J Ind Microbiol Biotechnol (2006) 33: 486–495
DOI 10.1007/s10295-005-0076-x

REVIEW

Arnold L. Demain

# From natural products discovery to commercialization: a success story

Received: 19 October 2005 / Accepted: 24 December 2005 / Published online: 10 January 2006


**Abstract** In order for a natural product to become a commercial reality, laboratory improvement of its production process is a necessity since titers produced by wild strains could never compete with the power of synthetic chemistry. Strain improvement by mutagenesis has been a major success. It has mainly been carried out by "brute force" screening or selection, but modern genetic technologies have entered the scene in recent years. For every new strain developed genetically, there is further opportunity to raise titers by medium modifications. Of major interest has been the nutritional control by induction, as well as inhibition and repression by sources of carbon, nitrogen, phosphate and end products. Both strain improvement and nutritional modification contribute to the new process, which is then scaled up by biochemical engineers into pilot scale and later into factory size fermentors.

**Keywords** Secondary metabolites · Primary metabolites · Fermentation · Biotechnology · Discovery · Genetic improvement · Process development · Natural products

## The birth of antibiotics

For thousands of years, moldy cheese, meat and bread were employed in folk medicine to heal wounds. It was not until the 1870s, however, that Tyndall, Pasteur, and Roberts separately observed the antagonistic effects of one microbe upon another. Pasteur, with his characteristic foresight, suggested the therapeutic potential of the phenomenon. For the next half-century, various microbial preparations were tested as medicines but they were either too toxic or inactive in animals. Finally, in 1929, Alexander Fleming published his historic observation that a contaminating mold, identified as *Penicillium notatum*, killed his bacterial culture of *Staphylococcus aureus*. He next found that the cell-free liquid, in which the mold had grown, could inhibit many bacterial species. He named the active substance penicillin. Attempts to isolate the antibiotic were made by British chemists in the 1930s, but the instability of the molecule frustrated their efforts. In 1939, a study was begun at Oxford University by Florey, Chain, Heatley, and their colleagues. This resulted in the successful preparation of a stable form of penicillin which showed remarkable antibacterial activity in animals and then in humans. With the help of the U.S. Department of Agriculture, several universities (especially the University of Wisconsin) and several American pharmaceutical companies, the production of penicillin by a related species, *Penicillium chrysogenum*, became a reality. The importance of Fleming's discovery was that it led to the first successful chemotherapeutic agent produced by a microbe, thus initiating the golden age of antibiotics, i.e., the "wonder drugs". The tremendous success attained in the battle against disease with penicillin not only led to the development of a new field of antibiotic research but also created an entirely new industry. It opened the way for the development of many other antibiotics, and yet penicillin still remains among the most active and least toxic of these miraculous compounds [18].

The advent of penicillin, which signaled the beginning of the antibiotic era, was closely followed by the discoveries of Professor SelmanWaksman at Rutgers University. This soil microbiologist and his students succeeded in isolating many new antibiotics from soil-inhabiting filamentous bacteria, i.e., the actinomycetes. The best known of these was streptomycin. However, even before streptomycin, Waksman and Woodruff published in 1940 on the discovery of the chromooligopeptides which they named the actinomycins. One such member of this group, actinomycin D, was used to combat the Wilms tumor in children and became an extremely important basic tool in the development of molecular biology, as an inhibitor of RNA polymerase.

A. L. Demain
Research Institute for Scientists Emeriti (R.I.S.E),
Drew University, Madison, NJ 07940, USA
E-mail: ademain@drew.edu

In 1945, Waksman, Schatz, and Bugie published their work on streptomycin, the first antibiotic active against the tuberculosis bacterium and also valuable in the fight against bacterial meningitis. Waksman received the Nobel Prize for this accomplishment in 1952, and with royalties turned over to the Rutgers University by a generous Merck administration, he was able to build the Waksman Institute of Microbiology. With his student Lechevalier, Waksman reported on the discovery of neomycin in 1948 and candicidin in 1953. Discovery of 25 additional antibiotics by the Waksman group followed. Neomycin, an aminoglycoside produced by *Streptomyces fradiae*, serves as a topical antibacterial agent, and the polyene candicidin, made by *Streptomyce griseus*, became a topical antifungal product. The discoveries of the Waksman group stimulated antibiotic drug discovery efforts internationally from 1948 until today. The selective action exerted on pathogenic bacteria and fungi by these microbial secondary metabolites ushered in the antibiotic era and for many years, we have benefited from this remarkable property of wonder drugs such as the penicillins, cephalosporins, tetracyclines, aminoglycosides, chloramphenicol, macrolides, and many others [13]. The successes were so impressive that these antibiotics were virtually the only drugs utilized for chemotherapy against pathogenic microorganisms. By 2002, over 22,000 bioactive compounds had been discovered from microbes. These included 20,000 antibiotics, mainly produced by the actinomycetes (45%), fungi (38%), and unicellular bacteria (17%, chiefly by *Pseudomonas* and *Bacillus*) [6]. Of the actinomycete antibiotics, about 80% are made by members of the genus *Streptomyces*. One microbe usually produces more than one compound. For example, a gentamicin-producing strain of *Micromonospora* forms 50 isolatable secondary metabolites [7].

## Pharmacological agents

Although the early emphasis on secondary metabolite discovery was mainly devoted to antibiotics, it was realized in the 1970s and 1980s that the compounds which possess antibiotic activity also possess other activities, that some of these had been quietly exploited in the past, and that such broadening of scope should be expanded. Thus, a broad screening of antibiotically active molecules for antagonistic activity against organisms other than microorganisms, as well as for activities useful for pharmacological applications, was proposed in order to yield new and useful lives for "failed antibiotics" and to isolate new bioactive compounds. A large number of in vitro laboratory tests were developed to help detect, isolate and purify useful compounds. Much of this emphasis was brought about by Hamao Umezawa [49, 50] who pointed out the potential importance of enzyme inhibitors as drugs. Fortunately, we entered into a new era in which microbial metabolites were applied to diseases heretofore only treated with synthetic compounds, i.e., diseases not caused by bacteria and fungi [2]. Since then, some of the greatest triumphs of secondary metabolite development have been with (1) enzyme inhibitors which are cholesterol-lowering agents, i.e., the statins [lovastatin, pravastatin (pravacol), the chemical derivative simvastatin (Zocor), and the completely synthetic atorvastatin (Lipitor)]; (2) immunosuppressants for organ transplantation (cyclosporine, sirolimus, tacrolimus, and mycophenolic acid); (3) antiparasitic agents [antihelmintics (avermectins) and the coccidiostats and ruminant growth promoters (polyethers: monensin, narasin, lasalocid, and salinomycin)]; (4) bioherbicides (bialaphos); (5) plant growth regulators (gibberellins); (6) biopesticides (kasugamycin, polyoxins); (7) bioinsecticides (spinosins and nikkomycin); and of great importance, (8) the antitumor agents (doxorubicin, daunorubicin, mitomycin, bleomycin, etc.). The statins became the most economically important group of products, exceeding $20 billion per year. Indeed, they constituted three of the top four selling drugs in recent years. Pravastatin's sales are over $3.6 billion, the annual market for Zocor is $7 billion, and Lipitor sales are $11 billion, making it the world's leading drug. Another type of enzyme inhibitor on the market is acarbose, a natural inhibitor of intestinal glucosidase, which is produced by an actinomycete of the genus *Actinoplanes*. It decreases hyperglycemia and triglyceride synthesis in adipose tissue, liver and the intestinal wall of patients suffering from diabetes, obesity and type IV hyperlipidemia.

