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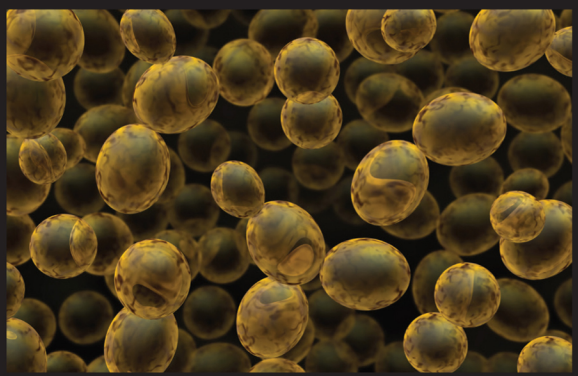
Principles of Fermentation Technology

Third Edition

Peter F. Stanbury, Allan Whitaker and Stephen J. Hall



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THIRD EDITION

Peter F. Stanbury

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This book is dedicated to all the staff, past and present,
of the Department of Biological and Environmental Sciences,
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CHAPTER

An introduction to fermentation processes

1

The term "fermentation" is derived from the Latin verb *fervere,* to boil, thus describing the appearance of the action of yeast on the extracts of fruit or malted grain. The boiling appearance is due to the production of carbon dioxide bubbles caused by the anaerobic catabolism of the sugar present in the extract. However, fermentation has come to have with different meanings to biochemists and to industrial microbiologists. Its biochemical meaning relates to the generation of energy by the catabolism of organic compounds, whereas its meaning in industrial microbiology tends to be much broader.

The catabolism of sugar is an oxidative process, which results in the production of reduced pyridine nucleotides, which must be reoxidized for the process to continue. Under aerobic conditions, reoxidation of reduced pyridine nucleotide occurs by electron transfer, via the cytochrome system, with oxygen acting as the terminal electron acceptor. However, under anaerobic condition, reduced pyridine nucleotide oxidation is coupled with the reduction of an organic compound, which is often a subsequent product of the catabolic pathway. In the case of the action of yeast on fruit or grain extracts, NADH is regenerated by the reduction of pyruvic acid to ethanol. Different microbial taxa are capable of reducing pyruvate to a wide range of end products, as illustrated in Fig. 1.1. Thus, the term fermentation has been used in a strict biochemical sense to mean an energy-generation process in which organic compounds act as both electron donors and terminal electron acceptors.

The production of ethanol by the action of yeast on malt or fruit extracts has been carried out on a large scale for many years and was the first "industrial" process for the production of a microbial metabolite. Thus, industrial microbiologists have extended the term fermentation to describe any process for the production of product by the mass culture of a microorganism. Brewing and the production of organic solvents may be described as fermentation in both senses of the word but the description of an aerobic process as a fermentation is obviously using the term in the broader, microbiological, context and it is in this sense that the term is used in this book.

THE RANGE OF FERMENTATION PROCESSES

There are five major groups of commercially important fermentations:

1. Those that produce microbial cells (or biomass) as the product.
2. Those that produce microbial enzymes.

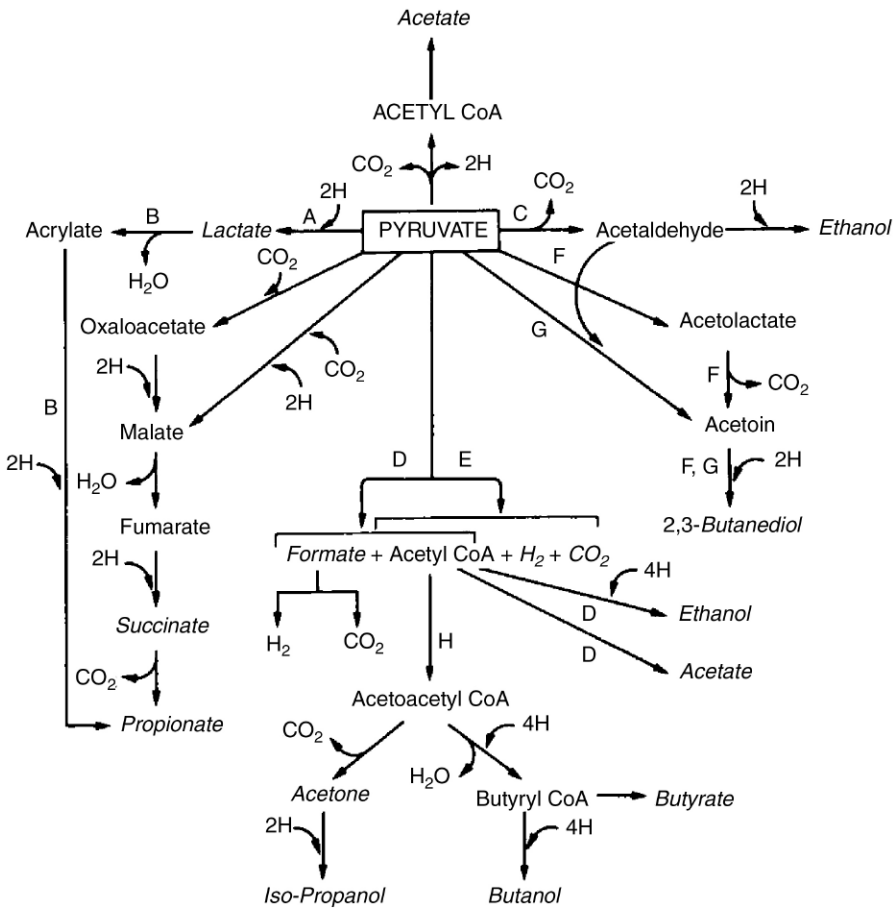

FIGURE 1.1 Bacterial Fermentation Products of Pyruvate

Pyruvate formed by the catabolism of glucose is further metabolized by pathways which are characteristic of particular organisms and which serve as a biochemical aid to identification. End products of fermentations are italicized (Dawes & Large, 1982). A, Lactic acid bacteria (*Streptococcus, Lactobacillus*); B, *Clostridium propionicum*; C, Yeast, *Acetobacter, Zymomonas, Sarcina ventriculi, Erwinia amylovora;* D, Enterobacteriaceae (coli-aerogenes); E, Clostridia; F, *Klebsiella*; G, Yeast; H, Clostridia (butyric, butylic organisms); I, Propionic acid bacteria.

3. Those that produce microbial metabolites.
4. Those that produce recombinant products.
5. Those that modify a compound that is added to the fermentation—the transformation process.

The historical development of these processes will be considered in a later section of this chapter, but it is first necessary to include a brief description of the five groups.

MICROBIAL BIOMASS

The commercial production of microbial biomass may be divided into two major processes: the production of yeast to be used in the baking industry and the production of microbial cells to be used as human food or animal feed (single-cell protein). Bakers' yeast has been produced on a large scale since early 1900s and yeast was produced as human food in Germany during the First World War. However, it was not until the 1960s that the production of microbial biomass as a source of food protein was explored to any great depth. As a result of this work, reviewed briefly in Chapter 2, a few large-scale continuous processes for animal feed production were established in the 1970s. These processes were based on hydrocarbon feedstocks, which could not compete against other high protein animal feeds, resulting in their closure in the late 1980s (Sharp, 1989). However, the demise of the animal feed biomass fermentation was balanced by ICI plc and Rank Hovis McDougal establishing a process for the production of fungal biomass for human food. This process was based on a more stable economic platform and has been a significant economic success (Wiebe, 2004).

