1.1

FROM GENE TO PRODUCT: THE ADVANTAGE OF INTEGRATIVE BIOTECHNOLOGY

FRANK-RANIER SCHMIDT
Sanofi-Aventis Deutschland, Frankfurt am Main, Germany

Chapter Contents

Handbook of Pharmaceutical Biotechnology, Edited by Shayne Cox Gad.

1.1.1 INTRODUCTION

Biotechnology and biotechnology-based methods are increasing in importance in medical therapies and diagnostics as well as in the discovery, development, and manufacture of pharmaceuticals. Biotechnologically manufactured pharmaceuticals will soon reach a market volume of more than $100 billion USD and, thus, some 20% of the total pharmaceutical market. The key step in their manufacture is the conversion of the genetic information into a product with the desired pharmacological activities by an appropriate selection, design, and cultivation of cells and microorganisms harboring the corresponding biosynthetic pathways and physiological properties.

The intention of this chapter is to give insights into the typical issues and problems encountered in the manufacture of biopharmaceuticals, to mediate general ideas and current strategies on how to proceed in the design and development of biotechnological processes, and to deliver the immediate theoretical backgrounds necessary for comprehension rather than to give detailed experimental instructions like a manual does. In focusing on gene recombinant proteins and peptidic antibiotics, the biotechnologically produced pharmaceuticals with the highest market share, representative aspects will be discussed (1) for process development and optimization approaches to increase product yield and process rentability and to ensure a consistent product quality, (2) for experimental approaches to design and to modify the molecular structure of compounds to meet specific medical needs, (3) for the replacement of chemical procedures by economically and ecologically advantageous biotechnological processes, (4) for critical issues of product purification, and (5) for specific demands in pharmaceutical production to conform to regulatory requirements. Finally, the advantage of an integrative biotechnology is emphasized, which designs the biosynthetic steps of the product in accordance with the requirements of product purification procedures already during early development stages.[1]

For further reviews comprehensively illustrating issues of biotechnological production processes, the reader is referred to further review articles [1–8]. To look up basic subjects of molecular and cellular biology, the reader is referred to textbooks [9, 10].

[1] Parts of this chapter were excerpted and modified from previously published reviews of the author [16, 86, 98, 113, 273] by courtesy of Springer-Verlag, Heidelberg.

1.1.2 PRODUCTION ORGANISMS AND EXPRESSION SYSTEMS

Design and development of all microbial production processes start with the selection of appropriate organisms, strains, and expression systems enabling high yields and high quality of a desired product with defined pharmacological properties.

1.1.2.1 Industrially Established Recombinant Expression Systems

Industrially established expressions systems for production of the marketed compounds are, besides inclusion, body-forming *Escherichia coli* strains, the yeast *Saccharomyces cerevisiae* and mammalian cells like CHO- and BHK-cells (Table 1.1-1). These systems were the genetically and physiologically most advanced and therefore mostly applied when recombinant production processes were starting to be developed in the mid-1980s and are now widely accepted by regulatory bodies. *E. coli* and *S. cerevisiae* can be grown cheaply and rapidly, are amenable to high cell density fermentations with biomasses of up to 130 g/L, possess short generation times, have high capacities to accumulate foreign proteins, are easy to handle, and are established fermentation organisms.

However, because gene recombinant pharmaceuticals continuously gain an increasing importance in medicine and are expected to help curing diseases that are not yet treatable today, new expression systems have to be exploited enabling the production of such pharmaceuticals with innovative properties that simultaneously meet key criteria like consistent product quality and cost effectiveness. Of particular interest in this regard are expression systems enabling the secretion of

TABLE 1.1-1. Industrially Used Recombinant Expression Systems

Product	Company	System
Blood coagulation factors (VII, VIII, IX)	Novo-Nordisk/Bayer/	BHK-Cells
	Centeon Genetics Baxter/ Centeon/Wyeth	CHO-Cells
Calcitonin	Unigene	*E. coli*/CHO-Cells
DNase (cystic fibrosis)	Roche	CHO-Cells
Erythropoetin	Janssen-Cilag/Amgen/ Boehringer	CHO-Cells
Darbepoetin	Amgen	CHO-Cells
Follicle stimulating hormone (follitropin)	Serono/Organon	CHO-Cells
Luteinisation hormone	Serono	CHO-Cells
Gonadotropin	Serono	CHO-Cells
Glucagon	Novo-Nordisk	*S. cerevisiae*
Glucocerebrosidase (Gaucher-disease)	Genzyme	CHO-Cells
Growth hormones (somatotropines)	Pharmacia & Upjohn/Lilly/	*E. coli*
	Novo-Nordisk/Ferring/ Genentech	
	Serono	Mouse Cell Line
	Serono/Bio-Technology General Corp	CHO-Cells

TABLE 1.1-1. *Continued*

Product	Company	System
Eutropin (Human growth hormone derivative)	LG Chemical	*S. cerevisiae*
Growth factors (GCSF u. GMCSF)	Novartis/Essex/Amgen/ Roche	*E. coli*
	Chugai Pharmaceuticals	CHO-Cells
Platelet-derived growth factor (PDGF)	Janssen-Cilag	*S. cerevisiae*
PDGF-Agonist	ZymoGenetics	*S. cerevisiae*
Hepatitis B vaccine	GlaxoSmithKline	*S. cerevisiae*
	Rhein Biotech	*H. polymorpha*
Hirudin	Sanofi-Aventis/Novartis	*S. cerevisiae*
Insulin and muteins	Sanofi-Aventis/Lilly/Berlin-Chemie	*E. coli*
Insulin	Bio-Technology General Corp	*E. coli*
	Novo-Nordisk	*S. cerevisiae*
Interferon alpha and muteins	Roche/Essex/Yamanouchi	*E. coli*
Interferon beta	Schering	*E. coli*
	Biogen/Serono	CHO-Cells
Interferon gamma (mutein)	Amgen/Boehringer	*E. coli*
Interleukin 2	Chiron	*E. coli*
Oprelvekin (interleukin 11-agonist)	Wyeth	Human Cell Line ROMI 8866
OP-1 (osteogenic, neuroprotective factor)	Curis/Striker	*E. coli*
Tissue plasminogen-activator	Genentech/Roche/Boehringer	CHO-Cells
Recombinant plasminogen-activator	Genentech/Roche/Boehringer	*E. coli*
Stem cell factor	Amgen	CHO-Cells
Tumor necrosis factor	Boehringer	*E. coli*

Note: Overview on the currently worldwide commercialized recombinant pharmaceuticals and the expression systems employed for their production. The substances are not listed strictly alphabetically but are partially grouped according to therapeutic areas. Antibodies, which are mostly manufactured by hybridoma cell line systems, are not considered. Data were extracted from the European patent database Esp@cenet (http://de.espacenet.com) and the IDdb3-database (http://www.iddb3.com). BHK = baby hamster kidney; CHO = chinese hamster ovary. (Taken from Ref. 16 © Springer-Verlag, Heidelberg)

correctly glycosylated and folded proteins into the culture broth. Such secretory systems offer advantages in terms of simple and fast product purification procedures and the avoidance of costly cell rupture, denaturation, and refolding processes (see Section 1.1.4) and thus conform to the requirements of an integrated production process.

Even though animal and plant systems (molecular pharming, [11]) and secretory plant cell culture systems have received a great deal of attention, their commercial feasibility is still under investigation, particularly with respect to their slightly different posttranslational modification modus leading to an altered pharmacological behavior and to allergenic properties [12, 13]. Established in the pharmaceutical

industry as production organisms are, besides the above-mentioned systems, further prokaryotic and yeast species as well as filamentous fungi, which are already employed for the manufacture of natural products (see Section 1.1.2.2).

The suitability of the most prominent secretory systems among these organisms from the viewpoint of an integrative process design for the manufacture of recombinant proteins will be evaluated in the next section by discussing their potential productivity and their physiological properties.

1.1.2.2 Evaluation of Secretory Expression Systems for Pharmaceutical Purposes

Escherichia Coli. As *E. coli* lacks fundamental prerequisites for efficient secretion, the marketed pharmaceuticals (Table 1.1-1) manufactured by *E. coli*-systems are mostly produced as inclusion bodies. Due to the membrane structure, the low chaperone and foldase level and the high periplasmatic protease concentration *E. coli*-secretion systems allow only comparably low product yields, making them suitable only for compounds marketed in small quantities like orphan drugs. Genentech (San Francisco, CA), for instance, has patented a secretory *E. coli*-system for the preparation of human growth hormone [14]. The secretory potential of *E. coli* is indicated by exceptional high product titers in the range of several grams per liter, which were reached in a system developed for secretion of hirudin using the alpha-cyclodextringlykosysl-transferase signal sequence as a leader und secretor mutants deficient in their membrane structure [15]. Titers of human-insulin-like-growth-factor or human-epidermal-growth-factor were reported to be as high as 900 mg/L and 325 mg/L, respectively [references in 16]. Most of the reached and published data, however, refers to processes leading to a periplasmatic product concentration (e.g., 2 g/L of a human antibody fragment, 700 mg/L of a monoclonal antibody) or stays below 100 mg/L, a value that generally is not considered to be in a competitive and economic range. Efforts are thus undertaken to condition *E. coli*-strains to efficient secreters. The main strategies to enhance secretion efficiency [17–19] comprise (1) employment of well-characterized secretion pathways like the alpha-hemolysin system [20] or components of such pathways like efficient signal sequences from efflux proteins [21] or outer membrane proteins [22], for instance, the maltose binding protein [23] or the TolC-protein [24]; (2) variation of the signal peptide; (3) cocloning of and coexpression of chaperones and foldases [25–27]; (4) enhancement of gene expression by employment of strong promotors and efficient transcription termination sequences; (5) generation of protease deficient mutants; (6) generation of cell wall lacking or cell wall deficient mutants [28, 29]; and (7) modulation of the protein primary structure that was found to exert a strong influence on productivity and secretion efficiency by influencing protease resistance, folding efficiency, and the tendency to form inclusion bodies. Details of the strategies for the design and development of secretory *E. coli* strains as well as for the controlled soluble cytoplasmatic expression of recombinant proteins may be taken from general reviews [17, 30–32].

Alternative Prokaryotic Expression Systems. In addition to conditioning *E. coli*-strains to efficient secreters, alternative species, which are considered to inherently possess a superior secretion capacity, are tried to be established as expression

systems. Comparably high product yields of 2 g/L and 1 g/L were reported for production of human calcitonin by *Staphylococcus carnosus* [33] and of proinsulin by *Bacillus subtilis* [34], an organism that is continuously characterized and improved as a cell factory for pharmaceutical proteins [8]. *Bacillus megaterium*, which is thought to be as efficient as *B. subtilis*, is currently developed as a secretory expression system by a Collaborative Research Center (SFB) of the German Research Community (DFG). For *Ralstonia eutropha* (formerly *Alcaligenes eutrophus*), employed at ICI and Monsanto for polyhydroxyalkanoate production at a scale of several 100 m^3 and genomically completely sequenced, 1,2 g/L of secreted organophosphohydrolase, a model enzyme proned to form inclusion bodies in *E. coli*, were reported [35]. *R. eutropha* displays a more efficient carbohydrate metabolism than *E. coli* and is easily amenable to high cell density fermentations with biomass concentration of more than 150 g/L dry weight. This permits a lower specific productivity that in turn reduces the inclinement to form inclusion bodies and thus enables a more efficient secretion. *Rhodococcus*, *Corynebacterium*, *Mycobacterium*, actinomycetes, and streptomycetes [36] are also considered to be potentially suitable for the development of efficient secretion systems. A comparative study with recombinant alpha-amylase demonstrated that final yields as well as enzyme activity were considerably higher when produced by *Streptomyces lividans*, by which it was completely secreted than by *E. coli* in which it was concentrated periplasmatically [37]. The yields reported so far, however, are still below cost-efficient ranges. A system developed by Hoechst/Aventis for insulin production yielded around 100 mg/L, and the yields of correctly folded human CD4-receptor sites are in the range of 200 mg/L. Attached as signal proteins were the prepeptide of the alpha-amylase inhibitor from *S. tendae* (tendamistat) and the signal sequence of a protease inhibitor (LTI) from *S. longisporus*. In the course of these studies, it was found that the choice of the linker and its length strongly influences secretion efficiency. The comparably low yields, however, demonstrate that still a lot of fundamental research is necessary to render streptomyces systems competitive. (Strategies and examples for enhancement of recombinant protein expression in *S. lividans* and the current status of the genetic and physiological development are given in Refs. 38 and 39.) A general focus of research will be the detailed exploration of the twin arginine translocation (TAT) pathway, which has been recently discovered in addition to the conventional prokaryotic secretory (sec) pathways and enables the export of proteins with cofactors in a fully folded conformation [40, 41]. It evidently plays a more important role in *Streptomyces* species [42] but might also be useable in other species.

As the potential and capacity of prokaryotes for prosttranslational modification appear to be quite limited and the knowledge about the pathways is quite scarce (reviews on bacterial protein glycosylation see Refs. 43 and 44), the employability of most of the known prokaryotes usually is restricted to the preparation of proteins that are naturally not glycosylated, such as insulin, hirudins, or somatotropins, or to natively glycosylated proteins that are pharmacologically also active without glycosylation, like various cytokines (tumor necrosis factor, interleukines, interferones). For production of proteins that are pharmacologically active only with an appropriate modification pattern, eukayotic cell systems are more suitable.

Yeasts. Besides possessing complex posttranslational modification pathways, they offer the advantage to be neither pyrogenic nor pathogenic and to secrete more

efficiently. Species established in industrial production procedures are *Saccharomyces cerevisiae*, *Kluyveromyces lactis, Pichia pastoris*, and *Hansenula polymorpha*, which will be dealt with more in detail in this section. Whereas *S. cerevisiae* is the best genetically characterized eukaryotic organism at all and still is the prevalent yeast species in pharmaceutical production processes (Table 1.1-1), *P. pastoris*, first employed by Phillips Petroleum for single-cell-protein production, is currently the most frequently used yeast species for heterologous protein expression in general. Whereas only just a few proteins were expressed by *Pichia* species at the beginning of the last decade [45], the expression of more than 400 proteins have been meanwhile reported now [46, 47]. *P. pastoris* is considered to be superior to any other known yeast species with respect to its secretion efficiency and permits the production of recombinant proteins without intense process development. The highest yields were reported for murine collagen (15 g/L), tetanus toxin fragment C (12 g/L compared with 1 g/L in *S. cerevisiae*), human serum albumin (10 g/L compared with 3 g/L in *K. lactis* and to 90 and 150 mg/L in *S. cerevisiae*), and human interleukin 2 (10 g/L). The highest reported yields for *H. polymorpha* relate to phytase (13,5 g/L) and to hirudin (g/L range) and for *K. lactis* to human serum albumin (3 g/L, see above). Even though *S. cerevisiae* offers a high secretory potential as evidenced by some 9-g/L secreted *Aspergillus niger* glucose oxidase [references in 16], such data document a general inferior secretory capacity, the reasons for which are numerous. For the methylotrophic species *Hansenula* and *Pichia* and the lactose using *K. lactis*, natively strong promoters are available that derive from the methanol and lactose assimilating pathways and their enzymes (e.g., alcohol- and methanol oxidase, lactose permease, galactosidase). As the enzymes of these pathways account for up to 30% of the total protein content, the metabolic efficiency with respect to the secreted protein is significantly higher as documented by Buckholz and Gleeson [48]: Only one or a few gene copies are sufficient in *P. pastoris* to gain the same yields as with 50 gene copies in *S. cerevisiae*. Furthermore, proteins with a molecular mass of above 30 kD are retained in the cytoplasma of *S. cerevisiae*, whereas *H. polymorpha* efficiently secretes proteins with a molecular mass of up to 150 kD, like the glucoamylase of *Aspergillus niger*. [Detailed overviews on the physiological properties of methylotrophic yeasts and *K. lactis* with respect to their use for recombinant protein production is given in Refs. 46, 47, and 49–53]. Further reasons for the differing secretion rates among the species are the specific proteolytic activities and the specific degrees and patterns of glycosylation. Besides having an impact on the protein's final pharmacological activity, glycosylation also exercises an influence on the folding and secretion efficiency. Among the discussed species, *S. cerevisiae* was shown to possess, besides a higher enzymatic activity in the secretion vesicles that leads to a reduced portion of intact secreted proteins [54], also the highest glycosylation capacity leading to a hyperglycosylation of the protein and a reduced secretion rate. Both the degree and the pattern of glycosylation are dependent on the genetic background of the species and strains employed as well as on the sequences of the expressed protein and adjacent regions. By employment of the natively highly glycosylated alpha-mating type factor as a secretion signal, the extent of the glycosylation of the product can be diminished or completely avoided as shown for human interleukin 6 [55]. NovoNordisk reported leader sequence-dependent insulin yields in *S. cerevisiae* [56] and in *S. cerevisiae* and *P. pastoris* [57]: The sequence and therewith the degree of glycosylation of the leader influences the

efficiency of the multistage cleavage and folding processes as well as the insulin glycosylation rate and secretability. Further enhancement of the secretion efficiency can be achieved by (1) mutating secretion enhancer genes [58], (2) suppressing secretion blocking functions, and (3) reducing proteolytic activities in secretion vesicles [54]. So-called supersecreter strains of *S. cerevisiae* have, for instance, been generated by inactivation of the PMR1 (SSC1) function and suppression of the secretion blocking ypt1-1 gene: the yields of non-glycosylated human pro-urokinase [59], of human serum albumin, and of human plasminogen-activator have been augmented to a factor of up to 10 [60]. The traditional approaches pursued for enhancement of gene expression are gene amplification, employment of strong promoters, and enhancement of the transcription and translation rate. A high amplification of the gene copy number [45, 46, 61] usually is achievable with episomal vectors, which however do not reach the mitotic stability of integrative systems like the transposon (e.g., Ty-element) mediated embedment of reiterative, dispers repetitive sequences in *S. cerevisae*. Transcription rates were reported to be enhanced up to 100 times through cotransformation with transcription activators and enhancers, which evidently are limiting factors for overexpression of foreign proteins [62, 63]. Translation efficiency can be enhanced by preventing an accelerated degradation of transcripts and the yeast typical random transcription termination through modulation of the recognition sequences. Prevention of the random transcription termination led to an increase of tetanus toxin fragment C yields in *S. cerevisiae* by a factor 2000–3000 to 1 g/L and 3% of the total soluble protein fraction [64].

