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Penicillin Acylase in the Industrial Production of β-Lactam Antibiotics

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Abstract:

Immobilized penicillin acylase is a biocatalyst suitable for the kinetically controlled industrial synthesis of semi-synthetic antibiotics in aqueous environments. Amoxicillin and ampicillin are obtained by condensing 6-aminopenicillanic acid with the amide or ester of D-(−)-4-hydroxyphenylglycine and D-(−)-phenylglycine, respectively. Similarly, the cephalosporin antibiotics cefadroxil and cephalexin can be obtained from 7-aminodesacetoxycephalosporanic acid.

Introduction

The industrial production of β-lactam antibiotics and their intermediates is undergoing a remarkable transformation. Traditional chemical conversions based on stoichiometry are being replaced by enzyme-catalyzed processes. Still, the starting materials for these transformations are fermentatively obtained chiral structures such as cephalosporin C, penicillin G, and penicillin V. The ever-increasing insight into biochemical pathways and the resulting optimization of fermentation processes will make this application of the chiral pool even more profitable in the years to come.

Currently, the majority of N-deacylations in β-lactam production processes is carried out enzymatically using enzymes of the group of penicillin acylases. Present developments indicate that the same enzymes can also be exploited successfully in a synthetic fashion. Consequently, processes for the large-scale enzyme-catalyzed production of valuable antibiotics such as amoxicillin, ampicillin, cefaclor, cefadroxil, and cephalexin, based on the condensation of the appropriate D-(−)-amino acid derivative with a β-lactam nucleus, are in a well-advanced stage.

In this paper, an overview is given of the current status of the development of environmentally benign production processes for β-lactam antibiotics. Illustrative examples are from our own efforts in the area of enzyme-catalyzed condensation reactions, which is part of a programme of Chemferm, the joint venture between DSM, producer of chiral intermediates, and Gist-brocades, manufacturer of β-lactam products.

Background

A general production chart of penicillin-derived antibiotics (semi-synthetic penicillins, SSPs, e.g., amoxicillin and ampicillin) and cephalosporin-derived antibiotics (semi-synthetic cephalosporins, SSCs, e.g., cefadroxil and cephalexin) is outlined in Scheme 1. In the traditional chemical approach, the fermentation product penicillin G is deacylated to 6-aminopenicillanic acid (6-APA) according to a procedure based on the formation of an imide chloride.[1] This procedure adequately discriminates between the secondary amide to be hydrolyzed and the highly labile tertiary amide of the β-lactam ring. On the other hand, 7-aminodesacetoxycephalosporanic acid (7-ADCA), an intermediate used in the production of two of the best-selling SSCs, can be obtained by oxidative ring expansion of penicillin G[2] followed by a similar deacylation. Subsequently, 6-APA and 7-ADCA are transformed into SSPs and SSCs respectively by condensation with a D-(−)-phenylglycine or a D-(−)-4-hydroxyphenylglycine derivative. Two of the most widely used approaches are Schotten−Baumann condensation with the acid chloride and mixed anhydride activated condensation of the Dane salt of the side chain.[3]

In the recent past, many companies have replaced chemical side chain hydrolysis, requiring hazardous chemicals and solvents such as phosphorus pentachloride and dichloromethane, by penicillin acylase catalyzed hydrolysis in an aqueous environment. Although microbial conversion of penicillin G into 6-APA has been known for almost five decades,[4] industrial application of the enzyme involved, penicillin acylase (penicillin amidase, penicillin amidohydrolase, EC 3.5.1.11), has been introduced successfully only in the past 8 years. This is primarily a result of the fact that efficient enzyme production and recovery was unavailable a decade ago. Generally, two types of enzymes can be distinguished. The penicillin acylases, of bacterial or fungal origin, display a preference for phenylacetic acid or phenoxyacetic acid and their derivatives bearing small substituents on the α-position (amino, hydroxy, methyl, chloro) and in the aromatic ring. Also other aromatic acetic acid derivatives are recognized.[5] Penicillin acylase is widely distributed among bacteria, yeast, and filamentous fungi. The enzyme is a heterodimer with a 20.5-kDa α-subunit and a 69-kDa β-subunit.[6] The crystal structure of *Escherichia coli* penicillin acylase at 1.9-Å resolution indicates the catalytically active centre to be the N-terminal serine residue of the