MICROBIAL ENZYMES

Enzymes have been produced commercially from plant, animal, and microbial sources. However, microbial enzymes have the enormous advantage of being able to be produced in large quantities by established fermentation techniques. Also, it is infinitely easier to improve the productivity of a microbial system compared with a plant or an animal one. Furthermore, the advent of recombinant DNA technology has enabled enzymes of animal origin to be synthesized by microorganisms (see Chapter 12). The uses to which microbial enzymes have been put are summarized in Table 1.1, from which it may be seen that the majority of applications are in the food and related industries. Enzyme production is closely controlled in microorganisms and in order to improve productivity these controls may have to be exploited or modified. Such control systems as induction may be exploited by including inducers in the medium (see Chapter 4), whereas repression control may be removed by mutation and recombination techniques. Also, the number of gene copies coding for the enzyme may be increased by recombinant DNA techniques. Aspects of strain improvement are discussed in Chapter 3.

MICROBIAL METABOLITES

The growth of a microbial culture can be divided into a number of stages, as discussed in Chapter 2. After the inoculation of a culture into a nutrient medium there is a period during which growth does not appear to occur; this period is referred as the lag phase and may be considered as a time of adaptation. Following a period during which the growth rate of the cells gradually increases, the cells grow at a constant maximum rate and this period is known as the log, or exponential, phase. Eventually, growth ceases and the cells enter the so-called stationary phase. After a further

Table 1.1 Commercial Applications of Enzymes

Industry	Application	Enzyme	Source
Baking and milling	Reduction of dough viscosity, acceleration of fermentation, increase in loaf volume, improvement of crumb softness, and maintenance of freshness	Amylase	Fungal
	Improvement of dough texture, reduction of mixing time, increase in loaf volume	Protease	Fungal/bacterial
Brewing	Mashing	Amylase	Fungal/bacterial
	Chill proofing	Protease	Fungal/bacterial
	Improvement of fine filtration	β-Glucanase	Fungal/bacterial
Cereals	Precooked baby foods, breakfast foods	Amylase	Fungal
Chocolate and cocoa	Manufacture of syrups	Amylase	Fungal/bacterial
Coffee	Coffee bean fermentation	Pectinase	Fungal
	Preparation of coffee concentrates	Pectinase, hemicellulase	Fungal
Confectionery	Manufacture of soft center candies	Invertase, pectinase	Fungal/bacterial
Cotton	Low temperature processing	Pectate lyase	Fungal
Corn syrup	Manufacture of high-maltose syrups	Amylase	Fungal
	Production of low D.E. syrups	Amylase	Bacterial
	Production of glucose from corn syrup	Amyloglycosidase	Fungal
	Manufacture of fructose syrups	Glucose isomerase	Bacterial
Dairy	Manufacture of protein hydrolysates	Protease	Fungal/bacterial
	Stabilization of evaporated milk	Protease	Fungal
	Production of whole milk concentrates, ice cream, and frozen desserts	Lactase	Yeast
	Curdling milk	Protease	Fungal/bacterial
Eggs, dried	Glucose removal	Glucose oxidase	Fungal
Fruit juices	Clarification	Pectinases	Fungal
	Oxygen removal	Glucose oxidase	Fungal
Laundry	Detergents	Protease, lipase	Bacterial
Leather	Dehairing, baiting	Protease	Fungal/bacterial
Meat	Tenderization	Protease	Fungal
Paper	Removal of wood waxes	Lipase	Fungal
Pharmaceutical	Digestive aids	Amylase, protease	Fungal

Table 1.1 Commercial Applications of Enzymes (*cont.*)

Industry	Application	Enzyme	Source
	Antiblood clotting	Streptokinase	Bacterial
	Various clinical tests	Numerous	Fungal/bacterial
	Biotransformations	Numerous	Fungal/bacterial
Photography	Recovery of silver from spent film	Protease	Bacterial
Protein hydrolysates	Manufacture	Proteases	Fungal/bacterial
Soft drinks	Stabilization	Glucose oxidase, catalase	Fungal
Textiles	Desizing of fabrics	Amylase	Bacterial
Vegetables	Preparation of purees and soups	Pectinase, amylase, cellulase	Fungal

Modified from Boing (1982).

period of time, the viable cell number declines as the culture enters the death phase. As well as this kinetic description of growth, the behavior of a culture may also be described according to the products that it produces during the various stages of the growth curve. During the log phase of growth, the products produced are either anabolites (products of biosynthesis) essential to the growth of the organism and include amino acids, nucleotides, proteins, nucleic acids, lipids, carbohydrates, etc. or are catabolites (products of catabolism) such as ethanol and lactic acid, as illustrated in Fig. 1.1. These products are referred as the primary products of metabolism and the phase in which they are produced (equivalent to the log, or exponential phase) as the trophophase (Bu'Lock et al., 1965).

