Chapter 1

Advancement of Biotechnology by Genetic Modifications

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Abstract

One of the greatest sources of metabolic and enzymatic diversity are microorganisms. In recent years, emerging recombinant DNA and genomic techniques have facilitated the development of new efficient expression systems, modification of biosynthetic pathways leading to new metabolites by metabolic engineering, and enhancement of catalytic properties of enzymes by directed evolution. Complete sequencing of industrially important microbial genomes is taking place very rapidly, and there are already hundreds of genomes sequenced. Functional genomics and proteomics are major tools used in the search for new molecules and development of higher-producing strains.

Key words Agriculture, Bioconversions, Biopharmaceuticals, Enzymes, Genetic engineering, Hosts, Metabolic engineering, Polymers, Primary metabolites, Organic acids, Alcohols, Secondary metabolites, Bioinsecticides, Recombinant proteins

1 Introduction

Advantages of microorganisms in the production of compounds, as compared to isolation from plants and animals or synthesis by chemists, include (a) rapid uptake of nutrients that supports high rates of metabolism and biosynthesis; (b) capability of carrying out a wide variety of reactions; (c) facility to adapt to a large array of different environments; (d) ease of genetic manipulation, both in vivo and in vitro, to increase production, to modify structures and activities, and to make entirely new products; (e) simplicity of screening procedures; and (f) a wide diversity.

Products from microbes are very diverse, ranging from very large molecules, such as proteins, nucleic acids, carbohydrate polymers, or even cells, to small molecules that are usually divided into primary metabolites, that is, those essential for vegetative growth, and secondary metabolites, that is, those nonessential for growth.

1.1 Primary Metabolites

Synthesis of microbial products during the exponential phase of growth is an integral part of the normal growth process. They are intermediates or end products of the pathways, are building blocks

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for essential macromolecules (e.g., amino acids and nucleotides), or are converted into coenzymes (e.g., vitamins). Other primary metabolites (e.g., organic acids and ethanol) result from catabolic metabolism; they are not used for building cellular constituents, but their production, which is related to energy production and substrate utilization, is essential for growth. Industrially, the most important primary metabolites are amino acids, nucleotides, vitamins, alcohols, and organic acids.

Production of a particular primary metabolite by deregulated organisms may inevitably be limited by the inherent capacity of the particular organism to make the appropriate biosynthetic enzymes. Recent approaches utilize the techniques of modern genetic engineering to correct such deficiencies and develop strains overproducing primary metabolites. There are two ways to accomplish after this (a) to increase the number of copies of structural genes coding for these enzymes and (b) to increase the frequency of transcription.

Novel genetic technologies are important for the development of overproducers. Ongoing genome-sequencing projects, involving hundreds of genomes, the availability of sequences corresponding to model organisms, new DNA microarray and proteomic tools, as well as the new techniques for mutagenesis and recombination described below, will no doubt accelerate strain improvement programs.

Genome-based strain reconstruction achieves the construction of a superior strain, which only contains mutations crucial to hyperproduction, but not other unknown mutations, which accumulate by brute-force mutagenesis and screening [1].

The directed improvement of product formation or cellular properties via modification of specific biochemical reactions or introduction of new ones with the use of recombinant DNA technology is known as metabolic engineering [2, 3]. Analytical methods are combined to quantify fluxes and to control them with molecular biological techniques to implement suggested genetic modifications. The overall flux through a metabolic pathway depends on several steps, not just a single rate-limiting reaction. Amino acid production is one of the fields with many examples of this approach [4]. Other processes improved by this technique include vitamins, organic acids, ethanol, 1,3-propanediol, and carotenoids.

Reverse (inverse) metabolic engineering is another approach that involves choosing a strain which has a favorable cellular phenotype, evaluating and determining the genetic and/or environmental factors that confer that phenotype, and finally transferring that phenotype to a second strain via direct modifications of the identified genetic and/or environmental factors [5, 6].

Molecular breeding techniques are based on mimicking natural recombination by in vitro homologous recombination [7]. DNA shuffling not only recombines DNA fragments but also introduces point mutations at a very low controlled rate [8]. Unlike site-directed mutagenesis, this method of pooling and recombining parts of similar genes from different species or strains has yielded remarkable improvements in a very short amount of time [9]. Whole-genome shuffling is another technique that combines the advantage of multiparental crossing allowed by DNA shuffling with the recombination of entire genomes through recursive protoplast fusion [10, 11].

Systems biology is an integrated, systemic approach to the analysis and optimization of cellular processes by introducing a variety of perturbations and measuring the system response [12]. Altered phenotypes are created by molecular biological techniques or by altering environments. Further characterization of the phenotype leading to maximal product formation is analyzed and quantified through the use of genome-wide high-throughput *omics* data and genome-scale computational analysis.

1.2 Amino Acids

Many of the techniques mentioned above have made a great impact on the production of amino acids. Different strategies include (a) amplification of a rate-limiting enzyme of pathway, (b) amplification of the first enzyme after a branchpoint, (c) cloning of a gene encoding an enzyme with more or less feedback regulation, (d) introduction of a gene encoding an enzyme with a functional or energetic advantage as a replacement for the normal enzyme, (e) amplification of the first enzyme leading from central metabolism to increase carbon flow into the pathway, and (f) isolation of a transport mutant decreasing amino acid uptake and intracellular feedback control while improving excretion.

Among the amino acids, L-glutamate and L-lysine, mostly used as feed and food additives, represent the largest products in this category. Produced by fermentation are 1.5 million tons of L-glutamate and 850,000 tons of L-lysine HCl. The total amino acid market was about 4.5 billion dollars in 2004 [13]. High titers of amino acids are shown in Table 1.

1.2.1 L-Glutamate

Glutamate was the first amino acid to be produced by fermentation because of its use as a flavoring agent (monosodium glutamate, MSG). The process employs various species of the genera *Corynebacterium* and *Brevibacterium*. Molar yields of glutamate from sugar are 50–60%, and broth concentrations have reached 150 g/L. Glutamic acid overproduction is feedback regulated. However, mutant strains with modifications in the cell membrane are able to pump glutamate out of the cell, thus allowing its biosynthesis to proceed unabated. Introduction of the *Vitreoscilla* hemoglobin gene *vgb* into *Corynebacterium glutamicum* increased cell growth

Table 1
High amino acid levels produced by fermentation

Amino acid	Titer (g/L)
L-Alanine	120
L-Arginine	96
L-Glutamate	150
L-Glutamine	49
L-Histidine	42
L-Hydroxyproline	41
L-Isoleucine	40
L-Leucine	34
L-Lysine HCl	170
L-Methionine	25
L-Phenylalanine	51
L-Proline	108
L-Serine	65
L-Threonine	124
L-Tryptophan	60
L-Tyrosine	55
L-Valine	51

and glutamic acid and glutamine production via increased oxygen uptake [14]. Workers at Ajinomoto Co., Inc. increased glutamate production from glucose by 9% by suppressing CO_2 liberation in the pyruvate dehydrogenase reaction [15]. They did this by cloning the *xfp* gene-encoding phosphoketolase from *Bifidobacterium animalis* into *C. glutamicum* and overexpressing it.

1.2.2 L-Lysine

Since the bulk of the cereals consumed in the world are deficient in L-lysine, this essential amino acid became an important industrial product. Although the lysine biosynthetic pathway is controlled very tightly in an organism such as *Escherichia coli* and in lysine-producing organisms (e.g., mutants of *C. glutamicum* and its relatives), there is only a single aspartate kinase (AK), which is regulated via concerted feedback inhibition by threonine plus lysine. Metabolic engineering has been used in *C. glutamicum* to improve L-lysine production [4]. A chimeric AK, composed of the N-terminal catalytic region from *Bacillus subtilis* AKII and the C-terminal region from *Thermus thermophilus*, was evolved

through random mutagenesis and then screened using a high-throughput synthetic RNA device which comprises an L-lysine-sensing riboswitch and a selection module. Of the three evolved aspartate kinases, the best mutant (BT3) showed 160% increased in vitro activity compared to the wild-type enzyme [16].

Comparative genome analysis between a wild-type strain and an L-lysine-producing *C. glutamicum* strain identified three mutations that increased L-lysine production when introduced into the wild-type strain. Introduction of the 6-phosphogluconate-dehydrogenase *gnd* mutation increased yield by 15% [17]. Further improvement was achieved by introducing the *mqo* mutation (malate: quinone oxidoreductase) resulting in an increased titer of 95 g/L in a fed-batch culture [18]. Transcriptome analysis revealed that strain B-6, as compared to the wild type, is upregulated in both the pentose phosphate pathway genes and the amino acid biosynthetic genes and downregulated in TCA cycle genes. Lysine HCl titers have reached 170 g/L [19]. Metabolic flux studies of wild-type *C. glutamicum* and improved lysine-producing mutants showed that yield increased from 1.2% to 24.9% relative to the glucose flux. Different approaches have been used to increase lysine production by *C. glutamicum* mutants including (1) deletion of different genes from the glycolytic pathway [20], (2) improving the availability of pyruvate by eliminating pyruvate dehydrogenase activity [21], (3) overexpression of pyruvate carboxylase or DAP dehydrogenase genes, and (4) overexpression of gene NCg10855 encoding a methyltransferase or the *amtA-ocd-soxA* operon [22]. A genetically defined *C. glutamicum* L-lysine overproducer strain was developed by system metabolic engineering of the wild type. Implementation of only 12 defined genome-based changes in genes encoding central metabolic enzymes redirected major carbon fluxes as desired toward the optimal pathway usage predicted by in silico modeling. The final engineered *C. glutamicum* strain was able to produce lysine with a high yield of 0.55 g per gram of glucose, a titer of 120 g/L of lysine, and a productivity of 4.0 g/L/h in fed-batch culture [23].

1.2.3 L-Threonine

Overproduction of L-threonine has been achieved in *Serratia marcescens* by transductional crosses, which combined several feedback control mutations in a single organism. This resulted in titers up to 25 g/L [24]. Combination in a single strain of another six regulatory mutations derived by resistance to amino acid analogs led to desensitization and derepression of the key enzymes of threonine synthesis. The resultant transductant produced 40 g/L of threonine [25]. The use of recombinant DNA technology led to strains that made 63 g/L threonine [26]. An *E. coli* strain was developed via mutation and genetic engineering and optimized by the inactivation of threonine dehydratase (TD) resulting in a process yielding 100 g/L of L-threonine in 36 h of fermentation [27].

