiae* by deleting the *ALD6* aldehyde dehydrogenase gene. *Yeast* 19:295–301.

Falcone PM, Chillo S, Giudici P, Del Nobile MA. 2007. Measuring rheological properties for applications in quality assessment of traditional balsamic vinegar: description and preliminary evaluation of a model. *J Food Eng* 80:234–40.

Falcone PM, Giudici P. 2008. Molecular weight and molecular weight distribution impacting traditional balsamic vinegar ageing. *J Agric Food Chem* 56:7057–66.

Falcone PM, Giudici P. 2009. A new perspective to study the quality of vinegars: weight distribution analysis. *Industrie delle Bevande* 38:29–37.

Falcone PM, Tagliazucchi D, Verzelloni E, Giudici P. 2010. Sugar conversion induced by the application of heat to grape must. *J Agric Food Chem* 58:8680–91.

Falcone PM, Verzelloni E, Tagliazucchi D, Giudici P. 2008. A rheological approach to the quantitative assessment of traditional balsamic vinegar quality. *J Food Eng* 86:433–43.

Fernandez L, Côrte-Real M, Loureiro V, Loureiro-Dias MC, Leão C. 1997. Glucose respiration and fermentation in *Zygosaccharomyces bailii* and *Saccharomyces cerevisiae* express different sensitivity patterns to ethanol and acetic acid. *Lett Appl Microbiol* 25:249–53.

Fleet GH, 1998. Microbiology of alcoholic beverages. In: Wood BJ, editor. *Microbiology of Fermented Foods*. 2nd ed. vol. 1. London: Blackie Academic & Professional. p 217–62.

Fleet GH, Heard GM. 1993. Yeasts-growth during fermentation. In: Fleet GH, editor. *Wine Microbiology and Biotechnology*. London: Harwood Academic. p 27–54.

Ganga MA, Martinez C. 2004. Effect of wine yeast monoculture practice on the biodiversity of non-*Saccharomyces* yeasts. *J Appl Microbiol* 96:76–83.

García-García I, Santos-Dueñas IM, Jiménez C, Jiménez-Hornero JE, Bonilla-Venceslada JL. 2009. Vinegar Engineering. In: Solieri L, Giudici P, editors. *Vinegars of the World*. Milan: Springer-Verlag. p 97–120.

Giudici P. 1990. Inibizione dell'acido acetico sull'attività dei lieviti osmofili isolati dall'aceto balsamico tradizionale. *Industrie delle bevande* 19:475–8.

Giudici P. 1993. Acido gluconico: criterio di genuinità dell'aceto balsamico tradizionale. *Industrie delle bevande* 22:123–5.

Giudici P, Altieri C, Cavalli R. 1992. Aceto balsamico tradizionale–preparazione del fermentato di base. *Industrie delle bevande* 21:478–83.

Giudici P, Barbagallo RN, Altieri C, Masini G. 1994. Origine ed evoluzione degli acidi organici durante l'invecchiamento dell'aceto balsamico tradizionale. *Industrie delle Bevande* 23:569–74.

Giudici P, Falcone PM. 2010. Aceto balsamico tradizionale: indicatori di invecchiamento e del rischio di solidificazione. *Industrie delle bevande* 39:19–36.

Giudici P, Falcone PM, Scacco A, Lanza MC. 2009c. Sensory analysis of the traditional balsamic vinegar. *Industrie delle bevande* 38:27–42.

Giudici P, Gullo M, Solieri L. 2009a. Traditional Balsamic Vinegar. In: Solieri L, Giudici P, editors. *Vinegars of the World*. Milan: Springer-Verlag. p 157–78.

Giudici P, Gullo M, Solieri L, De Vero L, Landi S, Pulvirenti A, Rainieri S. 2006. *Le fermentazioni dell'aceto balsamico tradizionale*. Reggio Emilia: Diabasis.

Giudici P, Gullo M, Solieri L, Falcone PM. 2009b. Technological and microbiological aspects of Traditional Balsamic Vinegar and their influence on quality and sensorial properties. In: Taylor SL, editor. *Advances in Food and Nutrition Research*. San Diego: Academic Press. p 137–82.

Giudici P, Pulvirenti A, De Vero L, Landi S, Gullo M. 2004. Cristallizzazione dell'aceto balsamico tradizionale *Industrie delle bevande* 193:426–9.

Giudici P, Rinaldi G. 2007. A theoretical model to predict the age of traditional balsamic vinegar. *J Food Eng* 82:121–7.

Giudici P, Zambonelli C, Passarelli P, Castellari L. 1995. Improvement of wine composition with cryotolerant *Saccharomyces* strains. *Am J Enol Vitic* 46:143–7.

Granchi L, Ganucci D, Messini A, Vincenzini M. 2002. Oenological properties of *Hanseniaspora osmophila* and *Kloeckera corticis* from wines produced by spontaneous fermentations of normal and dried grapes. *FEMS Yeast Res* 2:403–7.

Greenberg DE, Porcella SF, Zelazny AM, Virtaneva K, Sturdevant DE, Kupko JJ 3rd, Barbian KD, Babar A, Dorward DW, Holland SM. 2007. Genome sequence analysis of the emerging human pathogenic acetic acid bacterium *Granulibacter bethesdensis*. *J Bacteriol* 189:8727–36.

Gullo M, Caggia C, De Vero L, Giudici P. 2006. Characterization of acetic acid bacteria in "traditional balsamic vinegar." *Int J Food Microbiol* 106:209–12.

Gullo M, De Vero L, Giudici P. 2009. Succession of selected strains of *Acetobacter pasteurianus* and other acetic acid bacteria in traditional balsamic vinegar. *Appl Environ Microbiol* 75:2585–9.

Gullo M, Giudici P. 2006. Isolation and selection of acetic acid bacteria fro traditional balsamic vinegar. *Industrie delle Bevande* 35:334–50.

Gullo M, Giudici P. 2008. Acetic acid bacteria in traditional balsamic vinegar: phenotypic traits relevant for starter cultures selection. *Int J Food Microbiol* 125:46–3.

Gullo M, Giudici P. 2009 Acetic acid bacteria taxonomy from early descriptions to molecular techniques. In: Solieri L, Giudici P, editors. *Vinegars of the World*. Milan: Springer-Verlag. p 41–60.

Hazelwood LA, Daran JM, van Maris AJ, Pronk JT, Dickinson JR. 2008. The Ehrlich pathway for fusel alcohol production: a century of research on *Saccharomyces cerevisiae* metabolism. *Appl Environ Microbiol* 74:2259–66.

Henschke PA, Jiranek V. 1993. Yeast-Growth during fermentation. In: Fleet GH, editor. *Wine Microbiology and Biotechnology*. London: Harwood Academic. p 27–54.

Hidalgo C, Vegas C, Mateo E, Tesfaye W, Cerezo AB, Callejón RM, Poblet M, Guillamón JM, Mas A, Torija MJ. 2010. Effect of barrel design and the inoculation of *Acetobacter pasteurianus* in wine vinegar production. *Int J Food Microbiol* 141:56–62.

Jolly NP, Augustyn OPH, Pretorius IS. 2003 The effect of non-*Saccharomyces* yeasts on fermentation and wine quality. *S Afr J Enol Vitic* 24:55–2.

Joyeux A, Lafon-Lafourcade S, Ribereau-Gayon P. 1984. Evolution of acetic acid bacteria during fermentation and storage of wine. *Appl Environ Microbiol* 48:153–6.

Kalathenos P, Sutherland JP, Roberts TA. 1995. Resistance of some wine spoilage yeasts to combinations of ethanol and acids present in wine. *J Appl Microbiol* 78:245–50.

Kanchanarach W, Theeragool G, Inoue T, Yakushi T, Adachi O, Matsushita K. 2010. Acetic acid fermentation of *Acetobacter pasteurianus*: relationship between acetic acid resistance and pellicle polysaccharide formation. *Biosci Biotechnol Biochem* 74:1591–7.

Kittelmann M, Stamm WW, Follmann H, Truper HG. 1989. Isolation and classification of acetic acid bacteria from high percentage vinegar fermentations. *Appl Microbiol Biot* 30:47–52.

Kurtzman CP, Fell JW. 1998. *The Yeasts: A Taxonomic Study*. 4th ed. Amsterdam: Elsevier Science.

Leandro MJ, Sychrová H, Prista C, Loureiro-Dias MC. 2010. The osmotolerant fructophilic yeast *Zygosaccharomyces rouxii* employs two plasma-membrane fructose uptake systems belonging to a new family of yeast sugar transporters. *Microbiol* 157:601–8.

Lemmetti F, Giudici P. 2010a. Management of barrel set and quality of traditional balsamic vinegar. *Industrie delle Bevande*. 39(8):1–10.

Lemmetti F, Giudici P. 2010b. Mass balance and age of traditional balsamic vinegar. *Industrie delle Bevande* 39(2):18–28.

Leroy F, De Vuyst L. 2004. Lactic acid bacteria as functional starter cultures for the food fermentation industry. *Trends Food Sci Tech* 15:67–78.

Magyar I, Toth T. 2011. Comparative evaluation of some oenological properties in wine strains of *Candida stellata*, *Candida zemplinina*, *Saccharomyces uvarum* and *Saccharomyces cerevisiae*. *Food Microbiol* 28:94–100.

Masino F, Chinnici F, Bendini A, Montevecchi G, Antonelli A. 2008. A study on relationships among chemical, physical, and qualitative assessment in traditional balsamic vinegar. *Food Chem* 106:90–5.

Masino F, Chinnici F, Franchini GC, Ulrici A, Antonelli A. 2005. A study of the relationships among acidity, sugar and furanic compound concentrations in set of casks for aceto balsamico tradizionale of Reggio Emilia by multivariate techniques. *Food Chem* 92:673–9.

Matsushita K, Fujii Y, Ano Y, Toyama H, Shinjoh M, Tomiyama N, Miyazaki T, Sugisawa T, Hoshino T, Adachi O. 2003. 5-keto-D-gluconate production is catalyzed by a quinoprotein glycerol dehydrogenase, major polyol dehydrogenase, in *Gluconobacter* species. *Appl Environ Microbiol* 69:1959–66.

Matsushita K, Inoue T, Adachi O, Toyama H. 2005. *Acetobacter aceti* possesses a proton motive force-dependent efflux system for acetic acid. *J Bacteriol* 187:4346–52.

Matsushita K, Toyama H, Adachi O. 2004. Respiratory chains in acetic acid bacteria: Membrane-bound periplasmic sugar and alcohol respirations. In: Zannoni D, editor. *Respiration in Archaea and Bacteria*. Dordrecht: Springer. p 81–99.

Mazza S, Murooka Y. 2009. Vinegars through the age. In: Solieri L, Giudici P, editors. *Vinegars of the World*. Milan: Springer-Verlag. p 17–39.

Moreira N, Mendes F, Guedes de Pinho P, Hogg T, Vasconcelos I. 2008. Heavy sulphur compounds, higher alcohols and esters production profile of *Hanseniaspora uvarum and Hanseniaspora guilliermondii* grown as pure and mixed cultures in grape must. *Int J Food Microbiol* 124:231–38.

Muratore G, Licciardello F, Restuccia C, Puglisi ML, Giudici P. 2006. Role of different factors affecting the formation of 5-hydroxymethyl-2-furancarboxaldehyde in heated grape must. *J Sci Food Agr* 54:860–3.

Nanda K, Taniguchi M, Ujike S, Ishihara N, Mori H, Ono H, Murooka Y. 2001. Characterization of acetic acid bacteria in traditional acetic acid fermentation of rice vinegar (Komesu) and unpolished rice vinegar (Kurosu) produced in Japan. *Appl Envir Microbiol* 67:986–90.

Perez M, Luyten K, Michel R, Riou C, Blondin B. 2005. Analysis of *Saccharomyces cerevisiae* hexose carrier expression during wine fermentation: both low- and high-affinity Hxt transporters are expressed. *FEMS Yeast Res* 5:351–61.

Pina C, Goncalves P, Prista C, Loureiro-Dias MC. 2004. Ffz1, a new transporter specific for fructose from *Zygosaccharomyces bailii*. *Microbiol* 150:2429–33.

Piper P, Ortiz Calderon C, Hatzixanthis K, Mollapour M. 2001. Weak acid adaptation: the stress response that confers yeasts with resistance to organic acid food preservatives. *Microbiol* 147:2635–42.

Piva A, Di Mattia C, Neri L, Dimitri G, Chiarini M, Sacchetti G. 2008. Heat-induced chemical, physical and functional changes during grape must cooking. *Food Chem* 106:1057–65.

Plessi M, Monzani A, Coppini D. 1989. Quantitative determination of acids and derivatives in balsamic and other vinegars. *Sciences des Aliments* 9:179–83.

Pramateftaki PV, Lanaridis P, Typas MA. 2000. Molecular identification of wine yeasts at species or strain level: a case study with strains from two vine-growing areas of Greece. *J Appl Microbiol* 89:236–48.

Pronk JT, Steensma HY, van Dijken JP. 1996. Pyruvate metabolism in *Saccharomyces cerevisiae*. *Yeast* 12:1607–33.

Prust C, Hoffmeister M, Liesegang H, Wiezer A, Fricke WF, Ehrenreich A, Gottschalk G, Deppenmeier U. 2005. Complete genome sequence of the acetic acid bacterium *Gluconobacter oxydans*. *Nat Biotechnol* 23:195–2.

Radler F. 1993. Yeast: Metabolism of organic acids. In: Fleet GH, editor. *Wine Microbiology and Biotechnology*. London: Harwood Academic. p 165–82.

Rainieri S, Zambonelli C, Hallsworth JE, Pulvirenti A, Giudici P. 1998. *Saccharomyces uvarum*, a distinct group within *Saccharomyces sensu stricto*. *FEMS Microbiol Lett* 177:177–85.

Romano P, Fiore C, Paraggio M, Caruso M, Capece A. 2003. Function of yeast species and strains in wine flavour. *Int J Food Microbiol* 86:169–80.

Romano P, Marchese R, Laurita C, Salzano G, Turbanti L. 1999. Biotechnological suitability of *Saccharomycodes ludwigii* for fermented beverages. *World J Microbiol Biotechnol* 15:451–45.

Romano P, Suzzi G. 1993. Potential use for *Zygosaccharomyces* species in winemaking. *J Wine Res* 4:89–94.

Romano P, Suzzi G, Comi G, Zironi RJ. 1992. Higher alcohol and acetic acid production by apiculate wine yeasts. *J Appl Bacteriol* 73:126–30.

Romano P, Suzzi G, Comi G, Zironi R, Maifreni M. 1997. Glycerol and other fermentation products of apiculate wine yeasts. *J Appl Microbiol* 82:615–8.

Roustan JL, Sablayrolles JM. 2002. Modification of the acetaldehyde concentration during alcoholic fermentation and effects on fermentation kinetics. *J Biosci Bioeng* 93:367–75.

Sabate J, Cano J, Querol A, Guillamon JM. 1998. Diversity of *Saccharomyces* strains in wine fermentations; analysis for two consecutive years. *Lett Appl Microbiol* 26:452–5.

Saccani F, Ferrari Amorotti V. 1999. *Il balsamico della tradizione secolare. Storia, conoscenze tecniche ed esperienze vissute alle soglie del terzo millennio*. Spilamberto: Consorteria dell'Aceto Balsamico Tradizionale di Modena.

Sacchetti M. 1970. *Sull'Aceto Balsamico Modenese*. Edagricole, Bologna.

Saint-Prix F, Bonquist L, Dequin S. 2004. Functional analysis of the *ADL* gene family of *Saccharomyces cerevisiae* during anaerobic growth on glucose: The $NADP^+$-dependent Ald6p and Ald5p isoforms play a major role in acetate formation. *Microbiol* 150:2209–20.

Santamaria P, Garijo P, Lopez R, Tenorio C, Gutierrez AR. 2005. Analysis of yeast population during spontaneous alcoholic fermentation: effect of the age of the cellar and the practice of inoculation. *Int J Food Microbiol* 103:49–6.

Scanes KT, Hohmann S, Prior BA. 1998. Glycerol production by yeast *Saccharomyces cerevisiae* and its relevance to wine: a review. *S Afr J Enol Vitic* 19:17–24.

Schuller D, Valero E, Dequin S, Casal M. 2004. Survey of molecular methods for the typing of wine yeast strains. *FEMS Microbiol Lett* 231:19–26.

Schüller G, Hertel C, Hammes WP. 2000. *Gluconacetobacter entanii* sp. nov., isolated from submerged high-acid industrial vinegar fermentations. *Int J Syst Evol Microbiol* 50:2013–20.

Sellmer S, Sievers M, Teuber M. 1992. Morphology, virulence and epidemiology of bacteriophage particle isolated from industrial vinegar fermentations. *Syst Appl Microbiol* 15:610–6.

Shibata T, Ichikawa C, Matsuura M, Takata Y, Noguchi Y, Saito Y, Yamashita M. 2000. Cloning of a gene for D-sorbitol dehydrogenase from *Gluconobacter oxydans* G624 and expression of the gene in *Pseudomonas putida* IFO3738. *J Biosci Bioeng* 89:463–8.

Siau JE. 1984. *Transport Process in Wood*. Berlin: Springer.

Sievers M, Sellmer S, Teuber M. 1992. *Acetobacter europaeus* sp. nov., a main component of industrtial vinegar fermenters in central Europe. *Syst Appli Microbiol* 15:386–92.

Sievers M, Swings J. 2005. Family II. *Acetobacteraceae*. In: Garrity GM, editor. *Bergey's Manual of Systematic Bacteriology*. Vol. 2, Part C. The proteobacteria. New York: Springer-Verlag. p 41–54.

Sipiczki M, Romano P, Capece A, Parragio M. 2004. Genetic segregation of natural *Saccharomyces cerevisiae* strains derived from spontaneous fermentation of Aglianico wine. *J Appl Microbiol* 96: 1169–75.

Soden A, Iloakey H, Henschke PA. 2000. Effects of co-fermentation with *Candida stellata* and *Saccharomyces cerevisiae* on the aroma and composition of Chardonnay wine. *Aust J Grape Wine Res* 621–30.

Sokollek SJ, Hammes WP. 1997. Description of a starter culture preparation for vinegar fermentation. *Syst Appl Microbiol* 20:481–91.

Solieri L, Giudici P. 2005. Yeast starter selection for traditional balsamic vinegar. *Industrie delle Bevande* 34:526–31.

Solieri L, Giudici P. 2008. Yeasts associated to traditional balsamic vinegar: ecological and technological features. *Int J Food Microbiol* 125:36–45.

Solieri L, Giudici P. 2009. Vinegars of the world. In: Solieri L, Giudici P, editors. *Vinegars of the World*. Milan: Springer-Verlag. p 1–16.

Solieri L, Landi S, De Vero L, Giudici P. 2006. Molecular assessment of indigenous yeast population from traditional balsamic vinegar. *J Appl Microbiol* 101:63–71.

Sousa MJ, Miranda L, Côrte-Real M, Leão C. 1996. Transport of acetic acid in *Zygosaccharomyces bailii:* effects of ethanol and their implications on the resistance of the yeast to acidic environments. *Appl Environ Microbiol* 62:3152–7.

Sousa MJ, Rodrigues F, Côrte-Real M, Leão C. 1998. Mechanisms underlying the transport and intracellular metabolism of acetic acid in the presence of glucose in the yeast *Zygosaccharomyces bailii*. *Microbiol* 144:665–70.

Sousa-Dias S, Gonçalves T, Leyva JS, Peinado JM, Loureiro-Dias MC. 1996. Kinetics and regulation of fructose and glucose transport systems are responsible for fructophily in *Zygosaccharomyces bailii*. *Microbiol* 142:1733–8.

Stamm WW, Kittelmann M, Follmann H, Trüper HG. 1989. The occurrence of bacteriophages in spirit vinegar fermentation. *Appl Microbiol Biotechnol* 30:41–6.

Steels H, Bond CJ, Collins MD, Roberts IN, Stratford M, James SA. 1999. *Zygosaccharomyces lentus* sp. nov., a new member of the yeast genus *Zygosaccharomyces* Barker. *Int J Syst Bacteriol* 49:319–27.

Swiegers JH, Bartowsky EJ, Henschke PA, Pretorius IS. 2005. Yeast and bacterial modulation of wine aroma and flavor. *Aust J Grape Wine Res* 11:139–73.

Tagliazucchi D, Verzelloni E, Conte A. 2008. Antioxidant properties of traditional balsamic vinegar and boiled must model systems. *Eur Food Res Technol* 227:835–43.

Tagliazucchi D, Verzelloni E, Conte A. 2010. Contribution of melanoidins to the antioxidant activity of traditional balsamic vinegar during ageing. *J Food Biochem* 34:1061–78.

Torija MJ, Mateo E, Guillamón JM, Mas A. 2010. Identification and quantification of acetic acid bacteria in wine and vinegar by TaqMan-MGB probes. *Food Microbiol* 27(2):257–65.

Trcek J, Jernejc K, Matsushita K. 2007. The highly tolerant acetic acid bacterium *Gluconacetobacter europaeus* adapts to the presence of acetic acid by changes in lipid composition, morphological properties and PQQ-dependent ADH. Expression. *Extremophil* 11:627–35.

Trcek J, Toyama H, Czuba J, Misiewicz A, Matsushita K. 2006. Correlation between acetic acid resistance and characteristics of PQQ-dependent ADH in acetic acid bacteria. *Appl Microb Cell Physiol* 70:366–73.

Turtura GC. 1984. La microflora dell'Aceto Balsamico Naturale. *Industrie delle Bevande* 4:100–1.

Turtura GC. 1986. Microbiologia e chimica dell'Aceto Balsamico Naturale. In: Benedetti B, editor. *L'Aceto Balsamico*. Modena: Consorteria dell'Aceto Balsamico di Spilamberto. p 256.

Turtura GC, Benfanti L. 1988. Caratteristiche microbiologiche e chimiche dell'aceto balsamico naturale. Studio del prodotto. *Ann Microbiol* 38:51–74.

Vegas C, Mateo E, González A, Jara C, Guillamón JM, Poblet M, Torija MJ, Mas A. 2010. Population dynamics of acetic acid bacteria during traditional wine vinegar production. *Int J Food Microbiol* 138:130–6.

Versari GP, Parpinello F, Chinnici G, Meglioli G. 2011. Prediction of sensory score of Italian traditional balsamic vinegars of Reggio-Emilia by mid-infrared spectroscopy. *Food Chem* 125:1345–50.

Verzelloni E, Tagliazucchi D, Conte A. 2007. Relationship between the antioxidant properties and the phenolic and flavonoid content in traditional balsamic vinegar. *Food Chem* 105:564–71.

Verzelloni E, Tagliazucchi D, Conte A. 2010. Changes in major antioxidant compounds during aging of traditional balsamic vinegar. *J Food Biochem* 34:152–71.

Vilela-Moura A, Schuller D, Mendes-Faia A, Silva RD, Chaves SR, Sousa MJ, Côrte-Real M. 2011. The impact of acetate metabolism on yeast fermentative performance and wine quality: reduction of volatile acidity of grape musts and wines. *Appl Microbiol Biotechnol* 89:271–80.

Wu J, Gullo M, Chen F, Giudici P. 2010. Diversity of *Acetobacter pasteurianus* strains isolated from solid-state fermentation of cereal vinegars. *Curr Microbiol* 60:280–6.

Yakushi T, Matsushita K. 2010. Alcohol dehydrogenase of acetic acid bacteria: structure, mode of action, and applications in biotechnology. *Appl Microbiol Biotechnol* 86:1257–65.

Zeppa G, Giordano M, Gerbi V, Meglioli G. 2002. Characterization of volatile compounds in three acetification batteries used for the production of 'aceto balsamico tradizionale di Reggio Emilia.' *Ital J Food Sci* 14:247–66.

37

Palm Wine

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CONTENTS

37.1 Palm Wine: History and Traditional Significance

Traditional fermented beverages represent popular indigenous products that have formed an integral part of the diet since the early history of mankind. Generally, these beverages are prepared in the household or by cottage industries, using relatively simple techniques or equipment. As some of these products have undergone industrial development, they can also now be diffuse and be manufactured on a large scale (Wood 1998; Boekhout and Robert 2003; Hui et al. 2004).

Palm wine is an excellent example of a traditional fermented beverage, and it can be defined as a whitish, effervescent, alcoholic drink that is obtained from the spontaneous yeast and lactic acid bacteria (LAB) fermentation of the sugary sap extract from the tropical plants of the *Palmae* family. Indeed, the palm family is one of the most important and widespread plants economically, and it represents an important economical and ecological culture for many countries in the regions of north Africa (Ahmed et al. 1995). Effectively, the sap used to produce palm wine is most often taken from wild date palms, such as the silver date palm (*Phoenix sylvestris*), the palmyra and the jaggery palm (*Cariota urens*), the oil palm (*Elaeis guineensis*), the coconut palm (*Cocus nucifera*), the date palm (*Phoenix dactylifera*), the raffia palm (*Raphia hookeri* and *Raphia vinifera*), and the nipa palm, kithul palm, and lala palm (*Hyphaene coriacea*) (Faparusi 1971; Okafor 1972; Sanni and Looner 1993; Ejofor et al. 1994; Ayogu 1999; Nwachukwuet et al. 2006). The indigenous names used for this alcoholic beverage are widespread, such as *mimbo* in Cameroon, *toutou* in Gabon, *doga* and *nsafufuo* in Ghana, *emu* and *ogogoro* in Nigeria, *poyo* in Sierra Leone, *segero* in Papua New Guinea, and *lagbi* in Libya. In southern Africa, the palm wine that was called *ubusulu* is also produced in Maputaland, an area in the south of Mozambique between the Lobombo Mountains and the Indian Ocean. In parts of the central and western Democratic Republic of the Congo, palm wine is called *malafu*; indeed, there are four types of palm wine in the central and western Democratic Republic of the Congo: from the oil palm, there is *ngasi*, from the raffia palm, *dibondo*, from the coconut palm, *cocoti*, and from a short palm that grows in the savannah areas of western Bandundu and Kasai provinces, *mahasu*. In Tunisia, the date palm sap of *Phoenix dactylifera* L. is consumed directly as a fresh drink, called *legmi*, or used as an alcoholic beverage after its natural fermentation (Thabet et al. 2007). In India, there are three types of palm wine: from wild date palm (*Phoenix sylvestris*), there is *sendi*, from the palmyra palm (*Borassus flabellifer*) and the date palm (*Phoenix dactylifera*), *tari*, and from coconut palm (*Cocus nucifera*), *nareli* (Batra and Millner 1974).

Palm wine can also be distilled to create a stronger drink, which again goes by different names depending on the region (e.g., *arrock*, *village gin*, *charayam*, *country whiskey*). Throughout Nigeria, this

is commonly called *ogogoro*. In parts of southern Ghana, distilled palm wine is known as *akpeteshi* or *burukutu*. In Togo, it is famous as *sodabe*, while in the Philippines it is called *lambanog*.

Palm wine has important roles in many ceremonies in parts of Nigeria and elsewhere in central and western Africa. Guests at weddings, birth cerebrations and funeral wakes are served generous quantities. Indeed, as a token of respect to deceased ancestors at wakes, many drinking sessions begin with a small amount of palm wine being spilled onto the ground. Palm wine is also often infused with medicinal herbs to remedy a wide variety of physical complaints. Overall, palm wine is enjoyed by both men and women, although women usually drink it in less public venues.

Palm wine from the coconut palm is also known in other regions of the world. It is known as *nuoundua* in Vietnam, *tuak* in Malaysia, Indonesia, and North Sumatra, *tuba* in the Philippines, Borneo, and Mexico, and *goribon* in Sabah. Similarly, palm wine is consumed in Sri Lanka and Myanmar, where it is typically called *ra* and *htan yay*. Indeed, the production of palm wine has contributed to the endangered status of some palm species, such as the Chilean wine palm, *Jubaea chilensis* (Aidoo et al. 2006).

In some areas of India, palm wine can be served as the sweet unfermented sap, called *neera*, which is derived from the fresh sap, or as *kallu*, a sour beverage made from the fermented sap of the palm. Neera is collected, refrigerated, stored, and distributed by semigovernment agencies, with a little lime added to the sap to prevent the fermentation process. In others part of India, palm wine is evaporated to produce the unrefined sugar, called *jaggery*. In the state of Kerala in India, palm wine is used in the leavening of a local form of rice bread (as a substitute for yeast). Palm wine is mixed with rice dough and left overnight to aid the fermentation and allow the expansion of the dough overnight, making the bread soft when it is prepared. In the state of Andhra Pradesh in India, fermented palm sap is a popular drink in rural parts. The *kallu* is collected, distributed, and sold by the people of a particular caste. It is a big business in the cities of those districts, while in villages, people drink it every day after work.

37.2 Tapping and *In Situ* Fermentation

For a long time, the traditional tapping of the date palm, *Phoenix dactylifera* L., which belongs to the *Arecaceae*, was a common practice. The date palm sap is the store for the bulk of the reserve of photosynthetically produced carbohydrates of the palm, in the form of sucrose in solution in the vascular bundles of its trunk. When the central growing point or the upper part of the trunk is incised, this palm sap will exude as a fresh clear juice, which mainly consists of sucrose. Palm sap can be converted by the fermentation process into palm wine or vinegar (Mozingo 1989). Depending on the region and the palm species, many techniques for the tapping of the sap are used. This results in quantitative and qualitative variations in the collected sap. Similarly, the sap yield varies according to the collecting method, and the age and variety of the palm, although it is considered important, as a plant can provide up to 500 L during one tapping season (Barreveld 1993).

In various African countries and beyond, the sap of the palm tree is obtained by tapping the palm tree; this, and the palm sap collection, is carried out by the tapper. When the palm begins to blossom, the palm-sap tapper climbs up the tree and excises the new inflorescence, making a deep incision into the vessel of the tree. There is an art to the binding of the flower spates, and their pounding causes the sap to flow properly following the cutting of the spate tip, for the collection of the sap in earthen pitchers. Indeed, a container is fastened to the flower stump to collect the sap that is obtained from either the immature male inflorescence (inflorescence tapping) or from the stem (stem tapping). This is commonly practiced in Nigeria, Benin, and the Ivory Coast.

An alternative method is the felling of the entire tree. Where this is practiced, a fire is sometimes lit at the cut end to facilitate the collection of the sap. Here, the process of tapping the palm first involves felling or cutting down the tree, leaving the felled tree for a period of about 2 weeks to promote the concentration of the sap, followed by its tapping for up to 8 weeks.

Whatever the tapping techniques, the fermentation starts as soon as the sap flows into the pitcher. The fresh sap collected in the container contains a heavy suspension of yeasts and bacteria, giving the sap a milky white appearance (Okafor 1975). These microorganisms metabolize sugars to produce alcohol and various organic acids. If the palm wine is not consumed within a few days, it begins to develop a

vinegary taste, which is not acceptable to consumers. To be acceptable to most sugary tastes, in addition to the whitish aspect and this pleasant sugary taste, palm wine must also show vigorous effervescence (Okafor 1975). In African society, palm wine is extremely popular and has a significant role in many customary practices.

In this context, the microbiology and biochemistry of the palm wine fermentation process needs to be well understood to provide standardization of the production process and the final composition of the palm wine. However, like many other traditional fermented foods in Africa, the production of palm wine is conducted by the age-old method of chance inoculation and uncontrolled fermentation. Therefore, the usual variations in the quality and stability of the product are to be expected (Stringini et al. 2009).

37.2.1 Chemical Composition of Palm Sap

The chemical composition of date-palm sap (*P. dactylifera* L.) has shown that the dry matter represents 12.84%. For other species of palm trees, this value ranges from 10% to 15%, depending on the period of the sap collection and on the palm type. The protein content of palm sap is about 5.14% (based on the dry matter). Composition analysis of fresh sap from the date palm has shown that sugars are the major components, as 92% to 95% of dry matter. The sugar fraction is dominated by sucrose (73.46%), and in addition to sucrose, fructose (6.98%), glucose (5.91%), and myo-inositol (7.0%) have also been detected for the phloem sap of other plant species (Thabet et al. 2007, 2009). Moreover, the sap also contains 2.7% to 5.0% proteins and 2.3% to 2.6% minerals. With this composition representing a high concentration of nutrient components, palm sap is a good substrate for a lot of microorganisms, and it can be rapidly fermented by autochthonous microflora. These are essentially composed of yeasts, LAB, and acetic acid bacteria (AAB). Thus, this composition of the palm sap explains why it is highly susceptible to spontaneous fermentation.

Over recent decades, the consumption of this sweet beverage provided by the palm sap has increased, and it has become necessary and of interest to have an idea of its composition and toxicity. Different samples of palm wines have been randomly selected and examined. The results of these investigations have shown that as a beverage, as well as ethanol, palm wine contains minor volatile components, such as alcohols, aldehydes, carboxylic acids and esters, and trace of metals at low concentrations, which are below the internationally recognized maximal limits for alcoholic beverages, at <2 ng/L for cadmium, and <10 ng/L for arsenic (Mavioga et al. 2009).

37.2.2 Microbial Evolution and Main Fermentation Characters of Tapped Palm Wine

Because of the central role that alcoholic beverages have played in traditional society, it is important that the microbiology and biochemistry of the fermentation process are well understood. However, there have been a limited number of studies carried out on this topic. In particular, the earlier studies provided information for the international literature on the microbiological aspects of palm wine tapped from felled palm trees (Okafor 1972; Morah 1995; Agu et al. 1999; Ojimelukwe 2001).

On the other hand, in the last few years, some studies have been carried out on the different aspects of palm wine production, microbiological quality, and preservation (Amoa et al. 2007; Ogbulie et al. 2007; Stringini et al. 2008, 2009). These studies have revealed considerable microbial diversity. This biodiversity were present in the palms, the visiting insects, such as *Drosophila melanogaster*, and the tapping equipment used, as well as in the stalk, spate, and florets of these palm trees. This microbial diversity associated with the fruits, the crop growing, and the tree surfaces might have an important role during the spontaneous fermentation process (Fleet 2003). The various microorganisms that colonize this specific ecological niche include bacteria such as coliforms, LAB, AAB, yeasts, and molds (Mbugua 1985).

Generally, the microflora commonly found in coconut sap (*Cocos nucifera*), palmyra sap (*Borassus flabellifer*), and oil-palm sap (*Elaeis guineensis*) has been shown to include *Saccharomyces cerevisiae*, *Saccharomyces chevalieri*, *Kloeckera apiculata*, *Schizosaccharomyces pombe*, *Acetobacter aceti*, *Acetobacter rancens*, *Acetobacter suboxydans*, *Leuconostoc dextranicum*, *Micrococcus* sp., *Pediococcus* sp., *Bacillus* sp., and *Sarcina* sp. Of the yeasts, the predominant and best alcoholic

fermenter is *S. cerevisiae* (Theivendirarajah and Chrystopher 1987; Shamala and Sreekantiah 1988; Stringini et al. 2009).

The yeast species most often reported in African indigenous fermented foods and beverages is indeed *S. cerevisiae*. However, in indigenous fermentation, *S. cerevisiae*, often coexists with other microorganisms. LAB are recognized as having important roles in the fermentation and preservation of a great variety of food and feed, while the roles of AAB in the development of the vinegary taste in palm wine has also been investigated (Amoa-Awua et al. 2006). LAB and AAB are found at high levels, including for *Lactobacillus plantarum*, *Lactobacillus mesenteroides*, and others species of bacteria such as *Zymomonas mobilis* and *Acetobacter* spp., *A. aceti*, *A. rancens*, *A. suboxydans*, *Micrococcus* sp., *Pediococcus* sp., *Bacillus* sp., *Sarcina* spp., and *Leuconostoc dextranicum*. A novel LAB was recently isolated from among other LAB from palm wine and was named *Leuconostoc palmae* sp. nov. (Ehrmann et al. 2009). The yeast species *S. cerevisiae* and *S. pombe* have been reported to be the dominant yeast species (Odunfa and Oyewole 1998); however, other yeast species have also been found, including *S. chevalieri*, *Saccharomyces exigus*, *K. apiculata*, *Hanseniaspora occidentalis*, *Candida krusei*, other *Candida* spp., *Pichia pastoris*, *Pichia*. spp, *Saccharomycodes ludwigii*, and *Kodamaea ohmeri* (Atacador-Ramos 1996; Joshi et al. 1999; Amoa-Awua et al. 2006). A similar, marked, diversity of yeast genera has been reported in the context of traditional wine making and cider fermentation in Europe, even if the presence of nonfermenting bacteria are detected at low frequencies. The microbial evolution of the tapped palm wine observed in different studies has shown that after an initial lower presence, the yeast population showed a constant concentration of about 10^7 CFU/mL, starting from the second day. LAB and AAB were also abundant in the substrate from the initial samples (10^7 CFU/mL), and they maintained this concentration over the following days (Amoa-Awua et al. 2006; Stringini et al. 2009) in association with yeasts.

The process of tapping of palm wine from felled trees can be considered as semicontinuous fermentation. Indeed, samples are collected periodically (every day or twice a day) and added to the previous samples in a container placed near the tree. In this context, after an initial stabilization of the microbial population and chemical parameters (pH, residual sugar, ethanol, and other by-products), a constant condition develops. The main fermentation properties of the palm wine samples collected during the tapping confirm the progressive stabilization of the palm wine. After a first sample that shows a neutral pH, very high volatile acidity, and absence of ethanol, there is a progressive reduction in pH, which is probably due to the metabolism by LAB. At the same time, the ethanol concentration stabilizes at 3.5% to 4.5% vol, while the volatile acidity remains within acceptable values (Stringini et al. 2009). The microorganisms can convert the sucrose in the palm sap to glucose and fructose by invertase activity and finally to organic acids and alcohols (Naknean et al. 2010). It is generally known that the primary sources of invertase are the yeast, such as *S. cerevisiae*, *Saccharomyces carlsbergensis*, and the filamentous fungi, such as *Aspergillus oryzae* and *Aspergillus niger* (Takano 2005). Moreover, an increase in the total acidity and a decrease in the pH are also responsible for the inversion reaction (Naknean et al. 2010).

Understanding the biological processes of palm wine fermentation is essential for the improvement of palm wine production and the implementation of traditional technologies. As a consequence, assessing the diversity of the local yeast responsible for the alcoholic fermentation is a prerequisite. All taxonomic studies to date on palm wine yeasts have been performed using conventional yeast identification methods, which include the morphology, sporulation, and physiological activities of the yeasts. These classical methods based on plate counts and isolation and biochemical identification have been criticized, however, as only easily cultivatable microorganisms can be detected and members of microbial communities that need elective enrichment cannot be identified. Using such direct isolation procedures, *S. ludwigii* has been seen as the predominant species isolated from the first tapping, followed by *S. cerevisiae* and *Zygosaccharomyces bailii*. In the later sap, *S. cerevisiae* dominated this particular ecological niche, while *S. ludwigii* was only occasionally detected in the samples. This low yeast diversity is not surprising, since this particular environment is highly selective regarding pH, ethanol content, and anaerobic conditions, thus favoring the fermenting yeasts, and particularly *S. cerevisiae*, the dominant species seen here, as also seen in previous studies of tapped palm wine yeast flora (Olasupo and Obayori 2003; Amoa-Awua et al. 2006; Nwachukwu et al. 2006). Thus, investigations into the yeast populations using traditional microbiological methods have shown reduced species diversity, although in addition to *S. cerevisiae*, *Z. bailii*, and *S. ludwigii* have already been detected by these culture-based methods.

Recent developments in molecular techniques (PCR-based methods) have provided opportunities for yeast strain identification and has allowed substantial reductions in the time required, when compared with other molecular techniques (Esteve-Zarzoso et al. 1999). Similarly, culture-independent methods based on molecular biology techniques (e.g., denaturing gradient gel electrophoresis [DGGE] analysis) have been developed to study microbial population dynamics. Today, such culture-independent methods are particularly attractive, as they provide a useful and rapid strategy for yeast detection, and they represent a valid alternative to classical microbiological analyses. The results of these culture-independent methods such as PCR-DGGE analysis have shown broad biodiversity, as compared to results obtained using the culture-based methods. In addition, culture-independent analysis offers the possibility of detecting species that may be present in the habitat at viable, but nonculturable, levels (Head et al. 1997; Rappe and Giovannini 2003; Ercolini 2004). Indeed, DGGE analysis of yeast populations during tapping and fermentation of palm wine have shown the presence of *Hanseniaspora uvarum*, *Candida parapsilopsis*, *Candida fermentati*, *Pichia fermentans*. However, on the third day of palm wine fermentation, again *S. cerevisiae* were identified as the only species that remained in the substrate (Stringini et al. 2009). The yeast species found during the tapping of the palm sap are summarized in Table 37.1. These data obtained from this particular ecological niche appear to be in agreement with data from several other studies that have been carried out on the microbial ecology of wine fermentation (Egli et al. 1998; Beltrán et al. 2002; Santamaría et al. 2005; Blanco et al. 2006; Agnolucci et al. 2007), where the *S. cerevisiae* population can be composed of multiple strains, even in inoculated fermentations. However, only a few genetic studies have so far focused on yeast strains isolated from palm wine, which have revealed a degree of diversity with respect to genetic and physiological properties (Owama and Saunders 1990; Ezeronye and Okerentugba 2000, 2002, 2004). In contrast to the progressive selection and reduction of yeast species during the tapping process that favored *S. cerevisiae*, molecular characterization at the strain level has shown wide intraspecific biodiversity. Indeed, different biotypes were found in the samples collected across all of the fermentation phases of tapped palm wine, even after stabilization of the chemical conditions and the yeast species (Stringini et al. 2009).

To characterize the isolates at the strain level of a predominant yeast species such as *S. cerevisiae*, primers specific for delta sequences and minisatellites of genes encoding the cell wall have been used. Thus, intraspecific molecular characterization of the *S. cerevisiae* strains isolated from sap collected during the tapping of palm wine has revealed a broad spectrum of pattern profiles (Table 37.2), indicating that a multistarter fermentation takes place in this particular natural, semicontinuous fermentation process, confirming the results of a wide number of studies carried out during the past 20 years that

TABLE 37.1

Yeasts Species Found during the Tapping of the Palm Sap

	Methodology of Study	
Species	**Culture Dependent**	**Culture Independent (DGGE)**
Hanseniaspora uvarum (*Kloeckera apiculata*)	+	+
Hanseniaspora occidentalis	+	−
Candida parapsilosis	−	+
Candida fermentati	−	+
Candida krusei	+	−
Pichia fermentans	−	+
Pichia pastoris	+	−
Saccharomycodes ludwigii	+	+
Zygosaccharomyces bailii	+	+
Saccharomyces cerevisiae (*S. chevalieri*, *S. exigus*)	+	+
Schizosaccharomyces pombe	+	−
Kodamea ohmeri	+	−

Note: Data from different investigations.

TABLE 37.2

Frequency of Different Biotypes of *Saccharomyces cerevisiae* Collected during the Tapping of the Palm Sap

Tapping Time (days)	No. of Isolates	No. of Biotypes	Frequency (%)
0	2	2	100
1	10	6	60
2	10	5	50
3	10	3	30
5	8	5	62
5	11	5	45

Source: Stringini, M. et al., *Food Microbiol*, 26, 415–20, 2009, With permission.
Note: Total isolates: 51; total different biotypes: 15.

have also revealed the broad diversity of *S. cerevisiae* strains isolated during grape must fermentation (Versavaud et al. 1995; Esteve-Zarzoso et al. 2000; Sabate et al. 2002; Morrissey et al. 2004).

The use of yeast from palm wine has been evaluated in other different fermentation processes. Indeed, palm wine might also be an excellent alternative source of yeast (which can be easily isolated from this palm sap), in particular, in the evaluation of *S. cerevisiae* from pineapple fruits that have been used for wine production (Ayogu 1999; Nwachukwu 2006). In another investigation, high-ethanol-resistant yeast classified as *Saccharomyces* sp. that were isolated from Nigerian palm wine were used to brew a lager beer. The yeast isolates that survived the highest ethanol production were used to ferment the brewery wort and thus to produce 8.2% to 8.5% (v/v) ethanol; values that are almost double those of the control yeast from a local brewery (Agu et al. 1993, 1999). Some of the palm-wine-yeast isolates obtained from various localities in four provinces in southern Cameroon showed tolerance to both high sucrose and high ethanol concentrations, criteria that are indeed useful in fermentation.

37.3 Other Products from Palm Trees

Palm oil, coconut oil, and palm kernel oil are edible plant oils from the fruits of palm trees. Palm oil is extracted from the pulp of the fruit of the oil palm *Elaeis guineensis*, while palm kernel oil is derived from the kernel of the oil palm, and coconut oil is derived from the kernel of the coconut (*Cocos nucifera*). Palm oil contains a high amount of beta-carotene, which thus makes it naturally reddish in color. From a physicochemical point of view, palm oil, palm kernel oil, and coconut oil are three of the few highly saturated vegetable fats. In particular, palm oil is semisolid at room temperatures. Palm oil contains several saturated and unsaturated fats, in the forms of glyceryl laurate (0.1%, saturated), myristate (1%, saturated), palmitate (44%, saturated), stearate (5%, saturated), oleate (39%, monounsaturated), linoleate (10%, polyunsaturated), and linolenate (0.3%, polyunsaturated). Palm kernel oil and coconut oil are more highly saturated than palm oil, while like all vegetable oils, palm oil does not contain cholesterol, although saturated fat intake increases both LDL and HDL cholesterol. Palm oil is a common cooking ingredient in the tropical belt of Africa, Southeast Asia, and parts of Brazil. Nowadays, it is increasingly used in the commercial food industry in other parts of the world, which is also buoyed by its lower cost and the high oxidative stability (saturation) of the refined product when used for frying.

REFERENCES

Agnolucci M, Scarano S, Santoro S, Sassano C, Toffanin A, Nuti M. 2007. Genetic and phenotypic diversity of autochthonous *Saccharomyces* spp. strains associated to natural fermentation of 'Malvasia delle Lipari'. *Lett Appl Microbiol* 45:657–62.

Agu RS, Okenchi MC, Onyia AI, Onwumelu AH, Ajiwe VIE. 1999. Fermentation kinetic studies of Nigerian palm wine *Elaesis guineensis* and *Raphia hookeri* for preservation by bottling. *J Food Sci Technol* 205–9.

Agu RS, Onwumelu AH. 1993. Use of high-ethanol-resistant yeast isolates from Nigerian palm wine in lager beer brewing. *World J Microbiol Biotechnol* 6:660–1.

Ahmed ISA, Al-Gharibi KN, Daar AS, Kabir S. 1995. The composition and properties of date proteins. *Food Chem* 53:441–6.

Aidoo KE, Nout MJ, Sarkar PK. 2006. Occurrence and function of yeasts in Asian indigenous fermented foods. *FEMS Yeast Res* 6:30–9.

Amoa-Awua WK, Sampson E, Tano-Debrah K. 2006. Growth of yeasts, lactic and acetic bacteria in palm wine during tapping and fermentation from felled oil palm (*Elaeis guineensis*) in Ghana. *J Appl Microbiol* 47:1–8.

Atacador-Ramos M. 1996. Indigenous fermented foods in which ethanol in a major product. *Handbook of Indigenous Fermented Food*, 2nd ed. p. 363–508.

Ayogu TE. 1999. Evaluation of the performance of a yeast isolate from Nigerian palm wine in wine production from pineapple fruits. *Biores Technol* 69:189–90.

Barreveld WH. 1993. Date Palm Products. FAO Agricultural Services, Rome, bulletin No. 101.

Batra LR, Millner PD. 1974. Some Asian fermented foods and beverages and associated fungi. *Mycol* 66:942–50.

Beltran G, Torija M, Novo M, Ferrer N, Poblet M, Guillamon JM, Rozes N, Mas A. 2002. Analysis of yeast populations during alcoholic fermentation: A six years follow-up study. *Syst Appl Microbiol* 25:287–93.

Blanco P, Ramilo A, Cerdeira M, Orriols I. 2006. Genetic diversity of wine *Saccharomyces cerevisiae* strains in an experimental winery from Galicia (NW Spain). *Antonie van Leeuwenhoek* 89:351–7.

Boekhout T, Robert V. 2003. *Yeasts in Food: Beneficial and Detrimental Aspects*. Hamburg: Behr's Verlag.

Egli CM, Edinger WD, Mitrakul CM, Henick-Kling T. 1998. Dynamics of indigenous and inoculated yeast populations and their effect on the sensory character of Riesling and Chardonnay wines. *J Appl Microbiol* 85:779–89.

Ehrmann MA, Freiding S, Vogel RF. 2009. *Leuconostoc palmae* sp. nov., a novel lactic acid bacterium isolated from palm wine. *Int J Syst Evol Microbiol* 59:943–7.

Ejofor AO, Okafor N, Ugwueze EN. 1994. Development of baking yeast from Nigerian palm wine yeast. *World J Microbiol Biotechnol* 10:199–202.

Ercolini D. 2004. PCR-DGGE fingerprinting: novel strategies for detection of microbes in food. *J Microbiol Meth* 56:297–314.

Esteve-Zarzoso B, Belloch C, Uruburu F, Querol A. 1999. Identification of yeasts by RFLP analysis of the 5.8S rRNA gene and the two ribosomal internal transcribed spacers. *Int J Syst Bacteriol* 49:329–37.

Esteve-Zarzoso B, Gostincar A, Bobet R, Uruburu F, Querol A. 2000. Selection and molecular characterization of wine yeast isolated from the El Penedes area (Spain). *Food Microbiol* 17:532–53.

Ezeronye OU, Okerentugba PO. 2000. Genetic and physiological variants of yeast selected from palm wine. *Mycopathol* 152:85–9.

Ezeronye OU, Okerentugba PO. 2002. Flocculation and mating behaviour in *Saccharomyces* yeast from palm wine. *Niger J Exp Appl Biol* 3:83–8.

Ezeronye OU, Okerentugba PO. 2004. Nutrient utilization profile of *Saccharomyces cerevisiae* from palm wine in tropical fruit fermentation. *Antonie Van Leeuwenhoek* 86:235–40.

Faparusi SI. 1971. Microflora of fermenting palm sap. *J Food Sci Technol* 8:206–10.

Fleet GH. 2003. Yeasts in fruit and fruit products. In: Robert V, editor. *Yeasts in Food. Beneficial and Detrimental Aspects*. Hamburg: Behr's Verlag GmbH and Co. p. 267–87.

Head IM, Saunders JR, Pickup RW. 1997. Microbial evolution, diversity, and ecology; a decade of ribosomal RNA analysis of uncultivated microorganism. *Microbiol Ecol* 35:1–21.

Hui YH, Meunier-Goddik L, Hansen AS, Josephsen J, Nip WK, Stanfield PS, Toldra F. 2004. *Handbook of Food and Beverage Fermentation Technology*. New York: Marcel Dekker.

Joshi VK, Sandhu DK, Thakur NS. 1999. Fruit-based alcoholic beverages. In: *Biotechnology: Food Fermentation.* Ernakulam, India: Educational Publishers. p. 647–744.

Mavioga EM, Mullot JU, Frederic C, Huart B, Burnat P. 2009. Sweet little gobonese palm wine: A neglected alcohol. *West African J Med* 28:291–4.

Mbugua SK. 1985. Isolation and characterization of lactic acid bacteria during the traditional fermentation of uji. *East African Agric For J* 50:36–43.

Morah FN. 1995. Effect of metabisulphite on alcohol production in palm wine. *Food Chem* 53:153–6.
Morrissey WF, Davenport B, Querol A, Dobson ADW. 2004. The role of indigenous yeast in traditional Irish cider fermentations. *J Appl Microbiol* 97:647–55.
Mozingo HN. 1989. Palm. In: Holland DT, editor. *The Encyclopedia Americana*. Danbury Connecticut: Grolier Inc. p. 319–321.
Naknean P, Meenune M, Roudaut G. 2010. Characterization of palm sap harvested in Songkhla province, Southern Thailand. *Int Food Res J* 17:59–67.
Nwachukwu IN, Ibekwe VI, Nwabueze RN, Anyanwu, BN. 2006. Characterisation of palm wine yeast isolates for industrial utilisation. *Afr J Biotechnol* 5:1725–8.
Odunfa SA, Oyewole OB. 1998. African fermented foods. *Microbiol Fermen Foods* 2:713–52.
Ogbulie TE, Ogbulie JN, Njoku HO. 2007. Comparative study on the microbiology and shelf life stability of palm wine from *Elaeis guineensis* and *Raphia hookeri* obtained from Okigwe, Nigeria. *Afr J Biotechnol* 6:914–22.
Ojimelukwe PC. 2001. Effects of preservation with *Sacoglottis gabonensis* on the biochemistry and sensory attribute of fermenting palm wine. *J Food Biochem* 25:411–24.
Okafor N. 1972. Palm wine yeasts from parts of Nigeria. *J Sci Food Agric* 23:1399–407.
Okafor N. 1975. Microbiology of Nigerian palm wine with particular reference to bacteria. *J Appl Bacteriol* 38:81–8.
Olasupo NA, Obayori OS. 2003. Utilization of palm wine (*Elaeis guineensis*) for the improved production of Nigerian indigenous alcoholic drink, ogogoro. *J Food Proc Pres* 27:365–72.
Owama C, Saunders JR. 1990. Physiological variants of *Saccharomyces cerevisiae* and *Kloeckera apiculata* from palm wine and cashew juice. *J Appl Microb* 68:491–4.
Rappe MS, Giovannini SJ. 2003. The uncultured microbial majority. *Ann Rev Microbiol* 57:369–94.
Sabate J, Cano J, Esteve-Zarzoso B, Guillamòn JM. 2002. Isolation and identification of yeasts associated with vineyard and winery by RFLP analysis of ribosomal genes and mitochondrial DNA. *Microbiol Res* 157:267–74.
Sanni AI, Lonner C. 1993. Identification of yeasts isolated from Nigerian traditional alcoholic beverages. *Food Microbiol* 10:517–23.
Santamaría P, Garijo P, López R, Tenorio C, Gutierrez R. 2005. Analysis of yeast populations during spontaneous alcoholic fermentation: effect of the age of the cellar and the practice of inoculation. *Int J Food Microbiol* 103:49–56.
Shamala TR, Sreekantiah KR. 1988. Microbiological and biochemical studies on traditional Indian palm wine fermentation. *Food Microbiol* 5:157–62.
Stringini M, Comitini F, Taccari M, Ciani M. 2008. Yeast diversity in crop-growing environments in Cameroon. *Int J Food Microbiol* 127:184–9.
Stringini M, Comitini F, Taccari M, Ciani M. 2009. Yeast diversity during tapping and fermentation of palm wine from Cameroon. *Food Microbiol* 26:415–20.
Takano H. 2005. Investigation of chemical and physical properties of southwestern Wisconsin maple syrup. [MSc thesis]. USA: University of Wisconsin-Stout.
Thabet BI, Attia H, Besbes S, Deroanne C, Francis F, Drira NE, Blecker C. 2007. Physicochemical and functional properties of typical Tunisian drink: date palm sap (*Phoenix dactylifera* L.). *Food Biophys* 2:76–82.
Thabet BI, Besbes S, Attia H, Deroanne C, Francis F, Drira NE, Blecker C. 2009. Physicochemical characteristics of date sap "*lagmi*" from Deglet Nour palm (*Phoenix dactylifera* L.). *Int J Food Prop* 12:659–70.
Theivendirarajah K, Chrystopher RK. 1987. Microflora and microbial activity in palmyrah (*Borassus flabellifer*) palm wine in SriLanka. *MIRCEN J* 3:23–31.
Versavaud A, Courcoux P, Roulland C, Dulau L, Hallet JN. 1995. Genetic diversity and geographical distribution of wild *Saccharomyces cerevisiae* strain from the wine-producing area of Charentes, France. *Appl Environ Microbiol* 61:3521–9.
Wood BJB. 1998. *Microbiology of Fermented Foods*. 2nd ed. London, UK: Blackie Academic and Professional.

38

Brazilian Cachaça: Fermentation and Production

Fernanda Badotti, Fátima C. O. Gomes, and Carlos A. Rosa

CONTENTS

38.1 Cachaça History and Technology

The Portuguese settlers originally brought sugar cane from South Asia to Brazil. The climate of Brazil allowed for the adaptation and cultivation of this crop, which became the raw material in sugar production and contributed to the economic and social development at that time. Between 1532 and 1548, wine derived from sugar cane, known as acid sugar cane juice (*garapa azeda*), was produced using wooden vats. The fermented wine was distilled in clay stills to form a transparent liquid named *aguardente*, which is derived from the Greek phrase *acqua ardens*, meaning *the water that burns*. The resulting beverage was alternatively named cachaça, from the Castilian word *cachanza*, the name given to wines made from grape residue (*borra*). In 1756, the taxation of the beverage generated significant revenue for the reconstruction of Lisbon, which was devastated by a strong earthquake in 1755. After improvements in the production process, the Portuguese began to drink cachaça, which was previously only consumed by slaves. In 1819, cachaça was considered a typical Brazilian beverage, and since then, its production has become independent from the production of sugar (Associação Mineira dos Produtores de Cachaça de Qualidade [AMPAQ] 2010).

Until the end of the Second World War, traditional cachaça production remained predominant in Brazil. From 1945 to 1960, the first large-scale cachaça production companies began to appear, and cachaça production became increasingly industrialized. Currently, industrial distilleries are responsible for the production of about 1 billion of liters of cachaça per year, and the cachaça is distilled in stainless steel columns (Figure 38.1). These columns contain several plates with bubble caps and siphon tubes or other contact devices that function as a series of single-distillation apparatuses (Rosa et al. 2009). Traditional cachaça, however, is made through the distillation of sugar cane wine in copper alembics by rural producers, who produce an additional 300 million liters of cachaça per year (Rosa et al. 2009; Marini et al. 2009). The majority of traditional producers in Brazil use copper alembics for cachaça distillation (Figure 38.1). The copper found in the alembic still walls reacts with sulfides, such as demithyl sulfide, during the distillation process, lowering their concentrations. These reactions prevent taste defects that are typically associated with cachaças distilled in stainless steel columns (Cole and Noble 1995; Faria et al. 1993; Marini et al. 2009). Cachaça production occurs during the sugar cane harvest, from May to December, in most producing regions.

FIGURE 38.1 (a) Copper alembics for distillation of traditional cachaça, (b) *umburana* barrels to store cachaça, (c) stainless steel columns for industrial cachaça production, and (d) oak barrels for cachaça aging.

Cachaça is the most popular distilled beverage in Brazil and the third most popular distilled beverage worldwide. There are about 30,000 producers, most of them producing the beverage on a small scale, involved in the annual production of around 1.3 billion liters. The Brazilian government has recognized that there is a demand for the export of cachaça, and there are perspectives indicating a significant increase in sales in the coming years (AMPAQ 2010). There is some controversy about whether the traditional cachaça, which is distilled in alembics, or the industrial cachaça, which is distilled in columns, is of higher quality in terms of their sensory attributes (Marine et al. 2009). Comparisons of quality between the two distillation procedures are difficult to make because of the lack of standard fermentation and distillation procedures among producers (Marine et al. 2009; Rosa et al. 2009). Marine et al. (2009) demonstrated that cachaças distilled in either alembics or in stainless steel columns, using the same yeast strains in the fermentation process, did not show significant differences in their overall sensory attributes and were graded highly in sensory affective tests.

In the production of cachaça, the distillation in alembics is carried out in batches or discontinuously, where each load of wine undergoes distillation separately. This discontinuous process allows the various compounds in the wine to separate based on volatility. The distillation process has the aim to separate and concentrate the volatile compounds present in wine from the residual fraction called *vinhoto*, which is discarded. Compounds with greater volatility, such as methanol and acetaldehyde, are found primarily in the fraction obtained from the initial boiling, called *cabeça* (head), whereas the less volatile compounds, such as the higher alcohols, are found mainly in the *coração* (heart) and *cauda* (tail) fractions. The most important part of the distillate is the *coração* fraction, which is rich in ethanol and represents 80%–85% of total distillate. When the *cabeça* (head) and *cauda* (tail) fractions are not completely separated, the sensory quality of cachaça is degraded.

Cachaça is aged in wooden barrels, during which there is typically a loss of alcohol content. During this process, compounds in the distillate become oxidized, and the beverage becomes enriched with substances extracted from the wood. The colors and organoleptic characteristics are also enhanced during this time. Numerous chemical reactions are associated with the aging process. Among them are the reactions between secondary compounds from the distillation process, the direct extraction and incorporation of components from the wood into the beverage, and the reaction of the compounds from the wood with the original components of the distillate (Dias 2009). The aging periods of certain alcoholic beverages are established by legislation in some countries. The current legislation in Brazil requires cachaça to be aged for a minimum of one year (Brasil 2005). Aging reduces defects introduced during fermentation and distillation, improving the palatability of the beverage. The quality of the aging process

is dependent on the species of wood used, the size and pretreatment of the barrels, and the aging time. In Brazil, barrels used to age and store cachaça (Figure 38.1) are typically made from the following woods: *umburana* (*Amburana cearensis*), *bálsamo* (*Myroxylon peruiferum*), *jequitibá* (*Cariniana estrellensis*), European oak (*Quercus* spp.), *jatobá* (*Hymenaea* spp.), or *ipê* (*Tabebuia* spp.) (Mendes et al. 2009).

38.2 Microorganisms Associated with Fermentation

During the cachaça production process, a complex microbial community is responsible for the fermentation of sugar cane juice. The traditional fermentation process begins with yeasts that occur naturally in sugar cane juice (spontaneous fermentation), equipment, and water (Pataro et al. 2000; Campos et al. 2010). New populations of yeast and bacteria are added to the vats with the daily addition of water and sugar cane juice. As these microorganisms multiply, they modify the characteristics of the sugar cane must (Rosa et al. 2009). The natural microbial starter culture is usually prepared by a method known as *fermento caipira*. There is currently no standard method for the generation of these natural starter cultures (Pataro et al. 2000; Gomes et al. 2007; Silva et al. 2009); consequently, there are variations in the production, yield, and quality of cachaça in different regions. The starter culture corresponds to approximately 25% of the total volume of the vat. In general, the preparation of this start ferment includes the development of the fermentative microbiota in the sugar cane juice alone or mixing of sugar cane juice with crushed corn, rice, and other substrates. Distilleries producing industrial cachaça utilize yeasts normally used in commercial ethanol production or baking, and the fermentation is performed in 10,000-L vats. The fermentation vats used in the traditional process have a maximum production capacity of 1000 L (Figure 38.2) (Gomes et al. 2007; Rosa et al. 2009).

The different stress conditions encountered by yeasts during fermentation affect the overall survival of these microorganisms. These conditions create strong selective pressures on the strains present during fermentation (Guerra et al. 2001; Vianna et al. 2008; Rosa et al. 2009). Generally, the fermentation cycle for cachaça production involves a succession of yeast species, and eventually, *Saccharomyces cerevisiae* becomes the dominant species (Morais et al. 1997; Pataro et al. 2000; Schwan et al. 2001; Gomes et al. 2007). Throughout the season, a variety of *S. cerevisiae* strains appear, each with different genetic characteristics that influence the flavor of the cachaça. Depending on the predominant *S. cerevisiae* strain present in the vats during the season, the beverage quality can vary, even within the same distillery.

38.3 *Saccharomyces cerevisiae:* Molecular and Physiological Characterization of Indigenous Strains

There are many different species of yeast associated with sugar cane juice. *Saccharomyces cerevisiae* is responsible for the alcoholic fermentation of sugar cane must, and its population rises during the 24-hour fermentative cycle (Morais et al. 1997; Pataro et al. 2000). Most of the yeast species isolated during spontaneous fermentation are physiologically adapted to the environmental conditions observed

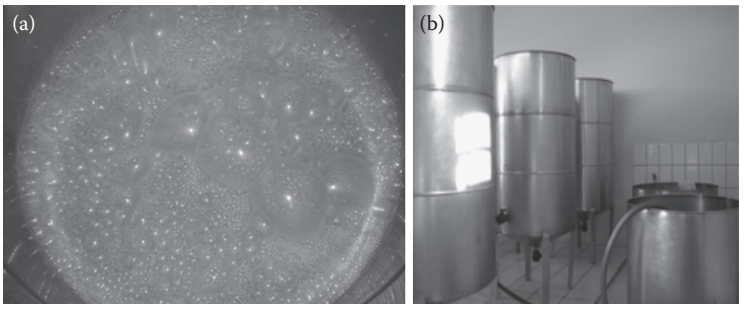

FIGURE 38.2 (a) Fermentation vat and (b) stainless steel vats used for traditional cachaça production.

in the fermentation vats. At the beginning of the process, yeast cells are affected by osmotic stress due to the high sucrose concentration in must, which can vary from 14% to 16%. Some of the unique features of cachaça fermentation, such as the short fermentative cycle with daily additions of sugar cane juice, high ethanol concentrations, and high fermentation temperatures, may be responsible for the selection of highly adapted yeast strains (Guerra et al. 2001; Pataro et al. 2002). Badotti et al. (2010) demonstrated that strains selected from the same cachaça-producing regions have significant variability in their fermentation characteristics, including strains that can grow well and strains that have difficulty growing under similar stress conditions. This indicates that the environmental conditions in the fermentation vat influence the selection of the yeast strains, and variations in the fermentation conditions result in a heterogeneous yeast population that carries out the majority of the fermentation. This heterogeneity leads to differences in the production of cachaça throughout the season as well as varying ethanol yields.

To determine the role of microorganisms during the different stages of fermentation, it is important to identify the yeasts involved because the quality of the beverage is strongly related to the microbial ecology during the processes of fermentation and maturation (Schwan et al. 2001). Frequently, morphological and physiological characteristics are used for identification. These methods are limited because phenotypic characterization is not sufficient for an unambiguous identification of yeast species. As a result, molecular techniques have been developed for the identification and characterization of yeasts. These molecular techniques can identify yeasts independently of gene expression (Sanz et al. 2005), and they allow for the accurate and rapid identification of yeast species or strains (Le Jeune et al. 2006).

There have been many studies on the genetics of *S. cerevisiae* strains that are involved in cachaça fermentation. Multiple techniques, such as pulsed field gel electrophoresis (PFGE), have detected the presence of different *S. cerevisiae* strains during the annual cycle of cachaça production, even in different fermentation vats from the same distillery (Pataro et al. 2000; Guerra et al. 2001). Although PFGE is a valuable method for differentiating yeast strains, it is complex and time-consuming, making the analysis of large numbers of samples difficult. Mitochondrial DNA restriction analysis (mtDNA-RFLP) has been widely used to characterize *S. cerevisiae* strains involved in cachaça fermentation (Araújo et al. 2007; Gomes et al. 2007; Marini et al. 2009; Silva et al. 2009; Badotti et al. 2010). mtDNA-RFLP is simple, reliable, fast, cost-effective, and can differentiate yeast strains isolated from the same must. This technique can also monitor the persistence and prevalence of specific yeast strains throughout the fermentative process. *Saccharomyces cerevisiae* cachaça populations analyzed by mtDNA-RFLP have been found to be highly diverse, differing from region to region and even among strains in vats at the same distillery (Badotti et al. 2010).

38.4 Use of Selected Starter Strains of *Saccharomyces cerevisiae*

In addition to spontaneous fermentation, or the use of baker and ethanol yeasts for cachaça production, selected *S. cerevisiae* starter strains obtained from the cachaça fermentation environment can be used. Starter cultures have been used for the production of distilled and fermented beverages (Cedeño 1995; Gutiérrez et al. 1997; Fahrasmane and Parfait 1998; Romano et al. 2003; Silva et al. 2006; Valero et al. 2007). The addition of yeast strains isolated from cachaça-fermenting environments to speed up the fermentative process may increase the levels of the desired metabolites while preventing the buildup of deleterious compounds produced by microbial contaminants (Gomes et al. 2007; Silva et al. 2009).

The use of selected strains for the fermentation of cachaça has several advantages over spontaneous fermentation. Desirable characteristics include rapid and efficient fermentation, stress tolerance, complete substrate fermentation, high sugar uptake and consumption, low acetic acid production, the production of desirable flavor compounds, efficient ethanol production, and lower variations in the flavor of the beverage (Oliveira et al. 2004 2005; Gomes et al. 2007).

The simple inoculation of a selected strain, or a dominant strain isolated from a certain distillery, is not enough to guarantee its predominance or an improvement in the efficiency of distilleries (Basso et al. 1993; Andrietta et al. 1997). It is important to note that dominant yeasts are not inherently dominant, but become dominant under certain conditions. It is widely accepted that yeast strains can be fully adapted to specific climates and can metabolize specific substrates.

Fermentation parameters such as productivity, ethanol yield, and the maximum specific velocity of cell growth are considered to be essential for good performance. In Brazil, these parameters, as well as some additional considerations, are frequently used to characterize yeasts for industrial alcohol production (Andrietta et al. 1999). There are a few selected starter strains of *S. cerevisiae* used for cachaça production in Brazil. These strains were selected based on their fermentative parameters and tests in distillery scale. Some of these strains are flocculent, which is advantageous for rural producers that do not use centrifugation to recover the starting fermentation culture. Other strains do not produce H_2S, a gas associated with undesirable flavors in various beverages (Silva et al. 2009).

38.5 Non-*Saccharomyces* Yeasts

Species of non-*Saccharomyces* yeasts, although present in lower numbers than *S. cerevisiae*, also contribute to the sensory composition of cachaça. Some species of non-*Saccharomyces* yeasts are present in the vats during the entire cachaça production period. These yeasts number between 10^3 and 10^6 CFU/mL, whereas strains of *S. cerevisiae* are generally present at 10^5 to 10^8 CFU/mL (Gomes et al. 2007; Marini et al. 2009). Non-*Saccharomyces* yeasts, such as *Schizosaccharomyces pombe*, may occasionally become the dominant yeast strain during fermentation (Gomes et al. 2002; Bernardi et al. 2008). Nevertheless, there is little information on the effect of non-*Saccharomyces* yeasts on the quality and aroma of cachaça (Dato et al. 2005).

Yeasts of the genera *Kloeckera*, *Candida*, *Debaryomyces*, *Kluyveromyces*, *Lachancea*, *Pichia*, *Torulaspora*, *Zygosaccharomyces*, and *Wickerhamomyces* are commonly isolated during cachaça fermentation (Morais et al. 1997; Pataro et al. 2000; Guerra et al. 2001; Schwan et al. 2001; Gomes et al. 2007; Marini et al. 2009; Vila Nova et al. 2009). These yeast species can be introduced daily with the addition of sugarcane juice, but they typically do not prevail due to the high alcohol concentrations generated by the end of the fermentation process. The growth of these yeasts during fermentation is influenced by factors such as the nutrient content of the must, temperature, pH, and overall cachaça production practices.

The flavor of alcoholic beverages is due to the presence of numerous volatile and nonvolatile compounds. Non-*Saccharomyces* yeasts can significantly contribute to the aromatic and chemical composition of many fermented beverages (Soden et al. 2000; Oliveira et al. 2005). These yeasts may affect fermentation directly, by producing unique flavors, and indirectly, by modulating the growth and metabolism of the dominant *S. cerevisiae* populations (Mills et al. 2002).

These contaminating yeasts are known to be resistant to adverse conditions, such as a high temperatures, substrate, and nutrient concentrations, and fermentation time. It is not yet known if the utilization of non-*Saccharomyces* yeast strains may improve the quality of cachaça, resulting in a beverage with a more robust content of secondary compounds.

38.6 Bacteria

Lactic acid bacteria deteriorate harvested cane sugar, reducing the overall sugar content, and they are also considered to be a contaminant during cachaça fermentation. These bacteria compete with yeasts for the sucrose in the sugar cane juice, which reduces the ethanol yield from fermentation (Narendranath and Brey 2009; Rosa et al. 2009; Campos et al. 2010; Gomes et al. 2010). During the production of traditional and industrial cachaça, contamination of the fermented must is a factor that leads to economic losses in the beverage manufacturing industry (Rosa et al. 2009; Gomes et al. 2010). High numbers of contaminating bacteria can damage the quality of the beverage by increasing the acidity of the sugar cane juice.

Lactic acid bacteria are able to grow under ethanol production conditions. Some species of Lactobacilli are able to grow in the presence of 15% (v/v) or more ethanol in substrates. Isolates of lactic acid bacteria from distilleries are well adapted to grow in fermentation conditions. Their growth is characterized by the presence of end products such as lactic acid and acetic acid, which inhibit yeast growth and metabolism (Narendranath and Brey 2009).

Schwan et al. (2001) demonstrated that bacterial growth was almost entirely suppressed during the production of cachaça in a traditional distillery and that the ratio of yeast to bacteria was never less than 80:1, even reaching 1052:1 after 4 hours of fermentation. These authors also showed that lactic acid bacteria were the most commonly isolated bacterial group, although they did not propagate during fermentation. The lactic acid bacteria may increase the level of volatile acids or produce compounds that negatively influence the taste and aroma of the beverage (Gomes et al. 2010). The presence of 1×10^7 CFU/mL of *Lactobacillus* in the initial must results in an approximately 1% (v/v) reduction of ethanol in the final product, depending on the strain of contaminant bacteria (Narendranath et al. 1997).

There are differences among the predominant species of bacteria in fermentation vats from different cachaça-producing regions, which may be due to the identification methods used. Carvalho-Netto et al. (2008) characterized the bacterial community in cachaça production by partial sequencing of the 16S rRNA gene, a cultivation-independent technique. The genus *Lactobacillus* accounted for approximately 66% of the sequences. *L. hilgardii* and *L. plantarum* were other species that were frequently identified. *Curtobacterium flaccumfaciens*, *L. casei*, *Leuconostoc mesenteroides*, and *L. citreum* sequences were found at low numbers. Similarly, a clone library produced from bacteria present in a cachaça fermentation vat also revealed a predominance of *Lactobacillus*. After 7 days of fermentation, the most prevalent species was *L. nagelli*, representing 76.9% of the total clones analyzed. The other clones were *Lactobacillus* sp. and *Lactobacillus paracasei* subsp. *paracasei*. After 21 days, *L. harbinensis* became the dominant species (75%). *L. paracasei* subsp. *paracasei*, *L. nagelli*, and *L. satsumensis* were also found at low numbers. After 35 days of fermentation, *L. buchneri* was represented in 57.9% of the clones analyzed, followed by *L. harbinensis* (21%) (Lacerda et al. 2011).

