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Microorganisms as producers of feedstock chemicals

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Microbial production of industrial chemicals from renewable resources

Feedstock chemicals are the raw materials of the chemical industry. They are used as starting materials for synthesizing a vast array of other chemicals - plastics and rubber, for example; as solvents; and for the manufacture of numerous products, including textiles and paper (Table 3.1).

Industrial chemicals were originally produced by extracting materials such as vegetable oils, starches, cellulose, lignins and waxes from plants. These carbon-containing raw materials are directly renewable by the photosynthetic reduction of carbon dioxide from the atmosphere. They can be chemically converted to other substances, thereby greatly expanding the number of products that can be derived from the plant kingdom.

Many of these conversions are carried out by microorganisms that can use the plant-derived materials as a source of energy for increasing the microbial cell mass, at the same time forming numerous by-products. The role of the microorganisms was not appreciated when they first began to be used for preserving foods and producing alcoholic beverages, however. The Dutch scientist Antony van Leeuwenhoek did not discover bacteria until the seventeenth century, and only later, in the nineteenth century, did the Frenchman, Louis Pasteur, convince the scientific world of the essential role of microorganisms in fermentation. The products of microbial metabolism were then identified at an increasingly rapid rate over the following decades.

At first the products came primarily from the energy-producing metabolic pathways of the microbes. For example, the glycolytic pathway provides ethanol for alcoholic beverages, carbon dioxide for leavening bread, and lactic acid for food preservation (Fig. 3.1). Other organic acids, such as acetic, propionic and citric acids, were identified as products of microbial metabolism by the end of the nineteenth century.

In the early twentieth century several additional organic chemicals were found to be products of microbial energy metabolism. The fermentation method for producing the solvents acetone and butanol was developed during World War I, when acetone was required for the production of the explosive cordite. A similar situation arose during World War II, when supplies of natural rubber from the Far East were disrupted. Researchers then found that 2,3-butanediol could be converted to 1,3-butadiene, which can be used to make synthetic rub-

Table 3.1. *Industrial chemicals produced by fermentation*

Organic chemical	Microbial sources	Selected uses
Formic acid	<i>Aspergillus</i>	Textile dyeing, leather treatment, electroplating, rubber manufacture
Ethanol	<i>Saccharomyces</i>	Industrial solvent, intermediate for vinegar, esters and ethers, beverages
Acetic acid	<i>Acetobacter</i>	Industrial solvent and intermediate for many organic chemicals, food acidulant
Glycolic acid	<i>Aspergillus</i>	Textile processing, pH control, adhesives, cleaners
Oxalic acid	<i>Aspergillus</i>	Printing and dyeing, bleaching agent, cleaner, reducing agent
Glycerol	<i>Saccharomyces</i>	Solvent, plasticizer, sweetener, explosives manufacture, printing, cosmetics, soaps, antifreezes
Propylene glycol	<i>Bacillus</i>	Antifreeze, solvent, synthetic resin manufacture, mould inhibitor
Isopropanol	<i>Clostridium</i>	Industrial solvent, cosmetic preparations, antifreeze, inks
Acetone	<i>Clostridium</i>	Industrial solvent and intermediate for many organic chemicals
Malonic acid	<i>Penicillium</i>	Manufacture of barbiturates
Lactic acid	<i>Lactobacillus</i> , <i>Streptococcus</i>	Food acidulant, dyeing, intermediate for lactates, leather treatment
Acrylic acid	<i>Bacillus</i>	Industrial intermediate for plastics
Butanol	<i>Clostridium</i>	Industrial solvent and intermediate for many organic chemicals
Butanoic acid	<i>Clostridium</i>	Manufacture of esters
2,3-Butanediol	<i>Aerobacter</i> , <i>Bacillus</i>	Intermediate for synthetic rubber manufacture, plastics and antifreeze
Methylethyl ketone	<i>Chlamydomonas</i>	Industrial solvent, intermediate for explosives and synthetic resins
Fumaric acid	<i>Rhizopus</i>	Intermediate for synthetic resins, dyeing, acidulant, antioxidant
Succinic acid	<i>Rhizopus</i>	Manufacture of lacquers, dyes and esters for perfumes
Malic acid	<i>Aspergillus</i>	Acidulant
Tartaric acid	<i>Acetobacter</i>	Acidulant, tanning, commercial esters for lacquers, printing
Itaconic acid	<i>Aspergillus</i>	Textile and paper manufacture, intermediate for plastics

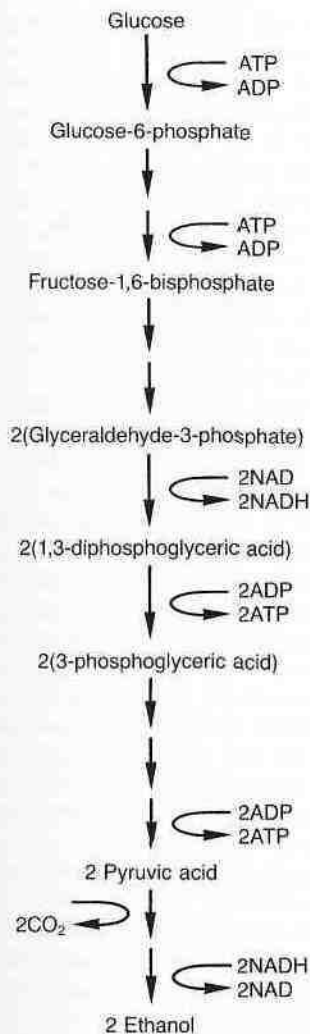


Fig. 3.1 The glycolytic pathway for alcoholic fermentations. In this pathway the sugar glucose is converted in several steps to pyruvic acid, which is then converted to ethanol with a release of carbon dioxide.

ber, and developed a fermentation route for making the 2,3-butanediol.

The modern pharmaceutical industry can trace its origins to 1928 when Alexander Fleming discovered penicillin, which was the first microbial antibiotic to be made commercially. Penicillin is one of the so-called secondary metabolites that are not products of microbial energy metabolism. After World War II the number of products made by microbial fermentations grew rapidly to include an expanding spectrum of antibiotics, amino

acids for animal feed, and enzymes. The wide range of products that can be made by microbial fermentation can be classified as shown in Table 3.2.

Throughout the nineteenth century and the first half of the twentieth, microbial production accounted for the bulk of the world's supply of organic chemicals. Raw materials are usually the key to the economics of commodity products, however, and this is true for organic chemicals as well. In 1920, Standard Oil of New Jersey initiated the production of organic chemicals from petroleum when the company began synthesizing isopropyl alcohol from propylene. Over the following decades, and especially after 1950, the use of petrochemical feedstocks for chemical manufacture grew rapidly because of the ready availability and low cost of petroleum and the rising costs of the plant-derived raw materials.

Despite the existence of numerous fermentation routes to the organic compounds, fermentation as a source of these chemicals declined rapidly although some are still obtained largely by microbial fermentations. These include ethanol, a number of organic acids, and the more complex organic chemicals, such as antibiotics, that require multiple synthetic steps.

However, the oil price shocks of the 1970s not only abruptly changed the relative costs of the raw materials for chemical and fermentation syntheses, but have also exposed the instability of the world petroleum market. As a result agricultural feedstocks once again became more attractive. In the United States, for example, fermentation facilities were rapidly constructed for producing ethanol from glucose that is obtained from cornstarch. Brazil also initiated a major programme for producing ethanol by fermentation methods, in this case using sucrose from molasses.

