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Synthesis of Cephalexin in Aqueous Medium with Carrier-bound and Carrier-free Penicillin Acylase Biocatalysts

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Abstract The use of very high substrate concentrations favors the kinetically controlled synthesis of cephalexin with penicillin acylase (PA) not only by Michaelian considerations, but also because water activity is depressed, so reducing the rates of the competing reactions of product and acyl donor hydrolysis. Commercial PGA-450, glyoxyl agarose immobilized (PAIGA) and carrier-free cross-linked enzyme aggregates of penicillin acylase (PACLEA) were tested in aqueous media at concentrations close to the solubility of nucleophile and at previously determined enzyme to nucleophile and acid donor to nucleophile ratios. The best temperature and pH were determined for each biocatalyst based on an objective function considering conversion yield, productivity, and enzyme stability as evaluation parameters. Stability was higher with PAIGA and specific productivity higher with PACLEA, but best results based on such objective function were obtained with PGA-450. Yields were stoichiometric and productivities higher than those previously reported in organic medium, which implies significant savings in terms of costs and environmental protection. At the optimum conditions for the selected biocatalyst, operational stability was determined in sequential batch reactor operation. The experimental information gathered is being used for a technical and economic evaluation of an industrial process for enzymatic production of cephalexin in aqueous medium.

Keywords Penicillin acylase · Enzyme immobilization · Cephalexin · Cross-linked enzyme aggregates · Multipoint covalent attachment

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Introduction

Penicillin acylase (penicillin amidohydrolase, E.C. 3.5.1.11) is a flexible enzyme that is able to catalyze several reactions of organic synthesis [1–6]. Among those reactions, the synthesis of semisynthetic penicillins and cephalosporins from the corresponding β-lactam nuclei and suitable acyl donors is of paramount importance to the pharmaceutical industry [7]. Synthesis can be conducted under thermodynamic [8, 9] or kinetic [10, 11] control. Under thermodynamic control, substrate conversion is limited by the equilibrium of the reaction. This is not so in the case of synthesis under kinetic control, where the formation of an acyl-enzyme complex generates a kinetic competition of nucleophilic attack either by the nucleophile substrate or water, which allows, in principle, to obtain substrates conversions well over the equilibrium. The conversion yield is the result of that competition. The drawback of synthesis under kinetic control is the extra cost represented by the use of an activated acyl donor and the precise control of the reaction required to avoid unnecessary product hydrolysis after maximum conversion yield is attained [12]; however, in most cases high conversion yields and productivities are obtained [13-15].

Cephalexin is a pharmaceutically relevant semisynthetic cephalosporin produced mainly by chemical synthesis; however, an industrial facility for producing cephalexin by a totally enzymatic process has entered into operation recently [16], with reduction of 50 to 15 kg waste/kg product being a major factor for success [17]. The kinetically controlled synthesis of cephalexin requires an activated acyl donor (phenylglycine methyl ester, PGME, or phenylglycine amide) to form an acyl-enzyme complex with the enzyme that is further attacked nucleophilically by the β-lactam nucleus, 7-amino3-desacetoxicephalosporanic acid (7ADCA) to yield the product, according to a well-reported mechanism [18–22]. Penicillin acylase is a moderately expensive enzyme so that stabilization is mandatory to increase the efficiency of utilization and reduce its impact on processing cost. Among the many strategies used for penicillin acylase stabilization [13], immobilization to solid supports [23–27] and autoimmobilization by aggregation are prominent [28–30]. Multipoint covalent attachment to activated agarose gels and cross-linked enzyme aggregates (CLEAs) are particularly promising in terms of enzyme stabilization and have been used successfully to immobilize penicillin acylase [31–36]. We have previously used glyoxyl-agarose immobilized penicillin acylase [37, 38] and CLEAs [39, 40], as well as the commercial biocatalyst PGA-450 [41], in the kinetically controlled synthesis of cephalexin in organic medium using ethylene glycol as cosolvent with PGME as acyl donor and obtained yields close to stoichiometric and fair productivities, being higher in the case of CLEAs. The presence of organic cosolvents was required to attain high yields at moderate substrates concentrations [37, 42–44], but the effect of organic solvent concentration was not significant when working at very high substrates concentrations [38, 40]. Therefore, the hypothesis was raised that at such conditions concentration yields as high as in organic medium and even higher productivities could be attained in a fully aqueous medium, with the additional advantages of cost savings in solvent and waste treatment and the environmental benefits associated. Very high substrates concentrations should favor the kinetically controlled synthesis of cephalexin with penicillin acylase (PA) not only by Michaelian considerations, but also because water activity is depressed reducing the rates of the competing reactions of product and acyl donor hydrolysis, and good synthesis to hydrolysis rates may be obtained by keeping the enzyme saturated by the nucleophile [45, 46].