Gomes et al. (2010) identified the populations of cultivable lactic acid bacteria involved in the fermentation of cachaça using physiological and molecular methods. One hundred sixty-nine isolates of lactic acid bacteria were identified. The counts of lactic acid bacteria were high during the fermentation process, where *L. casei* and *L. plantarum* were the most abundant species. Other lactic acid bacteria were found with low frequency, such as *L. ferintoshensis*, *L. fermentum*, *L. jensenii*, *L. murinus*, *Lactococcus lactis*, *Enterococcus* sp., and *Weissella confusa*.

Although lactic acid bacteria are considered contaminants in the fermentation of cachaça, a precise correlation between bacterial contamination and degradation of the fermentation process has not been demonstrated (Gallo 1992; Rosa et al. 2009; Gomes et al. 2010). The diversity of bacterial populations, as well as the role of these microorganisms during the cachaça production process, is still poorly understood.

38.7 Legislation and Secondary Compounds

According to the current legislation, cachaça refers exclusively to a beverage produced in Brazil at 20°C by the distillation of fermented sugar cane juice, with an alcohol content of 38% to 48% (v/v) and typical sensory characteristics (Brasil 2005). Fermentation of sugar cane juice produces ethanol and many other secondary components, including aldehydes, higher alcohols, acids, and esters. The flavor of alcoholic beverages is the result of numerous volatile and nonvolatile compounds that impart tastes and odors on the beverage. These compounds can be divided into various groups according to their chemical nature. Differences in flavor can be attributed to the yeast strains present during the fermentation process. The fermentation conditions and the type of inoculum may interfere with the formation of aromatic compounds and may have an influence on the production of other substances, including phenolic compounds and sulfur.

The Brazilian legislation has established that the sum of aldehydes, volatile acids, esters, furfural, and higher alcohols should be between 200 and 650 mg/100 mL of anhydrous alcohol in cachaça (Brasil 2005). A balance among volatile compounds is essential for the production of high-quality cachaça (Dato et al. 2005).

Distillation and aging are also important factors in the production of high-quality cachaça. Chemical reactions occur due to the high temperatures encountered during the distillation process, and chemical compounds may be formed or concentrated. Some compounds will become reduced, and new substances

can be generated during the distillation step (Boza and Horii 1998). During aging, the oxidation and esterification of compounds in the cachaça result in an improvement of the sensory experience associated with the beverage. Compounds derived from wood, such as volatile oils, tannic elements, sugars, glycerol, nonvolatile organic acids, and steroids, contribute to the changes in the flavor and color of cachaça (Cardello and Faria 1998).

Higher alcohols are derived from the metabolism of amino acids through decarboxylation and deamination reactions. The amino acids in the sugar cane originate from the hydrolysis of proteins from sugar cane cells and can be incorporated into the wine through must enrichment with rice flour or other organic material rich in protein. Additionally, yeasts are capable of reducing aldehydes to alcohols during fermentation. The primary higher alcohols produced by yeasts include aliphatic alcohols such as, *n*-propanol, isobutanol (2-methyl-1-propanol), amyl alcohol (2-methyl-1-butanol), isoamyl alcohol (3-methyl-1-butanol), hexanol, and aromatic alcohols such as 2-phenylethanol (Nykanen and Nykanen 1991). The Brazilian legislation limits the amount of each higher alcohol individually; however, it states that a maximum of 360 mg of higher alcohols/100 mL of anhydrous ethanol can be produced.

Esters are formed in reactions between acids and alcohols produced during fermentation and also by metabolic activity of microorganisms. Ethyl acetate is an ester primarily found in cachaça (Berry 1995). Esters are primarily responsible for the flavor of distilled beverages (Nykanen and Nykanen 1991). Ethyl, isobutyl, and isoamyl esters and short-chain fatty acid esters are primarily responsible for the aroma of cachaça, which possesses pleasant, fruity aromas (Suomalainen and Lehtonen 1979; Lehtonen and Jounela-Erikcsson 1983). Despite these characteristics, a single ester is rarely entirely responsible for a specific aroma (Nykanen et al. 1968). The aromas and tastes that cachaça acquires during aging are the result of the formation of esters, a process that occurs slowly and continuously (Pinheiro 1999). Brazilian legislation limits the total ester content of cachaça to 200 mg of total esters (ethyl acetate)/100 mL of anhydrous alcohol.

Aldehydes are also important in the overall flavor of alcoholic beverages, but high concentrations may be toxic to humans. Aldehydes are considered to be responsible for the negative affects associated with excessive drinking (Nascimento 1997). Like other short-chain aliphatic aldehydes, the pungent odor of aldehydes can increase the penetrating flavor of a beverage (Nykanen 1986; Nykanen and Nykanen 1991). It is thought that acetaldehyde-rich cachaças originate in distilleries that do not separate the head fraction (*cabeça*) during the distillation process (Chaves and Povoa 1992). Furfural is also an aldehyde and is associated with harvest conditions (previous burning of foliage) and distillation (residue from sugar cane). The maximum amount of acetaldehyde permitted in cachaça is 30 mg/100 mL anhydrous alcohol, according to Brazilian legislation.

Acetic acid is the primary organic acid excreted into the growth medium. Despite the fact that the concentration of acetic acid varies greatly between different cachaças, it is often responsible for 60% to 95% of the total acidity of the beverage (Nykanen and Nykanen 1991). The volatile acidity in cachaça is expressed as acetic acid content (maximum 150 mg/100 mL anhydrous alcohol). Excessive acidity negatively affects the flavor of cachaça.

Glycerol is the main secondary compound produced during fermentation and about 4%–5% of the sugar metabolized by yeast is converted to glycerol (Alves and Basso 1998). This compound directly affects the viscosity of alcoholic beverages, a property that plays an important role in the sensory experience associated with a particular beverage. The Brazilian legislation does not currently regulate the amount of glycerol that can be present in cachaça.

Finally, organic and inorganic contaminants may be present in cachaça at concentrations determined by the Brazilian legislation (Brasil 2005). The following compounds may be present at the following concentrations: methyl alcohol (20 mg/100 mL anhydrous alcohol), acrolein (5 mg/100 mL anhydrous alcohol), *sec*-butyl alcohol (10 mg/100 mL anhydrous alcohol), ethyl carbamate (150 mg/L anhydrous alcohol), *n*-butyl alcohol (3 mg/100 mL anhydrous alcohol), copper (5 mg/L anhydrous alcohol), lead (200 μg/L anhydrous alcohol), and arsenic (100 μg/L anhydrous alcohol). To improve the quality and commercial value of cachaça, it is important to understand its chemical composition and the effect that this composition has on the flavor and overall sensory experience of the beverage.

38.8 Final Considerations

The quality of cachaça, in terms of its sensory attributes, is difficult to determine because most of the producers do not use standard fermentation, distillation, or aging procedures. Currently, there is a tendency to use selected yeast strains at a large scale to improve the fermentation yield and quality of cachaça, which benefits cachaça producers. It is critical to determine the role of the individual microorganisms involved in the fermentation process, especially the bacteria and non-*Saccharomyces* yeasts, because little is known about the involvement of these organisms. It is also important to take steps to establish a standard procedure for the selection of starter strains with desirable characteristics. It will also be important to improve distillation conditions in both alembics and stainless steel columns to generate cachaças with high amounts of secondary compounds.

REFERENCES

Alves D, Basso L, editors. 1998. The effects of some variables on fuel ethanol fermentation by *Saccharomyces cerevisiae*. Nineteenth International Specialized Symposium on Yeast; 1998; Portugal.

Andrietta SR, Andrietta MGS, Serra GE. 1997. Leveduras não fermentativas—Processo de instalação e forma de detecção e eliminação. *STAB Açúcar Álcool Subprodutos* 15:32–5.

Andrietta SR, Migliari PC, Andrietta MGS. 1999. Classificação das cepas de levedura de processos industriais de fermentação alcoólica utilizando capacidade fermentativa. *STAB Açúcar Álcool e Subprodutos* 17:54–9.

Araújo RAC, Gomes FCO, Moreira ESA, Cisalpino PS, Rosa CA. 2007. Monitoring *Saccharomyces cerevisiae* populations by mtDNA restriction analysis and other molecular typing methods during spontaneous fermentation for production of the artisanal cachaça. *Braz J Microbiol* 38:217–23.

Associação Mineira dos Produtores de Cachaça de Qualidade (AMPAQ) [Acessed 2010 Nov 01] Available from: (http://www.ampaq.com.br).

Badotti F, Belloch C, Rosa CA, Barrio E, Querol A. 2010. Physiological and molecular characterization of *Saccharomyces cerevisiae* cachaça strains isolated from different geographic regions in Brazil. *World J Microbiol Biotechnol* 26:579–87.

Basso LC, Oliveira AJ, Orelli VFSM, Campos AA, Gallo CR, Amorin HV, editors. 1993. Dominância das leveduras contaminantes sobre as linhagens industriais avaliadas pela técnica de cariotipagem. 5th Congresso Nacional da STAB: Proceedings of a national workshop held at Águas de São Pedro, São Paulo, Brazil.

Bernardi TL, Pereira GVM, Cardoso PG, Dias ES, Schwan RS. 2008. *Saccharomyces cerevisiae* strains associated with the production of cachaça: identification and characterization by traditional and molecular methods (PCR, PFGE and mtDNA-RFLP). *World J Microbiol Biotechnol* 43:2705–12.

Berry DR. 1995. Alcoholic beverage fermentations. In: Lea AGH, Piggott JR, editors. *Beverage Production*. 1st ed. London: Blackie Academic and Professional. p 32–44.

Boza Y, Horii J. 1998. Influência da destilação sobre a composição e a qualidade sensorial da aguardente de cana-de-açúcar. *Ciênc Tecnol Aliment* 18:1–14.

Brasil. 2005. Ministério da Agricultura, Pecuária e Abastecimento. Instrução Normativa n.13 de 29 de junho de 2005. Aprova o Regulamento Técnico para Fixação dos Padrões de Identidade e Qualidade para Aguardente de Cana e Cachaça.

Campos CR, Silva CF, Dias DR, Basso LC, Amorim HV, Schwan RF. 2010. Features of *Saccharomyces cerevisiae* as a culture starter for the production of the distilled sugar cane beverage, cachaça in Brazil. *J Appl Microbiol* 108:1871–79.

Cardello HMAB, Faria JB 1998. Análise descritiva da aguardente de cana durante o envelhecimento em tonel de carvalho (*Quercus Alba L.*). *Cienc Tecnol Aliment* 18:169–75.

Carvalho-Netto OV, Rosa DD, Camargo LEA. 2008. Identification of contaminat bacteria in cachaça yeast by 16S RDNA gene sequencing. *Sci Agric* 65:508–15.

Cedeño M. 1995. Tequila production. *Crit Rev Biotechnol* 15:1–11.

Chaves JBP, Povoa MEB. 1992. A qualidade da aguardente de cana-de-açúcar. In: Mutton MJR, Mutton MA, editors. *Aguardente de Cana: Produção e Qualidade*. Jaboticabal: FUNEP. p 93–132.

Cole VC, Noble AC 1995. Flavor chemistry and assessment. In: Lea AGH, Piggot JR, editors. *Fermented Beverage Production*. 2nd ed. Glasgow: Blackie Academic and Professional. p 361–81.

Dato MCF, Pizauro Júnior JM, Mutton MJR. 2005. Analysis of the secondary compounds produced by *Saccharomyces cerevisiae* and wild yeast strains during the production of "cachaça." *Braz J Microbiol* 36:70–4.

Dias SMBC. 2009. Fatores que influenciam no processo de envelhecimento da cachaça. *Inf Agrop* 30:22–40.

Fahrasmane L, Parfait G. 1998. Microbial flora of rum fermentation media. *J Appl Microbiol* 84:921–8.

Faria JB, Deliz R, Rossi EA. 1993. Compostos sulfurosos e a qualidade das aguardentes de cana (*Saccharum officinarum* L.). *Bol Soc Bras Ciênc Tecnol Alim* 13:89–93.

Gallo CR. 1992. Identificação de bactérias contaminantes da fermentação alcoólica. *STAB* 2:30–4.

Gomes FCO, Pataro C, Guerra JB, Neves MJ, Corrêa SR, Moreira ESA, Rosa CA. 2002. Physiological diversity and trehalose accumulation in *Schizosaccharomyces pombe* strains isolated from spontaneous fermentations during the production of the artisanal Brazilian *cachaça Can J Microbiol* 48:399–406.

Gomes FCO, Silva CLC, Marini MM, Oliveira ES, Rosa CA. 2007. Use of selected indigenous *Saccharomyces cerevisiae* strains for the production of the traditional cachaça in Brazil. *J Appl Microbiol* 103:2438–47.

Gomes FCO, Silva CLC, Vianna CR, Lacerda ICA, Borelli BM, Nunes AC, Franco GR, Mourão MM, Rosa CA. 2010. Identification of lactic acid bacteria associated with traditional cachaça fermentations. *Braz J Microbiol* 41:486–92.

Guerra JB, Araújo RAC, Pataro C, Franco GR, Moreira ESA, Mendonça-Hagler LC, Rosa CA. 2001. Genetic diversity of *Saccharomyces cerevisiae* strains during the 24h fermentative cycle for the production of the artisanal Brazilian cachaça. *Lett Appl Microbiol* 33:106–11.

Gutiérrez AR, López R, Santamaría MP, Sevilla MJ. 1997. Ecology of inoculated and spontaneous fermentations in Rioja (Spain) musts, examined by mitochondrial DNA restriction analysis. *Int J Food Microbiol* 36:241–5.

Lacerda ICA, Gomes FCO, Borelli BM, Faria Jr CLL, Franco GR, Mourão MM, Morais PB, Rosa CA. 2011. Identifying the bacterial community responsible for traditional fermentation during the production of sour cassava starch, cachaça and Minas cheese by culture-independent 16S rDNA sequence analysis. *Braz J Microbiol* 42:650–7.

Le Jeune C, Erny C, Demuyter C, Lollier M. 2006. Evolution of the population of *Saccharomyces cerevisiae* from grape to wine in a spontaneous fermentation. *Food Microbiol* 23:709–16.

Lehtonen M, Jounela-Eriksson P. 1983. Volatile and non-volatile compounds in the flavour of alcoholic beverages. In: Piggott JR, editor. *Flavour of Distilled Beverages: Origin and Development*. Flórida: Verlag Chemie International Inc. p 64–78.

Marini MM, Gomes FCO, Silva CLC, Cadete RM, Badotti F, Oliveira ES, Cardoso CR, Rosa CA. 2009. The use of selected starter *Saccharomyces cerevisiae* strains to produce traditional and industrial cachaça: a comparative study. *World J Microbiol Biotechnol* 25:235–42.

Mendes LM, Mori FA, Trugilho PF. 2009. Potencial da madeira de agregar valor ä cachaça de alambique. *Inf Agrop* 30:41–8.

Mills DA, Johannsen EA, Cocolin L. 2002. Yeast diversity and persistence in Botrytis-affected wine fermentations. *Appl Environ Microbiol* 68:4884–93.

Morais PB, Rosa CA, Linardi VR, Pataro C, Maia ABRA. 1997. Characterization and succession of yeast populations associated with spontaneous fermentation for Brazilian sugar-cane 'aguardente' production. *World J Microbiol Biotech* 13:241–3.

Narendranath NV, Brey S. 2009. Bacterial contamination and control. In: Ingledew WM, editor. *The Alcohol Textbook*. 5th ed. Nottingham: Nottingham University Press. p 481–90.

Narendranath NV, Hynes SH, Thomas KC, Ingledew WM. 1997. Effects of lactobacilli on yeast-catalysed ethanol fermentations. *Appl Environ Microbiol* 63:4158–63.

Nascimento RF. 1997. Aldeídos em bebidas alcoólicas fermento-destiladas. *Rev Engarraf Moderno* 49:75–7.

Nykanen L, Nykanen I. 1991. Distilled beverages. In: Maarse H, editors. *Volatile Compounds in Food and Beverages*. New York: Dekker, Inc. p 548–80.

Nykanen L, Puputti E, Suomalainen H. 1968. Volatile fatty acids in some brands of whisky, cognac and rum. *J Food Sci* 33:88–92.

Oliveira ES, Cardello HNAB, Jerônimo EM, Souza ELR, Serra GE. 2005. The influence of different yeast fermentation, composition and sensory quality of cachaça. *World J Microbiol Biotechnol* 21:707–15.

Oliveira ES, Rosa CA, Morgano MA, Serra GE. 2004. Fermentation characteristics as criteria for selection of cachaça yeast. *World J Microbiol Biotechnol* 20:19–24.

Pataro C, Guerra JB, Gomes FCO, Neves MJ, Pimentel PF, Rosa CA. 2002. Trehalose accumulation, invertase activity and physiological characteristics of yeasts isolated from 24h fermentative cycles during the production of artisanal Brazilian cachaça. *Braz J Microbiol* 33:202–8.

Pataro C, Guerra JB, Petrillo-Peixoto ML, Mendonça-Hagler LC, Linardi VR, Rosa CA. 2000. Yeast communities and genetic polymorphism of *Saccharomyces cerevisiae* strains associated with artisanal fermentation in Brazil. *J Appl Microbiol* 88:1–9.

Pinheiro SHM. 1999. Perfil da qualidade da cachaça do Ceará. [Dissertação de Mestrado]. Departamento de Tecnologia de Alimentos. Fortaleza: Universidade Federal do Ceará. 113 p.

Romano P, Caruso M, Capece A, Lipani G, Paraggio M, Fiore C. 2003. Metabolic diversity of *Saccharomyces cerevisiae* strains from spontaneously fermented grape musts. *World J Microbiol Biotechnol* 19:311–5.

Rosa CA, Soares AM, Faria JB. 2009. Cachaça production. In: Ingledew WM, editor. *The Alcohol Textbook*. 5th ed. Nottingham: Nottingham University Press. p 481–90.

Sanz A, Martín R, Mayoral MB, Hernández PE, González I, Lacarra TG. 2005. Development of a PCR-culture technique for rapid detection of yeast species in vacuum packer ham. *Meat Sci* 71:230–7.

Schwan RF, Mendonça AT, Silva Jr JJ, Rodrigues V, Wheals AE. 2001. Microbiology and physiology of cachaça fermentations. *Antonie van Leeuwenhoek* 79:89–96.

Silva CLC, Rosa CA, Oliveira ES. 2006. Studies on the kinetic parameters for alcoholic fermentation by flocculent *Saccharomyces cerevisiae* strains and non-hydrogen sulfite-producing strains. *World J Microbiol Biotechnol* 22:857–63.

Silva CLC, Vianna CR, Cadete RM, Santos RO, Gomes FCO, Oliveira ES, Rosa CA. 2009. Selection, growth, and chemo-sensory evaluation of flocculent starter culture strains of *Saccharomyces cerevisiae* in the large-scale production of traditional Brazilian cachaça. *Int J Food Microbiol* 131:203–10.

Soden A, Francis IL, Oackey H, Henschke PA. 2000. Effects of co-fermentation with *Candida stellata* and *Saccharomyces cerevisiae* on the aroma and composition of Chardonnay wine. *Aust J Grape Wine Res* 6:21–30.

Suomalainen H, Lehtonen M. 1979. The production of aroma compounds by yeast. *J Inst Brew* 85:149–56.

Valero E, Cambon B, Schuller D, Casal M, Dequin S. 2007. Biodiversity of *Saccharomyces* yeast strains from grape berries of wine-producing areas using starter commercial yeasts. *FEMS Yeast Res* 7:317–29.

Vianna CR, Silva CLC, Neves MJ, Rosa CA. 2008. *Saccharomyces cerevisiae* strains from traditional fermentations of Brazilian cachaça: trehalose metabolism, heat and ethanol resistance. *Antonie van Leeuwenhoek* 93:205–17.

Vila Nova MX, Schuler ARP, Valente BTR, Morais MA. Yeast species involved in artisanal cachaça fermentation in three stills with different technological levels in Pernambuco, Brazil. *Food Microbiol* 26:460–6.

39

Brick Tea

Haizhen Mo and Xinhong Liang

CONTENTS

39.1 Introduction

Tea is one of the most consumed beverages worldwide. In fact, there are mainly three different kinds of tea. In China and many Southeast Asian countries, green tea is preferred. In Western countries and the rest of the world, the consumption of black tea is the highest. A fermentation process is necessary to produce black tea. Black tea can be further subdivided into naturally oxidized tea (although the term of *fermentation* is often used) and microbial fermented tea.

The fermentation process for producing most black teas is actually an oxidation process catalyzed by enzymes that are originally present in tea leaves (Fowler et al. 1998). Because no microorganisms are involved in this kind of enzyme-oxidized black tea, the use of the term *fermentation* is obviously not completely correct. Microbial fermentation in the production of black tea, however, is found to be carried out only in China (Chen 1992).

Brick tea, a kind of re-produced tea made from black tea, is an indispensable beverage for people living in the Yunnan, Sinkiang, and Tibet areas where there are few vegetables and fruits, and it is regarded as a nutritional supplement. Usually, it is as important as meat and milk for daily life. Its functions on digestive promotion, antidisease, and general health care were long believed by people living there, so indigene have brick tea every day, every meal (Chen 1992; Wu et al. 2010).

Brick tea is often made from microbially fermented black tea, steamed to soften the tea leaves, and then compressed into a brick shape for sale or storage. The tea soup has full body, with amber color and special flavor.

39.2 Brick Tea Processing

The production process is different from place to place; brick teas include Xiang Jian tea, black brick tea, dark green brick tea (Hubei laoqingzhuan), Liupu tea, and Puer brick tea.

Early processing of black tea and green tea is similar, but making microbial black tea needs a special process: pile fermentation. Pile fermentation is done at high temperature and humidity and involves a large number of microorganisms, leaving the tea compound drastically changed. Such produced black tea has a unique taste and color. In this section, two main brick teas, Puer and Fuzhuan, are introduced.

39.2.1 Puer Brick Tea Processing

Puer tea is produced mainly in the Yunnan district in China and consumed by a large part of the Chinese population and has recently become popular in Japan as well, as a functional beverage (Abe et al. 2008). Puer tea is a unique Chinese microbial fermented tea obtained through indigenous tea fermentation where microorganisms are involved in the manufacturing process. This tea is different from common black tea that undergoes a natural oxidation process through enzymes originally existing in tea leaves (Wood 1998). The process of Puer tea manufacture is characterized by solid-state fermentation with natural flora as inoculums, of which the fungus *Aspergillus niger* plays a key role (Mo et al. 2005, 2008; Xu et al. 2005).

A schematic description of Puer tea production is shown in Figure 39.1.

Puer tea is made from *Camellia sinensis* Kuntze. var. *assamica* Kitamura. During the Puer tea fermentation process, fresh leaves are fixed by heat in a drum to inactivate polyphenol oxidases. The fixed leaves are then rolled and partially dried under the sun. Pile fermentation is the critical procedure in Puer tea making. The partially dried leaves are piled up in humid conditions (moisture content about 40% at 25°C–60°C) for a few weeks, during which the tea polyphenols are more intensively oxidized by the action of microorganisms and oxygen than in the black tea fermentation process, resulting in low concentrations of tea polyphenols and tea catechins (Liang et al. 2005).

After pile fermentation, the pile is released, and the tea is aired, selected, and graded, resulting in loose Puer tea. Steaming and compressing this type of Puer tea using different compression utensils give different brick teas such as bowl tea, Puer brick tea, Chi Tse Bing, and so on.

Different from other types of tea, Puer tea becomes better with age, and the value of Puer tea will increase according to the length of time the tea is stored. Chinese call it "edible antique."

Often loose Puer tea will be compressed to different shapes. The first step is to accurately weigh and steam the tea to soften it and increase its viscosity. Then the hot and wet (17% moisture) tea leaves are loaded into a utensil and put the cyclostyle on top; cyclostyles are usually carved with a letter or flower pattern. After compression, the tea brick is loosened and cooled and the edge is modified and the ridge is cleared and trimmed using a mend knife. At the same time, the brick is checked if it is of suitable thickness and if the brand design is vivid.

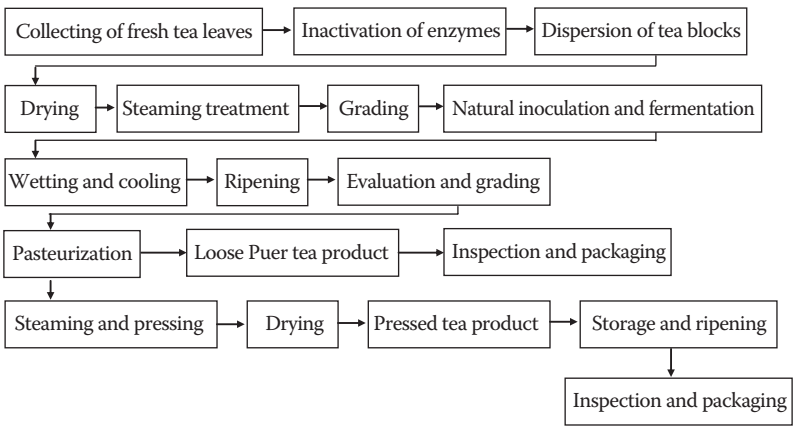

FIGURE 39.1 Schematic description of Puer tea processing chart. (Reprinted from Trends in *Food Science & Technology*, 19, Mo, H. Z. et al., Microbial fermented tea—a potential source of natural food preservatives, 124–30, Copyright (2008), with permission from Elsevier.)

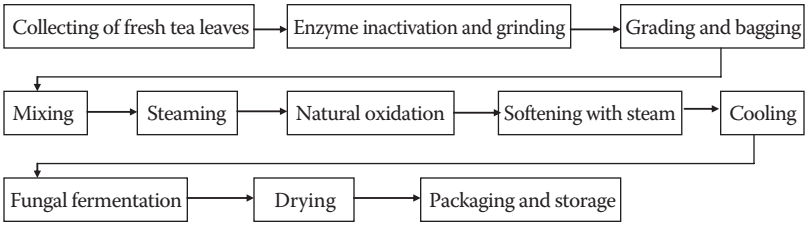

FIGURE 39.2 Schematic description of Fuzhuang brick tea processing chart. (Reprinted from Trends in *Food Science & Technology*, 19, Mo, H. Z. et al., Microbial fermented tea—a potential source of natural food preservatives, 124–30, Copyright (2008), with permission from Elsevier.)

The next step is drying the bricks, with the bricks neatly arranged on the oven rack and put into the drying room; in the first 3 days, the bricks are baked at 38°C, and every 8 hours the temperature is increased by 1°C, with 75°C as the maximum temperature. The usual baking time needed is 8 days, with the brick moisture content lower than 13%, and the bricks are then removed from the drying room and sealed with a trademark wrap, inserted into the sack, and stored.

39.2.2 Fuzhuan Brick Tea Processing

Fuzhuan brick tea is another kind of uniquely microbial fermented tea in China. Fu brick was first noted around 1860. It is a popular Chinese dark tea produced from Hunan Maocha. Fuzhuan brick was processed in summer, and is called Fu tea (in Chinese, *Fu* means hottest days in the summer). A schematic description of Fuzhuan brick tea production is given in Figure 39.2. The Fuzhuan brick tea manufacturing process includes steaming, piling, pressing, fungal fermentation, and drying; it is then compressed into a brick shape for sale or storage. The black tea is fermented by *Eurotium cristatum*, whose fungal growth is on the surface and inside and occurs as golden lush colonies that look like a golden fungal flora or golden flower, which is a symbol of high quality and it is a unique and special characteristic of Fuzhuan brick tea. Local residents believe that the more the golden flora, the higher the quality of tea (Wu 2010).

Fuzhuan brick tea has a special dark brown color, pure aroma, and yellow flora, and the tea extract is light amber and has a strong taste.

39.3 How to Drink Brick Tea

Compared with other kinds of tea, brick tea is consumed three different ways. First, before drinking, brick tea is compacted and a special knife is used to separate the brick to different layers or a sinker is used to smash the brick into smaller pieces. Second, except for Puer tea, most brick tea is not dipped into hot water but cooked or boiled for a few minutes and continuously churned to extract tea compounds. Third, during cooking, in most cases, seasonings, salt, or milk are added.

39.4 Compound Change and Dominating Microorganism during Fermentation

During black tea production, fresh tea leaves are cut and kneaded. At this stage, polyphenols and oxidation enzymes, which are stored in the different tissues of the leaf, are mixed together and the polyphenols are converted into a complex mixture of oxidation products.

The polyphenols from fresh tea leaf are quite unique. Their concentrations in the leaf, higher than those in other plants, are about 13%–25% of dry weight, and most of the polyphenols are composed of only four monomer catechins: (–)-epicatechin (EC), (–)-epicatechin-3-*O*-gallate (ECG), (–)-epigallocatechin (EGC), and (–)-epigallocatechin-3-*O*-gallate (EGCG) (Tanaka et al. 2010). Enzymatic oxidative

dimerization of pyrogallol-type polyphenols is important because of their high susceptibility to oxidation and abundance in the tea leaf. Production of unstable intermediates, such as dehydrotheasinensins and proepitheaflagallin, was demonstrated. However, degradation of the intermediates remained to be clarified, as the major degradation products, such as the theasinensins, only account for about half of the total degradation products. The unknown degradation products may hold the key to the solution of thearubigin formation in black tea chemistry. The enzymes play important roles in black tea manufacturing. The complex enzymatic reactions and fermentation metabolize compounds produces the color and flavor characteristic of each black tea brand.

Pile fermentation plays an important role in brick tea making. Mo et al. (2005) have done a microbiological analysis on samples from the indigenously fermented Puer tea. Microbial counting and identification revealed that *Aspergillus niger* was the dominant microorganism during fermentation and Abe et al. (2008) found that *Blastobotrys adeninivorans* also plays important roles in the nutritional enhancement of tea leaves during Puer tea fermentation.

The Fuzhuan tea microbial evolution during fungal fermentation showed that *Eurotium* spp., *Aspergillus* spp., and *Penicillium* spp. are the main microorganisms; different from Puer tea, the dominant fungus in Fuzhuan tea was *Eurotium* spp. (Zhang et al. 2010). Microbial fermentation resulted in the formation of its special aroma and taste. The carbonaceous, nitrogenous compounds and volatile compounds were identified to be important for Fuzhuan brick-tea's aroma and taste (Wu et al. 2010; Xu et al. 2007).

39.5 Biofunctional Effects

Although many kinds of brick tea are consumed in China, the research is only focused on the biofunctional effects of Puer tea.

39.5.1 Hypolipidemic Effects

Puer tea has not only a unique flavor but also has several health benefits, such as suppression of fatty acid synthesis expression, inhibitor of lipid and nonlipid oxidative damage, metal binding ability, and reduction of power and scavenging effect of free radicals (Duh et al. 2004; Jie et al. 2006). The hypolipidemic effects of tea have been well studied in animals and humans. The major components of tea demonstrated to be responsible for antiobesity and hypolipidemia are catechins, caffeine, and theanine (Chiang et al. 2006).

Kuo et al. (2005) reported that Puer tea and oolong tea can lower the levels of triglyceride more significantly than that of green tea and black tea, whereas Puer tea and green tea are more efficient than oolong tea and black tea in lowering the level of total cholesterol (Kuo et al. 2005). Liang et al. (2005) reported that Puer tea suppresses the genotoxicity induced by nitroarenes, lowers the atherogenic index, and increases high-density lipoprotein and total cholesterol ratio (Liang et al. 2005). Lu and Hwang (2008) collected 30 samples of Puer tea and assayed for cholesterol synthesis, inhibitory activity, and polyphenol composition. They found that all samples were able to inhibit the cholesterol biosynthesis in the Hep G2 cell model, and the inhibition ratios ranged from 7% to 35%. The inhibition abilities of tea polyphenol standards were in the order of gallocatechin gallate > EGCG > ECG > gallic acid > EGC > myricetin > quercetin > catechin > EC (Lu and Hwang 2008).

Lin et al. (2007) found that theaflavins attenuate hepatic lipid accumulation through activating AMP-activated protein kinase in human HepG2 cells, suggesting that theaflavins are bioavailable both in vitro and in vivo and may be active in the prevention of fatty liver and obesity. Way et al. (2009) also found that Puer tea attenuates hyperlipogenesis and induces hepatoma cell growth arrest through the activation of AMP-activated protein kinase in human HepG2 cells.

Fujita and Yamagami (2008a–c) also did many research on Puer tea, and they found Puer tea inhibits postprandial rise in serum cholesterol in mice and reduces serum cholesterol and low-density lipoprotein levels and renal fat weight in rats. Puer tea extraction has a specific antihypercholesterol effect in rodents. They also did examinations in humans and found that Puer tea extract significantly

reduced hypercholesterolemia and decreased total and low-density lipoprotein cholesterol levels accompanied by significant decreases in body weight, without affecting other biochemical parameters, and these findings indicate that Puer tea extract intake elicits a significant antihypercholesterolemic effect and might be useful for improving blood cholesterol levels in subjects at risk for heart disease or obesity.

Recently, statin has been identified in Puer tea extract. Clinical trials have confirmed that statin decreases the incidence of major coronary and cerebrovascular events, and this may be due to its hypolipidemic and anti-inflammatory effects (Jeng et al. 2007).

39.5.2 Antioxidation

Hou et al. (2009) examined the effects of the two commonly consumed forms of Puer tea on weight gain, serum levels of lipids and lipoprotein, lipid oxidation, and blood antioxidant enzymes in a rat hyperlipidemia model. Results suggest that Puer tea exerts strong antioxidant activity and lipid-lowering effects and therefore can be used to reduce the risk of cardiovascular disorders.

Jie et al. (2006) found that Puer tea ethyl acetate and *n*-butanol extracts and their fractions have superoxide anion– and hydroxyl radical–scavenging activity, with the ethyl acetate extract showing stronger effects than that of the typical green tea catechin EGCG and the raw Puer teas (natural microbially fermented) contained more antioxidant compounds compared to ripened Puer teas (inoculated microbial fermentation). EGCG, EGC, ECG, and quinic acid are the main antioxidant compounds (Ku et al. 2010).

39.5.3 Diabetes

Kao et al. (2006) reviewed the effects of tea catechins on obesity, and diabetes has received increasing attention. Tea catechins, especially EGCG, appear to have antiobesity and antidiabetic effects. These mechanisms may be related to certain pathways, such as the modulation of energy balance, endocrine systems, food intake, lipid and carbohydrate metabolism, redox status, and activities of different types of cells (i.e., fat, liver, muscle, and β-pancreatic cells).

Li et al. (2010) found two Puer tea components, EGCG and ECG, as potent α-glycosidase inhibitors and is a therapeutic approach for diabetes (Li et al. 2010). Neyestani et al. (2010) also found regular daily intake of black tea improves oxidative stress biomarkers and decreases serum C-reactive protein levels in type 2 diabetic patients.

39.5.4 Antibacterial Effect

A few studies reveal that extracts from microbial fermented teas contain natural antimicrobial components that have an inhibitive effect on several food-borne pathogen and spoilage bacteria (Greenwalt et al. 1998; Mo et al. 2005; Sreeramulu et al. 2001; Steinkraus et al. 1996). Antimutagenic and antimicrobial activities of Puer tea were also reported (Wu et al. 2007).

Mo et al. (2005) found that the antimicrobial activity of fermented Puer tea shows inhibitory effects on several food-borne bacteria, including spore-forming bacteria *Bacillus cereus*, *Bacillus subtilis*, *Clostridium perfringens*, and *Clostridium sporogenes*. The antimicrobial activity increases with the course of the fermentation. Mo et al. (2005, 2007) and Zhang et al. (2010) found that Fuzhuan brick tea also has antibacterial properties on the aforementioned spore-forming bacteria, but the activity is a bit lower then Puer tea.

39.6 Risks in Brick Tea Intake

Tea trees (*Camellia sinensis*) selectively absorb fluoride from the soil and air in the surroundings, and it accumulates mainly in the tea leaves in the form of AI and F complex. Long-term consumption of high-fluoride tea could result in chronic fluoride intoxication (Yi and Cao 2008). Fluoride intoxication is a popular problem in brick tea. Normally, brick tea is made with old tea leaves but not tender leaves,

so the fluoride content is higher than green tea and oolong tea. Ya'an and Yi'yang brick tea fluoride concentrations are 543–586 and ~365–400 mg/kg and are hundreds of times higher than the normal green tea (Chen 1992).

In some minority districts, the fluoride content of drinking water is <1 mg/L, but the fluorine content in herdsmen who drink brick tea can reach 1000 mg/kg. Adult herdsmen drink 2 L of water per day; most of it comes from milk tea or heavy tea. The survey about Mongolian children aged 8–12 years found that 90.14% of children experience dental fluorosis and 10% of children have severe dental fluorosis. Beverages and foods mixed with brick tea water are responsible for dental fluorosis in children.

In 1995, there was an investigation about the Tibetan and Han fluorosis status in the Daofu county of Ganzi Tibetan autonomous prefecture. A survey confirmed that local water had low fluoride, but the Tibetan herdsmen and monks used to drink large quantities of brick tea; adults take upto 12.46 mg of fluoride per day and young people take about 6.56 mg per day. The average brick tea consumption of Tibetans is 10.77 kg per person per year, with daily consumption of 29.3 g. In accordance with the brick tea fluoride content basis of 426 mg/kg, the Tibetans drinking brick tea of daily can absorb 1.25 mg of fluoride, which is more than 3 times the safe dose.

Cao et al. (2004) assayed the fluoride content in black tea in various commodities, packages, and preparation processes and evaluated the safety. They found the fluoride content of five stick-shaped black teas was 96.9–148 mg/kg, which suggests that for heavy black tea drinkers, the fluoride intake in areas with drinking water fluoridation and also other probable sources of fluoride may approach or reach the level of risk of chronic fluoride intoxication. They also compared Puer brick tea and Bianxiao brick tea fluoride content. The Bianxiao brick tea made in Hunan and Sichuan provinces are mainly consumed by minority ethnic groups in the border areas, with Bianxiao brick tea's water-soluble fluorine much greater than Puer brick tea's (mean levels 441 and 77 mg/kg, respectively). The difference in the fluorine levels of the two types of brick tea can be attributed to differences in the materials used to make them: Puer brick tea is made from tender leavesm whereas Bianxiao brick tea is made from old tough leaves in which fluorine has accumulated (Cao et al. 1998).

Thus, suitable brick tea intake is important to keep the fluoride in a safe amount. Wang et al. (2010) did a safety evaluation of Chinese Puer green tea extract and Puer black tea extract in Wistar rats and found that a dose of 1250 mg/kg/day for GTE and 5000 mg/kg/day for BTE following oral administration could be considered safe because it does not exceed the maximum recommended daily allowance of up to 4.0 mg for adults.

Pesticides and heavy metal are also possible health risks resulting from long-term drinking of brick tea. Cao et al. (2010) surveyed the aluminum, lead, cadmium. mercury, zinc, copper, and arsenic concentration in Puer tea and found that although the tea tree easily absorbs metal elements from soil, both the hazard quotient values for single elements and the hazard index value for all seven elements were far below the safety concentration limit in China. However, Fiedler et al. (2002) analyzed the polychlorinated dibenzo-*p*-dioxins and polychlorinated dibenzofurans (PCDD/PCDF), chlorinated pesticides, and PAH concentrations in brick tea and green tea. In tea leaves, DDT and its metabolites levels were within the safety limit of 0.2 mg/kg. Rather high levels of total PAH were obtained in brick tea (1048–1162 mg/kg), much higher than green tea (497–517 mg/kg). Minority ethnic groups in China drink a large amount of brick tea (>3 L per day) and consume more tea than Europeans or North Americans, thus resulting in a comparably higher intake of PCDD/PCDF.

Thus, large-scale epidemiological investigations and further studies on tea-type fluorosis are needed for those tea-drinking areas. In addition, it is urgent that governmental and international agencies adopt safe standards of fluoride content in tea commodities (Yi and Cao 2008).

Another safety problem in brick tea is that most microbially fermented teas undergo uncontrolled traditional indigenous fermentation. Not only the natural floras for inoculation but also the season of production strongly affects the product quality and consistency.

Therefore, a standardized fermentation process will not only ensure food safety but also the product quality. Furthermore, during the standardization and optimization of the process, more insight will be obtained for the metabolic mechanism of the fungi involved, how they produce favorable metabolites, and eventually an overproduction of these useful natural preservatives can be expected.

REFERENCES

Abe M, Takaoka N, Idemoto Y, Takagi C, Imai T, Nakasaki K. 2008. Characteristic fungi observed in the fermentation process for Puer tea. *Int J Food Microbiol* 124(2):199–203.

Cao HB, Qiao L, Zhang H, Chen JJ. 2010. Exposure and risk assessment for aluminium and heavy metals in Puerh tea. *Sci Total Environ* 408(14):2777–84.

Cao J, Luo SF, Liu JW, Li Y. 2004. Safety evaluation on fluoride content in black tea. *Food Chem* 88(2):233–6.

Cao J, Zhao Y, Liu JW. 1998. Safety evaluation and fluorine concentration of Pu'er brick tea and Bianxiao brick tea. *Food Chem Toxicol* 36(12):1061–3.

Chen ZM. 1992. *China Tea Encyclopedia*. Shanghai: Shanghai Cultural Publisher.

Chiang CT, Weng MS, Lin-Shiau SY, Kuo KL, Tsai YJ, Lin JK. 2006. Pu-erh tea supplementation suppresses fatty acid synthase expression in the rat liver through downregulating Akt and JNK signalings as demonstrated in human hepatoma HepG2 cells. *Oncol Res* 16(3):119–28.

Duh PD, Yen GC, Yen WJ, Wang BS, Chang LW. 2004. Effects of pu-erh tea on oxidative damage and nitric oxide scavenging. *J Agric Food Chem* 52(26):8169–76.

Fiedler H, Cheung CK, Wong MH. 2002. PCDD/PCDF, chlorinated pesticides and PAH in Chinese teas. *Chemosphere* 46(9–10):1429–33.

Fowler MS, Leheup P, Cordier J-L. 1998. Cocoa, coffee and tea. In Wood BJB, editor. *Microbiology of Fermented Foods*. London: Blackie Academic & Professional. p 128–47.

Fujita H, Yamagami T. 2008a. Antihypercholesterolemic effect of Chinese black tea extract in human subjects with borderline hypercholesterolemia. *Nutr Res* 28(7):450–6.

Fujita H, Yamagami T. 2008b. Efficacy and safety of Chinese black tea (Pu-Ehr) extract in healthy and hypercholesterolemic subjects. *Ann Nutr Metab* 53(1):33–42.

Fujita H, Yamagami T. 2008c. Extract of black tea (pu-ehr) inhibits postprandial rise in serum cholesterol in mice, and with long term use reduces serum cholesterol and low density lipoprotein levels and renal fat weight in rats. *Phytotherapy Res* 22(10):1275–81.

Greenwalt CJ, Ledford RA, Steinkraus KH. 1998. Determination and characterization of the antimicrobial activity of the fermented tea Kombucha. *Food Sci Technol-Lebensmittel-Wissenschaft Technol* 31(3):291–6.

Hou Y, Shao WF, Xiao R, Xu KL, Ma ZZ, Johnstone BH, Du YS. 2009. Pu-erh tea aqueous extracts lower atherosclerotic risk factors in a rat hyperlipidemia model. *Exp Gerontol* 44(6–7):434–9.

Jeng KC, Chen CS, Fang YP, Hou RCW, Chen YS. 2007. Effect of microbial fermentation on content of statin, GABA, and polyphenols in Pu-erh tea. *J Agric Food Chem* 55(21):8787–92.

Jie GL, Lin Z, Zhang LZ, Lu HP, He PM, Zhao BL. 2006. Free radical scavenging effect of Pu-erh tea extracts and their protective effect on oxidative damage in human fibroblast cells. *J Agric Food Chem* 54(21):8058–64.

Kao YH, Chang HH, Lee MJ, Chen CL. 2006. Tea, obesity, and diabetes. *Mol Nutr Food Res* 50(2):188–210.

Ku KM, Kim J, Park HJ, Liu KH, Lee CH. 2010. Application of metabolomics in the analysis of manufacturing type of pu-erh tea and composition changes with different postfermentation year. *J Agric Food Chem* 58(1):345–52.

Kuo KL, Weng MS, Chiang CT, Tsai YJ, Lin-Shiau SY, Lin JK. 2005. Comparative studies on the hypolipidemic and growth suppressive effects of oolong, black, pu-erh, and green tea leaves in rats. *J Agric Food Chem* 53(2):480–9.

Li DQ, Qian ZM, Li SP. 2010. Inhibition of three selected beverage extracts on alpha-glucosidase and rapid identification of their active compounds using HPLC-DAD-MS/MS and biochemical detection. *J Agric Food Chem* 58(11):6608–13.

Liang Y, Zhang L, Lu J. 2005. A study on chemical estimation of pu erh tea quality. *J Sci Food Agric.* p 381–90.

Lin CL, Huang HC, Lin JK. 2007. Theaflavins attenuate hepatic lipid accumulation through activating AMPK in human HepG2 cells. *J Lipid Res* 48(11):2334–43.

Lu CH, Hwang LS. 2008. Polyphenol contents of Pu-Erh teas and their abilities to inhibit cholesterol biosynthesis in Hep G2 cell line. *Food Chem* 111(1):67–71.

Mo HZ, Xu XQ, Yan MC, Zhu Y. 2005. Microbiological analysis and antibacterial effects of the indigenous fermented Puer tea. *Agro Food Ind Hi-Tech* 16(6):16–8.

Mo HZ, Zhu Y, Chen ZM. 2008. Microbial fermented tea—a potential source of natural food preservatives. *Trends Food Sci Technol* 19(3):124–30.

Neyestani TR, Shariatzade N, Kalayi A, Gharavi A, Khalaji N, Dadkhah M, Zowghi T, Haidari H, Shab-Bidar S. 2010. Regular daily intake of black tea improves oxidative stress biomarkers and decreases serum C-reactive protein levels in type 2 diabetic patients. *Ann Nutr Metab* 57(1):40–9.

Sreeramulu G, Zhu Y, Knol W. 2001. Characterization of antimicrobial activity in Kombucha fermentation. *Acta Biotechnol* 21(1):49–56.

Steinkraus KH, Shapiro KB, Hotchkiss JH, Mortlock RP. 1996. Investigations into the antibiotic activity of tea Fungus/Kombucha beverage. *Acta Biotechnol* 16(2–3):199–205.

Tanaka T, Matsuo Y, Kouno I. Chemistry of secondary polyphenols produced during processing of tea and selected foods. *Int J Mol Sci* 11(1):14–40.

Wang L, Xu RJ, Hu B, Li W, Sun Y, Tu YY, Zeng XX. 2010. Analysis of free amino acids in Chinese teas and flower of tea plant by high performance liquid chromatography combined with solid-phase extraction. *Food Chem* 123(4):1259–66.

Way TD, Lin HY, Kuo DH, Tsai SJ, Shieh JC, Wu JC, Lee MR, Lin JK. 2009. Pu-erh tea attenuates hyperlipogenesis and induces hepatoma cells growth arrest through activating AMP-Activated Protein Kinase (AMPK) in human HepG2 Cells. *J Agric Food Chem* 57(12):5257–64.

Wood BJB. 1998. *Microbiology of Fermented Foods*. London: Blackie Academic & Professional.

Wu YY, Ding L, Xia HL, Tu YY. 2010. Analysis of the major chemical compositions in Fuzhuan brick-tea and its effect on activities of pancreatic enzymes *in vitro*. *Afr J Biotechnol* 40(9):6748–54.

Xu X, Yan M, Zhu Y. 2005. Influence of fungal fermentation on the development of volatile compounds in the Puer tea manufacturing process. *Eng Life Sci* 5(4):382–6.

Xu XQ, Mo HZ, Yan MC, Zhu Y. 2007. Analysis of characteristic aroma of fungal fermented Fuzhuan brick-tea by gas chromatography/mass spectrophotometry. *J Sci Food Agric* 87(8):1502–4.

Yi J, Cao J. 2008. Tea and fluorosis. *J Fluorine Chem* 129(2):76–81.

Zhang H, Li H, Mo HZ. 2010. Microbial population and antibacterial activity in fuzhuan brick tea. *Food Sci (China)* 31(21):293–7.

40

Shalgam (Şalgam)

Huseyin Erten and Hasan Tanguler

CONTENTS

40.1 Introduction

Traditional foods and beverages have been an important part of our lives all over the world. Their production is one of the oldest manufacturing and preservation methods of human consumption, dating back to ancient times (Caplice and Fitzgerald 1999). Shalgam, a lactic acid–fermented product, is a traditional beverage highly popular in southern Turkey, particularly in the cities of Adana, Mersin, and Hatay. It has also become a popular drink in other parts of Turkey, especially in Istanbul, Ankara, and Izmir metropolises (Erten et al. 2008; Erten and Tanguler 2010).

Shalgam is a red, cloudy, and sour soft beverage that is produced by the lactic acid fermentation of black carrot, sourdough, salt, bulgur flour, turnip, and adequate water. It is traditionally made at home or at the home-scale level. Shalgam is also produced commercially on small and large scales. It is widely consumed with food and as a refreshing beverage, and its consumption has been increasing in recent years (Erten et al. 2008). Similar beverages to shalgam are *Kanji* and lactofermented carrot juice. Kanji, a traditional fermented beverage of northern parts of India, is produced by the natural fermentation of lengthwise sliced or grated black carrots mixed with requisite quantities of salt, crushed mustard, and red chili powder (Berry et al. 1989; Sethi 1990). The orange-rooted carrot is used for the production of lactofermented carrot juice. The starter microorganisms used for lactofermented carrot juice fermentation are homofermentative lactic acid bacteria, usually *Lactobacillus* (*L.*) strains (Demir et al. 2004, 2006).

This chapter describes the raw materials, production methods, and microbiological and chemical aspects of shalgam.

40.2 Shalgam Production

Shalgam production methods differ depending on the manufacturer. However, there are two main production methods for shalgam: traditional and direct. The essential process consists of preparation of raw materials, fermentation, and packaging.

40.2.1 Raw Materials

The main raw material used for the production of shalgam is roots of black carrot. The other raw materials are roots of turnip, bulgur flour, salt, sourdough, and drinkable water.

40.2.1.1 Black Carrot

The carrot, *Daucus carota* L., which has been cultivated for thousands of years, is one of the most important root vegetable plants cultivated worldwide. It is a member of Apiaceae (formerly Umbelliferae). There are two varieties of carrot: orange and black. For exact botanical classification, carrot cultivars are subdivided into two groups. First, *Daucus carota* ssp. *sativus* var. *sativus*, known as carotene (western) group, is grown worldwide, accounting for the majority of carrot production. This group is also known as orange-rooted carrots that are rich in carotenes (β-carotene is a precursor of vitamin A), vitamin C, and sugars (Pistrick 2001; Kammerer et al. 2004).

Second, *Daucus carota* ssp. *sativus* var. *atrorubens* Alef. belongs to the anthocyanin (eastern) group. It is traditionally grown in Turkey, Afghanistan, Egypt, Pakistan, and India. Black carrots are considered to be one of the most important sources of natural food colorants (Canbas 1985; Canbas 1991; Pistrick 2001; Kammerer et al. 2004).

In shalgam production, roots of black carrot are used as the main raw material for the formation of lactic acid as the predominant end product during sugar fermentation and for the red color of shalgam (Canbas and Deryaoglu 1993; Erten et al. 2008). In raw black carrot, the concentration of total sugar is 5.12%–7.09% (Canbas and Deryaoglu 1993; Gunes 2008; Utus 2008; Tanguler 2010). The principal soluble fermentable sugar of black carrot grown in Turkey is sucrose (49.95–331 g/kg), followed by glucose (6.66–56.4 g/kg), and fructose (6.65–43.6 g/kg) (Kammerer et al. 2004; Tanguler 2010).

The red color of shalgam derives from anthocyanins present in black carrots. The major anthocyanin compounds of black carrots are acylated and unacylated cyanidin derivates. The main compounds tentavitely identified are ferulic acid derivate of cyanidin 3-xylosyl-galactoside, cyanidin 3-xylosylglucosylgalactoside, caffeic acid derivate of cyanidin 3-xylosylglucosylgalactoside, cyanidin 3-xylosylglucosylgalactoside, *p*-hydroxybenzoic acid derivate of cyaniding 3-xylosylglucosylgalactoside, cinnamic acid derivate of cyanidin 3-xylosylglucosylgalactoside, and *p*-coumaric acid derivate of cyanidin 3-xylosylglucosylgalactoside (Kammerer et al. 2004; Turker et al. 2004; Tanguler 2010).

40.2.1.2 Turnip

Turnip, *Brassica rapa* L., is a member of the Cruciferae family (Sethi 1990). It is a minor raw material of shalgam production, and its roots could be used up to 2% if available on the market. Some shalgam makers do not use turnip root as an ingredient (Erten et al. 2008). The fermentable soluble sugars found in turnip are sucrose (0.21%–3.42%), glucose (0.20%–1.41%), fructose (0.14%–1.10%) (Rodríguez-Sevilla et al. 1999; Tanguler 2010).

40.2.1.3 Bulgur Flour

Bulgur (bulghur) flour (bran), which is used at the ratio of 3%, is a minor raw material for the production of shalgam. It also provides nutrients to microorganisms during the first stage of fermentation in the traditional production method as well as in the direct production method (Erten et al. 2008).

To obtain bulgur, wheat, *Tritucum aestivum* L. (Erbas et al. 2005), is parboiled, dried, dehusked, ground, and sieved. The product that remains on the upper layer of the sieves is called bulgur. The lower part (flour) (Turkish *setik*), which passes through sieves, is used in shalgam production. Apart from using its lower part as ingredients, bulgur can also be milled and used for shalgam making (TSE 2003). The amount of total sugar and starch in bulgur flour ranges from 2.2 to 3.30 g/100 g and from 4.44 to 5.84 g/100 g, respectively (Canbas and Fenercioglu 1984; Deryaoğlu 1990; Canbas and Deryaoglu 1993; Tanguler 2010).

40.2.1.4 Salt

Sodium chloride, also known as common salt, is an essential material in shalgam production. Salt added is commercial unrefined rock salt. Its concentration for shalgam fermentation is 1%–2% (Canbas and Deryaoglu 1993; Erten et al. 2008).

Salt serves several functions in the fermentation process. It inhibits spoilage and pathogenic organisms depending on its concentration, therefore favoring the growth of lactic acid bacteria. Its antimicrobial action involves a nonspecific a_w reduction effect and an additional inhibitory effect. In general, large levels of salt are required to achieve a significant adverse effect on microorganisms. Among the lactic acid bacteria, *Leuconostoc* spp. are the least tolerant to salt, while *Lactobacillus* spp. and *Pediococcus* spp. have similar salt tolerance (Nout and Rombouts 1992). Salt also provides salty flavor in the products (McFeeters and Pérez-Díaz 2010).

40.2.1.5 Sourdough

Sourdough, which is obtained by using bakers' yeast dough fermented at room temperature for overnight, is also used in shalgam production. Some producers who make shalgam by direct production method use only bakers' yeast *Saccharomyces cerevisiae* (Canbas and Deryaoglu 1993; Erten et al. 2008; Tanguler 2010). It is well-known that sourdough bread is made from sourdough in which fermentation involves mixed cultures of different lactic acid bacteria and yeasts. The most dominant lactic acid bacteria in sourdough for sourdough bread are *L. sanfranciscensis*, *L. pontis*, *L. brevis*, *L. plantarum*, *L. alimentarius*, *L. fructivorans*, *L. reuteri*, and *L. fermentum*. Besides *Lactobacillus*, as secondary microbiota of non-*Lactobacillus* lactic acid bacteria species exits at lower levels. The most frequently present yeasts are *Saccharomyces cerevisiae* and, to a lesser extent, *Saccharomyces exiguous*, *Candida krusei*, and *Candida milleri* (Gobbetti et al. 2005; Paramithiotis et al. 2006; Corsetti et al. 2007).

40.2.2 Shalgam Processing Methods

Shalgam is produced on an industrial scale. For shalgam production, a standard manufacturing technique is not available and processing varies from one plant to another. However, there are commercially two main processing methods for shalgam production: traditional and direct.

40.2.2.1 Traditional Production Method

Figure 40.1 outlines the traditional method employed in the production of shalgam. The traditional method consists of two distinct steps: first fermentation and second fermentation (Canbas and Deryaoglu 1993; Erten et al. 2008; Tanguler 2010).

First fermentation is also called sourdough fermentation. It is carried out for the enrichment of lactic acid bacteria and yeasts. Bulgur flour (3%), rock salt (0.2%), sourdough (0.2%), and adequate drinkable water are mixed and kneaded for the formation of dough. The dough is left for the first fermentation at room temperature for 3–5 days or more (Figure 40.1). The fermented mixture of bulgur flour and sourdough is extracted with adequate water for three to five times. The extracts obtained are used for second fermentation.

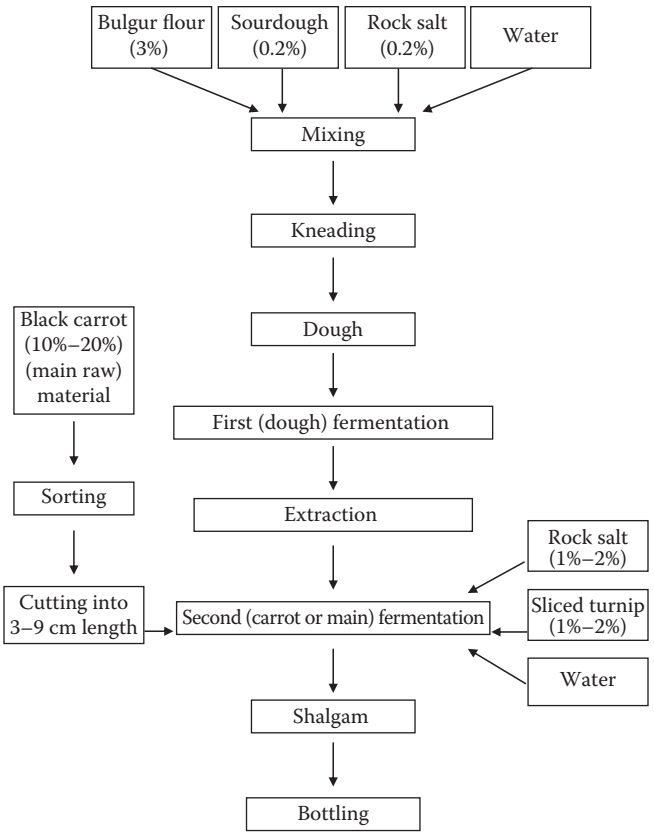

FIGURE 40.1 Flow diagram for shalgam production using traditional method.

During first fermentation, acid content considerably increases and consequently pH drops due to the important activities of mainly LAB and yeasts with less extent. Extract from first fermentation, which also includes sourdough flora, may help to give the second fermentation a good start (Canbas and Deryaoglu 1993; Erten et al. 2008; Tanguler 2010).

Some producers, however, do not extract the fermented mixture of bulgur flour and sourdough with water. After performing first fermentation, the dough is placed into a fabric bag and hung in the fermentation tank to perform second fermentation.

The second fermentation is also known as main or carrot fermentation (Figure 40.1). The extracts obtained from first fermentation are combined to perform the second fermentation with the roots of black carrot, rock salt (1%–2%), and, if available, sliced turnip (1%–2%) in a tank. Adequate drinkable water is added to fill the tank.

The black carrot added is washed if necessary. It is sorted to remove all the damaged and defected carrots. Then, it is cut into pieces of 3–9 cm length. Some shalgam producers also slice the roots of black carrot lengthwise. The level of black carrot added at the beginning of fermentation varies from 10% to 20% (Canbas and Deryaoglu 1993; Erten et al. 2008; Tanguler 2010). However, Gunes (2008) states that black carrot should be added at 15%–20% levels to achieve adequate acidity.

Second fermentation is naturally carried out for 3 to 10 days at ambient temperature, which can vary from 10°C to 35°C. It is performed in fiberglass, plastic, or stainless steel tanks. Microflora, chemical composition of raw materials, fermentation temperature, and salt as main parameters determine the duration and completeness of the fermentation. During the fermentation, color compounds (anthocyanins) are extracted. Total acidity is increased by the action of mainly lactic acid bacteria.

At the end of fermentation, a red and sour beverage is obtained. Clarification is not carried out in shalgam production. The fermented product is removed from the tank and marketed in sealed bottles and

plastic containers as well as loose. Fermented juice can also be seasoned by adding chili powder (Canbas and Deryaoglu 1993; Erten et al. 2008; Tanguler 2010).

40.2.2.2 Direct Production Method

Shalgam production by direct method is given in Figure 40.2. In the direct method, first fermentation (sourdough fermentation) is not performed. Black carrots are sorted out and cut into pieces. The chopped black carrots (10%–20%), salt (1%–2%), if available sliced turnip (1%–2%), bakers' yeast (*Saccharomyces cerevisiae*), or sourdough (0.2%) and adequate water are transferred to a tank to perform the lactic acid fermentation, which is called main (carrot) fermentation (Erten et al. 2008).

Fermentation takes place at ambient temperatures, ranging from 10°C to 35°C for 3–10 days. Fermented juice is removed from the vessel, and it is sold in sealed bottles and plastic containers as well as loose.

40.2.2.3 Fermentation Microbiology

The shalgam fermentation is a complex biochemical process involving mainly lactic acid bacteria and, to a lesser extent, yeasts. Lactic acid fermentation is the most important reaction (Erten et al. 2008). With regard to the pathways by which hexoses are fermented, lactic acid bacteria are briefly grouped as homofermentative and heterofermentative. Homofermentative lactic acid bacteria such as some *Lactobacillus*, *Pediococcus*, *Lactococcus*, and *Streptococcus* form lactic acid as the major or sole metabolic end product of glucose fermentation through the Embden-Meyerhof-Parnas pathway (glycolysis). Heterofermentative lactic acid bacteria such as *Leuconostocs*, *Oenococcus*, *Weissella*, and some *Lactobacillus* produce equimolar amounts of lactate, CO_2, and ethanol or acetate depending on air using pentose phosphate or phosphoketolase pathway (Holzapfel and Wood 1995; Caplice and Fitzgerald 1999; Blandio et al. 2003).

Microorganisms, mainly lactic acid bacteria and yeasts with less extent, responsible for the natural shalgam fermentations originate from the surfaces of raw materials, surfaces of vessels where product is produced and stored and from the sourdough extract (Erten et al. 2008). The counts of total mesophilic aerobic bacteria, total yeasts, and total non-*Saccharomyces* yeasts generally decrease during the second fermentation of shalgam. As pH drops rapidly, no coliforms are counted. In contrast, the number of lactic acid bacteria, which are the dominant microflora during the fermentation, increases (Tanguler 2010). The carbon dioxide formed during the vegetable fermentations replaces the air and inhibits undesirable

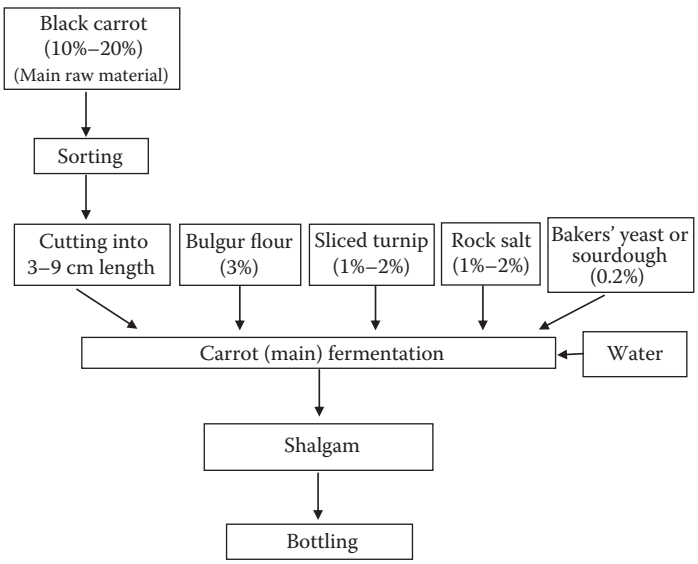

FIGURE 40.2 Flow diagram for shalgam production by direct method.

Gram-negative microorganisms in combination with added salt. It also helps the growth of lactic acid bacteria (Harris 1998). *L. plantarum* is believed to dominate shalgam fermentations using the traditional method. *L. paracasei* subsp. *paracasei* is also dominant bacterium but with less growth compared to growth of *L. plantarum* (Tanguler 2010). These bacteria have also been isolated by Erginkaya and Hammes (1992) and Arıcı (2004). Lactobacilli play an important role in several lactic acid fermentations of vegetables. They are generally most acid-tolerant of lactic acid bacteria. Lactic acid-fermented cucumber and olive fermentations are dominated and terminated primarily by *L. plantarum*, which is a facultatively heterofermentative lactobacilli (Harris 1998; Hutkins 2006). With much lower numbers, *L. brevis*, *L. fermentum*, and *Lactococcus lactis* are also isolated from shalgam fermentations depending on the production plants. The counts of *Saccharomyces* and non-*Saccharomyces* yeasts compared to lactic acid bacteria are low in shalgam production and their contribution to fermentation is not clear (Tanguler 2010). Harris (1998) states that fermentative (*Saccharomyces* spp., *Kluyveromyces* spp.) and oxidative (*Hansenula* spp., *Pichia* spp., *Candida* spp., *Debaryomyces* spp.) yeasts are found in vegetable fermentations. They can play a role in the sensory characteristics of shalgam, but yeasts can also be harmful by metabolizing the lactic acid formed and increasing the pH, which can cause spoilage.

Shalgam makers do not usually inoculate the fermentation tanks with selected starter cultures of lactic acid bacteria (Erten et al. 2008); however, a method of pitching up to 15% of shalgam from a previous healthy production batch is sometimes used (Canbas and Fenercioglu 1984). Tanguler (2010) studied 18 species of lactic acid bacteria isolated from shalgam fermentations to select the most promising bacterium for industrial usage as starter culture. He states that *L. plantarum*, *L. fermentum*, and *L. paracasei* subsp. *paracasei* can be used as shalgam starter cultures for commercial production.

40.2.2.4 Metabolic End Products

The principal end product of shalgam fermentation is lactic acid, which accounts for the majority of total acidity. Lactic acid formed during fermentation helps to preserve the beverage and enhances taste and aroma. Its concentration ranges from 5.18 to 8.05 g/L in shalgam (Canbas and Deryaoglu 1993; Tanguler 2010). Acetic acid, which is the principal volatile acidity formed probably by the action of heterofermentative lactic acid bacteria and yeasts during the shalgam fermentation, can be present at levels varying between 0.57 and 0.83 g/L (Tanguler 2010). As much as 6.41 g/L of ethyl alcohol can also be produced (Canbas and Deryaoglu 1993).

Forty-four volatile aroma compounds have been identified in shalgam including carbonyl compounds, volatile acids, higher alcohols, esters, terpenols, norisoprenoids, lactones, and volatile phenols. These minor products contribute the overall flavor of shalgam (Tanguler 2010).

Nearly all fermented foods including fermented vegetables can potentially contain biogenic amines. The level of biogenic amines formed depends on both the concentration of the amino acid substrates and the expression and activity of the amino acid decarboxylase enzymes (Hutkins 2006). Biogenic amine content of shalgam has also been investigated. Putrescine is the prevailing one, and its level was found 5.0–42.3 mg/L. Concentration of total biogenic amines varied from 26.7 to 134.3 mg/L. The concentrations present in shalgams that have been reported are quite below the maximum permissible limits (Ozdestan and Uren 2010).

40.2.2.5 Shelf Life of Shalgam

The shelf life of shalgam is limited to about 3–4 months during cold storage at 4°C in a sealed container (Erten et al. 2008; Tanguler 2010). Shalgam could be stored at 4°C and 20°C with sterile filtration for 6 months without any changes in its chemical, physical, microbiological, and sensory properties (Tanguler 2010).

Its shelf life could be extended by pasteurization (TSE 2003), but sensory characteristics of thermally treated shalgam due to cooked carrot flavor are not accepted (Canbas and Fenercioglu 1984). A maximum 200 mg/L of benzoic acid or its salts (sodium benzoate, potasium benzoate, calcium benzoate) as chemical additive may be used to increase its shelf life according to Turkish Food Codex Food Additives

TABLE 40.1

Specification of Shalgam

Specification	Minimum	Maximum
Titratable acidity expressed as lactic acid (g/L)	6.0	–
pH	3.3	3.8
Lactic acid (g/L)	4.5	5.5
Volatile acidity expressed as acetic acid (g/L)	0.7	1.2
Salt % (m/m)	–	2.0
Benzoic acid or its salts (mg/L)	–	200

Source: TSE, *TS 11149 Standard of Shalgam Beverage*, Turkish Standards Institute, Ankara, 2003, in Turkish. With permission. Anonymous, Turkish food codex food additives other than coloring and sweeteners, directive, *Turkish Food Codex Regulations, Regulation no: 2008/22*, Ministry of Agriculture and Rural Affairs, Ankara, 2008, in Turkish. With permission.

Other than Coloring and Sweeteners Directive (Anon 2008). The use of other preservatives is not permitted legally.

40.2.2.6 Turkish Specification

Turkish Standard Institution has the specification for shalgam (TSE 2003). Shalgam standards consists of the definition, classification, chemical, physical, microbiological, and sensory properties, sampling, analyses, packaging, labeling, storage, and others. Table 40.1 shows some chemical and physical specifications.

REFERENCES

Anonymous. 2008. Turkish food codex food additives other than coloring and sweeteners directive. Turkish food codex regulations, Regulation no: 2008/22. Ankara: Ministry of Agriculture and Rural Affairs. (In Turkish).

Arıcı M. 2004. Microbiological and chemical properties of a drink called shalgam. (Mikrobiologische und chemische eigenschaften von Shalgam), *Ernahrungs-Umschau* 51(1):10.

Berry SK, Manan JK, Joshi GJ, Saxena AK, Kalra CL. 1989. Preparation and evaluation of ready-to-serve (RTS) black carrot beverage (Kanji). *J Food Sci Technol* 26(6):327–8.

Blandio A, Al-Aseeri ME, Pandiella SS, Cantero D, Webb C. 2003. Cereal-based fermented foods and beverages. *Food Res Int* 36:527–43.

Canbas A. 1985. A study on the colour compounds of black carrot. *Turkish J Agric Forestry Seri* D_2 9(3):394–8, (In Turkish).

Canbas A, inventor; 1991. Recovery of anthocyanins from black carrot to be used in foodstuffs. *Eur Patent* 91,166,824.3.

Canbas A, Deryaoglu A. 1993. A research on the processing techniques and characteristics of shalgam beverage. *Turkish J Agric Forestry* 17:119–29.

Canbas A, Fenercioglu H. 1984. A study on shalgam beverage. *Gida (Food)* 9(5):279–86 (In Turkish).

Caplice E, Fitzgerald GF. 1999. Food fermentations: role of microorganisms in food production and preservation. *Int J Food Microbiol* 50:131–49.

Corsetti A, Settanni L, Valmorri S, Mastrangelo M, Suzzi G. 2007. Identification of subdominant sourdough lactic acid bacteria and their evolution during laboratory-scale fermentations. *Food Microbiol* 24:592–600.

Demir N, Acar J, Bahceci KS. 2004. Effects of storage on quality of carrot juices produced with lactofermentation and acidification. *Eur Food Res Technol* 218:465–8.

Demir N, Bahceci KS, Acar J. 2006. The effects of different initial *Lactobacillus plantarum* concentrations on some properties of fermented carrot juice. *J Food Proc Preserv* 30:352–63.

Deryaoglu A. 1990. A research on the processing techniques and characterictics of shalgam beverage. [MSc thesis]. Adana: Cukurova Univ. p 57 (In Turkish).

Erbas M, Certel M, Uslu MK. 2005. Microbiological and chemical properties of tarhana during fermentation and storage as wet-sensorial properties of tarhana soup. *LWT-Food Sci Tech* 38:409–16.

Erginkaya Z, Hammes WP. 1992. A study on the development of microorganisms during the shalgam fermentation and identification of isolated lactic acid bacteria. *Gida (Food)* 17(5):311–4 (In Turkish).

Erten H, Tanguler H. 2010. Fermented vegetable products. In: N Aran, editor. *Food Biotechnology*. Ankara: Nobel Yayin Dağitim: p 241–78. (In Turkish).

Erten H, Tanguler H, Canbas A. 2008. A traditional Turkish lactic acid fermented beverage: Shalgam (Salgam). *Food Rev Int* 24:352–9.

Gobbetti M, De Angelis M, Corsetti A, Di Cagno R. 2005. Biochemistry and physiology of sourdough lactic acid bacteria. *Trends in Food Sci Technol* 16:57–69.

Gunes G. 2008. A study on the determination of the most suitable quantity of black carrot (*Daucus carota*) for the production of shalgam. [MSc thesis]. Adana: Cukurova Univ. p 48 (In Turkish).

Harris LJ. 1998. The microbiology of vegetable fermentations. In: Wood BJB, editor. *Microbiology of Fermented Foods*. London: Blackie Academic & Professional. p 45–72.

Holzapfel WH, Wood BJB. 1995. Lactic acid bacteria in contemporary perspective. In: Wood BJB, Holzapfel WH, editors. *The Genera of Lactic Acid Bacteria*. Volume II. London: Blackie Academic & Professional. p 1–6.

Hutkins RW. 2006. *Microbiology and Technology of Fermented Foods*. Iowa: Blackwell Publishing IFT Press. p 473.

Kammerer D, Carle R, Schieber A. 2004. Quantification of anthocyanins in black carrot extracts (*Daucus carota* ssp. *sativus* var. *atrorubens Alef.*) and evaluation of their color properties. *Eur Food Res Tech* 219(5):479–86.

McFeeters RF, Pérez-Diaz I. 2010. Fermentation of cucumbers brined with calcium chloride instead of sodium chloride. *J Food Sci* 75(3):C291–6.

Nout MJR, Rombouts FM. 1992. Fermentative preservation of plant foods. *J Appl Bacteriol Symp* Supp 73:136S–47S.

