Section 24

Biochemical Engineering

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Nomenclature and Units

Symbol	Definition	SI units	U.S. customary units
A	Empirical constant	Dimensionless	Dimensionless
C	Concentration (mass)	kg/m^3	lb/ft^3
C	Concentration	mol/m^3	$(lb{\cdot}mol)/ft^3$
D	Diameter	m	ft
D	Effective diffusivity	m^2/s	ft^2/h
D	F/V	s^{-1}	h^{-1}
DRT	Decimal reduction time for sterilization	s	h
E	Activation energy	cal/mol	Btu/(lb·mol)
F	Flow or feed rate	m^3/s	ft^3/h
H	Concentration of host organisms	kg/m^3	lb/ft^3
K	Rate coefficient	Units dependent on order of reaction	Units dependent on order of reaction
K_M	Michaelis constant	kg/m^3	lb/ft^3
K_la	Lumped mass-transfer coefficient	s^{-1}	h^{-1}
K_d	Death-rate coefficient	s^{-1}	h^{-1}
K_s	Monod coefficient	kg/m^3	lb/ft^3
k	Kinetic constants	Dependent on reaction order	Dependent on reaction order
M	Coefficient for maintenance energy	Dimensionless	Dimensionless
N	Numbers of organisms or spores	Dimensionless	Dimensionless
P	Product concentration	kg/m^3	lb/ft^3
Q_{O_2}	Specific-respiration-rate coefficient	kg O_2/(kg organism·s)	lb O_2/(lb organism·h)
R	Universal-gas-law constant	8314 J/(mol·K)	0.7299 (ft^3)(atm)/(lb·mol·R)
r	Radial position	m	ft
S	Substrate concentration	kg/m^3	lb/ft^3
S	Shear	N/m^2	lbf/ft^2
S_o	Substrate concentration in feed	kg/m^3	lb/ft^3
T	Temperature	K	°F
t	Time	s	h
V	Velocity of reaction	mol/s	(lb·mol)/h
V_m	Maximum velocity of reaction	mol/s	(lb·mol)/h
V	Air velocity	m/s	ft/h
V	Fermenter volume	m^3	ft^3
VVM	Volume of air/volume of fermentation broth per minute	Dimensionless	Dimensionless
X	Organism concentration	kg/m_3	lb/ft^3
Y	Yield coefficient	kg/kg	lb/lb
		Greek symbols	
β	Dimensionless Michaelis constant	Dimensionless	Dimensionless
μ	Specific-growth-rate coefficient	s^{-1}	h^{-1}
$\hat{\mu}$ or μ_{max}	Maximum-specific-growth-rate coefficient	s^{-1}	h^{-1}
ω	Recycle ratio	Dimensionless	Dimensionless
ϕ	Thiele modulus	Dimensionless	Dimensionless

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INTRODUCTION TO BIOCHEMICAL ENGINEERING

The differences between biochemical engineering and chemical engineering lie not in the principles of unit operations and unit processes but in the nature of living systems. The commercial exploitation of cells or enzymes taken from cells is restricted to conditions at which these systems can function. Most plant and animal cells live at moderate temperatures and do not tolerate extremes of pH. The vast majority of microorganisms also prefer mild conditions, but some thrive at temperatures above the boiling point of water or at pH values far from neutrality. Some can endure concentrations of chemicals that most other cells find highly toxic. Commercial operations depend on having the correct organisms or enzymes and preventing inactivation or the entry of foreign organisms that could harm the process.

The pH, temperature, redox potential, and nutrient medium may favor certain organisms and discourage the growth of others. For example, pickles are produced in vats by lactobacilli well-suited to the acid conditions and with small probability of contamination by other organisms. In mixed culture systems, especially those for biological waste treatment, there is an ever shifting interplay between microbial populations and their environments that influences performance and control. Although open systems may be suitable for hardy organisms or for processes in which the conditions select the appropriate culture, many bioprocesses are closed and have elaborate precautions to prevent contamination. The optimization of the complicated biochemical activities of isolated strains, of aggregated cells, of mixed populations, and of cell-free enzymes or components presents engineering challenges that are sophisticated and difficult. Performance of a bioprocess can suffer from changes in any of the many biochemical steps functioning in concert, and genetic controls are subject to mutation. Offspring of specialized mutants that yield high concentrations of product tend to revert during propagation to less productive strains—a phenomenon called *rundown.*

This section emphasizes cell cultures and microbial and enzymatic processes and excludes medical, animal, and agricultural engineering systems. Engineering aspects of biological waste treatment are covered in Sec. 25.

Biotechnology has a long history—fermented beverages have been produced for several thousand years. But biochemical engineering is not yet fully mature. Developments such as immobilized enzymes and cells have been exploited partially, and many exciting advances should be forthcoming. Genetic manipulations through recombinant DNA techniques are leading to practical processes for molecules that could previously be found only in trace quantities in plants or animals. Biotechnology is now viewed as a highly profitable route to relatively valuable products. In the near future, costs of environmental protection may force more companies to switch from chemical processing that generates wastes that are costly to treat to biochemical methods with wastes that are easily broken down by biological waste treatment processes and that present much less danger to the environment. Some commercial bioprocesses could have municipal and industrial wastes as feedstocks, and the credits for accepting them should improve the economic prospects. When petroleum runs out and the

prices soar for petrochemicals, there will be large profits for fermentations that produce equivalent compounds.

BIOLOGICAL CONCEPTS

Cells The cell is the unit of life. Cells in multicellular organisms function in association with other specialized cells, but many organisms are free-living single cells. Although differing in size, shape, and functions, there are basic common features in all cells. Every cell contains cytoplasm, a colloidal system of large biochemicals in a complex solution of smaller organic molecules and inorganic salts. The cytoplasm is bounded by a semielastic, selectively permeable cell membrane that controls the transport of molecules into and out of the cell. There are biochemical transport mechanisms that spend energy to bring substances into the cell despite unfavorable concentration gradients across the membrane. Cells are protected by rigid cell walls external to the cell membranes. Certain bacteria, algae, and protozoa have gelatinous sheaths of inorganic materials such as silica.

Sequences of genes along a threadlike chromosome encode information that controls cellular activity. As units of heredity, genes determine the cellular characteristics passed from one generation to the next. In most cells, the chromosomes are surrounded by a membrane to form a conspicuous nucleus. Cells with organized nuclei are described as eukaryotic. Other intracellular structures serve as specialized sites for cellular activities. For example, photosynthesis is carried out by organelles called chloroplasts. In bacteria and cyanobacteria (formerly called blue-green algae), the chromosomes are not surrounded by a membrane, and there is little apparent subcellular organization. Lacking a discrete nucleus, these organisms are said to be prokaryotic.

Microorganisms of special concern to biochemical engineering include yeasts, bacteria, algae, and molds. The protozoa can feed on smaller organisms in natural waters and in waste-treatment processes but are not useful in producing materials of commercial value. Certain viruses called phages are also important in that they can infect microorganisms and may destroy a culture. A beneficial feature of microbial viruses is the ability to convey genetic materials from other sources into an organism. This is called transduction. Each species of microorganisms grows best within certain pH and temperature ranges, commonly between 20 and 40°C (68–104°F) and not too far from neutral pH.

Bacteria The bacteria are tiny single-cell organisms ranging from 0.5–20 μm in size, although some may be smaller, and a few exceed 100 μm in length. The cell wall imparts a characteristic round or ovoid, rod, or spiral shape to the cell. Some bacteria can vary in shape, depending on culture conditions; this is termed *pleomorphism.* Certain species are further characterized by the arrangement of cells in clusters, chains, or discrete packets. Some cells produce various pigments that impart a characteristic color to bacterial colonies. The cytoplasm of bacteria may also contain numerous granules of storage materials such as carbohydrates and lipids. Bacteria can contain plasmids that are pieces of genetic material existing outside the main genome. Plasmids can be used as vectors for introducing foreign genes into the bacteria that can impart new synthetic capabilities to an otherwise "wild" bacterial strain. Many bacteria exhibit motility by means of one or more hairlike appendages called flagella. Bacteria reproduce by dividing into equal parts, a process termed *binary fission.*

Under adverse conditions, certain microorganisms produce spores that germinate upon return to a favorable environment. Spores are a particularly stable form or state of bacteria that may survive dryness and temperature extremes. Some microorganisms form spores at a stage in their normal life cycle.

Many species may, under appropriate circumstances, become surrounded by gelatinous material that provides a means of attachment and some protection from other organisms. If many cells share the same gelatinous covering, it is called a slime; otherwise each is said to have a capsule.

Algae Algae are a very diverse group of photosynthetic organisms that range from microscopic size to giant kelp that may reach lengths of 20 m (66 ft). Some commercial biochemicals come from algal seaweeds, and algae supply oxygen and consume nutrients in some processes used for biological waste treatment. Although their rapid growth rates relative to other green plants offer great potential for producing biomass for energy or a chemical feedstock, there is little industrial use of algae. One proposed process uses Dunaliella, a species that grows in high salinity and accumulates glycerol internally to counter the high external osmotic pressure. Outdoor ponds are most suitable for growing algae because vast surfaces and high illumination are needed.

Fungi As a group, fungi are characterized by simple vegetative bodies from which reproductive structures are elaborated. All fungal cells possess distinct nuclei and produce spores in specialized fruiting bodies at some stage in their life cycles. The fungi contain no chlorophyll and therefore require sources of complex organic molecules for growth: Many species grow on dead organic material; others live as parasites.

Yeasts are one kind of fungi. They are unicellular organisms surrounded by a cell wall and possessing a distinct nucleus. With very few exceptions, yeasts reproduce by a process known as budding, where a small new cell is pinched off the parent cell. Under certain conditions, an individual yeast cell may become a fruiting body, producing spores.

Isolated Plant and Animal Cells Biotechnology includes recovery of biochemicals from intact animals and plants, but the care and feeding of them is beyond the scope of this section. Processes with their isolated cells have much in common with processes based on microorganisms. The cells tend to be much more fragile than microbial cells, and allowable ranges of pH and temperature are quite narrow. These cells occur in aggregates and usually require enzymes to free them. There is a strong tendency for the cells to attach to something, and cell cultures often exploit attachment to surfaces.

Plant and animal cells have numerous chromosomes. Growth rates are relatively slow. A typical nutrient medium will contain a large number of vitamins and growth factors in addition to complex nitrogen sources, because other specialized cells in the original structures supply these needs. A plant or animal cell is not like a microbial cell in its ability to function independently.

Viruses Viruses are particles of a size below the resolution of the light microscope and are composed mainly of nucleic acid, either DNA or RNA, surrounded by a protein sheath. Lacking metabolic machinery, viruses exist only as intracellular highly host-specific parasites. Many bacteria and certain molds are subject to invasion by virus particles. Those that attack bacteria are called bacteriophages. They may be either virulent or temperate (lysogenic). Virulent bacteriophages divert the cellular resources to the manufacture of phage particles; new phage particles are released to the medium as the host cell dies and lyses. Temperate bacteriophages have no immediate effect upon the host cell; they become attached to the bacterial chromosome. They may be carried through many generations before being triggered to virulence by some physical or chemical event.

Biochemistry All organisms require sources of carbon, oxygen, nitrogen, sulfur, phosphorus, water, and trace elements. Some have specific vitamin requirements as well. Green plants need only carbon dioxide, nitrate or ammonium ions, dissolved minerals, and water to manufacture all of their cellular components. Photosynthetic bacteria require specific sources of hydrogen ions, and the chemosynthetic bacteria must have an oxidizable substrate. Some microorganisms have the ability to "fix" atmospheric nitrogen by reduction. Organisms that use only simple inorganic compounds as nutrients are said to be *autotrophic* (self-nourishing).

Organisms that require compounds manufactured by other organisms are called *heterotrophs* (other-nourishing). Many heterotrophs secrete enzymes (exoenzymes) that hydrolyze large molecules such as starch and cellulose to smaller units that can readily enter the cell.

Proteins are macromolecules that play many roles such as serving as enzymes or components of cell membranes and muscle. The antibodies that protect against invasion by foreign substances are themselves proteins. There are twenty-odd amino acids found regularly in most naturally occurring proteins. Because of the great length of protein chains and the various sequences of amino acids, the theoretical number of possible proteins is astronomical. The amino acid sequence is referred to as the primary structure of a protein. The polypeptide

chain is usually coiled or folded to provide secondary structure to the molecule, and linkages through other functional groups (mainly disulfide bonds) form the tertiary structure. For some protein molecules, there may be spatial arrangement forming defined aggregates, known as the quarternary structure of proteins. For a polypeptide polymer to have biological activity a certain molecular arrangement is necessary. This requires not only the primary and secondary but also tertiary and sometimes quarternary structure. Such a strict structural requirement explains the high specificity of proteins. In the presence of certain chemical reagents, excessive heat, radiation, unfavorable pH, and so on, the protein structure may become disorganized. This is called *denaturation* and may be reversible if not too severe.

A special class of proteins, the enzymes, are biological catalysts that expedite reactions by lowering the amount of activation energy required for the reactions to go. An enzyme has an active site that may be thought of as an atomic vise that orients a portion of a molecule for its reaction. The rest of the enzyme is not just an inert glob. Regions that are recognized by antibodies enable living systems to identify and inactivate foreign proteins. Immunological reactions involving antibodies are a defense against such foreign proteins. Enzymes function in conjunction with another special class of compounds known as coenzymes. Coenzymes are not proteins; many of the known coenzymes include vitamins, such as niacin and riboflavin, as part of their molecular structure. Coenzymes carry reactant groups or electrons between substrate molecules in the course of a reaction. As coenzymes serve merely as carriers and are constantly recycled, only small amounts are needed to produce large amounts of biochemical product.

Hundreds of metabolic reactions take place simultaneously in cells. There are branched and parallel pathways, and a single biochemical may participate in several distinct reactions. Through mass action, concentration changes caused by one reaction may effect the kinetics and equilibrium concentrations of another. In order to prevent accumulation of too much of a biochemical, the product or an intermediate in the pathway may slow the production of an enzyme or may inhibit the activation of enzymes regulating the pathway. This is termed feedback control and is shown in Fig. 24-1. More complicated examples are known where two biochemicals act in concert to inhibit an enzyme. As accumulation of excessive amounts of a certain biochemical may be the key to economic success, creating mutant cultures with defective metabolic controls has great value to the production of a given product.

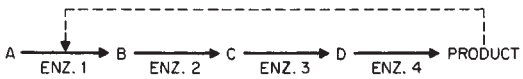

FIG. 24-1 Feedback control. Product inhibits the first enzyme.

Cell efficiency is improved by inhibiting or regulating the synthesis of unneeded enzymes, so there are two classes of enzymes—those that are constitutive and always produced and those that are inducible, i.e., synthesized when needed in response to an inducer, usually the initial substrate in a pathway. Enzymes that are induced in one organism may be constitutive in another.

Microorganisms exhibit nutritional preferences. The enzymes for common substrates such as glucose are usually constitutive, as are the enzymes for common or essential metabolic pathways. Furthermore, the synthesis of enzymes for attack on less common substrates such as lactose is repressed by the presence of appreciable amounts of common substrates or metabolites. This is logical for cells to conserve their resources for enzyme synthesis as long as their usual substrates are readily available. If presented with mixed substrates, those that are in the main metabolic pathways are consumed first, while the other substrates are consumed later after the common substrates are depleted. This results in diauxic behavior. A diauxic growth curve exhibits an intermediate growth plateau while the enzymes needed for the uncommon substrates are synthesized (see Fig. 24-2). There may also be preferences for the less common substrates such that a mixture shows a sequence of each being exhausted before the start of metabolism of the next.