Many products of primary metabolism are of considerable economic importance and are being produced by fermentation, as illustrated in Table 1.2. The synthesis of anabolic primary metabolites by wild-type microorganisms is such that their production is sufficient to meet the requirements of the organism. Thus, it is the task of the industrial microbiologist to modify the wild-type organism and to provide cultural conditions to improve the productivity of these compounds. This has been achieved very successfully, over many years, by the selection of induced mutants, the use of recombinant DNA technology, and the control of the process environment of the producing organism. This is exemplified by the production of amino acids where productivity has been increased by several orders of magnitude. However, despite these spectacular achievements, microbial processes have only been able to compete with the chemical industry for the production of relatively complex and high value compounds. In recent years, this situation has begun to change. The advances in metabolic engineering arising from genomics, proteomics, and metabolomics have provided new powerful techniques to further understand the physiology of "over-production" and to reengineer microorganisms to "over-produce" end products and intermediates of primary metabolism. Combined with the rising cost of petroleum and the desirability of environmentally friendly processes these advances are now facilitating the

Table 1.2 Some Primary Products of Microbial Metabolism and Their Commercial Significance

Primary Metabolite	Commercial Significance
Ethanol	"Active ingredient" in alcoholic beverages
	Used as a motor-car fuel when blended with petroleum
Organic acids	Various uses in the food industry
Glutamic acid	Flavor enhancer
Lysine	Feed supplement
Nucleotides	Flavor enhancers
Phenylalanine	Precursor of aspartame, sweetener
Polysaccharides	Applications in the food industry
	Enhanced oil recovery
Vitamins	Feed supplements

development of economic microbial processes for the production of bulk chemicals and feedstocks for the chemical industry (Otero & Nielsen, 2010; Van Dien, 2013). These aspects are considered later in this chapter and in Chapter 3.

During the deceleration and stationary phases, some microbial cultures synthesize compounds which are not produced during the trophophase and which do not appear to have any obvious function in cell metabolism. These compounds are referred to as the secondary compounds of metabolism and the phase in which they are produced (equivalent to the stationary phase) as the idiophase (Bu'Lock et al., 1965). It is important to realize that secondary metabolism may occur in continuous cultures at low growth rates and is a property of slow-growing, as well as nongrowing cells. When it is appreciated that microorganisms grow at relatively low growth rates in their natural environments, it is tempting to suggest that it is the idiophase state that prevails in nature rather than the trophophase, which may be more of a property of microorganisms in culture. The interrelationships between primary and secondary metabolism are illustrated in Fig. 1.2, from which it may be seen that secondary metabolites tend to be elaborated from the intermediates and products of primary metabolism. Although the primary biosynthetic routes illustrated in Fig. 1.2 are common to the vast majority of microorganisms, each secondary product would be synthesized by only a relatively few different microbial species. Thus, Fig. 1.2 is a representation of the secondary metabolism exhibited by a very wide range of different microorganisms. Also, not all microorganisms undergo secondary metabolism—it is common amongst microorganisms that differentiate such as the filamentous bacteria and fungi and the sporing bacteria but it is not found, for example, in the Enterobacteriaceae. Thus, the taxonomic distribution of secondary metabolism is quite different from that of primary metabolism. It is important to appreciate that the classification of microbial products into primary and secondary metabolites is a convenient, but in some cases, artificial system. To quote Bushell (1988), the classification "should not be allowed to act as a conceptual straitjacket, forcing the reader to consider all products