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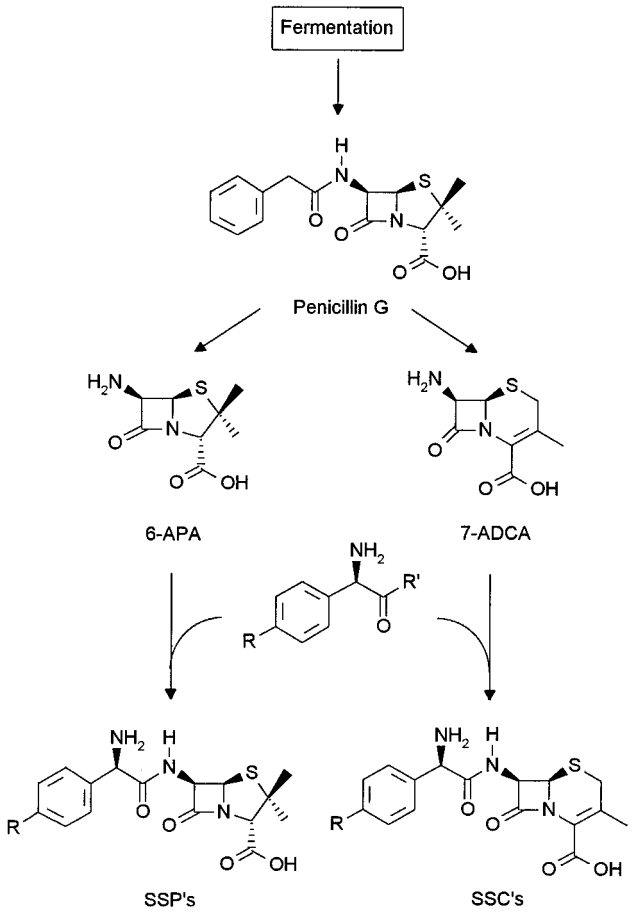
Scheme 1



β-subunit.[7] In contrast with other serine proteases, penicillin acylase does not appear to have a histidine residue in the vicinity of the active site that may act as base in the catalytic process. A more narrow substrate specificity is found in a second class of enzymes, the α-amino acid ester hydrolases, found in *Acetobacter* and *Xanthomonas* species.[8] As the name suggests, substrate specificity requires the presence of an amino group α to the carboxylic acid function.

In the production of cephalosporin antibiotics, not only is the above-mentioned penicillin G ring expansion strategy applied but also the cephalosporin nucleus can be obtained from the fermentation product cephalosporin C. However, the application of enzyme catalysis in the hydrolysis of the α-aminoadipyl side chain in cephalosporin C to give 7-aminocephalosporanic acid (7-ACA) is less well developed since extensive screening has not provided an enzyme capable of hydrolyzing the α-aminoadipyl side chain. Presently, a two-enzyme process can accommodate the removal of this side chain. A D-amino acid oxidase (EC 1.4.3.3) catalyzes oxidative deamination, the resulting α-keto acid spontaneously loses carbon dioxide in the presence of hydrogen peroxide, and finally the resulting glutaryl side chain is hydrolyzed using a glutaryl acylase.[9]

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Kinetically Controlled Enzyme-Catalyzed Synthesis

Use of penicillin acylase as catalyst in the synthetic direction was first demonstrated in 1960 by Kaufman and Bauer, who reported the *E. coli* penicillin acylase catalyzed formation of penicillin G from 6-APA and phenylacetic acid.[10] Since then, many more examples have been published in the scientific literature, of which those leading to therapeutically useful products are summarized in Table 1. The number of patent applications on the subject is at least as large.