## Microbial diversity

It is clear that the industrial microbiology field has utilized only a very minor portion of nature's microbial arsenal for the discovery of useful molecules. The reason is the inability of microbiologists to culture the vast majority of microbes in nature. It is estimated that only 1% of bacteria and 5% of fungi have been cultivated in the laboratory. This problem is being studied by a number of groups [32, 41, 45, 55], and some success has been achieved by using one or more of the following strategies: (1) very low nutrient concentrations, (2) signaling molecules, (3) inhibitors of undesired microbes, (4) long periods of incubation, (5) growth conditions resembling the natural environment, (6) protection of cells from exogenous peroxides, (7) addition of humic acid, (8) hypoxic (1–2% $O_2$) or anoxic atmospheres, (9) encapsulation of cells in gel microdroplets and detection of microcolonies by flow cytometry, and (10) high $CO_2$ along with high throughput polymerase chain reaction (PCR) technology.

Another technique used to tap into biodiversity is that of environmental DNA (metagenomic DNA) capture [28]. DNA is isolated from nature and cloned into *Escherichia coli* by using bacterial artificial chromosomes (BACs). The size of DNA isolated from soil is

usually up to 70 kb and that from water, up to 40 kb. BACs are capable of carrying inserts of up to 350 kb in size. Then, the *E. coli* clones are screened for biological activity. Of the secondary metabolites detected from metagenomic DNA, some were known molecules but a number of others were new compounds [27].

## Laboratory fermentation improvement

The most economically important compounds produced by microorganisms, other than enzymes and recombinant proteins, are the low molecular weight primary and secondary metabolites [15]. Primary metabolites are those compounds involved in growth of microbes whereas secondary metabolites are not. The most important primary metabolites of industry are the amino acids, purine nucleotides, vitamins and organic acids. With regard to secondary metabolites, the most economically valuable are antibiotics, antitumor agents, immunosuppressants, cholesterol-lowering agents, and anti-parasitic drugs.

Microbes isolated from nature usually produce extremely low levels of such metabolites. Overproduction must be achieved in the laboratory before pilot plant scale-up is attempted. Knowledge of microbial physiology is crucial for success since the environment presented to the microorganism is of the utmost importance. Virtually every industrial fermentation process runs at its optimum rate and efficiency when a nutritional limitation is imposed. Metabolic processes are controlled by sources of carbon, nitrogen, phosphorus, metals, induction, feedback regulation, growth rate, and enzyme decay. The areas of physiology that must be considered in order to develop successful industrial processes are nutrition, growth and death, transport, energy, building blocks, polymer synthesis, regulation of enzyme synthesis, action and degradation, as well as cellular differentiation.

When microbes from nature are brought into the laboratory, they are isolated away from contaminating microorganisms by streaking on an agar surface in a Petri dish. When purified, the original colony is often found to be made up of a mixed population of cells, all of the members belonging to the same strain but possibly showing somewhat different colonial morphologies. The individual isolates are then tested for production ability, and the best strain becomes the subject of a multi-year fermentation development and mutagenesis program. Although geneology diagrams may give the impression that the increased potency of each strain is strictly due to the result of genetic manipulation, much of it is actually due to fermentation development. Throughout the life of the commercial product, the mutation and fermentation parts of the improvement program go hand-in-hand. The reason is that mutants often respond to changes in fermentation medium and conditions in a positive way and perhaps differently than did their parent or grandparent cultures.

Fermentation development in the laboratory occurs as follows. First, the carbon, nitrogen, inorganic, and, if necessary, complex nutrients supporting growth must be determined and then modifications in the medium must be made to support product biosynthesis. Regulatory mechanisms that restrict the synthesis and activity of enzymes must be bypassed [43]. Such mechanisms evolved because it is usually detrimental for microbes to overproduce their precious metabolites in nature.

After finding the best conditions and nutrients for growth, requirements for product formation must be determined. It was the University of Wisconsin Professor Marvin Johnson and his student Jarvis who stated in their memorable 1947 paper on penicillin fermentation that "We have not been able to devise a medium on which rapid mycelium growth and rapid penicillin production occur simultaneously...An ideal medium should support two distinct fermentation rates: a rapid rate throughout the growth phase and a much slower rate during the remainder of the fermentation" [30]. This discovery was instrumental for further studies in which the requirements specific for product formation had to be determined. It was later established that limiting precursors have to be added for optimal production [12]. These included phenylalanine for production of gramicidin S, lysine for cephamycin C formation, and phenylacetic acid for penicillin G production. It was also Johnson who recognized that carbon sources optimal for growth may interfere with product formation, specifically that glucose was excellent for growth of the penicillin producer, *P. chrysogenum*, but very poor for penicillin production and that lactose was much better for overproduction. This led to many studies on carbon source repression of secondary metabolism [19]. Most such processes were found to be repressed by glucose whereas other carbon sources were found to be favorable, e.g., galactose for overproduction of actinomycin, citrate for bacitracin, sucrose for cephalosporin C. A surprising example was the novobiocin fermentation, where citrate was the interfering carbon source and glucose, the beneficial source. Nitrogen sources also have the capacity to interfere with fermentations [20]. Examples include interference by ammonium salts in the cephalosporin C, gibberellic acid, and penicillin G processes. Amino acids also can interfere, such as lysine in the penicillin and cephalosporin processes, and glutamine in the gibberellic acid fermentation. Non-interfering nitrogen sources include asparagine or arginine for cephalosporin production and glutamate for penicillin production. Although ammonium salts are usually repressive for secondary metabolite fermentations, an exception is the aflatoxin process where nitrate interferes but ammonium is non-interfering. Inorganic phosphorus is needed for growth but phosphate salts must be added in low concentration since they often interfere in secondary metabolism [21], e.g., in production of butirosin, candicidin, cephalosporin, clavulanic acid, ergot alkaloids, gentamicin, streptomycin, tetracycline, tylosin and vancomycin, among others. Care must also be taken

with other inorganic nutrients. As an example, iron deficiency is necessary for the riboflavin fermentation since iron represses GTP cyclohydrolase II and riboflavin synthetase. Mn deficiency is necessary for citric acid production since it increases intracellular ammonium levels by fivefold, which reverses citric acid inhibition of phosphofructokinase. Feedback control by the product of the fermentation must also be bypassed by removal of the product or by genetic manipulation (see below). Some examples of feedback in secondary metabolism are given in Table 1.