Table 3.2. Classification of fermentation products

Class	Examples
1. End-products of energy metabolism	Ethanol, acetic acid, lactic acid, butanol, acetone
2. Energy storage compounds	Glycerol, glycogen, other polysaccharides
3. Proteins	
Extracellular enzymes	Amylases, cellulases, proteases, amyloglucosidase, pectinase
Intracellular enzymes	Glucose isomerase, ligases, glucose oxidase, invertase
Foreign proteins	Insulin, human growth hormone, bovine growth hormone
4. Cell structures	Baker's yeast, bioinsecticides, single-cell protein, antigens
5. Intermediary metabolites	Amino acids, citric acid, vitamins, malic acid
6. Secondary metabolites	Antibiotics, gibberellins
7. Chemically modified substances	Steroids, sorbose, glucose

The advent of recombinant DNA technology, which also occurred in the 1970s, has added another new dimension to the manipulation of microbial metabolism. The range of products made by microbial cells can now be expanded; the organisms are being made to function as miniature chemical plants for synthesizing foreign proteins such as human insulin and growth hormone (see Chapter 4). Other genetic modifications may increase the efficiency of current fermentations or give the microbes the ability to grow on previously unusable energy sources.

Industrial solvents

Approximately 80 per cent of the ethanol produced in the world is still obtained from fermentations; the remainder comes largely by synthesis from the petroleum product, ethylene. The alcohol produced in the United States is primarily used in alcoholic beverages (Table 3.3), but this is not always the case elsewhere in the world. Brazil has embarked on a major programme to produce ethanol for fuel and thereby diminish petroleum imports. As of 1984, approximately 7.9 million tons of ethanol were produced by fermentation in Brazil, with sucrose from sugarcane as the carbon source.

The United States is also substantially increasing its fuel alcohol production, originally because of the rapid increase in petroleum costs during the 1970s and the subsequent need for developing alternative energy sources. More recently, as the petrol supply has increased, if only temporarily, the fuel alcohol programme has been sustained by the drastic, federally mandated reduction of the lead content of petrol and the value of ethanol as an octane enhancer.

A number of microorganisms can convert glucose in the absence of oxygen to by-products of energy metabolism, which typically include alcohols such as ethanol, isopropanol and butanol, and short-chained organic acids such as formic, acetic, lactic, propionic and butyric acids. Other products that can be produced are acetoin, 2,3-butanediol and acetone.

Most of the microorganisms that are capable of anaerobic metabolism can produce ethanol, but many are unable to make appreciable quantities because they can not tolerate the alcohol's

toxic effects on the cell membrane. Some microorganisms can accumulate high concentrations of ethanol, however. Historically, the most used microbe has been the yeast, *Saccharomyces cerevisiae* which can produce ethanol to give concentrations as high as 18 per cent of the fermentation broth. This yeast can grow both on simple sugars, such as glucose, and on the disaccharide sucrose, which is common table sugar. *Saccharomyces* is also generally recognized as safe as a food additive for human consumption and is therefore ideal for producing alcoholic beverages and for leavening bread.

In the metabolic pathway that produces ethanol, the yeast converts one molecule of glucose to two molecules of the alcohol plus two molecules of carbon dioxide (Fig. 3.1). The microorganism also gains two molecules of the energy intermediate adenine triphosphate (ATP), which it uses to maintain other cellular activities. If no cell mass were produced, 51 per cent of the glucose would be converted to alcohol. However, the actual yield is closer to 45 per cent because some of the glucose is converted to cell mass and to by-products, such as glycerol.

Although ethanol is primarily produced from glucose and sucrose, a wide range of other sugars can also be used. The sugars can be obtained from a variety of raw materials. Sugar-cane and sugar beets are the most common sources of sucrose, while any starch-containing plant material, depending on its cost and starch content, is a potential candidate for conversion to glucose. Fruits are also sources of glucose and fructose, as in the production of fruit wines. In the United States, corn is the preferred source of starch for the production of fuel alcohol, and blends of grains - wheat, corn, rye and barley - are used for making beer and distilled spirits. Molasses is the sugar source for rum production.

Although a great deal of research activity has been directed towards developing cellulose as a raw material for fermentation, it has yet to prove cost-competitive with the sugars and starches. Lactose from whey has also been used as a raw material, again with limited success. Not enough is available for large-scale operations nor are there yeast species that are both capable of using lactose as an energy source and tolerant of high alcohol concentrations. Moreover, whey contains high concentrations of dissolved minerals that further inhibit fermentation.

When corn is used as the starch source for fuel alcohol production, the germ is first separated out so that the valuable corn oil and protein can be recovered. In the corn wet-milling process, for example, the grain is first steeped in water for two days (Fig. 3.2). The softened corn is then broken in a shredding device, the degerminator, and the germ is separated by flotation. The remainder of the corn is wet ground, the fibre and hulls removed by screening, and the starch separated in centrifuges. The stream from the centrifuges, which contains the protein gluten, is dehydrated and the residue dried for sale as an animal feed. The starch is recovered by vacuum filtration and may either be dried before it is sold or further processed into fermentable sugars.

For ethanol production, the starch slurry is liquefied at a

Table 3.3. *US fermentation production of ethanol 1984*

Product	Annual production (million tons, as ethanol)
Beer	2.2
Fuel alcohol	1.6
Distilled spirits	1.1
Wine	0.5
<i>Total</i>	5.4

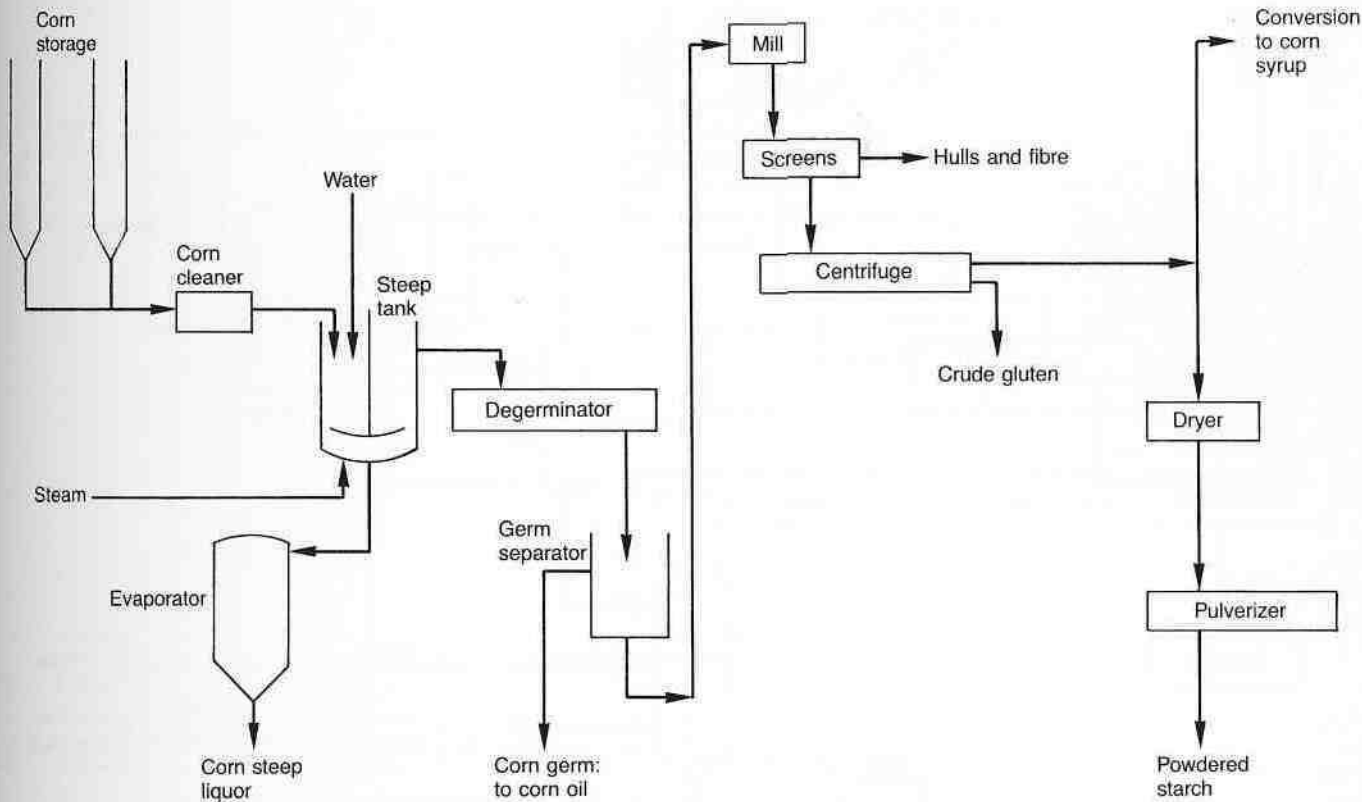


Fig. 3.2 Schematic diagram for the corn wet-milling process. The diagram is explained in the text.

temperature of 90 °C by alpha-amylase enzymes that can withstand this high temperature (Fig. 3.3). The conversion to sugars is completed at lower temperatures using glucoamylase enzymes. Approximately 98 per cent of the starch is converted to fermentable sugars by this treatment. The sugars are then fermented to produce ethanol by industrial strains of *Saccharomyces cerevisiae*.

