Industrial Biotechnology: Discovery to Delivery 30

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Introduction

Fermentation products have penetrated almost every sector of our daily lives. They are used in ethical and generic drugs, clinical and home diagnostics, defense products, nutritional supplements, personal care products, food and animal feed ingredients, cleaning and textile processing, and in industrial applications such as fuel ethanol production. Even before knowing about the existence of microorganisms, for thousands of years ancient people routinely used them for making cheese, soy sauces, yogurt, and bread. Although humans have used fermentation as the method of choice for manufacturing for a long time, it is only now being recognized for its potential towards sustainable industrial development.

Since the discovery of fermentative activity of microorganisms in the eighteenth century and its proof by the French scientist Louis Pasteur, fermentative production of alcohols, amino acids, enzymes (biocatalysts), organic acids, vitamins, and natural polymers for food, feed, and other industrial applications has become well established. Subsequently, microbes have been used as production workers in industry. The production of bakers' yeast in deep aerated tanks was developed towards the end of the nineteenth century. The German scientist Buchner discovered that active proteins, called enzymes, are responsible for ethanol fermentation by yeast. During World War I, Chaim Weizmann used a microbe to convert maize mash into acetone, which was essential in the manufacture of the explosive cordite. In 1923, Pfizer opened the world's first successful plant for citric acid fermentation. The process involved fermentation utilizing the mold *Aspergillus niger* whereby sugar was transformed into citric acid. Other industrial chemicals produced by fermentation were found subsequently, and the processes were reduced to commercial practice. These processes included production of butanol, acetic acid, oxalic acid, gluconic acid, fumaric acid, and many more.

The serendipitous discovery of penicillin in 1928 by Alexander Fleming, while researching agents that could be used to combat bacterial infections, opened the whole new world of antibiotics. American pharmaceutical companies such as Merck, Pfizer, and Squibb were the first in mass-producing penicillin in the early 1940s. Initially, *Penicillium notatum* was surface cultured in flasks. Later, a new strain, *Penicillium chrysogenum*, was cultured in deep aerated tanks in the presence of corn steep liquor medium, and gave 200 times more penicillin than did Fleming's mold. Streptomycin was next, an antibiotic that was particularly effective against the causative organism of tuberculosis. Today, the list of these antibiotics is long and includes among many others, such important antibiotics as chloramphenicol, the tetracyclines, bacitracin, erythromycin, novobiocin, nystatin, and kanamycin.

Besides the booming antibiotics industry, fermentative syntheses of amino acids such as L-lysine and L-glutamic acid became billion dollar businesses. Despite the long history of microbial fermentation processes, understanding of the molecular basis of biological systems has developed starting from the discovery of the double helix DNA only decades ago. The practice of modern biotechnology started when Boyer and Cohen recombined DNA, and Boyer and Swanson founded Genentech, the first biotechnology company. Soon, it became clear that novel biotechnological manipulations could create new cell systems capable of producing new molecules and modifying existing products and processes. By the 1980s, a number of new biotechnology companies such as Amgen, Biogen, Cetus, Genencor, and others started to develop products for healthcare, agricultural, and industrial applications. The promise of biotechnology has been high in terms of delivering new products through partnerships with established pharmaceutical, agricultural, and chemical companies such as Eli Lilly, Roche, Johnson & Johnson, Monsanto, Shell, and others.

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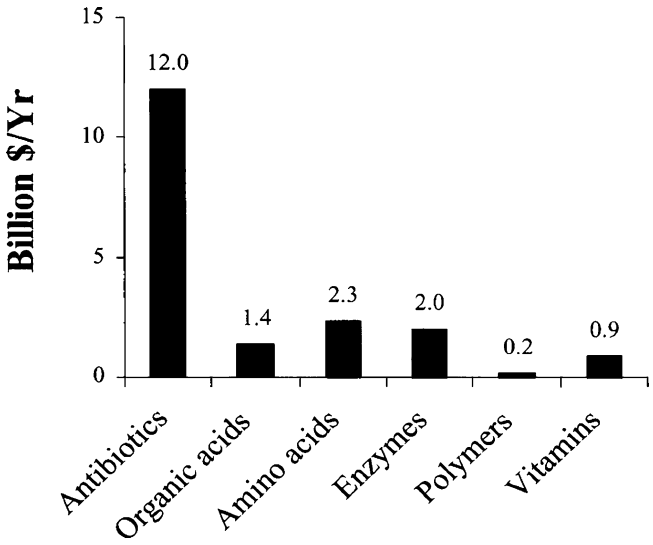


Fig. 30.1 Worldwide industrial biotechnology products market (except ethanol)

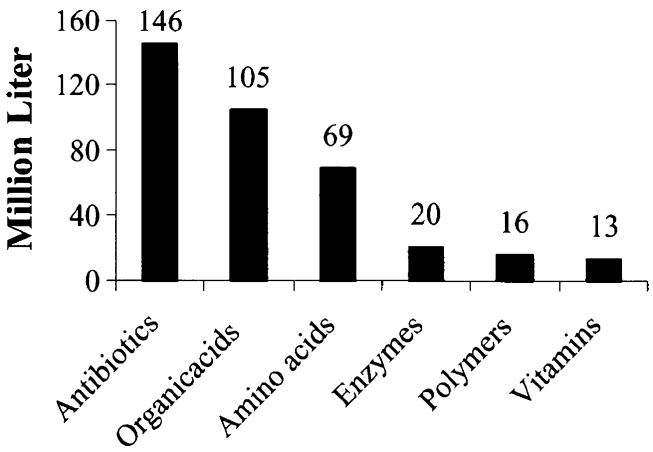


Fig. 30.2 Worldwide industrial biotechnology fermentation capacity distribution

By the end of the twentieth century, industrial applications of biotechnology started gaining momentum and proven laboratory techniques started to move into potentially huge markets. The field of industrial biotechnology (called "White Biotechnology" in Europe) was deemed the third wave of biotechnology. By definition, it is the application of scientific and engineering principles to the processing of materials by biological agents to provide goods and services. Industrial biotechnology continues to supply not only unique products, but is also considered synonymous with sustainable manufacturing processes. Some distinct advantages of industrial biotechnology include the use of renewable feedstocks (agricultural crop materials), generation of less industrial waste, lower cost for cleanup and disposal, and less pollution. The global market and fermentation capacity distribution (Figs. 30.1 and 30.2) for industrial biotechnology products, excluding ethanol, was estimated in 2002 at $17 billion. McKinsey & Company estimates that in the first decade of this century, biotechnology will affect up to 20% of the worldwide chemical market [1]. The major volumes of industrial biotechnology goods, such as alcohols, organic acids, amino acids, biopolymers, enzymes, antibiotics, vitamins, colorants, biopesticides, alkaloids, surfactants, and steroids, are expected to increase driven by innovative products, lower costs, renewable resources, and pollution reduction.

Discovery of Organisms and Molecules

Microbial Diversity

Microorganisms are chemically similar to higher plant and animal cells; they perform many of the same biochemical reactions. Generally, microorganisms exist as single cells, and they have much simpler nutrient requirements than higher life forms. Their requirements for growth usually are limited to air, a carbon source, generally in the form of sugars, a nitrogen source, and inorganic salts. Fermentation originally meant cellular activity/process without oxygen, but industrial biotechnology includes both aerobic and anaerobic fermentation processes. The nutrient source, complex (microbial, plant, or animal derived) or defined, not only provides carbon, nitrogen, and other elements (P, S, K, Mg, Ca, etc.) but also provides reducing and oxidizing agents [2]. Of course, in most of the cellular pathways, O_2 from air provides oxidation, releasing abundant metabolic energy from redox reactions. Depending on the physiological state of the cell, part of the metabolic energy escapes as heat that must be removed by the fermentor design. Sucrose, derived from sugarcane/beet and glucose derived from starch are often used as the carbon as well as energy sources for fermentation. Use of other carbon sources such as cellulose, hemicellulose, fats, etc. might increase in the near future. Proteins and fats are more reduced than saccharides and affect metabolism in terms of oxidation–reduction fluxes. The fluxes also depend on the endproduct(s) being more oxidized or reduced than the starting substrate(s).

Microbes occur in four main groups [3]:

- Bacteria and actinomycetales
- Viruses
- Fungi, including yeast
- Protozoa and algae

The chemical composition of microorganisms can be quite varied depending upon such factors as the composition of the growth medium, the age of the culture, and the cell growth rate. Table 30.1 lists the composition of a typical microbial cell [4]. All organisms contain the genetic information to produce a wide variety of enzymes and hence produce a great number of chemicals. Individual genes for

Table 30.1 Typical composition of microbial cell (about 70 % water)

Molecule	% Dry cell weight
DNA	3
RNA	20
Protein	55
Lipid	9
Liposaccharide	3
Peptidoglycan	3
Glycogen	3
Metabolites	3
Metal ions	1

specific enzymes can be organized into metabolic pathways that will define the biochemical metabolites that can be produced by the organism, which usually depends upon the environment in which the microorganism is growing. Inherent regulatory control processes allow cells to regulate their enzyme content in direct response to the environment. They prevent the formation of excess endproduct and superfluous enzymes. In this postgenomic era, the sequencing of a genome can be used for metabolic reconstruction of the microbial strain. Individual genes can be identified through homology to previously sequenced genes. Transcriptional analysis is another recent tool that allows the simultaneous measurement of all actively transcribed genes in a given organism at any given time. When combined with genomic information, attempts can be made to predict phenotypes or identify the organism.

For industrial processes, microbial strains with faulty regulation, altered permeability, enhanced enzymatic activities, or metabolic deficiencies are used to accumulate products. Such mutants have been changed so that their genetic mechanism is no longer sensitive to a particular controlling metabolite. Modern genetic engineering tools typically focus on creating such strains by making directed changes to the DNA of the organism.

Screening and Selection

Product discovery means identifying the molecular target (enzyme, pathway, metabolite) of the intended application. A very early phase in the discovery of a microbial product involves searching for microorganism(s) that synthesize the product of interest [5, 6]. In the past this meant screening for living microorganisms. However, by applying molecular biology tools [7], today it is possible to screen for the gene (s) of interest without culturing the organisms. Of course, the rich diversity of microorganisms in nature is often the starting point for the screening/selection process. This process requires a well-designed growth medium, catalyst function, and assay. For example, a novel enzyme screening and selection strategy is based on an understanding of the real-world conditions under which the enzyme must function. Many enzymes are used in applications, for example, laundry, cooking, and solvent, that are far removed from the natural physiological conditions. Such applications expose enzymes to pH, temperature, and/or chemicals that might inactivate or inhibit them.

The classical screening method has its roots in antibiotic discovery. It involves collection of a variety of samples of soils and organic matter, and isolation of their microbial population. Being laborious at times (i.e., finding a needle in a haystack), random screening methods have been replaced in some cases by more efficient selection techniques. These techniques subject a microbial source sample to a selection pressure (i.e., pulling a needle from a haystack selectively with a magnet). For example, the growth conditions are selected such that only microorganisms expressing the desired enzyme/pathway/metabolite are able to survive. Therefore, selecting an environment (pH, T, growth nutrient) that is optimal for the growth of the desired microorganism increases the success of such a discovery. Very often, for the source of enzymes, industrial biotechnology companies look for microbes called extremophiles that live in extreme conditions, because they generally contain enzymes that perform optimally under extreme conditions.

In the last decade, tremendous progress has been made in sequencing DNA from various organisms. It is possible to extract DNA directly from the microbial community present in a sample taken from the environment. As a result, thousands of gene sequences have been deposited in public databases. This information can be used to screen for proteins/enzymes by searching for similarity at the gene level. Once a homologous gene is identified, it can be transferred to a laboratory host organism such as *Escherichia coli* or *Bacillus subtilis*. This allows for the expression of the identified gene product without making the source organism overproduce the molecule of interest. However, application of this technique is limited to microorganisms that have been isolated, characterized, and deposited in culture collections. Ironically, a very small number of microorganisms that exist in nature have been isolated and those characterized are even fewer. There may be many useful reactions in organisms yet to be discovered. Faster sequencing methods have started to exponentially expand the genomic database. Several hundred genomes are in the database and the tools of bioinformatics are vastly improving for the discovery of genes of interest.

According to J. Craig Venter Institute (http://www.venterinstitute.org), it should be possible to reduce the cost of sequencing a human individual DNA to less than $1,000. At this low cost and high speed of sequencing, we expect to learn more about human genes by comparing the human

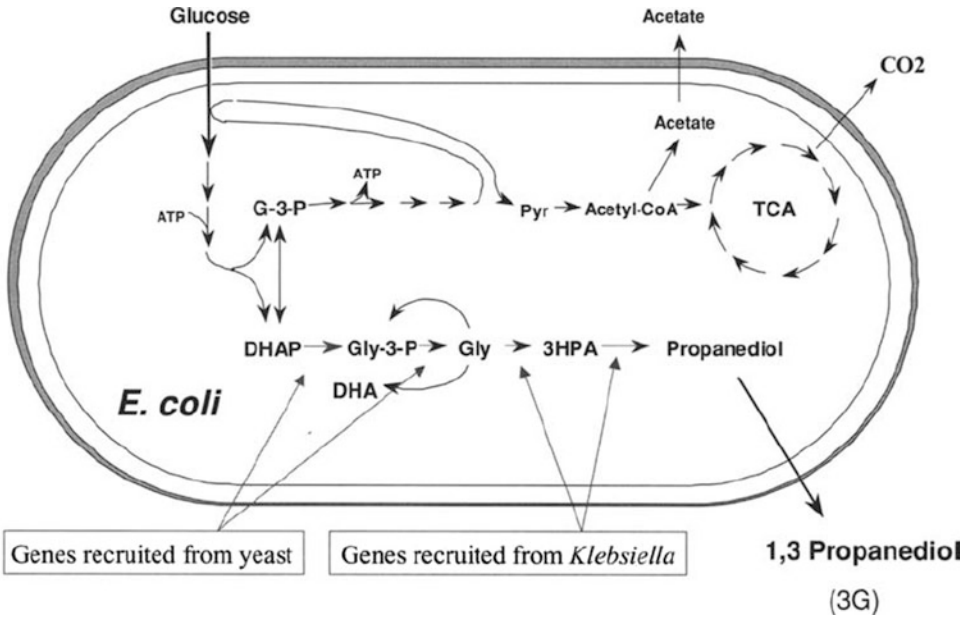


Fig. 30.3 Rational biocatalyst design strategy for 1,3-propanediol

genome sequence to genome sequences of other species that have been thoroughly studied. Such diverse information has the potential to aid in the development of new products, processes, and applications in medicine, agriculture, energy, and the environment.

Cell Engineering

Classical strain development was traditionally dependent on the evolutionary process of random mutation. In a normal bacterial population, one mutant arises in about 10^6 cells. This low rate of spontaneous mutation is unsatisfactory for strain improvement. However, mutagens, such as *N*-methyl-*N*′-nitro-*N*-nitrosoguanidine (NTG) and Ethyl Methane Sulfonate (EMS), are available that markedly increase the rate of mutation. Although it is relatively easy to produce mutants, strain improvement requires painstaking effort and ingenuity in devising screening tests. Knowledge of microbial physiology is essential in the development of many of these screens. Screening for resistance to metabolic analogues is probably the most important screening tool for devising means around normal metabolic control mechanisms. Although truly exemplary production organisms for important medical and industrial products were created using this process, the timeframe for many of these developments can be measured in decades.

In this era of genetic and metabolic engineering, the timeframe for strain development has been reduced, although it still is a major effort requiring substantial investment. As in classical strain development, knowledge of microbial physiology remains essential to the practice of metabolic engineering. Understanding the regulation of genes, in particular the interaction of promoters and regulatory proteins, is also essential. The five key strategies for metabolic engineering can be summarized as follows [8, 9].

1. Enhance committed step from central metabolism or branch point.
2. Eliminate transcriptional and allosteric regulation.
3. Identify and relieve rate-limiting step(s).
4. Prevent carbon and energy loss to competing pathways.
5. Production of foreign enzymes or metabolites.

Sophisticated metabolic models teamed with experimental data are finding widespread use in identifying strategic sites for metabolic engineering. For example, DesignPath® [10] is an approach based on the use of mathematical models and specifically generated algorithms that can be used to predict the right combination of genes that would enable a cell to overproduce a desired biomolecule, for example, 1,3-propanediol [11], and minimize the synthesis of undesired by-products, for example, acetic acid. Using such models, scientists have been able to combine in a single host (*E. coli*), a natural biological pathway present in yeast for the production of glycerol from dihydroxyacetone phosphate, and a pathway present in the bacterium *Klebsiella pneumoniae* to convert glycerol into 1,3-propanediol, to create a novel and unique process (for details see the section, "Delivery of Products"; Fig. 30.3).

Production strains, established through classical mutagenesis and screening to produce metabolites at a commercial level, were often started from strains known to overproduce at least some amount of the desired product. These strains had already overcome some of the natural regulation to keep from overproducing metabolites. However, this often led to strains that could overproduce more than one endproduct. To maximize production of the desired

endproduct alone, strains were selected that could not overproduce a sideproduct. This resulted in the development of an auxotrophic strain, which required the particular sideproduct in order to grow. The need for specialized growth media often resulted in added expense and downstream difficulties as the required component(s) were added as complex mixtures from animal or plant extracts. By using enzymes from different organisms, it is now possible to change gene regulation, prevent carbon loss to competing pathways, and eliminate auxotrophies.

The ability to use heterologous enzymes in a production host is one of the hallmarks of modern metabolic engineering. This can be accomplished by specific directed changes to the genome or by generating a high level of diversity. The goal of the effort is to improve a specific phenotype of the strain. This could be to produce a new metabolite not found in the original host or change the energy balance in the host or some other critical attribute. In any case, deep knowledge of the physiology of the organism is used to develop the appropriate screen to find the desired phenotype.

Molecular Engineering

Despite the diversity of enzymes in nature, it is rare that a screened or selected protein/enzyme might have all of the desired characteristics for a targeted application. Therefore, it may be necessary to engineer the starting protein by modifying its structure through mutation (deletion, insertion, or substitution) of one or more amino acids. Protein engineering can be carried out by genetic modification or by chemical means (acetylation, amidation, oxidation, covalent attachment of ligands). Compared to the chemical approach, site-directed genetic manipulation is more predictable. The specific changes in the gene sequence are made through the use of recombinant DNA technology. Because the structure of a protein determines its function, rational protein engineering starts with studying the three-dimensional structure of the protein either by X-ray diffraction, NMR, or molecular modeling. Often such studies take time and require access to special expertise; therefore, alternative approaches such as random mutagenesis and directed evolution have emerged.

Random mutagenesis has been used extensively as a tool for increasing the genetic variability and improving microbial strains as described earlier. However, random mutagenesis also means less control over the number and types of mutations made. A large number of clones (called a library) are first generated and then screened or selected for the targeted properties. Therefore, random mutagenesis methods tend to use high throughput or rapid and efficient screening techniques.

Directed evolution, a term that covers a wide range of techniques designed to quickly improve strain performance, uses repetitive rounds of mutagenesis along with screening/selection, until a series of mutations accumulate to give an enhanced phenotype. Once again, the key factors for success are understanding of the target gene, the method for generating variability, selection, and screening of the host. The technique makes use of the fact that enzymes retain their relevant tertiary structures and thereby function. The evolution through a range of acceptable molecular structure variations leads to better enzymes with which the organism can tolerate specific environmental stress.

Several in vivo as well as in vitro methods of molecular evolution have been developed based on the recent advances in high-throughput screening, functional genomics, proteomics, and bioinformatics. Molecular evolution technologies provide efficient tools for creating DNA libraries (random, directed, recombinational) and processes of selection for a desired function or characteristic of a target molecule. For example, "DNA shuffling" is a variation of the molecular evolution approach and involves recombination of closely related DNA sequences, for example, random fragmentation and reassembly of the fragments into genes. The method is useful to combine two or more properties, which have evolved separately, into the target molecule. Molecular engineering, optimization, evolution, and other tools enable the design of completely novel biomolecules and proteins for applications in agriculture, medicine, and industry.

Development of a Production Process

Strain

Strain development begins with identification or isolation of an organism capable of producing the molecule of interest. Invariably, the initial strain is improved by mutagenesis, screening, selection, and genetic engineering to meet the production process and economic requirements. The process requirements include handling of the strain, fermentation conditions, product recovery, formulation, and application. Not surprisingly, strain and process development take place in an integrated manner. For example, processes that are sensitive to the cost of fermentation raw materials look for carbon-efficient strains. Likewise, processes that aim to achieve high purity in product need to have fermentations that require the use of minimal medium and strains that produce minimal sideproducts. New genetic techniques allow the rational engineering of a production strain. A rational approach means that the genes defining the production of a particular metabolite are specifically engineered

through mutagenesis, deletion, or over-expression. Controlling expression of such genes determines the rate (productivity), yield (carbon conversion efficiency), and titer (concentration) of the product. The result is a production organism that is optimally tailored for the process and the product.

Some bacterial and fungal strains are capable of differentiation (sporulation, filamentation). This property can affect product formation and the physical properties of the fermentation broth. Some enzymes are synthesized as secondary products and their production does not appear to be growth associated. Production strains are distinguished on the basis of their fermentation behavior such as viscosity or recoverability. They can also be distinguished by patent or proprietary status. The complexity of product synthesis ranges from a relatively simple and well-understood induction and repression to very complex regulation mechanisms. Process development must thus deal with such complexity either by changing the genetic make-up or by optimizing process conditions.

Because classical approaches to strain development are labor intensive and offer limited knowledge, new strain development methods now start with a production host that is capable of rapid genetic manipulation and efficient product synthesis. Generic hosts and techniques allow for the construction of multiple strains for multiple products utilizing a baseline fermentation and recovery process. The so-called toolbox strategy of building strains applies to production of enzymes/proteins as well as biochemicals. Generic host-based strains (denoted GRAS, Generally Recognized As Safe) have been proven for safe use in industrial processes and have been approved by regulatory agencies such as the FDA, EPA, and USDA.

Once a desired production strain is developed, it can be maintained as a stock culture. In maintaining stock cultures, genetic changes must be minimized. This is best achieved by preventing nuclear divisions as most mutations occur as errors during DNA replication. The method of choice is to store cells or spores (if these are produced) in sealed ampoules at very low temperatures ($\leq$130°C) in liquid nitrogen. This method offers the great advantage that the culture can be stored almost indefinitely, thawed, and used immediately as an inoculum without loss of viability or diminution in metabolic rate. Cultures kept at −20 to −60°C are satisfactory but less active than those kept in liquid nitrogen. Although storage at 0–4°C allows some growth, this is better than storage at room temperature. Lyophilization (freeze-drying) is widely used and is very convenient because freeze-dried cultures retain viability without any genetic changes for years when stored at room temperature. It may be noted that all of these methods are, in effect, techniques to immobilize intracellular water and yet retain viability.

Fermentation Process

Analysis of many industrial fermentation processes shows that they are common reactions from a chemical, as well as a physical, viewpoint. Fermentation processes can be classified by the reaction mechanisms involved in converting the raw materials into products. These include reductions, simple and complex oxidations, substrate conversions, transformations, polymerizations, hydrolyses, complex biosyntheses, and the formation of cells.

Fermentation processes, except for sterilization, have in common many of the familiar chemical engineering unit operations. For example, aerobic fermentations involve the "mixing" of three heterogeneous phases: microorganisms, medium, and air. Other unit operations include "mass transfer" of oxygen from the air to the organisms and "heat transfer" from the fermentation medium.

Analysis of fermentations by the unit operation technique has added greatly to the understanding of their behavior. Of the operations auxiliary to those in the fermentor, engineers have made a major contribution towards the design of equipment to provide large volumes of sterile medium and air. Close cooperation between biologists and engineers has resulted in devising logical methods for screening large numbers of strains and translating the results of shake-flask and pilot-plant experiments to production vessels. The scale-up of fermentations, in some instances, is still empirical although sensitive oxygen probes and gas analysis techniques now available have enabled a rational approach to scaling-up aerated, Newtonian as well as non-Newtonian, fermentation processes.

Sterilization

In all fermentation processes, it is necessary to have contamination-free fermentation media and seed cultures. Liquid sterilization of the fermentation medium is conducted by two means, physical removal or inactivation by heat [12, 13]. Contaminating microorganisms can be removed from fluids by filtration. With improvements in membrane technology, sterile filtration is finding wider use, but can only be used with completely soluble media. The contaminants can also be destroyed by heat. Heat sterilization of media is the most common method used for sterilization of liquid media. This can be accomplished in either a batch or a continuous fashion.

Interest in continuous methods for sterilizing media is increasing, but for the successful operation of a continuous sterilizer, foaming of the media must be carefully controlled and the viscosity of the media must be relatively low. The advantages of continuous sterilization of media are as follows.

1. Increase in productivity because the short period of exposure to heat minimizes damage to media constituents.

2. Better control of quality.
3. Leveling of the demand for process steam.
4. Suitability for automatic control.

Design and operation of equipment for heat sterilization of media are based on the concept of thermal death of microorganisms. Consequently, an understanding of the kinetics of the death of microorganisms is important to the rational design of sterilizers.

The destruction of microorganisms by heat implies loss of viability, not necessarily destruction in the physical sense. The destruction of organisms by heat at a specific temperature follows a first order rate of reaction [14, 15]:

$$\frac{dN}{dt} = -KN = -(Ae^{-E/RT})N, \tag{30.1}$$

where K is the reaction rate constant with units of time^{-1}, N is the number of viable organisms/unit volume, t is the time, T is the absolute temperature, E is the energy of activation for death, R is the gas law constant, and A is the Arrhenius constant.

This equation can be integrated to give the design equation:

$$\ln\frac{N_0}{N_f} = A\int_0^{t_s} e^{-E/RT}\, dt, \tag{30.2}$$

where N_o is the number of contaminating organisms in the total fermentation medium to be sterilized, N_f is the level of contamination that must be achieved to produce the desired degree of apparent sterility, and t_s is the sterilization time.

In estimating the sterilization time for the medium, one must define the contamination, the desired degree of apparent sterility, and the time–temperature profile of the medium; that is, $T = f(t)$. For typical bacterial spore contaminants, the constants used in most designs have the following values.

$E = 68{,}700$ cal/g mol
$R = 1.987$ cal/g mol, K
$A = 4e^{+87.82}$, min^{-1}

Aerobic fermentation processes also require a continuous supply of large quantities of air, typically on the order of one volume of air per volume of liquid per minute, VVM. Sterilization of this air is mandatory in almost all fermentations. Absolute filter cartridges of polymeric membranes are now used almost exclusively in the fermentation industry. Relatively small units have replaced the large depth filters used in the past. Still, water and particulates pose a major problem for filters thus requiring the use of prefilters and traps to remove these contaminants before they reach the absolute filter. Parallel installation of the filters prevents a total shutdown of the fermentation process in the event of filter clogging.

Microbial Kinetics

Microbial kinetics [16, 17] can be separated in four distinct levels: at the molecular or enzyme, the macromolecular or cell component, cellular, and population level. Because each level has its own unique characteristics, different kinetic treatments are needed. Moreover, the environment in which these reactions take place also affects the kinetics. For example, reactions at the molecular/enzyme level involve enzyme-catalyzed reactions. When these reactions occur in solution, their kinetic behavior is similar to that of homogeneous catalyzed chemical reactions as described in Chap. 31. However, when enzymes are attached to inert solid supports or contained within a solid cell structure, their kinetics is similar to that of heterogeneous catalyzed chemical reactions.

As discussed in regard to microbial diversity, all microorganisms have basic requirements, those being water, a source of energy, carbon, nitrogen, salts, and trace metals, and possibly growth factors. However, the media used to isolate and screen production hosts are not necessarily those used in production fermentors. Statistical methods (Plackett–Burman, Box–Benkhen) are used for media screening and optimization. The media and conditions used may change from shake flask through the fermentor stages. The objective in developing a production medium is to maximize productivity and product quality, minimize sideproducts, and meet economics. Generally, large-scale fermentation media are made up of complex natural materials, supplemented with inorganic/organic salts [18, 19]. Development of a production medium combines understanding the physiology of growth and product formation developed through plate, shake flask, and fermentor studies.