In *C. glutamicum* ssp. *lactofermentum*, L-threonine production reached 58 g/L when a strain producing both threonine and lysine was transformed with a plasmid carrying its own *hom*, *thrB*, and *thrC* genes [28]. A recombinant isoleucine auxotrophic strain of *E. coli* (carrying extra copies of the *thrABC* operon, an inactivated *tdh* gene and mutated to resist high concentrations of L-threonine and L-homoserine) produced 80 g/L L-threonine in 1.5 days with a yield of 50% [29]. Cloning extra copies of threonine export genes into *E. coli* also increased threonine production [30].

Systems metabolic engineering was used to develop an L-threonine1-overproducing *E. coli* strain [31]. Feedback inhibition of aspartokinase I and III (encoded by *thrA* and *lysC*, respectively) and transcriptional attenuation regulation (*thrL*) were removed. Deletion of *tdh* and mutation of *ilvA* avoid threonine degradation. The *metA* and *lysA* genes were deleted to make more precursors available for threonine biosynthesis. Further target genes to be engineered were identified by transcriptome profiling combined with in silico flux response analysis. The final engineered *E. coli* strain was able to reach a high yield of 0.393 g of threonine per gram of glucose and 82.4 g/L in a fed-batch culture. With the use of an L-isoleucine leaky (ILE^L) and α-amino-β-hydroxyvaleric acid resistant (AHV^r) *E. coli* TRFC strain, carrying a pTHR101 plasmid containing a Thr operon, a threonine production of 118 g/L was achieved after 38 h with a productivity of 3.1 g/L/h (46% conversion ratio from glucose to threonine) [32]. A further increase in amino acid production to 124.57 g/L was obtained in TRFC strain by decreasing acetic acid production using combined feeding strategies [33]. Acetic acid is an inhibitor of growth and threonine production.

1.2.4 L-Valine

Combined rational modification, transcriptome profiling, and systems-level in silico analysis was used to develop an *E. coli* strain for the production of this amino acid [34]. The *ilvA*, *leuA*, and *panB* genes were deleted to make more precursors available for L-valine biosynthesis. This engineered strain, harboring a plasmid overexpressing the *ilvBN* genes, produced 1.3 g/L L-valine. Overexpression of the *lrp* and *ygaZH* genes (encoding a global regulator Lrp and L-valine exporter, respectively), and amplification of the *lrp*, *ygaZH*, and *lrp-ygaZH* genes enhanced production of L-valine by 21.6%, 47.1%, and 113%, respectively. Further improvement was achieved by using in silico gene knockout simulation, which identified the *aceF*, *mdh*, and *pfkA* genes as knockout targets. The VAMF strain (Val Δ*aceF* Δ*mdh* Δ*pfkA*) was able to produce 7.5 g/L L-valine from 20 g/L glucose in batch culture, resulting in a high yield of 0.378 g of L-valine per gram of glucose. Production by mutant strain VAL1 of *C. glutamicum* amounted to 31 g/L [35]. The mutant was constructed by overexpressing

biosynthetic enzymes via a plasmid, eliminating *ilvA* encoding threonine dehydratase, and deleting two genes encoding enzymes of pantothenate biosynthesis. The culture was grown with limiting concentrations of isoleucine and pantothenate. By applying metabolic engineering, a *C. glutamicum* strain (WCC003 harboring pJYW-4-*ilvBNC1-lrp1-brnFE*) deleted in the genes *aceE, alaT,* and *ilvA* and overexpressing the *ilvB, ilvN, ilvC, lrp1, brnF,* and *brnE* genes produced 51 g/L L-valine in fed-batch fermentation, with almost no detectable amino acid by-products such as L-alanine and L-isoleucine [36].

1.2.5 L-Isoleucine

Isoleucine processes have been devised in various bacteria such as *S. marcescens*, *C. glutamicum* ssp. *flavum*, and *C. glutamicum*. In *S. marcescens*, resistance to isoleucine hydroxamate and α-aminobutyric acid led to derepressed L-threonine deaminase (TD) and acetohydroxyacid synthase (AHAS) and production of 12 g/L of isoleucine [37]. Further work involving transductional crosses into a threonine overproducer yielded isoleucine at 25 g/L [24].

Feedback regulation in *C. glutamicum* was eliminated [38] by replacing the native threonine dehydratase gene *ilvA* with the feedback-resistant gene from *E. coli*. By introducing additional copies of genes encoding branched amino acid biosynthetic enzymes, lysine- or threonine-producing strains were converted into L-isoleucine producers with improved titers [39–41]. Amplification of the wild-type threonine dehydratase gene *ilvA* in a threonine-producing strain of *Corynebacterium lactofermentum* led to isoleucine production [42].

A threonine-overproducing strain of *C. glutamicum* was sequentially mutated to resistance to thiaisoleucine, azaleucine, and aminobutyric acid; it produced 10 g/L of isoleucine [43]. Metabolic engineering studies involving overexpression of biosynthetic genes were useful in improving isoleucine production by this species. Colon et al. [42] obtained an isoleucine-producing strain by cloning multiple copies of *hom* (encoding HDI) and wild-type *ilvA* (encoding TD) into a lysine overproducer and by increasing HK (encoded by *thrB*); a titer of 15 g/L isoleucine was obtained. Independently, Morbach et al. [44] cloned three copies of the feedback-resistant HD gene (*hom*) and multicopies of the deregulated TD gene (*ilvA*) in a deregulated lysine producer of *C. glutamicum*, yielding an isoleucine producer (13 g/L) with no threonine production and reduced lysine production. Application of a closed loop control fed-batch strategy raised production to 18 g/L [45]. Further metabolic engineering work involving amplification of feedback inhibition-insensitive biosynthetic enzymes converted lysine overproducers and threonine overproducers into *C. glutamicum* strains yielding 30 g/L of isoleucine [46].

C. glutamicum ssp. *flavum* studies employed resistance to α-amino-β-hydroxyvaleric acid, and the resultant mutant produced

11 g/L [47]. D-Ethionine resistance was used by Ikeda et al. [48] to yield a mutant producing 33 g/L in a fermentation continuously fed with acetic acid. Accumulated L-isoleucine in the cell is excreted via a two-component export system BrnFE, which is regulated by the global regulator Lrp. Overexpression of Lrp and BrnFE in *C. glutamicum* led to 27 g/L production in fed-batch fermentation [49]. A *C. glutamicum* strain overexpressing the threonine dehydratase (ilvA1) and acetohydroxy acid synthase (ilvBN1) feedback-resistant genes, as well as the ppnk1 gene, encoding NAD kinase (Strain IWJ001/pDXW-8-ilvBN1-ilvA1-ppnk1), led to a 32 g/L isoleucine production in a 72 h fed-batch fermentation [50]. Recently in a *Brevibacterium flavum* strain KM011 (Met^-+Eth^r+α-AB^r+Lys^L+AEC^r), betaine, vitamin B12, and vitamin B5 concentrations were optimized for isoleucine production in shake flasks. Under these conditions, a maximum of 13.35 g/L isoleucine was produced. However, a production of 35.26 g/L was obtained with optimized conditions using a 5-L fermenter [51].

1.2.6 L-Alanine

Lee et al. [52] introduced into an *E. coli* double mutant (lacking genes encoding a protein of the pyruvate dehydrogenase complex [*aceF*] and lactate dehydrogenase [*ldhA*]) a plasmid containing the *Bacillus sphaericus* alanine dehydrogenase gene (*alaD*). The strain produced L-alanine in 27 h with a yield on glucose of 0.63 g/g and a maximum productivity of 2 g/L/h. Further work has raised the titer to 114 g/L. A genetically engineered *E. coli* W (strain, XZ132), with a D-lactate dehydrogenase replaced by an alanine dehydrogenase from *Geobacillus stearothermophilus* and deletions in the methylglyoxal synthase (mgsA) and the catabolic alanine racemase (dadX) genes, produced 1279 mmol alanine [53]. Deletion of both enzymes reduced low levels of lactate and conversion of L- to D-alanine. A thermoregulated genetic switch, designed to dynamically control the expression of L-alanine dehydrogenase (alaD) from *G. stearothermophilus* on the *E. coli* B0016-060BC chromosome, led to an L-alanine titer of 120.8 g/L with higher overall and oxygen-limited volumetric productivities of 3.09 and 4.18 g/L/h, respectively, using glucose as the sole carbon source [54].

1.2.7 L-Proline

Proline-hyperproducing strains of bacteria, exhibiting reduced proline-mediated feedback inhibition of γ-glutamyl kinase (GK) activity (a result of single-base pair substitutions in the bacterial *proB* gene-coding region), have been isolated based on their resistance to toxic proline analogs (L-azetidine-2-carboxylic acid and 3,4-dehydro-DL-proline), compounds which inhibit GK activity while not interfering with protein synthesis. Cloning of the three genes of proline biosynthesis in *E. coli* on multicopy plasmids and selection of mutants of such plasmid-containing strains to

resistance to 3,4-dehydroproline led to a process producing 20 g/L proline [55].

A mutant of *S. marcescens* resistant to 3,4-dehydroproline, thiazolidine-4-carboxylate, and azetidine-2-carboxylate and unable to utilize proline produced 50–55 g/L L-proline [56]. Cloning of a gene bearing the dehydroproline-resistance locus on a plasmid yielded a recombinant strain of *S. marcescens* producing 75 g/L [57]. Further development work increased the production to over 100 g/L [58].

A sulfaguanidine-resistant mutant of *C. glutamicum* ssp. *flavum* produced 35 g/L L-proline [59]. When a glutamate-producing strain of *C. glutamicum* was grown under modified conditions, it made 48 g/L [60]. A strain of *Corynebacterium acetoacidophilum* produced 108 g/L proline when grown in the presence of glutamate [61].