The relationship among process improvements, commercial production levels and cost of product is given in Table 2. It is clear that as production goes up, costs go down. The titers of products made by industrial cultures are very high, 1,000-fold increases being achieved for many small metabolites. Of course, the higher the specific level of production, the simpler is the job of isolation. Consider the case of *Ashbya gossypii* which has been forced into making over 40,000 times more riboflavin than it needs for its own growth, or *Pseudomonas denitrificans* which produces a 100,000-fold excess of vitamin $B_{12}$. The original Oxford strain of *P. notatum* produced 5 mg of penicillin per l; today's *P. chrysogenum* strains make over 70 g/l, a figure higher than the dry weight of the cells in the fermentor! Titers of amino acids are given in Table 3. To create such overexpressing industrial strains, nutritional manipulations were clearly not enough and genetic techniques were required. Useful history is provided by returning to the penicillin story [18]. Although Fleming's original strain produced only traces of penicillin, ''brute force'' genetic manipulation made tremendous strides in production ability and led to a whole new technology known as ''strain improvement''. Strain selection began with *P. chrysogenum* NRRL 1951, the well-known isolate from a moldy cantaloupe obtained in a Peoria market. This strain was capable of producing 60 mg/l. Cultivation of spontaneous sector mutants and single-spore isolations led to higher-producing cultures from NRRL 1951. One of these, NRRL 1951–1325, produced 150 mg/l. It was next subjected to the X-ray treatment by Demerec of the Carnegie Institution at Cold Spring Harbor, New York, and mutant X-1612 was obtained; it yielded 300 mg/l. Workers at the University of Wisconsin obtained ultraviolet-induced mutants of Demerec's strain. One of these, Q-176, which produced 550 mg/l, is the ancestor of all strains used in industry today. The ''Wisconsin family'' of superior strains became well known all over the world, some producing over 1.8 g/l.

''Brute force'' genetics involves mutagenesis followed by screening of hundreds or thousands of survivors, or by selection for resistance to growth-inhibitory antimetabolites. Although tedious and labor-intensive, it is still used today in industry simply because IT WORKS! Mutagenesis is carried out with toxic chemicals and/or irradiation which kill the vast majority (70–99.99%) of the cells and allow the technologist to isolate an improved strain from the minor portion of cells surviving mutagenesis. The most useful mutagens include nitrosoguanidine, 4-nitroquinolone-1-oxide, methylmethane sulfonate, and ethylmethane sulfonate [5]. One cannot predict the frequency of a 10–15% improved strain but it lies between one in a thousand up to one in 100,000 survivors. Various types of procedures are used to examine the products of the mutagenic procedure: (1) random screening of survivors for improved production, (2) screening of (morphological) mutants with changes in colony morphology, (3) selection of mutants resistant to toxic analogues (antimetabolites) of pathway precursors or to the fermentation product itself, (4) screening of auxotrophic mutants (requiring for growth a primary metabolite such as an amino acid not required by the parent culture) and their revertants to prototrophy, and (5) revertants of non-producing mutants.

**Table 1** Feedback in secondary metabolism

| Idiolite | Enzyme | Mechanism |
|---|---|---|
| Bacitracin | Bacitracin synthetase | Inhibition |
| Chloramphenicol | Arylamine synthetase | Repression |
| Cycloheximide | Unknown | Unknown |
| Ergot alkaloid | Dimethylallyltryptophan synthetase | Inhibition |
| Erythromycin | SAM: erythromycin C O-methyltransferase | Inhibition |
| Gramicidin S | Gramicidin S synthetases | Inhibition |
| Indolmycin | Initial enzyme | Inhibition |
| Kanamycin | Acetyltransferase | Repression |
| Mycophenolic acid | O-methyltransferase | Inhibition |
| Puromycin | O-methyltransferase | Inhibition |
| Rubradirin | Unknown | Inhibition |
| Tetracycline | Anhydrotetracycline oxygenase | Inhibition |
| Tylosin | SAM: macrocin O-methyl-transferase | Inhibition |

**Table 2** Production of penicillins

| Year | Production (kg) | Cost ($/kg) |
|---|---|---|
| 1945 | 2,300 | 11,000 |
| 1963 | 3,000,000 | 150 |
| 1978 | 15,000,000 | 18.50 |
| 1992 | 22,000,000 | – |
| 1995 | 31,000,000 | 4.5 |

**Table 3** Titers of amino acid processes

| | |
|---|---|
| L-lysine HCl | 170 g/l |
| L-threonine | 100 |
| L-valine | 99 |
| L-arginine | 96 |
| L-glutamate | 88 |
| L-alanine | 75 |
| L-serine | 65 |
| L-tryptophan | 58 |
| L-phenylalanine | 51 |
| L-glutamine | 44 |
| L-histidine | 42 |
| L-isoleucine | 30 |

The aims of mutagenesis programs include (1) increasing titer, (2) allowing efficient assimilation of inexpensive, complex raw materials, (3) altering product ratios, (4) eliminating side products, (5) excretion of products, and (6) shortening of fermentation duration [39]. Titer increases often involve the isolation of auxotrophic mutants to limit intracellular accumulation of inhibitory or repressive compounds, e.g., threonine/methionine auxotrophs to eliminate feedback by these amino acids in the lysine process. They may also involve mutants which are resistant to antimetabolites of products and are no longer subject to inhibition or repression of enzymes of the biosynthetic pathway. In certain cases, these mutations result in gene amplification. Often, combining feedback-inhibition and feedback-repression resistant mutations in a single strain by recombinant genetics (see below) results in a synergy in the level of resistance obtained. The growth-inhibitory effects of antimetabolites can sometimes be increased by changing the carbon or nitrogen source or by adding a detergent. Sometimes, the product itself is used to select resistant mutants which no longer are killed or growth-inhibited by their own fermentation product [53]. Mutation can also be used to aid in the elucidation of the biosynthetic pathway used to make the product and also to discover new products and derivatives.