Ozdestan O, Uren A. 2010. Biogenic amine content of shalgam (Şalgam): A traditional lactic acid fermented Turkish beverage. *J Agric Food Chem* 58:2602–8.

Paramithiotis S, Gioulatos Tsakalidou E, Kalantzopoulos G. 2006. Interactions between *Saccharomyces cerevisiae* and lactic acid bacteria in sourdough. *Proc Biochem* 41:2429–33.

Pistrick K. 2001. Umbelliferae (Apiaceae). In: Harelt P, editor. *Mansfeld's Encyclopedia of Agricultural and Horticultural Crops*. 1st edn. Berlin: Springer. p 1259–67.

Rodríguez-Sevilla MD, Villanueva-Suárez MJ, Redondo-Cuenca A. 1999. Effects of processing conditions on soluble sugars content of carrot, beetroot and turnip. *Food Chem* 66:81–5.

Sethi V. 1990. Lactic fermentation of black carrot juice for spiced beverage. *Indian Food Packer* 44(3):7–12.

Tanguler H. 2010. Identification of predominant lactic acid bacteria isolated from shalgam beverage and improvement of its production technique. [DPhil thesis]. Adana: Cukurova Univ. p 367 (In Turkish).

TSE 2003. *TS 11149 Standard of Shalgam Beverage*, Ankara: Turkish Standards Institute. (In Turkish).

Turker N, Aksay A, Ekiz HI. 2004. Effect of storage temperature on the stability of athhocyanins of a fermented black carrot (*Daucus carota* var. L.) beverage: Shalgam. *J Agric Food Chem* 52:3807–13.

Utus D. 2008. The effect of black carrot (*Daucus carota*) size usage on the quality of shalgam production. [MSc thesis]. Adana: Cukurova Univ. p 55 (In Turkish).

41

Fermented Coconut Milk and Coconut Oil

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CONTENTS

41.1 Introduction

41.1.1 Coconut (*Cocos nucifera*)

The scientific name for coconut is *Cocos nucifera*. It is called coco, meaning "monkey face," by early Spanish explorers, as the three notches on the hairy nut resemble the head and face of a monkey. *Nucifera* means "nut-bearing." The native habitat of coconuts is not known, but they can traditionally be found in Asia's coastal regions and the Pacific Islands within 20° north and south latitudes. Ideally, coconuts grow in regions where the temperatures are between 75°F and 85°F and not less than 68°F. They require rainfall of 60 to 80 inches per year (O'Brien 2004).

Populations around the world use coconuts as their source of meat, juice, milk, and oil. Coconut is highly nutritious and is full of fiber, vitamins, and minerals. It also provides many health benefits beyond its nutritional content. Coconut has also been one of the sources of economy to nearly one third of the world's population. Among these cultures, the coconut has a long and respected history.

Coconut oil is an edible oil that is derived from the seeds of coconut palm. Nowadays, coconuts are grown in about 93 countries in an area of 11.8 million hectares (Bouaid et al. 2010). It is possibly the healthiest, most versatile, unprocessed dietary oil in the world. Coconut oil is extensively used in traditional medicine among Asian and Pacific populations. The Pacific Islanders called coconut the "tree of life."

41.1.2 Coconut Oil Composition

Coconut oil contains approximately 50% lauric acid. Lauric acid oils are unique in that they can pass abruptly from a brittle solid to a liquid, within a narrow temperature range. Coconut oil is highly oxidative stable, as more than 90% of its fatty acids are saturated (O'Brien 2004). It is rich in medium-chain triglycerides (MCTs). Medium-chain fatty acids (MCFAs) have 8 to 12 carbon atoms and are found mostly in butterfat and tropical oils. Like the short-chain fatty acids, these fats have antimicrobial properties, are absorbed directly for quick energy, and can contribute to the health of the immune system (Marten et al. 2006). Coconut oil is a major component of infant formulas and medical foods for people who cannot absorb long-chain fatty acids (O'Brien 2004).

The MCFAs of coconut oil such as lauric (C-12), caprylic (C-10), and mystic (C-14) acids are different from the common longer chain fatty acids (LCTs) found in other plant-based oils. LCTs are typically stored in the body as fat, while MCTs are preferably metabolized for energy (Kabara 1998). MCTs are solubilized in the aqueous phase of the intestinal contents, where they are absorbed, bound to albumin, and transported directly to the liver via the portal vein. MCTs are transported directly to the liver and enter the mitochondria without the benefit of carnitine cycle (Carandang 1998). Compared to long-chain fats, MCTs decrease protein catabolism in hypercatabolic states, raise thyroid function, are deposited less into adipose tissue, and do not form esters with cholesterol (Kabara 1998).

41.2 Coconut Oil Processing

Vegetable oils have been used by mankind for centuries for both food and nonfood applications. Before oil is obtained, oilseed such as coconut has to undergo either an expression or an extraction process to remove the entrapped oil. Expression is the process of mechanically pressing liquid out of the liquid-solid system (Mpagalilile et al. 2005; Brennan et al. 1990; Willems et al. 2008a,b). The oil obtained via these methods is of high quality, but the attainable yield is limited to roughly 80 wt% of the oil originally present. It is only in the last century that solvent extraction has been used in this field. The advantage of solvent extraction is the high yield that can be obtained economically with this method (>90 wt%) but at the expense of reduced oil quality (Willems et al. 2008a,b).

41.2.1 Virgin Coconut Oil Extraction

Several methods are currently practiced for removing oil from either fresh coconut meat or copra (dried coconut kernel). These techniques include

1. Solvent extraction
2. Dry process
3. Wet process

41.2.1.1 Solvent Extraction

Solvent extraction is the most frequently applied sample preparation procedure in plant material analysis. The method is limited by the compound solubility in the specific solvent used, and hence the quality and quantity of the extracted mixture are determined by the type of solvent applied. Although the method is relatively simple and quite efficient, it suffers from problems such as a long extraction time, relatively high solvent consumption, and often unsatisfactory reproducibility (Dawidowicz et al. 2008). Solvent extraction is possible with an appropriate solvent, such as benzene or *n*-hexane.

The determination of the content of either edible oil or total fat in oleaginous seeds is of paramount importance to the oil industry as the price of the raw material is a function of its richness in the commercial product. Traditionally, this determination has been based on leaching of ground seeds with an organic solvent and weighing the residue after solvent evaporation. The most widely used procedure for removing oil from the solid matrix remains conventional Soxhlet extraction, which is straightforward

and inexpensive but is also slow and tedious. The most severe shortcomings of Soxhlet extraction are the long time involved and the large volumes of organic solvents that are released into the atmosphere as the Soxhlet procedure is far from clean (Ayuso-García et al. 1999). Even though oil recovery is high, the process is rarely applied owing to its high risk and high investment cost (Mpagalile et al. 2005; Che Man et al. 1997).

A great variety of new approaches based on different principles such as supercritical fluid extraction, microwave irradiation, and closed system at high temperature and pressure (pressurized liquid extraction or accelerated solvent extraction) have been developed in the last few years to circumvent the shortcomings of conventional Soxhlet extraction. None surpasses it in the extraction of edible oils for reasons such as decreased efficiency of supercritical fluid extraction due to matrix effects, or the low polar nature of the solvent in microwave-assisted heating of the solvent and sample-solvent partitioning of the analytes in accelerated solvent extraction. The ideal approach would be one retaining the advantages of Soxhlet extraction (namely, sample—fresh solvent contact during the whole extraction step, no filtration step, simple manipulation) while circumventing its shortcomings by accelerating the process and minimizing environmental pollution (Ayuso-García et al. 1999).

41.2.1.2 Dry Process

The dry process is the present commercial technique for coconut oil extraction. Copra is cleaned, ground, steamed, and pressed through an expeller for coconut oil extraction. The effect of process parameters on oil expression efficiency from freshly dried coconut gratings was investigated by Mpagalile et al. (2005). The range of parameters investigated was: pressing time, particle size, pressure, moisture content, and temperature. The study indicated that oil expression from coconuts was highly dependent on the moisture content of the materials being pressed. Moisture content played a significant role in the improvement of oil expression efficiency under the two pressure levels investigated, namely low pressure (3%/13 MPa) and high pressure (13%/33 MPa). According to the results, optimum moisture content at low-pressure pressing was 11%. Other optimum pressing conditions for low-pressure coconut oil expression were a temperature of 60°C, fine gratings, and a pressing time of 8 min. The study has shown that oil release mechanisms tend to be highly influenced by the pressing conditions and the process variables.

41.2.1.3 Wet Process

The wet process is not subjected to initial drying of coconut (Hagenmaier et al. 1971). It can be carried out by crushing the coconut meat with water and filtering it to produce coconut milk or coconut cream. This emulsion contains a mixture of oil, water, protein, and sludge. Coconut oil can be separated through cold extraction, centrifuge, cooking, or fermentation.

The traditional fermentation method requires the mixture to be fermented for approximately 48 hours at a temperature of about 35°C. The oil is then heated at adequate temperature and timed to remove the access moisture to avoid rancidity. The purpose of the fermentation or enzymatic processes is to destabilize the emulsion and allow the separation of oil phase and water phase (carbohydrate and protein) (Soeka et al. 2008, Rahayu et al. 2008; Handayani et al. 2009).

However, coconut oil extraction by these wet process techniques has not been commercially successful. Usually, the recovery of coconut oil by the traditional wet process is low, about 30%–40%. Moreover, the oil obtained is of poor quality owing to the high moisture content (MC), dark color, and short shelf life. On the other hand, the traditional method is easy to handle, and the extracted coconut oil has a pleasant aroma and low free fatty acids.

Che Man et al. (1997) studied the use of 0.1%–0.4% acetic acid (25%) for coconut oil extraction and showed a recovery of 58.3%–60.3% of good-quality oil. In another study, Che Man et al. (1996) obtained a yield of 73.8% of good-quality oil with an enzyme mixture at 1% (w/w) each of cellulase, α-amylase, polygalacturonase, and protease at pH 7.0 and an extraction temperature of 60°C. pH was the most significant parameter in the enzymatic process. The process allowed initial purification by removal of the sludge in the aqueous phase. However, the stability of the lipoprotein emulsion limited the process efficiency of the oil separation (Sant'Anna et al. 2003).

41.2.1.3.1 Integrated Wet Process

The integrated wet process is a modified wet process introduced by the Institute of Bioproduct Development (IBD), Universiti Teknologi Malaysia, to obtain virgin coconut oil (VCO). This advance wet process involves cooling, churning, low heating, and centrifuging. Cooling is the mechanism used to de-emulsify the coconut milk. In the churning process, coconut milk needs to be converted into butter form. This method will remove as much water as possible from the coconut milk. The maximum permitted water content of butter (16%) is regulated by the International Standards set out by the Food and Agriculture Organization (WHO). The water content affects the quality of the butter, including its flavor and texture. The water content is affected by (Funahashi 2008):

a. Milk composition
b. Fat content of the cream
c. Cooling temperature of the cream
d. Physical ripening time of the cream
e. Cream feed temperature
f. Cream flow rate
g. Shear rate on the butter
h. Buttermilk draining

A previous study done by Funahashi (2008) showed that cream feed temperature showed the strongest influence on water content of the butter. Milk fat globules agglomerate more readily with increasing cream feed temperatures. This arises from the lower viscosity of the cream and the greater availability of free fat on the surface of the fat globules, leading to reduced churning time. The higher the temperature, the higher is the water holding capacity between the butter grains. The study showed that 10.5°C would be the best temperature for the churning process. A difference of 1°C in the temperature corresponded to a difference of 1% v/v in the water content of the butter.

The integrated wet process can produce high-quality product at high yield and shorter processing time. It can also produce coconut oil with the highest heat stability. The fermentation process needs to be carried out for 3 days to obtain VCO. On the other hand the integrated wet process only takes less than a day. Figure 41.1 shows the flow diagram for extracting VCO via the integrated wet process.

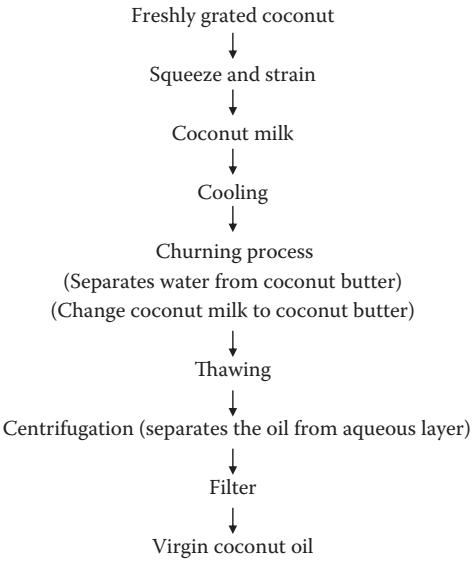

FIGURE 41.1 VCO extraction via integrated wet process.

41.3 Metabolite Profiling of Coconut Oil

Metabolites are quantifiable molecules that best reflect phenotype. Numerous studies have been carried out to profile coconut oil with various analytical approaches; however, technologies to quantify every metabolite in coconut oil simultaneously have yet to emerge. The biological informatics has yet to embrace the study of metabolites as aggressively as genomics. Because of the vast chemical diversity of metabolites and their wide variations, metabolomics research usually requires multiple techniques as certain classes of samples are more amenable to one analytical technique than others. With the present development of analytical equipment, the most common subclass of metabolites, namely lipids, can be profiled and a nearly complete database can be produced.

41.3.1 Lipid Profile of Coconut Oil

Researchers in IBD, UTM have profiled lipids in coconut oil using ultra-high performance liquid chromatography integrated with electrospray ionization tandem mass spectrometer. The lipid profiles of three different processing techniques, such as integrated process, wet process (fermentation), and dry process (cold press) have been developed and compared using the software LipidView™ 1.0 from Applied Biosystems/MDS Analytical Technologies. The software is a data processing tool that identifies and semiquantitates lipids in electrospray mass spectrometry data. The total amount of lipids detected in the fermentation method is the highest (141), followed by cold press (95) and integrated process (73). The presence of higher amount of lipids in coconut oil produced from fermentation might be contributed by the activities of enzymes during fermentation.

Lipids are an attractive subset of metabolites for the first practical steps in metabolomics applications (Watkins et al. 2001). Lipids reflect diet and metabolism in addition to their cellular functions as structural, energetic, and bioactive signaling molecules. In particular, fatty acids and sterols are the major components of lipids that survive digestion intact and enter cellular metabolic pathways. Therefore, profiling of lipid metabolites is the crucial step in understanding the role of coconut oil in modifying metabolism and ultimately in promoting health.

The lipid profile of coconut oil can be generally divided into four classes: glycerolipids, phospholipids, sterolipids, and sphingolipids. Phospholipids cover the highest percentage, which are about 50% of total lipids in coconut oil. The top three phospholipids are phosphatidic acid, phosphatidyletanolamine, and phosphatidylchlorine. No sterolipid is detected in coconut oil using the three mentioned processing approaches. The amount of sphingolipids in coconut oil is higher than glycerolipids. Glycerolipids consist of tri-, di-, and mono-acylglycerols, which are the most commonly studied lipid class. Among the three processing techniques, coconut oil from the integrated process contains the highest amount of triacylglycerols from carbon 40 to carbon 50. Triacylglycerols with the carbon number starting from 44 to 50 are detected from coconut oil produced from the dry process. The wet process produces coconut oil containing triacylglycerols with carbon number 46 only. In addition to triacylglycerols, diacylglycerols with carbon number from 42 to 44 are also detected in coconut oil obtained from the wet process, but not in the coconut oil from the dry process and the integrated process.

Young (1983) has also studied the analytical characteristics of coconut oil. He reported that coconut oil contains approximately 84% trisaturated glycerides, 12% disaturated-monosaturated glycerides, and 4% monosaturated-diunsaturated glycerides. These triglycerides can be divided into 13 groups from carbon 28 to carbon 52. These groups contain 79 individual triglycerides. Marina et al. (2009) reported that the major triacylglycerols in VCOs are LaLaLa (22.78%–25.84%), CLaLa (19.20%–21.38%), CCLa (14.43%–16.54%), LaLaM (13.62%–15.55%), and LaMM (7.39%–9.51%) with La, C, and M are lauric, capric, and myristic acids, respectively. Somehow, it is important to note that the triacylglycerol content of coconut oil is very much affected by the processing technique. Therefore, the selection of processing technique is essential as it would affect the triacylglycerol profile of coconut oil, which is indirectly influencing the application of the oil. Triacylglycerols have significant benefit in antiviral, antibacterial, anticaries, antiplaque, and antiprotozoal functions (German and Dillard 2004; Kabara 1978; Hierholzer and Kabara 1982).

41.3.2 Polyphenolics in Coconut Oil

Besides lipids, coconut oil also contains a lot of minerals, vitamins (provitamins A and E), phytosterols, and phenols. The presence of small quantities (less than 150 ppm) of δ- and γ-lactones affect the odor and taste of coconut oil. Phenolic substances are generally subdivided into simple phenols and polyphenols. The latter is characterized by the presence of more than one aromatic ring in a molecule. They are mostly secondary metabolites that exhibit a number of physiological effects in living organisms. Polyphenols have been reported to exert a variety of biological actions such as free radicals scavenging, metal chelation, modulation of enzyme activity, signal transduction, activation of transcription factors, and gene expression. These compounds are also capable of decreasing total cholesterol and LDL cholesterol in serum and tissues as well as increasing the antioxidant status.

Common plant phenols include phenolic acids (hydroxyl derivatives of benzoic and cinnamic acids) and their esters (chlorogenic and caftaric acids), chalcones (butein, okanin, licochalcone, and dihydrochalcones [phloridzin]), coumarins (aesculetin), flavonoids (flavonols, flavones, flavanonols, isoflavones, and/or anthocyanidins), and lignans. They can be found as free molecules (aglycones), in the form of conjugates (glycosides) or even as oligomers or polymers, for instance proanthocyanidins, lignans, or lignin.

Marina et al. (2009) reported that total sterol content in coconut oil averages around 0.096%. A wide spectrum of polyphenolics from the class of flavonoids can be found in coconut oil as antioxidative compounds (Nevin and Rajamohan 2006, 2008). Preliminary chemical analysis of polyphenols using UV-vis spectrometry method by Nevin and Rajamohan (2006) showed the presence of flavanones and flavonols in coconut oil.

In addition to flavonoids, phenolic acids can also be found in coconut oil. The identified phenolic acids are protocatechuic, vanillic, caffeic, syringic, ferulic, and *p*-coumaric acids (Marina et al. 2008; Seneviratne and Dissanayake 2008). The antioxidant activity in coconut oil could be due to these phenolic compounds. Phenolic antioxidants are abundantly present in plant sources. They can be in milligrams to grams per kilogram dry weight and approximately 4000 plant phenols are currently known (Vacel et al. 2010).

41.4 Applications of Coconut Oil

Coconut oil was recognized as a health oil in Ayurvedic medicine almost 4000 years ago. The findings of Kabara (1978) triggered a series of studies that led to the discovery of a number of applications of coconut oil from nutrition and health benefits to skincare.

41.4.1 Nutritional and Health Benefits of VCO

The nutritional benefit and health of coconut oil is believed to be derived from the chemistry of the medium-chain saturated fatty acids and its minor components. Coconut oil is easily digested, absorbed, and utilized by the cells.

Coconut oil will be converted to monolaurin when consumed in the body. Monolaurin has been shown to exhibit antimicrobial and antiviral properties. Its antiviral property is especially targeted to lipid-coated viruses such as HIV, herpes, cytomegalovirus, influenza, pathogenic bacteria, and protozoa (Enig 1993). The first clinical trial of monolaurin on HIV-infected patients was carried out at the San Lazaro Hospital in Manila (Dayrit 2008), and the result was very encouraging. According to Enig (1997), the AIDS organization "Keep Hope Alive" has documented the effectiveness of coconut oil in lowering the viral load of patients with HIV–AIDS.

Herpes virus and cytomegalovirus can result in the initial formation of atherosclerotic plaques and reclogging of arteries. According to Dayrit (2008), heart disease can be due to inflammatory process, microbial infection, and free radical injury to blood vessels. When there is no lauric acid source in the diet, the body cannot produce monolaurin to inhibit the viruses. Cholesterol is then triggered to oxidize in the presence of rancid food oils in the body, resulting in the formation of artery clogging material. The accumulation of atheromas would lead to the incidence of coronary heart disease.

Monolaurin is also present in human breast milk. It provides the protection from viral or bacterial or protozoal infections. This is the reason why breast-fed babies are usually healthier than bottle-fed babies. The medium-chain fats in coconut oil are similar to fats in mother's milk and have similar nutraceutical effects (Kabara 1998).

Monolaurin and the ether analogue of monolaurin have been shown to have the potential to dampen adverse reactions to glutamic acid. Monolaurin can induce proliferation of T-cells and inhibit the toxic shock syndrome toxin-1 mitogenic effects on T-cells (Nevin and Rajamohan 2006).

Many researchers have reported that coconut oil lowers cholesterol (Blackburn et al. 1988; Prior et al. 1981; Kurup and Rajmohan 1995). Nevin and Rajamohan (2004) reported that VCO lowered total cholesterol, triglycerides, phospholipids, low density lipoprotein (LDL), and very low density lipoprotein (VLDL) cholesterol levels but increased high density lipoprotein (HDL) cholesterol in serum and tissues. Since coconut oil tends to improve HDL profile, this oil can actually protect consumers from heart disease or stroke attack risk.

The fatty acids from the cholesterol esters are 74% unsaturated (38% polyunsaturated and 36% monounsaturated) and only 24% are saturated. None of the saturated fatty acids were reported to be lauric or myristic acids (Felton et al. 1994). Thus, the high intakes of coconut oil do not cause coronary artery disease. These MCFAs are easily digested because they go straight to the liver through the portal vein and are synthesized instantly into energy, thereby increasing body metabolism.

Isaac and Thormar (1990) reported that the antimicrobial effects of coconut oil are additive. The total concentration of the fatty acids and monoglycerides is critical for virus inactivation. The properties of antimicrobial action of lipids are related to their structure. Monoglycerides are active, whereas diglycerides and triglycerides are inactive. The fatty acids and monoglycerides produce their killing effect by lysing the plasma membrane lipid bilayer and solubilizing the lipids and phospholipids in the envelope of the virus (Isaac and Thormar 1991; Isaac, 1992). This antiviral action will cause the disintegration of the virus envelope. The inactivation and disintegration of the lipid covering pathogenic microorganisms will directly expose them to natural antibodies. Isaac and Schneidman (1991) reported that all members of the herpes virus family could be killed by saturated fatty acids and monoglycerides ranging from C6 to C14, which include approximately 80% of the fatty acids in coconut oil. However, Projan et al. (1994) proposed that the antimicrobial effect is related to the interference of monolaurin with signal transduction. Another hypothesis is that the antimicrobial effect is due to the interference of lauric acid with virus assembly and viral maturation (Hornung et al. 1994).

Coconut oil has a unique role in the diet as an important physiologically functional food. The term "functional foods" is defined as a food that provides health benefit over and beyond basic nutrients. As a functional food, coconut provides both energy and nutrients with its monoglycerides as its main functional components. For example, coconut oil is capable to stimulate thyroid function. In the presence of adequate thyroid hormone, LDL cholesterol will be converted to antiaging steroids, pregnenolone and progesterone. These substances are required in preventing the promotion of degenerative diseases and aging symptoms. Under certain in vitro conditions, medium-chain triacylglycerols exert proinflammatory effects but in vivo medium-chain triacylglycerols may reduce intestinal injury and protect from hepatotoxicity (Marten et al. 2006).

Compared to long-chain fatty acids, the chemical and physical properties of MCFAs show substantial metabolic differences (Marten et al. 2006). These distinct properties affect the way MCFAs are absorbed and metabolized. MCFAs have a lower melting point and have smaller molecule size, and thus coconut oil can easily permeate to cell membranes and be readily burned for energy. MCFAs also do not require binding to fatty acid binding protein, fatty acid transport protein, or fatty acid translocase (Marten et al. 2006). Long-chain fatty acids, on the other hand, are difficult to break down in the body and special enzymes are required for digestion (Lim-Sylianco 1987). Hence, long-chain fatty acids put more strain on the pancreas, liver, and the entire digestion system. Finally, long-chain fatty acids are stored in the body as fat and may be deposited within arteries as cholesterol (Lim-Sylianco 1987). Several aspects of MCFA metabolism affect features of the metabolic syndrome such as plasma lipid levels, insulin resistance, and inflammatory response, as well as weight management (Marten et al. 2006).

41.4.2 Coconut Oil in Cosmeceutical Applications

The essential cosmetic care for skin is moisturization. Moisturizers are expected to increase skin hydration and to modify the chemical nature of the surface to one that is smooth, soft, and pliable (Elsner and Maibach 2002). Moisturizers are externally applied compounds comprising multiple components, including occlusive ingredients and humectants. Occlusive moisturizing ingredients are oily substances that impair the evaporation of skin moisture by forming an epicutaneous greasy film that impedes water loss. Humectants are compounds that attract water from the dermis into the stratum corneum. Moisturization of the stratum corneum occurs from below, with the dermis contributing moisture to the skin (Flynn et al. 2001).

VCO is a popular ingredient in traditional skin care application especially in the South East Asia region. Norhayati et al. (2010) showed that a moisturizer incorporated with 40% of VCO-loaded solid-lipid microparticles (SLM) was most effective as a moisturizer compared to the others, both in terms of skin moisture and elasticity. Efficacy tests were conducted on 30 female volunteers with normal skin types. They were divided into two groups, and each group was given two types of moisturizing cream. Group 1 applied creams without VCO and creams with 20% VCO-SLMs. Group 2 applied creams with 30% VCO-SLMs and 40% VCO-SLMs. They were required to apply the cream on their volar forearm at specific areas twice a day (morning and night) for 28 days. All creams with VCO-SLMs as an active ingredient were more effective than the one without VCO-SLMs in retaining skin moisture. The highest increase in skin moistness (27.26%) from day 0 to day 28 was exhibited by volunteers using the moisturizer loaded with 40% VCO-SLMs. The lowest increase in moistness (19.40%) was exhibited by volunteers using the moisturizer without VCO-SLMs. The highest increase in skin elasticity (3.62%) from day 0 to day 28 was exhibited by volunteers using the moisturizer loaded with 40% VCO-SLMs. The lowest increase in skin elasticity (1.73%) was exhibited by volunteers using the moisturizer without VCO-SLMs.

Aromatherapy is an expanding complimentary therapy. One of the aromatherapy products commercially available is aromatherapy massage oil. The main ingredients of massage oils are carrier oils and essential oils. Essential oils are the active ingredients in aromatherapy, whereas carrier oils are used to dilute essential oils prior to skin application.

VCO is highly suitable to be used as a carrier oil for aromatherapy (Marin 2006). Songkro et al. (2010) characterized VCO-based aromatherapy massage oils. The VCO-based massage oils were clear and colorless. The only distinctive smell of the massage oil was from the blended essential oils. The results of an accelerated storage stability test showed no color change, no precipitation, no alteration in the aromatic scents but an increase in the peroxide and acid values of the massage oil compared to initial values.

41.5 Conclusions

Coconut oil is rich in MCFA. Various methods have been developed to extract coconut oil, either through dry or wet processing. Recently, there is a trend toward producing coconut oil that does not have to go through the RBD (refining, bleaching, and deodorizing). Rather than going to the normal dry process, this oil is obtained by wet processing, which entails the extraction of the cream from the fresh coconut milk and consequently breaking the cream emulsion. This process is more desirable, as no chemical or high heat treatment is imposed on the oil. The process consists of chilling, freezing, and thawing techniques.

Coconut oil has many applications. A large percentage of its uses are for edible and health purposes. Coconut oil is a well known functional food that provides health benefits over and beyond basic nutrients. Animal and human studies have shown that MCFA has no known toxicological properties orally, parenterally, or by dermal routes (Bach and Babayan 1982; CTFA 1980; Traul et al. 2000). Thus, coconut oil is safe to be consumed as a supplement. Traul et al. (2000) suggested that MCFA supplement can be consumed at levels of up to 15% of the dietary calories or approximately 50% of the dietary fat.

Apart from its edible uses, this versatile oil also has industrial applications. It is employed as one of the feedstock in the manufacturing of chemicals, synthetic detergents, soaps, and cosmetics (Rele et al.

2002; Carlsson 2009). Coconut oil has even been selected as an alternative base oil for industrial lubricants (Jayadas et al. 2006).

REFERENCES

Alonso-Salces RM, Korta E, Barranco A, Berrueta LA, Gallo B, Vicente F. 2001. Pressurized liquid extraction for the determination of polyphenols in apple. *J Chromatogr A* 933:37–43.

Ayuso-García LE, Luque de Castro MDL. 1999. A multivariate study of the performance of a microwave-assisted Soxhlet extractor for olive seeds. *Analytica Chimica Acta* 382:309–16.

Bach A, Babayan V. 1982. Medium chain tri-glycerides: an update. *Am J Clin Nutrit* 36:950–62.

Benthin B, Danz H, Hambuger M. 1999. Pressurized liquid extraction of medicinal plants. *J Chromatogr A* 873:211–9.

Blackburn GL, Kater G, Mascioli EA, Kowalchuk M, Babayan VK, Bistrian BR. 1988. A reevaluation of coconut oil's effect on serum cholesterol and atherogenesis. *J Philippine Med Assoc* 65:144–52.

Bouaid A, Martinez M, Aracil J. 2010. Biorefinery approach for coconut oil valorization: A statistical study. *Bioresource Technol* 101:4006–12.

Carandang EV, Gamboa GG. 1998. A preliminary study on the frequency of resistance development of *Staphylococcus aureus* to penicillin G in combination with monolaurin. *Philippine J Coconut Stud* 23(2):16–20.

Carlsson AS. 2009. Plant oils feedstocks alternatives to petroleum—A short survey of potential oil crop platforms. *Biochimie* 91:665–70.

Che Man Y, Suhardiyono, Asbi AB, Azudin MN, Wei LS. 1996. Aqueous enzymatic extraction of coconut oil. *JAOCS* 73:683–6.

Che Man YB, Abdul Karim MIB, Teng CT. 1997. Extraction of Coconut Oil with *Lactobacilius plantarum* 1041 IAM. *JAOCS* 74:1115–9.

Choi MPK, Chan KKC, Leung HW, Huie CW. 2003. Pressurized liquid extraction of active ingredients (ginsenosides) from medicinal plants using non-inonic surfactant solutions. *J Chromatogr A* 983:153–62.

CTFA. 1980. Expert report: Final report of the safety assessment of the caprylic/capric triglyceride. CTFA cosmetic ingredient review. Cosmetic, Toiletries and Fragrance Association. *J Environ Pathol Toxicol* 4:105–20.

Dayrit CS. 2008. Coconut oil in health and disease: Its and monolaurin's potential as cure for HIV/AIDS. Pharmacology University of the Philippines. Accessed on 8 Jan 2008.

Elsner P, Maibach HI. 2000. *Cosmeceuticals Drugs vs. Cosmetic*. Volume 23. Madison Avenue, N.Y.: Marcel Dekker, Inc.

Enig MG. 1993. Diet, serum cholesterol and coronary heart disease. In: Mann GV, editor. *Coronary Heart Disease: The Dietary Sense and Nonsense*. London: Janus Publishing. p 36–60.

Enig MG. 1997. Coconut oil: Anti-bacterial, Anti-viral ingredient for food, nutrition and health. AVOC Lauric Symposium. Oct 17, 1997; Manila, Philippines.

Felton CV, Crook D, Davies MJ, Oliver MF. 1994. Dietary polyunsaturated fatty acids and composition of human aortic plaques. *Lancet* 344:1195–6.

Flynn TC, Petros J, Clark RE, Viehman GE. 2001. Dry Skin and Moisturizers. *Clinics in Dermatology* 19:387–92.

Funahashi H, Horiuchi J. 2008. Characteristics of the churning process in continuous butter manufacture and modeling using an artificial neural network. *Int Dairy J* 18:323–8.

German JB, Dillard CJ. 2004. Saturated fats: What dietary intake? *Am J Clin Nutr* 80:550–9.

Hagenmaier R, Cater CM, Mattil KF. 1971. *Critical Unit Operations of the Aqueous Processing of Fresh Coconuts*. Texas: Food Protein Res Dev Center.

Handayani R, Sulistyo J, Rahayu RD. 2009. Extraction of coconut oil (*Cocos nucifera L.*) through fermentation system. *Biodiversitas* 10:151–7.

Hierholzer JC, Kabara JJ. 1982. *In vitro* effects of monolaurin compounds on enveloped RNA and DNA viruses. *J Food Safety* 4:1–12.

Hornung B, Amtmann E, Souer G. 1994. Lauric acid inhibits the maturation of vesicular stomatitis virus. *J Gen Virol* 75:353–61.

Isaac CE, Litov RE, Marie P, Thormar H. 1992. Addition of lipases to infant formulas produces antiviral and antibacterial activity. *J Nutr Biochem* 3:304–8.

Isaac CE, Schneidman K. 1991. Enveloped viruses in human and Bovine milk are inactivated by added fatty acids and monoglycerides. *FASEB J* 5:pA1288.

Isaac CE, Thormar H. 1990. Human milk lipids inactivated enveloped viruses. In: Atkinson SA, Hanson LA, Chandra RK, editors. *Breastfeeding, Nutrition, Infection and Infant Growth in Developed and Emerging Countries*. St. John's NF, Canada: Arts Biomedical Publishers and Distributors.

Isaac CE, Thormar H. 1991. The role of milk-derived antimicrobial lipids as antiviral and antibacterial agents in immunology of milk and the neonate. In: Mestecky J, and other editors. New York: Plenum Press.

Jayadas NH, Nair KP. 2006. Coconut oil as base for industrial lubricants-evaluation and modification of thermal, oxidative and low temperature. *Tribol Int* 39:873–8.

Kabara JJ. 1978. Fatty acids and derivatives as antimicrobial agents—A review. Symposium on the Pharmacological Effects of Lipids, AOCS 1–13.

Kabara JJ. 1998. *Nutritional and Health Aspects of Coconut Oil*. United States: Health Oils from the Tree of Life.

Kauffman B, Christen P. 2002. Recent extraction techniques for natural products: Microwave-assisted extraction and pressurised solvent extraction. *Phytochem Anal* 13:105–13.

Kurup PA, Rajmohan TII, editors. 1994. Consumption of coconut oil and coconut kernel and the incidence of atherosclerosis. Coconut and coconut oil in human nutrition, Symposium on coconut and coconut oil in human nutrition; 1994 March 27; India.

Lim-Sylianco CY. 1987. Anticarcinogenic effect of coconut oil. *Philippine J Coconut Stud* 12:89–102.

Marin I. 2006. *Aromatherapy for Massage Practitioners*. Sydney: Lippincott Williams & Wilkins, 149–58.

Marina AM, Che Man YB, Amin I. 2009. Virgin coconut oil: emerging functional food oil. *Trends Food Sci Technol* 20:481–7.

Marina AM, Che Man YB, Nazimah SAH, Amin I. 2008. Antioxidant capacity and phenolic acids of virgin coconut oil. *Int J Food Sci Nutr* 60:114–23.

Marten B, Pfeuffer M, Schrezenmeir, J. 2006. Medium-chain triglycerides. *Int Dairy J* 16:1374–82.

Mpagalile JJ, Clarke B. 2005. Effect of processing parameter on coconut oil expression efficiencies. *Int J Food Sci Nutr* 56(2):125–32.

Nevin KG, Rajamohan T. 2004. Beneficial effects of virgin coconut oil on lipid parameters and *in vitro* LDL oxidation. *Clin Biochem* 9(37):830–5.

Nevin KG, Rajamohan T. 2008. Influence of virgin coconut oil on blood coagulation factors, lipid levels and LDL oxidation in cholesterol fed Sprague-Dawley rats. E-SPEN. *Eur e-J Clin Nutr Metab* 3:e1-8.

Nevin KG, Rajamohan T. 2006. Virgin coconut oil supplemented diet increases the antioxidant status in rats. *Food Chem* 99:260–6.

Norhayati MN, Norhimdayah I, Azila AA, Mohd Amin SM, Mohamad Roji S, Ramlan A. The effect of virgin coconut oil loaded solid lipid microparticles (VCO-SLMs) on skin hydration and skin elasticity. In: Hesham AMAE, editor. *Proceedings of the 3rd International Conference on Biotechnology for the Wellness Industry; 2010 Oct. 8–9*; Kuala Lumpur, 47.

O'Brien RD. 2004. *Fats and Oils Formulating and Process for Application*. 2nd ed. Florida: CRC Press.

Prior IA, Davidson F, Salmond CE, Czochanska Z. 1981. Cholesterol coconuts and diet on Polynesian atolls: A natural experiment: The Pukapuka and Tokelau Island studies. *Am J Clin Nutr* 34:1552–61.

Projan SJ, Brown-Skrobot S, Schlievert PM, Vandenesch F, Norick RP. 1994. *J Bacteriol* 176:4204–9.

Rele AS, Mohile RB. 2002. Effect of mineral oil, sunflower oil, and coconut oil on prevention of hair damage. *J Cosmetic Sci* 54:175–92.

Sant'Anna BPM, Freitas SP, Coelho MAZ. 2003. Enzymatic aqueous technology for simultaneous coconut protein and oil extraction. *Fasc* 1(54):77–80.

Seneviratne KN, Dissanayake MS. 2008. Variation of phenolic content in coconut oil extracted by two conventional methods. *Int J Food Sci Technol* 43:597–602.

Songkro S, Sirikatitham A, Sungkarak S, Buaking K, Wungsintaweekul J, Maneenuan D, Oungbho K. 2010. Characterization of aromatherapy massage oils prepared from virgin coconut oil and some essential oils. *J Am Oil Chem Soc* 87:93–107.

Traul KA, Driedger A, Ingle DL, Nakhasi D. 2000. Review of the toxicologic properties of medium chain triglycerides. *Food Chem Toxicol* 38:79–98.

Vacel J, Ulrichova J, Klejdus B, Simanek V. 2010. Analytical methods and strategies in the study of plant polyphenolics in clinical samples. *Anal Method* 2:604–13.

Watkins SM, Hammock BD, Newman JW, German JB. 2001. Individual metabolism should guide agriculture toward foods for improved health and nutrition. *Am J Clin Nutr* 74:283–6.

Willems P, Kuipers NJM, De Haan AB. 2008a. Hydraulic pressing of oilseeds: Experimental determination and modeling of yield and pressing rates. *J Food Eng* 89:8–16.

Willems P, Kuipers NJM, De Haan AB. 2008b. Gas assisted mechanical expression of oilseeds: Influence of process parameters on oil yield. *J Supercrit Fluids* 45:298–305.

Young FVK. 1983. Palm kernel and coconut oils: Analytical characteristics, process technology and uses. *JAOCS* 2:374–9.

42

Coffee Fermentation

Rosane Freitas Schwan, Cristina Ferreira Silva, and Luis Roberto Batista

CONTENTS

42.1 Introduction

Originally, coffee was grown in the Kaffa region in Ethiopia; later, in the early 17th century, the Dutch spread it throughout Europe. Coffee reached North America in the beginning of the 17th century and South and Central America in 1825. The habit of drinking coffee was developed in Arab culture, and the coffee beverage was known only for its properties as a stimulant. Coffee fruit was consumed raw and fed to herds as a stimulant while they were traveling. In 1000 AD, the Arabs began to prepare an infusion of coffee cherries, boiling them in water. The roasting process was developed only in the 14th century, and coffee finally became similar to today's beverage (ABIC 2010).

As a beverage, coffee is characterized as energizing and nonalcoholic, with potential beneficial effects on human health; it mainly acts as an antioxidant (Andueza et al. 2004). There are different types of coffee beverages characterized by different nuances in terms of body, aroma, acidity, and astringency.

One hundred species are associated with the genus *Coffea*, but only two species are agroeconomically important, *Coffea arabica* and *Coffea canephora*. *Coffea dewevrei*, *C. congensis*, *C. eugenioides*, *C. kapakata*, *C. salvatrix*, *C. stenophyla*, *C. liberica*, *C. racemosa*, and others are primarily used in genetic crosses. *Coffea arabica*, known as Arabica in the coffee market, makes up 70% of total production volume, and *C. canephora* (Robusta coffee) is responsible for the remaining 30%. Currently, Brazil is the largest producer of arabica coffees, followed by Colombia, Paraguay, Venezuela, Indonesia, Ethiopia, India, Mexico, and 40 other countries. The United States, Brazil, and the European countries are the major consumer markets.

TABLE 42.1

Chemical Compounds and Their Respective Concentrations Present in Coffee Beans Used as Chemical Markers to Differentiate between *Coffea arabica* and *Coffea canephora*

Chemical Markers (Dry Weight)	*Coffea arabica*	*Coffea canephora*
Sucrose	9.3	5.45
Putrescine-free (μg/g)	47.9	11.1
Linoleic acid (%)	6.1	3.7
Oleic acid (%)	8.3	12.3
Chlorogenic acids	4.1	11.3
Caffeine (%)	1–2	>3
β-Tocopherol (μg/g)	58.46	13.1

42.2 *Coffea arabica* and *C. Canephora*

Coffea arabica was first described by Linnaeus in 1753. Its fruits are round, smooth, slightly bitter, and chocolaty in color, with a smooth crust and an intense aroma. Brazil is the leading producer and exporter of *Coffea arabica* (ABIC 2010) and the second largest consumer. There are currently 11 varieties most frequently planted in producing countries. These varieties are the result of breeding projects intended to achieve early maturity, a size suitable for mechanical harvesting, rust resistance, high-density planting, and improved beverage quality (Fazuoli 1999).

The *Coffea canephora* species, also known as Robusta or Conillon coffee, is stronger and more productive than Arabica coffee, but its flavor is less popular with consumers, and it is mainly used in the formulation of blends and espressos. Indonesia is the largest producer of this coffee, followed by Brazil, Vietnam, the Ivory Coast, and Uganda (ICO 2011).

The two species *Coffea arabica* and *C. canephora* feature differing levels of caffeine, chlorogenic acids, sucrose, and amines (putrescine) (Andueza et al. 2004; Casal et al. 2004, Martin et al. 2001, Gonzalez et al. 2001). Table 42.1 summarizes the different compounds and their compositions in Arabica and Robusta coffee.

42.3 Postharvest Processing of Coffee Fruits and Beverage Quality

The fruit consists of the peduncle, crown, exocarp (epicarp or crust), mesocarp (mucilage or pulp), endocarp (parchment), spermoderm (testa or outer coat), endosperm (albumen), and embryos with two cotyledons. The coffee bean used for beverage preparation is the endosperm. To obtain green beans, coffee fruits are subjected to three different types of postharvest processing. Figures 42.1, 42.2, and 42.3 show the three processes—dry, wet, and semidry, respectively—and their different stages. The choice of processing type is subject to the uniformity of fruit maturation, the local weather conditions at harvest time, and the availability of water at the production site. Yield is not a deciding factor because each different process produces equivalent yield. On average, from a 249.48 Kg batch of cherry fruits, 45.36 Kg of processed grains are obtained, which will decrease another 16% in weight after roasting (Hicks 2001).

42.3.1 Dry or Natural Process

In general, Robust and Arabic coffees grown in Brazil, Ethiopia, Haiti, Indonesia, and Paraguay are processed using the dry or natural method. This kind of process creates so-called natural or nonwashed coffees. During this process, the intact coffee fruits dry in terraces or platforms of concrete, asphalt, or packed dirt. The process involves few steps and entails low equipment demand (Figure 42.1). The fruit harvesting process, performed via manual stripping, yields fruit lots at different stages of maturation

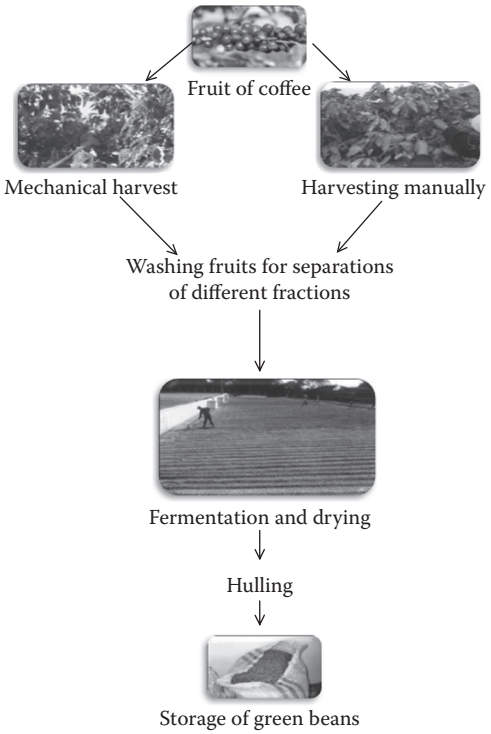

FIGURE 42.1 Main steps involved in dry or natural coffee fermentation.

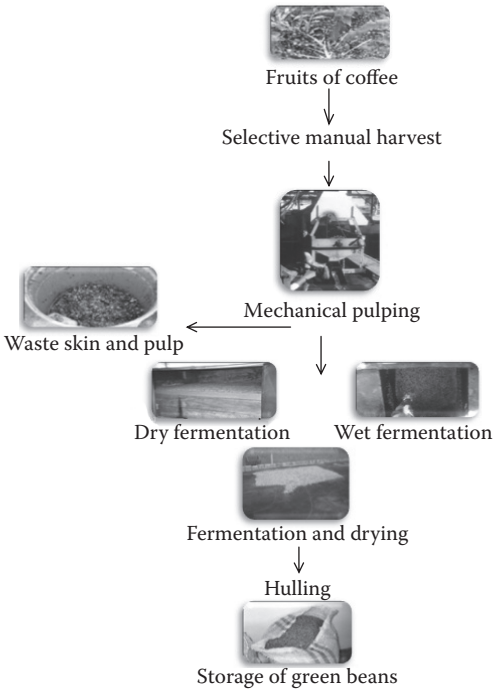

FIGURE 42.2 Main steps involved in wet coffee fermentation.

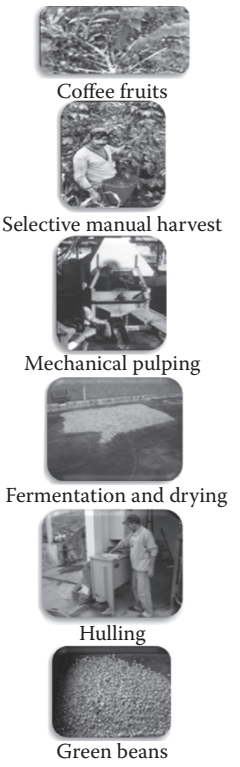

FIGURE 42.3 Main steps involved in semidry coffee fermentation.

(green, yellow green, cherry, dried). These types of fruit are separated by density during the fruit-washing phase. The fruits in the same maturity stage are placed on the threshing floor and remain there for about 15–20 days, depending on weather conditions. The fruits are raked daily so that they will dry evenly, and this process continues until they reach 11% and 12% humidity. This produces the so-called green beans, which are stored in burlap sacks until marketing.

Several physicochemical, microbiological, agricultural, cultural, and processing- and storage-related conditions can influence the final quality of the beverage. Natural coffees are more full-bodied and less acidic than coffees processed using the wet method. The drying and storage of coffee fruit is important to preserving the sensory and sanitary quality. Sanitary quality is preserved by reducing the water content and thus preventing colonization by toxigenic fungi during postprocessing. The drying temperature also affects cell integrity. When the cells of the grains are broken, leakage of cellular material occurs, and quality decreases (Borem et al. 2008). Natural fruits cannot be stored above 40°C (Sfredo et al. 2005) if they are to retain maximum quality, and the maximum possible storage time is 90 days (Coradi et al. 2007).

42.3.2 Wet Process

Wet processing gives rise to pulped, peeled, and demucilated coffees, depending on the processing procedure used. Countries such as Colombia, Kenya, countries in Central America, and Hawaii perform this type of processing. The steps involved are represented in Figure 42.2 and include the following:

1. Selective harvesting of fruits at the maximum maturity stage (cherry)
2. Mechanical depulping (demucilation)
3. Fermentation in tanks of water and subsequent drying

Roasted green beans will yield beverages that are softer, less viscous, and more acidic (Selmar et al. 2002) than natural coffees. The disadvantage of wet processing is the high consumption of water used to pulp, ferment, and wash beans after fermentation.

The washed coffee beans have a chemical composition different from that of nonwashed beans because the type of processing influences the metabolism of the seeds (Knopp et al. 2006). However, environmental factors and genetic factors related to treatment, agricultural practices, soil and climatic conditions, and technical procedures and postharvest fermentation time can influence the quality of the washed coffee beverage. This is because environmental factors such as shading and altitude influence the formation of aroma precursors such as proteins, amino acids, sucrose, triacylglycerols, chlorogenic acid, and caffeine, as suggested by Vaast et al. (2006). The end of fermentation is determined by the producers based on their tactile perceptions of the fruit. When the fruits are slippery, there is evidence of the presence of mucilage, indicating that fermentation should continue. However, tactile detection may fail, and as a result, Jackels and Jackels (2005) propose the periodic measurement of pH value as an alternative. When this parameter reaches a value of 4.6, this is indicative of the total release of mucilage and thus the end of fermentation.

42.3.3 Semidry Process

The semidry process is a variation of wet processing. It is an intermediate process between dry and wet processing (Figure 42.3) and results in what are called pulped natural coffees (Duarte et al. 2010). Some studies have focused on the chemical properties of coffee beans under semidry processing and the correlation between those properties and beverage quality (Duarte et al. 2010; Tarzia et al. 2010). No study has yet presented conclusive evidence of the level of influence of this process on the final quality of the beverage. However, it is possible to say that the qualities of coffee beverages made from pulped natural coffee lie somewhere between those obtained using dry and wet processing. The green beans produced via semidry processing are usually used in espresso blends (Duarte et al. 2010).

The concentration of aroma precursor compounds such as caffeine, trigonelline, sucrose, and chlorogenic acids can positively or negatively affect flavor. Chlorogenic acids are reported to be responsible for bitterness, and the concentration of these compounds in pulped natural coffees is lower than when wet processing is used. Trigonelline provides a pleasant flavor and is present in lower concentrations in semidry processing than in wet processed grains. Sucrose is the precursor to volatile compounds and corresponds, in dry weight, to 7.5 g/100 g. Aldehydes and carboxylic acids are desirable in high concentrations, as they are responsible for the production of furan. The concentration of sucrose is higher in semidry processing than in wet processing (Selmar et al. 2008).

42.3.4 Storage of Green Beans

After they are processed, the coffee fruits are hulled and stored until they are put on the market. After processing, the factors that affect the preservation of beverage quality are the physical storage conditions and the storage time (Coradi et al. 2007; Borem et al. 2008). Because the grains are stored for up to three years, they should be stored under constant temperature and relative humidity (RH) conditions to prevent the rehydration of the grain and postharvest reactions. The coffee bean embryos remain viable during processing but die after long periods of storage (usually after 6 months). After the death of the embryo, enzymes such as polyphenol oxidase and laccase can remain viable if the grain presents moisture above 20%; these would be inappropriate storage conditions and would allow the rehydration of the grains. The action of PPOs and laccase would cause the oxidation of phenolic compounds and decrease quality (Selmar et al. 2008).

In dry, semidry, and wet coffee processing in Brazil, storage temperatures above 40°C have damaged the cells of the grains, causing the leakage of intracellular material and decreasing beverage quality (Borem et al. 2008; Coradi et al. 2007). Selmar et al. (2008) suggest that the green beans must be stored (RH 63% and 22°C) with parchment because, regardless of the type of processing, the grains are of better quality when they are not hulled.

42.4 Microbiota of Dry Processed Coffee

Natural or dry processing is mainly used in Brazil, Ethiopia, Haiti, Indonesia, and Paraguay, where there is a low rainfall density during the fruit harvest. Studies focusing on microbial diversity in coffee fruits at different stages of maturity and during the overall drying process have been carried out by Silva et al. (2000, 2008a,b) and Vaughn (1958). These populations are diverse and include bacteria, yeasts, and filamentous fungi, all of which predominate in the fruits as they undergo constant physical and chemical changes. These changes are due to seed metabolism and to the gradual loss of water that occurs on the threshing floor. The drying period corresponds to the fruit fermentation period, in which the pulp and mucilage break down.

The species able to colonize the fruit must also be able to withstand the physicochemical changes that will occur (in pH, sugar content, and moisture), which will secrete pectinases on pectin pulp and mucilage. The depolymerization of pectin is the carbon source for microorganisms after the initial intake of simple sugars. This process is promoted by the 20-day period, during which the fruits remain on the threshing floor, with microbial growth sustained by pectin depolymerization. At the beginning of fermentation, the high water activity (approximately 0.9) facilitates the development and action of bacteria on the coffee fruit that are gradually replaced by yeasts and filamentous fungi at the end of the process (a_w 0.7). The largest bacterial population in the early stages of fermentation was recorded by Vaughn (1958), Silva et al. (2000, 2008a), and the largest population in samples of fresh coffee pulp was reported by Gaime-Perraud et al. (1993).

42.4.1 Bacteria

In natural coffee, some authors (Table 42.2) have identified the presence of Gram-negative and Gram-positive bacteria; in the first days of fermentation, the average population found is 10^6–10^9 CFU/g. Along with the high bacteria count in natural coffee fruit, there is also a great deal of species diversity, some native to the fruit, and so originating from the soil, agricultural tools, air, and water (Silva et al. 2008a). Thus, some species present in the fruit may not be part of the fermentation process.

All species identified in coffee fruits at different stages of natural maturation and processing are listed in the studies by Silva et al. (2000, 2008a,b). Among these species are *Tatumella ptyseos*, *Pseudomonas putrefaciens*, *P. mirabilis*, *Enterobacter aerogenes*, *Acinetobacter*, and *B. subtills* (Silva et al. 2000, 2008a), and *Paenibacillus amylolyticus* (Sakiyama et al. 2001), which is an endophytic species that presents pectin lyase activity and is therefore co-responsible for the fermentation of natural coffee, along with some yeast species.

TABLE 42.2

Bacteria Species Identified during the Period of Natural (Dry) Fermentation Coffee Fruits

Reference	Country	Bacteria
Silva et al. (2000, 2008b) Sakiyama et al. (2001) Vaughn (1958)	Brazil	Gram-positive: *Bacillus cereus*, *B. subtilis*, *B. macerans*, *B. megaterium*, *B. stearothermophilus*, *B. laterosporus*, *Cellulomonas. Arthrobacter*, *Microbacterium*, *Brochothrix*, *Dermabacter*, *Lactobacillus*, *Acinetobacter* sp., *B. polymyxa*, *Kurthia*, *Paenibacillus amylolyticus* Gram-negative: *Aeromonas*, *Enterobacter agglomerans*, *Pseudomonas*, *Serratia rubidea*, *S. plymutica*, *Hafnia*, *Tatumella ptyseos*, *Flavobacteriu*, *Klebsiella*, *Chromobacterium*, *Pasteurella*, *Acinetobacter*, *Cedecea*, *Citrobacter*, *Shigella*, *Providencia mirabilis*
De Bruyne et al. (2007)	Ethiopia	*Leuconostoc*
Van Pee and Castelein (1972)	Congo	*Erwinia dissolvens*, *Hafnia*, *Enterobacter aerogenes*, *E. cloacae*, *Klebsiella*

The presence of organic acid from fermentation metabolism (acetic, lactic, butyric, and propionic acids) confirms the microbial action in the fermentation process for natural coffees. In a study by Silva et al. (2008a), the production of organic acids from microbial metabolism and seed via high-performance liquid chromatography was assessed. In that study, it was observed that butyric acid is not detected in any of the samples analyzed. The presence of this acid in coffee beans generally gives an unpleasant flavor to the beverage and thus leads to quality loss. Acetic acid is detected mainly in the pulp and in parts of the mucilage. Acetic acid production is an aerobic metabolic process that can be of bacterial origin or the product of the oxidation of yeast-produced alcohol. The bacteria present on the surface of the coffee cherries synthesize the acid that can migrate to the pulp and mucilage and can interfere with the organoleptic quality of the beans. The negative influence of bacteria on the quality of the coffee beverage is linked to the species present and not to population density. In natural coffee, fruit rated very soft (i.e., as having optimal sensory quality), the bacterial population can represent up to 80% of total microbiota during this process.

42.4.2 Yeasts

The yeast population in natural coffee is usually lower than the bacterial population, presenting average values of 10^4 CFU/g; it becomes predominant with the reduction of water activity during the drying period and is detected in fruits with a_w of 0.6. *Pichia*, *Candida*, and *Arxula* are the most commonly found genera in fermented and dried fruits (Silva et al. 2000, 2008a). The species identified by Silva et al. (2000, 2008a) on the surface of coffee fruits included *Arxula adeninivorans*, *Blastobotrys proliferans*, *Candida auringiensis*, *C. glucosophila*, *C. incommunis*, *C. membranifaciens*, *C. paludigena*, *C. schatarii*, *C. saitoana*, *Candida fermentati*, *C. vartiovaarae*, *Citeromyces matritensis*, *Debaryomyces polymorphus*, *D. hansenii*, *Geotrichum fermentans*, *Pichia guilliermondii*, *Pichia acaciae*, *P. anomala*, *P. burtonii*, *P. ciferii*, *P. jadinii*, *P. lynferdii*, *P. ofunaensis*, *P. sydowiorium*, *P. subpelliculosa*, *Saccharomyces cerevisiae*, *Saccharomycopsis fermentans*, *S. fibuligera*, *Schizosaccharomyces cpombe*, *Sporopachydermia cereana*, *Stephanoascus smithiae*, *Trichosporonoides oedocephales*, and *Williopsis saturnus* var. *sargentensis.*

The involvement of yeast species in the fermentation process and bacterial action can be assumed based on their potential to produce pectin lyase and polygalacturonases. Of the identified species, *Debaryomyces hansenii*, *Pichia sydowiorium*, *Sthephanoascus smithiae*, and *Arxula adeninivorans* are those that have the capacity for pectin lyase production and that therefore cause degradation of the pulp and mucilage of natural coffee fruit (fermentation). The role of yeast may be related not just to the fermentation process but also to the control of filamentous fungi growth. Isolates belonging to the genera *Debaryomyces* and *Pichia* have demonstrated the ability to inhibit the growth of toxigenic fungi and may therefore have the potential for biological control (Ramos et al. 2010).

42.4.3 Filamentous Fungi

Studies of natural coffee microbial populations always emphasize filamentous fungi isolation and identification, but the microorganisms present during the fermentation and drying period are predominantly bacteria and yeasts. Therefore, the presence of filamentous fungi is not involved in the fermentation process; these are detected in the last days of fermentation and during storage. Various levels of fungal and toxin levels detected in processed dry beans can be found in studies by Batista et al. (2009), Batista and Chalfoun (2007), Silva et al. (2003), De Moraes and Luchese (2003), Chalfoun and Batista (2007), and Taniwaki et al. (2003).

The incidence of fungi in coffee fruits and beans is associated with a decrease in the sensory quality of the final coffee beverage, probably due to the production of metabolites such as superior acids. One exception is the presence of *Cladosporium cladosporioides*, which is usually found in high-quality beverages (Pereira et al. 2005; Chalfoun et al. 2007).

The species of filamentous fungi isolated in natural coffee are *Alternaria*, *Aspergillus flavus*, *A. niger*, *A. tamarii*, *A. ochraceus*, *A. wentii*, *A. versicolor*, *A. glaucus group Cladosporium herbarum*, *Fusarium stilboides*, *Penicillium chrysogenum*, *P. viridicatum*, *P. islandicum* (Mislivec et al. 1983—countries of

Central and South America, Asia, and Africa), *Aspergillus flavus*, *A. fumigatus*, *A. niger*, *A. parasiticus*, *A. sydowii*, *A. tamarii*, *A. terreus*, *Cladosporium cladosporioides*, *C. herbarum*, *C. macrocarpum*, *Fusarium oxysporum*, *Penicillium albidum*, *P. brevicompactum*, *P. chrysogenum*, *P. citrinum*, *P. cyclopium*, *P. funiculosum*, *P. jensenii* (Abdel-Hafez and El Maghraby 1992—Egypt), *Aspergillus fumigatus*, *A. niger*, *Fusarium graminearum*, *Penicillium granulatum* (Liardon et al. 1992—Brazil), *Mucor*, *Cercospora*, *Phoma*, *Penicillium* (Alves and Castro 1998—Brazil), *A. carbonarius*, *A. niger*, *A. ochraceus*, *Penicillium* sp. (Taniwaki et al. 2003—Brazil), *A. ochraceus*, *Aspergillus* section *Nigri*, *Rhizopus* (Pardo et al. 2004—Angola, Bolivia, Brazil, Costa Rica, Ecuador, Ethiopia, Guatemala, India, Indonesia, Ivory Coast, Java, Nicaragua, Uganda, and Vietnam), *A. tubingensis*, *A. carbonarius*, *A. ochraceus*, *A. sulphureus*, *A. tamari*, *A. niger*, *A. flavus* (Magnani et al. 2005—Brazil), *Cladosporium cladosporioides* (Chalfoun et al. 2007—Brazil), *A. ochraceus*, *A. flavus*, *A. sulphureus*, *A. niger*, *A. versicolor*, *A. sydowii*, *A. ostianus*, *A. melleus*, *A. dimorphicus*, *A. sclerotiorum*, *A. auricomus*, *A. foetidius*, *Eurotium amstelodami*, and *E. chevalieri* (Batista et al. 2001, 2007, 2009—Brazil), *A. flavus*, *A. niger*, *A. ochraceus*, *A. tamari*, *A. sydowii*, *A. foetidius*, *A. dimorphicus*, *Fusarium lateritium*, *F. solani*, *F. illudens*, *F. stilboides*, *F. semitectum*, *P. brevicompactum*, *P. crustosum*, *P. roqueforti*, *P. citrinum*, *Cladosporium cladosporioides*, *Paecelomyces* sp. (Silva et al. 2000, 2008a,b—Brazil).

42.4.3.1 Ochratoxin A–Producing Toxigenic Fungi

The toxigenic potential of a fungus is defined as the ability of a strain to produce toxic metabolites. Mycotoxins are characterized as secondary metabolites and are produced between the late exponential phase and early stationary phase of fungal growth; they are therefore not related to the biological role of microorganisms (Bars and Bars 2000). The conditions that enable the production of mycotoxins by a potentially toxigenic strain are more limited than those that permit the growth of fungi. Many strains of a species that is considered toxigenic may not possess this property. In potentially toxigenic species, the production of toxins can vary by up to 1000 times (Bars and Bars 2000).

Ochratoxin A present in coffee samples has mainly been attributed to the presence of species of the genus *Aspergillus* belonging to section *Circumdati* and section *Nigri* (Urban et al. 2001; Batista et al. 2003). Ochratoxin A is a secondary metabolite that is mainly produced by fungi belonging to the genera *Aspergillus*, *Petromyces*, *Neopetromyces*, and *Penicillium* (Frisvad and Samson 2000), but the genus *Aspergillus* is undoubtedly one of the most important for coffee. Among the species of the genus *Aspergillus* belonging to section *Circumdati*, the leading producer of ochratoxin A is *A. ochraceus*, but other species belonging to this section and producing ochratoxin A have been found in coffee fruit and beans, including *A. elegans*, *A. auricomus*, *A. ostianus*, *A. petrakii*, and *A. sclerotiorum sulphureus* (Batista et al. 2003). Frisvad et al. (2004) and Sartori et al. (2006) indicated that *A. westerdijkiae* can also contribute to the presence of ochratoxin A in coffee beans in Brazil. Studies have revealed that *A. carbonarius* is more common and represents the major source of ochratoxin A in section *Nigri*, whereas *A. niger* is rarely the producer (Heenan et al. 1998). Other studies indicate that other species including *A. steynii*, *A. westerdijkiae*, *A. lactofeatus*, and *A. sclerotioniger* (Frisvad et al. 2004; Samson et al. 2004) can also be producers of ochratoxin A in coffee. In the genus *Penicillium*, the only species considered producers of ochratoxin A are *Penicillium verrucosum* and *P. nordicum* (Larsen et al. 2001).

Both the environmental conditions of the geographic area and the types of grains influence the population of fungi and mycotoxin production. The study of the economic importance of *Aspergillus* species using polyphasic taxonomy is an innovative strategy that assists in characterizing the biodiversity of species and evaluating the risk of ochratoxin A contamination in coffee beans.

42.5 Microbiota of Wet Processed, Depulped, and Washed Coffee

The mechanical removal of pulp that occurs in wet processing allows mucilage to remain adhered to coffee beans. This mucilage is the substrate used by bacteria and yeast during fermentation. During fermentation, the precursors of flavor-enhancing compounds are naturally excreted by the fermentative microbiota present in coffee fruits (Gonzalez-Rios et al. 2007).

The microbial diversity of coffee beans in wet processing is lower than in dry processing due to fermentation conditions like reduced fermentation time (48 hours), faster decreases in pH, and pulp removal. The microbiota includes few species of bacteria and yeast and there have been no reports of filamentous fungi involved in the fermentation process. The greatest microbial diversity in washed coffee fruits can be recognized using molecular techniques that allow the detection of culturable and unculturable microorganisms. The molecular identification of microorganisms associated with coffee has been performed by Vilela et al. (2010) for semidry processing and Masoud et al. (2004) for wet processing. In semidry processing, microbial succession occurs, bacteria dominated at the beginning of the process, but after 72 hours, bacteria and yeast populations reach similar values (average 10^6 CFU/g), and the yeast dominate after 192 hours of fermentation (Vilela et al. 2010). This process is similar to the process of ecological succession described in Silva et al. (2008a), who observed that filamentous fungi were rarely found in semidry processes.

42.5.1 Bacteria

Studies dating back to the 1920s have isolated bacteria, fungi, and yeasts from coffee fruits, but do not make inferences about the involvement of microorganisms in mucilage degradation. Currently, there is still doubt about the apparent involvement of bacteria in coffee fruit fermentation, and since 2002, no results that address this issue have been published. An early study on the action of microorganisms present in coffee fermentation was published in 1946 by Pederson and Breed (1946) using samples of coffee from Colombia and Mexico. These authors isolated cocci and microaerophilic bacteria such as *Leuconostoc mesenteroides*, *Lactobacillus plantarum*, *Lactobacillus brevis*, and *Streptococcus faecalis* as facilitating the lysis of mucilage but not its detachment. Agate and Bhat (1966) isolate and identify *Streptococcus*, *Pseudomonas*, *Flavobacterium*, and *Proteus* from depulped coffees in India and state that these bacteria are not pectinolytic and are therefore not involved in the process of fermentation of depulped fruits.

Avallone et al. (1999, 2001, 2002) have isolated bacteria from wet processed fruits and detect the Gram-negative bacteria *Erwinia* and *Klebsiella* in a culture containing pectin, isolated from the coffee. They were characterized by low pectinolytic capacity and produced pectate lyase. The authors support the hypothesis that the presence of bacteria is facilitated by the consumption of simple sugars because the production of pectate lyase keeps these bacteria from affecting the fruit mucilage; the bacteria that produce polygalacturonase are rarely identified as *Lactobacillus brevis*. Microorganisms that facilitate the degradation of mucilage must be capable of secreting pectin lyase and polygalacturonase due to the methylated structure of pectin.

In semidry processing, 15 species, including *Acinetobacter*, *Bacillus cereus*, *B. macerans*, *B. megaterium*, *B. subtilis*, *Enterobacter agglomerans*, *Erwinia herbicola*, *Escherichia coli*, *Klebsiella pneumoniae*, *Lactobacillus brevis*, *L. plantarum*, *Lactococcus lactis*, *Leuconostoc mesenteroides*, and *Serratia* (Vilela et al. 2010), have been identified using traditional and molecular methods. Some of these species have also been identified in coffee cherries, possibly due to natural, common aspects of these two processes.

42.5.2 Yeasts

The population of yeasts in depulped coffees is greater than that of bacteria at the beginning of the fermentation period, with a density close to 10^4 CFU/g that increases during fermentation, reaching a value of 10^7 CFU/g (Masoud et al. 2004).

Of the studies that have been published on the isolation and the role of microorganisms in the fermentation of depulped coffee, only the research by Avallone et al. (2001, 2002) has not inferred the degradation of mucilage to be a product of the action of bacteria and/or yeast, which are responsible for the production of ethanol in overfermented coffees. However, pectinolytic yeasts have been isolated during the wet processing of coffee (Masoud and Jespersen 2006; Agate and Bhat 1966; Daivasikamani and Kannan 1986; Van Pee and Castelein 1971) in both Robust and Arabic coffees.

Agate and Bhat (1966) observed that the yeasts found in Robust coffee beans from India are predominant over the population of bacteria. The yeasts *Kluyveromyces*, *Saccharomyces* sp., *Saccharomyces marxianus* [*Kluyveromyces marxianus* (EC Hansen) van der Walt (1971)], *S. bayanus*, *S. cerevisiae*

var. *ellipsoideus* produce pectinases that degrade mucilage pectin. In that instance, it was impossible to confirm the degradation of mucilage because the degradation of mucilage was not observed when the depulped fruits were sterilized. Working with depulped coffees from the Congo, Van Pee and Castelein (1971) have identified yeasts of the genus *Candida*, with *C. guilliermondii* var. *membranaefaciens* found on the surface and mucilage of the grains; *C. parapsilosis*, *Saccharomyces cerevisiae*, *Torulopsis famata* [*Candida famata* (Harrison) SA Meyer & Yarrow (Yarrow and Meyer 1978)], *Saccharomyces marxianus* [*Kluyveromyces marxianus* (EC Hansen) van der Walt (1971)], *Candida tropicalis*, *Rhodotorula mucilaginosa*, and *Candida pelliculosa* were found on the surface of grains.

The different types of pectinase produced by organisms can affect the degree of depolymerization of the pectin components, which can then influence the selection of the dominant group of microorganisms (Jones and Jones 1984). The depolymerization of pectin can be performed using the enzymes pectin lyase, pectin methyl esterase, and polygalacturonase. Pectin from the fruit mucilage will be degraded via pectin lyase and polygalacturonase.

The presence of *Kluyveromyces marxianus* in wastewater for fermentation tanks and of *Pichia kluyvery*, *P. anomala*, *Hanseniaspora uvarum* with high pectinolytic capacity seems to enhance the action of yeast during the fermentation process (Serrat et al. 2002; Masoud and Jespersen 2006). The latter three species are capable of producing polygalacturonase in vitro under physicochemical conditions (pH 5.3–3.5 and 30°C) similar to those characteristic of the fermentation process. Thus, the authors conclude that yeasts use mucilage pectin as a carbon source with simple sugars (glucose and fructose), inducing polygalacturonase production.

Similarly, it has been observed that *Pichia kluyvery*, *P. anomala*, *Hanseniaspora uvarum* (Masoud and Kaltoft 2006) have an inhibitory effect on the growth of *Aspergillus ochraceus*, a fungus that is potentially toxigenic and is often isolated in coffee fruits and beans (Batista et al. 2009; Silva et al. 2008a,b). Thus, yeasts play a dual role during the processing of coffee fruits: they facilitate fermentation and biological control.

Species identified in washed and natural coffee fruits have also been found in semidry processing (Vilela et al. 2010). *Pichia anomala* have been shown to be similarly present in freshly harvested fruits (10^4 CFU/g) and have persisted through the 216-hour-long fermentation process. The persistence of this species may suggest the involvement of yeast in mucilage degradation. Other identified species include *Arxula* sp., *Candida ernobii*, *C. fukuyamaensis*, *C. membranifaciens*, *C. carpophila*, *Hanseniaspora uvarum*, *Kloeckera*, *Kluyveromyces* sp., *Pichia caribbica*, *Rhodotorula mucilaginosa*, *Saccharomyces bayanus*, and *Torulaspora delbrueckii*.

42.6 Final Considerations

Coffee is among the most consumed nonalcoholic beverages worldwide, and its production has significant financial ramifications in producing, exporting, and importing countries. The species *Coffea arabica* and *Coffea canephora* are the most used due to their cultural and sensory characteristics. Various chemical compounds are used to certify the coffee species used and the types of processing employed, as the beans have an aggregate value that is directly dependent on their quality. After harvesting, the fruits are processed via dry, semidry, and wet processing, which create different aromas and flavors, and it is up to the consumer to choose the type of beverage that he or she best likes. During processing, the fruits undergo a microbial fermentation process that is responsible for the spontaneous degradation of pulp and fruit mucilage. The microbiota involved in the fermentation process includes several species of bacteria, yeasts, and filamentous fungi. These seem not to participate in the fermentation process and are usually related to decreased beverage quality, with the exception of the *Cladosporium* species. The presence of filamentous fungi may create the risk of contamination by mycotoxins for fruits and coffee beans. The adoption of good agricultural practices and the hazard analysis and critical control points system will significantly reduce the risk of contamination by microorganisms and decrease the incidence of conditions in which the deterioration of the fruits or coffee beans may occur, for instance, by reducing ochratoxin A.

REFERENCES

Abdel-Hafez AII, El-Maghraby OMO. 1992. Fungal flora and aflatoxin associated with cocoa, roasted coffee and tea powders in Egypt. *Cryptogamie, Mycologie* 3(1):31–45.

ABIC. 2010. (www.abic.com.br). Acesso em 2 de outubro de 2010.

Agate AD, Bhat JV. 1966. Role of pectinolytic yeasts in the degradation mucilage layer of coffea robusta cherries. *Appl Environ Microbiol* 14(2):256–60.

Alves E, de Castro HA. 1998. Fungos associados ao café (*Coffea arabica* L) beneficiado e as fases pré e pós colheita em lavouras da região de Lavras. *Summa Phytopathol* 24:4–7.

Andueza S, Cid C, Nicoli MC. 2004. Comparison of antioxidant and pro-oxidant activity in coffee beverages prepared with conventional and "Torrefacto" coffee. *Lebensm-Wiss u-Technol* 37:893–7.

Avallone S, Brillouet J-M, Guyot B, Olguin E, Guiraud J-P. 2002. Involvement of pectolytic micro-organisms in coffee fermentation. *Int J Food Sci Technol* 37:191–8.