1.2.8 L-Hydroxyproline

Introduction of the proline 4-hydroxylase gene from *Dactylosporangium* sp. into recombinant *E. coli* producing L-proline at 1.2 g/L led to a new strain producing 25 g/L of hydroxyproline (*trans*-4-hydroxy-L-proline) [62]. When proline was added, the hydroxyproline titer reached 41 g/L, with a yield of 87% based on the amount of proline added. Optimization of the gene codons and vectors of proline-4-4-hydroxylase (*P4H*) in a recombinant *E. coli* BL21 strain and chemo-physical combination mutagenesis allowed isolation of strain NA45, able to grow in glycerol as a sole carbon source. With further systemic optimization of nutritional elements, the strain produced 25.4 g/L trans-4-hydroxy-L-proline at 48 h in fed-batch mode in a 5 L fermentor [63].

1.3 Nucleotides

Nucleotide fermentations became commercially important due to the activity of two purine ribonucleoside 5′-monophosphates, namely, guanylic acid (GMP) and inosinic acid (IMP), as enhancers of flavor. Titers of IMP and GMP have reached 30 g/L [64]. The techniques used to achieve such production are similar to those used for amino acid fermentations. In Japan, 2500 tons of GMP and IMP are produced annually with a market of $350 million. GMP can also be made by bioconversion of xanthylic acid (XMP). Genetic modification of *Corynebacterium ammoniagenes* involving transketolase, an enzyme of the nonoxidative branch of the pentose phosphate pathway, resulted in the accumulation of 39 g/L of XMP [65].

1.4 Vitamins

Riboflavin. Production of riboflavin (vitamin B_2) reached over 6000 tons per year and a titer of 20 g/L by overproducers such as the yeast-like molds, *Eremothecium ashbyii* and *Ashbya gossypii*. A bacterial process using *C. ammoniagenes* (previously *Brevibacterium ammoniagenes*) was developed by cloning and overexpressing the organisms own riboflavin biosynthetic genes [66] and its own

promoter sequences. The resulting culture produced 15 g/L riboflavin in 3 days.

Genetic engineering of a *Bacillus subtilis* strain, already containing purine analog-resistance mutations, led to improved production of riboflavin [67]. The industrial strain of *B. subtilis* was produced by [1] making purine analog-resistance mutations to increase guanosine triphosphate (GTP; a precursor) production and [2] using a riboflavin analog (roseoflavin)-resistance mutation in *ribC* that deregulated the entire pathway [68]. Resultant production was over 25 g/L. A genome-wide transcript expression analysis [69] was successfully used to discover new targets for further improvement of the fungus *A. gossypii* [70]. The authors identified 53 genes of known function, some of which could clearly be related to riboflavin production. A metabolic engineering approach overexpressing the *ribA* (encoding 3,4-dihydroxy-2-butanone 4-phosphate synthase) gene in *B. subtilis* RB50::[pRF69]n[pRF93] strain led to productions of 15 g/L riboflavin [71].

Biotin has traditionally been made by chemical synthesis, but recombinant microbes have approached a competitive economic position. Cloning of a biotin operon (*bioABFCD*) on a multicopy plasmid allowed *E. coli* to produce 10,000 times more biotin than did the wild-type strain [72]. Sequential mutation of *S. marcescens* to resistance to the biotin antimetabolite acedomycin (=actithiazic acid) led to mutant strain SB412, which produced 20 mg/L of biotin [73]. Further improvements were made by mutating selected strains to ethionine resistance (strain ET2, 25 mg/L), then mutating ET2 to S-2-aminoethylcysteine resistance (strain ETA23, 33 mg/L), and finally cloning in the resistant *bio* operon yielding a strain able to produce 500 mg/L in a fed-batch fermentation along with 600 mg/L of biotin vitamers. Later advances led to production by recombinant *S. marcescens* of 600 mg/L of biotin [74]. A process using an *E. coli* mutant resistant to β-hydroxynorvaline (a threonine antimetabolite) yielding 970 mg/L has been patented [75]. Biotin production was further increased to over 1 g/L by the use of a *B. subtilis* strain resistant to 5-(2-thenyl) pentanoic acid (a biotin analog) and overexpressing several *bio* genes.

Vitamin C (ascorbic acid) has traditionally been made in a five-step chemical process by first converting glucose to 2-keto-L-gulonic acid (2-KGA) with a yield of 50% and then converting the 2-KGA by acid or base to ascorbic acid (L-AA). Annual production is 110,000 tons generating revenue of over 600 million dollars. A novel process for vitamin C synthesis involved the use of a genetically engineered *Erwinia herbicola* strain containing a gene from *Corynebacterium* sp. The engineered organism converted glucose into 1 g/L of 2-KGA [76]. A better process was devised independently which converted 40 g/L of glucose into 20 g/L of 2-KGA [77]. This process involved cloning and expressing the gene

encoding 2,5-diketo-D-gluconate reductase from *Corynebacterium* sp. into *Erwinia citreus*. Another process used a recombinant strain of *Gluconobacter oxydans* containing genes encoding L-sorbose dehydrogenase and L-sorbosone dehydrogenase from *G. oxydans* T-100. The new strain was an improved producer of 2-KGA [78]. Further mutation to suppress the L-idonate pathway and to improve the promoter led to the production of 130 g/L of 2-KGA from 150 g/L of sorbitol. Further improvement of the strain was possible by suppressing the L-idonate pathway [79]. An *Erwinia herbicola*, transformed with the 2,5-diketo-D-gluconate reductase gene from *Corynebacterium* sp., produced up to 120 g/L 2-KGA in less than 120 h with the help of glucose dehydrogenase, gluconate dehydrogenase, ketogluconate dehydrogenase, and 2,5-diketo-D-gluconate reductase [80]. Similar to the chemical conversion of 2-KGA to vitamin C by lactonization, *X. campestris* probably lactonizes 2-KGA under oxidative stress to form L-AA. Using this method, 20.4 g/L L-AA were found in the extracellular broth which corresponds to the 14-fold amount of that detected in yeast cells directly synthesizing L-AA from D-glucose [81].

Vitamin B_{12} production depends on avoidance of feedback repression by vitamin B_{12}. The vitamin is industrially produced by *Propionibacterium shermanii* or *Pseudomonas denitrificans*. The early stage of the *P. shermanii* fermentation is conducted under anaerobic conditions in the absence of the precursor 5,6-dimethylbenzimidazole. These conditions prevent vitamin B_{12} synthesis and allow for the accumulation of the intermediate, cobinamide. Then, the culture is aerated, and dimethylbenzimidazole is added, converting cobinamide to the vitamin. In the *P. denitrificans* fermentation, the entire process is carried out under low levels of oxygen. A high level of oxygen results in an oxidizing intracellular environment, which represses the formation of the early enzymes of the pathway. Production of vitamin B_{12} has reached levels of 150 mg/L, 10 tons per year, and a world market of $71 million.

Other vitamins. Recombinant *E. coli*, transformed with genes encoding pantothenic acid (vitamin B_5) biosynthesis, and resistant to salicylic and/or other acids, produce 65 g/L of D-pantothenic acid from glucose using alanine as precursor [82]. A total of 7000 tons per year are made chemically and microbiologically. Thiamine (vitamin B_1) is produced synthetically at 4000 tons per year. Pyridoxine (vitamin B_6) is made chemically at 2500 tons per year.

1.5 Carotenoids

Carotenoid production processes have been extensively studied [83], but they have had difficulty in economically challenging chemical methods. Of over 600 microbial carotenoids, only β-carotene and astaxanthin are produced industrially by fermentation [84]. A semi-industrial β-carotene process was developed

using mated cultures of *Blakeslea trispora* plus and minus strains. β-Carotene was produced at 1 g/L in the early 1960s [85]. By the addition of carotogenic chemicals and antioxidants, the titer was raised to over 3 g/L [86]. Processes in development include those yielding β-carotene, lycopene, zeaxanthin, and astaxanthin. Some have been improved by metabolic engineering and directed evolution [87–89]. Metabolic engineering of *E. coli* has led to strains forming 0.2 g/L of lycopene [90]. Lutein, a xanthophyll carotenoid with antioxidant properties, had sales as a food colorant of $150 million in the USA [91]. It is thought that this carotenoid prevents age-related macular degeneration and cataracts. It is made from petals of marigold, but microalgae are a potential new source.

Chemical production of *trans*-astaxanthin has a selling price of $2,000 kg [92] and a market of over $100 million per year. It is mainly used for pigmentation of salmonids raised in aquaculture, a multibillion dollar industry [93]. Astaxanthin can be made by the yeast *Phaffia rhodozyma* (*Xanthophyllomyces dendrorhous*) and the microalga *Haematococcus pluvialis*. Genetically improved strains of *P. rhodozyma* produce 10 mg/g cells in industrial fermentors. Recent improvements in astaxanthin production have been published by de la Fuente et al. [94] and Rodríguez-Sáiz et al. [95] to get maximal astaxanthin titers of 420 mg/L when *X. dendrorhous* is fermented under continuous white light. Recently astaxanthin production has been reported with an engineered *C. glutamicum* strain. Volumetric productivities of up to about 0.4 mg/L/h reported in simple shaking flask cultures by the recombinant strain compare favorably with those reported for the commercially used production hosts such as the green microalga *H. pluvialis*, the red yeast *X. dendrorhous*, and the recombinant *E. coli* [96].

1.6 Organic Acids

Microbial production of organic acids is an excellent approach for obtaining building-block chemicals from renewable carbon sources [97]. Production of some organic acids started decades ago, and titers have been improved by classical mutation and screening/selection techniques as well as by metabolic engineering [98].

1.6.1 Citric Acid

Citric acid has a market of $2 billion. About 1 million tons per year are produced annually by *Aspergillus niger* and yeasts. The commercial process employs *A. niger* in media deficient in iron and manganese. Other factors contributing to high citric acid production are a high intracellular concentration of fructose 2,6-biphosphate, inhibition of isocitrate dehydrogenase, and low pH (1.7–2.0). In approximately 4–7 days, the major portion (80%) of the sugar (glucose or sucrose) provided is converted into citric acid. *A. niger* titers have reached over 200 g/L [99].