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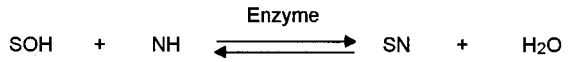
Table 1. **Enzymatic synthesis of β-lactam antibiotics**

product	enzyme[a]	source	ref
amoxicillin	AH	*Pseudomonas melanogenum*	11
amoxicillin	AH	*X. citri*	12, 13
amoxicillin	PA	*E. coli*	14
amoxicillin	PA	*Kluyveromyces citrophila*	15
ampicillin	AH	*Ps. melanogenum*	14, 16
ampicillin	AH	*Xanthomonas* sp.	17
ampicillin	PA	*E. coli*	13, 18−25
ampicillin	PA	*K. citrophila*	14, 26−28
cefadroxil	AH	*Ps. melanogenum*	14
cefadroxil	PA	*E. coli*	13
cefamandole	PA	*E. coli*	29
cefazolin	PA	*E. coli*	30, 31
cephalexin	AH	*A. pasteurianum*	32
cephalexin	AH	*Acetobacter turbidans*	11, 32, 33
cephalexin	AH	*Gluconobacter suboxydans*	32
cephalexin	AH	*Microbacterium dimorpha*	32
cephalexin	AH	*Proteus alboflavus*	32
cephalexin	AH	*Ps. melanogenum*	32
cephalexin	AH	*X. citri*	11, 32, 34−39
cephalexin	AH	*Xanthomonas compestris*	40
cephalexin	AH	*Xanthomonas oryzae*	32
cephalexin	AH	*Xanthomonas* sp.	16, 41
cephalexin	PA	*Bacillus megaterium*	42, 43
cephalexin	PA	*K. citrophila*	14, 28
cephalexin	PA	*E. coli*	11, 13, 44
cephalexin	?	*Achromobacter* sp.	45
cephaloglycin	AH	*A. pasteurianum*	32
cephaloglycin	AH	*A. turbidans*	32
cephaloglycin	AH	*G. suboxydans*	32
cephaloglycin	AH	*M. dimorpha*	32
cephaloglycin	AH	*Pr. alboflavus*	32
cephaloglycin	AH	*Ps. melanogenum*	32
cephaloglycin	AH	*X. citri*	32
cephaloglycin	AH	*X. oryzae*	32
cephaloglycin	AH	*Xanthomonas* sp.	16
cephaloglycin	PA	*E. coli*	46
cephaloglycin	PA	*K. citrophila*	28
cephalothin	AH	*K. citrophila*	47
cephalothin	PA	*E. coli*	46, 48

[a] AH: α-amino acid ester hydrolase. PA: penicillin acylase.

Scheme 2[a]

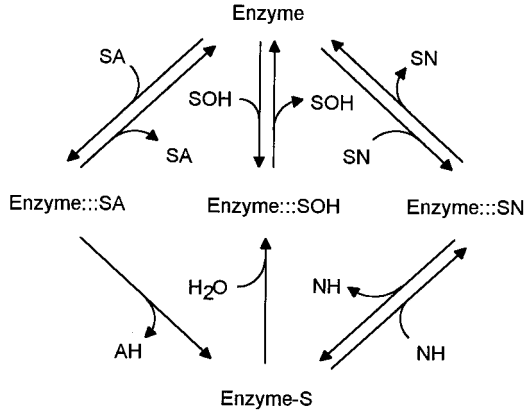


[a] Both the substrate SOH and the nucleophile NH are amino acids. In SOH the carboxylic acid takes part in the reaction with the amino group of NH. However, the favoured uncharged form of SOH (RCO_2H) can only be achieved with concurrent protonation of its amino group. The molecule thus obtained will not be recognized by the enzyme.