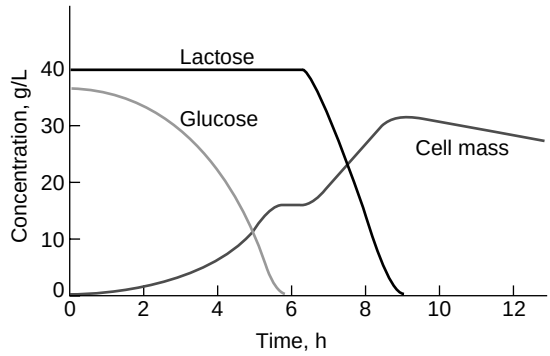

FIG. 24-2 Computer simulation of typical diauxic behavior.

Energy Many metabolic reactions, once activated, proceed spontaneously with a net release of energy. Hydrolysis and molecular rearrangements are examples of spontaneous reactions. The hydrolytic splitting of starch to glucose, for instance, results in a net release of energy. But a great many biochemical reactions are not spontaneous and therefore require an energy input. In living systems this requirement is met by coupling an energy-requiring reaction with an energy-releasing reaction. If a sufficient amount of energy is produced by a metabolic reaction, it may be used to synthesize a high-energy compound such as adenosine triphosphate (ATP). When the terminal phosphate linkage is broken, adenosine diphosphate (ADP) and inorganic phosphate are formed, and energy is provided. When sufficient energy becomes available, ATP is reformed from ADP.

In biological systems, the most frequent mechanism of oxidation is the removal of hydrogen, and conversely, the addition of hydrogen is the common method of reduction. Nicotinamide-adenine dinucleotide (NAD) and nicotinamide-adenine dinucleotide phosphate (NADP) are two coenzymes that assist in oxidation and reduction. These cofactors can shuttle between biochemical reactions so that one drives another, or their oxidation can be coupled to the formation of ATP. However, stepwise release or consumption of energy requires driving forces and losses at each step such that overall efficiency suffers.

Overall redox potential of a system determines the amount of energy that cells can derive from their nutrients. When oxygen is present to be the ultimate acceptor of electrons, complete oxidation of organic molecules yields maximum energy and usually results in the production of H_2O and CO_2. However, inside animals, in polluted waters, in the benthos (bottom region) of natural waters, and elsewhere, there is little or no free oxygen. In these environments, organisms develop that can partially oxidize substrates or can derive a small amount of energy from reactions where some products are oxidized while others are reduced. The pathways for complete oxidation may be absent and the presence of oxygen can disrupt the mechanisms for anaerobic metabolism so that the cell is quickly killed. The differences in efficiency are striking: Aerobic metabolism of one molecule of glucose can generate bond energy as much as 33 molecules of ATP, while anaerobic metabolism can yield as little as two molecules of ATP. Natural anaerobic processes accumulate compounds such as ethanol, acetoin, acetone, butanol, lactate, and malate. Products of natural aerobic metabolism are water and carbon dioxide, cell mass, and secondary metabolic products such as antibiotics.

Photosynthesis All living cells synthesize ATP, but only green plants and a few photosynthetic (or phototrophic) microorganisms can drive biochemical reactions to form ATP with radiant energy through the process of photosynthesis. All photosynthetic organisms contain one or more of the group of green pigments called chlorophylls. In plants, these are contained in organelles called chloroplasts. The number per cell of membrane-surrounded chloroplasts varies with species and environmental conditions. In higher plants, numerous chloroplasts are found in each cell of the mesophyll tissue of leaves, while an algal cell may contain a single chloroplast. A chloroplast has a sand-

wich of many layers alternating between pigments and enzymatic proteins such that electromagnetic excitation from light becomes chemical bond energy. Prokaryotic organisms have a unique type of chlorophyll and do not possess chloroplasts organelles. Instead, their photosynthetic systems are associated with the cell membrane or with lamellar structures located in organelles known as chromatophores. Chromatophores, unlike chloroplasts, are not surrounded by a membrane.

The net result of photosynthesis is reduction of carbon dioxide to form carbohydrates. A key intermediate is phosphoglyceric acid, from which various simple sugars are produced and disproportionated to form other carbohydrates.

Mutation and Genetic Engineering Exposing organisms to agents such as mustard chemicals, ultraviolet light, and x-rays increases mutation rate by damaging chromosomes. In strain development through mutagenesis, the idea is to limit the mutagen exposure to kill about 99 percent of the organisms. The few survivors of this intense treatment are usually mutants. Most of the mutations are harmful to the cell, but a very small number may have economic importance in that impaired cellular control may result in better yields of product. The key is to have a procedure for selecting out the useful mutants. Screening of many strains to find the very few worthy of further study is tedious and expensive. Such screening that was so very important to biotechnology a few decades ago is becoming obsolete because of genetic improvements based on recombinant DNA technology.

Whereas mutagenic agents delete or scramble genes, recombinant DNA techniques add desirable genetic material from very different cells. The genes may come from plant, animal, or microbial cells, or in a few instances they may be synthesized in the laboratory from known nucleic acid sequences in natural genes. Opening a chromosome and splicing in foreign DNA is simple in concept, but there are complications. Genes in fragments of DNA must have control signals from other nucleic acid sequences in order to function. Both the gene and its controls must be spliced into the chromosomes of the receiving culture. Bacterial chromosomes (circular DNA molecules) are cut open with enzymes, mixed with the new fragments to be incorporated, and closed enzymatically. The organism will acquire new traits. This technique is referred to as recombinant technology.

There are many tricks and some art in genetic engineering. Examples would be using bacteriophage infection to introduce a gene for producing a new enzyme in a cell. Certain strains of *E. coli*, *B. subtilis*, yeast, and streptomyces are the usual working organisms (cloning vectors) to which genes are added. The reason for this is that the genetics of these organisms is well understood and the methodology has become fairly routine.

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CELL AND TISSUE CULTURES

Mammalian Cells Unlike microbial cells, mammalian cells do not continue to reproduce forever. Cancerous cells have lost this natural timing that leads to death after a few dozen generations and continue to multiply indefinitely. Hybridoma cells from the fusion of two mammalian lymphoid cells, one cancerous and the other normal, are important for mammalian cell culture. They produce monoclonal antibodies for research, for affinity methods for biological separations, and for analyses used in the diagnosis and treatment of some diseases. However, the frequency of fusion is low. If the unfused cells are not killed, the myelomas will overgrow the hybrid cells. The myelomas can be isolated when there is a defect in their production of enzymes involved in nucleotide synthesis. Mammalian cells can produce the necessary enzymes and thus so can the fused cells. When the cells are placed in a medium in which the enzymes are necessary for survival, the myelomas will not survive. The unfused normal cells will die because of their limited life span. Thus, after a period of time, the hybridomas will be the only cells left alive.

A hybridoma can live indefinitely in a growth medium that includes salts, glucose, glutamine, certain amino acids, and bovine serum that provides essential components that have not been identified. Serum is expensive, and its cost largely determines the economic feasibility of a particular culture system. Only recently have substitutes or partial replacements for serum been found. Antibiotics are often included to prevent infection of the culture. The pH, temperature and dissolved oxygen, and carbon dioxide concentration must be closely controlled. The salt determines the osmotic pressure to preserve the integrity of the fragile cell.

Most glucose is metabolized to lactate because glycolysis is usually much faster than uptake rate of glycolytic intermediates. Glutamine acts as the primary source of nitrogen as well as providing additional carbon and energy. After glutamine is partially oxidized to glutamate, it can enter the TCA cycle and emerge as pyruvate. It has been estimated that between 30 and 65 percent of the cell energy requirement is derived from glutamine metabolism when both glucose and glutamine are available. Ammonia is produced in the deamination of glutamine to form glutamate and in the formation of alpha-ketoglutarate.

Plant Cells and Tissues It is estimated that today some 75 percent of all pharmaceuticals originate in plants. Typically, these compounds are derived from the secondary metabolic pathways of the cells. When plant or animal cells are cultured, concepts from microbiology come into play. Only specialized cells are used, and these can be improved with mutation, selection, and recombinant DNA techniques. One very major difference between cell and tissue cultures and most microbiological processes is very high susceptibility to contamination by foreign organisms. Most microorganisms grow rapidly and compete well; some are aided by their own changes to the environment. When a microbial process changes the pH to be far from neutrality or when the product such as ethanol is inhibitory to other organisms, growth of contaminants is discouraged. Cell and tissue cultures require rich media and are characterized by slow growth rates. There is seldom any protection by the products of the process. Optimum conditions for production of the secondary metabolites are not likely to be the same as for growth. Economics may hinge on a good balance of growing sufficient cells and favoring product formation.

Only a few biochemicals derived from plant cell and tissue cultures have high volume/low value products, but some have sizeable markets as specialty chemicals such as dyes, fragrances, insecticides, and pesticides. These differ from the low volume/very high value compounds that typify life-saving drugs and pharmaceuticals. Examples for both of these categories are listed in Table 24-1 along with the plant species of origin.

Because of cell specialization, some products are produced in cultures of those cellular types. Three main classifications of the types of plant cell and tissue cultures are:

Undifferentiated cell cultures. Aggregate clumps of cells on solid media (callus) or in liquid media (suspension)

Protoplast cultures. Cellular tissues devoid of cell wall material in culture

Organ cultures. Differentiated tissues of shoots, roots, anthers, ovaries, or other plant organs in culture

Primary Growth Requirements Primary growth is defined as the processes in a plant that are essential for the growth of the meristematic regions such as the shoot apex, root tip, and axillary meristems. Plant cell and tissue cultures have specific optima for their primary growth in terms of lighting, temperature, aeration, a nutrient medium that must supply a carbon source, vitamins, hormones, and inorganic constituents and with pH typically between 5.5 and 6.5. Aeration can be critical depending upon the species.

TABLE 24-1 Typical Products Derived from Plants

Compound	Application	Volume/ value	Source
Shikonin	Dye	High/low	*Lithospermum erthrorhizon*
Warfarin	Pesticide	High/low	Sweet clover
Gossypol	Pesticide/anti-fertility	Low/high	Cotton
Scopolamine	Antispasmodic	Low/high	*Daturastramonium*
Ajmalicine	Circulatory agent	Low/high	*Catharanthusroseus*
Taxol	Anticancer	Low/high	California yew tree

Although a few exceptions do exist where glucose, fructose, or galactose is preferred, the majority of the plant cultures use sucrose. Usual trace requirements are thiamine, niacin, riboflavin, pyridoxine, choline, ascorbic acid, and inositol. Hormones such as auxins and cytokinins promote an undifferentiated state or trigger differentiation into specific plant tissues. As with the whole plant, cellular groups, either differentiated or undifferentiated, require a set of inorganic elements such as nitrogen, phosphorus, potassium, magnesium, calcium, sulfur, iron, chlorine, boron, manganese, and zinc. The exact compositions and concentrations of these inorganic elements that are optimum for a particular plant species can be highly variable. However, prepackaged formulations of these salts that can even include the carbon source, vitamins, hormones, and pH buffers are commercially available.

Secondary Metabolic Requirements A difference between the growth and secondary metabolic phase is that the latter gains importance when approaching the reproductive stages. For example, many of the pigments of flowers are secondary metabolites (e.g., shikonin). Secondary mechanisms are typical responses to stress, such as change in pH (e.g., alkaloid production in *Hyocyamus muticus* cell cultures is optimum at pH 3.5, while growth is best at 5.0). Similarly, carbon-source concentrations affect *Morinda citrifolia* cell cultures that grow best at 5 percent sucrose but produce the anthraquinone secondary metabolites optimally at 7 percent. Temperature changes can cause flowering; several plants require a cold treatment to induce flowering. This is called *vernalization.* Secondary metabolic pathways in plant cell and tissue cultures seem to be highly controlled by the hormone level in the medium. Another method of eliciting secondary metabolites employs the natural defense mechanisms of the plants that have developed through evolution. For example, gossypol produced by *Gossypium hirsutum* (cotton) cells is a natural response of the plant when subjected to the infections of the wilt-producing fungus *Verticillium dahliae.*

Additional References: Lambert, K. J. and J. R. Birch, "Cell Growth Media" in *Animal Cell Biology,* vol. 1, 1985, pp. 85–122. van Wezel, A. L., C. A. M. van der Velden-de Groot, H. H. de Haan, N. van der Heuvel, and R. Schasfoort, "Large-Scale Animal Cell Cultivation for Production of Cellular Biologicals," *Dev. Bio. Stand.,* **60,** 229–236 (1985). Altman, D. W., R. D. Stipanovic, D. M. Mitten, and P. F. Heinstein, *In Vitro Cell. Dev. Biol.,* **21,** 659 (1985). Toivonen, L., M. Ojala, and V. Kauppinen, *Biotechnol. Bioeng.,* **37,** 673 (1991). Calcott, P. H., *Continuous Cultures of Cells,* vols. 1 and 2, CRC Press, 1981. Maramorosch, K. and A. H. McIntosh, *Insect Cell Biotechnology,* CRC Press, Boca Raton, 1994. Endress, R., *Plant Cell Biotechnology,* Springer-Verlag, Berlin, New York 1994. Morgan, S. J. and D. C. Darling, *Animal Cell Culture: Introduction to Biotechniques,* BIOS Scientific Pub, 1993. Goosen, M., A. Daugulis, and P. Faukner (eds.), *Insect Cell Culture Engineering,* M. Dekker, New York, 1993.

RECENT EMPHASES

Commercial use of cell and tissue culture continues to expand. Improvement of organisms through recombinant nucleic acid techniques has become commonplace. Formerly, a few laboratories were well ahead of most others, but now the methods have been perfected for routine use. Another technique that is widely practiced is culturing of cells that excrete high concentrations of just one antibody protein. The specificity of antibodies and antigens is exploited in medical testing procedures using these pure monoclonal antibodies.

Environmental issues are driving several aspects of biotechnology. Sites contaminated by toxic wastes can be cleaned by several alternative methods, but all are expensive. The most certain way to remove toxic materials from soil is to excavate it for incineration, but this requires much labor, energy, and money. Bioremediation in situ tends to be much less expensive on one hand but is slow and uncertain on the other. Microbial growth rates approach zero as nutrient levels fall to the low concentrations required for approval of the toxic site remediation. This means that rates tend to be unacceptable when striving for complete removal. Many toxic materials do not support growth of microorganisms. However, they may be degraded as the microorganisms grow on other nutrients. This is termed *cometabolism.*

Materials that are easily biodegraded could substitute for plastics and other organic chemicals that damage the environment. There has been some progress with natural surfactants produced by microorganisms; these would be used in detergents if properties were acceptable and costs were competitive. Biodegradable polymers such as poly-beta-hydroxybutyrate or its derivatives should eventually substitute for polyethylene and polypropylene, but costs are still too high.

BIOLOGICAL REACTORS

FERMENTERS

The term *fermentation* formerly distinguished processes from which air was absent, but the term has now been extended to aerobic processes. Bioprocessing is usually aseptic (free of unwanted organisms) in vessels held under positive pressure of sterile air to resist entry of contaminating microorganisms. A few processes such as the production of pathogenic organisms for medical purposes or for biological warfare operate below atmospheric pressure because safety of the plant operators is more important than the integrity of the product. Older processes such as manufacture of pickles had no special measures against contamination, but many of these have been converted to aseptic operations to prevent impairment of product quality by foreign organisms. Biological waste treatment employs elective cultures of microorganisms in relatively crude, open equipment.

