[12] N.H., Sigal, F.C. Dumont, *Ann. Rev. Immunol.* **1992**, *10*, 519.
[13] F.J. Dumont, M.J. Staruch, S.L. Koprak, M.R. Melino, N.H. Sigal, *J. Immunol.* **1990**, *144*, 251.
[14] J.J. Fung, S. Todo, A. Tzakis, A. Demetris, A. Jain, K. Abu-Elmagd, M. Allessiani, T.E. Starzl, *Transplant. Proc.* **1991**, *23* (1), 14.
[15] T.S. Chen, B.H. Arison, L.S. Wicker, E.S. Inamine, R.L. Monaghan, *J. Antibiotics* **1992**, *45* (1), 118.
[16] C. Ruby, U. S. Pat. **1989** 5,272,068.
[17] C. Ruby, U. S. Pat. **1992**, 5,324,644.
[18] A. Shafiee, T.S. Chen, P. Cameron, *Applied and Environmental Microbiology* **1995**, *61* (10), 3544.
[19] T.S. Chen, B.H. Arison, L.S. Wicker, E.S. Inamine, *J. Antibiotics* **1992**, *45* (4), 577.
[20] S.H. Vincent, B.V. Karanam, S.K. Painter, S-H.L. Chiu, *Archives of Biochemistry and Biophysics* **1992**, *294* (2), 454.
[21] A., Shafiee, H. Motamedi, T.S. Chen, *Eur. J. Bochem.* **1994**, *225*, 755.
[22] A. Shafiee, H. Motamedi, T.S. Chen, L. Kaplan, Fifth ASM Conference on Genetic and Molecular Biology of Industrial Microorganisms, **1992**, Oct. 11–15, Bloomington, Indiana.
[23] A. Shafiee, T.S. Chen, B.S. Arison, F.J. Dumont, L. Colwell, L. Kaplan, *J. Antibiotics* **1993**, *46* (9), 1397.
[24] T.S. Chen, B. Petuch, R. White, G. Dezeny, X. Li, B. Arison, T. Beattie, L. Colwell, F. Dumont, R. Monaghan, *J. Antibiotics* **1994**, *47* (12), 1557.
[25] B.R. Petuch, B. Arison, A. Hsu, R. Monaghan, F.J. Dumont, T.S. Chen, *J. Ind. Microbiology* **1994**, *13*, 131.
[26] J. Liu, J.D. Farmer, Jr., W.S. Lane, J. Friedman, I. Weissman, S.L. Schreiber, *Cell* **1991**, *66*, 807.
[27] J. Liu, M.W. Albers, T.J. Wandless, S. Luan, D.G. Alberg, P.J. Belshaw, P. Cohen, C. MacKintosh, C.B. Klee, S.L. Schreiber, *Biochemistry* **1992**, *31*, 3896.
[28] G.D. VanDuyne, R.F. Standaert, P.A. Karplus, S.L. Schreiber, J. Clardy, *Science* **1991**, *252*, 839.
[29] J.M. Moore, D.A. Peattie, M.J. Fitzgibbon, J.A. Thomson, *Nature* **1991**, *351*, 248.
[30] S.W. Michnick, M.K. Rosen, T.J. Wandless, M. Karplus, S.L. Schreiber, *Science* **1991**, *252*, 836.
[31] F.J. Dumont, M.J. Staruch, S.L. Koprak, M.R. Melino, N.H. Sigal, *J. Immunol.* **1990**, *144*, 251.

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For glutaryl-7-ACA acylase (*EC 3.5.1.3*), expressed in *Escherichia coli*, isolation and immobilization of the enzyme on a commercial carrier seems more attractive from a regulatory point of view. The immobilized enzyme shows very high operational stability, which may compensate for the costs of the carrier.

Despite its lower stability, cephalosporin C acetylesterase (*EC 3.1.1.41*), expressed in *E. coli*, was also immobilized on a commercial carrier for regulatory reasons. Moreover, extensive development of immobilized whole cells seemed economically not acceptable for this low-volume product.

A mathematical model for the enzymatic cleavage showed limitations of a combined application of two biocatalysts in a stirred tank reactor, *e.g.*, in terms of product yield.

1. Introduction

7-Aminocephalosporanic acid (7-ACA) is a key intermediate in the production of more than 50 semi-synthetic cephalosporin antibiotics, such as cefotaxime, cefpodoxime, cefazolin, ceftazidime, and ceftriaxone. The ever-increasing market volume of 7-ACA was estimated to reach almost 2,000 tons p.a. by the year 2000, representing a market value of approximately USD 400 million [1].

Starting with the fermentation, isolation and subsequent chemical cleavage of CephC in organic systems, solid 7-ACA can finally be obtained in *ca.* 95% purity by precipitation, filtration, and drying. The enzymatic cleavage can avoid the use of hazardous chemicals and solvents, and thus may have a positive impact on both the economics of the process and the environment. In addition, CephC does not necessarily have to be precipitated and dried, as it is typically required for the chemical cleavage in organic solvents. Finally, it opens a more economic way to prepare deacetyl-7-aminocephalosporanic acid (HACA), which is another key intermediate in cephalosporin derivatization.

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[13] F.J. Dumont, M.J. Staruch, S.L. Koprak, M.R. Melino, N.H. Sigal, *J. Immunol.* **1990**, *144*, 251.
[14] J.J. Fung, S. Todo, A. Tzakis, A. Demetris, A. Jain, K. Abu-Elmagd, M. Allessiani, T.E. Starzl, *Transplant. Proc.* **1991**, *23* (1), 14.
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A two-step enzymatic cleavage of CephC into 7-ACA (*Scheme 1*) has been developed, using immobilized D-amino acid oxidase (DAO) and glutaryl-7-ACA acylase (GAC) [2], whereas the optional addition of cephalosporin C acetylesterase (CAE) leads to HACA. *Biochemie* [3] will invest Euro 43 million in a new plant at its Frankfurt production site, which will have a yearly capacity of over 400 tons of 7-ACA. In order to obtain the highest quality, the enzymatic cleavage will be combined with a highly advanced purification method.

Rather than discussing biochemical issues, this paper gives a broad industrial view on economic, technical, and regulatory aspects considered during the development of the chemo-enzymatic process (CEP), and primarily focuses on the enzymes involved. The importance of these aspects, as well as their interrelations, are discussed with respect to the development of the process, but neither company-related specific issues, *e.g.*, availability of solvent-recovery systems and equipment, the capacity for development, the approval for investment, nor patent issues are considered.