Enzyme-catalyzed condensation processes can, in principle, be carried out under equilibrium control or under kinetic control. In the first case (Scheme 2), the enzyme catalyzes the establishment of the thermodynamic equilibrium between, on the one hand, substrate (SOH, i.e., an amino acid) and nucleophile (NH, i.e., a β-lactam nucleus) and, on the other hand, the product (SN, i.e., a semisynthetic β-lactam antibiotic) and water. The known penicillin acylases do not accept charged amino functions. If phenylglycine is to serve as substrate, the carboxyl function should be uncharged. However, when this situation is achieved, the amino group will be charged and enzymatic catalysis is not possible.[49,50]

In kinetically controlled synthesis, non-equilibrium concentrations of the product SN can be reached by activation of the substrate SOH to an amide, ester, or anhydride. When this is done, a reactive substrate is obtained that will have its amino group partly uncharged at pH values that are optimal for the enzyme (i.e., 6−8). In biological systems, the energy required for this activation is delivered by ATP; in industrial processes, chemical activation is needed for the conversion. Once an amide or ester (SA) is available, kinetically controlled formation of product SN can take place, accompanied by undesired processes such as hydrolysis of SA and SN to inactive SOH. The molar ratio of product SN to SOH is referred to as synthesis/hydrolysis ratio (S/H ratio) and is a function of reaction conditions and biocatalyst properties and formulation. The mechanism of kinetically controlled synthesis (Scheme 3) was established by Nam et al.[38] for the *Xanthomonas citri* catalyzed formation of cephalexin from D-(−)-phenylglycine methyl ester (D-(−)-PGM) and 7-ADCA and holds equally well for other products such as amoxicillin, ampicillin, cefaclor, and cefadroxil.

Scheme 3. **Kinetically-controlled synthesis**[a]



[a] The substrate SA is an amino acid amide ($RCONH_2$) or an amino acid ester (RCO_2R'). In contrast with equilibrium-controlled synthesis (Scheme 2), the enzyme−substrate complex can be formed. Unfortunately, this complex is subject not only to synthesis (attack of NH to give product SN) but also to hydrolysis (attack of water to give unreactive amino acid SOH).

The early work on enzymatic synthesis at Gist-brocades focused on the use of α-amino acid ester hydrolases from *Acetobacter pasteurianum* and *X. citri* in the synthesis of ampicillin (**2a** in Scheme 4) from 6-APA and PGM. First of all, it was found that ampicillin synthesis proceeds particularly smoothly at low temperatures (i.e., 5 °C), giving a conversion of 88% based on 6-APA when optically pure D-(−)-PGM was used. Secondly, ampicillin could also be obtained from the racemic form of PGM (77% conversion, ee 69%).[51] Although the ee values reached are remarkable compared to those for the penicillin acylases, where stereospecificity for the α-position of the side chain is only moderate, the idea of introducing optical activity in the side chain during the condensation reaction was eventually abandoned. For economic reasons, asymmetry should be introduced in a production pathway as early as possible so as to avoid the transport of isomeric ballast throughout the

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Scheme 4

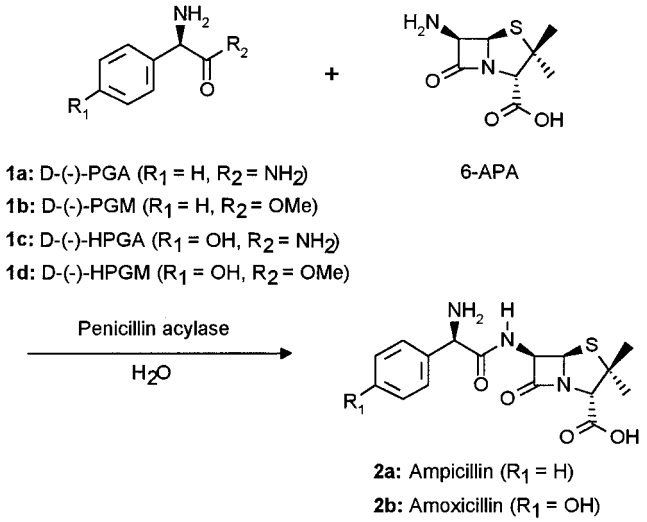

Table 2. **Influence of carrier composition on the S/H ratio in the enzymatic synthesis of ampicillin by immobilized *E. coli* penicillin acylase[a]**