An early example of the use of antimetabolites is the work of Supek et al. [47] on bacitracin formation. This group of peptide antibiotics contain from 4 to 12 branched amino acid residues and antibiotic formation is stimulated by leucine. Selection for resistance to the antimetabolite azaleucine led to the isolation of a mutant which was derepressed by five- to tenfold in transaminase B formation, had a 400% titer increase in chemically defined medium and a 20% improvement in a commercial type of complex medium. Another example involves the production of monensin A by *Streptomyces cinnamonensis*. The culture made this compound along with the undesirable monensin B in a 1:1 ratio. Monensin A has a butyrate moiety derived from valine but monensin B does not. Pospisil et al. [40] selected mutants resistant to the valine antimetabolites 2-amino-3-chlorobutyrate and norleucine and obtained mutants with a very desirable ratio of 9A:1B. A very popular strategy is the use of 2-deoxyglucose (DOG) to select for mutants resistant to carbon source repression. Anthracycline production by *Streptomyces peucetius* var *caesius* is repressed by glucose, sucrose, and xylose. DOG-resistant mutants were decreased in glucose uptake by 50% and in glucose kinase activity by 85%, and were no longer repressed by the sugars [24].

Usually, a single strain produces more than one secondary metabolite, e.g., *Streptomyces clavuligerus* forms clavulanic acid, other clavams, penicillin N, cephamycin C and holomycin. Mutation is often used to eliminate unwanted products and favor the production of the desired compound. This was accomplished with the lovastatin process of *Aspergillus terreus*, which included production of two other polyketides, sulochrin and asterric acid, along with lovastatin. Both undesirable products were eliminated by mutation [51]. Such a manipulation is much less costly than the removal of coproducts during downstream processing.

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'' (mutasynthesis) has been used for the discovery of many new aminoglycoside, macrolide, and anthracycline antibiotics. In this technique, a non-producing mutant (''idiotroph'') is isolated and then fed various analogs of the missing moiety. When such a procedure leads to a return of antibiotic activity, it is usually due to the formation of an analog of the original compound. Mutational biosynthesis was successfully employed in producing a novel commercial avermectin, called doramectin [46].

During the first 40 years of ''brute force'' genetics, genetic recombination was virtually ignored in strain development because of low frequencies of recombination, e.g., one recombinant in a million cells with *Streptomyces*. However, the development of protoplast fusion changed all that, with frequencies being increased from $10^{-6}$ to as high as $10^{-1}$. Mutant lines which were pursued independently were recombined genetically by the fusion of protoplasts to obtain a strain producing more than either parent. Soon, transformation, conjugation and recombinant DNA technologies became routine tools of the trade [16], being employed to achieve the following goals: (1) removing bottlenecks of rate-limiting reactions, (2) eliminating feedback regulation, (3) manipulating control genes, (4) perturbing central metabolism, (5) blocking competing pathways, (6) enhancing product excretion [26], and (7) decreasing the conversion of the desired product to a less active or inactive compound. An example of each strategy is as follows: (1) the expandase promoter of the penicillin biosynthetic pathway was replaced by the stronger ethanol dehydrogenase promoter [33]; (2) the threonine dehydratase gene *ilvA* of *Corynebacterium glutamicum* was replaced with a feedback-resistant *ilvA* from *E. coli* to increase isoleucine production [25]; (3) extra copies of positive control genes were inserted in actinomycete producers of actinorhodin, undecylprodigiosin and spiramycin whereas negative regulatory genes were deleted or inactivated in producers of methylenomycin, tetracenomycin, jadomycin, and daunorubicin [10]; (4) the first or second enzyme of the pentose phosphate pathway was deleted in *Streptomyces lividans* to increase actinorhodin production [9]; (5) tyrosine formation was blocked to increase phenylalanine titer [4]; (6) *lysE*, encoding the protein involved in lysine excretion, was overexpressed which increased lysine formation in *C. glutamicum* [52]; and (7) genes *dnrX, dnrH* and/or *dnrU*, involved in conversion of the desirable doxorubicin to other compounds, were disrupted, thus increasing doxorubicin titer by threefold [35]. Increasing the dosage of biosynthetic genes has become a popular

means to increase production. When this was done with early genes of the spinosyn pathway, production was increased by three- to fourfold [36].

In recent years, many novel genetic techniques have come upon the scene. These have been recently reviewed [1]. They include genome-based strain reconstruction, metabolic engineering, association analysis, massive parallel signature sequencing, directed evolution, DNA shuffling, transcriptional profiling, and whole genome shuffling (WGS). Transcriptional profiling was used to markedly improve an industrial strain of the lovastatin producer, *A. terreus*. The investigators [3] increased individually the dosage of seven biosynthetic and regulatory genes: *lovF, creA, fadA, ganA, gna1, gna3*, and *gpa1*. The *lovF* gene was found to be crucial for titer improvement. They fused the *lovF* promoter to the phleomycin resistance gene *ble* and used it as a reporter-based system. They then selected phleomycin-resistant mutants, one of which doubled the lovastatin production of the parent culture.

DNA shuffling was used to improve the doramectin process [46]. A parent strain of *Streptomyces avermitilis* produced avermectins B1 and B2 in a ratio of 0.6:1, respectively, B1 being the favorable member of the pair. By random mutation of gene *aveC* via PCR, a mutant was obtained with a more favorable ratio of 2.5:1. Finally, DNA shuffling was applied and the ratio was markedly increased to 15:1. WGS combines the advantage of multi-parental crossing allowed by the DNA shuffling with the recombination of entire genomes. This method was successfully applied to improve the tylosin production in *Streptomyces fradiae* [56]. Historically, 20 cycles of classical strain improvement at Eli Lilly and Co. carried out over 20 years employing about one million assays had improved the production sixfold. In contrast, two rounds of WGS with seven early strains each were sufficient to achieve similar results in 1 year and involved only 24,000 assays.

## Process development in the pilot plant and factory

S. J. Hochauser wrote in 1983 that "under the most rigorously defined conditions of temperature, pH, aeration, and nutrient concentrations, the organism will do whatever it damn well pleases." I am happy to say that this is no longer true. A large number of professionals have been trained in leading academic departments of biochemical engineering who are capable today of domesticating bacteria, fungi and even mammalian and insect cells. It used to be said that some processes were "unscalable"; this is a fable. Anything that can be developed in the laboratory by microbiologists and geneticists can now be scaled up to levels as high as a hundred thousand gallons in factory fermentors by trained "biological engineers". As a result, fermentation products are made today in multiton quantities around the world [14], resulting in very reasonable costs (Table 4).