Activities associated with bioreactors include gas/liquid contacting, on-line sensing of concentrations, mixing, heat transfer, foam control, and feed of nutrients or reagents such as those for pH control. The workhorse of the fermentation industry is the conventional batch fermenter shown in Fig. 24-3. Not shown are ladder rungs inside the vessel, antifoam probe, antifoam system, and sensors (pH, dissolved oxygen, temperature, and the like). Note that coils may lie between baffles and the tank wall or connect to the top to minimize openings below the water level, and bottom-entering mixers are used frequently. There is extensive process piping, and copper or brass fittings are taboo for some processes because of highly deleterious effects of copper (more than 50 percent reduction in yield has been noted in penicillin fermentations when a bronze valve was in a feed line). Cooling coils must be used for larger tanks because the heat-transfer area of a jacket is inadequate for cooling from sterilization temperature to operating temperature in a reasonable time. Some features of interest for a conventional fermenter are that (1) a bypass valve in the air system allows diversion of air so that foaming is not excessive and the redox potential is not too high during the early stage of fermentation when the inoculum is becoming established; (2) antifoam is added when excessive foam reaches a conductive or capacitive electronic probe; (3) all piping is sterilized by the use of steam and is protected by steam until put into use; (4) the level of liquid when filling the vessel is determined by reference to a calibration chart based on points in the tank such as a rung on the ladder; (5) the weight of the tank contents can be determined by the hydrostatic balance against air bubbled slowly through the sparger; and (6) pumps are very uncommon because it is so easy to force fluid from a pressurized vessel.

The need for highly cost-efficient oxygen transfer in fermentations such as those with hydrocarbon feedstocks has led to air-lift fermenters as shown in Fig. 24-4. The world's largest industrial fermenter was

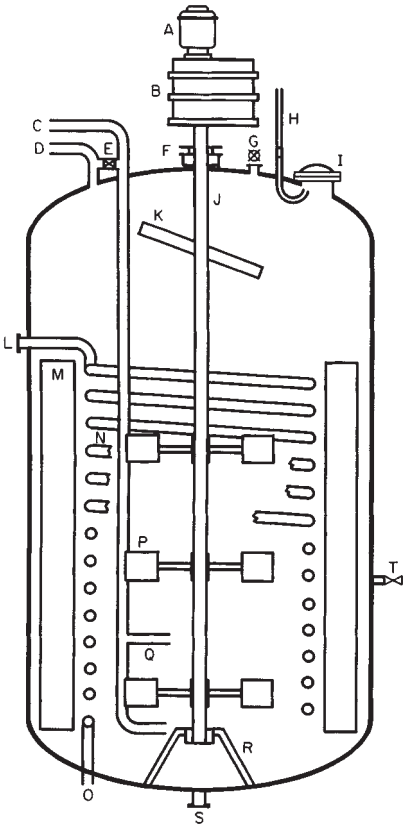

FIG. 24-3 Conventional batch fermenter. *A* = agitator motor; *B* = speed-reduction unit; *C* = air inlet; *D* = air outlet; *E* = air bypass valve; *F* = shaft seal; *G* = sight glass with light; *H* = sight-glass clean-off line; *I* = manhole with sight glass; *J* = agitator shaft; *K* = paddle to break foam; *L* = cooling-water outlet; *M* = baffle; *N* = cooling coils; *O* = cooling-water inlet; *P* = mixer; *Q* = sparger; *R* = shaft bearing and bracket; *S* = outlet (steam seal not shown); *T* = sample valve (steam seal not shown).

designed for producing single-cell protein from hydrocarbons at Billingham, England, U.K. Its dimensions are 100 m (328 ft) in height and 10 m (33 ft) in diameter.

A few variations on the standard fermenter have been attempted, but none has become popular. An obsolete design in which the fermenter was rotated to aerate the medium is shown in Fig. 24-5. Performance was unsatisfactory, and the units were turned on end, with spargers and agitation added. One of the largest fermenters used for antibiotics is a horizontal cylinder with several agitators, as in Fig. 24-6. Multiple agitator motors and shafts have also been used with vertical cylindrical vessels.

Another innovative design is the toroidal fermenter shown in Fig. 24-7. Motion in an axial direction allows intimate mixing with air. A special case of the air-lift fermenter is shown in Fig. 24-8. The feed enters at moderate pressure and is drawn downward to regions of very high hydrostatic pressure that provides a great driving force for gas transfer. In the other leg, lowering pressure allows gases to expand to induce circulation. Experimental units have been built in elevator shafts.

Ethanol fermentation is a particularly good example of product accumulation inhibiting the microbial culture. Most strains of yeast have a much slower alcohol production rate when ethanol reaches about ten percent, and the wine or saki strains that achieve over 20 percent by volume of ethanol are very, very slow. A system known as the Vacuferm for removal of alcohol by distillation as it is formed is

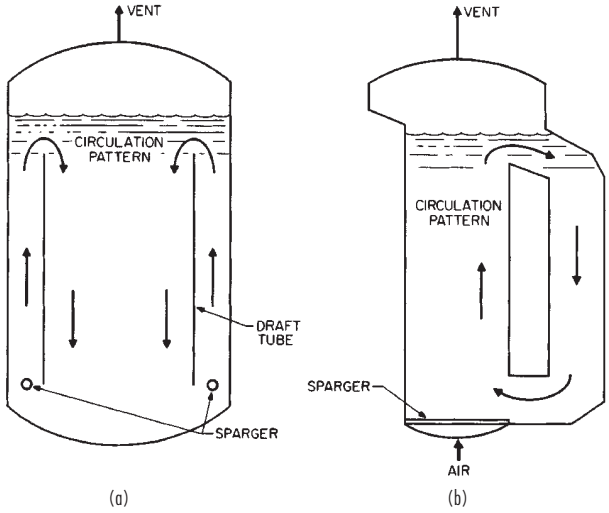

FIG. 24-4 Air-lift fermenters: (*a*) Concentric cylinder; (*b*) external recycle.

shown in Fig. 24-9. The vacuum is adjusted to the vapor pressure of the alcohol-water solution at the fermentation temperature, 30 to 40°C (86 to 104°F). Volumetric productivity is far better than that of a conventional fermenter, but there is a killing disadvantage of having to recompress large volumes of vapor so that alcohol can be condensed with normal cooling water instead of expensive cold brine. Furthermore, the large amounts of carbon dioxide generated by fermentation are evacuated and recompressed along with the alcohol and water vapors. A far better design is shown in Fig. 24-10, where the fermenter operates at normal pressure so that carbon dioxide escapes, and broth is circulated through the flash pot for vaporization of the ethanol. Although this system may seem to have attractive energy economy because metabolic heat is removed as the vapor flashes,

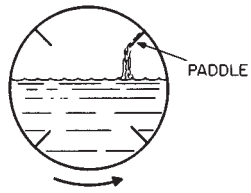

FIG. 24-5 Rotating fermenter.

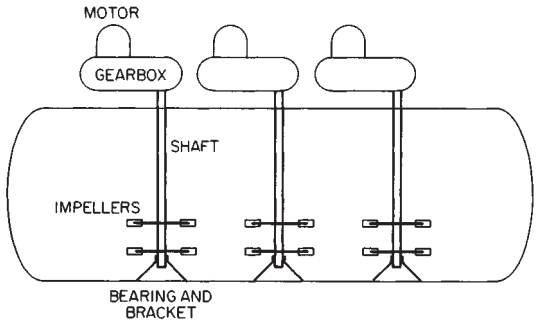

FIG. 24-6 Horizontal fermenter.

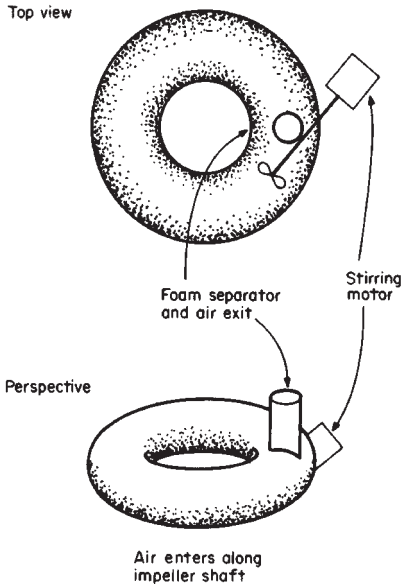

FIG. 24-7 Toroidal fermenter.

initial investment and the cost of pumping the vapors are high. Operating the ethanol fermentation at higher temperatures with thermophillic organisms has better economics in terms of milder vacuum and less recompression because of the higher vapor pressure, but evolving carbon dioxide can strip out product. Other alternatives for overcoming inhibition by the product are extraction from the fermentation broth with an immiscible solvent and/or operating with very dense cultures so that low productivity per cell is compensated by having many more cells.

Elevated cell concentrations can be achieved by separating cells from the effluent and recycling them to the fermenter. This has been

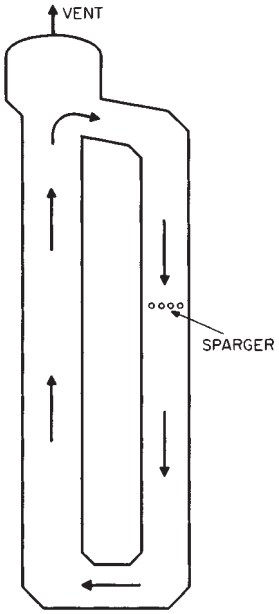

FIG. 24-8 Deep-shaft fermenter.

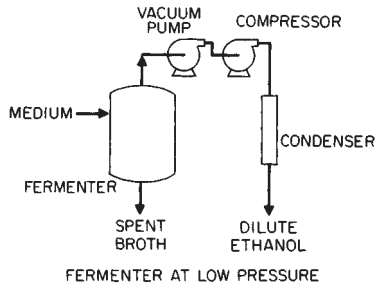

FIG. 24-9 Vacuferm.

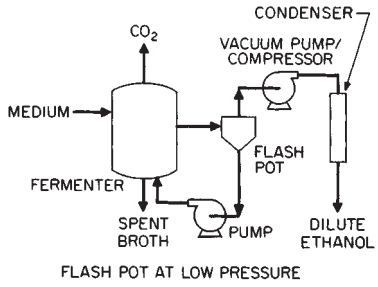

FIG. 24-10 Flash-pot fermenter.

standard practice for many years in biological waste treatment where dilute feed streams result in slow growth of the culture. Producing cell flocs that are collected easily by sedimentation is aided by recycling those cells that do settle. This is a selective advantage that may allow them to dominate. Industrial fermentations can afford more expense than can waste treatment, and centrifuges for collecting cells are not uncommon. Recycle of yeast cells can lower the fermentation time for ethanol significantly. Heavily coagulated cells can be retained in the fermenter; the tower fermenter shown in Fig. 24-11 uses this principle with yeast strains that flocculate naturally. Cells can also be retained in the reactor by attachment to a support. Vinegar is sometimes produced in a generator filled with wood shavings to which bacteria attach. Rocks or plastic support materials are used in trickling filters for waste treatment (see Sec. 25). Chemical agents can link cells to the support materials when simple adsorption does not hold them tightly enough. Gel entrapment can provide extremely high cell concentrations because the cells continue to multiply within the gel. Comparisons of results with fermentation of ethanol are in Table 24-2.

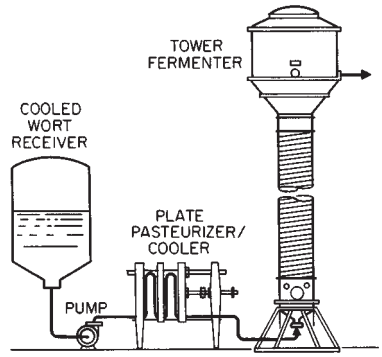

FIG. 24-11 Tower fermenter. (*Compliments of APV Corp.*)

TABLE 24-2 Comparison of Ethanol Fermenters

System	Typical time (h)	Typical ethanol concentration (%)
Conventional	72	10
Cell recycle	12	8
Tower fermenter	3	8
Gel immobilization	1	10

PROCESS CONSIDERATIONS

Fermentation can be combined with other operations. For example, feedback inhibition of enzymatic hydrolysis of cellulose can be relieved by removal of the product glucose by fermentation as it forms. This is termed *simultaneous-saccharification-fermentation* (SSF).

Valves and pumps that have a potential path for contaminating organisms are taboo for aseptic operations. Rising stem valves could bring organisms to the sterile side by the in and out motion as the valve operates. Diaphragm valves are still commonly used, but heating, cooling, and the abrasion by solids in the nutrient media are somewhat severe conditions leading to occasional rupture of a diaphragm and contamination of a run. Ball valves or plug valves do not have an absolute seal to the outside, but the direction of motion does not tend to bring organisms in. Contamination is seldom attributed to these valves; they are designed for easy maintenance in place, and there is the very nice human advantage that a glance at the handle tells easily whether the valve is open or closed. Many runs have been spoiled or impaired because a manual valve was left in the wrong position. For plant operations, pumps with diaphragms are satisfactory. In the lab or pilot plant, peristaltic pumps (also known as tubing squeezers) predominate.

Transfer of fluid in a fermentation plant usually makes use of air pressure differences. One or more manifold headers may interconnect many vessels. As transfers may have to be aseptic, headers are pressurized with steam until needed. A typical arrangement of steam seals is shown in Fig. 24-12.

Sample lines commonly have steam seals too. A typical layout is shown in Fig. 24-13. In the closed position, steam provides an absolute barrier to contamination. To take a sample, the steam line and the trap line are closed, and fermentation medium is flowed to waste until the pipes are cool to the touch so that sensitive products do not give false assays because of thermal destruction. Cooling takes up to 5 liters of medium if not done carefully, and bad practices can waste considerably more. Pilot-sized tanks have less massive fittings that are easier to cool; less medium is wasted, but oversampling to the point where the fermenter volume is low can be a problem. For this reason, alternate sampling methods have been devised. For example, a sterile syringe and needle may be used to sample through a rubber diaphragm in the wall of the tank. Although such methods appear reliable, there is a tendency to scrap all innovations and to return to tried and true steam seals when the factory encounters any period of contamination.

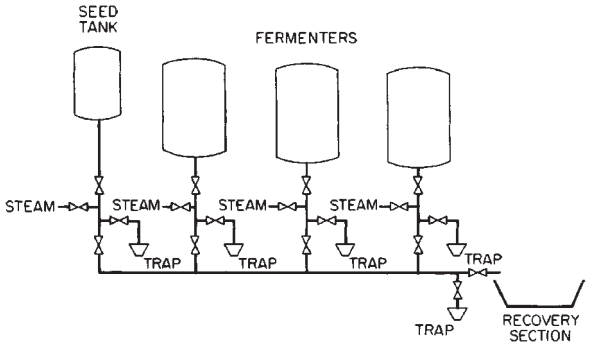

FIG. 24-12 Inoculation and harvest header.

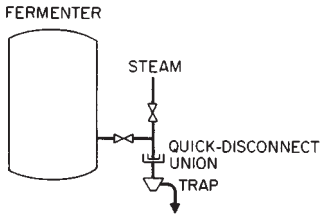

FIG. 24-13 Sample-line piping. (A valve to the sewer allows bypass of the trap while cooling the line.)

All piping to a fermenter is flushed with steam during the sterilization period. A clever means for sight-glass cleaning uses steam condensate that is naturally sterile in a dead leg. Steam pressure behind the condensate forces this water to the sight glass. Without cleaning, splashing and spray can quickly cover the sight glass with a thick coating of microorganisms and medium.

While it is easy to add materials to a fermentation, removal is difficult. Membrane devices have been placed in the fermenter or in external recycle loops to dialyze away a soluble component. Cells release wastes or metabolites that can be inhibitory; these are sometimes referred to as *staling factors*. Their removal by dialysis has allowed cell concentrations to reach ten to one hundred times that of control cultures.

