Available online at www.sciencedirect.com
ScienceDirect
Enzyme and Microbial Technology 40 (2007) 195–203
ENZYME AND MICROBIAL TECHNOLOGY
www.elsevier.com/locate/emt

# Production of cephalexin in organic medium at high substrate concentrations with CLEA of penicillin acylase and PGA-450

A. Illanes [a,*], L. Wilson [a], C. Altamirano [a], Z. Cabrera [a], L. Alvarez [a], C. Aguirre [b]

[a] *School of Biochemical Engineering, Pontificia Universidad Católica Valparaíso, P.O. Box 4059, Valparaíso, Chile*
[b] *Faculty of Sciences, Universidad Católica Ssma. Concepción, P.O. Box 297, Concepción, Chile*

Received 30 January 2006; received in revised form 10 March 2006; accepted 31 March 2006

**Abstract**

The kinetically controlled synthesis of cephalexin in ethylene glycol was previously optimized at moderate substrate concentrations obtaining yields close to stoichiometric. A study is now presented on the production of cephalexin at very high substrates concentrations, up to 750 mM acyl donor, with immobilized and cross-linked enzyme aggregates (CLEA) of penicillin acylase. Since conversion yield close to 100% was already obtained, attention was given to productivity under the hypothesis that increasing substrates concentration will produce a substantial increase in productivity without reducing yield. An increase of 29 times in volumetric productivity and 4.5 times in specific productivity was obtained with PGA-450 with respect to the results obtained at moderate substrates concentrations (below 100 mM acyl donor). Volumetric productivity was lower for CLEA than for PGA-450, but specific productivity was almost the same for both. Sequential batch reactor operations were conducted to assess the biocatalyst operational stability and global productivity, considering one half-life as biocatalyst life cycle. Under such criterion, 40.1 and 135.5 g of cephalexin/g of biocatalyst were obtained for PGA-450 and CLEA, respectively. Yields remained close to 100% during the whole cycle. These are very good values which can be improved by optimizing the biocatalyst replacement criterion.


*Keywords:* Penicillin acylase; CLEA; Cephalexin; Organic cosolvents

## 1. Introduction

Penicillin acylase is one of the few examples of successful application of enzymes for the large-scale production of valuable pharmaceuticals [1,2]. Its current industrial application refers to the production of 6 amino penicillanic acid (6APA) from either penicillin G or V; however, the enzyme has the potential for performing several reactions of organic synthesis, including the synthesis of derived penicillins and cephalosporins from the corresponding β-lactam nuclei and suitable acyl donors [3]. Synthesis can be performed under kinetic [4] or thermodynamic [5] control, but the former is preferred when high conversion yield is a process task [6,7]. In this strategy, the reaction of synthesis, that is the nucleophilic attack of the β-lactam nucleus to the enzyme-acyl complex, competes with the reactions of hydrolysis of both the antibiotic product and the activated acyl donor [8]. The reduction of water activity in the reaction medium is beneficial, since it depresses the competing hydrolytic reactions in favor of synthesis [9,10]. Water activity can be conveniently depressed by using cosolvents, or high concentration of substrates, or both.

Water miscible organic cosolvents, especially polyols, have proved to be suitable media for the synthesis of β-lactam antibiotics with immobilized penicillin acylase and in fact much higher yields can be obtained than in fully aqueous media [11,12]. However, initial rates are lower in organic cosolvents, so that productivity can be impaired. In addition, depending on the cosolvent, the enzyme can be less stable than in aqueous medium, so that robust biocatalysts are required. This problem has been thoroughly addressed and substantial improvements have been obtained recently for penicillin acylase, considering insolubilization with [13,14] and without inert matrices [15–17], directed immobilization and also genetic manipulations involving site-directed mutagenesis and protein engineering [18,19]. Comprehensive reviews on the subject have been published recently [20,21]. Cross-linking enzyme aggregates (CLEAs), are particularly promising novel biocatalysts for organic synthesis: they are robust enough to withstand harsh conditions,

* Corresponding author.
*E-mail address:* aillanes@ucv.cl (A. Illanes).

0141-0229/$ – see front matter © 2006 Elsevier Inc. All rights reserved.
doi:10.1016/j.enzmictec.2006.03.041

their production is simple and amenable for scale-up and they can have very high specific activities because there is no inert matrix involved [22,23]. CLEAs have been proposed as a novel form of biocatalyst for penicillin acylase for the synthesis of β-lactam antibiotics [15,17].

Other strategies for reducing product hydrolysis include in situ product removal [6,24] and product partition to a second phase [25]. The use of high substrates concentrations to the limit of solubility and beyond has been also shown to be a viable alternative for obtaining very high conversion yields in the synthesis of β-lactam antibiotics. Aqueous solution precipitate consists in keeping a saturated concentration of the nucleophile throughout the reaction by repetitive additions leading to product precipitation; it has been successfully applied to the synthesis of ampicillin with conversion yields as high as 95% [26–29]. All these strategies have succeeded in obtaining high yields of synthesis; not much attention has been paid, however, to productivity which is a very relevant process parameter to asses. Only recently some considerations on productivity have been made for the synthesis of ampicillin, amoxicillin and cephalexin in highly condensed aqueous systems under conditions of substrate supersaturation. Conversion yields were 98, 91 and 92%, respectively and productivities were significantly improved with respect to the aqueous solution precipitate strategy [29]. It is clear that, as a consequence of Michaelian kinetics, productivity should increase with increasing substrate concentrations [30], but the point is how many times can productivity be increased and how can the problems of heterogeneous reaction conditions be properly handled.

We have combined the favorable conditions of using cosolvents and high substrate concentrations to develop a sequential batch process for the synthesis of cephalexin raising the hypothesis that, under such conditions, productivity will be dramatically increased with respect to the control at moderate substrates concentrations, without reducing conversion yield. The process was developed with a commercial penicillin acylase (PGA-450) and a cross-linked enzyme aggregate from recombinant *Escherichia coli* penicillin acylase.

## 2. Materials and methods

### 2.1. Materials

(*R*)-(−)-2-phenylglycine methyl ester hydrochloride (97% pure) and cephalexin hydrate were from Sigma Chemical Company Inc. (St. Louis, MO, USA); (*R*)-(−)-2-phenylglycine (PG) was from Aldrich (Milwaukee, WI, USA). 7-Amino-3-desacetoxicephalosporanic acid (7ADCA) was kindly provided by Antibióticos S.A. (León, Spain) and penicillin G potassium salt (PenGK) was donated by Natsus S.A. (Lima, Perú). Ethylene glycol (EG) and all other reagents were analytical grade either from Sigma–Aldrich or Merck (Darmstadt, Germany).