**Table 4** Markets

| Product | t/year | $/t | $/£ |
|---|---|---|---|
| Fuel ethanol | 13,000,000 | 1700 | 0.85 |
| Citric acid | 9,000,000 | 1700 | 0.85 |
| MSG | 1,000,000 | 1900 | 0.95 |
| L-lysine | 450,000 | 2200 | 1.10 |
| L-lactic acid | 70,000 | 2100 | 1.05 |
| L-ascorbic acid | 60,000 | 1000 | 0.50 |
| Gluconic acid | 40,000 | 1700 | 0.85 |
| Xanthan | 30,000 | 8000 | 4.00 |
| Penicillin G | 25,000 | 20,000 | 10.00 |
| Aspartame | 15,000 | 40,000 | 20.00 |

One of the most important developments in the history of large-scale fermentations is the fed-batch process. Again, this derives from the work of Marvin Johnson at the University of Wisconsin during development of the penicillin fermentation over 50 years ago. Soltero and Johnson [44] wrote: "Glucose,...intermittently fed...to fermentations, has given penicillin yields on synthetic medium equal to, or even better than, those obtained...with lactose. Penicillin yields of twice those of lactose controls have been obtained...when glucose or sucrose is continuously added to the fermentations."

Another major development with respect to large-scale fermentation was the finding that culture morphology has a major effect on fermentor performance by filamentous organisms, especially molds. Generally speaking, the long filamentous type of growth is undesirable because it leads to high culture viscosity, thereby reducing oxygen transfer into the bulk culture and leading to oxygen starvation. This was seen in the citric acid fermentation where it was noted that small clumps of swollen filaments give the highest yields of citric acid. Such a desirable morphology was brought about by high aeration, low pH, phosphate limitation, and manganese limitation [38]. In general, factors found to be of importance in combating filamentous growth and yielding a desirable type of morphology such as small pellets are (1) increasing inoculum size, (2) modifying initial pH, (3) changing vessel type to reverse oxygen limitation, and (4) supplementing with additives [23]. Such additives include chelators, Tween 80, agar granules, or polymers. My group found two of these polymers, carboxymethylcellulose and carboxypolymethylene (Carbopol), to be especially good for *Aspergillus fumigatus* [54]. Genetics can also be of assistance here. It has been found that chitin synthetase genes *chsA* and *chsB* influence mycelial morphology of *Aspergillus oryzae*. Deletion of *chsB* modified morphology and decreased viscosity [37]. Another possible solution to the problem was generated by the finding that pulsed feeding of the carbon source (maltodextrin) to *A. oryzae*, rather than the usual type of fed-batch feeding, led to smaller fungal elements, reduced viscosity, higher dissolved oxygen, and increased nutrient uptake rates [8]. To my knowledge, pulsed feeding has not yet been tried in a secondary metabolite process. Foaming can also

become a serious problem in factory fermentors and is usually solved by the addition of various antifoaming agents. However, one group found that "foam-negative" mutants could indeed be obtained [29].

Metabolic engineering has become important in recent years. Its essence is the combination of analytical methods to quantify fluxes and the control of fluxes with molecular biological techniques to implement suggested genetic modifications. Flux is the focal point of metabolic engineering. Different means of analyzing flux are (1) kinetic based models, (2) control theories, (3) tracer experiments, (4) magnetization transfer, (5) metabolite balancing, (6) enzyme analysis, and (7) genetic analysis [22]. Metabolic control analysis revealed that the overall flux through a metabolic pathway depends on several steps, not just a single rate-limiting reaction [31]. Production of amino acids is one of the fields with many examples of this approach [42]. Metabolic flux studies of wild-type *C. glutamicum* and four improved lysine-producing mutants showed that the yield increased from 1.2 to 24.9% relative to the glucose flux. Metabolic engineering has also been applied to antibiotic production [34, 48]. The increases in metabolic flux were carried out by enhancing enzymatic activity, manipulating regulatory genes, enhancing antibiotic resistance, and heterologous expression of novel genes. There are many other successful applications of metabolic engineering for products such as 1,3-propanediol, carotenoids, organic acids, ethanol, vitamins, and complex polyketides in bacteria.

## The biopharmaceutical industry

The above paragraphs have dealt mainly with the contributions of applied microbiology to the pharmaceutical, nutrition, and flavor industries. However, the technology developed for the industrial production of primary and secondary metabolites impacted heavily on the development of the biopharmaceutical (biotechnology) industry. In the early 1970s, the era of modern biotechnology began with the development of recombinant DNA technology. Traditional industrial microbiology was merged with molecular biology to propel biology to new heights. It led to the establishment of a new industry, yielding many biopharmaceutical products manufactured with recombinant cultures [17]. The revolutionary exploitation of microbial genetic discoveries in the 1970s, 1980s, and 1990s depended upon the solid structure of industrial microbiology. Major microbial hosts for production of mammalian proteins became *E. coli*, *Bacillus subtilis*, *Bacillus megaterium, Saccharomyces cerevisiae*, *Pichia pastoris*, *Hansenula polymorpha*, and *Aspergillus niger*. With *E. coli*, the main obstacle to high cell density was acetate production which exerted toxicity. Success was attained when acetate production was limited by exponential feeding of glucose which kept the specific growth rate below that which brings on acetate production. Developments such as this, coming from traditional fermentation experience and knowledge, allowed the development of high cell density fermentation of microorganisms which reached over 200 g dry cell weight per l for bacteria and yeasts. Mammalian polypeptides are being produced in bacteria and yeasts at a level of 1 to 70% of cell protein and at concentrations as high as 15 g/l. The use of recombinant microorganisms provided the technology and experience necessary for the successful application of higher organisms such as mammalian cells, insect cells, and transgenic animals and plants as hosts for production of glycosylated recombinant proteins. The use of mammalian cell culture, chiefly immortalized Chinese hamster ovary (CHO cells) was mandated by the need for erythropoietin and tissue plasminogen activator production in the early 1980s. These glycosylated proteins could not be produced in *E. coli*. Mammalian cell cultures are usually CHO cells, but others have been used, e.g., N50 murine myeloma cells, baby hamster kidney cells, green monkey kidney cells, and human embryonic kidney cells. CHO cell processes have been developed that yield 3–5 g/l of recombinant protein. Insect cells in culture are also the hosts for recombinant protein production. Production of recombinant proteins in the baculovirus expression vector system in insect cells is at a level of 0.6–0.8 g/l.