Solid substrates such as pulverized wood cannot be stirred when slurry concentration exceeds about 5 percent. For saccharification prior to ethanol fermentation, keeping sugar concentration high can avoid an evaporation step. In a batch reactor, mixing limitations with the wood results in a dilute sugar solution. This has been circumvented by placing the wood in a column and percolating the solution through. As wood dissolves, more is added. Simultaneous fermentation of the sugars formed in the column is possible.

Oxygen Transfer Supplying sufficient oxygen can be a very challenging engineering problem for some aerobic fermentations. Oxygen is sparingly soluble in water; saturation with pressurized air at room temperature provides only 6 or 7 milligrams per liter of oxygen. A vigorous process can deplete the dissolved oxygen in several seconds when aeration is stopped. Mass transfer of gases to liquids is covered in Sec. 5. Emphasis is somewhat different for biological systems that commonly have bubble aeration. Because the number and size of bubbles is very difficult to estimate, transfer area is usually lumped with the mass-transfer coefficient as a $K_l a$ term. The "l" subscript in K_l signifies that liquid film resistance should greatly predominate for a sparingly soluble gas such as oxygen. The relationship between oxygen concentration and growth is of a Michaelis-Menten type (see Fig. 24-20). When a process is rate-limited by oxygen, the specific respiration rate (Q_{O2}) also increases steeply with dissolved oxygen concentration until a plateau is reached. The concentration below which respiration is severely limited is termed the critical oxygen concentration, which typically ranges from 0.5 to 2.0 ppm for well-dispersed bacteria, yeast, and fungi growing at 20 to 30°C (68 to 86°F). Above this critical concentration, the specific oxygen uptake increases only slightly with increasing oxygen concentrations.

A plot of the specific respiration rate Q_{O2} versus the specific growth rate coefficient μ is linear, with the intercept on the ordinate equal to the oxygen uptake rate for cell maintenance. A formulation of this is:

$$\text{Uptake rate} = \text{uptake for maintenance} + \text{uptake for growth}$$

or

$$Q_{O2}X = \frac{(Q_{O2})_M X + \mu X}{Y_g} \qquad (24\text{-}1)$$

where X is organism concentration, the subscript M denotes maintenance, Y_g is yield of cell mass per mass of oxygen, and the Q terms signify oxygen uptake rates in mass O_2 per mass of organisms.

This type of correlation applies to almost any substrate involved in cellular energy metabolism and is supported by experimental data and energetic considerations. However, it is based on assumptions true at or near the steady-state equilibrium conditions and may not be valid

during transient states. The oxygen-uptake equation should be modified when other cellular activities requiring oxygen can be identified. For example, use of oxygen for product formation would be represented by:

Uptake rate = maintenance uptake + growth uptake + product uptake

or
$$Q_{O2}X = (Q_{O2})_M X + \frac{dX}{dt}\frac{1}{Y_G} + \frac{dP}{dt}\frac{1}{Y_P} \qquad (24\text{-}2)$$

where P is the product concentration and Y is the yield of product per unit weight of limiting nutrient. Oxygen uptake is distributed between that for growth and that for cellular activities dependent on cell concentration.

As the oxygen transfer rate under steady-state conditions must equal oxygen uptake, $K_l a$ may be calculated:

$$K_l a = \frac{\text{overall oxygen uptake rate}}{(C^* - C)_{\text{mean}}} \qquad (24\text{-}3)$$

where C^* = concentration of oxygen in the liquid that would be in equilibrium with the gas-bubble concentration and $K_l a$ = the volumetric oxygen transfer rate.

A convenient method for measuring oxygen transfer rates in microbial systems depends on dissolved oxygen electrodes with relatively fast response times. Quite inexpensive oxygen electrodes are available for use with open systems, and steam-sterilizable electrodes are available for aseptic systems. There are two basic types: One develops a voltage from an electrochemical cell based on oxygen, and the other is a polarographic cell whose current depends on the rate at which oxygen arrives. See Fig. 24-14. Measurement of oxygen transfer properties requires only a brief interruption of oxygen supply. A mass balance for oxygen is:

$$\frac{dO}{dt} = \text{rate of supply} - \text{uptake rate} \qquad (24\text{-}4)$$

A tracing of the electrode signal during a cycle of turning aeration off and on is shown in Fig. 24-15. The rate of supply is zero (after bubbles have escaped) in the first portion of the response curve; thus, the slope equals the uptake rate by the organisms. When aeration is resumed, both the supply rate and uptake rate terms apply. The values for $C^* - C$ can be calculated from the data, the slope of the response curve at a given point is measured to get dC/dt, and the equation can be solved for $K_l a$ because all the other values are known.

Measurements of the rate of change in concentration of oxidizable chemicals in aerated vessels have questionable value for assessing rates with biological systems. Not only are flow patterns and bubble sizes different for biological systems, but surface active agents and

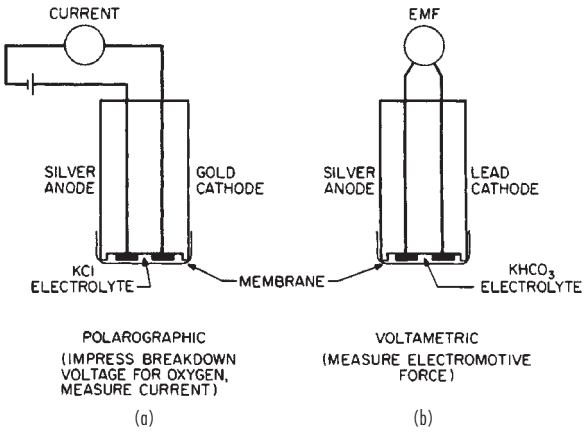

FIG. 24-14 Dissolved-oxygen electrodes: (*a*) polarographic (impress breakdown voltage for oxygen; measure current); (*b*) voltametric (measure electromotive force).

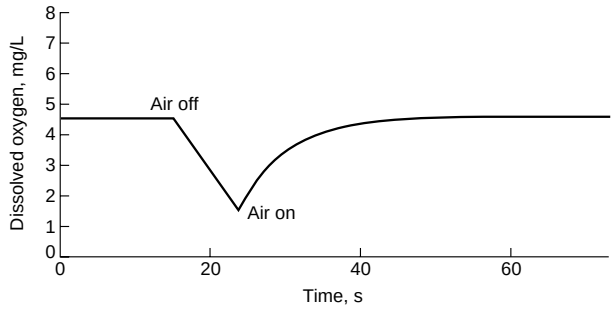

FIG. 24-15 Computer simulation of response for dynamic measurement of $K_l a$.

suspended particles can seriously impair gas transfer. Fig. 24-16 shows the effects of various particles. In general, spherical particles have a small effect, elongated particles have more effect, and entangled particles markedly impair transfer. As many mold cultures are intertwined and lipids and proteins are present with strong surface activity, oxygen transfer to a fermentation can be much slower than that to simple aqueous solutions.

Except as an index of respiration, carbon dioxide is seldom considered in fermentations but plays important roles. Its participation in carbonate equilibria affects pH; removal of carbon dioxide by photosynthesis can force the pH above 10 in dense, well-illuminated algal cultures. Several biochemical reactions involve carbon dioxide, so their kinetics and equilibrium concentrations are dependent on gas concentrations, and metabolic rates of associated reactions may also change. Attempts to increase oxygen transfer rates by elevating pressure to get more driving force sometimes encounter poor process performance that might be attributed to excessive dissolved carbon dioxide.

Sparger Systems Gas distributors in tanks are shown in Sec. 6. Large openings are desirable for spargers in industrial fermentations to avoid clogging by microbial growth, but the diameter is usually designed for the acoustic velocity that insures small bubbles. Relatively small holes or diffusers are used in activated sludge units for biological waste treatment, but there is commonly a means for swinging a section of the aerator out of the vessel for cleaning. Newer designs for fermenters were conceived as answers to the problems of oxygen transfer. The air lift fermenter (Fig. 24-4) creates intimate mixing of air and medium while using the buoyancy of the gas to mix the fluid.

Surface active substances also lead to foaming that can be so bad that most of the contents of the fermenter are lost. Mechanical antifoam devices are helpful but cannot function alone except when there is little propensity for foaming. The mechanical foam breakers rupture the large, weak bubbles while allowing tiny, rugged bubbles to accumulate. Surface active antifoam agents tend to reduce elasticity of the bubbles so that mechanical shocks are easily transmitted to encourage rupture. Several antifoam delivery systems are shown in Fig. 24-17. Some lipids used as antifoams are metabolized by the culture and must be replaced. The nutrition supplied by these oils may be beneficial, but they are much more expensive than their equivalents in carbohydrate nutrients. Furthermore, it is troublesome to have nutrition coupled to foam control. Several synthetic antifoam agents are not nutrients and tend to persist. Their tendency to be lost by coating solid surfaces in the fermenter means that more must be added occasionally. These synthetic antifoam agents are toxic to some organisms, but one of the many types is usually satisfactory.

Scale-Up Fermenters ranging from about two to over 100 liters (0.07–3.5 ft^3) have been used for research and development, but the smaller sizes provide too little volume for sampling and are difficult to replicate, while large vessels are expensive and use too much medium. Autoclavable small fermenters that are placed in a water bath for temperature control are less expensive than vessels with jackets or coils, but much labor is required for handling them. Pressure vessels that

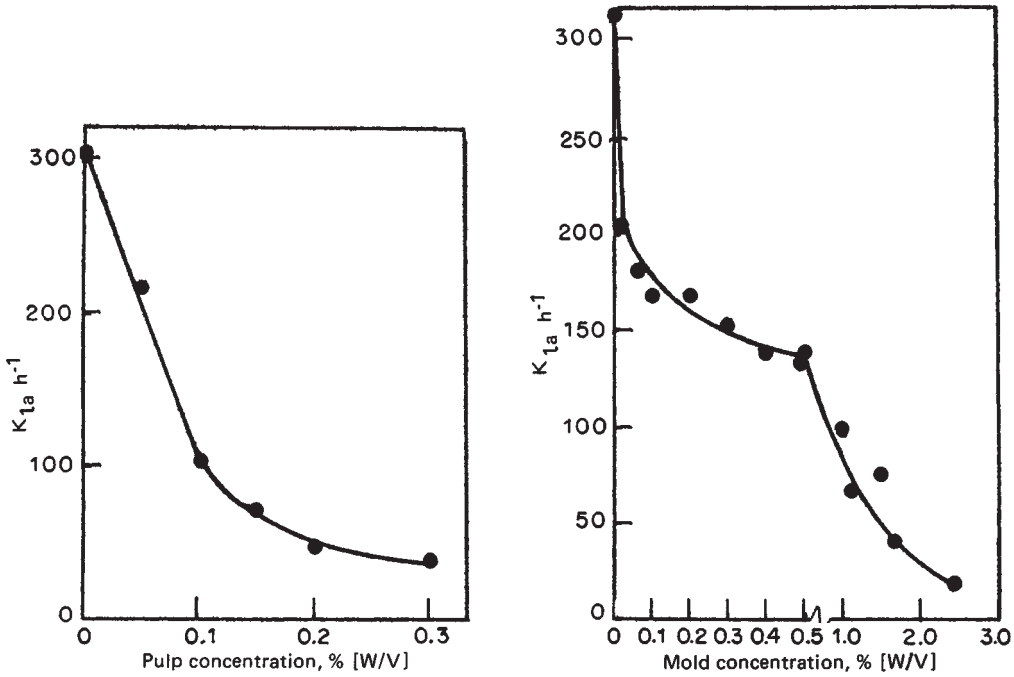

FIG. 24-16 Effect of solids on $K_l a$. Operating conditions: agitator speed, 800 r/min; air flow, 2.5 min. [*M. R. Brierly and R. Steel,* Appl. Microbiol., **7,** *57 (1959). Courtesy of American Society for Microbiology.*]

are sterilized in place are more convenient, but initial investment is high. Judgement is needed to select the most economical equipment and to plan for cost-effective experimentation.

A suitable means of scale-up for aerobic processes is to measure the dissolved oxygen level that is adequate in small equipment and to adjust conditions in the plant until this level of dissolved oxygen is reached. However, some antibiotic fermentations and the production of fodder yeast from hydrocarbon substrates have very severe requirements, and designers are hard-pressed to supply enough oxygen.

Older methods of fermentation scale up insisted on geometric similarity based on proportional physical dimensions. It was thought that applying the same power per unit volume as in the pilot equipment would give an equivalent process performance in large fermenters. Antibiotic fermentations aim for mixer power in the range of 0.2 to 4 Kw/m (0.1 to 2 HP/100 gal). As mixing devices have areas (dimensions squared) to supply a volume (dimensions cubed), methods based on dimensional similarity are fundamentally unsound. Scale-up based on equivalent oxygen transfer coefficient $K_l a$ has been reasonably successful.

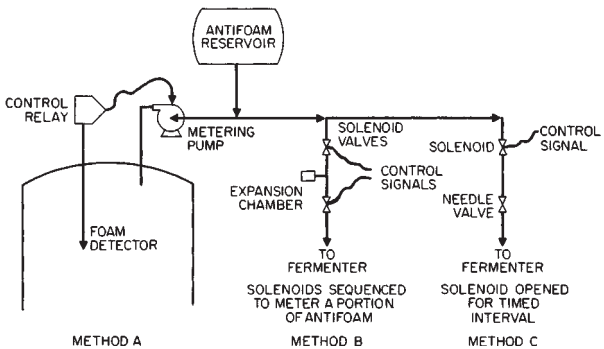

FIG. 24-17 Antifoam systems.

Impeller Reynolds number and equations for mixing power for particle suspensions are in Sec. 5. Dispersion of gasses into liquids is in Sec. 14. Usually, an increase in mechanical agitation is more effective than is an increase in aeration rate for improving mass transfer.

Other scale-up factors are shear, mixing time, Reynolds number, momentum, and the mixing provided by rising bubbles. Shear is maximum at the tip of the impeller and may be estimated from Eq. (24-5), where the subscripts *s* and *l* stand for *small* and *large* and *Di* is impeller diameter [R. Steel and W. D. Maxon, *Biotechnol. Bioengr.*, **4,** 231 (1962)].

$$S_s = S_l \left(\frac{Di_s}{Di_l} \right)^{1/3} \tag{24-5}$$

Some mycelial fermentations exhibit early sporulation, breakup of mycelium, and low yields if the shear is excessive. A tip speed of 250 to 500 cm/s (8 to 16 ft/s) is considered permissible. Mixing time has been proposed as a scale-up consideration, but little can be done to improve it in a large fermenter because gigantic motors would be required to get rapid mixing. Culturing cells from plants or animals is beset by mixing problems because these cell are easily damaged by shear.

Constant Reynolds number is not used for fermentation scale-up; it is only one factor in the aeration task. This is also true for considering the impeller as a pump and attempting scale-up by constant momentum. As mechanical mixing tends to predominate over bubble effects in improving aeration, scale-up equations including bubble effects have had little use.

Fermentation biomass productivities usually range from 2 to 5 g/(l·h). This represents an oxygen demand in the range of 1.5 to 4 g O/(l·h). In a 500-m fermenter, this means achievement of a volumetric oxygen transfer coefficient in the range of 250 to 400 h^{-1}. Such oxygen-transfer capabilities can be achieved with aeration rates of the order of 0.5 VVM (volume of air at STP/volume of broth) and mechanical agitation power inputs of 2.4 to 3.2 Kw/m (1.2 to 1.6 HP/100 gal).