Avallone S, Guiraud J-P, Guyot B, Olguin E, Brillouet J-M. 2001. Fate of mucilage cell wall polysaccharides during coffee fermentation. *J Agric Food Chem* 49:5556–9.

Avallone S, Guyot B, Michaux FN, Guiraud JP, Olguin PE, Brillouet JM, editors. Cell wall polysaccharides of coffee bean mucilage. Histological characterization during fermentation. In: *COLLOQUE Scientifique International sur le Café*; 1999 Aug 2–6 18; Helsinki, Finland: ASIC. c 1999. p 463–70.

Bars LL, Bars PL. 2000. Mycotoxigenic in grains application to mycotoxic prevention in coffee. 2000. In: *Seminario Internacional Sobre Biotecnologia Na Agroindústria Cafeeira*, 3. 2000, Londrina. Anais. Londrina: IAPAR/IRD. p 513.

Batista LR, Chalfoun SM. 2007. Incidência de ocratoxina a em diferentes frações do café (*Coffea arabica* L.): bóia, mistura e varrição após secagem em terreiros de terra, asfalto e cimento. Incidence of ochratoxin A in fraction diferents coffee beans (*Coffea arabica* L): boia mixes and varrição. *Ciênc Agrotec* 31(3):804–13.

Batista LR, Chalfoun SM, Prado G. 2001. Identificação de espécies toxigenicas de *Aspergillus* associadas aos grãos de café armazenados. *R Bras Armaz* 3:11–6.

Batista LR, Chalfoun SM, Prado G, Schwan RF, Wheals AE. 2003.Toxigenic fungi associated with processed (green) coffee beans. (*Coffea arabica* L.). *Int J Food Microbiol* 85(3):293–300.

Batista LR, Chalfoun SM, Silva CF, Cirillo M, Varga EA, Schwan RF. 2009. Ochratoxin A in coffee beans (Coffea Arabica L) processed by dry and wet methods. *Food Control* 20(9):784–90.

Borém FM, Marques ER, Alves E. 2008. Ultrastructural analysis of drying damage in parchment Arabica coffee endosperm cells. *Biosyst Eng* 99:62–6.

Casal S, Mendes E, Alves R, Alves RC, Beatriz M, Oliveira PP, Ferreira MA. 2004. Free and Conjugated Biogenic Amines in Green and Roasted Coffee Beans. *J Agric Food Chem* 52:6188–92.

Chalfoun SM, Batista LR. 2007. Incidencia de ocratoxina A em diferentes frações de grãos de café (*Coffea arabica* L.). *Coffee Sci* 1(1):28–35.

Chalfoun SM, Cunha RL, Carvalho VL, Nogueira DA. 2007. Seletividade de fungicidas cúpricos e sistêmicos sobre o fungo *Cladosporium cladosporioides* em cafeeiro. *Summa Phytopathol* 33(1): 93–5.

Coradi PC, Borém FM, Saath R, Marques ER. 2007. Effect of drying and storage conditions on the quality of natural and washed coffee. *Coffee Sci* 2(1):38–47.

Daivasikamani S, Kannan N. 1986. Studies on post-harvest mycoflora of coffee cherry robusta. Brief note. *J Coffee Res* 16(3/4):102–6.

De bryne K, Schillinger U, Caroline L, Boehringe B, Cleenwerck I, Vancannet M, De Vuyst L, Franz CMAP, Vandamme P. 2007. Leuconostoc holzapfelli sp. nov., isolated from Ethiopian coffee fermentation and assessment of sequence anlysis of housekeeping genes for delineation of Leuconostoc species. *Int J Syst Evol Microbiol* 57:2952–9.

De Moraes MHP, Luchese RH. 2003. Ochratoxin A on green coffee: Influence of harvest and drying processing procedures. *J Agric Food Chem* 51:5824–8.

Duarte GS, Pereira AA, Farah A. 2010. Chlorogenic acids and other relevant compounds in Brazilian coffees processed by semi-dry and wet post-harvesting methods. *Food Chem* 118:693–701.

Fazuoli LC. 1999. Cultivares IAC de café. *O Agronômico* 51(1):27–34.

Frisvad JC, Frank JM, Houbraken JAMP, Kuijpers AFA, Samson RA. 2004. New ochratoxin A producing species of *Aspergillus* section *Circumdati*. *Stud Mycol* 50:23–43.

Frisvad JC, Samson RA. 2000. *Neopetromyces* gen. nov. and an overview of teleomorphs of *Aspergillus* subgenus *Circumdati*. *Stud Mycol* 45:201–7.

Gaime-Perraud I, Roussos S, Martinez-Carrera D. 1993. Natural microorganisms of the fresh coffee pulp. *Micol Neotropical Aplic* 6:95–103.

Gonzáles AG, Pablos F, Martín MJ, Léon-Camancho M, Valdenebro MS. 2001. HPLC analysis of tocopherols and triglycerides in coffee and their use as authentication parameters. *Food Chem* 73:93–101.

Gonzalez-Rios O, Suarez-Quiroz ML, Boulanger R, Barel M, Guyot B, Guiraud J-P, Schorr-Galindo S. 2007. Impact of ''ecological'' post-harvest processing on the volatile fraction of coffee beans: I. Green coffee. *J Food Comp Anal* 20:289.

Heenan CN, Shaw KJ, Pitt JI. 1998. Ochratoxin A production by *Aspergillus carbonarius* and *A. niger* isolates and detection using coconut cream agar. *J Food Mycol* 1:67–72.

Hicks A. 2001. Post-harvest processing and quality assurance for speciality/organic coffee products. In: Chapman K, Subhadrabandhu S, editors. *The First Asian Regional Round-Table on Suitable, Organic and Specially Coffee Production, Processing and Marketing*; 2001 February 26–28; Chiangi-Mai, Thailand.

ICO. 2011. International Coffee Organization. Bean to Cup. Botanical aspects. www.ico.org. online access in 12.21.2011.

Jackels SC, Jackels CF. 2005. Characterization of the coffee mucilage fermentation process using chemical indicators: A field study in Nicaragua. *J Food Sci* 70:321–5.

Jones KL, Jones SE. 1984. Fermentations involved in the production of cocoa, coffee and tea. Amsterdam: Progress Industrial Microbiology 19:411–56.

Knopp S, Bytof G, Selmar D. 2006. Influence of processing on the content of sugars in green Arabica coffee beans. *Eur Food Res Technol* 223:195–201.

Larsen TO, Svendesen A, Smedsgaard J. 2001. Biochemical of ocratoxin A-producing strains of the genus *Penicillium*. *Appl Environ Microbiol* 67(8):3630–5.

Liardon R, Braendlin N, Spadone JC. Biogenesis of Rio flavour impact compound: 2,4,6-trichloroanisole. Quartozieme colloque scientifique international sur le café; 1992 14– 19Jul; San Francisco. Paris France: Association Scientifique Internacionale du Café. c1992.

Magnani M, Fernandes T, Prete CEC, Homechim M, Ono EYS, Vilas-Boas LA, Sartori D, Furlaneto MC, Fungaro MHP. 2005. Molecular identification of *Aspergillus* spp. isolated from coffee beans. *Sci Agric* 62(1):45–9.

Martín MJ, Pablos F, González AG, Valdenebro MS, Camacho ML. 2001. Fatty acid profiles as discriminant parameters for coffee varieties differentiation. *Talanta* 54:291–7.

Masoud W, Cesar LB, Jespersen L, Jakobsen M. 2004. Yeast involved in fermentation of *Coffea arabica* in East Africa determined by genotyping and by direct denaturating gradient gel electrophoresis. *Yeast* 21:549–56.

Masoud W, Jespersen L. 2006. Pectin degrading enzymes in yeasts involved in fermentation of *Coffea arabica* in East Africa. *Int J Food Microbiol* 110:291–6.

Masoud W, Kaltoft CH. 2006. The effects of yeasts involved in the fermentation of *Coffea arabica* in East Africa on growth and ochratoxin A (OTA) production by *Aspergillus ochraceus*. *Int J Food Sci Technol* 45:2167–75.

Mislivec PB, Bruce VR, Gibson R. 1983. Incidence of toxigenic and other molds in green coffee beans. *J Food Prot* 46(11):969–73.

Pardo E, Marin S, Ramos AJ, Sanchis V. 2004. Occurrence of ochratoxigenic fungi and ochratoxin a in green coffee from different origins. *Food Sci Technol Int* 10:45–9.

Perderson CS, Breed RS. 1946. Fermentation of coffee. *Food Res* 11(2):99.

Pereira RTG, Pfenning LH, Castro HA. 2005. Caracterização e dinâmica de colonização de *Cladosporium cladosporioides* (Fresen.) de vries em frutos do cafeeiro (*Coffea arabica* L.). Characterization and dynamic of colonization of *Cladosporium cladosporioides* (Fresen.) de Vries in coffee fruits (*Coffea arabica* L.). *Ciênc Agrotec* 29(6):1112–6.

Ramos DMB, Silva CF, Batista LR, Schwan RF. 2010. Inibição in vitro de fungos toxigênicos por *Pichia* e *Debaryomyces* SP isoladas de frutos de café (*Coffea arábica*). *Act Scient Agronomy* 32(3):397–402.

Sakiyama CCH, Paula EM, Pereira PC, Borges AC, Silva DO. 2001. Characterization of pectin lyase produced by an endophytic strain isolated from coffee cherries. *Lett Appl Microbiol* 33:117–21.

Samson RA, Houbraken JAMP, Kuijpers AFA, Frank JM, Frisvad JC. 2004. New ochratoxin or sclerotium producing species in *Aspergillus* section *Nigri*. *Stud Mycol* 50:45–61.

Sartori D, Furlaneto MC, Martins MK, Paula MRF, de Pizzirani-Kleiner AA, Taniwaki MH, Fungaro MHP. 2006. PCR method for the detection of potential ochratoxin-producing *Aspergillus* species in coffee beans. *Res Microbiol* 157:350–4.

Selmar D, Bytof G, Knopp SE. New aspects of coffee processing: the relation between seed germination and coffee quality. Dix-Neuviéme Colloque Scientifique International sur le Café; 2001 May 14–18; Paris, Trieste: ASIC. c2002.

Selmar D, Bytof G, Knopp SE. 2008. The storage of green coffee (*Coffea arabica*): Decrease of viability and changes of potential aroma precursors. *Ann Bot* 101:31–8.

Serrat M, Bermúdez RC, Villa TG. 2002. Production, purification, and characterization of a polygalacturonase from a new strain of *Kluyveromyces marxianus* isolated from coffee wet-processing wastewater. *Appl Biochem Biotechnol* 97:193–208.

Sfredo MA, Finzer JRD, Limaverde JR. 2005. Heat and mass transfer in coffee fruits drying. *J Food Eng* 70:15–25.

Silva CF, Batista LR, Abreu LM, Dias ES, Schwan RF. 2008a. Succession of bacterial and fungal communites during natural coffee (*Coffea arabica*) fermentation. *Food Microbiol* 25:951–7.

Silva CF, Batista LR, Schwan RF. 2003. Incidência de *Aspergillus* produtores de micotoxinas em frutos e grãos de café (*Coffea arabica* L.). *Rev Bras Armazo* (7):30–7.

Silva CF, Batista LR, Schwan RF. 2008b. Incidence and distribution of filamentous fungi during fermentation, drying and storage of coffee (*Coffea arabica* L.) beans. *Braz J Microbiol* 39:521–6.

Silva CF, Schwan RF, Dias ES, Wheals AE. 2000. Microbial diversity during maturation and natural processing of coffee cherries of *Coffea arabica* in Brazil. *Int J Food Microbiol* 60:251–60.

Taniwaki MH, Pitt JI, Teixeira AA, Iamanaka B. 2003. The source of ochratoxin A in Brazilian coffee and its formation in relation to processing methods. *Int J Food Microbiol* 82(2):173–9.

Tarzia A, Scholz MBS, Petkowicz CLO. 2010. Influence of the postharvest processing method on polysaccharides and coffee beverages. *Int J Food Sci Technol* 45:2167–75.

Urbano GR, Taniwaki MH, Leitão MFE, Vicentini MC. 2001. Occurrence of ochratoxin A-producing fungi in raw Brazilian coffee. *J Food Prot* 64(8):1226–30.

Vaast P, Bertrand B, Perriot J-J, Guyot B, Génard M. 2006. Fruit thinning and shade improve bean characteristics and beverage quality of coffee (*Coffea arabica* L.) under optimal conditions. *J Sci Food Agric* 86:197–204.

Van Pee W, Castelein JM. 1972. Study of the pectinolytic microflora, particularly the *Enterobacteriaceae*, from fermenting coffee in the Congo. *J Food Sci* 37 (2):171–4.

Van Pee W, Castelein JM. 1971. The yeast flora of fermenting robusta coffee. *E Afr Agric For J* 26:308–310.

Vaughn RH, Camargo R, Fallange H, Mello Ayres G, Sergedello A. 1958. Observations on the microbiology of the coffee fermentation in Brazil. *Food Technol* 12(Suppl.):12–57.

Vilela DM, Pereira GV de M, Silva CF, Batista LR, Schwan RF. 2010. Molecular ecology and polyphasic characterization of the microbiota associated with semi-dry processed coffee (*Coffea arabica* L.). *Food Microbiol* 27(8):1128–35.

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Pulque Fermentation

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CONTENTS

43.1 Introduction

Pulque is probably the oldest and most traditional Mexican alcoholic beverage, prepared, and consumed since pre-Hispanic times. Due to its great historical, religious, social, medical, and economical importance, it is the most widely studied beverage from the anthropological and scientific points of view (Loyola-Montemayor 1956; Godoy et al. 2003).

It is a milky white, viscous, slightly acidic alcoholic beverage traditionally produced by spontaneous fermentation of *aguamiel*, the sugary sap extracted from several *Agave* species that grow in the central Mexican plateau states (Loyola Montemayor 1956; Steinkraus 1996; García-Mendoza 1995, 1998; Blomberg 2000; Godoy et al. 2003). In pre-Hispanic times, pulque played an important role in the religion, rituals, divination, and curing of the indigenous communities (Bruman 2000). Currently, pulque persists as a typically popular beverage, and it is sold in *pulquerías* as well as in some restaurants. It is also produced at a small industrial scale using a mixed starter culture and controlling the quality and safety of the product, which is canned and exported (Ramírez et al. 2004).

In this chapter, a broad view of pulque is covered: a historical review is presented, from its discovery and relevance during the pre-Hispanic period, its role during colonial times, its consolidation, and production from the 15th to the 20th centuries, until the recent scientific and technological efforts to reincorporate pulque to the Mexican traditional cuisine. The different *Agave* species used in the production of pulque are mentioned as well as their actual distribution and density in the agave plantations. The traditional and industrial pulque production processes are described, including the microbiota involved as well as the biochemical changes that take place both in the spontaneous and the controlled fermentation. To understand the relevance and potential benefits of pulque among consumers, its main chemical and nutritional properties, including its health benefits, are discussed. Finally, the chapter concludes with a description of the actual status of pulque in Mexican society as well as the social, scientific, and technological challenges that have to be faced in order to achieve the rescue of this ancestral beverage.

43.2 History of Pulque

43.2.1 Origins and Pre-Hispanic Production

Pulque represents a Mexican icon and is probably the oldest and most traditional Mexican alcoholic beverage, as attested by archaeological vestiges. The ancient Aztecs called it *metoctli* or agave wine (from the nahuatl language *metl* = agave or maguey + *octli* = wine) as well as *iztacoctli* or white wine (from *izac* = white + *octli* = wine): they referred to the spoiled beverage with unpleasant odor and flavor as *poliuhquioctli* (from *poliuhqui* = spoiled or rotted + *octli* = wine), so it is probably from this last term that the word *pulque* was adopted by the Spaniards to designate the freshly produced beverage (Robelo 1948).

It is highly probable that ancient Otomíes were the first to prepare pulque toward the year 2000 BC, inheriting the elaboration process to almost all cultures of the central Mexican plateau (Martín del Campo 1938; Blomberg 2000). However, different legends attribute its origin to the Tolteca civilization (1021–990 BC) in the city of Tula. One of these legends considers that maguey and pulque are both a gift of the goddess *Mayahuel* (Gonçalves de Lima 1978; Ramírez Castañeda 1994). There is consensus among historians based on archeological evidences (200 BC–300 AD) and information from pre- and post-Hispanic codices from several important Mesoamerican cultures that pulque had a dominating presence in their daily life and a primordial role in religious and war rituals (Diguet 1928; Martin del Campo 1938; Sánchez-Marroquín 1949; Gonçalves de Lima 1978; Vallejo 1992).

Aztec civilization developed a full dominion of aguamiel and pulque production techniques incorporating to their diet its nutritional benefits and healing properties. This civilization considered pulque a divine food, a medicine, and a ritual element that allowed a closer relation with their cosmology. As the dominant culture in the Mexican plateau, the Aztecs established the moral guidelines for pulque consumption and religious use: only the children and youths, the elder, pregnant or nursing women to increase milk production, priests and warriors, or anyone before sacrifice were allowed to consume it, but excessive consumption was harshly punished, in some cases including the death penalty (Loyola-Montemayor 1956; Corcuera de Mancera 1991; Godoy et al. 2003). Aztecs' conquered cultures were forced to deliver aguamiel and pulque as tribute (Gonçalves de Lima 1978, 1990; Soberón-Mora 1998).

43.2.2 Pulque Production during the Spaniard Colonial Period

When the Aztec Empire fell, pulque lost its religious importance prevailing as a traditional popular beverage until today. At the beginning of the colonial period, the Spaniards promoted its consumption among the indigenous groups and recent immigrant slaves as a mean to control them (Corcuera de Mancera 1991; Ramirez et al. 2004). The fast growth of the productive and commercial activities introduced by the Spaniards during the 17th century gradually incorporated the production and commercialization of pulque. These activities flourished at the end of the 18th century with the development of the *haciendas pulqueras* (large farms producing pulque) devoted to the cultivation of *Agave* plants, extraction of aguamiel, and production of pulque, which was transported mainly to the cities of Mexico

and Puebla. These production organizations prospered in the actual central Mexican plateau states of Hidalgo (mainly in the Apan prairies), Tlaxcala, Puebla, Querétaro, Morelos, and Michoacán.

Since 1779, pulque production and commerce was strongly regulated, and special privileges were granted to Spaniards who controlled these agroindustry (Loyola-Montemayor 1956), becoming one of the most important economic activities in the New Spain. Taxes imposed to pulque became the fifth biggest income of the *Real Hacienda* and the Spanish Crown received from the pulque industry 4.3 million pesos in 1762 and 32 million from 1763 to 1809 (Segura 1901).

43.2.3 Production during the 19th and 20th Centuries

During the independence movement (1810–1821), pulque production suffered a drastic reduction. Once México became independent, government regulations regarding pulque production, quality, commerce, and taxes consolidated again the *haciendas pulqueras*, allowing them to cope with the increasing demand of the beverage by the rapidly growing population (Loyola Montemayor 1956). An important factor in this process was the introduction in 1867 of the railway system, which in particular connected the Apan prairies with the main consumption centers. In this context, the pulque agroindustry flourished and reached its best times at the beginning of the 20th century with an annual production of 500 million L obtained from around 140 million agave plants. In 1905, 350,000 L of pulque were consumed only in Mexico City. This agroindustry controlled the agave plantations and the production and retail sale of pulque, activities that originated great fortunes and the emergence of a new social economic class called the "pulque aristocracy" (Ramírez-Rancaño 2000; CECULTAH 2010).

In 1909, the owners of more than 50 haciendas created the *Compañía Expendedora de Pulques*, with the purpose of controlling production, distribution, and sale of pulque in Mexico City and to expand the hacienda activities to the elaboration, distribution and commerce of other agave products, including aguamiel syrups, glues, pulque distillates, and medical products, increasing the benefits of the agave industry (Gobierno del Estado de Hidalgo: Museo Nacional de Culturas Populares 1988).

By 1910, the pulque industry was affected again by the revolutionary movement (Soberón-Mora 1998). The social instability together with the introduction of the brewing companies and a strong campaign against pulque had a negative impact in this agroindustry. By 1914, *haciendas pulqueras* were seized and land reform started fractionating the agricultural fields. The elimination of *haciendas pulqueras* became a fact with the promulgation of a new constitution in 1917, which established in the 27th article the agricultural reform, which promoted the return and redistribution of the land to the peasants; this was the formal end of the three centuries of splendor of most of the *haciendas pulqueras* (Loyola Montemayor 1956; Ramírez-Rancaño 2000). Despite the land redistribution, the abundance of agave cultivars allowed the development of a new pulque industry that was able to cope with the decreased beverage demand. Therefore and until the 1920s, fresh pulque produced in several regions of the country was delivered daily by train to Mexico City. During 1934–1940, antialcoholic campaigns were established, particularly against pulque, as it was considered dirty and unhealthy, due to the lack of control in the production process and adulteration during its distribution after certification through the former pulque customs of Pantaco, Cuautepec, and Ticoman, where rigorous norms were applied until the seventies to authorize the entrance of pulque barrels to Mexico City (Gobierno del Estado de Hidalgo: Museo Nacional de Culturas Populares 1988). However, during the following decades the already mentioned problems, as well as the excessive and irrational exploitation of the agave plantations and the introduction of beer as an alternative beverage, considerably deteriorated the pulque industry (Loyola-Montemayor 1956; Ramírez-Rancaño 2000).

It was not until 1960 that considerable efforts were made in order to recover this important national heritage with the creation of the *Patronato del Maguey*, with the aim to improve the culture and exploitation of *Agave* through modifications in both the production and the distribution process. Canning was introduced (350 mL cans and commercialized under the registered trade name *Magueyin*) as an alternative way of distribution, efforts were made to improve the hygienic conditions of the beverage, and new uses of the *Agave* plants were developed to increase the income of the peasants (Gobierno del Estado de Hidalgo: Museo Nacional de Culturas Populares 1988; Steinkraus 1996). Unfortunately, canned pulque was not accepted by the traditional consumer as the product lacked the distinguishing sensorial

properties of fresh pulque and had a higher price. As a consequence, this industrialization attempt failed as well as subsequent efforts to commercialize other former patented pulque brands.

In the 20th century, according to the agronomical census, from 1998 to 2002, the surface devoted to *Agave* pulquero was reduced with the consequent decrease in pulque production. Nowadays, the most important agave pulquero production areas are located in the central states of Hidalgo, Tlaxcala, and Estado de México, and in some regions of Querétaro, Puebla, Morelos, and Michoacán. By the end of the last century, the depressed pulque industry situation was the consequence of the loss of cultivars, the lack of an efficient technology, and the low demand by consumers (Loyola-Montemayor 1956; Gobierno del Estado de Hidalgo: Museo Nacional de Culturas Populares 1988; Ramírez-Rancaño 2000).

43.2.4 Situation in the Early 21st Century

Currently, pulque is considered a traditional popular beverage that practically has disappeared from the urban market, only found in *pulquerías* located in popular districts of Mexico City. In the rural areas, near the production regions, pulque is still the preferred stimulating beverage of the low income class. It is consumed as part of their daily diet and is part of the tradition in all sorts of festivities, from births and baptisms to weddings and funerals. It is also common to find production for self-consumption among agave owners. Important efforts are being made to fight against its disappearance: it is being promoted in Mexican food restaurants and gastronomic festivals, while traditional *pulquerías* are recovered. In these traditional places pulque is now exclusively prepared with high quality raw materials and under hygienic conditions (Poblet 1995).

Nevertheless, pulque production remains as a predominantly traditional and small-scale industry that has not been able to massively reach local (other than Mexico City) or international markets (Ramírez et al. 2004). However, *Nectar del Razo*, in Tlaxcala, and *Desarrollo Agropecuario del Altiplano*, in Puebla, export their novel canned pulque product to different countries all around the world. In both companies, the traditional elaboration process has been modified to obtain a stable product with a long, shelf-life, which is sold white, that is to say, plain or added with fruits.

43.3 Agave Species Used for Aguamiel Extraction

Pulque is produced from aguamiel extracted from native *Agave* species, mainly *A. salmiana* var. *salmiana* (green or meek agave), *A. atrovirens* (white agave), and *A. mapisaga* (Mexican or long hand agave), and in lower proportion from *A. lehmannii* and *A. altisima* (Loyola-Montemayor 1956; García-Mendoza 1995, 1998), mostly distributed in the states of the central Mexican plateau where pulque has been mainly produced and consumed. These species grow in a vast region of arid, semiarid, and temperate zones, in sandy, poor, well-drained soils, with scare, and irregular precipitations. Preventing soil erosion, they can be found as single crop or protecting or limiting homes or farm lands (Loyola-Montemayor 1956; Steinkraus 1996; Ramírez et al. 2004; Ortiz-Basurto et al. 2008; Lappe-Oliveras et al. 2008).

Agave is a plant relatively easy to cultivate. Propagation may be carried out by transplanting young vigorous offsprings from adult plants, called *matecuates*. An adult plant can produce above 50 *matecuates* that grow around the mature plant. Young offsprings are harvested in the rainy season and transplanted into a new *magueyera* or agave plantation for aguamiel production where the young plants are arranged on parallel long rows called *melgas* or *metepnatle* (maguey wall), separated by 2–5 m. Alternatively, agave seeds can be grown in seedbeds under controlled conditions (Monterrubio 2007).

Aguamiel is extracted from 7- to 10-year-old mature agaves that are about to produce their inflorescence (*quiote*). Mature plants are castrated or broken by eliminating the floral bud, leaving a cavity (*cajete)* in the center of the agave stem. This operation is usually performed in early spring or late autumn. If the plant inflorescence grows, maguey will never produce aguamiel, and if *castration* is performed before agave maturation, the yield in aguamiel production will be scare. The resulting cavity is covered with agave leaves or with a sized stone to avoid the exposition of the cavity to the environment (Loyola-Montemayor 1956; Gobierno del Estado de Hidalgo: Museo Nacional de Culturas Populares 1988). An aging period follows castration to allow the maturation of the central leaves and the increase

of sap sugar content: 7%–14% w/v, depending of the agave species (Lappe-Oliveras et al. 2008). The healed cavity is scraped to open the vessels, promoting the sap flow and its accumulation in the cavity. The sap is collected twice a day (at daybreak and dusk) by oral suction through a dried gourd (*Lagenaria siceraria*) called *acocote*. Production of aguamiel lasts from 3 to 6 months until the plant dies, with an approximate production of 1000 L/plant (Loyola-Montemayor 1956; Gobierno del Estado de Hidalgo: Museo Nacional de Culturas Populares 1988; Steinkraus 1996). The volume of aguamiel produced varies throughout the harvesting period; at the beginning the plant produces around 0.4 L/plant/day, an amount that increases to 4 or 6 L/day during the next 3–6 months and then decreases to 0.4 L before the plant dies (Ortiz-Basurto et al. 2008). Once the plant dies, it is removed and substituted by an offspring, as already described (Monterrubio 2007; Lappe-Oliveras et al. 2008).

43.3.1 Aguamiel Composition and Properties

43.3.1.1 Physicochemical Characteristics of Aguamiel

Aguamiel is a milky white, lightly cloudy, thick, sweet, fresh-flavored, and lightly acid agave sap. It contains water, sugars, proteins, gums, and mineral salts as the most important components (Ramírez et al. 2004; Ortiz-Basurto et al. 2008; Escalante et al. 2008). The Mexican regulation NMX-V-022 (Banco de Normas Mexicanas 2010a) defines two types of aguamiel for pulque production. Type I refers to a best quality aguamiel (cleanest and with a highest sugar content, pH < 6.6–7.5 and < 0.9–1.03 mg lactic acid/100 mL) while type II includes slight acid aguamiel (pH < 4.5 and < 4 mg lactic acid/100 mL).

According to several sources, the main compounds present in the sap are 0.3 to 0.4 g/100 mL of all essential amino acids with exception of methionine, vitamins (mg/100 mL, 0.02 riboflavin, 0.06 thiamine, 0.45 niacin, 19.60 biotin, 21.64 *p*-aminobenzoic acid, 65.17 panthothenic acid, 22.98 pyridoxine, and 9.0 ascorbic acid), sugars (glucose, sucrose, fructose, fructo-oligosaccharides, and inulin), minerals (mg/100 mL, 25 sodium, 9 magnesium, 1.1 iron, 0.8 zinc, 0.3 copper, and 0.3 manganese). Aguamiel constitutes a water substitute or an alternative food in places where water is scare or of bad quality or where protein is not available (Sánchez-Marroquín and Hope 1953; Ortiz-Basurto et al. 2008; García-Garibay and López-Munguía 1993; Steinkraus 1996; Lappe-Oliveras et al. 2008).

The amount and composition of aguamiel during the production period varies depending on the *Agave* species, the cultivation conditions of the plant, the season of the year, the air relative humidity, and the soil properties (Sánchez-Marroquín 1970). However, in the physicochemical analysis of aguamiel from *A. mapisaga* harvested during 5 months, Ortiz-Basurto et al. (2008) found no significant changes in titrable acidity, pH (above 4.5), and dry matter content, but a slight decrement in fructose content was observed in samples collected after 3.5 months, concluding that the quality of aguamiel remains constant during all the harvesting period.

43.4 Pulque Production

The Mexican regulation NMX-V-037 (Banco de Normas Mexicanas 2010b) recognizes two types of pulque: type I includes *pulque de semilla* or *pulque pie de cuba* (seed or starter) and type II refers to commercial pulque. Pulque starter is prepared to increase the natural microbiota that determines the correct fermentation in the elaboration of the beverage in a tank exclusively devoted for this purpose. Type I sap is inoculated with pulque starter, and is used to establish the optimum biochemical equilibrium between the fermentable substrates and the microorganisms involved in fermentation; it is the base of commercial production (Loyola-Montemayor 1956; Ramírez et al. 2004; Lappe-Oliveras et al. 2008).

43.4.1 Traditional Pulque Production

The traditional process of pulque production has remained relatively unchanged. The process starts with the daily collection of aguamiel and its immediately transference to a container, traditionally a pork or goat skin bag called *cuero* (Figure 43.1 shows several stages and utensils used during aguamiel

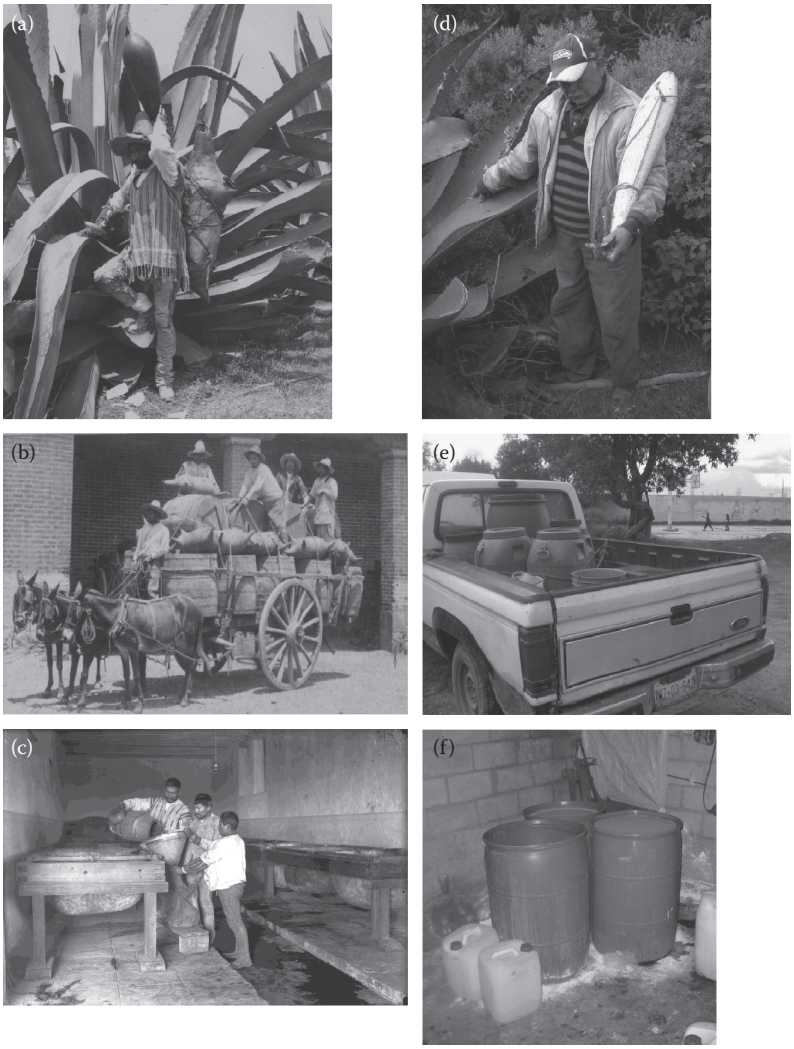

FIGURE 43.1 Aguamiel extraction, transport, and pulque fermentation during the early 20th century (a–c) and at present (d–e, from the aguamiel and pulque production location Las Mesas, Tlaxcala state). (a) Aguamiel extraction by the *tlachiquero* with an *acocote*, cajete scrapper tool (right hand), and skin bag to transport aguamiel to fermentation vats; (b) aguamiel and pulque transportation in skins bags and barrels; (c) former vats made with leather in a large tinacal; (d) modern *tlachiquero* with an *acocote* and scrapper tool; (e) aguamiel and pulque transport to small rural *pulquerías*; (f) large plastic barrels (200 L) in a small tinacal. (a–c, Reproduced with permission of Sistema Nacional de Fototecas, México. a, ©609476, Conaculta, INAH, Sinafo, FN, México; b, ©611009, Conaculta, INAH, Sinafo, FN, México; c, ©6535, Conaculta, INAH, Sinafo, FN, México.)

extraction and pulque production) or a wood container known as *castaña*; recently, plastic containers have been introduced. After sap collection, the cajete walls are scraped again to maintain the vessels open and a constant aguamiel flow.

Fermentation is carried out for several hours, depending on aguamiel quality, pulque starter maturity, the sap, and seed microbiota, and the season (environmental temperature changes). The degree of fermentation is adequate when a specific alcoholic degree and viscosity are reached (Loyola-Montemayor 1956; Steinkraus 1996).

Pulque fermentation is performed in containers made from different materials (leather, wood, plastic, glass fiber) placed in a closed room (*tinacal*, with small windows to control the temperature), as in the

haciendas pulqueras of the Spaniard colonial period. In pre-Hispanic times pulque fermentation was carried out in clay pots; during the colonial period these kind of containers were replaced by leather reservoirs that were used until 1900, when wood vats with higher capacities (800–1000 L) were introduced. In most *tinacales*, the fermentation tanks are generally arranged in parallel, while in some others they are placed around a circle (Bellingeri Martini 1980).

To produce the starter, a small amount of aguamiel (10–15 L) preferably with an adequate density and taste is poured into a devoted vat (located in a small room next to the *tinacal*) and spontaneously fermented until alcoholic and acetic tastes are detected or until a white layer called *zurron* is formed on the surface. This process is performed at room temperature and lasts several weeks depending on the season: 1 week in summer, while 3 or 4 weeks in winter (Loyola-Montemayor 1959; Bellingeri Martini 1980; Gobierno del Estado de Hidalgo: Museo Nacional de Culturas Populares 1988; Steinkraus 1996; Lappe-Oliveras et al. 2008).

Small amounts of fresh aguamiel are constantly poured until the vat is filled; this amount of seed should be enough to sustain commercial pulque production. Afterward, the starter is distributed in several vats, a process known as *tirar puntas*, and the fermentation is started with the addition of fresh aguamiel to the vats. An empirical rule applied to perform a good fermentation is to mix more starter than aguamiel during the cold season and less starter than aguamiel during the hot season. The fermentation finishes after 6 or 14 hours depending on seed and aguamiel quality, microbial content, and environmental conditions. As all these parameters affect the fermentation process it is not strange to find pulque with a wide variety of properties and compositions (Loyola-Montemayor 1956; Raminez et al. 2004). According to its quality, two types of pulque are recognized: fine or best-quality pulque obtained by fermentation of high-quality *aguamiel* from *A. salmiana* var. *salmiana*, *A. mapisaga*, and *A. atrovirens*, grown in cold weather and dried land, and poor quality pulque or *tlachique* produced by fermentation of poor quality aguamiel (low sugar content) from agaves grown in humid lands and temperate weather (Loyola-Montemayor 1956).

Due to the number and distribution of places where traditional pulque is produced, it is difficult to have a strict regulation and to avoid adulteration of the product, which is sometimes diluted with water to increase the volume of product, with gums to mask a deficient fermentation or with sugars to avoid offending flavors (Monterrubio 2007). Pulque is consumed alone or mixed with single or combined macerated fruits (strawberry, guava, maracuya, figs), vegetables (alfalfa, celery, tomatoes), nuts (pecans, almonds, peanuts, pine nuts, coconut), or condiments (cinnamon, black, or red pepper, coffee), beverages named *curados*.

43.5 Microbiology and Biochemistry of the Fermentation

43.5.1 Microbial Diversity in Pulque: Defining an Essential Microbiota?

Aguamiel constitutes a favorable medium for the proliferation of numerous microorganisms associated to the environment: from the cajete walls, incorporated with the scraping or extraction tools, from dust, or from insects such as *Drosophila* spp. This microbiota complements that of the starter present in the fermentation container to perform a series of distinctive fermentations processes that characterize pulque: acid (lactic and acetic), alcoholic (production of ethanol), and viscous (production of extracellular polysaccharides, EPS) (Sánchez-Marroquín and Hope 1953; Loyola-Montemayor 1956; Gonçalves de Lima 1990; García-Garibay and López Munguía 1993).

Early microbiological studies performed by Sánchez-Marroquín during 1946–1957, resulted in a description of the pulque microbiota and the proposal of *Lactobacillus* spp., *Leuconostoc mesenteroides*, *Zymomonas mobilis*, and the yeast *Saccharomyces cerevisiae* as the essential microorganisms in pulque fermentation. Using purified strains of these organisms, a mixed inoculum was developed and applied in controlled fermentation processes using sterilized aguamiel, demonstrating that the final fermented product was comparable in alcohol content, viscosity, and acidity to the traditional high-quality pulque produced commercially (Sánchez-Marroquín and Hope 1953; Sánchez-Marroquín et al. 1957). Although these results suggest the essential role of the selected microorganisms, recent studies on the

microbiology of aguamiel and pulque demonstrate the presence of a complex microbial diversity of bacteria and yeast (Escalante et al. 2004, 2008; Lappe-Oliveras et al. 2008).

Analysis of the bacterial diversity in pulque samples collected from Estado de México, Morelos, and Hidalgo demonstrate the presence of common species in all analyzed samples, but also unique species in pulque from each of the three regions. Lactic acid bacteria (LAB) related to *L. acidophilus* (homofermentative) was the most abundant group present in the samples, while the previously reported *L. mesenteroides* and *Z. mobilis* (considered as essential microorganisms) were detected in a minor proportion only in two of the studied samples (Escalante et al. 2004).

43.5.2 Microbial Dynamics of the Fermentation

Escalante et al. (2008) found in aguamiel an initial total count of 1.3×10^7 CFU/mL mesophilic bacteria, 3.2×10^8 CFU/mL LAB and 3.1×10^4 CFU/mL yeasts; in a sample taken after addition of the fresh aguamiel to the seed (corresponding to the initial fermentation time: T0) the total yeast count increased to 8.8×10^6 CFU/mL, while aerobic mesophilic bacteria and LAB counts were not modified: 1.2×10^7 and 1.5×10^8 CFU/mL, respectively. After 3 hours of fermentation, yeast increased to 1.4×10^7 CFU/mL, maintaining this level until the end of fermentation (6 hours); total bacteria counts remained constant (Escalante et al. 2008).

Bacterial diversity detected in aguamiel was composed mainly by *L. mesenteroides*, *L. citreum*, *L. kimchi*, and the Proteobacteria *Acinetobacter radioresistens*, *Erwinia rhapontici*, and *Enterobacter* spp. At T0, they were detected mainly some species observed in aguamiel and others present in the seed: *L. mesenteroides*, *L. citreum*, *Lactobacillus* spp. (related to *L. acidophilus*), and the Proteobacteria *E. agglomerans* (both in high proportion), *Kluyvera ascorbata*, *Serratia grimensis*, and the acetic acid bacteria (AAB) *Acetobacter malorum*. As fermentation proceed the population of *L. kimchi*, one of the most abundant LAB detected in aguamiel, decreased; those of *L. mesenteroides* and *L. citreum* remained relatively constant, while populations of *Lactococcus lactis* and *Z. mobilis* were detected in low proportion.

After 3 hours of fermentation, important changes in bacterial diversity were observed: *Lactobacillus* spp., *L. mesenteroides*, *E. agglomerans*, and *A. malorum* became the dominant species; *L. acidophilus* and *L. citreum* were detected in low proportion; *L. lactis*, *Z. mobilis*, and *A. radioresistens* remained relatively constant compared to T0, whereas *S. grimensis* and *K. ascorbata* were no longer found. At the end of the fermentation (6 hours), the bacteria diversity was mainly composed by the homofermentative *L. acidophilus* and *Lactobacillus* spp., the heterofermentative *L. mesenteroides*, *Lactococcus lactis* subsp. *lactis*, and *A. malorum*.

These results demonstrate that although no changes occurred in total bacteria counts during a 6-hour fermentation, important changes take place in species distribution.

Lactobacillus species have been previously detected in aguamiel and pulque fermentation, but no detailed information concerning the identity and role of this LAB group is available. Sánchez-Marroquín and Hope (1953) reported that lactobacilli are responsible for the assimilation of 53% of the sugars present in aguamiel. *L. acidophilus*, *L. hilgardii*, and the heterofermentative *L. sanfranciscensis* are capable to produce DL-lactic acid and to ferment glucose and fructose present in aguamiel, but only the former is able to ferment sucrose (Carr et al. 2002).

Lactobacilli are naturally associated to plants, fermented vegetables, fermented doughs, and alcoholic distilled beverages such as malt whiskey (van Beek and Priest 2000). The ability of LAB to grow in acid environments and tolerate high ethanol concentrations could explain their high abundance during pulque fermentation (final pH = 4.5 and 4%–6% ethanol, Escalante et al. 2008). The presence of the AAB *A. pomorum*, *A. malorum*, and *Gluconobacter oxydans* in different pulque samples and the high production of acetic acid in comparison to lactic acid after 6 hours of fermentation (Escalante et al. 2004, 2008), suggests an important role of these bacteria in pulque fermentation.

Yeast identified along pulque fermentation belong to *Saccharomyces* (*S. cerevisiae*, *S. bayanus*, *S. paradoxus*) and non-*Saccharomyces* (*Candida* spp., *C. parapsilosis*, *C. lusitaniae*, *Kluyveromyces marxianus*, *K. lactis*, *Hanseniaspora uvarum*, *Pichia* spp., *P. guilliermondii*, *Torulaspora delbrueckii*) are capable to produce ethanol from glucose, fructose, and sucrose and to synthesize nutritive amino acids and vitamins as well as flavor-volatile compounds that influence the quality and the aromatic

profile of the beverage. At early fermentation stages, the non-*Saccharomyces* yeasts develops, but as ethanol concentration increases, they are replaced by primary ethanol-producing yeasts such as *S. cerevisiae* and several *K. marxianus* ethanol-tolerant strains. Both yeasts species predominate throughout the fermentation until 6% of ethanol is reached (Lappe-Oliveras et al. 2008).

43.5.3 Physicochemical Changes during Pulque Fermentation

As already described, aguamiel is a sugar-rich sap containing sucrose, fructose, glucose, and fructo-oligosaccharides with a relatively acid pH (7.5–4.5), whereas pulque is an acidic (pH 4.3–3.5, total acidity of 0.75–0.4 g lactic acid/100 mL of pulque), alcoholic (4%–6%), and viscous beverage (Banco de Normas Mexicanas 2010b). Fresh aguamiel used to start fermentation with an initial pH of 6.0, decreases to 4.5 after mixing with the seed, and to 4.1 at the end of the process after 6 hours of fermentation. These environmental conditions favor the appearance of an acid- and ethanol-tolerant microbiota toward the end of the fermentation and probably affect the survival of non–acidic-resistant bacteria and yeast species that apparently disappear during the process as did the possible antimicrobial activity of LAB (production of bacteriocins and H_2O_2) and *Z. mobilis* subsp. *mobilis* (production of acetylmetylcarbinol) (Escalante et al. 2008; Lappe-Oliveras et al. 2008).

Fresh aguamiel has a mild temperature (17°C–20°C), which increases several degrees after inoculation with the seed as a consequence of the microbial activity. In a 6-hour fermentation carried out to assess the overall metabolic activity of the microbial community in aguamiel and pulque samples from the state of Morelos, the concentrations of sucrose, glucose, fructose, and fermentation products were determined during the process. Total sugars consumed after 6 hours of fermentation were 228.4 mM hexose equivalents (equivalent to 1370.5 mM of carbon, which was found as CO_2). It was assumed, in molar terms, that the amount of CO_2 produced is equal to the amount of acetic acid and ethanol. If 176.5 mM of carbon corresponding to 5.3 g/L of carbohydrates in EPS are also included, then 66.2% of total C was recovered in products. Ethanol reached a final value of 142.17 mM carbon equivalents, while 35.73 and 6.32 mM, respectively, of acetic and lactic acid concentrations were reached (Escalante et al. 2008).

Viscosity is one of the distinctive properties of pulque. This characteristic has been associated with EPS (dextran) production by *L. mesenteroides* strains (Sánchez-Marroquín and Hope 1953; Sánchez-Marroquín et al. 1957; Chellapandian et al. 1998; García-Garibay and López-Munguía 1993; Ramírez et al. 2004). However, after 6 hours of fermentation, Escalante et al. (2008) detected also a fructan EPS in pulque. Screening for EPS producing *Leuconostc* species from aguamiel and fermented pulque, allowed the isolation and identification of *L. citreum* as the unique EPS-producing bacteria (Conca 2008), questioning the essential role of *L. mesenteroides* during pulque fermentation.

43.6 Pulque: Nutritional and Traditional Medicine Aspects

Traditionally, pulque has been considered as a healthy beverage due to its nutrient content (described above). Based on a daily consumption, pulque can be considered as an important source of energy, vitamins, and essential amino acids, outstanding content of the essential amino acids lysine and tryptophan, which are deficient in the Mexican diet, which is based on maize. In some rural population in central Mexico where potable water supply is absent, pulque is consumed as a water substitute due to its low alcohol content and high availability (Vargas 1999). The modern indigenous Otomíes living in the Mezquital Valley include pulque as part of their basic diet; it represents the second source of energy (after tortillas) and the third source of protein. Moreover, pulque is their main source of vitamin C and second of thiamine, riboflavin, calcium, and bioavailable iron (Vargas et al. 1998). Backstrand et al. (2002) in a study of pulque consumption by several communities of the Estado de México highlands, established that a daily intake of 500 mL supplied 215 kcal, 45% of vitamin C, 10% of niacin, 7% of thiamine, 6% riboflavin, 15% of iron, and microbial protein (with an adequate proportion of lysine and tryptophan).

Considering that pulque is also an alcoholic beverage, it has the same problems associated to excessive consumption of nondistilled alcoholic beverages, but up to now, no toxicological problems other than those related to excessive consumption of any alcoholic beverage has been reported for pulque. Since

TABLE 43.1

Relevant Traditional Medical Uses and Beneficial Effects Associated with the Consumption of Pulque

Proposed Uses	Clinical Trial Evidence/Comments	References
Mixing with ground chili (*Capsicum annuum* L.) and pumpkin seeds (*Cucurbita* spp.) as a tonic	None	Traditional pharmacopoeia
Boiled with chichicpactli (bark of *Garrya laurifolia* Hartw. and *G. ovata* Benth.). Beneficial for chest, stomach, and back pains	None	Traditional pharmacopoeia
Control of: dyspepsia, stomach pain, diarrhea, anemia, anorexia, asthenia, vertigo, severe headache, and some neuralgias, urinal infections, typhus, pityriasis, and syphilis. Diuretic. It was consumed hot to favor diaphoresis, relieve cough, and facilitate expectoration. Sediments of pulque were used in plasters to heal wounds	None Effects probably associated to content of agave saponins (plants sterols with hypocholesterolemic, anti-inflammatory, and antibiotic activity). Specific effect of pulque remains unexplored	De la Cruz and Badiano 1964; Peana et al. 1997; Ramírez-Rancaño 2000
Increment of milk production during pregnancy and lactation	Possible galactogogue effect. Few clinical trials related to the content of ethanol in breast milk and effect on the weight and size of infant at moderate consumption	Argote-Espinoza et al. 1992; Flores-Huerta et al. 1992; Ortiz de Montellano 1993; Backstrand et al. 2001, 2004
Agaván (aguamiel syrup) against acute and chronic failure of kidney, bladder, and urethra; also for relief of cough and cold and control of anemia	None	Cook et al. 1995; Ramírez-Rancaño 2000
Control of anemia	Avoid ferritin and hemoglobin deficiencies due to a better absorption and iron bioavailability in the presence of ethanol and vitamin C. In particular, the consumption of pulque supplied the Otomí women population with 7% of their daily iron intake	Backstrand et al. 2001
Facilitate cellular respiration and absorption of carbohydrates and proteins	None	Vargas et al. 1998

the middle of the 20th century, several authors have tried to correlate the high incidence of cirrhosis and mortality in rural and indigenous communities in several central states of Mexico with pulque consumption. Nevertheless, it seems that there are other factors associated with the consumption of the beverage, as the presence of endotoxins produced by enterobacteria, the deficient nutrition of the consumers, which increase the rate of the disease or bad sanitary practices during its elaboration (Narro-Robles et al. 1992; Vargas et al. 1998; Kershenovich 1999).

Since the pre-Hispanic period, pulque has been used as traditional medicine to control several diseases (Table 43.1 summarizes principal traditional medical uses and beneficial effects associated to pulque consumption). During the colonial period several medical applications of maguey and its by-products were developed. Nevertheless, until very recently, the medical applications of pulque have been limited to traditional pharmacopoeia. Actually, there is an extensive application of pulque in the treatment of gastrointestinal disorders and intestinal infections that may be explained by pulque's prebiotic and probiotic activities.

43.6.1 Pulque as Probiotic and Prebiotic Agent

Aguamiel and pulque can be considered as probiotic products due to the presence of LAB, as *L. acidophilus* and *L. mesenteroides* (Escalante et al. 2004, 2008; Campos 2010), *Z. mobilis*, which has an

antagonist activity particularly against pathogenic bacteria and fungi species (Wuanick 1970; Gonçalves de Lima 1978) and yeast (Steinkraus 1996) and as prebiotics because of its fructan and oligofructan content (Ortiz-Basurto et al. 2008; Ramírez-Higuera 2009).

Several archeological evidences suggest that pulque has been used since pre-Hispanic times as an enema. It has recently been proven that this practice may have had beneficial effects on humans' health due to the enrichment of the digestive tract with potential probiotic bacteria (Lemus 2006). In support of the traditional applications of pulque in the treatment of several diseases, Campos (2010) reported the isolation of several *Leuconostoc* species from pulque, with potential probiotic activities such as resistance to acid and bile salts and antimicrobial activity *in vitro* and *in vivo* against pathogenic bacteria. Moreover, in recent experimental reports, Mancilla-Margalli and López (2006) and Ramírez-Higuera (2009) demonstrated the prebiotic activity of aguamiel inulin since it increases the growth of LAB.

43.7 Pulque Industrialization and Major Technological Challenges

43.7.1 Pulque Fermentation Medium Composition

The carbohydrate structure of agave plants and aguamiel is rather complex, combining the fructose polymer structure found in inulins and levans as described a few years ago by Mancilla-Margalli and Lopez (2006). Actually, the term *agavins* has been proposed by these authors due to the clear structural differences with inulin found in agave fructans. Most distilled agave fermented beverages rely on the total hydrolysis of sugars, while pulque fermentation properties are based in the amount of sucrose and monosaccharides found in aguamiel.

A modern production process requires the optimization and reduction in the agave production time. Each *agave pulquero* is capable to generate up to 1000 L of *aguamiel* in a daily extraction basis during 90 to 150 days, with a sugar content of 7% to 14%, depending on the agave species. For instance, in the case of *Agave tequilana* plants, the composition of water-soluble carbohydrates from plants of different ages revealed that 2-year plants exhibited the highest level of free monosaccharide and low-molecular-weight fructans (DP3–DP6) with potential application as prebiotics, while a maximum of fructan polymerization degree was achieved at 4 years with mean DP from 3 to 30 (Arrizon et al. 2009). According to these authors, as the type and amount of sugars accumulated in *A. tequilana* plants varies with age, harvesting time should be optimized in terms of productivity and product quality, a conclusion that could certainly be extrapolated to other agave species. This is in agreement with statements made previously by Sánchez-Marroquín (1970), who found the changes in aguamiel composition with collection time, agave region, and agave varieties an additional inconvenient for the process industrialization.

Surprisingly, Ortiz-Basurto et al. (2008) detected only minor differences in aguamiel composition among samples collected at different time intervals during the 3–6 months *aguamiel* harvest period of an *A. mapisaga* plant. Actually, the analyzed aguamiel contained 11.5 wt% of dry matter, which was composed mainly of sugars (75 wt%). Among these, 10 wt% were fructo-oligosaccharides. The variation of carbohydrate structure among the *agave pulqueros*, and its effect in the process and final product, is a research topic that has not yet been addressed, including the acceptability of industrialized pulque versions. On the other hand, industrialization has to be based in the planned and intensive production (propagation) of selected agave species from the actual wide variety of genetic diversity cultivated in México (García-Martín et al. 2007).

The introduction of hygienic aguamiel collecting and cajete-scraping techniques are also *conditio sine quibus* non for modernization. Aseptic mechanical extraction systems as well as the proper collecting and easy to handle reservoirs should be introduced. To avoid a preliminary fermentation, the agave juice must be pasteurized by microfiltration or cooled, as fermentation will occur even in the agave basin if not collected daily.

Up to now, no alternative to obtain aguamiel has been reported; however, it is always possible to obtain agave stem extracts by pressing or by extraction in diffusors of the agave stems. This alternative was reviewed previously (Steinkraus 1996), but no mention is made about the yields and sugar composition of the resulting extracts. This possibility may require the hydrolysis of fructans present in the agave stem as in

other agave fermentation procedures such as tequila, where the agave stems are either cooked (heat treated) or ground and subjected to water extraction to obtain fermentable sugars, mainly fructose from agavins. The 4–6 months required for aguamiel extraction could then be reduced to a few hours, but the resulting media although feasible for fermentation will no doubt differ from aguamiel and could not be referred as such.

43.7.2 Fermentation

The already described pioneer works of Alfredo Sánchez-Marroquín from 1953 to 1970 (Steinkraus 1996) are important not only in terms of the microbiological characterization of pulque, but also in terms of efforts in the modernization of the production process. Sánchez-Marroquín was the first to introduce a simplification of the enormous diversity of microorganisms isolated from the beverage to a minimum starter formula composed of *S. cerevisiae*, *Z. mobilis*, *Lactobacillus* spp., and *L. mesenteroides* (Sánchez-Marroquín et al. 1957; Sánchez-Marroquín 1970). Although the three types of fermentations (alcoholic, lactic, and viscous) have been generally recognized as an essential element of pulque production, the sensorial properties associated to the products of the three processes (alcohol, lactic acid, and viscosity) can be obtained by alternative microorganisms. For instance, EPS associated to pulque viscosity have always been recognized as dextrans (Sánchez-Marroquín and Hope 1953; Chellapandian et al. 1998) but may also include levans and/or inulins (Morales Arrieta et al. 2006; Olvera et al. 2007; Escalante et al. 2008). Isolated from pulque in 1924, *Z. mobilis*, recognized worldwide for its rapid alcohol fermentation, was included once among the essential pulque microbiota, but it is now considered as a "casual microorganism for pulque fermentation" (Escalante et al. 2004).

Besides its ethanol production properties, *Z. mobilis* is also known to produce levansucrase, an enzyme responsible for levan synthesis. Levan is a high-molecular-weight fructose polymer with β2–6 linkages among the fructose molecules, which also induces high viscosity. It is interesting to point out that no author has measured with precision the type and the amount of glycans (glucans and fructans) in pulque. However, and as already mentioned, it has been demonstrated that *Leuconostoc* spp. enzymatically produce dextrans, levans, and/or inulins from the equivalent extracellular or cell-associated enzymes (Chellapandian et al. 1998).

As early as 1967, Sánchez-Marroquín et al. reported that product quality as well as industrial operation would improve if the viscous fermentation is avoided. Actually, to avoid the synthesis of EPS, it is possible to invert all sucrose in aguamiel. Alternatively, glucose or fructose may also be added to support lactic and alcoholic fermentations. Finally, the presence of strains such as *L. mesenteroides* may be avoided. Nevertheless, efforts to preserve the prebiotic properties of fructo-oligosaccharides should be encouraged.

Although Sánchez-Marroquín et al. (1957) did not find differences in product quality following different inoculation strategies, parameters such as inoculation time and density of each of the selected strains remains to be defined in terms of growth rate (LAB grow much faster than yeast), final lactic acid and ethanol content, and eventually, EPS content. Kaffir (also known as Bantu), a traditional African beer, is also a complex traditional fermentation beverage that has been successfully produced on a large scale. In this case, the lactic acid fermentation of the saccharified sorghum starch takes place during one day, before alcoholic fermentation, which requires an additional 2 to 5 days.

Modern pulque versions will first have to solve the quest that represents the microbial definition of pulque, or alternatively, promote the product resulting from a selected microbial inoculum. Lappe-Oliveras et al. (2008) review the numerous reports dealing with pulque microbial studies in search for the optimal or essential microbial community; however, Escalante et al. (2008) demonstrated, using a polyphasic approach, to study bacterial diversity in aguamiel and pulque (including non–culture-dependent approaches and PCR-based molecular techniques) that the microbial definition of pulque remains as a difficult quest to solve.

43.7.3 Pulque Flavor

As far as flavor is concerned, Sánchez-Marroquín (1970) reports the measurement of an ester profile, but no reference is given regarding the composition and profile of the pulque bouquet. The total amount of esters (as ethylacetate) is reported as 20–30 mg/100 mL, aldehydes (as acetaldehyde) 2.5 mg/100 mL,

and higher alcohols (fusel oils) 80–100 mg/L. It has been proposed that the common tradition of combining pulque with fruits or vegetables (*curados*) derives from the intention to correct off flavors or fermentation defects. Offensive flavors derived from bacterial contaminations, frequent in traditional pulque, must absolutely be eliminated in a modern fermentation facility even if some traditional parts of the process are conserved. Some undesirable flavor compounds often result from a combination of metabolic pathways, particularly if a complex microbial diversity is involved. In a recent study, De León-Rodríguez et al. (2008) reported the aroma compounds of agave alcoholic beverages as determined by gas chromatography and headspace solid-phase microextraction–gas chromatography–mass spectrometry as well as a cluster analysis of the major components. There were 11 "major compounds" and around 17 minor compounds that could be used as authenticity markers since they were beverage-specific and provide an interesting fingerprint. It was observed that 3-methyl-thio-1-propanol and nonanoic acids are specific for pulque and may be used as authenticity markers. In this study, pulque is grouped separately from others agave beverages due to its low concentration of ethanol.

43.7.4 Pulque Conservation

The Mexican market in the United States and the ethnic food demands in the globalization era are the major driving forces to develop a stable pulque product produced at a larger scale. One of the main challenges in this direction lies in the difficulties associated to conservation. As pulque is usually consumed fresh after fermentation, mainly in rural areas, little efforts have been devoted to introduce stabilization procedures. Therefore, there are many decisions regarding pulque processing after fermentation that have to be taken into consideration, particularly if pulque production and commercialization continues to rise. These decisions involve the introduction of pasteurization and/or filtration steps as well as the addition of preservatives. If pasteurization had been introduced to pulque production at the same time as in beer we would probably be reading a different story about the evolution of both beverages in the 20th century.

It is almost impossible to bottle fermented pulque without pasteurization, as sugars are usually not exhausted; actually, a residual amount of agavin and fructo-oligosaccharides is desirable in order to increase the prebiotic attributes of the product. On the other hand, filtration or sterilization would increase pulque stability and shelf life but reduce its probiotic content, also recognized as one of pulque nutritional attributes; efforts to preserve or supplement its vitamin (ascorbic acid) and nonheme iron content should also have to be considered. Moreover, additives such as preservation agents, antioxidants, colorants, or texture agents will affect the image of pulque as a natural and, eventually, organic product. In the particular case of the pasteurized bottled brand "Pulque, Cool Passion" (Poliqui SA de CV), the product contains sodium benzoate as preservative, as well as color and sugars. The selection of an adequate packaging requires consideration as biological and photochemical oxidation may also be a problem in storage stability. The paradigm here is that to promote the industrialization of pulque, radical changes in the public perception of pulque have to be made, particularly if new markets are searched. An advantage is that the new pulque producers are probably offering a product that the large majority of the public (particularly outside the country) never tasted before. Actually, it is highly possible that new production processes will require modifications in the Mexican regulation system, as aguamiel as well as pulque are defined specifically in terms of composition and production norms (NMX-V-022 and NMX-V-037) approved in 1972.

According to the Del Razo group, which started canned pulque production in 1980 in the state of Tlaxcala to fulfill the pulque demand in the United States, the market had already grown to more than 100,000 L/month by the start of the 21st century. The Del Razo pulque canning process includes aseptic canning and a controlled process to preserve pulque's sensorial and physicochemical properties. After several unsuccessful efforts, the Del Razo group finally developed an effective process and now exports to the United States the brand "Pulque Hacienda 1881" in various flavor presentations as well as natural or plain pulque. According to Del Razo, the modification of their fermentation process has reduced the traditional off-flavor associated with pulque and also its viscosity, which means that neither *Z. mobilis* nor *L. mesenteroides* (nor at least the wild strains) are included in the process. Other trademarks include Jicara, Malinche, Pulquemex, and Pulque Azteca, also competing for the North American market.

After the paradigmatic work carried out by Sánchez-Marroquín, we are now witnessing a renewed interest in this ethnic beverage from both basic and applied research. Pulque is now fully recognized as rich in tradition, microbial diversity, and nutritional value. The recovery of "traditional pulque," as well as the birth of new alternatives of aguamiel processing are promoted by the demand of Mexicans living abroad as well as the curiosity of consumers looking for new gastronomic and nutritional experiences.

REFERENCES

Argote-Espinosa RM, Flores-Huerta S, Hernández-Montes H, Villalpando-Hernández S. 1992. Plasma clearance of ethanol and its excretion in the milk of rural women who consume pulque. *Rev Inv Clin* 441:31–6.

Arrizon J, Morel S, Gschaedler A, Monsan P. 2009. Comparison of the water-soluble carbohydrate composition and fructan structures of *Agave tequilana* plants of different ages. *Food Chem* 122:123–30.

Backstrand JR, Allen LH, Black AK, Mata M, Pelto G. 2002. Diet and iron status of non-pregnant women in rural central Mexico. *Am J Clin Nutr* 76:156–64.

Backstrand JR, Allen LH, Martínez E, Pelto G. 2001. Maternal consumption of pulque, a traditional central mexican alcoholic beverage: relationship to infant growth and development. *Pub Health Nutr* 4:883–91.

Backstrand JR, Goodman AH, Allen LH, Pelto GH. 2004. Pulque intake during pregnancy and lactation in rural Mexico: alcohol and child growth from 1 to 57 months. *Eur J Clin Nutr* 58:1626–34.

Banco de Normas Mexicanas [Internet]. Chapingo, Edo Mex: Colegio de Posgraduados de Chapingo; 2010 [Accesed 2010 Dec 2 a]. Available from: http://www.colpos.mx/bancodenormas/nmexicanas/NMX-V-022–1972.PDF.

Banco de Normas Mexicanas [Internet]. Chapingo, Edo Mex: Colegio de Posgraduados de Chapingo; 2010 [Accesed 2010 Dec 2 b]. Available from: http://www.colpos.mx/bancodenormas/nmexicanas/NMX-V-037–1972.PDF.

Bellingeri Martini, M. 1980. Las haciendas en México. México, D.F. Departamento de Investigaciones Históricas, Escuela Nacional de Antropología e Historia. 80 p.

Blomberg L. 2000. Tequila, mezcal y pulque, lo auténtico mexicano. México D.F.: Editorial Diana F. 284 p.

Bruman HJ. 2000. *Alcohol in Ancient Mexico*. Salt Lake City: The University of Utah Press. 158 p.

Campos I. 2010. Aislamiento e identificación de bacterias lácticas del pulque con capacidad probiótica. [MSc thesis]. México, D.F.: Universidad Nacional Autónoma de México. 88 p. Available from: Tesiunam database: www.dgbiblio.unam.mx/index.php/catalogos.

Carr FJ, Chill D, Maidas N. 2002. The lactic acid bacteria: a literature survey. *Crit Rev Microbiol* 28:281–370.

CECULTAH [Internet]. Pachuca, Hgo: Gobierno del Estado de Hidalgo; c 2010 [Accesed 2010 Nov 15]. Available from: http://cultura.hidalgo.gob.mx.

Chellapandian M, Larios C, Sánchez-González M, López-Munguía A. 1988. Production and properties of a dextransucrase from *Leuconostoc mesenteroides* IBT-PQ isolated from'pulque,' a traditional Aztec alcoholic beverage. *J Ind Microbiol Biotechnol* 21:51–6.

Conca R. 2008. Aislamiento e identificación de cepas de bacterias productoras de nuevos polisacáridos extracelulares del pulque. [MSc thesis]. México, D.F.: Universidad Nacional Autónoma de México. 85 p. Available from: Tesiunam database: www.dgbiblio.unam.mx/index.php/catalogos.

Cook JD, Reddy MB, Hurrel RF. 1995. The effect of red and white wines on non–heme-iron absortion in humans. *Am J Clin Nutr* 61:800–4.

Corcuera de Mancera S. 1991. *El fraile, el indio y el pulque. Evangelización y embriaguez en la Nueva España (1523–1548)*. México D.F.: Fondo de Cultura Económica. 309 p.

De la Cruz M, Badiano J. 1964. *Libellus de medicinalibus indorum herbis*. México, D.F.: IMSS. 394 p.

De León-Rodríguez A, Escalante-Minakata P, Jiménez-García MI, Ordoñez-Acevedo LG, Flores Flores JL, Barba de la Rosa AP. 2008. Characterization of volatile compounds from ethnic *Agave* alcoholic beverages by gas chromatography-mass spectrometry. *Food Technol Biotechnol* 46:448–55.

Diguet L. 1928. *Les cactacies utiles du Mexique. Archives D´Histoire Naturelle*. Paris: Societé Nationale D´Acclimatation de France. p 139–42.

Escalante A, Giles-Gómez M, Hernández G, Córdova-Aguilar MS, López-Munguía A, Gosset G, Bolívar F. 2008. Analysis of bacterial community during the fermentation of pulque, a traditional Mexican alcoholic beverage, using a polyphasic approach. *Int J. Food Microbiol* 124:126–34.

Escalante A, Rodríguez ME, Martínez A, López-Munguía A, Bolívar F, Gosset G. 2004. Characterization of bacterial diversity in Pulque, a traditional Mexican alcoholic fermented beverage, as determined by 16S rDNA analysis. *FEMS Microbiol Lett* 235:273–9.

Flores-Huerta S, Hernández-Montes H, Argote RM, Villalpando S. 1992. Effects of ethanol consumption during pregnancy and lactation on the outcome and postnatal growth of the offspring. *Ann Nutr Metab* 36:121–8.

García-Garibay M, López Munguía A. 1993. Bebidas alcohólicas no destiladas. In: García-Garibay M, Quintero-Ramírez R, López-Munguía A, editors. *Biotecnología Alimentaria*. México, D.F.: LIMUSA. p 263–311.

García-Martín C, Laarqué Saavedra A, Eguiarte JE, ZizumboVillarreal D. 2007. *En lo ancestral hay futuro: del tequila los mezcales y otros agaves*. México, D.F.: CICY, CONACYT, CONABIO, SEMARNAT, INE. 402 p.

García-Mendoza A. 1995. El maguey una planta maravillosa. In: *Cofradía en apoyo de la Mayora Mexicana A C. En torno al pulque y al maguey*. México D.F.: Grupo Editorial Siquisiri. 72 p.

García-Mendoza A. 1998. *Con sabor a maguey: Guía de la colección nacional de Agaváceas y Nolináceas del Jardín Botánico, Instituto de Biología-UNAM*. México D.F.: Jardín Botánico, Instituto de Biología-UNAM. 114 p.

Gobierno del Estado de Hidalgo: Museo Nacional de Culturas Populares. 1988. *El maguey: árbol de las maravillas*. Pachuca: Gobierno del Estado de Hidalgo. 178 p.

Godoy A, Herrera T, Ulloa M. 2003. *Más allá del pulque y del tepache. Las bebidas alcohólicas no destiladas de México*. México, D.F.: Instituto de Investigaciones Antropológicas, Universidad Nacional Autónoma de México. 107 p.

Gonçalves de Lima O. 1978. *El maguey y el pulque en los códices mexicanos*. México D.F.: Fondo de Cultura Económica. 278 p.

Gonçalves de Lima O. 1990. *Pulque, balché y pajauaru*. México, D.F.: Fondo de Cultura Económica. 405 p.

Kershenovich D. 1999. Endotoxinas y daño hepático. In: *Fundación de Investigaciones Sociales A. C. (FISAC). El pulque, la cultura y la salud*. México, DF: Cuadernos FISAC. p 29–37.

Lappe-Oliveras P, Moreno-Terrazas R, Arrizon-Gaviño J, Herrera-Suárez T, García-Mendoza A, Gschaedler-Mathis A. 2008. Yeasts associated with the production of Mexican alcoholic non distilled and distilled *Agave* beverages. *FEMS Yeast Res* 8:1037–52.

Lemus E. 2006. Los enemas prehispánicos como instrumentos para aplicar probióticos. *Tem Cien Tecnol* 10:17–26.

Loyola-Montemayor E. 1956. *La industria del pulque*. México, D.F.: Departamento de investigaciones industriales. Banco de México SA. 348 p.

Mancilla-Margalli NA, Lopez MG. 2006. Water-soluble carbohydrates and fructan structure patterns from *Agave* and *Dasylirion* species. *J Agric Food Chem* 54:7832–9.

Martín del Campo R. 1938. El pulque en el México precortesiano. *An Inst Biol UNAM* 9:5–23.

Monterrubio AL. 2007. *Las haciendas pulqueras de México*. México, D.F.: Universidad Nacional Autónoma de México. 257 p.

Morales Arrieta S, Rodríguez ME, Segovia L, López-Munguía A, Olvera Carranza C. 2006. Identification and functional charcaterization of *levS*, a gene coding for a levansucrase from *Leuconostoc mesenteroides* NRRL B-512 F. *Gene* 376:59–67.

Narro-Robles J, Gutiérrez-Ávila H, López-Cervantes M, Borges G, Rosovsky H. 1992. La mortalidad por cirrosis hepática en México II. Exceso de mortalidad y consumo de pulque. *Rev Sal Pub Mex* 34:388–405.

Olvera C, Centeno-Leija S, López-Munguía A. 2007. Structural and functional features of fructan sucrases present in *Leuconostoc mesenteroides* ATCC 8293. *Anton Leeuw Int J G* 92:11–20.

Ortiz de Montellano B. 1993. *Medicina, salud y nutrición aztecas*. México, DF: Siglo XXI Editores. 346 p.

Ortiz-Basurto RI, Pourcelly G, Doco T, Williams P, Dornier M, Belleville MP. 2008. Analysis of the main components of the aguamiel produced by the maguey-pulquero (*Agave mapisaga*) throughout the harvest period. *J Agric Food Chem* 56:3682–7.

Peana AT, Moretti MDL, Manconi V, Desole G, Pippia D. 1997. Antiinflammatory activity of aqueous extracts and steroidal sapogenins of *Agave americana*. *Plant Med* 63:199–204.

Poblet M. 1995. El pulque, una bebida que se extingue. In: Delgado A, editor. *En torno al pulque y al maguey*. México, D.F.: Grupo Editorial Siquisiri. 72 p.

Ramírez JF, Sánchez-Marroquín A, Álvarez MM, Valyasebi R. 2004. Industrialization of Mexican pulque. In: Steinkraus K, editor. *Industrialization of Indigenous Fermented Foods*. 2nd ed. New York: Marcel Dekker. p 548–75.

Ramírez Castañeda E. 1994. El maguey y el pulque. *Mex Desc* 18:10–9.

Ramírez-Higuera A. 2009. Evaluación del efecto prebiótico del aguamiel del maguey (*Agave salmiana*) en *Lactobacillus delbrueckii* spp. *bulgaricus*. [MSc thesis]. México, D.F.: Unidad Profesional Interdisciplinaria de Biotecnología (UPIBI), Instituto Politécnico Nacional. 77 p. Available from UPIBI Library.

Ramírez-Rancaño M. 2000. *Ignacio Torres Adalid y la industria pulquera*. México, D.F.: Instituto de Investigaciones Sociales, Universidad Nacional Autónoma de México. 796 p.

Robelo AC. 1948. *Diccionario de aztequismos*. México D.F.: Fuente Cultural. 548 p.

Sánchez-Marroquín A. 1949. Nuevos datos acerca de la microbiología del pulque. *Mem Rev Soc Cient Ant Alz* 56:505–17.

Sánchez-Marroquín A. 1970. Investigaciones realizadas en la Facultad de Química, UNAM, tendientes a la industrialización del agave XIV. *Rev Soc Quim Mex* 4:184–8.

Sánchez-Marroquín A, Hope PH. 1953. Agave juice. Fermentation and chemical composition of some species. *Agric Food Chem* 1:246–9.

Sánchez-Marroquín A, Terán J, Piso J. 1957. Estudios sobre la microbiología del pulque. XVIII. *Rev Soc Quim Mex* 1:167–74.

Segura JC. 1901. *El maguey. Memoria sobre el cultivo y beneficio de sus productos*. 3rd ed. México, D.F.: Oficina Tipográfica de la Secretaría de Fomento. 411 p.

Soberón-Mora A. 1998. El pulque: elixir milenario. In: Muría JM, editor. *Beber de tierra generosa. Historia de las bebidas alcohólicas en México*. México, D.F.: Fundación de Investigaciones Sociales AC. 249 p.