1.1. *Economic Aspects*

One of the main driving forces for the development of a CEP was the possibility to improve the economics of the 7-ACA/HACA production process. It seemed obvious that the overall costs of an enzymatic product are directly determined by the costs of biocatalyst preparation and its required quantity. Compared to the costs of enzyme purification and immobilization, those of fermentation and harvesting tend to represent a minor share. In contrast, the use of commercial carriers increases the costs of immobilization significantly, but a high specific activity of the resulting biocatalyst might compensate for this.

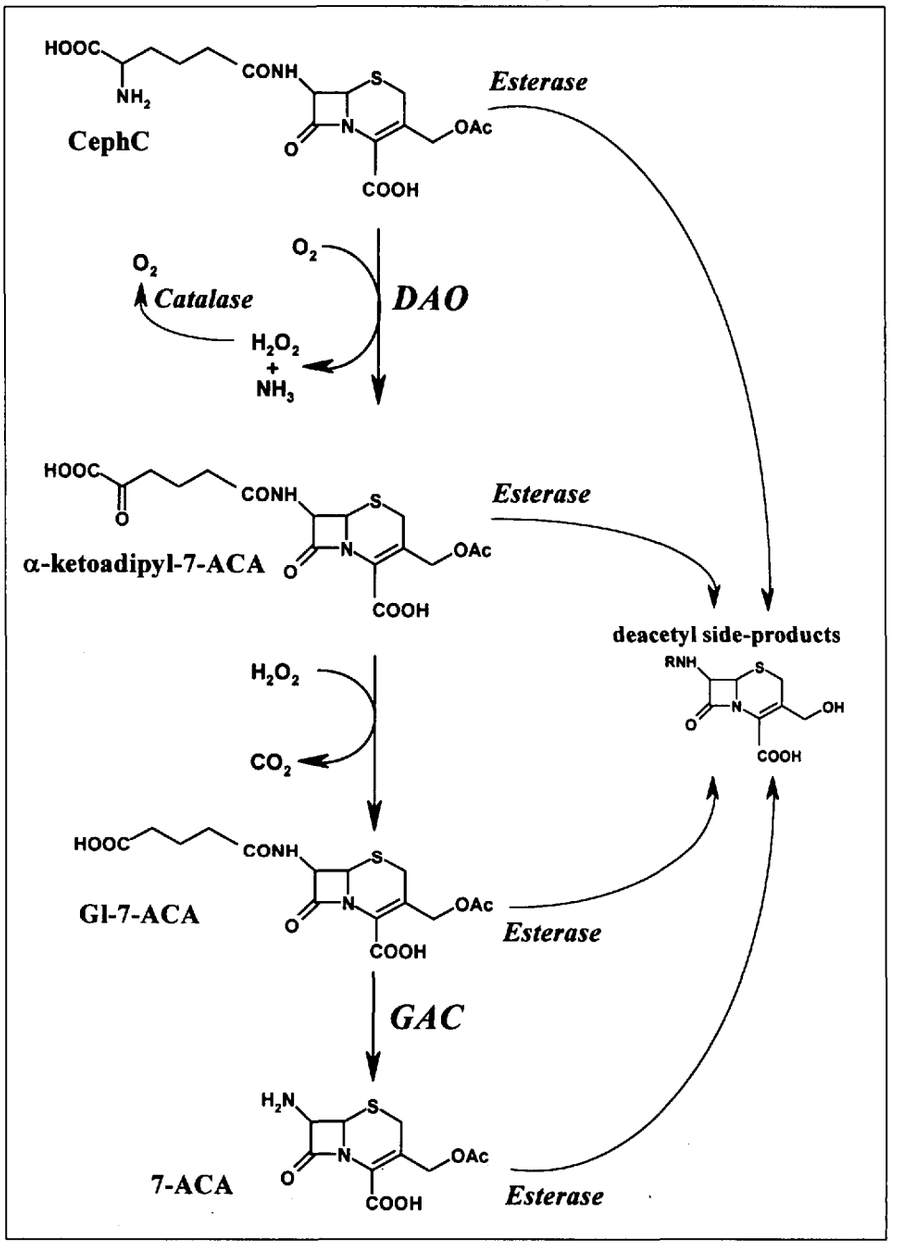
At the beginning of process development, the selection of the best route for biocatalyst preparation (see *Sect. 2*) was extremely difficult, as one must objectively consider costs and consumption of the enzyme, with the required reliable data on operational stability (*Sect. 3.3.*) being hard to come by. Finally, side-activities would lower the yield of conversion and thereby would have a direct negative impact on the economics of the process.

According to the literature [4–9] and our own observations, DAO seemed to have a low stability showing a life-time of only *ca.* 100 operational cycles in a stirred tank reactor (*Sect. 3.3.*). In order to develop a highly economic process, immobilization either of crude enzyme on a cheap carrier (*Sect. 2.3.2.*), or of whole cells (*Sect. 2.3.1.*) seemed to be favorable.

In contrast, GAC was regarded as a stable enzyme, which allowed more costly procedures for its isolation and the use of an advanced enzyme carrier. Tests under operational conditions with a prototype biocatalyst eventually confirmed this assumption.

Although preliminary data indicated a low stability of the CAE, the expression system and behaviour of the enzyme in purification and immobilization seemed so similar to that of GAC, that it made sense to use an analogous approach. Moreover, the expected product volume of HACA was estimated to be much lower than that of 7-ACA, so that it seemed more useful to keep costs and risks for process development low, rather than to develop a low-cost method for the preparation of CAE.

Scheme 1. *Desired and Undesired Products During the Enzymatic Cleavage of Cephalosporin C*



List of Abbreviations:

7-ACA	7-aminocephalosporanic acid
CAE	cephalosporin C acetylhydrolase = esterase (*EC 3.1.1.41*)
CEP	chemo-enzymatic process
CephC	cephalosporin C
DAO	D-amino acid oxidase = oxidase (*EC 1.4.3.3*)
GAC	glutaryl-7-ACA acylase = acylase (*EC 3.5.1.3*)
Gl-7-ACA	glutaryl-7-aminocephalosporanic acid
HACA	hydroxy-7-ACA = deacetyl-7-aminocephalosporanic acid
U	International Units = μmol per minute

1.2. *Technical Aspects*

Regarding large-scale applications of biocatalysts, features like specific activity and mechanical robustness were highly important. In a stirred tank reactor, the biocatalyst particles must withstand the mechanical stress of handling and stirring. Abrasion would lead not only to increased separation times during product isolation and to possible product contamination, but could also cause early replacement of the biocatalyst.

In order to enable fast and easy separation of product and biocatalyst, excellent hydrodynamic properties were aimed at. In this respect, larger biocatalyst particles (>300 μm) were preferred, as they also allow to drain off eventual microbial contaminants. On the other hand, the use of smaller particles (<300 μm) may cause a higher apparent specific activity [10], and consequently a significantly higher immobilization yield, which in turn lowers the costs of enzyme preparation.