Results are presented on the kinetically controlled synthesis of cephalexin from 7ADCA and PGME in aqueous medium at very high substrates concentration with three different

penicillin acylase biocatalysts: commercial PGA-450 and in-house produced glyoxyl agarose immobilized (PAIGA) and carrier-free cross-linked enzyme aggregates (PACLEA), both from recombinant *Escherichia coli* penicillin acylase. These biocatalysts, although based on a penicillin acylase of the same origin, are expected to behave differently as a consequence of the conformational changes produced during immobilization to solid supports [47–49] or aggregation [30, 50–51]. Best pH and temperature within pre-studied suitable ranges were determined for each biocatalyst, based on an objective function (F) considering maximum molar conversion yield (Y), volumetric productivity (P), and operational stability (S) as evaluation parameters. At the corresponding best operating conditions, the catalysts were compared on the basis of the same objective function to determine the most suitable for performing the kinetically controlled synthesis of cephalexin. The best conditions for the selected biocatalyst were validated and at such conditions its operational stability was assessed in a repeated batch reactor operation. Experimental data on the kinetics of synthesis of cephalexin and biocatalyst operational stability are being used for a preliminary technical and economic evaluation of an industrial process for the enzymatic production of cephalexin under a clean technology concept.

Materials and Methods

Chemicals and Reagents

Polyacrylamide gel surface bound penicillin acylase (PGA-450) from *E. coli* with 380± 20 IU/gram 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 with no loss of activity during the whole working period. PAIGA from recombinant penicillin acylase (Antibióticos S.A., León, Spain) with 220±20 IU/g was produced as already published [38]. PACLEA with 980±20 IU/g from the same source was prepared at optimized conditions as previously reported [39]. (R)-(-)-2-phenylglycine methyl ester hydrochloride (97% pure) and cephalexin hydrate were from Sigma Chemical (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); penicillin G potassium salt (PenGK) was donated by Natsus S.A. (Lima, Perú); agarose 10 BCL was from Iberagar (Coina, Portugal). All other reagents were of analytical grade and were either from Sigma-Aldrich or Merck (Darmstadt, Germany).

Analysis

Enzyme activity was determined from the initial slope of NaOH consumption to titrate the protons from phenylacetic acid produced by hydrolysis of penicillin G, using a pHstat (Mettler Toledo, DL50), as previously described [52]. One international unit of activity (IU) of penicillin acylase was defined as the amount of enzyme that hydrolyzes 1 μmol of penicillin G per minute from a 10-mM penicillin G solution in 0.1 M phosphate buffer pH 7.8 at 30 °C.

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} (300×3.9 mm) from Waters (Milford, MA, USA). Samples were eluted under gradient with a sonicated mixture of methanol and 10-mM phosphate buffer pH 7.0 at a flow rate of

1 mL/min and analyzed in the UV detector at 220 nm. Elution times were 2.9, 4.5, 5.7, and 7.4 minutes for 7ADCA, PG, cephalexin, and PGME, respectively. Concentration of substrates and products were calculated from calibration curves using stock solutions. High-performance liquid chromatography (HPLC) samples were always assayed in triplicate, the differences among them never exceeding 3%.

Synthesis of Cephalexin in Fully Aqueous Medium

Synthesis of cephalexin was 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. Samples were taken at intervals and were properly diluted before assay. At high substrate concentrations, the system is highly heterogeneous and a fraction of the substrates and the products are in solid state. Samples with solids in suspension were diluted before assay so that the solids were dissolved except for the biocatalyst particles that were filtered out (the volume occupied by the biocatalyst was insignificant).