Despite their physiologically advantageous properties and natively high expression and secretion capacity, just one industrial application is reported for each of the alternative yeast species: *H. polymorpha* is employed for hepatitis B vaccine production at Rhein-Biotech (Düsseldorf, Germany), *K. lactis* for bovine prochymosin production in a 40-m^3 scale at Gist-Brocades (Delft, Netherlands), and *P. pastoris* for production of recombinant carboxypeptidase B and trypsin at Roche (Basel, Switzerland). For pharmaceutical application, it has to be considerered that the methylotrophic yeasts in contrast to *S. cerevisiae* and *K. lactis* are not used in the production of foodstuffs and therewith have no GRAS (generally regarded as safe) status according to the U.S. Food and Drug Administration (FDA) criteria and have to be grown in expensive explosion-proofed equipments when the above-mentioned native induction systems are used. The discussed properties of the mentioned yeast species are compiled in Table 1.1-2 for comparison. The employ-

TABLE 1.1-2. Typical Sequence of Biotechnological Production Process Steps

Step	Method/Approach
Selection/design/engineering/ development of an appropriate species/strain/ expression system	**Criteria:** pharmacological activity and properties of the compound, productivity, process behavior, suitability for downstream processing steps, spectrum and pharmacological activity of side products to be removed, experimental experience with the respective system, biological and medical safety, acceptance by regulatory bodies

TABLE 1.1-2. *Continued*

Step	Method/Approach
Strain improvement	Mutation/selection, strain recombination (e.g., breeding/protoplast fusion), directed genomic alteration/metabolic engineering/enhancement of gene expression rates
Biosynthetic product structure modification	Amino acid exchange/combinatorial biosynthesis/precursor directed biosynthesis
Fermentation optimization	Empirical optimization of culture conditions (media components, pH, oxygen supply), clarification of the influence of process parameters on growth, productivity and side product formation
Fermentation scale-up	Detailed process characterization for reduction of stress exposure, insurance of homogenized reaction conditions, and identification of suitable scale-up parameters (e.g., with the help of chemometric modeling, computational fluid dynamics)
Downstream processing	
Cell separation and harvest, removal of particulate matters	Centrifugation (decanter, disk-stack separator, (semi)continuous centrifuges), filtration (dead-end, tangential flow filtration)
Cell rupture	High-pressure homogenization, bead mills, sonication, enzymatic treatments
Product capture	Filtration (micro-, ultra-, nano-filtration), precipitation, solvent extraction, ion exchange, size exclusion, affinity chromatography
Product purification/polishing	Hydrophobic interaction/reversed-phase chromatography
Clearance of contaminant agents (e.g., viruses, endotoxins)	Nano-filtration, heat, pH, chemical inactivation, ultraviolet, gamma irradiation
Drying	Heat, freezing, vacuum
Galenic preparation/filling	Addition of galenic excipients, supplements, stabilizers, and adjuvants

Notes: Comprehensive overview illustrating the typical steps of a biotechnological production process. Production starts with the selection of an appropriate species and strain and the engineering of an expression system that enable high product yields and permit easy handling during strain development, fermentation, and downstream processing. Classic measures for improvement of the selected strains and expression systems are mutation and selection runs, breeding, directed genomic alterations, metabolic engineering, and measures aimed at the enhancement of expression rates of genes involved in product biosynthesis. Cultivation of the cells occurs under suitable conditions permitting strong growth, high product yield, and high product quality and facilitating product recovery and purification during downstream processing, which usually starts with separation of the product from the biomass and the culture broth by centrifugation or filtration. Before the separation of intracellular occurring products, cells have to be ruptured by high-pressure homogenization (e.g., in a homogenizer, a Dyno mill, or a French press). For high product purity, high recovery rates and simultaneous maintenance of the pharmacological activity, product purification and polishing usually occurs by solvent and solid-phase extraction. The choice of suitable resins and solvents and their combinations is highly dependent on the chemical and physical properties of the product and the side metabolites to be removed.

The final step consists of product concentration and drying, e.g., by heat, freezing, and/or vacuum before the galenic preparation. The discrimination between product capture and polishing is somewhat arbitrary as is the assignment of the respective methods. Depending on the product and the individual product properties, the transitions between the steps are gliding and the methods can change accordingly.

ability of yeasts in some cases however might reach a limit, particularly when the pharmacological activity of the product is impaired by the glycosylation pattern. In *K. lactis*, for instance, which usually does not hyperglycosylate, an exceptional high glycosylation of human interleukin 1β has been observed, reducing the biological activity to 5% [65, 66]. In such cases, a postsynthetic chemical modification has to be considered or the employment of higher developed organisms.

Filamentous Fungi. Filamentous fungi are higher organized than yeasts and consequently have a more complex posttranslational modification apparatus more similar to mammals. Some proteins like t-PA in *Aspergillus nidulans* are produced with the natural human glycosylation pattern. For recombinant protein production, species are prefered, which are broadly employed in industry for production of enzymes, acids and antibiotics and thus possess GRAS-status: *A. nidulans*, *A. niger*, *A. sydowii*, *A. awamori*, various *Fusarium* and *Trichoderma* species, *Penicillium chrysogenum*, and *Acremonium chrysogenum*. Their productivity and secretion potential, which is in the range of 30–40 g/L for homologous enzymes like cellulases and amylases, is considered to be superior to any other system, but unfortunately could not be converted into corresponding yields of recombinant products, even when these were fused to such homologous enzymes. The highest yields are still obtained with heterologous fungal enzymes: 4-g/L *Fusarium* protease in *A. chrysogenum* and 4.6-g/L *A. niger* glucoamylase in *A. awamori*. The highest yield of a mammalian protein was reported for human interleukin 6 in *A. sojae* in a range of 300 mg/L [references in 16]. The yields of most human proteins like t-PA and various interferons, however, were reported to be below 1 mg/L [67–71]. Possible reasons for these incompetitive yields are restrictions in posttranslational metabolic steps like intracellular transport, folding, and processing. Even though filamentous fungi are industrially used now for decades, they are not adequately characterized on the physiological and genetic level. Little is known about details of the modification and secretion metabolism, and efficient gene transformation is hampered by degradation of foreign DNA, low transformation rate, and therefore, low copy numbers of the transferred genes and random genomic integration. Expression rates are restricted by a high RNA turnover, incorrect processing of the foreign messenger, and incomplete folding and secretion, which are, as in yeasts, both influenced by the glycosylation pattern. Proteins not completely or incorrectly glycosylated and excreted are rapidly degraded as shown for human interleukin 6 [71]. Currently, filamentous fungi cannot be regarded as a serious alternative for the production of pharmaceuticals. To fully exploit the potential of filamentous fungi, their physiology, particularly the glycosylation metabolism, thus has to be investigated and clarified in more detail.

Insect and Mammalian Cell Cultures. Animal cell cultures are the systems with highest similarity to human cells with respect to the pattern and capacity of posttranslational modifications. However, their cultivation is more complicated and costly and usually yields lower product titers. Among the known systems, insect cells transformed by baculovirus vectors have reached a comparable popularity as *Pichia* among yeasts because they are considered to be more stress resistant, easier to handle, and more productive compared with mammalian systems and are thus frequently employed for high-throughput protein expression. The highest reported

yields refer to human collagenase IV in *Trichoplusia* (300 mg/L). Yields reported for non-insect cultures were 80 mg/L for human apolipoprotein A1 in chinese hamster ovary (CHO)-cells and 1 mg/L for human laminin in human embryonal kidney cells [references in 16]. For commercial application scale-up, related questions have to be clarified, particularly concerning oxygen supply [72] and carbon dioxide accumulation [73], stability of the cell line, and the bioreactor type to be employed [74]. Further deficiencies of insect cells are observed in (1) an inefficient processing and an impairment of the folding and secretion capacity due to the baculovirus infection [75]; (2) the high, in part baculovirus encoded, protease activity and the resulting necessity to routinely employ protease inhibitors in the culture media or to develop protease deficient vectors [73]; (3) an insufficient expression strength; and (4) deviations of the posttranslational modification pattern, which could act immunogenic. For optimization, the construction of new innovative vectors and the coexpression of chaperones, foldases, and folding factors such as canexin [76] have been suggested as has been the broader development and application of alternative systems like *Drosophila* [75]. Preferably applied in pharmaceutical production processes are mammalian systems like CHO- and baby hamster kidney (BHK)-cells (Table 1.1-1). These systems are generally considered to be genetically more stable and easier to transform and to handle in scale-up processes, grow faster in adherent and submerged cultures, and are more similar to human cells and more consistent in their complete spectrum of modification [77], which minimizes the risk of the formation of structurally altered compounds with immunogenic properties. In some cases, mammalian systems can be the only choice for the preparation of correctly modified proteins. Studies of Tate et al. [78] comparing the expression of a rat serotonin transporter in various expression systems demonstrate that biologically active transporter was only synthesized in mammalian cells, whereas it was partially degraded in *E. coli*, not correctly folded in *Pichia* and not correctly glycosylated in insect cells. CHO- and BHK-cells further have the advantage to be recognized as safe regarding infectious and pathogenic agents and therefore to have a higher acceptance by regulatory bodies, which accelerates or at least does not delay approval procedures. [Issues related to the physiology and the technical handling of mammalian cell cultures in the manufacture of human therapeutics are discussed in detail in Refs. 74 and 79–81].

1.1.2.3 Criteria for the Choice of Recombinant Expression Systems

In summary, the criteria for the choice of an expression system in pharmaceutical production [82] are the existing expertise, the available physiological and genetic know-how and tools, the patent situation, and to avoid delays of product launch and commercialization, regulatory aspects like the acceptance by the approving authorities.

The overall decision criteria, however, is the pharmacological activity profile of the yielded protein in context with the posttranslational modification pattern followed by rentability.

For production of non-glycosylated proteins and proteins that are natively glycosylated but pharmacologically active also without glycosylation, prokaryotes, which usually lack metabolic pathways for glycosylation, theoretically are the most

suitable organisms, offering two alternatives: Either *E. coli*-strains are conditioned to efficient secreters, or efficient native secreters like *Bacillus*-species are accordingly developed. (The implications of the glycosylation pattern for the choice of expression systems and the physiology of glycosylation is reviewed and discussed in Ref. 83.) To fully exploit the secretion capacity of fungal species, a deeper understanding of their posttranslational modification physiology will be necessary to steer the degree and pattern of glycosylation, which influences both folding and secretion efficiency. Insect and mammalian cells display posttranslational modification patterns more similar or identical to humans, but in view of the entailed expenditures, their employment can only be justified if their modification machinery is required to ensure a desired pharmacological activity.

E. coli, *P. pastoris*, and Baculovirus-based systems are currently preferred in fundamental research for structural and functional analysis of proteins and are employed as high-throughput-expression systems but will certainly find their ways into production processes in the near future. None of the systems, however, can be considered to be generally superior to any other. For each product, the most suitable expression system has to be identified and optimized individually both on the genetic and on the fermentative level by taking into account the properties of the product, the organism, and the expression cassettes, which suggests to always have a set of accordingly developed expression systems available. Some key properties of the established expression systems are compiled in Table 1.1-3. A review on properties and prospects of further alternative systems is given in Ref. 84.

Once an expression system has been selected, the respective strains are continuously submitted to improvement programs to render fermentation processes more efficient by increasing strain productivity and by modifying physiological properties and process behavior to enhance process economy. This does not only apply to the fermentation process, but also to parts of downstream processing, thus requiring an integrated strain selection and process design. Strategies to improve productivity of secretory recombinant systems and of strains employed for the manufacture of natural products will be outlined in the next section.

TABLE 1.1-3. Features and Characteristics of Expression Systems

Property	Bacteria	Yeast	Insect and Mammalian Cell Cultures
Growth	Fast	Fast	Slow
Nutrient demand	Minimal	Minimal	Complex
Costs of media	Low	Low	High
Possible product yield	High	High	Low
Secretory capacity	Limtited	High	Medium
Glycosylation capacity	Limited	High	High
Modification capacity	Limited	High	High
Risk of retroviral Contamination	Low	Low	High
Risk of pyrogens	High	—	—
Scalability	Good	Good	Low
Process robustness	Good	Good	Low

1.1.3 ENHANCEMENT OF PRODUCTIVITY

1.1.3.1 Secretory Recombinant Expression Systems

In terms of downstream processing efficiency, secretory expression systems indeed offer potential advantages for production of recombinant proteins compared with inclusion body-forming cytosolic systems, but most of the potentially available secretory systems are not yet fully competitive for high-volume therapeutics like insulin and therefore still require intensive improvement efforts.

Current strategies to improve productivity and secretion efficiency comprise (1) enhancement of gene expression rates, (2) optimization of secretion signal sequences, (3) coexpression of chaperones and foldases, (4) creation of protease deficient mutants to avoid premature product degradation, and (5) subsequent breeding and mutagenesis.

1.1.3.2 Natural Products

Completely different is the situation faced in the production of the so-called natural products, which are mostly secondary metabolites and therewith the endproducts of complex biosynthetic pathways of filamentous bacteria and fungi and thus require different approaches. With few exceptions like the antidiabetic drug acarbose (Glucobay) [5] or the chosterol-lowering drug lovastatin (Mevicor) [85], such fermented pharmaceuticals of natural origin currently belong for the most part to the class of antibiotics [86].

Enhancement of the productivity is achieved at first by rounds of random mutation and selection and, if possible, by breeding, and/or by DNA injection or fusion techniques like protoplasting and at further stages, after a comprehensive characterization of the usually complex biosynthetic pathways, directed genomic alterations and metabolic engineering.

These approaches, developments, and states in industrial strain improvement and pathway characterization will be illustrated for some of the best characterized organisms, namely the two fungal species mainly employed in industrial β-lactam antibiotic production, the penicillin producing *Peni-cillium chrysogenum*, and the cephalosporin C producer *Acremonium chrysogenum*.

Characterization of Biosynthetic Pathways. The productivity of *Penicillium chrysogenum* could have been augmented impressively during the last decades: Penicillin titers have been increased by a factor of 50,000 from a very few milligrams/Liter in the 1940s to more than several 10 g/L by now and also some several 10 g/L cephalosporin are currently gained.

This development, however, has come to a halt during the last couple of years. As these titers have been reached through conventional mutation and selection [87–90] and no further significant increase in productivity could be noted, the tools of genetic engineering were more and more included into the improvement programs [91]. To gain a profound basis for directed genomic alterations, studies were conducted at various corporations and universities to elucidate the mechanisms and genes involved in β-lactam biosynthesis to characterize the respective biosynthetic pathways. As the results and achievements of these studies have already been

reviewed extensively and in detail elsewhere [92–97], the current knowledge will only shortly be summarized.

Cephalosporin C Synthesis in Acremonium Chrysogenum. Biosynthesis (Figure 1.1-1) starts with the polymerization of L-α-aminoadipic acid, L-cysteine, and L-valine to the linear tripeptide L-α-aminoadipyl-L-cysteinyl-D-valine (ACV-peptide). This reaction is catalyzed by the ACV-synthase (MW about 420 kD) through the following steps: (1) the ATP-dependent activation of these amino acids to bind them as thiolesters, (2) the epimerization of L-valine, and finally (3) the condensation by a thiotemplate mechanism [99].

Cyclization of the ACV-peptide to the bicyclic isopenicillin N (IPN) occurs under oxygen-, Fe^{2+}-, ascorbate-, and α-ketoglutarate-dependent action of the IPN-synthase (IPNS), which has an MW of about 38 kD. Inhibitory to IPNS activity are cobalt ions and glutathione.

Further pathway reactions are as follows:

- IPN-epimerization to penicillin N (IPN-epimerase).
- Penicillin N conversion to deacetoxycephalosporin C (DAOC) by expansion of the five-membered thiazolidine ring to a 6 C-dihydrothiazine-ring (DAOC-expandase).
- Formation of deactylcephalosporin C (DAC) by dehydroxylation and oxidation of the methylgroup in C3-position (DAC-hydroxylase).
- The acylation of DAC to cephalosporin C (DAC acetyltransferase).

DAOC-expandase and DAC-hydroxylase activities in *A. chrysogenum* are exerted by the same enzyme (MW 41 kD), which like the IPNS belongs to the group of α-ketoglutarate-dependent dioxygenases.

The first two enzymes, ACV- and IPN-synthase, are encoded by the *pcbAB*- and the *pcbC* gene, respectively. Both genes are linked to each other on chromosome VI by a 1.2-kb intergenic region carrying the putative promotor sequences from which they are divergently transcribed. The *pcbC*-promotor seems to be about five times stronger than the one of the *pcbAB*-gene [100]. The *cefEF*-gene and the *cefG*-gene encoding for the bifunctional expandase/hydroxylase and the DAC-acetyltransferase, respectively, are located adjacent to each other on chromosome II. Again the genes are separated by an intergenic region of 938 bp, which is supposed to harbor the promotors from which they are transcribed in opposite directions. The *cefG*-gene has been proven to contain two introns [101]. Due to an extreme *in vitro* lability, no information is yet available on the epimerase converting IPN to penicillin N as well as its putative *cefD* gene. Until recently, the structure of the *cefD* gene could have not been elucidated. Meanwhile, data could have been obtained indicating the existence of two reading frames (*cefD1* and *cefD2*) containing all characteristic motifs of mammalian acyl-CoA ligases and α-methyl-acyl-CoA racemases [93].

Regulatory studies demonstrated that the early functions are expressed simultaneously, whereas the later pathway genes *cefD* and *cefEF* seem to be induced sequentially.

In contrast, the penicillin biosynthesis genes in *P. chrysogenum* are considered to be expressed completely concomitantly.

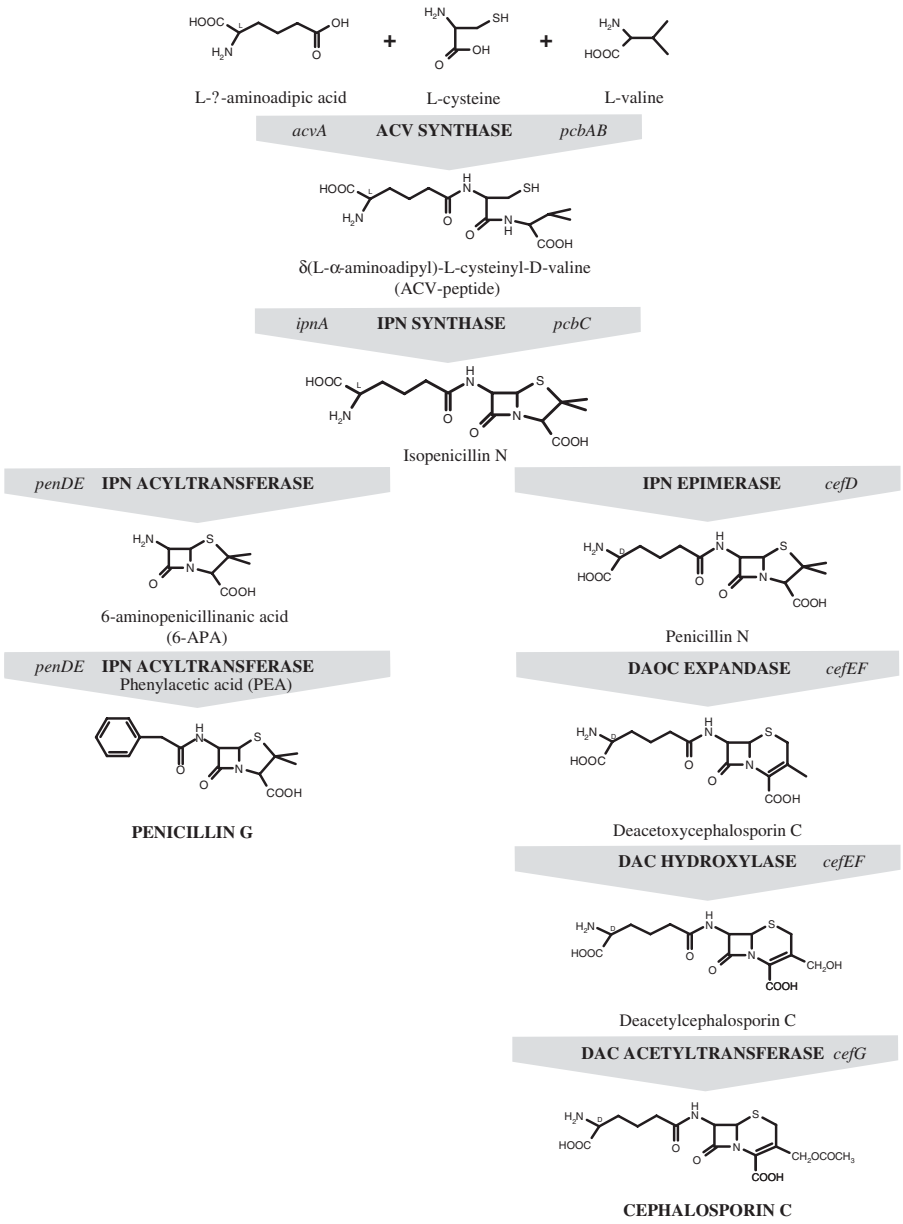

Figure 1.1-1. Biosynthetic pathways of penicillin G and cephalosporin C. In contrast to recombinant proteins, the structure and production of which can easily be modified by alterations of the encoding DNA sequence and the use of stronger promoters, respectively, structure modification and enhancement of expression rates of natural products by directed genomic alteration and metabolic engineering require a detailed characterization of the biosynthetic pathways as it has been performed for penicillin and cephalosporin biosynthesis in the filamentous fungi *Penicillium chrysogenum* (left) and *Cephalosporium acremonium* (right). Enzymes (bold) and genes are underlined by gray arrows. The first two reaction steps are common to both pathways. Cleavage of isopenicillin N to 6-APA and the subsequent acylation to penicllin G in *P. chrysogenum* and the expandase/hydroxylase activity in *A. chrsyogenum* are exerted by bifunctional enzymes. For details, see text. (Graph taken from Ref. 98, © Springer-Verlag, Heidelberg.)