Steinkraus K. 1996. Mexican pulque. In: Steinkraus K, editor. *Handbook of Indigenous Fermented Foods*. 2nd ed. New York: Marcel Dekker. 776 p.

Vallejo C. 1992. Los códices prehispánicos. *Mex Desc* 180:30–41.

van Beek S, Priest FG. 2000. Decarboxylation of substituted cinnamic acids by lactic acid bacteria isolated during malt whisky fermentation. *Appl Environ Microbiol* 66:5322–8.

Vargas LA. 1999. El pulque en la cultura de los pueblos indígenas. In: *Fundación de Investigaciones Sociales A. C. El pulque, la cultura y la salud*. México, D.F.: Cuadernos FISAC. 72 p.

Vargas LA, Aguilar P, Esquivel MG, Gispert M, Gómez A, Rodríguez H, Suárez C, Wacher C. 1998. Bebidas de la Tradición. In: Muría JM, editor. *Beber de tierra generosa. Historia de las bebidas alcohólicas en México*. México, D.F.: Fundación de Investigaciones Sociales AC. 249 p.

Wuanick MC. 1970. Cura de vaginitis de etiologia variada pelo emprego cultura de *Zymomonas mobilis* (Lindner). *Rev Inst Antibiot Rec* 10:47–50.

44

Probiotic Nondairy Beverages

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CONTENTS

44.1 Introduction

In recent years, alimentary habits have been influenced by several reports that relate diet and health. In this sense, functional foods have been outstanding since they are included in the food or food ingredients that promote beneficial effects on host health beyond nutritional functions. This market has been very promising and it is expected to reach US$ 8.6 billion by 2015, representing an increase of 21% compared to 2009 (Starling 2010). The functional products that have gained most popularity are energy drinks and fortified dairy products.

The incorporation of the probiotic strains in food and beverages is a global trend and the functional properties of these products have been demonstrated through scientific findings. New products containing probiotic strains have been launched, particularly drinks based on fruit and cereals. The last are interesting for the formulation of nondairy products, since they contain biologically active ingredients (e.g., β-glucan), which can act as prebiotics. Juices are already recognized as healthy food, consumed frequently by a large part of the world population. Besides, nondairy matrices do not contain dairy allergens (e.g., lactose). Also, the cholesterol level is lower than those found in dairy products.

Positive information about the consumption of probiotic products have been published in scientific reports emphasizing the health benefits when consumed regularly. Antimicrobial activity, improvement

in lactose metabolism, reduction in serum cholesterol, antimutagenic and anticarcinogenic properties, immune system stimulation, and prevention of gastrointestinal infections are some actions conferred by consumption of probiotics.

Fruit juices have been established into functional beverages segment through the commercialization of calcium- and vitamin-fortified juices. Despite that juices are suggested as ideal vehicles for delivering probiotic microorganisms, they present drawbacks such as low pH (between 2.5 and 3.7) and changes in sensory and nutritional aspects after processing. Besides, in many cases the supplementation is necessary. Also, vegetables could be used in probiotic fermentations because they have functional food components such as minerals, vitamins, dietary fibers, and antioxidants. Some studies have proposed, for example, the use of cabbage and tomato juices (Yoon et al. 2004, 2006).

Beverages containing probiotic strains represent a promising market. However, the development of this sector is confronted with challenges such as the appropriate processing and storage besides the factors that influence the consumer acceptability (i.e., appearance, aroma, and texture characteristics, age, gender, exposure time, information about health benefits). In this chapter, an overview of traditional and commercially available probiotic nondairy beverages as well as the technological aspects for successful production of these products is discussed.

44.2 Functional Foods

For thousands of years, microorganisms have been used to produce and preserve foods through the process of fermentation. Ancient peoples, such as the Babylonians, Egyptians, and Greeks, realized the benefits of fermentation, and the foods containing the lactic acid bacteria (LAB) and other microbial cultures were associated with the positive effects on human health such as yogurt, fermented milks, kefir, tempeh, soy sauce, cheese, fermented meat, and vegetables (Kanellaki et al. 2008). Foods that bring some benefit to health may be defined as functional foods. There are many definitions to functional foods, but a widely accepted definition describes functional food as a food that "affects beneficially one or more target functions of the body, beyond suitable nutritional effects, in a way that is relevant to either an improved state of health and well-being and/or reduction of risk of disease" (Diplock et al. 1999).

Functional foods with their specific health effects can indicate a new mode of thinking about the relationships between food and health in everyday life. Consumers are opposed to change the habits, even when these are known to be unhealthy (Williamson et al. 2000), but want to consume healthy foods (Brunso et al. 1996).

Previous research (Bech-Larsen and Grunert 2003) showed that the evaluation of health claims is partially determined by healthiness perceptions of the product. Consumers can only be expected to substitute conventional foods by functional foods if these are perceived as healthy (Ares and Gambaro 2007) and the promised health benefits are considered as relevant (Verbeke 2005). Consumers perceptions of the healthiness, processes, and enrichments involved in the production of functional foods are crucial to determine their acceptance (Bech-Larsen and Grunert 2003; Verbeke 2005).

Currently, bioprocess technology is an important tool using specific dietary microorganisms to produce certain desired fermented functional food products. Submerged fermentation as well as solid state fermentation is being employed to enrich various liquid, and solid substrates for the synthesis of several important biomolecules that brings several health benefits, which improve food functionality.

44.3 Probiotics

Microorganisms considered probiotics can be added to food and beverages or other products (e.g., supplements in the form of tablets, capsules, and freeze-dried preparations) resulting in probiotic products. Probiotics can be defined as "live microorganisms which when administered in adequate amounts confer a health benefit on the host" (FAO/WHO 2001). They can be found in dairy or nondairy matrices.

This group of microorganisms is economically relevant and represents a large part of sales of functional foods. The global market for probiotics was US$ 15.9 billion in 2008, and it is expected to reach US$ 19.6 billion by 2013 (Agheyisi 2008).

The mostly probiotic strains belong to the genera *Lactobacillus* and *Bifidobacterium*, with *Streptococcus*, *Enterococcus*, *Bacillus*, and *Pediococcus*, and some yeasts also are included. Some probiotic strains are presented in Table 44.1.

Ideally, probiotic strains should present benefits to the human health and technological properties such as resistance to bile and acid, adhesion to the human epithelial cells, colonization of the gastrointestinal system, production of antimicrobial system (e.g., bacteriocins), nonpathogenic and generally regarded as safe, low cost, maintain the viability during processing and storage and facility of the application in the product (Havenaar and Huis In't Veld 1992; Collins et al. 1998; Prado et al. 2008).

The regular consumption of probiotics is recommended to establish a positive balance of the useful or beneficial microbes in the intestinal flora (Soccol et al. 2010). Studies have demonstrated some therapeutical applications of the probiotics covering the prevention of urogenital diseases (Martinez et al. 2009), alleviation of constipation (Higashikawa et al. 2010), protection against gastrointestinal infections (Zocco et al. 2006; Iannitti and Palmieri 2010), reduction of hypercholesterolemia (Park et al. 2007), modulation of the immune system (Arunachalam et al. 2000), protection against some types of cancer (Lee et al. 2004), and prevention of allergies (Isolauri et al. 2000). However, for beneficial effects of probiotics are achieved is recommended intake of 7 log CFU/g at the consumption of 7 log CFU/g is recommended (FAO/WHO 2003).

44.3.1 Nondairy Probiotic Functional Foods

Nowadays, there are many functional foods and food products commercially available. Most of them are nondairy products that are produced by fermentation, which improves their functionality. Although dairy products are known as healthier, lactose intolerance and cholesterol content are two major drawbacks related to probiotic dairy products. Besides, traditions and economic reasons that limit the use of dairy fermented products in some developing countries promote the idea of reduction of dairy products as vehicles for probiotics and, in general, functional compounds (Prado et al. 2008). Cereals, fruits, vegetables, and soybeans are the main vehicles that have been used as nondairy and alternative food matrices to deliver probiotics (Table 44.2).

Although several food products are considered functional, they have improved their functionality and bioactivities after fermentation processes. Some examples have been demonstrated in literature,

TABLE 44.1

Microorganisms Regarded as Probiotics

***Lactobacillus* Species**	***Bifidobacterium* Species**	**Other Species**
Lb. acidophilus	*B. bifidum*	*Streptococcus thermophilus*
Lb. casei Shirota	*B. breve*	*Enterococcus faecalis*
Lb. delbrueckii spp. *Bulgaricus*	*B. infantis*	*Enterococcus faecium*
Lb. johnsonii	*B. longum*	*Pediococcus acidilactici*
Lb. reuteri	*B. adolescentis*	*Lactococcus lactis*
Lb. rhamnosus	*B. animalis*	*Leuconostoc mesenteroides*
Lb. plantarum	*B. lactis*	*Bacillus cereus*
Lb. salivarius		*Escherichia coli* Nissle 1917
Lb. crispatus		*Propionibacterium freudenreichii*
Lb. gasseri		*Saccharomyces boulardii*
Lb. gallinarum		*Saccharomyces cerevisiae*

Sources: Adapted from Holzapfel, W. H. et al., *Int J Food Microbiol*, 41(2), 85–101, 1998. Chow, J., *J Ren Nutr*, 12(2), 76–86, 2002. With permission.

TABLE 44.2

Alternative Nondairy Probiotic/Functional Foods

Product	Probiotic Strain	Cell Density	References
Maize flour and barley malt	*Lactobacillus reuteri*, *Lactobacillus acidophilus*, *Lactobacillus rhamnosus*	10^7–10^8 CFU/g	Helland et al. 2004
Malt Barley and wheat flours	*Lactobacillus reuteri*, *Lactobacillus acidophilus* *Lactobacillus plantarum*, *Lactobacillus fermentum*	10^8–10^{11} CFU/mL 10^7–10^9 CFU/mL	Charalampopoulos et al. 2002
Brown rice flour	*Bifidobacterium longum*	10^8–10^9 CFU/mL	Kabeir et al. 2005
Oat-based cereal bar	*Bifidobacterium lactis*	10^9 CFU/25g	Ouwehand et al. 2004
Maize or rice pudding	*Bifidobacterium animali*, *Lactobacillus acidophilus*, *Lactobacillus rhamnosus*	10^4 CFU/g	Helland et al. 2004b
Yosa (oat bran pudding)	*Bifidobacterium lactis*, *Lactobacillus acidophilus*	Not available	Salovaara 1996
Ogi (fermented cereal gruel processed from maize)	*Lactobacillus plantarum*	Not available	Okagbue 1995
Table olives	*Lactobacillus rhamnosus*, *Lactobacillus paracasei*, *Bifidobacterium bifidum*, *Bifidobacterium longum*	10^6–10^8 CFU/g	Lavermicocca et al. 2005
Artichokes	*Lactobacillus plantarum*, *Lactobacillus paracasei*	10^7–10^8 CFU/g	Valerio et al. 2006
Soy frozen dessert	*Lactobacillus acidophilus*, *Lactobacillus paracasei*, *Bifidobacterium lactis*, *Lactobacillus rhamnosus*, *Saccharomyces boulardii*	10^9 CFU/g	Heenan et al. 2004

and besides, many products are commercially available. Some of them are fermented soybeans, fruits, cereals, vegetables, and their health benefits are presented in Table 44.3.

44.4 Nondairy Probiotic Beverages

According to market researchers cited by Beveragedaily.com (2010), functional beverages make up one of the most dynamic parts of the beverage worldwide market. As a result of marketing and innovation, the level of consumer awareness of different types of probiotics has improved in the last years. This has driven research efforts into the development of alternative products that can appeal to a wide range of consumers. One emerging area of innovation has been probiotics and prebiotics in nondairy beverages. These types of products accounted for 10.7% of the global market for prebiotics and probiotics in 2008. Due to this value, several food companies have begun researching possible probiotic line extensions.

An increasing consumer demand for nondairy probiotic products push novel raw materials with regard to probiotics technology. The present technological innovations include finding solutions for the stability and viability problems of probiotics in new food matrices, such as fruits, cereals, and other vegetables (Farnworth et al. 2007). A total of 78% of current probiotic sales in the world today are delivered through fermented milk (Granato et al. 2010). Cereal, fruit juice, vegetable, and soybean beverages demonstrated to be a suitable nondairy alternative for delivering probiotics (Gobbetti et al. 2010; Rivera-Espinoza and Gallardo-Navarro 2010).

Probiotic beverages are usually marketed in the form of fermented milks and yogurts; however, with an increase in consumer vegetarianism throughout the developed countries, there is also a demand for

TABLE 44.3

Improvement of Functionality by Fermentation of Nondairy Products

Fermented Product	Benefits	Reference
Fermented soybean (tempeh, miso, sufu, soy sauce, shoyu)	Prevents certain hormone-dependent, cardiovascular diseases and various cancers Reduce blood lipids, oxidized LDL, homocysteine, and blood pressure Ameliorates symptoms of menopause Improves bone mass and reduce bone resorption, minimizing the osteoporosis effects	Dalais et al. 1997 Muhlbauer and Li 1997 Ridges et al. 2001 McCue and Shetty 2006
Soy sauce	Promotes digestion, enhances gastric juice secretion in humans, and increases the appetite Promotes histamine absorption as the causative agent of the blood pressure decrease effect Catalyzes the hydrolysis of angiotensin I resulting in the generation of the potent vasoconstrictor, which regulates arterial blood pressure Antimicrobial activity effect against pathogens Contains shoyuflavones produces histamine, a mediator of inflammation, allergy, and gastric acid secretion	Kojima 1954 Skeggs et al. 1956 Laragh et al. 1972 Tsuji et al. 1995 Kataoka et al. 2005
Natto, a fermented soybean by *Bacillus subtilis* (*natto*) spores and tap water	Beneficial effects on intestinal microflora and feed efficiency; effects on the immune system, antiallergy effect of subtilisin, fibrinolytic activity of subtilisin, role of vitamin K in the prevention of osteoporosis, presence of phytoestrogens against cancer and osteoporoses Reduces allergenic effect of soyabean	Hosoi and Kiuchi 2003
Tempeh, a soybean product fermented by *Rhizopus oligosporus*	Increases the nutritional values of some nutrients, development of vitamins, phytochemicals and antioxidative constituents—phytoestrogen concentration is high (0.3 mg/g soy protein) and is relatively high compared to other soybean products such as tofu and soy beverages Calcium-rich food Highest content of isoflavone	Astuti and Dalais 2000 Hutabarat et al. 2001 Bhathena and Velasquez 2002
Tofu	Digestion rate of 95% A good phytoestrogen concentration 0.1–2 mg/g soy protein	Shurtleff and Aoyagi 1999 Bhathena and Velasquez 2002
Grapes, specially red wines	Contains resveratrol, a polyphenolic compound responsible for acting as a cancer chemoprevention agent—anti-inflammatory factor and an antioxidant agent—inhibition of platelet aggregation and promotion of vasodilation, enhancing the production of a protective enzyme in the cardiovascular system—concentrations of phenolic compounds are 2–7 times higher than the concentrations in red grapes (Cabernet Sauvignon) and 3–12 times higher in Petite Sirah grapes Flavonoids (catechin and procyanidins)	Bhat et al. 2001 Orallo et al. 2002 Bradamante et al. 2004 Wolter et al. 2004
Vegetables (spinach, chicory, garlic, onion, mushrooms, cocoa)	Contains flavonoids, which prevent coronary heart disease and cancer mortality	Vattem and Shetty 2006
Fruits (grapes, black berries, cranberry, oranges, apples)	Contains catechins and procyanidins, which prevent coronary heart disease and cancer mortality Solid state fermentation of cranberry increases phenolic antioxidants, enrichment of ellagic acid by *Rhizopus oligosporus*. Fungal glycosidases mobilize some phenolic antioxidants in cranberry pomace and their activity by hydrolysis via β-glucosidase and releasing the aglycone, one of the functional phenolic phytochemicals found in fermented fruit products	Vattem and Shetty 2006

(*continued*)

TABLE 44.3 (Continued)

Improvement of Functionality by Fermentation of Nondairy Products

Fermented Product	Benefits	Reference
Green tea	In green tea there are four main cathechins, that is, epicatechin, epicatechin-3-gallate, epigallocatechin, and epigallocatechin-3-gallate, similar to flavonoids. The compounds found in both green and black teas inhibit a newly discovered chemical pathway involving an enzyme called JNK-2, appearing to play a key role in the development of tumors	Graham 1992 Park and Dong 2003
Black tea	Produced by fermentation of green tea (*Camellia sinensis*), contains polyphenols, flavonoids, and alkaloids (caffeine and theobromine), carbohydrates, tannins, and minerals High concentration of poplyphenolic gives the property of anticancer agent Contains theaflavins, which act as an antioxidant, cancer suppressor, and inhibitor of pathogenic microorganisms Antimicrobial compounds	Graham 1992 Tu et al. 2005 Cabrera et al. 2006 Wang and Li 2006 Mo et al. 2008
Fermented beans	Reduction of undigestible oligosaccharides such as stachyose and raffinose, hydrolyzed by α-galactosidases from acid lactic bacteria	Sanada et al. 2009 Yamaguishi et al. 2009
Vinegar from malt, cider, fruits, or even wine	Presence of phytochemicals, polysaccharides, protein phenolic compounds, which prevents hypertension, recovery from exhaustion, improves digestion, controls the blood glucose level, raises appetite, and promotes calcium absorption	Kondo et al. 2001 Xu et al. 2007
Cacao	Cocoa seeds (*Theobroma cacao* L.) are rich in phenolic compounds such as catechin, epicatehin, and the flavonoid procyanidins	Efraim and García 2004 Nazaruddin et al. 2006
Kimchi (fermented vegetables)	Korean fermented vegetables such as beachu, radish, green onion, red pepper powder, garlic, ginger, seafood by LAB *Leuconostoc mesenteroides* and *Lactobacillus sakei* High levels of vitamin C, β-carotene, dietary fiber, flavonoids, and chlorophylls, which are believed to be effective in preventing cancer Antitumoral activity Antimutagenity agents	Kwon and Kim 2007 Cho et al. 2009
Fermented meat	Hydrolysis of proteins into bioactive peptides, which would have some bioactivity. It was measured the ACE (angiostensin-I-converting enzyme) inhibitory activities of extracts of fermented sausages higher than not fermented products	Kato et al. 1994 Arihara 2006
Fermented fish (gyoshoyu)	Powerful antioxidative activity	Long et al. 2000
Cereals-based probiotic foods	In fermented cereals (breads and other products), improve texture and palatability, gluten-free products, stabilize or increase levels of various bioactive compounds, retard starch bioavailability (lower glycemic index products), improve mineral bioavailability Indicate for celiac disease population	Charalampopoulos et al. 2002, 2009 Arendt and Moore 2007
Prebiotics carbohydrates (inulin, oligofructose, fructooligosaccharides, acacia gum)	Their presence in foods stimulates the growth and activity of bifidobacteria and LAB in human intestine, bringing some health benefits: stimulation of immune factors, production of digestive enzymes, assistance in controlling the formation of free radicals	Gibson 1999 Gordon 2002

(*continued*)

TABLE 44.3 (Continued)

Improvement of Functionality by Fermentation of Nondairy Products

Fermented Product	Benefits	Reference
Microalgae	Produces antioxidants (carotenoids and xantophylls) Antimicrobial compounds such as polysaccharides or fatty acids (ω3), important role in mental health and neural function such as in visual development, depression, and schizophrenia Vitamins and vitamin precursors including α-tocopherol, β-carotene, niacin, thiamine, and ascorbic acid, phenolics such as polyphenolics, and hydroquinones and flavonoids, phospholipids particularly phosphatidylcholine, terpenoids, peptides, antioxidative substances, which contribute to the inhibition or suppression of cell oxidation processes Contains phenolic compounds, catechin, epigallocatechin, catechol, glycoside rutin, glycoside hesperidin, phlorotannins, and bomophenols Reduces risk of cardiovascular disease and cancers Contains polysaccharides in the form of alginates, fucans, lamininarans, cellulose, agar, and carrageenans. Also contributes to the antioxidant activity of marine algae	Chkhikvishvili and Ramazanov 2000 Ruperez et al. 2002 Takamatsu et al. 2003 Germany 2007 Yuan 2008

the vegetarian probiotic products. Furthermore, lactose intolerance, allergenic milk proteins, and cholesterol content are drawbacks related to the intake of dairy products, which makes the development of new nondairy probiotic foods essential (Granato et al. 2008). Technological advances have made possible to change some characteristics of matrices (e.g., fruits) by modifying food components in a controlled way (Betoret et al. 2003). This could make them good substrates for delivery probiotics. In addition, these substrates contain essential diet nutrients such as minerals, vitamins, dietary fibers, and antioxidants (Yoon et al. 2004), while lacking the drawbacks cited above (Luckow and Delahunty 2004; Sheehan et al. 2007).

Nowadays, around the world, there are a broad variety of traditional and commercial nondairy beverages with presumptive probiotic feature (Table 44.4). Much to them are nonalcoholic beverages produced with cereals, fruit, and vegetables as principal raw materials.

44.4.1 Traditional Beverages

Even though cereal grains constitute a major source of dietary nutrients all over the world, they are lacking in certain basic nutritional components (e.g., essential amino acids, B group vitamins). To overcome this limitation, fermentation might be the simplest way to improve nutritional value (Blandino et al. 2003; Gobbetti et al. 2010). Traditional fermented beverages (Table 44.4) have an important contribution to the human diet, especially in many African countries because fermentation is an inexpensive technology, which preserves the food and enhances its sensory properties (Gadaga et al. 1999).

Boza is a beverage consumed mainly in the Balkan region. It is a sweet colloid suspension made from wheat, rye, millet, maize, and other cereals mixed with carbohydrate. The microflora of *Boza* shows a rich source of presumable probiotic LAB and yeast (Todorov et al. 2008). The LAB isolated has been identified by Gotcheva et al. (2000) as *Lb. plantarum*, *Lb. acidophilus*, *Lb. fermentum*, *Lb. coprophilus*, *Leuconostoc raffinolactis*, *L. mesenteroides*, and *Leuconostoc brevis*. The yeasts isolated comprise *S. cerevisiae*, *Candida tropicalis*, *Candida glabrata*, *Geotrichum penicillatum*, and *Geotrichum candidum*. Strains of *Lactobacillus pentosus*, *Lactobacillus paracasei*, and *Lb. rhamnosus* that demonstrated active bacteriocins against pathogens were found in this product (Güven and Benlikaya 2005).

Bushera is the most common traditional cereal-based beverage prepared in the Western highlands of Uganda. The sorghum or millet flour from the germinated sorghum and millet grains is mixed with boiling water, and the mixture is left to ferment at ambient temperature for 1–6 days. The LAB isolated

TABLE 44.4

Potential Probiotic Traditional and Commercial Nondairy Beverages

Product	Microorganisms	Substrates	References
Traditional			
Boza	*Lactobacillus*, *Lactococcus*, *Pediococcus*, *Leuconostoc*, yeast	Cereals	Gotcheva et al. 2000; Todorov et al. 2008
Bushera	*Lactobacillus*, *Lactococcus*, *Leuconostoc*, *Enterococcus*, *Streptococcus*, *Weissella*	Sorghum and millet	Muyanja et al. 2003
Hardaliye	*Lactobacillus*, yeast, molds	Red grapes and mustard seeds	Arici and Coskun 2001
Kombucha	*Acetobacter*, *Gluconobacter*, *Lactobacillus*, yeast	Green or black tea	Teoh et al. 2004
Mahewu	*Lactobacillus*, *Lactococcus*	Maize	Blandino et al. 2003; MacMaster et al. 2005
Mangisi	LAB, yeast, molds	Millet	Zvauya et al. 1997
Pozol	*Lactobacillus*, *Lactococcus*, *Leuconostoc*, *Pediococcus*, yeast, molds	Cocoa and maize	Wacher et al. 2000
Togwa	LAB, yeast	Cereals	Willumsen et al. 1997; Molin 2001; Oi and Kitabatake 2003
Commercial®			
B.E. Wholegrain Liquid	*Lactobacillus*, *Saccharomyces*	Cereals	AGM Foods Pty, Grainfields Australia, Wynnum, Australia
Friscus	*Lb. plantarum* HEAL9 and *Lb. paracasei* 8700:2	Fruit juice	Skånemejerier, Bravo, Malmö, Sweden
Gefilus	*Lb. rhamnosus* ATCC 53103 (GG)	Fruit juice	Valio Ltd, Valio Gefilus, Helsinki, Finland
Goodbelly	*Lb. plantarum* 299v	Fruit juice	NextFoods, GoodBelly, Boulder, Colorado, USA
Lemon and ginger	*Lactobacillus*, *Saccharomyces*	Lemon juice, honey, and cereals	AGM Foods Pty, Grainfields Australia, Wynnum, Australia
Probiotic soymilk	Not available	Soymilk	Pulmuone Wildwood, Wildwood Organics, Fullerton, California, USA
Proviva	*Lb. plantarum* 299v	Fruit juice and cereals	Skånemejerier, Pro viva, Malmö, Sweden
Rela	*Lb. acidophilus*, *Lb. reuteri*, *B. lactis*	Fruit juice	Arla Ingman Ou Ab., Rela, Soderkulla, Finland
Silk Live	*Lb. bulgaricus*, *St. thermophilus*, *Lb. acidophilus*, *B. bifidum*, *Lb. casei*, *Lb. rhamnosus*	Soymilk	WhiteWave Foods, Silk, Broomfield, Colorado, USA
Vita Biosa	*Lb. acidophilus*, *Lb casei*, *B. bifidum*	Herbs	Biola Inc., Hamilton, Ontario, Canada

Note: ®Registered trademark. Available at online database of each manufacturer.

from bushera comprised of five genera, *Lactobacillus*, *Lactoccoccus*, *Leuconostoc*, *Enterococcus*, and *Streptococcus* (Muianja et al. 2003).

Hardaliye is a lactic acid fermented beverage found in Turkey that is produced from red grape with the addition of crushed mustard seeds and benzoic acid. During a *Hardaliye* fermentation process, Arici and Coskun (2001) found *lactobacillus* (*Lactobacillus paracasei* subsp. *paracasei* and *Lactobacillus casei* subsp. *pseudoplantarum*), yeast, and molds in the product.

Kombucha is a 2-week sweet fermented green or black tea consumed in Asia for more than two centuries. The microorganisms are a symbiotic cluster of acetic acid bacteria, LAB, and yeast (Chen and Liu 2000; Teoh et al. 2004). The metabolic and ecological interactions occurring during kombucha fermentation are still not well understood.

Mahewu also known as *amahewu* is a sour spontaneous fermented beverage made from corn meal, consumed in Africa. It is produced from maize porridge, which is mixed with water and sorghum, millet malt, or wheat flour. Generic mahewu is produced industrially, using *Lb. bulgaricus* subsp. *delbruecki* or *Lactobacillus brevis* (McMaster et al. 2005). The predominant microflora found in African mahewu is *Lactococcus lactis* subsp. *lactis* (Blandino et al. 2003).

Mangisi is a sweet-sour naturally fermented beverage much popular in Zimbabwe. The preparation of product involves malting of finger millet, milling, cooking, and a spontaneous fermentation. LAB, yeast, and molds were observed in *Mangisi* by Zvauya et al. (1997).

Pozol is a beverage produced in the south of Mexico, made by cooking maize in a 1% w/v lime solution, washing with water, grinding to make a dough known as *mixtamal*, shaping into blocks, wrapping in banana leaves, and leaving to ferment at ambient temperature for 1 to 4 days. The fermented product is suspended in water prior to drink. A heterogenous and potential probiotic microflora (BAL, *Enterobacteriaceae*, yeast, and molds) was isolated from pozol by Wacher et al. (2000).

Togwa is a starch-saccharified traditional beverage containing LAB and yeast, consumed by children and adults as refreshment in Africa, usually made from maize flour and millet malt (Oi and Kitabatake 2003). It is also produced using cassava flour and/or other cereals (Molin 2001). *Togwa* presented activity against enteropathogens (Kingamkono et al. 1998) and improved the barrier function of the intestinal mucosa in children with acute diarrhea (Willumsen et al. 1997).

44.4.2 Commercial Beverages

During the last years, probiotic microorganisms have been increasingly included mainly into commercial health promoting dairy products. Several worldwide companies have begun researching possible probiotic innovations. Recently, beverage and food industries innovated and moved to products with human wellness claims launching nondairy probiotic beverages in the market (Table 44.4).

For example, B.E. Wholegrain Liquid® (AGM Foods Pty, Grainfields Australia, Wynnum, Australia) is a fermented liquid that delivers LAB (*Lb. acidophilus* and *Lb. delbreukii* subsp. *bulgaricus*) and yeasts (*S. boulardii* and *S. cerevisiae*) as well as vitamins, amino acids, and enzymes. It is made from organic ingredients including grains, beans, and seeds such as the malted oats, maize, rice, alfalfa seed, pearl barley, linseed, mung beans, rye grain, wheat, and millet. The product claims wellness of the digestive system. Vita Biosa® (Biosa Inc., Hamilton, Ontario, Canada) is a mixture of aromatic herbs and other plants that are fermented by a combination of LAB cultures (*Lb. acidophilus* and *Lb. casei*) and *B. bifidum*. This beverage contains carbon dioxide and no sugar. Vita Biosa also contains a high number of antioxidants. The beverage claims ability to quickly restore the natural balance of microflora in the digestive system. Proviva® was launched in Sweden by Skånemejerier (Malmö, Sweden) in 1994. The active component comprises *Lb. plantarum* 299v fermented oatmeal gruel. Malted barely is added to enhance the liquefaction of the product. This fermented is used as the ingredient in the final product in which 5% of the oatmeal gruel is mixed with a fruit drink. The consumer product contains 10 log CFU/mL. The same company launched an extension of Proviva probiotic fruit juice beverage, based on clinical evidence that such microorganism can improve iron intake in women. The product differs from classic Proviva, containing higher amounts of iron. Valio Ltd. (Helsinki, Finland) began developing nondairy beverages with the strain *Lb. rhamnosus* GG in 1996. Gefilus® fruit beverages contain *Lb. rhamnosus* and have a shelf life of 5 weeks when refrigerated. Rela® is a fruit juice with *Lb. reuteri* MM53 manufactured by the Arla Ingman Ou Ab. (Soderkulla, Finland).

44.4.3 Scientific Finds

Current technological innovations in the field of probiotics include finding solutions for the stability and viability problems in new food environments, such as cereals, vegetables, and fruits. The development of nondairy probiotic beverages became a challenge for both scientific and applied research. Researches are important to develop new media and conditions for probiotic growth and development, increasing the number of products with established functionality. Key ingredients are necessary to obtain suitable sensory and physicochemical characteristics, probiotic stability, and shelf life. Cereals, vegetables, and fruit

juices are the main vehicles that have been used as nondairy alternative matrices to deliver probiotics in beverages. As fermentation process involves mixed cultures such as LAB, yeast, and fungi, traditional fermented foods are a plentiful source of potential probiotic microorganism. This section presents the current status of nondairy probiotic beverages.

44.4.3.1 Cereal-Based Beverages

Lei et al. (2006) studied a spontaneous fermented millet drink as a natural probiotic treatment for diarrhea in young children in Ghana. The authors concluded that no effect for the use of fermented millet drink was observed in the first week of intervention. The antibiotics used in the diarrhea treatment may affect the LAB, or the LAB in the fermented millet drink had no probiotic effects.

Molin (2001) reviewed some studies that check the probiotic characteristics of an oatmeal gruel fermented by a low pH resistant *Lb. plantarum* 299v mixed with a fruit juice. The product contains 12 log CFU/L and demonstrated an increase in the concentration of carboxylic acids (i.e., short-chain fatty acids in the colon are an energy source for the mucosal cells) in feces of healthy volunteers. This observed increase was due to a change in the composition of the colonic microflora (Mack et al. 1999). When the product was administrated to patients with irritable bowel syndrome, abdominal bloating, and pain were significantly lower in the treatment group than in the placebo group. The treated group also experienced better overall gastrointestinal function after 12 months of treatment (Nobaek et al. 2000). The beverage when administered during 6 weeks in a group of men with moderately elevated cholesterol showed significant reduction in the fibrinogen (produced during inflammatory process; linked to an increase risk factor for thrombosis) and LDL-cholesterol concentrations in serum (Bukowska et al. 1998). More studies are necessary to better understand what mechanisms are involved in the *Lb. plantarum* 299v intestinal and physiological effects because this strain demonstrated a good performance in an acidic system as fruit juice and in the human gastrointestinal system.

A whole-grain oat substrate was fermented with a previously studied probiotic strain *Lb. plantarum* B28 isolated from *Boza* (Gotcheva et al. 2000, 2002) by Angelov et al. (2006) to obtain a beverage with health benefits of the probiotics and oat prebiotic β-glucan. Several factors affecting the obtaining of a functional oat fermented beverage were established. The results demonstrated that the addition of aspartame, sodium cyclamate, saccharine, and Huxol as sweeteners had no effect on the dynamics of the fermentation process and on the viability of the starter culture during product storage (21 days).

Kedia et al. (2007) produced a malt potential probiotic substrate fermented by a mixed culture of *Lb. reuteri* 11951 and a yeast strain isolated from a traditional Chinese *Jiu Qü*, which is used as inoculum for the production of *Jiu Niang*, an alcoholic rice fermented food. Aroma, flavor, and pH were investigated. The *Lb. reuteri* 11951 growth was enhanced by the introduction of the yeast in the system. In mixed culture product, pH was lowered and the production of lactic acid and ethanol were increased in comparison against pure LAB culture.

Survivability of *Bifidobacterium pseudocatenulatum* G4 in fermented peanut milk supplemented with a prebiotic fructooligosaccharide and human grade yeast extract was evaluated for a period of 2 weeks by Kabeir et al. (2009). Under refrigeration storage the viability of the strain was maintained high at a level > 7 log CFU/mL. Lower accumulation of propionic and butyric acids in the product was detected, promoting the acceptability and preference of the final product.

Since a few decades ago, the soybean has received attention from researchers due to its protein quality and role in prevention of chronic disease such as menopausal disorder, cancer, atherosclerosis, and osteoporosis (Rivera-Espinoza and Gallardo-Navarro 2010). Because of their functional properties and vegetarian claim, it has a great application potential in the food industry. Probiotic soy-based fruit juice products became the new generation of beverages on the market (Granato et al. 2010). Fermentation has been a traditional option to increase digestibility of soy products and make them more flavored. Soy fermentation by probiotic LAB has the potential to reduce the levels of some carbohydrates possibly responsible for gas production in the intestinal system, increase isoflavone levels, and favor desirable changes in bacterial populations in the gastrointestinal tract (Champagne et al. 2009).

Soymilk is a good substrate suitable for growth of LAB. Several studies reported the production and use of fermented soymilk beverages as probiotic (e.g., Beasley et al. 2003; Shimakawa et al. 2003; Farnworth

et al. 2007; Champagne et al. 2009). Pham and Shah (2007) used two strains of *B. animalis* for a successful biotransformation of isoflavone glycosides to aglycones in soymilk. Donkor et al. (2007) assessed α-galactosidase activity and the release of bioactive peptides with ACE-inhibitory activities of *Lb. acidophilus* LAFTI® L10 and La4962, *B. lactis* LAFTI® B94, *B. longum* B1536, *Lb casei* LAFTI® L26 and Lc279, *Lb. delbrueckii* ssp. *bulgaricus* Lb1466, and *Streptococcus thermophilus* St1342 in fermented soymilk. All strains exhibited variable α-Galactosidase activity with *B. lactis* LAFTI® B94 showing the highest activity. *B. lactis* LAFTI® B94, *S. thermophilus* St1342, and *Lb. acidophilus* La4962 reduced raffinose (α-galactosides of sucrose nondigestible in the human gut to the absence of α-galactosidase) by more than 55%. All strains released bioactive peptides with ACE-inhibitory activities between 17% and 43%. Wang et al. (2006) concluded in their study that LAB and bifidobacteria can increase antioxidative activity in a fermented soymilk.

44.4.3.2 Vegetable-Based Beverages

Numerous natural food nutrients of particular interest that positively affect human health are phytonutrients from vegetables. Investigations have confirmed the antitumorous effects of vegetable extracts (Rakin et al. 2007). Highlighting this aspect, some studies presented in this section associated vegetable juices with probiotics to improve the nutritive quality of vegetables.

Some strains of LAB can convert starch into lactic acid, such as *Lactobacillus* and *Streptococcus* (Parada et al. 1996). For example, *Lb. plantarum* A6, isolated by Giraud et al. (1993), showed extracellular amylase activity.

Sour cassava starch is obtained by a natural fermentation, and this product is largely appreciated, for example, in Africa and South America. Santos (2001) developed a probiotic beverage with fermented cassava flour and (liquid phase plus solid) using a mixed culture of *Lb. plantarum* A6 and *Lb. casei* Shirota. The beverage maintained its microbiological (9 log CFU/mL) and physicochemical quality during 28 days of storage at 4°C.

The suitability of red beets as a vegetable-based substrate in the production of probiotic beet juice by beneficial bacteria (*Lb. acidophilus*, *Lb casei*, *Lb. delbrueckii* subsp. *bulgaricus*, and *Lb. plantarum*) was studied by Yoon et al. (2005). The study reported that all strains reduced the pH of beet juice due to their ability to produce lactic acid. Rakin et al. (2007) supplemented beetroot and carrot juices with brewers' yeast autolyzate before fermentation with *Lb. acidophilus* NCDO1748. The addition of the autolyzate favorably affected the increase of the number of LAB during the fermentation, reduction of fermentation time and enrichment of the vegetable juices with amino acids, vitamins, minerals, and antioxidants.

Nazzarro et al. (2008) developed a functional fermented carrot juice beverage with *Lb. rhamnosus* DSM20711 and *Lb. bulgaricus* ATCC 11842 supplemented with prebiotic components inulin and fructooligosaccharides. Both bacterial strains were capable of growing in carrot juice and survival at low pH during 4 weeks of storage at 4°C. *Lb. acidophilus* DSM 20079 was encapsulated in alginate-inulin-xanthan prebiotic gum and its ability to grow in carrot juice and survive 8 weeks of storage at 4°C and subsequent exposure to artificial gastrointestinal conditions were assessed by Nazzarro et al. (2009). Encapsulation significantly enhanced cell viability after fermentation and storage and protected *Lb. acidophilus* from exposure to simulated gastric conditions.

Yoon et al. (2006) developed a probiotic cabbage juice using LAB (*Lb. plantarum* C3, *Lb. casei* A4, *Lb. delbrueckii* subsp. *bulgaricus* D7). The cultures grew well on cabbage substrate and reached about 9 log CFU/mL after 48 hours of fermentation. After 4 weeks of the cold storage at 4°C, the viable cell of *Lb. plantarum* and *Lb. delbrueckii* were 7 and 5 log CFU/mL, respectively. *Lb. casei* did not survive at low pH and completely loose cell viability after 2 weeks of cold storage.

Shurkhna et al. (2006) modeled lactic acid fermentation of leguminous plant juices (fodder galega, red clover, and alfalfa) to provide a comparative efficiency assessment of the previously selected strains of LAB as potential components of starter cultures. LAB strains appeared efficient in fermenting (with reference to the rate and degree of acidogenesis, ratio of lactic and acetic acids, and dynamics of microflora) leguminous substrates.

Ferrari et al. (unpublished data) developed a functional probiotic fermented beverage using herbal mate extract as an organic ingredient which is also hypocholesterolemic and hepatoprotective. Among

different strains tested *Lb. acidophilus* ATCC 4356 demonstrated good fermentation of the substrate. Key ingredients in the extract included xanthines, polyphenols, and other antioxidants with potential benefits for the consumer. The addition of honey positively affected the development of *Lb. acidophilus* and the formulated beverage maintained microbial stability during shelf life.

44.4.3.3 Fruit-Based Beverages

Several years ago, juice manufacturers presented an interest into the development of fruit-based functional beverages with probiotics for gut benefit as line extensions of existing functional drinks. Fruit juice probiotic beverages became an important category because they have taste profiles that are appealing to all age groups and because they are perceived as healthy and refreshing foods (Rivera-Espinoza and Gallardo-Navarro 2010).

In the last years, many companies have released products, such as antioxidant-rich fruit juices and probiotics with digestive and immune health benefit claims. These functional beverages are dairy-free, soy-free, wheat-free, and vegan. Fruit juice can be certainly positioned as a healthy food product, due to its great amount of healthy components like vitamins, antioxidants, and polyphenols that exerts several positive benefits on the human health (Granato et al. 2010). Nowadays, Valio Ltd. (Finland) with the product Gefilus disputed the claim by Skånemejerier (Sweden) with the product Bravo that it was the world's first probiotic 100% fresh juice (Beveragedaily.com 2009).

The supplementation of probiotics to juices may require special technology because the microorganism can suffer in a number of processing situations (e.g., heat treatment or pasteurization) and matrix conditions (e.g., acidity) (Saxelin 2008). Chr Hansen (Denmark) launched in 2005 a flexible formulation system for adding probiotics directly to finished liquid products. The technology uses an aseptic dosing machine that allows the bacteria to be added just before they are filled into package. According to Sheehan et al. (2007), *Lactobacillus* and *Bifidobacterium* survived longer in orange and pineapple juice. *Lb. casei* DN-114001, *Lb. rhamnosus* GG, and *Lb. paracasei* NFBC43338 displayed robustness surviving in orange and pineapple juice for at least 12 weeks. However, after thermal pasteurization and high-pressure treatments, the strains were not capable of withstanding the treatments required to achieve a stable juice at levels > 6 log CFU/mL.

LAB has been considered a fastidious microorganism that demands essential amino acids and vitamins for growing. Several studies presented some probiotic strains that have the capability to develop in fruit matrices. Researchers have reported that cell viability depends of the strains used, the characteristics of the substrate, the oxygen content, and the final acidity of the product (Rivera-Espinoza and Gallardo-Navarro 2010).

The effect of potential probiotics and autochthonous LAB on health-promoting and sensory properties of tomato juice was studied by Di Cagno et al. (2009). Compared to nonfermented tomato juice or tomato juice fermented with allochthonous strains, the values of viscosity, color, total antioxidant activity, concentration of ascorbic acid, glutathione, and total free amino acid increased when the tomato juice was fermented with autochthonous starters.

Yoon et al. (2004) determined the suitability of the tomato juice as a raw material for the production of probiotic juice by *Lb. acidophilus* LA39, *Lb. plantarum* C3, *Lb. casei* A4, and *Lb. delbrueckii* subsp. *bulgaricus* D7. The LAB reduced the pH to 4.1, and the viable cell counts reached a range of 6 to 8 log CFU/mL after 4 weeks of cold storage at 4°C. Wang et al. (2009) assessed the feasibility of the fruit noni as a raw substrate for the production of probiotic juice by *Lb. casei*, *Lb. plantarum*, and *B. longum*. All tested strains grew well on noni juice. After 4 weeks of storage at 4°C, *B. longum* and *Lb. plantarum* survived under low pH conditions in fermented noni juice.

Soccol and Prado (2007) developed a green coconut probiotic water fermented by *Lb. plantarum* AC-1, *Lb. plantarum* B-7, and *B. animalis* subsp. *lactis* BFL-9 in the same proportion. The supplementation of the beverage with yeast extract, hydrolyzed soy protein, and sucrose produced maximum yield to mixed culture in 8 hours of fermentation. After 28 days of cold storage at 4°C, lactobacilli and *B. animalis* subsp. *lactis* presented 8 and 7 log CFU/mL, respectively. The fermented beverage added of sugar and coconut aroma presented good sensorial characteristics and acceptability from

potential consumers. Furthermore, the beverage could be classified as "low calorie" delivering only 33.5 kcal/100 mL.

Shah et al. (2010) examined the survival of *Lb. rhamnosus* HN001, *B. lactis* HN001, and *Lb. paracasei* Lpc 37 probiotic strains in a model fruit juice system pH 3.8 composed by trisodium citrate, citric acid, saccharose, water, vitamins, and antioxidants. The model juice containing vitamin C, grape extract, and green tea extract showed better survival of probiotic bacteria. The model juice containing grape seed extract, green tea extract, and vitamin C at the end of the 6 week storage period demonstrated an average viability of 4 log CFU/mL, 7 log CFU/mL, and 6 log CFU/mL, respectively.

Probiotic lactobacilli strains (*Lb. acidophilus* LB2, LB3, and LB45, *Lb. brevis* LB6, *Lb. rhamnosus* LB11 and LB24, *Lb. fermentum* LB32, *Lb. plantarum* LB42, and *Lb. reuteri* LB38) were evaluated for their ability to survive in a commercial fruit drink (mixture of pineapple, apple, orange, pear and/or grape, passion fruit, and lemon) stored at 4°C for up to 80 days by Champagne and Garder (2008). The viability observed was strain dependent. *Lb. acidophilus* LB3, *Lb. rhamnosus* LB11, *Lb. reuteri* LB38, and *Lb. plantarum* LB42 strains were selected to study their resistance to simulated gastrointestinal (GI) conditions. Viability in presence of 0.3% bile salts or of pancreatic enzymes was not affected by previous refrigerated storage.

Champagne et al. (2008) studied the viability during storage in conditions of simulating consumer use of *Lb. rhamnosus* R0011 inoculated in an apple pear raspberry juice. Study parameters were age of the juice, sampling size, incubation temperature, and atmosphere. Results showed that the strain lost in parts its viability in the juice when the product package was shaken, opened, and the cells were exposed to oxygen.

Saarela et al. (2006) investigated the stability of differently processed (fermentation time, pH during drying, and cryoprotectant) freeze-dried *B. animalis* subsp. E-2010 (Bb-12) cells in fruit juice and milk. Sucrose-protected cells survived better in juice, thus concluding that when choosing a cryoprotectant for a probiotic, the stability in target food applications should be also considered.

44.5 Technological Challenges

Nondairy beverages present a good potential for cultivation of probiotic microorganisms. However, few studies about the probiotics survival, criteria for fermentation, use as starters, and their relationship with other microorganisms have been reported (Kedia et al. 2007). The use of nondairy matrices is a challenge for technological performance since different species of microorganism present different sensitivities to the pH of substrate, postacidification in the final product, metabolism products, temperature, drying systems, and gastrointestinal conditions. Some factors have influenced the choice of substrate and strains and some measures to overcome these drawbacks are described below.

44.5.1 Choice of the Raw Material

Most of probiotic microorganisms are LAB, which are fastidious and need nutrients (e.g., amino acids, vitamins) to grow. Despite this requirement, some probiotic bacteria are able to grow in fruit matrices.

The cell viability depends on the strains used, characteristics of the substrate, oxygen content, and final acidity of the product (Shah 2001). The cranberry juice, for example, seems to be a less favorable medium compared to the orange and pineapple juices (Sheehan et al. 2007). It was demonstrated that red beets can be used as raw material in the preparation of probiotic beet juice. Among LAB tested, *Lb. plantarum* produced more lactic acid and was the more stable strain after 4 weeks at 4°C (Yoon et al. 2005).

When cereals are used in the probiotic fermentations, some parameters should be considered: composition and processing of cereal grains, substrate formulation, growth and productivity of the starter culture, stability of the probiotic strain, organoleptic properties, and nutritional value of the final product (Charalapompoulos et al. 2002). Malt has shown to support the growth of *Lb. plantarum*, *Lb. fermentum*,

Lb. acidophilus, and *Lb. reuteri*. In addition, wheat and barley extracts could exert protective effect on the viability of *Lb. plantarum*, *Lb. acidophilus*, and *Lb. reuteri* under acidic conditions (pH 2.5) (Charalapompoulos et al. 2003).

Oats represent the major source of β-glucan and have demonstrated great potential for growing *Lb. reuteri*, *Lb. acidophilus*, *Lb. plantarum*, and *B. bifidum* (Mårtenson et al. 2002; Angelov et al. 2006). β-Glucans are dietary fibers that can act as prebiotics and also lower the plasma cholesterol and postprandial serum glucose levels. It was demonstrated that these components were not affected by fermentation of oat based beverage with *Lb. plantarum* ATCC 8014, so they were available at the time of consumption (Gupta et al. 2010).

Several reports have indicated the soymilk as a good substrate for probiotic bacteria. Most studies use *Lactobacillus* and *Bifidobacterium* species for fermentative assays. This approach is interesting for increasing the consumption of soy-based products since they naturally present disagreeable flavor and their digestibility is affected by sugars such as raffinose and stachyose. The fermentation of soy and other grains by LAB have been proposed to solve these problems (Wang et al. 2003; Yamaguishi et al. 2009), and this has resulted in greater acceptance of soy products.

44.5.2 Synergy with Starter Microorganisms

Probiotics have been used as both starter and nonstarter cultures in dairy products, but their use as starters in fruits, vegetables, or grains is less common. In industrial processes, starter microorganisms are usually used with probiotic strains. It is important that strains involved in the fermentation do not operate antagonistically toward the probiotic strain.

The use of yeast has proved to be beneficial to the growth of LAB. In malt-based substrate fermentation, for example, the addition of yeast into the process increased the *Lb. reuteri* population. This may have been related to the production of vitamins, which promote a better growth of bacteria (Kedia et al. 2007).

It was demonstrated that probiotic bacteria grow slowly in cow milk. Whereas soy is a good substrate for growth in these bacteria, studies have suggested the formulation of soy yogurts using yogurt starter cultures (*S. thermophilus* and *Lb. delbrueckii* subsp. *bulgaricus*). It was found *Lb. rhamnosus* ATCC 53103 (GG) and *Lb. johnsonii* NCC533 (La-1) did not affect the growth of the starters strains and they were able to grow in soy beverage. In addition, it may be that the growth of probiotics is dependent on the liberation of amino acids by other bacteria (Farnworth et al. 2007). Also, Champagne et al. (2009) evaluated the behavior of the probiotic strains in commercial soybean beverage. They observed that *B. longum* R0175 and *Lb. helveticus* R0052 grew better in the presence of *S. thermophilus* against other strains tested.

44.5.3 Acidification

Naturally acidic substrates or low pH after fermentation are harmful to probiotic microorganisms because they lead to a decrease in the viability and stability. The tolerance to different acid conditions seems to be strain-specific.

Lb. plantarum C3 and *Lb. delbrueckii* subsp. *bulgaricus* D7 showed a better survival (8 log CFU/mL) in cabbage juice in comparison to *Lb. casei* A4 (not detected) when stored for 2 weeks at 4°C (Yoon et al. 2006).

Food carriers have influenced the survival of probiotic strains. It must be considered that during gastrointestinal passage, they are exposed to acid and bile conditions, which is interesting because they are protected by a microencapsulation system, thereby improving their stability.

The microencapsulation provides protection for probiotics through the entrapment of cells in hydrocolloid beads, which results in the increase of the viability and stability in food matrices as well as during the passage into the gastrointestinal tract. It can be performed by several techniques, including spray-drying, freeze-drying, and fluidized bed-drying. These techniques convert the cultures to a concentrated powdered form and allow the release of bacteria in the product (Krasaekoopt et al. 2003).

The supports usually used in microencapsulation are agar, polyacrylamide, chemically modified chitosan beads, and alginates (Kailasapathy 2002). Fruits like apple and quince have been suggested as immobilization supports and provided the maintenance of the biocatalyst by *Lb. casei* for the production of lactic acid after an extended storage time (129 days) a 4°C (Kourkoutas et al. 2005).

B. lactis DSM 10140 cells were immobilized in gellan/xanthan microcapsules and added to mahewu beverage. The microencapsulation reduced significantly the loss in viability culture during storage for 21 days at 4°C (McMaster et al. 2005). Also, the stability of the microencapsulated cells can be affected by the choice of different substrates. The survival of *B. animalis* spp. *lactis* E-2010 (Bb-12) was better in juice when cells were protected with sucrose compared with reconstituted skim milk (Saarela et al. 2006).

44.5.4 Heat Treatment

The most common thermal processing for assuring the safety and prolonging the shelf life is pasteurization. The treatment of juices becomes necessary since unpasteurized juices already have been involved in food-borne disease cases (Singh et al. 1996; Cook et al. 1998). Besides removing food pathogens, yeast, and mold, pasteurization is reliable and effective in the destruction/inactivation of enzymes. However, thermal processing can alter flavor and odor (especially in orange juice) characteristics and even comprise the nutritional value (e.g., decrease in the vitamin content).

Thermotolerant probiotic strains should be selected in order to assure the minimum viable level in the product (7–8 log CFU/g). In addition, pressure treatment (at least 400 MPa) could be used in combination with heat treatment (below 50°C) in order to inactivate the pectin methyl esterase, which causes instability of the citrus juices decreasing their shelf life (Bayindirli et al. 2006).

44.5.5 Perception of the Off-Flavors

It was known that several factors influenced the consumers' preference. First criterion for food selection is taste. Other factors are health considerations, exposure, age, and gender. Consumers relate foods to health are potential niche markets for functional foods. Health information is therefore of great relevance to the acceptance of new products.

The consumers have a natural tendency to prefer conventional fruit juices than functional juices. This is because functional juices frequently present an improper flavor known as off-flavor. Unpleasant aroma (perfumery, dairy) and flavors (sour, savory) have been observed in juices fermented with *Lb. plantarum* 299v (Luckow and Delahunty 2004).

Luckow and Delahunty (2004) compared probiotic black currant juices fermented by *Lb. plantarum* 299v with conventional black currant juices. The authors observed that consumers preferred the appearance of probiotic juice, but in relation to flavor, males preferred conventional juices and females like both. Also, the probiotic juice was more accepted by consumers over 40 years old, which could occur due to lower sensitivity in this group of people.

In order to reduce the sensations of aversive odors and flavors in food and beverages has been employed a technique called "masking," which consists in adding new substances or flavors to food (Reineccius 2000). The aim of this method is to mask negative sensory attributes from the juices fermented with probiotic cultures. Alternatively, tropical fruit juices can be used for this purpose contributing to the off-flavors are not perceived. Luckow et al. (2006) could mask orange juice fermented by *Lb. paracasei* through addition of 10% v/v of tropical fruit juices (pineapple, mango, passion fruit) reducing the perception of the off-flavors.

44.6 Perspectives

The application of probiotic cultures in nondairy products represents a great challenge. The consumer demand for nondairy probiotic beverages has increased in the last years because indirectly some

technical challenges were overcome by researches and industry. For example, the problem with the loss of probiotic viability by thermal treatment was in part resolved by the development of a system to add the probiotic bacteria directly into the finished food product. This innovation allowed the advent of the second generation of probiotic products where producers can inoculate a higher number of viable microorganisms in the product, and thus presumably increase its functionality. Probiotic microencapsulation technologies used, for example, in acidic fruit juice environmental could be cited in addition as another responsible for the increase of cells viability in the second-generation products.

After the EFSA (European Food safety Authority) article 13/5 claims regulation (European Community Regulation on nutrition and health claims) is issued, the commercial success of nondairy probiotic products depends mainly on scientific confirmed health claims through truthful strains. The consumers need to receive a comprehensible and reasonable message about the physiological effects of probiotics. Hundreds of products are marketed and labeled as probiotics but many strains have not been evaluated in vivo trials in humans. Frequently, strain identification is incomplete and unclear, and the viable CFU/mL are not mentioned on the labels. Too many producers worldwide are falsely labeling their products as probiotics. These bad practices could hinder the sector's expansion. In addition, safety issues must be considered in determining the dose connected with an effective daily intake. For these purposes, new studies must be carried out to test and explore more options of raw materials that have not yet been industrially utilized and reengineer products and processes.

Trends on the development of new functional fermented products offer an alternative in medicine substitution to the treatment of several human diseases. The use of natural ingredients are seen as healthy, and the demand for improved health and appearance is attracting fast grow segment on the fermented functional food market. Botanical sources, exotic and regional fruits, and vegetables from world biodiversity will be useful sources to produce new beverages and foods and isolate new potential probiotic strains.

Beverages would be the next food category where the healthy bacteria will make their mark. Likely candidates are chilled fruit juices, bottled water, fermented vegetable juices, or coconut water. There is great interest in expanding and diversifying this line of functional foods with the introduction of formulations nondairy.

REFERENCES

Agheyisi R. [Internet]. Wellesley, MA: BCC Research; c2008 [Accessed 2010 Sep 13]. The probiotics market: ingredients, supplements, foods. Report code: FOD035B. Available from: http://www.bccresearch.com/report/FOD035B.html.

Angelov A, Gotcheva V, Kuncheva R, Hristozova T. 2006. Development of a new oat-based probiotic drink. *Int J Food Microbiol* 112(1):75–80.

Arendt EK, Moore MM. 2007. Gluten-free cereal products as a functional food. In Hui YH, editor. *Handbook of Food Products Manufacturing*. New Jersey: John Wiley & Sons. p 1017–36.

Ares G, Gambaro A. 2007. Influence of gender, age and motives underlying food choice on perceived healthiness and willingness to try functional foods. *Apetite* 49:148–58.

Arici M, Coskun F. 2001. *Hardaliye*: fermented grape juice as a traditional Turkish beverage. *Food Microbiol* 18:417–21.

Arihara K. 2006. Functional properties of bioactive peptides derived from meat proteins, In: Nollet LML, Toldrá F, editors. *Advances Technologies for Meat Processing*. Boca Raton, FL: CRC Taylor & Francis. p 245–74.

Arunachalam K, Gill HS, Chandra RK. 2000. Enhancement of natural immune function by dietary consumption of *Bifidobacterium lactis* (HN019). *Eur J Clin Nutr* 54(3):263–7.

Astuti M, Dalais FS. 2000. Tempeh, a nutritious and healthy food from Indonesia. *Asia Pacific J Clin Nutr* 9:322–5.

Bayindirli A, Aplas H, Bozoğlu F, Hizal M. 2006. Efficiency of high pressure treatment on inactivation of pathogenic microorganisms and enzymes in apple, orange, apricot and sour cherry juices. *Food Control* 17(1):52–8.

Beasley S, Tuorila H, Saris PEJ. 2003. Fermented soymilk with a monoculture of *Lactococcus lactis*. *Int J Food Microbiol* 81:159–62.

Bech-Larsen T, Grunert KG. 2003. The perceived healthiness of functional foods: A conjoint study of Danish, Finnish and American consumers' perception of functional foods. *Appetite* 40:9–14.

Betoret N, Puente L, Díaz MJ, Pagán MJ, García MJ, Gras ML. 2003. Development of probiotic enriched dried fruits by vacuum impregnation. *J Food Eng* 56:273–7.

Beveragedaily.com [Internet]. Exploring drinks innovation for health and wellness. Montpellier, France: Decision News Media; [Accessed 2010 May 04]. Available from: http://www.beveragedaily.com/Formulation/Exploring-drinks-innovation-for-health-and-wellness.

Beveragedaily.com [Internet]. World first probiotic 100% juice launched. Montpellier, France: Decision news media; [Accessed 2009 Sep 02]. Available from: http://www.beveragedaily.com/Formulation/World-first-probiotic-100-juice-launched.

Bhat KPL, Kosmeder JW, Pezzuto JM. 2001. Biological effects of resveratrol. *Antiox Redox Signal* 3:1041–64.

Bhathena SJ, Velasquez MT. 2002. Beneficial role of dietary phytoestrogens in obesity and diabetes. *Am J Clin Nutr* 76:1191–201.

Blandino A, Al-Aseeri ME, Pandiella SS, Cantero D, Webb C. 2003. Cereal-based fermented foods and beverages. *Food Res Int* 36:527–43.

Bradamante S, Barenghi L, Villa A. 2004. Cardiovascular protective effects of resveratrol. *Cardiov Drug Rev* 22:169–88.

Brunso K, Grunert KG, Bredahl L. 1996. An analysis of national and crossnational consumer segments using the food related-lifestyle instrument in Denmark, France, Germany and Great Britain. Working Paper 35 MAPP Center der Aarhus School of Business.

Bukowska H, Pieczul-Mróz J, Jastrzebsk K, Chelstowski K, Naruszewicz M. 1998. Significant decrease in fibrinogen and LDL cholesterol levels upon supplementation of the diet with *Lactobacillus plantarum* (ProViva) in subjects with moderately elevated cholesterol concentrations. *Atherosclerosis* 137:437–8.

Cabrera C, Artacho R, Giménez R. 2006. Beneficial effects of green tea—A review. *J Am Col Nutr* 25:79–99.

Champagne CP, Gardner NJ. 2008. Effect of storage in a fruit drink on subsequent survival of probiotic lactobacilli to gastro–intestinal stresses. *Food Res Int* 41:539–43.

Champagne CP, Green-Johnson J, Raymond Y, Barrette J, Buckley N. 2009. Selection of probiotic bacteria for the fermentation of a soy beverage in combination with *Streptococcus thermophilus*. *Food Res Int* 42(5–6):612–21.

Champagne CP, Raymond Y, Gagnon R. 2008. Viability of *Lactobacillus rhamnosus* R0011 in an apple–based fruit juice under simulated storage conditions at the consumer level. *J Food Sci* 73:221–6.

Charalapompoulos D, Pandiella SS, Webb C. 2002. Growth studies of potentially probiotic lactic acid bacteria in cereal-based substrates. *J Appl Microbiol* 92(5):851–9.

Charalapompoulos D, Pandiella SS, Webb C. 2003. Evaluation of the effect of malt, wheat and barley extracts on the viability of potential probiotic lactic acid bacteria under acidic conditions. *Int J Food Microbiol* 82(2):133–41.

Charalampopoulos D, Wang R, Pandiella SS, Webb C. 2002. Application of cereals and cereal components in functional foods: a review. *Int J Food Microbiol* 79:131–41.

Charalampopoulos D, Vázquez JA, Pandiella SS. 2009. Modelling and validation of *Lactobacillus plantarum* fermentations in cereal-based media with different sugar concentrations and buffering capacities. *Biochem Eng J* 44:96–105.

Chen C, Liu BY. 2000. Changes in major components of tea fungus metabolites during prolonged fermentation. *J Appl Microbiol* 89:834–9.

Chkhikvishvili ID, Ramazanov ZM. 2000. Phenolic substances of brown algae and their antioxidant activity. *Appl Biochem Microbiol* 36:289–91.

Cho KM, Renukaradhya KM, Islam SMA, Lim WJ, Hong SY, Kim JM, Yun MH, Cho JJ, Yun HD. 2009. Novel multiplex PCR for the detection of lactic acid bacteria during kimchi fermentation. *Mol Cell Probes* 23:90–4.

Chow J. 2002. Probiotics and prebiotics: a brief overview. *J Ren Nutr* 12(2):76–86.

Collins JK, Thornton G, O'Sullivan GO. 1998. Selection of probiotic strains for human applications. *Int Dairy J* 8(5–6):487–90.

Cook KA, Dobbs TE, Hlady WG, Wells JG, Barrett TJ, Puhr ND, Lancette GA, Bodager DW, Toth BL, Genese CA, Highsmith AK, Pilot KE, Finelli L, Swerdlow DL. 1998. Outbreak of *Salmonella* serotype Hartford infections associated with unpasteurized orange juice. *JAMA* 280(17):1504–9.

Dalais FS, Rice GE, Murkies AL, Bell RJ, Wahlqvist ML. 1997. Effects of dietary phytoestrogens in postmenopausal women. *Maturitas* 27:214. Ireland: Elsevier Science.

Di Cagno R, Mazzacane F, Rizzello CG, De Angelis M, Giuliani G, Meloni M, De Servi B, Gobbetti M. 2009. Synthesis of γ-aminobutyric acid (GABA) by *Lactobacillus plantarum* DSM19463 in grape must and its effect on the level of expression of human β-defensin-2. *Appl Microbiol Biotechnol* 86(2):731–8.

Diplock AT, Aggett PJ, Ashwell M, Bornet F, Fern EB, Roberfroid MB. 1999. Scientific concepts of functional foods in Europe: consensus document. *Br J Nutr* 81:1–27.

Donkor ON, Henriksson A, Vasiljevic T, Shah NP. 2007. α-Galactosidase and proteolytic activities of selected probiotic and dairy cultures in fermented soymilk. *Food Chem* 104:10–20.

Efraim P. 2004. Estudo para minimizar as perdas de flavonóides durante a fermentação de sementes de cacau para produção de chocolate. [MSc thesis]. São Paulo: Base Alimentarium, FEA Unicamp.

Farnworth ER, Mainville I, Desjardins MP, Gardner N, Fliss I, Champagne C. 2007. Growth of probiotic bacteria and bifidobacteria in a soy yogurt formulation. *Int J Food Microbiol* 116(1):174–81.

Food and Agriculture Organization/World Health Organization (FAO/WHO) [Internet]. Codex standard for fermented milks, CODEX STAN 243; 2003 [Accessed 2010 Dec 20]. Available from: http://www.codexalimentarius.net/download/standards/400/CXS_243e.pdf.

Food and Agriculture Organization/World Health Organization (FAO/WHO) [Internet]. Health and nutritional properties of probiotics in food including powder milk with live lactic acid bacteria, Report of a Joint FAO/WHO Expert Consultation on Evaluation of Health and Nutritional Properties of Probiotics in Food including Powder Milk with Live Lactic Acid Bacteria, Córdoba, Argentina; 2001 [Accessed 2010 Set 29]. Available from: http://www.who.int/foodsafety/publications/fs_management/en/probiotics.pdf.

Gadaga TH, Mutukumira AN, Narvhus JA, Feresu SB. 1999. A review of traditional fermented foods and beverages of Zimbabwe. *Int J Food Microbiol* 53:1–11.

Germany. 2007. Spirulina microalgae: the new functional food option in our diet, Innovations report. Nachrichten & Berichte, Biowissenschaften Chemie.

Gibson GR. 1999. Dietary modulation of the human gut microflora using the prebiotics oligofrutose and inulin. *J Nutr* 129:1439–41.

Giraud E, Grosselin L, Parada JL, Raimbault M. 1993. Purification and characterization of an extracellular amylase from *Lactobacillus plantarum* strain A6. *J Appl Bacteriol* 75:276–82.

Gobbetti M, Di Cagno R, De Angelis M. 2010. Functional microorganisms for functional food quality. *Crit Rev Food Sci Nutr* 50:1–12.

Gordon DT. 2002. Intestinal health through dietary fibre, prebiotics and probiotics. *Food Technol* 56:23.

Gotcheva V, Hristozova E, Hristozova TS, Guo M, Roshkova Z, Angelov A. 2002. Assessment of potential probiotic properties of lactic acid bacteria and yeast strains. *Food Biotechnol* 16(3):211–25.

Gotcheva V, Pandiella SS, Angelov A, Roshkova ZG, Webb C. 2000. Microflora identification of the Bulgarian cereal-based fermented beverage boza. *Process Biochem* 36:127–30.

Graham HN. 1992. Green tea composition, consumption and polyphenol chemistry. *Prev Med* 21:334–50.

Granato D, Branco GF, Nazzaro F, Cruz AG, Faria JAF. 2010. Functional foods and nondairy probiotic food development: Trends, concepts, and products. *Comp Rev Food Sci Food Technol* 9:292–302.

Gupta S, Cox S, Abu-Ghannam N. 2010. Process optimization for the development of a functional beverage based on lactic acid fermentation of oats. *Biochem Eng J* 52(2–3):199–204.

Güven K, Benlikaya N. 2005. Acid pH produced by lactic acid bacteria prevent the growth of *Bacillus cereus* in *boza*, a traditional fermented Turkish beverage. *J Food Saf* 25:98–108.

Havenaar R, Huis in't Veld, JHJ. 1992. Probiotics: a general view. In: Wood BJB, editor. *The Lactic Acid Bacteria in Health and Disease*. Vol. 1. New York: Chapman & Hall Press. p 209–24.

Helland MH, Wicklund T, Narvhus A. 2004a. Growth and metabolism of selected strains of probiotic bacteria, in maize porridge with added malted barley. *Int J Food Microbiol* 91:305–13.

Helland MH, Wicklund T, Narvhus JA. 2004b. Growth and metabolism of selected strains of probiotic bacteria in milk– and water–based cereal puddings. *Int Dairy* J 14:957–65.

Higashikawa F, Noda M, Awaya T, Nomura K, Oku H, Sugiyama M. 2010. Improvement of constipation and liver function by plant-derived lactic acid bacteria: a double-blind, randomized trial. *Nutrition* 26(4):367–74.

Holzapfel WH, Haberer P, Snel J, Schillinger U, Huis in't Veld, JHJ. 1998. Overview of gut flora and probiotics. *Int J Food Microbiol* 41(2):85–101.

Hosoi T, Kiuchi K. 2003. Natto—A food made by fermenting cooked soybeans with *Bacillus subtilis* (*natto*), In. Farnworth, ER editor. *Handbook of Fermented Functional Foods*. CRC Press. p 228–44.

Hutabarat LS, Greenfield H, Mulholland H. 2001. Isoflavones and coumestrol in soybeans and soybean products from Australia and Indonesia. *J Food Compos Anal* 14:43–58.

Iannitti T, Palmieri B. 2010. Therapeutical use of probiotic formulations in clinical practice. *Clin Nutr* 29(6):701–25.

Isolauri E, Arvola T, Sütas Y, Moilanen E, Salminen S. 2000. Probiotics in the management of atopic eczema. *Clin Exp Allergy* 30(11):1604–10.

Kabeir BM, Abd–Aziz S, Muhammad K, Shuhaimi M, Yazid AM. 2005. Growth of *Bifidobacterium longum* BB536 in media (fermented cereal porridge) and their survival during refrigerated storage. *Lett Appl Microbiol* 41:125–31.

Kabeir BM, Yazid AM, Hakim MN, Khahatan A, Shaborin A, Mustafa S. 2009. Survival of *Bifidobacterium pseudocatenulatum* G4 during the storage of fermented peanut milk (PM) and skim milk (SM) products. *African J Food Sci* 3(6):150–5.

Kailasapathy K. 2002. Microencapsulation of probiotic bacteria: technology and potential application. *Curr Iss Intest Microbiol* 3(2):39–48.

Kanellaki M, Bosnea LA, Koutinas AA. 2008. Advances Fermentation Technology. In Pandey A, Larroche C, Soccol CR, Dussap GG, editors. *Production of Fermented Dairy Products*. New Delhi: API Asiatech. p 406–28.

Kataoka S. 2005. Functional effects of japanese style fermented soy sauce (Shoyu) and its components. *J Biosci Bioeng* 100:227–34.

Kato T, Matsuda T, Tahara T, Sugimoto M, Sato Y, Nakamura R. 1994. Effects of meat conditioning and lactic fermentation on pork muscle protein degradation. *Biosci Biotechnol Biochem* 58:408–10.

Kedia G, Wang R, Patel H, Pandiella SS. 2007. Used of mixed cultures for the fermentation of cereal-based substrates with potential probiotic properties. *Process Biochem* 42(1):65–70.

Kingamkono RR, Sjogren E, Svanberg U. 1998. Inhibition of enterotoxin production by, and growth of enteropathogens in a lactic acid-fermenting cereal gruel. *World J Microbiol Biotechnol* 14:661–7.

Kojima T. 1954. Effect of soy sauce on gastric juice secretion. *Rinsho Shoukakibyogaku Zasshi* 2:728–32.

Kondo S, Tayama K, Tsukamoto Y, Ikeda K, Yamori Y. 2001. Antihypertensive effects of acetic acid and vinegar on spontaneously hypertensive rats. *Biosci Biotechnol Biochem* 65:2690–4.

Kourkoutas Y, Xolias V, Kallis M, Bezirtzoglou E, Kanellaki M. 2005. *Lactobacillus casei* cell immobilization on fruit pieces for probiotic additive, fermented milk and lactic acid production. *Process Biochem* 40(1):411–6.

Krasaekoopt W, Bhandari B, Deeth H. 2003. Evaluation of encapsulation techniques of probiotics for yogurt. *Int Dairy J* 13(1):3–13.

Kwon EA, Kim MH. 2007. Microbial evaluation of commercially packed kimchi products. *Food Sci Biotechnol* 16:615–20.

Laragh JH, Baer L, Brunner HR, Buhler FR, Sealey JE, Vaughan ED. 1972. Renin, angiotensin and aldosterone system in pathogenesis and management of hypertensive vascular disease. *Am J Med* 52:633–52.

Lavermicocca P, Valerio F, Lonigro SL, De Angelis M, Morelli L, Callegari ML, Visconti A. 2005. Study of the adhesion and survival of Lactobacilli and Bifidobacteria on table olives with the aim of formulating a new probiotic food. *Appl Environ Microbiol* 71:4233–40.

Lee JW, Shin JG, Kim EH, Kang HE, Yim LB, Kim JY, Joo HG, Woo HJ. 2004. Immunomodulatory and antitumor effects in vivo by the cytoplasmic fraction of *Lactobacillus casei* and *Bifidobacterium longum*. *J Vet Sci* 5(1):41–8.

Lei V, Friis H, Michaelsen KF. 2006. Spontaneously fermented millet product as a natural probiotic treatment for diarrhoea in young children: An intervention study in Northern Ghana. *Int J Food Microbiol* 110:246–53.

Long LH, Kwee DC, Halliwell B. 2000. The antioxidant activities of seasonings used in Asian cooking. Powerful antioxidant activity of dark soy sauce revealed using the ABTS assay. *Free Radic Res* 32: 181–6.

Luckow T, Delahunty C. 2004. Which juice is 'healthier'? A consumer study of probiotic non-dairy juice drinks. *Food Qual Prefer* 15(7–8):751–9.

Luckow T, Sheehan V, Fitzgerald G, Delahunty C. 2006. Exposure, health information and flavour-masking strategies for improving the sensory quality of probiotic juice. *Appetite* 47(3):315–25.

Mack DR, Michail S, Wei S, McDougall L, Hollingsworth MA. 1999. Probiotics inhibit enteropathogenic *E. coli* adherence *in vitro* by inducing intestinal mucin gene expression. *Am J Physiol* 276:941–50.

Mårtensson O, Öste R, Holst O. 2002. The effect of yogurt culture on the survival of probiotic bacteria in oat-based, non-dairy products. *Food Res Int* 35(8):775–84.

McCue PP, Shetty K. 2006. Potential health benefits of soybean isoflavonoids and related phenolic antioxidants, In Shetty K, Paliyath G, Pometto A, Levin RE, editors. *Food Biotechnol*. 2nd ed. Boca Raton, FL: CRC Press. p 772–80.