Kinetically controlled synthesis of cephalexin was conducted with the three biocatalysts at temperatures of 10°, 14°, and 20 °C and pH 6.5, 7.0, and 7.4, ranges that cover the adequate conditions for synthesis in ethylene glycol medium (30% and 40% *v*/*v*) as previously reported [38, 40, 41]. Substrates concentrations were near the solubility limit of 7ADCA in the reaction mixture to ensure initially homogeneous conditions at all pH and temperatures studied. Conditions were 200 mM 7ADCA and 600 mM PGME. The acyl donor to nucleophile molar ratio could not be reduced below 3 as yield was significantly reduced [43, 52]. Enzyme to limiting substrate ratio was 125 IU/mmol 7ADCA as suggested by previous results in organic medium [41].

Kinetics of cephalexin synthesis was evaluated in terms of the following parameters:

- Molar conversion yield (Y), defined as the maximum molar conversion of the limiting substrate (7ADCA) into product (cephalexin):

$$Y = \frac{[\text{Ceph}]_{\max}}{[\text{7ADCA}]_0} \cdot 100 \tag{1}$$

- Volumetric productivity (P), defined as the amount of cephalexin produced per unit time and unit of reaction volume at the time of maximum conversion yield (mM/h):

$$P = \frac{[\text{Ceph}]_{\max}}{t_{\max}} \tag{2}$$

- Specific productivity (P_{sp}), defined as the amount of cephalexin produced per unit time and unit of biocatalyst mass at the time of maximum conversion yield (mmol/h g biocatalyst):

$$P_{sp} = \frac{[\text{Ceph}]_{\max} \cdot V_{rxn}}{t_{\max} \cdot m_{cat}}, \tag{3}$$

where: $[\text{Ceph}]_{máx}$ is the concentration (millimolar [mM]) of cephalexin at maximum conversion yield, $[\text{7-ADCA}]_0$ is the initial concentration (mM) of 7ADCA, V_{rxn} is the volume of reaction, $t_{máx}$ is the time at which maximum conversion yield is attained, and m_{cat} is the biocatalyst mass (gram).

Stability of the biocatalysts was assessed in terms of the half-life values under nonreactive conditions at the corresponding pHs and temperatures and at the same enzyme concentration as the one used in the reactions of synthesis.

An objective function (F) was defined where the above parameters were weighed according to:

$$F = \frac{w_Y \cdot f_Y + w_P \cdot f_P + w_S \cdot f_S}{w_Y + w_P + w_S} \tag{4}$$

where f_Y, f_P, and f_S are the relative values of the respective parameters (fractions of the highest value obtained arbitrarily taken as 1, and w_Y, w_P, and w_S are the corresponding weight coefficients. Weight coefficients are hard to establish as it is quite difficult to evaluate *a priori* their relative incidence in the cost structure of the process. Therefore, they were initially considered equal ($w_Y = w_P = w_S = 1$) but then were varied to see how robust is the determination of the best values. This objective function was used to determine the best pH and temperature for each biocatalyst but also to discriminate among them at their corresponding best operating conditions. Experiments of synthesis were done in duplicate with differences below 5%. All data points were assayed in duplicate, or in triplicate, if the difference between samples exceeded 3% (only in a few cases); reported data are the average of two (or three) samples.

Operational Stability of the Selected Biocatalyst

Operational stability of the selected biocatalyst at its optimum conditions was determined by measuring the activity at the beginning and at the end of each batch. Stability was also determined by measuring the variation of the initial rate of synthesis in each sequential batch. Batches were conducted for a time (90 min) at which the maximum conversion yield is easily achieved in the first batch. As enzyme deactivates, this yield is not achievable at that time, but for simplicity, all batches were conducted for 90 minutes irrespective of the final yield attained, as the purpose was to determine operational stability. In all cases, maximum yield was attainable by increasing the reaction time, which was experimentally checked. Reactors were the same as those described in the previous section; at the end of each batch the reactor was emptied through its bottom filter to recover the biocatalyst, which was then thoroughly washed to remove the precipitated products (PG and eventually cephalexin). The hydrolytic activity was measured at the beginning and at the end of each batch, and the activity of synthesis was assayed at the beginning of each batch by measuring the initial rate of cephalexin synthesis; its variation throughout the batches was used to assess the operational stability of the biocatalyst in terms of its potential of synthesis.

