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REVIEW ARTICLE

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Fungal biotechnology

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Abstract Fungi are used in many industrial processes, such as the production of enzymes, vitamins, polysaccharides, polyhydric alcohols, pigments, lipids, and glycolipids. Some of these products are produced commercially while others are potentially valuable in biotechnology. Fungal secondary metabolites are extremely important to our health and nutrition and have tremendous economic impact. In addition to the multiple reaction sequences of fermentations, fungi are extremely useful in carrying out biotransformation processes. These are becoming essential to the fine-chemical industry in the production of single-isomer intermediates. Recombinant DNA technology, which includes yeasts and other fungi as hosts, has markedly increased markets for microbial enzymes. Molecular manipulations have been added to mutational techniques as a means of increasing titers and yields of microbial processes and in the discovery of new drugs. Today, fungal biology is a major participant in global industry. Moreover, the best is yet to come as genomes of additional species are sequenced at some level (cDNA, complete genomes, expressed sequence tags) and gene and protein arrays become available.

Keywords Yeasts ecology · Pectic enzymes · Food technology

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Fungi as cell factories

Since prebiblical times, fungi, including both true filamentous fungi and yeasts, have been used to produce products such as beer, wine, bread, and cheese. The twentieth century, a golden age of industrial microbiology, yielded a myriad of products made by fermentation processes: solvents, antibiotics, enzymes, vitamins, amino acids, polymers, and many other useful compounds [30]. The development of molecular biology techniques provided new ways to use yeasts and molds as microbial cell factories for the production of homologous and heterologous (especially mammalian) proteins as well as other metabolites, such as antibiotics, pigments, and fatty acids. The choice of the strain is made on the basis of production yields and regulatory issues, especially for fungi used in the food industry. Host strains are usually chosen from among those which have attained the so-called GRAS (Generally Recognized As Safe) status by the U.S. Food and Drug Administration (FDA). Several species of fungi have that status and are currently being used for large-scale production of recombinant proteins and metabolites [84].

Production of recombinant polypeptides

Pharmaceutical proteins

Since it is a food organism, *Saccharomyces cerevisiae* is considered to be a safe host for the production of pharmaceutical proteins. This yeast can be grown rapidly and to a high cell density, can secrete heterologous proteins into the extracellular broth, and knowledge of its genetics is more advanced than that of any other eukaryote [86]. Mammalian genes have been cloned and expressed in *S. cerevisiae*, including human interferon [52], human epidermal growth factor [14], and human hemoglobin [100]. The most commercially important yeast recombinant process has been the production of genes encoding surface antigens of the hepatitis B virus,

resulting in the first safe hepatitis B vaccine [75, 104]. Despite these successful examples, *S. cerevisiae* is sometimes regarded as a less than optimal host for large-scale production of mammalian proteins because of certain drawbacks, such as hyperglycosylation, the presence of α-1,3-linked mannose residues that may cause antigenic responses in patients, and the absence of strong and tightly regulated promoters.

For these reasons, *Pichia pastoris* has become one of the most extensively used expression systems [51, 86, 87]. Among the advantages of this methylotrophic yeast over *S. cerevisiae* are: (1) an efficient and tightly regulated methanol promoter (*AOX1*) which yields alcohol oxidase at 30% of soluble protein, (2) less extensive glycosylation, due to shorter chain lengths of N-linked high-mannose oligosaccharides, usually up to 20 residues lacking the terminal α-1,3-mannose linkages [15, 27, 85], (3) integration of multiple copies of foreign DNA into chromosomal DNA yielding stable transformants [42, 86], (4) the ability to secrete high levels of foreign proteins, (5) high-density growth and straightforward scale-up [85, 87]. There are many examples of intracellular or extracellular recombinant products that have been made in *P. pastoris* [16, 26, 51, 85, 86]. Nonetheless, one of the main drawbacks to this excellent expression system is its non-GRAS status, although some products made by this yeast are being evaluated in phase III clinical trials. For example, the production of recombinant hirudin, a thrombin inhibitor from the medicinal leech *Hirudo medicinalis*, results in yields of 1.5 g secreted product/l [96].

Heterologous gene expression in the methylotrophic yeast *Hansenula polymorpha* is similar to that of *P. pastoris*. The promoter of the methanol oxidase gene is used to express foreign genes. As with *AOX1* in *P. pastoris, MOX* in *H. polymorpha* is also highly expressed and tightly regulated, giving enzyme levels up to 37% of total cell protein [43]. One major difference is that expression of *MOX* is significantly derepressed in the absence of glucose or during glucose limitation [34] and therefore tight regulation of the *MOX* promoter is lost under the high-glucose conditions usually used for high-biomass fermentations [41].

The development of molecular techniques for the production of recombinant heterologous proteins in filamentous fungi is laborious and has contrasted markedly with the success achieved in yeasts. Some advances in transformation have been recently reported, e.g., restriction enzyme-mediated integration [95] and *Agrobacterium tumefaciens* Ti-plasmid-mediated transformation [46]. Levels of production of non-fungal proteins are lower than those of homologous proteins. This is due to factors that influence production, i.e., transcription, translation, secretion, and extracellular degradation [4, 47, 84, 108]. Different strategies have been developed to overcome these problems, including the construction of protease-deficient strains [73, 105], the introduction of a large number of gene copies [5, 46], the use of strong fungal promoters, efficient secretion signals [47, 76, 108], and fusions with a gene that encodes part of or an entire well-secreted protein [47, 84]. Gene fusion is the first choice in attempting to produce non-fungal proteins in fungal hosts. Fusion has resulted in levels of secreted proteins of 5 mg human interleukin-6/l [17, 23], 2 mg human lysozyme/l [8] and 250 mg human lactoferrin/l [114]. Higher concentrations have been obtained for some of these proteins after mutagenic treatment of high-producing strains, e.g., human lactoferrin at 5 g/l [113].

Recent studies have shown the fungal secretory pathway to be a limiting factor in heterologous enzyme production. Studies on screening for mutant strains with altered secretion properties using green fluorescent protein as reporter [45], elucidation of the role of secretion-related chaperones and foldases [22, 61, 90, 112], kinetic studies on protein secretion [78], and the effects of hyphal branch frequency [11] are examples of the work being carried out to understand this complex process.