For example, for deciding on the type of carbon source, the phenomenon of catabolite repression must be considered. Similarly, regulation of nitrogen and sometimes phosphorus metabolism are important factors to consider. Sometimes high concentrations of salts and free amino acids necessary for high cell density are inhibitory to product formation. One area often overlooked is the elimination of unwanted impurities, derived either from the raw material or produced via metabolism. These impurities represent not only a waste of the carbon source, but their accumulation at high levels may lead to inhibition of growth or even cell death.

At a population level, a material balance on cells growing by binary fission can be described by:

$$\frac{dX}{dt} = \mu \cdot X, \tag{30.3}$$

where X is the cell concentration and μ is the specific growth rate of the microbial culture.

Monod [20] suggested that cells growing on a limiting substrate were controlled by a limiting enzymatic reaction. Enzymatic reactions had been modeled using Michaelis–Menten kinetics and Monod applied the same methodology to microbial growth. He described the specific growth rate by:

$$\mu = \mu_{\max} \cdot \frac{S}{K_s + S}, \tag{30.4}$$

where $\mu_{\max}$ is the maximum growth rate of the particular strain being studied, s is the concentration of the limiting substrate, and K_s is the so-called affinity constant for the limiting substrate and is equal to the concentration of the substrate resulting in one-half the maximum growth rate. Combining the above equations results in the cell balance equation:

$$\frac{dX}{dt} = \mu_{\max} \cdot \frac{S \cdot X}{K_s + S}. \tag{30.5}$$

A similar material balance on the limiting substrate requires knowledge of the relationship between cell growth and substrate utilization. The simplest relationship would assume that a fixed amount of cells could be produced from a given amount of substrate, or the yield of biomass on substrate:

$$Y = \frac{dX/dt}{-(dS/dt)} = -\frac{dX}{dS}. \tag{30.6}$$

The linearity between cell growth and substrate consumption does not always hold true, especially at low specific growth rate. Pirt [3] first suggested the concept of maintenance. He described maintenance as the substrate required to generate energy for cell functions independent of growth rate. Examples of cell functions include maintenance of ion gradients and the turnover of macromolecules. The resulting material balance on the limiting substrate is:

$$\frac{dS}{dt} = -\left(\frac{\mu}{Y^{\max}} + m\right)X, \tag{30.7}$$

where $Y^{\max}$ is the maximum growth yield and m is the maintenance coefficient. The term in parentheses is often called the specific substrate consumption rate, q_s.

Because substrate can also be consumed to make a microbial product, the concept of specific rate can also be used for product formation,

$$\frac{dS}{dt} = -(q_s + q_p)X, \tag{30.8}$$

where q_p is the specific product formation rate. The simplest expressions relate product formation to either cell growth or substrate consumption:

$$q_p = Y_{p/s} \cdot q_s \tag{30.9a}$$

$$q_p = Y_{p/x} \cdot \mu \tag{30.9b}$$

The last equation is an example of growth-associated product formation, often observed for primary metabolites. However, for many secondary metabolites, much of the product formation takes place when growth rate slows. This is described as nongrowth-rate associated kinetics. Leudeking and Piret [21] first described a mixture of the two by the following equation, which takes into account both growth-rate associated and nongrowth-rate associated forms of production,

$$q_p = \alpha \cdot \mu + \beta. \tag{30.10}$$

Ideal Types of Fermentors

There are a large number of different types of fermentation processes that are used commercially, which are selected based on several different factors [22–24]. Depending on the strain to be used, the fermentation could be aerobic or anaerobic, and the desired product could be either the biomass itself or a metabolite or polymer produced by the biomass. The kinetics of product formation, whether growth associated or nongrowth associated, also influences the process. Often procedures downstream of the fermentation unit operation have a major control of the overall process and determine how the fermentation is conducted.

Although there are a multitude of possible different fermentation process designs, most of them can be lumped under the following four [23], shown in Fig. 30.4a–d,

- Batch
- Fed-batch
- Continuous
- Continuous with recycle

The batch process is the simplest fermentation to perform. It is a closed system with constant volume. The only addition after the start of the fermentation is air, if it is needed, resulting in a constant volume. All components are in a constant state of change as the substrate is consumed and biomass and products are produced. Figure 30.4a shows the typical batch fermentor characteristic: nutrient is present from the start of the process; nothing is fed and no steady state can be reached. In other words, characteristic volume of culture (V) is constant, denoted by $V = c$. But specific growth rate (μ) and cell mass (X) are not constant. Batch

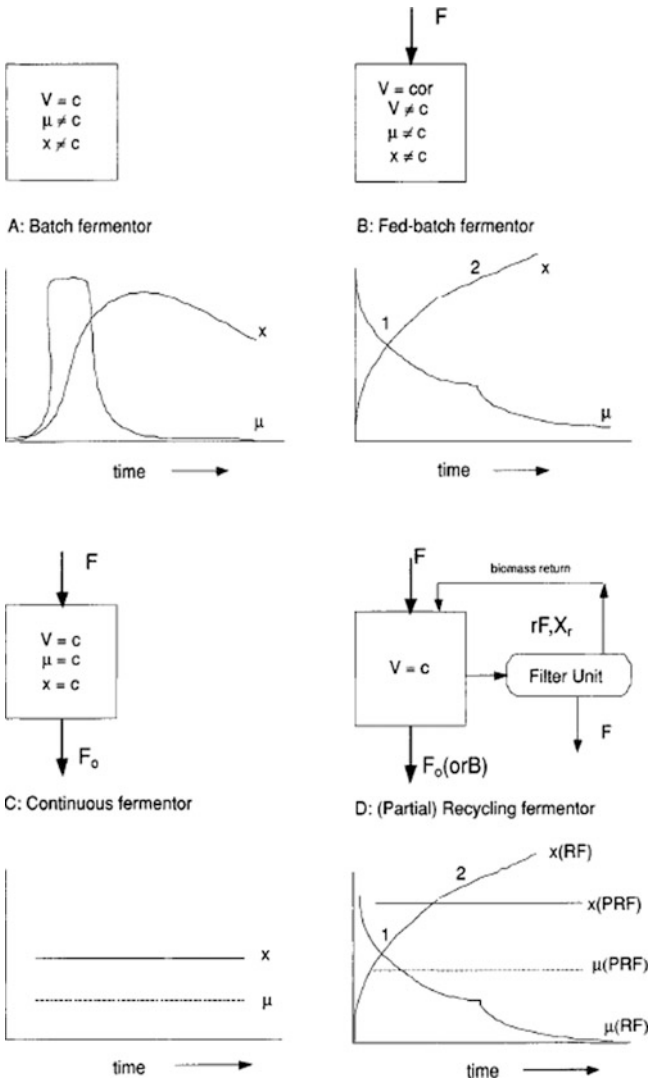


Fig. 30.4 Characteristics of major fermentor types

fermentations are often used for highly mutated strains that are developed by classical mutagenesis and selection. Such strains are easily taken over by faster-growing, less-productive strains in prolonged cultures. Batch processes are still quite common in the antibiotic, organic acid, and ethanol production industries.

In the fed-batch fermentation, nutrients can be added making it an open system for substrates, but still a closed system for biomass and biomass-derived products. As shown in Fig. 30.4b, in this type of process, the volume is not constant. The flow rate of nutrients can vary during the course of the fermentation. Fed-batch operation is very commonly used in industrial processes for the production of baker's yeast, enzymes, amino acids, and many other metabolites.

In the continuous system, as shown in Fig. 30.4c, by constantly removing contents from the fermentor, the process is now open with respect to all components. Typically, the addition of feed and the removal of broth are equal, resulting in a constant volume. Under these conditions, a steady state is achieved wherein all parameters become constant. Such a system is widely used for physiological studies. However, industrial uses are typically limited to the production of biomass (single cell protein) for food or feed, and waste treatment plants.

The final system, shown in Fig. 30.4d, is the continuous system with a partial (PRF) or complete (RF) cell recycle. It is similar to the continuous system, but cells are returned to the fermentor by means of a biomass separation device. Tangential flow-filtration units, centrifuges, and settling tanks have all been used for biomass separation [25]. In the partial cell recycle fermentor, a steady state is achieved as in the continuous system. This process is typically used to increase the productivity of the system and is used commonly in wastewater treatment and ethanol production type applications.

The differential equations shown in the Microbial Kinetics section are used to describe the batch fermentation mode. These can be solved to describe the increase in biomass and product concentrations and the decrease in substrate concentration. The fed-batch system takes into account the addition of substrate during the fermentation. The mass balance on substrate is described by

$$\frac{\mathrm{d}}{\mathrm{d}t}(V \cdot S) = F(t) \cdot S_{\mathrm{f}} - V(q_{\mathrm{s}} + q_{\mathrm{p}})X. \tag{30.11}$$

A total mass balance on the reactor is:

$$\frac{\mathrm{d}}{\mathrm{d}t}(\rho \cdot V) = \rho_{\mathrm{F}} \cdot F(t), \tag{30.12}$$

where ρ is the density of the fermentation broth, ρ_{F} is the density of the feed solution with S_{f} as the substrate concentration, and $F(t)$ describes the rate of addition of feed as a function of fermentation time. As in the batch fermentation, these equations can be solved to show the concentration–time profiles of substrate, biomass, and product. The last equation assumes a negligible change in the reactor mass due to air addition (humidity, oxygen) and gas removal (carbon dioxide, water). Such assumptions, in practice, must be checked.

For a typical single-stage continuous fermentor at steady state, a biomass material balance around the reactor yields the following relationship,

$$\frac{\mathrm{d}X}{\mathrm{d}t} = 0 = -\frac{F}{V}X + \mu X, \tag{30.13}$$

from which:

$$\mu = \frac{F}{V} = D. \tag{30.14}$$

This means that the dilution rate in the fermentor sets the growth rate of the biomass, and a change in the dilution rate will cause a change in the growth rate.

Because most single-stage continuous fermentors are used to produce biomass, they are usually operated to optimize biomass productivity. The unit volume biomass productivity of such a reactor is defined as DX. This unit volume productivity can be expressed as

$$DX = DY\left(S_0 - \frac{K_s D}{\mu_{max} - D}\right). \tag{30.15}$$

By taking the first derivative of the productivity expression with respect to the dilution rate and setting it equal to zero, the dilution rate of maximum productivity, D_m, can be found as

$$D_m = \mu_{max}\left(1 - \sqrt{\frac{K_s}{K_s + S_0}}\right) \tag{30.16}$$

and the maximum productivity, $D_m X$, as

$$D_m X = \mu_{max}\left(1 - \sqrt{\frac{K_s}{K_s + S_0}}\right) \cdot Y \cdot \left(S_0 - \frac{K_s D_m}{\mu_{max} - D_m}\right). \tag{30.17}$$

Biomass recycle as sketched in Fig. 30.4d is frequently used in fermentors as a way of increasing the biomass productivity. The increased biomass productivity obtained with a recycle fermentor is a function of the recycle ratio, r, and the cell concentration factor, $C = X_r/X$, achieved in the concentrator. Equations expressing the recycle system behavior are derived from material balances around the reactor. For the cell biomass balance at steady state:

$$\frac{dX}{dt} = 0 = \frac{F}{V}(0) + \frac{rF}{V}X_r - \frac{F}{V}(1 + r)X + \mu X, \tag{30.18}$$

from which:

$$\mu = D\left(1 + r - r\frac{X_r}{X}\right) = D(1 + r - rC) \tag{30.19}$$

Because $(1 + r - rC) < 1$, it is possible to operate the system at dilution rates greater than the maximum growth rate. It is this stability imparted by the cell recycle fermentor system that makes it useful, especially in waste treatment applications.

Oxygen Transfer Considerations

In aerobic fermentations, oxygen is a basic substrate that must be supplied for growth. As described earlier for other substrates, the relationship between oxygen concentration and growth is of a Michaelis–Menton type and can be described by a specific rate relationship (see (30.5)). The K-value for oxygen typically ranges from 0.5 to 2.0 ppm for well-dispersed bacteria, yeast, and fungi growing at 20–30°C. For growth at temperatures greater than 30°C; the specific oxygen uptake increases only slightly with increasing oxygen concentration [14].

Under steady-state conditions, the oxygen transfer rate must be equal to the oxygen uptake rate:

$$Q_{O_2} X = k_L a (C^* - C)_{mean}, \tag{30.20}$$

where C^* is the concentration of oxygen in the liquid that would be in equilibrium with the gas-bubble concentration. k_L is the oxygen mass transfer coefficient and a is the bubble interfacial area. In small, well-mixed systems, the gas-bubble concentration can be assumed to be equal to that in the gas escaping from the fermentor. However, in large reactors, the log-mean average between the inlet and outlet air concentrations is more appropriate. The oxygen mass transfer coefficient and interfacial area are typically lumped together as $k_L a$. It is not practical to try to separate these two terms.

Utilizing this relationship, the lumped oxygen mass transfer coefficient can be estimated as

$$k_L a = \frac{Q_{O_2} X}{(C^* - C)_{mean}}. \tag{30.21}$$

Scale-Up/Down and Control

There are usually problems scaling up new fermentations as well as with translation of process-improvement data for well-established fermentations from laboratory operations to existing plant equipment [26]. In general, fermentations are scaled up on the basis of achieving similar oxygen transfer capabilities in the plant equipment that proved to be optimal at the bench scale.

The oxygen transfer capability required for processes can vary greatly. Organisms with a low specific growth rate may require as little as 25 mmol/L/h whereas high specific growth rate cultures could require ten times that amount. The range of airflow required can vary from as little as 0.1 VVM to >1 VVM. Likewise, mechanical power requirements can vary from less than 0.5 kW/m^3 to >5 kW/m^3.

There are various correlations between $k_L a$ and power inputs. Some design engineers prefer to scale-up/down on the following basis,

$$k_L a = k\left(\frac{P_m}{V}\right)^{\alpha} (v_s)^{\beta}, \tag{30.22}$$

where P_m is the motor power and v_s is the superficial gas velocity. The coefficients α and β are typically in the range of 0.5 for large-scale plant equipment. Mixing is one of the most critical factors of large-scale fermentors and among agitator designs, Rushton turbine type impeller has most often been used and studied [27].

Unaerated mechanical power input can be estimated from:

$$P_u \propto \rho N^3 D^5, \tag{30.23}$$

where N is the rotation speed of the impeller shaft (rpm) and D is the diameter of the impeller.

For scale-up, however, it is usually the removal of heat that causes design problems. With the previously mentioned mechanical agitation power inputs, up to 5 kW/m^3 of energy are needed to remove mechanical heat. The peak metabolic heat load for aerobic fermentation of glucose at ~250 mmol O_2/L h uptake rate is generally greater than 35 kW/m^3. Even with a fermentation temperature as high as 37°C and cooling water temperature as low as 18°C, it is difficult to remove the heat in large fermentors without external heat exchange or extensive cooling coils in the fermentor. Internal spiral cooling coils can be undesirable because they could interfere with the mixing patterns. Thus, vertical coils that also act as mixing baffles are finding more widespread use. Chilling of the cooling water can also be used to increase heat removal.

Once a plant is built, the conditions of agitation, aeration, mass (oxygen) transfer, and heat transfer become set. Therefore, those environmental conditions achievable in plant scale equipment should be scaled down to the pilot plant and laboratory-type equipment (shake flask, micro-reactor) to ensure that the earlier studies are carried out under conditions that can be duplicated.

With the rise of the genomic and postgenomic era, high-throughput gene sequencing, proteomics, metabolomics, and systems biology have created a wealth of biological data. This has given rise to the need for higher throughput fermentation technology to screen libraries of natural and in vitro generated compounds. Initially, screens of new organisms and metabolic pathways were performed in shake flasks and throughout the last decade increasingly in microtiter plates. Although these methods are straightforward and well proven, they are laborious and do not allow complex manipulation of growth conditions. Furthermore, they do not provide a full complement of data for the knowledge-based selections of organisms or desired physiological properties.

Within the past 5 years, novel technologies have been developed that allow more directed and controlled experiments to screen organisms, fermentation, and media conditions at small scales. A number of academic and private institutions are developing machines that are capable of growing cells at the microliter and sometimes nanoliter scale. They are able to supply aeration, oxygen transfer, pH control, and online data output similar to a pilot-scale fermentor. These devices require minute amounts of cells and media and thus offer time, cost, and environmental advantage and ultimately enhance productivity. Very small scales have been described for cell growth and cell-based assays in microfluidic devices, such as the compact disk (CD) format technology by Gyros Microlabs (http://www.gyrosmicro.com). Intermediate scale microfabricated devices include the bioprocessors platform (PCT WO 2002/083852), the Micro Reactor device (http://www.gener8.com), and a number of devices by academic laboratories, most prominently, from the University of Maryland at Baltimore (http://www.umbc.edu/cbe/rao.html). For instance, the Micro Reactor device is based on a 24-well plate format with several milliliters fermentation capacity in each well. Airflow, pH, temperature, and agitation can be controlled electronically. The machine provides online readouts for pH, dissolved oxygen, and other parameters. Another larger intermediate scale machine offered by DASGIP AG, Fedbatch-Pro®, is a modular system that delivers a high degree of flexibility to perform a number of microbiological applications (http://www.dasgip.com). It remains to be seen if these machines will eventually be capable of replacing the classical scale-up versions of stainless steel fermentors.

Instrumentation and Control

In successfully scaling up/down any fermentation, knowledge of the regulatory mechanisms of metabolic pathways that synthesize the desired product and the instrumentation to detect the regulatory metabolites is necessary. To optimally run a fermentation process, it is essential to perform the initial fermentation research on fully monitored environmental systems, then correlate the environmental observations with existing knowledge of cellular control mechanisms, and finally reproduce the desired environmental control conditions through continuous computer monitoring, analysis, and feedback control of the fermentation environment.

As a result of advances made in sensor development, today more so than in the past, it is possible to rely on environmental control in order to gain economical fermentation results. Until recently fermentation control was limited to that of temperature, pH, and aeration. With the development of numerous sensors and inexpensive computing systems, the engineer can think in terms of sophisticated control systems for fermentation processes. Figure 30.5 shows how a highly instrumented fermentor is designed to secure basic information on almost all the parameters of the fermentation process. For advanced information, both

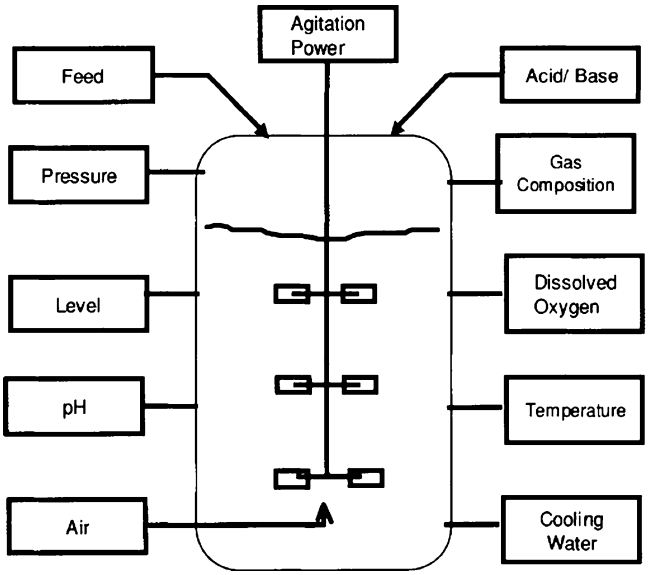


Fig. 30.5 Highly instrumented industrial fermentor

offline intracellular (e.g., messenger RNA and protein arrays, metabolites) and extracellular (e.g., metabolites, proteins/peptides by LC/GC) analyses methods are readily available.

One of the most important sensors needed is one that reliably monitors cell density. An IR fiber-optic cell density probe has been used for this because it can directly monitor cell growth (without dilution) in high-cell-density bacterial fermentations. The ability to do an online sample filtration through the use of hollow fibers or rotating filters has made possible continuous, online measurement of glucose, lactate, and other metabolites. However, glucose, nitrogen substrate, and phosphate sensors that can withstand repeated system sterilization are still needed.

Repeated sampling for measurement during a fermentation process can be tedious and thus indirect measurement via computers appears to be a viable alternative. Combined with other information obtained from sensors, these measurements make possible the calculation of several fermentation parameters, as given in Table 30.2. Computer simulation can also be used to indirectly measure a given component based on a mass balance equation of that component for the fermentation process. Besides these uses, the computer has applications in fermentation processes for continuous automated monitoring and feedback control and dynamic optimization of the process [16, 28].

Recovery of Fermentation Products

The isolation and purification of fermentation products is often collectively referred to as downstream processing. The early part of the separation of a bioproduct is the primary recovery process, whereas the elements further downstream may include purification, concentration, and formulation. The overall goal of downstream processing and formulation is to recover the product of interest cost effectively at high yield, purity, and concentration, and in a form that is stable, safe, and easy to use in a target application.

Table 30.2 Gateway measurements for fermentor monitoring and control

Parameter	Calculation
pH	Acid/base uptake or formation
Air flow rate, in and out O_2 concentration	O_2 uptake rate
Air flow rate, in and out CO_2 concentration	CO_2 production rate
O_2 uptake rate, CO_2 production rate	Respiratory quotient, specific metabolic rate
Power input, air velocity	O_2 transfer rate
Integrated CO_2 produced	Cumulative metabolic activity

Fermentation products include the cells themselves, solvents and chemicals, organic and amino acids, antibiotics, polysaccharides, lipids, RNA and DNA, vaccines, bulk and fine diagnostic and therapeutic proteins, food and feed ingredients, and enzymes. These biomolecules can differ substantially in nature and require a large variety of methods to separate and purify them. They are often produced in low concentrations in complex media together with many other components.

In the development of manufacturing processes for the production of biological molecules, much emphasis is placed on fermentor design and scale-up, thus one might assume that the recovery processes of fermentation products are rather straightforward and relatively simple. Nothing could be further from the truth. A point in case is an example of an antibiotic production plant. The investment for the recovery facilities is claimed to be about four times greater than that for the fermentor vessels and their auxiliary equipment. In organic and amino acid fermentations, as much as 60% of the fixed costs of fermentation plants is attributable to the recovery unit operations.

Figure 30.6 shows a typical recovery process for antibiotics, and a schematic overview of a typical downstream process in an enzyme plant is given in Fig. 30.7. From these diagrams it is apparent that most recovery processes involve combinations of the following procedures.

- Fermentor harvest
- Mechanical separations of cells from fermentation broth (clarification)
- Optional disruption of cells
- Extraction of the compound of interest
- Preliminary fractionation procedures
- High-resolution separation steps
- Concentration
- Formulation
- Optional drying

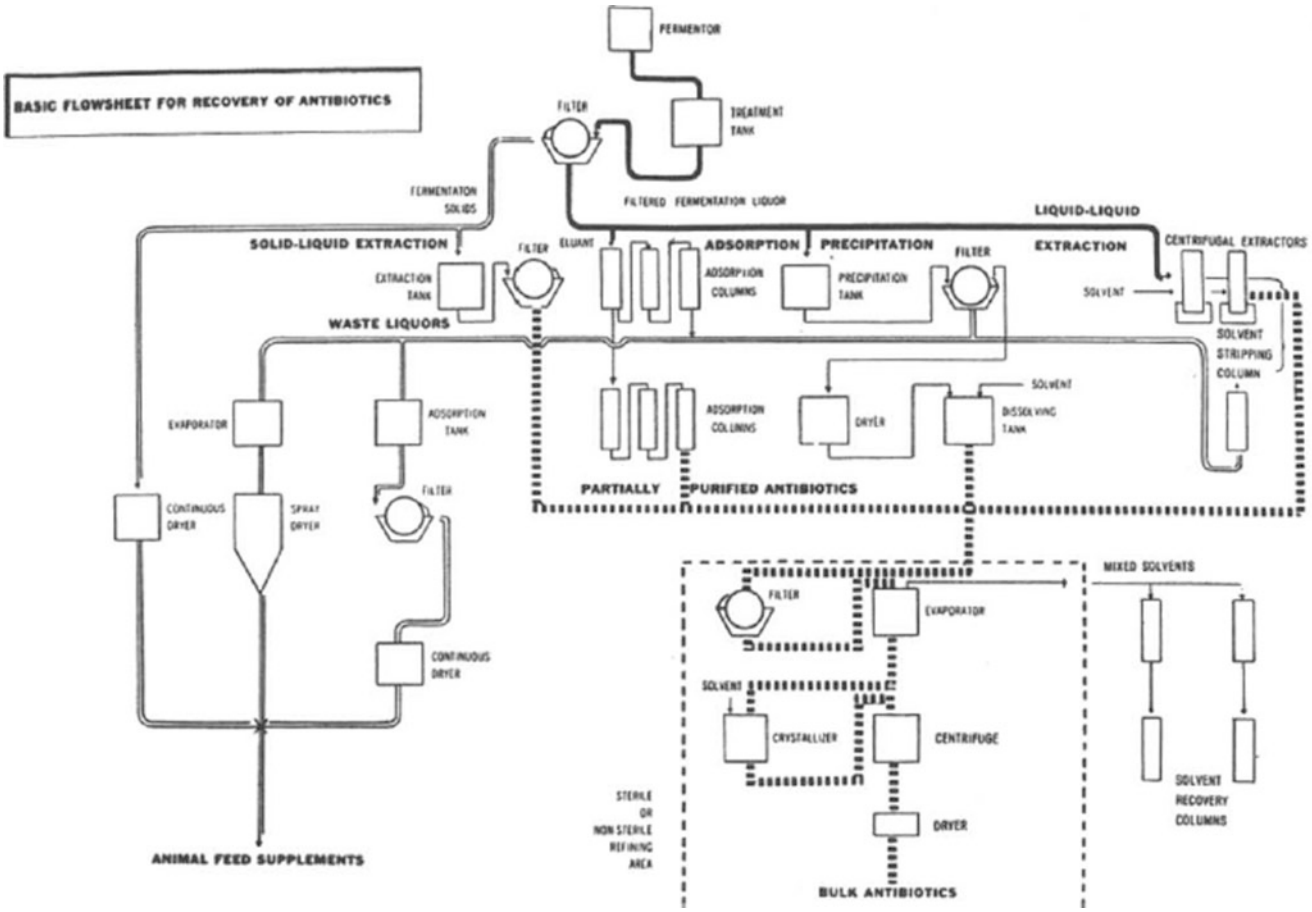


Fig. 30.6 Basic flow sheet for the recovery of antibiotics (Ind Eng Chem 49:1494, 1957. Copyright American Chemical Society and used with the permission of the copyright owner)

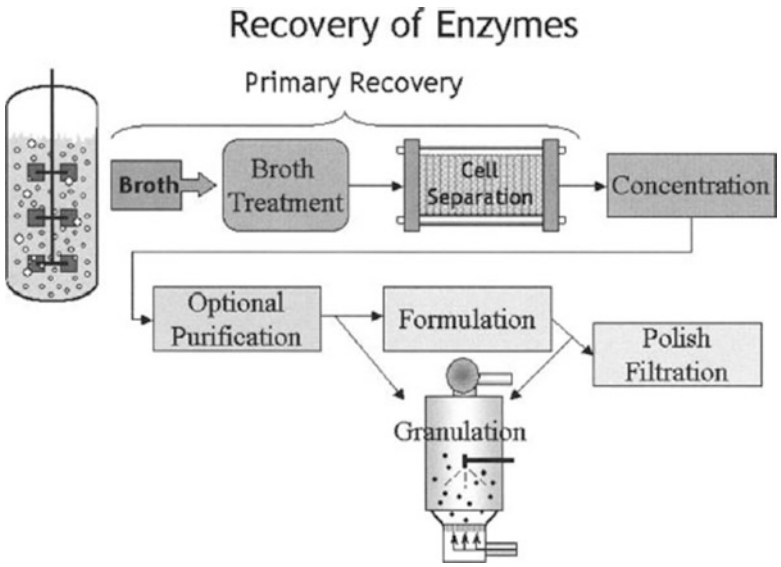


Fig. 30.7 Typical recovery process for industrial enzymes

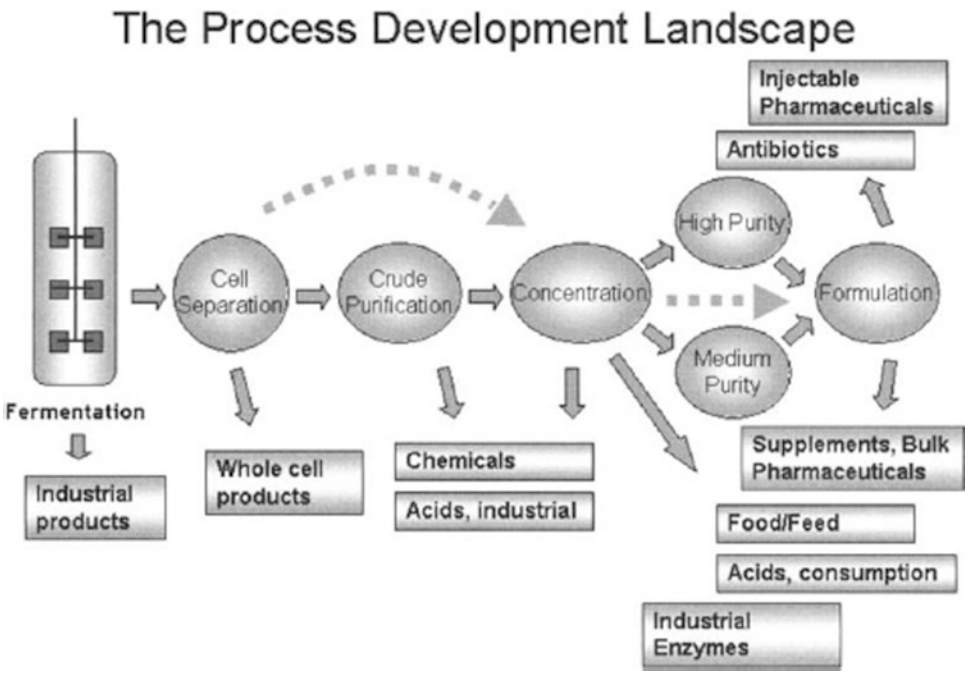


Fig. 30.8 The downstream processing cascade arranged by final product requirement

In contrast to fermentation processes, where typically one format (i.e., a fermentor vessel) is used, a number of widely differing techniques are necessary to accomplish purification and formulation of biological products. The variety of available separation technologies is large and the order and permutations of sequential processing steps are countless. Although this may hamper standardization of downstream processes, it also provides the benefit of flexibility because different unit operations can be tailored to the greatest efficiency in a cost-effective manner. Possibly a better way to illustrate a downstream process design is shown in Fig. 30.8. Here it is shown that recovery is not a linear sequential process but rather the downstream process development follows a palette of options that can be applied depending on the required specifications of the endproduct and as such, it is a modular approach.