Biotechnology has had a major effect on health care, diagnostics and agriculture, and made inroads in the practices of other industries such as energy, mining, foods, and chemicals. Within 4 years of the discovery of recombinant DNA technology, genetically engineered bacteria were making human insulin and human growth hormone. Within 10 years, recombinant insulin was being produced commercially. This led to an explosion of investment activity in new companies, mainly dedicated to innovation via genetic approaches; this is still in progress. Today, drugs from the biotechnology industry represent 38% of all approved drugs.

The modern biotechnology industry has come up with important blood products, infectious disease combatants, immunotherapy products, growth factors for mammalian cells, and monoclonal antibodies. Approximately 4,600 biotechnology companies exist in the world. Over 140 biopharmaceuticals are on the market and hundreds more are in the clinic against over 200 diseases (AIDS, cardiovascular, diabetes, arthritis, cancer, etc.).

The market for therapeutic proteins amounts to $39 billion within a total biopharmaceutical market of $40–50 billion. The most well-known products of the modern biotechnology industry are the mammalian polypeptides such as erythropoietin (EPO) with an $11 billion market; human insulin (Novolin and Humulin), $5.4 billion; interferon α (Intron A), $2.7 billion; the white blood cell stimulant, Neupogen (filgrastim), $2.5 billion; interferon β-1a (Avonex for multiple sclerosis), $2.2 billion; human growth hormone (HGH), $1.8 billion; recombinant hepatitis B vaccine ($0.7 billion); somatropin (Huma-

trope and Neutropin), $0.7 billion; and cerezyme/ceredase (alglucerase), $0.6 billion.

Monoclonal antibodies are the fastest growing therapeutic protein class, already with a market of $5.4 billion. ReoPro was the first successful therapeutic monoclonal antibody, being approved in 1994 for inhibition of platelet aggregation (blood clotting); it had a market of $384 million by 2002. It successfully prevented complications of angioplasty such as death, heart attack, and need for repeat angioplasty. Rituxan, used for non-Hodgkins lymphoma, has achieved sales of $2.8 billion. Enbrel (etanercept), Remicade (infliximab), and Humira (adalimumab) are for rheumatoid arthritis; Remicade is also useful for Crohn's disease. Sales of Enbrel and Remicade are approximately $1.5 billion each. Enbrel is a monoclonal antibody which binds TNF, a protein involved in inflammation. Herceptin (trastuzumab) is prescribed for metastatic breast cancer in a subgroup of 25–30% of women suffering from this disease. These women have tumors overexpressing the epidermal growth factor receptor protein, HER2. Sales of Herceptin were $745 million in 2002. Syagis (palivizumab; MEDI-493) prevents lower respiratory tract disease caused by the respiratory syncytial virus and was the first monoclonal antibody used for infectious disease; it netted $800 million in 2003. Today, over 20 monoclonal antibodies are on the market, and over 100 more are in clinical trials for allergy, asthma, autoimmune diseases, cancer, cardiovascular, transplantation, and viral infection. A unique application has been the "magic bullet" approach which utilizes monoclonal antibodies specific for tumor cells to bring a toxic agent into intimate contact with the tumor. The toxic microbial enediyne antitumor drug calicheamicin was attached to a humanized monoclonal antibody and found to be active against acute myeloid leukemia (AML). The conjugate, called Mylotarg (gemtuzumab ozogamicin), became a marketed product in 2001. The monoclonal antibody directs the antitumor drug to the CD33 antigen which is a protein expressed by myeloid leukemic cells. Production of monoclonal antibodies has reached 1–2 g/l in a fed-batch process conducted for a 2-week period.

A major change in the biopharmaceutical industry is the imminent entry of generic firms. Between 2001 and 2006, biopharmaceuticals with a total market of over $13 billion are going off patent.

Molecular biology has been the major driving force in biopharmaceutical research. Although the industry mainly dealt with production of natural proteins in recombinant organisms for many years, today it encompasses ribozymes, antisense molecules, monoclonal antibodies, gene and cell therapy, genomics, proteomics, pharmacogenomics, drug delivery, combinatorial chemistry and biology, developmental biology, high throughput screening, and bioinformatics. It is clear that genetic engineering of microbes and other forms of life has made major impacts in the world and has changed the face of medicine and industry.

## Final comments

Microbial products have been an overwhelming success in our society. It has been stated that the doubling of our life span in the twentieth century is mainly due to the use of secondary metabolites. They have reduced pain and suffering, and revolutionized medicine by allowing for the transplantation of organs. Natural products from microbes and plants are the most important anti-cancer and anti-infective agents. Over 60% of approved and pre-NDA candidates are either such products or related to them, not including biologicals such as vaccines and monoclonal antibodies [11]. Almost half of the best selling pharmaceuticals are microbial or plant products, or are related to them. Often, the natural molecule has not been used itself but served as a lead molecule for manipulation by chemical means, or by the genetic techniques such as combinatorial biosynthesis.

The path from discovery through scale-up of microbial processes has been a huge commercial success. By 1996, the world market for antimicrobials amounted to $23 billion and involved some 150–300 products, either natural, semi-synthetic or synthetic. The $8 billion US antimicrobial market in 1995 included cephalosporins (45%), penicillins (15%), quinolones (11%), tetracyclines (6%) and macrolides (5%). Consider what has occurred since the discovery of the first antibiotic, penicillin. Following on the heels of penicillin production by *P. chrysogenum* came the discoveries of cephalosporin formation by *Cephalosporium acremonium*, cephamycin, clavam and carbapenem production by actinomycetes, and monocyclic $\beta$-lactam production by actinomycetes and unicellular bacteria. Each one of these groups has yielded medically useful products. Currently, sales of $\beta$-lactam compounds, which amount to $21 billion per year, form the largest share of the world's $32 billion per year anti-infective market. The $\beta$-lactam antibiotics include penicillins such as penicillin G, penicillin V, ampicillin, cloxacillin, and piperacillin; cephalosporins such as cephalothin, cephaloridine, cephalexin, and cefaclor; and cephamycins such as cefoxitin. In addition, $\beta$-lactam antibiotics include the more recently developed nonclassical structures such as (1) monobactams, including aztreonam; (2) carbapenems, including thienamycin, which is chemically transformed into imipenem, a component of the combination drug primaxin; and (3) clavulanic acid, a $\beta$-lactamase inhibitor which is used in combination with penicillins (Augmentin™ is amoxicillin + clavulanic acid and Timentin™ is ticarcillin + clavulanic acid). Clavulanic acid has a market of over $1 billion.