For many proteins that have pharmaceutical applications, N-glycosylation is necessary for stability, proper folding, e.g., erythropoietin and human chorionic gonadotropin (hGC), and pharmacokinetics [57]. Although O-linked glycosylation in yeast is quite different from that in higher eukaryotes, N-linked glycosylation is more conserved [86]. In yeast recombinant proteins, as well as in mammalian polypeptides, a core oligosaccharide unit ($GlcNAc_2Man_9Glc_3$) is present at the endoplasmic reticulum [68]. The three glucose residues and one mannose are removed and processing of the core oligosaccharide continues in the Golgi, where there is divergence between yeasts and higher eukaryotes. Recombinant yeast proteins usually show high-mannose side chains ($GlcNAc_2Man_{2-6}$) where elongation may take place in further addition steps. Mammalian proteins show two different types of oligosaccharide side chains: low-mannose residues ($GlcNAc_2Man_3$) plus additional residues of galactose, fucose, and sialic acid or a mixture of high-mannose and complex type oligosaccharides [64]. Little research has been carried out on glycosylation in molds although hyperglycosylation does not seem to occur and low-mannose side chains are formed [35, 72, 91]. The glycosylation of a protein can be different depending on factors such as the medium in which the cells are grown.

Commercial recombinant enzymes

Recombinant fungi are one of the main sources of enzymes for industrial applications. The industrial enzyme market reached $1.6 billion in 1998 [99] for the following application areas: food, 45%; detergents, 34%; textiles, 11%; leather, 3%; pulp and paper 1.2%. This does not include diagnostic and therapeutic enzymes. The market for these non-pharmaceutical proteins reached $2 billion in 2000. Over 60% of the enzymes used in the detergent,

food, and starch processing industries are recombinant products [24]. Although the number of heterologous fungal enzymes approved for food applications is not very large, the list is continuously increasing [http://www.enzymetechnicalassoc.org]. Due to the low yields achieved with non-fungal proteins (see above), many recombinant food-grade proteins are of fungal origin [4, 80]. There is one exception in which the donor strain is not another fungus, i.e., calf rennin (chymosin), which is used for cheese making. Production of this bovine protein in recombinant *Aspergillus niger* var *awamori* amounted to about 1 g/l after nitrosoguanidine mutagenesis and selection for 2-deoxyglucose resistance [33]. Further improvement was done by parasexual recombination, resulting in a strain producing 1.5 g/l from parents producing 1.2 g/l [12]. A recombinant strain of *Aspergillus oryzae* producing an aspartic proteinase from *Rhizomucor miehei* has been approved by FDA for cheese production [http://vm.cfsan.fda.gov, 80] (Fig. 1).

Microbial lipases have a huge potential in areas such as food technology, biomedical sciences, and chemical industries since they are: (1) stable in organic solvents, (2) possess broad substrate specificity, (3) do not require cofactors, and (4) exhibit high enantioselectivity [55, 56, 93]. In the food industry, lipases are commonly used in the production of fruit juices, baked foods, desirable flavors in cheeses, and interesterification of fats and oils to produce modified acylglycerols. There are three fungal recombinant lipases currently used in the food industry, *Rhizomucor miehi*, *Thermomyces lanuginosus* and *Fusarium oxysporum*, all of which are produced in *A. oryzae* [http://vm.cfsan.fda.gov, 80].

Lipases are extremely important in the detergent industry. They are extensively used in household detergents, industrial cleaners, and leather processing, where they can be combined with proteases, oxidases, and peroxidases [79]. To be suitable, lipases should be alkalophilic, able to work at temperatures above 45 °C and at pH values of about 10, and capable of functioning in the presence of the various components of wash-product formulations, such as oxidants and surfactants. In 1994, Novo Nordisk introduced Lipolase, the first commercial recombinant lipase for use in a detergent, by cloning the *Humicola lanuginose* lipase gene into the *A. oryzae* genome [20, 79].

Fungal secondary metabolites

Antibiotics

Of the 12,000 antibiotics known in 1995, about 22% could be produced by filamentous fungi [10, 100]. These include the natural penicillin G and the biosynthetic penicillin V, with a combined market of $4.4 billion, many semisynthetic penicillins, and the semisynthetic cephalosporins, which have a market of $11 billion.

Immunosuppresive agents

Cyclosporin A was originally discovered as a narrow-spectrum antifungal peptide produced by the mold *Tolypocladium nivenum* (previously *Tolypocladium inflatum)*[13]. Discovery of the drug's immunosuppressive activity led to its use in heart, liver, and kidney transplants and thus to the overwhelming success of the organ-transplant field. A very old broad-spectrum fungal antibiotic produced by several species of *Penicillium*, mycophenolic acid, was never commercialized as an antibiotic, but its 2-morpholinoethylester was approved as a new immunosuppressant for kidney transplantation in 1995 and for heart transplants in 1998. The ester is called mycophenolate mofetil (CellCept) and is a prodrug that is hydrolyzed to mycophenolic acid in the body.

Hypocholesterolemic agents

Fungal statins (lovastatin, pravastatin and others [37]), which act as inhibitors of 3-hydroxy-3-methylglutaryl-coenzyme A reductase, the regulatory and rate-limiting enzyme of cholesterol biosynthesis in liver, have a market of $15 billion. The first member of this group, compactin, was an antibiotic product of *Penicillium brevicompactum* [18] and *Penicillium citrinum* [38]. Later on, Endo and Alberts et al. [2, 36] independently discovered the more active methylated form of compactin, lovastatin, in broths of *Monascus ruber* and *Aspergillus terreus*, respectively. Lovastatin was approved by the FDA in 1987.

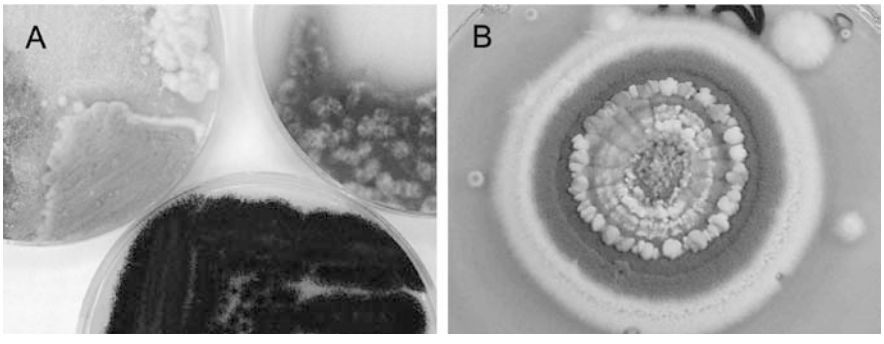

Fig. 1A, B Fungal strains from the Puleva Biotech culture collection. (A) *Aspergillus niger, Aspergillus oryzae* and *Monascus prupurea*. (B) *Penicillium* sp.