Penicillin Formation by Penicillium Chrysogenum. The first reactions of the penicillin biosynthetic pathway are identical to the ones in *A. chrysogenum* (Figure 1.1-1). IPN, however, is not epimerized to penicillin N; instead it is converted to 6-aminopenicillanic acid (6-APA) by removal of the L-α-aminoadipic acid side chain, which is substituted by a hydrophobic acyl group. Both steps are catalyzed by the same enzyme, the acyl coenzyme A: IPN acyltransferase (IAT). The enzymatic activity of IAT is believed to be the result of the processing of a 40-kD monomeric precursor into a dimeric form consisting of two subunits with MWs of 11 and 29 kD. Due to the broad substrate specifity of IAT, various penicillin derivatives are synthesized naturally by attachment of different acyl-CoA derivatives to the 6-APA-core. For industrial purposes, to facilitate extraction by organic solvents, synthesis usually is directed to the less hydrophilic penicillin V or penicillin G. This is by addition of phenoxyacetic acid or phenylacetic acid, respectively, as precursors to the culture broth.

The IAT encoding *penDE*-gene (also named *aatA*) is located on chromosome I and organized as a cluster together with the ACV-synthase and IPN-synthase encoding genes *pcbAB* (also named *acvA* in *Penicillium*) and *pcbC* (*ipnA*).

The enzymes involved in penicillin biosynthesis are distributed at different sites of the cell: ACV-activity was found to be bound to vacuole membranes, IPN-synthase occurs dissolved in the cytoplasm and IAT-activity is microbody associated.

1.1.3.3 Approaches and Goals for Further Strain Improvement

Analysis and Comparison of Strains. To get hints for more rational strain improvement approaches, (1) highly mutated production strains were genetically and physiologically compared with their less-productive ances-tors, (2) concentrations of pathway intermediates were determined to identify potential pathway bottlenecks, and (3) regulatory mechanisms were investigated.

In the course of such studies, high-performance strains of *P. chrysogenum* turned out to possess amplified copies of single genes like the *pcbC* gene or even copies of the whole cluster as well as increased steady-state transcript levels of pathway genes [102–104]. In some strains, the amplifications were shown to be organized in tandem repeats, which presumably were generated by a hot-spot TTTACA hexanukleotide [105]. Comparison of promotor strengths of these genes from high and low productive strains did not reveal any differences. This indicates the involvement of additional unknown trans-acting factors [106], as the amounts of increased mRNA did not correlate with the degree of gene amplification. Also a high specific activity of IPN synthase was reported in a more evolved *Penicillium* strain, which was independent from transcript amounts and probably due to a higher enzyme stability [102, 107].

In *A. chrysogenum*, no amplification of the relevant genes could be detected. Nevertheless, transcript amounts in production strains are significantly increased [108].

Also chromosome rearrangements could be detected in high titer strains [109–111], but their causative influence on productivity remains unclarified for the moment.

Directed Genomic Alterations. *Enhancement of Gene Expression.* Con-sidering these findings, the experiments conducted so far to improve productivity mainly concentrated on enhancing the pathway gene dosages and on enhancing promotor strengths to remove presumed pathway bottlenecks. For instance, the *cefEF*-activity was generally believed to constitute a potential rate-limiting step in view of the high accumulation of penicillin N in various strains of *A. chrysogenum* and accordingly amplified.

Such approaches resulted in partially significant increases of productivity in low and medium titer strains: Improvements of the final yields by 50% have been reported [96].

However, all improvements achieved so far with high-performance production strains remained with a 5% maximum in the range of normal statistical deviations.

Also more exotic approaches like the (presumed) improvement of the intracellular oxygen supply through cloning and expressing of a bacterial hemoglobin gene from *Vitreoscilla* [112] failed to increase productivity in high titer strains.

The reasons for these failures are manifold:

1. As both direction and site of integration of the imported DNA cannot be controlled sufficiently and transformation efficiency is still quite low, the probability of finding new, higher producing mutants even in a large-scale screening is rare.
2. Most of the data available are from studies performed at academic institutions with original strains. Even when sharing the same ancestral strain, the high-performance strains employed in industries have an individual genealogy and mutation-selection history, so that their physiological behavior and properties differ drastically. Data and knowledge obtained with a particular strain thus cannot always be generalized and transfered to other strains. Even among industrial strains, significant differences have been revealed. For instance, the expression of the *cefG*-gene and the acetyl transferase activity was reported to constitute a possible rate-limiting step in Panlabs-strains [113]. Studies conducted on other industrial strains, however, could not confirm this observation.
3. The genetic instability and drift rises with the degree of genomic alterations, particularly during long-term vegetative propagation with numerous generation cycles in industrial large-scale fermentations.
4. The complex interdependence with other metabolic areas is not yet completely investigated. Consequently, after removal of an obvious β-lactam pathway bottleneck, unknown reaction steps of the linked and preceding pathways might become flux limiting. To overcome such flux limitations, one of the most exciting recent discoveries in molecular biology, namely RNA interference (RNAi), might play a major role in the future.

The Possible Impact of RNAi in Strain Improvement. The mechanisms of p osttranscriptional gene silencing, generally summed up as RNAi [114] offer potential approaches for the specific shut down of any gene of interest: Native genes can be silenced by triggering the RNAi cascade through introduction of homologous genetic sequences. As a result of these possibilities, RNAi has already

revolutionized fundamental research in molecular biology, particularly in functional genomics, and will substantially contribute to novel therapeutic approaches in medicine [115, 116]. For fungi, which evidently were among the first organisms in which RNAi and associated phenomena were observed [117–122], this RNAi triggered cosuppression phenomenon has been termed "quelling" and bears an enormous potential with respect to the study of biosynthetic pathways and the improvement of metabolic productivity.

On the one hand, the implication of RNAi may supply an explanation for observations made in the course of strain improvement programs of *Penicillium chrysogenum* and *Acremonium chrysogenum*, according to which antibiotic productivity can decrease drastically upon amplification of genes involved in antiobiotic biosynthesis.

The characterization and inactivation of these cosuppression mechanisms thus might help to overcome such limitations as does an application of fungal RNAi tools for silencing of pathway blocking or metabolic flux limiting genes, e.g., by the use of a novel vector system consisting of dsRNA viruses [114].

To fully realize the potential for further enhancement of productivity, which is generally assumed to be in the range of some 300% and to fully exploit the potential of RNAi, current and future studies thus aim at filling the actual gaps of knowledge and will be focused on

- The exploration of regulatory mechanisms and circuits on the transcriptional, translational, and posttranslational level.
- The determination of the specific intermediate turnover rates resulting from specific enzyme activities, enzyme titers, and enzyme stabilities.
- The detailed investigation of linked and preceding pathways.
- The investigation of product secretion mechanisms.

However, as no great leaps in productivity are currently reached, strains emerging from the development programs are also selected for beneficial alterations of genetic, physiological, and morphological properties that contribute to an enhanced process economy.

Improvement of Process Behaviour. Among the desired alterations are:

- A decrease of side products, which (1) consume metabolic energy at the expense of the desired main product, (2) hamper the final product purification, or (3) even can be inhibitory to the production organism. For instance, a significant reduction of DCPC concentrations has been reached through knocking out esterases that hydrolyze cephalosporin C to DCPC during fermentation. Esterase inactivation was by conventional mutagenesis as well as through gene-disruption and by introduction of anti-sense genes.
- Increase of the tolerance toward toxic side products and fed precursors like phenyl- or phenoxyacetic acid (see above).
- Further reduction of feedback inhibition by endproducts and metabolites (see below).
- Further reduction of catabolite repression (see below).

- Enhancement of genetic stability.
- Enhancement of viability, stress resistance, and life span.
- Accelerated product formation and enhancement of time-specific productivity to shorten fermentation time.
- Enhancement of the strain-specific productivity to reduce the amount of biomass to be processed and disposed.
- Increase of product secretion rate.
- Improvement of the fermentation behavior like (1) higher efficiency of substrate/precursor consumption, (2) diminished oxygen demand, and (3) diminished shear sensitivity.
- Improvement of filterability.

The in-depth characterization of physiological properties and biosynthetic pathways not only aims at improving process behavior and process economy, but also it relates to modifications of the inherent biosynthetic pathways (pathway engineering) to create innovative, novel products with improved properties and enhanced therapeutic values, e.g., to overcome the antibiotic resistance problem, a major global health-care problem of today [86].

1.1.4 BIOSYNTHETIC STRUCTURE MODIFICATION

Although the metabolic engineering of beta-lactams by combining β-lactam encoding genes from various sources [95, 123] is still in an explorative state, the study of polyketide pathways and the creation of polyketide antibiotic derivatives is more advanced.

1.1.4.1 Combinatorial Biosynthesis

Compounds emerging from the polyketide pathways are the classic subject of metabolic engineering [124] and for creation of novel structures. To create novel antibiotics, the natural biodiversity of polyketides is trying to be enhanced artificially by modifying and newly combining the biosynthetic pathway steps and enzymes involved [125, 126]. Polyketide synthases [127, 128] are multienzyme systems, which due to their modular structure and the occurrence of their encoding genes as clusters, are considered to be most amenable for directed alterations, genetic intervention, and heterologous expression in foreign hosts with different metabolic pathways. Concepts and strategies that are currently being pursued for creating novel and un-natural polyketides [129–132] comprise (1) creating block mutants at various biosynthetic levels to accumulate intermediate metabolites; (2) elimination of unwanted groups; (3) modification of polyketide synthases to further broaden substrate range, eventually combined with a precursor-directed biosynthesis (see below); (4) formation of new structures by deleting or introducing additional modules; (5) modification, addition, or elimination of postsynthetic modification steps like glycosylation, lactonization, and amidation; (6) the cloning and combination of various polyketide synthase genes in heterologous hosts to construct hybrid pathways and antibiotics [130]; and (7) linking and combining routes of polyketides

with pathways of nonribosomal peptide synthesis to create hybrid peptide–polyketide compounds [131–135]. As the microbial nonribosomal peptide synthases share many structural and catalytic properties [136] with polyketide synthases like their organization into catalytic modules and domains [137], they are also envisaged as ideal subjects for metabolic engineering by creating large sets of modules and subsequently combining them artificially [95, 138, 139]. The rapidly growing body of patents makes evident that an increasing number of companies are working on the field of combinatorial biosynthesis. However, it also becomes evident that, despite their promising prospects, these strategies of combinatorial biosynthesis are still in an initial state and need further intense research to elucidate the required genetic and physiological details.

This is just in contrast to another approach of modifying the molecular structure of peptidic compounds on the biosynthetic level, namely the directed biosynthesis of the desired compound structures by feeding of appropriate biosynthetic precursors or stimulating agents during fermentation, as it is routinely performed for production of penicillin V und G by the addition of phenoxyacetic acid or phenylacetic acid, respectively, as precursors to facilitate penicillin extraction from the culture broth.

1.1.4.2 Precursor Directed Biosynthesis

Microbial peptidic compounds can be modified not only in their peptide structure by direct incorporation of amino acids supplemented during fermentation but also, if present, in their fatty acid moiety [140]. The possibilities of precursor-directed derivatization of cyclic peptides cores and fatty acid moieties by amino acids will be illustrated for three compound complexes (Figure 1.1-2) with unique modes of action and outstanding spectra of activity against multidrug resistant germs, which were developed by Aventis, Eli Lilly, and Cubist Pharmaceuticals. The complex A1437 of Aventis is synthesized by *Actinoplanes friulensis* [141], which originally produced a mixture of altogether eight lipopeptides (A–H), classified according to the exocyclic amino acid position and the type of their fatty acid chains [142] (Figure 1.1-2). Through addition of L-valine and asparagines, the biosynthesis could be directed toward the production of the desired D-peptide [143], which exhibited superior pharmacological properties in preclinical trials and is characterized by an exocyclic asparagine and an iso-C14 fatty acid moiety (Figure 1.1-2). The A54145 complex from Eli Lilly [144] exhibits the noteworthy characteristic, that a built-up resistance was immediately lost in the absence of this compound [145]. It consists of eight lipopeptides produced by *Streptomyces fradiae* containing four similar peptide nuclei in combination with three different fatty acid acyl side chains. The nuclei differ in their valine/isoleucine and glutamate/3-methylglutamate substitutions at one or both of two locations of the peptide ring. The composition of the peptide nucleus as well as the percentage of branched-chain fatty acid acyl substituents could be directed by supplementation of either valine or isoleucine [144] (Figure 1.1-2). Likewise, the complex A21978 from *Streptomyces roseosporus*, also developed at Eli Lilly, can be influenced in its composition by modification of the fatty acid chain. Precursing with valine results in an enhanced formation of the compound C2, whereas the compound C1 was preferably built upon feeding of isoleucine [146] (Figure 1.1-2). Compound C1 was inlicensed as

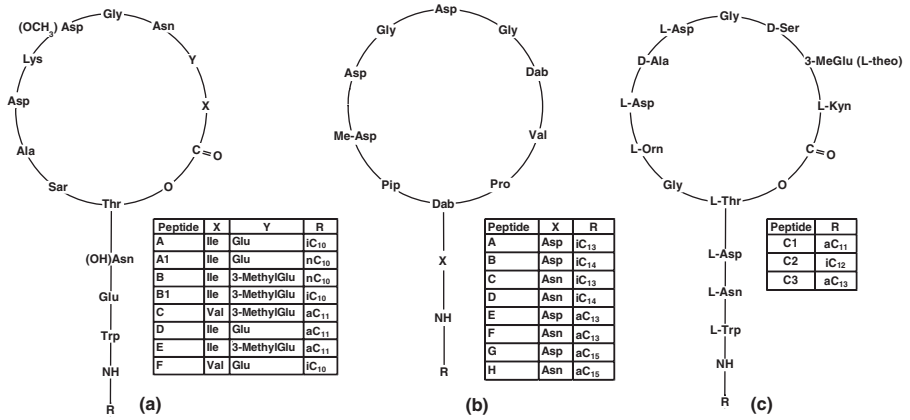

Figure 1.1-2. Structure modification of peptide antibiotics by precursor directed biosynthesis. Biosynthetic structure modification can be performed by metabolic engineering requiring an in-deph pathway characterization or by feeding of appropriate precursor compounds incorporated into the product. Examples are the lipopeptide complexes A 54145 from Eli Lilly (a), A 1437 from Aventis (b), and A 21978 developed by Eli Lilly and Cubist Pharmaceuticals (c) synthesized by *Streptomyces fradiae, Actinoplanes friulensis*, and *S. roseosporus*, respectively. The composition of both the peptide cores as well as the fatty acid moieties can be modulated *in vivo* by supplementation of amino acids like valine, leucine, isoleucine, and asparagine. The addition of valine leads to the preferable formation of peptides A 54145 C and F. The biosynthetic pathways of *A. friulensis* could be directed toward a more specific production of the desired peptide A 1437 D by feeding of valine and asparagines, whereas the preferred compound A 21978 C1 (daptomycin) could be stimulated by the addition of isoleucine. For the further details and references, see text. (Graph taken from Ref. 84, © Springer-Verlag, Heidelberg.)

daptomycin by Cubist Pharmaceuticals for clinical development. However, due to rhabdomyolytic activities and a slight haemolytic potential [138, 147, 148], it has been approved by the U.S. Food and Drug Administration in 2003 as Cubicin® salve for topical use only. Despite such disadvantageous properties, peptides in general, including those that are from nonmicrobial sources and ribosomally synthesized, are currently considered to be among the most promising compound classes for development of novel antibacterials [86, 149, 150].

However, it has to be taken into account that amino acid and precursor incorporation as well as regulation mechanisms of biosynthetic pathways also depend on growth phases, on environmental influences, and thus on the conduction of the fermentation process during which the cells are propagated.

A prerequisite for a successful fermentation are controlled and homogenous environmental conditions for the cellular reactions. However, the ensurance of controlled reaction conditions, particularly in large production scales, has been turned out to be one of the most critical issues of industrial biotechnology, as will be illustrated in the next section. For a detailed discussion of problems specifically related to the fermentation of β-lactam antibiotics, the reader is referred to the review by Schmidt [98].

1.1.5 FERMENTATION OPTIMIZATION AND SCALE-UP

The scale-up of fermentation processes as a central problem in biotechnology was first recognized and described during industrial penicillin production at the beginning of the 1940s and has been studied in more detail in *E. coli* and recombinant protein production.

1.1.5.1 Reduced Mixing Quality and Enhanced Stress Exposure

Fermentation scale-up is aimed at the manufacture of larger product quantities, if at all possible, with a simultaneous increase or at least consistency of specific yields and product quality. The changed geometric and physical conditions in larger scales, however, has lead to a less favorable mixing behavior and to impaired physiological reaction conditions, which in turn may lead to a decreased process constance and reproducibility, to reduced specific yields, to an increase of unwanted side products, and thus ultimately, to a diminished batch-to-batch consistency and product quality, which are all key issues in industrial production processes.

The problem of reduced mixing quality in larger scales is aggravated with increasing vessel sizes: The opposite substrate and oxygen gradients along the vessel height that are formed as a result of the conventional fermenter design, according to which substrate feed usually occurs from the top and aeration from the bottom, are more pronounced in larger reactors due to (1) longer distances to be covered leading to larger substrate and oxygen depletion zones, (2) larger volumes of culture broth to be stirred and therewith longer mixing times, and (3) stronger hydraulic pressure gradients influencing the oxygen transfer rate (OTR; Equations 14 and 15, Table 1.1-4) [151–155]. Cells at the fermenter top are exposed to excess glucose concentrations and simultaneously suffer from oxygen limitations, whereas those at the bottom are exposed to glucose starvation. Excess glucose concentrations (threshold value for *E. coli* at 30 mg/L) result in acetate overproduction (overflow-metabolism), and a simultaneous oxygen limitation further induces the formation of ethanol, hydrogen, formiate, lactate, and succinate (mixed acid fermentation: Refs. 154, 156, and 157). The produced acids can become reassimilated in oxygen-rich zones but in any case first lead to a temporary acidification of the microenvironment and later eventually of larger regions. Combined with a decreased transportation and elimination of carbon dioxide, detrimental metabolites, and surplus heat generated by agitation and metabolic processes, resulting in zonal overheating, the lower mixing rates in large scales thus lead to the formation of zones with enhanced stress conditions. The subsequent activation of stress genes (a survey on bacterial stress proteins is given in Ref. 158) only partially protects the cell against detrimental stress effects. For instance, despite activation of heat stress genes like *E. coli* dnaK and clpB, reducing misfolding and aggregation of heat-sensitive proteins [159], metabolic changes and damages such as translocation of proteins and membranes have been observed upon heat exposure [160]. The evidently unavoidable detrimental effects of the repeated and cyclic passing of different stress zones and the subsequent continuous activation and shut down of the corresponding stress genes are believed to lead to a completely altered physiology [155, 161] with metabolic shifts, which ultimately reduce growth and productivity and increase byproduct formation.