carrier composition	S/H ratio[b]
gelatin/alginate amine (1%)	3.5
gelatin/alginate amine (3%)	2.9
gelatin/chitosan (1%)	2.6
gelatin/chitosan (2%)	2.1
gelatin/chitosan (3%)	3.4
gelatin/pectin (2%)	1.9
gelatin/pectin (3%)	2.7
gelatin/polyethylene imine (1%)	2.4
gelatin/polyethylene imine (2%)	2.8
gelatin/polyethylene imine (3%)	2.5
polyacrylamide[c]	2.8
polyacrylamide[d]	2.4

[a] Condensations were carried out in water at 20 °C and pH 7.5 (maintained with HCl) using 6-APA (300 mM) and D-(−)-PGA (500 mM). The amount of enzyme used was 1 unit mL^{-1}. [b] The S/H ratio was determined at 10% conversion using reversed-phase HPLC. [c] Enzygel; commercially available from Boehringer Mannheim GmbH, Germany. [d] Commercially available from Recordati, Milan, Italy.

whole process. Furthermore, in order to reach sufficient optical purity, conversions must be kept below 50%. This implies a very complicated downstream processing since, besides product isolation and enzyme recovery, unreacted L-(+)-PGM must be racemized and reused, and the sensitive β-lactam nucleus must be recovered with high efficiency.

With the development of immobilization techniques, the economical feasibility of enzymatic condensation reactions became within reach. Covalent attachment of *E. coli* penicillin acylase on a gelatin-based carrier yielded a water-insoluble catalyst that could be recycled manyfold. By tuning of the composition of the carrier material, the S/H ratio in synthesis reactions could be optimized as outlined in Table 2 in the case of ampicillin synthesis.[52] Initial S/H ratios at 10% conversion indicate that gelatin-based carriers display high S/H ratios as compared to commercially available immobilized penicillin acylases.

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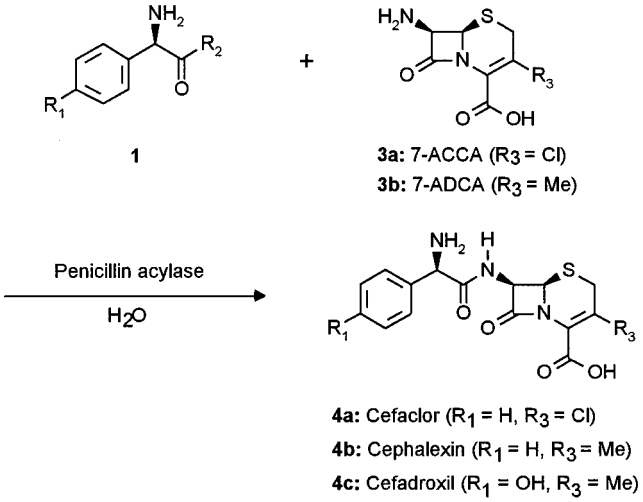
Scheme 5



Enzyme-Catalyzed Synthesis of D-(−)-Phenylglycine-Derived Antibiotics

Cephalexin. The first successful application of the catalyst mentioned above was achieved in the production of cephalexin (**4b** in Scheme 5) from 7-ADCA and D-(−)-PGA or D-(−)-PGM.[53] As outlined above, kinetically controlled synthesis requires delicate optimization of reaction conditions towards the optimal conversion. The main problem resides in the formation of unwanted D-(−)-phenylglycine (D-(−)-PG). In principle, a very high yield based on the β-lactam nucleus can be obtained when a high molar excess of D-(−)-PG derivative is used, as was the general strategy in most of the references mentioned in Table 1. Major drawbacks of this approach are the necessity for recycling excess D-(−)-PGA or D-(−)-PGM, and the recovery of D-(−)-PG. An elegant solution for the recovery of D-(−)-PGA was found by treating the reaction mixture, following removal of immobilized enzyme and solid products, with benzaldehyde.[54] The Schiff base of D-(−)-PGA thus obtained crystallizes from the reaction mixture and can be recovered by filtration.