Several general considerations limit the range of practical choices for a process design. The type of production organism (bacteria vs. fungi or mammalian cells) has an impact on the downstream process design. The fermentation media for these organisms can differ substantially and the media themselves can play an important role for the recovery strategy. Defined media often deliver a purer product but lower concentrations, whereas complex media contain more impurities but also generates more product. The physical, chemical, and biochemical properties of the desired product have an impact on the recovery strategy, as do the microbial cell's physiological states and properties. Proteins and enzymes require more "gentle" conditions to avoid unfolding or chemical denaturation than those required for small molecules. General parameters to observe are large swings in or extreme changes in pH and temperatures. Both proteins and small molecules can have hydrophobic, hydrophilic, or limited solubility properties that need to be considered when devising a recovery and purification strategy.

The principles and equipment discussed below are mostly focused on proteins and enzymes. Several other products, such as small molecules, metabolites, vitamins, and acids can require more specialized methods. However, many of the unit operations described here are also used in the recovery of these latter compounds.

Separation of Proteins and Peptides

Biological products can be inside the cells (intracellular), loosely associated with the cells, or secreted (extracellular). Each of these products requires a different approach towards the purification strategy. Intracellular and membrane-bound proteins and enzymes are more difficult to recover than the secreted ones, requiring physical or chemical disruption of cells and the challenge of separating the desired compound from viscous or entraining substances such as nucleic acids and cell wall debris. Fermentations are typically not harvested until the cell mass and the product are concentrated, often requiring some postharvest dilution to avoid entrainment losses and low equipment throughput. Continuous fermentations, on the other hand, generate a more dilute stream with a hydraulic load that can add significantly to the cost of subsequent concentration.

The simplest downstream processes include direct use of the entire fermentation broth, cells and all. This is suitable for low-cost industrial products. Another cost-effective method is the lysis of cells in the crude broth and direct use of the lysate. Yet another method that avoids lengthy purification steps is in situ extractive fermentation, whereby a product is directly extracted from the broth by use of solvents. However, many industries require more sophisticated purification methods due to safety and environmental considerations. For instance, it is imperative that live genetically modified organisms (GMO) and their DNA are not present in a released product.

As can be seen from Fig. 30.8, the nature of its final application will dictate how pure the product needs to be and which raw materials may be used in its manufacture. Typically, high purity is not required of industrial enzymes. In contrast, therapeutic proteins and small drug molecules require the highest level of purification. The purity range for food and feed compounds is somewhere in the middle. Raw materials must meet regulatory requirements for the application and must often be tested for their toxicological properties. Finally, economic considerations constrain both the range of raw materials and separations processes that can be used. Although multiple chromatographic steps are routinely included in the downstream processing of human therapeutic proteins, few industrial proteins include even a single chromatographic step [29]. Purification, if any, of industrial products is accomplished by less expensive techniques such as extraction or crystallization.

Fermentor Harvest and Primary Recovery

Extracellular products. In order to prevent cell lysis, removal of cells from a fermentation broth is usually started within hours after harvest. After cell separation, the clarified fermentation broth is more stable and can be stored refrigerated for days. Upon harvest, most primary recovery steps involve some pretreatment of the fermentation broth, which can range from simple cooling and dilution for fungal broths to pH adjustments, addition of salts, stabilizers, or flocculation agents in order to minimize degradation of the desired product and facilitate further processing. For labile compounds, such as protease enzymes, control of temperature, pH, oxidants, inhibitors, and activators is essential at harvest and throughout the recovery process. For example, it is important to maintain a molar excess of calcium ion to ensure the thermal stability of certain *Bacillus* proteases and amylases that contain calcium-binding sites.

Industrial products are mostly of the extracellular variety. They can be recovered directly from the fermentation broth. The primary recovery involves removing the cells from the broth, aptly called cell separation. Three different techniques are commonly used to achieve this goal: filtration, microfiltration, and centrifugation.

Filtration. Filtration can include filter presses, rotary drum vacuum filters (RDVFs), belt filters, and variations on synthetic membrane filtration equipment, such as filter cartridges, pancake filters, or plate and frame filter presses. These processes typically operate in a batch mode: when the filter chamber is filled up or the vacuum drum cake is exhausted, a new batch must be started. This type of filtration is also called dead-end filtration because the only fluid flow is through the membrane itself. Due to the small size of cells and their compressible nature, typical cell cakes have low permeability and filter aids, such as diatomaceous earths, perlite, or other mined materials are added to overcome this limitation. Moreover, the presence of high solids and viscous polymeric fermentation by-products can limit filtration fluxes without the use of filter aids.

In dead-end filtration, a cake forms on the surface of the pad as the filtration proceeds. The cake permeability is the most important physical property of a porous medium and the hydraulic properties of the flow and the specific cake resistance are described by Darcy's Law:

$$J = \frac{\mathrm{d}V}{A\,\mathrm{d}t} = \frac{\Delta p}{v_0(R_\mathrm{m} + R_\mathrm{c})}, \tag{30.24}$$

where R_m is the resistance of the membrane, R_c is the resistance of the filter cake, Δp is the transmembrane pressure, A is the area available for filtration, and v_0 is the permeate viscosity. Cake resistance can be expressed in terms of the specific cake resistance:

$$R_\mathrm{c} = \alpha \rho_\mathrm{c} \frac{V}{A}, \tag{30.25}$$

where α is the specific cake resistance and ρ_c is the mass of dry filter cake per unit volume of permeate. The combination of equations (30.24) and (30.25) and integration gives:

$$\frac{tA}{V} = \frac{\rho_\mathrm{c}\alpha v_\mathrm{o}}{2\Delta_\mathrm{p}}\left(\frac{V}{A}\right) + \frac{v_\mathrm{o}R_\mathrm{m}}{\Delta p}, \tag{30.26}$$

where specific cake resistance can be obtained from the slope of a plot of tA/V vs. V/A.

Fungal fermentations, such as those of *Trichoderma* or *Aspergillus* sp., lend themselves particularly well to cell separation by filtration through a RDVF because of the ease with which the fungal mat can be shaved off by the drum's knife, renewing the filter cake surface to maintain high filtration flux.

Bacterial fermentation broths can be processed either by filtration or centrifugation, but the much smaller size of bacteria generally requires the addition of a polymeric flocculant. Most flocculants are cationic and function by bridging the negative surface charges on neighboring cells to increase the particle size and facilitate either sedimentation rate or filtration flux and clarity. The choice of flocculant and optimization of dosage is a delicate balance among obtaining good separation quality, yield, cost, and minimization of residual excess polymer in the product.

Microfiltration. Microfiltration, the use of tangential flow anisotropic membranes to permeate the product of choice while retaining solids, can be an attractive cell separation technique because it does not require the use of flocculants or filter aids. It is, in principle, a more technically sophisticated version of classic dead-end filtration processes. Microfiltration yields can be low due to progressive fouling of membranes. Advanced engineering has overcome many of the early problems and microfiltration is now used widely in the recovery of therapeutic proteins and in the food, beverage, dairy, and water treatment industries.

Microfiltration units can be configured as plate and frame flat sheet equipment, hollow fiber bundles, or spiral wound modules. The membranes are typically made of synthetic polymers such as polyethersulfone (PES), polyamide, polypropylene, or cellulosic mats. Alternate materials include ceramics, stainless steel, and carbon. Each of these comes with its own set of advantages and disadvantages. For instance, ceramic membranes are often recommended for the filtration of larger particles such as cells because of the wider lumen of the channels. However, it has been shown that spiral wound units can also be used for this purpose, provided appropriate spacers are used.

Together with related technologies such as ultrafiltration, nanofiltration, and reverse osmosis, microfiltration relies on membranes with a defined pore size. The operation mode is as "cross flow" or "tangential flow" in which filtrate is pumped parallel to the membrane surface. Cells or cell debris and large molecules are retained on one side of the membrane and the biological product solution passes through the membrane. The primary advantage of tangential flow vs. dead-end is that membrane fouling only affects the membrane pores themselves, whereas particulate matter on top of the membrane is continuously swept away. In that manner, no filter cake is created. Microfiltration is complex and much research has been done to describe the theoretical underpinnings of the process. Equipment and membrane replacement costs are high. Capital and operating costs of filters and centrifuges of equal production capacity can be more advantageous. Nevertheless, microfiltration equipment, when designed and operated properly, has the potential to deliver high throughputs due to its continuous operation, reduction of process steps, and reduction of the waste generated. It also enables the use of recycle streams to increase product yields and decrease the cost of raw materials.

Centrifugation. Centrifugation is another viable method for removing microbes from fermentation broth. Three types of centrifuges are commonly used: (a) disk-stack centrifuges (clarifiers), (b) tubular and basket centrifuges, and (c) decanter centrifuges that contain solid removing scrolls. All three can be operated in a continuous mode for higher throughput. Less common are tubular centrifuges, which generate the highest centrifugal forces but have limited throughput. The main challenge in centrifugation is to identify a suitable flocculant or process conditions whereby the cell mass is easily conveyed or discharged from the centrifuge bowl without breakup or carryover into the centrate. Although centrifugation can handle a high concentration of cell solids, filtration can provide more complete removal of trace solids that can interfere with downstream concentration or purification steps. The presence of flocculants and filter aids in cell wastes can cause added disposal costs. Often, the centrate cannot be completely clarified, thus requiring combinations of a final filtration step and centrifugation to achieve a nonturbid product.

Intracellular products. Intracellular production of bioproducts is less preferable but sometimes the only way to produce certain compounds in appreciable amounts. In this case, cell disruption is required for recovery. High-pressure homogenization, bead mills, and chemical or enzymatic disruption of the cell wall with lysozyme or similar enzymes can be used to achieve cell breakage. In the case of small molecules, organic solvent extraction has also been described. If cell debris remains in the centrate, it must be removed by methods described earlier, thus adding extra steps to the process.

Purification. Purification of the product can follow or precede the step of product concentration. Biological product purification is often and quite mistakenly equated with column chromatography. Undoubtedly, this technology is widely applied and has advanced rapidly in recent decades. Chromatography is a separation technique that includes various separation principles, such as gel filtration, size exclusion, ion exchange, hydrophobic interaction, and affinity binding/elution. A major advance has been the development of cross-linked pressure-sustaining support matrices for high volume applications. Continuous moving bed technologies, expanded bed methods, and displacement chromatography have all contributed to greater versatility and cost effectiveness of the technique and have greatly expanded the tools available for high-resolution separation. More recently, high-pressure liquid chromatography (HPLC), originally an analytical tool, has been refined for preparative separations.

Although chromatography offers the greatest potential and diversity of mechanisms for separation and purification, with some notable exceptions, it is not cost effective in the manufacture of industrial commodities due to the expense, low throughput, and low binding capacity of chromatography resins. Furthermore, the complexity and control required for reproducible operation can be cost prohibitive for very large-scale production systems. It is also uneconomical for concentration of the product. A large body of literature exists for the chromatography of biological molecules bolstered by its extensive use in the biopharmaceutical industry.

Purification can also be achieved in other ways, through precipitation with salts, crystallization, and through aqueous two-phase extraction. Some of these methods are associated with substantial capital cost, low throughput, low yields, or waste issues. Fractional precipitation, one of the oldest protein separation technologies, can be surprisingly effective to separate a compound of interest from a complex broth. For instance, the process of fractional solvent precipitation of blood plasma components has been used since World War II.

It is sometimes necessary to remove an undesirable side activity from proteins and enzymes, particularly when it is not feasible to delete the side activity genetically. A case in point is the presence of co-secreted proteases. Even if minor protease contamination from the host organism does not cause significant degradation of the product during processing, it is likely to damage the product in the final formulation over several months of storage. In other cases, side activities must be removed because they interfere in the final application. Incremental additions of chaotropic salts, such as ammonium sulfate, water-binding polymers, such as polyethylene glycol, or organic solvents are added to a solution containing the enzyme, and the relative amount of each enzyme vs. other protein impurities in each fraction is monitored. Similar to the pooling of chromatographic elution fractions, the fractional "cuts," which have the most favorable balance of net enzyme purity and recovered yield, are combined.

Crystallization is related to precipitation in that it is governed by a compound's physicochemical properties. For example, the second virial coefficient, B_{22}, which characterizes the two-body interactions between protein molecules in dilute solutions:

$$B_{22} = \frac{2\pi}{M^2} \int_0^\infty r^2 (1 - e^{-u(r)/kT})\, dr \tag{30.27}$$

where M is the protein molecular weight, r is the intermolecular separation distance, $u(r)$ is the interaction potential, k is the Boltzmann constant, and T is the absolute temperature. The interaction potential $u(r)$ describes the interaction forces between the two protein molecules.

Proteins have long been crystallized for commercial production. This process is highly scalable, reproducible, and can be cost effective. As is the case with precipitates, crystals can be recovered via centrifugation or filtration processes. The purity achieved can be quite high, even though it is often not comparable to multiple chromatographic steps due to crystalline entrapment of impurities. Overall, it often meets the requirements for a number of industrial and food applications. A major challenge can be the time-consuming screening of appropriate precipitants, pH conditions, and temperatures and the mapping of the solubility phase diagrams. Recently developed high-throughput techniques for screening crystallization parameters have lessened the laborious nature of identifying appropriate conditions. Both precipitation and crystallization are excellent methods to concentrate the molecule of choice. A further advantage is that proteins in crystalline or precipitated form display good storage stability. Industrial biotechnology companies, such as former Genencor International, have developed large-scale crystallization methods for enzymes [30].

In the last one to two decades, aqueous two-phase extraction has become an attractive purification and concentration technique. It provides the selectivity of classical solvent extraction without its denaturing potential. Utilizing incompatible two-polymer and polymer–salt combinations and adjustments in pH and ionic strength, this technique separates proteins based on differences in hydrophobicity, surface charge, and molecular weight. Segregation of the separated molecule is governed by the partitioning coefficient (α):

$$\alpha = \frac{C_s}{C_w}, \tag{30.28}$$

where C_s is the concentration in the solvent phase and C_w is the concentration in the water phase. One application of the technique has been purification of genetically engineered chymosin from multiple side activities produced by the fungal host background.

An important step in the manufacture of biopharmaceutical injectables is the removal of endotoxin and viral contamination. Numerous methods have been described and include chromatographic steps and the use of filter cartridges [31].

Purification of bioproducts from fermentation, although necessary, comes with a major drawback. Biological compounds evolved in an environment of complex components are adapted to be most stable in these conditions. Highly pure forms of bioproducts have a tendency towards lower chemical and physical stability. For instance, proteases are prone to autolysis in the absence of impurities that inhibit degradation. This is one of the major challenges faced by the formulator of biological products (see section "Formulation").

Concentration. Clarified filtrates, centrates, or column eluates are usually too dilute for use in their specific applications, thus, substantial amounts of water must be removed. This can be achieved by evaporation or by ultrafiltration. Concentration methods used in industrial settings, such as evaporation, which is done under vacuum, and solvent extraction, may or may not be suitable for dewatering proteins because of their potential for thermal or chemical denaturation, and due to high energy costs associated with evaporation. The benefit of evaporation is that nonvolatile compounds that may stabilize the proteins are retained.

Ultrafiltration (UF), which can be a viable alternative, has membrane pore sizes that are much smaller than those in microfiltration, allowing only water and small molecules to travel through the membrane. Thus, proteins, peptides, and other large molecules can be retained as water is forced through the membrane. Typical molecular weight cut-off sizes for UF membranes are 5–100 kDa. This technology has emerged to become one of the most common methods for concentration of large biomolecules. UF is an attractive method because of its low energy consumption. It can be combined with diafiltration whereby repeated additions and removal of water lead to a cleaner product. UF fluxes and yields are often significantly enhanced by upstream removal or omission of potential membrane foulants, such as polysaccharides or antifoams. In the case of smaller molecules, nanofiltration can be used.

Nanofiltration membranes have a molecular weight cut-off ranging from 100 to 1,000 Da and with such small pore sizes are able to retain molecules such as humic acids and certain salts. This allows for production of parasite- and solids-free water without the need of chemicals.

Precipitation, crystallization, and extraction can also be used for concentration, but are more typically utilized as purification techniques. The latter methods also may cause problems with their environmental impact in as much as they require substantial additions of chemicals.

Decolorization and finishing. Decolorization is sometimes required for certain applications, mostly as an aesthetic preference. It is always desirable to solve these issues upstream. For example, color can be minimized by choice of fermentation medium components and control of the sterilization cycle so as to lessen the Maillard reactions between nitrogen and sugars. Color can also be reduced by treatment with activated carbon, use of antioxidants, and by diafiltration with membranes. Carbon-impregnated filter pads can be used to combine polish filtration with a decolorization step.

"Finishing," sometimes also called "polishing" refers to one or more filtration steps at the end of the downstream process that have the goal of clarifying and reducing the microbial load of the product. Solids are most conveniently removed using a filter press loaded with cellulosic, synthetic polymer pads, or disposable submicron gauge filter cartridges. Precoating pads with filter aids prevent premature fouling. All these methods can be used to remove microbes to the point of sterility if required. Often, formulation ingredients are added to the product concentrates before the final decolorization and finishing steps are performed.

Formulation

Formulation of a product depends on how the endproduct will be used and the ability to satisfy safety requirements. Many bioproducts are supplied in liquid form, as it is convenient to meter and use. If the compound is slated for dry products, the concentrate can either be stored for later use or applied directly for granulation. In many industries, proteins, peptides, and other bioproducts are supplied as encapsulates or granules in order to meet safety standards.

As with fermentation and downstream processing, precise formulation recipes are often kept as closely guarded trade secrets. Comparatively few publications on new approaches or new technologies are available in the literature [32]. However, the intellectual property landscape is filled with composition of matter patents.

Proteins are large molecules comprised of tens to hundreds of amino acid subunits. Their unique properties are associated with specific conformations of the amino acid chain. The active conformations are thermodynamically quite fragile, typically only a 5–15 kcal/mol difference in free energy between the stable and the unfolded conformations. Additionally, the amino acid subunits that define the protein contain a variety of reactive groups that are subject to chemical degradation pathways resulting in the loss of structure and/or activity. Thus, ensuring the physical and chemical integrity of a protein's native structure is a primary concern when using proteins or enzymes in applications where its structure is important. Many proteins are only soluble up to 20–30% (w/w) and therefore considerations for keeping the compounds well solubilized are of importance.

Dry formulations. Because of the long-term instability of proteins in aqueous solution, enzyme producers and formulators have attempted to produce stable solid formulations since enzymes were first used. Proteases tend to degrade via autolysis and can be incompatible with surfactants. These problems are easily overcome by storing the enzyme in the solid state. Initially, commercially produced enzymes for laundry detergents were spray-dried or sprayed onto salt cores without coating. Spray-drying provides a fast and cost-effective way of compartmentalizing

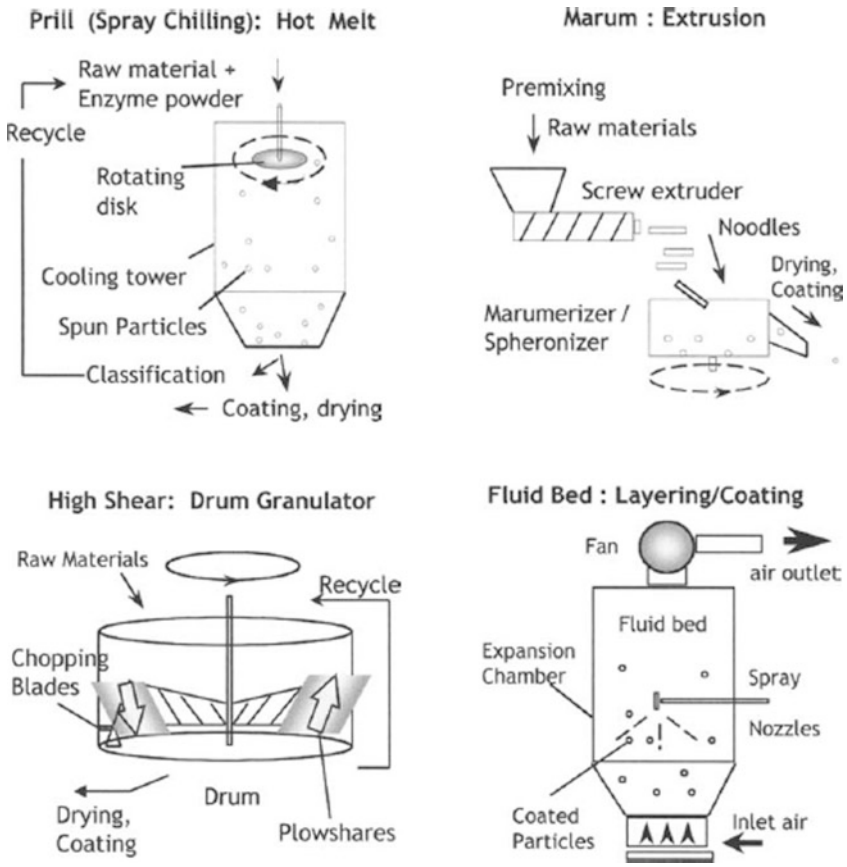


Fig. 30.9 Common granulation method in enzyme manufacturing

two incompatible ingredients and maintaining enzyme activity over a product's typical shelf life. However, noncoated granules have led to cases of allergic reactions in formulating plant personnel and resulted in a setback for the enzyme industry in 1970s. Thus, the technologies used to produce modern dry products have evolved far beyond simple spray-drying.

Product safety is an important consideration as many bioproducts can cause adverse responses in humans and many therapeutic fermentation products are very potent bioactive molecules. Negative effects can include irritation and allergic reactions, which are due to the possible immunogenic nature of many biological compounds. Lyophilization, or freeze-drying, which is used in therapeutic applications results in a powdered product that is contained in vials as an additional safety measure to prevent direct exposure. It offers a gentle way of protecting a compound's fragile nature because the drying temperature is kept below freezing. However, these technologies are expensive.

Many of today's bioproducts are supplied in a granular form. Granulation is a generic term for particle size enlargement. Granules in consumer products need to provide a tough barrier to prevent release of the bioactive molecules in airborne dust, while providing quick release once used in the final application. Enzyme granules contain coatings that are designed to withstand the physical impact and shear forces typically encountered during powder processing, maintaining excellent flowability throughout the process.

Many ways of producing enzyme granules have evolved, with just a handful of methods being currently in common use. These include prilling (spray-chilling), marumerization/spheronization, drum and high shear granulation, and fluid-bed coating (Fig. 30.9). The latter two techniques are superior in producing low dust granules. An overview of the different technologies is provided in Table 30.3. In general, fluid-bed technology (Fig. 30.10) is the most flexible approach, giving granules with the most uniform appearance and smooth coatings (Fig. 30.11). Other technologies may have an edge in either cost or throughput, as they are more amenable to continuous operation. The advantage of the fluidized-bed method is that the entire granulation process can be carried out within a single, contained piece of equipment. The spray-coating process allows the sequential application of layers of different thicknesses and compositions with almost infinite flexibility. In addition, spray coating allows one-step application of controlled release coatings.

A less commonly used method is spray-chilling, whereby the product is incorporated into meltable cores. This method is straightforward and inexpensive but has the drawback of being limited by the meltability of its ingredients. Moderate

Table 30.3 Comparison of powdered enzyme production methods

Technology	Equipment	Advantages	Disadvantages
Prilling (hot melt)	Prilling tower	Continuous process	High product dust
		High capacity	Low melting point
		Easy rework	Requires predried enzyme
Extrusion/spheronization (marum)	Screw extruder, Marumerizer, fluid bed coater	Inexpensive raw materials	Complex multistep process
		Continuous process	High dust during process and in product
			Requires highly concentrated enzyme
Drum granulation (high shear)	Lödige mixer	High capacity	Dusty, multistep process
	Littleford mixer	Continuous process	Requires highly concentrated enzyme
	Fluid bed or drum coater	Tough granules	Wide particle size distribution
Fluid bed (layering/coating)	Fluid bed coater	Single, contained reactor	Batch process
		Flexible formulation	Difficult rework
		Tough uniform granules	

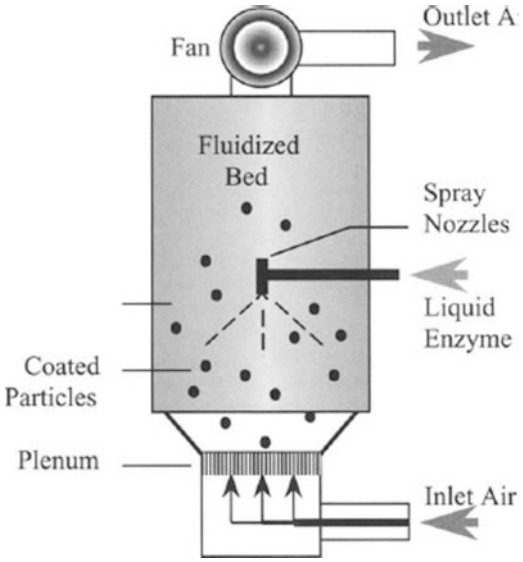


Fig. 30.10 Schematic of a fluidized-bed coater

to high temperatures during the shipping or during use of the product can lead to agglomeration of the capsules.