TABLE 1.1-4. Fermentation parameters, coefficients and terms, implicated in mixing, aeration, oxygen, and heat transfer, suitable as scale up variables to be kept constant alone or combined with each other or other process relevant variables, preferably but not necessarily, as dimensionless groups as described and discussed e.g., by Hubbard [228] and Wang and Cooney [230]. For instance, the k_La—value as the most frequently applied physical scale up variable has been combined with [1] the vessel backpressure *p* by maintaining the product of $k_La \times p$ constant through variation of pressure, agitation, and aeration for scale up of *Bacillus thuringiensis* fermentations (Flores et al. [232]) or [2] the aeration rate *vvm* under variation of power input, working volume and aeration according to the Wang-Cooney-equation [217]. For details and further explanations see text (taken from Ref 273 © Springer-Verlag, Heidelberg).

Parameter / coefficient	Mathematical characterization	Symbol explanation
Power input (P); volumetric power input (P/V)	(1) $P = 2\,\pi\,n\,M = N_{Po}\,\rho\,n^3\,d_I^5$ [kg m^2 s^{-2} = W]	P = power input; n = stirrer speed; M = momentum; N_{Po} = dimensionless power number (impeller specific);
Dimensionless power number (N_{Po})	(2) $N_{Po} = P / \rho\,n^3\,d_I^5$	ρ = density of the medium; n = stirrer speed; d_I = impeller diameter
Impeller tip speed (v_{tip})	(3) $v_{tip} = 2\,\pi\,n\,d_I$ [m / sec]	n = stirrer speed; d_I = impeller diameter
Reynolds number (Re)	(4) $Re = n\,d_I^2\,\rho / \eta$	Re = Reynolds number; n = stirrer speed; d_I = impeller diameter; ρ = density of the medium; η = dynamic viscosity
Mixing time (T_m)	(5) $T_m = f\,(n, d_I, \upsilon) = V / Q = V / N_{fl}\,n\,d_I^3$ [sec]	T_m = mixing time; n = stirrer speed; d_I = impeller diameter; υ = kinematic viscosity; V = volume; Q = volumetric flow rate; N_{fl} = pumping number
Dimensionless mixing time ($T_m n$)	(6) $T_m\,n = f\,(Re, N_{Po})$	T_m = mixing time; n = stirrer speed; T_m n = dimensionless mixing time; Re = Reynolds number N_{Po} = dimensionless power number
Modified dimensionless power number	(7) $N'_{Po} = N_{Po}\,Re^3\,d_F / d_I = P\,d_F\,\rho^2 / \eta^3$	N'_{Po} = modified dimensionless power number T'_m n = modified dimensionless mixing time
Modified dimensionless mixing time	(8) $T'_m\,n = n\,T_m / [(d_F / d_I)^2\,Re] = T_m\,\eta / d_F^2\,\rho$	N_{Po} = dimensionless power number; Re = Reynolds number; d_F = inside vessel diameter; d_I = impeller diameter; P = power input; n = stirrer speed; t_m = mixing time; ρ = fluid density; η = dynamic viscosity

TABLE 1.1-4. *Continued*

Parameter / coefficient	Mathematical characterisation	Symbol explanation
Aeration rate (volume per volume per minute, vvm)	(9) $A_R = F_G / V_R$ [m^3/ m^3 min]	A_R = aeration rate; F_G = volumetric gas flow rate V_R = fermenter reaction volume
Superficial gas velocity (v_s)	(10) $v_s = F_G / A$ [m / sec]	v_s = superficial gas velocity F_G = volumetric gas flow rate A = fermenter cross section
Gas hold up (τ)	(11) $\tau = V_R / F_G$	τ = gas hold up; V_R = fermenter reaction volume; F_G = volumetric gas flow rate; ε = fractional gas hold up; V_G = dispersed gas volume;
Fractional gas hold up (ε)	(12) $\varepsilon = V_G / V_R$	
Gassing number (N_{Qg})	(13) $N_{Qg} = f\,(Fr, Re, S, (\rho_g / \rho_0), (\eta_g / \eta_0)) = q_g / n\, d_I^3$	N_{Qg} = gassing number; Fr = Froude number; Re = Reynolds number; S = fluid constant ρ = density; η = dynamic viscosity $_g$ = aerated; $_0$ = not aerated; $q_{g=}$ gas throughput n = stirrer speed; d_I = impeller diameter
Oxygen transfer rate (OTR)	(14) $OTR = k_La\,(C_G - C_L) = k_La\,L_{O2}\,(pO_{2G} - pO_{2L})$ [kg O_2 /m^3 h] with (15) $C_G = 0{,}526\,p_i / 36 + T$ [mg / l]	OTR = oxygen transfer rate from gas to liquid phase k_L = mass transfer coefficient; a = specific interfacial surface area; C_G = oxygen saturation concentration in the gas phase; C_L = measured oxygen saturation concentration in the liquid phase; L_{O2} = oxygen solubility in the liquid phase; pO_{2G} = partial pressure of oxygen in the gas phase; pO_{2L} = partial pressure of oxygen in the liquid phase; p_i = vessel back pressure [bar]; T = temperature [°C]
Volumetric oxygen mass transfer coefficient (k_La)	(16) $k_La = a'\,(P / V_R)^b\,v_s^c$ [s^{-1}]	k_La = oxygen transfer coefficient; P = power input; V_R = fermenter reaction volume; v_s = superficial gas velocity; a′, b and c as specific fluid constants to be determined experimentally

k_La scale dependent according to Wang-Cooney	(17) $k_La = k'(P_g/V_R)^a(v_s)^b(B/6)^{0,8}(j/d_I)^{0,3}$ [s^{-1}]	k', a, b = vessel specific coefficients; P_g = power input in aerated vessel; V = volume of culture broth; V_s =superficial gas velocity; B = number of stirrers j = baffle width; d_I = impeller diameter
Respiratory quotient (RQ)	(18) $RQ = CER / OUR = pCO_{2E} - pCO_{2i} / pO_{2i} - pO_{2E}$ [mol CO_2 / mol O_2]	RQ = respiratory quotient; CER = carbon dioxide emission rate; OUR = oxygen uptake rate; pCO_2 = partial pressure of carbon dioxide; pO_2 = partial pressure of oxygen; $_i$ = air feed; $_E$ = exhaust air
Energy balance (Q_{ges})	(19) $Q_t = Q_{met} + Q_{ag} + Q_{aer} - Q_{evap} - Q_{hxch}$ [kWh = kJ] with (20) $Q_{met} = Y_{ho} R_o$ [kWh = kJ]	Q_t = total energy; Q_{met} = energy generated by metabolic activities; Q_{ag} = energy generated by agitation; Q_{aer} = energy generated by aeration; Q_{evap} = energy losses through evaporation; Q_{hxch} = energy losses through cooling; Y_{ho} = approximate metabolic constant (460 kJ/mol O_2); R_o = molar oxygen uptake rate (mol O_2 / s)
Heat transfer coefficient	(21) $\alpha = (dQ/dt) / A\ dT$	α = heat transfer coefficient; dQ/dt = heat transfer; A = surface area; dT = temperature difference

Altered physiology. Changes in respiratory states like the switch from aerobic to anaerobic conditions and the imposed stress conditions, increased by induction and overproduction of the recombinant protein, lead to shifts in the coupled amino acid biosynthesis, an altered composition of the amino acid pools and changes in the protein biosynthesis machinery (162, 162a, b, c). Even though translation processes are considered to be generally performed precisely [163], the resulting shifts in the amino acid pool in turn lead, due to the inherent inaccuracy of the translation machinery (unspecificity of aminoacyl-tRNA-synthases, "wobbling" of tRNA), to aminoacid misincorporations in freshly synthesized proteins, particularly in phases of high protein production rates, which are characterized by amino acid shortages. Muramatsu et al. [164] report the incorporation of β-methylnorleucin instead of isoleucin into recombinant *E. coli* hirudin in positions 29 and 59. A misincorporation of norvalin instead of leucin and methionine into human recombinant hemoglobin in *E. coli* has been published by Apostol et al. [165] and Kiick et al. [166]. Amino acid misincorporations are thus a cause for an increase of byproducts at the expense of the desired main product yields.

Reduced Plasmid Stability. An essential prerequisite for high product yields particularly in larger scales, in which cultures pass a higher number of generations due to larger culture broth volumes and longer inoculation chains from the cell bank to the production stage, is the stable propagation of plasmids to daughter cells. Plasmid stability is influenced by the plasmid properties, including size and nucleotide sequence; by the genetic background of the host [167, 168]; as well as by process parameters like temperature, growth rates, and substrate concentrations. Lin and Neubauer [169] show that rapid glucose oscillations favor plasmid stability and recombinant protein production rate, whereas high glucose concentrations diminish plasmid stability [170]. Plasmid stability and plasmid numbers are thus negatively influenced, more difficult to control, and less easy to be maintained in larger scales. A concept to render plasmid stability and expression rates more independent from such physiological influences is the application of runaway-plasmids, the replication of which can be induced separately from growth in the desired fermentation phase [171, 172], enabling copy numbers of up to 1000 in the expression phase and, due to the separation of their replication and expression from the growth phase, simultaneously a more precise replication and amino acid incorporation. Further possible measures to enhance the accuracy of metabolic processes are a reduction of the generation time and numbers throughout the process and a deceleration of the metabolic speed, e.g., through reduction of temperature, and the development of more stress-resistant microbial strains.

A prioritized goal of process optimization and scale-up thus consists of an appropriate process design that improves the physiological conditions and the metabolic accuracy by minimizing microbial stress exposure.

1.1.5.2 Process Characterization

To identify process-specific stress factors and to understand the physiological responses to the vessel-specific physical conditions, the mutual influences and interactions of the various physical and physiological parameters have to be analyzed in detail. An overview on the analytical methods currently applied and yet in development is given in the following.

Analytical Methods. Among the physiologically most relevant parameters are, besides the pH as discussed, biomass, cell viability, the concentrations of substrates, metabolites and products, the partial oxygen and carbon dioxide pressures in culture broth, and the composition of the exhaust gas, giving information about respiratory states as indicated, e.g., by the respiratory quotient (RQ; Equation 18, Table 1.1-2). Established analytical methods and tools are colorimetric procedures, chromatography [173], mass spectroscopy [174, 175], enzymatic and electrophoretic methods [176–178], hybridizing techniques, biochips [155, 161, 179], or flow cytometry. Multiparameter flow cytometry permits, in addition to the analysis of usual metabolites, also the analysis of the cellular DNA-, RNA-, and protein content, cell viability, membrane potential, intracellular pH, cell size, and cell development stages [180–183]. In general, the data are, if possible, preferably captured as real-time values by in situ online measurements as occurring for physical parameters [184] to avoid falsified or gappy data due to time differences between sampling and analysis. *Ex situ* methods can be designed as real-time procedures in combination with robust automated sampling and sample preparation methods like ultra-filtration [173, 175, 185] and flow injection analysis (FIA) [173, 186–190]. An online FIA glucose analysis method of the native culture broth without prior sampling and filtration has been presented by Arndt and Hitzmann [191] and Kleist et al. [192]. For their robustness, *in situ* measurements of biological parameters are preferably performed by optical probes (optodes), working either on a physical base, e.g., by refractional or spectroscopic measurements, chemically as by ion exchange reactions [193] or enzymatically by gel-embedded biocatalysts, the activity of which can be measured by means of pH-changes or by fluorophores (chemo- and biosensors) [194, 195]. The hitherto employed enzymes have restricted their application mostly to the analysis of sugar compounds [190], whereby the most frequent applications are reported for glucose [187]. However, despite intensive research, only a few concepts practically applicable for industrial purposes could have been developed. Broader applications are physical optodes enabling the simultaneous measurement and quantification of several parameters and metabolites through continuous scans or excitations and subsequent absorption and scattering measurements within defined wavelength ranges like (1) 2-D-fluorescence spectroscopy [196–198] as shown for the determination of NAD(P)H concentrations through excitation at 350 nm and the fluorescence measurements at 450 nm [190]; (2) near-infrared-spectroscopy (NIR) [199–201], which has been employed as a 700–2500-nm scan in the antibiotic fermentation at Pfizer [202, 203] for the measurement of nutrient and product as well as side product concentrations; or (3) measurements in infrared [204], ultraviolet, or visible wavelength spectra [190]. By means of capacitance measuring electrodes (Aber Instruments Ltd., U.K.), viable cell counts can be performed by seizing the electric capacitance and conductivity of cells with intact membranes in a generated electric field. To gain a picture as comprehensive as possible by enhancing the amount of analyzable substances, multichannel arrays with parallel employment of various analytical procedures monitoring several parameters simultaneously have been established and new concepts like artificial noses [205] and electronic tongues [206] currently being integrated into fermentation technology, allowing the analysis of a large number of analytes by cross-talking semi-specific sensors that act in analogy to the human olphactory sensoric system. The realization of this concept will be facilitated by a

further miniaturization of the sensors and chips employed [207] as it has been achieved with biochips developed for the pharmaceutical natural product screening, onto which the needed number of reactants is fixed in smallest space or is brought together in microchannels and the analytes are separated in microcapillars (lab-on-a-chip-technology) [208–210].

1.1.5.3 Process Optimization

For analysis, interpretation and correlation of the obtained signals and data through chemometric modeling [195, 197], neural network—tools (e.g., Unscrambler 9.0, Camo, Norway)—seem to have gained an increasingly important role [211]. Despite inherent limitations [212, 213], adequately trained neural networks are capable of recognizing and revealing nonlinear, highly complex, and even nonobvious, hidden relations among a multitude of various physical, biochemical, and physiological process parameters and to design the according process models. Hooked up to expert systems, they allow immediate and short-term reactions to process deviations by anticipating physiological drifts and their influences on product yield and quality and to change the according set points and process profiles. In this way, neural network tools help to optimize process control and to facilitate the identification of suitable strategies for process optimization and scale-up, e.g., with respect to an optimized glucose–oxygen equilibrium.

As the maximum glucose and oxygen uptake rates are not constant but depend on growth phases and rates and get reduced by induction of product synthesis [170], Lin et al. [214] established an integrative kinetic model combining these parameters with the aid of the simulation program SCILAB (Inria, F) enabling the determination of the maximum glucose and oxygen uptake rates through determination of the time that passes between glucose pulses and subsequent changes of partial oxygen pressure (pO_2) and biomass concentration. Such a model thus allows the alignment of the glucose feed meeting the maximum uptake capacity and therefore avoiding overflow metabolism. The observed pattern of glucose oscillation even can serve as a key parameter condition in later scale-up stages [169]. The equilibration of carbohydrate and oxygen supply thus constitutes a key component of process control and optimization. Further strategies aiming at the avoidance of oxygen limitation and glucose overflow metabolism consist of the employment of alternative carbon sources like glycerol [215] or galactose [216] and of a drastic glucose restriction. Wong et al. [217] report that minimal concentrations of glucose and yeast extract yielded the highest concentrations of 0.5 and 1 g/L of a recombinant K99 antigen. Yim et al. [218] achieved constant yields of 4.4 g/L of human granulocyte colony stimulating factor during scale-up from 2.5 to 30 L by limiting glucose feed and keeping the growth rate at the minimum ($\mu = 0.116\,h^{-1}$). Besides the abovementioned parameters like the maximum uptake capacities, the various different physiological or directly measurable physical process parameters like pH, ammonium consumption, pO_2, OTR, or the growth parameters can be applied as a reference parameter for glucose feed in a closed-loop design. Dantigny et al. [219] describe a biomass controlling feed depending on the ethanol formation rate and RQ for *S. cerevisiae*. To achieve a more uniform glucose distribution in large-scale tanks, Larsson et al. [151] and Bylund et al. [152] suggested a glucose feed at the dynamic zones of the fermenter bottom together with the injected air. A step ahead

in this regard would be a further enhancement of the mixing quality by a reactor and process design permitting a multilevel injection of both air and substrates into high turbulence zones.

A supportive approach for comprehension of the large-scale hydrodynamic and reaction conditions to ensure homogenous reaction conditions and to reduce both the size of stress zones and the zonal residence times is the depiction of these zones through transfer into small reactors (scale-down) or through high-performance computing computational fluid dynamics (CFD) [220]. Whereas the sort and amount of parameters that can be simulated by the scale-down approach appear to be restricted, CFD is meanwhile broadly applied, e.g., the flow modeling software tools of Fluent Inc., Lebanon, NH (Fluent 4,5,6 and MixSim). A simulation of the trajectories and distributions of gas bubbles and mass transports enables the determination of zonal pO_2 values and oxygen transfer rates. The combination with parameters like substrate concentrations and gradients, the residence times in the respective zones, population dynamics, and metabolic fluxes (structured metabolic models) leads to integrative models (integrated fluid dynamics, IFD), which permit the prediction of physiological effects and reactions. Even though it is emphasized that the integration of CFD and structured biokinetics currently requires a deeper understanding of the dynamics of metabolic and regulatory networks and cascades of signal transduction triggered by microenvironmental fluctuations and thus further research [221], it will most likely contribute long term to a realistic modeling of the interplay between physics and physiology and thus will facilitate the identification of key parameters influencing product yield and quality the most and therewith of suitable scale-up parameters and strategies. Problems related to the scale-up of physical parameters are illustrated in the next section.

1.1.5.4 Physical Scale-Up Parameters

Suitable for employment as physical scale-up parameters are all known process parameters and coefficients exerting known physiological effects, particularly those affecting oxygen supply; heat transportation and mixing, such as power input (Equation 1; Table 1.1-4), aeration, and agitation rate (Equations 3, 9, and 10; [222]); mixing time (Equation 5; [223]); pO_2; OTR (Equations 14, 15; [224, 225]); oxygen mass transfer coefficient (k_La, Equations 16, 17); and biocalorimetric variables like heat fluxes and heat transfer coefficients (Equations 19, 20, and 21). Biocalorimetric measurements were found to be in definite correlation with metabolic activities and with OTR and are employable as growth phase indicators [226, 227]; the calculations, however, require a precise measurement and knowledge of heat fluxes and sources, energy inputs, and the temperature distribution in the vessel. In most cases, however, it is, depending on the scale-up factor, not, or only restrictedly, possible from the physical viewpoint to keep physical parameters constant throughout the scale-up. Constance of even a single specific parameter mostly leads to an uncontrolled and unpredictable change of other variables into dimensions that are technically not realizable. Classic examples are (1) the mixing time, which inevitably increases in larger vessels due to the larger volumes to be stirred and that cannot be unlimitedly compensated and kept constant by increasing stirrer speeds and energy inputs, and (2) the volumetric energy input P/V. A constant volumetric power input has indeed been successfully applied as a scale-up

parameter for the early industrial penicillin fermentations (1 hp per gallon, equivalent to 1.8 kW per 1 m^3) and in fermentations with low energy inputs [216], but it is limited in fermentations requiring high energy inputs, like recombinant *E. coli* cultures.

For this reason, mathematically driven approaches are pursued for both process and reactor scale-up by forming dimensionless coefficients (e.g., by the Buckingham-Pi method as cited by Hubbard [228]), which are kept constant by an appropriate choice and adjustment of the relevant influencing process and fermenter parameters. Well-established examples are, among many others, the dimensionless power number (Equation 2), Reynolds number (Equation 4), gassing number (Equation 13), and the modified dimensionless power number (Equation 7) and the modified dimensionless mixing time (Equation 8). The comparison of the latter two coefficients and of the corresponding curves enables the identification of appropriate types of stirrers capable of exerting the desired mixing performance at a given stirrer speed with a minimum of energy consumption and therewith to compensate the above-mentioned limitations of volumetric energy inputs at larger scales. (A quantum leap in stirrer technology in this regard seems to be the Visco-Jet of Inotec, which possesses cone-formed short tubes instead of blades and pretends to exhibit the best mixing performance with a minimum of power input and shear stress without foam generation.) The performance of further various types and designs of stirrers is presented and discussed by Junker et al. [229]. Stirrer performance coefficients and curves are thus crucial scale-up aids.