In a large, efficiently run facility the carbon dioxide produced by the fermentation reaction is recovered for sale as a by-product. After the reaction is complete, the yeast and other solids are separated from the fermentation broth by centrifugation and dried for sale as an animal feed supplement. The clarified broth is sent to a beer still to recover and partially concentrate the ethanol. Further distillation in a rectifying distillation column brings the ethanol concentration to 95 per cent, the maximum that can be achieved by distillation of a water solution. The remaining water is then removed by distillation with another solvent such as benzene, thus producing pure alcohol that can readily be blended into high-octane petrol or used as an industrial solvent.

The acetone-butanol fermentation converts various raw materials into two valuable industrial chemicals with ethanol

as a by-product. Hydrogen and carbon dioxide may also be recovered as by-products in a sufficiently large production facility. The bacterial strain most frequently used for acetone and butanol production is the strict anaerobe *Clostridium acetobutylicum*, although *C. butylicum* may be used instead. Consequently, the fermentation can be run only in the complete absence of oxygen.

The acetone-butanol fermentation may start with such raw materials as molasses, starches, cellulose, whey or the sulphite liquor from paper manufacture, as sources of glucose. The bacteria are aided by the availability of complex nitrogen sources that contain proteins and vitamins, in addition to a glucose source. Although corn meal is ideal as a provider of starches, proteins and vitamins, molasses and corn steep are preferred for commercial operations because of their lower prices.

Ethanol production by *Saccharomyces cerevisiae* requires that starches first be hydrolysed enzymatically to glucose. However, *Clostridium* species can do this conversion themselves because during cell growth they produce and secrete amylase enzymes. Once the glucose is released from the raw material by the bacterial enzymes it enters the glycolytic pathway where it produces pyruvate and acetyl CoA, with a release of carbon

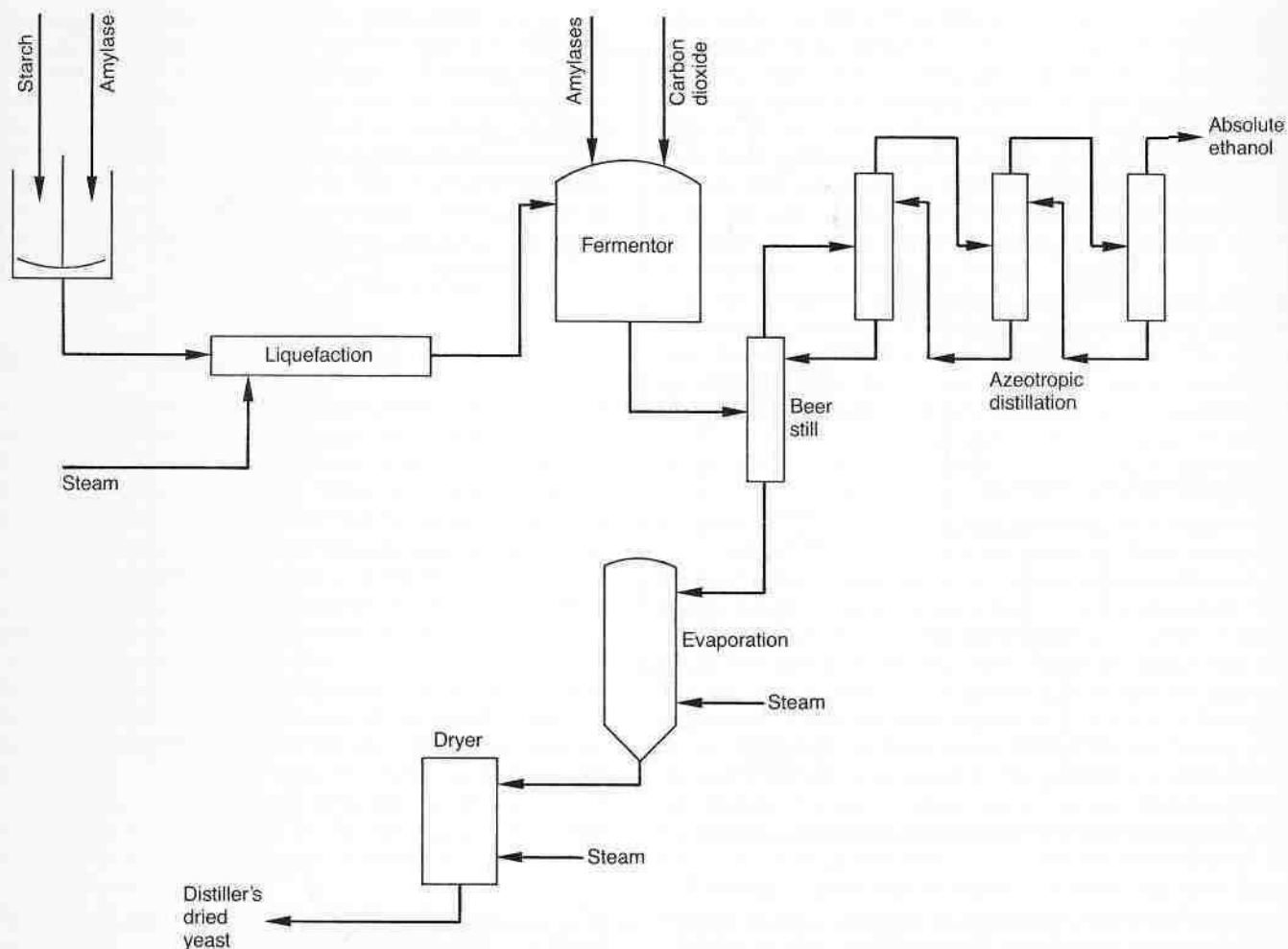


Fig. 3.3 Schematic diagram for the industrial fermentation of sugar to ethanol. The diagram is explained in the text.

dioxide and hydrogen gases (Fig. 3.4). The first phase of the fermentation is complete with the conversion of the pyruvate and acetyl CoA to butyric and acetic acids.

In the second phase, the mixed acids are converted to the mixed solvents butanol, acetone and ethanol, which are present in final ratios of approximately 60 to 30 to 10. The fermentation is over in about 36 hours when the butanol concentration becomes high enough to disrupt the cell membrane and stop the reactions. This occurs at a concentration of approximately 12 grams of butanol per litre of fermentation broth. At the end of the fermentation, conversion of the intermediates to butanol is not complete, but a spectrum of intermediates remains (Table 3.4). Because the residual solids from the fermentation broth are rich in the vitamin riboflavin, it is economic to recover them for sale as a protein-rich supplement for animal feed.

Table 3.4. End-products of the acetone-butanol fermentation^a

Product	<i>C. acetobutylicum</i>	<i>C. butylicum</i>
Butanol	23	24
Acetone	7	—
Ethanol	2	—
Carbon dioxide	54	50
Hydrogen	2	1
Acetic acid	5	6
Butyric acid	2	8
Acetoin	3	—
Isopropanol	—	4

^aYields are expressed as grams product formed per 100 grams of glucose consumed.
(Data converted from that of Doelle, H.W. (1975) *Bacterial Metabolism*, 2nd edn. Academic Press, New York.)