Antitumor agents

Taxol, a natural anticancer agent, was originally discovered in plants [111] but can also be produced by the fungus *Taxomyces andreanae* [95]. It is approved for the treatment of breast and ovarian cancer and is the only commercial antitumor drug known to act by blocking depolymerization of microtubules. In 2000, taxol sales amounted to over $1 billion for Bristol Myers-Squibb, representing 10% of the company's pharmaceutical sales and its third largest selling product [102].

Mycotoxins

Ergot alkaloids produced by different species of *Claviceps* are used for the treatment of many pathologies such as migraine headache, cerebral circulatory disorder, uterine contraction, bleeding after childbirth, and also for prevention of implantation in early pregnancy [9, 109]. Among their physiological activities are the inhibition of the action of adrenalin, noradrenalin, and serotonin and the contraction of smooth muscles of the uterus. Some of the ergot alkaloids also possess antibiotic activity.

Zearelanone, produced by *Gibberella zeae* (syn. *Fusarium graminearum*) [50], is an estrogen and its reduced derivative zeranol is used as an anabolic agent in cattle and sheep, increasing both growth and feed efficiency. Gibberellic acid, a member of the phytotoxic mycotoxin group known as the gibberellins, is produced by *Gibberella fujikuroi* . Gibberellins are used to speed up barley malting, improve malt quality, increase the yield of vegetables, and cut in half the time required to obtain lettuce and sugar beet seed crops. They are isoprenoid growth regulators controlling flowering, seed germination, and stem elongation [103].

Pigments

Fermentation of *Monascus purpureus* on rice to prepare koji or ang-kak (red rice) has been used as a traditional Chinese food and medicine since 800 A.D. [71]. The water-soluble red pigments monascorubramine and rubropunctamine are produced by reaction of the orange pigments monascorubrin and rubropunctatin with amino acids present in the fermentation media [60]. The fungus is used for preparing red rice, wine, soy bean cheese, meat, and fish and is authorized for food use in China and Japan.

The yeast *Phaffia rhodozyma* has become the most important microbial source for the production of the carotenoid astaxanthin [3]. This pigment is responsible for the orange to pink color of salmonid flesh and the reddish color of boiled crustacean shells. Feeding of pen-reared salmonids with a diet containing this yeast induces pigmentation of the white muscle [58, 59].

Blakeslea trispora has been used for the industrial production of β-carotene in Russia for years. In this fermentation, a fungal mated culture is used with a preferred ratio of minus and plus mating strains [28]. The accumulation of β-carotene is strongly linked to sexual interaction between the two mating types. A hormone-like substance produced during mating, the major component of which is trisporic acid, stimulates pigment production.

Polyunsaturated fatty acids

Morteriella isabellina and *Mucor circinelloides* can accumulate up to 5 g γ-linoleic acid/l in a medium based on molasses or glucose [28]. *Morteriella alpina* is the best choice for the production of arachidonic acid. Within the last 4 years, the cloning of all desaturases required for the synthesis of this polyunsaturated fatty acid (PUFA) has been described [88, 89], although a Δ17 desaturase for synthesis of longer PUFAs and a fatty acid elongase remain elusive. This fungus is also able to accumulate eicosapentanoic acid when cultured at low temperature.

Regulation of fungal secondary metabolism

Most secondary metabolites are formed via enzymatic pathways rather than by a ribosomal mechanism. The enzymes occur as individual proteins, free or complexed, or as parts of modules of large multifunctional polypeptides carrying out a multitude of enzymatic steps, e.g., in the cases of polyketide synthases and peptide synthetases. Whether chromosomal or plasmid-borne, the secondary metabolism genes are often clustered, but not necessarily as single operons. Clusters of fungal biosynthetic genes have been found encoding enzymes for the production of penicillins, cephalosporins [1], and sterigmatocystin [19] by *Aspergillus nidulans*, and trichothecenes [53] by *Fusarium sporotrichiodes*.

Regulation by carbon source

Glucose, usually an excellent carbon source for growth, often interferes with the formation of secondary metabolites. Instead, polysaccharides (e.g., starch), oligosaccharides (e.g., lactose) and oils (e.g., soybean oil, methyloleate) are often preferable for fermentations yielding secondary metabolites [29]. In media containing a mixture of a rapidly used and a slowly used carbon source, the former is utilized first to produce cells but little to no secondary metabolites are formed. After the rapidly assimilated compound is depleted, the "second-best" carbon source is used for the production phase, known as the "idiophase."

Regulation by nitrogen source

Nitrogen regulation affects both primary and secondary metabolism [29]. The control of enzyme synthesis is

generally exerted by the intracellular nitrogen pool. Many secondary metabolic pathways are negatively affected by nitrogen sources favorable for growth, e.g., ammonium salts. As a result, a slowly assimilated amino acid is often used as the nitrogen source to encourage high production of secondary metabolites. Information concerning the mechanism(s) underlying the negative effect(s) of ammonium and certain amino acids on industrial processes is scarce.

A more specific type of control takes place when a particular amino acid (or biosynthetic group of amino acids) represses and/or inhibits production of a secondary metabolite because the primary metabolite(s) and the idiolite are derived from the same branched pathway, and the amino acid(s) exerts negative feedback regulation on the biosynthetic pathway before the branch point. An example is the negative effect of lysine on penicillin synthesis which is caused by lysine inhibiting homocitrate synthase [31], an enzyme involved in the formation of the penicillin precursor, L-α-aminoadipic acid.

Regulation by phosphorus source

A rather specific negative effect of inorganic phosphate arises from its ability to inhibit and/or repress phosphatases. Because biosynthetic intermediates of certain pathways are phosphorylated whereas the ultimate product is not, phosphatases are sometimes required in biosynthesis. Although only little is known about the mechanism of general phosphate control of secondary metabolism, there is a strong possibility that phosphate regulation also works by affecting enzyme activities, such as phosphorylation by protein kinases and dephosphorylation by phosphoprotein phosphatases [65]. Phosphate also appears to interfere in many secondary metabolic pathways not known to have phosphorylated intermediates.