Solid formulations can provide some significant advantages, such as enhanced stability, delayed or controlled release, and protection against deactivation during harsh applications. One example of the latter is the use of granules to encapsulate cellulase enzymes against deactivation in the steam-pelleting process that is used in producing animal feed pellets. The use of effective stabilizers and coatings can prevent or retard exposure to moisture under high heat, yet allow release of the enzyme in the subsequent feed application.

Many therapeutic molecules can be encapsulated using other techniques, such as coacervation, micro- or nanospheres, microcapsules, or liposomes. Coacervation, often used for flavor encapsulation, can be a simple aqueous phase separation of immiscible droplets, or in the case of complex coacervation, the use of opposite electrostatic charges. It is one of the basic processes of capsule wall formation. Other biochemicals, such as flavors, vitamins, or citric acid are sold directly in the crystallized forms and can be incorporated directly into the final product, such as tablets, capsules, granules, and so on.

Liquid formulations. The successful application of enzymes in liquid formulations presents several technical obstacles that are not encountered in powders and granules. These problems stem from the fact that liquid products are complex aqueous solutions, and physical separation of enzymes from other potentially inactivating ingredients is impractical. Necessary ingredients for a viable endproduct can affect the physical and chemical stability of enzymes. In addition, as other types of enzymes are added to formulations that already contain proteases, proteolytic degradation of enzymes is a concern.

Liquid formulations can be used as pastes or slurries, dispersions or emulsions (in aqueous or other solvents) to physically separate the compound to be protected. Dispersions, pastes, or slurries are not commonly used but are a feasible approach when highly concentrated products are needed. For instance, many early analytical enzymes were sold as crystal slurries to preserve stability. Dispersions can be prepared from dry materials. Most of the dispersions are visually cloudy in appearance due to the particle size of the dispersed compounds and are thus undesirable in most endproducts.

Many catalytically active proteins are hydrolases and are subject to three principal means of deactivation: denaturing or unfolding, catalytic site inactivation, and proteolysis. Denaturation is best minimized by controlling temperature and pH and by avoiding the presence of chemical denaturants. Catalytic site inactivation is prevented by supplying sufficient levels of cofactor, typically a metal cation, and preventing oxidation of the active site, for example, by formulating with antioxidants. Alternatively, oxidative

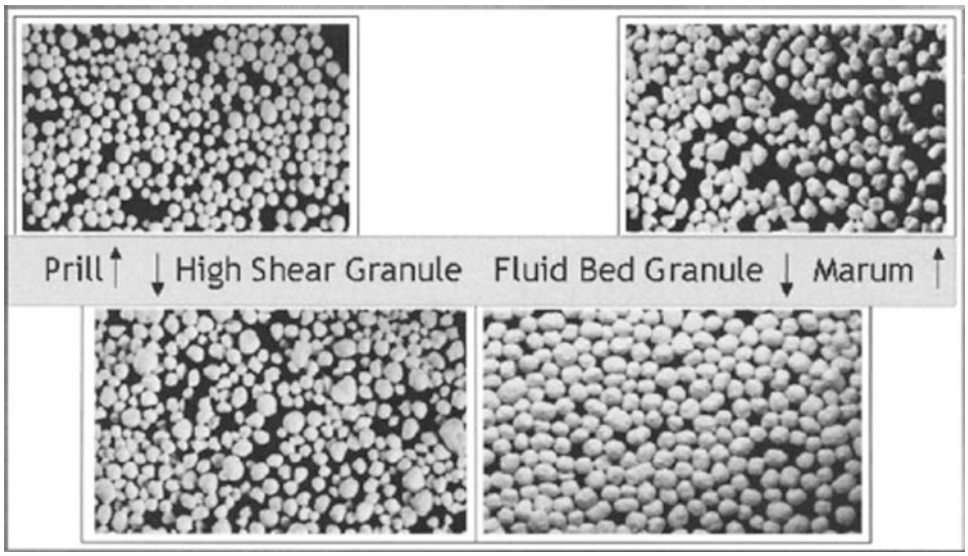


Fig. 30.11 Commercially available enzyme granules (Courtesy Genencor International)

resistance can be engineered into the protein structure. Finally, in the case of proteases, proteolysis or autodigestion can be minimized by reducing water activity or by addition of inhibitors. Thus, the final liquid formulation includes stabilizers, antimicrobial substances, and osmolytes to reduce the water activity and increase thermodynamic stability. Useful water-sequestering compounds include sugars and other polyols, such as glycerol, sorbitol, and propylene glycol. Useful inhibitors include substrate analogues such as peptides and acid salts. Once the formulation excipients are added, the last step includes a final polish filtration to provide a clear liquid.

Different industries pose different challenges to the protein formulator. Many feed enzymes are sold as formulated liquid concentrates. In this case, the major requirements for a liquid formulation are enzymatic stability and preservation against microbial growth. It is sometimes not appreciated that the dominant factor affecting enzyme stability is the intrinsic stability of the enzyme itself; formulation can do very little to correct for a structurally labile protein. Therefore, it is advisable to make stability an important criterion of the initial enzyme screening process.

An important case is the application of enzymes in laundry detergents. Market trends in the United States show that consumers prefer liquids to powder detergents by a ratio of 2 to 1. These products are stored with no temperature control on shelves in the presence of harsh surfactants, such as linear alkylbenzyl sulfonate (LAS) and require extraordinary measures for stabilization. LAS, by its nature as an effective cleaning agent, causes surfactant-induced unfolding in proteins. There are countless examples of the development of stabilization systems in the intellectual property space. A common theme is to reduce the water activity and to use borate/glycol stabilizers that bind to the active site of proteases.

Microbial growth is fairly easy to control by the addition of food grade antimicrobials such as sodium benzoate, methylparaben, and various other commercial preparations. In addition, reduction of the water activity through use of sequestering agents aids in controlling microbial growth. The prevention of precipitation and hazes is often a highly empirical challenge and one that is very specific to the enzyme and the specific raw materials used in the process. It is best to use ingredients with high solubility and to screen potential formulations by extended storage at high and low temperatures. In the case of proteins, an additional objective is to keep the pH value as far away as possible from the isoelectric point of the protein, at which the protein is least soluble.

Emulsions are the most commonly used type of formulation in the food, paint, and personal care industries. The typical emulsions are "oil in water" or "water in oil" preparations. Proteins, peptides, metabolites, and other fermentation products can be incorporated into emulsions. Covalent attachment of hydrophobic compounds, such as fatty acids or polyethylene glycols, to proteins or peptides lends these hydrophilic molecules to reside in the lipid phase.

Whole Cell Recovery

The recovery of whole cells is best explained by the manufacturing procedure for baker's yeast. This process is almost identical to the early stage of protein recovery, except that the final product is the cell instead of the filtrate. After fermentation, the cells are spun out with a centrifuge, washed with water, and recentrifuged to yield a yeast cream with a solids concentration of approximately 18%. Cream yeast can be loaded directly into tanker trucks and delivered to customers equipped with an appropriate cream

yeast handling system. Alternatively, the yeast cream can be pumped to a plate and frame filter press or an RDVF and dewatered to a cake-like consistency with 30–32% yeast solids content. The press cake yeast is crumbled into pieces and packed or spray-dried for dry products. After packaging, the yeast is ready for shipping to retail.

Separation of Small Molecules and Metabolites

Metabolites, vitamins, organic and amino acids, and specialty chemicals are commonly referred to as small molecules, particularly in the therapeutic world to differentiate these compounds from proteins or peptides, commonly named biopharmaceuticals or biologicals. Many of the same unit operations are applied for the recovery and purification of small molecules as described earlier. Purification of bulk products has cost constraints, whereas pharmaceuticals are subject to strict purity requirements.

Possibly the largest class of molecules produced in microorganisms are acids. This group includes acetic acid, citric acid, gluconic acid, lactic acid, and, of course, the amino acids. Citric acid is recovered (Fig. 30.14) after the cells have been removed by RDVF. It is then solvent extracted or precipitated as a calcium salt by adding hydrated lime [$Ca(OH)_2$]. The precipitate is recovered by a second RDVF and then acidified with sulfuric acid where it forms citric acid and calcium sulfate (gypsum). A third RDVF removes the gypsum. Lactic acid is usually produced as an alkali or earth alkali salt.

Another excellent example is given by penicillin recovery, a weak acid. Penicillin can be recovered by solvent extraction and its partitioning coefficient into solvent can be steered by the pH value. In the dissociated form it is more hydrophilic, whereas in the protonated form it is more hydrophobic. Thus, at low pH, penicillin partitions into the solvent phase.

A number of relatively new methods are being investigated to improve the recovery of small molecules. These methods include electrokinetic separators with bipolar membranes, simulated moving-bed chromatography, and supercritical fluid extraction. The latter is practiced for food components. It has also been described for proteins but has not yet found wide acceptance in this field. A fast-growing field is the production of bioethanol via fermentation processes either from milled corn or from recycled biomass. The fermentation and saccharification processes can occur simultaneously in the fermenting tank by means of saccharification enzymes (amylases, cellulases).

Once the mash is fermented, it is transferred to a distilling unit. The distilling system is designed to produce greater than 90% combustible bioethanol from fermented grain mash. The system can be arranged in an array of columns: mash columns, aldehyde-extraction columns, and rectifying columns. The remaining alcohol-free liquid is drawn off from the bottom of the rectifying column. This liquid can be blended with the watery meal from the mixing tank before cooking.

For the dehydration step, molecular sieves made of zeolite can be used. The alcohol is extracted from the molecular sieve at 99.75%, dehydrated as vapor and captured in condensers. New research focuses on alternate aspects of this separation process to improve efficiency such as developing membranes for ethanol recovery. Low-cost pervaporation, an energy efficient combination of membrane permeation and evaporation, for ethanol extraction and bioethanol production holds much promise.

The liquid left after distillation is subjected to centrifugation, where most of the suspended solids are separated. The clear liquid can be recycled by adding it back to the starch conversion stage. The moist cake released by the centrifuges is mixed with the syrup produced by the evaporator to form a homogenous mixture and is dewatered in dryers. The dryers produce a Distillers Dried Grains with Solubles (DDGS) meal, which can be formed into pellets. These are used in many applications, most prominently in animal feed.

It is evident from the discussion earlier that downstream processing and formulation depend heavily on the industry and the product. There are countless possibilities on how a molecule can be recovered, purified, and formulated. More importantly, the fermentation, recovery, and formulation processes are intimately intertwined. Depending on the type of production organism and fermentation process used, the downstream processing has to adjust accordingly. It is thus imperative that process development is highly integrated. In the modern fermentation industry, this is usually achieved with process development and formulation departments working closely to deliver the best product at a competitive cost. Moreover, optimal integration of these departments with the strain development teams is also seen. Current industry trends also include reduction in the number of process steps because each step results in a yield loss. Sustainable technologies such as reuse of water, ingredients, and minimal by-product generation are also being adopted. Ultimately, all these efforts are beneficial for improving the production economics.

Regulatory Considerations

Bioproducts are regulated according to their application, for example, whether enzymes are used in food, feed, detergents, textile processing, and so on. Regulations also differ country by country. Irrespective of legislation, it is prudent for the product manufacturer to ensure that the

production process, the potential product, and its intended use are safe prior to introduction.

Along with transfer of the strain, fermentation, recovery, and formulation process to the manufacturing plant, validated quality control assays, which are suitable for ensuring product quality, must also be provided. The EPA, FDA, and other agencies throughout the world are all involved in regulation of products derived from naturally occurring organisms and GMO. Essentially the regulations require: (1) the use of nonpathogenic organisms, (2) the absence of endotoxin or other toxins, (3) a well-documented and reproducible fermentation and downstream process, and (4) use of safe and well-characterized organisms and processes. In response to the latter, not only are the GMO well characterized, they are designed to barely survive in the general environment. Because recombinant organisms can be grown under contained good large-scale industry practices, safety issues relating to environmental release are minimized.

For control and containment of waste, regulatory requirements dictate appropriate containment that is related to the risk presented by the organism. Most of the processes are contained and hence subject to guidelines for industrial applications of recombinant DNA. Containment may be achieved biologically on the basis of inherent properties of the organisms, for example, their survival in the general environment is limited. Also, operation and design of the manufacturing facility minimizes potential releases of the recombinant organism to the environment. The degree of physical containment is matched to the risk presented by the organism. The same considerations apply to products made by recombinant organisms. Air monitoring is performed in production environments to evaluate levels of protein. This is important for assessing the efficiency of containment, respiratory protection requirement, and ensuring that environment levels meet occupational exposure limits.

Delivery of Products

Figure 30.12 represents a cell factory model to produce several categories of products via fermentation and biocatalysis processes. Carbon conversion in a cell factory into product(s) is essential to maintain the necessary energy (ATP, NAD(P)H, FADH) flow for the cell to replicate and maintain itself. The production of biochemicals is strictly linked to cell functions like cell synthesis (reduction–oxidation balance, cofactor regeneration, charge neutralization), and substrate turnover. Maximization of conversion yield, therefore, requires minimization of wasteful functions. Likewise, maximization of conversion rate requires maintenance of maximum driving force, e.g., ATP per conversion, key intracellular metabolite concentration, biomass per ATP, sufficient enzymatic activities, etc. Such principles continue to evolve in developing synthetic biology based processes, but it is too early to call them "laws of biotechnology." Synthetic cell engineering continues to significantly improve beyond what nature has already made possible, reaching 85–95% of what's theoretically possible. Accordingly, most favorable hosts, enzymes, raw materials, and process steps should be optimally integrated to achieve the best product economics and production efficacies.

Synthetic cell engineering is primarily the integration of four components: mining of genetic diversity and sequencing, cell physiology analysis and system modeling, synthetic pathway design and optimizing construction, integrated process design and engineering (see Fig. 30.13). Such integration requires several upfront tools outlined below:

1. Expression vectors to clone genes and adjust their copy numbers, and multiple markers to clone multiple genes
2. Transformation methods for above, gene deletion and integration systems

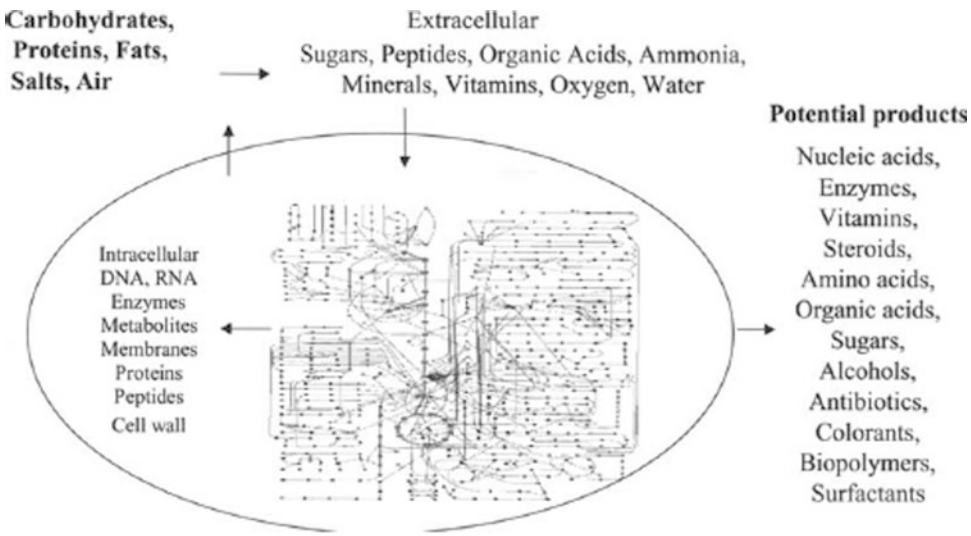


Fig. 30.12 A cell factory model

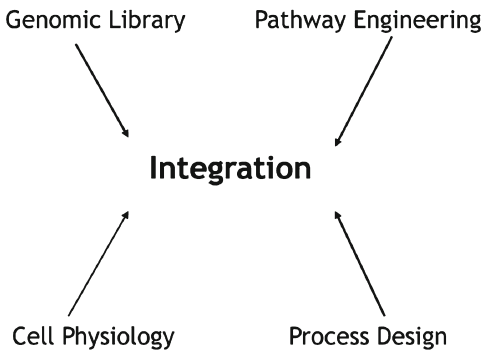


Fig. 30.13 A cell factory model depicting the four components that need to be integrated to produce several categories of products via fermentation and biocatalysis processes

3. Codon optimization of heterologous genes for higher expression
4. Mutagenesis of genes for in vivo regulation of enzymes
5. Transcriptional control, i.e., change promoter sequences to achieve different mRNA levels
6. Engineering of ribosomal sequences for translation modulation
7. Mutagenesis of pathway genes to change enzyme activity, specificity of substrate, product or inhibitor

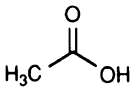
To develop cost-effective products for the biobased industry, the field of synthetic biology is continually developing a holistic understanding of microbial metabolism, and the effect of end-product on cell growth and maintenance. For example, functional genomic techniques now can monitor cell-wide transcription, translation, levels of metabolites and their interactions. By integrating the cell system-wide data and models, cell metabolism can be analyzed. The system knowledge can then be used to design/assemble cell components for specific functions and eventually build a cell factory platform.

Also, the size of the worldwide industrial chemicals business is ever increasing from $1 to $2 trillion, and global companies like DuPont, Genencor, Goodyear, BASF, DSM, Monsanto, Dow, Cargill, ADM, Tate & Lyle, Roquette, Mitsubishi, Braskem, Rohm & Haas and even new companies like Gevo, Amyris, Genomatica, Verdezyne, Ceres, and more continue to invest in industrial biotechnology to create high-value biochemicals, biomaterials, and biofuels. These companies are all interested in commercializing the biomaterial building blocks like propane diol, isoprene, succinic acid, acrylic acid, levulinic acid, isosorbide, butane diol, butylenes, ethylene glycol, ethylene, lysine, caprolactem, terephthalic acid, adipic acid, furan dicarboxylic acid, and of course natural sugar polymers like starch, cellulose, and xylose and their derivatives (see Fig. 30.14). But sustained growth, improved economics, and renewable resources will have to go hand in hand to enable replacement of petroleum derived chemicals and processes. Industrial-scale manufacturing aspects such as uses, synthesis methods, and costs of some of these bioproducts are reviewed below as well as in Chap. 31.

Organic Acids and Polymers

There are many organic acids that can be produced by microbial or biochemical means. However, at present, only acetic acid (as vinegar), citric acid, itaconic acid, gluconic acid, 2-keto-gulonic acid, and lactic acid are produced industrially by fermentation. Other organic acids, such as fumaric, gallic, malic, and tartaric acids, once produced by fermentation or enzyme processes, are now produced commercially, predominantly by the more economic means of chemical synthesis.

Acetic acid. Acetic acid as a chemical feedstock is manufactured by chemical synthesis. Acetic acid in the form of vinegar (at least 4% acetic acid by law) is produced largely via the oxidation of ethanol by bacteria of the *Acetobacter* genus [33].



Vinegar is one of the oldest known fermentation products, predated only by wine and possibly by certain foods from milk. First derived from the spoilage of wine, vinegar has been used as a condiment, food preservative, medicinal agent, primitive antibiotic, and even today as a household cleansing agent. Today, vinegar is produced almost entirely for use in foods. Vinegar may be defined as the product of a double fermentation: an alcoholic fermentation of a sugar source (fruits and their juices, cereals, syrups) usually by a selected strain of the yeast *Saccharomyces cerevisiae* or *ellipsoidens* and a second fermentation to oxidize the alcohol (including synthetic) to acetic acid by a suitable culture of *Acetobacter* organisms. The theoretical maximum yield of acetic acid on glucose by this route is 67% (two moles of acetic acid produced from every mole of glucose consumed). A homofermentative culture, *Clostridium thermoaceticum*, is known to be capable of fixing CO_2 and yielding three moles of acetic acid from one mole of glucose under anaerobic conditions. However, the technology for this process has not been commercialized.

Several vinegar-manufacturing processes are commercially used, including the following:

1. Trickling-bed reactor
2. Submerged cell reactor
3. Tower reactor

The circulating, trickling generator, which is still widely used, is a large tank generally of wood, such as redwood or

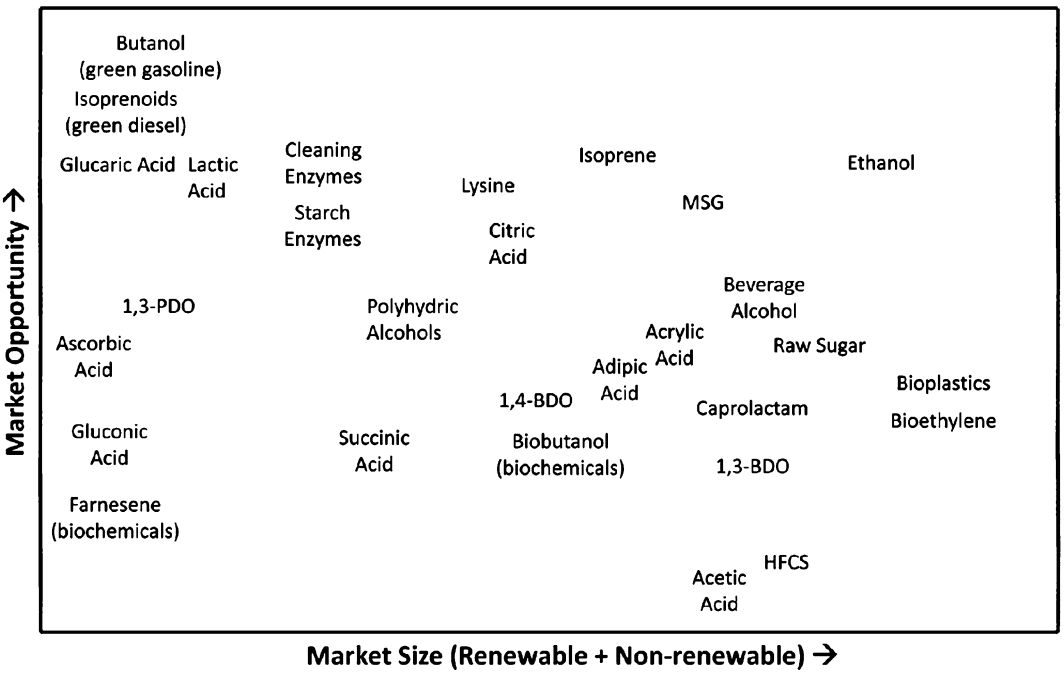


Fig. 30.14 Market opportunity (growth potential) vs. current market size of biochemicals and petrochemicals

fir but preferably cypress. Air is circulated in the generator by a number of equally spaced inlets. A pump circulates the ethanol–water–acetic acid mixture from the collection reservoir up through a cooler to the top of the tank. The liquid trickles down through the packing and returns to the bottom reservoir. The temperature of the generator is about 29°C at the top and 35°C at the bottom. A portion of the finished vinegar is periodically withdrawn from the reservoir and replaced with the ethanol-containing charge to maintain ethanol concentration in 0.2–5% range. If ethanol is depleted in the generator, the *Acetobacter* die, and the generator becomes inactive.

The Frings Acetator (produced by the Heinrich Frings Company of Bonn, Germany) is a submerged batch fermentor. It consists of a stainless steel tank with internal cooling coils, a high-speed, bottom-entering agitator, and a centrifugal foam breaker. The unique feature of this Acetator is its highly efficient method of supplying air, accomplished by high-velocity self-aspirating rotor that pulls air in from the room to the bottom of the tank. When the ethanol content falls to 0.2% by volume about 35–40% of the finished product is removed. Fresh feed is pumped in to restore the original level, and the cycle starts again. Cycle time for 12% vinegar is about 35 h, and the rate of production can be 10× that of the trickling generator. The yield of acetic acid on ethanol is higher as well, for example, 94 for the Acetator compared to 85% for the trickling generator. However, much more extensive refining equipment is necessary for filtering vinegar produced by the submerged process because the mash contains both the vinegar and the bacteria that produced it.

The tower fermentor is a relatively new aeration system applied to vinegar production. The fermentor is constructed of polypropylene reinforced with fiberglass. Aeration is accomplished through a plastic perforated plate covering the cross-section of the tower and holding up the liquid. The cost of the tower fermentor is said to be approximately half that of a Frings Acetator of equivalent productive capacity. It has been reported that the tower fermentor is satisfactory for producing all types of vinegar.

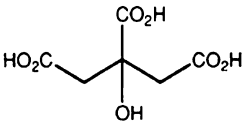
Since 1993, a two-stage submerged fermentation has been used to produce vinegar of more than 20% acetic acid. In the first fermentor, alcohol is added slowly to a total concentration of about 18.5%. After the acetic acid concentration has reached 15%, about 30% of the liquid from the first fermentor is transferred to a second fermentor. In the second fermentor, the fermentation is carried out at a reduced temperature of 18–24°C and continues until the alcohol is almost depleted.

Recombinant strains of *Acetobacter aceti*, cloned with either alcohol dehydrogenase or aldehyde dehydrogenase, have been tested for vinegar production. The bacteria with the aldehyde dehydrogenase gene produced acetic acid more rapidly than those with the alcohol dehydrogenase and were more resistant to high concentrations of acetic acid.

Vinegar clarification is accomplished by filtration, usually with the use of filter aids such as diatomaceous earth or bentonite. After clarification, vinegar is bottled, sealed tightly, pasteurized at 60–65°C for 30 min, and then cooled to 22°C. Vinegar can be concentrated by freezing or by a reverse osmosis membrane process.

The world production (excluding China and Russia) of 10% vinegar is estimated to be about two billion liters a year or about 200,000 metric tons of acetic acid. Price depends on the source (fruit, malt, grain, etc.).

Citric acid. Citric acid, whose structure is shown below, is the most important organic acid produced by means of fermentation.



Citric acid is used in soft drinks, candies, wines, desserts, jellies, jams, as an antioxidant in frozen fruits and vegetables, and as an emulsifier in cheese. As the most versatile food acidulant, citric acid accounts for about 70% of the total food acidulant market. It provides effervescence by combining the citric acid with a biocarbonate/carbonate source to form carbon dioxide. Citric acid and its salts are also used in blood anticoagulants to chelate calcium, block blood clotting, and buffer the blood. Citric acid is contained in various cosmetic products such as hair shampoos, rinses, lotions, creams, and toothpastes. More recently, citric acid has been used for metal cleaning, substituted for phosphate in detergents, for secondary oil recovery, and as a buffer/absorber in stack gas desulfurization. The use of sodium citrate in heavy-duty liquid laundry detergent formulations has resulted in a rapid increase in the use of citric acid.

Wehmet first described citric acid as a product of mold fermentation in 1893. However, it was only in 1919 that commercial fermentation processes based on sucrose were developed [34]. Although many organisms have been shown to produce citric acid from carbohydrates, *A. niger* has been the best organism for industrial production. Figure 30.15 summarizes the reactions leading to citric acid from glucose. It is worthwhile to note that one mole of glucose yields one mole of citric acid with no consumption of oxygen. The overall reaction is actually energy yielding. It yields one mole of ATP and two moles of NADH per mole of citric acid, and minimal growth is needed during production. This aspect makes fermentative production of citric acid a good candidate for the process of cellular immobilization.