Furthermore, any of these parameters and coefficients can be combined with other variables to set up and create new, process-specific parameter correlations, coefficients, terms, groups, and characteristic curves (examples given, e.g., by Wang and Cooney [230]).

This relates also to the k_La—value (Equation 16), which currently is the most applied physical scale-up variable because it includes the relevant parameters influencing oxygen supply like agitation (via energy input) and aeration as superficial gas velocity (Equation 10) and that, as a component of dimensionless terms, is also frequently employed for reactor scale-up [231].

Taking into account the limitation of a k_La–oriented scale-up in the form of a limited power input, OTR, and tolerable shear stress, Flores et al. integrated the backpressure p by maintaining the product of $k_La \times p$ constant through a variation of pressure, agitation, and aeration for scale-up of *Bacillus thuringiensis* fermentations [232]. Although the specific growth was reduced and the biomass production and sporulation efficiency remained constant, fermentation time could be shortened and toxin yields were increased. Wong et al. successfully scaled-up *E. coli* fermentations from 5 L to 200 L by keeping constant the product of k_La and aeration rate (vvm) under variation of power input, working volume, and aeration according to the Wang–Cooney equation (Equation 17; [230]), which reflects these parameters in dependence of the fermentation scale [217]. Diaz and Acevedo [224] argue that the oxygen transfer capacity, indicated by the k_La value, is not the most process-relevant parameter, but the effective oxygen transfer rate as a product of k_La and mass transfer potential is, i.e., the difference between the pO_2-values in the gas and the liquid phase, and suggest an OTR-based scale-up strategy (Equations 14 and 15).

A common, simple and robust method is the maintenance of a constant pO_2 in the culture broth by variation of stirrer speed and aeration rate. To avoid shear

stress and to keep the energy input (P) at the lowest possible level, the pO_2 is steered at the minimum limit. Riesenberg et al. employ a stirrer speed and glucose-feed steered pO_2 of 20% for interferon α–*E. coli*-fermentations in 30-L and 450-L scales [233]. These examples demonstrate that, for each process, appropriate variables and coefficients and therewith scale-up strategies have to be identified individually.

1.1.5.5 Development of Fermentation Models and Strategies

Despite the central role of the scale-up issue in biotechnology and the comparably large body of literature, no common, generally applicable strategy seems to be established. For each product, process, and facility, a suitable scale-up strategy has to be elaborated.

A wholistic scale-up strategy consists of a comprehensive and detailed process characterization to identify key stress factors and key parameters influencing product yield and quality the most, and of an appropriate process control and process design ensuring optimum mixing and reaction conditions, supported by appropriate knowledge and data-driven models [234, 235] as well as computational tools.

It should be kept in mind, however, that any approach and any model will always be approximative and that a compromise in the process-related knowledge will always be gappy and that the known mathematical methods and relations cannot completely reflect the highly complex interactions and relationships of the physical conditions governing the fermentation process [236].

As a matter of fact, it seems, that, in view of the high complexicity of the fermentation parameters influencing each other and the only rudimentary and fragmentary reflection of the reality any model can deliver and finally the different layouts of vessels and facilities that rarely are designed according to strict scale-up criteria, successful scale-up in most cases will not be the result of a conclusive and straight-lined experimental strategy but will be the outcome of an independent optimization on each scale that highly depends on the experience, skill, and last, but not least, intuition of the experimentalist.

To a lesser extent, this also holds true for the design of procedures aimed at the isolation and purification of the compounds from the culture broth. As these purification steps follow upon the biosynthetic steps and the upstream processing in the bioreactor, they are usually referred as "downstream processing."

1.1.6 DOWNSTREAM PROCESSING

1.1.6.1 Product Recovery and Purification

Biopharmaceutical products consist of a multitude of compounds and structures with most having different physical and chemical properties and derive from a large variety of sources like human and animal tissues, body fluids, plant material [237], and as illustrated above, microbial fermentations. Accordingly, purification strategies have to be developed individually and empirically that reflect the physicochemical properties of the product, of the product source, and of potential contaminants by finding appropriate sorts, sequences, combinations, and operation

modes of the respective downstream processing steps to finally achieve high purities and high recovery rates while maintaining the pharmaceutical activity of the molecule.

Traditionally, downstream processing steps are roughly subgrouped into operations related to cell harvest, cell rupture in case of intracellularly occurring products, product capture, product purification, and product polishing to manufacture a drug ready for galenic preparation and consist of a sequence of solid–liquid separation and solvent and solid-phase extraction steps. Large-scale cell harvest usually occurs by preparative centrifugation, e.g., in a decanter or in disk stack separator or by filtration to simultaneously separate existing suspended particles from the surrounding broth. Fragile mammalian cells are usually separated by filtration methods only.

If the value product occurs intracellularly, the slurry is taken up in washing buffers of appropriate ionic strength for preparation of the cell rupture, which occurs, e.g., by high-pressure homogenization, sonification, bead milling, or enymatic procedures.

Product capture is defined as the first product extraction and purification step aimed at a product concentration by volume reduction and partial purification, whereas polishing constitutes the final purification step, which removes persistent residual minor impurities like denatured, aggregated, or nonfunctional isoforms of the product, and which follows the preceding (intermediate) purification steps aimed at the removal of contaminating solutes like host cell proteins, DNA, and media components. Product capture usually starts with precipitation, solvent extraction, and/or a cascade of ultra-filtration and nano-filtration steps before the first ion exchange or size exclusion chromatography. Antibodies and antibody derivatives, which account for about 20% of the biopharmaceutical products currently in development, are preferably bound to affinity matrices [238] by natural immunoglobulin-binding ligands such as the *Staphylococcus aureus* membrane and cell wall protein A [239] or the streptococci surface protein G. To broaden the availability of specific structures with suitable binding properties, synthetic ligands with enhanced stability and resistance to chemical and biochemical degradation are continuously attempting to be developed, supported, e.g., by computer-aided design. The development of synthetic ligands with enhanced selectivity and stability, tailored to specific biotechnological needs and product structures, will lead to more efficient, less expensive, and safer procedures not only for purification of antibodies but also for other proteins at manufacturing scales. Also, antibodies can be employed as a means for protein purification in the form of immunoaffinity chromatography [240]. A novel emerging technology is the use of synthetic single-stranded nucleic acid molecules (aptamers) as affinity ligands [241], which fold up into unique three-dimensional structures specifically binding to the desired target structure as it has been shown for the purification of thyroid transcription factor or selectin receptor globulin [242, 243].

The subsequent purification steps including polishing are mostly performed on hydrophobic interaction resins and/or on reversed-phase matrices.

1.1.6.2 Downstream Processing Optimization and Economization

Mostly due to the tremendous costs of sometimes several thousand € per liter for chromatographic resins and production scales of up to several hundred liters column

volume and due to the prices for the columns themselves of up to hundreds of thousands of U.S. dollars, downstream processing expenses account for the largest part (50–90%) of the total production costs and are therewith a focal area for process economization attempts. One of the many criteria for the choice of a resin is its shelf life. Bearing in mind that every downstream processing step coincides with a minimum product loss of 5–10%, the removal of a particular step not only helps to save the respective capital investment, but concomittantly to increase the final product yields, and thus to render the whole production process more economical in several respects. To reduce the number of required steps, a selection has to be made for resins exhibiting excellent separation, resolution, and yield with respect to a given product and the contaminants, i.e., for resins exhibiting excellent product binding selectivity and capacity. Likewise, the operation mode of the column has to be optimized with respect to the specific product load, adjustment of the physico-chemical operational conditions like pressure, temperature, pH(-shift), ionic strength, and sort of buffers and eluents (e.g., evaluation of continuous vs. step gradients).

A further approach is the combination of the solid–liquid separation and product recovery into a single step, e.g., by expanded bed adsorption [244, 245], which permits the direct initial purification from culture broths without the need of prior removal of suspended solids. In contrast to the conventional column operation, the mobile phase is pumped upward through the column bed from beneath. The bed thus starts to expand at a liquid flow rate above a critical value, and the gaps between the sorbent beads expand. The controlled distribution of bead size and bead weight results in a stable expanded bed in which the beads oscillate around a steady position and thus avoid clogging of the column. Whereas preparative liquid chromatography methods [246] have become a well-established separation and purification method in the pharmaceutical industry, techniques like two-phase system partitioning [247] and its variant reverse micellar extraction [248], which also potentially offer advantages like the possibility of a direct extraction from the culture broth through partitioning of the compounds into two immiscible phases, need further developmental activities for economic large-scale applications in the production of biopharmaceuticals and are less common.

A further criterion for the choice and design of a downstream processing procedure is thus its scalability.

1.1.6.3 Downstream Processing Scale-Up

Comparable with the scale-up of fermenters and fermentation processes, the scale-up of downstream processes, particularly of column and column operation, is not simply a matter of increasing size. Increasing column diameters, reducing the stabilizing wall effects, and increasing resin bed heights may lead to an altered settling behavior of the beads, to channel formation through shrinking and swelling of the packed matrix, to column clogging with a concomittant reduction of the flow rates and an increase of the column back pressure, to altered hydrodynamic behavior and residence times of the process fluids, and to an altered pattern for the product/contaminants adsorption/desorption pattern [249]. To avoid the resulting quality impairments and deviations, resins with a suitable scale-up behavior have to be selected and the resin bed height is tried to be kept constant as far as possible as

is the flow rate as key scale-up parameters, which requires an appropriate experimental design in early laboratory development stages. As this, however, is only possible to a limited extent, downstream processing scale-up also is the result of an optimization of the respective operation conditions (see above) on each scale.

A procedure that has been optimized for decades and scaled-up to dimensions of several cubed meters is the commercial β-lactam purification.

(For further general articles dealing with problems and issues of downstream processing, the reader is referred to the reviews in Refs. 249–255).

1.1.6.4 Downstream Processing of β-Lactam Compounds

As most of the product is secreted and is thus concentrated in the culture broth, the recovery process starts with filtration, usually with a rotary vacuum filter, followed by a cascade of solvent and solid-phase extraction steps.

Common penicillin extraction solvents are amyl- and butyl-acetate or methylisobutylketone. As penicillin is extracted as a free acid at pH 2–2.5 and the molecule is instable at this pH, extraction occurs in a counter-flow using Podbielniak- and Luwesta-centrifugal extractors to shorten the contact time with the solvent and to prevent product decomposition.

Purification is through subsequent and repeated crystallization from an aqueous solution after alkalization.

In contrast to penicillin, the hydrophilic cephalosporin is more suitable for solid-phase extraction. For high yield and purity extraction, a combination of several, different chromatographic steps is used.

Hydrophobic interaction chromatography on neutral polyaromatic resins like Amberlite XAD 4, 16, 1180, or Diaion HP20 is widely used in combination with weak basic anion exchangers like Diaion WA-30 or strong acidic cation exchangers like Amberlite XAD 2000 and Diaion SK-1B [256, 257]. The mentioned resins are recognized for their high sorption capacity and their long shelf life.

Whereas the improvements on the extraction level are less spectacular, significant progress over the last few years can be noticed with respect to side-chain cleavage to 6-aminopenicillanic acid (6-APA) and 7-aminocephalosporanic acid (7-ACA) in switching from chemical to enzymatic procedures.

1.1.7 POSTSYNTHETIC STRUCTURE MODIFICATION

Most antibiotics in therapeutic use are synthesized or modified exclusively by the means of chemistry and are derived from the established compound classes, which have been known for decades. Chemical derivatization thus has also been applied to substances that are of microbial origin and that therefore are termed semi-synthetic.

Among the most recent semi-synthetic antimicrobials are Aventis' streptogramin derivative Synercid and the erythromycin derivatives clarithromycin (Klacid) from Abbott (Abbott Park, Illinois) and roxithromycin (Rulid) and the brand new ketolide telithromycin (Ketek), both from Aventis (Paris, France) [86].

Despite these recent successes and increasing efforts, the yields of therapeutically useful entities emerging from such chemical derivation programs are continuously decreasing.

For this reason, large-scale derivation methods (combinatorial chemistry) are integrated in the search for innovative antibiotics as are biotechnological approaches for structure modification. Biocatalytic procedures, which have been shown to be useful for generating novel antibiotic structures like Loracarbef, a novel β-lactam compound from Eli Lilly (Indianapolis, Indiana) highly active against various β-lactamase producing species [258, 259], are also about to replace continuously chemical production processes. At several universities and corporations, screening studies have been initiated to find appropriate enzymes for compound conversions, as it has been successfully established in production processes for penicillin and cephalosporin. (A review discussing criteria like resource and energy consumption, emissions, health risk potential, area use, and environmental effects for the evaluation of the eco-efficiency of biotechnological processes is given by Saling [260]).

1.1.7.1 β-Lactam Side-Chain Cleavage

6-APA and 7-ACA as the key intermediates for the production of semisynthetic penicillins and cephalosporins (see Section 1.1.3.2) are obtained by removal of the acyl side chains.

There is a large body of patents existing for chemical and enzymatic splitting procedures, but enzymatic processes have been more successful for economical as well as for ecological reasons.

Enzymatic 7-ACA splitting procedures [for general review, see 261] have been developed and commercialized by companies like Asahi Chemical, Hoechst, and Novartis. The replacement of the hitherto employed chemical deacylation processes like the imino ether (Figure 1.1-3) or the nitrosyl chloride method [262] resulted in a cost reduction of 80% and a decrease of the waste volume by a factor 100 from 31 t to 0.3 tons per 1-ton 7-ACA. Chlorinated hydrocarbons like dimethyl aniline and methylene cloride as well as heavy metal ions can be completely avoided. Instead of zinc salt formation, multiple silylation, formation of the imino chloride, imino ether, and finally an imino ether hydrolysis, the side chain is removed in two enzymatic steps (Figure 1.1-3).

Cephalosporin C is first oxidized and deaminated by a D-amino acid oxidase (DAO), which can be obtained from various fungal species, like the yeasts *Trigonopsis variabilis* and *Rhodotorula gracilis* or the ascomycete *Fusarium solani*. The resulting α-keto-adipyl-7-ACA, upon decarboxylation, is converted into glutaryl-7-ACA (G-7-ACA). DAO is a flavoenzyme containing flavin adenine dinucleotide as the prosthetic group and catalyzes oxidation of D-amino acids to their corresponding keto acids. In a second step, the glutaryl side chain of G-7-ACA is deacylated by a glutarylamidase from *Pseudomonas diminuta* [263]. The molecular data of other potentially suitable enzymes and genes from various sources are given by Isogai [264]. It is noteworthy that the enzymatic splitting process could have only been rendered economical and therefore commercially employable through a significant increase of glutarylamidase yield on the fermentation level by using a gene-recombinant *E. coli*-strain.

A recombinant amidase from *E. coli* is also the most commonly employed enzyme for 6-APA production by deacylation of penicillin G [265], a process that has been established now for decades [266]. With an annual turnover of 30 tons, the *E. coli* penicillin amidase is one of the most widely used biocatalysts, despite

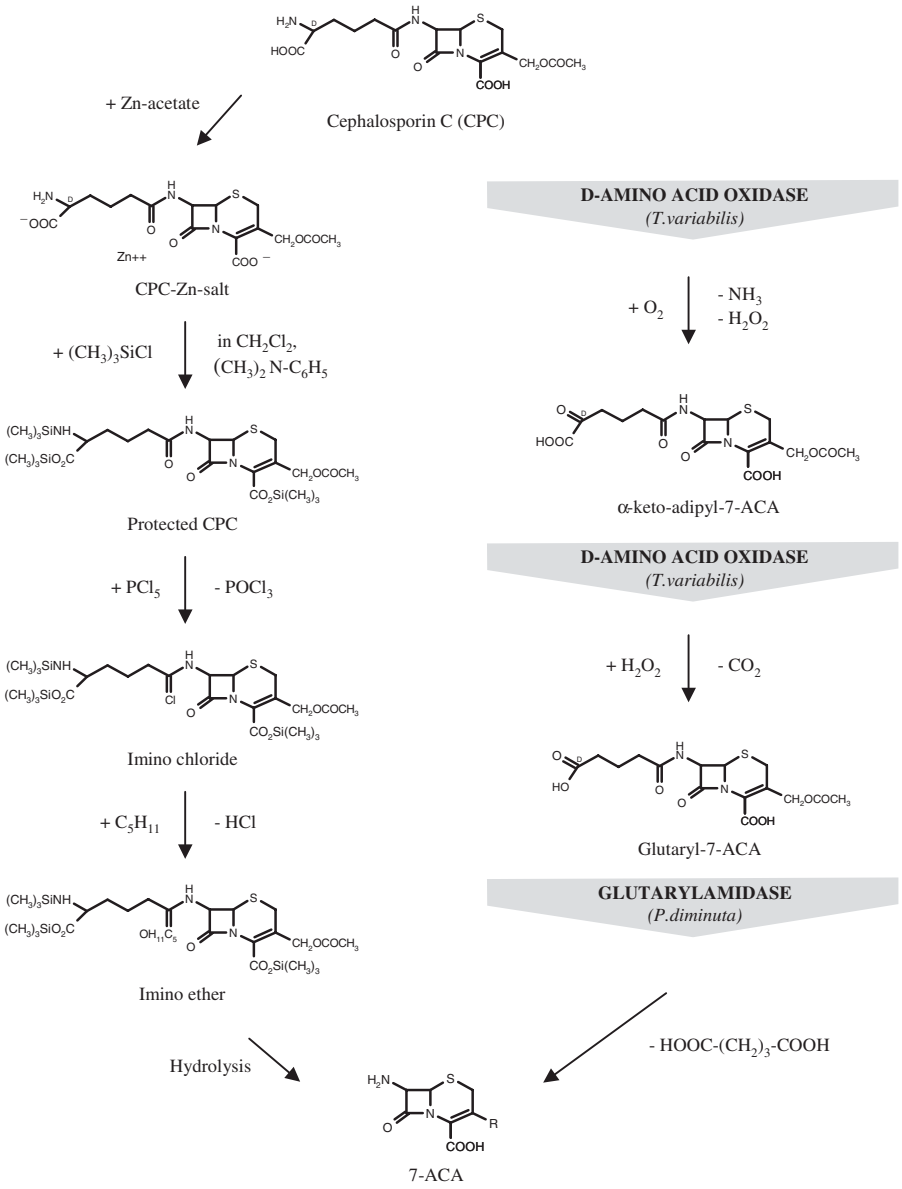

Figure 1.1-3. Replacement of the chemical cephalosporin C splitting procedure by a biotechnological process. Biotechnological production and derivatization procedures are continuously being developed to replace chemical processes for both economical and ecological reasons. An established industrial process is the enzymatic splitting of cephalosporin C to 7-ACA by D-amino acid oxidase of the yeast *Trigonopsis variabilis* and a glutarylamidase of *Pseudomonas diminuta* (right) replacing the chemical imino ether method (left). For further details, see text. (Graph taken from Ref. 98, © Springer-Verlag.)

the discovery of further similar acting enzymes from various microbial sources, including penicillin V acylases [267] from the basidiomycetes *Bovista plumbea* [268, 269] and *Pleurotus ostreatus* [270]; from the ascomycete *Fusarium* [271]; or from the yeast *Cryptococcus.*

A significant milestone in economization of enzymatic β-lactam production has been reached by enzyme immobilization permitting a preservation and multiple use of the cleavage enzymes. The currently most employed resins in industries for this purpose are epoxyacrylic acids (Eupergit) and silica gel derivatives (Deloxan). The general economic criterion for the preparation of a biocatalyst is production costs in relation to yield, turnover rate, storage stability, and operational/mechanical stability. Further criteria are outlined in the *Guidelines for the Characterization of Immobilized Biocatalysts* by the European Federation of Biotechnology. Application criteria are filterability, sedimentation velocity, and particle firmness. Operational and storage stability as well as the activity of this biocatalyst and the quality of 6-APA with respect to color are improved by sulfur reducing compounds [272]. As pointed out, the ensurance of product quality and drug safety is a key issue in pharmaceutical production processes.

