The business of biotechnology

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Abstract

Industrial microbiology and industrial biotechnology have enormous versatility involving microbes, mammalian cells plants, and animals. It encompasses the microbial production of primary and secondary metabolites and small and large molecules from plants and animals. Amino acids, nucleotides, vitamins, solvents, and organic acids comprise the primary metabolites. Multibillion-dollar markets are involved in the production of amino acids. Fermentative production of vitamins is replacing many synthetic vitamin-production processes. In addition to the multiple reaction sequences of fermentations, microorganisms are extremely useful in carrying out biotransformation processes. Multibillion-dollar markets exist for the medically useful microbial secondary metabolites, i.e., 160 antibiotics and derivatives such as the β-lactam peptide antibiotics, glycopeptides, lipopeptides, polyketides, aminoglycosides, and others. The anti-infective market amounts to 55 billion dollars. Secondary and primary metabolites are of great importance to our health, nutrition, and economics. Enzymatic and cell-based bioconversions are becoming essential to the fine chemical industry, especially for the production of single-isomer intermediates. Microbes also produce hypocholesterolemic agents, enzyme inhibitors, immunosuppressants, and antitumor compounds, some having markets of several billion dollars per year. They also make agriculturally important secondary metabolites such as coccidiostats, animal growth promotants, antihelmintics, and biopesticides. Recombinant DNA technology has served to improve the production of all of the above products. Molecular manipulations have been added to mutational techniques as a means of increasing titers and yields of microbial processes and in discovery of new drugs, but have made a major impact in creating a viable biopharmaceutical industry. This industry has made a fantastic impact in the business world, yielding biopharmaceuticals (recombinant protein drugs, vaccines, and monoclonal antibodies) having markets of many billions of dollars. It also produced a revolution in agriculture and has markedly increased the markets for microbial enzymes. Today, microbiology is a major participant in global industry and will be a major player in the new bioenergy industry, hopefully to replace petroleum within the next 50 years.

Keywords
Biotechnology; primary metabolites; secondary metabolites; economics; microbiology; biopharmaceuticals

1. Introduction

Products such as bread, beer, wine, distilled spirits, vinegar, cheese, pickles, and other fermented materials have been with us for centuries, being provided by bacteria and fungi. Originally, these processes were used for the preservation of fruits, vegetables, and milk, but these developed into more sophisticated products satisfying the palate and psyche of humans. World War I brought on a second phase of biotechnology which resulted in a quantum leap in the economic importance of microbes. The acetone-butanol fermentation was developed in England by Weizmann, and in Germany, Neuberg developed the glycerol fermentation. Both acetone and glycerol were needed for manufacture of munitions to support the war efforts of the respective opposing nations. Following these events were fermentations, bioconversions, and enzymatic processes yielding many useful products with large annual markets such as amino acids, nucleotides, vitamins, organic acids, solvents, vaccines, and polysaccharides. Of tremendous importance was the discovery in England of penicillin by Fleming, its development by Florey, Heatley, Chain, and Abraham, and the discovery of actinomycins, streptomycin, and other antibiotics by Waksman and his students in the USA. This yielded, after World War II, a revolution in discovery and production of secondary metabolites such as antibiotics. These molecules have had major beneficial effects on human and animal health. Often secondary metabolites with antibiotic activity were used for purposes other than the killing or growth-inhibition of bacteria and/or fungi. These commercial products include hypocholesterolemic agents, other enzyme inhibitors, immunosuppressants, anticancer agents, bioherbicides, bioinsecticides, coccidiostats, animal growth promotants, and ergot alkaloids. Other important secondary metabolites which do not have any antibiotic activity include the antihelmintic ivermectin, the bioinsecticide spinosad, and the

plant growth stimulants, the gibberellins.

In the early 1970s, a phenomenal third phase began with the birth of recombinant DNA technology. Traditional industrial microbiology became industrial biotechnology by merging with molecular biology to yield many new products of the modern biotechnology era. Recombinant DNA technology impacted the production of primary and secondary metabolites, bioconversions, and the enzyme industry. Of major significance was the establishment of the biopharmaceutical industry which, although ignored in the 1970s by the pharmaceutical industry, has become an important part of the latter. The recent decline in the pipeline of the major companies of the pharmaceutical industry is being reversed by products such as mammalian proteins and monoclonal antibodies, developed by the 35-year-old biopharmaceutical industry.

2. Why microorganisms are used in industry

Microorganisms are important to us for many reasons, but one of the principal ones is that they produce things of value. These may be very large materials such as proteins, nucleic acids, carbohydrate polymers, or even cells, or they can be smaller molecules which we usually separate into metabolites essential for vegetative growth, and those inessential—i.e., primary and secondary metabolites, respectively. The power of the microbial culture in the competitive world of commercial synthesis can be appreciated by the fact that even simple molecules, i.e., L-glutamic acid and L-lysine, are made by fermentation rather than by chemical synthesis. Although a few products have been temporarily lost to chemical synthesis (e.g., solvents like acetone and butanol), it is obvious that most natural products are made by fermentation technology. Despite the efficiency of the chemical route to riboflavin, commercial production of this compound is carried out by fermentation. Multistep chemical processes to vitamin C and steroids still employ microbial bioconversion steps. Most natural products are so complex and contain so many centers of asymmetry (i.e., containing a carbon atom to which four different groups are attached) that they probably will never be made commercially by chemical synthesis.

The importance of the fermentation industry resides in five important characteristics: (i) microorganisms' high ratio of surface area to volume, which facilitates the rapid uptake of nutrients required to support high rates of metabolism and biosynthesis; (ii) a tremendous variety of reactions which microorganisms are capable of carrying out; (iii) a facility to adapt to a large array of different environments, allowing a culture to be transplanted from nature to the laboratory flask, then to the factory fermentor, where it is capable of growing on inexpensive carbon and nitrogen sources and producing valuable compounds; (iv) the ease of genetic manipulation, both in vivo and in vitro, to increase formation of products, to modify structures and activities, and to make entirely new products; and (v) microorganisms' ability to make specific enantiomers, usually the active ones, in cases where normal chemical synthesis yields a mixture of active and inactive enantiomers.

The main reason for the use of microorganisms to produce compounds that can otherwise be isolated from plants and animals or synthesized by chemists is the ease of increasing production by environmental and genetic manipulation. Although microbes are extremely good in presenting us with an amazing array of valuable products, they usually produce them only in amounts that they need for their own benefit; thus they tend not to overproduce their metabolites. Regulatory mechanisms have evolved in microorganisms that enable a strain to avoid excessive production of its metabolites so that it can compete efficiently with other forms of life and survive in nature. The fermentation microbiologist, however, desires a "wasteful" strain which will overproduce and excrete a particular compound that can be isolated and marketed. During the screening stage, the microbiologist is searching for organisms with weak regulatory mechanisms. Once a desired strain is found, a development program is begun to improve titers by modification of culture conditions, mutation, and recombinant DNA technology. The microbiologist is actually modifying the regulatory controls remaining in the original culture so that its "inefficiency" can be further increased and the microorganism will excrete tremendous amounts of these valuable products into the medium.