Microbiological production of citric acid can be implemented by three techniques:

- Solid-state fermentation
- Liquid surface fermentation
- Submerged culture fermentation

In solid-state, or Koji, fermentation, *A. niger* is grown on moist wheat bran (70–80% water) and produces citric acid in 5–8 days. This process is practiced only in Japan and accounts for about one-fifth of Japanese citric acid production. In liquid surface, or shallow tray, fermentation, beet

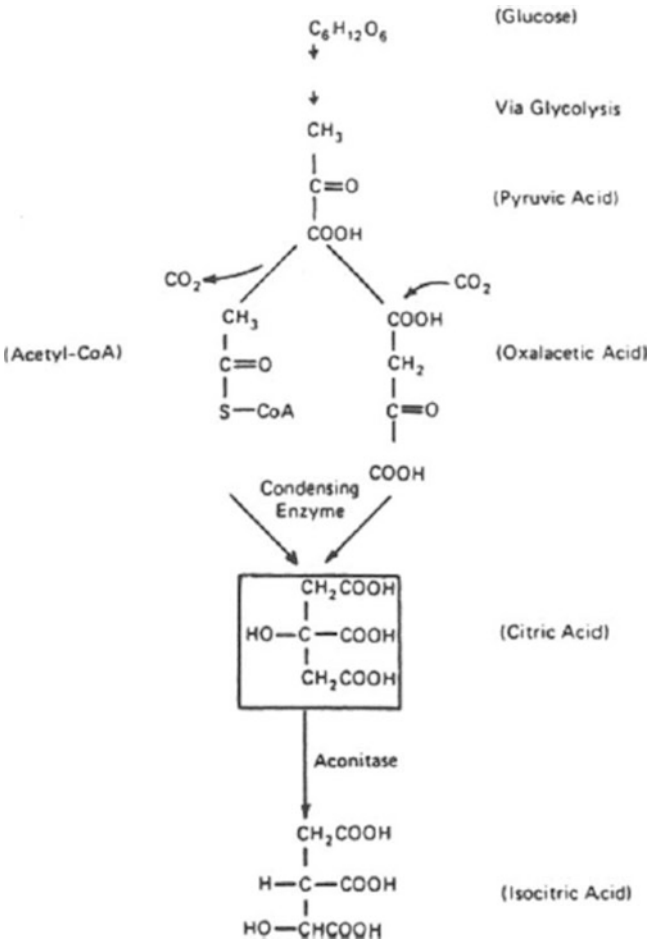


Fig. 30.15 Pathway leading to citric acid from glucose

molasses (containing 48–52% sugar) or cane molasses of blackstrap (containing 52–57% sugar) or high test (containing 70–80% sugar) are introduced into a mixer. Dilute sulfuric acid is added to adjust the pH to about 6.0. Phosphorus, potassium, and nitrogen in the form of acids or salts are added as nutrients for proper mold growth and optimal citric acid production. The mix is then sterilized and finally diluted with water to a 15–20% sugar concentration. The medium flows by gravity into shallow aluminum pans or trays arranged in tiers in sterile chambers. Most chambers have provisions for regulation and control of temperature, relative humidity, and air circulation. Each tray holds about 400 L of solution at a depth of 76 mm. When the medium has cooled to about 30°C, it is inoculated with spores of *A. niger*. The tray fermentation requires 8–12 days. The pH drops to about 2 at the end of the fermentation, and the acid content varies from 10 to 20%. Some oxalic and gluconic acids are also formed. The temperature is maintained at 28–32°C during the fermentation. Sterile air is circulated through the chambers, and the relative humidity is controlled between 40 and 60%.

Most newly constructed plants have adopted the submerged culture or deep fermentation process. The fermentation medium consists of sucrose (around 200 g/L) and mineral salts to provide a balanced supply of iron, zinc, copper, magnesium, manganese, and phosphate. The provision of a suitable culture medium is the most critical factor in obtaining a high yield of citric acid. The fermentation is carried out at 25–27°C. Oxygen is provided by bubbling air at a rate of 0.5–1.5 volumes of air/volume of solution/minute without mechanical agitation. It is generally accepted that the formation of pellets between 1 and 2 mm in diameter in the fermentation mash is most desirable. Pelleting reduces broth viscosity, increases oxygen transfer, and simplifies mycelium separation in the recovery scheme. The submerged fermentation has a time cycle of 6–9 days. The yield of citric acid on sugar varies from producer to producer but the theoretical maximum is 112% on sucrose. The liquid surface fermentation yield is high (90–95%) but the submerged culture fermentation is a bit lower. Reducing the formation of by-products, mainly oxalic acid, has resulted in improvements in the yield of the submerged culture process, nearly reaching those of the surface culture process.

The fermentation broth from the solid state, surface culture, or submerged culture process is treated similarly for recovery and refining of citric acid. Two recovery methods are used: precipitation and filtration, and extraction. A process flowsheet including the fermentation and refining section using the first method is shown in Fig. 30.16. The mycelium, which is initially filtered out of the fermentation liquor, may be used as fertilizer after proper weathering and processing. The clarified liquor flows to precipitating tanks fitted with stirrers where it is heated to a temperature of 80–90°C. The oxalic acid present is separated by preferential precipitation through the addition of a small amount of hydrated lime. The resulting calcium oxalate is purified separately in a manner similar to the process described for citric acid recovery.

Approximately one part of hydrated lime for every two parts of liquor is added slowly over a 1-h period while the temperature is raised to about 95°C. The precipitated calcium citrate is filtered on a vacuum filter, and the filtrate, free of citrate, is discarded as waste. The calcium citrate cake is moved to acidulation tanks, where it is acidified with dilute sulfuric acid. It is then filtered, and the citric acid mother liquor is decolorized by a charcoal treatment. The purified liquor is concentrated in a vacuum evaporator, run into a crystallizer where, upon cooling, citric acid crystallizes, generally in the form of the monohydrate. The resulting acid is of USP grade.

The extraction method treats the filtered fermentation liquor with a highly selective solvent, tri-*n*-butyl phosphate in kerosene, and then recovers free citric acid by

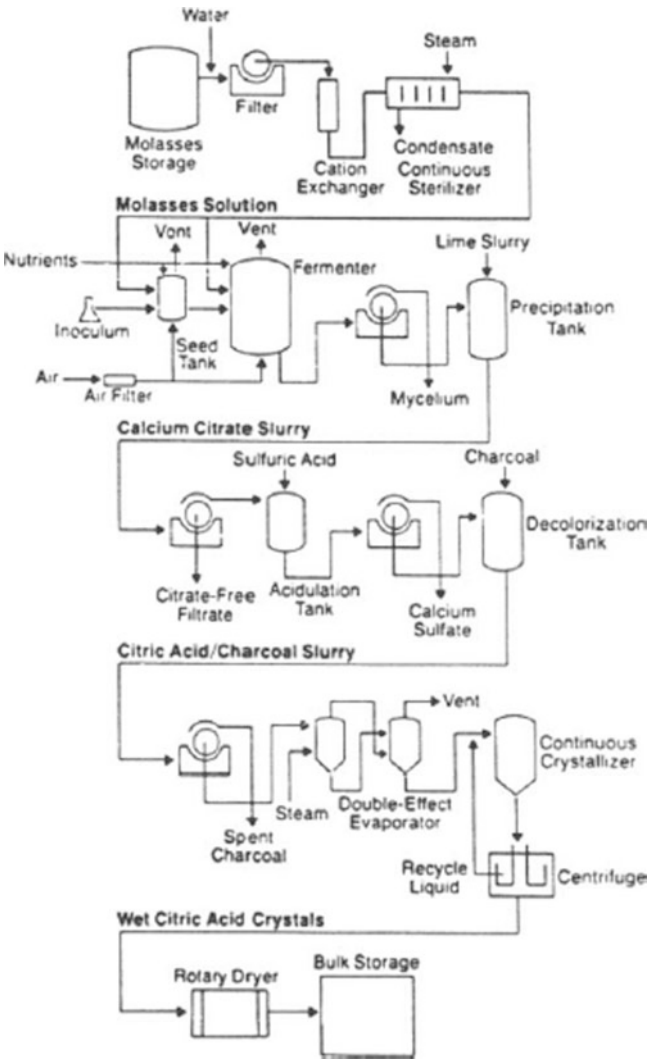


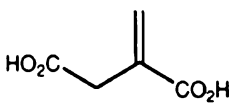
Fig. 30.16 Citric acid flow sheet

counterextraction with water. The aqueous solution, which is further concentrated and crystallized, yields 92% citric acid with 8% soluble impurities.

In the last two decades, the citric acid industry has seen some major changes in ownership and expansions. The worldwide production stands at about one million ton a year. The major producers include the Swiss-based Jungbunzlauer, La Citique Belge/Roche in Belgium, Bharat Starch Industries in India, and ADM, Cargill, and Tate & Lyle in the United States.

The list price of citric acid (anhydrous) has been in the range of 60–90 cents/lb, USP, 100-lb bags.

Itaconic acid



Itaconic acid (methylene succinic acid) is an unsaturated dibasic acid. It is a structurally substituted methacrylic acid, and therefore primarily used as a copolymer in acrylic or methacrylic resins. Because pure acrylic fibers are dye-resistant, it is necessary to include other components to make the fibers susceptible to dyes. An acrylic resin containing 5% itaconic acid offers superior properties in taking and holding printing inks and in bonding. In addition to its main application as a component of acrylic fibers, itaconic acid is used in detergents, food ingredients, and food shortenings.

Previously, itaconic acid was isolated from pyrolytic products of citric acid or produced by converting aconitic acid present in sugarcane juice. It is now produced on a commercial basis predominantly by direct fermentation of sugars. The biosynthesis of itaconic acid follows the metabolic sequence shown in Fig. 30.17.

Although both *Aspergillus itaconicus* and *Aspergillus terreus* are known producers of itaconic acid, the latter is superior and is used industrially with either surface (shallow-pan) or submerged (deep-tank) fermentation [35]. The medium contains a sugar source, corn steep liquor, ammonium sulfate, and mineral salts of calcium, zinc, magnesium, and copper. The fermentation, similar to that of citric acid, is very sensitive to concentrations of copper and iron. The fermentation is carried out at 39–42°C, pH of 2.0–4.0, and under vigorous agitation. Moderate, but continuous, aeration is critical. The batch cycle time is 3–6 days. The highest known product concentration is 180–200 g/L from a medium containing 30% sugar. Therefore, the yield of itaconic acid on sugar is typically 50–70%. The itaconic acid recovery scheme involves the following:

1. Acidification of itaconic precipitates, if present
2. Filtration to remove mycelium and other suspended solids
3. Activated carbon treatment (not necessary for industrial grade product)
4. Filtration to remove carbon
5. Evaporation and crystallization

If a high-purity acid is desired, further purification steps such as solvent extraction, ion exchange, and carbon decolorization are used.

The price of itaconic acid in 2002 was about $2/lb, and the world production is estimated to be about 20,000 t/year. The major producers include Cargill (using Pfizer technology) in the United States, San Yuan in Taiwan, Rhodia in France, and Merck in Germany.

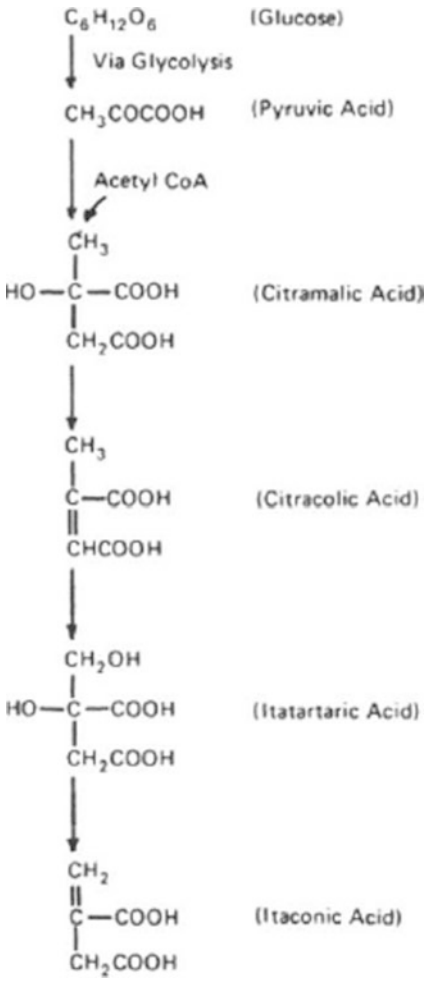
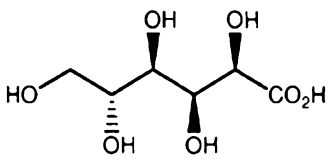
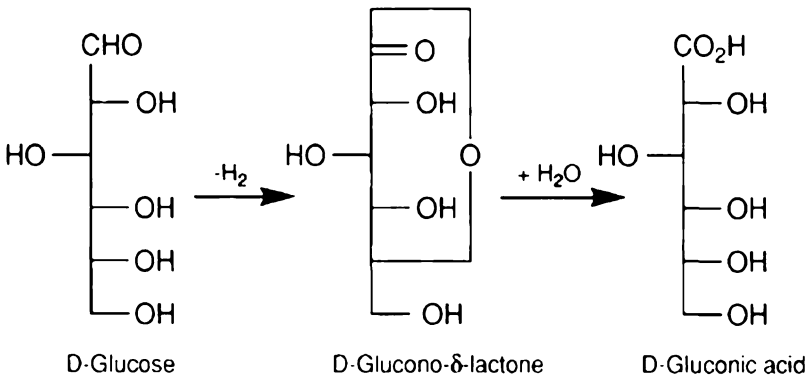


Fig. 30.17 Proposed metabolic sequence for biosynthesis of itaconic acid

Gluconic acid. Gluconic acid is produced by the oxidation of the aldehyde group of glucose to a carboxylic acid.





Gluconic acid may be prepared from glucose by oxidation with a hypochlorite solution, by electrolysis of a solution of sugar containing a measured amount of bromine, or by fermentation/enzymatic conversion (glucose oxidase, catalase) of glucose by fungi or bacteria (U.S. Patent 5,897,995). For economic reasons, the biological methods are now preferred.

Gluconic acid is marketed in the form of a 50% aqueous solution, calcium gluconate, sodium gluconate, and glucono-δ-lactone. Gluconic acid finds use in metal pickling, as an acidulant in foods, as a protein coagulant in tofu (soybean curd) manufacture, as a calcium sequestrant in detergent formulations, in the pharmaceutical area in mineral (calcium and iron) supplements, and as a cement viscosity modifier in the construction area. Calcium gluconate is widely used for oral and intravenous therapy. Sodium gluconate, a sequestering agent in neutral or alkaline solutions, finds use in the cleansing of glassware. Glucono-δ-lactone is used as a food flavor and an acidulant in making cheese and tofu, baking powders, and effervescent products.

Commonly used organisms for gluconic acid fermentation are *A. niger* and *Gluconobacter suboxydans*. The larger volume production uses the fungal process. Most of the gluconic acid produced from the *Gluconobacter* process is marketed as glucono-δ-lactone.

During gluconic acid fermentation, glucose is first oxidized (or, more correctly, dehydrogenated) to glucono-δ-lactone. This is carried out by glucose oxidase. Hydrogen peroxide is also produced in this step, but is decomposed by catalase. The fermentation can be by either surface or submerged culture, the latter more generally practiced in industry. Horizontally rotating fermentors have also been used. Calcium gluconate fermentation, in which calcium carbonate is used for neutralization of the product, is limited to an initial glucose concentration of approximately 15% because of the low solubility of calcium gluconate in water (4% at 30°C). Neutralization by sodium hydroxide instead allows the use of up to 35% glucose in the medium.

The recovery of calcium gluconate from fermentation broth involves the following:

1. Filtration to remove mycelium and other suspended solids
2. Carbon treatment for decolorization
3. Filtration to remove carbon
4. Evaporation to obtain a 15–20% calcium gluconate solution
5. Crystallization at a temperature just above 0°C

In sodium gluconate fermentation, the medium also contains corn steep liquor, urea, magnesium sulfate, and some phosphates. The pH is controlled above 6.5 by addition of sodium hydroxide. One to 1.5 volumes of air per volume of solution per minute (vvm) are supplied for efficient oxygenation and a high back-pressure, up to 30 psig, is desirable. The fermentation cycle is 2–3 days. Continuous fermentation is used in Japan to convert a 35% glucose solution to sodium gluconate with a yield higher than 95%. The continuous process doubles the productivity of the usual batch system.

Sodium gluconate can be recovered from the fermentation filtrate by concentrating it to 42–45% solids, adjusting the pH to 7.5 with sodium hydroxide, followed by drum-drying. In glucono-δ-lactone fermentation, *G. suboxydans* converts a 10% glucose solution to glucono-δ-lactone and free gluconic acid in about 3 days. Approximately 40% of the gluconic acid is in the form of glucono-δ-lactone. Aqueous solutions of gluconic acid are in equilibrium with glucono-δ-lactone. Crystals that separate out of a supersaturated solution below 30°C are predominantly of free gluconic acid; from 30 to 70°C, the crystals are principally glucono-δ-lactone; and above 70°C, they are mainly of γ-lactone.

Sodium gluconate sells for 30–70 cents/lb, depending on the liquid or dry form. Dry sodium gluconate is the main form of gluconic acid/gluconate consumed in the United States. The world market of gluconic acids and salts is about 60,000 t/year. The major producers include Glucona

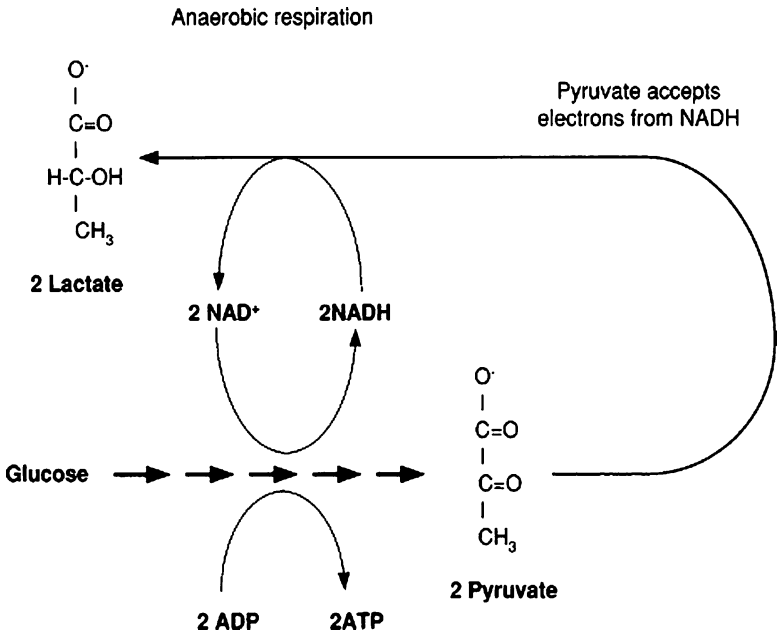
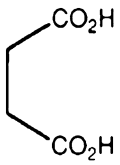


Fig. 30.18 Lactic acid from glucose fermentation

America (a subsidiary of Aveba B.A.) and PMP Fermentation Products (acquired by Fujisawa) in the United States; Benckister, Jungbunzlauer, and Roquette Freres in Europe; and Fujisawa and Kyowa Hakko in Japan. An isolated enzyme system (glucose oxidase-catalase) has recently become cost-competitive with the fermentation process and is therefore likely to expand its use.

Succinic acid

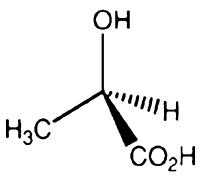


Succinic acid, as an intermediate in the chemical synthesis of 1,4-butanediol, tetrahydrofuran, and adipic acid, has a very large market potential [36]. It is a common intermediate in the metabolic pathway of several anaerobic microorganisms including *Anaerobiospirillum succiniproducens* and *Actinobacillus succinogenes*. However, succinate is produced by mixed-acid fermentations in low yields and concentrations along with several by-products. As the sole product of fermentation, it is possible to produce succinic acid at titers >100 g/L and yield of 1.2 moles per mole of glucose. In such a process, organisms use the phosphoenolpyruvate (PEP) carboxykinase pathway to make succinic acid. Other key enzymes include PEP carboxylase, malate dehydrogenase, fumarase, and fumarate dehydrogenase. Carbon dioxide concentration has been shown to regulate the levels of these enzymes for production of succinate. Carbon dioxide functions as an electron acceptor and modulates the flux of PEP.

An *E. coli* strain has also been engineered by overexpressing PEP carboxylase. Metabolic engineering of the strain was also performed in which genes encoding pyruvate:formate lyase and lactate dehydrogenase were inactivated. PEP flux was also increased through inactivation of the glucose-specific phosphotransferase system (PTS) system. Ideally, for minimizing salt formation in an acid fermentation, the pH should be lower than the pK_a. Otherwise, the conversion of the salt to the free acid adds significant costs in the final purification.

According to http://www.nrel.gov/docs/fy04osti/35523.pdf, there is a significant market opportunity for biobased C4 building block diacids. However, in order to be competitive with petrochemical-derived products, the fermentation cost needs to be below $0.25/lb. Although much progress has been made in the engineering of organisms for succinic acid production, at this point it remains commercially challenging.

Lactic acid



Lactic acid has been used as a food preservative and food-flavoring compound. Recent attention on lactic acid has been for its use in making polylactic acid (PLA), a biodegradable polymer. As a result, the market for lactic acid is rapidly growing. Under batch fermentation conditions, homolactic fermentative Lactic Acid Bacteria (LAB) make lactic acid as

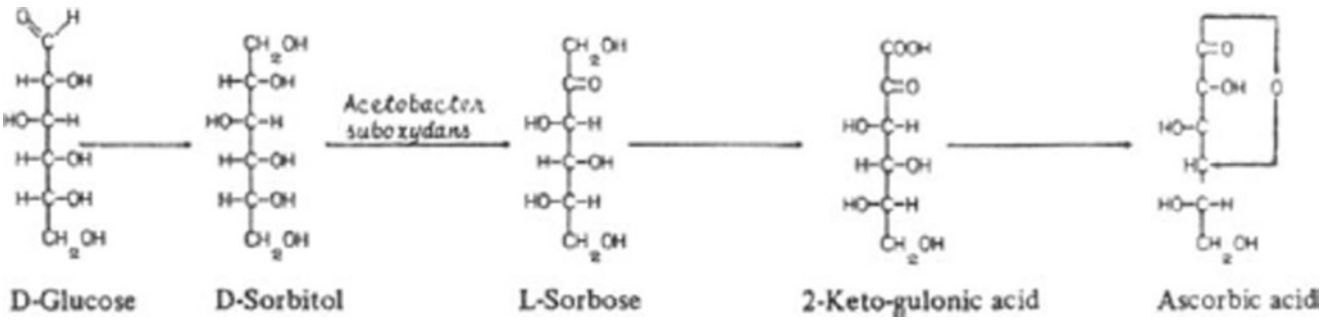


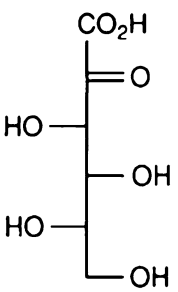
Fig. 30.19 Reactions leading to vitamin C from glucose

their endproduct. As shown in Fig. 30.18, 2 moles of lactate and 2 moles of ATP are formed per mole of glucose used. Although free lactic acid is preferred for most industrial processes, the anaerobic fermentation operates optimally at pH 5 where the salt of the acid is formed. To obtain lactic acid in its free form, the fermentation process must be carried out well below its pK_a of 3.87. This challenge has been met by cloning the bovine LDH gene into yeast, which has resulted in 1.19 mole lactate production per mole of glucose during fermentation at low pH. The fermentor titer reached is >100 g/L with a productivity of 0.8 g/L/h. A strain of *S. cerevisiae* overexpressing the lactate–proton symporter *JEN1* resulted in increased titer and yield.

Recovery of lactic acid is complicated by the high solubility of its salt. The traditional method of recovering calcium lactate is being replaced by membrane, electrodialysis, or ion-exchange purification methods. Producers such as Purac, Cargill, ADM, and Jiangxi sell lactic acid for $0.50–3.00 per kg depending on whether the product is industrial, feed, food, or pharma grade.

NatureWorks® LLC has set up a $300 million plant at Blair, NE, which is capable of producing about 140,000 t/year of polylactide polymers from corn sugar. It employs a fermentation process to produce two chiral isomers of lactic acid from glucose, which are then cracked to form three lactide isomers. The isomers are subsequently polymerized to polylactide.

2-Keto-L-gulonic acid



2-Keto-L-Gulonic acid (2-KLG) is the precursor for Vitamin C (L-Ascorbic acid). Vitamin C is used on a large scale as an antioxidant in food, animal feed, beverages, pharmaceutical formulations, and cosmetic applications. About one half of the vitamin C is used in vitamin supplements and multivitamin preparations, one quarter in food additives, 15% in beverages, and 10% in animal feed. When ascorbic acid is used in cosmetics, it is phosphorylated to prevent its oxidation.

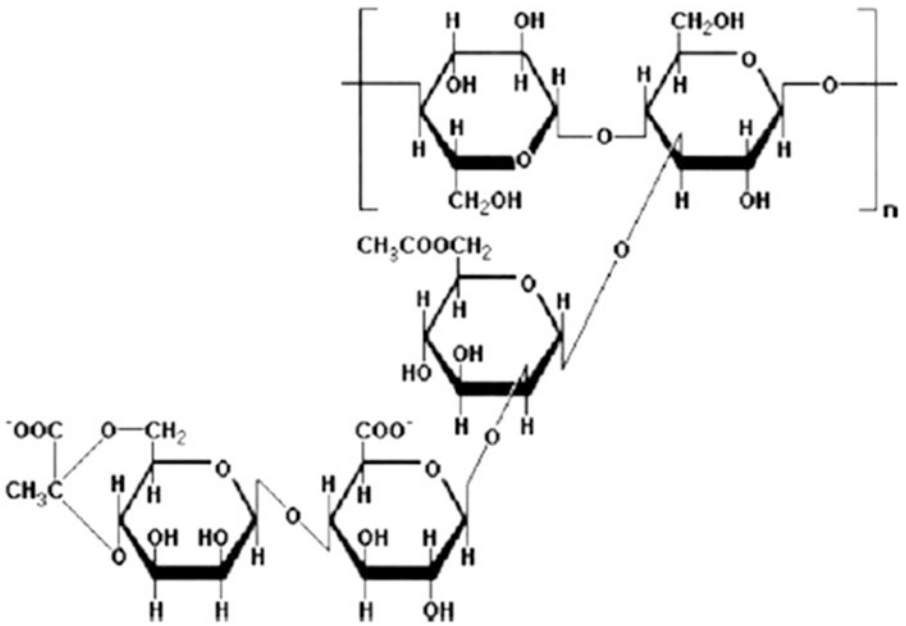
Figure 30.19 shows the reactions involved in the combined microbiological and chemical conversions of glucose, via sorbitol, to vitamin C, called the Reichstein and Grussner process. Sorbitol is made by catalytic hydrogenation of glucose and L-Sorbose is produced from sorbitol by the action of several species of bacteria, the most commonly used being *Acetobacter suboxydans*. Because this organism is very sensitive to nickel ions, it is important that the medium and fermentor be free of nickel. The medium normally consists of 100–200 g/L sorbitol, 2.5 g/L cornsteep liquor, and an antifoam agent such as soybean oil. The medium is sterilized and cooled to 30–35°C, about 2.5% inoculum is added, and the tank is aerated and sometimes stirred. This is the only step based on fermentation, and the step yield is 80–90% in 20–30 h. The chemical steps in the conversion of sorbose to ascorbic acid involve the preparation of the diacetone derivative, which is then oxidized, the acetone groups are removed, and the resultant 2-KLG is isomerized to the enediol with ring closure. It is believed that this chemical process currently operates at an overall yield of about 50% vitamin C on glucose.

Groups all around the world have pursued alternative vitamin C production technologies. A one-step fermentation process starting from glucose to produce 2-keto-L-gluconic acid was reported to be practiced in China, a microalgae process was developed in the United States and a two-stage fermentation process via the intermediate 2,5-diketo-D-gluconic acid (2,5-DKG) was developed in Japan. Genentech [37] succeeded in cloning the 2,5-DKG reductase gene from a *Corynebacter* sp. and expressing it in an *Erwina* sp. that naturally produces 2,5-DKG via the fermentative oxidation of glucose. The recombinant *Erwina* culture is thus capable of carrying out the complex oxidation and reduction steps to form 2-KLG in a single fermentation.

Genencor International, in partnership with Eastman Chemical Co. has developed such a single fermentation process using genetically engineered *Pantoea citrea*. To maximize the product yield on glucose, detailed analysis of the steps in the formation of 2-KLG was carried out. In the periplasm of this organism, glucose is first converted to gluconic acid by a membrane-bound PQQ-dependent glucose dehydrogenase. This is followed by the oxidation of gluconic acid to 2-keto-D-gluconate (2-KDG) by a cytochrome C coupled enzyme, gluconate dehydrogenase. In a third reaction, 2-KDG is further oxidized to 2,5-DKG by 2KDG dehydrogenase, another cytochrome C coupled enzyme. Purification, characterization, and determination of the enzyme structure of 2,5-DKG reductase have added significantly to the understanding and development of this production process.