Induction of secondary metabolite synthases

In a number of secondary metabolic pathways, primary metabolites increase production of the final product. These effectors are often precursors and one has to determine whether the effect is merely due to an increase in precursor supply and/or includes induction of one or more synthases of the biosynthetic pathway. Stimulatory precursors that are also inducers include tryptophan for dimethylallyltryptophan synthetase in ergot alkaloid biosynthesis [66], phenylalanine in benzodiazapene alkaloid formation [70], methionine for δ-(L-α-aminoadiphyl)-L-cysteine-L-valine synthetase (ACVS), cyclase and expandase in the cephalosporin pathway of *Acremonium chrysogenum* [107, 116], and phenylacetate for the phenylacetate uptake system involved in penicillin G formation in *Penicillium chrysogenum* [40].

Fungal regulatory genes

Clustering of fungal genes is not common except in cases of assimilation of certain nutrients (e.g. proline, quinate, ethanol, nitrate) and production of secondary metabolites [63]. Regulation of pathways in fungi (mainly studied in *A. nidulans*) can be narrow or broad-domain regulation [92]. Narrow -domain regulation usually involves a positively acting pathway-specific regulatory protein containing a zinc binuclear cluster: CX2CX6CX6CX2CX6CX2. Broad-domain control employs the positively acting nitrogen regulatory gene, *areA*, [67] and/or a negatively acting carbon repressor gene, *creA* [32].

Feedback regulation

The role of feedback regulation in controlling secondary metabolism is well-known. Many secondary metabolites inhibit or repress their own biosynthesis, usually acting on one key enzyme of their biosynthetic pathway.

Strain improvement

Production of new fungal metabolites by application of recombinant DNA technologies is of great interest. Continued progress in the area of metabolic engineering has led to overproduction of limiting enzymes of important biosynthetic pathways, thus increasing production of the final products.

Brewing yeasts have been engineered in order to overcome several problems. Thus, cloning an endoglucanase from *Trichoderma reesei* [81] led to a strain able to hydrolyze the barley β-glucans, which reduce the filterability of beer and lead to precipitates and haze in the final product. Similar technology was used to create starch-utilizing *S. cerevisiae* strains producing lower acidity and enhanced flavor. Recombinant *A. niger* amyloglucosidase is able to break down unfermentable dextrins for light-beer production [49]. Brewing yeasts have been engineered to produce acetolactate decarboxylase from *Enterobacter aerogenes* and *Acetobacter aceti*. This enzyme eliminates diacetyl and the requirement for the 3- to 5-week flavor maturation period that normally follows a 1-week fermentation stage [97]. The resulting beer suffers no loss of quality and flavor. Lower acidity and enhanced flavor in wine has been achieved by transformation of wine yeast with the gene encoding the malolactic conversion enzyme from *Lactobacillus delbrueckii*. Some studies using DNA chip technology have already been carried out to understand and overcome many technical problems facing winemakers [82].

Replacement of the native promoter of the ACVS-encoding gene in *A. nidulans* increased penicillin production 30-fold [62]. Expression of *cefE* from *Streptomyces clavuligerus* or *cefEF* from *A. chrysogenum*

in *P. chrysogenum* led to recombinant strains able to produce the cephalosporin intermediates adipyl-7-ACA and adipyl-7-ADCA [25]. Disruption of the gene *cefEF* of *A. chrysogenum* yielded strains accumulating high titers of penicillin N that was subsequently converted to deacetoxycephalosporin C (DAOC) after cloning *cefE* from *S. clavuligerus* into the high-producing strains [106].

Thaumatin, a protein from the plant *Thaumatococcus danielli* with an intense sweetness (about 3,000 times more than sucrose), has been recently approved as a food-grade ingredient. Successful expression of thaumatin was achieved in *Penicillium roqueforti* and *A. niger* var *awamori* [39] at titers of 2–7 mg/l. Recently, an impressive improvement in yield (up to 14 mg/l) has been obtained in *A. niger* var *awamori* by use of stronger promoters and higher gene dosage [76]. Production of the sweetener xylitol has also been improved by transforming the *XYL1* gene of *Pichia stipitis* encoding a xylose reductase into *S. cerevisiae* [48].

Production of lactic acid in *S. cerevisiae* has been achieved by cloning and expression of a muscle bovine lactate dehydrogenase gene, reaching productivities of 11 g/l h [83]. Development of fermentation processes for the production of β-carotene in the food-grade yeast *Candida utilis*, containing the carotenoid biosynthetic genes from the bacteria *Erwinia uredovora* and *Agrobacterium aurantiacum*, is in progress [74, 94]. Using a similar strategy, cloning of two desaturases from *Morteriella alpina* led to a recombinant yeast strain able to produce γ-linoleic acid [54].

Combining heterologous gene expression of a single plant enzyme and eight mammalian proteins, as well as four targeted gene deletions, led to a recombinant *S. cerevisiae* strain able to produce hydrocortisone, the major adrenal glucocorticoid of mammals and an important intermediate of steroidal drug synthesis [101].

Future prospects

The last few years have been a period of great progress using fungi as cell factories. There are four major fronts in which work is currently underway. The first is the development of alternative hosts, especially those that have already been given GRAS status by the FDA and can be used in the food industry. Research is being focused on species such as *Aspergillus sojae*, *Aspergillus japonicus*, *Mortierella alpina* and *Fusarium veneratum*, among others [84]. The second front is the development of better molecular techniques to improve expression and secretion of non-fungal proteins in filamentous fungi. The third major front involves the use of these molecular techniques to carry out metabolic engineering in order to modify and improve particular biosynthetic pathways. The final front will utilize the techniques dealing with the overall analysis of gene expression, i.e., genomics, proteomics and metabolomics. Four fungal genomes have already been sequenced, *S. cerevisiae* [44], *Schizosaccharomyces pombe* [115], *A. niger* [http://www.dsm.com] and *Neurospora crassa* [http://www-genome.wi.mit.edu], and sequencing of four others are in progress (*A. fumigatus, A. nidulans, Candida albicans* and *Ustilago maydis*) [http://www.tigr.org]. Initial steps on filamentous fungal genomics [7] and proteomics have recently been published [21, 69], and undoubtedly much more will become available in the years ahead.