Often heat removal causes design problems for scale-up. Mechanical agitation coupled with a metabolic heat from the growing biomass

overwhelms cooling capacity of a large fermenter with only a jacket. External circulation through a heat exchanger or extensive coils inside the fermenter must be used. In highly viscous fermentations, internal cooling coils are usually not desirable because of interference with mixing patterns. Numerous schemes exist for heat removal in large fermenters such as half-coil baffles (plate coils) and draft tubes. Heat removal limits the size of packed-cell bioreactors. There is also a serious problem of gas evolution for bioreactors when the cells are immobilized with a membrane or by retention in a gel because the gas can rupture the structure.

Evaporation of medium provides a little cooling. The inlet air to particulate filters must not be near saturation because condensation of moisture on the filter medium mobilizes contaminating microorganisms so that their chances of penetration are greatly increased. Sometimes humidified air is used, and the filter unit is heated to prevent condensation. However, this is common only for small equipment where the extra operations are relatively easy to install and maintain.

Once a plant is built, the conditions of agitation, aeration, oxygen transfer, and heat transfer are more or less set, and sterilization cycles are defined. Those environmental conditions achievable in plant-scale equipment should be scaled down to the pilot plant and laboratory equipment (shaken flasks) to insure that results can be translated.

Sterilization Some old, traditional fermentations such as those for alcohol and pickles are conducted by organisms that are hardy and help their own cause by creating conditions that are unfavorable for competitors. Yeasts, for example, lower pH by producing acids from sugars, and tolerance to alcohol is another powerful advantage that allows their domination. Nevertheless, modern factories use aseptic techniques or extreme care to minimize contamination that can jeopardize product quality by affecting taste, texture, aroma, or appearance. Bioprocesses such as tissue culture to produce vaccines are very easily contaminated because there is an abundance of nutrients and no inherent protection against foreign organisms. Thus, bioprocesses range from relatively good self-protection to practically none; the value of the product and the need for quality control determine the extent to which precautions must be taken. Contamination in the practical sense is statistical in that foreign organisms can be present but may not propagate rapidly enough to damage the run. Good defense against contamination is relatively inexpensive, while absolute protection is impossible and attempts to achieve it can be inordinately costly. The production of agents for biological warfare takes extreme pains to keep the organisms away from the workers, yet people operating the fermentations are occasionally killed. The most expensive and best protected industrial fermentations are sometimes contaminated. Sterilization and aseptic techniques to keep bioprocesses uncontaminated can be crucial.

Common ways of sterilization are removal of microorganisms by filtration or killing them with heat or chemicals. Sterilization by filtration follows a standard unit operation that is covered in Sec. 18. The differences for biochemical engineering are: (1) the filter medium and the downstream lines are steamed at a pressure where the temperature kills all organisms and spores; (2) the sizes of the particles being removed are in the micrometer range; and (3) the filter medium should be reusable and not degraded by repeated heating.

Sterilization by Filtration Air is almost always sterilized by filtration. The alternative of heating to sterilize has been successful for small installations, but large equipment for heating an air stream to sterilizing temperatures has not been sufficiently reliable. While it seems simple enough to maintain a section of the air-supply pipeline at high temperature, automatic control is needed to adjust for varying heat transfer as flow rate changes, and an air cooling section is needed to prevent excessive heat load on the fermenter. Furthermore, energy costs are now much higher than when heating seemed a promising alternative to filtration. For exit gases from a fermentation that has hazardous organisms, heating is a reasonable precaution, and cost is not the key factor. A clever heating method failed in full-scale testing. The air was compressed to raise its temperature by the Joule-Thompson effect, but the method was abandoned because several batches became contaminated. Any flow system for heat sterilization is crippled by process upsets because a slug of material can have inadequate temperature or exposure time for killing.

Sterilization of liquids by filtration has performed very well since the advent of membrane filters of small pore size. When heat can damage the ingredients, filtration is an ideal choice. However, the extra handling and equipment mitigate against filtration because heat sterilization of a batch is easy and relatively inexpensive. A tank must be steam-sterilized anyway, so it is convenient to fill it first and sterilize the contents as well. When some constituents must not be subjected to heat, it is customary to sterilize the rest of the medium with heat and to filter concentrated solutions of the delicate ingredients. Very large vessels can have insufficient heat-transfer surface; the tank is sterillized empty, and the medium is sterilized by flow through a continuous sterilizer.

The magnitude of the air sterilization problem is seen from the usual needs of a highly aerobic fermentation where roughly 1 volume of air per volume of medium per minute may be used. For a factory with 20 fermenters of 100,000 l (3500 ft^3) each, 2 million l/m (70,000 ft^3/m) of air is handled. Very large compressors are used, and at least two are required so that one can be down for maintenance.

Fibrous or particulate filters are not important anymore because membrane filters are relatively compact and perform very well. For filtration by straining, there is an intermediate air velocity at which filtration efficiency is a minimum because different collection mechanisms predominate at different ranges of velocity. At low velocities, diffusional and electrostatic forces on the particle are important, and increased velocity shortens the time for them to operate. At high velocities, inertial forces that increase with air velocity come into play; below a certain air velocity, their effect on collection is zero. Surges or brief power failures could change velocity and collection efficiency.

Membrane filters for air sterilization are reliable and relatively small. Membranes are quite efficient for filtering air and tend to capture particles larger than the pore size. To ensure safety, a pore size of 0.2 to 0.3 micrometers is recommended. Hydrophilic membranes should not be used because moisture is held tightly in their pores and not dislodged unless quite high pressure drops are created across the membrane. Moisture tends to drain from hydrophobic membranes and collect in a sump. Sizing of a membrane unit for air filtration is based on the number of cartridges needed. Only 60 percent of the available pressure drop should be used in the calculation to allow for increased resistance as particles collect on the membrane. Figure 24-18 shows membrane cartridges in parallel in a housing.

Sterilization of Media First-order kinetics may be assumed for heat destruction of living matter, and this leads to a linear relationship when logarithm of the fraction surviving is plotted against time. However, nonlogarithmic kinetics of death are quite often found for bacterial spores. One model for such behavior assumes inactivation of spores via a sensitive intermediate state by the mechanism:

$$C_r \rightarrow C_s \rightarrow C_d \tag{24-6}$$

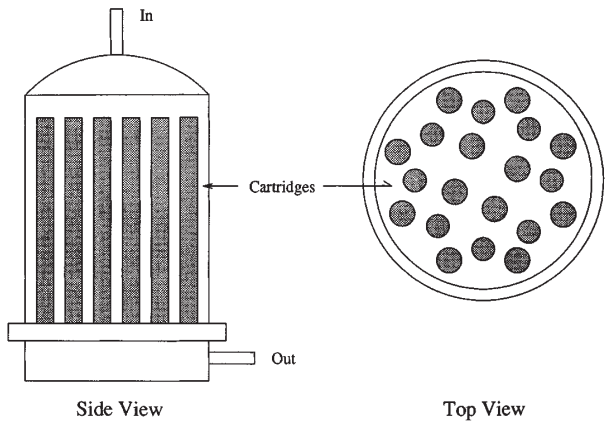

FIG. 24-18 Sketch of housing and membrane cartridges for air filtration. Typical cartridges are 76 cm long and 7.36 cm in diameter of polyvinylidene difluoride with 0.22-μm pores.

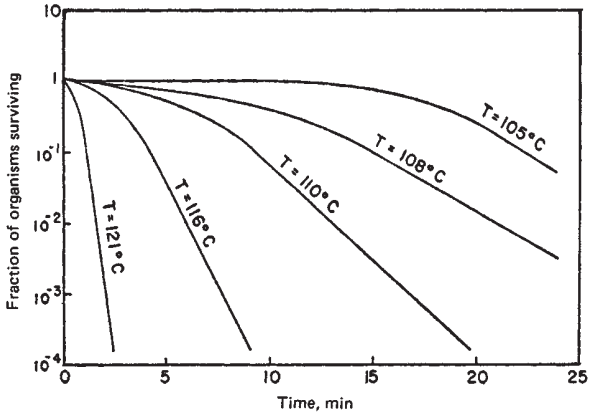

FIG. 24-19 Typical death-rate data for bacterial spores (*B. stearothermophilus*). To convert °C to °F, multiply by 1.8 and add 32 (*Wang et al.*, Fermentation and Enzyme Technology, *Wiley-Interscience, New York, 1979, p. 140.*)

where C_r, C_s, and C_d are concentrations of resistant, sensitive, and dead spores, respectively. Typical plots are in Fig. 24-19.

Relative thermal resistance for the different types of microorganisms encountered in typical environments associated with fermentation broths is shown in Table 24-3. Bacterial spores are far more resistant to moist heat than are any other type of microbial contaminants; thus, a sterilization cycle based on the destruction of bacterial spores should destroy all life.

TABLE 24-3 Relative Resistance to Killing by Moist Heat

	Relative resistance
Vegetative bacteria or yeast	1.0
Bacterial spores	3,000,000
Mold spores	2 to 10
Virus and bacteriophage	1 to 5

As predicted by the Arrhenius equation (Sec. 4), a plot of microbial death rate versus the reciprocal of the temperature is usually linear with a slope that is a measure of the susceptibility of microorganisms to heat. Correlations other than the Arrhenius equation are used, particularly in the food processing industry. A common temperature relationship of the thermal resistance is *decimal reduction time* (DRT), defined as the time required to reduce the microbial population by one-tenth. Over short temperature intervals (e.g., 5.5°C) DRT is useful, but extrapolation over a wide temperature interval gives serious errors.

The activation energy (E) associated with microbial death is larger than the thermal inactivation of chemical compounds in fermentation broths (see Table 24-4). Thus by sterilizing at high temperatures for short times (HTST), overcooking of nutrients is minimized.

TABLE 24-4 Various Activation Energies for Thermal Destruction

	Activation energy, cal/mol
Folic acid	16,800
d-Panthothenyl alcohol	21,000
Cyanocobalamin	23,100
Thiamine hydrochloride	22,000
Bacillus stearothermophilus	67,700
Bacillus subtilus	76,000
Clostridium botulinum	82,000
Putrefactive anaerobe NCA 3679	72,400

To convert calories per mole to British Thermal Units, multiply by 1.8.

Batch Sterilization Assuming that the presence of one single contaminating organism could cause ultimate failure of the desired fermentation, it is necessary to assign some probability of success. For example, if one contaminated fermentation per thousand can be tolerated, the design calculation for the sterilization cycle should use 0.001 organisms per fermentation. However, in batch sterilization, the heating, holding, and cooling portions of the cycle all contribute toward the reduction of the microbial contaminants. Furthermore, the specific death-rate constant varies because the temperature of the medium changes. Therefore, the design criterion total is composed of

$$\nabla \text{ total} = \nabla \text{ heating} + \nabla \text{ holding} + \nabla \text{ cooling}$$

$$\nabla \text{ heating} = \ln \frac{N_0}{N_1} = A \int_0^{t_1} \exp\left(-\Delta E/RT\right) dt \tag{24-7}$$

$$\nabla \text{ holding} = \ln \frac{N_1}{N_2} = A \int_0^{t_2} \exp\left(-\Delta E/RT\right) dt \tag{24-8}$$

$$\nabla \text{ cooling} = \ln \frac{N_2}{N} = A \int_0^{t_3} \exp\left(-\Delta E/RT\right) dt \tag{24-9}$$

Other parameters affecting the temperature profile include the viscosity of the medium and amount of suspended solids or insoluble materials such as vegetable oils that can foul heat-transfer surfaces. Releasing part of the pressure after the holding period gives flash cooling. The temperature-time profile during the cooling portion of the sterilization cycle includes effects of cooling coils or the fermenter jacket and evaporative cooling if sterile air is injected to remove heat. Most of the sterilization is derived from the holding portion of the cycle; the cooling cycle contributes little to the overall process. An extremely long heating cycle should be avoided because its contribution towards microbial destruction is far outweighed by its detrimental biochemical effects.

Continuous Sterilization Continuous sterilization permits short detention times at high temperature to avoid overcooking and has potential for improvement in yield. Tubular or plate-and-frame heat exchangers in a system for continuous sterilization provide economical heat exchange between process streams. However, direct injection of steam offers almost instantaneous heating to the sterilization temperature but wastes energy because heating and cooling are not integrated. Steam injection has no heating surface to foul.

In the holding section of a continuous sterilizer, correct exposure time and temperature must be maintained. Because of the distribution of residence times, the actual reduction of microbial contaminants in the holding section is significantly lower than that predicted from plug flow assumption. The difference between actual and predicted reduction in viable microorganisms can be several orders of magnitude; therefore, a design based on ideal flow conditions may fail.

Cell Culture Single plant and animal cells are much larger than microbial cells and are easily damaged by shear due to intense agitation. Standard stirred tank bioreactor designs tend not to work well for undifferentiated plant and animal cells. Attempts to substitute paddle and spiral agitators has proven successful in some cases, but shear can still cause a morphological change in the structure of the cells. Usually, single cells and cellular aggregates tend to be smaller and produce significantly less secondary metabolites with improper agitation. Airlift reactors may work well, and cultured cells tend not to have severe oxygen requirements. There has been a thrust for new systems that can maintain absolute sterility without depending on antibiotics that can mask the slow development of a low-grade infection in a culture and can affect the metabolism of cultured cells in subtle ways that cannot always be predicted.

Cells that must attach to a surface can be grown on spongy polymers, arrays of thin tubing or hollow fibers, stacks of thin plates, or microscopically small beads called microcarriers. Damage from shear can be a serious problem; thus, aeration systems cannot employ the vigorous mixing that aids mass transfer in microbial processes. Airlift bioreactors have been developed that are tapered instead of being cylindrical with a central draft tube. Above a zone of intense aeration are the suspended cells circulating with little more mixing than would result from Brownian motion.

Improvements in fermentation include microcarriers that not only provide support for anchorage-dependent cells but also aid in harvesting at the end of a run. While microcarriers based on dextran, polystyrene and polyacrylamide beads have been widely used in the past, new materials for microcarriers make separating cells from their growth medium easier. Some are made of collagen that is detached from the cells by immersion in dilute collagenase. However, since some collagen can be left in solution, downstream processing can be made difficult. An alternative is to use plastic beads coated with collagen. The plastic can be easily separated after the cells are released enzymatically. Another alternative is glass-coated particles that induce attachment of the cells' long slender filopodia. At the end of a process, a brief incubation in dilute trypsin gently removes the cells from the beads.

Supplying sufficient oxygen can be difficult when dealing with differentiated plant tissues such as root cultures that can reach lengths of several decimeters and can be highly branched and complex structurally. In this case, mechanical agitation is impractical because the mass of roots can occupy approximately 50 percent of the reactor volume. Alternatives are bubble columns, rotating drums, and trickle bed reactors where the medium is recycled and sprayed over the column of roots.

Because of the differences in primary and secondary metabolism, a reactor may have a dual-stage fed-batch system. In other words, fed-batch operation optimizes growth with little or no product formation. When sufficient biomass has accumulated, a different fed-batch protocol comes into play.

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PRODUCT RECOVERY

Although most of the purification equipment in a large biotechnological factory is the same as that used throughout the chemical process industries, there are fewer separations in which the product reaches elevated temperatures. Most biochemicals are destroyed if heated. Recovery of products from the bioprocess fluid can be more difficult and expensive than all of the previous steps. The ratio of recovery costs to cost of creating the product can range from about one to more than ten because the investment for the recovery facilities may be several times that for the fermenter vessels and their auxiliary equipment. As much as 60 percent of the fixed costs of fermentation plants for organic acids or amino acids is attributable to the recovery section. The costs for recovery of proteins based on recombinant DNA techniques are particularly high.