Alternative processes have been developed with *Candida* yeasts, especially from hydrocarbons. Such yeasts are able to convert *n*-paraffins to citric and isocitric acids in extremely high yields

(150–170% on a weight basis). Titers as high as 225 g/L have been reached [100]. Anastasiadis and Rehm [101] reported production levels of 250 g/L with *Candida oleophila* ATCC 20177 under submerged continuous fermentation, with glucose as carbon source.

1.6.2 Acetic Acid

Titers of acetic acid reached 53 g/L with genetically engineered *E. coli* [102] and 83 g/L with a *Clostridium thermoaceticum* mutant [103]. Cloning of the aldehyde dehydrogenase gene from *Acetobacter polyoxogenes* on a plasmid vector into *Acetobacter aceti* subsp. *xylinum* increased the rate of acetic acid production by over 100% (1.8–4 g/L/h) and the titer by 40% (68–97 g/L) [104].

1.6.3 Lactic Acid

Whole-genome shuffling was used to improve the acid tolerance of a commercial lactic acid-producing *Lactobacillus* sp. [105]. Further approaches using this recursive protoplast fusion technique yielded strains of *Lactobacillus rhamnosus* ATCC 11443 with improved glucose tolerance (160–200 g/L glucose) while simultaneously enhancing L-lactic acid production by 71% as compared to the wild type. Shuffling of a mutant strain of *Lactobacillus delbrueckii* NCIM 2025 and *Bacillus amyloliquefaciens* ATCC 23842 produced a fusant that could utilize liquefied cassava bagasse starch directly to yield a titer of 40 g/L of lactic acid with a 96% conversion of starch to lactic acid [106].

Although lactobacilli make more lactic acid than *Rhizopus oryzae*, they produce mixed isomers. The fungus, however, produces L-(+) lactic acid exclusively. The yield is about 60–80% of added glucose, the remainder going to ethanol. By increasing lactic dehydrogenase levels via plasmid transformation with *ldhA*, more lactate could be made from pyruvate, and production was increased to 78 g/L, whereas the undesirable coproduct ethanol was reduced from 10.6 to 8.7 g/L [107]. A recombinant *Saccharomyces cerevisiae* strain containing six copies of bovine L-lactate dehydrogenase produced 122 g/L from sugar cane with an optical purity of 99.9% or higher [108]. Expression of the same bovine enzyme and a deletion of the pyruvate decarboxylase gene in *Kluyveromyces lactis* produced 109 g/L [109].

A recombinant *E. coli* strain was constructed that produced optically active pure D-lactic acid from glucose at virtually the theoretical maximum yield, for example, two molecules from one molecule of glucose [110]. D-Lactic acid has also been produced at 61 g/L by a recombinant strain of *S. cerevisiae* containing the D-lactic dehydrogenase gene from *Leuconostoc mesenteroides* [111].

1.6.4 Succinic Acid

Sanchez et al. [112] used metabolic engineering to create an *E. coli* strain, which had three deactivated genes of the central metabolic pathway, that is, *adhE*, *ldhA*, and *act-pta*, and an inactivated *iclR*

gene, which resulted in activation of the glyoxylate pathway. The strain produced 40 g/L of succinate. Metabolic engineering of *Mannheimia succiniciproducens* led to a strain producing 52 g/L of succinic acid at a yield of 1.16 mol/mol glucose and a productivity of 1.8 g/L/h in fed-batch culture [113]. A metabolically engineered succinate-producing strain of *E. coli* yielded 58 g/L succinate in a 59 h fed-batch fermentation under aerobic conditions [114]. The average succinate yield was 0.94 mol/mol of glucose, the average productivity was 1.08 g/L/h, and the average specific activity was 90 mg/g/h. A titer of 99 g/L with a productivity of 1.3 g/L/h has been reached with recombinant *E. coli* [115].

1.6.5 Other Organic Acids

Metabolic engineering of *Clostridium tyrobutyricum* created a fermentation strain yielding 80 g/L butyric acid and a yield on glucose of 0.45 g/g [116]. *S. cerevisiae* normally produces 2 g/L of malic acid from fumaric acid. However, a recombinant strain containing a cloned fumarase gene was able to produce 125 g/L with a yield of almost 90% [117]. Microbial fermentation titers of some other organic acids are 135 g/L pyruvic acid, 107 g/L fumaric acid, 90 g/L shikimic acid, 69 g/L dehydroshikimic acid, 85 g/L itaconic acid, 504 g/L gluconic acid, 106 g/L propionic acid, 68 g/L oxalic acid, and 136 g/L glyceric acid. An oxidative bioconversion of saturated and unsaturated linear aliphatic 12–22 carbon substrates to their terminal dicarboxylic acids was developed by gene disruption and gene amplification [118]. Product concentrations reached 200 g/L, and problematic side reactions such as unsaturation, hydroxylation, and chain-shortening did not occur.

1.7 Alcohols

Ethanol. Fermentation of sugars by *S. cerevisiae* in the case of hexoses, and *Kluyveromyces fragilis* or *Candida* species with lactose or a pentose, results in the production of ethanol. Under optimum conditions, approximately 120 g/L ethanol can be obtained. Such a high concentration slows down growth and the fermentation ceases.

A *S. cerevisiae* fusant library obtained by genome shuffling was screened for growth at 35, 40, 45, 50, and 55 °C on agar plates containing different concentrations of ethanol [119]. After three rounds of genome shuffling, a strain was obtained which was able to grow on plates up to 55 °C, completely utilized 20% (w/v) glucose at 45–48 °C, produced 99 g/L ethanol, and tolerated 25% (v/v) ethanol stress.

In silico metabolic models have been used to overcome the redox imbalance in *S. cerevisiae* engineered with the *Xyl1* and *Xyl2* genes from *Pichia stipitis* [120, 121]. Overexpression of both genes led to an accumulation of NADH and a shortage of NADPH. Deletion of $NADP^+$-dependent glutamate dehydrogenase (GGH1) and overexpression of NAD^+-dependent GDH2 led to an increase in ethanol production using xylose as fermentation

substrate. An in silico genome-scale gene insertion strategy was used to improve ethanol production and decrease the production of by-products glycerol and xylitol [122]. Introduction of glyceraldehyde-3-phosphate dehydrogenase in *S. cerevisiae* led to a 58% reduction in glycerol, a 33% reduction in xylitol, and a 24% increase in ethanol production.

When biomass is used as a carbon source for ethanol production, its breakdown results in liberation of acetic acid. The acid interferes with ethanol production. Tolerance of *Candida krusei* GL560 to acetic acid was improved by genome shuffling [123]. A mutant, S4-3, which was isolated and selected after four rounds, had a higher viability in different media containing acetic acid than did the parent strain GL560. The mutant also improved its multiple stress tolerance to ethanol, H_2O_2, heat, and freeze-thawing.

E. coli was converted into an ethanol producer (43 g/L) by cloning the alcohol dehydrogenase II and pyruvate decarboxylase genes from *Zymomonas mobilis* [124]. By cloning and expressing the same two genes in *Klebsiella oxytoca*, the recombinant was able to convert crystalline cellulose to ethanol in high yield when fungal cellulase was added [125]. Maximum theoretical yield was 81–86%, and a titer of 47 g/L of ethanol was produced from 100 g/L of cellulose.

Recombinant strains of *E. coli*, *Zymomonas*, and *Saccharomyces* can convert corn fiber hydrolysate to 21–35 g/L ethanol with yields of 0.41–0.50 ethanol per gram of sugar consumed [126]. For a recombinant *E. coli* strain making 35 g/L, the time was 55 h, and the yield was 0.46 g ethanol per gram of available sugar, which is 90% of the attainable maximum.

1,3-Propanediol. A strain of *Clostridium butyricum* converts glycerol to 1,3-propanediol (PDO) at a yield of 0.55 g/g glycerol consumed [127]. A major metabolic engineering feat was carried out in *E. coli* leading to a culture growing on glucose and producing PDO at 135 g/L, with a yield of 51% and a rate of 3.5 g/L/h [128]. To do this, eight new genes were introduced to convert dihydroxyacetone phosphate (DHAP) into PDO. Production was further improved by modifying 18 *E. coli* genes, including regulatory genes. PDO is the monomer used to chemically synthesize industrial polymers such as polyurethanes and the polyester fiber Sorono™ by DuPont. This bioplastic is polytrimethylene terephthalate (3GT polyester) made by reacting terephthalic acid with PDO [129]. PDO is also used as a polyglycol-like lubricant and as a solvent.

D-*Mannitol* is a naturally occurring polyol, widely used in the food, chemical, and pharmaceutical industries. A whole-cell bioconversion of D-fructose to D-mannitol was developed by metabolic engineering of *E. coli* [130]. The *mdh* gene encoding mannitol dehydrogenase from *Leuconostoc pseudomesenteroides* and the *fdh* gene encoding formate dehydrogenase from *Mycobacterium*

vaccae were coexpressed in *E. coli* along with the *glf* gene encoding the glucose facilitator protein of *Z. mobilis*. The process yielded 75–91 g/L of D-mannitol, a specific productivity of 3.1–4.1 g/g/h and a molar yield of 84–92% with no by-products. An improved bioconversion process was developed with a recombinant *E. coli* strain in the presence of added glucose isomerase yielding 145 g/L of D-mannitol from 180 g/L glucose [131]. Supplementation of the medium used for mannitol production by *Candida magnolia* with Ca^{2+} and Cu^{2+} increased production up to 223 g/L [132].