Yet another complicating factor is the relatively low solubility of D-(−)-PG under the conditions applied for synthesis. Since also the required product has a low solubility, complex downstream processing is unavoidable. Three solids (i.e., the desired antibiotic, D-(−)-PG, and immobilized enzyme) need to be separated from the reaction mixture. Enzyme separation was achieved by workers from Novo Nordisk who developed an immobilized enzyme with a density below unity.[55] When stirring is stopped, the immobilized enzyme will float whereas solid products can be removed from the bottom of the reactor. Alternatively, a sieving technique as outlined in Figure 1 may be applied. When an enzyme immobilized on sufficiently large particles (i.e., diameter > 150 μm) is used, the catalyst can be retained during drainage of the reactor. By designing an appropriately spaced sieve, crystalline material can be transported together with the effluent.[56]

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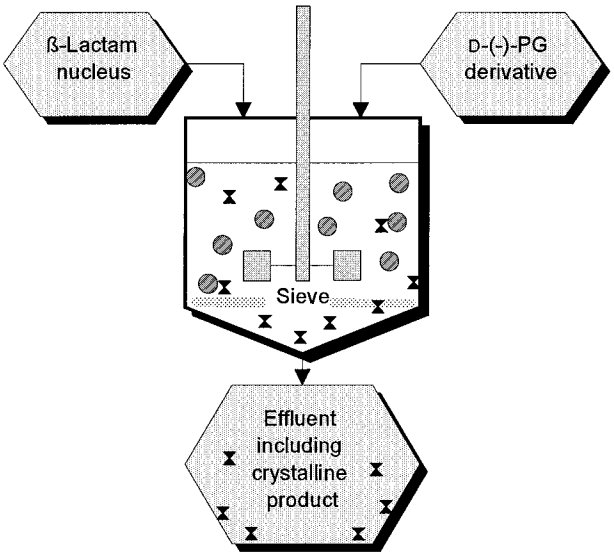

Figure 1.

One way to overcome the problem of excessive production of D-(−)-PG is to use near-equimolar amounts of substrates and design a process in which the degree of conversion is kept relatively low. As a result, an efficient recycling of β-lactam substrate must be realized. This may be done in situ, as suggested by Kasche,[57] in the case of D-(−)-PGM, where a constant recycling of D-(−)-PG to D-(−)-PGM is thought to take place as a result of the formation of methanol, which can act as a nucleophile. Should this be the case, the effect may be further enhanced by supplying additional methanol. Indeed, Kim et al.[25] have reported a 2-fold increase in yield in the case of ampicillin synthesis at a methanol concentration of 40% (v/v). A drawback of this method is a 3-fold drop in the initial rate. In our hands, this approach gave only moderate improvements. Alternatively, the unreacted β-lactam nucleus may be recovered by crystallization after isolation of the product. For the cephalexin process, this may be a fruitful strategy since the solubility of 7-ADCA at its isoelectric point is very low and, thus, losses in mother liquor may be neglected. However, the fragile β-lactam nucleus requires careful handling when losses resulting from degradation are to be kept minimal. This is even more so the case with penicillin derivatives.