1.1.8 QUALITY ISSUES

According to regulatory requirements, pharmaceutical production facilities and processes have to be proven to function properly across the entire range of process critical parameters by a qualification of the facilities and equipment, which consists of the steps design qualification (DQ), installation qualification (IQ), operational qualification (OQ), and performance qualification (PQ) and which results in a validation of the production process to substantiate that the complete system is capable of manufacturing the respective product reproducibly in a consistent quality as expected within preset specifications and in compliance with all laws and guidelines, like the cGMP (current good manufacturing practice) regulations, among others, issued by regulating authorities like the FDA and European Medicines Agency (EMEA).

The products have to be sterile and free of any contaminations that might derive from process material, residues of preceding production campaigns (cross-contaminations) in the case of product changes, and of the agents used for facility cleaning. To restrict the introduction of such contaminations into the repective production steps, the production facilities, equipments, and solutions are usually cleaned according to validated cleaning procedures (e.g., with 1 M NaOH) and sterilized by heat or filtration before their use. Product sterility is achieved by aseptical performance of the last manufacturing steps and by micro-filtration (0.2 μm) or, if possible, by terminal heating of the product before filling. Residues of chemical cleaning agents are removed according to validated procedures by rinsing with purified water.

As biopharmaceuticals are potentially contaminanted with possibly harmful viruses and allergenic and pyrogenic acting endotoxins as residual components of their host cell, guidelines and directions have been issued by the FDA's Center for Biologics Evaluation and Research (CBER) requesting validated procedures for virus and endotoxin removal and inactivation.

1.1.8.1 Virus and Endotoxin Removal

Endotoxins, which consist of the lipopolysaccharide fraction present in the cell wall of gram negative germs and which tend to adhere to equipment surfaces and to persist in products and product solutions, as do viral contaminants deriving from cell culture processes or from starting plasma or tissue material, can also be efficiently removed by final filtration steps. Whereas ultra-filtration with a cut-off of or below 10 kDa is sufficient for endotoxin removal, viruses need to be removed by nano-filtration steps complemented by a variety of inactivation measures like pasteurization, pH inactivation, solvent/detergent treatment, or ultraviolet and gamma ray irradiation. Usually, endotoxins and viruses are already deconcentrated to a significant extent by the chromatographic steps and the solvent exposure in the course of the normal purification procedures [251].

1.1.9 CONCLUSION

Marching in step with the technical and methodological progresses, biotechnology is gaining in increasing importance in pharmaceutical production processes by replacing chemical production procedures for economical and ecological reasons and in the development and commercialization of novel therapeutic principles.

To fully exploit the potential of biotechnological production methods, an integrated process design will be necessary considering the downstream processing requirement during the design of upstream operations and vice versa (A comprehensive overview illustrating the complete biotechnological production chain is given in Table 1.1-3.) As the fate of a compound as a pharmaceutical also depends on the prospective production costs already estimated in the development phase and production processes cannot be significantly changed after approval without running the risk of having to perform ad-ditional clinical trials, it is of utmost importance to choose and design production organisms, strains, vectors, expression cassettes, and fermentation procedures as early as possible in compliance with the prospective harvest and purification methods also to be chosen and designed according to cost criteria. Integrative biotechnology thus is not only a key prerequisite for the development of competitive and economical production processes to relieve the downward price pressure applied by generic drug makers after patent expiration and for the acceleration of market approval for new drugs in the highly competitive environment of the biopharmaceutical industry, but also it is a key technology to save and liberate capital for the development of novel drugs for the benefit of mankind.

REFERENCES

1. Choi J H, Lee S Y (2004). Secretory and extracellular production of recombinant proteins using *Escherichia coli*. *Appl. Microbiol. Biotechnol.* (online).
2. Li Y, Wei G, Chen J (2004). Glutathione: a review on biotechnological production. *Appl. Microbiol. Biotechnol.* 66:233–242.
3. Leathers T D (2003). Biotechnological production and application of pullulan. *Appl. Microbiol. Biotechnol.* 62:468–473.

4. Hermann T (2003). Industrial production of amino acids by coryneform bacteria. *J. Biotechnol.* 104:155–172.
5. Wehmeier U F, Piepersberg W (2004). Biotechnology and molecular biology of the alpha-glucosidase inhibitor acarbose. *Appl. Microbial. Biotechnol.* 63:613–625.
6. Pulz O, Gross W (2004). Valuable products from biotechnology of microalgae. *Appl. Microbiol. Biotechnol.* 65:635–648.
7. Lee P C, Schmidt-Dannert C (2002). Metabolic engineering towards biotechnological production of carotenoids in microorganisms. *Appl. Microbiol. Biotechnol.* 60:1–11.
8. Westers L, Westers H, Quax W J (2004). Bacillus subtilis as cell factory for pharmaceutical proteins: A biotechnological approach to optimize the host organism. *Biochim. Biophys. Acta.* 1694:299–310.
9. Miesfeld R (1999). *Applied Molecular Genetics*, Wiley, New York.
10. Weaver R F (1994). *Molecular Biology of the Cell*, Garland Publishing, New York.
11. Faye L, Boulaflous A, Benchabane M, Gomord V, Michaud D (2005). Protein modifications in the plant secretory pathway: Current status and practical implications in molecular pharming. *Vaccine* 23:1770–1778.
12. Hellwig S, Drossard J, Twyman R M, Fischer R (2004). Plant cell cultures for the production of recombinant proteins. *Nat. Biotechnol.* 22:1415–1422.
13. Goldstein D A, Thomas J A (2004). Biopharmaceuticals derived from genetically modified plants. *QJM* 97:705–716.
14. Gregory G, Heyneker H L (1988). Secretion of correctly processed human growth hormone in *E. coli* and *Pseudomonas*. U.S. Patent 4755465.
15. Habermann P, Bender R (2001). Signal sequences for the production of leu-hirudin via secretion by *E. coli* in a culture medium. International Patent WO 01/21662.
16. Schmidt F R (2004a). Recombinant expression systems in the pharmaceutical industry. *Appl. Microbiol. Biotechnol.* 65(4):363–372.
17. Choi J H, Ryu Y W, Seo J H (2005). Biotechnological production and applications of coenzyme Q_{10}. *Appl. Microbiol. Biotechnol.* (epub ahead of print).
18. Weickert M J, Doherty D H, Best E A, Olins P O (1996). Optimization of heterologous protein production in *Escherichia coli*. *Curr. Opin. Biotechnol.* 7:494–499.
19. Makrides S C (1996). Strategies for achieving high-level expression of genes in *Escherichia coli*. *Microbiol. Rev.* 60:512–538.
20. Gentschev I, Dietrich G, Goebel W (2002). The *E. coli* alpha-hemolysin secretion system and its use in vaccine development. *Trends Microbiol.* 10:39–45.
21. Ward A, Hoyle C, Palmer S, O'Reilly J, Griffith J, Pos M, Morrison S, Poolman B, Gwynne M, Henderson P (2001). Prokaryote multidrug efflux proteins of the major facilitator superfamily: Amplified expression, purification and characterization. *J. Mol. Biotechnol.* 3:193–200.
22. Pines O, Inouye M (1999). Expression and secretion of proteins in *E. coli*. *Mol. Biotechnol.* 12:25–34.
23. Bassford P J, Silhavy T J, Beckwith J R (1979). Use of gene fusion to study secretion of maltose-binding protein into *Escherichia coli* periplasm. *J. Bact.* 139:19–31.
24. Yamanaka H, Nomura T, Fujii Y, Okamoto K (1998). Need for TolC, an *Escherichia coli* outer membrane protein, in the secretion of heat-stable enterotoxin I across the outer membrane. *Microb. Pathogen.* 25:111–120.
25. Lund P A (2001). Microbial molecular chaperones. *Adv. Microb. Physiol.* 44:93–140.
26. Hartl F U (1996). Molecular chaperones in cellular protein folding. *Nature* 381:571–580.

27. Collier D N (1993). SecB: A molecular chaperone of *Escherichia coli* protein secretion pathway. *Adv. Protein. Chem.* 44:151–193.
28. Kujau M J, Hoischen C, Riesenberg D, Gumpert J (1998). Expression and secretion of functional miniantibodies McPC603scFvDhlx in cell-wall-less L-form strains of *Proteus mirabilis* and *Escherichia coli*: A comparison of the synthesis capacities of L-form strains with an *E. coli* producer strain. *Appl. Microbiol. Biotechnol.* 49:51–58.
29. Rippmann J F, Klein M, Hoischen C, Brocks B, Rettig W J, Gumpert J, Pfizenmaier K, Mattes R, Moosmayer D (1998). Procaryotic expression of single-chain variable-fragment (scFv) antibodies: Secretion in L-form cells of *Proteus mirabilis* leads to active product and overcomes the limitations of periplasmic expression in *Escherichia coli*. *Appl. Environ. Microbiol.* 64:4862–4869.
30. Mergulhao F J, Summers D K, Monteiro G H (2005). Recombinant protein secretion in *Escherichia coli*. *Biotechnol. Adv.* 23:177–202.
31. Swartz J R (2001). Advances in *Escherichia coli* production of therapeutic proteins. *Curr. Opin. Biotechnol.* 12:195–201.
32. Sorensen H P, Mortensen K K (2005). Soluble expression of recombinant proteins in the cytoplasm of *Escherichia coli*. *Microb. Cell. Fact.* 4:1–8.
33. Dilsen S, Paul W, Sandgathe A, Tippe D, Freudl R, Thömmes J, Kula M R, Takors R, Wandrey C, Weuster-Botz D (2000). Fed-batch production of recombinant human calcitonin precursor fusion protein using *Staphylococcus carnosus* as an expression-secretion system. *Appl. Microbiol. Biotechnol.* 54:361–369.
34. Olmos-Soto J, Contreras-Flores R (2003). Genetic system to overproduce and secrete proinsulin in *Bacillus subtilis*. *Appl. Microbiol. Biotechnol.* 62:369–373.
35. Srinivasan S, Barnard G C, Gerngross T U (2002). A novel high-cell-density protein expression system based on *Ralstonia eutropha*. *Appl. Env. Microbiol.* 68:5925–5932.
36. Nakashima N, Mitani Y, Tamura T (2005). Actinomyctes as host cells for production of recombinant proteins. *Microb. Cell. Fact.* 4:7.
37. Pierce J J, Robinson S C, Ward J M, Keshavarz-Moore E, Dunnill P (2002). A comparison of the process issues in expressing the same recombinant enzyme periplasmatically in *Escherichia coli* and extracellularly in *Streptomyces lividans*. *J. Biotechnol.* 92:205–215.
38. Anné J, van Mellaert L (1993). *Streptomyces lividans* as host for heterologous protein production. *FEMS Microbiol. Lett.* 114:121–128.
39. Paradkar A, Trefzer A, Chakraburtty R, Stassi D (2003). *Streptomyces* genetics: a genomic perspective. *Crit. Rev. Biotechnol.* 23:1–27.
40. Berks B C, Sargent F, Palmer T (2000). The Tat protein export pathway. *Molec. Microbiol.* 35:260–274.
41. Teter S A, Klionsky D J (1999). How to get a folded protein across a membrane. *Trends Cell. Biol.* 9:428–431.
42. Schaerlaekens K, van Mellaert L, Lammertyn E, Geukens N, Anne J (2004). The importance of the Tat-dependent protein secretion pathway in Streptomyces as revealed by phenotypic changes in tat deletion mutants and genome analysis. *Microbiol.* 150:21–31.
43. Upreti R K, Kumar M, Shankar V (2003). Bacterial glycoproteins: functions, biosynthesis and applications. *Proteomics* 3:363–379.
44. Benz I, Schmidt A (2002). Never say never again: Protein glycosylation in pathogenic bacteria. *Molec. Microbiol.* 45:267–276.

45. Russel C, Mawson J, Yu P L (1991). Production of recombinant products in yeasts: A review. *Aust. J. Biotechnol.* 5:48–55.
46. Lin Cereghino J L, Cregg J M (2000). Heterologous protein expression in the methylotrophic yeast *Pichia pastoris*. *FEMS Microbiol. Rev.* 24:45–66.
47. Lin Cereghino G P, Lin Cereghino J, Ilgen C, Cregg J M (2002). Production of recombinant proteins in fermenter cultures of the yeast *Pichia pastoris*. *Curr. Opin. Biotechnol.* 13:329–332.
48. Buckholz R G, Gleeson M A (1991). Yeast systems for the commcercial production of heterologous proteins. *Bio/technol.* 9:1067–1072.
49. Gellissen G (2000). Heterologous protein production in methylotrophic yeasts. *Appl. Microbiol. Biotechnol.* 54:741–750.
50. Gellissen G, Weydemann U, Strasser A W M, Piontek M, Janowicz Z A, Hollenberg C P (1992). Progress in developing methylotrophic yeasts as expression systems. *Trends Biotechnol.* 10:413–417.
51. Cregg J M, Lin Cereghino J L, Shi J, Higgins D R (2000). Recombinant protein expression in *Pichia pastoris*. *Mol. Biotechnol.* 16:23–52.
52. Swinkels B W, van Ooyen A J J, Bonekamp F L (1993). The yeast *Kluyveromyces lactis* as an efficient host for heterologous gene expression. *Antonie van Leeuwenhoek* 64:187–201.
53. Schaffrath R, Breuning K D (2000). Genetics and molecular physiology of the yeast *Kluyveromyces lactis*. *Fungal Genet. Biol.* 30:173–190.
54. Gellissen G, Hollenberg C P (1997). Application of yeasts in gene expression studies: A comparison of *Saccharomyces cerevisiae*, *Hansenula polymorpha* and *Kluyveromyces lactis*—a review. *Gene* 190:87–97.
55. Guisez Y, Tison B, Vandekerckhove J, Demolder J, Bauw G, Haegeman G, Fiers W, Contreras R (1991). Production and purification of recombinant human interleukin-6 secreted by the yeast *Saccharomyces cerevisiae*. *Eur. J. Biochem.* 198:217–222.
56. Kjeldsen T (2000). Yeast secretory expression of insulin precursors. *Appl. Microbiol. Biotechnol.* 54:277–286.
57. Kjeldsen T, FrostPettersson A, Hach M (1999). Secretory expression and characterization of insulin in *Pichia pastoris*. *Biotechnol. Appl. Biochem.* 29:79–86.
58. Chow T Y, Ash J J, Dignard D, Thomas D Y (1992). Screening and identification of a gene, PSE-1, that affects protein secretion in *Saccharomyces cerevisiae*. *J. Cell Sci.* 101:709–719.
59. Turner B G, Avgerinos G C, Melnick L M, Moir D T (1991). Optimization of pro-urokinase secretion from recombinant *Saccharomyces cerevisiae*. *Biotechnol. Bioengin.* 37:869–875.
60. Sleep D, Belfield G P, Ballance D J, Steven J, Jones S, Evans L R, Moir P D, Goodey A R (1991). *Saccharomyces cerevisiae* strains that overexpress heterologous proteins. *Bio/technol.* 9:183–187.
61. Fleer R (1992). Engineering yeast for high level expression. *Curr. Opin. Biotechnol.* 3:486–496.
62. Poletti A, Weigel N L, McDonnell D P, Schrader W T, O'Malley B W, Conneely O M (1992). A novel, highly regulated, rapidly inducible system for the expression of chicken progesterone receptor, cPR_A, in *Saccharomyces cerevisiae*. *Gene* 114:51–58.
63. Purvis I J, Chotai D, Dykes C W, Lubahn D B, French F S, Wilson E M, Hobden A N (1991). An androgen-inducible expression system for *Saccharomyces cerevisiae*. *Gene* 106:35–42.
64. Romanos M A, Makoff A J, Fairweather N F, Beesley K M, Slater D E, Rayment F B, Payne M M, Clare J J (1991). Expression of tetanus toxin fragment C in yeast: Gene

synthesis is required to eliminate fortuitous polyadenylation sites in AT-rich DNA. *Nucl. Ac. Res.* 19:1461–1467.

65. Fleer R, Chen X J, Amellal N, Yeh P, Fournier A, Guinet F, Gault N, Faucher D, Folliard F, Fukuhara H, Mayaux J F (1991a). High-level secretion of correctly processed recombinant human interleukin-1β in *Kluyveromyces lactis*. *Gene* 107:285–295.
66. Fleer R, Yeh P, Amellal N, Maury I, Fournier A, Bacchetta F, Baduel P, Jung G, L'Hôte H, Becquart J (1991b). Stable multicopy vectors for high level secretion of recombinant human serum albumin by *Kluyveromyces lactis*. *Bio/technol.* 9:968–975.
67. Jeenes D J, Mackenzie D A, Roberts I N, Archer D B (1991). Heterologous protein production by filamentous fungi. *Biotechnol. Genet. Eng. Rev.* 90:327–367.
68. Radzio R, Kück U (1997). Synthesis of biotechnologically relevant heterologous proteins in filamentous fungi. *Proc. Biochem.* 32:529–539.
69. Gouka R J, Punt P J, van den Hondel C (1997). Efficient production of secreted proteins by *Aspergillus*: Progress limitations and prospects. *Appl. Microbiol. Biotechnol.* 47:1–11.
70. Conesa A, Punt P J, van Luijk N, van den Hondel C (2001). The secretion pathway in filamentous fungi: A biotechnological view. *Fungal Genet. Biol.* 33:155–171.
71. Punt P J, van Biezen 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.
72. Reiter M, Blüml G (1994). Large-scale mammalian cell culture. *Curr. Opin. Biotechnol.* 5:175–179.
73. Ikonomou L, Schneider Y J, Agathos S N (2003). Insect culture for industrial production of recombinant proteins. *Appl. Microbiol. Biotechnol.* 62:1–20.
74. Birch J R, Froud S J (1994). Mammalian cell culture system for recombinant protein production. *Biologicals* 22:127–133.
75. McCarroll L, King L A (1997). Stable insect cultures for recombinant protein production. *Curr. Opin. Biotechnol.* 8:590–594.
76. Betenbaugh M J, Ailor E, Whiteley E, Hinderliter P, Hsu T A (1996). Chaperone and foldase coexpression in the baculovirus-insect cell expression system. *Cytotechnology* 20:149–159.
77. Gervais A, Hammel Y A, Pelloux S, Lepage P, Baer G, Carte N, Sorokine O, Strub J M, Koerner R, Leize E, Van Dorsselaer A (2003). Glycosylation of human recombinant gonadotropins: Characterization and batch-to-batch consistency. *Glycobiology* 13:179–189.
78. Tate C G, Haase J, Baker C, Boorsma M, Magnani F, Vallis Y, Williams D C (2003). Comparison of seven different heterologous protein expression systems for the production of the serotonin transporter. *Biochim. Biophys. Acta.* 1610:141–153.
79. Grampp G E, Sambanis A, Stephanopoulos G N (1992). Use of regulated secretion in protein production from animal cells: An overview. *Adv. Biochem. Engin. Biotechnol.* 46:35–62.
80. Wurm F, Bernard A (1999). Large-scale transient expression in mammalian cells for recombinant protein production. *Curr. Opin. Biotechnol.* 10:156–159.
81. Hesse F, Wagner R (2000). Developments and improvements in the manufacturing of human therapeutics with mammalian cell cultures. *Trends Biotechnol.* 18:173–180.
82. Vapnek P (1991). Choosing a production system for recombinant proteins. *Biol. Rec. Microorg. Anim. Cells* 34:1–14.