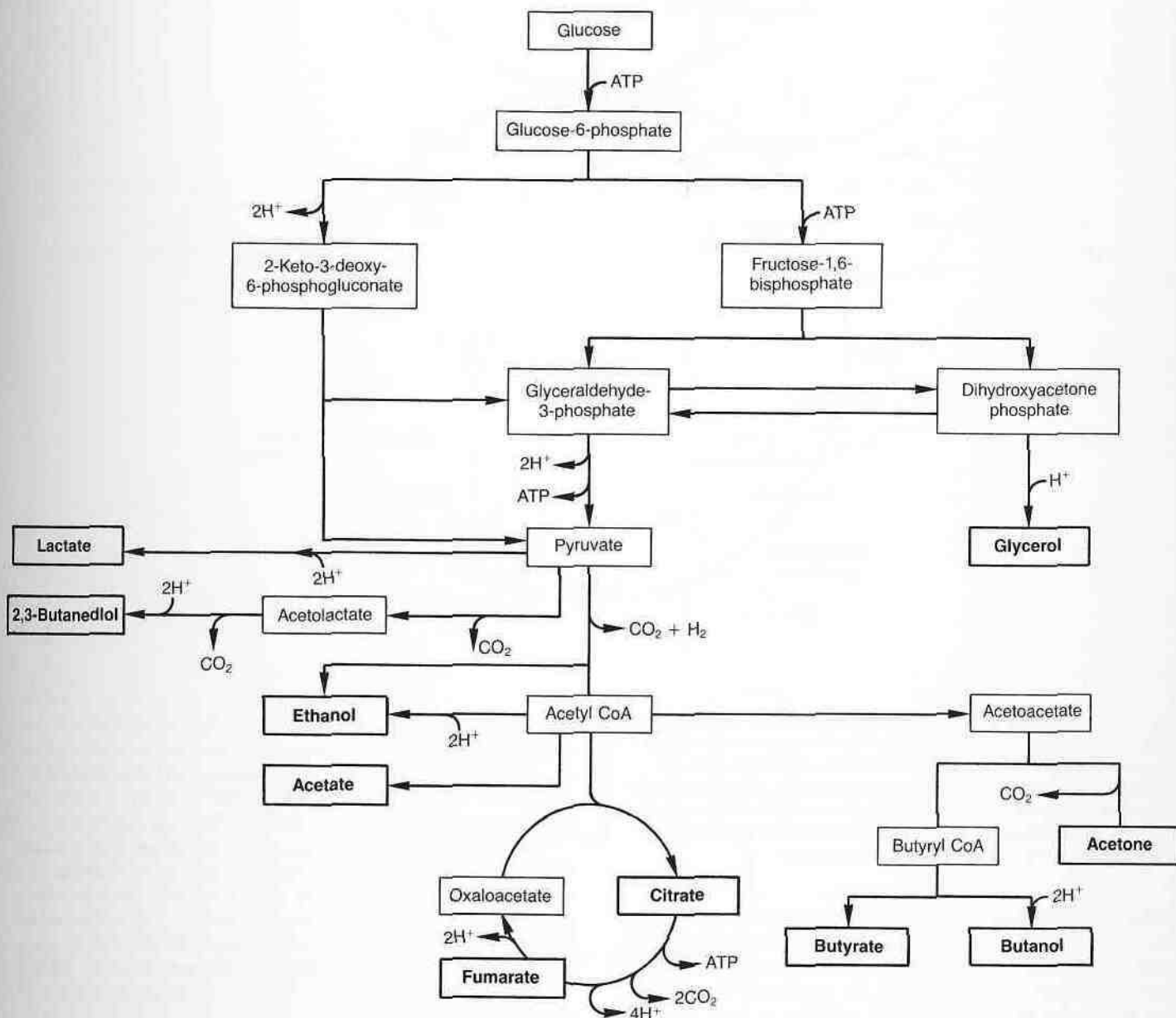


Fig. 3.4 The reactions involved in the fermentation of glucose to butanol and acetone. The glycolytic pathway converts the glucose to pyruvate which is then converted to acetyl CoA. The acetyl CoA in turn is converted to a variety of products, including acetone and butanol, as well as several organic acids.

Organic acids

A wide variety of organic acids can be produced by microbial fermentations. Except for alcohol, these were the earliest fermentation products. Acetic acid in the form of vinegar is the

classic example. During the mid-nineteenth century the acid began to be produced industrially for use as a chemical intermediate. This was followed by the development of fermentation processes for making propionic, gluconic, citric and lactic acids, and during the early twentieth century fumaric, malic, itaconic and oxogluconic acids were added to the list of fermentation products. A tartaric acid fermentation process has also been developed but this acid is commercially produced as a by-product of wine fermentations.

Of the other organic acids, the major fermentation product today is citric acid, although significant quantities of lactic,

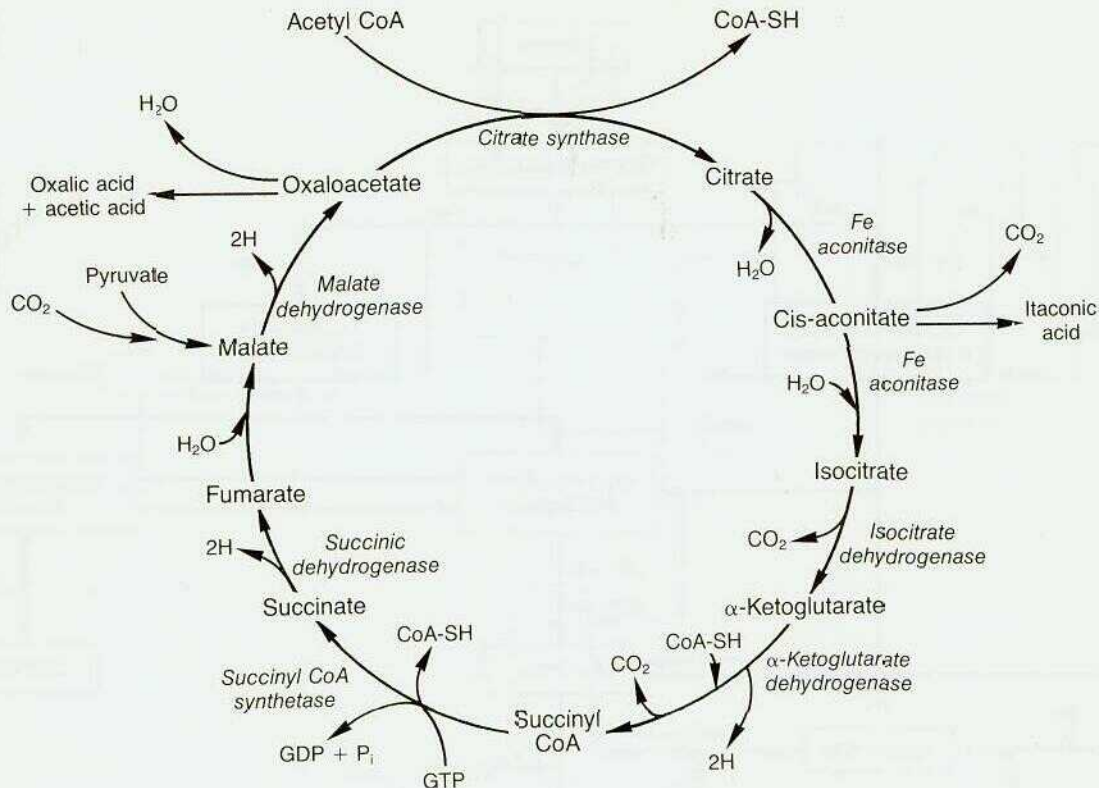


Fig. 3.5 The tricarboxylic acid cycle. This cycle is one of the cell's major pathways for energy production. The first step is the synthesis of citrate from acetyl CoA and oxaloacetate. The next step, the conversion of citrate to *cis*-aconitate, requires the cofactor iron (Fe). In the industrial production of citrate steps are usually taken to remove iron ions from the fermentation mixture to prevent *cis*-aconite formation and thereby improve the yields of citric acid.

gluconic and itaconic acids are also still produced by fermentation. Fumaric and malic acids can be obtained either by fermentation or by organic synthesis, depending on the costs of the feedstocks. Currently, these acids are produced by isomerization and hydration of maleic acid, which is in turn made by the catalytic oxidation of petroleum-based hydrocarbons. Because of regulatory requirements, acetic acid in the form of vinegar is still produced by fermentation, but the industrial grade of the acid is made by catalytic oxidations of low molecular weight petrochemicals or by catalytic carbonylation of methanol.