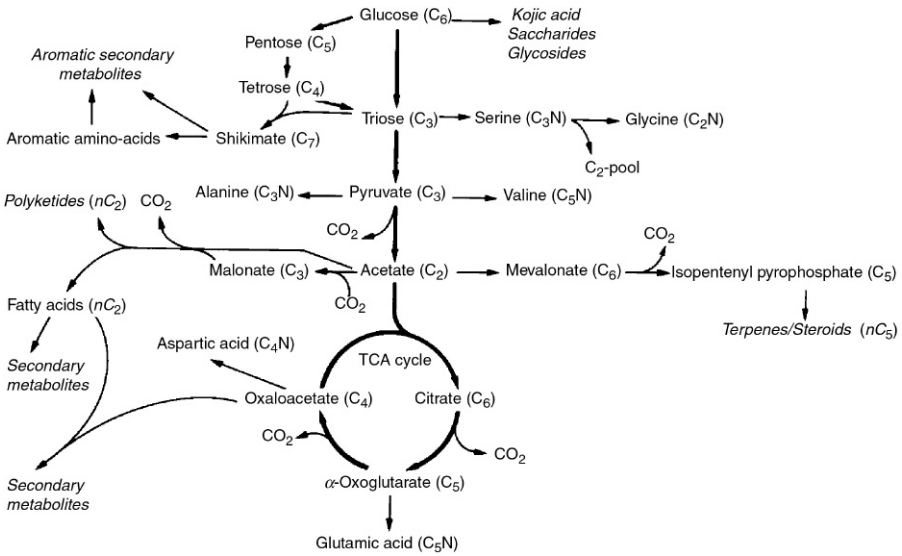

FIGURE 1.2 The Interrelationships Between Primary and Secondary Metabolism

Primary catabolic routes are shown in heavy lines and secondary products are italicized (Turner, 1971).

as either primary or secondary metabolites." It is sometimes difficult to categorize a product as primary or secondary and the kinetics of synthesis of certain compounds may change depending on the cultural conditions.

The physiological role of secondary metabolism in the producer organism in its natural environment has been the subject of considerable debate and their functions include effecting differentiation, inhibiting competitors, and modulating host physiology. However, the importance of these metabolites to the fermentation industry is the effects they have on organisms other than those that produce them. Many secondary metabolites have antimicrobial activity, others are specific enzyme inhibitors, some are growth promoters and many have pharmacological properties (Table 1.3). Thus, the products of secondary metabolism have formed the basis of a major section

Table 1.3 Some Secondary Products of Microbial Metabolism and Their Commercial Significance

Secondary Metabolite	Commercial Significance
Penicillin, cephalosporin, streptomycin	Antibiotics
Bleomycin, mitomycin	Anticancer agents
Lovastatin	Cholesterol-lowering agent
Cyclosporine A	Immunosuppressant
Avermectins	Antiparasitic agents

of the fermentation industry. As in the case for primary metabolites, wild-type microorganisms tend to produce only low concentrations of secondary metabolites, their synthesis being controlled by induction, quorum sensing, growth rate, feedback systems, and catabolite repression, modulated by a range of effector molecules (van Wezel & McDowall, 2011). The techniques which have been developed to improve secondary metabolite production are considered in Chapters 3 and 4.

RECOMBINANT PRODUCTS

The advent of recombinant DNA technology has extended the range of potential fermentation products. Genes from higher organisms may be introduced into microbial cells such that the recipients are capable of synthesizing "foreign" proteins. These proteins are described as "heterologous" meaning "derived from a different organism." A wide range of microbial cells has been used as hosts for such systems including *Escherichia coli, Saccharomyces cerevisiae,* and filamentous fungi. Animal cells cultured in fermentation systems are also widely used for the production of heterologous proteins. Although the animal cell processes were based on microbial fermentation technology, a number of novel problems had to be solved—animal cells were considered extremely fragile compared with microbial cells, the achievable cell density is very much less than in a microbial process and the media are very complex. These aspects are considered in detail in Chapters 4 and 7. Products produced by such genetically engineered organisms include interferon, insulin, human serum albumin, factors VIII and IX, epidermal growth factor, calf chymosin, and bovine somatostatin. Important factors in the design of these processes include the secretion of the product, minimization of the degradation of the product, and control of the onset of synthesis during the fermentation, as well as maximizing the expression of the foreign gene. These aspects are considered in more detail later in this chapter and in Chapters 4 and 12.

