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Optimization of cephalexin synthesis with immobilized penicillin acylase in ethylene glycol medium at low temperatures

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Abstract

Organic cosolvents, and among them, polyols, are suitable media to perform the enzymatic synthesis of β-lactam antibiotics with immobilized penicillin acylase, because they effectively reduce water activity, depressing hydrolytic reactions in favor of synthesis. Among polyols, ethylene glycol has proven to be particularly suited as reaction medium for their synthesis. Previous studies have shown that pH, temperature, and cosolvent concentration are the most relevant variables in the kinetically controlled synthesis of cephalexin from 7-amino-3-deacetoxy cephalosporanic acid and phenylglycine methyl ester, conversion yield increasing at low temperatures and high cosolvent concentrations. The objective of this work is the optimization of temperature, pH, and ethylene glycol concentration in the kinetically controlled synthesis of cephalexin with immobilized penicillin acylase at lower than ambient temperature in terms of substrate molar conversion yield. Phenylglycine was used as acyl donor and 7-amino-3-deacetoxy cephalosporanic acid was the limiting substrate at 30 mM. Optimization was performed using surface of response methodology, optimum conditions being 12 °C, pH 6.8, and 60% (v/v) ethylene glycol, at which cephalexin yield was close to stoichiometric with respect to the limiting nucleophile, which is unattainable in aqueous medium. Stability of the biocatalyst at optimum conditions for cephalexin synthesis was very high, with a projected half-life of 1500 h, making it a suitable catalyst for the large-scale production of cephalexin.

Keywords: Penicillin acylase; Cephalexin; β-Lactam antibiotics; Enzymatic synthesis; Immobilized enzymes

1. Introduction

Semi-synthetic cephalosporins are a family of antibiotics of considerable therapeutic and commercial relevance [1]. They are produced, mostly chemically, from leading molecules, like cephalosporin C [2,3] and penicillin G [4] and can arise either from 7-amino-3-deacetoxy cephalosporanic (7ADCA), like cephalexin [5], cefadroxil [6], and cefaclor [7], or from 7-amino cephalosporanic (7ACA), like cephalotin [8], cefamandole [9], and cefazolin [10].

Biocatalysis can be considered a viable alternative to replace the chemical route in use for the production of semi-synthetic β-lactam antibiotics [3]. Higher specificity and lower environmental burden of biocatalytic processes are paving the way for such substitution [11], but better biocatalysts and higher yields than actually obtained are required [12]. Substantial advances have been made in penicillin acylase biocatalyst design by directed immobilization [13,14], aggregation [15,16] and derivatization [17] and fruits are still to come from protein engineering techniques, like site-directed mutagenesis and directed evolution [18,19]. On the other hand, solvent engineering has been a major breakthrough in enzyme biocatalysis [20,21]. The use or organic solvents as reaction medium for synthesis with penicillin acylase is appealing, because reduced water activity depresses water driven reactions in favor of synthesis [22], while increasing the proportion of reactive non-ionized species [23]. However, hydrophobic solvents are hardly compatible with penicillin acylase and the process conditions required for synthesis, so that in practice more hydrophilic organic cosolvents are to be preferred, even though they are reportedly deleterious for

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enzyme activity [24]. There are, however, notable exceptions like polyols, where penicillin acylase is as active and even more stable than in aqueous medium [25], which has been consistently proven in the case of ethylene glycol [26–28].

Synthesis of derived cephalosporins with penicillin acylase can be conducted under thermodynamic [29–31] or kinetic control [32–34].

The first strategy considers the displacement of equilibrium from hydrolysis to synthesis, this is, the direct condensation of the nucleophile and the acyl donor. Non-ionic forms of the substrates are required for the condensation reaction, so that pH will be an important variable in this strategy by altering the ionic equilibria of the nucleophile and the acyl donor substrates. Cosolvents may be helpful in this strategy by altering the p*K* of the carboxylic acids and by reducing the amount of water that pulls equilibrium in favor of hydrolysis of the condensation product. In this strategy, the yield is determined by the thermodynamic equilibrium of the reaction (which is largely independent of the biocatalyst used) and drastic conditions are usually required to displace it in favor of synthesis, so that very robust biocatalysts are required to perform adequately [44].