Independent of their size, the surface characteristics of the particles may favor the adhesion and proliferation of microbial contamination, thus leading to decreased operational stability and higher costs of the biocatalyst.

1.3. *Quality and Regulatory Aspects*

As *Biochemie* has been one of the leading suppliers of 7-ACA on the world market, one more driving force for a CEP was the policy of production of the highest quality. Enzyme-quality features, such as minimal side-activities, excellent mechanical properties, and resistance towards microbial contamination were known to be highly relevant regarding large-scale applications.

During enzymatic cleaving of CephC, typical side-products and intermediates, such as 7-ACA-sulfoxide, deacetyl-cephalosporin derivatives, Gl-7-ACA, and α-ketoadipyl-7-aminocephalosporanic acid may be formed, whereas others may result from the precipitation procedure of 7-ACA or from the quality of the starting CephC.

7-ACA shows some instability in alkaline aqueous environment, needed for optimal enzymatic cleavage, which, to a minor extent, causes the non-enzymatic side-reaction of 7-ACA into HACA. Moreover, DAO and GAC do not distinguish between deacetyl-, deacetoxy- and cephalosporin C, so that these substrate impurities are all converted into their 7-ACA derivatives, which are hard to eliminate without additional purification steps.

In the planning of an enzymatic production of 7-ACA, the regulatory requirements of the final pharmaceutically active cephalosporin derivatives has to be considered. Even in the case of genetically modified microorganisms, this means that no enzymes originating from pathogenic organisms should be used, and that the inserted DNA sequence must not contain any sections, which are irrelevant to the protein to be expressed. Furthermore, to avoid pyrogenic contamination, *e.g.*, originating from the application of *E. coli*-based enzymes, an ultrafiltration step and/or an effective crystallization of 7-ACA is recommended. The use of biocatalysts originating from fungi or yeasts is regarded to be less pretentious in this respect.

2. Enzyme Preparation

2.1. *Strains and Fermentation*

Fermentation of *Trigonopsis variabilis* seemed the best way for obtaining DAO with high activity (>5 kU/kg broth), although, for example, the aeration and the nitrogen-source have to be limited, and enzyme production has to be induced by expensive D-methionine or D-alanine [4][8].

For the production of GAC and CAE, the cloning and heterologous expression in *E. coli* was more useful, although additional safety and regulatory aspects had to be considered. Besides the increased fermentation yields (>10 kU/kg broth), recombinant enzymes can be expressed without special media needs, making fermentation easier than with the native microorganism. However, by using recombinant organisms for large-scale fermentation, problems might arise from uncontrolled over-expression of the enzyme or from plasmid instability, which, of course, would have a negative impact on the economics of the process.

2.2. *Harvesting, Pre-Treatment, Storage*

On a technical scale, fermentation broth of 1–30 m^3 volume, which is the starting material for preparation of both immobilized whole cells (*Sect. 2.3.1.*) and immobilized enzymes (*Sect. 2.3.2.*), required enough stability to allow storage for several days. To reduce the volume for storage and to improve stability, the fermentation broth can be concentrated about 5- to 10-fold by means of centrifugation or microfiltration. In the case of *Trigonopsis variabilis* or *E. coli*, the cell slurry may be either frozen as ice-flakes, allowing storage at –18° without loss of activity for months, or just chilled to 5–10°, which imposes a direct use for immobilization within a few days, but saves on investment and energy.

For immobilization of the whole cells (*Sect. 2.3.1.*), additional pre-treatments might be necessary to preserve the target activity or to inactivate undesirable side-activities selectively. The DAO-activity in *Trigonopsis variabilis*, for example, can be stabilized by addition of glutardialdehyde (0.3% *w/w*) to the fresh fermentation broth [11].

2.3. *Immobilization*

2.3.1. *Immobilization of Whole Cells*

Bioconversions with cells have routinely been used in analytical screening for new enzyme activities, but for technical applications, the suitability of cell preparations might be limited by activity and stability. For example, their specific activity depends on the quality of the starting cell slurry, and is usually lowered during addition of a polymer for immobilization, which increases mass and limits diffusion. Furthermore, the operational stability of whole cells may be eventually limited by its tolerance towards mechanical stress.

In the case of recombinant bacteria expressing GAC and CAE, there is an additional safety issue, especially in the production of pharmaceutical compounds, originating from possible bleeding of pyrogenics and from partial detachment of cells, as well as from loss of DNA fragments thereof.

In the special case of application of *free cells* [12], additional problems – like product contamination with soluble and insoluble cell-decomposition products – have to be solved, *e.g.*, by investing on ultrafiltration equipment, which in turn would lead to an increased consumption of energy and to a loss of product. Consequently, to avoid a cell-disruption procedure, *i.e.*, an expensive homogenizer or *Dyno*™ mill, eventual enzyme-purification steps, as well as the use of costly carriers (*Sect. 2.3.2.*), the approach of *immobilization of cells* may be recommended.

Since yeasts do not have pyrogenics and their cell wall is known to be very stable, a process for the immobilization of DAO based on whole cells of the yeast *Trigonopsis variabilis* seemed preferable.

Although several immobilizations of whole cells have been published [13–16], not many seemed to meet the requirements for large-scale application, especially with respect to successful multicyle use. However, the immobilization of whole cells by co-polymerization with acrylamide may lead to biocatalysts suitable for industrial use [17], but the radical

polymerization mechanism of acryl amide caused a significant decrease of DAO activity in *Trigonopsis variabilis*. Finally, milder systems of cell-embedding, *e.g.*, co-polymerization with polyethylene imine after suspension in organic solvent, showed to be successful (*Textbox 1*). The resulting biocatalyst had a specific DAO activity of *ca.* 100 U/g dry weight (*Sect. 3.1.*) and was used in a stirred tank reactor for *ca.* 100 cycles (*Sect. 3.3.*).

It is known from literature that, in the case of diffusion limitations caused by the cell wall, problems with poor apparent specific activity of whole cells might be overcome by permeabilization with agents such as organic solvents, alkaline solutions, detergents, *etc.* [11][18–20]. Usually, this is performed *before* the immobilization of the cells, but here the combined interaction with polyethylene imine at pH 9 and with the organic solvent during immobilization resulted in an improved biocatalyst. Compared to the *apparent* activity of the starting material (cell slurry), the biocatalyst was obtained with *ca.* 70% (apparent) activity yield by this method.