McMaster LD, Kokott SA, Reid SJ, Abratt VR. 2005. Use of traditional African fermented beverages as delivery vehicles for *Bifidobacterium lactis* DSM 10140. *Int J Food Microbiol* 102(2):231–7.

Mo H, Zhu Y, Chen Z. 2008. Microbial fermented tea—A potential source of natural food preservatives. *Trends Food Sci Technol* 19:124–30.

Molin G. 2001. Probiotics in foods not containing milk or milk constituents, with special reference to *Lactobacillus plantarum* 299v. *Am J Clin Nutr* 73:380–5.

Muhlbauer RC, Li F. 1997. Frequency of food intake and natural dietary components are pontent modulators of bone resorption and bone mass in rats. *Biomed Pharmacother* 51:360–3.

Muianja CMBK, Narvhus JA, Treimo J, Langsrud T. 2003. Isolation, characterization and identification of lactic acid bacteria from bushera: A Ugandan traditional fermented beverage. *Int J Food Microbiol* 80:201–10.

Nazaruddin R, Seng LK, Hassan O, Said M, Lim KS. 2006. Effect of pulp preconditioning on the content of polyphenols in cocoa beans (*Theobroma cacao*) during fermentation. *Ind Crop Prod* 24:87–94.

Nazzaro F, Fratinni F, Coppola R, Sada A, Orlando P. 2009. Fermentative ability of alginate-prebiotic encapsulated *Lactobacillus acidophilus* and survival under simulated gastrointestinal conditions. *J Funct Foods* 1:319–23.

Nazzaro F, Fratinni F, Sada A, Orlando P. 2008. Synbiotic potential of carrot juice supplemented with *Lactobacillus* spp. and inulin or fructooligosaccharides. *J Sci Food Agric* 88:2271–6.

Nobaek S, Johansson ML, Molin G, Ahrné S, Jeppsson B. 2000. Alternation of intestinal microflora is associated with reduction in abdominal bloating and pain in patients with irritable bowel syndrome. *Am J Gastroenterol* 95:1231–8.

Oi Y, KIitabatake N. 2003. Chemical composition of an East African traditional beverage, *togwa*. *J Agric Food Chem* 51:7024–8.

Okagbue RN. 1995. Microbial biotechnology in Zimbabwe: current status and proposal for research and development. *J Appl Sci South Africa* 1:148–58.

Orallo F, Alvarez E, Camina M, Leiro JM, Gomez E, Fernandez P. 2002. The possible implication of trans-resveratrol in the cardioprotective effects of long-term moderate wine consumption. *Mol Pharmacol* 61:294–302.

Ouwehand AC, Kurvinen T, Rissanen P. 2004. Use of a probiotic *Bifidobacterium* in a dry food matrix, an *in vivo* study. *Int J Food Microbiol* 95:103–6.

Parada JL, Zapata LE, De Frabrizio SV, Martinez A. 1996. Microbiological and technological aspects of cassava starch fermentation. *World J Microbiol Biotech* 12:53–6.

Park AM, Dong Z. 2003. Signal transduction pathways: targets for green and black tea polyphenols. *J Biochem Mol Biol* 36:66–77.

Park YH, Kim JG, Shin YW, Kim SH, Whang KY. 2007. Effect of dietary inclusion of *Lactobacillus acidophilus* ATCC 43121 on cholesterol metabolism in rats. *J Microbiol Biotech* 17(4):655–62.

Pham TT, Shah NP. 2007. Biotransformation of isoflavone glycosides by *Bifidobacterium animalis* in soymilk supplemented with skim milk powder. *J Food Sci* 72(8):316–24.

Prado FC, Parada JL, Pandey A, Soccol CR. 2008. Trends in non-dairy probiotic beverages. *Food Res Int* 41:111–23.

Rakin M, Vukasinovic M, Siler-Marinkovic S, Maksimovic M. 2007. Contribution of lactic acid fermentation to improved nutritive quality vegetable juices enriched with brewer's yeast autolysate. *Food Chem* 100:599–602.

Reineccius GA. 2000. Flavouring systems for functional foods. In: Schmidl MK, Labuza TB, editors. *Essentials of Functional Foods*. Gaithersburgh, MD: Aspen Publishing. p 89–97.

Ridges L, Sunderland R, Moernlan K, Meyer B, Astheimer L, Howe P. 2001. Cholesterol lowering benefits of soy and linseed enriched foods. *Asia Pacific J Clin Nutrit* 10:204–11.

Rivera-Espinoza Y, Gallardo-Navarro Y. 2010. Non-dairy probiotic products. *Food Microbiol* 27:1–11.

Ruperez P, Ahrazem O, Leal JA. 2002. Potential antioxidant capacity of sulfated polysaccharides from the edible marine brown seaweed *Fucus vesiculosus*. *J Agric Food Chem* 50:840–5.

Saarela M, Virkajärvi I, Alakomi HL, Sigvart-Mattila P, Mättö J 2006. Stability and functionality of freeze-dried probiotic *Bifidobacterium* cells during storage in juice and milk. *Int Dairy J* 16(12):1477–82.

Salovaara H. 1996. The time of cereal based functional food is here: introducing yosa, a vellie. In Skrede G, Magnus EM, editors. *The Nordic Cereal Industry Towards Year 2000*. Norway: Haugesund. p 195–202.

Sanada CTN, Karp SG, Spier MR, Portella AC, Gouvêa PM, Yamaguishi CT, Vandenberghe LPS, Pandey A, Soccol CR. 2009. Utilization of soybean vinasse for α-galactosidase production. *Food Res Int* 42(4):476–83.

Santos MCR. 2001. Desenvolvimento de bebida e farinha láctea fermentada de ação probiótica a base de soro de leite e farinha de mandioca por cultura mista de *Lactobacillus plantarum* A6, *Lactobacillus casei* Shirota e *Lactobacillus acidophilus*. [MSc thesis]. Curitiba, PR: Fed Univ Paraná. 106 pp.

Saxelin M. 2008. Probiotic formulations and applications, the current probiotics market, and changes in the marketplace: A European perspective. *Clin Infect Dis* 46:76–9.

Shah NP. 2001. Functional foods from probiotics and prebiotics. *Food Technol* 55(11):46–53.

Shah NP, Ding WK, Fallourd MJ, Leyer G. 2010. Improving the stability of probiotic bacteria in model fruit juices using vitamins and antioxidants. *J Food Sci* 75(5):278–82.

Sheehan VM, Ross P, Fitzgerald GF. 2007. Assessing the acid tolerance and the technological robustness of probiotic cultures for fortification in fruit juices. *Innov Food Sci Emerg Technol* 8(2):279–84.

Shimakawa Y, Matsubara S, Yuki N, Ikeda M, Ishikawa F. 2003. Evaluation of *Bifidobacterium breve* strain Yakult-fermented soymilk as a probiotic food. *Int J Food Microbiol* 81:131–6.

Shurkhna RA, Validov SZ, Boronin AM, Naumova RP. 2006. Modeling of lactic acid fermentation of leguminous plant juices. *Appl Bioch Microbiol* 42:204–9.

Shurtleff W, Aoyagi A. 1999. *The Book of Tofu*. Tokyo, Japan.

Singh BR, Kulshreshtha SB, Kapoor KN. 1996. An orange juice-borne outbreak due to enterotoxigenic *Escherichia coli*. *J Food Sci Technol India* 32:504–6.

Skeggs LT, Kahn JR, Shumway NP. 1956. The preparation and function of the hypertension-converting enzyme. *J Exp Med* 103:295–9.

Soccol CR, Prado FC, inventors; 2007. Processo tecnológico para produção de uma bebida fermentada a base de água de coco com propriedades probióticas. Brazil patent PI0703244–7.

Soccol CR, Vandenberghe LP de S, Spier MR, Medeiros ABP, Yamaguishi CT, De Dea Lindner J, Pandey A, Soccol VT. 2010. The potential of probiotics: a review. *Food Technol Biotech* 48(4):413–34.

Starling S [Internet]. US functional foods market to grow 21 per cent by 2015; 2010 [Accessed 2010 Dec 08]. Available from: http://www.nutraingredients-usa.com/ Consumer-Trends/ US-functional-foods-market-to-grow-21-per-cent-by-2015.

Takamatsu S, Hodges TW, Rajbhandari I, Gerwick WH, Hamann MT, Nagle DG. 2003. Marine natural products as novel antioxidant prototypes. *J Nat Prod* 66:605–8.

Teoh AL, Heard G, Cox J. 2004. Yeast ecology of *Kombucha* fermentation. *Int J Food Microbiol* 95:119–26.

Todorov SD, Botes M, Guigas C, Schillinger U, Wiid I, Wachsman MB, Holzapfel WH, Dicks LMT. 2008. *Boza*, a natural source of probiotic lactic acid bacteria. *J Appl Microbiol* 104:465–77.

Tsuji H, Okada N, Yamanishi R, Bando N, Kimoto M, Ogawa T. 1995. Measurement of Gly m Bd 30K, a major soybean allergen, in soybean products by a sandwich enzyme linked immunosorbent assay. *Biosci Biotechnol Biochem* 59:150–1.

Tu Y, Xia H, Watanabe N. 2005. Changes in catechins during the fermentation of green tea. *Appl Biochem Microbiol* 41:574–7.

Valerio F, De Bellis P, Lonigro ST, Morelli L, Visconti A, Lavericocca P. 2006. *In vitro* and *in vivo* survival and transit tolerance of potentially probiotic strains carried by artichokes in the gastrointestinal tract. *Appl Environ Microbiol* 72:3042–5.

Vattem DA, Shetty K. 2006. In: Shetty K, Paliyath G, Pometto A, Levin RE, editors. *Food Biotechnol*. Florida: CRC Taylor & Francis. p 789–823.

Verbeke W. 2005. Agriculture and the food industry in the information age. *Euro Rev Agric Econ* 32:347–68.

Wacher C, Barzana E, Lappe P, Ulloa M, Owens JD. 2000. Microbiology of Indian and Mestizo *pozol* fermentation. *Food Microbiol* 17:251–6.

Wang C, Li Y. 2006. Research progress on property and application of theaflavins. *African J Biotechnol* 5:213–8.

Wang C, Ng CC, Tzeng W, Shyu YT. 2009. Probiotic potential of noni juice fermented with lactic acid bacteria and bifidobacteria. *Int J Food Sci Nutr* 1:1–9.

Wang YC, Yu RC, Yang HY, Chou CC. 2003. Sugar and acid content in soymilk fermented with lactic acid bacteria alone or simultaneously with bifidobacteria. *Food Microbiol* 20(3):333–8.

Wang YC, Yu RC, Yang HY, Chou CC. 2006. Antioxidatives activities of soymilk fermented with lactic acid bacteria and bifidobacteria. *Food Microbiol* 23:128–35.

Williamson MS, Hunt AE, Pope JF, Tolman NM. 2000. Recommendations of dietians for overcoming barriers to dietary adherence in individuals with diabetes. *The Diabetes Educator* 26:272–9.

Willumsen JF, Darling JC, Kitundu JA. 1997. Dietary management of acute diarrhoea in children: effect of fermented and amylase-digested weaning foods on intestinal permeability. *J Pediat Gastroenterol Nutr* 24:235–41.

Wolter F, Ulrich S, Stein J. 2004. Molecular mechanisms of the chemopreventive effects of resveratrol and its analogs in colorectal cancer: key role of polyamines. *J Nutr* 134:3219–22.

Xu Q, Tao W, Ao Z. 2007. Antioxidant activity of vinegar melanoidins. *Food Chem* 102:841–9.

Yamaguishi CT, Sanada CT, Gouvêa PM, Pandey A, Woiciechowski AL, Parada JL, Soccol CR. 2009. Biotechnological process for producing black bean slurry without stachyose. *Food Res Int* 42(4):425–9.

Yoon KY, Woodams EE, Hang YD. 2004. Probiotication of tomato juice by lactic acid bacteria. *J Microbiol* 42(4):315–8.

Yoon KY, Woodams EE, Hang YD. 2005. Fermentation of beet juice by beneficial lactic acid bacteria. *Lebensm-Wiss u -Technol* 38(1):73–5.

Yoon KY, Woodams EE, Hang YD. 2006. Production of probiotic cabbage juice by lactic acid bacteria. *Bioresource Technol* 97(12):1427–30.

Yuan YV. 2008. Marine algal constituents. In Barrow C, Shahidi F, editors. *Marine Nutraceuticals and Functional Foods*. Boca Raton, FL: CRC Press.

Zocco MA, dal Verme LZ, Cremonini F, Piscaglia AC, Nista EC, Candelli M, Novi M, Rigante D, Cazzato IA, Ojetti V, Armuzzi A, Gasbarrini G, Gasbarrini A. 2006. Efficacy of *Lactobacillus* GG in maintaining remission of ulcerative colitis. *Aliment Pharmacol Ther* 23(11):1567–74.

Zuauya R, Mygochi T, Parawira W. 1997. Microbial and biochemical changes occurring during production of *masvusvu* and *mangisi*, traditional Zimbabwean beverages. *Plant Food Human Nutr* 51:43–51.

Part VII

Fermentation and Food Ingredients

45

Fresh and Fermented Vegetables as a Source of Proteolytic Bacteria

Catalina Elena Kotlar, María Victoria Agüero, Alejandra Graciela Ponce, Olinda Pérez Borla, and Sara I. Roura

CONTENTS

45.1 Sources of Commercial Proteolytic Bacteria and Applications

Microbial proteases are among the most important hydrolytic enzymes and have been studied extensively since the advent of enzymology. There is renewed interest in the study of proteolytic enzymes, which not only play an important role in the cellular metabolic process but have also gained considerable attention in the industrial community. Moreover, proteases are the most important category of industrial enzymes, accounting for more than 65% of the total industrial enzyme market (Banik et al. 2004). Microbial proteases are a group of enzymes that can have application in numerous industries (Gulrajani et al. 2000; Gupta et al. 2002; Najafi et al. 2005; Prakash et al. 2005), and they are important tools in medical and pharmaceutical processes (Bhaskar et al. 2007; In et al. 2002). In the food-processing industry, the proteolytic activity of microorganisms is associated with a wide variety of processes such as fermented foods (Casaburi et al. 2008; Hayashi et al. 1990; Mauriello et al. 2002; Oneca et al. 2007), oil extraction (Kashyap et al. 2007), bakery (Lauer et al. 2000), clarification of juices (Dawes et al. 1994), and waste treatments (Sudeepa et al. 2007; Wang et al. 1997). Bacteria, molds, and yeast are some of the microorganisms that are able to produce proteases. Microorganisms elaborate a large array of proteases, which are intracellular and/or extracellular. Intracellular proteases are important for various cellular and metabolic processes, such as sporulations, differentiation protein turnover, maturation of enzymes and hormones, and maintenance of the cellular protein pool. The extracellular ones are important for protein hydrolysis in cell free environments and able the cell to absorb and utilize hydrolytic products. At the same time, these extracellular proteases have also been commercially exploited to assist protein degradation in various industrial processes (Salem et al. 2009). Microbes represent an excellent protease source due to their broad biochemical diversity (Godfrey and West 1996), their rapid growth, the limited space,

and the low cost that growing substrates required for their cultivation (Gupta et al. 2002). Each organism or strain has its own special conditions for maximum enzyme production. Extracellular protease production by microorganisms is highly influenced by media components, variation in carbon/nitrogen ratio, presence of some easily metabolizable sugars, such as glucose (Beg et al. 2003), and presence of metal ions (Secades et al. 1999). Several others factors, such as aeration, inoculums density, pH, temperature, and incubation time also affect the amount of protease production and their interaction plays an important role in the synthesis of these enzymes (Puri et al. 2002).

Vegetables are renewable raw materials associated with diverse microbial populations (Gómez et al. 2002; Ponce et al. 2008), therefore they could be an interesting and cheap source of proteolytic bacteria. Among vegetables cabbage (*Brassica oleracea* var. *capitata*) is a biennial sturdy and inexpensive leafy plant. It is widely cultivated throughout the world and stored so well that it is available all the year round.

The aim of the present chapter is to describe the isolation and characterization of extracellular producing bacteria from fresh and fermented cabbage. To select profitable bacteria to apply on different industries, the proteolytic substrate specificity of the isolated bacteria was assayed using different protein sources. Taken into account the uses and the high demand of proteases, there is a need for the research of new strains of bacteria that produce enzymes with novel properties and the development of low cost industrial medium formulations. In commercial practice, the optimization of medium composition is done to maintain a balance between the various medium components, minimizing the amount of unutilized components at the end of fermentation. In the light of the above facts, another objective of this study was to optimize the fermentation medium in order to enhance the protease production from two of the strains previously isolated from fresh and fermented cabbage. The optimization of culture medium and cultivation was performed using both conventional methods and the Plackett-Burman (PB) experimental design that is usually applied as a preoptimization stage looking for the study of the statistical factors with significance on the dependent variable. Once the critical components and operating conditions to the protease production were screened, the second stage of the optimization procedure was to find the optimum for maximum product formation. RSM was employed in this second stage to optimize the fermentation medium for enhancing protease production.

45.2 Proteolytic Activity of Bacteria Isolated from Fresh and Fermented Vegetables

45.2.1 Isolation and Characterization of Proteolytic Bacteria from Fresh and Fermented Cabbage

The raw materials used to isolate proteolytic bacteria were thoroughly chopped leaves of fresh cabbage and thoroughly chopped and shredded leaves of cabbage fermented under two conditions: condition A: 22°C ± 1°C for 3 days plus 15°C–20°C for 20 days and condition B: 22°C ± 1°C for 3 days plus 15°C–20°C for 20 days plus 15°C–20°C for a year and fresh cabbage consisted for thoroughly chopped leaves. The pH values varied from 6.5–6.7 for fresh cabbage juice to 3.6–3.7 for fermented material, without significant differences between pH of the cabbage fermented in A and B conditions.

Previous studies have shown that culture media composition influences the expression of extracellular proteases (Nicodème et al. 2005; Wang et al. 2008). This work proposed the use of a basal medium (brain heart infusion, M) and a modified basal medium (minimal medium, MM), both supplemented with milk. The MM was limited in energy source from sugars and relatively poor in protein content, including yeast extract as a unique source of both vitamins and nucleic acids. This poor growing condition enhanced the microbial extracellular proteases production, so it was chosen as the medium on which to grow the bacteria for characterization and examination of the proteolytic specificity of bacteria from fresh and fermented cabbage. As clearing zones surrounding the caseinolytic colonies were observed without trichloroacetic acid addition, the bacteria remain viable allowing to be grown on other protein substrates. Conventional methods used to investigate the occurrence in food of proteolytic bacteria require the study of a large number of organisms selected at random. The utilization of the simple and reliable above-described method to look for proteolytic bacteria allowed the isolation of seven bacteria (Table 45.1), which can be potentially useful in different industries.

TABLE 45.1

Source, Morphological, and Physiological Characteristics of Exoprotease-Producing Bacteria

Bacteria[a]	Source	Gram	Catalase	Cocci	Bacillus
Enterococcus hirae	Fresh cabbage	+	−	+	
Pseudomonas not classified[b]	Fresh cabbage	−	+		+
Bacillus cereus	Fresh cabbage	+	+		+
Enterococcus hirae	Fermented cabbage (A)	+	−	+	
Lactococcus lactis	Fermented cabbage (A)	+	−	+	
Bacillus cereus	Fermented cabbage (A)	+	+		+
Bacillus cereus	Fermented cabbage (A)	+	+		+

[a] Caseinolytic bacteria of cabbage juices were studied in MM supplemented with 6.2 g/L protein of skimmed milk (about 5 g/L of casein) medium. Bacteria were characterized on incubation day 2.

[b] *Pseudomonas* sp. 93% identity with other not classified *Pseudomonas*.

The morphological and physiological characteristics of the proteolytic bacteria isolated from fresh and fermented cabbage are shown in Table 45.1. The proteolytic bacteria are characterized by ribosomal DNA genes analysis.

Proteolytic bacteria isolated from fresh cabbage are typical in soil, water, and vegetables. Fermented cabbage contained proteolytic bacteria, which are resistant to acid grown media (Setlow 2006) or are able to survive in that environmental condition (Foulquié Moreno et al. 2006). Some noncaseinolytic bacteria (exhibited nonclearing halo during the whole incubation period) from fresh and fermented cabbage were selected at random to determine their proteolytic activity on other protein substrates.

Table 45.2 shows the proteolytic substrate specificity of caseinolytic and noncaseinolytic bacteria isolated as described above. All the proteolytic bacteria exhibited positive response in the second or third day of the whole incubation period (seven days). *Bacillus cereus* showed proteolytic activity on all the seven proposed proteins, thus they could have different applications in diverse industries (Horikoshi et al. 1999). The caseinolytic *Enterococus hirae* hydrolyzed gelatin, but no other proteins offered as substrates. Some enterococci carry potential virulence factors and have been associated with a number of human infections. Gelatinase (Gel) is an extracellular metalloendopeptidase involved in the hydrolysis of gelatin, collagen, hemoglobin, and other bioactive peptides (Su et al. 1991). Singh et al. (1998) demonstrated that Gel is a virulence factor of enterococci. Hence the utilization of this strain in food industry requires more studies on the actual presence of Gel. The six proteins other than bovine serum albumin were hydrolyzed by *Pseudomonas* not classified. On the other hand, solely one of the selected noncaseinolytic bacteria, *Pseudomonas putida*, showed positive proteolytic activity only against ovalbumin. *Pseudomonas* sp. are involved in industrial, agricultural, bioremediation, and other processes (Wang

TABLE 45.2

Proteolytic Specificity of the Selected Bacteria of Fresh Cabbage in MM Medium Supplemented with Different Proteins at 32°C

	MM plus × g/L Protein of						
Bacteria	Milk 6.2 g/L Milk Protein (casein 5 g/L)	Hemoglobin 5 g/L	Gluten 5 g/L	Gelatin 5 g/L	Milk Whey 5 g/L	Ovalbumin 5 g/L	Bovine Serum Albumin 5 g/L
Enterococcus hirae	+	−	−	+	−	−	−
Pseudomonas not classified[a]	+	+	+	+	+	+	−
Bacillus cereus	+	+	+	+	+	+	+
Pseudomon putida	−	−	−	−	−	+	−

[a] *Pseudomonas* sp. 93% identity with other not classified *Pseudomonas*.

et al. 1997; Najafi et al. 2005). Exploring the properties of the extracellular proteases of *Pseudomonas putida* and *Pseudomonas* not classified isolated in this work from fresh cabbage is an important objective in order to know the future applications of those bacteria. *Lactococcus lactis* subsp. *lactis* hydrolyzed gluten, gelatin, and ovalbumin; therefore, it could be interesting exploring its capacity as processing aids in bakeries (Rollán et al. 2001). Furthermore, determining the ability of the strain isolated in this work to hydrolyze the gliadin polypeptides identified as gluten-allergen compound (Gerez et al. 2006; Rizzello et al. 2007) is an important future objective. The isolated caseinolytic *Ent. hirae* exhibited proteolytic activity on none of the other offered proteins. Fortunately, the presence in enterococci of virulence factors as Gel is strain-dependent (Foulquié Moreno et al. 2006). As the *Ent. hirae* strain isolated from fermented cabbage not shown gelatinolytic nor hemoglobinolytic activity, looking for its potential industrial applications seems a valuable future objetive. Both *B. cereus* strains isolated from fermentation conditions A and B showed the same proteolytic substrate specificity as the *B. cereus* strain isolated from fresh cabbage.

Through the utilization of a simple and reliable method, our study was successful in isolating extracellular protease–producing bacteria from an economic and easily available source: fresh and fermented cabbage. These bacteria were able to hydrolyze from low-molecular-weight proteins (hemoglobin, bovine serum albumin) to high-molecular-weight proteins (gelatin, gluten). From a commercial point of view it is important to obtain strains that are resistant to the adverse situations that occur during some industrial processes (Lale et al. 2007). The microorganisms isolated from fermented cabbage in this work are exposed to various harsh conditions: the end of fermentation imposes a hostile environment on the microorganisms that involves low pH, low concentration of nutrients and, possibly, unfavorable osmotic environment. Therefore, it is possible that those microorganisms perform the former condition. In addition, based on their protein hydrolysis profiles, the characterized proteolytic bacteria could be useful in some industries like textile or leather, in laundry detergents, in medical and pharmaceutical processes, in bioremediation, in agriculture, or in food processing. However, further knowledge of the pH and temperature resistance and other properties of the extracellular proteases of those strains will be necessary in order to know their real commercial aptitude.

45.2.2 Characterization of *Bacillus cereus* Isolated from Fermented Cabbage and Conventional Optimization of Extracellular Protease Production

Among bacteria, *Bacillus* strains are the most important producers of commercial proteases (Ghorbel-Frikha et al. 2005), specifically extracellular ones. Strains of *Bacillus cereus* possess high proteolytic potential, expressed by their ability to synthesize and excrete protease into the medium. This spore forming, mesophilic, and relatively anaerobic rod is potent in terms of biosynthesis of various hydrolytic enzymes, including proteases, α- and β-glucanases, or lipases. Proteolytic enzymes containing serine proteinases (Shaheen et al. 2008), Ca^{2+}- and Zn^{2+}-dependent metalloproteinases (Karbalaei-Heidari et al. 2007), keratinases (Brandelli et al. 2005; Ghosh et al. 2008), and collagenases (Nip et al. 2006) are known to be produced by *B. cereus*. One of the several challenges faced by industrial application of microbial proteases is to obtain its optimal activity and stability. Each organism or strain has its own special conditions for maximum enzyme production. In commercial practice, the optimization of medium composition is done to maintain a balance between the various medium components, thus minimizing the amount of unutilized components at the end of fermentation.

The effect of physicochemical conditions on microorganism growth such as temperature and pH was investigated. Figure 45.1 shows the time course of patterns of cell growth for *Bacillus cereus* spp. grown at different temperatures: 10°C, 20°C, 32°C, and 40°C. *B. cereus* spp. was able to grow at 20°C, 32°C, and 40°C, showing typical growth profiles with an exponential phase that began in a range of the third and fourth hour and ended between the sixth and ninth hours. For these temperatures, the exponential phase lasted about 3 hours with maximum specific growth rates. During this period, the cell growth curve presented a linear profile. The time course of *Bacillus cereus* spp. at 10°C did not show significant cell growth. The maximum optical density (0.9) was observed at 32°C, so this temperature was considered optimal for the cell growth. To establish the optimum pH growth, *Bacillus cereus* spp. was incubated at 32°C (the optimal temperature) in minimal broth (MB), an agarless MM (Perez Borla et al.

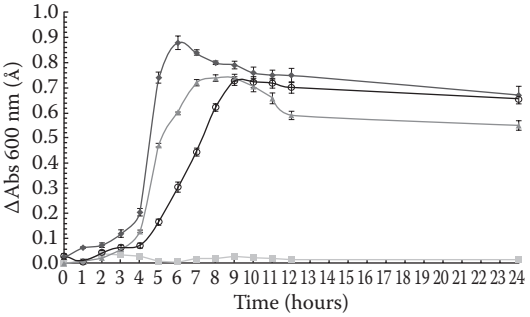

FIGURE 45.1 Kinetic growth profiles of *Bacillus cereus* spp. at different temperatures: 10°C (■) 20°C (○), 32°C (◆), and 40°C (▲) in MB buffered at pH 7.0 on a rotary shaker. Each point is the mean of three determinations; standard errors were less than 10% of the means.

2009) containing 0.1% bacteriological glucose (w/v) and 0.25% yeast extract (w/v) buffered at five different pH levels. Figure 45.2 shows the kinetic growth profiles in MB at pH 5.7, 7.0, and 8.0. Cell growth at pH 7.0 showed the maximum absorbance values and their lag phase was extended until the third hour. The lowest absorbance values were observed at pH 8.0 starting the acceleration phase at the fourth hour. The bacteria growth at pH 5.7 was intermediate between both pH 7.0 and 8.0. Similar cell growth profiles were obtained in the latent phase for pH 7.0 and 5.7 until the third hour.

Figure 45.3 shows the effect of different modifiers in MB at optimal conditions of temperature and pH (32°C and 7.0, respectively). The use of glucose as modifier showed an increase in the turbidity of the cell culture, reaching the maximum in the seventh hour of growth without significant change in the rate of growth. This result indicated that the exponential phase was prolonged compared to control sample (only MB). The other modifiers (Ca^{2+}, K^{+}, and peptone) presented an inhibitor effect in the cell growth compared to control sample. The effect of these modifiers on the cell growth in MB at 10, 20, and 40°C is present in Figure 45.4.

Extracellular proteolytic activity of the *Bacillus cereus* strain was determined through quantitative (using azocasein as the substrate) and qualitative enzymatic assays (in MM supplemented with 6.2 g/L protein of skimmed milk plates with aliquot of the growth culture placed into wells). Protease activity obtained in MB was measured at regular intervals of 0, 1, 2, 5, 6, 7, 8, and 24 hours. Relative protease activity measured as the increase of one absorbance unit at 335 nm, 90 min of incubation at 37°C per microbial growth (absorbance of culture measured at 600 nm) of *B. cereus* spp. was shown in Figure 45.5a. The higher activities were obtained within the acceleration phase (Figure 45.5a). Nonproteolytic activity, expressed as relative activity, was observed during the stationary phase. Figure 45.5b shows the absolute proteolytic activity (amount of enzyme that caused a change of absorbance of 0.01 at 335 nm in 90 min at 37°C) during *Bacillus* growth. The protease activity began at the lag phase extending its protease action during all the exponential phase. The highest absolute proteolytic activity was found at the

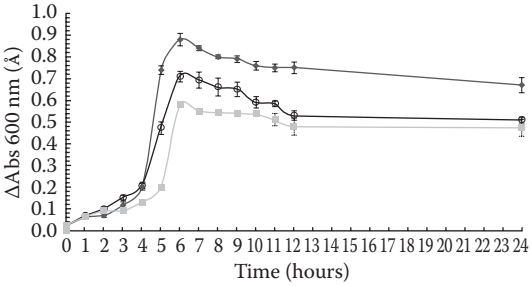

FIGURE 45.2 Kinetic growth profiles of *Bacillus cereus* spp. grown at 32°C in MB at buffer pH 5.7 (○), pH 7.0 (◆), and pH 8.0 (■) on a rotary shaker. Each point is the mean of three determinations; standard errors were less than 10% of the means.

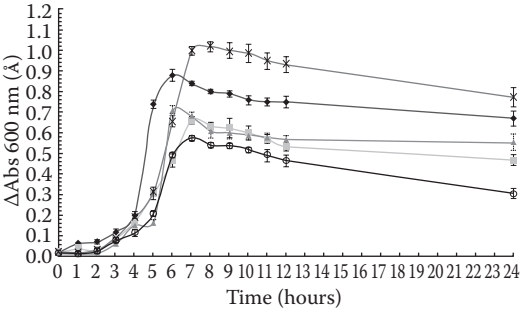

FIGURE 45.3 Kinetic growth profiles of *Bacillus cereus* spp. grown in MB buffered at pH 7.0 and 32°C on a rotary shaker enriched with different modifiers: MB (◆), MB + Ca^{2+} (■), MB + K^{+} (▲), MB + Glu (x), and MB + peptone (○). Each point is the mean of three determinations; standard errors were less than 10% of the means.

exponential phase. As was expected, nonabsolute proteolytic activity was observed during the stationary phase. The maximum cell concentration was at 6 hours of incubation (Figure 45.1), and the highest enzyme activity was at 5 hours with values of absorbance (600 nm) of 0.879 and 11.84 UA, respectively. Parallel to these measures, the proteolytic activity halos in MM plates supplemented with milk were determined (Figure 45.5c). Aliquots of cell culture incubated in MB were placed in MM plates wells. Under these conditions, the maximum proteolytic halos observed took place during the lag and exponential phases. Notorious small halos were detected in the stationary phase, inducing on that a lower and poor protease activity was present at this phase. When spores were inoculated again in MM agar plates supplemented with milk, they found adequate conditions to begin their vegetative growth producing proteolytic enzymes, breaking proteins, and obtaining energy. To support this hypothesis, the halo diameter

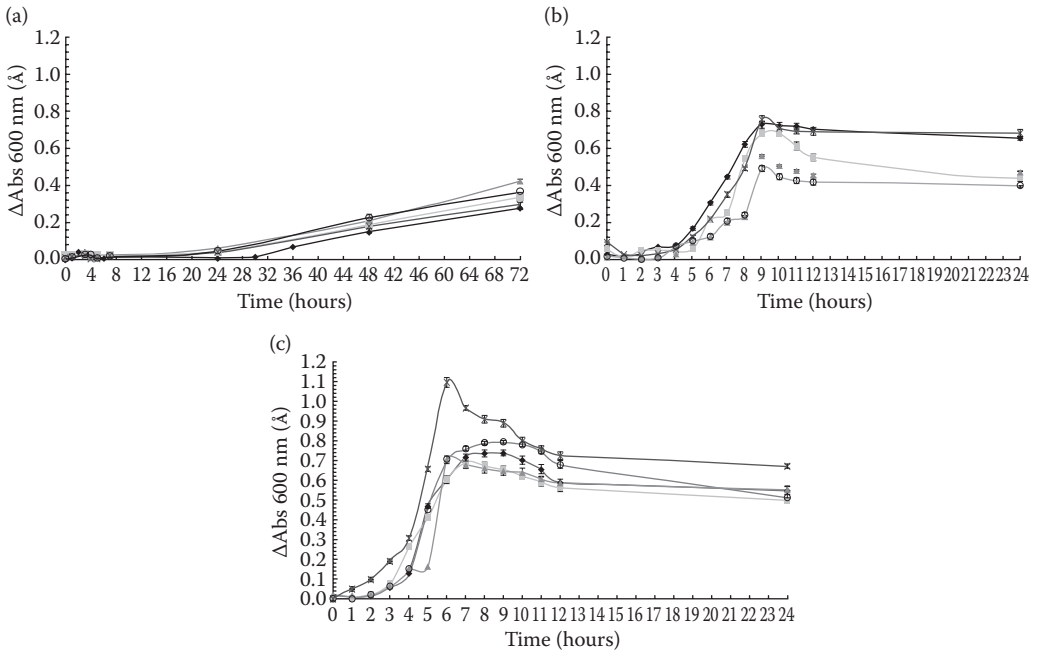

FIGURE 45.4 Kinetic growth profiles of *Bacillus cereus* spp. grown in MB (pH 7) enriched with different modifiers and incubated at different temperatures: MB (◆), MB + Ca^{2+} (■), MB + K^{+} (▲), MB + Glu (×), and MB + peptone (○). Cells were grown at 10°C (a), 20°C (b), and 40°C (c) on a rotary shaker. Each point is the mean of three determinations; standard errors were less than 10% of the means.

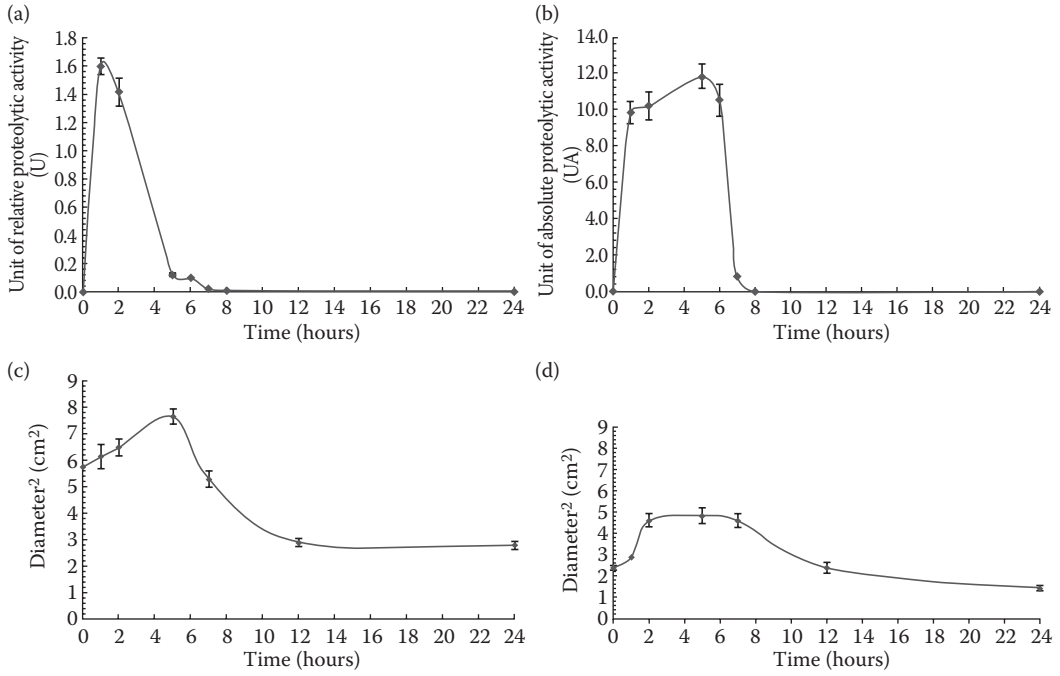

FIGURE 45.5 Time course of protease production by *B. cereus* of enzyme isolated from culture grown in MB at pH 7 and 32°C. Relative proteolytic activity (a), absolute proteolytic activity (b), diameter square of proteolytic halos from culture grown in MM (c), and diameter square of proteolytic halos from crude enzyme (d). Standard deviations are represented by error bars.

square was quantified but using only cell free culture supernatants (protease source) obtained at the same *Bacillus* growth times as Figure 45.5c. The values in terms of the diameter square of the clear zone surrounding the protease source were lower than those that appeared by inoculation of the active culture (Figure 45.5d). As a constant, the highest activity of protease was found from the lag phase to the end of the exponential phase. In the stationary growth period, a minimal halo was detected, and it was smaller compared to the halo found when culture was inoculated. The halo that appeared in the stationary phase could be present by the enzymatic hydrolysis of the nitrogen source present in the MB (yeast extract).

The results on the pH effect on protease activity were shown in Figure 45.6a,b, establishing the medium pH at 5.7, 7, and 8. As was mentioned, 7.0 was the optimum pH for *Bacillus cereus* spp. growth (Figure 45.2). However, for the proteolytic activity expression, the optimum pH was 8.0 despite a reduced

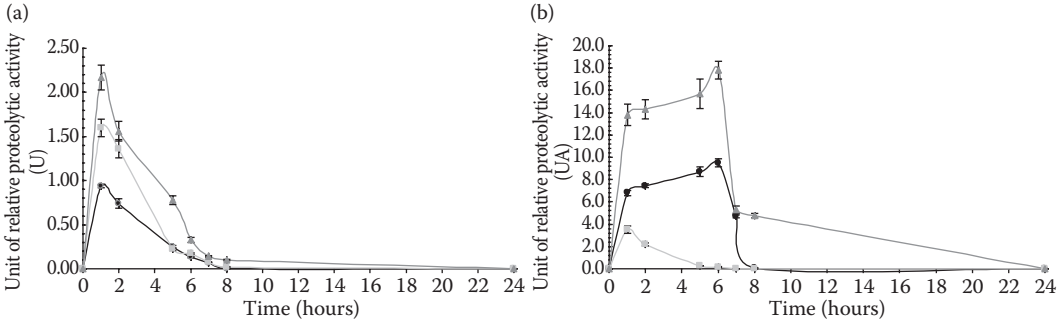

FIGURE 45.6 Effect of pH on protease activity of *B. cereus* spp. Relative proteolytic activity (a) and absolute proteolytic activity (b). Cells growth in MB at 32°C buffered at pH 5.7 (●), 7.0 (■), and 8.0 (▲). Each point is the mean of three determinations; standard errors were less than 10% of the means.

microbial growth at this pH value (Figure 45.2). For the three pH analyzed, the higher extracellular proteolytic activity occurred during the lag and exponential phases (Figure 45.6a,b). If activity was expressed as absolute values, it is noticeable that during the stationary phase (pH 8.0), a residual extracellular proteolytic activity was detected. As at pH 8.0 *B. cereus* spp. cells grew slowly, they deplete the substrate gradually, thus express longer extracellular protease activity (Figure 45.6b).

The effect of growth temperature on specific extracellular proteolytic activity is shown in Figure 45.7a,b. Different information was obtained if the activity was expressed in a relative or absolute way. At 10°C, *Bacillus* grew significantly slower showing the maximum relative extracellular proteolytic activity compared with *Bacillus* growing at 32°C (Figure 45.7a). Once again, at 10°C, the microorganisms could deplete the substrate nutrients gradually, showing a remaining proteolytic activity during the stationary phase.

If absolute activity was recorded, the maximum extracellular proteolytic activity was observed at 32°C and 40°C, while at 32°C, the activity was expressed early in the lag phase, at 40°C the maximum activity expression was during the exponential phase.

The effect of different modifiers on the extracellular protease production from *Bacillus cereus* spp., expressed as absolute extracellular proteolytic activity, was determined for the different assayed temperature (10°C, 20°C, 32°C, and 40°C) (Figure 45.8a–d). As can be seen, each modifier affected extracellular protease expression differently related with incubation temperature. At 10°C, all modifiers affected the extracellular protease expression, increasing its activity between 3 and 6 times compared with the control sample (MB). However, with the addition of peptone and glucose, the activity was expressed in the lag phase, but the addition of K^+ and Ca^{2+} delayed the protease expression to the late exponential phase. At 20°C, and similar to the modifier effects observed at 10°C, a positive effect in the protease expression was observed. The activity increase ranged between 7 and 10 times compared with the control sample (MB). All modifiers expressed the protease activity during the lag and exponential phase. With glucose addition, the maximum activity was present in the late exponential phase, maintaining a high protease activity (14–15 UA) during the stationary phase. It was probably that microorganisms use the glucose as a fast carbon source of MB. When an important biomass was reached, the microorganisms use its enzyme battery to hydrolyze the protein substrate of MB toward to obtain energy. At 32°C, only the MB enrichment with peptone appears to cause an accelerated protease expression, reaching at 2 hours its maximum activity (19 UA); after that, the activity decreased gradually during the assayed time. K^+ improved also the proteolytic expression compared with MB reaching the highest value in the late exponential phase. During the stationary phase and comparing with MB, all the modifiers added produced a significant protease activity; with an increase between 5 and 15 orders with the addition of glucose and peptone as modifiers, respectively. At 40°C, any of the modifiers improved the proteolytic expression. For the control culture (MB) and with modifiers, the activity expression occurred during the

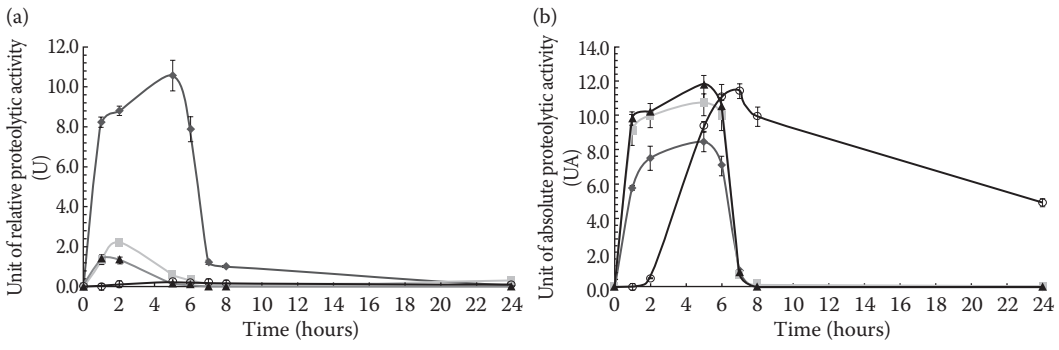

FIGURE 45.7 *Bacillus cereus* spp. protease activity in MB at buffer pH 7.0 at different temperature: 10°C (◆), 20°C (■), 32°C (▲), and 40°C (○). Relative proteolytic activity (a) and absolute proteolytic activity (b). Each point is the mean of three determinations; standard errors were less than 10% of the means.

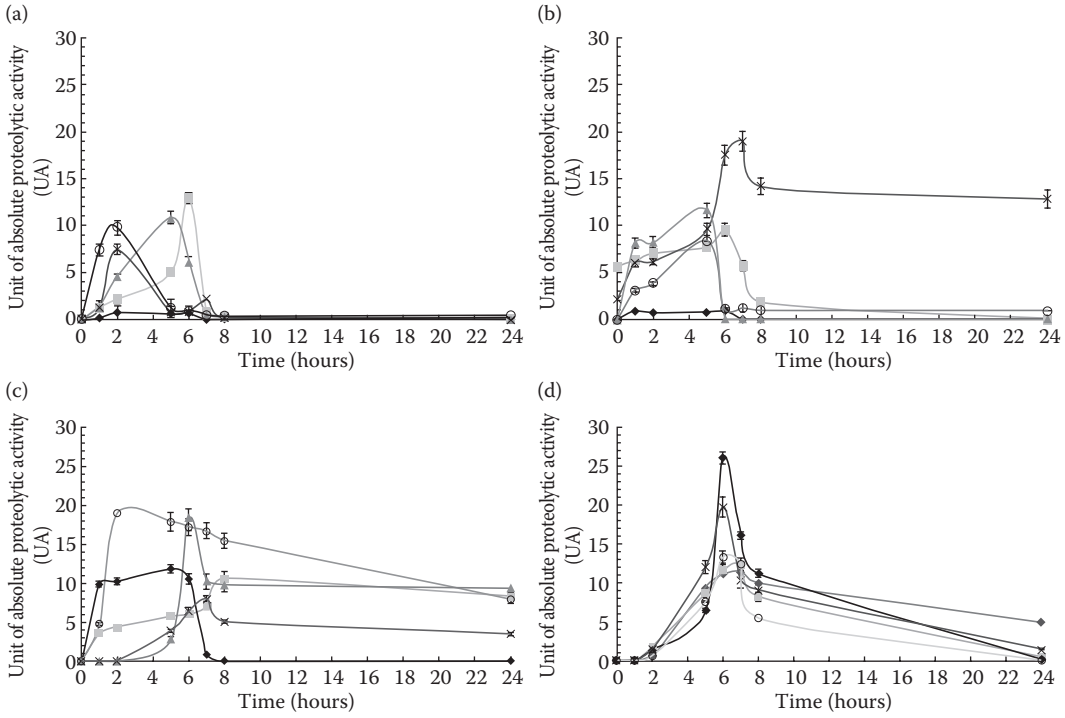

FIGURE 45.8 Effect of different modifiers on the protease activity from *Bacillus cereus*, incubated in MB buffered at pH 7: MB (◆), MB + Ca^{2+} (■), MB + K^{+} (▲), MB + Glu (×), and MB + peptone (○), at 10°C (a), 20°C (b), 32°C (c), and 40°C (d). Results were expressed as absolute proteolytic activity. Each point is the mean of three determinations; standard errors were less than 10% of the means.

late exponential phase (Figure 45.8d). In general, Ca^{2+} ions did not exert an enzyme stabilization role without effects in the protease activity at any of the assayed temperatures.

The strain of *B. cereus* presented a typical kinetic growth profile, showing a short lag phase and marked exponential and stationary phases. The decline phase was not observed probably due to the cell sporulation that produced a significant decrease in the absorbance values because of the property of the spores to absorb light at the selected wavelength (600 nm). The phase contrast microscopy observation of spores with Schaeffer-Fulton stain supported these results. The optimal pH for microorganism growth was 7, while the optimal pH for the enzyme production was found at pH 8. This result is in accordance with several earlier reports showing pH optima for *Bacillis cereus* spp. protease expression being alkaline (Bani et al. 2004). Despite the strain isolated from fermented cabbage, whose juice has an acid pH between 3 and 4 (Perez Borla et al. 2010), these pH ranges were not optimum for *B. cereus* sp. growth and its extracellular protease production (data not shown). This behavior could indicate that *B. cereus* strain survives in this acid juice, acquiring a sporulated form. Then the spores were well reactivated in BHI, leading to the spore's germination and therefore dominating the vegetative cells into the medium. Generally, food preservation processes expose bacteria to both lethal and sublethal stresses. Bacteria may have different mechanisms for surviving these extreme environmental stresses (Davidson et al. 2002). In this context, the formation of endospores in response to acid stress during cabbage fermentation is a survival strategy for *Bacillus cereus* spp. Our experimental results showed that the maximum amount of enzyme was produced by the bacterium in its exponential growth phase, and a reduction in enzyme expression was noted beyond this period, precisely in the stationary phase. Similar results were reported previously by other authors (Gopal et al. 2007). Although the rapid proteolytic activity expression constituted a technologic advantage, in Figure 45.5a when proteolytic activity was expressed relative to the bacterial growth, the reduced number of microorganisms could be responsible for the

enhanced proteolytic activity and this could not be a benefit. Since a technological point of view, an elevated enzyme concentration was desirable. The enzyme presented a significant absolute extracellular proteolytic activity at the beginning of the fermentation during the lag and exponential phases; following a diminution of the absolute extracellular protease activity took place. Two independent facts could explain this behavior: the extracellular protease production is controlled by the level of a repressor, which is directly or indirectly an intermediate in many pathways related to a feedback inhibition, or the low level of nutrients in the medium provokes a depletion in the enzyme activity, due to the morphological changes from vegetative to sporulated cell forms that do not produce extracellular proteases. The proteolytic activity of culture assayed in MM enriched with milk showed that the enzyme presented activity also in the stationary phase, indicating that if the MM is properly complemented with any nitrogen source, there was no drain of protease production during the stationary phase. This fact could indicate that the limited substrate conditions observed during the stationary phase (Figure 45.5a,b) could be responsible for the absence of proteolytic activity in this period. Also, the influence of pH on *B. cereus* spp. growth supports the hypothesis previously proposed about substrate exhaustion as the main cause of the absence of proteolytic activity in the stationary phase. Based on the obtained results, the effect of the MB supplementation with different modifiers on the protease activity showed a variation with the incubation temperature. The results indicated that the production of protease in the presence of the assayed modifiers is a function of temperature. At 10°C and 20°C, all modifiers affected positively the protease expression, but at 32°C and 40°C, the effect of temperature prevailed over the inducers effects. In contrast, other microorganisms showed only very low level of specific activity in nonsupplemented synthetic medium (Nicodeme et al. 2005). In a previous work, Ghorbel-Frinkha et al. (2005) reported that the production of protease by a *B. cereus* strain is absolutely calcium-dependent. So the fact that the *Bacillus cereus* strain isolated from fermented cabbage produced alkaline proteolytic enzymes in minimally substrate conditions that does not require either ions (Ca^{2+} or K^{+}) or addition of nutrients (peptone or glucose) could represent a novel type of enzyme produced at optimal temperature (32°C) of *Bacillus* growth. Growth temperature controlled the production of the protease by *Bacillus cereus* spp. In the range from 10°C to 40°C, the proteolytic activity rapidly increased with temperature, and it drastically decreased at and below 20°C. On the basis of our results, we suggest that the optimal temperature of protease production was coincidental with the optimal growth temperature. The effect of extreme temperature on the enzymatic activity has to be analyzed in further studies. *B. cereus* strains used for this study could have different applications in diverse industries (Horikoski 1999) due to their ability to hydrolyze many protein sources such as casein, hemoglobin, gluten, gelatin, milk whey, ovalbumin, and bovine serum albumin (Pérez-Borla et al. 2010). This strain may be regarded as a potential starting culture due to its high proteolytic activity and hence rapid fermentation would be expected (Chantawannakul et al. 2002). A combination of different nitrogen sources could be assay in a future study. Yeast extract and peptone was found to be the optimum nitrogen source for protease production for a *B. cereus* strain in previous study (Ghorbel-Frikha et al. 2005). In future works, we will emphasize the search of microorganisms that resist several technological stress conditions during the different process and could produce desirable metabolic products. Proteases produced by *Bacillus* species are the most important group of secondary metabolites that are widely exploited (Ferrari et al. 1993). Important advantages would obtain if undesirable microorganisms, like *B. cereus* spp., could act in favor of the biotechnological process mainly in the food industry where such microorganisms are frequently involve in undesired contamination of equipments and products.

45.2.3 Simultaneous Optimization of Biomass and Protease Biosynthesis by a Local Isolated *Pseudomonas* sp.

45.2.3.1 Selection of Main Medium Components and Operating Conditions

In general, medium optimization by the traditional "one factor at a time" technique was extensively used in the past. This method is not only laborious and time-consuming but also often leads to an incomplete understanding of the system behavior, resulting in confusion and a lack of predictive ability (Zhang 2007). Several statistical designs have been developed to overcome this drawback. Among them, the PB design is highly recommended when more than five factors have to be investigated. This design is useful

to detect the main factors economically, assuming all interactions are negligible when compared with the few important main effects (Dhandhukia 2007). In this way, PB is usually applied as a preoptimization stage, with the primary objective of studying the statistical significance of factors on the dependent variable and to eliminate nonsignificant factors. Thus, efforts may be concentrated upon most significant variables selected for further optimization. In recent years, PB design was extensively applied to investigate the significance of different medium components on the production of lactic acid by *Lactobacillus* sp. KCP01 (Chauhan 2007) and by *Lactobacillus amylophilus* GV6 (Naveena 2005a,b), chitinase by *Pantoea dispersa* (Gohel 2006) and by *Alcaligenes xylosoxydans* (Vaidya 2003), transglutaminase by *Bacillus circulans* BL32 (Volken de Souza 2008), biosurfactant by a marine bacterium (Mukherjee 2008), poly-β-hydroxybutyrate by methylotrophic bacterium (Mokhtori-Hosseini 2009), among others.

One significant feature is to establish the upper and lower limits for each variable to be analyzed by PB design. Most studies fix these limits through bibliographic data (Guangrong 2009); however, when a new microorganism was characterized, the selection of the appropriate limits for each variable could be critical.

The microorganism used in this work was previously isolated from fresh cabbage by Pérez Borla et al. (2010) and identified in CERELA center (CONICET, Tucumán, Argentina; 2008) as *Pseudomonas* sp.

Table 45.3 resumes the composition and the incubation conditions utilized by modifications on the MM supplemented with 6.2 g/L protein of skimmed milk (5 g/L of casein). Different plates, each of one with two halos, with the corresponding modified MM were used to assay the ability of the microorganism to grow and to liberate extracellular protease and hydrolyze the skimmed milk (Pérze Borla et al. 2010). Wells 5 mm in diameter were cut under sterile conditions into these agar plates, filled with

TABLE 45.3

Prescreening of Nutrients and Physical Parameters Effects on the *Pseudomonas* sp. Growth and Protease Production

	Activated Modifiers					Incubated Conditions	
No.	NaCl (% w/v)	Glucose (g/L)	Peptone (g/L)	Ca^{2+} (mM)	Mg^{2+} (mM)	pH	Temperature (°C)
1	1.00	0.00[a]	0.00	0.00	0.00	7	30
2	2.00	0.00	0.00	0.00	0.00	7	30
3	3.00	0.00	0.00	0.00	0.00	7	30
4	0.00	1.00	0.00	0.00	0.00	7	30
5	0.00	4.00	0.00	0.00	0.00	7	30
6	0.00	10.00	0.00	0.00	0.00	7	30
7	0.00	0.00	1.00	0.00	0.00	7	30
8	0.00	0.00	3.00	0.00	0.00	7	30
9	0.00	0.00	5.00	0.00	0.00	7	30
10	0.00	0.00	0.00	1.00	0.00	7	30
11	0.00	0.00	0.00	3.00	0.00	7	30
12	0.00	0.00	0.00	7.00	0.00	7	30
13	0.00	0.00	0.00	0.00	1.00	7	30
14	0.00	0.00	0.00	0.00	3.00	7	30
15	0.00	0.00	0.00	0.00	7.00	7	30
16	0.00	0.00	0.00	0.00	0.00	4	30
17	0.00	0.00	0.00	0.00	0.00	7	30
18	0.00	0.00	0.00	0.00	0.00	11	30
19	0.00	0.00	0.00	0.00	0.00	7	0
20	0.00	0.00	0.00	0.00	0.00	7	10
21	0.00	0.00	0.00	0.00	0.00	7	20
22	0.00	0.00	0.00	0.00	0.00	7	30

[a] 0.00 indicates no modifications for the MM original composition.

20 µL aliquot of the growth culture, and incubated statically and aerobically during 48 hours. Bacterial proteolytic activity was observed daily. Five nutrients (NaCl, glucose, peptone, Ca^{2+}, and Mg^{2+}) and two physical parameters (pH and temperature) were modified at three levels (except temperature, which was modified in four levels) embracing a roomy profile (Table 45.3). At each level, the effect of the physicochemical modifications was verified by the radial bacterial grow around the well (Hope 1985) and color and size of the clear zones, due to the proteolytic activity, around wells. For each condition (Table 45.3), a positive control, corresponding to MM without inoculums, was added into the wells. The experiment was done three times.

PB design was used to screen the variables (k_i) that significantly influence the protease production and microorganism growth. This design assumes that there are no interactions between the different media constituents, x_i, in the range of the variables taken under consideration and a linear approach is considered sufficient for screening:

$$Y = \beta_0 + \sum \beta_i x_i; i = 1,2,...,8$$

where Y is the estimated function for protease production or microbial growth, β_i are the regression coefficients, and x_1, x_2, x_3, x_4, x_5, x_6, x_7, and x_8 are the temperature, agitation speed, pH, glucose, peptone, NaCl, Ca^{2+}, and Mg^{2+} factors, respectively.

In this work, eight factors and three dummy variables were screened to evaluate the relative importance of them on both proteolytic and biomass production. Each factor was represented at two levels, high and low, which were denoted by (+1 or 1) and (−1), respectively. The coded levels for each factor were determined in the pre screening test and are shown in Table 45.4. The screening was carried out through 12–24 trial experimental run, and the level of each factor within each run was determined by the PB matrix (Table 45.5). Each row in Table 45.5 represents a trial, and each column represents an independent (assigned) or dummy (unassigned) variable. All experiments were carried out in duplicates, and the average values of protease unit (Y_1) and microorganism growth, measured as the absorbance at 600 nm (Y_2), were taken as responses.

The effect $E_{(x_i)}$ of each factor (x_i) was determined by the following equation:

$$E_{(x_i)} = \frac{2\left(\sum M_{i+} - \sum M_{i-}\right)}{N}$$

TABLE 45.4

Threshold Limits Established by the Prescreening Assay to Be Applied to the PB Design

			Levels	
Variables			**Low (−1)**	**High (+1)**
x_1	Temperature	°C	10	30
x_2	Agitation speed	rpm	0	100
x_3	pH		5	9
x_4	Glucose	g/L	0[a]	4
x_5	Peptone	g/L	1	5
x_6	NaCl	% w/v	1	3
x_7	Ca^{2+}	mM	1	7
x_8	Mg^{2+}	mM	1	7

[a] The low level of glucose indicated that the medium was not supplemented with exogenous glucose, but the MB contains 1 g/L of bacteriological glucose.

TABLE 45.5

Experimental Design Using the PB Design for Medium Component Screening Together with the Protease Activity and Microorganism Growth Obtained for Each Run

	Variables											Observed Response	
Run	x_1^a	x_2	x_3	x_4	x_5	x_6	x_7	x_8	d_1^b	d_2	d_3	Protease Activity	Microbial Growth
1	1	−1	1	−1	−1	−1	1	1	1	−1	1	0.1089	0.3063
2	1	1	−1	1	−1	−1	−1	1	1	1	−1	0.1450	0.3397
3	−1	1	1	−1	1	−1	−1	−1	1	1	1	0.0047	0.8392
4	1	−1	1	1	−1	1	−1	−1	−1	1	1	0.1136	0.6743
5	1	1	−1	1	1	−1	1	−1	−1	−1	1	0.0985	0.3578
6	1	1	1	−1	1	1	−1	1	−1	−1	−1	0.0424	1.1117
7	−1	1	1	1	−1	1	1	−1	1	−1	−1	0.0054	0.1880
8	−1	−1	1	1	1	−1	1	1	−1	1	−1	0.1033	0.1300
9	−1	−1	−1	1	1	1	−1	1	1	−1	1	0.0274	0.0387
10	1	−1	−1	−1	1	1	1	−1	1	1	−1	0.1834	0.7218
11	−1	1	−1	−1	−1	1	1	1	−1	1	1	0.0046	0.3328
12	−1	−1	−1	−1	−1	−1	−1	−1	−1	−1	−1	0.0707	0.0988

[a] x_i are the variables.

[b] d_i are the dummy (unassigned) variables.

M_{i+} and M_{i-} correspond to the protease activity from the trials when the factor x_i was present at high and low levels, respectively. Experimental error was estimated by calculating the variance among the dummy variables as follows:

$$V_{\text{eff}} = \frac{\sum E_{\text{d}}^2}{n}$$

where V_{eff} is the variance of the concentration effect, E_{d} is the effect for the dummy variable, and n is the number of dummy variables.

45.2.3.1.1 Prescreening Assay

To establish the threshold limits for *Pseudomonas* sp., a rapid qualitative prescreening assay was performed changing nutrient concentration and physical parameters. Glucose concentration affected in different ways the *Pseudomonas* sp. growth and its protease activity. The maximum proteolytic halos were found at the lower assayed concentration (1.00 g/L); instead, the largest zone of bacterial growth was observed at intermediate assayed glucose concentration (5.00 g/L). At the three NaCl concentrations, external proteolytic halos were observed with little changes of its diameters. However, for bacterial growth, the effect was greater at the highest NaCl concentration assayed (3%). Proteolytic halos were observed in all the plates supplemented with peptone. However, while variations in the peptone concentration of the MM no significant changed the halo sizes, the zone of bacterial growth increase as increase the concentration of this nutrient in the assayed range (1.00, 3.00, and 5.00 g/L). Mg^{2+} ions affected differently the evaluated characteristics of the *Pseudomonas* sp. strain. At the higher Mg^{2+} concentration, the zone of bacterial growth was smaller, but the proteolytic activity was the greatest. However, neither the zone of bacterial growth nor the halo presented a marked variation in the size and in the diameter, respectively, compared with the other assayed variables. Similar to Mg^{2+}, an increase in the Ca^{2+} concentration caused a decrease in the bacterial growth but an increase in the proteolytic halo diameter.

For the variable pH, three levels in the range from 4 to 10 were assayed. Both the bacterial growth and the proteolytic hydrolyses were favored at pH 7. At pH 4, none colonies formed around the halo; hence, nonhalo was observed. At pH 4, a partial precipitation of the casein was observed in the plate. At pH 10,

no proteolytic halo was also observed, and a marked brown coloration was developed in the MM, probably due to Maillard reaction.

Temperature effects were assayed at four levels: 0°C, 10°C, 20°C, and 30°C. At 0°C, neither zone of bacterial growth nor protease activity was observed. At 20°C and 30°C, the bacterial growth zone and the proteolytic halos were practically similar in magnitude and greater than at 10°C.

Taking into account the prescreening assay and analyzing the obtained results described previously, the boundaries were set delimiting the range in which the proteolytic activity and microbial growth were significant. Thus, the defined levels for the PB design were presented in Table 45.2.

45.2.3.1.2 PB Design Applied to Protease Activity

Pseudomonas sp. produced a high yield of protease in trial 10 (0.1834 PU) followed by trials 2 and 4 (0.1450 and 0.1136, respectively) (Table 45.5). The common factor between these three experiments is the high temperature level (30°C), while the other factors were set indistinctly at its lower or upper level.

Table 45.6 presents the statistical parameters (the effect, parameter estimate, sum of squares [SS], contribution to SS, *t* value, *p* value, and confidence level) obtained from protease activity and microorganism growth results. One advantage of the PB design is its ranking of the different variable effects on the measured response independent of its nature (either nutrient or physical factor) or sign (whether contributes positively or negatively). Another useful plot for identifying the main factors is the Pareto chart of *t* values that shows the obtained statistically *t* values (absolute value) for each factor ranked from highest to lowest (independent on its positive or negative contribution). In the Pareto chart, the largest *t* values (most important factors) are presented in the left portion and then progress down to the smallest *t* values (least important factors.) Figure 45.9 shows the effects (a) and the Pareto chart (b) for protease activity. The most important factor determining protease production was the temperature, followed by stirring speed, NaCl concentration, and pH (Figure 45.2b). These last three factors have negative effects on protease activity (Figure 45.9a). Analyzing the confidence level obtained for each factor, it was found that the variables x_1 (temperature) resulted significantly ($p < 0.1$) for the protease production with confidence levels

TABLE 45.6

Parameter Estimates and Hypothesis Tests for Proteolytic Activity and Microorganism Growth Responses

Coded Variable	Variable	Effect	Parameter Estimate	SS	Contribution to Total SS (%)	*t* Value	*p* Value	Confidence Level
Proteolytic activity								
x_1	Temperature	0.08	0.0396	0.019	58.69	2.93	0.0612	93.9
x_2	Agitation speed	–0.05	–0.0256	0.008	24.41	–1.89	0.1557	84.4
x_3	pH	–0.03	–0.0126	0.002	5.95	–0.93	0.4204	58.0
x_4	Glucose	0.01	0.0065	0.001	1.59	0.48	0.6628	33.7
x_5	Peptone	0.00	0.0009	0.000	0.03	0.07	0.9488	5.1
x_6	NaCl	–0.03	–0.0129	0.002	6.20	–0.95	0.4123	58.8
x_7	Ca^{2+}	0.03	0.0084	0.001	2.61	0.62	0.5808	41.9
x_8	Mg^{2+}	0.19	–0.0037	0.000	0.52	–0.27	0.8013	19.9
Microbial growth								
x_1	Temperature	0.31	0.1570	0.296	25.79	3.34	0.0443	95.6
x_2	Agitation speed	0.20	0.0999	0.120	10.45	2.13	0.1332	86.7
x_3	pH	0.23	0.1133	0.154	13.43	2.41	0.0947	90.5
x_4	Glucose	–0.28	–0.1402	0.236	20.56	–2.99	0.0584	94.2
x_5	Peptone	0.21	0.1049	0.132	11.52	2.23	0.1115	88.9
x_6	NaCl	0.17	0.0830	0.083	7.20	1.77	0.1755	82.5
x_7	Ca^{2+}	–0.18	–0.0888	0.095	8.25	–1.89	0.1550	84.5
x_8	Mg^{2+}	–0.10	–0.0517	0.032	2.80	–1.10	0.3510	64.9

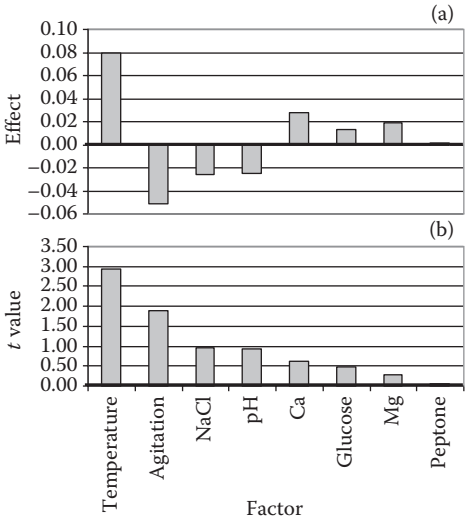

FIGURE 45.9 (a) Effect of operating and nutritional factors on protease production by *Pseudomonas* sp. in batch culture. (b) Pareto plot for *t* values.

of 93.88%. Temperature presented a positive effect indicating that going from the low level (0°C) to the high level (30°C) the PU (Table 45.4) increases. Another useful statistical parameter is the contribution of each factor to the total SS. It can be observed in Table 45.6 that the significant factor (temperature) contributed in a 58.69% to the SS, stirring speed (with a partial SS of 24.41%, giving both more significant factors a total contribution of 83.10%), NaCl (with a partial SS of 6.20%, giving the three factors a total contribution of 89.30%) and pH (with a partial SS of 5.95%, giving a total contribution of 95.25%).

The other factors (glucose, peptone, Ca^{2+}, and Mg^{2+}) contributed in the 4.75% residual SS. Considering the terms that were significant for protease activity (temperature) and the additional terms that contribute to the SS (stirring speed, NaCl concentration, and pH), the linear regression equation with influence on protease activity from PB design could be written as

$$Y_1 = 0.07566 + 0.03964\, x_1 - 0.02556\, x_2 - 0.01262\, x_3 - 0.01287\, x_6,$$

where Y_1 is the predicted response (protease activity) and x_1, x_2, x_3, and x_6 are the coded variables for temperature, agitation speed, pH, and NaCl, respectively. This model contributes to the SS with a value of 95.25%.

45.2.3.1.3 PB Design Applied to Microbial Growth

Table 45.5 also presents the results of PB experiment with respect to microbial growth. *Pseudomonas* sp. showed the highest growth in trial 6 (1.1117) followed by trials 3 and 10 (0.8392 and 0.7183, respectively). Table 45.6 presents the statistical parameters (the effect, parameter estimate, sum of square (SS), contribution to SS, *t* value, *p* value, and confidence level) obtained from microbial growth results. Analyzing the confidence level obtained for each factor, it was found that the variables x_1 (temperature), x_4 (glucose), and x_3 (pH) resulted significant ($p < 0.1$) for the microbial growth (with confidence levels of 95.57, 94.16, and 90.53%, respectively). Figure 45.10 shows the effects of factors on microbial growth (a) and the Pareto chart for this variable (b). The factor presenting the highest effect is temperature, followed by glucose concentration and pH. The other factors tested in this design contributed differently on the isolated *Pseudomonas* sp. growth, which means that some of them affected positively and others negatively (Figure 45.10b). Similar to the effect observed with the measured response PU, temperature also presented a positive effect on microbial growth (Figure 45.3a), indicating that going from the low level (0°C) to the high level (30°C) increases microorganism growth. Moreover, pH has a positive effect too indicating that pH near 9 could be required for further optimization processes, suggesting that *Pseudomonas*

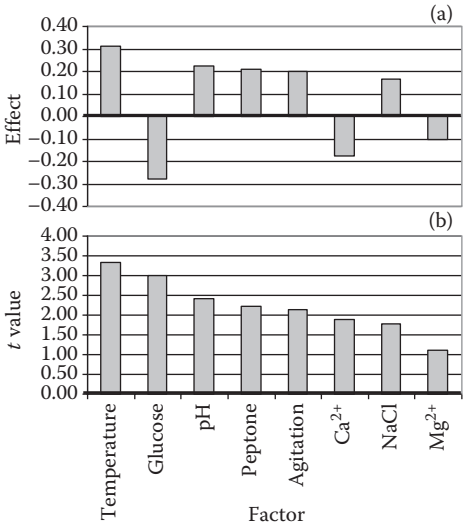

FIGURE 45.10 (a) Effect of operating and nutritional factors on *Pseudomonas* sp. growth in batch culture. (b) Pareto plot for *t* values.

sp. is an alkalophilic microorganism. The glucose concentration effect presented a negative sign. It must be remembered that MB contains 1 g/L of glucose, and then zero level correspond to this minimal concentration. That means that lower glucose concentration (<1 g/L) will be advisable to microorganism growth. Analyzing the contribution of each factor to the total SS, it was found that the significant factors (temperature, glucose, and pH) contributed in a 59.78% to the SS (Table 45.6). Other factors that significantly contribute to the SS are the peptone concentration (with a partial SS of 11.52%, giving the four factors a total contribution of 71.30%) and stirring speed (with a partial SS of 10.45%, giving a total contribution of 81.75%). The other factors (Ca^{2+}, NaCl, and Mg^{2+}) contributed in the 18.25% residual SS. Considering only the terms that mostly contribute to microbial growth (on the basis of contribution in the SS), the linear regression equation that had an important influence on this independent variable from PB design could be written as

$$Y_2 = 0.4283 + 0.1570\,x_1 + 0.0999\,x_2 + 0.11332\,x_3 - 0.1402\,x_4 + 0.1049\,x_5,$$

where Y_2 is the predicted response (microorganism growth) and x_1, x_2, x_3, x_4, and x_5 are the coded variables for temperature, agitation speed, pH, glucose, and peptone, respectively. This model contributes to the SS with a value of 81.75%.

45.2.3.2 Response Surface Optimization Using Box–Behnken Design

Once the components critical to the enzyme production are screened, the second stage of the media optimization is to find the optimum level of each variable that most affects the response with the aim of maximizing the product formation. RSM can be employed in this second stage (Agüero 2010). RSM is a powerful and efficient mathematical approach widely applied in the optimization of the fermentation process, e.g., media components on enzyme production (Agüero 2010; El Enshay 2008; Srinu Babu 2007), production of other metabolites (Abha Mishra 2008; Lu-E 2007; Waldir 2007), spore production, bacteriocin production, and biomass production optimization. It can give information about the interaction between variables, provide data necessary for design and process optimization, and give multiple responses at the same time (Zhang 2007). Several studies reported that media components have great influence on extracellular protease production (Adinarayana 2002; Guangrong 2002). Also,

each microorganism presents particular optimal conditions for the protease secretion (Lei Yu 2008). Therefore the optimization of protease production by a new strain requires defining the constituents and their specific concentrations accordingly. In the next part of the work, the aims were to compare the protease production under two different situations: (a) when major nutrients and culture conditions for biomass production are optimized and (b) when major nutrients and culture conditions for protease production are optimized.

45.2.3.2.1 Experimental Designs and Statistical Analysis Using Box–Behnken Methodology

RSM with a Box–Behnken (BB) design was used to optimize the process, finding interactions among the significant factors obtained from PB design (Agüero 2010). BB is a spherical resolving design that consists of the central and middle points of the edges (Abha Mishra 2008). In the BB experimental design, the total number of experimental combinations is $N = k^2 + k + c_p$, where k is the number of independent variables and cp is rotatable number of central point. For 3 level factor BB experimental designs, a total of 15 experimental runs are needed. Tables 45.1 and 45.2 represent the 15-trial experimental designs for the protease and biomass optimization, respectively, in which each variable was tested in three different coded levels: low (−1), middle (0), and high (+1). In developing the regression equation the factors were coded according to the equation:

$$X_i = (x_i - x_{0i})/\Delta x_i,$$

where X_i is the coded value of the ith independent variable, x_i is the natural value of the ith independent variable, x_{0i} is the natural value of the ith independent variable at the center point, and Δx_i is the steep change value.