Results and Discussion

Synthesis of Cephalexin in Fully Aqueous Medium

Syntheses of cephalexin under kinetic control with PGA-450, PAIGA, and PACLEA were carried out at 200 mM 7ADCA, 600 mM PGME, and 125 IU/mmol 7ADCA at the pH and temperature ranges established. Reactions were conducted to the point of maximum yield and beyond, and Y, P, and P_{sp} were determined according to Eqs. 1, 2, and 3. Results are summarized in Tables 1, 2, and 3 for PGA-450, PAIGA, and PACLEA, respectively.

Table 1 Effect of pH and temperature in the synthesis of cephalexin with PGA-450 at 200 mM 7ADCA, 600 mM PGME and 125 IU per mmol 7ADCA.

T (°C)	pH	Y (%)	P (mM/h)	$t_{1/2}$ (h)	f_Y	f_P	f_S	F_{111}	F_{211}
10	7.0	87.8	422	2770	0.915	0.774	0.902	0.864	0.876
	7.4	84.8	339	**3070**	0.883	0.622	1	0.835	0.847
14	7.0	86.8	417	2670	0.904	0.765	0.870	0.846	0.861
	7.4	**96.0**	384	2720	1	0.705	0.886	**0.864**	**0.898**
20	7.0	88.7	533	1480	0.924	0.978	0.482	0.795	0.827
	7.4	90.9	**545**	1020	0.947	1	0.332	0.760	0.808

Numbers in bold correspond to the results obtained at the best conditions, determined according to the proposed objective function, considering $w_Y = w_P = w_S = 1$ (F_{111}) and $w_Y = 2$; $w_P = w_S = 1$ (F_{211})

Stability of biocatalysts was determined in separate experiments under nonreactive conditions (without substrates), but maintaining the rest of the conditions used in the experiments of synthesis. Inactivation of biocatalysts through time was followed until reaching their corresponding half-lives and those values were used as stability parameters as recorded in Tables 1, 2, and 3. Enzyme inactivation (data not shown) did not follow one-stage first-order kinetics, data being better represented by a two-stage series mechanism of inactivation [53]. However, for the sake of comparison, half-life was considered adequate, although in this case there is no direct correlation between half-life and inactivation rate constants. The stability of all three biocatalysts decreased with temperature, but all of them were quite stable at the conditions studied, PAIGA being the most stable, as expected for an enzyme immobilized by multipoint covalent attachment [48]. The effect of pH on stability varied, however, from one biocatalyst to another. The effect was rather mild for PGA-450 and for PAIGA, but was strong in the case of PACLEA where the stability was considerably higher at pH 7.0 than at 7.4, which might be the consequence of partition in the protein network within the CLEA. In most cases, Y was higher at pH 7.4. Below pH 7.0, Y decreased sharply as what happened in the synthesis of cephalexin when using ethylene glycol as cosolvent medium [37, 41] where this effect has proven to be the consequence of the decrease in the ratio of synthesis to hydrolysis rates below pH 7.0 [54]. At pH 6.5 (data not shown), Y was below 70% in all cases so that was not further considered. There was no definite trend of Y with temperature within the range studied and varied from one biocatalyst to another, being somewhat higher at 14 °C for PGA-450, at 20 °C for PAIGA, and at 10 °C for PACLEA, but only at pH 7.0; in this latter case, there was a strong interaction between the variables pH and temperature. As expected, P increased with

Table 2 Effect of pH and temperature in the synthesis of cephalexin with PAIGA at 200 mM 7ADCA, 600 mM PGME and 125 IU per mmol 7ADCA.