Research and development for better recovery of existing products may provide diminishing returns as time passes, and some companies focus on new products. Government regulations are different for drugs that are sold in very nearly pure state and for biologicals that may be ill-defined. Little paperwork is required for a process improvement for a pure drug. For biologicals intended for humans, securing government approval makes it unwise to modify the recovery process except when the potential savings are very great. The extensive and expensive testing and validation of such therapeutic agents is keyed to the processes for making and purifying them. Only trivial changes are permitted; otherwise the testing must be repeated. Market forces make it important to have a new product tested and ready for sale as quickly as possible, but this usually means that the process has not been optimized. Whereas competition and process improvements resulted in remarkable lowering of prices in the past, the regulations now discourage investment in process development other than at the early stages prior to submission of the documents for government approval.

Certain products (e.g., inclusion bodies) are contained inside the cells and are not released or only partially released to the medium. It may be possible to flush impurities from the cells before breaking them to get the product. Cell disruption is a unit operation peculiar to biochemical engineering. The equipment, however, may be borrowed from other industries. Colloid mills and shear devices used for manufacturing paint and other products effectively rupture walls of many types of cells. Cells with high resistance to shear can be passed through the unit several times, but heat generation from the process can cause loss of product. Shear alone can denature sensitive proteins. Ultrasonic energy is commonly used on a small scale for cell disintegration but is impractical for large batches. Grinding with sand or beads, high-pressure pumping through a tiny orifice, freezing and thawing, dessication, adding lytic enzymes, inducing autolysis with a chemical such as chloroform, and various means of creating shear are alternative or synergistic means of rupturing cells. There are encouraging results with special mutants of cells with impaired ability to form cell walls at temperatures slightly above their normal growth temperatures. When shifted to the elevated temperature, cell division gives damaged walls and lets the cell contents leak out. The bioprocess operates at its optimum temperature until the temperature is raised shortly before harvest. Cells that form at the elevated temperature release their contents easily.

Whenever possible, the fermentation fluid goes directly to ion exchange, solvent extraction, or some other step. However, prior removal of biological cells and other solids is usually necessary. Centrifugation can be considered, but rotary drum filters with string discharge are commonly used. In the past, large amounts of filter aid were added because many fermentation broths are slimy and hard to filter. Present practice employs polymeric bridging agents to agglomerate the solids. This allows good filtration with only small amounts of filter aid.

The most popular steps for recovery are ion exchange or solvent extraction because selectivity is good, costs are reasonable, and large scale is feasible. Unfortunately, some biochemicals neither exchange ions nor extract well. These and other purification steps can be affected by modifications in fermentation. Adding excess lipids or antifoam oils to a fermentation can aggravate emulsion problems for solvent extraction or impair ion exchange by coating the resin. Stability of biochemical products can be troublesome. For example, penicillin fermentation broth is acidified just prior to contact with the extracting solvent because low pH causes very rapid destruction of penicillin in water. The most popular immiscible solvent is methylisobutylketone (MIBK); halogenated hydrocarbons are avoided because of their hazard for humans. Penicillin is extracted back into an aqueous phase at pH 7.5 to 8 using bicarbonate as a buffer because harsher agents are difficult to control. The Podbelniak design of centrifuge (see Sec. 15) is widely used in the United States for extraction of fermentation broths, while the Westphalia design is common in Europe. Countercurrent flow through a centrifuge should result in more than one equilibrium contact, but emulsions may carry product into the phase being wasted. Lead-and-trail operation of the centrifuges can improve yields. Extraction back into water is seldom troubled by emulsions, and DeLavalle separators work well.

Some products are precipitated from the fermentation broth. The insoluble calcium salts of some organic acids precipitate and are collected, and adding sulfuric acid regenerates the acid while forming gypsum (calcium sulfate) that constitutes a disposal problem. An early process for recovering the antibiotic cycloserine added silver nitrate to the fermentation broth to precipitate an insoluble silver salt. This process was soon obsolete because of poor economics and because the silver salt, when dry, exploded easily.

Of the various types of purification steps based on sorption to a

solid phase, ion exchange is the most straightforward. See Sec. 22 for a discussion of techniques. Ion-exchange resins actually adsorb Vitamin B_{12} well instead of exchanging it. Carbon adsorption has considerable importance to biochemical engineering, primarily for the removal of traces of colored impurities. When neither solvent extraction nor ion exchange can be used as the primary concentration/purification step because of the chemical properties of the desired product, an alternative may be adsorption on carbon. Although carbon adsorption may be unselective and low in capacity, it can provide a roughing step to get the purification scheme started.

Isolation procedures for many biochemicals are based on chromatography. Practically any substance can be selected from a crude mixture and eluted at relatively high purity from a chromatographic column with the right combination of adsorbent, conditions, and eluant. For bench scale or for a small pilot plant, such chromatography has rendered alternate procedures such as electrophoresis nearly obsolete. Unfortunately, as size increases, dispersion in the column ruins resolution. To produce small amounts or up to tens of kilograms per year, chromatography is an excellent choice. When the scale-up problem is solved, these procedures should displace some of the conventional steps in the chemical process industries.

Affinity chromatography uses ligands with high specificity for certain compounds. There are several types of affinity that can be employed: antigen-antibody, enzyme-substrate, enzyme-cofactor, chelation with metal ions, or special biochemical attractions such as the protein avidin for the vitamin biotin. Numerous purifications have been devised wherein affinity chromatography is able to isolate quite pure product from a very crude mixture. Expensive affinity agents are regenerated and reused many times. In some cases, the attraction is so strong that the adsorbent can be added batchwise. This scales up well but is less convenient than column operations in terms of collection, elution, and regeneration of the affinity agent.

Conventional elution chromatography has the serious disadvantage of dilution, and usually a concentration step must follow. The technique of displacement chromatography circumvents dilution and may even result in an eluant more concentrated than the feed. A displacer compound breaks the desired product from the chromatographic material sharply, and a column heavily loaded with several biochemicals will release them one at a time depending on their adsorption equilibria. However, the displacers tend to be expensive and can be troublesome to remove from the product.

A number of water-soluble polymers will cause phase separation when present together at concentrations of a few percent. The most widely used polymers are polyethylene glycol (PEG) and dextran. Proteins, other macromolecules, and cell components such as mitochondria distribute in the phases or collect at the interface. Proteins are destabilized at organic solvent/water interfaces, but when each solvent is water, the interfacial tension is negligible. Some salts such as potassium phosphate will also induce phase separation when a polymer is present, but the salt concentration must be high. Two-phase aqueous systems provide a mild method for purification of proteins, and scale-up to large volumes presents no engineering problems. The polymers can have functional groups that improve distribution coefficients of the biochemical products, but the costs for these polymers are high. Although highly promising, two-phase aqueous methods are used only for valuable products because the cost of the polymers is too high and they are not easily recovered for reuse. Another drawback is distribution coefficients not far from 1 for most proteins; several extraction stages are needed to get acceptable yields when the distribution coefficients are unfavorable.

Surface-active agents and liquids immiscible in water can form tiny dispersed units called reverse micelles. These can extract biochemicals from water or permit complexing or reacting in ways not possible in simple aqueous systems.

Crystallization is the preferred method of forming many final products because very high purification is possible. High purity antibiotic crystals can be produced from colored, rather impure solutions if the filter cake is uniform and amenable to good washing to remove the mother liquor. When a sterile pharmaceutical product is desired, crystals are formed from liquid streams that have been sterilized by filtration.

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PROCESS MODELING

It is generally assumed that properties of very large numbers of cells can be treated as continuous functions having average properties because there are so many cell divisions occurring that the overall rates follow smooth curves. There is an exception in which the cells can all be induced to divide at the same time because events such as illumination or temperature changes slow or halt a step in division. The cells can be triggered to proceed together from that point with overall numbers that are stepwise with time. This is termed a *synchronous culture;* the steps are seldom distinct for more than a few generations unless the triggering event continues to be applied periodically.

Mass balances for common, unsynchronized batch culture give:

$$\frac{dX}{dt} = \mu X - K_d X \tag{24-10}$$

$$\frac{dS}{dt} = -\frac{\mu X}{Y} \tag{24-11}$$

$$\mu = f(S) \tag{24-12}$$

Various functional relationships between μ and S have been proposed, but the Monod equation is used almost exclusively:

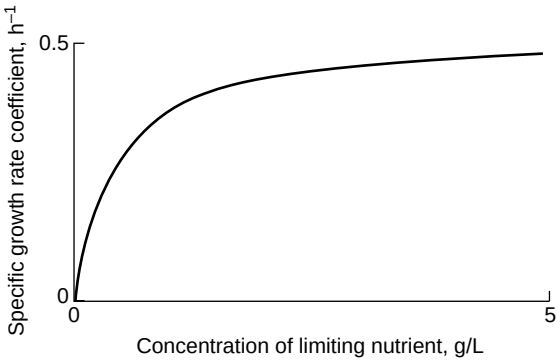

FIG. 24-20 Plot of the Monod equation.

$$\mu = \mu_{max} \times \frac{S}{K_s + S} \tag{24-13}$$

A graph of the Monod equation is shown as Fig. 24-20.

The death rate coefficient is usually relatively small unless inhibitory substances accumulate, so Eq. (24-10) shows an exponential rise until S becomes depleted to reduce μ. This explains the usual growth curve (Fig. 24-21) with its lag phase, logarithmic phase, resting phase, and declining phase as the effect of K_d takes over.

Structured Models Meaningful detail can be added to culture models in several ways. Cells can be compartmentalized according to biochemical functions, and the components can interact. For example, there can be a group of equations for carbohydrate metabolism, a group for protein synthesis, another for nucleic acid synthesis, and so on. This permits a much more intricate description of cell activities but at the expense of having so many rate constants that assigning values to them may end up as guesswork. For cells with distinct life cycles, a structured model may have compartments corresponding to each stage in the cycle. In addition, each compartment may be subdivided into the biochemical functions mentioned above. Such complicated models have had limited practical use but have great value for directing research toward areas where information is lacking.

Continuous Culture Continuous culture has been a goal of bioengineers for several decades because batch culture has inherent down time for cleaning and sterilization and long lags before the organisms enter a brief period of high productivity. Continuous runs can last many weeks, but there must be stoppages for cleaning and maintenance. Bacteria may foul surfaces to a small extent, but molds tend to form thick coatings on the shaft, coils, and any protuberances in the fermenter after several weeks of continuous cultivation, that seriously impair mixing and mass transfer.

The nutrition and the product mix can be advantageously manipulated as functions of dilution rate. A serious problem, however, is instability of the culture itself. There is a tendency to revert to less productive strains that quickly replace the finely tuned mutants that achieve high titers of product. The main successes with continuous fermentation have been with rugged strains that are producing either cell mass for cattle feed or a simple enzyme or metabolite. When a single stage is used for a product that is elaborated from cells that are not growing, it is difficult to optimize simultaneously cell growth, product production, and efficient use of substrates.

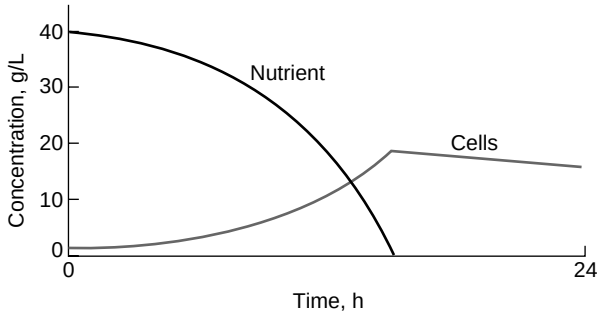

FIG. 24-21 Microbial growth curve. $dx/dt = \mu X - K_d X$; $ds/dt = -\mu X/Y$; $\mu = \mu_{max} S/(K_s + S)$; $\mu_{max} = 0.35$; $K_d = 0.025$; $K_s = 12.0$ mg/L; $Y = 0.48$.

In view of its few industrial applications, continuous culture gets a disproportionate amount of attention from academicians. As a research tool, batch culture suffers from changing concentrations of products and reactants; varying pH and redox potential; and a complicated mix of growing, dying, and dead cells. Data from continuous cultures are much easier to interpret because steady states are achieved or there are repeatable excursions from steady state. The usual explanations for limited use of continuous culture in industry are: culture instability, difficulty of maintaining asepsis, insufficient knowledge of microbial behavior, and reluctance to convert existing factories. Overall cost savings can be relatively small for continuous cultivation because productivity of the bioreator is not very important compared to high product concentration. Another factor is the cost of each research station. Rapid progress in research and development requires multiple vessels for screening many variables, but there are usually only one or two continuous fermenters in the lab or pilot plant because the cost of pumps, reservoirs, sterilizers, and controls is relatively high.

Conventional means for continuous culturing are the chemostat in which nutrient is fed to a reactor at constant rate and the turbidostat that employs feedback control of pumping rate to maintain a fixed turbidity of the culture. Another alternative with feedback control of a nutrient or product concentration has been termed *auxostat, nustat,* or *nutristat.* Proportional control of the pumping rate is desirable because continuous cultures can have oscillatory responses induced by turning the feed pump on or off. A chemostat tends to be unstable and erratic at dilution rates that approach the maximum specific growth rate of the organisms. This is explained by the adjustment of growth rate to nutrient concentration in the region where a small change in dilution rate equates to a big change in nutrient concentration. An auxostat has little advantage over a chemostat at moderate dilution rates but is stable at the high dilution rates at which the chemostat is unreliable.

MATHEMATICAL ANALYSIS

The concept of a limiting nutrient is essential to the theory of continuous culture. There will only be exact stoichiometric balance of all the ingredients going into the cells when a very deliberate and time-consuming effort has been made to determine the details of cell nutrition. Even then, there may be a different balance if the growth rate is changed or kinetic rather than stoichiometric limitations may apply. The ingredient in short supply relative to the other ingredients will be exhausted first and thus limit cellular growth or product synthesis. The other ingredients may exhibit toxicity or influence cellular activities, but there will not be acute shortage as in the case of the limiting nutrient.

Mass balances for one vessel in a series of continuous fermenters give:

Rate of change = rate in − rate out + rate of production

or
$$\frac{V dX_n}{dt} = FX_{n-1} - FX + V\mu_v X_n \tag{24-14}$$

Dividing through by V and substituting $D = F/V$:

$$\frac{dX_n}{dt} = D(X_{n-1} - X_n) + \mu_n X_n \tag{24-15}$$

and
$$\frac{V dS_n}{dt} = FS_{n-1} - FS_n - \frac{V\mu_n X_n}{Y} - VMX_n \tag{24-16}$$

$$\frac{dS_n}{dt} = D(S_{n-1} - S_n) - \frac{\mu_n X_n}{Y} - MX_n \tag{24-17}$$

For a single vessel with sterile feed, this reduces to:

$$\frac{dX}{dt} = \mu X - DX \tag{24-18}$$

$$\frac{dS}{dt} = D(S_o - S) - \frac{\mu X}{Y} - MX \tag{24-19}$$

This is an old, familiar analysis that applies to any continuous culture with a single growth-limiting nutrient that meets the assumptions of perfect mixing and constant volume. The fundamental mass balance equations are used with the Monod equation, which has no time dependency and should be applied with caution to transient states where there may be a time lag as μ responds to changing *S*. At steady state, the rates of change become zero, and $\mu = D$. Substituting:

$$D = \mu_{max} \frac{S}{K_s + S} \tag{24-20}$$

and

$$S = \frac{DK_s}{\mu_{max} - D} \tag{24-21}$$

Solving for *X* gives:

$$X = DY \times \frac{S - S_o}{D + MY} = Y(S_o - S) \tag{24-22}$$

From these equations, the behavior of *X* and *S* as functions of dilution rate can be plotted as in Fig. 24-22.