Textbox 1. Preparation of DAO by Immobilization of Cells of *Trigonopsis variabilis* [2]

Glutardialdehyde stabilization: Cells of *Trigonopsis variabilis* ATCC 58536 (DAO activity: 25970 U) are pre-treated by adding glutardialdehyde (21 g, 25% in water) to the fermentation broth (2.7 kg, containing 100 g dry cell mass) and allowed to stand 1 h at r.t. and pH 7.5. The cells are harvested by centrifugation. DAO Activity in the concentrated cell mass is 26350 U
Polymer treatment: The cell mass (735 ml = 100 g cell dry weight) is suspended with β-mercaptoethanol (5 mM), ethylenediaminetetraacetic acid (EDTA, 2 mM) and polyethylene imine solution (40 g, 50% in water, 600–1000 kDa) in water (*ca.* 800 ml total volume) and stirred slowly for approx. 90 min at pH 11, which is adjusted by the addition of a NaOH solution at r.t., whereafter the pH of the mixture is adjusted to pH 9 by the addition of H_3PO_4. DAO Activity: 24360 U (94% of pre-treated DAO activity).
Cross-linking: In a stirring vessel, the cell/polymer mixture is added to *n*-tributylphosphate (3000 ml) at r.t. A homogeneous dispersion of the cell/polymer droplets (100–600 μm) in n-tributylphosphate is obtained within *ca.* 30 min, and glutardialdehyde solution (40 g, 25% in water) is added. Cross-linking starts immediately and is completed after 60 min. The solid, spherical particles are isolated and washed with tap water. After draining, the particles are hardened by slowly stirred incubation in glycerol (2000 g) for 1 h.
After removal of glycerol by draining and washing, moist, solid spherical particles (580 g) are obtained, corresponding to 121 g of dry weight. Specific DAO activity in the solid spherical particles is 32 U/g moist weight (153 U/g dry weight), *i.e.*, total DAO activity is 18560 U (71% of the pre-treated DAO activity) (*Sect. 2.3.1.*).
Phenylmethylsulfonyl fluoride (PMSF) treatment: Glycerol-hardened particles (270 g) are suspended in phosphate buffer pH 7.0 (1 l, 20 mM), and phenylmethylsulfonyl fluoride solution (100 ml EtOH, 10 mg/ml) is added under stirring (*Sect. 2.4.*).
After incubation at r.t. for *ca.* 3 h, the particles are removed.
The DAO activity in the solid spherical particles is 148 U/g dry weight (*i.e.*, approx. 7400 U).

2.3.2. *Immobilization of Isolated Enzymes*

The use of immobilized enzymes as partly purified proteins on carriers seemed to have some clear technical advantages over immobilized cells, as *a*) the mechanical and chemical properties of the carrier are specified and guaranteed by the supplier, *b*) the quality of biocatalyst is less dependent on the quality of the fermentation broth as a result of enzyme purification steps, and *c*) it enables a faster and less expensive development of enzyme binding and stabilization, due to a simple development strategy.

Textbox 2. Isolation of GAC from *E. coli* [2]

A cell pellet (10 g) of the recombinant *E. coli* strain CCM 4229 (GAC activity: 720 U, *i.e.*, approx. 300 U/g dry weight), is isolated from the fermentation broth, washed, re-suspended in phosphate buffer pH 7.0 (50 ml, 50 mM), and homogenized under pressure (700 bar). The resulting cell homogenate is mixed with a flocculation agent (1 ml of *Sedifloc™ CL 900-18/40*) and incubated for 1 h at 40° under stirring, after which the pH is adjusted to 5.2 by the addition of AcOH. After a further hour at 10°, the mixture is clarified by centrifugation and the GAC-containing cell-free supernatant (GAC activity: 615 U) is adjusted to pH 7.5, mixed with phenylmethylsulfonyl fluoride solution (0.5 ml EtOH, 10 mg/ml) and incubated for 3 h. GAC activity of the cell-free extract obtained after phenylmethylsulfonyl fluoride treatment is determined with 622 U (section 2.4.).

However, these benefits require the establishment of a process for cell break-down and protein purification, although in the case of technical enzymes, quality demands are low, so that in most cases elaborated purification methods, *e.g.*, by columns, are avoidable. A coagulation with polyethylene imine at a slightly acidic pH and increased temperature may lead to a cell-debris-free enzyme-containing supernatant after centrifugation, subsequent to cell break-down (*Textbox 2*). This 'crude soluble enzyme' preparation of GAC, CAE, or DAO can be easily bound on commercial carriers and be used successfully for the CephC cleavage process. Still, homogenizers for bacteria or *Dyno*™ mills for yeasts are needed to extract the soluble enzyme mechanically, since methods of chemical or enzymatic cell lysis seem not satisfactory on a technical scale in this case. This equipment for cell break-down and separation on a large scale is expensive and may represent a significant part of the investment costs.

Several ways of immobilizing soluble enzymes were published, but not all of them might be successfully used on a technical scale. One of the first established carriers is *Eupergit*™ *C*, which has been widely used in industrial biocatalysis, since its oxirane groups easily covalently bind proteins, but the price of *Eupergit*™ *C* largely influences the costs of the biocatalysts.

Alternatively, it is known that glutardialdehyde can link amino groups of weak anion exchangers with the amino groups of proteins. For example, the resins *Amberlite*™ *MB1* [21] and *Duolite*™ *A365* [22] only require a pre-treatment with glutardialdehyde, whereafter the enzyme solution is added to the resin. The whole process of activation and binding takes only a few hours, whereas oxirane carriers have recommended incubation times of more than 24 hours.

Recently, binding of enzymes on strong anion exchangers [23] showed to be highly valuable for technical use. First, the enzyme is incubated with the ion exchanger for 1–6 hours at pH 7–8 to allow complete binding, without previous chemical treatment. Finally, cross-linking with glutardialdehyde is believed to build large protein clusters on the surface of the resin, fixing the enzymes irreversibly.

All carriers described above may be suitable for GAC, CAE, and DAO, but compared with oxirane carriers, ion-exchanger resins are usually much cheaper, and their production and application have been established for many decades. More-

over, procedures for immobilization on strong anion exchangers are relatively simple.

Apart from the costs of the carrier, the economics of a CEP is also determined by the specific activity (*Sect. 3.1.*) and the operational stability (*Sect. 3.3.*) of the biocatalysts. In this respect, there seemed to be no significant differences between the above-mentioned carriers.

2.4. *Reduction of Side-Activities*

From an economic point of view, a process route with only a few simple procedures resulting in a crude soluble-enzyme solution for immobilization seemed to be preferable. Although expensive and/or low-yield purification steps, *e.g.*, enzyme crystallization or chromatography, may contribute significantly to the total costs of biocatalysts, high-purity preparations (>90%) were known to be technically effective as well [10].