Ampicillin. Ampicillin synthesis is hampered by the fact that the product solubility is high (compared to that of, e.g., amoxicillin). Thus, degradation percentages can amount to unacceptable levels unless special care is taken. Since penicillanic acid derivatives are more sensitive towards degradation than cephalosporanic acid derivatives at almost all pH values, recycling techniques as advocated in the case of cephalexin are not attractive. A solution must be found in a design in which all 6-APA present is converted to product, which, in turn, is rapidly recovered by means of crystallization. Thus, in a typical procedure, ampicillin is synthesized from 6-APA and excess D-(−)-PGA or D-(−)-PGM during which process both crystalline ampicillin and crystalline D-(−)-PG are formed. The biocatalyst is removed by sieving, and all crystals are dissolved at acidic pH. After concentration, pure ampicillin can be obtained by crystallization at its isoelectric point. In a second concentration and crystallization stage, D-(−)-PG can be obtained (Figure 2).

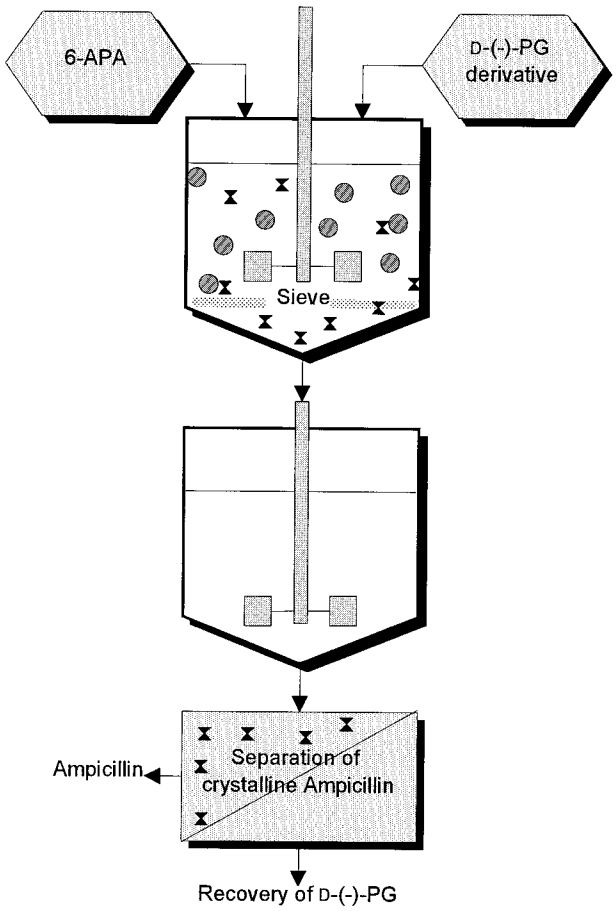

Figure 2.

Cefaclor. As outlined above, it has become clear that enzyme-catalyzed synthesis of antibiotics requires the development of separate condensation and downstream processing strategies for each individual product of interest. Recent preliminary research into the synthesis of other antibiotics has emphasized this theory.

Cefaclor (**4a** in Scheme 5) is a D-(−)-PG-derived antibiotic based on the nucleus 7-aminodesacetoxymethyl-3-chlorocephalosporanic acid (7-ACCA) which can be obtained by ozonolysis and chlorination of 3-methylene cephams.[58] Cefaclor is unstable at pH values above 6.5 whilst the solubility of 7-ACCA is very low at pH levels below 6.5. Consequently, it is impossible to design an economically feasible high-yielding enzymatic conversion at any pH value. In developing enzymatic condensation processes based on the labile and relatively expensive nucleus 7-ACCA, complexation of the final product is a promising strategy, as already shown for cephalexin.[59] By coadministration of a complexing agent (i.e., β-naphthol) to the reaction mixture, the 2/1 cefaclor−β-naphthol complex crystallizes and very