Once the protease production as protease unit (PU) and biomass as optical density (OD) at 600 nm were measured for each trial, two independent second-order polynomial models were fitted to the response data obtained from the design. The general polynomial equation is in the following form:

$$Y = \beta_0 + \Sigma\beta_i X_i + \Sigma\beta_{ij} X_i X_j + \Sigma\beta_{ii} X_1^2,$$

where Y is the predicted response (protease or biomass production), β_0 is the model constant, β_i is the linear coefficient, β_{ii} is the quadratic coefficient, β_{ij} is the coefficient for the interaction effect, and X_i is a dimensionless coded value of x_i (independent variable).

In the work previously mentioned (Section 45.2.2), using PB design, it was found that the most significant variables for the protease production by the *Pseudomonas* sp. strain were temperature, agitation speed, NaCl, and pH. pH also plays an important role in the fermentation process. Most substrates used in solid-state fermentation are known to possess excellent buffering capacity, and this explains the fairly constant pH during fermentation process. The bacterial enzyme produced under optimal conditions will be applied from a technological approach to an agro industrial waste such as exhausted malted barley. This unbuffered industrial byproduct presented typically acid pHs, and this fact conditioned the enzyme selection in the present study. Thus, the acid character of the enzyme was exploited setting the pH in 5. Hence, for industrial applications, only temperature, NaCl content, and agitation speed have to be optimized (Table 45.7).

On the other hand, by analyzing the confidential level obtained for each studied factor, it was found that the variables temperature, glucose, and pH resulted significant ($p < 0.1$) for the microbial growth (with confidential levels of 95.57%, 94.16%, and 90.53%, respectively). Thus, these three variables were used in the BB design for the study of the optimal bacterial growth conditions (Table 45.8).

45.2.3.2.2 Biomass Production Optimization

Table 45.8 presents the BB experimental design matrix together with the results of the experiments (observed and predicted results) for studying the effects of temperature, glucose content, and pH on biomass production. Figure 45.11b shows that the contour lines are nearly symmetric and appear (at different extents) as circles displaced to the higher values of the variables, suggesting that high temperature,

TABLE 45.7

BB Experimental Design Matrix for Protease Production

	Factor			Protease Unit (PU)	
Run	x_1	x_2	x_3	**Observed**	**Predicted**
1	−1 (10°C)	−1 (0 rpm)	0 (3 g/L)	0.053009058	0.09123898
2	−1 (10°C)	1 (100 rpm)	0 (3 g/L)	0.046183477	0.07582198
3	1 (30°C)	−1 (0 rpm)	0 (3 g/L)	0.151758373	0.08221694
4	1 (30°C)	1 (100 rpm)	0 (3 g/L)	0.091746446	0.09064594
5	0 (20°C)	−1 (0 rpm)	−1 (1 g/L)	0.108588789	0.10412598
6	0 (20°C)	−1 (0 rpm)	1 (5 g/L)	0.10610676	0.15075194
7	0 (20°C)	1 (100 rpm)	−1 (1 g/L)	0.045651613	0.13983198
8	0 (20°C)	1 (100 rpm)	1 (5 g/L)	0.08208426	0.10805794
9	−1 (10°C)	0 (50 rpm)	−1 (1 g/L)	0.085364085	0.07766799
10	1 (30°C)	0 (50 rpm)	−1 (1 g/L)	0.094139832	0.08180997
11	−1 (10°C)	0 (50 rpm)	1 (5 g/L)	0.048665506	0.08633497
12	1 (30°C)	0 (50 rpm)	1 (5 g/L)	0.120112497	0.08799491
13	0 (20°C)	0 (50 rpm)	0 (3 g/L)	0.11381878	0.19114096
14	0 (20°C)	0 (50 rpm)	0 (3 g/L)	0.112843697	0.19114096
15	0 (20°C)	0 (50 rpm)	0 (3 g/L)	0.108500145	0.19114096

Note: x_1, Temperature; x_2, agitation speed; x_3, NaCl content.

pH, and glucose content were favorable for cell growth. The maximum response falls within the design boundary. Circular contour plot indicated that the interactions between the variables are negligible.

The analysis determined that the maximum response of biomass production was 1.64 (OD at 600 nm) with corresponding optimum to uncoded variables at 25.29°C, 3.35 g glucose/L, and pH 7.46. This optimal medium pH (7.45) for cell growth was significantly higher than the optimal pH range of 5.5 to 6.0 mentioned by Hussein et al. (2004), who worked with different *Pseudomonas* species resistant

TABLE 45.8

BB Experimental Design Matrix for Biomass Production

	Factor			Biomass Production	
Run	x_1	x_2	x_3	**Observed**	**Predicted**
1	−1 (10°C)	−1 (0[a] g/L)	0 (7)	0.0280	–0.4975
2	−1 (10°C)	1 (4g/L)	0 (7)	0.0287	0.1762
3	1 (30°C)	−1 (0 g/L)	0 (7)	0.8400	0.6925
4	1 (30°C)	1 (4g/L)	0 (7)	1.7153	1.4688
5	0 (20°C)	−1 (0 g/L)	−1 (5)	0.2073	0.1595
6	0 (20°C)	−1 (0 g/L)	1 (9)	0.0.8097	0.7584
7	0 (20°C)	1 (4g/L)	−1 (5)	0.6090	0.6603
8	0 (20°C)	1 (4g/L)	1 (9)	0.8877	0.9355
9	−1 (10°C)	0 (2 g/L)	−1 (5)	0.0083	–0.1905
10	1 (30°C)	0 (2 g/L)	−1 (5)	0.0283	0.2236
11	−1 (10°C)	0 (2 g/L)	1 (9)	0.0007	–0.1946
12	1 (30°C)	0 (2 g/L)	1 (9)	0.9030	1.1018
13	0 (20°C)	0 (2 g/L)	0 (7)	1.4190	1.4412
14	0 (20°C)	0 (2 g/L)	0 (7)	1.4657	1.4412
15	0 (20°C)	0 (2 g/L)	0 (7)	1.4390	1.4412

Note: x_1, Temperature; x_2, glucose content; x_3, pH.

[a] 0.00 indicates no modifications for the MM original composition.

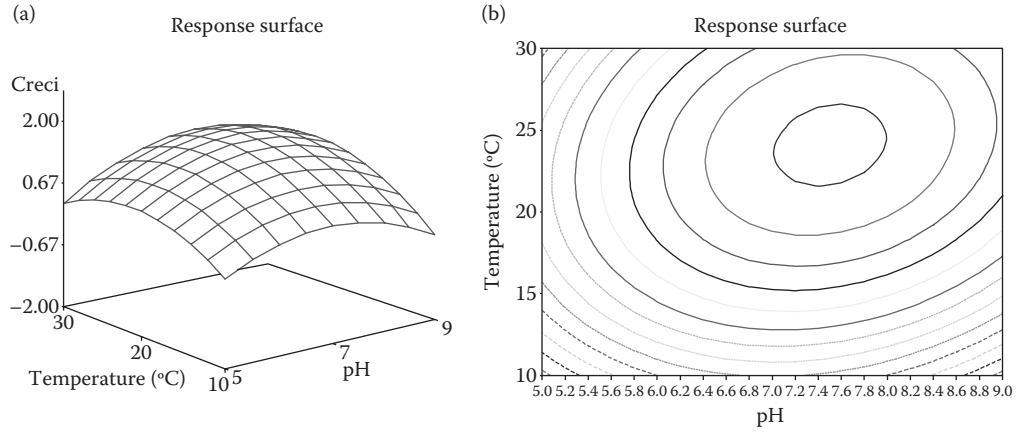

FIGURE 45.11 (a) Response surface plot and (b) corresponding contour plot showing the effect on temperature, pH, and their mutual effect on growth (OD at 600 nm) from the *Pseudomonas* not classified strain. Glucose content was held 2 g/L.

to different metal ions and that were isolated from mixed industrial and domestic wastewater. Figure 45.11a shows the kinetic profiles of biomass production resulting of the *Pseudomonas* sp. strain incubation under both optimal conditions (biomass and protease production).

45.2.3.2.3 Protease Production Optimization

Table 45.7 presents the BB experimental design matrix together with the results of the experimental runs (predicted and observed results) showing the effects of temperature, NaCl content, and agitation speed on protease production. Figure 45.12 shows three-dimensional surface representing the response (protease production) as a function of the two factors: temperature vs. agitation speed and temperature vs. salinity for the experimental range considered. It was evident that the temperature has the main effect on protease production, showing a maximum of PU between 22°C and 28°C, independently of the value that adopted the other assayed variables. The influence of both the agitation speed and the salinity on

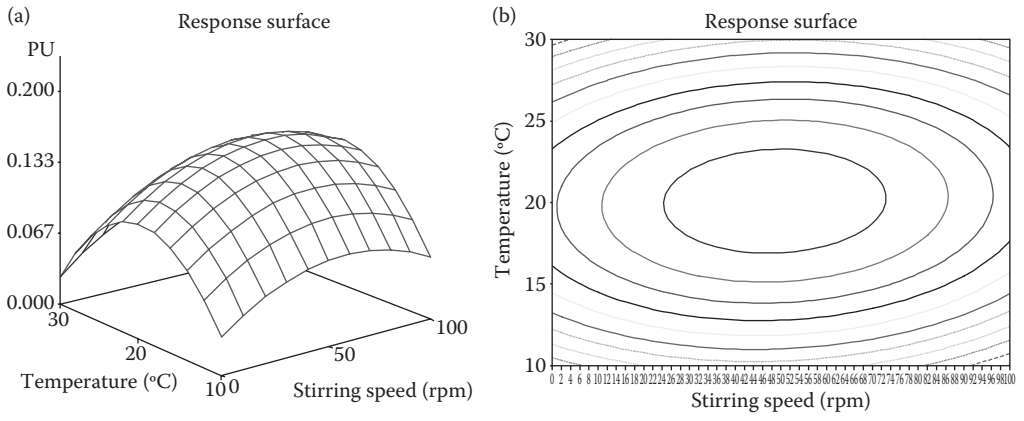

FIGURE 45.12 (a) Response surface plot and (b) corresponding contour plot showing the effect on temperature, agitation speed, and their mutual effect on protease production (PU) from the *Pseudomonas* not classified strain. NaCl content was held at 1% w/v.

the protease production expressed as PU was not constant, changing the maximum response according to the values of agitation speed and salinity. Each response surface and contour plot for protease production represented the different combinations of two tested variables at one time while the third one was keeping at a determinate level (–1, 0, or +1). There are pairs of response surfaces and the corresponding contour plots. Figure 45.12 presents two typical ones.

The optimum points corresponded to 20.07°C, 47.61 rpm, and 2.07% w/v NaCl. The predicted protease production in the optimum, obtained by applying regression analysis on model using SAS package software, was of 0.1913 PU.

Agitation speed also played an important role in protease production, probably due to its importance in terms of oxygen and nutrient transfer into the liquid medium, especially for the growth of an aerobic bacteria like *Pseudomonas* sp.; similar results were found in other studies. Romsonma et al. (2010) considered the shaker speed as one of the major factors for the improvement of silk degumming protease production from *Bacillus subtilis* C4 by statistical approaches in submerged culture. In contrast with an earlier report, which confirms that the addition of NaCl to the growth medium of *P. aeruginosa* did not increase extracellular protease production (Häse 2003), higher levels of protease activity by the isolated *Pseudomonas* strain were observed in 2 g/L salt medium.

Figure 45.13 shows the profiles resulted of the *Pseudomonas* sp. strain cultivation under the optimized protease production conditions and the optimal ones for microorganism growth.

All these studies clearly showed that the statistical design RSM was very powerful in the optimization of bioprocesses. Optimizing the media formulation requirement for maximum enzyme production was extremely important in industrial scale for economic reasons. In many studies, it was common to have found optimization procedures for protease production of different microbial source. In these kinds of studies, the biomass production was not mentioned. We recognized that it was important to consider not only the protease production, but the amount of microorganism presented in the submerged fermentation also had to be taken into account. That means that it could be possible to obtain the same proteolytic activity through low microorganism load but with important levels of protease production and also with many organisms with low levels of protease production. This last condition could be not considered if only the proteolytic production was taken into account. This research established an optimized fermentation process for both biomass and protease production separately and concluded that the maximum protease production was achieved during protease production optimization. Maximum protease production was obtained at lower temperatures than the optimum for growth (5°C lower). The model indicates that temperature has the major effect on protease production by the *Pseudomonas* strain. Other RSM studies also showed that temperature is a very relevant factor for microbial protease production (Thys 2006) and also for biomass production (Ramkrihna 2005).

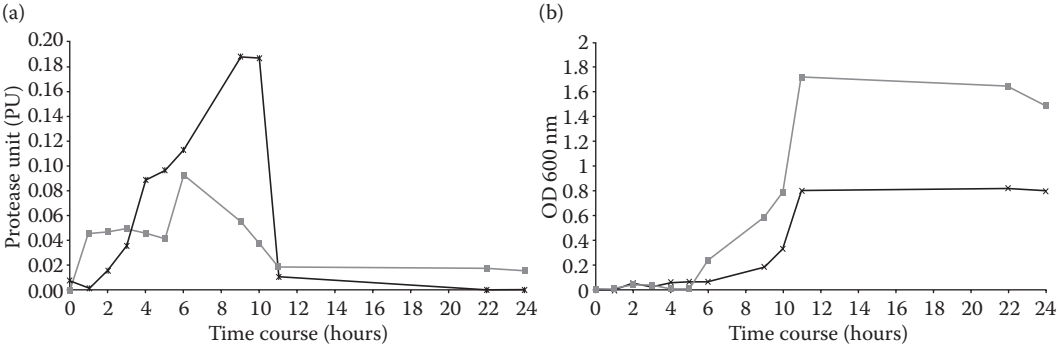

FIGURE 45.13 (a) Protease production kinetic and (b) biomass kinetic in batch cultures using optimized media for microorganism growth (■) and for protease production (×).

45.2.4 Conclusions

Many researchers are interested in the synthesis of peptides or amino acids using proteases extracted from diverse sources. Microorganisms are a potentially important source of enzyme production. The relationship between the kinetic growth of the microorganisms and the culture conditions cannot be extended proportionally to the proteolytic activity. Therefore, optimization of the protease production implicates the well knowledge of the physical and chemical factors that influence the growing of the microorganisms and the studied of the factor that affect directly or indirectly the protease activity. Despite several studies referring to *Bacillus cereus* as a food spoilage and food poisoning organism, which are not desirable in the food industry (Durick et al. 2005), we recognize the use of *B. cereus* enzymes as an important resource for this industry. In future studies, we will center on the technological importance of the application of these enzymes for the obtaining of wished products.

PB designs resulted to be an excellent exploratory technique that allowed to statistically select the most significant factor affecting *Pseudomonas* sp. growth and protease production, using this statistical tool, the total number of factors (usually large) is reduced to a smaller amount, which can be subjected to further optimization. This simple and rapid methodology presents the difficulty in the selection of the appropriate lower and higher levels for each factor. Therefore a prescreening assay was proposed as an efficient and effective tool to begin the experimental design process. It is noteworthy that the main factors affecting the protease unit were different than those from the bacterial growth. For both, the main important factor was temperature. The enzymatic activity was increased at high temperatures, resulting in high protease production. Also, microorganism growth was promoted at the higher temperature level. As is well known the pH is another variable that plays a main roll in bacterial growth and also in reactions catalyzed by enzymes. In this case, an opposed behavior was found, while acidic pH benefited enzymatic activity, microorganism growth was favored in an alkaline medium. The negative effect of glucose in microorganism growth was probably caused by the direct energy source disposable in the MM (without further added glucose), which could be sufficient for the basic metabolic functions and to produce high growth rates. The effect of glucose demands for protease production was not significant, resulting almost indifferent to the change in glucose concentrations in the fermentation medium to the protease activity.

The relative effects of Ca^{2+} and Mg^{2+} were almost negligible. Similar result were found by Nicodème et al. (2005), who found *Pseudomonas* strains that were able to grow in minimal salt medium. Salinity negatively affected protease production. Instead, NaCl concentration played a positive role on *Pseudomonas* sp. growth. It was reported that *Pseudomonas* sp. were grown in a minimal salt medium (Nicòdeme et al. 2005). The positive effect from the stirring speed could be explained with the rupture of the biofilm that *Pseudomonas* sp. formed in the medium surface, inducing multiple formation of smaller nucleus for microorganism growth (Drenkard 2002). Agitation is important for adequate mixing, mass transfer, and heat transfer. Good mixing between gas and liquid phases could be achieved by means of aeration and agitation. On the other hand, agitation at higher stirring speeds may cause disruption of cells in the fermentation by shear forces and formation of vortex, which may result in poor mass transfer for both oxygen and substrate (El Enshay 2008). Thus, a balance between aeration and agitation should be considered during the cultivation of aerobic microorganisms in submerged culture. Stirring speed has a negative effect on the protease production, indicating that if a stirring speed near the minimum level (0 rpm) or directly, enzyme immobilization could be required for further optimization processes. On the one hand, agitation favors the effective coalitions in enzymatic reactions; on the other hand, it could adversely affect the proper space orientation of the enzyme in its active sites, which were highly modified by orbital shaking movement. This could probably result in a negative response of stirring speed on protease activity. The interpretation of the sign of each effect must be well established. Some authors interpreted that when the sign is positive, the influence of the factor upon the response is greater at high concentration, and when negative, the influence is greater at a low concentration (El-Nagger 2006). Other authors analyzed the positive signs as the corresponding factors that favor the response, while negative signs indicate that the factor inhibit the response (Ahmad 2009). Finally, Gohel et al. (2006) and Dhandukia et al. (2007) established that if the effect is positive, a higher concentration than the indicated high (+) concentration in PB experiment is required to increase the response, and if the effect is negative, a lower concentration than the indicated low

(–) concentration in PB experiment is necessary. We found that this last interpretation could not be applied to explain the sign of variables such as stirring speed, which due to the low level was defined as 0 rpm, and there is no sense to set a speed lower than this low level because it would be negative. Taking into account the above arguments and in agreement with the results observed in the present work, we based on the following interpretation: a negative sign means that going from low level to high level for a factor decreases the response and a positive sign means that going from the low level to the high level increases the response. The effect for a factor is always described as the change in the response in going from the low level of that factor to the high level. The significant factors on the biomass and the protease production identified by PB will be considered for the next stage in the medium optimization by using response surface optimization techniques and the studies in bioreactor.

45.3 Use of Proteolytic Bacteria in Food Processing

Proteases have a wide range of applications: meat tenderization, beer, cheese, and bread elaboration, production of modified food proteins, lead manufacture, detersive industry, industrial wastes treatment, pharmaceuticals, etc. In keeping with up to date information, Argentina imports the bulk of the proteases used by the industry (Natalucci 2000).

It is well known that there has been progress in developing technologies to convert raw materials into food products, oils, flours, and derivatives, but it is also verifiable that as a technology becomes more industrialized, the greater the volume of generated wastes. Today, these are subexploited because of the difficulty in finding a direct application and their problems with storage or elimination, some of which may cause environmental problems.

Advances in industrial biotechnology offer potential opportunities for economic utilization of agroindustrial residues such as defatted soy flour, defatted sunflower flour, and barley spent grain (byproduct of the brewing industry) as substrate for many biochemical reactions. Due to their rich organic nature, they can serve as an ideal substrate for the production of value-added products such as proteases.

The biodiversity of natural resource existing in Argentina is of great significance and has large industrial demands because of their application ranging from food, to paper, and to the pharmaceutical industry. Today, microbial proteases have completely replaced chemical hydrolysis in several processing industries.

One research line of the Research Group on Food Engineering (GIIA, Faculty of Engineering, National University of Mar del Plata) is based on the use of regional agroindustrial waste with low added value for obtaining protein hydrolyzates with high added value. This project proposes to isolate and characterize microorganisms with proteolytic activity isolated from divers agroindustrial waste or vegetables available in the region, emphasizing those microorganisms that have withstood severe conditions of stress during the various industrial operations applied (acidification, osmotic stress, mechanical pressing, extraction with organic solvents, use of high temperatures, etc.), using tools from the traditional microbial biotechnology, and to optimize its use in products with higher added value from agroindustrial waste. From the industrial point of view, it is important to select microorganisms capable of adequately performing different processes and is resistant to adverse conditions occurring during these processes (Lale et al. 2007). For example, some subspecies of *Lactococcus lactis* are exposed to hostile conditions imposed by industrial processes in the production of dairy products and nisin (Kim et al. 1999) and some *Bacillus* species that are resistant to high pH and/or high temperatures, conditions that make them useful for use in the detergent industry, leather, and other (Najafi et al. 2005).

It should be noted that Argentina is a purely agricultural and livestock country with abundant generation of agroindustrial waste, most of them with a high protein content, that are underutilized in the form of low value-added products. To this end, various products are used for the extraction of proteins present in them. The protein in the form of protein isolates, in itself a product of high added value, is used for obtaining protein hydrolyzates that can be used in specialized food.

Based on the sources of enzymes of bacterial origin isolated and studied in GIIA, it has progressed in the implementation of substrates and found that barley spent grain can be a raw material of interest for this application, among others. Nowadays, the barley spent grain, like many other agroproducts, is

intended primarily used as an animal feed. Traditionally, this product has received little attention in terms of marketing. However, it is a potential source of protein and fiber for use in processed foods.

REFERENCES

Abha Mishra, Sunil Kumar, Sudhir Kumar. 2008. Application of Box–Behnken experimental design for optimization of laccase production by Coriolus versicolor MTCC138 in solid state fermentation. *J Sc Industrial Research* 67:1098–107.

Adinarayana K, Ellaiah P. 2002. Response surface optimization of the critical medium components for the production of alkaline protease by a newly isolated *Bacillus* sp. *J Pharm Pharmaceut Sci* 5(3):272–8.

Agüero MV, Kotlar CE, Roura SI. 2010. Simultaneous optimization of biomass and protease biosynthesis by a local isolated *Pseudomonas* sp. Part 1. Selection of main medium components and operating condition. *Ind Biotechnology* IND-2010-0012. In press.

Ahmad MM, Nomani MS, Panda BP. 2009. Screening of nutrient parameters for red pigment production by *Monascus purpureus* MTCC 369 under submerged fermentation using Plackett-Burman design. *Chiang Mai J Sci* 36(1):104–9.

Babu GS, Kiran RS, Lokeswari N, Raju JK. 2007. Optimization of protease production from Aspergillus oryzae sp. using Box–Behnken experimental design. *E-J Chem* 4(2):145–53.

Banik RM, Prakash M. 2004. Laundery detergent compatibility of the alkaline protease from *Bacillus cereus*. *Microbiol Res* 159(2):135–40.

Beg QAK, Sahai V, Gupta R. 2003. Statistical media optimization and alkaline production from *Bacillis mojavensis* in a bioreactor. *Process Biochem* 39(2):203–9.

Bhaskar N, Sudeepa ES, Rashmi HN, Tamil Selvi A. 2007. Partial purification and characterization of protease of *Bacillus proteolyticus* CFR3001 isolated from fish processing waste and its antibacterial activities. *Bioresource Technol* 98:2758–64.

Brandelli A, Riffel A. 2005. Production of an extracellular keratinase from *Chryseo bacterium* sp. growing on raw leathers. *Electron J Biotechnol* 8:1.

Casaburi A, Di Monaco R, Cavella S, Toldrá F, Ercolini D, Villani F. 2008. Proteolytic and lipolytic starter cultures and their effect on traditional fermented sausages ripening and sensory traits. *Food Microb* 25(2):335–47.

Chantawannakul P, Oncharoen A, Klanbut K, Chukeatirote E, Lumyong S. 2002. Characterization of proteases of *Bacillus subtilis* strain 38 isolated from traditionally fermented soybean in Northern Thailand. *Sci Asia* 28:241–5.

Chauhan KU, Trivedi, Patel KC. 2007. Statistical screening of medium components by Plackett–Burman design for lactic acid production by *Lactobacillus* sp. KCP01 using date juice. *Bioresource Technol* 98:98–103.

Davidson PM, Harrison MA. 2002. Resistance and adaptation to food antimicrobials, sanitizers and other process controls. *Food Technol* 56(11):69–78.

Dawes H, Struebi P, Keene J. 1994. Kiwifruit Juice clarification using a fungal proteolytic enzyme. *J Food Sci* 59(4):858–61.

Dhandhukia PC, Thakkar VR. 2007. Significant medium components for enhaced jasmonic acid production by Lasidiplodia theobromae using Plackett-Burman. *Curr Trends Biotechnol Pharm* 1(1):79–86.

Drenkard E, Ausubel FM. 2002. Pseudomonas biofilm formation and antibiotic resistance are linked to phenotypic variation. U.S. National Library of Medicine. National Institutes of Health. *Nat Biotechnol* 416(6882):740–3.

Durick K, Van Coillie E, Swiecicka I, Meyfroidt G, Devlieger H, Meulemans A. 2005. Fatal family outbreak of Bacillus cereus–associated food poisoning. *J Clin Microbiol* 4277–9.

El Enshay H, Abuoul-Enein A, Helmy S, Azaly Y. 2008. Optimization of the industrial production of alkaline protease by *Bacillus licheniformis* in different production scales. *Aust J Basic Appl Sci* 2(3):583–93.

El-Naggar MY, El-Assar S, Youssef AS, El-Sersy NA, Beltagy EA. 2006. Extracellular β-mannanase production by the immobilization of the locally isolated *Aspergillus niger*. *Int J Agric Biol* 8(1):57–62.

Ferrari E, Jarnagin AS, Schmidt BF. 1993. *Commercial Production of Extracellular Enzymes*. Washington, DC: American Society for Microbiology. p 917–37.

Foulquié-Moreno MR, Sarantinopoulos P, Tsakalidou E, de Vuyst L. 2006. The role and application of enterococci in food and health. *Int J Food Microbiol* 106:1–24.

Gerez CL, Rollán GC, de Valdez GF. 2006. Gluten breakdown by lactobacilli and pediococci strains isolated from sourdough. *Lett Appl Microbiol* 42(5):459–64.

Ghorbel-Frikha B, Sellami-Kamoun A, Fakhfakh A, Haddar A, Manni L, Narsi M. 2005. Production and purification of calcium-dependent protease from *Bacillus cereus* BG1. *J Ind Microbiol Biotechnol* 32:186–94.

Ghosh A, Chakrabarti K, Chattopadhyay D. 2008. Degradation of raw feather by a novel high molecular weight extracellular protease from newly isolated *Bacillus cereus* DCUW. *J Ind Microbiol Biotechnol* 35:825–34.

Godfrey T, West S. 1996. *Industrial Enzymology*. 2nd ed. New York: Macmillan Publishers, Inc. 3 p.

Gohel V, Chaudhary T, Vyas P and Chhatpar HS. 2006. Statistical screening of medium components for the production of chitinase by the marine isolate *Pantoea dispersa*. *Biochem Eng J* 28:50–6.

Gómez R, Muñoz M, de Ancos B, Cano MP. 2002. New procedure for the detection of lactic acid bacteria in vegetables producing antibacterial substances. *LWT* 35(3):284–8.

Gopal J, Sarvesh K, Vinay S. 2007. Production of moderately halotolerant, SDS stable alkaline protease from *Bacillus cereus* MTCC 6840 isolated from Lake Nainital, Uttaranchal State, India. *Braz J Microbiol* 38:773–9.

Guangrong H, Dai D, Hu W, Juiang J. 2002. Optimization of medium composition for thermostable protease production by *Bacillus* sp. HS08 with a statistical method. *Afr J Biotechnol* 7(8):1115–22.

Gulrajani ML, Agarwal R, Grover A, Suri M. 2000. Degumming of silk with lipase and protease. *Indian J Fibre Textile Res* 25:69–74.

Gupta R, Beg QK, Lorenz P. 2002. Bacterial alkaline proteases: molecular approaches and industrial applications. *Appl Microbiol Biotechnol* 59(1):15–32.

Häse CC. 2003. Ion motive force dependence of protease secretion and phage transduction in *Vibrio cholerae* and *Pseudomonas aeruginosa*. *FEMS Microbiol Lett* 227(1):65–71.

Hayashi K, Revell DF, Law BA. 1990. Accelerated ripening of cheddar cheese made with the aminopeptidase of Brevibacterium linens and a commercial neutral proteinase. *J Dairy Sci* 57:571–7.

Hope CFA, Burns RG. 1985. The barrier-ring plate technique for studying extracellular enzyme. *J Gen Microbiol* 131:1237–43.

Horikoski K. 1999. Alkaliphiles: some application of their products for biotechnology. *Microbiol Mol Biol Rev* 63(4):735–50.

Hussein H, Moawad H, Farag S. 2004. Isolation and characterization of Pseudomonas resistant to heavy metals contaminants. *Arab J Biotechnol* 7(1):13–22.

In MJ, Chae HJ, Oh NS. 2002. Process development for heme-enriched peptide by enzymatic hydrolysis of haemoglobin. *Bioresource Technol* 84(1):63–8.

Karbalaei-Heidari HR, Ziaee A, Schaller J, Amozegar MA. 2007. Purification and characterization of an extracellular haloalkaline protease produced by the moderately halophilic bacterium *Salinivibrio* sp. strain AF-2004. *Enzyme Microbial Technol* 40:266–72.

Kashyap MC, Agrawal YC, Ghosh PK, Jayas DS, Sarkar BC, Singh BPN. 2007. Oil extraction rates of enzymatically hydrolysed soybeans. *J Food Eng* 81:611–7.

Kim W, Ren J, Dunn N. 1999. Differentiation of Lactococcus lactis subspecies lactis and subspecies cremoris strains by their adaptive response to stresses. *FEMS Microbiol Lett* 171(1):57–65.

Lale R, Tukel C, Akcelik M. 2007. Protein profile and plasmid content of Lactococcus lactis subsp lactis LL5Z and *Lactococcus lactis subsp cremoris* LC79 strains under several stress conditions. *T J Vet An Sci* 31(4):247–52.

Lauer I, Bonnewitz B, Meunier A, Beverini M. 2000. New approach for separating *Bacillus subtilis* metalloprotease and alpha-amylase by affinity chromatography and for purifying neutral protease by hydrophobic chromatography. *J Chromatogr B Biomed Appl* 737:277.

Lu ES, Guo-Qing Y, Xiao-Ying Z, Zhen-Xing T, Jian-Shu C, Wen-Yue X. 2007. Medium optimization for 5′-phosphodiesterase production from *Penicillium citrinum* using response surface methodology. *Food Technol Biotechnol* 45(2):126–33.

Mauriello G, Casaburi A, Villani F. 2002. Proteolytic activity of *Staphylococcus xylosus* strains on pork myofibrillar and sarcoplasmic proteins and use of selected strains in the production of 'Naples type' salami. *J Appl Microbiol* 92(3):482–90.

Mokhtari-Hosseini ZB, Vasheghani-Farahani E, Heidarzadeh-Vazifekhoran A, Abbas Shojaosadati S, Karimzadeh R, Khosravi Darani K. 2009. Statistical media optimization for growth and PHB production from methanol by a methylotrophic bacterium. *Bioresource Technol* 100(8):2436–43.

Mukherjee S, Das P, Sivapathasekaran C, Sen R. 2008. Enhanced production of biosurfactant by a marine bacterium on statistical screening of nutritional parameters. *Biochem Eng J* 42(3):254–60.

Najafi MF, Deobagkar D, Deobagkar CC. 2005. Purification and characterization of an extracellular amylase from *Bacillus subtilis* AX20. *Protein Exp Purif* 41:349–54.

Najafi MF, Deobagkar D, Deobagkar D. 2005. Potential application of protease isolated from *Pseudomonas aeruginosa* PD100. *Electron J Biotechnol* 8:2.

Natalucci CL, Priólo NS, Arribare MC, López LMI, Brullo A, Cqffini NO. 2000. Fitoproteasas: Un recurso natural renovable no explotado en Argentina. Anales de Sociedad Argentina para la Investigación de Productos Aromáticos IX Congreso Nacional de Recursos Naturales Aromáticos y medicinales 16:35–43.

Naveena BJ, Altaf M, Bhadrayya K, Reddy G. 2005a. Direct fermentation of starch to L(+) lactic acid in SSF by *Lactobacillus amylophilus* GV6 using wheat bran as support and substrate: medium optimization using RSM. *Process Biochem* 40(2):681–90.

Naveena BJ, Altaf M, Bhadrayya K, Reddy G. 2005b. Selection of medium components by Plackett-Burman design for production of L(+) lactic acid in SSF by *Lactobacillus amylophilus* GV6 in SSF using wheat bran. *Bioresource Technol* 96:485–90.

Nicodème M, Grill JP, Humbert G, Gaillard JL. 2005. Extracellular protease activity of different *Pseudomonas* strains: dependence of proteolytic activity on culture conditions. *J Appl Microbiol* 9(3):641–8.

Nip WK, Lan CY, Moy JH. 2006. Partial characterization of a collagenolytic enzyme fraction from the hepatopancreas of the freshwater prawn, Macrobrachium rosenbergii. *J Food Sci* 50(4):1187–8.

Oneca M, Ortigosa M, Irigoyen A, Torre P. 2007. Proteolytic activity of some *Lactobacillus paracasei* strains in a model ovine-milk curd system: determination of free amino acids by RP-HPLC. *Food Chem* 100(4):1602–10.

Pérez Borla O, Davidovich LA, Roura SI. 2010. Isolation and characterization of proteolytic microorganisms from fresh and fermented cabbage. *LWT - Food Sci Technol* 43(2):298–301.

Ponce A, Roura S, Del Valle C, Moreira M. 2008. Antimicrobial and antioxidant activities of edible coatings enriched with natural plant extracts: *in vitro* and *in vivo* Studies. *Postharv Biol Technol* 49:294–300.

Prakash M, Banik RM, Koch-Brandt C. 2005. Purification and characterization of *Bacillus cereus* protease suitable for detergent industry. *Appl Biochem Biotechnol* 127:143–56.

Puri S, Beg QK, Gupta R. 2002. Optimization of alkaline protease from *Bacillus* sp. by response surface methology. *Curr Microbiol* 44(4):286–90.

Sen RK, Babu S. 2005. Modeling and optimization of the process conditions for biomass and sporulation of a probiotic culture. *Process Biochem* 40:2531–8.

Rizzello CG, De Angelis M, Di Cagno R, Camarca A, Silano M, LositoI, De Vincenzi M, De Bari MD, Palmisano F, Maurano F, Gianfrani C, Gobbetti M. 2007. Highly efficient gluten degradation by lactobacilli and fungal proteases during food processing: new perspectives for celiac disease. *Appl Environ Microbiol* 73:4499–507.

Rollan G, de Valdez F. 2001. The peptide hydrolase system of *Lactobacillus reuteri*. *Int J Food Microbiol* 70:303–7.

Romsomsa N, Chim-anagae P, Jangchud A. 2010. Optimization of silk degumming protease production from *Bacillus subtilis* C4 using Plackett-Burman design and response surface methodology. *Science Asia* 36:118–24.

Salem SR, Shabeb MSA, Amara AA. 2009. Optimization of thermophilic protease production in *Bacillus* mixed cultrues under mesophilic conditions. *World J Agric Sci* 5(3):375–83.

Secades P, Guijarro JA. 1999. Purification and characterization of an extracellular protease from the fish pathogen *Yersinia ruckeri* and effect of culture conditions on production. *Appl Environ Microbiol* 65(9):3969–75.

Setlow P. 2006. Spores of *Bacillus subtilis*: their resistance to and killing by radiation, heat and chemicals. *J Appl Microbiol* 101:514–25.

Shaheen M, Shah A, Hameed A, Hasan F. 2008. Influence of culture conditions on production and activity of protease from *Bacillus subtilis* BS1. *Pak J Bot* 40(5):2161–9.

Singh KV, Coque TM, Weinstock GM, Murray BE. 1998. *In vivo* testing of an Enterococcus faecalis efaA mutant and use of efaA homologs for species identification. *FEMS Immunol Med Microbiol* 21:323–31.

Su YA, Sulavik MC, He P, Makinen KK, Makinen PL, Fiedler S. 1991. Nucleotide sequence of the gelatinase gene (gelE) from *Enterococcus faecalis subsp. Liquefaciens*. *Infect Immunity* 59(1):415–20.

Sudeepa ES, Tamil Selvi A, Bhaskar N. 2007. Antimicrobial activity of a lipolytic and hydrocarbon degrading lactic acid bacterium isolated from fish processing waste. *J Food Sci Technol* 44:417–21.

Thys RCS, Guzzon SO, Caldero-Olivera F, Brandelli A. 2006. Optimization of protease production by Micobacterium sp. In feather meal using response surface methodology. *Process Biotechnol* 41:67–73.

Vaidya R, Vyas P, Chhatpar HS. 2003. Statistical optimization of medium components for the production of chitinase by *Alcaligenes xylosoxydans*. *Enzyme Microb Technol* 33(1):92–6.

Volken de Souza CF, Costa Rodrigues R, Heck JX, Záchia Ayub MA. 2008. Optimization of transglutaminase extraction produced by *Bacillus circulans* BL32 on solid-state cultivation. *J Chem Technol Biotechnol* 83(9):1306–13.

Waldir E, Rychtera M, Melzoch K, Quillama E, Egovil E. 2007. Producción de ácido láctico por *Lactobacillus plantarum* L10 y cultivos batch y continuo. *Rev Perú Biol* 14(2):271–5.

Wang SL, Chio SH, Chang WT. 1997. Production of chitinase from shellfish wastes by *Pseudomonas aeruginosa* K-187. *Proc Nat Sc Council, Rep China. Part B* 21:71–9.

Wang SL, Yang CH, Liang TW, Yen YH. 2008. Optimization of conditions of protease production by *Chryseobacterium taeanense* TKU001. *Biores Technol* 99:3700–7.

Yu L, Lei T, Ren X, Pei X, Feng Y. 2008. Response surface optimization of L-(+)-lactic acid production using corn steep liquor as an alternative nitrogen source by Lactobacillus rhamnosus CGMCC 1466. *Biochem Eng J* 39(3):496–502.

Zhang J, Gao N. 2007. Application of response surface methodology in medium optimization for pyrivic acid production of Torulopsis glabrata TP19 in batch fermentation. *J Zhejiang Univ Sci* 8(2):98–114.

46

Soymilk Fermentation and Enzymes Production

Marisa S. Garro and Graciela Savoy de Giori

CONTENTS

46.1 Introduction

Soymilk is a substrate used for the production of a number of fermented products (fermented beverages, culture drinks, yogurt-like products, nondairy frozen desserts, cheese substitutes, tofu, sauces, and other typical Asian products). Different edible microorganisms (bacteria, yeast, and fungi) whose enzymes, particularly glycosidases, amylases, proteases, and lipases, hydrolyze the sugars, polysaccharides, proteins, and lipids to nontoxic products with flavors, aromas, and textures pleasant and attractive to the human consumer are used in the preparation of these fermented foods.

In this chapter, we described the action of different lactic acid bacteria and related microorganism (as bifidobacteria) on soymilk.

The action of these microorganisms and its enzymes play different roles in food processing:

- Reduction of flatulence effects
- Improvement of protein digestibility and release of biopeptides with different healthy effects
- Isoflavone bioconversion
- Flavor improvement
- Lactic acid production as preservative

46.2 Soymilk and Fermented Soymilks

Soymilk is produced from the aqueous extraction of soybean (*Glycine max*). The first evidence of soymilk is in China, extending to the Asian continent, and appearing in the 17th to 18th centuries in Europe. It was introduced in the United States in the 19th century. During the 1930s, it mainly had its greater expansion in America, and several factories that industrialized soymilk appeared. We can find several processes (both patented and unpatented) for soymilk production (Brown et al. 1982; Gupta and Gupta 1988; Nelson et al. 1976; Steinkraus 1973; Wilkens et al. 1967). In general, the basic methods of preparation are soaking the soybeans, grinding them with water, cooking, and filtering. The liquid that results after filtration is called soymilk. The nutritional composition, appearance, and flavor of good quality

soymilk are remarkably similar to that of cow's milk. All traditional soymilks are filtered, whereby the okara (insoluble soybean pulp) was removed. Some modern soymilks are suspended and contain the entire original soybean except its hull, whereas others are made from soy protein isolates (Tsangalis et al. 2002; Toda et al. 2007). Traditionally, soymilk has a "beany" taste, which is well accepted by the Chinese but less so by the Western palate. The "beany" off-flavor is predominantly because of the presence of aldehydes, ketones, and alcohols compounds (Rackis et al. 1979; Takasahashi et al. 1979). The formation of these compounds mainly results from the hydroperoxidation of polyunsaturated fatty acids, catalyzed by lipoxygenase (Wilkens et al. 1967; Wilkens and Lin 1970). Oriental methods of soymilk manufacture establish conditions for this oxidation to occur during the initial soaking and grinding of soybeans (heat treatments). Today, commercial methods implement steps that either prevent the formation of objectionable volatiles (inactivation of lipoxygenase by heating) or remove the residual off-flavors using deodorization techniques (Liu 1997; Mizutani and Hashimoto 2004); other techniques include acid treatments, enzymatic treatments, supercritical carbon dioxide extraction, genetic alteration of soybeans, and addition of flavor compounds (Che Man et al. 1989; Maheshwari et al. 1995; Srinivas et al. 1992). Using correct processing techniques, this "beany" taste can be reduced or eliminated. Recently, with the recognition of its health benefits and with its improved flavor and texture, soymilk has now a high and increasing acceptance. However, soybeans have antinutritional factors, some of which are heat-labile. Trypsin inhibitors, phytates, hemagglutinins, and other minor factors can be eliminated by proper processing of the soybean before being used. To eliminate or reduce flatulence factors, lysinoalanines, estrogens, or saponins, different strategies are necessary. Some of them will be discussed in this chapter.

The proximal composition of soymilk is protein (3.0%–3.6%), sugars (2.9%–3.5%), fat (2.0%–2.5%), ash (0.5%), pH 6.8–7.0. In summary, soymilk has about total solids 8.4%–10%, and water 88%–92%, depending on the water/bean ratio in its processing. The main soluble sugars present are saccharose (2.1%–2.3%), raffinose (0.1%–0.3%), and stachyose (0.7%–0.9%); in addition, soymilk has insoluble carbohydrates such as cellulose, hemicelluloses, pectin, and trace amounts of starch. It also contains other minor components, including minerals, vitamins, phytate, and phenolic compounds (isoflavones). Soymilk has a high nutritional value, is an excellent source of good quality protein and B vitamins, and is inexpensive. However, it is not a rich source of calcium, and most commercial soymilk products are fortified with calcium. More information about physicochemical composition and different techniques of soymilk preparation have been well documented (Liu 1997).

The development of "beany" flavor during the elaboration of soymilk has limited its use. As described earlier, diverse efforts were given to eliminate disagreeable flavors due to the degradation of lipid oxidation, which inactivates the lipoxygenase system in the initial processing. Another alternative method to eliminate disagreeable flavors is fermentation, which is successfully done with fungal inoculations, using *Rhizopus oligosporus* (Martinelli et al. 1964; Steinkraus 1974), *Neurospora sitophila* (Steinkraus 1974), and *Aspergillus oryzae* (Umeda et al. 1969), and bacterial inoculations, such as *Bacillus natto* (Hesseltine et al. 1972). The action of these organisms on the soybean components was studied in detail by numerous investigators since they are involved in fermented traditional Asian foods. In 1970, Gray published a complete review on the use of fungi fermentations in foods processing, and at present different researchers continuously studying the properties of these organisms in soybean products (Hu et al. 2009). In regards to the use of lactic acid bacteria, Kellogg (1934) was first in preparing a fermented food base soybean similar to butter using *Lactobacillus acidophilus*. Later, Geherke and Weiser (1947, 1948) found that the substrate soybean was adapted for the growth of bacteria. These authors observed that lactic cultures such as *Leuconostoc cremoris*, *Lc. dextranicum*, and *Streptococcus lactis* approximately produced half of acid with respect to cow's milk. Despite this, there were no significant differences in the acidity of both substrates. Ariyama (1963) developed a technological process for obtaining a product similar to yogurt using *L. bulgaricus* and yogurt cultures that used soymilk supplemented with 15% of saccharose. The acid production in the product obtained by Ariyama is the result of the activity of the microorganisms of yogurt. *S. fecalis* was used to prepare a cheese-like product (Kenkyusho 1965). In this case, the soymilk was supplemented with casein, glucose, fat from cow's milk, and calcium chloride. The resulting curd was processed using conventional cheese preparation method. The use of *S. thermophilus* in the preparation of other cheese-like product made from soymilks was studied by Hang and Jackson (1967a,b) with satisfactory results. The obtained product was superior in body

and texture, than those elaborated by addition of acetic acid or precipitation with salt. The skimmed milk incorporation, rennin, and lactic cultures improve the flavor of the product. The growth of the microorganisms in soymilk improved with the addition of skimmed milk, which contributes lactose. Yamanaka and Furukawa (1970) used a mixed culture of *S. thermophilus* and *L. bulgaricus* to prepare a fermented drink based on proteins of soybean, cow milk, and saccharose. They also added a mixture of amino acids (alanine, arginine, aspartic acid, sodium glutamate, lysine, methionine, and glycine) to mask the characteristic flavor of the soybean protein. The same authors informed that using only proline or proline and alanine also yielded satisfactory results. These same authors realized a study on the capacity of the lactic bacteria to form acid on soymilk and combinations soya–skimmed milk. They determined that the amount of acid produced by *S. thermophilus*, *L. bulgaricus*, *S. fecalis*, *L. acidophilus*, and *L. casei* is greater in the mixture of soymilk and skimmed milk (70%:30%) than in single skimmed milk. They also observed that the addition of glucose to the mixture stimulated this activity, whereas the addition of saccharose tapeworm solely affects the speed of acidification of *L. acidophilus*. Also, it was concluded that the texture of the curd becomes firmer when the proportion of soymilk increased. Lypolytic and proteolytic activities of lactic cultures on soymilk were studied by Angeles and Marth (1971c,d). According to these authors, the majority of the tried microorganisms do not produce hydrolysis of soybean lipids. Nevertheless, *L. casei*, *L. delbrueckii*, and *S. thermophilus* released some of present fatty acids in of soymilk, as well as in culture media enriched with 2% of soybean lipids. *L. delbrueckii* and *S. thermophilus* presented weak proteolytic activity in soymilk compared with *Bacillus cereus* and *Micrococcus conglomeratus*.

Acid production by lactic bacteria is affected by the processing of the soybean grain and the different methods of soymilk preparation. During the soaking of the seeds, water drains and eliminated the carbohydrates. With this practice, its concentration is diminished and therefore acid production falls. Lo et al. (1968) observed that prolonging the soaking time increase the soluble water solids and its loss (approximately one third of the total). Bandenhop and Hackler (1974) observed that when the beans are soaked in 0.05 N sodium hydroxide, an alkaline (pH 7.37) extract is obtained, which does not favor the development of the lactic bacteria. Mital et al. (1974), using another method (crushing of the grain of soybean and water in boiling), showed that all the tried cultures exhibit major lactic acid growth and production. These authors conclude that the differences in behavior of the microorganisms in both types of milk would have to the lost of one of the growth factors during the extraction of fats. Angeles and Marth (1971a,b) studied the effect of heating the soymilk on lactic acid production and verified that heat treatment with a temperature of 60°C for 15 minutes has a favorable effect, whereas a temperature of 80°C for 5–60 minutes produces an adverse effect. It is attributed to the increase of inhibitory substances, such as sulfydryl groups and toxic sulfide, and to the decrease of oxide reduction potential by effect of the acid production. The majority of the studies related to soymilk fermentation concentrated on supplementing the substrate soybean with glucose, lactose, or whey milk powder, with the intention of facilitating the growth of the microorganisms (Fuchs et al. 2005; Tsangalis et al. 2003; Wei et al. 2007). Garro et al. (1994a) evaluated the capacity of lactic acid bacteria to grow in soymilk without the addition of sugars. Strains of *L. acidophilus, L. casei*, and *S. salivarius* subsp. *thermophilus* only use the sucrose present in soymilk with a good lactic acid production; however, it was dependent on the strain used. *L. plantarum* and *L. fermentum* have the ability to use sucrose, melibiose, raffinose, and stachyose. These lactic cultures demonstrate a good capacity for growth and lactic acid formation in soymilk. Selected strains of *S. salivarius* subsp. *thermophilus*, *L. casei*, *L. fermentum*, and *Bifidobacterium longum* were evaluated as single culture in soymilk substrate. These cultures demonstrate more that 70% of the carbohydrates were used until the sixth hour of fermentation, with good amounts of lactic acid formed (Garro et al. 1998, 1999a). Microbial survival and metabolic activity are important properties of starter cultures used in fermented foods. These properties were evaluated in fermented soymilk product using different lactic acid cultures and bifidobacterium (Chou and How 2000; Garro et al. 1999b; Wang et al. 2002). Using conductimetry to measure metabolic changes in lactic acid bacteria present in fermented soymilk products is advantageous with respect to time and cost compared with traditional methods (Garro et al. 2002). Mixed cultures of *B. longum* and *L. fermentum* were studied in soymilk in comparison with pure cultures (Garro et al. 2004). Several fermentation parameters such as changes in bacterial population and acidification properties of soymilk fermented with these cultures at different temperatures were analyzed

to improve the performance of these microorganisms in probiotic products. The use of carbohydrates as well as enzymatic activity was determined. A coculture with *L. fermentum* could offer nutrients or conditions that somehow maintain the viability of *B. longum* in the mixed culture. Soymilk fermented using selected strains of lactic acid bacteria (able to grow in soy) were evaluated in vivo using a murine experimental model. The effect of heat treatment on the fermented SM product was also determined (LeBlanc et al. 2004a).

Strains of lactic acid bacteria or bifidobacteria that are able to use sucrose, raffinose, and stachyose can be grown in soymilk substrate. Therefore, an alternative to the use of fermented soybean products, which has low raffinose and stachyose content and has other healthy properties, could be realized with the use of these microorganisms. In our country, although there is an increase in the use and production of soybean in the last 10 years, only a small percentage (~5%) is used for food. Until 1985, the majority of the production was exported as whole soybeans, but, in the recent years, industrialization increased it considerably, with a 66% increased use in 1991–1993 (texturized products, soybean meal, soybean oil, subproducts, and soybean beverages). These products began to spread in the last decade and the market practically is unknown. Argentina exports whole soybeans to developing countries and soybean meal and oil to developed countries. It is of great interest, therefore, to develop new fermented food from soybean using lactic acid bacteria and/or bifidobacteria, which would take advantage of the important nutritional and healthy properties of the microorganisms and substrate.

46.3 α-Galactosidase and Soymilk

The main sugars present in soybean are stachyose, raffinose, and saccharose. The first two sugars (soluble galacto-oligosaccharides, or GOS) are not digested by the intestinal tract in humans and can cause flatulence and digestive problems (Hartwig et al. 1997; Rackis et al. 1970; Suarez et al. 1999). Therefore, it is necessary to eliminate or reduce these sugars from soymilk using suitable treatments. Enzymatic processes or fermentation with selected microorganisms could be a good alternative.

Raffinose and stachyose are α-galactosides of sucrose comprising three and four sugar moieties, respectively, and are nondigestible due to the absence of α-galactosidase in the small intestinal mucosa. Consequently, intact oligosaccharides pass directly into the lower intestine where they are metabolized by bacteria that possess the necessary enzyme, resulting in the production of gases (Cristofaro et al. 1974). Nevertheless, oligosaccharides have been shown to possess prebiotic properties, as they increase the populations of indigenous bifidobacteria in the colon (Hayakawa et al. 1990) and suppress the growth and activity of putrefactive bacteria (Masai et al. 1987).

α-Galactosidase (α-D-galactoside galactohydrolase EC 3.2.1.22) is a homodimeric glycoprotein (glycoside hydrolase enzyme). It hydrolyzes the α-1,6-linked α-galactoside residues from simple oligosaccharides such as melibiose, raffinose, and stachyose and from polymeric galactomannans (Dey and Pridham 1972). α-Galactosidase is widely distributed in nature, mainly in microorganisms, plants, and animals (Bahl and Agrawal 1969; Church et al. 1980; Dey et al. 1993; Garro et al. 1993, 1994b; Suzuki et al. 1970). This enzyme is of particular interest because of its biotechnological applications. Microbial or plant α-galactosidases can be added to soybean products (meal, soymilk, or molasses) to hydrolyze GOS (raffinose/stachyose) to digestible carbohydrates and thereby moderate the flatulence-causing property of soybean products. The enzyme isolated from fungi and yeast has been studied and documented for many years (Cruz et al. 1981; Viana et al. 2006; Thananunkul et al. 1976); however, in other microorganisms such as lactic acid bacteria, the studies are recent and scarce (Garro et al. 1993, 1996a,b; Tsangalis and Shah 2004; Yoon and Hwang 2008). The presence of this enzyme has been reported in the *Bifidobacterium* genus as constitutive, whereas in lactic acid bacteria, it is produced only by some strains of *Lactobacillus*, *Leuconostoc*, and *Pediococcus* (Desjardins et al. 1990; Garro et al. 1993, 1994a,b; Garro 1995; Gonzalez and Kunka 1986; Mital and Steinkraus 1975). Purification and characterization of α-galactosidases from several sources have been reported, and their properties differ markedly depending on their source (Garro et al. 1996a; Puchart et al. 2000; Suzuki et al. 1970; Zapater et al. 1990). Most fungal α-galactosidases reach their maximum activity at the extremely acid pH range (2.7–4.0) and at temperatures above 50°C (Bahl and Agrawal 1969, 1972; Li and Shetlar 1964; Suzuki et al. 1970). The

enzyme isolated from *L. fermentum* was characterized and purified (Garro et al. 1993, 1996a). This enzyme is localized in the cytoplasm and exhibited its maximum activity at pH 5.8 and at temperatures between 35°C and 45°C. The molecular weight of the enzyme was 195,000 Da. This value was lower than the one reported for α-galactosidase from *Aspergillus saitoi* (290,000 Da; Sugimoto and Van Buren 1970) but higher than those obtained for *A. awamori* (130,000 Da; McGhee et al. 1978) and *Pichia guilliermondii* (143,000 Da; Church et al. 1980). Studies of the effect of environmental conditions on the production of α-galactosidase showed that the level of the enzyme was affected by growth conditions and carbon source. It was stimulated by galactose and showed catabolic repression by glucose (Garro et al. 1996b). Molecular aspects of α-galactosidase genes from lactic acid bacteria have been well documented (Kim et al. 2005; Savoy de Giori et al. 2010; Schuler et al. 1985).

The production of this enzyme from lactic acid bacteria and bifidobacteria was evaluated in soymilk substrate and raffinose medium (Garro et al. 1999a, 2004, 2006; LeBlanc et al. 2004b). Enzyme production in soymilk was lower than in culture media. The production of α-galactosidase in *L. fermentum* and *B. longum* growth in culture media was evaluated under different pH conditions. The maximum enzyme production was observed at the start of the stationary growth phase, the increase of α-galactosidase activity followed the same time course of stachyose/raffinose (α-GOS) degradation. Different studies were done to elucidate *in vitro* and *in vivo* action of α-galactosidase enzyme from *L. fermentum* in the prevention of digestive disorders associated with soymilk or stachyose/raffinose consumption (Leblanc et al. 2004c, 2005, 2008). These studies were the first to demonstrate the in situ effect of this enzyme, paving the way for other investigations that elucidate the composition of the product that produces flatulence.

Strains of lactic acid bacteria or bifidobacteria, such as *L. fermentum* or *B. longum*, have the ability to hydrolyze the stachyose or raffinose present in soymilk, reducing the level of this oligosaccharide by 60%–85%. Using these strains can produce fermented soy products with a low level of nondigestible GOS. These processes are used to minimize or remove such problems and enhance sensorial acceptability as well as nutritional content of fermented soy foods, which have greater consumer acceptability.

46.4 Proteases, Peptidases, and Soymilk

Soymilk is one of the popular and traditional soy products and is consumed in China and other Asian countries (Shun-Tang et al. 1997) as a nutritious and economical protein food (Matsuura et al. 1989). In Western countries, the consumption of soymilk was generated as an important replacement of cow milk due to lactose intolerance or allergic reaction to cow's milk and as a low-cost source of good quality protein and energy (Kanthamani et al. 1978; Kwok and Niranjan 1995; Liu 1997; Rosenthal et al. 2003). In the recent years, with the promotion of its healthful components, its use has increased, not only as a good source of protein and most consumers now associate soy with a healthy lifestyle due to its potential health benefits.

In a whole soybean, about 40% of total dry matter is protein. On the basis of solubility, the main soy proteins are globulins (are soluble in salt solutions). In soybean, these globulins represent around 65%–80% of the total storage seed proteins and are commonly known as glycinin and conglycinin. Soy protein exhibits four fractions after ultracentrifugation, designated as 2S, 7S, 11S, and 15S (Liu 1997). The β-conglycinin is the most abundant protein of the fraction 7S, with its biological function being the protein reserve of the seed. It approximately represents 30% of the total protein. It is a glycoprotein with a molecular weight ranging from 140 to 200 kDa and is constituted by three subunits α, α′, and β. The glycinin, whose function is also being one of the reserve, is the majority protein of the fraction 11S and constitutes 40% of the total protein of the seed. It is a hexameric protein with a molecular weight ranging from 320 to 360 kDa. Subunits of glycinin are composed of acidic (A1a, A1b, A2, A3, A4, A5) and basic (B1a, B1b, B2, B3, B4) polypeptides linked by a disulfide bond and are not glycosylated (Hou and Chang 2004a,b; Maruyama et al. 2003; Nielsen 1985).

Soy protein is a nourishing protein with high biological quality that contributes to essential amino acids such as phenylalanine, isoleucine, leucine, lysine, threonine, tryptophan, valine, and, in low concentration, the sulphur-containing amino acids (methionine/cystein). Soy protein is rich in lysine, and

this amino acid is limited in cereals, which are generally rich in sulfur-containing amino acids. Hence, the soy protein is an ideal protein source to complement cereals. The nutritive value of food proteins depend on amino acid bioavailability or protein digestibility. The improvements in digestibility produce small chains of peptides and release bioactive peptides.

In the recent years, the interest in functional foods increased; in this context, soy protein has been recognized for its good functional properties (Kinsella 1979). Different strategies to modify the structure and physicochemical characteristic of the protein are necessary. These can be physical, chemical, or enzymatic methods (Feeney and Whitaker 1977; Hamada and Marshall 1989; Puski 1975; Wang and Gonzalez de Mejia 2005). Protein hydrolysates are physiologically better than the intact proteins because their intestinal absorption appears to be more effective (Ziegler et al. 1998). In general, heat treatment improves the digestibility of soy protein due to reduction of trypsin inhibitors and other antinutritional factors. In addition, disulfide bonds play a major role in maintaining the native protein structure and stability. Chemical modifications in general are expensive and may be not suitable for food uses due to lack of specificity and toxicity (Liu 1997). The enzymatic process constitutes the third valid alternative. These process involves commercial enzymes or enzymes from microorganism such as *Mucor* sp., *Apergillus oryzae*, *Rhizopus* sp., *Bacillus* (B.) *natto*, and *B. subtilis* (Gibbs et al. 2004; Wang and Gonzalez de Mejia 2005). One of the simplest ways of producing food-grade hydrolyzed proteins is to use lactic acid bacteria, which are generally recognized as safe and are traditionally used to ferment raw materials of vegetable and animal origin. In this manner, protein breakdown occurs during the food manufacture with lactic acid bacteria. The proteolytic system of the lactic acid bacteria is one of the most studied nowadays, not only because of the impact of the physiology of the lactic acid bacteria but also because of its relation with the texture and flavor of the fermented products (Savijoki et al. 2006; Smit et al. 2005). The recent analysis of the genome of the lactic acid bacteria has contributed to one complete characterization of the proteolytic system in different species (Liu et al. 2010). The structural components of the proteolytic systems can be divided in three groups on the basis of their function: (1) cell envelopment proteinase that degrades the protein into oligopeptides; (2) peptide transport systems that move hydrolysis products through the cytoplasmic membrane; and (3) intracellular peptidases that degrade the peptides into shorter peptides and free amino acids. These amino acids will be degraded thoroughly by dependent metabolic routes strains to generate volatile compounds responsible for the aroma profile of fermented products (Christensen et al. 1999; Kunji et al. 1996). Several studies were undertaken to explain the main pathways involved in the industrial production and excellent reviews have covered these aspects of the most extensively utilized dairy starters (Kok and de Vos 1994; Poolman et al. 1995; Savijoki et al. 2006). On the other hand, the action of the proteolytic system of lactic acid bacteria on soybean proteins lacks detailed information in the literature. Probably, the cause is the weak action of lactic acid bacteria enzymes on soy proteins in comparison with the strong proteolytic systems from fungi and yeast. The capacity of 45 strains of *Lb. fermentum*, *Lb. plantarum*, *Lb. reuteri*, *Lb. helveticus*, *Lactococcus lactis*, *Streptococcus thermophilus*, *Pediococcus acidilactici*, *Pediococcus pentosaceous*, and *Leuconostoc* were screened for their proteolytic activity on soybean protein extracts in our laboratory. Only 17% of the studied strains displayed proteolytic activity, being α′- and α- of β conglycinin the preferably degraded soy protein fractions. Strains of *Lb. reuteri* were the most active, whereas *Leuc. cremoris* showed little hydrolytic action (70% and 2% of reduction, respectively). Acidic fractions of glycinin were hydrolyzed in greater extension by *Leuc. cremoris* (50% of reduction). Nevertheless, the basic fractions of the glycinin were not degraded by any of the analyzed strains. The obtained results allow select microorganisms that can hydrolyze soybean proteins, which would increase digestibility and diminish the allergenic fractions present in this substrate. Aguirre et al. (2008) published the first evidence of the action of enzymatic system of whole lactic acid bacteria cell on soy protein extract. Eleven strains of different lactobacilli and one *Pediococcus* was screened. Three strains of lactobacilli exerted some action against the basic subunit of glycinin (*L. paracasei* subsp. *paracasei*, *L. delbrueckii* subsp. *lactic*, and *L. helveticus*). This study gives detailed information on the breakdown of the individual soy protein fractions by proteolytic enzymes from lactic acid bacteria. Furthermore, these studies allowed gain better knowledge of their hydrolytic ability and the nature of the generated products in relation to soy food. In our laboratory, studies using the selected proteolytic lactic acid strain in soymilk are being carried out. In the fermented soy beverage, we observed similar

patterns of soy protein degradation through whole-cell suspensions of the same lactobacilli. In addition, the release of new peptides with potential biological activities was observed by Aguirre et al. (2008) and confirmed using fermented soymilk. In the last years, the release of peptides with recognized biological activity took much attention. The main source of biopeptide is milk protein, but soy protein is an interesting alternative. The majority of the studies used commercial enzymes or microorganisms as starters in traditional Asian soy foods (fungi or yeast), and the main source was soy protein isolate or whole soy beans (Chen et al. 1996, 2003; Gibbs et al. 2004; Jung et al. 2005; Gouda et al. 2006; Kodera and Nio 2006; Zhong et al. 2007). The works using enzymes from lactic acid cultures are few (Aguirre et al. 2008; Donkor et al. 2005; Tsai et al. 2006; Wang et al. 2006). Some of these researchers used yogurt starter (*Streptococcus thermophilus* and *Lactobacillus delbrueckii* ssp. *bulgaricus*) combined with probiotic strains (*L. acidophilus*, *L. casei*, and *Bifidobacterium lactis*) grown in soymilk to prepare a soy-based beverage (Donkor et al. 2005). In this drink, they determined proteolytic activities assessed by measuring liberated amino acids and peptides using the *o*-phthaldialdehyde (OPA) methods and ACE inhibitory activity. Other more complete studies used lactic fermentation (*L. casei*, *Lactobacillus acidophilus*, *Streptococcus thermophilus*, *Lactobacillus bulgaricus*, and *Bifidobacterium*) combined with commercial proteases to accelerate the production of bioactive peptides in fermented soymilk (Tsai et al. 2005). The new techniques developed in this experiment allow release two bioactive substances of peptides (800–900 Da) with ACE inhibitory activity and γ-aminobutyric acid. They were produced simultaneously in this experiment by protease-facilitated lactic acid bacteria fermentation of soymilk. These bioactive substances contribute to lower blood pressure of spontaneously hypertensive rat (SHR). Antioxidant activity was determined in fermented soymilk prepared with *Lactobacillus acidophilus* CCRC 14079 and *Streptococcus thermophilus* CCRC 14085, *Bifidobacterium infantis* CCRC 14633 (ATCC 27920), and *B. longum* B6 (Wang et al. 2006). However, in this paper, the precursors of this biological activity were not isolated. The importance of study and knowledge on the action of proteolytic system of lactic acid bacteria and bifidobacteria in soymilk is still limited and further studies are necessary.

46.5 β-Glucosidase and Soymilk

β-Glucosidase is another important enzyme that exerts an effect on soymilk micronutrients (isoflavones). β-Glucosidase (EC 3.2.1.21) hydrolyzes the β-(1–4)-glucosidic bonds with the release of glucose. Soybean and unfermented soybean products only contain glucoside forms, which comprise 80% to 95% of total isoflavones. The composition and content of isoflavones in soybean products are associated with certain processing techniques, such as fermented hydrolysis, fermentation, and removal of foam (Aderson and Wolf 1995; Wang and Murphy 1994a,b, 1996). Isoflavones exist in different types of aglycone forms (daidzein, genistein, and glycitein), glucoside forms (daidzin, genistin, and glycitin), and malonyl glucoside and acetyl glucoside forms (Kudou et al. 1991). The glucoside conjugates of isoflavones are converted to aglycones by β-glucosidase during soybean processing (Toda et al. 2001; Yin et al. 2004). Soy isoflavones are reported to play a role in the prevention of osteoporosis, cardiovascular disease, and several hormone dependent cancers (Adlercreutz 2002; Omoni and Aluko 2005; Setchell and Cassidy 1999). The biologically active (estrogen-like) and bioavailable aglycone forms of daidzein, genistein, and glycitein are linked to the prevention and potential treatment of the above mentioned diseases. The biochemistry, chemistry, and physiology of the isoflavones in soybeans and their food products were recently reviewed by Barnes (2010). The β-glucosidase activity produced by some strains of lactic acid bacteria (principally *Lactobacillus*) and *Bifidobacterium* hydrolyzes isoflavone glucosides into bioactive aglycones during soymilk fermentation (Tochikura et al. 1986; Hendrich 2002). The use of lactic acid bacteria or bifidobacteria starters on soymilk in turning isoflavones into absorbable aglycones was studied by several researchers (Chun et al. 2007; Otieno et al. 2005, 2006; Pham and Shah 2008a,b, 2009; Tsangalis et al. 2002; Wei et al. 2007). Marazza et al. (2009) screened bacterial strains with high β-glucosidase activity as well as determined partial biochemical characterization of this enzyme of one strain of *L. rhamnosus*. The effect of selected strain on the hydrolysis of isoflavone to aglycones, residual sugars, and organic acids produced in soymilk fermented at 37°C for 24 h was also determined. The

data presented in this study add new knowledge to current literature. On the other hand, this strain was able to increase bioactive isoflavones during soymilk fermentation to achieve 100% of bioconversion at 12 h. The selected microorganism will be used as a starter culture to produce different aglycone-rich functional fermented soymilk.

46.6 Conclusion

In summary, soymilk is a low-cost source of protein that has some antinutritional factors that can be reduced or eliminated using different enzymes or fermentative processes with selected microorganisms.

Some strains of lactic acid bacteria and related microrganisms (as bifidobacteria) need specific enzymes to produce fermented or pretreated soymilk with improved general properties (low flatulence, good digestibility, enrichment in isoflavones aglicones) besides potential probiotic effects of each strain.

α-Galactosidase contributes to the production of soymilk with low conten α-GOS, which produces intestinal disturbance in humans.

Proteases and peptidases contribute to soy products that has improved digestibility and bioactive peptides which has positive health contribution in cancers, improvement in bone health, hypocholesterolemic effect, and antiaging/antioxidant effects.

β-Glucosidases contribute in the bioconversion of isoflavones in aglycones, which can be assimilated by humans and has beneficial effects on bone mineralization, alleviation of menopausal symptoms, hypocholesterolemic effect, and antiaging/antioxidant effects.

Those foods whose health benefits are supported by sufficient scientific studies have the potential to be an increasingly important component of a healthy lifestyle, and fermented products with health benefits are increasingly gaining credibility and attention.

REFERENCES

Aderson R, Wolf W. 1995. Composition changes in trypsin inhibitor, phytic acid, saponins and isoflavones related to soybean processing. *J Nutr* 125:581–8.

Adlercreutz H. 2002. Phytoestrogens and breast cancer. *J Steroid Biochem. Mol Biol* 83(1–5):113–8.

Aguirre L, Garro M, Savoy de Giori G. 2008. Enzymatic hydrolysis of soybean protein using lactic acid bacteria. *Food Chem* 111:976–82.

Angeles AG, Marth EH. 1971a. Growth and activity of lactic acid bacteria in soy milk. I. Growth and acid production. *J Milk Food Technol* 34:30–6.

Angeles AG, Marth EH. 1971b. Growth and activity of lactic acid bacteria in soy milk. II. Heat treatment of soy milk and culture activity. *J Milk Food Technol* 34:63–8.

Angeles AG, Marth EH. 1971c. Growth and activity of lactic acid bacteria in soy milk. III. Lipolytic activity. *J Milk Food Technol* 34:69–75.

Angeles AG, Marth EH. 1971d. Growth and activity of lactic acid bacteria in soy milk. III. Proteolytic activity. *J Milk Food Technol* 34:69–75.

Ariyama H. 1963. Process for the manufacture of a synthetic yogurt from soybean. Application: USA 3096177, 2 Jul 1963.

Bahl OP, Agrawal KML. 1969. Glycosidases of *Aspergillus niger*. I. Purification and characterization of α- and β-galactosidases and β-N-acetylglucosaminidase. *J Biol Chem* 244:2970–8.

Bahl OP, Agrawal KML. 1972. α-Galactosidase β-galactosidases and β-N-acetylglucosaminidase from *Aspergillus niger*. In: Ginsburg V, editor. *Methods in Enzymology*. Complex Carbohydrates (PartB). Vol XXVIII. New York, London: Academic Press. p 728–34.

Bandenhop AF, Hackler LR. 1974. Effects of soaking soybeans in sodium hydroxide solution as pretratament for soymilk production. *Cereal Sci Today* 15:84–8.

Barnes S. 2010. The biochemistry, chemistry and physiology of the isoflavones in soybeans and their food products. *Lymphatic Res Biol* 8(1):89–98. doi:10.1089/lrb.2009.0030.

Brown BD, Wei LS, Steinberg MP, Villota R. 1982. Minimizing protein insolubilization during thermal inactivation of lipoxygenase in soybean cotyledons. *J Am Oil Chem Soc* 59:88–92.

Che Man YB, Wei LS, Nelson AI. 1989. Acid inactivation of soybean lipoxygenase with retention of protein solubility. *J Food Sci* 54(4):963–7.

Chen HM, Muramoto K, Yamauchi F, Nokihara K. 1996. Antioxidant activity of designed peptides based on the antioxidative peptide isolated from digests of a soybean peptide. *J Agric Food Chem* 44:2619–23.

Chen J, Okada T, Muramoto K, Suetsuna K, Yang, S. 2003. Identification of angiotensin I-converting enzyme inhibitory peptides derived from the peptic digest of soybean protein. *J Food Biochem* 26:543–54.

Chien HL, Huang HY, Chou CC. 2006. Transformation of isoflavone phytoestrogens during the fermentation of soymilk with lactic acid bacteria and bifidobacteria. *Food Microbiol* 23:772–8.

Chou CC, Hou JW. 2000. Growth of bifidobacteria in soymilk and survival in the fermented soymilk drink during storage. *Int J Food Microbiol* 56:113–21.

Christensen JE, Dudley EG, Pederson JA, Steele JL. 1999. Peptidases and amino acid catabolism in lactic acid bacteria. *Antonie Van Leeuwenhoek* 76:217–46.

Chun J, Kim G, Lee K, Choi D, Kwon GH, Park JY, Jeong SJ, Kim JS, Kim JH. 2007. Conversion of isoflavone glucosides to aglycones in soymilk by fermentation with lactic acid bacteria. *J Food Sci* 72(2):M39–44.

Chun J, Kim JS, Kim JH. 2008. Enrichment of isoflavone aglycones in soymilk by fermentation with single and mixed cultures of Streptococcus infantarius 12 and Weissella sp. 4. *Food Chem* 109:278–84.

Church FC, Meyers SP, Srinivasan VR. 1980. Isolation and characterization of alpha-galactosidase from *Pichia guilliermondii.* In: Underkofler LA, Wulf ML, editors. *Developments in Industrial Microbiology*. Vol. 21. Arlington, VA: Society for Industrial Microbiology. p 339–48.

Cristofaro E, Mottu F, Wuhrmann JJ. 1974. Involvement of the raffinose family of oligosaccharides in flatulence. In: Sipple HL, McNutt KW, editors. *Sugar in Nutrition*. Chapter 20. New York: Academic Press.

Cruz R, Batistela JC, Wosiaki G. 1981. Microbial α-Galactosidase for Soymilk Processing. *J Food Sci* 46:1196–200.

Desjardins ML, Roy D. 1990. Growth of bifidobacteria and their enzyme profiles. *J Dairy Sci* 73:299–307.

Dey PM, Patel S, Brownleader MD. 1993. Induction of α-galactosidase in *Penicillium ochrochlorn* by guar (*Cyanopsis tetragonobola*) gum. *Biotechnol Appl Biochem* 17:361–71.

Dey PM, Pridham JB. 1972. Biochemistry of α-galactosidases. *Adv Enzymol* 36:91–130.

Donkor ON, Henriksson A, Vasiljevic T, Shah NP. 2005. Probiotic strains as starter cultures improve angiotensin-converting enzyme inhibitory activity in soy yogurt. *J Food Sci* 70:M375–81.

Feeney RE, Whitaker JR. 1977. Food proteins: improvement through chemical and enzymatic modification. *Adv Chem Ser* 160. Washington, DC: American Chemists Society. p. 312.

Fuchs RHB, Borsato D, Bona E, Hauly MCO. 2005. "Iogurte" de soja suplementado com oligofrutose e inulina. *Ciencia e Tecnol Alim* 25:175–81.