The future of fungal biotechnology is encouraging when one considers that all the contributions that have been made already by fungi have been done with less than 5% of the fungal species present in nature. Soils and marine environments contain thousands of unknown microbial species, many of them fungi. New methods are being used to harness "environmental DNA" and to bring about the cultivation of so-called unculturable microorganisms. About 30–50% of known proteins have no known function. As more functions are revealed by functional genomics and bioinformatics, new targets will become available for screening fungal products.

Fungal enzymes will be improved in activity, specificity, and stability by directed evolution [6, 77]. Secondary metabolite pathways of fungi will be enhanced by directed evolution of whole cells ("whole genome shuffling") in concert with metabolic engineering. New secondary metabolites will be created by combinatorial biosynthesis in fungi.

References

1. Aharonowitz Y, Cohen G, Martín JF (1992) Penicillin and cephalosporin biosynthetic genes: structure, organization, regulation and evolution. Annu Rev Microbiol. 46:461–495
2. Alberts AW, Chen J, Kuron G, Hunt V, Huff J, Hoffman C, Rothrock J et al (1980) Mevinolin: A highly potent competitive inhibitor of hydroxymethylglutaryl-coenzyme A reductase and a cholesterol-lowering agent. Proc Natl Acad Science USA 77:3957–3961
3. Andrewes AG, Phaff HJ, Starr MP (1976) Carotenoids of *Phaffia rhodozyma,* a red-pigmented fermenting yeast. Phytochemistry 15:1003–1007
4. Archer D (2000) Filamentous fungi as microbial cell factories for food use. Curr Opin Biotechnol 11:478–483
5. Archer D, Jeenes DJ, MacKenzie DA (1994) Strategies for improving heterologous protein production from filamentous fungi. Anton van Leeuwenhoek 65:245–250
6. Arnold FH (2001) Combinatorial and computational challenges for biocatalyst design. Nature 409:253–257
7. Askenazi M, Driggers EM, Holtzman DA, Norman TC, Iverson S, Zimmer DP, Boers ME et al (2003) Integrating transcriptional and metabolite profiles to direct the engineering of lovastatin-producing fungal strains. Nat Biotechnol 21:150–156
8. Baron M, Tiraby G, Calmels T, Parriche M, Durand H (1992) Efficient secretion of human lysozyme fused to the Sh ble phleomycin resistance protein by the fungus *Tolypocladium geodes*. J Biotechnol 24:253–266
9. Bentley R (1997) Microbial secondary metabolites play important roles in medicine: prospects to discovery of new drugs. Perspect Biol Med 40:364–394
10. Berdy J (1995) Are actinomycetes exhausted as a source of secondary metabolites? In: Proceedings of 9th international symposium on the biology of actinomycetes. Part I. Allerton, New York, pp 3–23