### 2.2. Biocatalysts

Polyacrylamide gel surface bound penicillin acylase (PGA-450) from *E. coli* with $320 \pm 20\,IU_H/g$ and $230\,IU_S/g$ was from Roche Molecular Biochemicals (Mannheim, Germany). Immobilized biocatalyst spherical particles were around 0.1 mm in diameter. The biocatalyst was stored wet at 5 °C and remained fully active during the whole working period.

Penicillin acylase from *E. coli*, with $220 \pm 20\,IU_H/mL$ and $21 \pm 2\,mg/mL$ protein, was a product from Antibióticos S.A. (León, Spain), kindly provided by Dr. José Manuel Guisán (Instituto de Catálisis, CSIC, Madrid, Spain). The enzyme was centrifuged and dialysed prior to use and remained fully active at 5 °C during the whole working period.

Cross-linked enzyme aggregates of recombinant penicillin acylase (PACLEA) were produced as already reported [31]. The process for the production of PACLEA consists of four steps: the first one is precipitation, in which the enzyme is contacted with the precipitating agent; the second one is cross-linking of the precipitated protein with a bifunctional reagent, glutaraldehyde in this case; the third one is reduction, to stabilize the Schiff base formed by the reaction of the aldehyde groups of the reagent and the amino groups in the protein (mainly ε-amino group from lysine) forming a stable secondary amine; the final step is a thorough washing of the biocatalyst to remove any adsorbed protein and reagents. Ammonium sulfate was selected as the best precipitating agent in terms of recovery and specific activity of the biocatalyst [31]. The PACLEA produced had a specific activity of $1130\,IU_H/g$ and $640\,IU_S/g$ and was stored wet at 5 °C, remaining fully active during the whole working period. CLEAs were of approximately 0.1 μm in diameter (as determined by electronic microscopy; unpublished results) with a water content of 84%.

### 2.3. Analysis

Hydrolytic activity of penicillin acylase was determined from initial rate data, analyzing 6APA formation as reported by Shewale et al. [32]. One international unit of hydrolytic activity ($IU_H$) was defined as the amount of penicillin acylase that catalyzes the hydrolysis of 1 μmol of PGK/min from 134 mM PGK in 0.1 M phosphate buffer pH 7.8 at 30 °C and 250 rpm. Synthetic activity of penicillin acylase was determined from the initial slope of the reaction. One international unit of synthetic activity ($IU_S$) was defined as the amount of penicillin acylase that produces 1 μmol of cephalexin (Cex) per minute under the conditions of reaction. Substrates and products of synthesis were identified and analyzed by HPLC using a Shimadzu delivery system LC-10AS with a Shimadzu UV SPD-10AV UV–vis detector and a CBM-101 Shimadzu HPLC/PC integrator. The column used was a μ-Bondapack $C_{18}$ (300 mm × 3.9 mm) from Waters (Milford, MA, USA). Samples were eluted isocratically with a sonicated mixture of 70% (v/v) 20-mM phosphate buffer pH 7.0 and 30% (v/v) methanol at a flow rate of 1 mL/min, and analyzed in the UV detector at 214 nm. Elution times were 2.8, 3.5, 6.3 and 12.7 min for 7ADCA, PG, Cex and phenylglycine methyl ester (PGME), respectively. Concentration of substrates and products were calculated from calibration curves using stock solutions. HPLC samples were always assayed in triplicate, differences among them never exceeding 3%.

### 2.4. Synthesis of cephalexin

Syntheses were performed batch-wise, with temperature and pH control, in 50 mL Pyrex glass reactors with a working volume of 30 mL, equipped with a paddle impeller at a stirring speed of 200 rpm to keep biocatalyst particles in suspension, and a bottom sintered glass filter to recover the biocatalyst after each run. Samples were taken at intervals and were properly diluted prior to be assayed by HPLC. Two kinds of samples were taken: one with the whole content of the reaction medium, including the eventual solids, and one through a filter to keep solids out. Samples with solids in suspension were diluted prior to assay so that the solids were dissolved except for the biocatalyst particles that were filtered out (the volume occupied by the biocatalyst was insignificant). At high substrate concentrations the system is highly heterogeneous and a fraction of the product is precipitated. In this case, the Cex produced is partly dissolved and partly in the form of a precipitate. The total amount and the soluble amount of Cex are determined by Eqs. (1) and (2), respectively:

$$M_T = C_T V_R \frac{(MW)_P}{1000} \quad (1)$$

$$M_F = C_F f V_R \frac{(MW)_P}{1000} \quad (2)$$

where $M_T$ is the total mass of product (g); $M_F$ the total mass of product in the filtrate (liquid phase) (g); $C_T$ the total concentration of product in the reaction medium (mM); $C_F$ the concentration of product in the filtrate (liquid phase)

(mM); $(MW)_P$ the molecular weight of product (g/g mol); $V_R$ the total volume of reaction (liquid plus solids) (L); $f$ is the volumetric fraction of the liquid phase (experimentally determined).

The determination of product in the precipitate was calculated by a material balance (Eq. (3)), according to Eq. (4):

$$M_T = M_{PPT} + M_F \tag{3}$$

$$M_{PPT} = (C_T - C_F \times f)V_R \frac{(MW)_P}{1000} \tag{4}$$

where $M_{PPT}$ is the total mass of product in the precipitate (g).

Syntheses of Cex with PGA-450 were conducted under kinetic control at 30% (v/v) EG medium using PGME as acyl donor and 7ADCA as the limiting substrate at pH 7.4 and 14 °C, at the previously selected PGME–7ADCA molar ratio of 3 and at 125 $IU_H$/mmol 7ADCA [7]. That temperature, pH and EG concentration were determined as the best conditions from a weighed objective function in which conversion yield [7] and substrate solubility [30] were considered as independent variables. Substrates concentrations ranged from 30 mM 7ADCA and 90 mM PGME (used as control) to 250 mM 7ADCA and 750 mM PGME. PGME was always soluble, so that the initial condition of homogeneity or heterogeneity in the reaction medium was determined by the concentration of 7ADCA. At higher concentrations, the medium was hardly tractable. Once the best conditions of substrate concentrations were established in terms of conversion yield and productivity, the enzyme to substrate ratio was reduced to the point in which the conversion yield was significantly reduced with respect to the maximum value obtained.

Syntheses with PACLEA were done under the same conditions as above.

Molar conversion yield was defined as the maximum molar conversion of 7ADCA into Cex. Volumetric productivity (mM/h) was determined as the amount of Cex produced per unit time and unit reaction volume at maximum yield. Specific productivity (mmol/h g) was determined as the amount of Cex produced per unit time and unit mass of biocatalyst at maximum yield. Experiments were done in duplicate and samples assayed in triplicate with variations below 5% among them. In order to determine maximum conversion yield, reactions were monitored up to the point in which product concentration levelled off or began to decline.