Genetics has had a long history of contributing to the production of microbial metabolites[1]. Thousandfold increases have been recorded for small metabolites. Of course, the higher the specific level of production, the simpler is the job of product isolation. The tremendous increases in fermentation productivity and the resulting decreases in costs have come about mainly by mutagenesis and screening for higher-producing microbial strains. Mutation has also served to (i) shift the proportion of metabolites produced in a fermentation broth to a more favorable distribution; (ii) elucidate the pathways of secondary metabolism; and (iii) yield new compounds. With regard to new compounds, the medically useful products demethyltetracycline and doxorubicin (adriamycin) were discovered by simple mutation of the cultures producing tetracycline and daunorubicin (daunomycin), respectively. The technique of "mutational biosynthesis" has been used for the discovery of many new aminoglycoside, macrolide, and anthracycline antibiotics. It was successfully employed in the development of a new commercial antiparasitic avermectin, called doramectin[2]. Today, modern methods of

genetics and metabolic engineering are contributing to further increases in microbial production.

3. Production of primary metabolites

Primary metabolites are the small molecules of all living cells that are intermediates or end products of the pathways of intermediary metabolism, or are building blocks for essential macromolecules, or are converted into coenzymes. The most industrially important are the amino acids, nucleotides, vitamins, solvents, and organic acids. Primary metabolites vary in size from hydrogen gas (2 Da) to vitamin B_{12} (1,355 Da). It is not surprising to us that amino acids and vitamins are used in human and animal nutrition, that ethanol, acetone, and butanol are used as fuels and/or solvents, and that citric and acetic acids are used as acidulants. However, many of these general metabolites are used in novel ways: the sodium salts of glutamic, 5′-inosinic and 5′-guanylic acids as flavor enhancers, sodium gluconate as a sequestering agent to prevent the deposition of soap scum on cleaned surfaces, and fumarate in the manufacture of polyester resins. Organisms used to produce primary metabolites are often fantastic in their degree of overproduction after being genetically and physiologically manipulated by industrial scientists.

3.1. AMINO ACIDS

The amino acid market is over $6 billion (US) and has been growing at 5–10% per year[3]. Production amounts to 3 million tons per year. World production of amino acids is shown in *Table 1*.

Monosodium glutamate, a potent flavor enhancer, is the major amino acid in terms of tonnage. It is made by fermentation using various species of the genera *Corynebacterium* and *Brevibacterium*, e.g., *Corynebacterium glutamicum, Brevibacterium flavum*, and *Brevibacterium lactofermentum*. Today, the latter two glutamate-producing species are classified as subspecies of *C. glutamicum*, e.g., *C. glutamicum* ssp. *flavum* and *C. glutamicum* ssp. *lactofermentum*.

In amino acid production, feedback regulation is often bypassed by isolating an auxotrophic mutant and partially starving it of its requirement. A second means to bypass feedback regulation is to produce mutants resistant to a toxic analogue of the desired metabolite, i.e., an antimetabolite. Combinations of auxotrophic and antimetabolite-resistance mutations are common in the development of primary metabolite-producing microorganisms. The genome of *C. glutamicum* and a related species was sequenced in 2003 by Japanese scientists at Kyowa Hakko Kogyo Co., Ltd.[11], the Ajinomoto Co., Inc.[12], and also by a German group from various institutes and Degussa AG[13]. These achievements are assisting in the improvement of strains overproducing amino acids.

Table 1. Worldwide production of amino acids[4–10]

AMINO ACID	PROCESS	TONS/YEAR	MARKET ($)
L-Alanine	Enzymatic	500	–
L-Arginine	Fermentation	1,200	150 million
L-Aspartic acid	Enzymatic	10,000	43 million
L-Cysteine	Enzymatic	3,000	4.6 million
L-Glutamic acid	Fermentation	1,600,000	1.5 billion
L-Glutamine	Fermentation	1,300	–
Glycine	Chemical	22,000	–
L-Histidine	Fermentation	400	–
L-Isoleucine	Fermentation	400	–
L-Leucine	Fermentation	500	–
L-Lysine-HCl	Fermentation	850,000	1.5 billion
DL-Methionine	Chemical	400,000	2.3 billion
L-Phenylalanine	Fermentation	12,650	198 million
L-Proline	Fermentation	350	–
L-Serine	Fermentation	300	–
L-Threonine	Fermentation	70,000	270 million
L-Tryptophan	Enzymatic	3,000	150 million
L-Tyrosine	Fermentation	165	50 million
L-Valine	Fermentation	500	–

Recombinant DNA techniques have made their way into the amino acid production area. Microbial strains have been constructed with plasmids bearing amino acid biosynthetic operons.

Genetic engineering has made an impact by use of the following strategies: (i) amplification of a gene encoding the rate-limiting enzyme of a pathway; (ii) amplification of the gene encoding the first enzyme after a branch-point; (iii) cloning of a gene encoding an enzyme with greater or less feedback regulation; (iv) introduction of a gene encoding an enzyme with a functional or energetic advantage as replacement for a normal enzyme; (v) amplification of the gene encoding the first enzyme leading from central metabolism to increase carbon flow into the pathway followed by sequential removal of bottlenecks caused by accumulation of intermediates.

Transport mutations have become very useful. Mutations decreasing amino acid uptake allow for improved excretion and lower intracellular feedback control. This has been especially important in production of tryptophan and threonine. In cases where excretion is carrier-mediated, increase in activity of these carrier enzymes increases production of the amino acid. Exporter genes in *C. glutamicum* are

known for lysine, isoleucine, and threonine.

As a result of genetic and physiological manipulations, fermentation titers have reached the levels shown in *Table 2*. Despite the high fermentation titers shown in the table, L-phenylalanine and L-aspartic acid are produced enzymatically and used mainly for manufacture of the sweetener, aspartame.

3.2 NUCLEOTIDES AND NUCLEOSIDES

Commercial interest in nucleotide fermentations is due to the activity of two purine ribonucleoside 5′-monophosphates, namely guanylic acid (5′-GMP) and inosinic acid (5′-IMP) as enhancers of flavor[14,15]. Some 2,500 tons of GMP and IMP were produced in Japan in 1998 with a combined market of $350 million per year[5]. Three main processes are used: (i) hydrolysis of yeast RNA by fungal nuclease to AMP and GMP, followed by enzymatic deamination of AMP to IMP; (ii) fermentative production of the nucleosides inosine and guanosine by *Bacillus subtilis* mutants followed by chemical phosphorylation, and (iii) direct fermentation of sugar to IMP by *C. glutamicum* mutants plus conversion of guanine to GMP by salvage synthesis using intact cells of *Brevibacterium ammoniagenes*. Titers of IMP by direct fermentation reached 27 g per L in the mid-1990s[15]. The key to effective purine accumulation is the limitation of intracellular AMP and GMP. This limitation is best effected by restricted feeding of purine auxotrophs[16]. Thus, adenine-requiring mutants lacking adenylosuccinate synthetase accumulate hypoxanthine or inosine that results from breakdown of intracellularly accumulated IMP. These strains are still subject to GMP repression of enzymes of the common path. To minimize the severity of this regulation, the adenine auxotrophs are further mutated to eliminate IMP dehydrogenase. These adenine-xanthine double auxotrophs show a twofold increase in specific activity of some common-path enzymes and accumulate up to 15 g inosine per L under conditions of limiting adenine and xanthine (or guanosine). Further deregulation is achieved by selection of mutants resistant to purine analogues. Mutants requiring adenine and xanthine and resistant to azaguanine produce over 20 g inosine per L. Insertional inactivation of the IMP dehydrogenase gene in another *B. subtilis* strain yielded a culture producing 35 g inosine per L[17]. Genetic engineering of the inosine monophosphate dehydrogenase gene in a *B. subtilis* strain, which was producing 7 g per L guanosine and 19 g per L inosine, changed production to 20 g per L guanosine and 5 g per L inosine[18]. Other *B. subtilis* mutants produce as much as 30 g per L guanosine. With regard to pyrimidine production, a recombinant strain of *B. subtilis* produces 18 g per L of cytidine, and a mutant lacking homoserine dehydrogenase (which increased the concentration of the precursor aspartate in the cell) produces 30 g per L[19].