Citric acid is used widely in foods and beverages as an acidulant and flavour enhancer and industrially as a component that improves detergent action, as a chelator for metals, and as an antioxidant. Until 1930 the acid was produced from citrus fruits, primarily in Italy, although an alternative source, namely fermentation by moulds, had been discovered in the late

nineteenth century. Over the following decades of development of the fermentation process numerous genera of moulds, including *Aspergillus*, *Penicillium*, *Paecilomyces*, *Mucor* and *Ustilina*, were found to produce this acid. More recently, most yeasts and many bacteria were also found to be good producers.

The fermentation production of citric acid was improved by Pfizer, Inc., of Groton, Connecticut, and commercialized in 1923, after which fermentation rapidly became the lowest cost process and dominated the world market. Today, the world demand for citric acid is approximately 250 000 metric tons, approximately one-half of which is still produced by Pfizer. Miles Laboratories, Inc., of Elkhart, Indiana, makes about one-quarter of the total.

Citric acid is synthesized naturally as an intermediate in the tricarboxylic acid cycle, one of the cell's major pathways for energy production (Fig. 3.5). Carbon feeds into the cycle in the form of acetyl CoA from the glycolytic pathway. Consequently, any carbon source that can enter glycolysis can be used to produce citric acid. The most common carbon sources have been low-cost sugars such as those in beet and cane molasses. More recently, hydrocarbons that are oxidized by the *Candida* species of yeast to acetyl CoA have been shown to be economically attractive sources of carbon for the fermentation.

Citric acid was originally produced in tray cultures with the moulds growing on the surface of the fermentation mixture

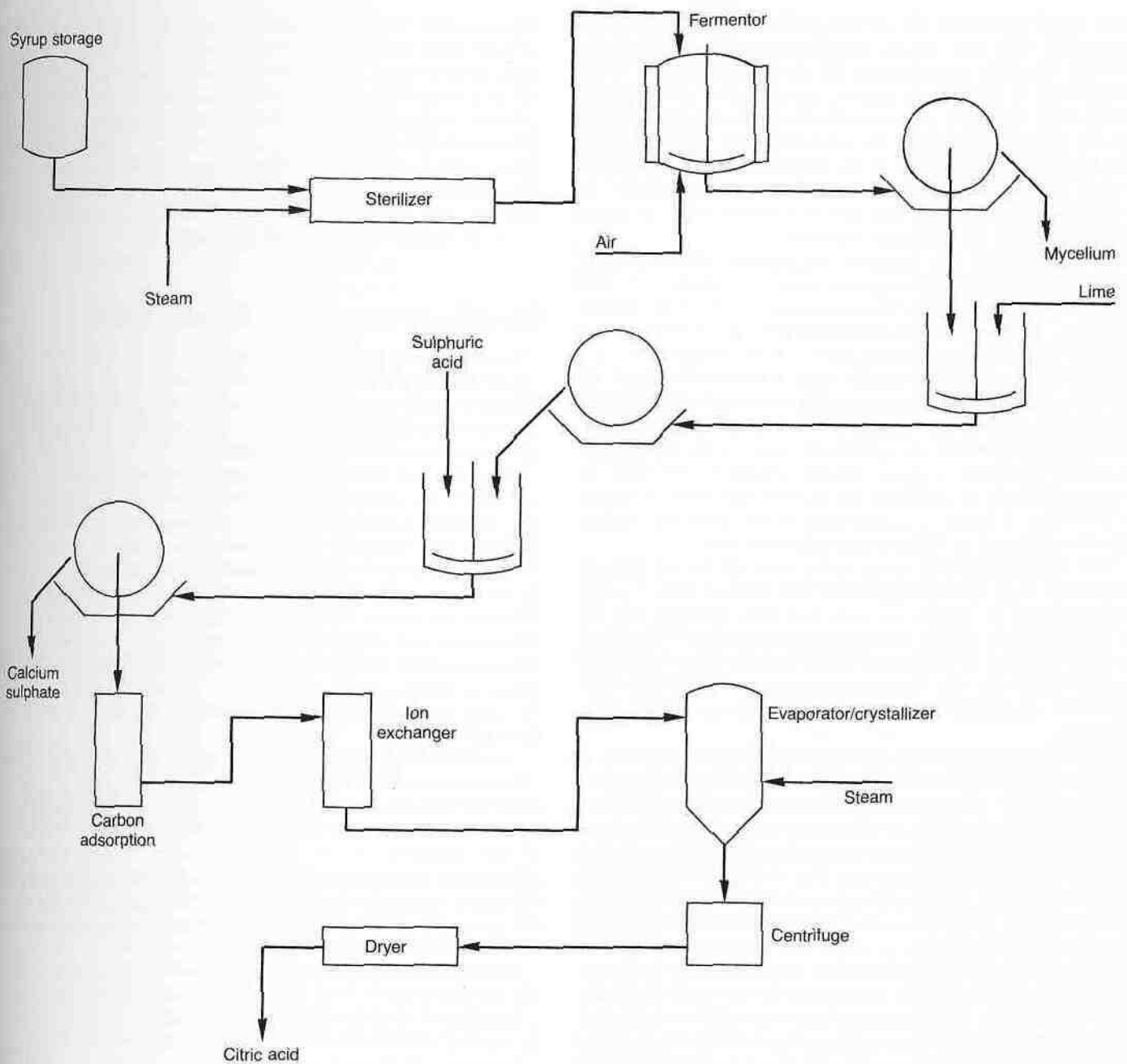


Fig. 3.6 Schematic diagram of citric acid manufacture. The diagram is explained in the text.

and oxygen provided by free diffusion. However, submerging the mould in the broth in an aerated fermentor tank gives higher production rates and is now the preferred method. *Asper-*

gillus niger, which was used from the beginning of citric acid production, is still the mould of choice when the substrate is a sugar.

Early work on the development of the fermentation process showed that pure substrates are required to attain high citric acid production. The metal ions that usually contaminate the substrates have an adverse effect. This is especially true for

iron, which stimulates the growth of the microbial cells at the expense of citric acid production. Iron is required for growth, but the optimum concentration for the fermentation is in the range of 0.1 to 10 parts per million, depending on the microbial strain used. Citrate is not normally overproduced and released by the microorganism and the iron deficiency serves to block the tricarboxylic acid cycle at the step in which citrate is converted to d⁺-aconitate. As a result, citrate accumulates. To enhance citric acid yields the iron is removed from the sugars by means of an ion exchange treatment.

Other additives can mitigate the negative effects of iron, however. Potassium ferrocyanide forms precipitates of trace metals and reduces their concentrations in the fermentation medium. The optimum concentration of potassium ferrocyanide for citric acid production is in the range of 0.1 to 1 gram per litre, depending on the initial trace metal content. In addition, alcohols such as methyl, ethyl and isopropyl stimulate yields when used in the concentration range of 1 to 4 per cent. Higher concentrations are sufficiently toxic to the cell growth to reduce the yields. Copper can also counteract the effect of iron, presumably by inhibiting the enzyme that converts citrate to aconitate. A copper concentration of 500 parts per million can counteract up to 10 parts per million of iron.