The major manufacturers include DSM, BASF, and four Chinese producers, viz. Northeast Pharmaceutical, North China Pharmaceutical, Shijiazhong Pharmaceutical, and Jiangshan Pharmaceutical. The current world supply of ascorbic acid is more than 80,000 metric tons per year with annual revenues in excess of US $700 million.

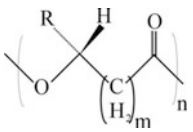
Xanthan gum



Xanthan gum is an anionic polymer of β-(1,4)-D-glucopyranose glucan with side chains of 1,3-α-D-mannopyranose and (1,2)-β-D-glucuronic acid residues [38]. It is produced naturally by bacteria to enable them to stick to plants. The negatively charged carboxyl groups on the side chains generate viscosity when the xanthan gum is mixed with water. The glucan backbone is protected by the side chains, thus making it relatively stable to acids, alkalis, and cellulase enzymes. Xanthan gum is used as a thickener, stabilizer, emulsifier, and foaming agent in products such as salad dressings, cosmetics, pharmaceuticals, paints, lubricants, and ice cream. Its key property is high viscosity at low shear and thinning character at high shear. Xanthan gum is not affected by ionic strength over a wide pH, shear or temperature range.

Xanthan gum is produced commercially via aerobic submerged fermentation of sugar by *Xanthomonas campestris*. Different strains or fermentation conditions produce differing degrees of acetylation, pyruvylation, and hence functionalities. The production strain is grown in medium containing carbohydrate, nitrogen, and salts. The production process of xanthan gum is energy intensive and costly, and product titer and productivity are relatively low due to high viscosity. In addition, the broth is difficult to filter due to cells in the broth. After fermentation, the broth is sterilized to inactivate cells; the product is precipitated or coagulated, separated by centrifuges, dried, and milled. About 20,000 t of xanthan are produced each year with a market value more than US $160 million.

Poly(3-hydroxyalkanoates)



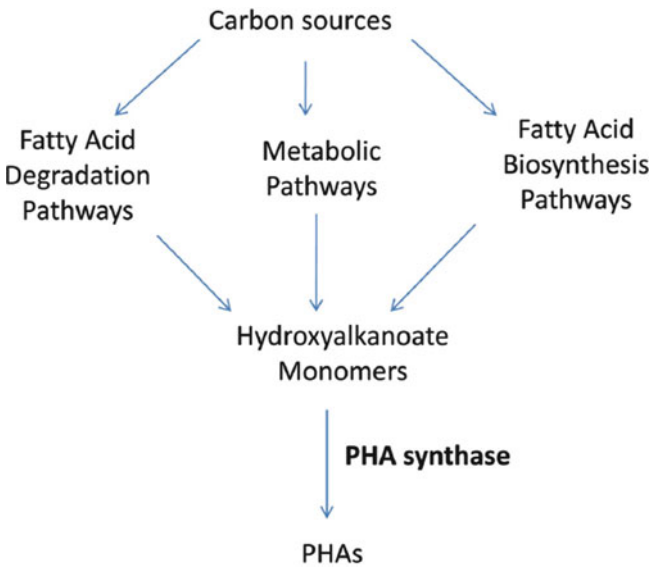


Fig. 30.20 Pathway for synthesis of poly(3-hydroxyalkanoates) using a variety of substrates

Among a wide variety of biobased polymers [39], poly(3-hydroxyalkanoates) (PHAs) also known as microbially produced polyesters are diverse in terms of constituents and biosynthetic pathways, and therefore desired as starting materials for a variety of conventional and thermo-plastics applications. Some microorganisms produce polyesters naturally, e.g., Ralstonia eutropha formally known as *Alcaligenes eutrophus*, a gram negative bacteria that produces PHAs as inclusion bodies or granules in the cytoplasm and serve as storage of carbon and energy. Natural organisms accumulate PHAs under excess carbon or some nutrient limitation, and use them later under carbon starvation. But engineered micro-organisms can accumulate up to 80–95% of the cell's dry weight, and large scale fermentors can grow >100 g/L dry cell weight. PHA is the most common polyester made in nature, and poly-3-hydroxybutyrate (PHB) was the first to be discovered. Other PHAs have since been discovered, including those containing hydroxyvalerate units, as in poly-3-hydroxyvalerate (PHV). Because the structure and monomeric composition of PHAs determine the applications for each type of polymer, a variety of polymers have been synthesized by co-feeding of various substrates or by metabolic engineering of the production organism (see Fig. 30.20).

Production organisms are fed carbon sources like glucose, sucrose, oils, propionic acid, etc. After fermentation, the cells are lysed, the cell debris is separated, and the PHA is extracted with a solvent, precipitated from water as powder, purified, and converted to pellets. Production of PHA could take up to 5 kg of raw material per kg product, and genetic improvements in microorganisms are necessary for higher PHA yield, use of cheaper feedstocks, and lower capital.

PHA commercial production has been considered by several companies like W. R. Grace, ICI, DSM, Metabolix, Monsanto, Proctor & Gamble (P&G), Kaneka, Meridian, Tianan, Tianjin, and others. According to P&G/Kaneka, PHA/PHB (Nodax^{TM}) production cost has to be <1.5 USD per kg for high volume applications, and alkaline digestibility and flushability are convenience factors of interest to the production of single-use consumer goods.

Alcohols

The organic chemicals that fall into this category and can be produced by fermentation include ethanol, 1,3-propanediol, butanol, acetone, 2,3-butanediol, and glycerol. Butanol and acetone have been produced industrially by fermentation, but currently for economic reasons, chemical synthesis is the manufacturing method of choice. However, as price and availability of ethylene and propylene as feedstocks for the synthetic processes become subjects of concern, there is a renewed interest in examining the fermentation processes. As the cost of crude oil continues to go up while the price of renewable resource such as agricultural crops remains relatively stable, there has been an increasing interest in producing chemical feedstocks from renewable resources by biological means.

1,3-Propanediol

1,3-Propanediol is a monomer with potential utility in the production of polyester fibers and in the manufacture of polyurethanes and cyclic compounds. A variety of chemical routes to 1,3-propanediol are known. For example, (1) ethylene oxide may be converted to 1,3-propanediol over a catalyst in the presence of phosphine, water, carbon monoxide, hydrogen, and an acid; (2) a catalytic solution phase hydration of acrolein followed by reduction; or (3) from glycerol, reacted in the presence of carbon monoxide and hydrogen over catalysts having atoms from group VIII of the periodic table. Although it is possible to generate 1,3-propanediol by these methods, they are capital intensive and/or generate waste streams that contain environmental pollutants.

The biological production of 1,3-propanediol from glycerol has been known for a number of years [11]. The use of natural organisms (e.g., enteric bacteria such as *K. pneumoniae*, *Citrobacter freundi*, and Clostridia, such as *C. butyricum*) to produce 1,3-propanediol from glycerol has been well studied. Continued optimization of the basic anaerobic glycerol fermentation process has produced promising results. A number of batch or fed-batch

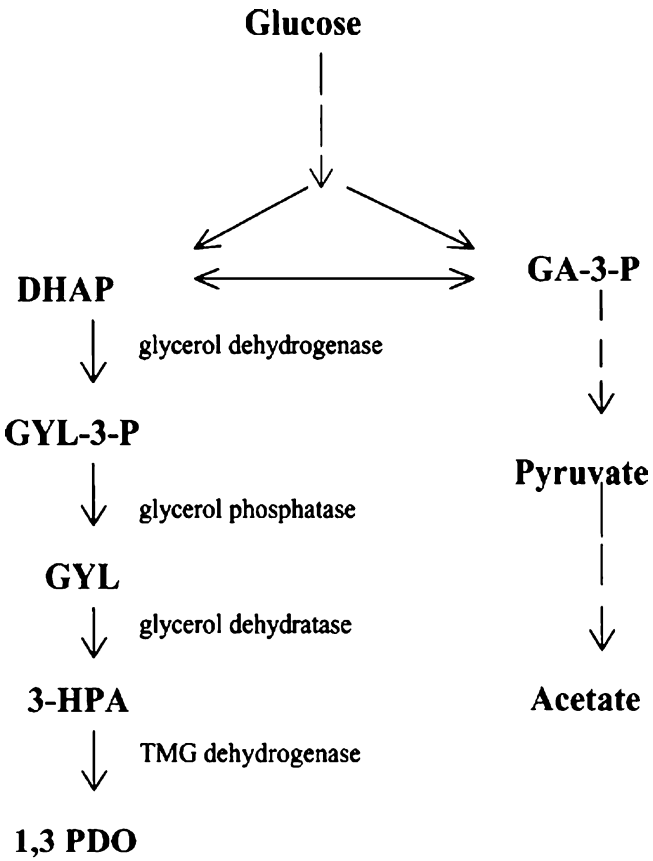


Fig. 30.21 1,3-PDO pathway from glucose

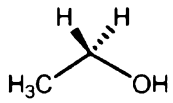
fermentations using *K. pneumoniae* or *C. butyricum* have produced titers of 50–75 g/L and a yield of 0.44–0.69 mol/mol 1,3-propanediol from glycerol. However, the complete conversion of glycerol to 1,3-propanediol is not possible due to the requirement of an additional reducing power in the form of NAD(P)H.

Neither the chemical nor the biological methods described earlier for the production of 1,3-propanediol are well suited for industrial scale. The chemical processes are energy intensive and the biological processes require the expensive starting material, glycerol. A more desirable process would be to develop a microorganism that would have the ability to convert basic carbon sources such as carbohydrates or sugars to the desired 1,3-propanediol. Such a single organism approach developed by Genencor International and DuPont overcomes this problem by generating reducing cofactors from the glucose conversion to CO_2. The ability to control both carbon and energy flow in the single organism allows for the more efficient use of the input carbon source.

As shown in Fig. 30.21, the conversion of glucose to 1,3-propanediol requires the combination of two natural pathways: glucose to glycerol and glycerol to 1,3-propanediol. The two enzymes involved in the conversion of glycerol to 1,3-propanediol have been cloned and characterized from several organisms, viz. *Klebsiella*, *Citrobacter*, and *Clostridium*. The first enzyme in the pathway is glycerol dehydratase. The dehydratase has been shown to undergo catalytic inactivation and requires the addition of a reactivation complex of two additional proteins. The second enzyme in the pathway is a NAD-linked tri-methylene glycol (TMG) dehydrogenase. Both of these enzymes have been cloned and expressed in *E. coli*. The pathways for the production of glycerol are found in the yeast, *S. cerevisiae* that produces glycerol from the glycolytic intermediate dihydroxyacetone-3-phosphate using two enzymes: dihydroxyacetone-3-phosphate dehydrogenase and glycerol-3-phosphate phosphatase. To construct a single organism to produce 1,3-propanediol from glucose, one could clone the glycerol pathway into a natural 1,3-propanediol producer, or the 1,3-propanediol pathway into a natural glycerol producer. Although either of these approaches seems simple and direct, there are problems involving natural regulation of the pathways. However, building both pathways into *E. coli* is advantageous as it is the most completely studied organism. It provides a rich set of genetic tools (sequenced genome, vectors, promoters, well-characterized metabolism, and physiology) and a large number of metabolic mutants that have been constructed and analyzed [40]. Moreover, *E. coli* has been used in large-scale fermentation for production of industrial and healthcare products, does not naturally overproduce glycerol or 1,3-propanediol, and thus has a strong likelihood of no natural regulation to overcome. Modifications of more than 70 *E. coli* genes were analyzed and the final production organism had 18 genes modified. Some of these genes were regulatory elements that indirectly affected the function of a few hundred other genes. The optimized strain of *E. coli* produced 135 g/L of 1,3-propanediol, at 3.5 g/L/h, and 51% weight yield on glucose.

The joint venture between DuPont and Tate & Lyle has demonstrated the large-scale feasibility to produce 1,3-propanediol by fermentation (bio-PDO™). The process uses the genetically engineered organism developed jointly by former Genencor and DuPont. The joint venture has announced that in 2006 they will commercially produce bio-PDO™ from corn to replace petrochemicals in the production of their polymer Sorona®, for many end-use applications, including fibers. The bio-PDO™ will be produced at a new plant in Loudon, TN, USA. The bioprocess for making 1,3-propanediol is claimed to require 40% less capital and has a 25% lower operating cost than the chemical process for making the same product. Because of a different impurity profile, the polymer made from the biological-based 1,3-propanediol has improved properties and actually creates a superior product compared to the chemically based 1,3-propanediol (http://www.dupont.com/sorona/faqs.html).

Ethanol



Ethanol is a versatile chemical being used in industrial solvents, thinners, detergents, toiletries, cosmetics, and pharmaceuticals. It is also used as an intermediate for manufacturing other chemicals such as glycol ethers, ethyl chloride, amines, ethyl acetate, vinegar, and acetaldehyde. With the ever-increasing price and dwindling supply of crude oil, ethanol fermented from grains and other renewable organic resources is becoming an economical fuel supplement. The U.S. fermentation ethanol capacity is now about four billion gallons a year, a result of efforts by the ethanol industry to expand its use in fuels, as a replacement for the oxygenate, methyl *tert*-butyl ether.

Ethanol is produced by fermentation from sugar-containing materials such as grain products, fruits, molasses, whey, and sulfite waste liquor. Yeast, particularly strains of *S. cerevisiae*, is almost exclusively used in industrial ethanol fermentation. It tolerates ethanol concentrations up to about 20% (by volume), and under anaerobic conditions converts over 85% of the available carbohydrates to ethanol and carbon dioxide. Air or oxygen suppresses the formation of ethanol from sugar (the Pasteur effect). Under aerobic conditions, a major portion of the carbohydrates goes towards cell growth. Ethanol is formed via glycolysis (the Embden–Meyerhof–Parnas pathway). The overall reaction starting from glucose can be written as follows:

$$C_6H_{12}O_6 \rightarrow 2C_2H_5OH + 2CO_2 + 18\ \text{kcal}$$

The ethanol yield on glucose is thus 51% by weight. Because the carbohydrate is also used for cell growth and respiration, the overall yield of ethanol from total carbohydrate consumed is about 42–46 wt.% on a glucose equivalent basis.

Ethanol fermentation can be conducted on any carbohydrate-rich substrate. Molasses, which is the waste mother liquor that remains after the crystallization of sucrose in sugar mill operations, is widely used. Blackstrap molasses contains 35–40% sucrose and 15–20% invert sugars (glucose and fructose). High-test molasses contains 22–27% sucrose and 50–55% invert sugars. Most of the blackstrap molasses do not require the addition of other nutrients for ethanol fermentation. However, high-test molasses requires ammonium sulfate and other salts such as phosphates. In the molasses process, blackstrap or high-test molasses is charged into a mixing tank and diluted with warm water to give a sugar concentration of 15–20%. The pH is adjusted to 4–5, the mash pasteurized, cooled, and charged into fermentor tanks, and 1–5% yeast inoculum is added. The fermentation is carried out nonaseptically at 23–32°C. Antibiotics may be added to control possible bacterial contaminations. Because the overall reaction is exothermic, cooling is necessary. The fermentation takes 28–72 h to produce an ethanol concentration of 8–10%. Carbon dioxide normally is vented, but if it is to be recovered, then the vent gas is scrubbed with water to remove entrained ethanol and then purified using activated carbon. The bubbling due to carbon dioxide generation by fermentation could provide sufficient agitation for small tanks. Mechanical agitation may be necessary for large fermentors. The fermentation may be conducted batchwise or continuously, with or without recycling yeast. Although continuous fermentation and/or cell recycle can significantly improve productivity and thus reduce required capital investment, it may have only a limited impact on lowering product costs in as much as a significant portion of the total cost comes from raw materials.

Ethanol can also be produced by fermentation of starch, whey, sulfite waste liquor, cellulose, and hemicellulose. Potato or tapioca can also be converted to ethanol. Grain fermentations [41] require additional pretreatment because yeast cannot metabolize starch directly. The grain (corn, sorghum, barley, wheat, rice, or rye) is ground and heated in an aqueous slurry to gelatinize or solubilize the starch. Starch-liquefying enzymes (thermostable alpha amylase) are added in this step. The liquefied starch is then cooled to 60–65°C. Yeast is then added to carry out fermentation simultaneously with saccharification by amyloglucosidase (or glucoamylase). The subsequent fermentation and refining procedures are the same as those using molasses as raw materials.

The grain-based ethanol process, also called dry milling was developed and optimized for the beverage industry utilizing starch-based feedstock and energy-intensive processes. Such processes tend to produce some nonfermentable sugars during cooking of starch. Salts added for the needed pH adjustments of the enzymatic treatments. Both of these result in extra process costs. A granular starch hydrolyzing enzyme (GSHE) technology is likely to replace the traditional energy intensive process of liquefaction/saccharification (http://www.biorefineryworkshop.com/presentations/Dean.pdf). The GSHE is a new enzyme product to reduce energy usage for the production of inexpensive glucose from granular starch, which replaces the energy-intensive liquefaction/saccharification. The ethanol industry needs continued energy, cost, and conversion efficiency improvements. An ethanol production process that has the following key features is much desired.

- Energy savings—elimination of jet cooking
- Capacity increase—high solids and increased ethanol yield

- Reduction of yeast growth inhibitors—high glucose concentration, Maillard reaction products, and so on.
- Operational cost reduction—labor, time, and chemicals
- Value-added co-product, Dried Distiller's Grains and Solubles—higher protein content
- Process simplification—fewer unit operations

GSHE-like novel enzyme-processing technologies with reduced total operating cost will provide a dramatic acceleration in the timelines for the establishment of the biobased economy. This developing technology will also have a major positive impact on the life cycle costs of biobased products and address the growing concerns among policy makers and environmentalists.

The GSHE is a blend of enzymes with synergistic activity on granular starch. The key enzymes of the GSHE blend include acid stable alpha amylases and glucoamylases that "drill" holes in the starch granules. Thus the GSHE is capable of hydrolyzing insoluble granular (uncooked) starch into fermentable sugars by enabling depolymerization of starch to glucose without the need for the energy-intensive jet-cooking step, in a Simultaneous Saccharification and Fermentation (SSF) process for alcohol production. Genencor International has achieved high level of expression of these enzymes in industrial production strains resulting in an acceptable cost structure.

Research to improve ethanol fermentation has also focused on the development of a high solids, continuous feeding process as well as improved yeast strains. Other related developments include processes for the use of biomass feedstocks, such as cellulosic waste material [42–46]. The former Genencor and NREL collaboration funded by the U.S. DOE has (a) put in place the tools required for continual improvement of biomass-derived cellulases, (b) built a suite of enzymes with enhanced thermostability and improved specific performance at elevated temperatures, (c) developed an improved production strain for the enhanced cellulase components and demonstrated an enhanced production process. Therefore, plans are in place to provide a developmental product(s) in support of continued industry development.

Developments in genetic engineering have made possible the development of new organisms (yeasts and bacteria) that can survive in higher concentrations of ethanol, tolerate higher sugar concentrations, grow at higher temperatures, and utilize starch or cellulose feedstock directly (http://www.farmfoundation.org/projects/03-18_biofuels.pdf).

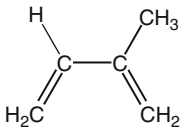
Ingram's [44] pathway engineering program has created a production organism patented by the University of Florida, with improved efficiency of ethanol production from C5 and C6 sugars (xylose, arabinose, galactose, mannose, glucose). Gelunol holds the rights to the patented engineered bacteria and is building a large-scale biomass-to-ethanol plant in Jennings, LA. The plant is expected to be operational in 2007 and will use bagasse waste from sugarcane to produce up to 30 million gallons a year of ethanol. According to the USDA and DOE, production of one billion ton of biomass per year is possible on a sustainable basis. This scale of biomass has the conversion potential of producing 100 billion gallons of ethanol per year.

In order to prevent the diversion of industrial alcohol to potable uses, it is denatured by the addition of some material that renders the treated alcohol unfit for use as a beverage. A fuel mixture such as 10% alcohol and 90% gasoline has become the most important use of fermentation ethanol. Ethanol from grain fermentations has been made competitive as a liquid fuel in the United States because of improvements in technology, especially in the area of efficient enzymes and energy usage in production plants, and various government subsidies designed to reduce the nation's dependence on imported foreign oil. Other countries in Europe as well as Brazil, India, and China also have substantial production of fermentation ethanol for use as fuel. In the United States, ADM, Tate & Lyle, and Cargill are the biggest among a large number of producers (for ethanol recovery, see "Recovery section").

Hydrocarbons

Hydrocarbons that can be produced by fermentation are many, from hydrogen and methane typically produced in anaerobic waste treatment, to ethylene and isoprene produced naturally by a variety of micro- and macro-organisms (plants, trees, animals). Very recent advancement of pathway engineering of isoprene and farnesene production systems has drawn significant attention.

Isoprene



Isoprene is an important commodity chemical used in a wide range of industrial applications ranging from the production of synthetic rubber for tires and coatings to use in adhesives and development of specialty elastomers. Styrene-isoprene-styrene block copolymers form an essential part of most hot melt pressure sensitive adhesive formulations and *cis*-polyisoprene or synthetic rubber is used in tire manufacture. All of the world's isoprene is produced from petroleum-derived feedstocks and is subject to volatility in pricing and supply linked to oil.

A first of its kind biobased process to produce isoprene (BioIsoprene™) from renewable raw materials is in

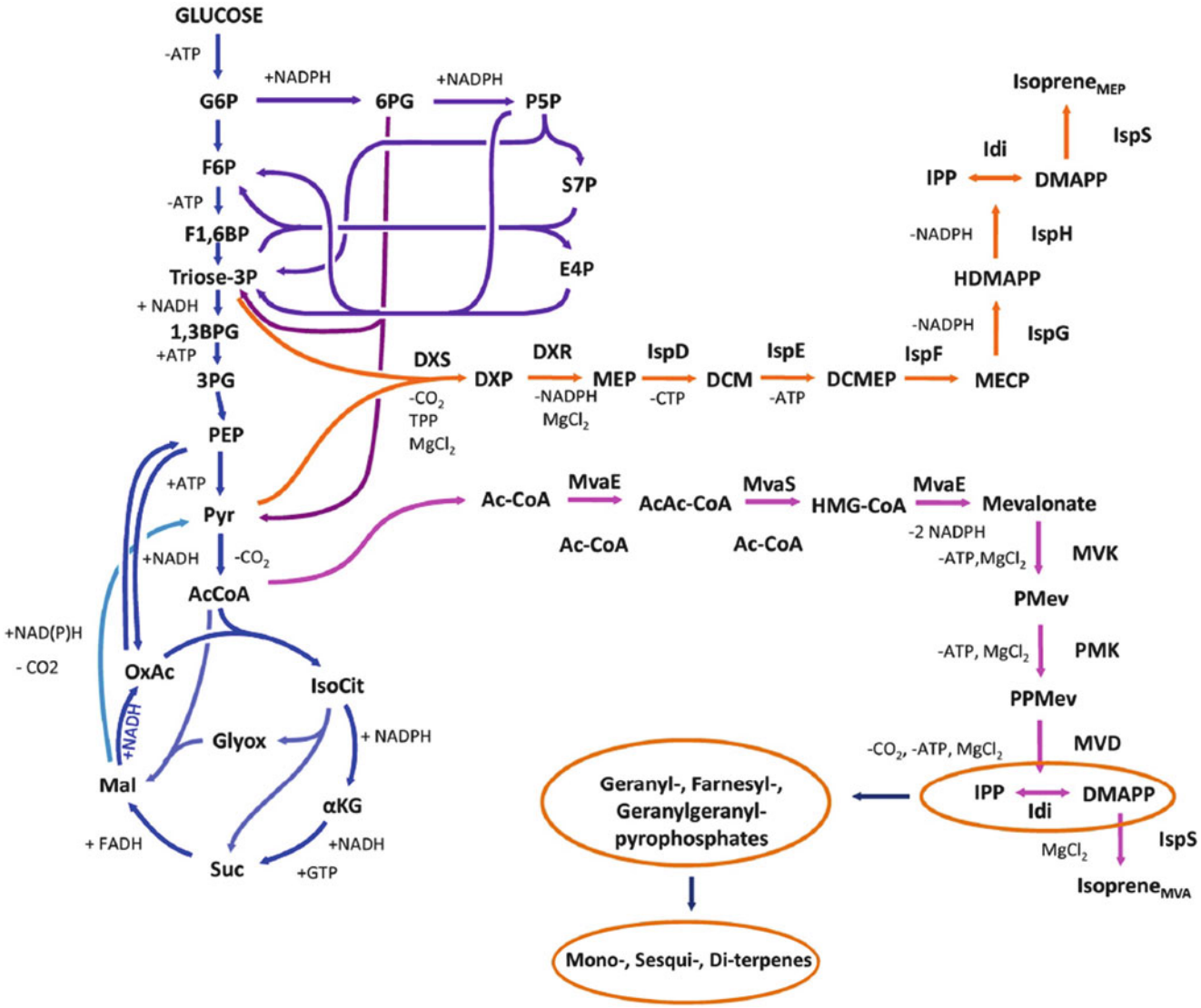


Fig. 30.22 Isoprene production pathways: mevalonic acid (MVA) and methyl erythritol phosphate (MEP)

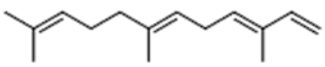
development by former Genencor and The Goodyear Tire & Rubber Company. Production system for BioIsoprene™ is based on microbial fermentation of renewable sugars converted to dimethylallyl pyrophosphate (DMAPP), the key substrate of isoprene synthase, the key plant enzyme engineered into the production organism. DMAPP and its isoform, isoprenyl pyrophosphate (IPP), are essential compounds in all living organisms and are produced via one of two known pathways, the mevalonic acid (MVA) pathway present in eukaryotes and some prokaryotes, and the 5-methyl erythritol phosphate pathway (MEP, also known as deoxyxylulose phosphate [DXP] pathway) present in prokaryotes and plants [47] (Fig. 30.22). Carbon flux into both pathways stems from intermediates derived from sugar metabolism via central metabolism, acetyl-CoA for MVA; pyruvate and glyceraldehyde 3-phosphate for MEP. These pathways are amenable to molecular biology manipulations and metabolic pathway engineering in both prokaryotes (*E. coli*) and eukaryotes (*S. cerevisiae*). Both biosynthetic isoprenoid pathways, MEP and MVA, are being optimized towards this end. The key to successful process is engineering a microbial cell factory that is streamlined in physiology to drive sugar conversion through engineered biosynthetic pathways to produce isoprene at near theoretical yields. Unlike other biobased systems to produce biochemicals, BioIsoprene™ is produced as a gas-phase product and released into the off gas of the fermentor without any noticeable negative physical or inhibitory impacts on the production organism. The product comes out of the fermentor continuously in the gas phase and is recovered from the fermentation off gas in a continuous process. Current state-of-the-art technology has resulted in production, recovery,

polymerization, and manufacture of tires with the isoprene component produced via fermentation. Polymer-grade BioIsoprene™ is recovered from the integrated process. Potential benefits of the gas-phase nature of the product include: (a) reduction and/or elimination of feedback inhibition by the isoprene product on further synthesis; (b) efficient recovery and purification of polymer-grade BioIsoprene™ from the fermentation; and (c) possibility to use crude, low cost feedstocks.

The development of BioIsoprene^{TM} represents a major achievement for industrial biotechnology because it has the potential to enable production of isoprene from renewable raw materials to deliver commercial quantities of a basic C5 hydrocarbon that, in principle, can be used as a feedstock for a large number of value-added products. One of these products is a synthetic *cis*-polyisoprene (synthetic rubber), which for decades has been recognized as a suitable replacement for natural rubber in many applications. Additionally, there is potential for creating polyisoprenes with improved material properties. The BioIsoprene^{TM} process offers a real possibility for obtaining the quantities of low-cost isoprene needed to produce a meaningful large-volume alternative to Hevea natural rubber and petroleum-derived isoprene.