Despite the above successes, microbiologists know that much work remains and that antimicrobial technology alone will not permanently win the war against infectious microorganisms due to resistance development in pathogenic microbes. We will have to be satisfied to merely stay one step ahead of the pathogens for a

long time to come; thus, the search for new antibiotics must not be stopped. New entities are continually needed because of (1) the development of resistant pathogens; (2) the emergence of some 30 new diseases since 1980 including AIDS, Hanta virus, Ebola virus, *Cryptospiridium*, Legionnaire's disease, Lyme disease, and *E. coli* 0157:H7; (3) the existence of naturally resistant bacteria, e.g., *Pseudomonas aeruginosa* causing fatal wound infections, burn infections and chronic and fatal infections of lungs in cystic fibrosis patients, *Stenotrophomonas maltophilia*, *Enterococcus faecium, Burkholderia cepacia* and *Acinetobacter baumanni* (some enterococci are resistant to all known commercial antibiotics); and (4) the toxicity of some of the current compounds. Other organisms exist which are not normally virulent but do infect immunocompromised patients. Fungal infections are a real problem, having doubled from the 1980s to the 1990s. There is an increasing incidence of candidiasis, cryptococcosis, and aspergillosis, especially in AIDS patients; aspergillosis failure rates exceed 60%. Fungal infections occur often after lung, kidney, heart, and lung transplant operations, usually by *Candida* and *Aspergillus* spp. Pulmonary aspergillosis is the main factor involved in death of recipients of bone marrow transplants and *Pneumocystis carinii* is the number one cause of death in patients with AIDS from Europe and North America. Current treatments include the synthetic azoles (e.g., fluconazole and flucytosine) or the natural polyene amphotericin B. However, usage is becoming limited by resistance development to the azoles and toxicity of amphotericin B. Fortunately, a new antifungal, caspofungin, has appeared on the scene recently. It is clear that the future success of the pharmaceutical industry depends on the combination of complementary technologies such as microbial natural product discovery, high throughput screening, genomics and proteomics, combinatorial chemistry and combinatorial biosynthesis.

## References


1. Adrio JL, Demain AL (2005) Genetic improvement of processes yielding microbial products. FEMS Microbiol Rev (in press)
2. Adrio JL, Demain AL (2005) Microbial cells and enzymes; a century of progress. In: Barredo JL (ed) Methods in biotechnology, vol 17. Microbial enzymes and biotransformations. Humana, Totowa, New Jersey, pp 1–27
3. Askenazi M, Driggers EM, Holtzman DA, Norman TC, Iverson S, Zimmer DP, Boers ME, Blomquist P., Martinez EJ, Monreal AW, Feibelman TP, Mayorga ME, Maxon ME, Sykes K, Tobin JV, Cordero E, Salama SR, Trueheart J, Royer JC, Madden KT (2003) Integrating transcriptional and metabolite profiles to direct the engineering of lovastatin-producing fungal strains. Nat Biotechnol 21:150–156
4. Backman K, O'Connor MJ, Maruya A, Rudd E, McKay D, Balakrishnan R, Radjai M, DiPasquantonio V, Shoda D, Hatch R, Venkatsubramanian K (1990) Genetic engineering of metabolic pathways applied to the production of phenylalanine. Ann NY Acad Sci 589:16–24
5. Baltz RH (1986) Mutation in *Streptomyces*. The Bacteria 9: 61–94
6. Berdy J (2005) Bioactive microbial metabolites. A personal view. J Antibiot 58:1–26
7. Berdy J, Kadar-Pauncz J, Mehesfalvi-Vajna Z, Horvath G, Gyimesi J (1977) Metabolites of gentamicin producing *Micromonospora* species. Part 1. Isolation and identification of metabolites. J Antibiot 30:945–953
8. Bhargava S, Wenger KS, Marten MR (2003) Pulsed feeding during fed-batch *Aspergillus oryzae* fermentation leads to improved oxygen mass transfer. Biotechnol Prog 19:1091–1094
9. Butler MJ, Bruheim P, Jovetic S, Marinelli F, Postma PW, Bibb MJ (2002) Engineering of primary carbon metabolism for improved antibiotic production in *Streptomyces lividans*. Appl Environ Microbiol 68:4731–4739
10. Chater KF, Bibb MJ (1997) Regulation of antibiotic production. In: Rehm H-J, Reed G, Kleinkauf H, von Doehren H (eds) Biotechnology, vol 7. 2nd edn. Products of secondary metabolism. VCH, Weinheim pp 57–105
11. Cragg GM, Newman DJ, Snader KM (1997) Natural products in drug discovery and development. J Nat Prod 60:52–60
12. Demain AL (1998) Induction of microbial secondary metabolism. Int Microbiol 1:259–264
13. Demain AL (1999) Pharmaceutically active secondary metabolites of microorganisms. Appl Microbiol Biotechnol 52:455–463
14. Demain AL (2000) Small bugs, big business: the economic power of the microbe. Biotechnol Adv 18:499–514
15. Demain AL (2000) Microbial biotechnology. Trends Biotechnol 18:26–31
16. Demain AL (2001) Molecular genetics and industrial microbiology—30 years of marriage. J Ind Microbiol Biotechnol 27:352–356
17. Demain AL (2004) The biopharmaceutical revolution. Chim Oggi/Chem Today 22(11–12):41–44
18. Demain AL, Elander RP (1999) The $\beta$-lactam antibiotics: past, present, and future. Ant v Leeuwenhoek 75:5–19
19. Demain AL, Vaishnav P (2002) Regulation of $\beta$-lactam antibiotic biosynthesis by carbon sources. Chim Oggi/Chem Today 20(11–12):46–51
20. Demain AL, Vaishnav P (2003) Nitrogen regulation of biosynthesis of antibiotics and other secondary metabolic products. Pharma Chem 2(10):92–96
21. Demain AL Vaishnav P (2004) Secondary metabolism in microbes and its control by phosphate and metals. SIM News 54:104–113
22. Eggeling L, Sahm H, de Graaf AA (1996) Quantifying and directing metabolic flux: application to amino acid overproduction. Adv Biochem Eng Biotechnol 54:1–30
23. Elmayergi H (1975) Mechanism of pellet formation of *Aspergillus niger* with an additive. J Ferm Technol 53:722–729
24. Escalante L, Ramos I, Imriskova I, Langley E, Sanchez S (1999) Glucose repression of anthracycline formation in *Streptomyces peucetius* var. *caesius*. Appl Microbiol Biotechnol 52:572–578
25. Guillouet S, Rodal AA, An G-H, Lessard PA, Sinskey AJ (1999) Expression of the *Escherichia coli* catabolic threonine dehydratase in *Corynebacterium glutamicum* and its effect on isoleucine production. Appl Environ Microbiol 65:3100–3107
26. Han L, Parekh S (2004) Development of improved strains and optimization of fermentation processes. In: Barredo JL (ed) Microbial processes and products. Humana, Totowa, New Jersey, pp 1–23
27. Handelsman J (2004) Soils—the metagenomics approach. In: Bull AT (ed) Microbial diversity and bioprospecting. ASM Press, Washingtopn, DC pp 109–119
28. Handelsman J, Rondon MR, Brady SF, Clardy J, Goodman RM (1998) Molecular biological access to the chemistry of unknown soil microbes: a new frontier for natural products. Chem Biol 5:R245-R249
29. Ishizuka H, Wako K, Kasumi T, Sasaki T (1989) Breeding of a mutant of *Aureobasidium* sp. with high erythritol production. J Ferm Bioeng 68:310–314