Table 2. Titers of amino acid fermentations[9,10]

AMINO ACID	TITER (G PER L)
L-Alanine	75
L-Arginine	96
L-Glutamic acid	85
L-Histidine	42
L-Isoleucine	40
L-Leucine	34
L-Lysine-HCl	170
L-Phenylalanine	51
L-Proline	100
L-Serine	65
L-Threonine	100
L-Tryptophan	58
L-Tyrosine	26
L-Valine	99

3.3 VITAMINS

More than half of vitamins produced commercially are fed to domestic animals[20]. The vitamin market was $2.3 billion in 2003. Microbes produce seven vitamins or vitamin-like compounds commercially: beta-carotene, vitamin B_{12}, vitamin B_{13}, riboflavin, vitamin C, linolenic acid, vitamin F, and ergosterol. Production figures are shown in *Table 3*.

Riboflavin (vitamin B_2) was produced commercially for many years by both fermentation and chemical synthesis[24], but today, fermentation is the major route. Six years after BASF acquired the Merck *Ashbya gossypii* process, they shut down chemical production in favor of the fermentation process, in 1996. Riboflavin overproducers include two yeast-like molds, *Eremothecium ashbyii* and *Ashbya gossypii*, which synthesize riboflavin in concentrations greater than 20 g per L. A riboflavin-overproducer such as *A. gossypii* makes 40,000 times more vitamin than it needs for its own growth. The biochemical key to riboflavin overproduction appears to involve insensitivity to the repressive effects of iron. Riboflavin formation by *A. gossypii* is stimulated by precursors hypoxanthine and glycine. A newer process using a recombinant *B. subtilis* strain yields 20–30 g riboflavin per L. Resistance to purine analogs has improved production in *Candida flareri* and *B. subtilis*, as has resistance to roseoflavin, a riboflavin antimetabolite. Mutation of *A. gossypii* to resist-

Table 3. Production of vitamins and related compounds by fermentation and other means[21-23]

COMPOUND	METHOD*	TONS/ YEAR	MARKET $MILLION	ORGANISM
Biotin (vitamin H)	C	88	64	
β-Carotene (provitamin A)	C, E, F	100	—	*Blakeslea trispora, Dunaliella salina, Dunaliella bardawil*
Folic acid	C	534	17	
γ-Linoleic acid	F	1,000	—	*Mortierella isabellina*
Niacin	C	28,000	133	
Orotic acid (vitamin B_{13})	F	100	—	*Corynebacterium glutamicum*
Pantothenate	C, F	10,000	156	
Provitamin D3	C, E	500	—	
Pyridoxine (vitamin B_6)	C	3,800	70	
Riboflavin (vitamin B_2)	F	4,600	134	*Ashbya gossypii, Bacillus subtilis*
Thiamine (vitamin B_1)	C, F	3,700	67	
Tocopherol	C, E	10,000	—	
Vitamin A (retinol)	C	2,800	308	
Vitamin B_{12} (cyanocobalamin)	F	25	105	*Propionibacterium shermanii, Pseudomonas denitrificans*
Vitamin C (ascorbic acid)	C + B	107,000	486	*Gluconobacter oxydans*
Vitamin E	C, E	30,000	89	
Vitamin F (polyunsat. fatty acids)	E, F	1,000	—	Fungi
Vitamin K_2	C	2	—	

*C=chemical synthesis; E=extraction; F=fermentation; B=bioconversion

ance to itaconic acid and aminomethylphosphonic acid (glycine antimetabolite) has yielded improved riboflavin producers.

Vitamin B_{12} (cyanocobalamin) is produced industrially with *Propionibacterium shermanii* and *Pseudomonas denitrificans*[25,26]. Such strains make about 100,000 times more vitamin B_{12} than they need for their own growth. The key to the fermentation is avoidance of feedback repression by vitamin B_{12}. Of major importance in the *P. denitrificans* fermentation is the addition of betaine. Vitamin B_{12} overproduction is totally dependent upon betaine but the mechanism of control is unknown. *Propionibacterium freudenreicheii* can produce 206 mg per L but is not yet a major industrial producing organism. It is thought that *P. denitrificans* produces about 300 mg per L.

In production of biotin, feedback repression is caused by the enzyme acetyl-CoA carboxylase biotin holoenzyme synthetase, with biotin 5-adenylate acting as corepressor[27]. Strains of *Serratia marcescens* obtained by mutagenesis, selected for resistance to biotin antimetabolites and subjected to molecular cloning, produce 600 mg per L in the presence of high concentrations of sulfur and ferrous iron[28]. Traditionally, biotin has been produced chemically but new biological processes are becoming economical.

Vitamin C (L-ascorbic acid) has been produced almost completely by chemical synthesis (Reichstein process) for many years. This otherwise chemical process utilizes one bioconversion reaction, the oxidation of D-sorbitol to L-sorbose. It has been shown to proceed at the theoretical maximum, i.e., 200 g per L of D-sorbitol can be converted to 200 g per L of L-sorbose, when using a mutant of *Gluconobacter oxydans* selected for resistance to high sorbitol concentration. Vitamin C is used for nutrition of humans and animals as well as a food antioxidant. Global production of L-ascorbic acid has a market of $600 million and an annual growth rate of 3-4%[29]. The Reichstein process will probably have to compete with commercial fermentation approaches in the next few years[30]. A novel process involves the use of a genetically engineered *Erwinia herbicola* strain containing a gene from *Corynebacterium* sp. The engineered organism converts glucose to 2-ketogulonic acid, which can be easily converted by acid or base to ascorbic acid[31]. Another process devised independently converts 40 g per L glucose into 20 g per L 2-keto-L-gulonate[32]. This process involves cloning of the gene encoding 2,5-diketo-D-gluconate reductase from *Corynebacterium* sp. into *Erwinia citreus*. Plasmid cloning of the genes encoding L-sorbose dehydrogenase and L-sorbosone dehydrogenase from *G. oxydans* back into the same organism yielded a strain capable of converting 150 g per L of D-sorbitol into 130 g per L of 2-keto-L-gulonate[33].

3.4. ORGANIC ACIDS

Microbes have been widely used for the commercial production of organic acids. Citric, acetic, lactic, gluconic, and itaconic acids are the main organic acids with commercial application[34]. Other valuable organic acids are malic, tartaric, pyruvic, and succinic acids.