### 2.5. Production of cephalexin in sequential batch reactor operation

These experiences were done at the best conditions determined previously. The experimental setup was the same as above, but 100 mL reactors with 60 mL of working volume were used instead to reduce the impact of sampling in its operation. After each batch, the reactor was emptied and the solids retained by the bottom filter. Precipitates were dissolved by thorough washings with water until no PG was detectable, and the biocatalyst was retained inside the reactor for the next batch. The residual activities of hydrolysis and synthesis were determined at each batch to assess the operational stability of the biocatalyst. Some biocatalyst was removed by sampling and this was taken into account by reducing the volume in the next batch so as to maintain always the same concentration of biocatalyst (and the same enzyme to substrate ratio) in the reaction medium. Experiments were prolonged for one half-life of the biocatalyst, tentatively considered as replacement criterion, so defining one cycle of biocatalyst use. Maximum conversion yield and time to attain it were recorded in each batch. Volumetric productivity in batch $i$, $P_i$ (mM/h), specific productivity in batch $i$, $P_{sp,i}$ (mmol/h g), product produced in batch $i$, $M_i$ (g) and accumulated product within one cycle of biocatalyst use, $M$ (g), were determined as:

$$P_i = \frac{C_{T,i}}{t_i} \tag{5}$$

$$P_{sp,i} = \frac{C_{T,i}V_{R,i}}{t_i m_{cat}} \tag{6}$$

$$M_i = \frac{C_{T,i}V_{R,i}(MW)_P}{1000} \tag{7}$$

$$M = \sum_{i=1}^{n} M_i \tag{8}$$

where $n$ is the number of batches in one cycle of biocatalyst use, $V_{R,i}$ the volume of reaction in batch $i$ (L), $C_{T,i}$ the concentration of product (mM) at maximum conversion yield in batch $i$ and $t_i$ is the time (h) to attain it.

### 2.6. Operational stability of biocatalysts

The operational stability of the biocatalysts was determined by measuring the residual activity of hydrolysis at the end of one batch and the residual activity of synthesis at the beginning of the following batch. Since biocatalyst activities did not decay significantly in one batch, measurements were made every six batches. The time recorded in this case corresponds to the real time that the biocatalyst was exposed to the conditions of reactions and not to the time to attain maximum yield, since the reaction has to be continued beyond that point to determine it.

## 3. Results and discussion

### 3.1. Synthesis of cephalexin with PGA-450 and PACLEA

Syntheses of Cex with PGA-450 were conducted at increasing substrates concentrations at the previously determined optimum conditions for the synthesis at moderate substrate concentrations: pH 7.4, 14 °C, 200 rpm, 30% (v/v) EG, PGME/7ADCA molar ratio of 3 and 125 $IU_H$/mmol 7ADCA [7]. Time course of the reaction of synthesis is illustrated for the case of 200 mM 7ADCA concentration in Fig. 1A. Results for the whole range

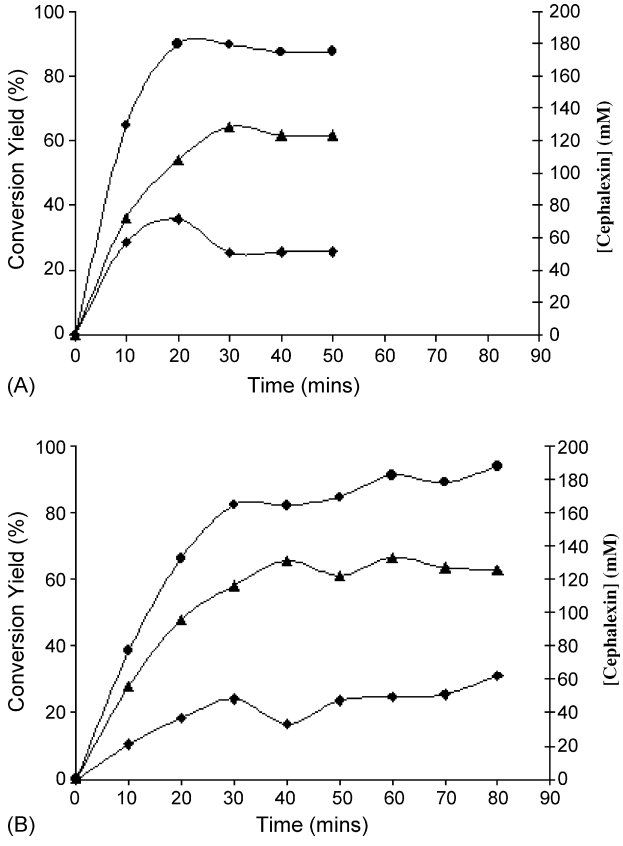


Fig. 1. Time course of the reaction of synthesis of cephalexin with PGA-450 at pH 7.4, 14 °C, 30% (v/v) EG, 200 mM 7ADCA and 600 mM PGME. (A) 125 $IU_H$/mmol 7ADCA; (B) 62.5 $IU_H$/mmol 7ADCA; (●) total concentration of product in the reaction medium; (▲) concentration of product in the filtrate (liquid phase) (mM); (♦) concentration of product in the precipitate (solid phase).

Table 1
Effect of substrate concentration on conversion yield ($Y$), volumetric productivity ($P$) and specific productivity ($P_{sp}$) of synthesis of cephalexin with PGA-450 at increasing substrates concentration at pH 7.4, 14 °C, 30% (v/v) EG, PGME/7ADCA molar ratio of 3 and 125 $IU_H$/mmol 7ADCA

| Biocatalyst mass (g) | [7ADCA] (mM) | Time for $Y_{max}$ (h) | [Cex] (mM) | $Y$ (%) | $P$ (mM/h) | $P_{sp}$ (mmol/h g) |
|---|---|---|---|---|---|---|
| 0.36 | 30 | 1.5 | 25.8 | 85.9 | 17.2 | 1.43 |
| 1.07 | 90 | 1 | 83.5 | 92.73 | 83.5 | 2.34 |
| 1.79 | 150 | 0.67 | 138.2 | 92.1 | 207.3 | 3.47 |
| 2.38 | 200 | 0.5 | 179.4 | 89.7 | 358.7 | 4.52 |
| 2.97 | 250 | 0.92 | 235.0 | 94.0 | 256.4 | 2.59 |

Dotted line indicates the boundary between initially homogeneous and initially heterogeneous reaction conditions.