The sugar-containing syrup to be used in the submerged process of citric acid production is first treated with a cation exchange resin to remove the iron. It is then sterilized and fed to the fermentor, which has already been sterilized (Fig. 3.6). A water solution of nutrients, which has also been sterilized, is introduced into the fermentor to supply the reaction with ammonia, phosphate and other minerals that are missing from the syrup.

The pH in the fermentor is adjusted to the range of 5 to 6 when molasses is the substrate or below 3 if purer forms of sugars are used. After the temperature has been brought up to 30 °C the tank contents are inoculated with *Aspergillus niger*. The fermentation is allowed to proceed for 4 to 5 days, during which time 120 to 150 grams per litre of sugar are converted to 100 to 140 grams per litre of citric acid. To achieve the highest yields sufficient aeration must be maintained to prevent oxygen starvation of the microbe.

At the end of the fermentation the broth is filtered and then treated with lime to raise the pH and precipitate out the calcium salt of citric acid. The precipitate is recovered from the slurry by filtration and redissolved with sulphuric acid, thus producing a calcium sulphate precipitate, which is removed by another filtration step. The acidified citric acid is treated with activated carbon to remove coloured impurities and by ion exchange to remove residual calcium and other cations. The citric acid crystallizes when it is concentrated in an evaporator, after which the crystals are recovered in a centrifugal filter and dried in a rotary kiln drier.

Lactic, itaconic and gluconic acids, the remaining commercial acids that are still made in significant quantities by fermentation, are all produced in a fashion similar to the citric acid

fermentation. Species of *Aspergillus* are used except in the case of lactic acid, which is produced instead by species of *Lactobacillus* or *Streptococcus*. The fermentations are run either with molasses as a source of sucrose or starch as a source of glucose. The product concentrations reached in organic acid fermentations generally fall in the range of 5 to 10 per cent by weight. Although lactic acid is produced in the absence of oxygen, the other fermentations all require oxygen for high productivity. Lactic, itaconic and gluconic acids are recovered and purified by methods similar to those used for citric acid. •

Economic aspects of fermentation

Microorganisms can be considered as microscopic chemical plants that assimilate raw materials and synthesize chemical products while reproducing. The efficiency of the processes within the microbes is often near 100 per cent, with little production of unnecessary by-products.

Over the years the technology that uses these microbial chemical plants has changed a great deal. Through the first half of the twentieth century it was fairly simple. Early fermentations involved the anaerobic production of solvents in large vats. Even today, potable alcohol is frequently produced in 20 000 gallon wood tanks with open tops and no instrumentation to monitor the course of the fermentation reaction. Aerobic fermentations were originally carried out in tray cultures with the microorganisms growing on the surface of a layer of grain or grain products. Acetic and gluconic acids were produced by bringing the fermentation broth into contact with air in beds of wood shavings.

However, the need to produce yeast in large volumes led to the development of large fermentation vessels into which air could be introduced. Once the importance of penicillin was established during World War II, the commercial production of this antibiotic led to the infusion of modern chemical engineering into fermentation technology. Fermentation vessels were then routinely designed for sterilization and control of pH and temperature, although fermentations were still carried out in the 'batch mode' (Fig. 3.7).

In this type of operation the vessel and its nutrient contents are sterilized, and a fresh inoculum of rapidly growing cells is introduced so that the cell volume amounts to 1 to 10 per cent of the total vessel contents. For aerobic fermentations the vessel is agitated and filtered air is introduced. After 2 to 5 days of growth, the fermentation broth is pumped out and filtered to remove the cell mass and solids before the product is recovered and purified. More recently, fermentation operations have been extended to include the continuous type, either with or without recycling of the cells. This permits the processing of more material per vessel with a corresponding reduction in capital costs. The production of high-volume chemicals such as ethanol has been greatly facilitated by the use of the continuous system with cell recycling.

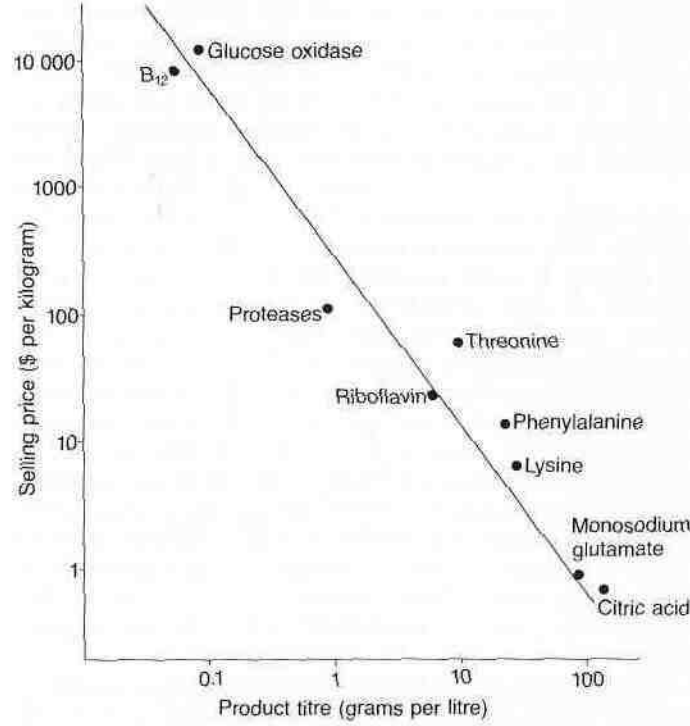
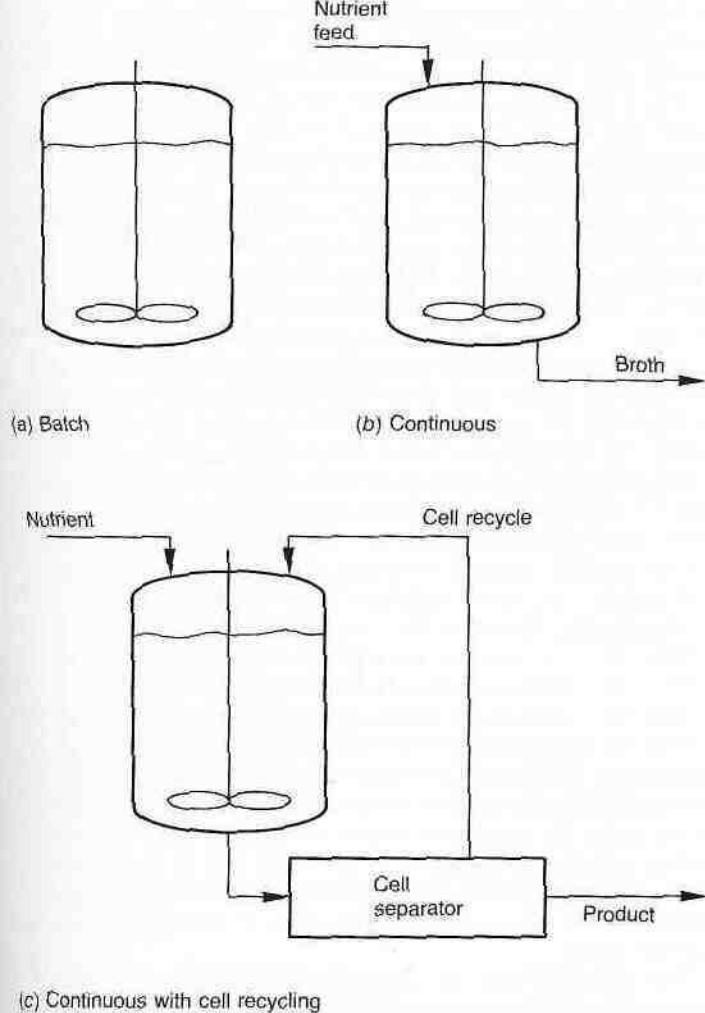


Fig. 3.8 Effect of product titre on the selling price. The higher the concentration of the product in the fermentation broth, the lower the selling price will be.

products can be directly related to the product concentration in the fermentation broth (Fig. 3.8). The effect is particularly strong for concentrations below 0.1 per cent. Above a 1 per cent concentration the production costs are more dependent on the special requirements of the individual product. An example is the fermentation cost for the production of an amino acid. As the titre increases above 10 grams per litre it has a diminishing effect on the production cost (Fig. 3.9).