11. Bocking SP, Wiebe MG, Robson GD, Hansen K, Christiansen LH, Trinci AP (1999) Effect of branch frequency in *Aspergillus oryzae* on protein secretion and culture viscosity. Biotechnol Bioeng 65:638–648
12. Bodie EA, Armstrong GL, Dunn-Coleman NS (1994) Strain improvement of chymosin-producing strains of *Aspergillus niger* var *awamori* using parasexual recombination. Enzyme Microb Technol 16:376–382
13. Borel JF, Feurer C, Gabler HU, Stahelin H (1976) Biological effects of cyclosporin A: a new antilymphocytic agent. Agents Action 6:468–475
14. Brake AJ, Merryweather JP, Coit DG, Heberlein UA, Masiarz FR, Mullenbach GT, Urdea MS et al (1984) α-Factor-directed synthesis and secretion of mature foreign proteins in *Saccharomyces cerevisiae*. Proc Natl Acad Sci USA 81:4642–4646
15. Bretthauer RK, Castellino FJ (1999) Glycosylation of *Pichia pastoris*-derived proteins. Biotechnol Appl Biochem 30:193–200
16. Brierley RA (1998) Secretion of recombinant human insulin-like growth factor I (IGF-I). In: Higgins DR, Cregg JM (eds) *Pichia* Protocols. Methods in Molecular Biology, vol 103. Humana, Totowa, New Jersey, pp 149–177
17. Broekhuijsen MP, Mattern IE, Contreras R, Kinghorn JR, van den Hondel CAMJJ (1993) Secretion of heterologous proteins by *Aspergillus niger*: production of active human interleukin-6 in a protease deficient mutant by KEX2-like processing of a glucoamylase-HIL6 fusion protein. J Biotechnol 31:135–145
18. Brown AG, Smale TC, King TJ, Hasenkamp R, Thompson RH (1976) Crystal and molecular structure of compactin: a new antifungal metabolite from *Penicillium brevicompactum*. J Chem Soc Perkin Trans I:1165–1170
19. Brown DW, Yu JH, Kelkar HS, Fernandes M, Nesbitt TC, Keller NP, Adams TH et al (1996) Twenty-five coregulated transcripts define a sterigmatocystin gene cluster in *Aspergillus nidulans*. Proc Natl Acad Sci USA 93:1418–1422
20. Carlsen S (1990) Molecular biology in research and production of industrial enzymes. In: Wolnak B, Scher M (eds) Industrial use of enzymes; technical and economic barriers. Brenard Wolnak and Associates, Chicago, Illinois, pp 52–69
21. Chambergo FS, Bonaccorsi ED, Ferreira AJ, Ramos AS, Ferreira JR, Abrahao-Neto J, Farah JP et al (2002) Elucidation of the metabolic fate of glucose in the filamentous fungus *Trichoderma reesei* using expressed sequence tag (EST) analysis and cDNA microarrays. J Biol Chem 277:13983–13988
22. Conesa A, Punt PJ, van Luijk M, van den Holden CAMJJ (2001) The secretion pathway in filamentous fungi: a biotechnological view. Fungal Genet Biol 33:155–171
23. Contreras R, Carrez P, Kinghorn JR, van den Hondel CAMJJ, Fiers W (1991) Efficient KEX2-like processing of a glucoamylase-interleukin-6 fusion protein by *Aspergillus nidulans* and secretion of mature interleukin-6. Bio/Technology 9:378–380
24. Cowan D (1996) Industrial enzyme technology. Trends Biotechnol 14:177–178
25. Crawford L, Stephan AM, McAda PC, Rambosek JA, Conder MJ, Vinci VA, Reeves CD (1995) Production of cephalosporin intermediates by feeding adipic acid to recombinant *Penicillium chrysogenum* strains expressing ring expansion activity. Bio/Technology 13:58–62
26. Cregg JM, Vedvick TS, Raschke WC (1993) Recent advances in the expression of foreign genes in *Pichia pastoris*. Bio/Technology 11:905–910
27. Dale C, Allen A, Fogarty S (1999) *Pichia pastoris:* a eukaryotic system for the large-scale production of biopharmaceuticals. BioPharm 12:36–42
28. De Baets S, Vandedrinck S, Vandamme EJ (2000) Vitamins and related biofactors, microbial production. Encyclopedia of microbiology. Academic, London, 4:837–853
29. Demain AL (1996) Fungal secondary metabolism: regulation and functions. In: Sutton B (ed) A century of mycology. Cambridge University Press, Cambridge, USA, pp 233–254
30. Demain AL (1999) Metabolites, primary and secondary. In: Flickinger MC, Drew SC (eds) Encyclopedia of Bioprocess Technology: Fermentation, Biocatalysis and Bioseparation. Wiley, New York, pp 1713–1732
31. Demain AL, Masurekar PS (1974) Lysine inhibition of in vivo homocitrate synthesis in *Penicillium chrysogenum*. J Gen Microbiol 82:143–151
32. Dowzer CE, Kelly JM (1991) Analysis of the *creA* gene, a regulator of carbon catabolite repression in *Aspergillus nidulans*. Mol Cell Biol 11:5701–5709
33. Dunn-Coleman NS, Bloebaum P, Berka R, Bodie E, Robinson R, Armstrong G, Ward M et al (1991) Commercial levels of chymosin production by *Aspergillus*. Bio/Technology, 9:976–981
34. Egli T, van Dijken JP, Veenhuis M, Harder W, Feichter A (1980) Methanol metabolism in yeasts: regulation of the synthesis of catabolite enzymes. Arch Microbiol 124:115–121
35. Elbein AD, Mitchell M, Molyneux RJ (1985) Effect of castanospermine on the structure and secretion of glycoprotein enzymes in *Aspergillus fumigatus*. J Bacteriol 160:67–75
36. Endo A (1979) K Monacolin, a new hypocholesterolemic agent produced by *Monascus* species. J Antibiot 32:852–854
37. Endo A (1985) Compactin (ML-236B) and related compounds as potential cholesterol-lowering agents that inhibit HMG-CoA reductase. J Med Chem 28:401–405
38. Endo A, Kuroda M, Tsujita Y (1976) ML-236B and ML-236C, new inhibitors of cholesterolgenesis produced by *Penicillium citrinin*. J Antibiot 29:1346–1348
39. Faus I (2000) Recent developments in the characterization and biotechnological production of sweet-tasting proteins. Appl Microbiol Biotechnol 53:145–151
40. Fernandez-Canon JM, Reglero A, Martinez-Blanco H, Luengo JM (1989) Uptake of phenylacetic acid by *Penicillium chrysogenum* Wis 54–1255: a critical regulatory point in benzylpenicillin biosynthesis. J Antibiot 42:1398–1409
41. Gellissen G, Janowicz JA, Merckelbach A, Piontek M, Keup P, Weydemann U, Hollenberg CP, Stasser WM (1991) Heterologous gene expresión in *Hansenula polymorpha*: efficient secretion of glucoamylase. Bio/Technology 9:291–295
42. Gellissen G, Janowicz ZA, Weydemann U, Melber K, Strasser AWM, Hollenberg CP (1992) High-level expression of foreign genes in *Hansenula polymorpha*. Biotech Adv 10:179–189
43. Giuseppin M, van Eijk HM, Bes BC (1988) Molecular regulation of methanol oxidase activity in continuous cultures of *Hansenula polymorpha*. Biotechnol Bioeng 32:577–583
44. Goffeau A, Barrel BG, Bussey R, Davis RW, Dujon B, Feldmann H, Galibert F et al (1996) Life with 6000 genes. Science 274:563–567
45. Gordon CJ, Khajal V, Ram AF, Archer D, Brookman JL, Trinci AP, Jeenes DJ et al (2000) Glucoamylase:green fluorescent protein fusion to monitor protein secretion in *Aspergillus niger*. Microbiology 146:415–426
46. Gouka RJ, Gerk C, Hooykaas PJJ, Bundock P, Musters W, Verrips CT, de Groot MJA (1999) Transformation of *Aspergillus awamori* by *Agrobacterium tumefaciens*-mediated homologous recombination. Nature Biotechnol 6:598–601
47. Gouka RJ, Punt PJ, van den Hondel CAMJJ (1997) Efficient production of secreted proteins by *Aspergillus*: progress, limitations and prospects. Appl Microbiol Biotechnol 47:1–11
48. Hallborn J, Walfridsson M, Airaksinen U, Ojamo H, Hahn-Hagerdal B, Penttila ME, Keranen S (1991) Xylitol production by recombinant *Saccharomyces cerevisiae*. Bio/Technology 9:1090–1095
49. Hammond JRM (1988) Brewery fermentation in the future. J Appl Bacteriol 65:169–177
50. Hidy PH, Baldwin RS, Greasham RL, Keith CL, McMullen JR (1977) Zearelenone and some derivatives: production and biological activities. Adv Appl Microbiol 22:59–82