Table 2
Effect of substrate concentration on conversion yield ($Y$), volumetric productivity ($P$) and specific productivity ($P_{sp}$) of synthesis of cephalexin with PGA-450 at increasing substrates concentration at pH 7.4, 14 °C, 30% (v/v) EG, PGME/7ADCA molar ratio of 3 and 62.5 $IU_H$/mmol 7ADCA

| Biocatalyst mass (g) | [7ADCA] (mM) | Time for $Y_{max}$ (h) | [Cex] (mM) | $Y$ (%) | $P$ (mM/h) | $P_{sp}$ (mmol/h g) |
|---|---|---|---|---|---|---|
| 0.18 | 30 | 2.67 | 27 | 89 | 10.13 | 1.69 |
| 0.54 | 90 | 1.33 | 85.7 | 95 | 64.28 | 3.57 |
| 0.89 | 150 | 1.17 | 150 | 100 | 128.57 | 4.33 |
| 1.19 | 200 | 0.67 | 198.8 | 99.4 | 298.2 | 7.52 |
| 1.48 | 250 | 0.92 | 229 | 91 | 249.82 | 5.06 |

Dotted line indicates the boundary between initially homogeneous and initially heterogeneous reaction conditions.

of substrates concentrations are summarized in Table 1. Volumetric and specific productivities increased significantly with substrates initial concentrations up to the value of 200 mM 7ADCA and 600 mM PGME, but decayed from then on. Yield was not affected, being even somewhat higher than in the control at 30 mM 7ADCA and 90 mM PGME. Increases in volumetric and specific productivities were 21 and 3 times, respectively. Stirring speed of 200 rpm was determined to keep solids in suspension throughout the reaction. Results did not change significantly over 200 rpm.

The system was then forced to lower enzyme to substrate ratio. Time course of the reaction of synthesis at 62.5 $IU_H$/mmol 7ADCA is illustrated for the case of 200 mM 7ADCA in Fig. 1B. Results for the whole range of substrates concentrations are summarized in Table 2. Again, specific productivities increased significantly with substrate initial concentration up to the value of 200 mM 7ADCA and 600 mM PGME, and decayed from then on, but in this case yields were higher (close to stoichiometric) and specific productivities were also higher than at 125 $IU_H$/mmol 7ADCA. Increases in volumetric and specific productivities with respect to the control at 30 mM 7ADCA and 90 mM PGME were 29 times and 4.5 times, respectively.

The system was forced again to even lower enzyme to substrate ratio. Results at 200 mM 7ADCA and 600 mM PGME for substrates concentrations in the range from 15.6 to 125 $IU_H$/mmol 7ADCA are summarized in Table 3. Even though $Y$ is still stoichiometric at 31.25 $IU_H$/mmol 7ADCA, volumetric and specific productivities are reduced by 69 and 38% with respect to 62.5 $IU_H$/mmol 7ADCA. Then, the best conditions for synthesis were established as 200 mM 7ADCA, 600 mM PGME (initially heterogeneous conditions) and 62.5 $IU_H$/mmol 7ADCA. Under these conditions a dramatic increase in productivity was obtained with no compromise in yield, being it close to stoichiometric.

The synthesis of Cex with PACLEA was then conducted at the best conditions determined above for PGA-450. Time course of the reaction of synthesis with PACLEA is shown in Fig. 2. As in the case with PGA-450, $Y$ was close to 100%, but volumetric productivity was only 21% of the value obtained with PGA-450. This is a consequence of the lower rate of reaction which produced a considerable increase in the time required to attain maximum yield, as can be seen from Figs. 1 and 2. Lower reaction rates in the case of PACLEA are a reflection of diffusional restrictions, which is worthwhile to determine in quantitative terms.

The results obtained in the synthesis of Cex at initially heterogeneous conditions compare quite favorably with those recently reported in similar systems. Under the strategy of aqueous solution precipitate, Youshko et al. [27], working at initially

Table 3
Effect of the enzyme to substrate ratio ($E/S$) on conversion yield ($Y$), volumetric productivity ($P$) and specific productivity ($P_{sp}$) of synthesis of cephalexin with PGA-450 at 200 mM 7ADCA and 600 mM PGME

| Biocatalyst mass (g) | $E/S$ ($IU_H$/mmol 7ADCA) | Time for $Y_{max}$ (h) | $Y$ (%) | [Cex] (mM) | $P$ (mM/h) | $P_{sp}$ (mmol/h g) |
|---|---|---|---|---|---|---|
| 0.3 | 15.6 | 3.17 | 88.1 | 176.2 | 55.6 | 5.56 |
| 0.6 | 31.25 | 2.17 | 100 | 200 | 92.3 | 4.62 |
| 1.19 | 62.5 | 0.67 | 99.4 | 198.8 | 298.2 | 7.52 |
| 2.38 | 125 | 0.5 | 89.7 | 179.4 | 358.7 | 4.52 |

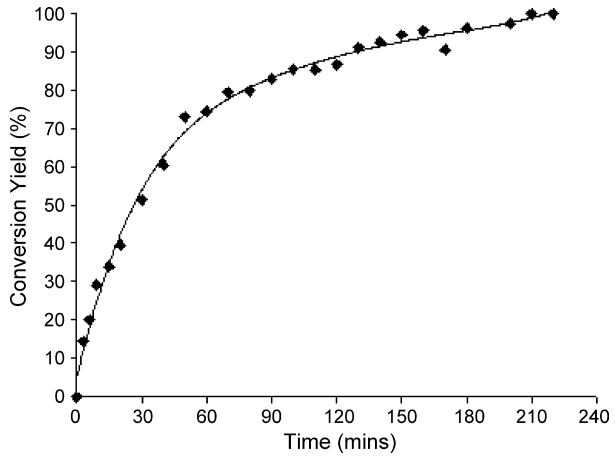


Fig. 2. Time course of the reaction of synthesis of cephalexin with PACLEA at pH 7.4, 14 °C, 30% (v/v) EG, 200 mM 7ADCA and 600 mM PGME and 62.5 $IU_H$/mmol 7ADCA.

homogeneous conditions, obtained a productivity of 130 mM/h at a conversion yield of 75% with 300 mM 6APA and 500 mM phenylglycine amide in the synthesis of ampicillin. The conversion yield increased to 87% by keeping a high concentration of nucleophile throughout the synthesis and precipitation of the product ampicillin via supersaturation. However, a longer reaction time was required to attain such high yield so that productivity was lowered. When they forced the system to initially heterogeneous conditions at 450 mM nucleophile to ensure saturation with the nucleophile during a substantial part of the synthesis, productivity of 167 mM/h was obtained at a conversion yield of 93%, which represents an improvement with respect to the previous condition. The same authors obtained a productivity of 220 mM/h at a yield slightly over 90% when working under initially heterogeneous conditions with 600 mM 6APA and 900 mM PGME and they forced the system even more by developing a semi-continuous process with repetitive additions of the substrates 6APA and PGME, obtaining a conversion yield of 97%, the highest reported to date for ampicillin; however, productivity fell to 83 mM/h [28]. Even though such volumetric productivities are quite lower than the one reported in this work, comparison is not strictly fair since it is a well reported fact that 7ADCA is a better nucleophile than 6APA for penicillin acylase [33]. The synthesis of ampicillin, amoxicillin and Cex in highly condensed aqueous system under conditions of kinetic substrate supersaturation has been recently reported [29]. Conversion yields were 98, 91 and 92%, respectively. Volumetric productivity for amoxicillin was around 60 mM/h, which is rather modest but, unfortunately, no information is given to