83. Brooks S A (2004). Appropriate glycosylation of recombinant proteins for human use: implications of choice of expressions system. *Mol. Biotechnol.* 28:241–255.
84. Sodoyer R (2004). Expression systems for the production of recombinant pharmaceuticals. *BioDrugs* 18:51–62.
85. Sutherland A, Auclair K, Vederas J C (2001). Recent advances in the biosynthetic studies of lovastatin. *Curr. Opin. Drug Discov. Devel.* 4:229–236.
86. Schmidt F R (2004b). The challenge of multidrug resistance: Actual strategies in the development of novel antibacterials. *Appl. Microbiol. Biotechnol.* 63:335–343.
87. Parekh S, Vinci V A, Strobel R J (2000). Improvement of microbial strains and fermentation processes. *Appl. Microbiol. Biotechnol.* 54:287–301.
88. Penalva M A, Rowlands R T, Turner G (1998). The optimization of penicillin biosynthesis in fungi. *TIBTECH* 16:483–489.
89. Rowlands R T (1992). Strain improvement and strain stability. In: Finkelstein D B, Ball C (eds.), *Biotechnology of Filamentous Fungi, Technology and Products*, CRC Press, Boca Raton, FL, pp. 41–64.
90. Rowlands R T (1984). Industrial strain improvement: mutagenesis and random screening procedures. *Enzyme Microb. Technol.* 6:3–10.
91. Lal R, Khanna R, Kaur H, Khanna M, Dhingra N, Lal S, Gartemann K H, Eichenlaub R, Ghosh P K (1996). Engineering antibiotic producers to overcome the limitations of classical strain improvement programs. *Crit. Rev. Microbiol.* 22:201–255.
92. Brakhage A A, Sprote P, Al-Abdallah Q, Gehrke A, Plattner H, Tuncher A (2004). Regulation of penicillin biosynthesis in filamentous fungi. *Adv. Biochem. Eng. Biotechnol.* 88:45–90.
93. Schmitt E K, Hoff B, Kück U (2004). Regulation of cephalosporin biosynthesis. *Adv. Biochem. Eng. Biotechnol.* 88:1–43.
94. Brakhage A A (1998). Molecular Regulation of β-lactam biosynthesis in filamentous fungi. *Microbiol. Mol. Biol. Rev.* 62:547–585.
95. Martin J F (1998). New aspects of genes and enzymes for β-lactam antibiotic synthesis. *Appl. Microbiol. Biotechnol.* 50:1–15.
96. Skatrud P L (1992). Genetic engineering of β-lactam antibiotic biosynthetic pathways in filamentous fungi. *TIBTECH* 10:324–329.
97. Brakhage A A, Turner G (1995). Biotechnical genetics of antibiotic synthesis. In: Kück U (ed.), *The Mycota II, Genetics and Biotechnology*, Springer, Berlin, pp. 263–285.
98. Schmidt F R (2002). Beta-Lactam Antibiotics: Aspects of Manufacture and Therapy. In Osiewacz H D (ed.), *The Mycota X; Industrial Applications*, Springer, Berlin, pp. 69–91.
99. Zhang J, Wolfe S, Demain A L (1988). Phosphate repressible and inhibitible β-lactam synthetases in *Cephalosporium acremonium* strain C-10. *Appl. Microbiol. Biotechnol.* 29:242–247.
100. Menne S, Walz M, Kück U (1994). Expression studies with the bidirectional pcbAB-pcbC promotor region from *Acremonium chrysogenum* using reporter gene fusions. *Appl. Microbiol. Biotechnol.* 42:57–66.
101. Guiterrez S, Velasco J, Fernandez F J, Martin J F (1992). The cefG gene of *Cephalosporium acremonium* is linked to the *cefEF* gene and encodes a deacetylcephalosporin C acetyltransferase closely related to homoserin O-acetyltransferase. *J. Bacteriol.* 174:3056–3064.
102. Smith D J, Bull J H, Edwards J, Turner G (1989). Amplification of the isopenicillin N synthase gene in a strain of *Penicillium chrysogenum* producing high levels of penicillin. *Mol. Gen. Genet.* 216:492–497.

103. Smith D J, Burnham M R K, Bull J H, Hodgson J E, Ward J M, Browne P, Brown J, Barton B, Earl A J, Turner G (1990). β-lactam antibiotic biosynthetic genes have been conserved in clusters in prokaryotes and eukaryotes. *EMBO J.* 9:741–747.
104. Barredo J L, Diez B, Alvarez E, Martin J F (1989). Large amplification of 35-kb DNA fragment carrying two penicillin biosynthetic genes in high penicillin producing strains of *P. chrysogenum*. *Curr. Genet.* 16:453–459.
105. Fierro F, Barredo J L, Diez B, Guiterrez S, Fernandez J, Martin J F (1995). The penicillin cluster is amplified in tandem repeats linked by conserved hexanucleotide sequences. *Proc. Natl. Acad. Sci. USA* 92:6200–6204.
106. Newbert R W, Barton B, Greaves P, Harper J, Turner G (1997). Analysis of a commercially improved *Penicillium chrysogenum* strain series: involvement of recombinogenic regions in amplification and deletion of the penicillin biosynthesis gene cluster. *J. Ind. Microbiol. Biotechnol.* 19:18–27.
107. Brakhage A A, Browne P, Turner G (1994). Analysis of penicillin biosynthesis and the expression of penicillin biosynthesis genes of *Aspergillus nidulans* by targeted disruption of the acvA gene. *Mol. Gen. Genet.* 242:57–64.
108. Kück U, Walz M, Mohr G, Mracek M (1989). The 5′-sequence of the isopenicillin N-synthetase gene (pcbC) from *Cephalosporium acremonium* directs the expression of the prokaryotic hygromycin B phosphotransferase gene (hph) in *Aspergillus niger*. *Appl. Microbiol. Biotechnol.* 31:358–365.
109. Walz M, Kück U (1991). Polymorphic karyotypes in related *Acremonium* strains. *Curr. Genet.* 19:73–76.
110. Skatrud P L, Queener S W (1989). An electrophoretic molecular karyotype for an industrial strain of *Cephalosporium acremonium*. *Gene* 79:331–338.
111. Smith A W, Collis K, Ramsden M, Fox H M, Peberdy J F (1991). Chromosome rearrangements in improved cephalosporin C producing strains of *Acremoinium chrysogenum*. *Curr. Genet.* 18:235–237.
112. DeModena J A, Gutierrez S, Velasco J, Fernandez F J, Fachini R A, Galazzo J L, Hughes D E, Martin J E (1993). The production of cephalosporin C by *Acremonium chrysogenum* is improved by the intracellular expression of a bacterial hemoglobin. *Bio/Technology* 11:926–929.
113. Mathison L, Soliday C H, Stepan T, Aldrich T, Rambosek J (1993). Cloning, characterization, and use in strain improvement of the *Cephalosporium acremonium* gene *cefG* encoding acetyl transferase. *Curr. Genet.* 23:33–41.
114. Schmidt F R (2005a). About the nature of RNA interference. *Appl. Microbiol. Biotechnol.* 67:429–435.
115. Hannon G J, Rossi J J (2004). Unlocking the potential of the human genome with RNA interference. *Nature* 431:371–378.
116. Wassenegger M (2002). Gene silencing-based disease resistance. *Transgenic. Res.* 11:639–653.
117. Schmidt F R, Davis N D, Diener U L, Lemke P A (1983). Cycloheximide induction of aflatoxin synthesis in a nontoxigenic strain of *Aspergillus flavus*. *Bio/technology (Nature)* 1:794–795.
118. Schmidt F R, Lemke P A, Esser K (1986). Viral influences on aflatoxin formation by *Aspergillus flavus*. *Appl. Microbiol. Biotechnol.* 24:248–252.
119. Schmidt F R (2004c). RNA interference detected 20 years ago? *Nat. Biotechnol.* 22:267–268.
120. Pandit N N, Russo V E (1992). Reversible inactivation of a foreign gene, hph, during the asexual cycle in *Neurospora crassa* transformants. *Mol. Gen. Genet.* 234:412–422.

121. Romano N, Macino G (1992). Quelling: transient inactivation of gene expression in *Neurospora crassa* by transformation with homologous sequences. *Mol. Microbiol.* 6:3343–3353.
122. Pickford A S, Catalanotto C, Cogoni C, Macino G (2002). Quelling in *Neurospora crassa*. *Adv. Genet.* 46:277–303.
123. Liras P, Rodriguez-Garcia A, Martin J F (1998). Evolution of the clusters of genes for beta-lactam antibiotics: A model for evolutive combinatorial assembly of new beta-lactams. *Int. Microbiol.* 1:271–278.
124. Nielsen J (2001). Metabolic engineering. *Appl. Microbiol. Biotechnol.* 55:263–285.
125. Weber T, Welzel K, Pelzer S, Vente A, Wohhleben W (2003). Exploiting the genetic potential of polyketide producing streptomycetes. *J. Biotechnol.* 106:221–232.
126. Kantola J, Kunnari T, Mantsala P, Ylihonkoa K (2003). Expanding the scope of aromatic polyketides by combinatorial biosynthesis. *Comb. Chem. High Throughput Screen* 6:501–512.
127. Hutchinson C R, Fujii I (1995). Polyketide synthase gene manipulation: A structure-function approach in engineering novel antibiotics. *Annu. Rev. Microbiol.* 49:201.
128. Katz L, Donadio S (1993). Polyketide synthesis: prospects for hybrid antibiotics. *Annu. Rev. Microbiol.* 47:875–912.
129. Floss H G (2001). Antibiotic synthesis: from natural to unnatural products. *J. Ind. Microbiol. Biotechnol.* 27:183–194.
130. Pfeifer B A, Khosla C (2001). Biosynthesis of polyketides in heterologous hosts. *Microbiol. Mol. Biol. Rev.* 3:106–118.
131. Staunton J, Wilkinson B (2001). Combinatorial biosynthesis of polyketides and non-ribosomal peptides. *Curr. Opin. Chem. Biol.* 5:159–164.
132. McDaniel R, Katz L (2001). Genetic engineering of novel macrolide antibiotics. In: Lohner K (ed.), *Development of Novel Antimicrobial Agents: Emerging Strategies.* Horizon Scientific Press Norwich, U.K., pp. 45–60.
133. Du L, Shen B (2001). Biosynthesis of hybrid peptide–polyketide natural products. *Curr. Opin. Drug. Discov. Dev.* 4:215–228.
134. Du L, Sanchez C, Shen B (2001). Hybrid peptide—polyketide natural products: Biosynthesis and prospects towards engineering novel molecules. *Metab. Eng.* 3:78–95.
135. Kleinkauf H, von Döhren H (1995). Linking peptide and polyketide biosynthesis. *J. Antibiot.* 48:563–567.
136. Cane D E, Walsh C T (1999). The parallel and convergent universes of polyketide synthases and non-ribosomal peptide synthases. *Chem. Biol.* 6:R319–325.
137. Marahiel M A, Stachelhaus T, Mootz H D (1997). Modular peptide synthases involved in nonribosomal peptide synthesis. *Chem. Rev.* 97:2651–2673.
138. Mankelow D P, Neilan B A (2000). Non-ribosomal peptide antibiotics. *Expert Opin. Ther. Pat.* 10:1583–1591.
139. Trauger J W, Walsh C T (2000). Heterologous expression in *Escherichia coli* of the first module of nonribosomal peptide synthase for chloroeremomycin, a vancomycin-type glycopeptide antibiotic. *Proc. Natl. Acad.* 97:3112–3117.
140. Kaneda T (1977). Fatty acids of the genus Bacillus: an example of branched chain preference. *Bacteriol. Rev.* 41:391–418.
141. Aretz W, Meiwes J, Seibert G, Vobis G, Wink J (2000). Friulimicins: Novel lipopetide antibiotics with peptidoglycan synthesis inhibiting activity from *Actinoplanes friulensis* sp. Nov. I. Taxonomic studies of the producing microorganism and fermentation. *J. Antibiotics* 53:807–815.

142. Vertesy L, Ehlers E, Kogler H, Kurz M, Meiwes J, Seibert G, Vogel M, Hammann P (2000). Friulimicins: Novel lipopetide antibiotics with peptidoglycan synthesis inhibiting activity from *Actinoplanes friulensis* sp. nov. II. Isolation and structural characterization. *J. Antibiotics* 53:816–827.

143. Vertesy L, Aretz W, Decker H, Ehlers E, Kurz M, Schmidt F R, Knauf M, Kogler H (1999). Lipopetide calcium salts: Process for their preparation and their use. *EP* 1068223.

144. Boeck L D, Wetzel R W (1990). A54145, A new lipopeptide antibiotic complex: factor control through precursor directed biosynthesis. *J. Antibiotics* 43:607–615.

145. Counter F T, Allen N E, Fukuda D S, Hobbs J N, Ott J, Ensminger P W, Mynderse J S, Preston D A, Wu C Y E (1990). A54145 A new lipopetide antibiotic complex: microbiological evaluation. *J. Antibiotics* 43:616–622.

146. Zmijewski M J, Briggs B, Occolowitz J (1986). Role of branched chain fatty acid precursosrs in regulating factor profile in the biosynthesis of A21978 complex. *J. Antibiot* 39:1483–1485.

147. Stephenson J (2001). Researchers describe latest strategies to combat antibiotic resistant microbes. *JAMA* 285:2317–2318.

148. Tally F P, DeBriun F (2000). Development of daptomycin for Gram-positive infections. *J. Antimicrob. Chemo.* 46:523–526.

149. Hancock R E W, Patrzykat A (2002). Clinical development of cationic antimicrobial peptides: From natural to novel antibiotics. *Curr. Drug Targets Infect. Disord.* 2:79–83.

150. Mor A (2000). Peptide–based antibiotics: a potential answer to raging antimicrobial resistance. *Drug Dev. Res.* 50:440–447.

151. Larsson G, Törnkvist M, Wernersson E S, Trägard C, Noorman H, Enfors S O (1996). Substrate gradients in bioreactors: origin and consequences. *Bioproc. Engin.* 14:281–289.

152. Bylund F, Collet E, Enfors E O, Larsson G (1998). Substrate gradient formation in the large scale lowers cell yield and increases byproduct formation. *Bioproc. Eng.* 18:171–180.

153. Bylund F, Guillard F, Enfors S O, Trägardh C, Larsson G (1999). Scale down of recombinant production: a comparative study of scaling performance. *Bioproc. Engin.* 20:377–289.

154. Bylund F, Castan A, Mikkola R, Veide A, Larsson G (2000). Influence of scale-up on the quality of recombinant human growth hormone. *Biotechnol. Bioengin.* 69:119–128.

155. Enfors S O, Jahic M, Rozkov A, Xu B, Hecker M, Jürgen B, Krüger B, Schweder T, Hamer G, O'Beirne D, Noisommit-Rizzi N, Reuss M, Boone L, Hewitt C, McFarlane C, Nienow A, Kovacs T, Trägardh C, Fuchs L, Revstedt J, Friberg P C, Hjertager B, Blomsten G, Skogman H, Hjort S, Hoeks F, Lin H J, Neubauer P, van der Lans R, Luyben K, Vrabel P, Manelius A (2001). Physiological responses to mixing in large scale bioreactors. *J. Biotechnol.* 85:175–185.

156. Xu B, Jahic M, Bomsten G, Enfors S O (1999). Glucose overflow metabolism and mixed acid fermentation in aerobic large-scale fed-batch processes with *Echerichia coli*. *Appl. Microbiol. Biotechnol.* 51:564–571.

157. Castan A, Enfors S O (2001). Formate accumulation due to DNA release in aerobic cultivations of *Escherichia coli*. *Biotechnol. Bioengin.* 77:324–328.

158. Kwint K, Nachin L, Diez A, Nyström T (2003). The bacterial universal stress protein: Function and regulation. *Curr. Opin. Microbiol.* 6:140–145.

159. Mogk A, Tomoyasu T, Goloubinoff P, Rudiger S, Roder D, Langen H, Bukau B (1999). Identification of thermolabile *Escherichia coli* proteins: Prevention and reversion of aggregation by DnaK and ClpB. *EMBO J.* 18:6934–6949.

160. Umakoshi H, Kuboi R, Komaswa I, Tsuchido T, Matsumura Y (1998). Heat-induced translocation of cytoplasmic beta-galactosidase across inner membrane of *Escherichia coli*. *Biotechnol. Prog.* 14:210–217.

161. Schweder T, Krüger E, Xu B, Jürgen B, Blomsten G, Enfors S O, Hecker M (1999). Monitoring genes that respond to process-related stress in large-scale bioprocesses. *Biotechnol. Bioengin.* 65:151–159.

162. Fenton D, Lai P H, Lu H, Mann M, Tsai L (1997). Control of norleucine incorporation into recombinant proteins. U.S. Patent 5599690.

162a. Martinez-Force E, Benitez T (1992). Changes in yeast amino acid pool with respiratory versus fermentative metabolism. *Biotechnol. Bioengi.n* 40:843–849.

162b. Hondorp ER, Matthews RG (2004). Oxidative stress inactivates cobalamin-independent methionine synthase (MetE) in Escherichia coli. *PLoS Biology* 2:1138–1158.

162c. Hoffmann F, Rinas U (2004). Stress induced by recombinant protein production in *Escherichia coli*. *Adv. Biochem. Eng. Biotechnol.* 89:73–92.

163. Weickert M J, Apostol I (1998). High-fidelity translation of recombinant human hemoglobin in *Escherichia coli*. *Appl. Env. Microbial.* 64:1589–1593.

164. Muramatsu R, Negishi T, Mimoto T, Miura A, Miosawa S, Hayashi H (2002). Existence of β-methylnorleucine in recombinant hirudin produced by *Escherichia coli*. *J. Biotechnol.* 93:131–142.

165. Apostol I, Levine J, Lippincott J, Leach J, Hess E, Glascock C B, Weickert M J, Blackmore R (1997). Incorporation of norvaline at leucine positions in recombinant human hemoglobin expressed in *Escherichia coli*. *J. Biol. Chem.* 272:28980–28988.

166. Kiick K L, Weberskirch R, Tirell D A (2001). Identification of an expanded set of translationally active methionine analogues in *E. coli*. *FEBS Lett.* 502:25–30.

167. Friehs K (2003). Plasmid copy number and plasmid stability. *Adv. Biochem. Engin. Biotechnol.* 86:47–82.

168. Thiry M, Cingolani D (2002). Optimizing scale-up fermentation processes. *Trends Biotechnol.* 20:103–105.

169. Lin H Y, Neubauer P (2000). Influence of controlled glucose oscillations on a fed-batch process of recombinant *Escherichia coli*. *J. Biotechnol.* 79:27–37.

170. Neubauer P, Lin H Y, Mathiszik B (2003). Metabolic load of recombinant protein production: Inhibition of cellular capacities for glucose uptake and respiration after induction of a heterologous gene in *Escherichia coli*. *Biotechnol. Bioengin.* 83:53–64.

171. Ansorge M B, Kula M R (2000). Production of recombinant L-leucine dehydrogenase from Bacillus cereus in pilot scale using the runaway replication system *E. coli* (pIET98). *Biotechnol. Bioengin.* 68:557–562.

172. Nordstrom K, Uhlin B E (1992). Runaway-replication plasmids as tools to produce large quantities of proteins from cloned genes in bacteria. *Biotechnology* 6:661–666.

173. van de Merbel N C (1997). The use of ultra-filtration and column liquid chromatography for an on-line fermentation monitoring. *Trends. Anal. Chem.* 16:162–173.

174. Buchholz A, Takors R, Wandrey C (2001). Quantification of intracellular metabolites in *Escherichia coli* K12 using liquid chromatographic electrospray ionization tandem mass spectrometric techniques. *Anal. Biochem.* 295:129–137.