Citric acid is easily assimilated, palatable, and has low toxicity. Consequently, it is widely used in the food and pharmaceutical industry. It is employed as an acidifying and flavor-enhancing agent, as an antioxidant for inhibiting rancidity in fats and oils, as a buffer in jams and jellies, and as a stabilizer in a variety of foods. The pharmaceutical industry uses approximately 15% of the available supply of citric acid. About 1.75 million tons of citric acid are produced per year, with a major market of $1.6 billion.

Citric acid is produced via the Embden-Meyerhof pathway and the first step of the tricarboxylic acid cycle. The major control of the process involves the feedback inhibition of phosphofructokinase by citric acid. The commercial process employs the fungus *Aspergillus niger* in media deficient in iron and manganese. Manganese deficiency has two beneficial effects in the citric acid fermentation: (i) it leads to high levels of intracellular NH_4 which reverses citric acid inhibition of phosphofructokinase; and (ii) it brings on the formation of small mycelial pellets which are the best morphological form for citric acid production. The morphological effect is due to a change in cell wall composition caused by growth in low Mn^+. A high level of citric acid production is also associated with an elevated intracellular concentration of fructose 2,6-biphosphate, an activator of glycolysis[35]. Other factors contributing to high citric acid production are the inhibition of isocitrate dehydrogenase by citric acid, and the low pH optimum (1.7 - 2.0). Higher pH levels (e.g., 3.0) lead to production of oxalic and gluconic acids instead of citric acid. The low pH inactivates glucose oxidase which normally would yield gluconic acid[36]. In approximately 4 to 5 days, the major portion (80%) of the sugar is converted to citric acid, titers reaching over 100 g per L.

High concentrations of citric acid can also be produced by *Candida oleophila* from glucose[37]. In chemostats, 200 g per L can be made and more than 230 g per L can be produced in continuous repeated fed-batch fermentations. This compares to 150–180 g per L by *A. niger* in industrial batch or fed-batch fermentations for 6–10 days. The key to the yeast fermentation is nitrogen limitation coupled with an excess of glucose. The citric acid is secreted by a specific energy-dependent transport system induced by intracellular nitrogen limitation. The transport system is selective for citrate over isocitrate. Processes have also been developed with *Candida* species growing on hydrocarbons or oils. Such yeasts are able to convert n-paraffins to citric and isocitric acids in extremely high yields. Production of citric acid instead of isocitric acid is favored by selecting yeast mutants which are deficient in the enzyme aconitase. Titers as high as 225 g per L have been reached with these yeasts[36].

Vinegar has been produced since 4,000 BCE. A solution of ethanol is converted to acetic acid in which 90–98% of the ethanol is attacked, yielding a solution of vinegar containing 12–17% acetic acid. Vinegar formation is best carried out with species of *Gluconacetobacter* and *Acetobacter*[23]. In 2001, acetic acid production amounted to 7.5 million tons[38]. An interesting application of genetic engineering in the acetic acid fermentation was the cloning of the aldehyde dehydrogenase gene from *Acetobacter polyoxogenes* on a plasmid vector into *Acetobacter aceti* subsp. *xylinum*. This manipulation increased the rate of acetic acid production by over 100% (from 1.8 to 4 g per Lh) and the titer by 40% (from 68 to 97 g per L)[39].

Fermentation has virtually eliminated chemical synthesis of lactic acid. Whereas lactobacilli produce mixed isomers, *Rhizopus* makes L-(+)-lactic acid solely. *Rhizopus oryzae* is favored for production since it makes only the stereochemically pure L-(+)-lactic acid. It is produced anaerobically with a 95% (w/w) yield based on charged carbohydrate, a titer of over 100 g per L, and a productivity of over 2 g per Lh. This is comparable to processes employing lactic acid bacteria. Global production is 250,000 tons per year. Lactic acid sells for $1.22 per pound[4]. It is polymerized into polylactide which is a new environmentally favorable bioplastic. The polylactide process was developed by a joint effort of Dow Chemical and Cargill. Also of importance is the non-chlorinated environmentally benign solvent, ethyl lactate.

Production of gluconic acid amounts to 150 g per L from 150 g per L glucose plus corn steep liquor in 55 hours by *A. niger*[40]. Titers of over 230 g per L have been obtained using continuous fermentation of glucose by yeast-like strains of *Aureobasidium pullulans*[41]. Fifty thousand to 60,000 tons are made per year, with a market of about $125 million.

Itaconic acid is used as a co-monomer in resins and synthetic fibers and also in coatings, adhesives, thickeners, and binders[42]. It is made by *Aspergillus terreus* at 16,500 tons per year and sells for $4 per kg. Productivity is 1 g per L h and its concentration reaches 80 g per L. Synthetic processes are not competitive with the fungal process. Certain *Candida* species produce 42 g per L. Yield from sucrose in molasses is 70%. Itaconic acid has an annual market of $68 million[5].

Although microbial processes exist for the other acids, they have not been exploited commercially on a large scale. Succinic acid can be produced by the rumen organism *Actinobacillus succinogenes* at 110 g per L[43]. The projected price at a hypothetical 75,000 tons per year level is $0.55 per kg. However, present production is only 15,000 tons per year, all made synthetically from petroleum at a price of $2.70–4.00 per lb ($1.22–$1.81 per kg). Pyruvic acid production amounts to 69 g per L at 56 h, with a yield of 0.62 g per g glucose using *Torulopsis glabrata*[44].

3.5 ALCOHOLS

Ethyl alcohol is a primary metabolite that can be produced by fermentation of a sugar, or a polysaccharide that can be depolymerized to a fermentable sugar. Yeasts are preferred for these fermentations, but the species used depends on the substrate employed. *Saccharomyces cerevisiae* is employed for the fermentation of hexoses, whereas *Kluyveromyces fragilis* or *Candida* species may be utilized if lactose or pentoses, respectively, are the substrates. Under optimum

conditions, approximately 10–12% ethanol by volume is obtained within 5 days. Such a high concentration slows down growth and the fermentation ceases. Ethanol is produced in Brazil from cane sugar at 12.5 billion liters per year and is used as a 25% fuel blend or as a pure fuel. With special yeasts, e.g., sake yeasts, the fermentation can be continued to alcohol concentrations of 20% by volume. However, these concentrations are attained only after months or years of fermentation. With regard to beverage ethanol, some 60 million tons of beer and 30 million tons of wine are produced each year.

Although synthetic ethanol production from the petrochemical ethylene was once the predominant source of industrial ethanol, today ethanol is mainly manufactured in the U.S. by fermentation of corn. Because of the elimination of lead from gasoline, ethanol is being substituted as a blend to raise gasoline's octane rating. The steady increase in consumption is also due to phasing out of the use of methyl tert-butyl ether (MTBE) as gasoline oxygenate, as legislated by many states in the US. Ethanol is now being used as an oxygenate to reduce CO_2 emissions by improving overall oxidation of gasoline. It is a more efficient oxygenated fuel than MTBE; only half the volume is necessary to produce the same effect as that of MTBE. Furthermore, ethanol is biodegradable in contrast to MTBE.