Table 4
Production of cephalexin under sequential batch operation with PGA-450 at pH 7.4, 14 °C, 30% (v/v) EG, 200 mM 7ADCA, 600 mM PGME and 62.5 $IU_H$/mmol 7ADCA

| Batch number | $V_R$ (mL) | $M_C$ (g) | $t_B$ (h) | $t_{ac}$ (h) | $Y$ (%) | [Cex] (mM) | $P$ (mM/h) | $P_{sp}$ (mmol/h g) | $M_{Cex}$ (g) | $M_{Cex,ac}$ (g) |
|---|---|---|---|---|---|---|---|---|---|---|
| 1 | 60 | 2.380 | 0.67 | 0.67 | 100 | 200 | 300 | 7.53 | 4.17 | 4.17 |
| 2 | 59.19 | 2.348 | 0.58 | 1.25 | 100 | 200 | 342.9 | 8.69 | 4.11 | 8.28 |
| 3 | 58.38 | 2.316 | 0.67 | 1.92 | 100 | 200 | 300 | 7.52 | 4.06 | 12.34 |
| 4 | 57.57 | 2.284 | 0.67 | 2.59 | 100 | 200 | 300 | 7.52 | 4.00 | 16.34 |
| 5 | 56.76 | 2.252 | 0.67 | 3.26 | 98 | 196 | 294 | 7.37 | 3.86 | 20.20 |
| 6 | 55.95 | 2.220 | 0.67 | 3.93 | 100 | 200 | 300 | 7.52 | 3.89 | 24.09 |
| 7 | 64.64 | 2.168 | 0.92 | 4.85 | 100 | 200 | 218.2 | 6.48 | 4.49 | 28.58 |
| 8 | 53.02 | 2.104 | 0.83 | 5.68 | 100 | 200 | 240 | 6.07 | 3.68 | 32.26 |
| 9 | 52.21 | 2.072 | 0.92 | 6.60 | 100 | 200 | 218.2 | 5.48 | 3.63 | 35.89 |
| 10 | 51.4 | 2.040 | 1.00 | 7.6 | 96 | 192 | 192 | 4.84 | 3.43 | 39.32 |
| 11 | 50.59 | 2.010 | 1.00 | 8.6 | 96 | 192 | 192 | 4.83 | 3.37 | 42.70 |
| 12 | 49.78 | 1.976 | 1.00 | 9.6 | 100 | 200 | 200 | 5.04 | 3.46 | 46.15 |
| 13 | 48.47 | 1.924 | 1.00 | 10.6 | 100 | 200 | 200 | 5.04 | 3.37 | 49.52 |
| 14 | 46.85 | 1.860 | 0.92 | 11.52 | 100 | 200 | 218.2 | 5.48 | 3.26 | 52.78 |
| 15 | 46.04 | 1.828 | 1.00 | 12.52 | 100 | 200 | 200 | 5.04 | 3.20 | 55.98 |
| 16 | 45.23 | 1.796 | 1.08 | 13.6 | 100 | 200 | 184.6 | 4.66 | 3.14 | 59.12 |
| 17 | 44.42 | 1.764 | 1.08 | 14.68 | 97 | 194 | 179.1 | 4.52 | 2.99 | 62.11 |
| 18 | 43.61 | 1.732 | 1.08 | 15.76 | 100 | 200 | 184.6 | 4.66 | 3.03 | 65.14 |
| 19 | 42.3 | 1.680 | 0.92 | 16.68 | 99 | 198 | 216 | 5.42 | 2.91 | 68.05 |
| 20 | 40.68 | 1.616 | 1.08 | 17.76 | 97 | 194 | 179.1 | 4.52 | 2.74 | 70.79 |
| 21 | 39.87 | 1.584 | 1.25 | 19.01 | 96 | 192 | 192 | 3.87 | 2.66 | 73.45 |
| 22 | 39.06 | 1.552 | 1.42 | 20.43 | 100 | 200 | 141.2 | 3.54 | 2.71 | 76.17 |
| 23 | 38.25 | 1.520 | 1.42 | 21.85 | 100 | 200 | 141.2 | 3.54 | 2.66 | 78.82 |
| 24 | 37.44 | 1.488 | 1.33 | 23.18 | 100 | 200 | 150 | 3.78 | 2.60 | 81.43 |
| 25 | 36.13 | 1.436 | 1.42 | 24.6 | 100 | 200 | 141.2 | 3.54 | 2.51 | 83.94 |
| 26 | 34.51 | 1.372 | 1.33 | 25.93 | 100 | 200 | 150 | 3.78 | 2.40 | 86.33 |
| 27 | 33.7 | 1.340 | 1.33 | 27.26 | 100 | 200 | 150 | 3.78 | 2.34 | 88.67 |
| 28 | 32.89 | 1.308 | 1.42 | 28.68 | 100 | 200 | 141.2 | 3.54 | 2.29 | 90.96 |
| 29 | 32.08 | 1.276 | 1.33 | 30.01 | 100 | 200 | 150 | 3.78 | 2.23 | 93.19 |
| 30 | 31.27 | 1.244 | 1.42 | 31.43 | 98 | 196 | 138.4 | 3.47 | 2.13 | 95.32 |

$V_R$: volume of reaction; $M_C$: biocatalyst mass; $t_B$ time of batch; $t_{ac}$: accumulated time; $M_{Cex}$: cephalexin produced in each batch; $M_{Cex,ac}$: accumulated cephalexin produced.

Table 5
Production of cephalexin under sequential batch operation with PACLEA at pH 7.4, 14 °C, 30% (v/v) EG, 200 mM 7ADCA, 600 mM PGME and 62.5 $IU_H$/mmol 7ADCA