Synthesis under kinetic control requires an activated acyl donor, in the form of an ester [35] or an amide [36]. It is usually a better strategy when product yield is the main issue, since product concentration is not limited by the equilibrium of the reaction [37]. In the kinetically controlled synthesis of β-lactam antibiotics, the reaction of synthesis (synthetase activity) will occur simultaneously with the hydrolysis of both the activated acyl donor (esterase activity) and the antibiotic product (amidase activity) [32]. Yields will be then favored by reducing water activity, since the rates of the hydrolytic reactions will be reduced [22]. Because of the shape of its sorption isotherm, ethylene glycol is particularly effective in reducing water activity [38]. Previous studies of cephalexin synthesis with immobilized penicillin acylase, showed a strong impact of ethylene glycol on product yield, as a consequence of the increase in the ratio of synthesis to hydrolysis [39]. Temperature (in the range above ambient) and pH were relevant variables in the synthesis of cephalexin in ethylene glycol medium with another penicillin acylase biocatalyst, yields increasing at lower temperature [40].

This article presents the optimization of the kinetically controlled synthesis of cephalexin with immobilized penicillin acylase in ethylene glycol medium in the range of temperature below ambient. Temperature, pH, and ethylene glycol concentration were selected as the most relevant variables, and optimum conditions were determined using response surface methodology, having cephalexin molar yield as objective function. The hypothesis underlying is that yield should increase at low temperatures and high ethylene glycol concentrations at levels significantly higher than those obtained in aqueous medium and temperatures above ambient.

2. Materials and methods

2.1. Materials

7-Amino-3-desacetoxicephalosporanic acid (7ADCA), (R)-(−)-2-phenylglycine methyl ester hydrochloride (PGME) 97% pure, D-α-phenylglycine (PG) and cephalexin hydrate were from Sigma Chemical Company Inc. (St. Louis, MO, USA). Penicillin G potassium salt was a donation of Natsus S.A. (Lima, Perú). Ethylene glycol (EG) and all other reagents and solvents were analytical grade either from Sigma-Aldrich (St. Louis, MO, USA) or Merck (Darmstadt, Germany). Immobilized penicillin acylase (PGA-450) from *Escherichia coli* with 320 ± 20 IU/g was from Roche Molecular Biochemicals (Mannheim, Germany). One international unit of activity (IU) was defined as the amount of PGA-450 that hydrolyses 1 μmol PenG/min from 134 mM PenG in 0.1 M phosphate buffer pH 7.8 at 30 °C and 250 rpm.

2.2. Analysis

Substrates (7ADCA and PGME) and products (cephalexin and PG) 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 6.0 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 3.7, 7.0, and 11.6 min for 7ADCA, cephalexin, and PGME, respectively. Concentration of substrates and products were calculated from calibration curves using stock solutions. Hydrolytic activity of PGA-450 was determined from initial rate data, analyzing 6APA formation as reported elsewhere [41].

2.3. Synthesis of cephalexin with PGA-450

Synthesis of cephalexin was performed batch-wise in pH and temperature-controlled reactors with 50 mL of reaction medium. A paddle impeller was used to maintain biocatalyst particles suspended. Substrate concentrations were 30 mM 7ADCA, and 90 mM PGME and the enzyme to limiting substrate ratio was 125 IU/mmol 7ADCA, as suggested from previous studies with ampicillin [28]. The amount of enzyme added was adjusted according to temperature to provide an equal catalytic potency (at 12 °C, this corresponds to 26 mg of PGA-450/mL of reaction).

Temperature (*T*), pH, and EG concentration (C_{EG}) varied according to the experimental design below. During synthesis, pH and temperature were monitored and samples were taken to analyze product and substrates to determine yield and volumetric productivity. Yield was defined as the

maximum molar conversion of 7ADCA into cephalexin (%) and productivity as the amount of cephalexin produced per unit time and unit reaction volume at maximum yield (mM/h). Specific productivity was determined per gram of biocatalyst.