The dependence on petroleum for energy in the US has become a major problem, with annual consumption of 137 billion gallons of gasoline[45]. In 2006, 4.8 billion gallons of bioethanol were made from corn in the US. There is thus not enough corn in the US to make an impact in the energy problem, and it is thought that other types of biomass will have to be used, e.g., cellulosic/hemicellulosic biomass from agriculture and forestry. To convert such material into fermentable substrates, chemical pretreatment (e.g., mild acid hydrolysis) will be necessary, and many enzymes, such as cellulases, hemicellulases, etc., will be required. Fuel ethanol produced from biomass would provide relief from air pollution caused by the use of gasoline and would not contribute to the greenhouse effect.

The main types of microbes being considered are recombinant yeasts, recombinant Gram-negative bacteria such as *Escherichia coli* and *Klebsiella oxytoca*, and the celluloytic anaerobic bacteria such as the clostridia. *E. coli* has been converted, by recombinant DNA technology, into an excellent ethanol producer[46]. Genes encoding alcohol dehydrogenase II and pyruvate decarboxylase from *Zymomonas mobilis* were inserted in *E. coli* and became the dominant system for NAD regeneration. Ethanol represents over 95% of the fermentation products in the genetically engineered strain, whereas the original *E. coli* strain carried out a mixed acid fermentation. Recombinant *E. coli* produced 46 g per L ethanol from rice hulls pretreated by dilute acid[47]. Bacteria such as clostridia and *Zymomonas* are being reexamined for ethanol production after years of neglect. *Clostridium thermocellum*, an anaerobic thermophile, can convert waste cellulose directly to ethanol[48]. Other clostridia produce acetate, lactate, acetone, and butanol and will be utilized as petroleum becomes depleted in the world. Butanol is very attractive since it (i) contains 1/3 higher energy content than ethanol; (ii) does not require modification of automobile engines until its content in a blend with gasoline reaches 40% (whereas the modification required with ethanol is at the 15% level); and (iii) is easier to ship than is ethanol.

Production of glycerol is usually done by chemical synthesis from petroleum feedstocks, but good fermentations processes are available[49]. Osmotolerant yeast strains (*Candida glycerinogenes*) can produce up to 130 g per L with yields of 63% and productivity of 32 g per Ld. The price of synthetic glycerol is $0.56/lb. Six hundred thousand tons of glycerol are produced annually by (i) recovery as a by-product of the fat and oil industries; (ii) synthesis from propylene; and (iii) to a small extent, by glucose fermentation using *S. cerevisiae*[50]. A number of studies are being carried out using physiological control and genetic engineering in the hopes of making the fermentation process competitive with synthesis.

Mannitol is not metabolized by humans and is about half as sweet as sucrose[51]. It is considered as a low-calorie sweetener. Its production has reached 213 g per L from 250 g per L fructose after 110 h by *Candida magnoliae*. Mannitol has a market of $100 million and sells for $3.32 per pound[52].

3.6 MISCELLANEOUS PRIMARY METABOLITES

Polysaccharides are important commercial products made by microorganisms. The most well-known is xanthan gum, produced at 30,000 tons per year using *Xanthomonas campestris*, with a market of $408 million[4,5]. It has many uses in the food, pharmaceutical, and other industries and sells for $4.90 per pound. Dextran is produced by *Leuconostoc mesenteroides* and sells for $49 per pound. It is used as a therapeutic agent to restore blood volume after casualties, as a blood plasma substitute, as iron dextran to alleviate iron-deficiency anemia, and as an adsorbant. Production titer of pullulan, a neutral water-soluble polysaccharide made by *A. pullulans*, amounts to 37 g per L[53].

There are many other microbial polymers including scleroglucan, curdlan, alginate, galactomannan, glucomannan, mannans, galactans, phosphomannangellan, succinoglycan, hyaluronic acid, glycan, emulsan, chitosan, tremellan, and the biodegradable group of plastics known as polyhydroxyalkanoates. They are either being used in industry or medicine for various applications or are awaiting future application.

Microalgae, e.g., species of *Rhodophyta* and *Phaeophyta*, are used to produce phytocolloids such as agar, alginates, and carrageenan[54]. The world market is $6 billion, and production is at 7.5 million tons per year. Microalgal biomass amounts to 5,000 tons per year with a market of $1.25 billion. This does not include processed products such as phycocyanin, *Spirulina* biomass, *Chlorella* biomass, carotenoids including ß-carotene and astaxanthin, fatty acids, lipids, polysaccharides, and immune modulators. Estimates of the number of microalgal species are 200,000 to several million, compared to 250,000 species of higher plants. A major group in the microalgae are the cyanobacteria, of which 2,000 species are known.

Fusarium venenatum A 3/5 (formerly *Fusarium graminearum*) has been used for producing microbial protein for human consumption since 1985[55]. Its use was determined after screening about 3,000 different fungi. The filamentous nature of the fungus is important to impart texture in the foods. Mycoprotein is the largest-selling substitute for meat in the UK. It is also sold in five other European countries. Sales in 2000 were $135 million.

DuPont's new environmentally friendly bioplastic is polytrimethylene terephthalate (3GT polyester), a fiber made by chemically reacting terephthalic acid with fermentation-derived 1, 3-propanediol. DuPont teamed up with Genencor International to develop a metabolically engineered strain of *E. coli* which could make 1,3-propanediol economically from corn starch.

4. Production of secondary metabolites

Microbially produced secondary metabolites are extremely important to our health and nutrition[56]. A group that includes antibiotics, other medicinals, toxins, pesticides, and animal and plant growth factors, they have tremendous economic importance. In batch or fed-batch culture, secondary metabolites are produced usually after growth has slowed down. They have no function in growth of the producing cultures, are produced by certain restricted taxonomic groups of organisms, and are usually formed as mixtures of closely related members of a chemical family. In nature, secondary metabolites are important for the organisms that produce them, functioning as (i) sex hormones; (ii) ionophores; (iii) competitive weapons against other bacteria, fungi, amoebae, insects, and plants; (iv) agents of symbiosis; (v) effectors of differentiation[57]; and (vi) agents of communication between microbial cells.

4.1 ANTIBIOTICS

The best known of the secondary metabolites are the antibiotics. This remarkable group of compounds form a heterogeneous assemblage of biologically active molecules with different structures and modes of action. They attack virtually every type of microbial activity such as synthesis of DNA, RNA, and proteins, membrane function, electron transport, sporulation, germination, and many others. Since 1940, we have witnessed a virtual explosion of new and potent antibiotic molecules which have been of great use in medicine, agriculture, and basic research. However, the rate of discovery drastically dropped after the 1970s. The search for new antibiotics must continue in order to combat evolving pathogens, naturally resistant bacteria and fungi, and previously susceptible microbes that have developed resistance. In addition, new molecules are needed to improve pharmacological properties; combat tumors, viruses, and parasites; and develop safer and more potent compounds. About 6,000 antibiotics have been described, 4,000 from actinomycetes. Certain species and strains are remarkable in their ability to make a multiplicity of compounds. *Streptomyces griseus* strains produce over 40 different antibiotics and strains of *B. subtilis* make over 60 compounds. Strains of *Streptomyces hygroscopicus* make almost 200 antibiotics. One *Micromonospora* strain can produce 48 aminocyclitol antibiotics. The antibiotics vary in size from small molecules like cycloserine (102 daltons) and bacilysin (270 daltons) to polypeptides such as nisin, which contains 34 amino acid residues.