T (°C)	pH	Y (%)	P (mM/h)	$t_{1/2}$ (h)	f_Y	f_P	f_S	F_{111}	F_{211}
10	7.0	88.4	112	**7500**	0.952	0.554	1	0.835	0.865
	7.4	83.2	105	7250	0.897	0.520	0.967	0.795	0.820
14	7.0	87.7	140	6900	0.945	0.693	0.92	0.853	0.876
	7.4	89.5	165	6900	0.965	0.817	0.92	**0.900**	**0.917**
20	7.0	89.7	179	2225	0.967	0.886	0.297	0.717	0.779
	7.4	**92.8**	**202**	1780	1	1	0,237	0.745	0.809

Numbers in bold correspond to the results obtained at the best conditions, determined according to the proposed objective function, considering $w_Y = w_P = w_S = 1$ (F_{111}) and $w_Y = 2$; $w_P = w_S = 1$ (F_{211})

Table 3 Effect of pH and temperature in the synthesis of cephalexin with PACLEA at 200 mM 7ADCA, 600 mM PGME and 125 IU/mmol 7ADCA.

T°C)	pH	*Y* (%)	*P* (mM/h)	$t_{1/2}$ (h)	f_Y	f_P	f_S	F_{111}	F_{211}
10	7.0	**84.0**	183.3	**3010**	1	0.645	1	**0.882**	**0.911**
	7.4	72.4	158.1	1970	0.862	0.556	0.655	0.691	0.734
14	7.0	79.5	212.0	2670	0.946	0.746	0.887	0.860	0.881
	7.4	77.8	186.8	1400	0.926	0.657	0.465	0.683	0.744
20	7.0	74.8	260.6	2240	0.890	0.917	0.744	0.850	0.860
	7.4	82.9	**284.2**	850	0.987	1	0.282	0.756	0.814

Numbers in bold correspond to the results obtained at the best conditions, determined according to the proposed objective function, considering $w_Y = w_P = w_S = 1$ (F_{111}) and $w_Y = 2$; $w_P = w_S = 1$ (F_{211})

temperature for all biocatalysts, which is a consequence of the increase in activity. There was no definite trend with respect to the effect of pH on *P* and in most cases the effect was not significant, which is a reflection of the little difference in activity at the two pH values tested. *P* at pH 6.5 (data not shown) was considerably lower in all cases as a consequence of the significant decrease in enzyme activity below pH 7.0. In the case of PGA-450 and PAIGA, the best pH was 7.4, which differs somewhat with the results obtained at moderate substrates concentrations in ethylene glycol medium where the pH optima were close to 7.0 [35, 39].

According to the objective function (Eq. 4), if the parameters are weighed equally ($w_Y = w_P = w_S = 1$), the best pH and temperatures are 7.4 and 14 °C both for PGA-450 and PAIGA, and 7.0 and 10 °C for PACLEA. The relative weights were varied to $w_Y = 2$ and $w_P = w_S = 1$, which can be considered reasonable as *Y* has a profound influence in the quality of product and downstream operations. This is quite relevant in the case of pharmaceuticals [55], but the best conditions remained unchanged in all cases. In fact, the better conditions were further reinforced for the case of PGA-450 and PACLEA, and in the case of PAIGA, *Y* has to be weighed fivefold ($w_Y = 5$; $w_P = w_S = 1$) to modify the best conditions of pH and temperature determined. Therefore, the criterion for determining the best pH and temperature based on such objective function can be considered robust.

The kinetics of cephalexin synthesis with the three biocatalysts at their corresponding best temperature and pH values is presented in Fig. 1. Decrease in yield after maximum is smooth, as in the case of synthesis at high concentrations of substrates in ethylene glycol