30. Jarvis GF, Johnson MJ (1947) The role of the constituents of synthetic media for penicillin production. J Amer Chem Soc 69:3010–3017
31. Kacser H, Acerenza L (1993) A universal method for achieving increases in metabolite production. Eur J Biochem 216:361–367
32. Kaeberlein T, Lewis K, Epstein SS (2002) Isolating "uncultivable" microorganisms in pure culture in a simulated natural environment. Science 296:1127–1129
33. Kennedy J, Turner G (1996) δ-(L-α-aminoadipyl)-L-cysteinyl-D-valine synthetase is a rate limiting enzyme for penicillin production in *Aspergillus nidulans*. Mol Gen Genet 253:189–197
34. Khetan A, HuW-S (1999) Metabolic engineering of antibiotic biosynthetic pathways. In: Demain AL, Davies JE (eds) Manual of industrial microbiology and biotechnology. 2nd edn. ASM Press, Washington, DC pp 717–724
35. Lomovskaya N, Doi-Katayama Y, Filippini S, Nastro C, Fonstein L, Gallo M, Colombo AL, Hutchinson CR (1998) The *Streptomyces peucetius dpsY* and *dnrX* genes govern early and late steps of daunorubicin and doxorubicin biosynthesis. J Bacteriol 180:2379–2386
36. Madduri K, Waldrom M, Matsushima P, Broughton MC, Crawford K, Merlo DJ, Baltz RH (2001) Genes for the biosynthesis of spinosyns: applications for yield improvement in *Saccharopolyspora spinosa*. J Ind Microbiol Biotechnol 27:399–402
37. Mueller C, Hansen K, Szabo P, Nielsen J (2003) Effect of deletion of chitin synthase genes on mycelial morphology and culture viscosity in *Aspergillus oryzae*. Biotechnol Bioeng 81:525–534
38. Papagianni M (2004) Fungal morphology and metabolite production in submerged mycelial processes. Biotechnol Adv 22:189–259
39. Parekh S, Vinci VA, Strobel RJ (2000) Improvement of microbial strains and fermentation processes. Appl Microbiol Biotechnol 54:287–301
40. Pospisil S, Peterkova M, Krumphanzl V, Vanek Z (1984) Regulatory mutants of *Streptomyces cinnamonensis* producing monensin A. FEMS Microbiol Lett 24:209–213
41. Rappe MS., Giovannoni SJ (2003) The uncultured microbial majority. Annu Rev Microbiol 57:369–394
42. Sahm H, Eggeling L, de Graaf AA (2000) Pathway analysis and metabolic engineering in *Corynebacterium glutamicum*. Biol Chem 381:899–910
43. Sanchez S, Demain AL (2002) Metabolic regulation of fermentation processes. Enzyme Microb Technol 31:895–906
44. Soltero FV, Johnson MJ (1953) Effect of the carbohydrate nutrition on penicillin production by *P. chrysogenum* Q-176. Appl Microbiol 1:52–57
45. Stevenson BS, Eichorst SA, Wertz JT, Schmidt TM, Breznak JA (2004) New strategies for cultivation and detection of previously uncultured microbes. Appl Environ Microbiol 70:4748–4755
46. Stutzman-Engwall K, Conlon S, Fedechko R, McArthur H, Pekrun K, Chen Y, Jenne S, La C, Trinh N, Kim S, Zhang Y-X, Fox R, Gustafsson C, Krebber A (2005) Semi-synthetic DNA shuffling of *aveC* leads to improved industrial scale production of doramectin by *Streptomyces avermitilis*. Metab Eng 7:27–37
47. Supek V, Gamulin S, Delic V (1985) Enhancement of bacitracin biosynthesis by branched-chain amino acids in a regulatory mutant of *Bccillus licheniformis*. Folia Microbiol 30:342–348
48. Thykaer J, Nielsen J (2003) Metabolic engineering of β-lactam production. Metab Eng 5:56–69
49. Umezawa H (1972) Enzyme Inhibitors of Microbial Origin. University Park Press, Baltimore
50. Umezawa H (1982) Low-molecular-weight inhibitors of microbial origin. Annu Rev Microbiol 36:75–99
51. Vinci VA, Hoerner TD, Coffman AD, Schimmel TG, Dabora RL, Kirpekar AC, Ruby CL, Stieber RW (1991) Mutants of a lovaststin-hyperproducing *Aspergillus terreus* deficient in the production of sulochrin. J Ind Microbiol 8:113–120
52. Vrljic M, Sahm H, Eggeling L (1996) A new type of transporter with a new type of cellular function: L-lysine export from *Corynebacterium glutamicum*. Mol Microbiol 22:815–826
53. Woodruff HB (1966) The physiology of antibiotic production: the role of the producing organism. Symp Soc Gen Microbiol 16:22–46
54. Yang W, Hartwieg EA, Fang A, Demain AL (2003) Effects of carboxymethylcellulose and carboxypolymethylene on morphology of *A. fumigatus* NRRL 2346 and fumagillin production. Curr Microbiol 46:24–27
55. Zengler K, Toledo G, Rappé M, Elkins J, Mathur E J, Short J M, Keller M (2002) Cultivating the uncultured. Proc Natl Acad Sci USA 99:15681–15686
56. Zhang Y-X, Perry K, Vinci VA, Powell K, Stemmer WPC, del Cardayre SB (2002) Genome shuffling leads to rapid phenotypic improvement in bacteria. Nature 415:644–646