Sorbitol, also called D-glucitol, is 60% as sweet as sucrose and is used in the food, pharmaceutical, and other industries. Its worldwide production is estimated to be higher than 500,000 tons per year, and it is made chemically by catalytic hydrogenation of D-glucose or syrup with a 50:50 mixture of glucose and fructose. It is also produced by extraction from seaweed as a by-product of alginate and iodine manufacture. However, excellent microbial processes have been developed [133]. Toluenized (permeabilized) cells of *Z. mobilis* produce 290 g/L of sorbitol and 283 g/L of gluconic acid from a glucose and fructose mixture in 16 h with yields nearly 95% for both products [134]. Other leading organisms are recombinant *C. glutamicum* at 285 g/L, *Lactobacillus intermedius* at 227 g/L, *C. magnoliae* at 223 g/L, and many others producing between 100 and 200 g/L. Metabolic engineering of *Lactobacillus plantarum* for high sorbitol production was successfully achieved by a simple two-step strategy overexpressing the two sorbitol-6-phosphate dehydrogenase genes (*srlD1* and *srlD2*) identified in the genome sequence [135].

n-Butanol is a good alternative fuel additive as it has two more carbons than ethanol, which results in an energy content about 40% higher. Also, automobile engines do not require modification until the percentage of butanol reaches over 40% of the total automobile fuel. In contrast, modification is required when ethanol is added to gasoline at levels exceeding 15%. Butanol can be obtained from the acetone–butanol–ethanol fermentation of *Clostridium beijerinckii* or *Clostridium acetobutylicum*. Butanol-resistant mutants showed increased production of butanol and acetone [136]. Biochemical engineering modifications were able to increase total acetone, butanol, and ethanol production (ABE) to 69 g/L [137]. A mutant in the presence of added acetate was able to produce almost 21 g/L butanol and 10 g/L of acetone from glucose [138].

Because butanol's octane number is lower than that of ethanol and the octane number increases with methyl branching and double bonds, other higher alcohols are also being considered as biofuels, for example, branched C 4 and C 5 alcohols. They are also desirable because of their higher energy density, lower vapor pressure, and lower hygroscopicity as compared to ethanol [139]. They include isopropanol, 1-propanol, 1-butanol (*n*-butanol), isobutanol (2-methyl-propanol), 3-methyl-1-butanol,

2-methyl-1-butanol, isopentanol (3-methyl-1 butanol), and isopentenol (3-methyl-3-buten-1-ol). A novel screening method based on overcoming the toxicity associated with the accumulation of prenyl diphosphate was used to screen a library of 19,000 clones harboring fragments of *Bacillus* genomic DNA [140]. Two genes, *yhfR* and *nudF*, coding for proteins capable of overcoming the toxicity associated with accumulating IPP and DMAPP were isolated. Both protein products have an affinity for IPP and DMAPP, converting them into isopentenol. *Clostridium beijerinckii* (*Clostridium butylicum*) produces 20 g/L of 1-butanol and 2 g/L of isopropanol as part of a mixed product. Recombinant *E. coli* can produce 4.9 g/L of isopropanol. Recently, a new strategy for the production of these alcohols has been reported [141]. This approach is based on the diversion of 2-keto acid intermediates from the endogenous amino acid pathway to alcohol biosynthesis especially that of isobutanol. As a result, engineered *E. coli* can produce 22 g/L of isobutanol in 110 h with a yield of 86% of the theoretical maximum.

Other alcohols. The noncariogenic, noncaloric, and diabetic-safe sweetener erythritol has 70–80% of the sweetness of sucrose. It can be produced by *Aureobasidium* sp. (165 g/L), an acetate-negative mutant of *Yarrowia lipolytica* (170 g/L), a *C. magnoliae* osmophilic mutant (187 g/L), the osmophile *Trichosporon* sp. (88 g/L), *Torula* sp. (200 g/L), and the yeast *Pseudozyma tsukubaensis* (245 g/L) [142].

Xylitol is a naturally occurring sweetener with anticariogenic properties, which is used for some diabetes patients. It can be produced by chemical reduction of D-xylose or by fermentation. Xylitol production at 150 g/L was obtained with *Candida guilliermondii* 2581 at pH 6.0 and shaking at 60 rpm [143].

2 Secondary Metabolites

As a group that includes antibiotics, pesticides, pigments, toxins, pheromones, enzyme inhibitors, immunomodulating agents, receptor antagonists and agonists, pesticides, antitumor agents, immunosuppressants, cholesterol-lowering agents, plant protectants, and animal and plant growth factors, these metabolites have tremendous economic importance. This remarkable group of compounds is produced by certain restricted taxonomic groups of organisms and is usually formed as mixtures of closely related members of a chemical family.

2.1 Antibiotics

Antibiotics are the most well-known secondary metabolites and have a tremendous importance. The most well known are the β-lactams, tetracyclines, aminoglycosides, chloramphenicol, macrolides, and other polyketides, polyenes, and glycopeptides, among

others. They have been crucial in the increase in average life expectancy in the USA from 47 years in 1900 to 74 for males and 80 for women in 2000 [144]. More than 350 agents have reached the market as antimicrobials. The global market for finished antibiotics has reached $35 billion.

2.1.1 β-Lactams

The β-lactams are the most important class of antibiotics in terms of use. Included are the penicillins, cephalosporins, cephamycins, clavulanic acid, and carbapenems. Many of the current penicillins and cephalosporins are semisynthetic. All of the above are of great importance in chemotherapy of bacterial infections.

β-Lactamases of pathogenic bacteria are the major cause of resistance development, and there are over 450 such enzymes. However, β-lactams are still very useful due to the discovery of β-lactamase inhibitors. Although clavulanic acid is a β-lactam compound, it has only low antibacterial activity but is used widely as an inhibitor of β-lactamase, in combination with β-lactam antibiotics. Conventional strain improvement by protoplast fusion of auxotrophic strains yielded a fusant producing 30-fold more clavulanic acid than the wild type [145]. Inactivation of two G-3-P dehydrogenases, encoded by *gap1* and *gap2* by targeted gene disruption, doubled clavulanic acid production [146]. Also, increased dosage of biosynthetic genes *ceas* and *cs2* [147] or overexpression of positive regulatory genes increased production two- to threefold [148, 149].

Yield improvements have been achieved through different strategies. Thus, protoplast fusion between strains of *Penicillium chrysogenum* yielded a higher-producing strain of penicillin G [150]. Metabolic engineering was used to replace the normal promoter with the ethanol dehydrogenase promoter [151], increasing penicillin production up to 30-fold.

Protoplast fusion was also been carried out with strains of *Acremonium chrysogenum* to obtain a strain that produced 40% more cephalosporin C than the parent [152]. Production was also improved by cloning multiple copies of cyclase [153] or the *pcbC* and the *cefEF* genes [154].

Two improved cephamycin C-producing strains from *Nocardia lactamdurans* were fused to obtain cultures, which produced 10–15% more antibiotic [155]. Overexpression of *lat*, encoding lysine-aminotransferase, also led to an overproducing strain [156]. High-level expression of *ccaR*, a positive regulatory gene in *Streptomyces clavuligerus* [157], led to a two- to threefold increase in antibiotic production.

Chemical methods had traditionally been used to produce 7-aminocephalosporanic acid (7-ACA) and 7-aminodeacetoxycepalosporanic acid (7-ADCA), chemical precursors of semisynthetic cephalosporins. These processes are being replaced by safer microbiological processes. Transformation of *P.*

chrysogenum with bacterial *cefD* and *cefE* genes allowed the production of deacetoxycephalosporin C (DAOC) [158], another key intermediate in the commercial production of semisynthetic cephalosporins. Also, cloning of *cefE* from *S. clavuligerus* or *cefEF* and *cefG* from *Acremonium chrysogenum* into *P. chrysogenum* fed with adipic acid as side-chain precursor [159] resulted in the formation of several adipyl-6/7-intermediates. Enzymatic removal of the adipoyl side chain led to the production of 7-ADCA.

Disruption and one-step replacement of the *cefEF* gene of an industrial strain of *A. chrysogenum* yielded strains accumulating up to 20 g/L of penicillin N. Cloning and expression of the *cefE* gene from *S. clavuligerus* into those high-producing strains yielded recombinant strains producing high titers of DAOC [160]. An *E. coli* strain containing the D-amino acid oxidase gene from *Trigonopsis variabilis* and the glutaryl-7-aminocephalosporanic acid acylase gene from *Pseudomonas* sp. was able to convert cephalosporin C directly to 7-ACA [161].

Natural carbapenems, such as thienamycin, are made by *Streptomyces cattleya*, *Erwinia carotovora* subsp. *carotovora*, *Serratia* sp., and *Photorhabdus luminescens* [162]. Carbapenems are resistant to attack by most β-lactamases. The commercial carbapenems are made synthetically and include imipenem, meropenem, and ertapenem. Thienamycin is one of the most potent and broadest in spectrum of all antibiotics known today. Although a β-lactam, it is not a member of the penicillins or cephalosporins. It is active against aerobic and anaerobic bacteria, both Gram positive and Gram negative, including *Pseudomonas*. This novel structure was isolated in Spain from a new soil species, which was named *S. cattleya* [163]. Interestingly, this culture also produces penicillin N and cephamycin C. Also used to combat β-lactamase containing pathogens are new carbapenems, such as the recently approved doripenem (S-4661) which has broad-spectrum activity against resistant bacteria including *Pseudomonas aeruginosa*.

2.1.2 Other Antibiotics

The biosynthesis of polyketide macrolides has been subjected to genetic engineering [164]. This group of compounds includes antibiotics such as erythromycin, oleandomycin, pikromycin, tylosin, and amphotericin B. Reverse metabolic engineering increased erythromycin production by *Aeromicrobium erythreum* [165]. The technique is also known as inverse metabolic engineering and as combinatorial engineering. Tylosin production was increased up to 60% in *Streptomyces fradiae* by transposing a second copy of *tylF*, encoding macrocin *O*-methyltransferase, into a neutral site on its chromosome [166].

Genetic engineering of the nystatin biosynthetic pathway yielded polyenes with high antifungal activity, which are less toxic than amphotericin B [167]. The production of antibiotics in heterologous hosts via combinatorial biosynthesis is becoming very

popular in antibiotic production and discovery [168]. New derivatives of antibiotics have been obtained after the biosynthetic paths were elucidated and the biosynthetic genes isolated [169]. Over 200 new polyketides have been made by combinatorial biosynthesis [170, 171]. The discovery of new antibiotics has also been achieved by genetic recombination between producers of different or even the same antibiotics [172–174].