In the case of the production of pharmaceuticals, however, the approach of crude soluble enzyme is likely to cause problems regarding regulatory issues and may thus be less feasible (*Sect. 1.3.*), because the effective removal of enzymatic side-activities might be necessary in order to avoid side-products in the CEP.

Simple methods, like pH- or heat-treatment [13][24][25], selective inhibitors, or organic solvents can occasionally allow a sufficient decrease in side-activities, while the target enzyme is recovered almost completely. These one-step procedures may avoid the need for expensive technical equipment and seem very attractive for industrial applications.

Concerning the enzymatic cleavage of CephC, two side-activities are crucial (*Scheme 1*): *a*) esterase activity in GAC and DAO, and *b*) catalase activity in DAO. In general, β-lactamases only play a role in the case of microbial infections, and their appearance can be avoided by special hygienic handling or by addition of an antimicrobial agent.

Esterase in GAC and DAO may finally lead to an extensive accumulation of HACA, which lowers the yield and quality of the 7-ACA. But this side-activity might be removed easily by chromatography [4][26] or enzyme crystallization [27]. Selective coagulation of the homogenized cells of GAC-expressing *E. coli* with polyethylene imine at acidic pH and increased temperature may also be effective in the inactivation of esterase, but conditions are so harsh, that significant losses of GAC activity may occur. Therefore, specific inactivation of esterase by irreversible chemical inhibition with phenylmethylsulfonyl fluoride (PMSF) seems to be preferable. Applied in a low concentration for 3 h, this well-known agent completely blocks the active site of esterase, leaving the GAC fully intact (*Textbox 2*).

In the case of DAO, the selective inactivation of esterase in intact cells of *Trigonopsis variabilis* can be performed by chemical inhibition with Cu^{2+} [19], acetone [28], or heat treatment [29], but surprisingly, phenylmethylsulfonyl fluoride (PMSF) is also highly successful in inactivating this esterase (*Textbox 1*). The use of esterase-free GAC and DAO avoids the formation of up to 4% additional deacetyl derivatives relative to the starting concentration of CephC in both reaction steps, enabling an 8% overall increase of 7-ACA and an improved product quality.

Catalase in DAO preparations lowers the reaction yield in CephC cleavage. As shown in *Scheme 1*, hydrogen peroxide – produced during the oxidative deamination of CephC – is crucial for the subsequent decarboxylation of α-ketoadipyl-7-aminocephalosporanic acid into Gl-7-ACA. The competitive degradation of hydrogen peroxide by catalase (>150 U/g dry weight) can be so strong, that – even after the addition of extra hydrogen peroxide – the conversion of α-ketoadipyl-7-aminocephalosporanic acid into Gl-7-ACA is no longer complete. Chemical inactivation of catalase [30][31] with inhibitors such as aminotriazole, azide, ascorbate, or perborate seems insufficient with *Trigonopsis variabilis*, whereas physical methods like increased pH, organic solvents, or heat treatment result in catalase-free cells [32]. However, the effectiveness of inactivation may depend on the *Trigonopsis variabilis* strain.

Heat treatment for *Trigonopsis variabilis* seems preferable, because esterase and catalase are simultaneously inactivated by very short heating at alkaline pH, whereas the DAO activity is well preserved and the resulting cell slurry is suitable for immobilization of cells and for isolation of the enzyme as well [29].

2.5. *Ecological and Safety Aspects*

The increasing importance of ecology and occupational health in chemical industry is not only governed by ethical insight, but also by legal and administrative regulations. Apart from regulatory aspects concerning the pharmaceutical compound, safety issues for the preparation and handling of genetically modified organisms have to be considered. After intense debate during the last few years in Europe, the production and the use of recombinant process enzymes are now considered to be safe by the public and administration bodies [32].

In certain cases, technical means to reduce the output of living recombinant cells and the administrative permit for the production of enzymes using genetically modified organisms are still needed. However, by using strains of the lowest safety requirements (S1 level), these are met relatively easily and do not considerably limit the choice of enzyme source.

In contrast, emissions into the working area or into the environment, resulting from a chemical process, would lower the public acceptance for a facility, which would cause public reactions calling for a delay or even a shutdown of the production. Thus, the implementation of a CEP is an essential help in order to ensure the ecological improvement and the positive image of the company.

In the case of the enzymatic cleavage of CephC, some typical safety and environmental issues of biocatalyst preparation had to be considered during development. For example, the use of *Eupergit*™ *C* in immobilizations according to the recommendations of the manufacturer leads to an output of up to 0.5 kg inorganic phosphate per kg carrier in the wastewater. On a laboratory scale, sodium azide is a very helpful antimicrobial agent, yet for large-scale application such toxic substances may lead to problems in terms of storage, occupational health, and wastewater treatment. Thus, the possibilities of low-risk antimicrobial agents or procedures and equipment which keep the microbial contamination low should be considered at the beginning of a biocatalyst development.

A potential safety and environmental issue in the immobilization of cells (*Sect. 2.3.1.*) is the use of flammable solvents, which also requires an effective solvent recovery and even a special waste-water treatment process.

3. Enzyme Characterization

Essential for the economic and technical success of a CEP was the fulfilment of all the relevant biocatalyst-quality criteria, for which adequate analytical methods had to be established before or during the development.

The final operational conditions of an optimized CEP may significantly differ from the ones chosen at the beginning of development. Therefore, when optimizing a process, one has to determine the relevant empirical factors and to make sure that there is a correlation between the

results from analytical methods and the observed process data.

3.1. *Specific Activity*

DAO Activity can be assayed from the reaction products of CephC oxidation, either by HPLC analysis of α-ketoadipyl-7-aminocephalosporanic acid and Gl-7-ACA, by the oxygen consumption, or by the determination of hydrogen peroxide. Alternatively, D-alanine can be used as a substrate for DAO [8], but in this case, the correlation of the results of analysis with process data seems to be more problematic. Hydrolytic activities are normally determined by titration of glutaric acid during cleavage of Gl-7-ACA (GAC), or of acetic acid during the cleavage of CephC (CAE). This latter substrate is preferred as it does not involve the more allergenic 7-ACA.

To meet the specified conversion time at operational conditions, the amount of biocatalyst to be loaded to the CEP is calculated from its specific activity. This makes the reliability of the activity-assay crucial. However, the apparent specific activity of a biocatalyst is not a function of the intrinsic activity of the enzyme alone, but may likely be influenced by the diffusion limitations of substrates and products. During development of the biocatalyst and the CEP, these effects may vary strongly. Therefore, it may be difficult to interpret the results from the activity assay alone, without feed-back from the CEP or from additional investigations.