A second important cost factor is the yield of product from the carbon source, which typically accounts for 20 to 60 per cent of the total production cost. High yields are therefore imperative to keep those costs down. This is particularly true for the less expensive industrial chemicals. Breakdown of the fermentation costs for a typical organic acid at two different yields illustrates that the cost of the raw material drops significantly as the product yield and titre rise (Table 3.5). The percentage of the total remains almost unchanged, however. The utility costs also drop very significantly because the higher yield permits a large decrease in the fermentor volume.

The recovery and purification costs can be highly variable, depending on the product. Some products, the hydrolytic enzyme amylase, for example, are recovered simply by removing the cell mass from the fermentation broth; further purification

Fig. 3.7 Three types of fermentor operation. In the batch mode (a) the microorganisms and the necessary nutrients are simply mixed in the fermentor until the reaction has reached the desired stage of completion. At that time the contents of the vessel are withdrawn and the product recovered. In the continuous mode, (b) a nutrient solution is fed slowly into the fermentor while the broth with the product is slowly withdrawn. Some continuous operations allow for the cells that are removed with the broth to be recycled back into the fermentor (c).

The key factors that influence production costs are the fermentation productivity, that is, the product's titre (concentration in the fermentation broth) per cycle time; the yield from the carbon source; and the ease of recovery and purification of the product. Because the cycle times of most fermentations are 2 to 3 days, the productivity is most affected by product titre. Over four log cycles the prices of a wide range of fermentation

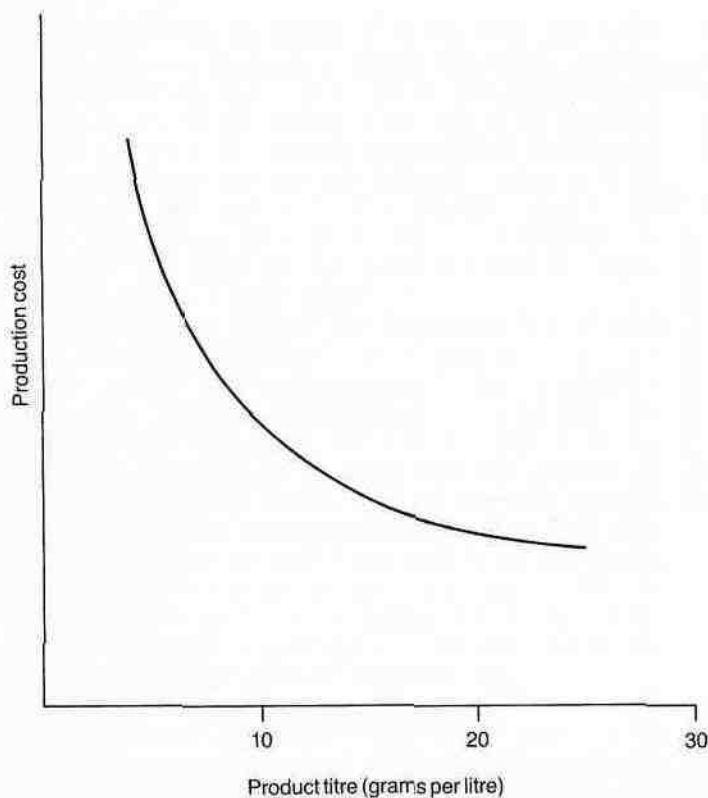


Fig. 3.9 Effect of the product titre on the production costs of an amino acid. The price comes down rapidly until the titre reaches 10 grams per litre, after which further increases in the concentration have a diminishing effect on price.

Table 3.5. Fermentation product cost breakdown

Annual production: 5 million pounds Carbon source: glucose				
Titre:	10 grams/litre		40 grams/litre	
Product yield ^a :	30%		63%	
	\$/lb	%	\$/lb	%
Raw materials	1.55	45	0.93	43
Utilities	0.61	17	0.20	9
Labour	0.79	23	0.79	36
Capital	0.53	15	0.27	12
Total	3.48		2.19	

^aProduct produced per unit of glucose consumed, as percentage of theoretical maximum.

is not needed. Other products, such as the proteins that are intended for therapeutic use in human patients, require careful purification to remove contaminating agents.

For a fairly simple commodity product, such as an organic acid, the recovery and purification costs amount to approximately one-half of the capital cost investment and about one-half of the operating costs. Other industrial chemicals, including absolute alcohol, which must be distilled, also have approximately one-half of the total cost tied up in the recovery and purification operations. These processes are very sensitive to the size of the facility. Substantial cost savings can therefore be realized as the production capacity increases.

A number of problems have plagued the fermentation industry from the outset. One of the most pervasive and troublesome is microbial contamination. Once a competing microorganism infects the fermentation batch, the product yield drops and the product quality may decline to unacceptable levels. The result may be a complete loss of the contaminated fermentation batch. This could represent up to 1 per cent of the annual production of the product, because there are usually no more than 100 to 150 batches per year. Although many fermentations become contaminated, few result in a total loss.

The bacterial viruses called phages can produce a second type of contamination. Phage infection usually results in a rapid lysis of the entire population of production cells and a complete loss of the fermentation. The bacteria used to produce organic and amino acids are particularly susceptible to this type of contamination and must be protected by operating the fermentation under the strictest of aseptic conditions. Once a contamination occurs, the facility is at increased risk of further problems. The source of the contaminant must be quickly determined and all infected areas sterilized to protect against repeat contaminations.

The mutants that may spontaneously appear in microbial populations constitute a third type of contaminant. The larger the number of generations during the fermentation cycle, the more likely will be the appearance of a less productive mutant subpopulation. This is one of the principal reasons why continuous fermentation processes have not been adopted for many products. The more the producing organism has been modified and selected for high product formation, the greater is the selection pressure for a non-producing subpopulation of the microorganism to take over the batch.

A second production constraint is the need to provide sufficient oxygen transfer rates to aerobic fermentations to maintain growth rates at high cell densities. As the microbial population grows, nutrients may be supplied at high rates simply by using pumps, but the means of oxygen introduction may be less efficient. For rapidly growing bacterial populations in vessels of less than 1000 litre capacity, oxygen availability is the most likely limitation on cell growth. As the vessel capacity is increased beyond 1000 litres, however, heat transfer may become a more significant limiting factor because increased vessel size means that there is less surface area per unit of

volume for heat release. In that event, oxygen transfer may be sufficient, but the heat removal insufficient to maintain the correct fermentation temperature.

The viscosity of the fermentation broth constitutes a third possible production constraint. Some of the fungal microorganisms form networks of filaments called mycelia. Although these organisms, if slow-growing, may not be limited by the oxygen supply, the viscosity of the broth may become sufficiently high as the cell population increases for the broth to become unpumpable. As this point the fermentation must be halted, because the broth needs to be transferred by pumps to subsequent processing steps.

A fourth constraint is the need to dispose of the unused components of the fermentation broth. The largest waste component is usually the cell mass itself. Before environmental pollution was identified as a major problem, the cell mass was simply dumped, but once regulatory agencies prohibited this practice, wastes were instead processed through treatment facilities at sites available from the local municipalities. This expense encouraged the development of the solid waste matter as a high-protein additive for animal feed. Today most commercial microorganisms have been approved as feed additives. The exceptions to this include *Escherichia coli*, which is one of the principal bacterial species used for making foreign proteins, and other bacteria that contain toxins. Instead of obtaining a 5 to 10 cent per pound credit for selling the residual cell mass from these organisms, the manufacturer instead has to pay out 10 to 20 cents a pound to dispose of the material in a landfill.