The interesting features are: (1) *X* goes to zero and *S* reaches S_o as *D* approaches μ_{max}; (2) *S* is not a function of S_o when *D* is less than μ_{max}; (3) the maintenance coefficient is very important at low dilution rate but has little effect afterwards; and (4) S_o is never so high that μ_{max} can be reached, thus washout always occurs before μ_{max} and is a function of S_o.

Mixing has been shown to be critical at low dilution rates because uptake of substrate is extremely rapid for cells in a starved condition. Vigorous agitation is required in small vessels to insure homogeneous distribution of the feed; such intense agitation is probably impractical in large vessels, but good dispersion has been achieved by distributing the feed from many fine openings throughout the vessel.

It is easy to postulate advantages for multistage continuous culture but very difficult to conduct all of the research and development of the many parameters that should be optimized. Each stage could have its feed streams, control of pH and other conditions, and recycle of cells or fluids from other steps in the process. Not only are there many parameters to study for each stage, but changes in one stage can markedly affect other stages. It can be quite troublesome to get representative conditions and cultures in a given stage to begin research because of the complicated interactions with other stages. Time delays in lines and separators for recycling plus complexities from nonideal flow regimes cause a theoretical analysis to be faulty. An optimized multistage continuous fermentation system with recycle and control is a most difficult engineering feat, and the dynamics of microbial responses to upsets are poorly understood. Nevertheless, a three-stage continuous fermentation process is used in industry for the manufacture of a vitamin.

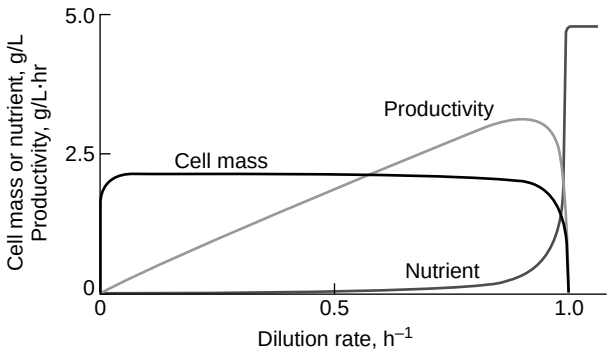

FIG. 24-22 Computer analysis of steady states in continuous culture; $\mu = 2.38$ h^{-1}; $S_o = 1100$ mg/l; $Y = 0.45$; $K_s = 35$ mg/l; $M = 0.05$.

COMPUTER AIDS FOR ANALYSIS AND DESIGN

Specialized programs make layout easy in the form of diagrams of the individual components and their interconnections. Often there are databases for thermodynamic properties as well as routines that will calculate approximate values for properties of the compounds handled in the factory. The programs save the engineer from many tedious calculations, and it is practical to investigate options for equipment sizes and operating conditions. Advice on selecting modeling software is available [Chan, W. K., J. F. Boston, and L. B. Evans, "Select the Right Software for Modeling Separation Processes," *Chem. Engr. Prog.*, **87,** 63–69 (1991)]. A partial list of programs is:

PRO/II from Simulation Sciences Inc., Fullerton CA

HYSIM from Hyprotech, Ltd., Calgary, Alberta; Houston TX; Whittier CA

CHEMCAD from Coade Engr. Software, Houston TX

DesignPFD from ChemShare, Houston TX

Aspen/SP from JSD Simulation Service Co., Denver CO

ELECTROSIM (processes dealing with dissociation and chemical reactions), from Real Time Simulation

POWERTRAN-PC, from Bigelow Systems

DataLogiX, from DataLogiX Formula Systems

G2, a bioprocess expert program with simulation and control from GENSYM, Cambridge, MA

BioPro Designer from Intelligen, Inc., Scotch Plains, NJ

PLANT CELL AND TISSUE CULTURES

Monod kinetics where the growth is limited by a single substrate can apply but only for specific stages of growth and not for the entire growth cycle of plant cells. An example of this is cells grown in a 14 l (0.5 ft^3) fermenter where the latter stages of growth were adequately described by the Monod equation but the initial stages were not. It is understandable that differentiated plant tissues with their distinct cellular characteristics may not be suited to a simple growth-rate expression. For example, root tissues have a meristematic stage where cells are actively dividing. This is followed by cells expanding and maturing to transport nutrients. Older cells may sequester certain nutrients until required by the younger cells and thus alter the availability of substrates.

A kinetic model originally derived by Nyholm is distinguished from Monod's model by the fate of a limiting substrate. Instead of immediate metabolism, the substrate in Nyholm's model is sequestered. The governing equations are:

$$\frac{dC_i}{dt} = v - \mu C_i \tag{24-23}$$

$$\mu = f(C_i) \tag{24-24}$$

$$v = \frac{1}{x}\frac{dS_i}{dt} \tag{24-25}$$

In these equations, μ = specific growth rate coefficient, *v* = specific rate of substrate uptake, *t* = time, *x* = biomass concentration, S_i = intracellular substrate, and C_i = concentration of intracellular substrate. Several examples where these equations can be applied include nitrogen limitations in *M. citrifolia* cultures and phosphate limited growth in *C. roseus*, *N. tabacum*, and *Papaver somniferum*.

The production of secondary metabolites has often been characterized using the classical equations of Leudeking and Piret. However, the complexities of plant cell and tissue cultures have led to revisions to this equation to include fresh cell weight and viability, cell expansion, and culture death phase. Therefore, the production model is written as the following:

$$\frac{dP}{dt} = \alpha V \frac{dX_d}{dt} + \beta V X_d + \frac{P}{V}\frac{dV}{dt} \tag{24-26}$$

where P = total intracellular product concentration, t = time, V = fraction of viable cells, and X_d = total dry cell weight. Furthermore, a and b represent the usual growth and nongrowth associated production constants, respectively. This type of model has been successfully applied to the production of ajmalicine and serpentine from *C. roseus* cell cultures. Similarly, the production of gossypol by *G. hirsutum* cell cultures used this type of production model but took into account the further metabolism of the phytoalexin due to the growth of new cells.

Because of the differences in primary and secondary metabolism, a reactor may have a dual-stage fed-batch system. In other words, fed-batch operation optimizes growth with little or no product formation. When sufficient biomass has accumulated, a different fed-batch protocol comes into play.

When the production of the secondary metabolites coincides with the death and general lysis of the cells, the recovery of the product is simply a matter of separation from the spent production solution downstream of the reactor. An example of this type of operation was initially used in Japan during the production of shikonin. However, if the secondary metabolites are stored in the vacuole of the cells and the cells remain viable but dormant during the production phase, then a permeabilizing agent such as dimethylsulfoxide (DMSO), detergents, proteins, and antibiotics may be employed in some cases in concentrations that make the cells leak product out but maintain cell viability. Success for this type of product recovery has been reported in *C. roseus, Datura innoxia,* and *Daucus carota* cell cultures.

ADDITIONAL REFERENCES: Nyholm, N., *Biotechnol. Bioeng.* **18,** 1043 (1976). Bailey, C. M. and H. Nicholson, *Biotechnol. Bioeng.* **34,** 1331 (1989). Cazzulino, D. L., H. Pedersen, C. K. Chin, and D. Styer, *Biotechnol. Bioeng.* **35,** 781 (1990). Staba, E. J., *Plant Tissue Cultures as a Source of Biochemicals,* CRC Press, 1980. Thorpe, T. A., *Plant Tissue Culture: Methods and Applications in Agriculture,* Academic Press, 1981. Payne, G., V. Bringi, C. Prince, and M. Shuler, *Plant Cell and Tissue Culture in Liquid Systems,* Hanser Publishers, 1992. Brodelius, P. and K. Nilson, *Eur. J. Appl. Microbiol. Biotechnol.,* **17,** 275 (1983). Rehm, H.-J. and G. Reed, *Biotechnology,* vol. 6b, VCH Verlagsgesellschaft, 1988.

Recycle Separation and recycle of cells results in much longer residence times for the cells than for the fluid and permits relatively high cell concentrations. In waste treatment, the dilute feed leads to slow growth rates, so more rapid processing is attained through cell recycle to establish a higher population. A higher percentage of the cells may be dead in a recycle system because all are in a starved state. High rates of production are also important in industrial fermentations with cell recycle, and there is the added advantage of reusing cells instead of diverting expensive substrate to producing more cells.

In waste treatment with activated sludge units, organisms not associated with flocs are not collected and tend to leave the system as recycle increases the proportions of flocculating types. Recycle of collectible algae to outdoor ponds has profound influence on the population, but seasonal changes can develop small algae despite retention of large algae; thus, recycle fails as the few large algae die or escape collection.

Recycle of fermentation fluids has quite different objectives than those for cell recyle that aims for population control or greater productivity. Spent broths have leftover nutrients, so the recycling process can save on costs of nutrients and make up water while greatly reducing the volumes sent to waste treatment. Of course, total recycle is bad because undesirable materials build up in concentration and can poison the fermentation. This buildup determines the amount of recycle, but there may be purification steps to remove toxic substances. For example, glycerol is a by-product of alcohol fermentation, but its low volatility means that much is in the stillage from alcohol recovery. Its removal by a physical or chemical step would prevent accumulation in the fermenter by recycling. Alternatively, it could be metabolized by a special strain or mixed culture. Glycerol recovery from the recycle stream in the bioproduction of ethanol now competes with older processes for recovery in the oil and fat industries.

Figure 24-23 is a sketch of continuous culture with recycle. The symbols for flow rates and organism concentrations are F and X, respectively. Assuming perfect mixing and steady state so that the derivatives can be set to zero, mass balances lead to:

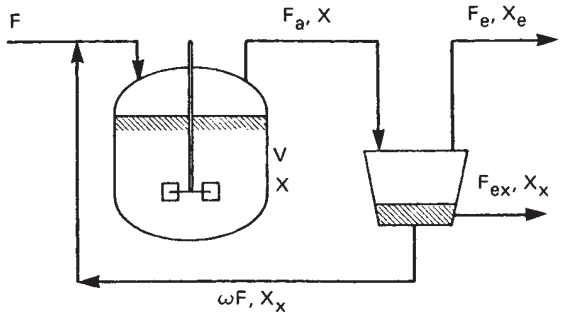

FIG. 24-23 Continuous culture with recycle. (*A. E. Humphrey, "Biochemical Engineering" in* Encyclopedia of Chemical Processing and Design, *vol. 4, July 1977, pp. 359–394.*)

$$\mu = D\left[1 + \omega\left(1 - \frac{1+\omega}{1+\omega-\frac{F_e}{F}}\right)\right] \tag{24-27}$$

Without recycle, washout occurs when D is greater than μ_{max}, but recycle permits operation with D far greater than μ_{max}. A family of curves is shown in Fig. 24-24 for concentrations of cell mass and nutrient at different recycle ratios. The distinct differences from Fig. 24-22 with no recycle are obvious.

Mixed Cultures Mixtures of microorganisms characterize the processes shown in Table 24-5.

Processes for biological waste treatment have elective cultures, and the proportions of different species can shift dramatically in response to changing nutrition or physiological conditions. There is an interesting area of research on defined mixtures of microorganisms, but there has been little practical application of the results. The definitions of various interactions are in Table 24-6. These definitions are difficult to apply to real systems where there is highly complicated interplay among organisms that play various roles with respect to each other.

Two types of interaction, competition, and predation are so important that worthwhile insight comes from considering mathematical formulations. Assuming that specific growth-rate coefficients are different, no steady state can be reached in a well-mixed continuous culture with both types present because, if one were at steady state with $\mu = D$, the other would have μ unequal to D and a rate of change unequal to zero. The net effect is that the faster-growing type takes over while the other declines to zero. In real systems—even those that approximate well-mixed continuous cultures—there may be profound

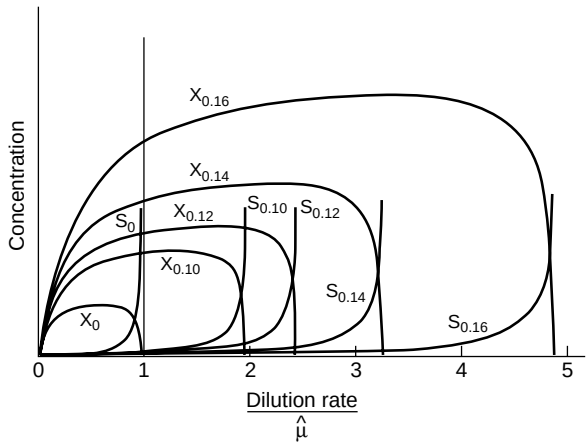

FIG. 24-24 Effect of recycle on steady-state concentrations of cell mass and limiting nutrient. 5-fold increase in cell concentration in separator. Subscripts denote fraction of cell concentrate recycled.

TABLE 24-5 Mixed Culture Processes

Process	Types of organisms
Commercial	
Alcoholic beverages	Various yeasts, molds, and bacteria
Sauerkraut	*L. plantarum* plus other bacteria
Pickles	*L. plantarum* plus other bacteria
Cheeses	Propionibacteria, molds, and possibly many other microorganisms
Lactic acid	Two lactobacillus species
Waste treatment	
Trickling filters	Zoogloea, protozoa, algae, fungi
Activated sludge	Zoogloea, *Sphaerotilus,* yeasts, molds, protozoa
Sludge digestion	Cellulolytic and acid-forming bacteria, methanogenic bacteria
Sewage lagoons	Many types from most microbial families

TABLE 24-6 Some Definitions of Microbial Interactions

Competition	A race for nutrients and space
Predation	One feeds on another
Commensalism	One lives off another with negligible help or harm
Mutualism	Each benefits the other
Synergism	Combination has cooperative metabolism
Antibiosis	One excretes a factor harmful to the other

changes in relative numbers of the various organisms present, but complete takeover by one type is extremely uncommon. Survival of a broad range of species is highly advantageous in natural systems because a needed type will be present should an uncommon nutrient (pollutant?) be added or the conditions change.

Prey-predator or host-parasite systems can be analyzed by mass balance equations:

$$\frac{dH}{dt} = \mu_H H - DH - KHP \tag{24-28}$$

$$\frac{dP}{dt} = \mu_P P - DP \tag{24-29}$$

$$\frac{dS}{dt} = D(S_o - S) - \frac{\mu_H H}{Y} \tag{24-30}$$

where H = the concentration of hosts (prey)
P = the concentration of predators
S = substrate concentration (food for prey)
K = a coefficient for killing

and μ_H and μ_P are Monod functions of S and H respectively.

Computer simulation of these equations is shown in Fig. 24-25. Real systems do have this type of oscillating behavior, but frequencies and amplitudes are erratic.

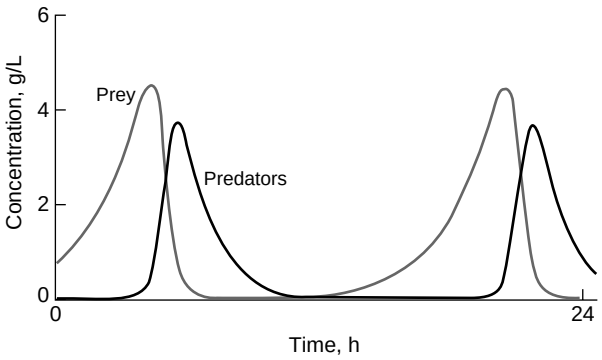

FIG. 24-25 Computer simulation of prey-predator kinetics.