3.2. *Mechanical Features*

Usually, the abrasion and mechanical strength of a commercial carrier are specified by the supplier and do not need much attention by the user during development. A mechanical characterization of immobilized enzymes seems thus to be unnecessary.

In the case of immobilization of cells, mechanical and hydrodynamic features of the biocatalyst have to be carefully determined in assays especially developed for this purpose. Among these parameters, the particle-size distribution, the microscopic texture, and the behavior under compression, obtained from computer-controlled extrusion of the biocatalyst through a nozzle [33][34], seem to be crucial. The combined data of these parameters allow a simple and effective prediction of the robustness of a biocatalyst in the stirred tank reactor. Alternatively, measuring mechanical behavior by simulating operational conditions would need at least 100 hours of experimental work.

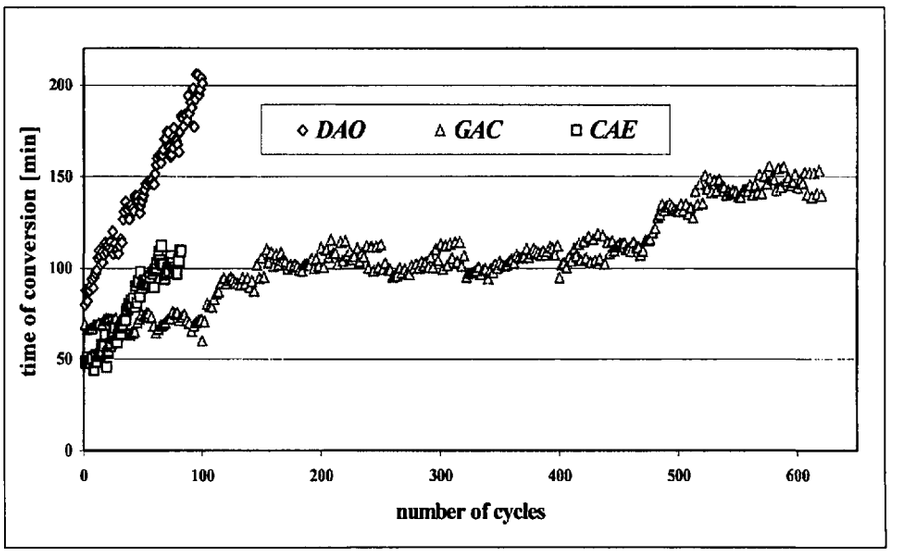


Fig. *Typical operational stability curves of immobilized DAO, GAC, and CAE*

Textbox 3. Test under Operational Conditions

On laboratory scale, a well-characterized sample of biocatalyst with known *analytical activity*, is applied to a temperature-controlled stirred tank reactor with bottom sieve. Before starting, the biocatalyst is conditioned and finally suspended in water. Within one reaction cycle, substrate solution is applied, and the control of process parameters (pH, pressure, dissolved oxygen) is started. The reaction can be monitored by HPLC or alternatively by on-line measurement of consumed NaOH solution in the case of GAC and CAE, or of oxygen-consumption in the case of DAO. After complete conversion of the substrate (after 100–200 min), the reactor is drained using a bottom valve. Each reaction cycle is finished by re-suspending the biocatalyst in water.

Enzyme consumption can be calculated from a plot of reaction time *vs.* cycle number (*Fig.*). In order to obtain reliable numbers, the tests are terminated not before the reaction time is twice the one of the starting cycle. Assuming a yield of 90% for each enzymatic step, the total product quantity can be easily calculated based on the number of cycles. The *enzyme consumption* is defined as the initially applied *analytical activity* divided by the total product quantity.

Especially in the case of the GAC, where the reaction is extended over 600 cycles (*i.e.*, >1000 h), special care should be taken concerning microbial contamination and growth, storage during shut-down (weekend), and reliability of the equipment (for instance pH control).

3.3. *Operational Stability*

Enzyme consumption strongly determines the economics of a CEP (*Sect. 1.1.*), and therefore has to be estimated during process development. One should take into account, however, that both the sensitivity of the enzyme to pH-, temperature-, and mechanical stress, and the operational circumstances like stirring, 'uncontrolled' pH-changes, microbial contamination, and exposure of the biocatalyst to air simultaneously may play a role in degradation of the biocatalyst.

So it may be distinguished between *a*) a *test under operational conditions* (repeated batch evaluation), which allows a more precise prediction of the behavior during production and of process economics, and *b*) 'analytical' methods which are more suited in optimization of biocatalyst preparation, *e.g.*, by indicating the optimal carrier or immobilization method.

Moreover, during *tests under operational conditions*, detailed information about conversion, yield, intermediate and by-products may be directly derived from the in-process-control data (HPLC), whereas the reactor effluent allows isolation and analysis of the product. But the latter procedures are very laborious and time consuming.

3.3.1. Test under Operational Conditions

In principle, a *test under operational conditions* is meant to simulate production conditions during a repeated batch evaluation in order to determine the enzyme consumption [4]. Under operational process conditions, defined amounts of

biocatalysts are applied on laboratory scale to automated stirred tank reactors with a bottom sieve, representing a scale-down model of the planned production facilities. The biocatalysts are repeatedly used until the initial conversion time is doubled. Subsequently, the enzyme consumption is calculated from the initially applied activity and the calculated total product yield from all the cycles (*Fig.*, *Textbox 3*).

In the case of DAO, approximately 100 cycles, *i.e.*, two weeks of testing were achieved, whereas the tests under operational conditions for GAC usually went on for more than 600 cycles. It seems obvious that during enzyme development or quality assurance such lengthy procedures are not acceptable. However, during the implementation of the enzymes into the CEP, tests under operational conditions are very important, also for the acquisition of data on process economics.

3.3.2. *Analytical Tests at Elevated Temperature*

In order to obtain comparable stability data on various carriers or procedures more quickly, an accelerated stability test at an elevated temperature (35–45°), shortening the life-time of the biocatalyst 4- to 8-fold, may be recommendable.

Moreover, the data of enzyme decay at various operating temperatures enables one to calculate the activation energy for enzyme decomposition, which in turn contributes to the mathematical modelling of the absolute enzyme productivity. The data needed can be collected in separated batch series at elevated fixed temperatures [35–37], or in a continuously working chemostat with gradual temperature shift [38][39]. Using this *Short Method for Activity and Stability*, a quick comparison of samples of GAC and CAE is possible within a few days per sample, independent of the stability of the biocatalyst.

During development of CAE, this method also allowed to select the optimal route for immobilization of the enzyme, and its predictions were later confirmed by *tests under operational conditions*. However by analytical tests at elevated temperatures, only the thermodynamic characteristics of the immobilized enzyme, but no long-term effects, such as microbial growth, are taken into account.