The antibiotic market includes about 160 antibiotics and derivatives such as the β-lactam peptide antibiotics, the macrolide polyketides and other polyketides, tetracyclines, aminoglycosides, and others[58,59]. The global market for anti-infective antibiotics is $55 billion. The anti-infective market is made up of 62% antibacterials, 13% sera, immunoglobulins and vaccines, 12% anti-HIV antivirals, 7% antifungals, and 6% non-HIV antivirals[60]. Prices of bulk antibiotics in 2003 were $92 per lb and for specialty antibiotics about $1,000 per lb[7]. The market for *Streptomyces* antibiotics is over $25 billion[61], and that for antifungal drugs more than $4 billion[62].

In the pursuit of more-effective antibiotics, new products are made chemically by modification of natural antibiotics; this process is called semisynthesis. The most striking examples are the semisynthetic penicillins and cephalosporins, erythromycins (e.g., azithromycin, clarithromycin), and the recently introduced tetracycline, tigecycline. Thousands of penicillins, cephalosporins, tetracyclines, and rifamycins have been prepared semisynthetically over the years. For the discovery of new or modified products, recombinant DNA techniques are being used to introduce genes coding for antibiotic synthetases into producers of other antibiotics or into non-producing strains to obtain modified or hybrid antibiotics[63-65].

The global market for penicillins G and V is $8.2 billion, that for cephalosporins $11 billion and for other ß-lactams $1.5 billion, making a total of over $20 billion for ß-lactam antibiotics. Quinolones

have a market of $6.4 billion, including fluoroquinolones at $3.2 billion. Macrolides sell for $6.0 billion, aminoglycosides for $1.8 billion, tetracyclines for $1.4 billion[66], the glycopeptides vancomycin and teicoplanin for $1 billion combined[67], and the azole antifungals for $2 billion. After antibiotics, the next largest anti-infective market is $10.2 billion for antivirals, not including vaccines.

Antibiotics with markets over $1 billion dollars include Augmentin (amoxicillin plus clavulanic acid) at $2.1 billion; azithromycin at $2.0 billion, ciprofloxacin at $ 1.8 billion, Biaxin (clarithromycin) at $1.16 billion, Rocephin (ceftriaxone) at $1.07 billion, and Levaquin/Floxin (levofloxacin/ofloxacin) at $1.07 billion. Clavulanic acid, an actinomycete β-lactam, is an important β-lactamase inhibitor and is sold in combination with penicillins. Over 60,000 tons of penicillins G and V are produced annually, of which 25,000 tons represent the bulk products used for direct medical use. The rest is converted to 6-APA (for semisynthesis of ampicillin, amoxicillin, and other penicillins), and to 7-ADCA (for production of semisynthetic cephalosporins). Although cephalosporin C is not used directly in medicine, it is converted to 7-ACA, another intermediate for semisynthesis of cephalosporins, which sells for $100–200/kg. There are over 50 such antibiotics on the market today.

Titers of penicillin with *Penicillium chrysogenum* have reached 70 g per L, whereas those of cephalosporin C by *Acremonium chrysogenum* are over 30 g per L. Published data on clavulanic acid production by *Streptomyces clavuligerus* indicate the titer to be above 3 g per L[68].

Oxytetracycline titer is almost 100 g per L[69] and that of chlortetracycline is over 33 g per L in a 156 h process[70]. Production of erythromycin is 10–13 g per L[71], produced by fermentation at about 4,000 tons per year. Less than 1,000 tons annually are used as erythromycin A; the rest is semisynthetically converted to 1,500 tons of azithromycin, 1,500 tons of clarithromycin, and 400 tons of roxithromycin.

A recently approved antibacterial is daptomycin, a lipopeptide produced by *Streptomyces roseosporus*. It acts against Gram-positive bacteria including vancomycin-resistant enterococci, methicillin-resistant *Staphylococcus aureus*, and penicillin-resistant *Streptococcus pneumoniae*[72]. It kills by disrupting plasma membrane function without penetrating into the cytoplasm.

Caspofungin acetate (pneumocandin, L-743,872, MIC 991, Cancidas), which inhibits cell wall formation via inhibition of β-1,3-glucan synthase, was approved in 2000. It is a parenteral candin type of antifungal. It is administered as an aerosol for prophylaxis against *Pneumocystis carinii*, a major cause of death in HIV patients from North America and Europe. It is also active against *Candida, Aspergillus,* and *Histoplasma*. Other echinocandin derivatives are Astellas Pharma's micafungin (FK-463) and Versicor's (now, Pfizer) anidulafungin (Vechinocandin, LY-303366).

4.2 ANTITUMOR AGENTS

Ever since the discovery of the actinomycins by Waksman and Woodruff[73] in 1941 and the use of actinomycin D against the Wilms tumor in children, microbes have served as a prime source of anticancer agents. The important microbial molecules are mitomycin C, bleomycin, daunorubicin, doxorubicin, etoposide, and calicheamicin, all made by actinomycetes. Taxol (paclitaxel) is a very effective agent against breast and ovarian cancer, and although it can be made by endophytic fungi[74] it is actually made by plant cell culture or from pine needles of the yew tree. Another plant product is camptothecin (CPT), which is a modified monoterpene indole alkaloid produced by certain angiosperms, which is active against type I DNA topoisomerase. Its water-soluble derivatives irinotecan and topotecan are used against cancer with a total 2003 market of $1 billion[75]. It also can be made by endophytic fungi[76].

Plant cell culture processes are expensive. Only two processes are in commercial use, one for shikonin (a cosmetic ingredient) and the other for Taxol. Taxol had sales amounting to about $1.6 billion and was Bristol Myers-Squibb's third-largest selling product in 1999.

4.3 PHARMACOLOGICAL AGENTS

Many microbial products with important pharmacological activities were discovered by screening for inhibitors using simple enzymatic assays[77]. One huge success was the discovery of the fungal statins, including compactin, lovastatin (mevinolin), pravastatin (Pravacol, Mevalotin) and others which act as cholesterol-lowering agents[78]. Lovastatin is produced by *A. terreus*. Pravastatin is bioconverted from compactin. Zocor (simvastatin) is a semi-synthetic product made from lovastatin. Lipitor (atorvastatin) is a synthetic compound devised by consideration of the structure of the fungal statins. The statins are potent competitive inhibitors of 3-hydroxy-3-methylglutaryl-coenzyme A reductase in liver. The largest segment of the pharmaceutical business is for these cholesterol-lowering compounds. In 2001, the statins constituted three of the four best-selling drugs. In order of decreasing markets, they were Zocor (first), Lipitor (second), and pravastatin (fourth). Sales in 2002 of Zocor reached $7.2 billion, while pravastatin's sales were over $3.6 billion[79]. Lipitor had achieved sales of $13 billion in 2005[79]. Sales of cholesterol- and triglyceride-lowering drugs reached $32 billion in 2005[80].