Combinatorial biosynthesis has been used to construct macrolides with new sugar moieties [175, 176]. Methymycin and pikromycin, produced by a gene cluster of *Streptomyces venezuelae* and normally containing the sugar desosamine, were modified by cloning of a gene from the calicheamicin producer, *Micromonospora echinospora* spp. *calichensis*. The gene encodes TDP-glycerohexulose aminotransferase. Transfer of a 12.6 kb DNA fragment from the tetracenomycin C-producing *Streptomyces glaucescens* to *Streptomyces lividans* resulted in tetracenomycin C production by the latter [177]. The fragment contains 12 genes of biosynthesis and resistance. Novel hybrid tetracenomycins were produced by introducing a 25 kb cosmid from the elloramycin biosynthetic pathway of *Streptomyces olivaceus* into the polyketide synthase (PKS)-deleted mutant of the urdamycin producer, *S. fradiae*, and into the mithramycin producer, *Streptomyces argillaceus* [178]. The cosmid contains a glycosyltransferase gene whose enzyme has broad substrate specificity and thus produces hybrid products containing different D- and L-sugars. For more than 35 years, vancomycin and teicoplanin were the only antibiotics active against multidrug-resistant Gram-positive bacteria. Their use became severely limited by an increase in multidrug resistance. One group of narrow-spectrum compounds are the streptogramins which are synergistic pairs of antibiotics made by a single microbial strain. The pairs are constituted by a (Group A) polyunsaturated macrolactone containing an unusual oxazole ring and a dienylamide fragment and a (Group B) cyclic hexadepsipeptide possessing a 3-hydroxypicolinoyl exocyclic fragment. Such streptogramins include virginiamycin and pristinamycin [179]. Pristinamycin, made by *Streptomyces pristinaespiralis*, is a mixture of a cyclodepsipeptide (pristinamycin I) and a polyunsaturated macrolactone (pristinamycin II). Increasing resistance to pristinamycin in the pristinamycin producer *S. pristinaespiralis* was combined with genome shuffling to increase pristinamycin production ninefold [180].

An important new strategy to improve the discovery of new antibiotics is genome mining, which has come about due to advances in microbial genomics [181]. Mining of whole-genome sequences and genome scanning allows the rapid identification of more than 450 clusters of genes in antibiotic-producing cultures encoding biosynthesis of new bioactive products and the prediction of structure based on gene sequences [182, 183]. These efforts include mining of whole-genome sequences, genome

scanning, heterologous expression, and discovery of novel chemistry. Genomics will also provide a huge group of new targets against which natural products can be screened [184]. Up to February 2017, more than 16,000 complete microbial genome sequences were available in one or two scaffolds. Of them, 411 belong to fungi and 699 to actinobacteria. Currently, 69 streptomycete genomes have been sequenced [185]. The smaller genome corresponds to *Streptomyces xiamenensis* with 5.95 Mb size, and the biggest is *Streptomyces rapamycinicus* with 12.7 Mb.

Consensus sequences can be obtained through alignments from the enzyme domains and used to construct Hidden Markov Models (HMM) of the chemical structure to predict SMILES. For this purpose, many online and stand-alone sources are freely available. Some of them include antiSMASH 2.0 [186], which detects 24 types of secondary metabolites. CLUSEAN, NaPDoS, NP.search, and Bagel2 are used for lantipeptides, while PKS/NRPS analysis, SBSPKS [187], and SMURF pipeline were developed for fungal systems. AntiSMASH is one of the most popular softwares since it is friendly and easy going.

2.2 Antitumor Agents

Microorganisms have played a crucial role in identifying compounds with therapeutic benefit against cancer [188]. Most of the important compounds used for chemotherapy of tumors are microbially produced antibiotics mainly made by actinomycetes. Among the most well known are actinomycin D (dactinomycin), anthracyclines (including daunorubicin, doxorubicin, epirubicin, pirarubicin, idarubicin, valrubicin, and amrubicin), glycopeptolides (bleomycin and phleomycin), mitomycin C, anthracenones (mithramycin, streptozotocin, and pentostatin), the enediyne calicheamicin attached to a monoclonal antibody (Mylotarg®), and, recently, the epothilones.

Novel anthracyclines have been produced by metabolic engineering, that is, cloning genes from antitumor-producing species into other producing or nonproducing strains, or by blocking deoxysugar biosynthesis. A new anthracycline, 11-hydroxyaclacinomycin A, was produced by cloning the doxorubicin resistance gene and the aklavinone 11-hydroxylase gene *dnrF* from the doxorubicin producer, *Streptomyces peucetius* subsp. *caesius*, into the aclacinomycin A producer [189]. The hybrid molecule showed greater activity against leukemia and melanoma than aclacinomycin A. Another hybrid molecule produced was 2″-amino-11-hydroxyaclacinomycin Y, which was highly active against tumors [190]. Additional new anthracyclines have been made by introducing DNA from *Streptomyces purpurascens* into *Streptomyces galilaeus*, both of which normally produce known anthracyclines [191]. Novel anthracyclines were produced by cloning DNA from the nogalomycin producer, *Streptomyces nogalater*, into *S. lividans* and into an aclacinomycin-negative mutant of *S.*

galilaeus [192]. Cloning of the actI, actIV, and actVII genes from *Streptomyces coelicolor* into the 2-hydroxyaklavinone producer, *S. galilaeus* 31,671, yielded novel hybrid metabolites, desoxyerythrolaccin and 1-*O*-methyl-desoxyerythrolaccin [193]. Similar studies yielded the novel metabolite aloesaponarin II [194].

Epirubicin (4′-epidoxorubicin) is a semisynthetic anthracycline with less cardiotoxicity than doxorubicin [195]. Genetic engineering of a blocked *S. peucetius* strain provided a new method to produce it [196]. The gene introduced was *avrE* of the avermectin-producing *Streptomyces avermitilis* or the *eryBIV* genes of the erythromycin producer, *Saccharopolyspora erythrea*. These genes and the blocked gene in the recipient are involved in deoxysugar biosynthesis.

Taxol®, a diterpene alkaloid, is approved for breast and ovarian cancer and acts by blocking depolymerization of microtubules. In addition, Taxol promotes tubulin polymerization and inhibits rapidly dividing mammalian cancer cells [197]. Taxol was originally isolated from the bark of the Pacific yew tree (*Taxus brevifolia*), but it took six trees of 100 years of age to treat one cancer patient [198]. It is now produced by plant cell culture or by semisynthesis from taxoids made by *Taxus* species. Early genetic engineering of *S. cerevisiae* yielded no taxadiene (the taxol precursor) because too little of the intermediate, geranylgeranyl diphosphate, was formed. When the *Taxus canadensis* geranylgeranyl diphosphate synthase gene was introduced, 1 mg/L of taxadiene was obtained [199]. More recent metabolic engineering studies [200] yielded a *S. cerevisiae* strain producing over 8 mg/L taxadiene and 33 mg/L geranylgeraniol. Taxol has sales of $1.6 billion per year.

Epothilones are an important group of new anticancer agents produced by the myxobacterium, *Sorangium cellulosum* [201]. Rounds of classical mutation and screening followed by recursive protoplast fusion resulted in fusants able to produce 130-fold more epothilone B compared to the starting strain. Epothilones have a mode of action similar to Taxol and, very importantly, are active against Taxol-resistant tumors.

2.3 Cholesterol-Lowering Agents

The largest segment of the pharmaceutical business is that of cholesterol-lowering drugs, amounting to about 30% of global sales. The first member of the fungal statins, that is, compactin, was discovered in cultures of *Penicillium brevicompactum* [202] and *Penicillium citrinum* [203]. A few years later, the more active methylated form of compactin, known as lovastatin (monacolin K, mevinolin, Mevacor™), was isolated from broths of *Monascus ruber* and *Aspergillus terreus* [204, 205]. Simvastatin (Zocor™), a semisynthetic derivative of lovastatin, reached a market of over $7 billion. Pravastatin, a product of compactin bioconversion, attained sales of $5 billion. The synthetic statin,

atorvastatin (Lipitor™), became the world's leading drug at $12 billion per year.

Recently, association analysis, a strategy that integrates transcriptional and metabolic profiles, led to an improvement in lovastatin production of over 50% [206]. Improvement was achieved by increasing the dosage of lovastatin biosynthetic genes and of regulatory genes for secondary metabolism.

2.4 Antihelmintics

Microbially produced polyethers such as monensin, lasalocid, and salinomycin dominate the coccidiostat market.

The avermectins, a family of secondary metabolites having both antihelmintic and insecticidal activities, produced by *S. avermitilis*, have a market of over 1 billion dollars per year. Despite their macrolide structure, avermectins lack antibiotic activity and do not inhibit protein synthesis, nor are they ionophores; instead, they interfere with neurotransmission in many invertebrates.

Although the Merck Laboratories had earlier developed a commercially useful synthetic product, thiobenzole, they had enough foresight to also examine microbial broths for antihelmintic activity and found a nontoxic fermentation broth which killed the intestinal nematode, *Nematospiroides dubius*, in mice. The *S. avermitilis* culture, which was isolated by Omura and coworkers at the Kitasato Institute in Japan [207], produced a family of secondary metabolites having both antihelmintic and insecticidal activities. These were discovered by Merck scientists and named "avermectins." They are disaccharide derivatives of macrocyclic lactones with exceptional activity against parasites, that is, at least ten times higher than any synthetic antihelmintic agent known. They have activity against both nematode and arthropod parasites in sheep, cattle, dogs, horses, and swine.

Incorporation of multiple copies of *afsR2*, a global regulatory gene, from *S. lividans* into wild-type *S. avermitilis* increased avermectin production by 2.3-fold [208]. Transposon mutagenesis was used to eliminate production of the troublesome toxic oligomycin in *S. avermitilis* [209]. DNA shuffling of the *ave C* gene of *S. avermitilis* gave an improved ratio of the undesirable CHC-B2 to the desirable CHC-B1 of 0.07:1. This was an improvement of 21-fold over the ratio with the starting strain [210].

A semisynthetic derivative, 22,23-dihydroavermectin B1 (ivermectin), is 1000 times more active than thiobenzole and is a commercial veterinary product. Ivermectin is made by hydrogenation at C22–C23 of avermectin B1a and B1b with rhodium chloride acting as catalyst. By genetic engineering of *S. avermitilis*, in which certain PKS genes were replaced by genes from the PKS of *S. venezuelae* (the pikromycin producer), ivermectin could be made directly by fermentation, thus avoiding semisynthesis [211].

A new avermectin, called Doramectin (=cyclohexyl avermectin B1), was developed at Pfizer by the technique of mutational biosynthesis [212]. Indeed, it was the first commercially successful example of mutational biosynthesis.