4. Enzyme Application

4.1. *Reactor Design*

A stirred tank reactor (2–10 m^3) with bottom sieve allows easy operation and maintenance and an efficient separation of product and biocatalyst. Moreover, it may save on investment costs compared with alternatives such as 'continuous-cascade-reactors' and 'column-reactors'.

Also, stirred tank reactors may well function in the case of GAC and CAE, which have high specific activity and excellent mechanical features resulting from the commercial carrier. However, poor mechanical properties and low specific activity of the biocatalyst would result in reduced filtration rates, *i.e.*, prolonged process time, which in turn would lower the production capacity and product yield. These parameters turned out to be crucial during the development of immobilized cells.

For the oxidative deamination of CephC, the aeration with pure oxygen at atmospheric pressure or with air under pressure requires an effective system for gas distribution at the inlet, as well as effective stirring. In addition, recycling of the gas might reduce emissions (*e.g.*, ammonia) and the need for the pH adjustment. To avoid microbial contamination, it is recommended to clean all gases used for the tank drain-off and the oxygen supply by sterile filters.

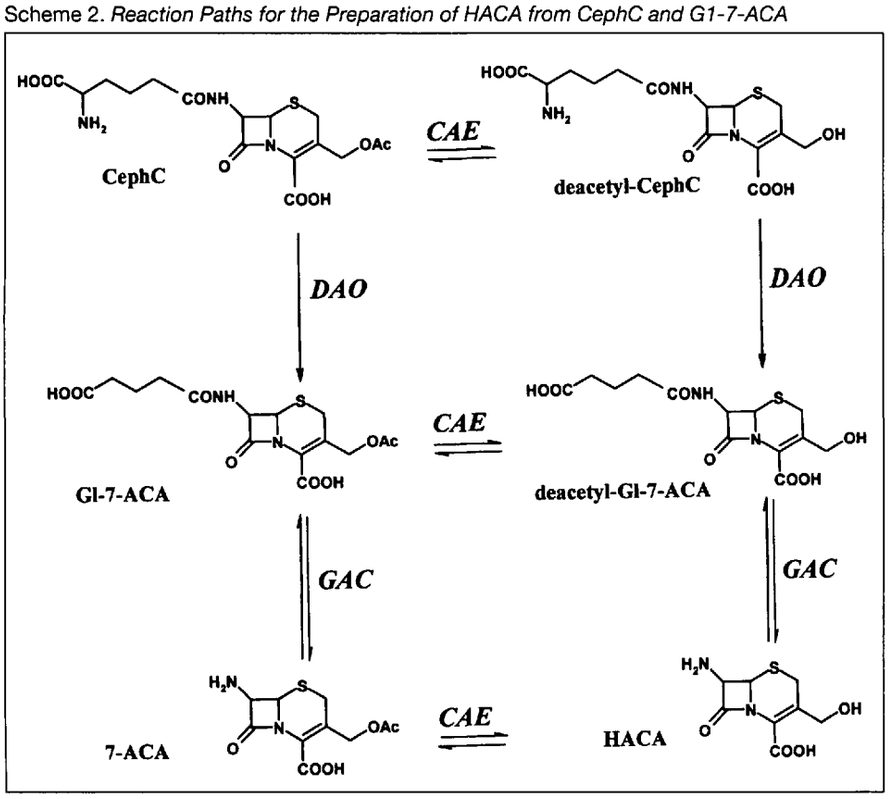
4.2. *Process Design*

Since at constant gas flow, CephC finally becomes the rate-limiting substrate, the steep increase of dissolved oxygen might be used to determine the end of the oxidation reaction with DAO. In the case of GAC and CAE, the consumption of alkaline solution for the neutralization of glutaric and acetic acid usually indicates the end of the reaction. Also, on-line HPLC is known to be a reliable method for reaction monitoring, but this requires more investment and personnel.

One important feature of biocatalysts is the ever-decreasing activity during prolonged use, which gradually increases the time required for conversion. In order to avoid hold-up of CephC from fermentation, regular addition of small portions of fresh biocatalyst would compensate for a decrease in activity. Moreover, the remaining activity may be exploited more efficiently in this way.

A typical problem that might arise during the repeated use of a biocatalyst is the excessive growth of microorganisms, as the reaction conditions are not sterile. Besides product contamination, this can lead to a decrease in apparent activity in general. In the case of DAO, an incomplete conversion (accumulation of α-ketoadipyl-7-aminocephalosporanic acid) due to the catalase activity of the contaminants is even likely to occur. Antimicrobial agents would cause regulatory and waste-water problems, hence, microbial contamination should be avoided by hygienic precaution during preparation and application of the biocatalysts.

Scheme 2. *Reaction Paths for the Preparation of HACA from CephC and GI-7-ACA*



Finally, a smooth downstream processing of the product is a prerequisite for high yield and quality, because the aqueous 7-ACA solution is not very stable at the alkaline cleavage conditions.

4.3. *Combined Reactions*

The combined application of DAO and GAC [5][6][31] or alternatively GAC and CAE in a single reactor (*Scheme 2*) seemed to be an attractive way to save on equipment and to reduce product losses due to the shortened overall process duration. For the conversion of Gl-7-ACA into HACA, a mathematical model was developed, that allows calculation of reaction yields in the combined or the separate application of GAC and CAE (*Textbox 4*).

In the special case of equally low enzyme inputs, the calculated yield of HACA is higher using the combined application, but at higher enzyme inputs, the separate performance of GAC followed by CAE is an advantage at all simulated substrate concentrations (range 10–100 mM Gl-7-ACA), although total conversion time is shorter for the combined application.

It turned out experimentally that both glutaric and acetic acid are inhibitors of CAE and GAC (K_i between 40–60 mM), which may lead to a higher accumulation of the relatively unstable 7-ACA resulting in increased decay thereof and, finally, in a lower yield of HACA.

By this model, it is demonstrated that the combined use of enzymes may lead to sub-optimal product yields as compared with separate application, but that, on the other hand, this might be overcome by careful optimization of the ratio of GAC to CAE activity.

Although such interrelations of DAO and GAC in the cleavage of CephC into 7-ACA are not expected – as these enzymes are dissimilar in their inhibition behavior – disadvantages of their combined application in general may arise, *i.e.*, *a*) DAO may easily become rate-limiting for the GAC reaction, as the fine-tuning of enzyme input seems difficult, and *b*) DAO and GAC are known to show different operational stabilities [40].

Nevertheless, the combined application of DAO and CAE – which to our knowledge has not yet been published – seems still interesting for the transformation of CephC into HACA, since *a*) the operational stability of CAE and DAO is similar, *b*) both enzymes act independently, and *c*) since Gl-7-ACA and deacetyl-Gl-7-ACA are much more stable than 7-ACA, the process yield is not very sensitive to reaction conditions and duration.