Of great importance in human medicine are the immunosuppressants such as cyclosporin A, sirolimus (rapamycin), tacrolimus (FK506), and mycophenolate mofetil (CellCept). They are used for heart, liver, and

kidney transplants and were responsible for the establishment of the organ transplant field. Cyclosporin A, which has a market of $1.5 billion, is made by the fungus *Tolypocladium nivenum* (previously *Tolypocladium inflatum*). Mycopenolate mofetil is a semisynthetic product of the oldest known antibiotic, mycophenolic acid, and is also made by a fungus. Sirolimus and tacrolimus are products of streptomycetes.

Many pharmacological agents were first isolated as antibiotics (e.g., cyclosporins, rapamycin, mycophenolic acid, statins) or as mycotoxins (ergot alkaloids) before they were put to work as drugs.

The cost of bringing a new drug to market has been increasing rapidly. In 2003, it took 13.3 years from its first patent application, on the average, for a new chemical entity (NCE) to reach the market. The cost of bringing a drug to market rose to $1.7 billion[81], whereas in 1999, it was only $600 million[82]. Most drug candidates fail in clinical development because of inadequate efficacy, poor pharmacokinetics, metabolic instability, low aqueous solubility, immunogenicity, or toxicity. During 1978–1980, the average number of NCEs launched by the pharmaceutical industry was 43. In 1998–2000, the number had dropped to 33. The number launched in 2003 was 30—the lowest in over 20 years. Virtually no targets from genomics have yielded candidates. Much of this problem has been caused by the trend among large pharmaceutical companies to merge and to desert natural products. R&D investment in the drug industry in the US in 2002 was $31 billion. Although this is a large sum, the industry is not spending enough of it on the detection, isolation, and screening for new natural products and is unfortunately spending a disproportionate amount on promotion—some of the major pharmaceutical companies are spending almost twice as much on promotion as on R&D[83].

In contrast to the shrinking pipeline of the major pharmaceutical companies, the progress of the biotechnology (biopharmaceutical) companies has been remarkable *(see Section 6)*. Between 1994 and 2003, 30% to 55% of the NCEs introduced into medicine came from biotechnology companies. In the early years of the new century, the five largest pharmaceutical companies in-licensed from 6 to 10 products from biotechnology or specialty pharmaceutical companies (yielding 28–80% of their revenue). The new biopharmaceutical industry had two drug/vaccine approvals in 1982, none in 1983–84, and only one in 1985. However, this figure rose to 32 in 2000. In 2004, seven of the top 30 drugs were biopharmaceuticals from the biotech industry. The number of patents granted to biotechnology companies rose from 1,500 in 1985 to 9,000 in 1999.

4.4 AGRICULTURAL AND ANIMAL HEALTH PRODUCTS

In commercial use are microbiologically produced (i) biopesticides including fungicides (e.g., kasugamycin, polyoxins); (ii) bioinsecticides (*Bacillus thuringiensis* crystals, nikkomycin, spinosyns); (iii) bioherbicides (bialaphos); (iv) antihelmintics and endectocides; (v) coccidiostats which are also ruminant growth promoters; (vi) plant growth regulators (gibberellins); and (vii) anabolic agents in farm animals (zearelanone). Microbially produced polyethers[84], such as monensin, lasalocid and salinomycin, dominate the coccidiostat market and are also the chief growth promotants in use for ruminant animals; they are produced by species of *Streptomyces*. Among the antihelmintics and endectocides are the avermectins (ivermectin, doramectin), a group of streptomycete products having high activity against helminths (e.g., worms) and arthropods (e.g., lice, ticks, mites)[85]. The history of this amazing animal drug, which became an important human agent against river blindness disease (onchocerciasis) in the tropics, has been published[86,87]. The 1998 market for avermectins was over $1 billion divided among livestock ($750 million) and pets ($330 million)[88]. The activity of avermectin is an order of magnitude greater than that of previously discovered antihelmintic agents, the vast majority of which were produced synthetically.

Some of the above compounds have antibiotic activity either too weak or too toxic for medical use (e.g., monensin) or were discovered as mycotoxins (e.g., ergot alkaloids, gibberellins, zearelanone) before they found agricultural usage. The gibberellins are isoprenoid growth regulators controlling flowering, seed germination, and stem elongation[89]. They are produced at a level of over 25 tons per year with a global market of $125 million. The protein crystal of *B. thuringiensis* has a bioinsecticide market of $120 million[90] but its major importance lies in its gene used to render recombinant plants insect-resistant. In 2000, the world market for biopesticides was $450 million[91].

The animal health market involves 3.3 billion livestock, 16 billion poultry, and 1 billion pets. Of the five leading drugs for pets, at least two are made from fermentation products: ivermectin and milbemycin oxime. The animal health industry had sales of $11.3 billion in 2003, divided among antimicrobials (26%), biologicals (23%), parasiticides (32%), and other pharmaceuticals (19%).

5. Enzymes and bioconversions

Experiencing immediate impact from the developments in recombinant DNA technology was the industrial enzyme industry, which had been supplying enzymes with a market of about $300 million in the 1980s. Enzyme companies, realizing that their products were encoded by single genes, rapidly adopted recombinant DNA techniques to increase enzyme production and to make new enzymes. Much of the public is not aware that virtually all laundry detergents contain genetically engineered enzymes and that much cheese is made with a genetically engineered enzyme (chymosin, or rennin).

The industrial enzyme market has annual sales of $2.3 billion with applications in detergents (34%), foods (27%), agriculture and feeds (16%), textiles (10%), and leather, chemicals, and pulp and paper (10%). One hundred thousand tons of glucose isomerase, 40,000 tons of penicillin amidase, and 30,000 tons of nitrilase are made annually. The protease subtilisin, which is used in washing powders, accounts for $200 million of this market. The market for the animal-feed supplement phytase is $135 million. Over 60% of manufactured enzymes are recombinant products[92].

The world markets for some major products of enzymatic reactions are as high as $1 billion. *Streptomyces* glucose isomerase is used to isomerize D-glucose to D-fructose, to make 15 million tons per year of high fructose corn syrup, valued at $1 billion[93]. The high-intensity sweetener market, comprising aspartame, saccharin, cyclamate, neohesperidine DC, acesulfame-K, and thaumatin, amounts to $1 billion[94], with aspartame accounting for $800 million. *Pseudomonas chlorapis* nitrile hydratase is produced at 100,000 tons per year[95] and employed to produce 30,000 tons/year of acrylamide (valued at $300 million) from acrylonitrile[96]. *E.coli* penicillin amidase is used to prepare the ß -lactam intermediates 6-APA and 7-ADCA, valued at $200 million[96]. Some 40,000 tons of 6-APA are produced per year. Significant markets exist for specialty enzymes such as recombinant chymosin for cheese making ($140 million)[97], restriction enzymes for molecular techniques ($100 million)[98], and Taq polymerase for PCR applications ($80 million)[99]. Taq polymerase is the most popular of all reagents requested on NIH grants. A huge market ($2.3 billion) exists for therapeutic enzymes[100].