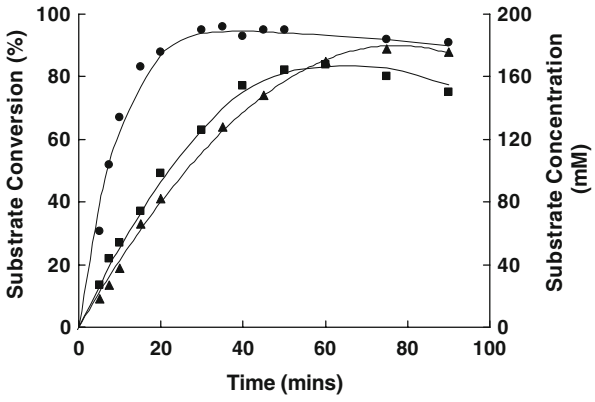

Fig. 1 Synthesis of cephalexin at 200 mM 7ADCA, 600 mM PGME, and 125 IU/mmol 7ADCA. (*Filled circle*): PGA-450; (*filled triangle*): PAGA; (*filled rectangle*): PACLEA at the best conditions of pH and temperature for each biocatalyst (see Tables 1, 2, and 3)

medium [38], and quite smoother than in the case of synthesis at moderate substrates concentration in such medium [37, 41, 56], reflecting that the use of very high substrates concentration effectively depress the hydrolytic reactions. Conversion yields correlate well with the synthesis to hydrolysis ratio of the antibiotic, but the effect is determined mainly by the initial rates of synthesis (higher for PGA-450 and lower for PAIGA); the rates of antibiotic hydrolysis were quite similar for PGA-450 and PAIGA and somewhat higher for PACLEA. A summary of results obtained in these reactions is in Table 4. The stability of PAIGA was much higher than in the other two biocatalysts, which is a definite advantage of immobilization by multipoint covalent attachment. P_{sp} was significantly higher for PACLEA, which is an advantage of carrier-free biocatalysts, as the activity per unit mass is considerably higher compared with carrier-bound biocatalysts. P was much higher for PGA-450 as a reflection of the much higher reaction rates at comparable IU per millimole 7ADCA; the lower P values were obtained with PAIGA. The value close to 400 mM/h obtained with PGA450 is to our knowledge the highest reported for the enzymatic synthesis of cephalexin [14]. Y was also significantly higher for PGA-450, the lower Y values being obtained with PACLEA.

Despite being the less stable biocatalyst, PGA-450 was somewhat superior to the others when compared in terms of the F function at the corresponding best pH and T values, as shown in Table 4. This difference increases as w_Y is weighed higher because the highest Y was obtained with this biocatalyst. Synthesis of cephalexin at the pH and temperature values determined has been further optimized for PGA-450 with respect to the enzyme to substrate ratio and acyl donor to nucleophile ratio. Although the enzyme-to-substrate ratio could be reduced to one-half without reduction in Y (at the expense of the corresponding reduction in P), reduction of the acyl donor to nucleophile ratio below 3 reduced Y significantly [52]. Despite the lower Y and P values obtained, PAIGA is an interesting alternative to commercial immobilized penicillin acylase because it is a very stable biocatalyst, so that global productivity may be quite high. At the end of the reaction with PGA-450, 61% of the cephalexin was soluble and the remaining 39% had precipitated out. On the other hand, 75% of the PG produced as a byproduct of the reaction of synthesis and also by hydrolysis of PGME was in the form of a precipitate, whereas only 25% remained dissolved in the medium; 71% of the remaining 7ADCA was soluble and the other 29% was in the precipitate. Most of the PGME has been already hydrolyzed to PG at the end of the reaction.

Operational Stability of the Selected Biocatalyst

The operational stability of PGA-450 was determined at the best conditions previously established: pH 7.4, 14 °C, 125 IU/mmol 7ADCA under initially homogeneous conditions

Table 4 Comparison of the biocatalysts at their best pH and T in the synthesis of CEX at 200 mM 7ADCA, 600 mM PGME and 125 IU per mmol 7ADCA at the corresponding best values of pH and temperature.