| Batch number | $V_R$ (mL) | $M_C$ (g) | $t_B$ (h) | $t_{ac}$ (h) | $Y$ (%) | [Cex] (mM) | $P$ (mM/h) | $P_{sp}$ (mmol/h g) | $M_{Cex}$ (g) | $M_{Cex,ac}$ (g) |
|---|---|---|---|---|---|---|---|---|---|---|
| 1 | 60.00 | 0.571 | 3.17 | 3.17 | 100 | 200 | 63.16 | 6.64 | 4.17 | 4.17 |
| 2 | 59.20 | 0.563 | 3.33 | 6.50 | 1009 | 200 | 59.97 | 6.31 | 4.11 | 8.28 |
| 3 | 58.40 | 0.556 | 3.67 | 10.17 | 100 | 200 | 54.55 | 5.73 | 4.06 | 12.34 |
| 4 | 57.60 | 0.548 | 4.00 | 14.17 | 99.7 | 199 | 49.83 | 5.24 | 3.99 | 16.33 |
| 5 | 56.80 | 0.541 | 4.00 | 18.17 | 100 | 200 | 50.00 | 5.25 | 3.95 | 20.27 |
| 6 | 56.00 | 0.533 | 4.00 | 22.17 | 100 | 200 | 50.00 | 5.25 | 3.89 | 24.16 |
| 7 | 53.10 | 0.505 | 4.00 | 26.17 | 100 | 200 | 50.00 | 5.26 | 3.69 | 27.85 |
| 8 | 51.50 | 0.490 | 4.00 | 30.17 | 100 | 200 | 50.00 | 5.26 | 3.58 | 31.43 |
| 9 | 50.70 | 0.483 | 4.17 | 34.33 | 98.0 | 196 | 47.02 | 4.94 | 3.45 | 34.88 |
| 10 | 49.90 | 0.475 | 4.50 | 38.83 | 99.9 | 200 | 44.40 | 4.66 | 3.46 | 38.35 |
| 11 | 49.10 | 0.467 | 4.50 | 43.33 | 100 | 200 | 44.44 | 4.67 | 3.41 | 41.76 |
| 12 | 48.30 | 0.460 | 4.67 | 48.00 | 100 | 200 | 42.86 | 4.50 | 3.36 | 45.11 |
| 13 | 45.40 | 0.432 | 4.83 | 52.83 | 99.6 | 199 | 41.21 | 4.33 | 3.14 | 48.25 |
| 14 | 43.80 | 0.417 | 5.00 | 57.83 | 100 | 200 | 40.00 | 4.20 | 3.04 | 51.30 |
| 15 | 43.00 | 0.409 | 5.33 | 63.17 | 100 | 200 | 37.50 | 3.94 | 2.99 | 54.29 |
| 16 | 42.20 | 0.402 | 5.67 | 68.83 | 98.6 | 197 | 34.79 | 3.65 | 2.89 | 57.17 |
| 17 | 41.40 | 0.394 | 5.83 | 74.67 | 100 | 200 | 34.29 | 3.60 | 2.88 | 60.05 |
| 18 | 40.60 | 0.387 | 6.17 | 80.83 | 100 | 200 | 32.43 | 3.40 | 2.82 | 62.87 |
| 19 | 37.70 | 0.359 | 6.50 | 87.33 | 96.0 | 192 | 29.54 | 3.10 | 2.51 | 65.39 |
| 20 | 36.10 | 0.344 | 7.00 | 94.33 | 100 | 200 | 28.57 | 3.00 | 2.51 | 67.90 |
| 21 | 35.30 | 0.336 | 7.17 | 101.50 | 99.2 | 198 | 27.68 | 2.91 | 2.43 | 70.33 |
| 22 | 34.48 | 0.329 | 7.17 | 108.67 | 100 | 200 | 27.91 | 2.93 | 2.40 | 72.72 |
| 23 | 33.66 | 0.321 | 7.50 | 116.17 | 100 | 200 | 26.67 | 2.80 | 2.34 | 75.06 |
| 24 | 32.84 | 0.313 | 7.67 | 123.83 | 100 | 200 | 26.09 | 2.74 | 2.28 | 77.34 |

$V_R$: volume of reaction; $M_C$: biocatalyst mass; $t_B$ time of batch; $t_{ac}$: accumulated time; $M_{Cex}$: cephalexin produced in each batch; $M_{Cex,ac}$: accumulated cephalexin produced.

determine productivity in the case of Cex. Results are hardly comparable in terms of specific productivity because very different types of enzyme preparations are being used and in many cases not enough information is given to calculate it. In most cases soluble penicillin acylase preparations have been used, which precludes enzyme recovery.

### 3.2. Production of cephalexin in sequential batch reactor operation

At the best conditions determined above for PGA-450, the production of Cex was now conducted in sequential batch operation mode. The same conditions established for PGA-450 were used with PACLEA. The process was prolonged for one biocatalyst half-life, which occurred after 30 batches in the case of PGA-450 and 24 batches in the case of PACLEA.

Results are summarized in Table 4 for PGA-450 and in Table 5 for PACLEA. As seen in Table 4, in the case of PGA-450 yield remained constant and close to 100% throughout all the cycle, but productivity in the last batch decreased to 46% of the initial value as a consequence of the increase in the time required to attain maximum yield, which is a reflection of enzyme inactivation. As seen in Table 5, yield also remained constant and close to 100% with PACLEA and in this case productivity in the last batch decreased to 41% of the initial value, as a consequence of enzyme inactivation. However, the time of operation was significantly higher in the case of PACLEA, so that its operational stability was higher as it will be analyzed in the next section.

Total specific productivity and the amount of product per unit mass of biocatalyst after one cycle of biocatalyst used were 3.67 mmol/h g and 40.1 (g/g), respectively for PGA-450 and 3.15 mmol/h g and 135.5 (g/g), respectively for PACLEA. The values of total specific productivity are quite similar for both biocatalysts, since the higher reaction times obtained with PACLEA are compensated by its higher specific activity. However, the amount of product per unit mass of biocatalyst is much higher in the case of PACLEA, which is a consequence of its very high specific activity, a distinctive advantage of CLEAs. On the other hand, PGA-450 has the advantage of a much higher volumetric productivity than PACLEA. Amount of product per unit of activity of hydrolysis was 0.125 g/$IU_H$ for PGA-450 and 0.119 g/$IU_H$ for PACLEA. Amount of product per unit activity of synthesis was 0.18 g/$IU_S$ for PGA-450 and 0.21 g/$IU_H$ for PACLEA. As expected, these latter values are similar for both biocatalysts.

### 3.3. Operational stability of biocatalysts

Stability of PGA-450 and PACLEA during sequential batch reactor operation was determined by measuring the residual activity of hydrolysis and synthesis. The residual hydrolytic activity was determined after each batch and the residual synthetic activity was determined from the initial slope of the production curve in the following batch. Measurements were taken after six batches since the biocatalysts were stable enough not to appreciate significant enzyme decay between two con-

Table 6
Operational stability of PGA-450 and PACLEA at pH 7.4, 14 °C, 30% (v/v) EG, 200 mM 7ADCA, 600 mM PGME and 62.5 $IU_H$/mmol 7ADCA, determined in terms of hydrolytic activity and synthetic activity

| Parameter | PGA-450 | | PACLEA | |
|---|---|---|---|---|
| | Hydrolytic activity | Synthetic activity | Hydrolytic activity | Synthetic activity |
| $A$ | 0.7403 | 0.727 | 1.36 | 1.1951 |
| $k_1$ ($h^{-1}$) | 0.574 | 0.3940 | 0.050 | 0.01928 |
| $k_2$ ($h^{-1}$) | 0.0052 | 0.0132 | 0.014 | 0.0165 |
| $t_{1/2}$ (h) | 77.20 | 30.91 | 93.77 | 105.50 |
| $R$ | 0.89 | 0.97 | 0.99 | 0.99 |

$A$: ratio of specific activity of the intermediate and initial enzyme species; $k_1$: inactivation rate constant in the first stage of enzyme inactivation; $k_2$: inactivation rate constant in the second stage of enzyme inactivation; $t_{1/2}$: enzyme half-life.

secutive batches. Results are presented in Fig. 3 for PGA-450 and PACLEA. In all cases, kinetics of enzyme inactivation was well modeled according to series biphasic mechanism [34,35]. Values of the inactivation parameters and half-life under operation conditions are presented in Table 6. As seen, PACLEA was significantly more stable than PGA-450, but this difference is higher when the residual activity is determined as synthetic activity. This is to be expected since the apparent stability is higher for a diffusion controlled reaction, as it occurs in the synthesis of Cex with PACLEA [36]. Enzyme losses throughout the sequential batch operation were due to inactivation, as shown in Fig. 3. No mechanical losses were produced since the reaction was conducted in a closed system with full retention of the biocatalyst particles. Microscopic examination after the series of batches showed no apparent damage or attrition of the biocatalyst particles.