In addition to the multiple reaction sequences of fermentations, microorganisms are extremely useful in carrying out biotransformation processes, in which a compound is converted into a structurally related product by one or a small number of enzymes contained in cells[101]. Bioconverting organisms are known for practically every type of chemical reaction. Transformed steroids have been very important products for the pharmaceutical industry. One of the earliest and most famous is the biotransformation of progesterone to 11-α-hydroxyprogesterone. The reactions are stereospecific, the ultimate in specificity being exemplified by the steroid bioconversions. This specificity is exploited in the resolution of racemic mixtures, when a specific isomer rather than a racemic mixture is desired. Bioconversion has become essential to the fine chemical industry, in that customers are demanding single-isomer intermediates[102]. These reactions are characterized by extremely high yields, i.e., 90–100%. Other attributes include mild reaction conditions and the coupling of reactions using a microorganism containing several enzymes working in series. There is a tremendous interest in immobilized cells to carry out such processes. These are usually much more stable than either free cells or enzymes and are more economical than immobilized enzymes. Recombinant DNA techniques have been useful in developing new bioconversions. For example, the cloning of the fumarase-encoding gene in *S. cerevisiae* improved the bioconversion of malate to fumarate from 2 g per L to 125 g per L in a single manipulation[103]! The conversion yield using the constructed strain was near 90%.

6. Recombinant DNA and the rise of the biopharmaceutical industry

The biopharmaceutical industry has made a major impact in the business world. In 2002–2003, there were revenues of about $36 billion in the US and $40–50 billion in the world. By 2004, over 197 approved biotechnology drugs and vaccines had been developed by biotechnology companies, and revenues reached $63 billion. Over 5,000 companies exist in the world, and thousands of employees work in these firms.

The most well-known products of the modern biotechnology industry are the mammalian polypeptides. Peptide drugs have disadvantages of low bioavailability, thus requiring injection, and high cost, but their advantages of high specificity and low toxicity far outweigh the negative aspects. Drugs for cancer, blood clotting products used for hemophelia, colony stimulating factors for neutropenia, interferons, monoclonal antibodies, and metabolic products make up the major types of biopharmaceuticals on the market and in development. The best-selling biopharmaceuticals from 2002 to 2004 are shown in *Table 4*[104].

Other important products include GM-CSF (granulocyte-macrophage colony-stimulating factor), a hormone that activates the immune system to recognize and kill cancer cells and is used for bone marrow transplants ($1.5 billion), Gleevec from Novartis for chronic myeloid leukemia ($1.1 billion), Serono and Organon's follicle stimulating hormone for in vitro fertilization ($1 billion), Amgen's TNF receptor-binding protein for arthritis and other inflammatory diseases ($860 million), Genzyme's glucocerebrosidase (Cerezyme) for Gaucher's disease ($740 million), Bayer's Factor VIII for hemophelia ($670 million), Genentech's Activase and other TPAs (tissue plasminogen activators) for thrombotic disorders ($640 million), Novo-Nordisk's Factor VIIA ($630 million) for hemophelia, Serono's luteinizing hormone for in vitro fertilization ($590 million), and Chiron's (now Novartis) interleukin 2 (Proleukin) for metastatic kidney cancer and immunostimulation ($200 million).

Monoclonal antibodies are the fastest-growing therapeutic protein class. Over 20 monoclonal antibodies are on the market. Sales of therapeutic antibodies increased rapidly, from 1995, when they were in the

Table 4. Best-selling biopharmaceuticals

NAME	CLASS	INDICATION	COMMERCIAL NAMES	COMPANIES	MARKET ($ B)
Erythropoietin (EPO)	Hormone	Anemia	Epogen, Procrit, Eprex, Epogin, NeoRecormon, Aranesp	Amgen, Johnson & Johnson, Roche, Kirin, Sankyo	13.1
Interferon-α, interferon-β	Cytokines	Interferon-α: cancer, hepatitis Interferon-β: multiple sclerosis, hepatitis	PEG intron, Pegasys, Avonex, Rebif, Betaseron	Schering-Plough, Roche, Biogen, Serono, Schering AG, Chiron	6.0
Human insulin	Hormone	Diabetes	Novulin, Humalin, Humalog	Novo Nordisk, Eli Lilly	5.6
Granulocyte-colony stimulating factor (G-CSF)	Hormone	Neutropenia	Neupogen, Neulasta, Filgrastim, pegFilgrastim	Amgen, Roche, Schering	3.0
Rituximab	Monoclonal antibody	Non-Hodgkin's lymphoma	Rituxan	Genentech/Idec	2.8
Etanercept	Receptor fusion protein	Rheumatoid arthritis	Enbrel	Amgen, Wyeth	4.1
Infliximab	Monoclonal antibody	Crohn's disease	Remicade	Johnson & Johnson	2.1
Human growth hormone (HGH)	Hormone	Growth disorders and renal insufficiency	Saizen, Humatrope, Protopin, Neutropin	Serono, Genentech, Biogen Idec, Novo Nordisk, Akzo Nobel, Eli Lilly	1.8
Trastuzumab	Monoclonal antibody	Breast cancer	Herceptin	Roche	1.8
Palivizumab	Monoclonal antibody	Prevention against respiratory syncytial virus	Synagis	Medimmune	1.0

low millions, to $2 billion in 2000, $3.5 billion in 2001, $4.3 billion in 2002, $5.5 billion in 2003 and $6.8 billion in 2004. Monoclonal antibodies have moved from 100% of mouse origin to 30% mouse (chimeric), to 5% murine (humanized), to 100% human (fully human), with resulting increases in effectiveness. The first commercial monoclonal antibody was ReoPro for prevention of complications during coronary angioplasty[105]. It has a market of $400 million. Monoclonals shown in *Table 4* include rituximab, infliximab, trastuzumab, and palivizumab. Another is adalimumab (Humira) for rheumatoid arthritis, which targets tumor necrosis factor (TNF-α) and has a market of $1 billion. Titers of monoclonal antibodies have reached over 3 g per L.

7. Final comments

During the last few years, an expanded view of the cell has been possible due to the impressive advances in all the "omics" techniques (genomics, proteomics, metabolomics) and high-throughput technologies for measuring different classes of key intracellular molecules. "Systems biology" has recently emerged as a term and a scientific field to describe an approach that considers genome-scale and cell-wide measurements in elucidating process and mechanisms. Progress in strain development will depend not only on all the technologies mentioned above, but also on the development of mathematical methods that facilitate the elucidation of mechanisms and identification of genetic targets for modification. Such technologies and mathematical approaches will all contribute to the generation and characterization of microorganisms able to synthesize large quantities of commercially important metabolites. The ongoing sequencing projects involving hundreds of genomes, the availability of sequences corresponding to model organisms, new DNA microarray and proteomics tools, as well as new techniques for mutagenesis and recombination will accelerate strain improvement programs.

Today, microbiology is a major participant in modern global industry. It is hard to believe that it all started less than 70 years ago with the citric acid fermentation. The doubling of life expectancy in the developed countries is, in a large way, due to the discovery and exploitation of antibiotics. The discoveries of modern genetics and molecular biology led to the establishment of Cetus Corporation, the first biotechnology company, only 36 years ago. Today, this biopharmaceutical industry is making spectacular advances in medicine. The best is yet to come, as microbes move into the environmental and energy sectors. As stated many years ago by Louis Pasteur, "The microbe will have the last word."

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