BioIsoprene™ will have broader commercial applications beyond the biochemical uses of isoprene in synthetic rubber, adhesives, and specialty elastomers. As a C5 hydrocarbon, BioIsoprene™ has inherent fuel properties and represents a key biobased intermediate that can be converted to a drop-in transportation fuel additive using chemical catalysis to C10 and C15 biobased hydrocarbon fuels, thus addressing performance gasoline, jet fuel, and biodiesel markets.

Farnesene



Amyris has engineered yeasts that convert sugars into a hydrocarbon molecule called farnesene [48], a sesquiterpene which can be processed into cosmetics, lubricants, and diesel and jet fuel. If hydrogenated, the hydrocarbon can be turned into a diesel fuel that burns cleaner than conventional diesel. Amyris' process uses engineered yeast to convert sugar into beta-farnesene, in oil form, easy to separate from aqueous fermentation broth.

As shown in Isoprene production pathway (Fig. 30.22), farnesene, like many isoprenoids, is assembled using the five-carbon monomer IPP and its isomer DMAPP. IPP and DMAPP could also be condensed by IPP synthases into geranyl pyrophosphate (GPP, C10), farnesyl pyrophosphate (FPP, C15), or geranylgeranyl pyrophosphate (GGPP, C20). Terpene synthases convert these long chain pyrophosphate molecules into branched chain or cyclic alkenes, e.g., farnesene. Isoprenoid tailoring enzymes can then oxidize the alkenes into alcohols or reduce them to alkanes. Alternatively, isoprenyl phosphatases or IPP synthase mutants can generate long-chain alcohols such as farnesol, geraniol, and geranylgeraniol from GPP, FPP, or GGPP, respectively.

While the Amyris industrial synthetic biology platform can use a wide variety of feed stocks, their initial focus is on sugarcane due to its abundance, generally low cost, and favorable carbon footprint. That's why Amyris' initial production efforts are in Brazil, leveraging the infrastructure of existing sugar and ethanol mills. However, their success depends on increasing the sugar conversion efficiency (15–16% kg farnesene per kg sugar reported). Other success factors include productivity (less than 1 g/L/h reported), farnesene concentration in broth (about 100 g/L reported), and large-scale operation (200,000 L size fermentors reported).

Advanced biofuels

Ethanol has become a valuable additive to liquid transportation fuels. During 2010, 13 billion gallons were produced in the United States with most of this being fermented using grains as starting material. Ethanol provides high octane, but the energy density is only about 70% that of traditional gasoline. The hydroscopic property of ethanol also renders it difficult to transport in existing pipelines without resulting in corrosion. For these and other reasons, there has been increased interest in producing fuels besides ethanol from renewable resources [49, 50].

Higher Alcohols

Production of higher alcohols can be further broken out into two key groups: (1) fermentative pathways to isopropanol and *n*-butanol and (2) nonfermentative pathways to a larger diversity of branched chain alcohols.

Fermentative pathways to alcohols are typically based upon the acetone-butanol-ethanol (ABE) fermentation classically found in Clostridia. Acetone can be reduced in a single step to form isopropanol. For economic reasons, native production of these compounds in Clostridia is not currently practiced. But through synthetic biology and metabolic engineering, it has been possible to optimize heterologous pathways to maximize yield and productivity in industrial microorganisms. Considerable work has focused especially on production of butanol especially in *E. coli* and *S. cerevisiae*. Pathways for three different butanol species are shown in Fig. 30.23a. For *n*-butanol, two molecules of acetyl-CoA are condensed to form acetoacetyl-CoA, which then undergoes reduction and dehydration to generate *n*-butanol. 2-butanol is formed through acetolactate by decarboxylation, dehydration, and reduction. Isobutanol is formed by a nonfermentative pathway through 2-keto-isovalerate.

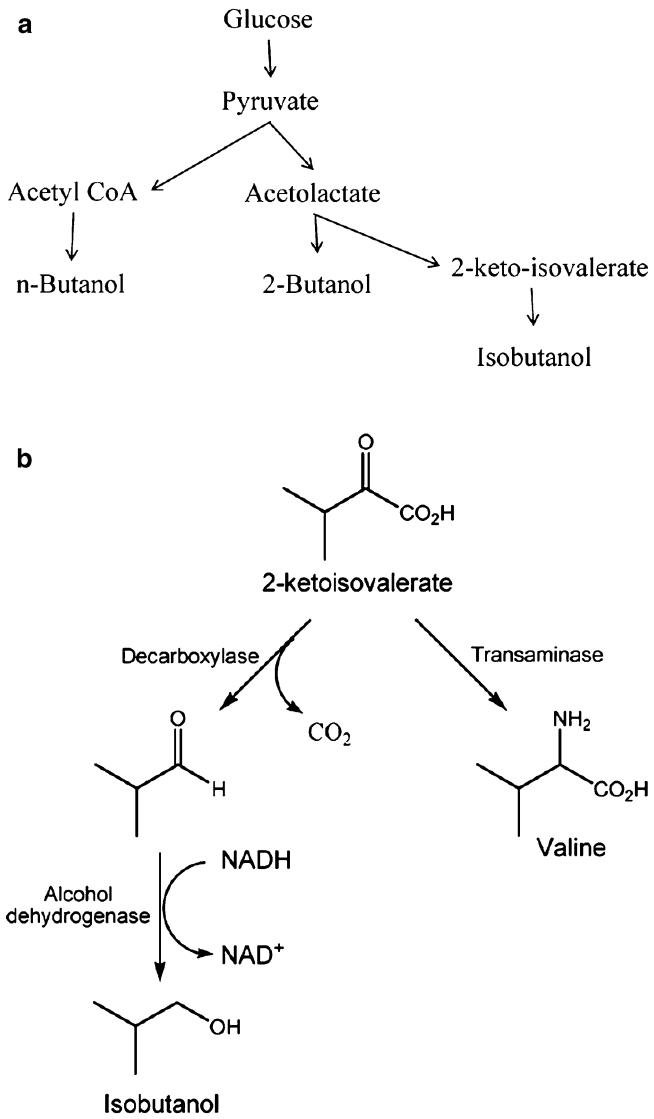


Fig. 30.23 (**a**) Pathways for three different butanol species, (**b**) pathway for the production of isobutanol

Nonfermentative pathways can result in a wider range of higher alcohols. The key steps in all these pathways are the decarboxylation of 2-keto acids and reduction of the resulting aldehyde to the corresponding alcohol. Many amino acid biosynthesis pathways have these 2-keto-acids as intermediates. As an example, 2-keto-isovalerate is the product of the penultimate step in valine biosynthesis. But instead of being transaminated to valine, it can also be decarboxylated and reduced to make isobutanol. This is shown in Fig. 30.23b. There is a wide range of alcohols that can be formed from these naturally occurring 2-keto acids. However, the range of alcohols that can be formed has been increased even further by engineering completely new pathways to make nonnatural 2-keto acids [51]. These then undergo the same decarboxylation and reduction to form alcohols. Once again, enzymes from the branched chain amino acid pathways were used. The leuABCD operon of *E. coli* acts upon 2-keto-isovalerate to add a carbon leading to 2-keto-isocaproate and then to leucine. Two enzymes were protein engineered to allow conversion of nonnative substrates. Making changes to leuA and 2-keto-isovalerate decarboxylase resulted in the formation of a large number of nonnative alcohols with carbon range of C5–C8. This work indicates that the types of molecules that can be produced are not limited by what is observed by native biosynthetic reactions.

Biodiesel Production

Biodiesel production from plant oils has been practiced. Natural triacylglycerols in oils undergo transesterification with methanol or ethanol to make fatty acid methyl and ethyl esters (FAME and FAEE). Think of modified diesel vehicles whose exhaust smells like French fries. However, the availability of plant oils limits the potential use as fuels. And the transesterification reaction can be costly. Acyltransferases can be used to perform the reaction in vivo by feeding fatty acids in strains making ethanol [52]. Conversely, deregulation of fatty acid biosynthesis has allowed for FAEE formation with alcohol feeding, but without fatty acid feeding [53]. It is only a matter of time before complete FAEE synthesis is made directly from carbohydrates.

Isoprenoid-based biofuels

Isoprenoids are naturally occurring hydrocarbons that carry out a number of biological functions. For instance, electron transport is mediated by ubiquinones and membrane structure and function are controlled by sterols [54]. Much of the recent work on the production of isoprenoids was initiated by the production of the antimalarial drug, artemisinin [55]. The key intermediates leading to all isoprenoids are the C5 isomers IPP and DMAPP. These are formed by two different pathways, the MVA pathway from acetyl-CoA and the MEP(DXP) pathway from pyruvate and glyceraldehye-3-phosphate. These can be joined together in many ways by terpene synthases to generate C10, C15, and higher hydrocarbons. Phosphatases can directly convert many of these compounds to fuel-like molecules.

Carbon sources for biofuels production

Simple sugars have been the starting point for first-generation ethanol production. Sucrose from sugarcane has driven Brazilian production while starch from grains has been enzymatically converted to glucose in the rest of the world. However, this typically uses only a portion of the plant material for incorporation into product. Agricultural residues or dedicated energy crops could greatly increase the

amount of available substrate for conversion to biofuels. But the use of these lignocellulosic sources is not without issues [56]. The presence of lignin, a heterogeneous polymer of aromatic alcohols, can interfere with the hydrolysis of the desired carbohydrates. A large number of pretreatment processes have been developed to separate or modify the lignin to make the carbohydrates more accessible. Pretreatments are also required to modify the structure of recalcitrant cellulose to make it more amenable to enzymatic digestion. These processes include physical, thermal, and chemical treatments. Cellulose is then converted to fermentable glucose, just as in the starch-based processes. The final component of lignocelluloses is hemicelluloses. Hemicellulose is another heterogeneous polymer made up of a number of different sugar monomers. The most abundant of these is xylose. Different from six-carbon glucose, xylose is a five-carbon sugar that is metabolized by different pathways than glucose. Metabolic engineering has been employed extensively to combine simultaneous fermentation of both hexoses and pentoses to ethanol in a variety of organisms including *Zymomonas mobilis* [57], *S. cerevisiae* [58], and *E. coli* [59]. These strains are still being improved and in addition to ethanol, can be the starting point for the generation of advanced biofuels. Adding new pathway elements, modifying transport mechanisms, and alleviating native control hierarchy have all been utilized in the building of these strains.

Next generation processes: fuels from carbon dioxide

The use of sucrose from sugarcane, starch from corn, and even lignocellulosic biomass are all examples of utilizing chemical energy produced by photosynthetic organisms from solar energy and CO_2. Producing advanced biofuels directly from these photosynthetic organisms could ultimately result in the largest reduction of atmospheric CO_2 [60]. The most advanced systems are those producing biodiesel directly from photosynthetic microalgae. Some strains can generate lipids, used as energy storage compounds, at nearly 40% of the total dry biomass. These lipids can be extracted and undergo the same transesterification process as from plant-derived oils.

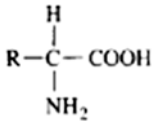
Photosynthetic organisms such as microalgae generate energy and reducing power from light. Photons are captured by specialized light harvesting complex proteins and the energy is used to split water into protons, electrons, and oxygen. Electrons are passed along an electron transport chain and eventually to NADPH. Protons are released across an organelle membrane creating a proton gradient used to generate ATP. These biological energy compounds are then used in the Calvin cycle to fix CO_2. CO_2 is added to ribulose-1,5-bis-phosphate, which is then split into two molecules of 3-phosphoglycerate, a key 3-carbon metabolic intermediate.

Metabolic engineering approaches are being applied to microalgae just as they have been applied to other organisms to generate biofuels. Here, focus has been in two main areas: (1) increasing oil production and (2) increasing photosynthetic efficiency. Most probably nonintuitive, reducing the amount of light harvesting complex proteins has led to increases in photosynthetic efficiency.

Photosynthetic organisms lead to a new problem in large-scale production. In order to function, the culture needs both abundant light and CO_2. Open ponds are one obvious choice for cultivation. However, closed bioreactor designs have numerous advantages over open ponds. These include, among others, high surface area per unit volume, ability to keep out contaminating organisms, and the more efficient use of water.

Amino Acids

Amino acids, in general, can be represented by the formula:



Because the amino group is on the α-carbon, the amino acids with this general formula are known as α-amino acids. The α-carbon atom becomes asymmetric when R is not an H atom. Naturally occurring amino acids have an L-configuration. Amino acids are the building blocks of proteins, and the elementary composition of most proteins is similar; the approximate percentages are:

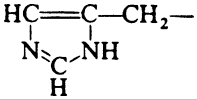
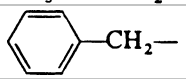
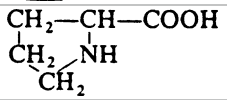
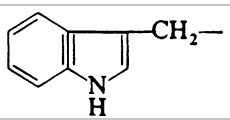
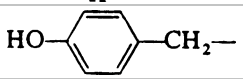
$$C = 50 - 55, \quad N = 15 - 18,$$

$$H = 6 - 8, \quad S = 0 - 4, \quad O = 20 - 23$$

Table 30.4 gives the structure of R, molecular weight, and elementary composition of each of the 20 amino acids commonly found in proteins.

Of the natural amino acids, only methionine is manufactured chemically as the racemic mixture. All other natural types are produced by fermentation as the L-enantiomer. Commercially, the most common amino acids manufactured are the L-forms of glutamic acid, mostly mono-sodium glutamate (MSG), lysine, phenylalanine, aspartic acid, threonine, tryptophan, arginine, citrulline, glutamine, histidine, isoleucine, leucine, ornithine, proline, tyrosine, and cysteine [61]. The biological methods vary (e.g., fermentation, extraction from natural sources, and enzymatic synthesis). Amino acids obtained from purified proteins are derived from chemical or enzymatic hydrolysis. They can also be isolated from industrial by-products; extracted from plant or animal tissues; or synthesized by

Table 30.4 Twenty common amino acids

Amino acids	R^-	Elemental Composition (%wt.)					
		M.W.	C	H	O	N	S
Alanine	CH_3-	89	40	8	36	16	0
Arginine	$H_2N-C(=NH)-NH-CH_2-CH_2-$	174	41	8	18	32	0
Asparagine	$H_2N-C(=O)-CH_2-$	132	36	6	36	21	0
Aspartic acid	$HO-C(=O)-CH_2-$	133	36	5	48	11	0
Cysteine	$HS-CH_2-$	121	30	6	26	12	26
Glutamic acid	$HO-C(=O)-CH_2-CH_2-$	147	41	6	44	10	0
Glutamine	$H_2N-C(=O)-CH_2-CH_2-$	146	41	7	33	19	0
Glycine	$H-$	75	32	7	43	19	0
Histidine		155	46	6	21	27	0
Isoleucine	$CH_3-CH_2-CH(CH_3)-$	131	55	10	24	11	0
Leucine	$CH_3-CH(CH_3)-CH_2-$	131	55	10	24	11	0
Lysine	$H_2N-CH_2-CH_2-CH_2-$	146	49	10	22	19	0
Methionine	$CH_3-S-CH_2-CH_2-$	149	40	7	22	9	2
Phenylalanine		165	66	7	19	8	0
Proline		115	52	8	27	12	0
Serine	$HOCH_2-$	105	34	7	46	13	0
Threonine	$CH_3-CH(OH)-$	119	40	8	40	12	0
Tryptophan		204	65	6	16	14	0
Tyrosine		181	60	6	26	8	0
Valine	$CH_3CH(CH_3)-$	117	51	9	12	27	0

organic, enzymatic, or microbiological means. All these methods yield the L-isomer.

Alanine and aspartic acid are produced commercially utilizing enzymes. In the case of alanine, the process of decarboxylation of aspartic acid by the aspartate decarboxylase from *Pseudomonas dacunhae* is commercialized. The annual world production of alanine is about 200 t. Aspartic acid is produced commercially by condensing fumarate and ammonia using aspartase from *E. coli*. This process has been made more convenient with an enzyme immobilization technique. Aspartic acid is used primarily as a raw material with phenylalanine to produce aspartame, a noncaloric sweetener. Production and sales of aspartame have increased rapidly since its introduction in 1981. Tyrosine, valine, leucine, isoleucine, serine, threonine, arginine, glutamine, proline, histidine, citrulline, L-dopa, homoserine, ornithine, cysteine,

tryptophan, and phenylalanine also can be produced by enzymatic methods.

The worldwide amino acids market amounts to about $5 billion. MSG and the animal feed additives, methionine and lysine, account for about 75% of this sales value. The other amino acids are used as precursors in pharmaceuticals, food additives, and animal feed. The worldwide demand for glutamic acid is about 800,000 t/year, 300,000 t/year for methionine, and 500,000 t/year for lysine. Other significant amino acids such as aspartic acid, phenylalanine, threonine, and glycine, each have a worldwide market of about 10,000–20,000 t/year. Tryptophan and cysteine command a global market in the thousands of tons as well.

Glutamic acid. MSG is an important flavor enhancer for natural and processed foods. It is also good for protecting the flavor and color of preserved foods and suppressing off-flavors. Glutamic acid is not an essential amino acid. However, it has some pharmaceutical uses and also improves the growth of pigs.

A number of glutamic acid-producing bacteria are known, for example,

- *Corynebacterium glutamicum* (synonym *Micrococcus glutamicus*)
- *Brevibacterium flavum*
- *Brevibacterium divaricatum*

Among them, *C. glutamicum* is used most commonly in the industry. Kyowa Hakko has recently completed a genome map of its glutamic acid producing organism, and hopes to improve glutamic acid fermentation efficiency and create a next-generation production system through pathway engineering.

The fermentation medium contains a carbon source (glucose or molasses), a nitrogen source (urea, ammonium sulfate, corn steep liquor, or soy hydrolyzate), mineral salts to supply potassium, phosphorus, magnesium, iron, and manganese, and less than 5 μg of biotin per liter depending upon the strain. The biotin requirement is the major controlling factor in the fermentation. When too much biotin is supplied for optimal growth, the organism produces lactic acid. Under conditions of suboptimal growth, glutamic acid is excreted. The metabolic pathway involved in the biosynthesis of glutamic acid from glucose limits α-ketoglutarate dehydrogenase activity, which is a notable phenotype of glutamic acid overproducing microorganisms.

The fermentation is conducted aerobically in tanks with k_La value in 300 mmol O_2/L/h/atm range. If aeration is not adequate, lactic acid is produced, and the yield of glutamic acid is poor. If aeration occurs in excess, even more lactic acid in addition to some α-ketoglutaric acid is produced. The fermentation temperature is 28–33°C, and the optimal pH is 7–8. Continuous feeding of liquid or gaseous ammonia controls pH and supplies ammonium ions to the fermentation. The fermentation cycle is 24–48 h, and the final concentration of glutamic acid is about 120–150 g/L. The overall yield of glutamic acid on sugar is about 65% on a weight basis. A portion of sugar is used for cellular growth; otherwise glutamic acid yield on sugar is 86% according to the equation:

$$C_{12}H_{22}O_{11} + 2NH_3 + 3O_2 \rightarrow 2C_5H_9O_4N + 2CO_2 + 5H_2O$$

For recovery of glutamic acid after fermentation, the broth is clarified by adding acid to pH 3.4, heating to 87°C, holding for a sufficient time to coagulate suspended solids, and filtering the coagulated solids. The clarified broth is concentrated by evaporation followed by glutamic acid crystallization. Other recovery schemes such as membrane filtration or ion-exchange can be used.

MSG sells for about $2/kg, and glutamic acid (99.5% pure) for about $4/kg. The major manufacturers of these are Ajinomoto, Asahi Foods, Kyowa Hakko, Takeda (Japan), Orsan (France), Biacor (Italy), Cheil Sugar, Mi-Won (S. Korea), and Tung Hai Fermentation Industry, Ve Wong (Taiwan).

Lysine. Lysine, biologically active in its L-configuration, is an essential amino acid in human and animal nutrition. The richest sources of lysine are animal proteins such as meat and poultry, but it is also found in dairy products, eggs, and beans. On the other hand, cereal grains, such as corn, wheat, and rice, are usually low in lysine. For applications in human food, lysine in its salt forms can be added to cookies and bread, and in solution it can be used to soak rice. As most animal feed rations are based on corn and other grains, supplementing the feedstuffs with lysine (plus methionine) significantly improves their nutritional value for breeding poultry and pigs.

A fermentation process for producing lysine was made possible by using mutants of *C. glutamicum* or *B. flavum*. Both auxotrophic and regulatory mutants have been obtained for overproduction of lysine. Figure 30.24 shows the biosynthetic pathway of lysine and its metabolic controls in *C. glutamicum*.

Molasses was the most common carbon source until being replaced by glucose for reasons of easier downstream processing. Sufficient amounts (over 30 μg/L) of biotin must be included in the medium to prevent the excretion of glutamic acid. This biotin requirement was previously met by using molasses as the carbon source and must now be added exogenously. The fermentation runs at temperatures of about 28–33°C and pH 6–8. High aeration is desirable. The final product concentration is around 100–140 g/L, and the fermentation time is 48–72 h. The yield of lysine on carbohydrate is about 40–50%. The formation of lysine from sucrose can be represented as follows:

$$C_{12}H_{22}O_{11} + 2NH_3 + 5O_2 \rightarrow C_6H_{14}O_2N_2 + 6CO_2 + 7H_2O$$

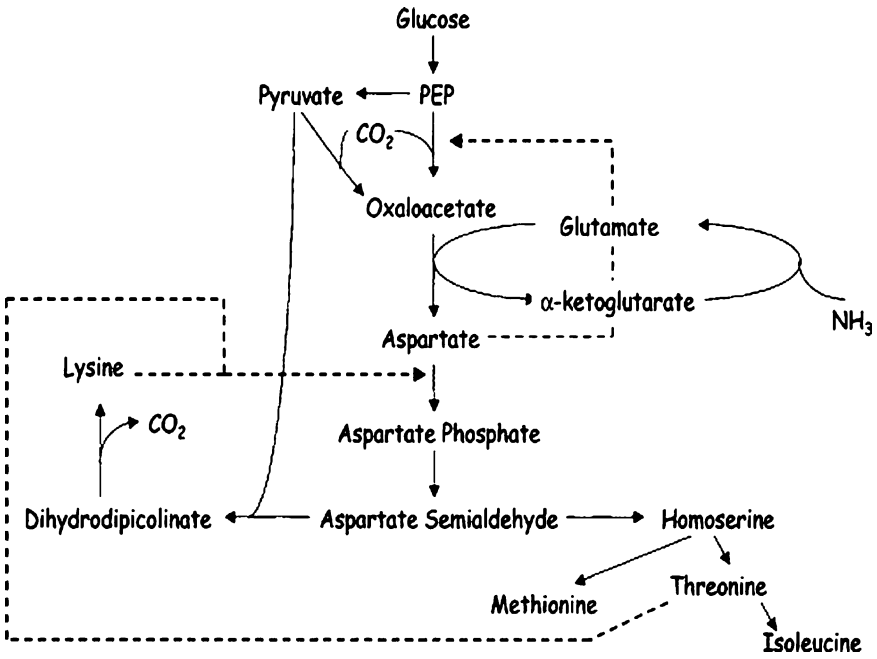


Fig. 30.24 Lysine biosynthesis from glucose in *Corynebacteria*

Ion-exchange resins are used for isolation of lysine from fermentation broths. The eluted lysine is then crystallized from water. The most common commercial form of lysine used in animal feed is 98% lysine monohydrochloride. For lower purity, the entire fermentation broth may be evaporated and syrup or the dried product is used as an animal feed supplement.

Standard commercial food-grade lysine (as 98.5% lysine monohydrochloride) is about $5/kg, and lysine as a feed supplement is about $2/kg. The major producers include Ajinomoto and Kyowa Hakko of Japan, Sewon and Chiel Sugar of South Korea, and ADM, BioKyowa (a subsidiary of Kyowa Hakko), Heartland Lysine (a subsidiary of Ajinomoto), and Degussa (formerly joint venture of Cargill and Degussa in the United States). China's Global Bio-Chem has been increasingly selling protein–lysine (protein with enhanced lysine content), as well. The world demand for lysine is expected to increase continually. In fact, the increasing ethanol fermentation capacity for bioethanol production provides an opportunity for increased lysine production. Distillers' Dried Grains (DDG), a by-product of ethanol fermentation used as a protein source in animal feeds, is deficient in lysine and other essential amino acids. Therefore, it needs to be supplemented with lysine for full-value use.

Aromatic amino acids. The aromatic amino acids, phenylalanine and tryptophan, provide some of the first examples of chemical production using microorganisms through the use of pathway engineering [62, 63]. Intermediates in the aromatic amino acid pathway can also be used as precursors to other biosyntheses with genes recruited from different organisms. Examples include catechol, adipic acid, shikimic, and quinic acids. In general, the aromatic pathway illustrates the potential of multiple product opportunities from one pathway and provides a great leveraging factor with respect to technical and commercial development costs.

Initially, work on the aromatic amino acid pathway of *E. coli* was focused on the construction of a strain for the overproduction of natural amino acids (phenylalanine, tryptophan, tyrosine). These efforts have focused on:

1. Cloning and optimization of complete primary aromatic pathway with an emphasis on removal of transcriptional and allosteric regulation, that is, removal of rate-limiting steps.
2. Enhancement of carbon flux to the aromatic pathway through modification of gene activities within central metabolism.

For the aromatic pathway (Fig. 30.25), the critical control points are the condensation of PEP and erythrose-4-phosphate to 3-deoxy-D-arabinoheptulosonate 7-phosphate, DAHP, by DAHP synthase. For tryptophan, the formation of anthranilic acid from chorismic acid by anthranilate synthase is the second critical control point. The transcriptional regulation was overcome through the use of alternative promoters and allosteric regulation was circumvented by the classical technique of selection for feedback-resistant mutants using toxic analogues of the repressing compounds.

For tryptophan production in *E. coli*, the natural regulation controlling production of tyrosine and phenylalanine was

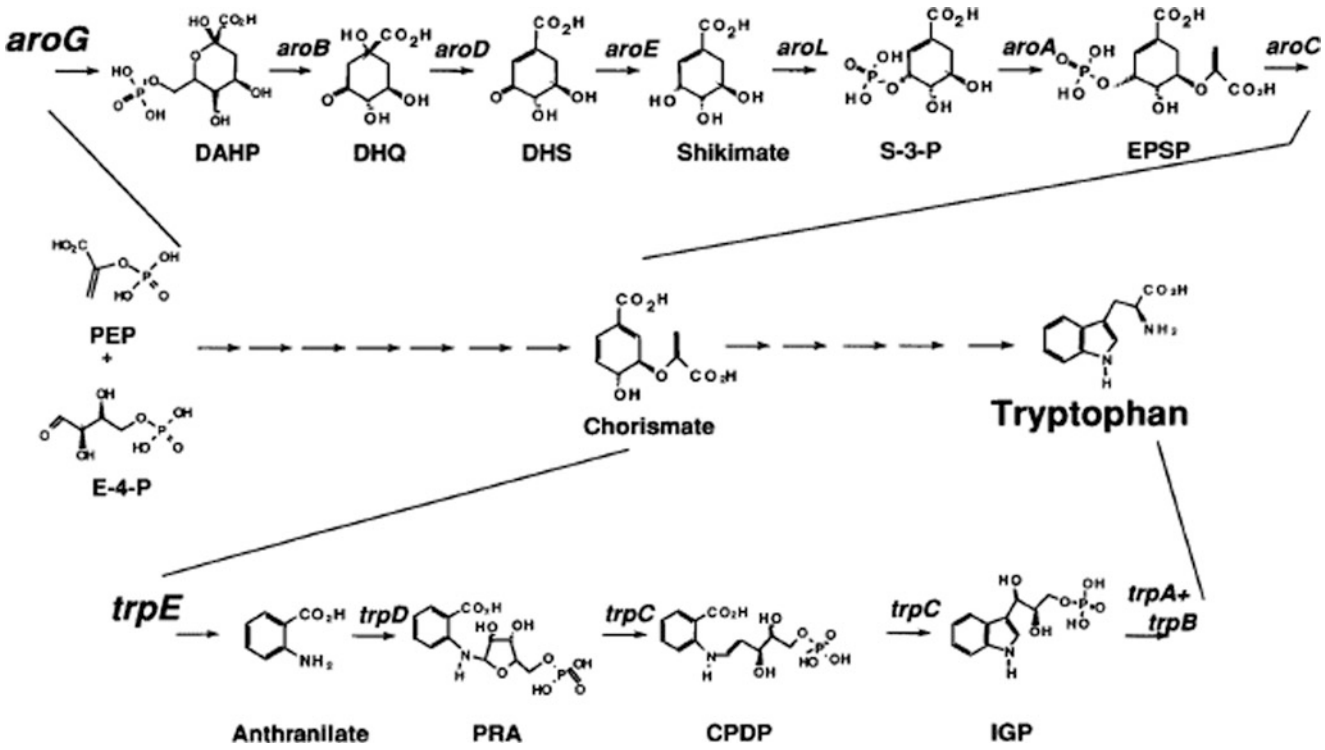


Fig. 30.25 *Escherichia coli* aromatic pathway to tryptophan

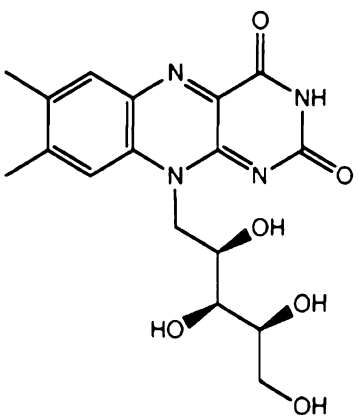
sufficient to keep carbon flowing specifically to tryptophan. This eliminated the need for addition of auxotrophic compounds to the growth medium. The major industrial producers of tryptophan are ADM, Kwoya Hakko, and Ajinomoto.