Another interaction with grave consequences is attack on a species by a phage (microbial virus) that is usually highly specific. Infection of a cell by a virulent phage results in the production of 10 to several hundred new phage particles as phage nucleic acid takes over control of cellular activities. The cell disintegrates and releases phage that infect other cells to reach high phage titers quickly. A few cells of the host species may be resistant to phage; such resistance can be acquired through mutation. These cells have fewer competitors and may thrive. However, mutations also occur in phage, so highly complicated behavior occurs as the hosts mutate and mutate further as the phage mutates to counter host resistance.

Commercial fermentation groups usually maintain different strains of cultures suitable for production so that phage attacks can be thwarted by substituting a nonsusceptible culture. After a period of time for the phage to dissipate, it may be possible to return the most desirable production strain.

Bioprocess Control An industrial fermenter is a fairly sophisticated device with control of temperature, aeration rate, and perhaps pH, concentration of dissolved oxygen, or some nutrient concentration. There has been a strong trend to automated data collection and analysis. Analog control is still very common, but when a computer is available for on-line data collection, it makes sense to use it for control as well. More elaborate measurements are performed with research bioreactors, but each new electrode or assay adds more work, additional costs, and potential headaches. Most of the functional relationships in biotechnology are nonlinear, but this may not hinder control when bioprocess operate over a narrow range of conditions. Furthermore, process control is far advanced beyond the days when the main tools for designing control systems were intended for linear systems.

Many of the sensor problems such as those with steam-sterilizable pH electrodes and dissolved oxygen probes have been solved. Perhaps the most important factor for bioprocessing is the concentration of organisms, but there is no practical method for continuous measurement. Samples of the process fluid must be filtered and dried to get the mass concentration of cells. Numbers can be obtained by direct counting with a microscope or by counting the colonies that form when samples are cultured with nutrient medium in Petri dishes. In lieu of direct measurement, many other ways to estimate cell concentration are tried. Turbidity of the culture fluid can be correlated with cell concentration, but properties and calibration change during the process, and the optical surfaces of the sensors tend to become fouled. Alternatives such as measuring electrical conductivity or capacitance of the fluid sometimes are useful but often are suited only to specific cases. Indirect methods such as measuring protein produced by cells or monitoring nucleic acids are reported, but their proportionality to cell mass may vary during the fermentation. An important advance was made by developing computer models that can interpret measured variables to calculate cell mass or product concentration that may be difficult or impractical to measure on-line.

Mounting electrodes in a bioreactor is costly, and there is an additional contamination risk for sensitive cell cultures. Some other sensors of practical importance are those for dissolved oxygen and for dissolved carbon dioxide. The analysis of gas exiting from a bioreactor with an infrared unit that detects carbon dioxide or a paramagnetic unit that detects oxygen (after carbon dioxide removal) has been replaced by mass spectrophotometry. Gas chromatographic procedures coupled with a mass spectrophotometer will detect all the volatile components.

A useful index of process performance is the oxygen uptake rate, OUR, that is calculated from the difference in oxygen concentration of the inlet air and the exiting gas. Also important is the respiration ratio defined as the carbon dioxide evolved divided by the oxygen consumed.

Although dynamic responses of microbial systems are poorly understood, models with some basic features and some empirical features have been found to correlate with actual data fairly well. Real fermentations take days to run, but many variables can be tried in a few minutes using computer simulation. Optimization of fermentation with models and real-time dynamic control is in its early infancy; however, bases for such work are advancing steadily. The foundations for all such studies are accurate material balances.

The common indices of the physical environment are: temperature, pressure, shaft power input, impeller speed, foam level, gas flow rate, liquid feed rates, broth viscosity, turbidity, pH, oxidation-reduction potential, dissolved oxygen, and exit gas concentrations. A wide variety of chemical assays can be performed; product concentration, nutrient concentration, and product precursor concentration are important. Indices of respiration were mentioned with regard to oxygen transfer and are particularly useful in tracking fermentation behavior. Computer control schemes for fermentation can focus on high productivity, high product titer, or minimum cost. Computer systems may perform on-line optimization of fermentation. Progress has been slow by empirical methods because there is a multiplicity of variables and because statistical techniques suffer from the relatively poor reproducibility of fermentations. Careful attention to preparation of inoculum, time/temperature factors of sterilization, and the timing of inoculation and feeding can greatly reduce variability of bioprocess performance.

ENZYME ENGINEERING

ENZYMATIC REACTION KINETICS

Enzymes are excellent catalysts for two reasons: great specificity and high turnover rates. With but few exceptions, all reactions in biological systems are catalyzed by enzymes, and each enzyme usually catalyzes only one reaction. For most of the important enzymes and other proteins, the amino-acid sequences and three-dimensional structures have been determined. When the molecular structure of an enzyme is known, a precise molecular weight could be used to state concentration in molar units. However, the amount is usually expressed in terms of catalytic activity because some of the enzyme may be denatured or otherwise inactive. An international unit (IU) of an enzyme is defined as the amount capable of producing one micromole of its reaction product in one minute under its optimal (or some defined) reaction conditions. Specific activity, the activity per unit mass, is an index of enzyme purity.

Although the mechanisms may be complicated and varied, some simple equations can often describe the reaction kinetics of common enzymatic reactions quite well. Each enzyme molecule is considered to have an active site that must first encounter the substrate (reactant) to form a complex so that the enzyme can function. Accordingly, the following reaction scheme is written:

$$E + S \underset{2}{\overset{1}{\rightleftharpoons}} ES \overset{3}{\rightarrow} P + E \tag{24-31}$$

where E = enzyme, S = substrate, ES = enzyme-substrate complex, and P = product.

Reactions 1 and 2 may be assumed to be in equilibrium soon after the enzyme is exposed to its substrate. Rate equations for these reactions are:

$$\frac{d(S)}{dt} = k_2(ES) - k_1(E)(S) \tag{24-32}$$

$$\frac{d(ES)}{dt} = k_1(E)(S) - (k_1 + k_3)(ES) \tag{24-33}$$

$$\frac{d(P)}{dt} = k_3(ES) \tag{24-34}$$

where k_1, k_2, k_3 = kinetic constants shown with the arrows in Eq. (24-31). Analysis leads to the Michaelis-Menten equation:

$$\frac{d(P)}{dt} = \frac{V_{max}\,(S)}{K_M + (S)} \tag{24-35}$$

where K_M = Michaelis Constant and V_{max} = maximum rate of reaction.

This equation successfully describes the kinetic behavior of a surprisingly large number of reactions of different enzymes. Taking reciprocals of both sides gives:

$$\frac{dt}{d(P)} = \frac{K_M}{(S)\,V_{max}} + \frac{1}{V_{max}} \tag{24-36}$$

A linear plot of the reciprocal of the reaction rate versus $1/(S)$ will allow the determination of K_M and V_{max} from experimental data.

Kinetic behavior becomes complicated when there are two chemical species that can both complex with the enzyme molecules. One of the species might behave as an inhibitor of the enzyme reaction with the other as the substrate. Depending upon the nature of the complex, different inhibition patterns will yield different kinetic equations. For example:

$$E + S \rightleftharpoons ES \rightarrow P + E \tag{24-37}$$

$$E + I \rightleftharpoons EI \tag{24-38}$$

Since the EI complex does not yield product P, and I competes with S for E, there is a state of *competitive inhibition.* By analogy to the Michaelis-Menten equation:

$$\frac{dt}{d[P]} = \frac{K_M}{V_{max}}\frac{1}{S}\left(1 + \frac{[I]}{K_i}\right) + \frac{1}{V_{max}} \tag{24-39}$$

where I = concentration of the competitive inhibitor and K_i = inhibition constant.

Enzyme reactions are also sensitive to pH and temperature changes. In characterizing an enzyme, its optimal pH and optimal temperature are conditions at which the enzyme has its highest catalytic activity.

For a somewhat more extensive exposure to enzyme reaction kinetics, consult standard biochemistry texts and also Dixon, M. and E. C. Webb, *Enzymes,* 2d ed., Academic Press, 1964; Segal, I. H., *Enzyme Kinetics,* Wiley, 1975; Gacesa, P. and J. Hubble, *Enzyme Technology,* Open University Press, England, 1987.

Immobilized Enzymes One factor that usually impedes the development of wide industrial application of enzymes is high cost. Immobilization is a technique to retain enzyme molecules for repeated use. The method of immobilization can be adsorption, covalent bonding, or entrapment. Semipermeable membranes in the form of flat sheets or hollow fibers are one way to restrain the enzyme while allowing smaller molecules to pass. Polyacrylamide gel, silica gel, and other similar materials have been used for entrapment of biologically active materials including enzymes. Encapsulation is another means of capture by coating liquid droplets containing enzymes with some semipermeable materials formed in situ. Generally speaking, entrapment does not involve a chemical or physical/chemical reaction directly with the enzyme molecules; and the enzyme molecules are not altered. Physical adsorption on active carbon particles and ionic adsorption on ion-exchange resins are important for enzyme immobilization. A method with a myriad of possible variations is covalent bonding of the enzyme to a selected carrier. Materials such as glass particles, cellulose, silica, and so on, have been used as carriers for immobilization. Enzymes immobilized by entrapment and adsorption may be subject to loss due to leakage or desorption. On the other hand, the chemical treatment in forming the covalent bond between an enzyme and its carrier may permanently damage some enzyme molecules. In enzyme immobilization, two efficiency terms are often used. Immobilization yield can be used to describe the percent of enzyme activity that is immobilized,

$$\%\ \text{yield} = 100 \times \frac{\text{activity immobilized}}{\text{starting activity}}$$

Immobilization efficiency describes the percent of enzyme activity that is observed:

$$\text{percent efficiency} = 100 \times \frac{\text{observed activity}}{\text{activity immobilized}}$$

When an enzyme molecule is attached to a carrier, its active site might be sterically blocked and thus its activity becomes unobservable (inactivated).

One of the most important parameters of an immobilized-carrier complex is stability of its activity. Catalytic activity of the complex diminishes with time because of leakage, desorption, deactivation, and the like. The half-life of the complex is often used to describe the activity stability. Even though there may be frequent exceptions, linear decay is often assumed in treating the kinetics of activity decay of an immobilized complex.

Immobilization by adsorption or by covalent bonding often helps to stabilize the molecular configurations of an enzyme against alternations including those that may cause thermal deactivation. Immobilized enzymes tend to be less sensitive to pH changes than are free enzymes. Although careful choice of the immobilization chemistry can result in stabilized activity, there are some enzymes that are much less stable after immobilization. Most carriers are designed to have high porosity and large internal surface areas so that a relatively large amount of enzymes can be immobilized onto a given volume or given weight of the carrier. Therefore, in an immobilized enzyme-carrier complex, the enzyme molecules are subject to the effect of the microenvironment in the pores of the complex. Surface charges and other microenvironmental effects can create a shift up or down of optimal pH of the enzyme activity.

An immobilized enzyme-carrier complex is a special case that can employ the methodology developed for evaluation of a heterogeneous catalytic system. The enzyme complex also has external diffusional effects, pore diffusional effects, and an effectiveness factor. When carried out in aqueous solutions, heat transfer is usually good, and it is safe to assume that isothermal conditions prevail for an immobilized enzyme complex.

The Michaelis-Menten equation and other similar nonlinear expressions characterize immobilized enzyme kinetics. Therefore, for a spherical porous carrier particle with enzyme molecules immobilized on its external as well as internal surfaces, material balance of the substrate will result in the following:

$$2\frac{D_e}{r}\frac{dS}{dr} + D_e\frac{d^2S}{dr^2} = \frac{V_{max}S}{K_M + S} \qquad (24\text{-}40)$$

with also the usual boundary conditions, at $r = R$, $S = S$ and at $r = 0$, $dS/dr = 0$ where R = radius of the sphere, r = distance from sphere center, S = substrate concentration, and D_e = effective diffusivity. Normalizing results in:

$$\frac{d^2y}{dx^2} + \frac{2}{x}\frac{dy}{dx} - \phi^2\beta\left(\frac{y}{\beta} + y\right) = 0 \qquad (24\text{-}41)$$

where y is dimensionless concentration, x is dimensionless distance, and ϕ and β are dimensionless constants; ϕ is sometimes referred to as the Thiele modulus of the immobilized enzyme complex. The boundary conditions are $x = 1$, $y = 1$ and at $x = 0$, $dy/dx = 0$. Graphical solutions are available in standard tests. Two meaningful asymptotic conditions have analytical solutions. In one extreme, $\beta \to 0$, meaning $S >> K_m$, and accordingly the Michaelis-Menten equation reduces to a zero-order reaction with $V = V_{max}$. This is the condition of saturation (i.e., the substrate supply is high and saturates all of the active sites of the enzyme molecules). In the other extreme, $\beta \to \infty$, meaning $K_m >> S$, and accordingly the Michaelis-Menten equation approaches that of a first-order reaction with $V = V_{max}S/K_m$. This is the condition of a complete substrate control.

Enzymatic Reactors Adding free enzyme to a batch reactor is practical only when the value of the enzyme is relatively low. With expensive enzymes, reuse by retaining the enzyme with some type of support makes great economic sense. As some activity is usually lost in tethering the enzyme and the additional operations cost money, stability is very important. However, many enzymes are stabilized by immobilization; thus, many reuses may be possible.

Methods of immobilization have already been discussed, and various reactor configurations are possible. An enzyme immobilized on beads of a support material or captured in a gel droplet is essentially a catalytic particle. Mounted in a packed column, there may be upflow or downflow of the feed solution, and a fluidized bed may be feasible except that particle collision often endangers stability of the enzyme. A serious problem is growth of microorganisms on the particles because enzymes are proteins that are nutritious. As immobilized enzymes often have more thermal stability than do free enzymes, the columns can be run at elevated temperatures (50 to 65°C, or 122 to 149°F) to improve reaction rate and to inhibit most but not all contaminating organisms. Sterile feed solutions and aseptic technique can minimize contamination, but, more commonly, antiseptics are added to the feed, and there is occasional treatment with a toxic chemical to wash organisms from the column. Particles with immobilized enzymes are sometimes added to a reactor and recovered later by filtration or by some trick such as using magnets to collect enzymes attached to iron.

Cellulose is hydrolyzed by a complex of several enzymes. The mix of enzyme activities produced by mold cultures can have insufficient amounts of the enzyme beta-glucosidase to maintain a commercially acceptable hydrolysis rate. This enzyme can be produced with a different microbial culture and used to supplement the original enzyme mix, but the cost is high. It is logical to immobilize the beta-glucosidase for multiple use. Handling is minimized by circulating fluid from the main reactor through an external packed column of immobilized enzyme.

Enzymes can be immobilized in sheets. One design had discs of enzymes fastened to a rotating shaft to improve mass transfer, and an alternate design had the feed stream flowing back and forth through sandwiches of sheets with enzyme. However, volumetric efficiency of such reactors is low because sheets with finite spacing offer less area than that of packed particles.

It is possible to add free enzyme and recover it by ultrafiltration, but sufficient membrane surface to get good rates and the required auxiliary equipment are expensive. A hollow fiber device packs a vast amount of membrane area into a small volume. Enzyme may be immobilized inside or outside of the fiber, and it is easy to flush and replace the enzyme. Drawbacks to this design are: (1) The stability of the enzyme is not affected—activity that survives the immobilization step can have enhanced long-term stability; (2) there are two mass-transfer steps, as the substrate must diffuse through the fiber to reach the enzyme and the product must diffuse back; and (3) diffusion is poor on the outside of packed fibers. There is a scheme with the enzyme immobilized on or in the membrane to provide excellent contact as the feed is forced through. Although not yet commercialized, this method appears quite attractive. Recent Russian research with quick freezing has produced gels of enzymes that have high activity, good stability, and a temperature range up to the point where the gel collapses.

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