175. Matz G, Lennemann F (1996). On-line monitoring of biotechnological processes by gas chromatographic—mass spectrometric analysis of fermentation suspensions. *J. Chromatogr.* 750:141–149.

176. Klyushnichenko V (2004). Capillary electrophoresis in the analysis and monitoring of biotechnological processes. *Methods Mol. Biol.* 276:77–120.
177. Nishi H, Tsumagari N, Kakimoto T, Terabe S (1989). Separation of β-lactam antibiotics by micellar electro-kinezic chromatography. *J. Chromatogr.* 477:259–270.
178. Nishi H, Fukuyama T, Matsuo M (1990). Separation and determination of aspoxicillin in human plasma by micellar electrokinetic chromatography with direct sample injection. *J. Chromatogr.* 515:245–255.
179. Gabig-Ciminska M, Holmgren A, Andresen H, Bundvik Barken K, Wumpelmann M, Albers J, Hintsche R, Breitenstein A, Neubauer P, Los M, Czyz A, Wegrzyn G, Silfversparre G, Juregn B, Schweder T, Enfors S O (2004). Electric chips for rapid detection and quantification of nucleic acids. *Biosens. Bioelectron.* 15:537–546.
180. Hewitt C J, Nebe-von Caron G (2001). An industrial application of multiparameter flow cytometry: Assessment of cell physiological state and its application to the study of microbial fermentations. *Cytometry* 44:179–187.
181. Hewitt C J, Nebe-Von-Caron G (2004). The application of multi-parameter flow cytometry to monitor individual microbial cell physiological state. *Adv. Biochem. Eng. Biotechnol.* 89:197–223.
182. Hewitt C J, Nebe-von Caron G, Axelsson B, McFarlane C M, Nienow A W (2000). Studies related to the Scale-up of high-cell-density *E. coli* fed-batch fermentations using multiparameter flow cytometry: Effect of a changing microenvironment with respect to glucose and dissolved oxygen concentration. *Biotechnol. Bioengin.* 70:381–390.
183. Kell D B, Ryder H M, Kaprelyants A S, Westerhoff H V (1991). Quantifying heterogenity—flow cytometry of bacterial cultures. *Anton. van Leeuwen. Int. J. Gen. Mol. Microbiol.* 60:145–158.
184. Harms P, Kostov Y, Rao G (2002). Bioprocess monitoring. *Curr. Opin. Biotechnol.* 13:124–127.
185. Schaefer U, Boos W, Takors R, Weuster-Bootz D (1999). Automated sample device for monitoring intracellular metabolite dynamics. *Anal. Biochem.* 270:88–96.
186. Rocha I, Ferreira E C (2002). On-line simultaneous monitoring of glucose and acetate with FIA during high cell density fermentation of recombinant *E. coli*. *Bioelectrochem.* 56:127–129.
187. Tothill I E, Newman J D, White S F, Turner A P F (1997). Monitoring of the glucose concentration during microbial fermentation using a novel mass-producible biosensor suitable for on-line use. *Enzyme Microb. Technol.* 20:590–596.
188. Schügerl K, Brandes T, Dullau K, Holzhauer-Rieger K, Hotop S, Hübner U, Wu X, Zhou W (1991). Fermentation monitoring and control by online flow injection and liquid chromatography. *Anal. Chim. Acta.* 249:87–100.
189. Huang Y L, Foellmer T J, Ang K C, Khoo S B, Yap M G S (1995). Characterization and application of an on-line flow injection analysis/wall-jet electrode system for glucose monitoring during fermentation. *Anal. Chim. Acta.* 317:223–232.
190. Vaidyanathan S, Macaloney G, Vaughan J, McNeil B, Harvey L M (1999). Monitoring of submerged bioprocesses. *Crit. Rev. Biotechnol.* 19:277–316.
191. Arndt M, Hitzmann B (2004). Kalman filter based glucose control at small set points during fed-batch cultivation of Saccharomyces cerevisiae. *Biotechnol. Prog.* 1:377–383.
192. Kleist S, Miksch G, Hitzmann B, Arndt M, Friehs K, Flaschel E (2003). Optimization of the extracellular production of bacterial phytase with *Escherichia coli* by using different fed-batch fermentation strategies. *Appl. Microbiol. Biotechnol.* 61:456–462.

193. Spichiger-Keller U E (1997). Ion- and substrate specific optode membranes and optical detection modes. *Sensors and Actuators, B: Chemical* B38:68–77.

194. Wolfbeis O S (2002). Fiber-optic chemical sensors and biosensors. *Anal. Chem.* 74:2663–2678.

195. Griffiths D, Hall G (1993). Biosensors—what real progress is being made. *Trends Biotechnol.* 11:122–130.

196. Boehl D, Solle D, Hitzmann B, Scheper T (2003). Chemometric modeling with two-dimensional fluorescence data for Claviceps purpurea bioprocess characterization. *J. Biotechnol.* 105:179–188.

197. Solle D, Geissler D, Stark E, Scheper T, Hitzmann B (2003). Chemometric modeling based on 2D-fluorescence spectra without a calibration measurement. *Bioinformatics* 19:173–177.

198. Stark E, Hitzmann B, Schügerl K, Scheper T, Fuchs C, Koster D, Märkl H (2002). In-situ-fluorescence probes: a useful tool for non-invasive bioprocess monitoring. *Adv. Biochem. Eng. Biotechnol.* 74:21–38.

199. Hall J W, McNeil B, Rollins M J, Draper I, Thompson B G, Macaloney G (1996). Near-infrared spectroscopic determination of acetate, ammonium, biomass and glycerol in an industrial *Escherichia coli* fermentation. *Appl. Spectroscopy* 50:102–108.

200. Vaccari G, Dosi E, Campi A L, Gonzalez-Vara A, Matteuzi D, Mantovani G (1994). A near infrared spectroscopy technique for the control of fermentation processes: an application to lactic acid fermentation. *Biotechnol. Bioengin.* 43:913–917.

201. Macaloney G, Hall J W, Rollins M J, Draper I, Thompson B G, McNeil B (1994). Monitoring biomass and glycerol in an *Escherichia coli* fermentation using near-infrared spectroscopy. *Biotechnol. Techn.* 8:281–286.

202. Hammond S V (1992). NIR analysis of antibiotic fermentations. In: Murray I, Cowe I A (eds.), *Making Light Work: Advances in Near Infrared Spectroscopy*, vol 1. VCH Publishers, New York, pp. 584–589.

203. Hammond S V, Brookes I K (1992). Near infrared spectroscopy—a powerful technique for at-line and on-line analysis of fermentations. In: Ladisch M R, Bose A (eds.), *Harnessing Biotechnology for the 21st Century: Proceedings of the 9th International Symposium and Exhibition*, *Am. Chem. Soc.*, Washington, DC, pp. 325–333.

204. Pollard D, Buccino R, Connors N, Kirschner T, Olewinski R, Saini K, Slamon P (2001). Real-time analyte monitoring of a fungal fermentation, at a pilot scale, using in mid-infrared spectroscopy. *Bioproc. Biosyst. Engin.* 24:13–24.

205. Dickinson T A, White J, Kauer J S, Walt D R (1998). Current trends in artificial nose technology. *TIBTECH* 16:250–258.

206. Turner C, Rudnitskaya A, Legin A (2003). Monitoring batch fermentations with an electronic tongue. *J. Biotechnol.* 103:87–91.

207. Moser I, Jobst G, Urban G A (2002). Biosensor array for simultaneous measurement of glucose, lactate, glutamate, and glutamine. *Biosens. Bioelectron.* 17:297–302.

208. Collins G E, Wu P, Lu Q, Ramsey J D, Bromund R H (2004). Compact, high voltage power supply for the lab-on-chip. *Lab. Chip.* 4:408–411.

209. Neils C, Tyree Z, Finlayson B, Folch A (2004). Combinatorial mixing of microfluidic streams. *Lab. Chip.* 4:342–350.

210. Farinas J, Chow A W, Wada H G (2001). A microfluidic device for measuring cellular membrane potential. *Anal. Biochem.* 295:138–142.

211. Ferreira L S, de Souza M B, Folly R O M (2001). Development of an alcohol fermentation control system based on biosensor measurements interpreted by neural networks. *Sens. Act B: Chem.* 75:166–171.

212. Chen V C P, Rollins D K (2000). Issues regarding artificial neural network modeling for reactors and fermenters. *Bioproc. Biosyst. Engin.* 22:85–93.
213. Trelea I C, Titica M, Landaud S, Latrille E, Corrieu G, Cheruy A (2001). Predictive modeling of brewing fermentation: From knowledge-based to black box models. *Math Comp. Sim.* 56:405–424.
214. Lin H Y, Mathiszik B, Xu B, Enfors S O, Neubauer P (2001). Determinantion of the maximum specific uptake capacities for glucose and oxygen in glucose-limited fed-batch cultivations of *Escherichia coli*. *Biotechnol. Bioengin.* 73:347–357.
215. Lee S Y (1996). High-cell density culture of *Escherichia coli*. *Trends Biotechnol.* 14:98–105.
216. Kim C H, Rao K J, Youn D J, Rhee S K (2003). Scale-up of recombinant hirudin production from *Saccharomyces cerevisiae*. *Biotechnol. Bioproc. Engin.* 8:303–305.
217. Wong I, Hernandez A, Garcia M A, Segura R, Rodriguez I (2002). Fermentation scale-up for recombinant K99 antigen production cloned in *Escherichia coli* MC1061. *Proc. Biochem.* 37:1195–1199.
218. Yim S C, Jeong K J, Chang H N, Lee S Y (2001). High-level secretory production of human granulocyte-colony stimulating factor by fed-batch culture of recombiant *E. coli*. *Bioproc. Biosyst. Engin.* 24:249–254.
219. Dantigny P, Ninow J L, Lakrori M (1991). A new control strategy for yeast production based on the L/A approach. *Appl. Microbiol. Biotechnol.* 36:352–357.
220. Cant S (2002). High-performance computing in computational fluid dynamics: Progress and challenges. *Philosoph. Transact.: Mathem. Phys. Engin. Sci.* 360:1211–1225.
221. Schmalzriedt S, Jenne M, Mauch K, Reuss M (2003). Integration of physiology and fluid dynamics. *Adv. Biochem. Eng. Biotechnol.* 80:19–68.
222. Alves S S, Vasconcelos J M T (1996). Optimization of agitation and aeration in fermenters. *Bioproc. Biosyst. Engin.* 14:119–123.
223. Oniscu C, Galaction A I, Cascaval D, Ungureanu F (2001). Modeling of mixing in stirred bioreactors 1. Mixing time for non-aerated simulated broths. *Roum. Biotechnol. Lett.* 6:119–129.
224. Diaz A, Acevedo F (1999). Scale-up strategy for bioreactors with Newtonian and non-Newtonian broths. *Bioproc. Engin.* 21:21–23.
225. Russel A B, Thomas C R, Lilly M D (1995). Oxygen transfer measurements during yeast fermentations in a pilot scale airlift fermenter. *Bioproc. Biosyst. Engin.* 12:71–79.
226. Türker M (2004). Development of biocalorimetry as a technique for process monitoring and control in technical scale fermentations. *Thermochimica Acta.*, In Press.
227. Anderson R K I, Jayaraman K, Voisard D, Marison I W, von Stockar U (2002). Heat flux as an on-line indicator of metabolic activity in pilot scale bioreactor during the production of *Bacillus thuringiensis* var *galleriae*-based pesticides. *Thermochimica Acta.* 386:127–138.
228. Hubbard D W (1987). Scaleup strategies for bioreactors containing non-newtonian broths. *Ann. NY Acad. Sci.* 506:600–607.
229. Junker B H, Stanik M, Barna C, Salmon P, Buckland B C (1998). Influence of impeller type on mass transfer in fermentation vessels. *Bioproc. Biosyst. Engin.* 19:403–413.
230. Wang D I C, Cooney C L (1997). Translation of laboratory, pilot, and plant scale data. In: Wang D I C, et al. (eds.), *Fermentation and enzyme technology.* Wiley, New York, pp. 194–211.
231. Yawalkar A A, Heesink A B M, Versteeg G F, Vishwas G P (2002). Gas-liquid mass transfer coefficient in stirred tank reactors. *Can. J. Chem. Engin.* 80:840–848.

232. Flores E R, Perez F, de la Torre M (1997). Scale-up of *Bacillus thuringiensis* fermentation based on oxygen transfer. *J. Ferm. Bioengin.* 83:561–564.
233. Riesenberg D, Menzel K, Schulz V, Schumann K, Veith G, Zuber G, Knorre W A (1990). High cell density fermentation of recombinant *Escherichia coli* expressing human interferon alpha 1. *Appl. Microbiol. Biotechnol.* 34:77–82.
234. Berkholz R, Guthke R (2001). Model based sequential experimental design for bioprocess optimization—an overview. *Focus Biotechnol.* 4:129–141.
235. Berkholz R, Rohlig D, Guthke R (2000). Data and knowledge based experimental design for fermentation process optimization. *Enzyme Microb. Technol.* 27:784–788.
236. Liden G (2002). Understanding the bioreactor. *Bioproc. Biosyst. Engin.* 24:273–279.
237. Drossard J (2004). Downstream processing of plant-derived recombinant therapeutic proteins. In: Fischer R, Schillberg S (eds.), *Molecular Farming.* Wiley-VCH, Weinheim, Germany.
238. Roque A C, Lowe C R, Taipa M A (2004). Antibodies and genetically engineered related molceules: Production and purification. *Biotechnol. Prog.* 20:639–554.
239. Jungbauer A, Hahn R (2004). Enegineering protein A affinity chromatography. *Curr. Opin. Drug Discov. Devel.* 7:248–256.
240. Burgess R R, Thompson N E (2002). Advances in gentle immunoaffinity chromatography. *Curr. Opin. Biotechnol.* 13:304–308.
241. Hage D S (1999). Affinity chromatography: A review of clinical applications. *Clin. Chem.* 45:593–615.
242. Murphy M B, Fuller S T, Richardson P M, Doyle S A (2003). An improved method for the in vitro evolution of aptamers and applications in protein detection and purification. *Nucl. Ac. Res.* 31:18–110.
243. Romig T S, Bell C, Drolet D W (1999). Aptamer affinity chromatography: combinatorial chemistry applied to protein purification. *J. Chromatogr. B Biomed. Sci. Appl.* 731:275–284.
244. Hubbuch J, Thommes J, Kula M R (2005). Biochemical engineering aspects of expanded bed adsorption. *Adv. Biochem. Eng. Biotechnol.* 92:101–123.
245. Anspach F B, Curbelo D, Hartmann R, Garke G, Deckwer W D (1999). Expanded bed chromatography in primary protein purification. *J. Chromatogr. A* 865:129–144.
246. Guiochon G (2002). Preparative liquid chromatography. *J. Chromatogr. A* 965:129–161.
247. Cunha T, Aires-Barros R (2002). Large-scale extraction of proteins. *Mol. Biotechnol.* 20:29–40.
248. Krishna S H, Srinivas N D, Raghavarao K S, Karanth N G (2002). Reverse micellar extraction for downstream processing of proteins/enzymes. *Adv. Biochem. Eng. Biotechnol.* 75:119–183.
249. Walter J (2000). Scale-up of downstream processing. *J. Chromatogr. Libr.* 61:765–783.
250. Kadir F, Hamers M (2002). Production and downstream processing of biotech compounds. In: Crommelin D J A, Sindelar R D (eds.), *Pharmaceutical Biotechnology* (2nd ed.). Taylor and Francis Ltd, London, U.K., pp. 53–71.
251. Kalyanpur M (2002). Downstream processing in the biotechnology industry. *Mol. Biotechnol.* 22:87–98.
252. Chisti Y (1998). Strategies in downstream processing. In: Subramanian G (ed.), *Bioseparation and Bioprocessing* (2nd ed.). Wiley-VCH, Weinheim, Germany, pp. 3–30.

253. Desai M A (2002). *Downstream Processing of Proteins.* Humana Press, Totowa, NJ.
254. Bhat S T, et al. (2005). *Chemistry of natural products.* Springer, Berlin, Germany.
255. Verrall M S (1996). Downstream processing of natural products. A practical handbook. Wiley, New York.
256. Gosh A C, Bora M M, Dutta N N (1996). Developments in liquid membrane separation of beta-lactam antibiotics. *Bioseparation* 6:91–105.
257. Gosh A C, Mathur R K, Dutta N N (1997). Extraction and purification of cephalosporin antibiotics. In: Scheper T (ed.), *Advances in Biochemical Engineering/ Biotechnology*, vol 56. Springer-Verlag, Berlin, pp. 111–145.
258. Bandak S I, Tumak M R, Allen B S, Bolzon L D, Preston D A (2000). Assessment of the susceptibility of Streptococcus pneumoniae to cefaclor and loracarbef in 13 countries. *J. Chemother.* 12:299–305.
259. Troxler R, von Grävenitz A, Funke G, Wiedemann B, Stock I (2000). Natural antibiotic susceptibility by *Listeria* species: *L. grayi, L. innocua, L. ivanovii, L. monocytogenes, L. seeligeri* and *L. welshimeri* strains. *Clin. Microbiol. Infect.* 6:525–535.
260. Saling P (2005). Eco-efficiency analysis of biotechnological processes. *Appl. Microbiol. Biotechnol.* 68:1–8.
261. Parmar A, Kumar H, Marwaha S S, Kennedy J F (1998). Recent trends in enzymatic conversion of cephalosporin C to 7-aminocephalosporanic acid (7-ACA). *Cri. Rev. Biotechnol.* 18:1–12.
262. Bianchi D, Bortolo R, Golini P, Cesti P (1998). Application of immobilized enzymes in the manufacture of beta-lactam antibiotics. *Chim. In. (Milan)* 80:875–885.
263. Binder R G, Numata K, Lowe D A, Murakami T, Brown J L (1993). Isolation and characterization of a *Pseudomonas* strain producing glutaryl-7-aminocephalosporanic acid acylase. *Appl. Env. Microbiol.* 59:3321–3326.
264. Isogai T (1997). New processes for production of 7-aminocephalosporanic acid from cephalosporin. *Drugs Pharm. Sci.* 82:733–751.
265. Gomez A, Rodriguez M, Ospina S, Zamora R, Merino E, Bolivar F, Ramirez O T, Quintero R, Lopez-Munguia A (1994). Strategies in the design of a penicillin acylase process. In: Galindo R, Ramirez OT (eds.), *Advances in Bioprocess Engineering.* Kluwer, Dodrecht, pp. 29–40.
266. Bruggink A, Roos E C, de Vroom E (1998). Penicillin acylase in the industrial production of beta-lactam antibiotics. *Org. Process Res. Dev.* 2:128–133.
267. Shewale J G, Sudhakaran V K (1997). Penicillin V acylase: Its potential in the production of 6-aminopenicillanic acid. *Enzyme Microb. Technol.* 20:402–410.
268. Stoppok E, Wagner F, Zadrazil F (1981). Identification of a penicillin V acylase processing fungus. *Eur. J. Appl. Microbiol. Biotechnol.* 13:60–61.
269. Schneider W J, Roehr M (1976). Purification and properties of penicillin acylase of *Bovista plumbea*. *Biochimica. Biophysica. Acta*. 452:177–185.
270. Brandl D, Kleiber W (1972). Verfahren zur Herstellung von 6-Aminopenicillansäure. Austrian Patent no. 297923, Vienna, Austria.
271. Singh K, Sehgal S N, Vezina C (1967). Conversion of penicillin V to 6-aminopenicillanic acid by the use of spores. U.S. Patent no. 3.305.453.
272. Wilms J, de Vroom E (1998). Preparation of β-lactam antibiotics. European Patent no. 0201688.
273. Schmidt F R (2005b). Optimization and scale-up of industrial fermentation processes. *Appl. Microbiol. Biotechnol.* 68:425–435.