2.5 Immunosuppressive Agents

Cyclosporin A was originally discovered as a narrow-spectrum antifungal peptide produced by the mold, *Tolypocladium niveum* (previously *Tolypocladium inflatum*). Discovery of immunosuppressive activity led to its use in heart, liver, and kidney transplants and to the overwhelming success of the organ transplant field. Sales of cyclosporin A have reached $1.5 billion. Although cyclosporine A had been the only product on the market for many years, two other products, produced by actinomycetes, provided new opportunities. These are rapamycin (=sirolimus) [213] and the independently discovered FK-506 (tacrolimus) [214]. They are both narrow-spectrum polyketide macrolide antifungal agents, which are 100-fold more potent than cyclosporin as immunosuppressants and less toxic. FK-506 and rapamycin have been used clinically for many years. FK-506 had a market of $2 billion in 2007. Mutants developed by increasing resistance to FK-506 produce higher titers [215].

Genome shuffling using mutants of the rapamycin producer, *Streptomyces hygroscopicus*, as well as interspecies fusion of protoplasts of *S. hygroscopicus* D7-804 and *Streptomyces erythreus* ZJU325, generated improved rapamycin-producing strains [216]. Two genes of the rapamycin biosynthetic cluster in *S. hygroscopicus*, that is, *rap G* and *rap H*, encode positive regulatory proteins for rapamycin production [217]. Overexpression of either gene increased rapamycin formation, whereas their deletions eliminated rapamycin biosynthesis. They act by affecting the promoter of the operon.

2.6 Bioinsecticides

The insecticidal bacterium, *Bacillus thuringiensis* (BT), owes its activity to its crystal protein produced during sporulation. Crystals plus spores had been applied to plants for years to protect them against lepidopteran insects. BT preparations are highly potent, some 300 times more active on a molar basis than synthetic pyrethroids and 80,000 times more active than organophosphate insecticides. In 1993, BT represented 90% of the biopesticide market and had annual sales of $125 million.

A very important insecticide is Spinosad (Naturalyte®) produced by *Saccharopolyspora spinosa* and used for protection of crops and feedstock animals. Spinosad is a mixture of two tetracyclic macrolides containing forosamine and tri-*O*-methyl rhamnose with different levels of methylation on the polyketide moiety. The two components are spinosyns A and D, which differ by a methyl group at position 6 of the polyketide. Spinosad is an excellent nontoxic

agricultural insecticide. Genome shuffling has been used for strain improvement [218].

3 Recombinant Proteins: Biopharmaceuticals

Biopharmaceutical proteins can be categorized into four major groups: (1) protein therapeutics with enzymatic activity (e.g., insulin), (2) protein vaccines, (3) protein therapeutics with special targeting activity (e.g., monoclonal antibodies), and (4) protein diagnostics (e.g., biomarkers) [219]. Biologics accounted for over $80 billion in sales in 2008. Six of these therapeutic proteins were among the best selling drugs in the USA in that year. Monoclonal antibodies and Fc-fusion proteins made up 43% of this market value.

By means of genetic engineering, desired proteins are massively generated to meet the copious demands of industry [220]. Protein quality, functionality, production speed, and yield are the most important factors to consider when choosing the right expression system for recombinant protein production.

Non-glycosylated proteins are usually made in *E. coli* or yeasts, and they constitute 55% of the therapeutic protein market (39% by *E. coli*, 1% by other bacteria, and 15% by yeasts) [221]. On the other hand, N-glycosylated proteins are usually made in mammalian cells, which mimic human glycosylation. Chinese hamster ovary (CHO) cells provide about 35% of the therapeutic protein market, but the process is very expensive, and the glycoproteins made are not exactly of the human type. Although yeasts, molds, and insect cells are generally unable to provide mammalian glycosylation, the methylotrophic yeast, *Pichia pastoris*, has been genetically engineered to produce a human type of glycosylation [222].

Directed evolution of proteins has been reviewed by Yuan et al. [223]. Strategies include DNA shuffling, whole-genome shuffling, heteroduplex, random chimeragenesis of transient templates, assembly of designed oligonucleotides, mutagenic and unidirectional reassembly, exon shuffling, Y-ligation-based block shuffling, nonhomologous recombination, and the combining of rational design with directed evolution.

3.1 Bacteria

Bacterial systems are used to make somatostatin, insulin, bovine growth hormone for veterinary applications, α-1 antitrypsin, interleukin-2, tumor necrosis factor, β-interferon, and γ-interferon. *E. coli* is one of the earliest and most widely used hosts for the production of heterologous proteins [224]. As early as 1993, recombinant processes of *E. coli* were responsible for almost $5 billion worth of products, that is, insulin, human growth hormone, interferons, and G-CSF. Advantages of *E. coli* include rapid growth, rapid expression, ease of culture and genome modifications, low

cost, and high product yields [225]. It is used for massive production of many commercialized proteins. This system is excellent for functional expression of non-glycosylated proteins.

E. coli genetics are far better understood than those of any other microorganism. Recent progress in the fundamental understanding of transcription, translation, and protein folding in *E. coli*, together with the availability of improved genetic tools, is making this bacterium more valuable than ever for the expression of complex eukaryotic proteins. Its genome can be quickly and precisely modified with ease, promotor control is not difficult, and plasmid copy number can be readily altered. This system also features alteration of metabolic carbon flow, avoidance of incorporation of amino acid analogs, formation of intracellular disulfide bonds, and reproducible performance with computer control. *E. coli* can accumulate recombinant proteins up to 80% of its dry weight and survives a variety of environmental conditions. Recombinant protein production in *E. coli* can be increased by mutations which eliminate acetate production [226]. Avecia Biologics achieved a titer of 14 g/L of recombinant protein using *E. coli* [227].

The value of transcriptome analysis in process improvement was shown by Choi et al. [228]. They analyzed an *E. coli* process yielding human insulin-like growth factor 1 fusion protein (IGF-If) in a high-density culture. Of 200 or so genes whose expression was downregulated after induction, the ones involved in biosynthesis of amino acids or nucleotides were studied. Amplification of two of these, *prsA* (encoding PRPP synthetase) and *glpF* (encoding the glycerol transporter), raised product formation from 1.8 to 4.3 g/L.

Bacilli have yields as high as 3 g/L. The organisms used are usually *Bacillus megaterium*, *B. subtilis*, and *Bacillus brevis*. *Staphylococcus carnosus* can produce 2 g/L of secreted mammalian protein.

An improved Gram-negative host for recombinant protein production has been developed using *Ralstonia eutropha* [229]. The system appears superior to *E. coli* with respect to inclusion body formation. Organophosphohydrolase, a protein prone to inclusion body formation with a production of less than 100 mg/L in *E. coli*, was produced at 10 g/L in *R. eutropha*. Another useful bacterium is *Pseudomonas fluorescens* MB101 developed by Dowpharma [230]. This system has produced 4 g/L of TNF-α.

3.2 Yeasts

Yeasts, the single-celled eukaryotic fungal organisms, are often used to produce recombinant proteins that are not produced well in *E. coli* because of problems dealing with folding or the need for glycosylation. The major advantages of yeast expression systems are listed in Table 2. The yeast strains are genetically well characterized and are known to perform many posttranslational modifications. They are easier and less expensive to work with than insect

Table 2
Advantages of yeast expression systems

Stable strains
High density of growth
Durability
High production titers and yields
Protein glycosylation
Reasonable cost
Product processing similar to that of mammalian cells
Suitable for isotopically labeled proteins
Suitable for S–S-rich proteins
Protein folding assisted
Chemically defined media supporting rapid growth

or mammalian cells and are easily adapted to fermentation processes.

The two most utilized yeast strains are *S. cerevisiae* and the methylotrophic yeast *P. pastoris*. Glucose oxidase from *A. niger* is produced by *S. cerevisiae* at 9 g/L. Recombinant products on the market which are made in *S. cerevisiae* are insulin, hepatitis B surface antigen, urate oxidase, glucagons, granulocyte macrophage colony-stimulating factor (GM-CSF), hirudin, and platelet-derived growth factor.

P. pastoris has the desirable qualities of dense growth and methanol-induced expression and secretion of recombinant proteins. It is used for the commercial production of non-glycosylated human serum albumin and glycosylated vaccines. Strains have been developed which are capable of human-type N-glycosylation, and such products are already in clinical testing. High recombinant protein yields can be obtained with *P. pastoris*, for example, 10 g/L of tumor necrosis factor [231], 14.8 g/L of gelatin, 15 g/L of mouse collagen [232], and *E. coli* phytase and *Candida parapsilosis* lipase/acetyltransferase at 6 g/L. Bacterial proteins such as intracellular tetanus fragment C were produced as 27% of protein with a titer of 12 g/L [233]. Production of serum albumin in *S. cerevisiae* amounted to 0.15 g/L, whereas in *P. pastoris*, the titer was 10 g/L [234]. Indeed, claims have been made that *P. pastoris* can make 20–30 g/L of recombinant proteins [235].

Heterologous gene expression in another methylotroph *Hansenula polymorpha* yielded 13.5 g/L of phytase, and other proteins were made at levels over 10 g/L. Among the advantages of methylotrophic yeasts over *S. cerevisiae* as a cloning host are the following: (1) higher protein productivity, (2) avoidance of

hyperglycosylation, (3) growth in reasonably strong methanol solutions that would kill most other microorganisms, (4) a system that is cheap to set up and maintain, and (5) integration of multicopies of foreign DNA into chromosomal DNA yielding stable transformants [236].

3.3 Filamentous Fungi (Molds)

Filamentous fungi are attractive hosts for recombinant DNA technology because of their ability to secrete high levels of bioactive proteins with posttranslational processing such as glycosylation. *A. niger* excretes 25 g/L of native glucoamylase [99, 237]. Foreign genes can be incorporated via plasmids into chromosomes of the filamentous fungi where they integrate stably into the chromosome as tandem repeats providing superior long-term genetic stability. An excellent comprehensive review of heterologous expression in *Aspergillus* has been published by Lubertozzi and Keasling [238]. Like mammalian cells, fungi possess the cellular machinery for translation of proteins, protein folding, and posttranslational modification. Like bacteria, they are easy to culture. Genetic development of aspergilli has included (1) transformation systems; (2) expression constructs; (3) targeted integration and copy number manipulation; (4) promoters; (5) improved gene design; (6) engineering of proteases, secretion, and glycosylation; and (7) tools for tagging, targeting, and silencing of genes.