51. Higgins DR, Cregg JM (1998) Introduction to *Pichia pastoris*. In: Higgins DR, Cregg JM (eds) *Pichia* protocols. Methods in molecular biology, vol 103. Humana, Totowa, New Jersey, pp 1–15
52. Hitzeman RA, Leung DW, Perry LJ, Kohr WJ, Levine HL, Goeddel DW (1983) Secretion of human interferons by yeast. Science 219:620–625
53. Hohn TM, McCormick SP, Desjardins AF (1995) Evidence for a gene cluster involving trichothecene-pathway biosynthetic genes in *Fusarium sporotrichioides*. Curr Genet 24:291–295
54. Huang YS, Chaudhary S, Thurmond JM, Bobik EG, Yuan L, Chan GM, Stobart AK et al (1999) Cloning of Δ^{12} and Δ^{6} desaturases from *Mortierella alpina* and recombinant production of γ-linoleic acid in *Saccharomyces cerevisiae*. Lipids 34:649–659
55. Jaeger KE, Dijkstra BW, Reetz MT (1999) Bacterial biocatalysts: molecular biology, three-dimensional structures, and biotechnological applications of lipases. Ann Rev Microbiol 53:315–351
56. Jaeger KE, Reetz MT (1998) Microbial lipases form versatile tools for biotechnology. Trends Biotechnol 16:396–403
57. Jenkins N, Curling EM (1994) Glycosylation of recombinant proteins: problems and prospects. Enzyme Microb Technol 16:354–364
58. Johnson EA, Conklin DE, Lewis MJ (1977) The yeast *Phaffia rhodozyma* as a dietary pigment source for salmonids and crustaceans. J Fish Res Bd Canada 34:2417–2421
59. Johnson EA, Villa TG, Lewis MJ (1980) *Phaffia rhodozyma* as an astaxanthin source in animal diets. Aquaculture 20:123–134
60. Juzlova P, Martinkova L, Kren V (1996) Secondary metabolites of the fungus *Monascus*: a review. J Indust Microbiol 16:163–170
61. Kasuya T, Nakajima H, Kitamoto K (1999) Cloning and characterization of the *bipA* gene encoding ER chaperone BiP from *Aspergillus oryzae*. J Biosci Bioeng 88:472–478
62. Kennedy J, Turner G (1996) delta-(L-alpha-aminoadipyl)-L-cysteinyl-D-valine synthetase is a rate limiting enzyme for penicillin production in *Aspergillus nidulans*. Mol Gen Genet 253:189–197
63. Keller NP, Hohn TM (1997) Metabolic pathway gene clusters in filamentous fungi. Fungal Genet Biol 21:17–29
64. Kornfeld R, Kornfeld S (1985) Assembly of asparagine-linked oligosaccharides. Ann Rev Biochem 54:631–664
65. Krebs EG, Beavo JA (1979) Phosphorylation-dephosphorylation of enzymes. Ann Rev Biochem 48:923–959
66. Krupinski VM, Robberts JE, Floss HG (1976) Physiological study of ergot: induction of alkaloid synthesis by tryptophan at the enzymatic level. J Bacteriol 125:158–165
67. Kudla B, Caddick MX, Langdon T, Martinez-Rosi NM, Bennett CF, Sibley S, Davies RW et al (1990) The regulatory gene *areA* mediating nitrogen metabolite repression in *Aspergillus nidulans*. Mutations affecting specificity of gene activation alter a loop residue of a putative zinc finger. EMBO J 9:1355–1364
68. Kukuruzinska MA, Bergh ML, Jackson BL (1987) Protein glycosylation in yeast. Ann Rev Biochem 56:915–944
69. Lim D, Hains P, Walsh B, Nevalainen H (2001) Proteins associated with the cell envelope of *Trichoderma reesei*: a proteomic approach. Proteomics 1:899–909
70. Luckner M, Nover L, Böhm H (1977) Secondary metabolism and cell differentiation. Mol Biol Biochem Biophys 23:57
71. Ma J, Li Y, Ye Q, Li J, Hua Y, Ju D, Zhang D et al (2000) Constituents of red yeast rice, a traditional Chinese food and medicine. J Agric Food Chem 48:5220–5225
72. Maras M, van Die I, Contreras R, van den Holden CAMJJ (1999) Filamentous fungi as production organisms for glycoproteins of bio-medical interest. Glycoconjugates J 16:99–107
73. Mattern EI, van Noort JM, Berg P, Archer D, Roberts IN, van den Hondel CAMJJ (1992) Isolation and characterization of mutants of *Aspergillus niger* deficient in extracellular proteases. Mol Gen Genet 234:332–336
74. Miura Y, Kondo K, Saito T, Shimada H, Fraser P, Misawa M (1998) Production of the carotenoids lycopene, β-carotene, and astaxanthin in the food yeast *Candida utilis*. Appl Environ Microbiol 64:1226–1229
75. Miyanohara A, Toh-e A, Nozai C, Hamada F, Ohtomo N, Matsubara K (1983) Expression of hepatitis B surface antigen gene in yeast. Proc. Natl Acad Sci USA 80:1–5
76. Moralejo FJ, Cardoza RE, Gutierrez S, Martin JF. Thaumatin production in *Aspergillus awamori* by use of expression cassettes with strong fungal promoters and high gene dosage. Appl Environ Microbiol 65:1168–1174
77. Ness JE, Del Cardayre SB, Minshull J, Stemmer WP (2000) Molecular breeding: the natural approach to protein design. Adv Protein Chem 55:261–292
78. Pakula TM, Uusitalo J, Saloheimo M, Salonen K, Aarts RJ, Penttila ME (2000) Monitoring the kinetics of glycoprotein synthesis and secretion in the filamentous fungus *Trichoderma reesei*: cellobiohydrolase I (CBHI) as a model protein. Microbiology 146:223–232
79. Pandey A, Benjamin S, Soccol CR, Nigam P, Krieger N, Soccol VT (1999) The realm of microbial lipases in biotechnology. Biotechnol Appl Biochem 29:119–131
80. Pariza MW, Johnson EA (2001) Evaluating the safety of microbial enzyme preparations used in food processing: ''update for a new century. Regul Toxicol Pharmacol 33:173–186
81. Penttila ME, Andre L, Saloheimo M, Lehtovaara P, Knowles JK (1987) Expression of two *Trichoderma reesei* endoglucanases in the yeast *Saccharomyces cerevisiae*. Yeast 3:175–185
82. Perez-Ortin JE, Garcia-Martinez J, Alberola TM (2002) DNA chips for yeast biotechnology. The case of wine yeasts. J Biotechnol 98:227–241
83. Porro D, Brambilla L, Ranzi M, Martegani E, Alberghina L (1995) Development of metabolically engineered *Saccharomyces cerevisiae* cells for the production of lactic acid. Biotechnol Prog 11:294–298
84. Punt PJ, van Viesen N, Conesa A, Albers A, Mangnus J, van den Hondel C (2002) Filamentous fungi as cell factories for heterologous protein production. Trends Biotechnol 20:200–206
85. Romanos MA (1995) Advances in the use of *Pichia pastoris* for high-level expression. Curr Opin Biotechnol 6:527–533
86. Romanos MA, Scorer CA, Clare JJ (1992) Foreign gene expression in yeast: a review. Yeast 8:423–488
87. Rosenfeld SA (1999) Use of *Pichia pastoris* for expression of recombinant proteins. Methods Enzymol 306:154–169
88. Sakuradani E, Kobayashi M, Ashikari T, Shimizu S (1999 a) Identification of Δ^{12} fatty acid desaturase from arachidonic acid-producing *Mortierella* fungus by heterologous expression in the yeast *Saccharomyces cerevisiae* and the fungus *Aspergillus oryzae*. Eur J Biochem 261:812–820
89. Sakuradani E, Kobayashi M, Shimizu S (1999 b) Δ^{6} fatty acid desaturase from arachidonic acid-producing *Mortierella* fungus – gene cloning and its heterologous expression in a fungus, *Aspergillus*. Gene 238:445–453
90. Saloheimo M, Lund M, Penttila ME (1999) The protein disulphide isomerase gene of the fungus *Trichoderma reesei* is induced by endoplasmic reticulum stress and regulated by the carbon source. Mol Gen Genet 262:35–45
91. Salovouri I, Makarow M, Rauvala H, Knowles J, Kääriäinen L (1987) Low molecular weight high-mannose type glycans in a secreted protein of the filamentous fungus *Trichoderma reesei*. Bio/Technology 5:152–156
92. Scazzocchio C (1992) Control of gene expression in the catabolic pathways of *Aspergillus nidulans*: a personal and biased account. J Biotechnol 23:43–68
93. Schmid RD, Verger R (1998) Lipases: interfacial enzymes with attractive applications. Angew Chem Int Ed 37:1608–1633
94. Shimada H, Kondo K, Fraser P, Miura Y, Saito T, Misawa M (1998) Increased carotenoid production by the food yeast *Candida utilis* through metabolic engineering of the isoprenoid pathway. Appl Environ Microbiol 64:2676–2680