Catalyst	T (°C)	pH	Y%	r[a]	P mM/h	P_{sp} mmol/h g_{cat}	$t_{1/2}$ h	f_Y	f_P	f_S	F_{111}	F_{211}
PGA-450	14	7.4	**96.0**	**66.2**	**384**	4.92	2720	1	1	0.394	**0.798**	**0.849**
PAGA	14	7.4	89.5	18.9	165	3.39	**6900**	0.932	0.430	1	0.787	0.823
PACLEA	10	7.0	84.0	8.8	183.3	12.76	3010	0.875	0.477	0.436	0.596	0.666

Numbers in bold correspond to the results obtained at the best conditions, determined according to the proposed objective function, considering $w_Y = w_P = w_S = 1$ (F_{111}) and $w_Y = 2$; $w_P = w_S = 1$ (F_{211})

[a] Ratio of initial rate of synthesis and hydrolysis of cephalexin

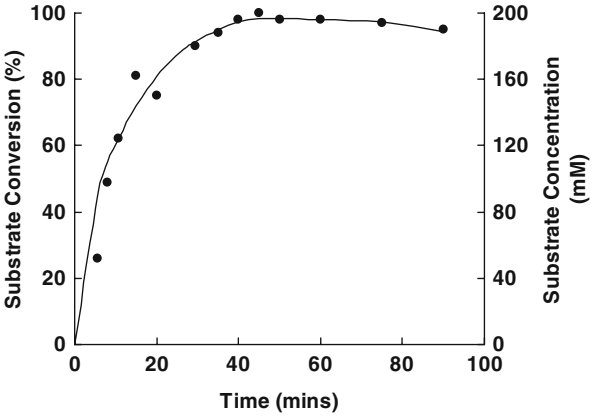

Fig. 2 Synthesis of cephalexin with PGA-450 at 125 IU/mmol 7ADCA at pH 7.4, 14 °C, 200 mM 7ADCA, 600 mM PGME and 125 IU/mmol 7ADCA in the first batch of a series of sequential batches

with 200 mM 7ADCA and 600 mM FGME. Time course of cephalexin synthesis in the first batch is presented in Fig. 2. Results are quite similar to those reported in Fig. 1 for PGA-450, which validates the process. Results for 10 consecutive batches with biocatalyst recovery are summarized in Table 5. No enzyme make-up was considered between batches, except for the amounts withdrawn for sampling. Although production batches were prolonged for 90 minutes (more than the time required to attain maximum yield in the first batch), after a few batches the yield at that time began to slowly decline as the biocatalyst was progressively inactivated. Although the mode of operation will be the one in which the activity lost will be replenished by the addition of fresh biocatalyst (to better reproduce the performance of the first batch), this mode of operation was adopted by the reasons given in "Materials and Methods". Between batches, the recovered biocatalyst had to be stored for some time, so that final mass activity at the end of one batch differed somewhat from the initial in the following batch. From the data in Table 5, an average of 2% loss of enzyme activity per batch is observed, whether expressed as hydrolytic or synthetic activity. Figure 3 shows the time course of PGA-450 inactivation during the synthesis of cephalexin with PGA-450 at 125 IU/mmol 7ADCA, 200 mM 7ADCA, and 600 mM PGME at pH 7.4 and 14 °C. Profiles of enzyme decay are

Table 5 Synthesis of cephalexin with PGA-450 at 125 IU/mmol 7ADCA with 200 mM 7ADCA, 600 mM PGME in a series of sequential batches.

Batch Nr	Mass hydrolytic activity (IU_H/g)		Mass activity of synthesis (IU_S/g)[a]	Conversión Yield (%)	Accumulated time (h)
	Initial	Final			
1	294.0	290.0	167.2	98.0	90
2	305.8	295.4	165.0	98.3	180
3	289.5	286.4	162.6	96,7	270
4	267.1	265.8	158.1	96.3	360
5	276.9	277.7	157.0	92.3	450
6	256.0	247.1	146.3	90.5	540
7	245.3	239.8	137.3	88.8	630
8	235.0	233.8	138.8	89.9	720
9	235.1	239.5	135.4	85.8	810
10	237.3	233.9	133.9	83.1	900