A 1000-fold increase in phytase production was achieved in *A. niger* by the use of recombinant technology [239]. Recombinant *Aspergillus oryzae* can produce 2 g/L of human lactoferrin [240] and 3.3 g/L of *Mucor* rennin [241]. Production of human lactoferrin [242] by *Aspergillus awamori* via rDNA technology and classical strain improvement amounted to 2 g/L of extracellular protein [240]. *A. niger* glucoamylase was made by *A. awamori* at 4.6 g/L.

The fungus *Chrysosporium lucknowense* has been genetically converted into a nonfilamentous, less viscous, low-protease-producing strain that is capable of producing very high yields of heterologous proteins [243]. Dyadic International, Inc., the company responsible for the development of the *C. lucknowense* system, claims protein production levels of up to 100 g/L of native extracellular protein.

3.4 Mammalian Cells

CHO cells constitute the preferred system for producing monoclonal antibodies and some other recombinant proteins. Other cell types include (1) various mouse myelomas such as NS0 murine myeloma cells [244], (2) baby hamster kidney (BHK) cells for production of cattle foot-and-mouth disease vaccine, (3) green monkey kidney cells for polio vaccine [245], and (4) human cell lines such as human embryonic kidney (HEK) cells. NSO is a nonsecreting subclone of the NS-1 mouse melanoma cell line. By 2006,

production of therapeutic proteins by mammalian systems reached $20 billion [246].

Animal-free, protein-free, and even chemically defined media with good support of production have been developed [247]. Protein production by mammalian cells (CHO) went from 5–50 mg/L in 1985 to 50–500 mg/L in 1995 and 1–5 g/L in 2005 [248]. A number of mammalian processes are producing 3–5 g/L of recombinant protein [249], and in some cases, protein titers have reached 10 g/L in industry [250], including antibodies [251]. A rather new system is that of a human cell line known as PER.C6 of Crucell Holland BV, which, in cooperation with DSM Biologics, was reported to produce 15 g/L [252] and, then later, 27 g/L of a monoclonal antibody [253]. Protein production of over 20 g/L has been achieved in serum-free medium, but the production of 2–3 g/L in such media is more usual.

3.5 Insect Cells

Insect cells are able to carry out more complex posttranslational modifications than can be accomplished with fungi. They also have the best machinery for the folding of mammalian proteins and are therefore quite suitable for making soluble proteins of mammalian origin [254]. The most commonly used vector system for recombinant protein expression in insects is the baculovirus, especially the nuclear polyhedrosis virus (*Autographa californica*), which contains circular double-stranded DNA, is naturally pathogenic for lepidopteran cells, and can be grown easily in vitro. The usual host is the fall armyworm (*Spodoptera frugiperda*) in suspension culture. A larval culture can be used which is much cheaper than mammalian cell culture.

Baculovirus-assisted insect cell expression offers many advantages as follows:

1. Eukaryotic posttranslational modifications without complication, including phosphorylation, N- and O-glycosylation, correct signal peptide cleavage, proper proteolytic processing, acylation, palmitoylation, myristoylation, amidation, carboxymethylation, and prenylation [255, 256].
2. Proper protein folding and S–S bond formation, unlike the reducing environment of *E. coli* cytoplasm.
3. High expression levels. The virus contains a gene encoding the protein polyhedron, which is made at very high levels normally and is not necessary for virus replication. The gene to be cloned is placed under the strong control of the viral polyhedrin promoter, allowing expression of heterologous protein of up to 30% of cell protein.
4. Easy scale-up with high-density suspension culture.
5. Safety, expression vectors are prepared from the baculovirus which can attack invertebrates but not vertebrates or plants.
6. Lack of limit on protein size.

7. Efficient cleavage of signal peptides.
8. Simultaneous expression of multiple genes [257].

Production of recombinant proteins with the baculovirus expression vector system in insect cells reached 600 mg/L in 1988 [258]. Later information indicated that the baculovirus insect cell system can produce 11 g/L of recombinant protein [259]. Recombinant insect cell cultures have yielded over 200 proteins encoded by genes from viruses, bacteria, fungi, plants, and animals [260].

4 Enzymes

Over the years, high titers of enzymes were obtained using "brute-force" mutagenesis and random screening of microorganisms. Recombinant DNA technology acted as a boon for the enzyme industry in the following ways [261]: (1) plant and animal enzymes could be made by microbial fermentations, for example, chymosin; (2) enzymes from organisms difficult to grow or handle genetically were now produced by industrial organisms such as species of *Aspergillus* and *Trichoderma*, and *K. lactis*, *S. cerevisiae*, *Y. lipolytica*, and *Bacillus licheniformis* (e.g., thermophilic lipase was produced by *A. oryzae* and *Thermoanaerobacter* cyclodextrin glycosyl transferase by *Bacillus*); (3) enzyme productivity was increased by the use of multiple gene copies, strong promoters, and efficient signal sequences; (4) production of a useful enzyme from a pathogenic or toxin-producing species could now be done in a safe host; and (5) protein engineering was employed to improve the stability, activity, and/or specificity of an enzyme.

Genes encoding many microbial enzymes have been cloned, and the enzymes expressed at levels hundreds of times higher than those naturally produced. Over 60% of the enzymes used in different applications including detergent, food, and starch processing industry are recombinant proteins [262]. Recombinant molds are one of the main sources of enzymes for industrial applications. Yields as high as 4.6 g/L have been reached for several hosts including *A. niger*, *A. oryzae*, *A. awamori*, *C. lucknowense*, and *A. chrysogenum*.

Plant phytase [263], produced in recombinant *A. niger*, was used as a feed for 50% of all pigs in Holland. A 1000-fold increase in phytase production was achieved in *A. niger* by the use of recombinant technology [239]. Mammalian chymosin was cloned and produced by *A. niger* or *E. coli*, and recombinant chymosin was approved in the USA; its price was half that of natural calf chymosin.

Three fungal recombinant lipases are currently used in the food industry. They are from *Rhizomucor miehei*, *Thermomyces lanuginosus*, and *Fusarium oxysporum* and are produced in *A. oryzae*. They are used for laundry cleaning, interesterification of lipids, and esterification of glucosides producing glycolipids which have

applications as biodegradable nonionic surfactants for detergents, skin care products, and contact lenses and as food emulsifiers. Washing powders have been improved in activity, and low-temperature operation has been achieved by the application of recombinant DNA technology and site-directed mutagenesis of genes encoding proteases and lipases [264, 265]. The first commercial recombinant lipase used in a detergent was from *Humicola lanuginosa*. The gene was cloned into the *A. oryzae* genome.

A multicopy plasmid of *B. subtilis* was used to increase by 2500-fold the production of an α-amylase from *B. amyloliquefaciens* [266]. An exoglucanase from *Cellulomonas fimi* was overproduced in *E. coli* to a level of over 20% of cell protein [267]. The same host has also been used to clone the endo-β-glucanase components from *Thermomonospora* and *Clostridium thermocellum* as well as the cellobiohydrolase I gene of *Trichoderma reesei* [268]. *P. pastoris* was engineered to produce and excrete *S. cerevisiae* invertase into the medium at 100 mg/L [269]. Self-cloning of the xylanase gene in *S. lividans* resulted in a sixfold overproduction of the enzyme [270].

The properties of many enzymes have been modified by random mutagenesis and screening of microorganisms over the years leading to changes in substrate specificity, feedback inhibition, kinetic parameters (V_{max}, K_m or K_i), pH optimum, thermostability, and carbon source inhibition. Based on this information, more rational techniques such as site-directed mutagenesis were used to introduce single changes in amino acid sequences yielding similar types of changes in a large variety of enzymes. Modification of eight amino acids increased heat tolerance and temperature stability at 100 °C of a protease from *Bacillus stearothermophilus* [271]. Interestingly, all mutations were far from the active site of the enzyme.

Molecular breeding techniques (e.g., *DNA shuffling* and *DNA family shuffling*) are being currently used to generate enzymes with improved properties such as activity and stability at different pH values and temperatures [272], increased or modified enantioselectivity [273], altered substrate specificity [274], stability in organic solvents [275], novel substrate specificity and activity [276], increased biological activity of protein pharmaceuticals and biological molecules [277], as well as novel vaccines [278, 279]. Two proteins from directed evolution work were already on the market by year 2000 [280]. These were green fluorescent protein of Clontech [281] and Novo Nordisk's LipoPrime® lipase.

5 Closing Remarks

The fermentation industry developed slowly from the beginning of the twentieth century to the early 1970s using brute-force mutagenesis followed by screening or selection. However, the birth of

the era of recombinant DNA (rDNA) in 1971–1973 catalyzed a major change in the way useful processes could be developed. Production of primary metabolites was markedly improved by modern genetic techniques. Environmentally friendly fermentations replaced chemical synthesis to a great extent. Of great interest has been the application of rDNA technology to the production of secondary metabolites and to the elucidation of their biosynthetic pathways. Tools include transposition mutagenesis, targeted deletions, genetic recombination via combinatorial biosynthesis, transcriptome analysis, proteomics, metabolomics, metabolic engineering, etc. Genes encoding many enzymes have been cloned, and the enzymes have been expressed at levels hundreds of times higher than those naturally produced. Random redesign techniques have generated enzymes with improved properties including activity, stability, increased or modified enantioselectivity, altered substrate specificity, etc. An entirely new field of industrial microbiology has arisen out of rDNA, that is, the biopharmaceutical industry which is the most rapidly expanding segment of the biological industry, especially that of monoclonal antibodies. The best is yet to come from the fantastic combination of industrial microbiology and rDNA technology. Many of the new techniques are carried out by small companies and academic groups who could play a major role in rescuing us from the antibiotic crisis that we are now experiencing. Furthermore, we look forward to its role in eventually replacing the environmentally dangerous energy sources that we live with today, that is, petroleum, coal, etc., with future biofuels made from agricultural and forest biomass.

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