95. Shuster JR, Connelley MB (1999) Promoter-tagged restriction enzyme-mediated insertion mutagenesis in *Aspergillus niger*. Mol Gen Genet 262:27–34
96. Sohn JH, Kang HA, Rao KJ, Kim CH, Choi ES, Chung BH, Rhee SK (2001) Current status of the anticoagulant hirudin: its biotechnological production and clinical practice. Appl Microbiol Biotechnol 57:606–613
97. Sone H, Fujii T, Kondo K, Shimizu F, Tanaka JI, Inoue T (1988) Nucleotide sequence and expression of *Enterobacter aerogenes* α-acetolactate decarboxylase gene in brewers' yeast. Appl Environ Microbiol 54:38–42
98. Stierle A, Strobel G, Stierle D (1993) Taxol and taxane production by *Taxomyces andreanae,* an endophytic fungus of Pacific yew. Science 260:214–216
99. Stroh WH (1998) Industrial enzymes market. Gen Eng News 18:11–38
100. Strohl WR (1997) Industrial antibiotics: today and the future. In: Strohl WR (ed) Biotechnology of antibiotics, 2nd edn. Marcel Dekker, New York, pp 1–47
101. Szczebara FM, Chandelier C, Villeret C, Masurel A, Bourot S, Duport C, Blanchard S et al (2003) Total synthesis of hydrocortisone from a simple carbon source in yeast. Nature Biotechnol 21:143–149
102. Thayer AM (2000) Busting down a blockbuster drug. Chem Eng News 78:20–21
103. Tudzinski B (1999) Biosynthesis of gibberellins in *Gibberella fujikuroi*: biomolecular aspects. Appl Microbiol Biotechnol 52:298–231
104. Valenzuela P, Medina A, Rutter WJ, Ammerer G, Hall BD (1982) Synthesis and assembly of hepatitis B virus surface antigen particles in yeast. Nature 298:347–350
105. van der Hombergh JP, van de Vondervoort PJ, van der Heijden NC, Visser J (1997) New protease mutants in *Aspergillus niger* result in strongly reduced in vitro degradation of target proteins; genetic and biochemical characterization of seven complementation groups. Curr Genet 28:299–308
106. Velasco J, Adrio JL, Moreno MA, Diez B, Soler G, Barredo JL (2000) Environmentally safe production of 7-aminodeacetoxycephalosporanic acid (7-ADCA) using recombinant strains of *Acremonium chrysogenum*. Nature Biotechnol 18:857–861
107. Velasco J, Gutierrez S, Fernández FJ, Marcos AT, Arenos C, Martín JF (1995) Exogenous methionine increases levels of mRNAs transcribed from *pcbAB*, *pcbC*, and *cefEF* genes, encoding enzymes of the cephalosporin biosynthetic pathway, in *Acremonium chrysogenum*. J Bacteriol 176:985–991
108. Verdoes JC, Punt PJ, van den Hondel CAMJJ (1995) Molecular genetic strain improvement for the overproduction of fungal proteins by filamentous fungi. Appl Microbiol Biotechnol 43:195–205
109. Vining LC, Taber WA (1979) Ergot alkaloids. In: Rose AH (ed) Economic microbiology, vol 3. Secondary products of metabolism. Academic, London, pp 389–420
110. Wagenbach M, O'Rourke K, Vitez L, Wieczorek A, Hoffman S, Durfee S, Tedesco J et al (1991) Synthesis of wild type and mutant hemoglobins in *Saccharomyces cerevisiae*. Bio/Technology 9:57–61
111. Wall ME, Wani MC (1995) Campothecin and taxol: discovery to clinic. Cancer Res 55:753–760
112. Wang H, Ward M (2000) Molecular characterization of a PDI-related gene *prpA* in *Aspergillus niger* var *awamori*. Curr Genet 37:57–64
113. Ward P, Cunningham GA, Conneely OM (1997) Commercial production of lactoferrin, a multifunctional iron-binding glycoprotein. Biotechnol Genet Eng Rev 14:303–319
114. Ward P, Piddington CS, Cunningham GA, Zhou X, Wyatt RD, Conneely OM (1995) A system for production of commercial quantities of human lactoferrin, a broad spectrum natural antibiotic. Bio/Technology 13:498–503
115. Wood V, Gwilliam R, Rajandream MA, Lyne M, Lyne R, Stewart A, Sgouros J et al (2002) The genome sequence of *Schizosaccharomyces pombe*. Nature 415:871–880
116. Zhang JY, Wolfe S, Demain AL (1987) Effect of ammonium as nitrogen source on production of δ-(L-α-aminoadipyl)-L-cysteinyl-D-valine synthetase by *Cephalosporium acremonium* C-10. J Antibiot 40:1746–1750