*Measured as the initial reaction rate in each batch

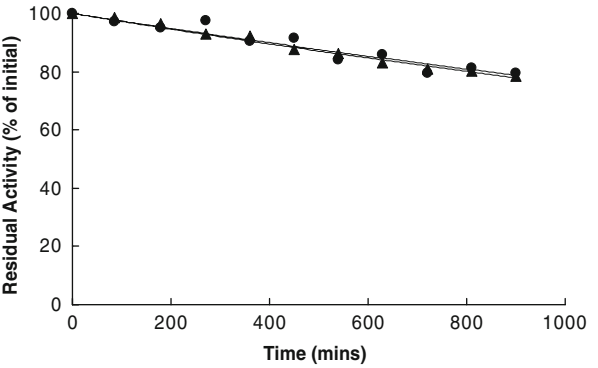

Fig. 3 Time course of PGA-450 inactivation during the synthesis of cephalexin at pH 7.4, 14 °C, 200 mM 7ADCA, 600 mM PGME and 125 IU/mmol 7ADCA. (*Filled circle*): hydrolytic activity; (filled triangle): activity of synthesis

about the same when measuring residual hydrolytic activity and residual activity of synthesis. Enzyme inactivation was modeled according to one-stage first-order kinetics (k_D=0.0175 h^{-1}) and the extrapolated operational enzyme half-life was 40 h. This value is lower than the one reported under nonreactive conditions, but the difference is mainly the consequence of the manipulation of the biocatalyst between batches rather than proper biocatalyst inactivation, these being an artifact at laboratory scale that can be avoided at production scale with a sound procedure of biocatalyst recovery. With data extrapolated from Fig. 3, a projected cycle of enzyme considering one and two half-lives as criteria for biocatalyst replacement is of 27 and 53 productive batches, respectively.

Based on the experimental results obtained in the synthesis of cephalexin with PGA-450, including the distribution of substrates and products between liquid phase and solid phase (precipitate) at the end of reaction, and the operational stability of the biocatalyst, an industrial process is being designed for the production of 12,650 kg of cephalexin per year under a clean technology concept. The objective market of 7ADCA-derived cephalosporins in Chile is being considered in this production process. Preliminary results indicate that under a reasonable scenario of costs of raw materials and price of product, the project is profitable with an internal return rate of investment (IRR) close to 80%. However, because of the uncertainties in the values considered, a sensitivity analysis was performed with respect to the cost of PGME and the price of the product, which are the most critical items in the economic profile of the project. The profitability appears robust with respect to the price of the product (reduction in the selling price of 40% reduces the IRR from 80 to 60%), but not as much with respect to the estimated cost of the PGME (an increase of 40% in the cost of PGME reduces the IRR from 80 to 40%). The cost of the biocatalyst is not critical because of its very efficient utilization.

Conclusions

Two carrier-bound penicillin acylase biocatalysts (PGA-450 and PAIGA) and one carrier-free biocatalyst (PACLEA) have been evaluated for the kinetically controlled synthesis of cephalexin at high substrate concentrations in aqueous medium. All of them performed well, but according to an objective function based on conversion yield, productivity, and biocatalyst stability, PGA-450 was determined as the best when comparing at the corresponding best conditions for each biocatalyst, mainly because of the higher yield and productivity obtained. However, PAIGA is a very stable biocatalyst and PACLEA has a

very high mass activity that turns into a high, specific productivity, so that it is interesting to keep on working with them.

Yields obtained with PGA-450 are almost stoichiometric and a volumetric productivity close to 400 mM/h is the highest reported for cephalexin to our knowledge. These results are better than those previously reported in ethylene glycol medium, with the additional advantages of costs savings in solvent and waste treatment, and the environmental benefits associated.

Operational stability of PGA-450 was determined in sequential batch operation for 10 consecutive batches with an average activity loss of less than 2% per batch, which allows using the enzyme for a cycle of about 60 batches before biocatalyst replacement (considering a criterion of use of two half-lives).

A preliminary evaluation of an industrial process for the production of cephalexin based on the experimental data obtained is underway. The high cost of PGME and the excess required to drive the reaction to completion is the main obstacle that remains to be addressed. We have not succeeded in reducing the excess of acyl donor below 3 mol/mol of nucleophile, which appears as the key issue for improving the economics of the process. The development of better biocatalysts not requiring such excess, as the consequence of a better synthesis to hydrolysis ratio, and the reduction in the cost of PGME or its replacement by a cheaper acyl donor (i.e, phenylglycine amide) are key issues that remain to be solved.

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