Garro MS. 1995. Behavior of lactic acid bacteria and related micro-organisms on soymilk. [DPhil thesis]. Argentina: National University of Tucumán (UNT).

Garro MS, Aguirre L, Savoy de Giori G.2006. Biological activity of *Bifidobacterium longum* in response to environmental pH. *Appl Microbiol Biotechnol* 70:612–7.

Garro MS, de Giori GS, de Valdez GF, Oliver G. 1994a. Utilization of soymilk galactosaccharides by lactobacilli. *Microbiologie-Aliments-Nutr* 12:61–6.

Garro MS, de Valdez GF, de Giori GS. 2002. Application of conductimetry for evaluation of lactic starter cultures in soymilk. *J Food Sci* 67:1175–8.

Garro MS, de Valdez GF, de Giori GS. 2004. Temperature effect on the biological activity of *Bifidobacterium longum* CRL 849 and *Lactobacillus fermentum* CRL 251 in pure and mixed cultures grown in soymilk. *Food Microbiol* 21:511–8.

Garro MS, de Valdez GF, Oliver G, de Giori GS. 1996b. Influence of carbohydrates on the α–galactosidase activity of *Lactobacillus fermentum*. *Current Microbiol* 33:302–5.

Garro MS, de Valdez GF, Oliver G, de Giori GS. 1999b. Starter culture activity in refrigerated fermented soymilk. *J Food Prot* 62:808–10.

Garro MS, Font de Valdez G, Oliver G, Savoy de Giori G. 1996a. Purification of α-galactosidase from *Lactobacillus fermentum*. *J Biotechnol* 45:103–9.

Garro MS, Font de Valdez G, Oliver G, Savoy de Giori G. 1998. Growth characteristics and fermentation products of *Streptococcus salivarius* subs. *thermophilus*, *Lactobacillus casei* and *L. fermentum* in soymilk. *Z Lebensm Unters Forsch A* 206:72–5.

Garro MS, Font de Valdez G, Oliver G, Savoy de Giori G. 1999a. Hydrolysis of soya milk oligosaccharides by *Bifidobacterium longum* CRL 849. *Z Lebensm Unters Forsch A* 208:57–59.

Garro MS, Savoy de Giori G, Font de Valdez G, Oliver G. 1993. Characterization of alpha-galactosidase from Lactobacillus fermentum. *J Appl Bacteriol* 75:485–8.

Garro MS, Savoy de Giori G, Font de Valdez G, Oliver G. 1994b. α-D-Galactosidase (EC 3.2.1.22) from *Bifidobacterium longum*. *Lett Appl Microbiol* 19:16–9.

Gehrke C, Weiser HH. 1947. Comparatives studies on growth and biochemical features of microorganisms grown in cow's milk and soybean milk. *Food Res* 12:360–4.

Gehrke C, Weiser HH. 1948. Comparatives studies of the biochemical activity of *Streptococcus lactis, Streptococcus citrovorus*, and *Streptococcus paracitrovorus* when grown in cow's milk and in soybean milk. *J Dairy Sci* 31:213–22.

Gibbs BF, Zougman A, Masse R, Mulligan C. 2004. Production and characterization of bioactive peptides from soy hydrolysate and soyfermented food. *Food Res Int* 37:123–31.

Gonzalez CF, Kunka BS. 1986. Evidence for plasmid linkage of raffinose utilization and associated α-galactosidase and sucrose hydrolase activity in *Pediococcus pentosaceous*. *Appl Env Microbiol* 51:105–9.

Gouda KGM, Gowda LR, Appu Rao AG, Prakash V. 2006. Angiotensin I-converting enzyme inhibitory peptide derived from Glycinin, the 11S globulin of soybean (Glycine max). *J Agric Food Chem* 54:4568–73.

Gray W. 1970. The use of fungi as food in food processing. *Crit Rev Food Technol* 1:225–329.

Gupta RP, Gupta RR, inventors; 1988. Equipment for making no-beany flavour soymilk. U.S. Patent 4744524–32538.

Hamada JS, Marshall WE. 1989. Preparation and functional properties of enzymatically deamidated soy proteins. *J Food Sci* 54:598–601.

Hang YD, Jackson H. 1967a. Preparation of soybean cheese using lactic starter organisms. I General characterisitics of the finished cheese. *Food Technol* 21:95–6.

Hang YD, Jackson H. 1967b. Preparation of soybean cheese using lactic starter organisms. II Effects of addition of rennet extract and skim milk. *Food Technol* 21:97–100.

Hartwig EE, Kuo TM, Kenty MM. 1997. Seed protein and its relationship to soluble sugars in soybeans. *Crop Sci* 37:770–3.

Hayakawa K, Mizutani J, Wada K, Masai T, Yoshihara I, Mitsuoka T. 1990. Effects of soybean oligosaccharides on human faecal microflora. *Microbial Ecology in Health and Disease* 3:293–303.

Hendrich S. 2002. Bioavailability of isoflavone. *J Chromatogr* B 777:203–10.

Hesseltine CW, Wang HL. 1972. Fermented soybean food products. In: Smith AK, Circle SJ, editors. Soybeans. chemistry and technology. *Proteins*. Vol 1. Westport CT: Avi Publishing Co. Inc. p 389–419.

Hou HJ, Chang KC. 2004a. Structural characteristics of purified glycinin from soybeans stored under various conditions. *J Agric Food Chem* 52:3792–800.

Hou HJ, Chang KC. 2004b. Structural characteristics of purified β-conglycinin from soybeans stored under four conditions. *J Agric Food Chem* 52:7931–7.

Hu FL, He YQ, Huang B, Li CR, Fan MZ, Li ZZ. 2009. Secondary metabolites in a soybean fermentation broth of Paecilomyces militaris. *Food Chem* 116:198–201.

Jung S, Murphy PA, Johnson LA. 2005. Physicochemical and functional properties of soy protein substrates modified by low levels of protease hydrolysis. *J Food Sci* 70:C180–7.

Kanthamani S, Nelson AI, Steinberg MP. 1978. Home preparation of soymilk: a new concept. Whole soybean foods for home and village use. *Intsoy* 14(5):5–9.

Kellogg JH, inventor; 1934 Dec 4. Method of making acidophilus milk. U.S. Patent 1982941.

Kenkyusho CM, inventor; 1965 Jul 30. Preparation of soy cheese. Japanese Patent 16737.

Kim JH, Park JY, Jeong SJ, Chun JY, Lee JH, Chung DK, Kim JH. 2005. Characterization of the α-Galactosidase gene from *Leuconostoc mesenteroides* SY1. *J Microbiol Biotechnol* 15(4):800–8.

Kinsella JE. 1979. Functional properties of soy protein. *J Am Oil Chem Soc* 56:242–58.

Kodera T, Nio N. 2006. Identification of an angiotensin I-converting enzyme inhibitory peptides from protein hydrolysates by a soybean protease and the antihypertensive effects of hydrolysates in spontaneously hypertensive model rats. *J Food Sci* 71:C164–73.

Kok J, de Vos WM. 1994. The proteolytic system of lactic acid bacteria. In: Gasson M, De Vos W, editors. *Genetics and Biotechnology of Lactic Acid Bacteria*. Glasgow: Blackie Academic & Professional. p 169–210.

Kudou S, Fleury Y, Welti D, Magnulato D, Uchida T, Kitamura K. 1991. Malonyl isoflavone glycosides in soybean seeds (*Glycine max* Merill). *Agric Biol Chem* 55:2227–33.

Kunji ER, Mierau I, Hagting A, Poolman B, Konings WN. 1996. The proteolytic systems of lactic acid bacteria. *Antonie Van Leeuwenhoek* 70:187–221.

Kwok K, Niranjan K. 1995. Review: Effect of thermal processing on soymilk. *Int J Food Sci Technol* 30:263–95.

Leblanc J, Clier F, Bensaada M, Savoy de Giori G, Guerekobaya T, Sesma F, Juillard V, Rabot S, Piard JC. 2008. Ability of *Lactobacillus fermentum* to overcome host alpha-galactosidase deficiency, as evidenced by reduction of hydrogen excretion in rats consuming soya alpha-galacto-oligosaccharides. *BMC Microbiol* 8:22–31.

LeBlanc JG, Garro MS, de Giori GS, de Valdez GF. 2004a. A novel functional soy-based food fermented by lactic acid bacteria: Effect of heat treatment. *J of Food Sci* 69(8):M246–50.

LeBlanc JG, Garro MS, Savoy de Giori G. 2004b. Effect of pH on Lactobacillus fermentum growth, raffinose removal, alpha-galactosidase activity and fermentation products. *Appl Microbiol Biotechnol* 65:119–23.

LeBlanc JG, Garro MS, Silvestroni A, Connes C, Piard, JC, Sesma F, de Giori GS. 2004c. Reduction of alpha-galactooligosaccharides in soyamilk by *Lactobacillus fermentum* CRL 722: *in vitro* and *in vivo* evaluation of fermented soyamilk. *J Appl Microbiol* 97(4):876–81.

LeBlanc JG, Piard JC, Sesma F, de Giori GS. 2005. *Lactobacillus fermentum* CRL 722 is able to deliver active alpha-galactosidase activity in the small intestine of rats. *FEMS Microbiol Lett* 248(2):177–82.

Li YT, Shetlar MR. 1964. Occurrence of α-galactosidase in higher fungi: Isolation of α-galactosidase from *Calvatia cyathiformis*. *Arch Biochem Biophys* 108:523–30.

Liu KS. 1997. *Soybeans: Chemistry, Technology and Utilization*. New York: Chapman and Hall.

Liu M, Bayjanov JR, Renckens B, Nauta A, Siezen RJ. 2010. The proteolytic system of lactic acid bacteria revisited: a genomic comparison. *BMC Genom* 11:36. doi:10.1186/1471-2164-11–36. http://www.biomedcentral.com/1471-2164/11/36.

Lo WY, Steinkraus KH, Hand DB, Hackler LR, Wilkens WF. 1968. Soaking soybeans before extraction as it affects chemical composition and yield of soy milk. *Food Technol* 33:1188–90.

Maheshwari P, Ooi ET, Nikolov ZL. 1995. Off-flavor removal from soy-protein isolate by using liquid and supercritical carbon dioxide. *J Am Oil Chem Soc* 72(10):1107–15.

Marazza JA, Garro MS, Savoy de Giori G. 2009. Aglycone production by *Lactobacillus rhamnosus* CRL981 during soymilk fermentation. *Food Microbiol* 26:333–9.

Martinelli AF, Hesseltine CW. 1964. Tempeh fermentations. Package and tray fermentations. *Food Technol* 18:167–71.

Maruyama N, Fukuda T, Saka S, Inui N, Kotoh J, Miyagawa M, Hayashi M, Sawada M, Moriyama T, Utsumi S. 2003. Molecular and structural analysis of electrophoretic variants of soybean seed storage proteins. *Phytochemistry* 64:701–8.

Masai T, Wada K, Hayakawa K, Yoshihara I, Mitsuoka T. 1987. Effects of soybean oligosaccharides on human intestinal flora and metabolic activities. *Jpn J Bacteriol* 42:313–25.

Matsuura M, Obata A, Fukushima D. 1989. Objectionable flavor of soymilk developed during the soaking of soybeans and its control. *J Food Sci* 54:602–5.

McGhee JE, Silman R, Bagley EM. 1978. Production of α-galactosidase from *Aspergillus awamori:* properties and action of p-NPG (-D) and galactooligosaccharides of soymilk. *J Am Oil Chem* 55:244–7.

Mital BK, Steinkraus KH. 1975. Utilization of oligosaccharides by lactic acid bacteria during fermentation of soymilk. *J Food Sci* 40:114–8.

Mital BK, Steinkraus KH, Naylor HB. 1974. Growth of lactic acid bacteria in soy milk. *J Food Sci* 39:1018–22.

Mizutani T, Hashimoto H. 2004. Effect of grinding temperature on hydroperoxide and off-flavor contents during soymilk manufacturing process. *J Food Sci* 69:112–6.

Nelson AI, Steinberg MP, Wei LS. 1976. Illinois process for preparation of soymilk. *J Food Sci* 41:57–61.

Nielsen NC. 1985. The structure and complexity of the 11S polypeptides in soybeans. *J Am Oil Chem Soc* 62:1680–6.

Omoni AO, Aluko RE. 2005. Soybean foods and their benefits: potential mechanisms of action. *Nutr Rev* 63(8):272–83.

Otieno DO, Ashton JF, Shah NP. 2005. Stability of b-glucosidase activity produced by *Bifidobacterium* and *Lactobacillus* spp. in fermented soymilk during processing and storage. *J Food Sci* 70(4):236–41.

Otieno DO, Ashton JF, Shah NP. 2006. Evaluation of enzymic potential for biotransformation of isoflavones phytoestrogen in soymilk by *Bifidobacterium animalis*, *Lactobacillus acidophilus* and *Lactobacillus casei*. *Food Res Int* 39:394–407.

Pham TT, Shah NP. 2008a. Effect of lactulose on biotransformation of isoflavone glycosides to aglycones in soymilk by Lactobacilli. *J Food Sci* 73(3):M158–65.

Pham TT, Shah NP. 2008b. Effects of lactulose supplementation on the growth of bifidobacteria and biotransformation of isoflavone glycosides to isoflavone aglycones in soymilk. *J Agric Food Chem* 56(12):4703–9.

Pham TT, Shah NP. 2009. Hydrolysis of isoflavone glycosides in soymilk by betagalactosidase and beta-glucosidase. *J Food Biochem* 33:38–60.

Poolman B, Kunji E, Hagting A, Juillard V, Konings W. 1995. The proteolytic pathway of Lactococcus lactis. *J Appl Bacteriol* 79:65–75.

Puchart V, Vrsanská M, Bhat MK, Biely P. 2000. Purification and characterization of α-galactosidase from a thermophilic fungus *Thermomyces lanuginosus*. *Biochem Biophys Acta* 1524:27–37.

Puski G. 1975. Modification of functional properties of soy proteins by proteolytic enzyme treatment. *Cereal Chem* 52:655–62.

Rackis JJ, Honig DH, Sessa DJ, Steggerda FR. 1970. Flavor and flatulence factors in soybean protein products. *J Agric Food Chem* 18:977–82.

Rackis JJ, Sessa DJ, Honig DH. 1979. Flavor problems of vegetable food proteins. *J Am Oil Chem Soc* 56(3):262–71.

Rosenthal A, Deliza R, Cabral LMC, Cabral LC, Farias CAA, Domingues AM. 2003. Effect of enzymatic treatment and filtration on sensory characteristics and physical stability of soymilk. *Food Control* 14:187–92.

Savijoki K, Ingmer H, Varmanen P: Proteolytic systems of lactic acid bacteria.2006. *Appl Microbiol Biotechnol* 71:394–406.

Savoy de Giori G, Aguirre L, Marazza J, Garro MS. 2010. An overview of lactic acid bacteria applications for healthful soy foods development. In: Mozzi F, Raya RR, Vignolo GM, editors. *Biotechnology of Lactic Acid Bacteria. Novel Applications*. Iowa USA: Wiley Blackwell. p 289–99.

Schuler R, Mudgett R, Mahoney RR. 1985. Kinetic properties of α-D-galactosidase from *Lactobacillus fermenti*. *Enzyme Microb Technol* 7:207–11.

Setchell KDR, Cassidy A. 1999. Dietary isoflavones: biological effects and relevance to human health. *J Nutr* 129:758S–67S.

Shun-Tang G, Ono T, Mikami M. 1997. Interaction between protein and lipid in soybean milk at elevated temperatures. *J Agric Food Chem* 45:4601–5.

Smit G, Smit BA, Engels WJM. 2005. Flavour formation by lactic acid bacteria and biochemical flavour profiling of cheese products. *FEMS Microbiol Rev* 29:591–610.

Srinivas H, Swamylingappa B, Chand N. 1992. Secondary extraction of soybeans using hexane-acetic acid: effect on beany flavor removal and physicochemical properties. *J Agric Food Chem* 40(2):276–9.

Steinkraus KH, inventor; 1973. Defatted soybean meal. U.S. Patent 3,721,569.

Steinkraus KH. 1974. Research on traditional oriental and India fermented foods. In: *Fermented foods. Current Science and Technology*. New York State Agric. Exp. Sta. Geneva Eight Annual Symposium Special Report N 16. April 1974. p 10–13.

Suarez FL, Springfield J, Furne JK, Lohrmann TT, Kerr PS, Levitt MD. 1999. Gas production in humans ingesting a soybean flour derived from beans naturally low in oligosaccarides. *Am J Clin Nutr* 69:135–9.

Sugimoto H, Van Buren JP. 1970. Removal of oligosaccharides from soymilk by an enzyme from *Aspergillus saitoi. J Food Sci* 35:655–60.

Suzuki H, Li SC, Li YT. 1970. α-Galactosidase from *Mortierella vinacea*. *J Biol Chem* 245:781–6.

Takahashi N, Sasaki R, Chiba H. 1979. Enzymatic improvement of food flavor. IV. Oxidation of aldehydes in soybean extracts by aldehyde oxidase. *Agric Biol Chem* 43(12):2557–62.

Thananunkul D, Tanaka M, Chichester CO, Lee TC. 1976. Degradation of raffinose and stachyose in soybean milk by α-galactosidase from *Mortierella vinacea*. Entrapment of α-galactosidase within polyacrylamide gel. *J Food Sci* 41:173–5.

Toda K, Chiba K, Ono T. 2007. Effect of components extracted from okara on the physicochemical properties of soymilk and tofu texture. *J Food Sci* 72:C108–13.

Toda T, Sakamoto A, Takayanagi T, Yokotsura K. 2001. Changes in isoflavone compositions soybean during soaking process. *Food Sci Technol Res* 6:314–9.

Tokikura T, Sakai K, Fujiyoshi T, Tachiki T, Jumagai H. 1986. p-Nitrophenyl glycoside-hydrolyzing activity in bifidobacteria and characterization of β-D-galactosidase of *Bifidobacterium longum*. *Agric Biol Chem* 50(9):2279–86.

Tsai JS, Lin YS, Pan BS, Chen TJ. 2006. Antihypertensive peptides and γ-aminobutyric acid from prozyme 6 facilitated lactic acid bacteria fermentation of soymilk. *Process Biochem* 41:1282–8.

Tsangalis D, Ashton JF, McGill AEJ, Shah NP. 2002. Enzymic transformation of isoflavone phytoestrogens in soymilk by α-glucosidase-producing bifidobacteria. *J Food Sci* 67:3104–13.

Tsangalis D, Ashton JF, McGill AEJ, Shah NP. 2003. Biotransformation of isoflavones by bifidobacteria in fermented soymilk supplemented with D-glucose and L-cysteine. *J Food Sci* 68:623–31.

Tsangalis D, Shah NP. 2004. Metabolism of oligosaccharides and aldehydes and production of organic acids in soymilk by probiotic bifidobacteria. *Int J Food Sci Technol* 39:541–54.

Umeda I, Nakamura K, Yamato M, Nakamur Y. 1969. Investigations on comparative production of shoyu (soy sauce) from defatted soybean meals obtained from United States and Japanese soybeans and processed by United States and Japanese methods. U.S. Dept Agric Final Tech Rep PL 480 UR-A-11 40:21.

Viana PA. F, Rezende ST, Marques VM, Trevizano LM, Passos FML, Oliveira MGA, Bemquerer MP, Oliveira JS, Guimaraes VM. 2006. Extracellular α-galactosidase from *Debaryomyces hansenii* UFV-1 and its use in the hydrolysis of raffinose oligosaccharides. *J Agric Food Chem* 54:2385–91.

Wang H, Murphy PA. 1994a. Isoflavone composition of American and Japanese soybean in Iowa: effects of variety; crop year and location. *J Agric Food Chem* 42:1674–7.

Wang H, Murphy PA. 1994b. Isoflavone content in commercial soybean foods. *J Agric Food Chem* 42:1666–73.

Wang H, Murphy PA. 1996. Mass balance study of isoflavone during soybean processing. *J Agric Food Chem* 44:2377–83.

Wang W, Gonzalez de Mejia E. 2005. A new frontier in soy bioactive peptides that may prevent age-related chronic diseases. *Comprehensive Rev Food Sci Food Safety* 4:63–78.

Wang YC, Yu RC, Chou CC. 2002. Growth and survival of bifidobacteria and lactic acid bacteria during the fermentation and storage of cultured soymilk drink. *Food Microbiol* 19:501–8.

Wang YC, Yu RC, Chou CC. 2006. Antioxidative activities of soymilk fermented with lactic acid bacteria and bifidobacterias. *Food Microbiol* 23:128–35.

Wei QK, Chen TR, Chen JT. 2007. Using of *Lactobacillus* and *Bifidobacterium* to production of isoflavone aglycones in fermented soymilk. *Int J Food Microbiol* 117:120–4.

Wilkens WF, Mattick LR, Hand DB. 1967. Effect of processing method on oxidative off-flavors of soybean milk. *Food Technol* 21:1630–3.

Wilkens WF, Lin FM. 1970. Gas chromatographic and mass spectral analysis of soybean milk volatiles. *J Agric Food Chem* 18:333–6.

Yamanaka K, Furukawa N. 1970. Studies on utilization of soybean protein for food manufacturing. II Influence of soy milk added to skim milk on the acidity and the hardness of curd produced by lactic bacteria for dairy use. *J Food Sci Technol* 17:456–61.

Yin LJ, Li LT, Li ZG, Tatsumi E, Saito M. 2004. Changes in isoflavone content and composition of sufu (fermented tofu) during manufacturing. *Food Chem* 87:587–92.

Yoon MY, Hwang HJ. 2008. Reduction of soybean oligosaccharides and properties of α-D-galactosidase from *Lactobacillus curvatus* R08 and *Leuconostoc mesenteriodes* JK55. *Food Microbiol* 25: 815–23.

Zapater IG, Ullah AHJ, Wodzinski RJ. 1990. Extracellular α-galactosidase (EC 3.2.1.22) from *Aspergillus ficuum* NRRL 3135: Purification and characterization. *Prep Biochem* 20:263–96.

Ziegler F, Nitenberg G, Coudray-Lucas C, Lasser P, Giboudeau J, Cynober L. 1998. Pharmacokinetic assessment of an oligopeptide-based enteral formula in abdominal surgery patients. *Am J Clinical Nutr* 67:124–8.

Zhong F, Zhang X, Ma J, Shoemaker CF. 2007. Fractionation and identification of a novel hypocholesterolemic peptide derived from soy protein Alcalase hydrolysates. *Food Res Int* 40:756–62.

47

*Microorganisms and Microbially Derived Ingredients Used in Foods**

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CONTENTS

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47.1 Introduction

Some of the food ingredients used in this country are described by the U.S. Food and Drug Administration (FDA) in their regulations; some are not. The latter group of ingredients, although not listed in official regulations, is used in food under various legal parameters promulgated by the FDA. Many of such ingredients are microorganisms and microbially derived food ingredients. All uses are limited to nonpathogenic and nontoxicogenic strains of the respective organisms and comply with current good manufacturing practices (GMP). In most cases, fermentation occurs in varying degrees. The lists provided by the FDA in its website (www.FDA.gov) are listed below, where 21 CFR 172, an example of a legal citation, refers to Volume 21, United States Code of Federal Regulations, Section 172.

1. Food additives derived from microorganisms listed in 21 CFR 172 and 173
2. Substances derived from microorganisms (including seaweed) affirmed by FDA as generally recognized as safe, or GRAS, in 21 CFR184
3. Substances derived from microorganisms affirmed by FDA as GRAS for indirect uses in foods in 21 CFR186
4. Substances (enzymes) derived from microorganisms recognized by FDA as GRAS in Opinion Letters issued by the FDA
5. Foods for human consumption that may contain or be derived from microorganisms listed in 21 CFR 131, 133, 136, and 137

We will discuss each of the above five categories or listings. All information in this chapter has been modified from documents available at the website of the FDA (www.FDA.gov).

47.2 Microorganisms and Food Additives Listed in 21 CFR 172, 173

These substances are listed in Table 47.1. A brief description is presented here.

TABLE 47.1

Food Additives Derived from Microorganisms Listed in 21 CFR 172 and 173

Regulation in 21 CFR	Ingredient
§172.155	Natamycin derived from *Streptomyces natalensis* and *Streptomyces chattanoogensis*
§172.325	Bakers yeast protein derived from *Saccharomyces cerevisiae*
§172.590	Yeast-malt sprout extract, derived from *Saccharomyces cerevisiae*, *Saccharomyces fragilis*, *Candida utilis*
§172.620	Carrageenan, a hydrocolloid extracted from the following members of the families *Gigartinaceae* and *Soliericeae* of the class *Rodophyceae* (red seaweed): *Chondrus crispus*, *Chondrus ocellatus*, *Eucheuma cottonii*, *Eucheuma spinosum*, *Gigartina acicularis*, *Gigartina pistillata*, *Gigartina radula*, *Gigartina stellata*
§172.655	Furcelleran, the refined hydrocolloid extracted from *Furcellaria fastigiata* of the class *Rodophyceae* (red seaweed)
§172.695	Xanthan gum derived from *Xanthomonas campestris*
§172.725	Gibberellic acid derived by fermentation from *Fusarium moniliforme*
§172.896	Dried yeasts, *Saccharomyces cerevisiae*, *Saccharomyces fragilis*, and dried torula yeast, *Candida utilis*
§172.898	Bakers yeast glycan from *Saccharomyces cerevisiae*
§173.110	Amyloglucosidase derived from *Rhizopus niveus* for use in degrading gelatinized starch into constituent sugars
§173.120	Carbohydrase and cellulase derived from *Aspergillus niger* for use in clam and shrimp processing
§173.130	Carbohydrase derived from *Rhizopus oryzae* for use in the production of dextrose from starch
§173.135	Catalase derived from *Micrococcus lysodeikticus* for use in the manufacture of cheese
§173.140	Esterase-lipase derived from *Mucor miehei* var. Cooney *et* Emerson as a flavor enhancer in cheeses, fats and oils, and milk products
§173.145	α-Galactosidase derived from *Morteirella vinaceae* var. *raffinoseutilizer* for use in the production of sucrose from sugar beets
§173.150	Milk-clotting enzymes, microbial for use in the production of cheese (milk-clotting enzymes are derived from *Endothia parasitica*, *Bacillus cereus*, *Mucor pusillus* Lindt and *Mucor miehei* and *Aspergillus oryzae* modified to contain the gene for aspartic proteinase from *Rhizomucor miehei* var. *Cooney et Emerson*)
§173.160	*Candida guilliermondii* as the organism for fermentation production of citric acid
§173.165	*Candida lipolytica* for fermentation production of citric acid
§173.280	A solvent extraction process for recovery of citric acid from *Aspergillus niger* fermentation liquor

47.2.1 Natamycin (Pimaricin, 21 CFR 172.155)

Natamycin, also known as pimaricin, is a polyene macrolide antimycotic substance possessing an empirical formula of $C_{33}H_{47}NO_{13}$ and a molecular weight of 665.7. The additive may be applied on cheese, as an antimycotic, in amounts not to exceed 20 mg/kg (20 ppm) in the finished product.

47.2.2 Bakers Yeast Protein (21 CFR 172.325)

Bakers yeast protein may be safely used in food in accordance with the following conditions. It is the insoluble proteinaceous material remaining after the mechanical rupture of yeast cells of *Saccharomyces cerevisiae* and removal of whole cell walls by centrifugation and separation of soluble cellular materials. The viable microbial content of the finished ingredient is

1. Less than 10,000 organisms/gram by aerobic plate count
2. Less than 10 yeasts and molds/gram
3. Negative for *Salmonella*, *Escherichia coli*, coagulase-positive *Staphylococci*, *Clostridium perfringens*, *Clostridium botulinum*, or any other recognized microbial pathogen or any harmful microbial toxin

The ingredient is used in food as a nutrient supplement.

47.2.3 Yeast-Malt Sprout Extract (21 CFR 172.590)

Yeast-malt sprout extract may be safely used in food in accordance with the following prescribed conditions:

1. The additive is produced by partial hydrolysis of yeast extract (derived from *Saccharomyces cereviseae*, *Saccharomyces fragilis*, or *Candida utilis*) using the sprout portion of malt barley as the source of enzymes
2. The additive may be used as a flavor enhancer in food at a level not in excess of that reasonably required to produce the intended effect

47.2.4 Carrageenan (21 CFR 172.620)

The food additive carrageenan may be safely used in food in accordance with the following prescribed conditions:

1. It is the refined hydrocolloid prepared by aqueous extraction from the following members of the families Gigartinaceae and Solieriaceae of the class Rodophyceae (red seaweed):
   a. *Chondrus crispus*
   b. *Chondrus ocellatus*
   c. *Eucheuma cottonii*
   d. *Eucheuma spinosum*
   e. *Gigartina acicularis*
   f. *Gigartina pistillata*
   g. *Gigartina radula*
   h. *Gigartina stellata*
2. It is a sulfated polysaccharide, the dominant hexose units of which are galactose and anhydrogalactose
3. It is used or intended for use in the amount necessary for an emulsifier, stabilizer, or thickener in foods, except for those standardized foods that do not provide for such use
4. The label and labeling of the additive should bear the name of the additive, carrageenan

47.2.5 Furcelleran (21 CFR 172.655)

The food additive furcelleran may be safely used in food in accordance with the following prescribed conditions:

1. It is the refined hydrocolloid prepared by aqueous extraction of *Furcellaria fastigiata* of the class Rodophyceae (red seaweed)
2. It is a sulfated polysaccharide, the dominant hexose units of which are galactose and anhydrogalactose
3. It is used or intended for use in the amount necessary for an emulsifier, stabilizer, or thickener in foods, except for those standardized foods that do not provide for such use
4. The label and labeling of the additive should bear the name of the additive, furcelleran

47.2.6 Xanthan Gum (21 CFR 172.695)

The food additive xanthan gum may be safely used in food in accordance with the following prescribed conditions:

1. The additive is a polysaccharide gum derived from *Xanthomonas campestris* by a pure-culture fermentation process and purified by recovery with isopropyl alcohol. It contains D-glucose,

D-mannose, and D-glucuronic acid as the dominant hexose units and is manufactured as the sodium, potassium, or calcium salt.
2. The strain of *Xanthomonas campestris* is nonpathogenic and nontoxic in man or other animals.
3. The additive is produced by a process that renders it free of viable cells of *Xanthomonas campestris.*
4. The additive is used or intended for use in accordance with GMP as a stabilizer, emulsifier, thickener, suspending agent, bodying agent, or foam enhancer in foods for which standards of identity established do not preclude such use.
5. The label of its container should bear, in addition to other required information, the name of the additive and the designation "food grade."
6. The label or labeling of the food additive container should bear adequate directions for use.

47.2.7 Gibberellic Acid and Its Potassium Salt (21 CFR 172.725)

The food additives gibberellic acid and its potassium salt may be used in the malting of barley in accordance with the following prescribed conditions:

1. The additives meet the following specifications
2. The gibberellic acid is produced by deep-culture fermentation of a suitable nutrient medium by a strain of *Fusarium moniliforme* or a selection of this culture
3. The empirical formula of gibberellic acid is represented by $C_{19}H_{22}O_6$
4. Potassium gibberellate is the potassium salt of the specified gibberellic acid
5. The gibberellic acid or potassium gibberellate may be diluted with substances GRAS in foods or with salts of fatty acids as permitted under regulations
6. They are used or intended for use in the malting of barley under conditions whereby the amount of either or both additives present in the malt is not in excess of 2 ppm expressed as gibberellic acid and the treated malt is to be used in the production of fermented malt beverages or distilled spirits only, whereby the finished distilled spirits contain none and the finished malt beverage contains not more than 0.5 ppm of gibberellic acid
7. The label of the package should bear, in addition to the other required information
   a. The name of the additive, "gibberellic acid" or "potassium gibberellate," whichever is appropriate
   b. An accurate statement of the concentration of the additive contained in the package
   c. Adequate use directions to provide not more than 2 ppm of gibberellic acid in the finished malt
   d. Adequate labeling directions to provide that the final malt is properly labeled as required

The label of the treated malt should bear, in addition to the other required information, the statements:

1. "Contains not more than 2 ppm ___," the blank being filled in with the words "gibberellic acid" or "potassium gibberellate," whichever is appropriate
2. "Brewer's malt—To be used in the production of fermented malt beverages only" or "Distiller's malt—To be used in the production of distilled spirits only," whichever is appropriate

47.2.8 Dried Yeasts (21 CFR 172.896)

Dried yeast (*Saccharomyces cerevisiae* and *Saccharomyces fragilis*) and dried torula yeast (*Candida utilis*) may be safely used in food provided the total folic acid content of the yeast does not exceed 0.04 mg per gram of yeast (approximately 0.008 mg of pteroyglutamic acid per gram of yeast).

47.2.9 Bakers Yeast Glycan (21 CFR 172.898)

Bakers yeast glycan may be safely used in food in accordance with the following conditions:

1. Bakers yeast glycan is the comminuted, washed, pasteurized, and dried cell walls of the yeast, *Saccharomyces cerevisiae*. It is composed principally of long chain carbohydrates, not less than 85% on a dry solids basis. The carbohydrate is composed of glycan and mannan units in approximately a 2:1 ratio.
2. The viable microbial content of the finished ingredient is
   a. Less than 10,000 organisms/gram by aerobic plate count.
   b. Less than 10 yeasts and molds/gram.
   c. Negative for *Salmonella*, *E. coli*, coagulase positive *Staphylococci*, *Clostridium perfringens*, *Clostridium botulinum*, or any other recognized microbial pathogen or any harmful microbial toxin.

The additive is used or intended for use in the foods presented in Table 47.2 when standards of identity do not preclude such use. The label and labeling of the ingredient should bear adequate directions to assure that use of the ingredient complies with regulation.

47.2.10 Amyloglucosidase Derived from *Rhizopus niveus* (21 CFR 173.110)

Amyloglucosidase enzyme product, consisting of enzyme derived from *Rhizopus niveus*, and with diatomaceous silica as a carrier, may be safely used in food in accordance with the following conditions:

1. *Rhizopus niveus* is classified as follows: class, Phycomycetes; order, Mucorales; family, Mucoraceae; genus, *Rhizopus*; species, *niveus*
2. The strain of *Rhizopus niveus* is nonpathogenic and nontoxic in man or other animals
3. The enzyme is produced by a process which completely removes the organism *Rhizopus niveus* from the amyloglucosidase
4. The additive is used or intended for use for degrading gelatinized starch into constituent sugars, in the production of distilled spirits and vinegar
5. The additive is used at a level not to exceed 0.1% by weight of the gelatinized starch

47.2.11 Carbohydrase and Cellulase Derived from *Aspergillus niger* (21 CFR 173.120)

Carbohydrase and cellulase enzyme preparation derived from *Aspergillus niger* may be safely used in food in accordance with the following prescribed conditions:

1. *Aspergillus niger* is classified as follows: class, Deuteromycetes; order, Moniliales; family, Moniliaceae; genus, *Aspergillus;* species, *niger*

TABLE 47.2

Use of Bakers Yeast Glycan in Foods and Limitations

Use	Limitations
In salad dressings as an emulsifier and emulsifier salt, stabilizer, and thickener or texturizer	Not to exceed a concentration of 5% of the finished salad dressing.
In frozen dessert analogs as a stabilizer and thickener or texturizer	Not to exceed GMP
In sour cream analogs as a stabilizer and thickener or texturizer	Same as above
In cheese spread analogs as a stabilizer and thickener or texturizer	Same as above
In cheese- and sour cream–flavored snack dips, as a stabilizer and thickener or texturizer	Same as above

2. The strain of *Aspergillus niger* is nonpathogenic and nontoxic in man or other animals
3. The additive is produced by a process that completely removes the organism *Aspergillus niger* from the carbohydrase and cellulase enzyme product
4. The additive is used or intended for use as follows:
   a. For removal of visceral mass (bellies) in clam processing
   b. As an aid in the removal of the shell from the edible tissue in shrimp processing
5. The additive is used in an amount not in excess of the minimum required to produce its intended effect

47.2.12 Carbohydrase Derived from *Rhizopus oryzae* (21 CFR 173.130)

Carbohydrase from *Rhizopus oryzae* may be safely used in the production of dextrose from starch in accordance with the following prescribed conditions:

1. *Rhizopus oryzae* is classified as follows: class, Phycomycetes; order, Mucorales; family, Mucoraceae; genus, *Rhizopus;* species, *Rhizopus oryzae*
2. The strain of *Rhizopus oryzae* is nonpathogenic and nontoxic
3. Carbohydrase is produced under controlled conditions to maintain nonpathogenicity and nontoxicity, including the absence of aflatoxin
4. Carbohydrase is produced by a process which completely removes the organism *Rhizopus oryzae* from the carbohydrase product
5. Carbohydrase is maintained under refrigeration from production to use and is labeled to include the necessity of refrigerated storage

47.2.13 Catalase Derived from *Micrococcus lysodeikticus* (21 CFR 173.135)

Bacterial catalase derived from *Micrococcus lysodeikticus* by a pure culture fermentation process may be safely used in destroying and removing hydrogen peroxide used in the manufacture of cheese, in accordance with the following conditions.

1. The organism *Micrococcus lysodeikticus* from which the bacterial catalase is to be derived is demonstrated to be nontoxic and nonpathogenic
2. The organism *Micrococcus lysodeikticus* is removed from the bacterial catalase before use of the bacterial catalase
3. Bacterial catalase is used in an amount not in excess of the minimum required to produce its intended effect

47.2.14 Esterase-Lipase Derived from *Mucor miehei* (21 CFR 173.140)

Esterase-lipase enzyme, consisting of enzyme derived from *Mucor miehei* var. *Cooney et Emerson* by a pure culture fermentation process, with maltodextrin or sweet whey as a carrier, may be safely used in food in accordance with the following conditions:

1. *Mucor miehei* var. *Cooney et Emerson* is classified as follows: class, Phycomycetes; subclass, Zygomycetes; order, Mucorales; family, Mucoraceae; genus, *Mucor*; species, *miehei*; variety *Cooney et Emerson*
2. The strain of *Mucor miehei* var. *Cooney et Emerson* is nonpathogenic and nontoxic in man or other animals

3. The enzyme is produced by a process which completely removes the organism *Mucor miehei* var. *Cooney et Emerson* from the esterase-lipase
4. The enzyme is used as a flavor enhancer
5. The enzyme is used at levels not to exceed current GMP in the following food categories: cheeses, fat and oils, milk products
6. Use of this food ingredient is limited to nonstandardized foods and those foods for which the relevant standards of identity permit such use
7. The enzyme is used in the minimum amount required to produce its limited technical effect

47.2.15 α-Galactosidase Derived from *Mortierella vinaceae* var. *raffinoseutilizer* (21 CFR 173.145)

The food additive α-galactosidase and parent mycelial microorganism *Mortierella vinaceae* var. *raffinoseutilizer* may be safely used in food in accordance with the following conditions:

1. The food additive is the enzyme α-galactosidase and the mycelia of the microorganism *Mortierella vinaceae* var. *raffinoseutilizer*, which produces the enzyme
2. The additive is used or intended for use in the production of sugar (sucrose) from sugar beets by addition as mycelial pellets to the molasses to increase the yield of sucrose, followed by removal of the spent mycelial pellets by filtration
3. Enzyme removal is such that there are no enzyme or mycelial residues remaining in the finished sucrose

47.2.16 Milk-Clotting Enzymes, Microbial (21 CFR 173.150)

Milk-clotting enzyme produced by pure-culture fermentation process may be safely used in the production of cheese in accordance with the following prescribed conditions:

1. Milk-clotting enzyme is derived from one of the following organisms by a pure-culture fermentation process
2. *Endothia parasitica* classified as follows: class, Ascomycetes; order, Sphaeriales; family, Diaporthacesae; genus, *Endothia*; species, *parasitica*
3. *Bacillus cereus* classified as follows: class, Schizomycetes; order, Eubacteriales; family, Bacillaceae; genus, *Bacillus*; species, *cereus* (Frankland and Frankland)
4. *Mucor pusillus* Lindt classified as follows: class, Phycomycetes; subclass, Zygomycetes; order, Mucorales; family, Mucoraceae; genus, *Mucor*; species, *pusillus*; variety, Lindt
5. *Mucor miehei Cooney et Emerson* classified as follows: class, Phycomycetes; subclass, Zygomycetes; order, Mucorales; family, Mucoraceae; genus, *Mucor*; species, *miehei*; variety, *Cooney et Emerson*
6. *Aspergillus oryzae* modified by recombinant DNA techniques to contain the gene coding for aspartic proteinase from *Rhizomucor miehei* var. *Cooney et Emerson*, classified as follows: class, Blastodeuteromycetes (Hyphomycetes); order, Phialidales (Moniliales); genus, *Aspergillus*; species *oryzae*
7. The strains of organism identified are nonpathogenic and nontoxic in man or other animals
8. The additive is produced by a process that completely removes the generating organism from the milk-clotting enzyme product
9. The additive is used in an amount not in excess of the minimum required to produce its intended effect in the production of those cheeses permitted by standards of identity established

47.2.17 *Candida guilliermondii* (21 CFR 173.160)

The food additive *Candida guilliermondii* may be safely used as the organism for fermentation production of citric acid in accordance with the following conditions:

1. The food additive is the enzyme system of the viable organism *Candida guilliermondii* and its concomitant metabolites produced during the fermentation process
2. The organism *Candida quilliermondii* is made nonviable and is completely removed from the citric acid during the recovery and purification process

47.2.18 *Candida lipolytica* (21 CFR 173.165)

The food additive *Candida lipolytica* may be safely used as the organism for fermentation production of citric acid in accordance with the following conditions:

1. The food additive is the enzyme system of the organism *Candida lipolytica* and its concomitant metabolites produced during the fermentation process
2. The additive is used or intended for use as a pure culture in the fermentation process for the production of citric acid from purified normal alkanes

47.2.19 Solvent Extraction Process for Citric Acid (21 CFR 173.280)

A solvent extraction process for recovery of citric acid from conventional *Aspergillus niger* fermentation liquor may be safely used to produce food-grade citric acid in accordance with the following conditions:

1. The solvent used in the process consists of a mixture of *n*-octyl alcohol, synthetic isoparaffinic petroleum hydrocarbons, and tridodecyl amine.
2. The component substances are used solely as a solvent mixture and in a manner that does not result in formation of products not present in conventionally produced citric acid.
3. Residues of *n*-octyl alcohol and synthetic isoparaffinic petroleum hydrocarbons are removed in accordance with GMP. Current GMP results in residues not exceeding 16 ppm (ppm) *n*-octyl alcohol and 0.47 ppm synthetic isoparaffinic petroleum hydrocarbons in citric acid.

47.3 Microorganisms and Food Additives Listed in 21 CFR 184

This lists covers substances derived from microorganisms (including seaweed) affirmed by FDA as GRAS in 21 CFR184 (see Table 47.3).

47.3.1 Acetic Acid (21 CFR 184.1005)

Acetic acid ($C_2H_4O_2$) is known as ethanoic acid. It occurs naturally in plant and animal tissues. It is produced by fermentation of carbohydrates or by organic synthesis. The principal synthetic methods currently employed are oxidation of acetaldehyde derived from ethylene, liquid phase oxidation of butane, and reaction of carbon monoxide with methanol derived from natural gas.

1. The ingredient is used as a curing and pickling agent, flavor enhancer, flavoring agent, and adjuvant; pH control agent; as a solvent and vehicle; and as a boiler water additive.
2. The ingredient is used in food at levels not to exceed current (GMP). Current GMP results in a maximum level as served, of 0.25% for baked goods; 0.8% for cheeses and dairy product analogs; 0.5% for chewing gum; 9.0% for condiments and relishes; 0.5% for fats and oils; 3.0%

TABLE 47.3

Substances Derived from Microorganisms Affirmed by FDA as GRAS in 21 CFR184

Section in 21 CFR	Ingredient or Substance
§184.1005	Acetic acid may be produced by fermentation
§184.1011	Alginic acid made from certain brown algae
§184.1012	α-Amylase enzyme preparation from *Bacillus stearothermophilus* used to hydrolyze edible starch to produce maltodextrin and nutritive carbohydrate sweeteners
§184.1027	Mixed carbohydrase and protease enzyme product derived from *Bacillus licheniformis* for use in hydrolyzing proteins and carbohydrates in the preparation of alcoholic beverages, candy, nutritive sweeteners, and protein hydrolysates
§184.1061	Lactic acid may be produced by fermentation
§184.1081	Propionic acid from bacterial fermentation
§184.1115	Agar-agar, extracted from a number of related species of red algae class Rhodophyceae
§184.1120	Brown algae, to be used dried as a flavor enhancer, are seaweeds of the species: *Analipus japonicus*, *Eisenia bicyclis*, *Hizikia fusiforme*, *Kjellmaniella gyrata*, *Laminaria angustata*, *Laminaria longirruris*, *Laminaria Longissima*, *Laminaria ochotensis*, *Laminaria claustonia*, *Laminaria saccharina*, *Laminaria digitata*, *Laminaria japonica*, *Macrocystis pyrifera*, *Petalonia fascia*, *Scytosiphon lome*
§184.1121	Red algae, to be used dried as a flavor enhancer, are seaweeds of the species: *Gloiopeltis furcata*, *Porphyra crispata*, *Porhyra deutata*, *Porhyra perforata*, *Porhyra suborbiculata*, *Porphyra tenera*, *Rhodymenis palmata*
§184.1133	Ammonium alginate from certain brown algae
§184.1187	Calcium alginate from certain brown algae
§184.1318	Glucono delta-lactone, by oxidation of D-glucose by microorganisms that are nonpathogenic and nontoxicogenic to man or other animals. These include but are not restricted to *Aspergillus niger* and *Acetobactor suboxydans*
§184.1372	Insoluble glucose isomerase enzyme preparations are derived from recognized species of precisely classified, nonpathogenic, and nontoxicogenic microorganisms, including *Streptomyces rubiginosus*, *Actinoplane missouriensis*, *Streptomyces olivaceus*, *Streptomyces olivochromogenes*, and *Bacillus coagulans* grown in a pure culture fermentation that produces no antibiotic
§184.1387	Lactase enzyme preparation from *Candida pseudotropicalis* for use in hydrolyzing lactose to glucose and galactose
§184.1388	Lactase enzyme preparation from *Kluyveromyces lactis* (previously called *Saccharomyces lactis*) for use in hydrolyzing lactose in milk
§184.1420	Lipase enzyme preparation from *Rhizopus niveus* used in the interesterfication of fats and oils
§184.1538	Nisin preparation from *Lactococcus lactis* Lancefield Group N for use as an antimicrobial agent to inhibit the outgrowth of *Clostridium botulinum* spores and toxin formation in pasteurized cheese spreads
§184.1610	Potassium alginate, the potassium salt of alginic acid, derived from certain brown algae
§184.1685	Rennet (animal-derived) and chymosin preparation from *Escherichia coli* K-12, *Kluyveromyces marxianus* var. *lactis* or *Aspergillus niger* var. *awamori* to coagulate milk in cheeses and other dairy products
§184.1695	Riboflavin biosynthesized by *Eremothecium ashbyii*
§184.1724	Sodium alginate, the sodium salt of alginic acid, derived from certain brown algae
§184.1848	Butter starter distillate from milk cultures of *Streptococcus lactis*, *Streptococcus cremoris*. *Streptococcus lactis* subspecies *diacetylactis*, *Leuconostoc citovorum*, *Leuconostoc dextranicum*
§184.1924	Urease enzyme preparation from *Lactobacillus fermentum* for use in the production of wine
§184.1945	Vitamin B_{12} from *Streptomyces griseus*
§184.1950	Vitamin D, produced by ultraviolet irradiation of ergosterol isolated from yeast and related fungi
§184.1983	Bakers yeast extract from *Saccharomyces cerevisiae*
§184.1985	Aminopeptidase enzyme preparation from *Lactococcus lactis* used as an optional ingredient for flavor development in the manufacture of cheddar cheese

for gravies and sauces; 0.6% for meat products; and 0.15% or less for all other food categories. The ingredient may also be used in boiler water additives at levels not to exceed current GMP.

47.3.2 Alginic Acid (21 CFR 184.1011)

Alginic acid is a colloidal, hydrophilic polysaccharide obtained from certain brown algae by alkaline extraction. The ingredient is used in food only within the following specific limitations:

1. Category of food: soup and soup mixes
2. Maximum level of use in food (as served): not to exceed current GMP
3. Functional use: emulsifier, emulsifier salt, formulation aid, stabilizer, and thickener

47.3.3 α-Amylase Enzyme Preparation from *Bacillus stearothermophilus* (21 CFR 184.1012)

1. α-Amylase enzyme preparation is obtained from the culture filtrate that results from a pure culture fermentation of a nonpathogenic and nontoxicogenic strain of *Bacillus stearothermophilus*. Its characterizing enzyme activity is α-amylase (1,4 α-D-glucan glucanohydrolase, EC 3.2.1.1).
2. The ingredient is used in food with no limitation other than current GMP. The affirmation of this ingredient as GRAS as a direct human food ingredient is based upon the following current GMP conditions of use:
   a. The ingredient is used as an enzyme in the hydrolysis of edible starch to produce maltodextrins and nutritive carbohydrate sweeteners.
   b. The ingredient is used at levels not to exceed current GMP.

47.3.4 Mixed Carbohydrase and Protease Enzyme Product (21 CFR 184.1027)

1. Mixed carbohydrase and protease enzyme product is an enzyme preparation that includes carbohydrase and protease activity. It is obtained from the culture filtrate resulting from a pure culture fermentation of a nonpathogenic strain of *B. licheniformis.*
2. The ingredient is used as an enzyme to hydrolyze proteins or carbohydrates.
3. The ingredient is used in the following foods at levels not to exceed current GMP: alcoholic beverages, candy, nutritive sweeteners, and protein hydrolyzates.

47.3.5 Lactic Acid (21 CFR 184.1061)

1. Lactic acid ($C_3H_6O_3$), the chemical 2-hydroxypropanoic acid, occurs naturally in several foods. It is produced commercially either by fermentation of carbohydrates such as glucose, sucrose, or lactose, or by a procedure involving formation of lactonitrile from acetaldehyde and hydrogen cyanide and subsequent hydrolysis to lactic acid.
2. The ingredient is used in food with no limitation other than current GMP. The affirmation of this ingredient as GRAS as a direct human food ingredient is based upon the following current GMP conditions of use:
   a. The ingredient is used as an antimicrobial agent; a curing and pickling agent; a flavor enhancer, a flavoring agent, and adjuvant; a pH control agent; and a solvent and vehicle.
   b. The ingredient is used in food, except in infant foods and infant formulas, at levels not to exceed current GMP.

47.3.6 Propionic Acid (21 CFR 184.1081)

1. Propionic acid ($C_3H_6O_2$) is an oily liquid having a slightly pungent, rancid odor. It is manufactured by chemical synthesis or by bacterial fermentation.
2. The ingredient is used in food with no limitation other than current GMP. The affirmation of this ingredient as GRAS as a direct human food ingredient is based upon the following current GMP conditions of use:
3. The ingredient is used as an antimicrobial agent and a flavoring agent.
4. The ingredient is used in foods at levels not to exceed current GMP.

47.3.7 Agar-Agar (21 CFR 184.1115)

1. Agar-agar is a dried, hydrophyllic, colloidal polysaccharide extracted from one of a number of related species of red algae (class Rhodophyceae)
2. The ingredient is used in food under conditions indicated in Table 47.4

47.3.8 Brown Algae (21 CFR 184.1120)

1. Brown algae are seaweeds of the species *Analipus japonicus*, *Eisenia bicyclis*, *Hizikia fusiforme*, *Kjellmaniella gyrata*, *Laminaria angustata*, *Laminaria claustonia*, *Laminaria digitata*, *Laminaria japonica*, *Laminaria longicruris*, *Laminaria longissima*, *Laminaria ochotensis*, *Laminaria saccharina*, *Macrocystis pyrifera*, *Petalonia fascia*, *Scytosiphon lomentaria*, and *Undaria pinnatifida*. They are harvested principally in coastal waters of the northern Atlantic and Pacific oceans. The material is dried and ground or chopped for use in food.
2. The ingredient is used in food only within the following specific limitations:
   a. Category of food: spices, seasonings, and flavorings
   b. Maximum level of use in food (as served): not to exceed current GMP
   c. Functional use: flavor enhancer, flavor adjuvant

47.3.9 Red Algae (21 CFR 184.1121)

1. Red algae are seaweeds of the species *Gloiopeltis furcata*, *Porphyra crispata*, *Porphyra deutata*, *Porphyra perforata*, *Porphyra suborbiculata*, *Porphyra tenera*, and *Rhodymenia palmata*. *Porphyra* and *Rhodymenia* are harvested principally along the coasts of Japan, Korea, China, Taiwan, and the East and West coasts of the United States. *Gloiopeltis* is harvested principally in southern Pacific coastal waters. The material is dried and ground or chopped for use in food.
2. The ingredient is used in food only within the following specific limitations:
   a. Category of food: spices, seasonings, and flavorings
   b. Maximum level of use in food (as served): not to exceed current GMP
   c. Functional use: flavor enhancer

TABLE 47.4

Foods and Usage Levels for Agar-Agar

Foods (as served)	Maximum Usage Levels Permitted (%)	Functions
Baked goods and baking mixes	0.8	Drying agent, flavoring agent, stabilizer, thickener
Confections and frostings	2.0	Flavoring agent, stabilizer, thickener, surface finisher
Soft candy	1.2	Stabilizer and thickener
All other food categories	.25	Flavoring agent, formulation aid, humectant, stabilizer, thickener

TABLE 47.5

Ammonium Alginate: Food, Level, and Functions

Category of Food	Maximum Level of Use in Food (as Served) (%)	Functional Use
Confections and frostings	0.4	Stabilizer, thickener, §170.3(o)(28) of this chapter
Fats and oils	0.5	Same as above
Gelatins and puddings	0.5	Same as above
Gravies and sauces	0.4	Same as above
Jams and jellies	0.4	Same as above
Sweet sauces	0.5	Same as above
All other food categories	0.1	Humectant, stabilizer, thickener

47.3.10 Ammonium Alginate (21 CFR 184.1133)

Ammonium alginate is the ammonium salt of alginic acid, a natural polyuronide constituent of certain brown algae. Ammonium alginate is prepared by the neutralization of purified alginic acid with appropriate pH control agents. The ingredient is used in food only within the specific limitations in Table 47.5.

47.3.11 Calcium Alginate (21 CFR 184.1187)

1. Calcium alginate is the calcium salt of alginic acid, a natural polyuronide constituent of certain brown algae. Calcium alginate is prepared by the neutralization of purified alginic acid with appropriate pH control agents, or from sodium alginate by metathesis with appropriate calcium salts.
2. The ingredient is used in food only within the specific limitations in Table 47.6.

47.3.12 Glucono delta-Lactone (21 CFR 184.1318)

1. Glucono delta-lactone ($C_6H_{10}O_6$), also called *D*-gluconic acid delta-lactone or *D*-glucono-1,5-lactone, is the cyclic 1,5-intramolecular ester of *D*-gluconic acid. It is prepared by direct crystallization from the aqueous solution of gluconic acid. Gluconic acid may be produced by the oxidation of *D*-glucose with bromine water, by the oxidation of *D*-glucose by microorganisms that are nonpathogenic and nontoxicogenic to man or other animals, or by the oxidation of *D*-glucose with enzymes derived from these microorganisms.
2. The ingredient is used in food with no limitation other than current GMP. The affirmation of this ingredient as GRAS as a direct human food ingredient is based upon the following current GMP conditions of use:

TABLE 47.6

Calcium Alginate: Foods, Levels, and Functions

Category of Food	Maximum Level of Use in Food (as Served) (%)	Functional Use
Baked goods	0.002	Stabilizer, thickener
Alcoholic beverages	0.4	Same as above
Confections and frostings	0.4	Same as above
Egg products	0.6	Same as above
Fats and oils	0.5	Same as above
Gelatins and puddings	0.25	Same as above
Gravies and sauces	0.4	Same as above
Jams and jellies	0.5	Same as above
Sweet sauces	0.5	Same as above
All other food categories	0.3	Same as above

a. The ingredient is used as a curing and pickling agent, leavening agent, pH control agent, and sequestrant.
b. The ingredient is used at levels not to exceed current GMP.

47.3.13 Insoluble Glucose Isomerase Enzyme Preparations (21 CFR 184.1372)

1. Insoluble glucose isomerase enzyme preparations are used in the production of high-fructose corn syrup. They are derived from recognized species of precisely classified nonpathogenic and nontoxicogenic microorganisms, including *Streptomyces rubiginosus*, *Actinoplanes missouriensis*, *Streptomyces olivaceus*, *Streptomyces olivochromogenes*, and *Bacillus coagulans*, that have been grown in a pure culture fermentation that produces no antibiotics. They are fixed (rendered insoluble) for batch production with GRAS ingredients or may be fixed for further immobilization with either GRAS ingredients or approved materials.
2. The ingredient is used in food with no limitation other than current GMP. The affirmation of this ingredient as GRAS as a direct human food ingredient is based upon the following current GMP conditions of use:
   a. The ingredient is used as an enzyme to convert glucose to fructose.
   b. The ingredient is used in high-fructose corn syrup, at levels not to exceed current GMP.

47.3.14 Lactase Enzyme Preparation from *Candida pseudotropicalis* (21 CFR 184.1387)

1. This enzyme preparation is derived from the nonpathogenic, nontoxicogenic yeast *C. pseudotropicalis*. It contains the enzyme lactase (β-D-galactoside galactohydrolase, EC 3.2.1.23), which converts lactose to glucose and galactose. It is prepared from yeast that has been grown by a pure culture fermentation process.
2. The ingredient is used in food with no limitations other than current GMP. The affirmation of this ingredient as GRAS as a direct human food ingredient is based upon the following current GMP conditions of use:
   a. The ingredient is used as an enzyme to convert lactose to glucose and galactose.
   b. The ingredient is used in food at levels not to exceed current GMP. Current GMP is limited to use of this ingredient to reduce the lactose content in milk and milk-derived food products where food standards do not preclude such use.

47.3.15 Lactase Enzyme Preparation from *Kluyveromyces lactis* (21 CFR 184.1388)

1. This enzyme preparation is derived from the nonpathogenic, nontoxicogenic yeast *Kluyveromyces lactis* (previously named *Saccharomyces lactis*). It contains the enzyme β-galactoside galactohydrase, which converts lactose to glucose and galactose. It is prepared from yeast that has been grown in a pure culture fermentation and by using materials that are GRAS or are food additives that have been approved for this use by the FDA.
2. The ingredient is used as an enzyme to convert lactose to glucose and galactose.
3. The ingredient is used in food at levels not to exceed current GMP. Current GMP is to use this ingredient in milk to produce lactase-treated milk, which contains less lactose than regular milk, or lactose-reduced milk, which contains at least 70% less lactose than regular milk.

47.3.16 Lipase Enzyme Preparation Derived from *Rhizopus niveus* (21 CFR 184.1420)

1. Lipase enzyme preparation involves a lipase enzyme obtained from the culture filtrate resulting from a pure culture fermentation of a nonpathogenic and nontoxigenic strain of *Rhizopus niveus.* The enzyme preparation also contains diatomaceous earth as a carrier. The character-

izing activity of the enzyme, which catalyzes the interesterification of fats and oils at the 1- and 3-positions of triglycerides, is triacylglycerol lipase (EC 3.1.1.3).

2. The ingredient is used in food with no limitation other than current GMP. The affirmation of this ingredient as GRAS as a direct human food ingredient is based upon the following current GMP conditions of use:
   a. The ingredient is used as an enzyme for the interesterification of fats and oils.
   b. The ingredient is used in food at levels not to exceed current GMP.

47.3.17 Nisin Preparation (21 CFR 184.1538)

1. Nisin preparation is derived from pure culture fermentations of certain strains of *Streptococcus lactis* Lancefield Group N. Nisin preparation involves nisin, a group of related peptides with antibiotic activity.
2. The ingredient is used as an antimicrobial agent to inhibit the outgrowth of *Clostridium botulinum* spores and toxin formation in pasteurized cheese spreads and pasteurized process cheese spreads; pasteurized cheese spread with fruits, vegetables, or meats; pasteurized process cheese spread; pasteurized process cheese spread with fruits, vegetables, or meats.
3. The ingredient is used at levels not to exceed GMP. The current GMP level is the quantity of the ingredient that delivers a maximum of 250 ppm of nisin in the finished product.

47.3.18 Potassium Alginate (21 CFR 184.1610)

Potassium alginate is the potassium salt of alginic acid, a natural polyuronide constituent of certain brown algae. Potassium alginate is prepared by the neutralization of purified alginic acid with appropriate pH control agents.

The ingredient is used in food only within the specific limitations in Table 47.7.

47.3.19 Rennet (Animal-Derived) and Chymosin Preparation (Fermentation-Derived) (21 CFR 184.1685)

1. Rennet and bovine rennet are commercial extracts containing the active enzyme rennin, also known as chymosin (EC 3.4.23.4). Rennet is the aqueous extract prepared from cleaned, frozen, salted, or dried fourth stomachs (abomasa) of calves, kids, or lambs. Bovine rennet is the product from adults of the animals listed above. Both products are called rennet and are clear amber to dark brown liquid preparations or white to tan powders.
2. Chymosin preparation is a clear solution containing the active enzyme chymosin (EC 3.4.23.4). It is derived, via fermentation, from a nonpathogenic and nontoxigenic strain of *Escherichia coli* K-12 containing the prochymosin gene. The prochymosin is isolated as an insoluble aggregate that is acid-treated to destroy residual cellular material and, after solubilization, is acid-treated to form chymosin. It must be processed with materials that are GRAS or are food additives that have been approved by the FDA for this use.

TABLE 47.7

Potassium Alginate: Foods, Levels, and Functions

Category of Food	Maximum Level of Use in Food (as Served) (%)	Functional Use
Confections and frostings	0.1	Stabilizer and thickener
Gelatins and puddings	0.7	Same as above
Processed fruits and fruit juices	0.25	Same as above
All other food categories	0.01	Same as above

3. Chymosin preparation is a clear solution containing the active enzyme chymosin (EC 3.4.23.4). It is derived, via fermentation, from a nonpathogenic and nontoxigenic strain of *Kluyveromyces marxianus* variety *lactis*, containing the prochymosin gene. The prochymosin is secreted by cells into fermentation broth and converted to chymosin by acid treatment. All materials used in the processing and formulating of chymosin must be either GRAS or be food additives that have been approved by the FDA for this use.
4. Chymosin preparation is a clear solution containing the active enzyme chymosin (EC 3.4.23.4). It is derived, via fermentation, from a nonpathogenic and nontoxigenic strain of *Aspergillus niger* van Tieghem variety *awamori* (Nakazawa) Al-Musallam (synonym *A. awamori* Nakazawa) containing the prochymosin gene. Chymosin is recovered from the fermentation broth after acid treatment. All materials used in the processing and formulating of chymosin preparation must be either GRAS or be food additives that have been approved by the FDA for this use.
5. The ingredient is used in food with no limitation other than current GMP. The affirmation of this ingredient as GRAS as a direct human food ingredient is based upon the following current GMP conditions of use:
   a. The ingredient is used as an enzyme, a processing aid, and a stabilizer and thickener.
   b. The ingredient is used in the following foods at levels not to exceed current GMP: cheeses; frozen dairy desserts and mixes; gelatins, puddings, and fillings; and milk products.

47.3.20 Riboflavin (21 CFR 184.1695)

1. Riboflavin ($C_{17}H_{20}N_4O_6$) occurs as yellow to orange-yellow needles that are crystallized from 2 N acetic acid, alcohol, water, or pyridine. It may be prepared by chemical synthesis, biosynthetically by the organism *Eremothecium ashbyii or* isolated from natural sources.
2. The ingredient is used in food with no limitation other than current GMP. The affirmation of this ingredient as GRAS as a direct human food ingredient is based upon the following current GMP conditions of use:
   a. The ingredient is used as a nutrient supplement.
   b. The ingredient is used in foods at levels not to exceed current GMP. The ingredient may also be used in infant formula.

47.3.21 Sodium Alginate (21 CFR 184.1724)

1. Sodium alginate is the sodium salt of alginic acid, a natural polyuronide constituent of certain brown algae. Sodium alginate is prepared by the neutralization of purified alginic acid with appropriate pH control agents.
2. The ingredient is used in food only within the following specific limitations:

   Table 47.8 describes the foods, levels, and functions for use of sodium alginate.

47.3.22 Starter Distillate (21 CFR 184.1848)

1. Starter distillate (butter starter distillate) is a steam distillate of the culture of any or all of the following species of bacteria grown on a medium consisting of skim milk usually fortified with about 0.1% citric acid: *Streptococcus lactis*, *S. cremoris*, *S. lactis subsp. diacetylactis*, *Leuconostoc citrovorum*, and *L. dextranicum*. The ingredient contains more than 98% water, and the remainder is a mixture of butterlike flavor compounds. Diacetyl is the major flavor component, constituting as much as 80% to 90% of the mixture of organic flavor compounds. Besides diacetyl, starter distillate contains minor amounts of acetaldehyde, ethyl formate, ethyl acetate, acetone, ethyl alcohol, 2-butanone, acetic acid, and acetoin.

TABLE 47.8

Sodium Alginate: Foods, Levels, and Functions

Category of Food	Maximum Level of Use in Food as Served (%)	Functional Use
Condiments and relishes except pimento ribbon for stuffed olives	1.0	Texturizer, formulation aid, stabilizer, thickener
Pimento ribbon for stuffed olives	6.0	Same as above
Confections and frostings	0.3	Stabilizer, thickener
Gelatins and puddings	4.0	Firming agent, flavor adjuvant; stabilizer, thickener
Hard candy	10.0	Stabilizer, thickener
Processed fruits and fruit juices	2.0	Formulation aid and texturizer
All other food categories	1.0	Emulsifier, firming agent, flavor enhancer, flavor adjuvant, processing aid, stabilizer and thickener, surface active agent

2. The ingredient is used in food with no limitation other than current GMP. The affirmation of this ingredient as GRAS as a direct human food ingredient is based upon the following current GMP conditions of use:
   a. The ingredient is used as a flavoring agent and adjuvant.
   b. The ingredient is used in food at levels not to exceed current GMP.

47.3.23 Urease Enzyme Preparation from *Lactobacillus fermentum* (21 CFR 184.1924)

1. This enzyme preparation is derived from the nonpathogenic, nontoxicogenic bacterium *Lactobacillus fermentum*. It contains the enzyme urease, which facilitates the hydrolysis of urea to ammonia and carbon dioxide. It is produced by a pure culture fermentation process and by using materials that are GRAS or are food additives that have been approved for this use by the FDA.
2. The ingredient is used in food with no limitation other than current GMP. The affirmation of this ingredient as GRAS as a direct human food ingredient is based upon the following current GMP conditions of use:
   a. The ingredient is used in wine as an enzyme to convert urea to ammonia and carbon dioxide.
   b. The ingredient is used in food at levels not to exceed current GMP. Current GMP is limited to use of this ingredient in wine to inhibit formation of ethyl carbamate.

47.3.24 Vitamin B_{12} (21 CFR 184.1945)

1. Vitamin B_{12}, also known as cyanocobalamin ($C_{63}H_{88}CoN_{14}O_{14}P$), is produced commercially from cultures of *Streptomyces griseus*.
2. The ingredient is used in food with no limitation other than current GMP. The affirmation of this ingredient as GRAS as a direct human food ingredient is based upon the following current GMP conditions of use:
   a. The ingredient is used as a nutrient supplement.
   b. The ingredient is used in food at levels not to exceed current GMP. Vitamin B_{12} also may be used in infant formula.

47.3.25 Vitamin D (21 CFR 184.1950)

Vitamin D is added to food as the following food ingredients:

1. Crystalline vitamin D_2 ($C_{28}H_{44}O$), also known as ergocalciferol, is the chemical 9,10-seco(5*Z*,7*E*,22*E*)-5,7,10(19),22-ergostatetraen-3-ol. The ingredient is produced by ultraviolet irradiation of ergosterol isolated from yeast and related fungi and is purified by crystallization.
2. Crystalline vitamin D_3 ($C_{27}H_{44}O$), also known as cholecalciferol, is the chemical 9,10-seco(5*Z*,7*E*,)-5,7,10(19)-cholestatrien-3-ol. Vitamin D_3 occurs in, and is isolated from, fish liver oils. It is also manufactured by ultraviolet irradiation of 7-dehydrocholesterol produced from cholesterol. It is purified by crystallization. Vitamin D_3 is the vitamin D form that is produced endogenously in humans through sunlight activation of 7-dehydrocholesterol in the skin.
3. Vitamin D_2 resin and vitamin D_3 resin are the concentrated forms of irradiated ergosterol (D_2) and irradiated 7-dehydrocholesterol (D_3) that are separated from the reacting materials. The resulting products are sold as food sources of vitamin D without further purification.
4. The ingredients are used in food as the sole source of added vitamin D only within the specific limitations in Table 47.9.
5. Vitamin D may be used in infant formula.
6. Vitamin D may be used in margarine.

47.3.26 Bakers Yeast Extract (21 CFR 184.1983)

Bakers yeast extract is the food ingredient resulting from concentration of the solubles of mechanically ruptured cells of a selected strain of yeast, *Saccharomyces cerevisiae*. It may be concentrated or dried. The ingredient is used as a flavoring agent and adjuvant at a level not to exceed 5% in food.

47.3.27 Aminopeptidase Enzyme Preparation Derived from *Lactococcus lactis* (21 CFR 184.1985)

1. Aminopeptidase enzyme preparation is derived from the nonpathogenic and nontoxicogenic bacterium *Lactococcus lactis* (previously named *Streptococcus lactis*). The preparation contains the enzyme aminopeptidase (EC 3.4.11.1) and other peptidases that hydrolyze milk proteins. The preparation is produced by pure culture fermentation.
2. The ingredient is used in food with no limitations other than current GMP. The affirmation of this ingredient as GRAS as a direct human food ingredient is based upon the following current GMP conditions of use:
3. The ingredient is used as an enzyme, and as an optional ingredient for flavor development in the manufacture of cheddar cheese, and in the preparation of protein hydrolysates.
4. The ingredient is used at levels not to exceed current GMP.

TABLE 47.9

Vitamin D: Foods, Levels, and Functions

Category of Food	Maximum Levels in Food (as Served)	Functional Use
Breakfast cereals	350 (IU/100 g)	Nutrient supplement
Grain products and pastas	90 (IU/100 g)	Same as above
Milk	42 (IU/100 g)	Same as above
Milk products	89 (IU/100 g)	Same as above

TABLE 47.10

Substances Derived from Microorganisms Recognized by FDA as GRAS in Opinion Letters

Enzymes
Carbohydrase, cellulase, glucose oxidase-catalase, pectinase, and lipase from *Aspergillus niger*
Carbohydrase and protease from *Aspergillus oryzae*
Carbohydrase and protease from *Bacillus subtilis*
Invertase from edible baker's yeast or brewer's yeast (*Saccharomyces cerevisiae*)

47.4 Microorganisms and Food Additives Issued in FDA Opinion Letters

See Table 47.10.

47.5 Microorganisms and Food Additives Listed in 21 CFR 186

These are substances derived from microorganisms affirmed by FDA as GRAS for indirect uses in foods in 21 CFR186.

47.5.1 Dextrans (21 CFR 186.1275)

Dextrans are high-molecular-weight polysaccharides produced by bacterial fermentation of sucrose. Commercially available dextrans are synthesized from sucrose by *Leuconostoc mesenteroides* strain NRRL B-512(F). Partial depolymerization and purification of the fermented mixture should produce a product that is free of viable microorganisms. The ingredient is used or intended for use as a constituent of food-contact surfaces. The ingredient is used at levels not to exceed GMP.

47.5.2 Sorbose (21 CFR 186.1839)

Sorbose (L-sorbose, sorbinose) is an orthorhombic, bisphenoidal crystalline ketohexose. It was originally identifed in the juice of mature berries from the mountain ash (*Sorbus aucuparia*) where it occurs as the result of microbial oxidation of sorbitol. It also occurs naturally in other plants. Sorbose can be synthesized by the catalytic hydrogenation of glucose to D-sorbitol. The resulting sorbitol can be oxidized by *Acetobacter xylinum* or by *Acetobacter suboxydans*. The ingredient is used or intended for indirect food use as a constituent of cotton, cotton fabrics, paper, and paperboard in contact with dry food. The ingredient migrates to food at levels not to exceed GMP.

47.6 Microorganisms and Food Additives Listed in 21 CFR 131, 133, 136, and 137

The list is provided in Table 47.11. Previous sanctions by the FDA were granted for the use of harmless lactic acid producing bacteria, such as *Lactobacillus acidophilus*, as optional ingredients in specified standardized foods. These bacteria are permitted for use in cultured milk (which includes buttermilk) (21 CFR 131.112), sour cream (21 CFR 131.160), cottage cheese (21 CFR 133.128), and yogurt (21 CFR 131.200), provided that the mandatory cultures of *Lactobacillus bulgaricus* and *Streptococcus thermophillus* are also used in the yogurt.

For more details,

1. Refer to the legal citations
2. Refer to the appropriate chapters in the two volume texts
3. Use the index of the two volume texts to locate the information

TABLE 47.11

Foods for Human Consumption That May Contain or Be Derived from Microorganisms Listed in 21 CFR Parts 131, 133, 136, and 137

Section in 21 CFR	Standardized Food
§131.111	Acidified milk, with or without the addition of characterizing microbial organisms, and aroma-, and flavor-producing microbial culture. Conditions for their use are prescribed in the referent regulations
§131.200	Yogurt made by the lactic acid–producing bacteria *Lactobacillus bulgaricus* and *Streptococcus thermophilus*
§133.106	Blue cheese, characterized by the presence of the mold *Penicillium roquefortii*
§133.113	Cheddar cheese, subjected to the action of a lactic acid producing bacterial culture and clotting enzymes of animal, plant, or microbial origin used in curing or flavor development
§136.110	Bread, rolls, and buns may contain as optional ingredients lactic acid–producing bacteria
§137.105	Flour may contain α-amylase obtained from the fungus *Aspergillus oryzae*