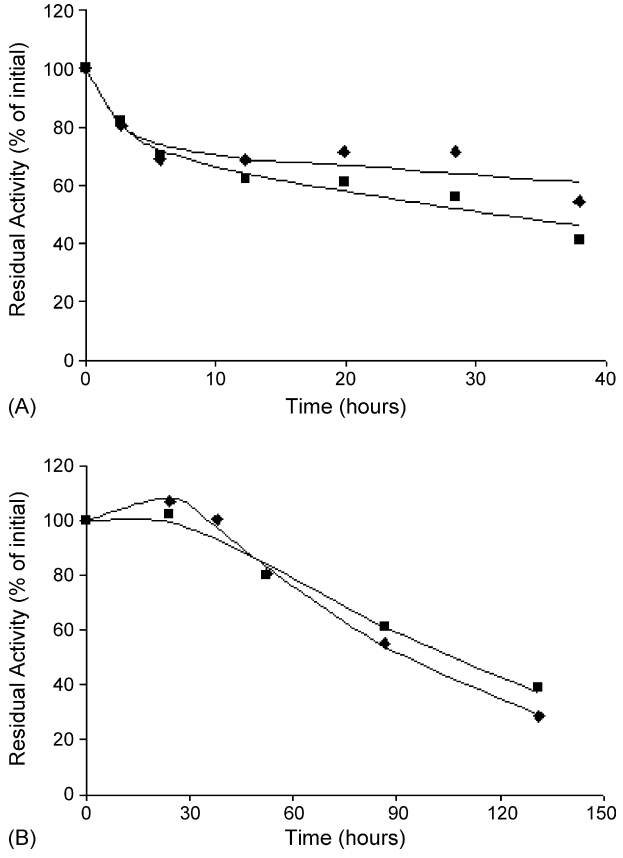


Fig. 3. Time course of enzyme inactivation during sequential batch reactor operation at pH 7.4, 14 °C, 30% (v/v) EG, 200 mM 7ADCA and 600 mM PGME and 62.5 $IU_H$/mmol 7ADCA. (A) PGA-450; (B) PACLEA; (♦) residual hydrolytic activity; (■) residual synthetic activity.

## 4. Conclusions

The kinetically controlled synthesis of Cex with PGA-450 and PACLEA was studied at very high substrates concentrations to the limit of nucleophile solubility and beyond, forcing then the system to lower enzyme to substrate ratio. The hypothesis has been confirmed that a substantial increase in productivity can be obtained at such conditions without reducing conversion yield. Actually, an increase of almost 30 times in volumetric productivity was obtained with respect to the control at 30 mM 7ADCA and 90 mM PGME, keeping conversion yield close to 100%.

The synthesis of Cex with PACLEA was conducted at the best conditions determined above for PGA-450 and stoichiometric conversion yields were also obtained, although productivity was significantly reduced as a consequence of the lower reaction rates, which reflects the presence of diffusional restrictions.

The production of Cex with PGA-450 and PACLEA was evaluated in sequential batch reactor operation at the above conditions to the point of 50% biocatalyst inactivation (one half-life). A much higher volumetric productivity was obtained with PGA-450, but PACLEA had a higher stability and higher specific activity, which reflects in a much higher mass of product obtained per unit mass of biocatalyst. The experiments were conducted on an equal hydrolytic activity basis (62.5 $IU_H$/mmol 7ADCA); even though this is reasonable from a practical point of view (hydrolases are usually marketed in terms of their hydrolytic activity), this is not strictly fair for CLEAs, since the hydrolytic to synthetic activity ratio was higher for PACLEA than for PGA-450. This type of information is seldom reported, even though it is quite relevant to assess the production process. It still remains to be established which biocatalyst is the most suitable for industrial production. A cost-based objective function is being developed to give further insight in this respect. It has to be pointed out that, even though results are very good and compare quite favorably with those previously reported in similar systems, there is still room for improvement, especially

in the case of PACLEA, where optimization of relevant variables such as pH, temperature and EG concentration remains to be done. Production parameters can still be improved by optimizing the biocatalyst replacement criterion, based on a cost objective function.

PACLEA is a very promising penicillin acylase biocatalyst for the production of β-lactam antibiotics, since it performs equally well and it should compare quite favorably in terms of cost with current carrier-bound penicillin acylases. However, handling of the biocatalyst within the reactor in a heterogeneous system might pose some problems when scaling-up the process to industrial level.

## Acknowledgements

This work was funded by Grants 1040748 and 1050303 from Fondecyt, Chile. The authors wish to thank Ms. Rosa Arrieta for her valuable analytical support.