Textbox 4. Model for Simultaneous Application of GAC and CAE

The model is based on *Michaelis-Menten* assumptions, but also considers the reversibility of all enzyme reactions (*i.e.*, the reaction equilibrium), the appearance of an additional product-enzyme-complex, as well as competitive inhibition by glutaric and acetic acid. The complete kinetic parameters for the GAC and CAE reactions are experimentally measured, verified, and finally combined in order to simulate the hydrolysis of Gl-7-ACA into HACA by the enzyme mixture at 20° and pH 8.0. Furthermore, the chemical degradation parameters of all participating cephalosporin derivatives are determined and considered in the model, which seems a powerful tool to predict not only reaction rates, but also side-products and yields of hydrolysis (product isolation was not considered).

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[1] F. Mirasol, *Chemical Market Reporter*, 31 August 1998.
[2] A. Reichert, W. Riethorst, F. Knauseder, N. Palma, *World Patent* 99/13058, 1999.
[3] *Biochemie* is part of *Novartis* and ranks among the world's leading manufacturers of antibiotics (*e.g.*, penicillins and cephalosporins) and biotechnologically active ingredients. *Biochemie* offers a wide variety of essential medicines and active drug substances in more than 120 countries. The *Biochemie Group*, which had sales of >650 million in 1998, employs about 3000 people at production sites in Austria, Indonesia, Italy, Germany, and Spain. The Frankfurt facility, which was affiliated into *Biochemie* in 1998, is the group's third fermentation base and employs a workforce of approximately 350 people. The production program encompasses the manufacture of various intermediates and active ingredients for antibiotic pharmaceuticals.
[4] W. Tischer, U. Giesecke, G. Lang, A. Roeder, F. Wedekind, *Ann. N.Y. Acad. Science* **1992**, *672*, 502.
[5] H.D. Bianchi, R. Bortolo, P. Golini, P. Cesti, *Appl. Biochem. Biotechnol.* **1998**, *73*, 257.
[6] B.L. Wong; Y.Q. Shen, *European Patent* 0 465 600 B1,1995.
[7] T. Schräder, J.R. Andreesen, *Arch. Microbiol.* **1996**, *45*, 458.
[8] T. Schräder, J.R. Andreesen, *Arch. Microbiol.* **1996**, *165*, 41.
[9] F. Alfani, M. Cantarella, N. Cutarella, A. Gallifuoco, P. Golini, D. Bianchi, *Biotechnol. Letters* **1997**, *19*, 175.
[10] W. Tischer, V. Kaschke, *Tibtech* **1999**, *17*, 326.
[11] E.S. Dey, S. Flygare, K. Mosbach, *Appl. Biochem. Biotechnol.* **1990**, *27*, 239.
[12] J.T. Vinzenzi, G.J. Hansen, *Enzyme Microb. Technol.* **1993**, *15*, 281.
[13] S.M. Gastrelius, *Enz. Engin.* **1980**, *5*, 439.
[14] S.M. Gastrelius, *United States Patent* 4,288,552, 1981.
[15] V.I. Loznsky, A.L. Zubov, E.F. Titow, *Enzyme Microb. Technol.* **1996**, *18*, 561.
[16] L. Gajar, A. Singh, R. S. Dubey, R. C. Srivasta, *Appl. Biochem. Biotechnol.* **1997**, *66*, 159.
[17] L.T. Hsiau, W.C. Lee, F.S. Wang, *Appl. Biochem. Biotechnol.* **1996**, *62*, 303.
[18] B.H. Arnold, R.A. Fildes, J.R. Potts, J.E. Farthing, *US Patent* 3,801,458, 1974.
[19] Asahi Kasei, *Austrian Patent* 401 269 B, 1996.
[20] S.K. Mujawar, V.K. Sudharkan, J.G. Shewale, *Biocatalysis and Biotransformation* **1995**, *12*, 215.
[21] A. Anita, C.A. Sastry, M.A. Hashim, *Bioprocess Engineering* **1997**, *17*, 355.
[22] D. Bianchi, P. Golini, R. Bortolo, P. Cesti, *Enzyme Microb. Technol.* **1996**, *18*, 592.
[23] *Fujisawa Pharmaceuticals, World Patent* 98/06829 (japan.), 1998.
[24] Y.-H. Lee, W.-S. Chu, W.-H. Hsu, *Biotechnol. Letters* **1994**, *16*, 467.
[25] R. Binder, J. Brown, G. Romanick, *Appl. Environ. Microbiol.* **1994**, *60*, 1805.
[26] A. Deshpande, K. Sankaran, S.F. D'Souza, G.B. Nadakarini, *Biotechnology Techniques* **1987**, *1*, 55.
[27] S. Ichikawa, Y. Shibuya, R. Kodaira, *Agric. Biol. Chem.* **1981**, *45*, 2231.
[28] J.T. Vincenzi, *European Patent* 0 409 521 B1, 1990.
[29] T. Bayer, U. Holst, U. Wirth, *European Patent* 0 600 247 A2, 1993.
[30] R.A. Fildes, J.R. Potts, J.E. Farthing, *United States Patent* 3,801,458, **1974.**
[31] A. Nikolov, B. Danielsson, *Enzyme Microb. Technol.* **1994**, *16*, 1037.
[32] B. Diez, E. Mellado, M. Rodriguez, R. Fouces, J. L. Barredo, *Biotechnol. Bioengin.* **1997**, *55*, 216.
[33] V.A.P. Martins dos Santos, E.J.T.M. Leenen, M.M. Ripoll, C. van der Sluis, T. van Viliet, J. Tramper, R.H. Wijffels, *Biotechnol. Bioengin.* **1997**, *56*, 518.
[34] O. Ariga, H. Takagi, W.A. Nishiziwa, Y. Sano, *J. Ferment. Technol.* **1987**, *65*, 651.
[35] A. Llanes, L. Wilson, C. Altamirano, A. Aillapà, *Progress in Biotechnology* **1998**, *15*, 27.
[36] A. Llanes, C. Altamirano, M. E. Zuniga, *Biotechnol. Bioengin.* **1995**, *50*, 609.
[37] J. Konecny, M. Sieber, *Biotechnol Bioeng.* **1980**, *22*, 2013.
[38] H. Voss, *German Patent* 195 46 192 A1, 1995.
[39] M. Boy, A. Dominik, H. Voss, *Progress in Biotechnology* **1998**, *15*, 35.
[40] D. Bianchi, R. Bortolo, P. Goloni, P. Cesti, *La Chimia e l'Industria*, **1999**, *80*, 879.