The impact of recombinant DNA technology

The early use of microorganisms for fermentations relied on the selection of specific, naturally occurring strains that produced the desired end-products. These included the alcohol-producing yeasts and the naturally occurring moulds that yielded the organic acids. Although the fundamental metabolic pathways of these organisms are essentially the same, the various species evolved so that they could grow under differing environmental conditions of oxygen concentration, pH and temperature. The organic acids, for example, lower the pH of the surrounding environment to the level preferred by the microorganism, and antibiotics kill foreign bacteria that might be competitors. The production of by-product solvents such as ethanol allows microorganisms to maintain the correct balance between the reduced and oxidized forms of the molecules that participate in energy metabolism. The ability to use different substrates as energy sources also provided competitive advantages for the microbes.

Because the microorganisms evolved to survive and multiply rapidly in specific environments, they became very efficient, producing very little waste. This means, among other things, that the cells maintain very tight control over the numerous

biochemical pathways that they carry out, and that it is therefore necessary to upset these internal controls to increase product yields. Often the control is exerted by blocking expression of the genes that code for the products themselves or for the enzymes needed to make or degrade the products. The cell can also exert a finer level of regulation by blocking the activity of the enzymes after they are made.

One way of disrupting these internal controls is to use various mutating agents, such as chemicals or radiation, that alter the DNA of the cell at random locations. Most frequently, the mutants formed are crippled and of little value. By mutating and screening a large population, however, mutants can be found in which the DNA is disrupted at specific locations that cause the cell to lose its control either over the synthesis of an important enzyme in the pathway that makes a desired product or over the enzyme's activity. As a result, the cell produces increased concentrations of the product and secretes it into the surrounding medium. The process of mutation followed by selection was, and still is, a tedious and imprecise procedure for obtaining a specific deregulated microorganism.

Recombinant DNA technology offers a more direct way of manipulating the cell's metabolism for the production of specific biochemical products. The technology has proved extremely useful for determining the nucleotide sequences of the DNA in and around genes and identifying those segments that are needed for the control of the genes or their products. These segments constitute prime targets that might be specifically mutated or deleted to alter the control of the genes. New genes might also be introduced into bacterial cells to give them

- novel synthetic capacities.

The first impact of recombinant DNA technology on microbial production of industrial chemicals will most probably be improvement in the yields. Most carbon sources currently consist of starches that must be hydrolysed to simple sugars before they can be used by the microorganism. The hydrolysis does not go to completion but leaves 1 to 2 per cent of the starch as limit dextrans, which are short-chained polysaccharides containing the branch points of the original molecule. The hydrolysed starches also contain unhydrolysed cellulose amounting to as much as 10 per cent of the total carbohydrate.

If new genes that encode enzymes for digesting the limit dextrans and cellulose could be introduced into industrial microorganisms, their carbon source utilization might increase by 2 to 10 per cent. Such a strategy might also obviate the need for the chemical and enzymatic hydrolysis that is now required to convert polysaccharides such as starch into sugars. Introduction of other genes into the microbes might allow carbon sources such as lactose from whey or pentose sugars from the paper and pulp industry to be substituted for hydrolysed starch. By adding new genetic capabilities to commercial microorganisms, additional low-cost raw materials could become available for chemical manufacture.

One of the major goals of fermentation process development is to increase the product yields while reducing the complexity

and costs of the raw materials. Using the maximum amount of the raw material for direct production of the end-product with minimal by-product accumulation is most desirable. The primary by-product is the cell mass that accumulates as the microorganisms grow. The best approach to minimizing this accumulation is to uncouple growth from product formation so that the biomass can be used for extended times for the sole purpose of converting the raw materials to the end-product. This has been done for amino acid production by genetically modifying the microorganism to increase the concentrations and activities of the intracellular enzymes that synthesize the product.

For those syntheses that require energy-coupled reactions, a low level of cell growth may be necessary to maintain the oxidation-reduction balance in the cell. The credibility of the strategy of uncoupling growth from product formation is amply demonstrated by such examples as the acetone-butanol fermentation described above, in which the theoretical limit of the product yield from the intermediate organic acid is approached.

Carbon dioxide is a second major by-product of fermentations. The stoichiometry of the reactions within the cell places a limit on the amount of carbon lost to carbon dioxide. This amount can in some cases be reduced by redirecting the carbon flow within the cell. This might be done by adding new genes for alternative pathways that allow different raw materials to be used. This will be particularly effective if the alternative carbon source feeds into the synthetic pathway at a site closer to the end-product than the entry site for the original raw material. The result may be a significant improvement in the yields of traditional industrial chemicals. Modern genetic engineering techniques also permit high-yielding processes for new products to be developed in a small fraction of the time required by the traditional techniques of mutation and selection.

Reducing the length of the fermentation cycle is another approach to improving the biosynthesis of industrial chemicals. Frequently the microorganisms used for fermentations grow slowly and accumulate large quantities of end-products only after a high cell density is reached. The overall process time is typically 2 to 4 days, during which the fermentation is at risk for contamination by phage and foreign or mutant bacteria. Any reduction in cycle time therefore provides two benefits: higher productivity and, as a result, lower capital costs per unit product; and a reduction in the number of fermentation batches lost to contamination.

Growth rates might be increased by switching to a faster-growing microorganism, such as *Escherichia coli*, and engineering into it the capability of producing the desired product. Or, as already mentioned, the growth phase may be uncoupled from the production phase. This should permit the cell to grow unencumbered by the demand to synthesize large quantities of product. Once the proper cell density is reached, the microorganism can be genetically switched from the growth phase to rapid product production by activating a key enzyme within the synthetic pathway. By growing unencumbered, the microor-

ganism should be better able to compete with foreign bacteria and mutants, thereby decreasing the number of batches lost to contamination. When the end-product is toxic to cell growth, this strategy has the additional benefit of allowing the cells to reach a high density before harmful concentrations of product accumulate.

A key constraint in large-scale industrial fermentations is heat generation. Because most commercial fermentations operate near ambient temperatures, heat removal requires the use of refrigerated water to cool the fermentors and large surfaces for heat transfer. The temperature of the operations could be increased, and the problem of heat removal greatly reduced, if strains of producer microorganisms could be developed to withstand high temperatures. This might permit fermentation temperatures to be raised from approximately 35 °C to near 100 °C. Thermophilic microorganisms exist that can grow at such high temperatures.

Protein engineering is another major thrust of research on genetic manipulation. The tools of recombinant DNA techniques can be used to change the nucleotide sequence of a gene and therefore the structure of the corresponding protein. This approach is currently being used to alter the activities of new therapeutic proteins, but it also has great potential for other industrial proteins. Some of the most valuable commodities of the industrial world are fibres and adhesives. Silk, which is all protein, is one of the strongest fibres known to man. It may be possible to use genetic engineering to alter silk's structure to improve its already useful properties. Incorporation of the genes for silk production into alternative organisms may lead to its production at a reduced cost.

The protein-based adhesive made by barnacles is one of the strongest known. Genetic modification of the gene for the adhesive protein could permit the material's application in fields ranging from surgery to manufacturing. Isolation of the gene and its placement in appropriate microorganisms would allow large-scale commercial production of the protein.

In summary, the modern tools of recombinant DNA technology have now opened up an immense potential for manipulating the miniature chemical plants of microorganisms. The first products of the new technology have been therapeutic proteins, such as human insulin and growth hormone, for the health care industry. Improvements in the microbial production of amino acids, industrial organic acids and ethanol will follow, as will further improvements that will allow new raw materials to be used and lower production costs.

Once petroleum prices rise again, the application of recombinant DNA technology to the production of industrial chemicals such as those listed in Table 3.1 will be pursued with even greater vigour. The next area of major impact will be the modification of proteins and polysaccharides to create new products for industrial applications. The large amount of scientific effort now being expended on an international scale virtually guarantees these developments during the remaining years of this century.

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