TRANSFORMATION PROCESSES

Microbial cells may be used to convert a compound into a structurally related, financially more valuable, compound. Because microorganisms can behave as chiral catalysts with high positional specificity and stereospecificity, microbial processes are more specific than purely chemical ones and enable the addition, removal, or modification of functional groups at specific sites on a complex molecule without the use of chemical protection. The reactions, which may be catalyzed include dehydrogenation, oxidation, hydroxylation, dehydration and condensation, decarboxylation, animation, deamination, and isomerization. Microbial processes have the additional advantage over chemical reagents of operating at relatively low temperatures and pressures without the requirement for potentially polluting heavy-metal catalysts. Although the production of vinegar is the oldest established microbial transformation process (conversion of ethanol to acetic acid), the majority of these processes involve the production of high-value compounds including steroids, antibiotics, and prostaglandins.

However, the conversion of acetonitrile to acrylamide by *Rhodococcus rhodochrous* is an example of the technology being used in the manufacturing of a bulk chemical—20,000 metric tons being produced annually (Demain & Adrio, 2008).

A novel application of microbial transformation is the use of microorganisms to mimic mammalian metabolism. Humans and animals will metabolize drugs such that they may be removed from the body. The resulting metabolites may be biologically active themselves—either eliciting a desirable effect or causing damage to the organism. Thus, in the development of a drug it is necessary to determine the activity of not only the administered drug but also its metabolites. These studies may require significant amount of the metabolites and while it may be possible to isolate them from tissues, blood, urine, or faeces of the experimental animal, their concentration is often very low resulting in such approaches being time-consuming, expensive, and far from pleasant. Sime (2006) discussed the exploitation of the metabolic ability of microorganisms to perform these biotransformations. Thus, drug metabolites have been produced in small-scale fermentation, facilitating the investigation of their biological activity and/or toxicity.

The anomaly of the transformation fermentation process is that a large biomass has to be produced to catalyze a single reaction. Thus, many processes have been streamlined by immobilizing either the whole cells, or the isolated enzymes, which catalyze the reactions, on an inert support. The immobilized cells or enzymes may then be considered as catalysts, which may be reused many times.

THE CHRONOLOGICAL DEVELOPMENT OF THE FERMENTATION INDUSTRY

The chronological development of the fermentation industry may be represented as five overlapping stages as illustrated in Table 1.4. The development of the industry prior to 1900 is represented by stage 1, where the products were confined to potable alcohol and vinegar. Although beer was first brewed by the ancient Egyptians, the first true large-scale breweries date from the early 1700s when wooden vats of 1500 barrels capacity were introduced (Corran, 1975). Even some process control was attempted in these early breweries, as indicated by the recorded use of thermometers in 1757 and the development of primitive heat exchangers in 1801. By the mid-1800s, the role of yeasts in alcoholic fermentation had been demonstrated independently by Cagniard-Latour, Schwann, and Kutzing but it was Pasteur who eventually convinced the scientific world of the obligatory role of these microorganisms in the process. During the late 1800s, Hansen started his pioneering work at the Carlsberg brewery and developed methods for isolating and propagating single yeast cells to produce pure cultures and established sophisticated techniques for the production of starter cultures. However, use of pure cultures did not spread to the British ale breweries and it is true to say that many of the small, traditional, ale-producing breweries still use mixed yeast cultures at the present time but, nevertheless, succeed in producing high quality products.