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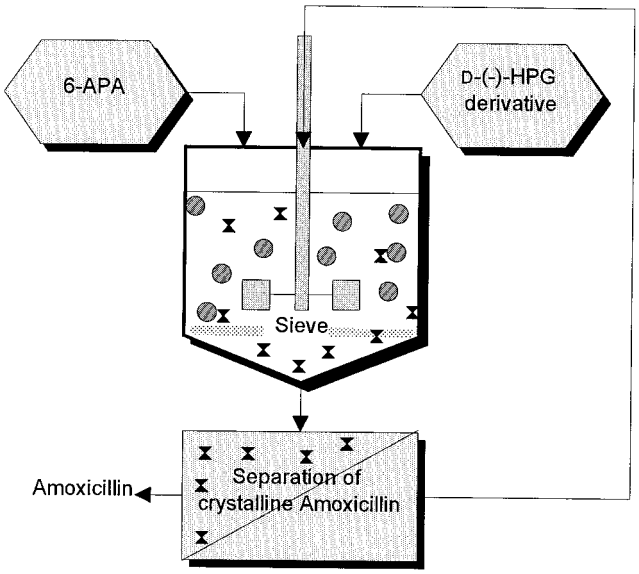

Figure 3.

high conversions (>90%) are easily reached. As a result of the low solubility of the complex, losses in mother liquors may be neglected. Decomplexation is normally effected at low pH in a two-phase system. This implies the introduction of an organic solvent into the process, which may be considered a drawback of complexation technology.

Enzyme-Catalyzed Synthesis of D-(−)-4-Hydroxyphenylglycine-Derived Antibiotics

Amoxicillin. In the enzyme-catalyzed synthesis of amoxicillin (**2b** in Scheme 4) from 6-APA and D-(−)-4-hydroxyphenylglycine amide (D-(−)-HPGA) or D-(−)-4-hydroxyphenylglycine methyl ester (D-(−)-HPGM), advantage can be taken of the very low solubility of the product at conversion conditions. Due to this phenomenon, the S/H ratio is high since, in solution, almost no amoxicillin is available for hydrolysis. Furthermore, for the same reason, degradation of amoxicillin is minimal. Since amoxicillin is the first compound to precipitate, a semi-continuous reactor system for the production of amoxicillin at high substrate concentrations could be developed successfully (Figure 3).[60]

Cefadroxil. Cefadroxil (**4c** in Scheme 5) is a semisynthetic antibiotic combining the side chain of amoxicillin (D-(−)-HPG) and the nucleus of cephalexin (7-ADCA).

There are several drawbacks in enzymatic cefadroxil synthesis when compared to enzymatic amoxicillin synthesis. First of all, it is difficult to pinpoint a pH value at which both the side chain derivative and the nucleus are sufficiently soluble. At pH values above 7, 7-ADCA readily dissolves a high concentration but the side chain derivative has only moderate solubility. At pH values below 7, the situation is completely reversed. Thus, careful investigation is required to establish a pH at which both reactants are present in acceptable concentrations, i.e., both the reactivity and the S/H ratio are acceptable. Secondly, the solubility of cefadroxil under enzymatic conversion conditions is relatively high in comparison with amoxicillin solubility. For this reason, chemical degradation and enzymatic hydrolysis of cefadroxil are unwanted events that may not be neglected. Thirdly, despite the above-mentioned difficulties, a high conversion must be achieved in the condensation reaction in order to obtain a mixture from which cefadroxil can be recovered by crystallization. If the conversion is too low, the product will be contaminated with unacceptably high levels of β-lactam nucleus and/or side chain derivative.

As already mentioned in the case of cefaclor, the situation can be drastically improved by employing β-naphthol as a complexing agent during the enzymatic reaction. By crystallization of the cefadroxil formed as its β-naphthol complex, the product is protected against chemical as well as enzymatic degradation during the enzymatic conversion and recovery procedure. In this way, the above-mentioned drawbacks may be overcome, as this process results in a higher conversion and S/H ratio.[59]

Conclusion

The aqueous enzymatic synthesis of β-lactam antibiotics is a rapidly evolving technique which has proven its feasibility. The keystones for this technique are the development of a biocatalyst that can be recycled manyfold and the product-dedicated design of downstream processes. Yields as high as or better than those obtained in traditional chemical condensations are achievable, and thus, economically attractive processes are within reach.

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