The same could not be said for the construction of a strain for the overproduction of phenylalanine. Here the control mechanisms for tyrosine were not sufficient to keep a significant amount of carbon from being diverted. However, instead of using an auxotrophic strain, a technology was developed to keep the gene for chorismate mutase and prephenate dehydrogenase present during the growth phase of the fermentation and then have it excised from the chromosome during the production phase. Due to the sweetener Aspartame, the market for phenylalanine has expanded to 10,000 metric tons per year with an average price of $10 per kg. The major producers are Nutrasweet and Ajinomoto.

Vitamins and Neutraceuticals

In the commercial market for vitamins, the most significant are ascorbic acid (Vitamin C), the D group (D2, D3, ergosterol), folic acid, and alpha-tocopherol (Vitamin E). The next important group of vitamins is biotin, cyanocobalamin (B_{12}), inositol, pantothenic acid, and riboflavin (B_2). Microorganisms can synthesize many vitamins of medical importance. Vitamin B_2 (riboflavin) and vitamin B_{12} (cyanocobalamin) are products of fermentation. Vitamin C (ascorbic acid) precursor, 2-keto-L-gulonic acid (2-KLG) is produced microbiologically as well as chemically as discussed in the "Organic Acids and Polymers" section.

Riboflavin. Riboflavin is used as a dietary supplement in both human food and animal feed. The yellow-orange riboflavin crystals are only sparingly soluble in water. To include riboflavin in water-soluble formulations, sodium riboflavin-5′-phosphate is used.



The first organism employed primarily for riboflavin production was *Clostridium acetobutylicum*, the anaerobic bacterium used for the microbial production of acetone and butanol. Riboflavin was purely a by-product and was found in the dried stillage residues in amounts ranging from 40 to 70 μg/g of dried fermentation solids. Later investigations disclosed that riboflavin could be produced by yeast such as *Candida flareri* or *C. guilliermondi*, and the yield was as high as 200 mg/L.

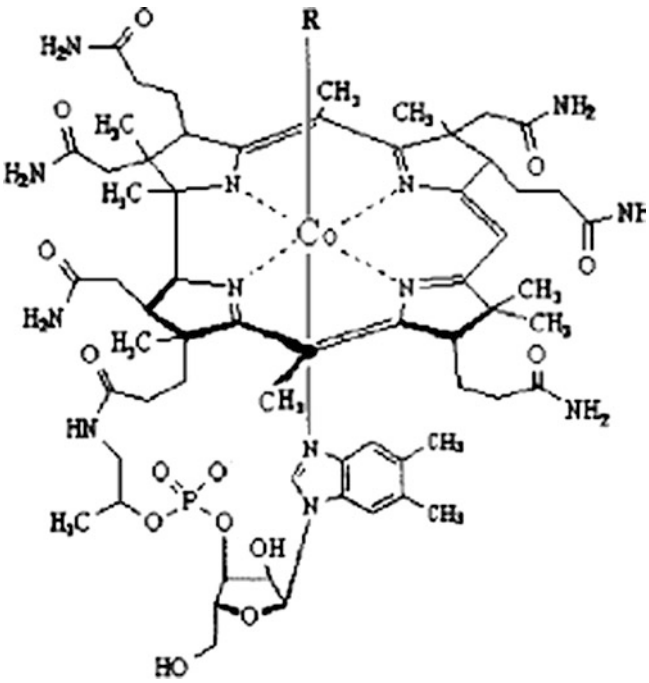
Other studies on a fungus, *Eremothecium ashbyii*, and a closely related organism known as *Ashbya gossypii* resulted in the production of much larger amounts of riboflavin. Yields as high as 10–15 g/L were possible. Then, major fermentation strain and process improvements were made with the *A. gossypii* strain. The fermentation lasts 8–10 days. Cell growth occurs in the first 2 days, and enzymes catalyzing riboflavin synthesis are formed during the growth period. Glycine and edible oil stimulate the formation of riboflavin, but they are not its precursors. The additions of carbohydrate and oil permit the overproduction of riboflavin [64]. The riboflavin carbon yield is about 50% on carbohydrate and about 100% on oil.

Upon completion of the fermentation, the solids are dried to a crude product for animal feed supplement or processed to an USP-grade product. In either case, the pH of the fermented medium is adjusted to pH 4.5. For the feed-grade product, the broth is concentrated to about 30% solids and dried on double-drum driers.

When a crystalline product is required, the fermented broth is heated for 1 h at 121°C to solubilize the riboflavin. Insoluble matter is removed by centrifugation, and riboflavin recovered by conversion to the less soluble form. The precipitated riboflavin is then dissolved in water, polar solvents, or an alkaline solution, oxidized by aeration, and recovered by recrystallization from the aqueous or polar solvent solution or by acidification of the alkaline solution.

The major producers of riboflavin include DSM and BASF. There have been significant improvements in both of the production processes. DSM opened a new riboflavin production facility in Grenzach, Germany in 2000, based on a fermentation process with a genetically modified *B. subtilis*. On the other hand, BASF, working with the University of Salamanca, Spain, engineered an improved *A. gossipii* that produces a larger amount of enzymes for riboflavin synthesis, and increased its production capacity by 20% at its Ludwigshafen facility without capital investment. The annual production of riboflavin by ADM, BASF, and DSM is about 4,000 metric ton. The feed-grade product sells for about $30/kg and the USP-grade product $50/kg.

Cyanocobalamin



Vitamin B_{12}, cyanocobalamin, is an important biologically active compound. It serves as a hematopoietic factor in mammals and as a growth factor for many microbial and animal species. Its markets are divided into pharmaceutical (96–98% pure) and animal feed (80% pure) applications. All vitamin B_{12} is now made commercially by fermentation [65].

Spent liquors from streptomycin and other antibiotic fermentations contain appreciable amounts of vitamin B_{12}. Bacterial strains producing high amounts have been specially selected for commercial production. Today vitamin B_{12} is obtained from fermentations using selected strains of *Propionibacterium* or *Pseudomonas* cultures. A full chemical synthesis process for vitamin B_{12} is known. However, it requires some 70 steps and for all practical purposes is of little value.

The *Pseudomonas denitrificans* strain is most often used for commercial production of vitamin B_{12} [66, 67]. It requires medium containing sucrose, yeast extract, and several metallic salts. Dimethylbenzimidazole (10–25 mg/L) and cobaltous nitrate (40–200 mg/L) must be supplemented at the start of the culture in order to enhance vitamin production. Betaine (tri-methyl glycine) stimulates the biosynthesis of vitamin B_{12}, even though the organism does not metabolize it. Similarly, choline also has favorable effects, either by activating some biosynthetic steps or altering the membrane permeability. Glutamic acid, on the other hand,

stimulates cellular growth. Because of high betaine and glutamic acid contents, beet molasses (60–120 g/L) is preferentially used in industrial fermentations of vitamin B_{12}. The fermentation is conducted with aeration and agitation. Optimal temperature is about 28°C and pH near 7.0. The yield reported in the literature was 59 mg/L in 1971, using a *Pseudomonas* strain. A yield of 200 mg/L was reported for vitamin B_{12} fermentations using *Propionibacteria* in 1974. It is believed that yields of vitamin B_{12} have been significantly improved since then.

About 80% of the vitamin produced is outside the cells and 20% inside the cells. For recovery, the whole broth is heated at 80–120°C for 10–30 min at pH 6.5–8.5 and treated with cyanide or thiocyanate to obtain cyanocobalamin. B_{12} isolation can then be accomplished by adsorption on a cation-exchange resin, such as Amberlite IRC 50. Extraction can also be done by using phenol or cresol alone or in a mixture with benzene, butanol, carbon tetrachloride, or chloroform; or separation by precipitation or crystallization upon evaporation with appropriate diluents such as cresol or tannic acid. Using the extraction method, 98% pure cyanocobalamin can be obtained with a 75% yield. The total world market for cyanocobalamin is estimated to be in excess of 10,000 kg/year and sells for about $25/g. The leading producers include DSM, Wockhardt, and Merck.

Docosahexaenoic and arachidonic acids. Docosahexaenoic acid, DHA, is a long-chain polyunsaturated omega-3 (22:6 ω3) fatty acid whereas arachidonic acid, ARA, is a long-chain polyunsaturated omega-6 (20:4 ω6) fatty acid. Both DHA and ARA are found in human milk and are important nutrients in infant development, and especially for brain development. DHA is a major structural and functional fatty acid in the gray matter of the brain, the retina of the eye, and the heart cell membranes. DHA can be obtained through the diet in foods such as fatty fish (accumulated via natural algae) and organ meat. ARA is the principal omega-6 fatty acid found in the brain as well. Adults obtain ARA readily from the diet in foods such as meat, eggs, and milk. The DHA- and ARA-rich oil can also be used in supplements and functional foods for older children and adults for improvement in cardiovascular health.

Martek (http://www.martekbio.com) and DSM have developed processes to produce oils rich in DHA and ARA. The DHA is extracted from fermented microalgae (*Cryptecodinium cohnii*) and the ARA is extracted from soil fungus (*Mortierelle alpina*). In the DHA production process, the microalgae are grown in fermentors (80,000–260,000 L scale). The cell mass is then harvested by centrifugation, and the concentrated cell mass is spray-dried. The dry cell mass is then broken down into smaller particles and extracted by a solvent for the oil-containing DHA. The solvent is then evaporated from the oil, leaving crude oil. The crude oil is refined, bleached, and deodorized to cleanse it of any impurities. The final product is rich in DHA. Likewise, DSM ferments the fungus, and extracts the crude ARA oil by the DHA downstream type process. Crude oils can be extracted with the traditional hexane extraction method or with new solvents such as isopropanol or supercritical CO_2. The final cell mass, total fermentation time, and the lipid content of the organism are very important for the economic feasibility, as these factors largely determine the productivity. Media components such as the amount of sugars and nitrogen have a significant effect on growth and lipid accumulation.

The market for DHA, ARA, and other essential neutraceuticals used in dairy, beverages, cereals, and breads, is multibillion dollars and growing as the health benefits are determined for prenatal to elderly care.

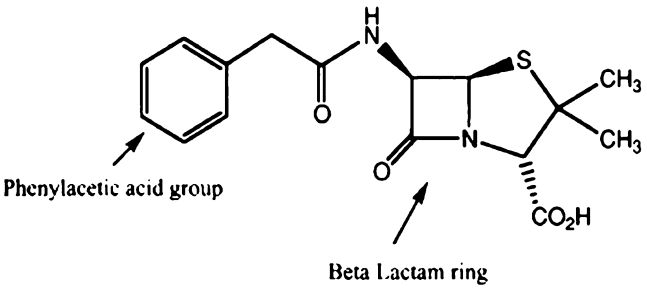
Table 30.5 Examples of natural antibiotics and producing organisms

Natural antibiotic	Producing organism
Amphotericin B	*Streptomyces nodosus*
Bleomycin	*Streptomyces verticillus*
Cephalosporin	*Cephalosporium* sp.
Erythromycin A	*Saccharopolyspora erythraea*
Gentamycin	*Micromonospora purpurea*
Lovastatin	*Aspergillus terreus*
Neomycin	*Streptomyces fradiae*
Penicillin G	*Pencillium chrysogenum*
Spiramycin	*Streptomyces ambofaciens*
Tetracycline	*Streptomyces aureofaciens*
Vancomycin	*Streptomyces orientalis*

Antibiotics

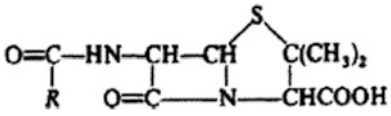
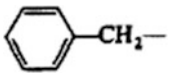
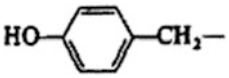
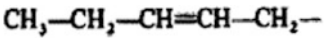
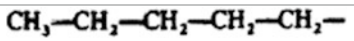
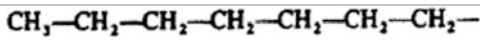
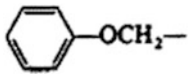
Since the early 1940s, an intensive search for new and useful antibiotics has taken place throughout the world. More than 10,000 antibiotics have been discovered from microbial sources [68–73]. *Streptomyces* spp., filamentous fungi, nonactinomycete bacteria, and non-Streptomycete strains of actinomycetales are major sources of antibiotics, examples of which are listed in Table 30.5. Of the antibiotics in clinical use, 50% are produced by microbial fermentation and others by a combination of microbial synthesis and subsequent modification.

Penicillins



Penicillin G

Table 30.6 Structural formulae of natural penicillins

Formula	
Type of penicillin	*Side chain R substitutions*
(G) Benzyl	
(X) *p*-Hydroxybenzyl	
(F) 2-Pentenyl	
(Dihydro F) *n*-Pentyl	
(K) *n*-Heptyl	
(V) Phenoxy	

The original mold observed and preserved by Alexander Fleming was a strain of *P. notatum*, a common laboratory contaminant. Later, cultures of *P. chrysogenum* were found to be better producers of penicillin, and the present industrial strains have been derived from this species. The original strains produced the antibiotic only by surface fermentation methods and in very low yields. Improved media and productive strains under submerged aerobic fermentation conditions led to dramatic yield increases. Subsequent improvements, principally in culture selection and mutation, further improved yields, reaching 20–30 g/L.

Table 30.6 gives the structural formulae of the "natural penicillins," which are comprised of several closely related structures with aliphatic and aromatic substitutions to the common nucleus. The early impure product contained mixtures of these penicillins; however, penicillin G has become the preferred type and the crystalline product of commerce. Phenylacetic acid or its derivatives are used as precursors in the fermentation medium to enhance penicillin biosynthesis.

Nonpigmented mutants of *P. chrysogenum*, for example, Wisconsin 49-133 derivatives, are universally employed in the industrial process. The desired culture is propagated from a laboratory stock in small flasks and transferred to seed tanks. A typical production medium is as shown below.

Components	Grams/liter
Corn steep liquor	30
Lactose	30
Glucose	5.0
$NaNO_3$	3.0
$MgSO_4$	0.25
$ZnSO_4$	0.044
Phenyl acetamide (precursor)	0.05
$CaCO_3$	3.0

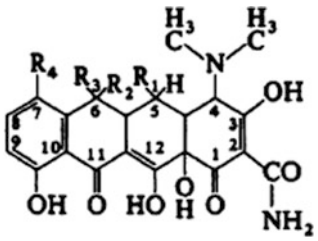
(*continued*)

The medium is sterilized, cooled to 24°C, and inoculated. After 24-h seed growth, larger fermentors are seeded. Sterile air is sparged through the tank, at about one volume per volume per minute. The time of the production stage varies from 60 to 200 h.

For recovery, the broth is clarified by means of rotary vacuum filters. The penicillin, being acidic, is extracted from the aqueous phase into a solvent, such as methyl isobutyl ketone or amyl acetate, at a pH of 2.5 by means of a continuous countercurrent extractor, such as a Podbielniak. The penicillin is then re-extracted with an aqueous alkaline solution or a buffer at a pH of 6.5–7.0. About 90% recovery yield is typical at this step. The aqueous solution is chilled, acidified, and extracted again with a solvent, such as ether or chloroform. Finally, penicillin is re-extracted into water at a pH of 6.5–7.0 by titration with a solution of base. The base used depends on which salt of penicillin is desired. The popular forms are sodium or potassium salts. A typical flow sheet for antibiotic recovery is shown in Fig. 30.6.

Cephalosporins. Microbiological processes for production of cephalosporin C resemble in many respects those used for penicillin production. Special strains of *Cephalosporium* have been selected that produce more cephalosporin C and less cephalosporin N than the parent culture. The growth of these strains in certain special fermentation media has resulted in higher antibiotic titers. Even with these improvements in processing, the antibiotic concentration, averaging 10–20 g/L, is much lower than those reported for the penicillins.

Table 30.7 Structure of clinically important tetracyclines



	R_1	R_2	R_3	R_4
Tetracycline	H	OH	CH_3	H
7-Chlortetracycline (Aureomycin®)	H	OH	CH_3	Cl
5-Oxytetracycline (Terramycin®)	OH	OH	CH_3	H
6-Demethyl-7-chlortetracycline (Declomycin®)	H	OH	H	Cl
6-Deoxy-5-oxytetracycline (Vibramycin®)	OH	H	CH_3	H
6-Methylene-6-deoxyl-6-demethyl-5-oxytetracycline (Rondomycin®)	OH	H	CH_2	H

Tetracyclines. In 1948, a broad-spectrum antibiotic, chlortetracycline (Aureomycin), was introduced by the Lederle Laboratories, a Division of American Cyanamid Company. This antibiotic is produced by *Streptomyces aureofaciens* when grown under submerged aerobic conditions on media composed of sugar, corn steep liquor, and mineral salts. The crystalline compound has a golden yellow color, which suggested the trade name. A related antibiotic, oxytetracycline (Terramycin), a product of *Streptomyces rimousus*, is produced by Pfizer Inc. It is chemically and biologically similar to chlortetracycline. Independent research by both companies eventually provided the structure of these two important chemotherapeutic agents. Both the compounds may be regarded as derivatives of a nucleus known as tetracycline. Their structures along with those of other clinically important tetracyclines are given in Table 30.7. Tetracycline can also be produced by fermentation of *S. aureofaciens* under special conditions, such as chloride starvation or by use of special strains of the organism that fail to halogenate efficiently. Tetracycline possesses many of the chemotherapeutic properties of chlortetracycline and oxytetracycline. Mutations of tetracycline-producing organisms have led to other tetracycline analogues, of which 6-demethyl-7-chlortetracycline (Declomycin) has clinical use. Chemical modifications of oxytetracycline have generated two other useful members of the family, known as Vibramycin and Rondomycin. Tetracyclines are active in vivo against numerous gram-positive and gram-negative organisms, and some of the pathogenic Rickettsiae and large viruses.

Dramatic price reduction has come with improved production. For instance, a million-unit vial of penicillin (1,667 units = 1 mg of potassium penicillin G) had a wholesale price of $200 in 1943. Today, a million units of potassium penicillin G sell for as little as $0.50 or approximately $20/kg of free acid. Cephalosporins sell for around $250/kg. Tetracyclines, used as animal feed supplements, sell for about $60/kg. The antibiotic market is over $12 billion, and volume continues to increase simultaneous with price drop. Cephalosporins, macrolides, penicillins, quinolones, and tetracyclines account for the majority of the sales of antibiotics.

In the last 20 years, it has become evident that the contribution of microorganisms does not have to be limited to the realm of infectious diseases. Metabolites of microorganisms have been found to have many other interesting therapeutic applications. Particularly as our understanding of disease processes at the biochemical and genetic levels becomes more advanced, enzymes or receptors have increasingly served as specific targets for therapeutic intervention. Recombinant DNA technology, in particular, has helped to produce reagents as tools for the development of practical and high-throughput screening methodologies based on mechanism of action. Automation and miniaturization have also allowed a rapid increase in the throughput of the screening process.

Several of these bioactive natural products have been successfully developed as therapeutics for clinical use. For example, Cyclosporin A is a fungal decapeptide principally used to suppress immune rejection in organ transplant patients. Mevinolin and compatin both control cholesterol synthesis in human. The search for enzyme- or receptor-targeted microbial products does not limit itself to medical use. Several commercially important "antibiotics" such as Nikkomycin and Avermectin have been found for agricultural applications in recent years.

Biopharmaceuticals

The term "biopharmaceuticals" has been generally accepted to distinguish the large molecule pharmaceuticals (mainly proteins) that have emerged as a result of the modern "biotechnology" from the traditional small molecule drugs.

Table 30.8 Examples of biopharmaceutical products, applications, and manufacturers

Biopharma product	Application	Companies
Insulin	Diabetes	Eli Lilly, Novo Nordisk
Erythropoietin	Anemia	Amgen, Johnson & Johnson
Human growth hormone	Growth deficiency	Genentech, Pharmacia
Interferon α	Hepatitis	Schering, Roche
Interferon β	Multiple sclerosis	Chiron, Biogen
Factor VIII	Hemophilia	Bayer
Tissue Plasminogen Activator	Blood clot	Genentech
Glucocerebrosidase	Gaucher's disease	Genzyme
Therapeutic antibodies	Cancer	Glaxo, Amgen, Genentech
GCSF	White blood cell	Amgen, Sankyo

Biopharmaceuticals that are industrially produced by microbial (bacterial or yeast) fermentation include insulin, human growth hormone, hepatitis B surface antigen vaccine, alpha interferon, beta interferon, gamma interferon, granulocyte colony stimulating factor, and interleukin-2. Table 30.8 lists some of the major biopharma products, some of which are produced by mammalian cell culture.

Insulin is the first human biopharmaceutical product that was commercialized using the recombinant DNA technology. Its fermentative expression in *E. coli*, downstream recovery, and purification serves as a good example for the large-scale production of recombinant proteins. Insulin, given its rather small size, is expressed as N-terminal extended proinsulin using the *trp* promoter. The fermentation is carried out at about 37°C and near neutral pH. It is a fast, aerobic fermentation. The *trp* operon is turned on when the fermentation runs out of its natural repressor, tryptophan. The chimeric protein as it is expressed accumulates very rapidly inside the cells as insoluble aggregates (inclusion or refractile bodies). The formation of inclusion bodies prevents proteolysis and facilitates product recovery. The recombinant *E. coli* fermentation usually runs for 18–24 h. At the end of fermentation, the inclusion bodies account for about 10–30% of total dry cell mass. Following purification from the inclusion bodies, the C-peptide is removed from proinsulin using trypsin and carboxypeptidase, and the properly folded and cleaved insulin is purified further. Fermentatively or microbially produced recombinant proteins can also be expressed as soluble proteins inside the cells or secreted outside the cells.

The biopharmaceuticals sales are about $45 billion and represent 10% of the total pharma market. Today, 25% of new drugs are biopharmaceuticals. Since the introduction of insulin over 25 years ago, 160 biopharma products, ranging from proteins, monoclonal antibodies, and nucleic acid-based products, have been approved for use. In 2004 alone, 12 new biopharmaceuticals have been approved, of which only 3 are produced by microbial fermentation, as cell culture is the preferred method for production of biopharma drugs.

Drug development, in general, is a slow, lengthy, and expensive process, requiring an average investment of $0.8 billion over 12–15 years for discovery, development, preclinical testing, Phase I, II, and III trials, and FDA approval prior to marketing. Given the complications of biopharma drug discovery and development, microbial fermentation and biocatalysis may aid in introducing rapid and innovative processes to produce potential drug candidates. It may thus be worth exploring the possibility of producing biopharmaceuticals by microbial fermentation and demonstrating that they can be made reproducibly to meet the right purity, concentration, and dose at the required market cost and volume targets.

Enzymes

Microorganisms used for production of enzymes range from prokaryotic systems including both the gram-negative and gram-positive bacteria to eukaryotic systems such as yeasts and fungi. For most of the history of enzymes, their production has occurred predominantly in strains known to make the enzyme of interest [30, 74–77]. Thus, many different types of microorganisms have been employed to make enzymes for different types of uses, as discussed in Chap. 31.

For example, an alkaline protease naturally secreted by *Bacillus licheniformis* to break down proteinaceous substrates, resulted in one of the first commercially produced enzymes, Subtilisin Carlsberg, for use in detergents. Similarly, α-amylase was produced from *B. licheniformis* because it naturally secretes a highly thermo-stable α-amylase capable of breaking down starch to more easily digestible oligosaccharides. Strains of *Bacillus* have been some of the workhorse strains for enzyme production for decades, based mainly upon their ability to overproduce subtilisin and α-amylase. Those strains and the promoters of these genes are also used to express other enzymes, wild type and engineered.

Glucoamylase, which completely breaks down oligosaccharides to glucose, is produced by strains of the fungal genus, *Aspergillus*. Strains that overproduce glucoamylase have been isolated over the years. Another carbohydrase, an acid lignocell enzyme complex, found in the fungus *Trichoderma*, is capable of breaking down cellulosic substrates to glucose, similar to the starch-degrading enzymes. This particular application has turned out to be much more challenging to commercialize but has found applications in treatment of textiles, feed, and food. New programs initiated by the U.S. Department of Energy have helped improve the expression and activity of the lignocell enzyme complex.

One of the enzymes that has been used on a large scale is glucose/xylose isomerase, which catalyzes the rearrangement of glucose to fructose, to yield a product with a sweet taste like sucrose. The enzyme is present in *Streptomyces* spp. as well as a few other organisms. Once again, the native host strain has been improved for production of glucose isomerase.

Future: Biorefineries

Biorefineries integrate biobased industries/processing facilities that utilize mostly plant materials as feedstocks to produce food, feed, fuel, chemicals, materials, and intermediates for making varieties of products. Fermentative processes discussed here and biocatalysis discussed in Chap. 31 can be applied for a wide variety of processes where renewable resource-based materials are used and produced. Bioprocesses are continually becoming more efficient through better understanding and controlling of metabolic pathways. Today, industrial biotechnology companies such as DuPont Industrial Biosciences and Novozymes sell enzymes produced via microbial fermentation processes for a wide variety of bioprocessing applications. Chemical producers are seeking new and renewable feedstocks as well as environmentally friendly and sustainable production processes, in the face of escalating nonrenewable raw material prices. In addition, there is a need for development of interesting new chemistry and products. Major chemical companies—including Dow Chemical, DuPont, BASF, Degussa, and Celanese—have invested heavily to explore these opportunities, often through alliances with small technology firms that offer specific expertise (http://pubs.acs.org/email/cen/html/060804150713.html). However, most products made today are relatively low-volume, high-value types, for example, drugs and specialty chemicals. Only a few products, such as ethanol, are produced by fermentation at a million-ton scale per year.

Improvements in fermentation organisms and processes will have to be achieved in order to raise productivity and conversion efficiency and lower production costs (http://www.nrel.gov/docs/fy04osti/35523.pdf). Enzymatic processing is also expected to play a major role in refining biofeedstocks into sugars, protein, oil, and other by-products. Such advances alone will enable the vision of biorefineries to become a reality. Analogous to conventional refineries, progress in three areas will be key for the successful development of biorefineries: low energy milling of biofeedstocks to its components, efficient bioconversion of mixed sugars to products, and the utilization of by-products. These improvements will require integration of all major areas of industrial biotechnology: novel enzymes and microorganisms, functional genomics, pathway engineering, protein engineering, biomaterial development, bioprocess design, product development, and applications.

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