## References

[1] Bruggink A. Synthesis of β-lactam antibiotics. Dordrecht: Kluwer Academic Publishers; 2001, 335 pp.
[2] Wegman M, Janssen M, van Rantwijk F, Sheldon R. Towards biocatalytic synthesis of β-lactam antibiotics. Adv Synth Catal 2001;343:559–76.
[3] Arroyo M, de la Mata I, Acebal C, Castillón M. Biotechnological applications of penicillin acylases: state of the art. Appl Microbiol Biotechnol 2003;60:507–14.
[4] Hernández-Justiz O, Terreni M, Pagani G, García J, Guisán J, Fernández-Lafuente R. Evaluation of different enzymes as catalysts for the production of β-lactam antibiotics following a kinetically controlled strategy. Enzyme Microb Technol 1999;25:336–43.
[5] Nierstrasz V, Schroën C, Bosma R, Kroon P, Beeftink H, Janssen A, et al. Thermodynamically controlled synthesis of cefamandole. Biocatal Biotransform 1999;17:209–23.
[6] Schroën C, Nierstrasz V, Bosma R, Kroon P, Tjeerdsma P, de Vroom E, et al. Integrated reactor concept for the enzymatic kinetic synthesis of cephalexin. Biotechnol Bioeng 2002;80:144–54.
[7] Illanes A, Anjarí S, Altamirano C, Aguirre C. Optimization of cephalexin synthesis with immobilized penicillin acylase in ethylene glycol medium at low temperatures. J Mol Catal B: Enzym 2004;30:95–103.
[8] Giordano RC, Ribeiro M, Giordano RL. Kinetics of β-lactam antibiotics synthesis by penicillin acylase (PGA) from the viewpoint of the industrial enzymatic reactor optimization. Biotechnol Adv 2006;24:27–41.
[9] Aguirre C, Toledo M, Medina V, Illanes A. Effect of cosolvent and pH on the kinetically controlled synthesis of cephalexin with immobilised penicillin acylase. Process Biochem 2002;38:351–60.
[10] Wei D, Yang L. Effect of ethylene glycol on the synthesis of ampicillin using immobilized penicillin acylase. J Chem Technol Biotechnol 2003;78:431–6.
[11] Illanes A, Fajardo A. Kinetically controlled synthesis of ampicillin with immobilized penicillin acylase in the presence of organic cosolvents. J Mol Catal B: Enzym 2001;11:587–95.
[12] Arroyo M, Torres-Guzmán R, de la Mata I, Castillón M, Acebal C. A kinetic examination of penicillin acylase stability in water-organic solvent systems at different temperatures. Biocatal Biotransform 2002;20:53–6.
[13] Terreni M, Pagani G, Ubiali D, Fernández-Lafuente R, Mateo C, Guisán J. Modulation of penicillin acylase properties via immobilization techniques: one pot chemoenzymatic synthesis of cephamandole from cephalosporin C. J Bioorg Med Chem Lett 2002;11:2429–32.
[14] Rocchietti S, San Vicente-Urrutia A, Pregnolato M, Tagliani A, Guisán J, Fernández-Lafuente R, et al. Influence of the enzyme derivative preparation and substrate structure on the enantioselectivity of penicillin G acylase. Enzyme Microb Technol 2002;31:88–93.
[15] Cao L, van Langen L, van Rantwijk F, Sheldon R. Cross-linked aggregates of penicillin acylase: robust biocatalysts for the synthesis of β-lactam antibiotics. J Mol Catal B: Enzym 2001;11:665–70.
[16] Wilson L, Illanes A, Abián O, Pessela B, Fernández-Lafuente R, Guisán J. Co-aggregation of penicillin G acylase and polyionic polymers: an easy methodology to prepare enzyme biocatalysts. Biomacromolecules 2004;5:852–7.
[17] Wilson L, Illanes A, Pessela B, Abián O, Fernández-Lafuente R, Guisán J. Encapsulation of crosslinked penicillin G acylase aggregates in Lentikats: evaluation of a novel biocatalysts in organic media. Biotechnol Bioeng 2004;86:558–62.
[18] Yang S, Zhou L, Tang H, Pan J, Wu X, Huang H, et al. Rational design of a more stable penicillin G acylase against organic cosolvent. J Mol Catal B: Enzym 2002;18:285–90.
[19] Gabor E, de Vries E, Janssen D. A novel penicillin acylase from the environmental gene pool with improved synthetic properties. Enzyme Microb Technol 2005;36:182–90.
[20] Rajendhran J, Gunasekaran PJ. Recent biotechnological interventions for developing improved penicillin G acylases. Biosci Bioeng 2004;97:1–13.
[21] Kallenberg A, van Rantwijk F, Sheldon R. Immobilization of penicillin G acylase: the key to optimum performance. Adv Synth Catal 2005;347:905–26.
[22] Cao L, van Rantwijk F, Sheldon R. Cross-linked enzyme aggregates: a simple and effective method for the immobilization of penicillin acylase. Org Lett 2000;2:1361–4.
[23] López-Serrano P, Cao L, van Rantwijk F, Sheldon R. Cross-linked enzyme aggregates with enhanced activity: application to lipases. Biotechnol Lett 2002;24:1379–83.
[24] Yang L, Wei Z. Effect of temperature on the enzymatic synthesis of cofactor with in situ product removal. J Mol Catal B: Enzym 2003;26:99–104.
[25] Wei D, Zhu J, Cao X. Enzymatic synthesis of cephalexin in aqueous two-phase systems. Biochem Eng J 2002;11:95–9.
[26] Youshko M, Švedas V. Kinetics of ampicillin synthesis catalyzed by penicillin acylase from *E. coli* in homogeneous and heterogeneous systems. Quantitative characterization of nucleophile reactivity and mathematical modelling of the process. Biochemistry (Moscow) 2000;65:1624–33.
[27] Youshko M, van Langen L, de Vroom E, Moody H, van Rantwijk F, Sheldon R, et al. Penicillin acylase catalyzed synthesis of ampicillin in "aqueous solution precipitate" systems. High substrate concentration and supersaturation effect. J Mol Catal B: Enzym 2000;10:509–15.
[28] Youshko M, van Langen L, de Vroom E, van Rantwijk F, Sheldon R, Švedas V. Highly efficient synthesis of ampicillin in an "aqueous solution precipitate" system: repetitive addition of substrates in a semicontinuous process. Biotechnol Bioeng 2001;73:426–30.
[29] Youshko M, Moody H, Bukhanov A, Boosten W, Švedas V. Penicillin acylase-catalyzed synthesis of β-lactam antibiotics in highly condensed aqueous systems: beneficial impact of kinetic substrate supersaturation. Biotechnol Bioeng 2004;85:323–9.
[30] Illanes A, Altamirano C, Fuentes M, Zamorano F, Aguirre C. Synthesis of cephalexin in organic medium at high substrate concentrations and low enzyme to substrate ratio. J Mol Catal B: Enzym 2005;35:45–51.
[31] Illanes A, Wilson L, Caballero E, Fernández-Lafuente R, Guisán J. Cross-linked penicillin acylase aggregates for the synthesis of β-lactam antibiotics in organic medium. Appl Biochem Biotechnol 133 (2006), in press.
[32] Shewale J, Kumar K, Ambedkar G. Evaluation of determination of 6-aminopenicillanic acid by *p*-dimethylamino benzaldehyde. Biotechnol Tech 1987;1:69–72.

[33] Alkema W, de Vries E, Floris R, Janssen D. Kinetics of enzyme acylation and deacylation in the penicillin acylase-catalyzed synthesis of β-lactam antibiotics. Eur J Biochem 2003;270:3675–83.

[34] Henley J, Sadana A. Series-type enzyme deactivation: influence of intermediate activity on deactivation kinetics. Enzyme Microb Technol 1984;6:35–41.

[35] Illanes A, Altamirano C, Zuñiga M. Thermal inactivation of immobilized penicillin acylase in the presence of substrate and products. Biotechnol Bioeng 1996;50:609–16.

[36] Naik S, Karanth N. Effect of internal and external diffusion on the apparent stability of immobilised enzyme catalysts. J Appl Chem Biotechnol 1978;28:569–80.