2.4. Experimental design for optimization

The effects of pH, T, and C_{EG} on the synthesis of cephalexin, at the established substrates concentrations and enzyme to limiting substrate (7ADCA) ratio, were determined using response surface methodology [42]. At the above defined conditions, a master combination of pH, T, and C_{EG} was set according to a full 2^k factorial design (three factors at two levels), considering also three central points to evaluate the experimental error. The design was further expanded to a circumscribed central composite design, introducing $2k$ additional runs, being 17 the total number of experiments required for this experimental design, as seen in Table 1. Range of variation of pH and C_{EG} was determined from a previous study at higher than ambient temperature with another immobilized penicillin acylase [40]. The range of temperature was set below ambient. A software package (Modde 4.0 for Windows, Umetri, Umeå, Sweden) was employed to fit, using multiple linear regression, a (tentatively) second order model for the response (yield) with respect to T, pH, and Cs, according to:

$$Y = a + bX_T + cX_{pH} + dX_{C_{EG}} + eX_T^2 + fX_{pH}^2 + gX_{C_{EG}}^2 + hX_T X_{pH} + iX_T X_{C_{EG}} + jX_{pH} X_{C_{EG}} \quad (1)$$

where Y represents yield, X represents the variables in their coded form and a to j are the corresponding coefficients. The determination coefficient (R^2) is the fraction of variation of the response explained by the model. The prediction coefficient (Q^2) is the fraction of variation of the response that can be predicted by the model and provides the best summary of the fit of the model. R^2 is an overestimate and Q^2 an underestimate of the goodness of fit of the model. Optimum reaction conditions predicted from such models, in terms of T, pH, and C_{EG}, were experimentally validated. At those optimum conditions, the effect of acyl donor to nucleophile molar ratio was also studied at values higher and lower than the value of 3 (90 mM:30 mM) used in the optimization. The objective was to evaluate the option of reducing the excess of PGME to reduce costs of substrate and product recovery from the reacted medium.

Table 1
Experimental design and yields obtained in the kinetically controlled synthesis of cephalexin with PGA-450 considering temperature (T) ethylene glycol concentration (C_{EG}) and pH as variables

Experiment number	pH	T (°C)	C_{EG} (% v/v)	Yield (%)
1	6.0	0	40	46
2	6.0	0	60	78
3	6.0	25	40	59
4	6.0	25	60	63
5	7.5	0	40	44
6	7.5	0	60	86
7	7.5	25	40	73
8	7.5	25	60	67
9	6.75	12.5	40	66
10	6.75	12.5	60	99
11	6.75	0	50	87
12	6.75	25	50	70
13	6.0	12.5	50	87
14	7.5	12.5	50	80
15	6.75	12.5	50	91
16	6.75	12.5	50	92
17	6.75	12.5	50	92

2.5. Operational stability of PGA-450 in the synthesis of cephalexin

Stability of PGA-450 during cephalexin synthesis, at the previously determined optimum conditions, was determined under sequential batch operation in reactors similar to those described above, but provided with a bottom screen to recover the biocatalyst. After each batch, the product was removed and the biocatalyst recovered and washed thoroughly to remove some precipitated PG. Each batch was conducted until the maximum yield was obtained. The time required to attain it was recorded in each batch and the residual activity after each one was also determined. Since the time of each batch at the enzyme concentration used in the optimization was short and too many batches would be required to appreciate enzyme inactivation, the concentration of biocatalyst was reduced to 1/8 of the previous value, so that the time to attain maximum yield increased accordingly.

3. Results and discussion

3.1. Optimization of synthesis of cephalexin with PGA-450

Optimization of cephalexin synthesis under kinetic control was done considering temperature (in the range below ambient), pH, and C_{EG} as variables. Ratio of PGME to 7ADCA was kept in 3 and enzyme to limiting substrate in 125 IU/mmol 7ADCA. Experimental design and results, in terms of Y, are summarized in Table 1. Representative time-course of cephalexin syntheses are illustrated in Fig. 1. As expected for a kinetically controlled reaction, cephalexin concentration increased up to a maximum, after which decreased as product hydrolysis outweighs synthesis. During synthesis, the acyl donor PGME is also hydrolyzed as revealed by the continuous increase in PG concentration (not shown). The same pattern of synthesis was observed in all experimental conditions tested. Initial rates are somewhat lower at higher C_{EG}, which is a common observation when using organic cosolvents as reaction medium [43], but hydrolysis of product is reduced as revealed by a much smoother decay of yield after maximum.

A quadratic model (Eq. (1)) was fitted from the experimental data in Table 1, which, after eliminating

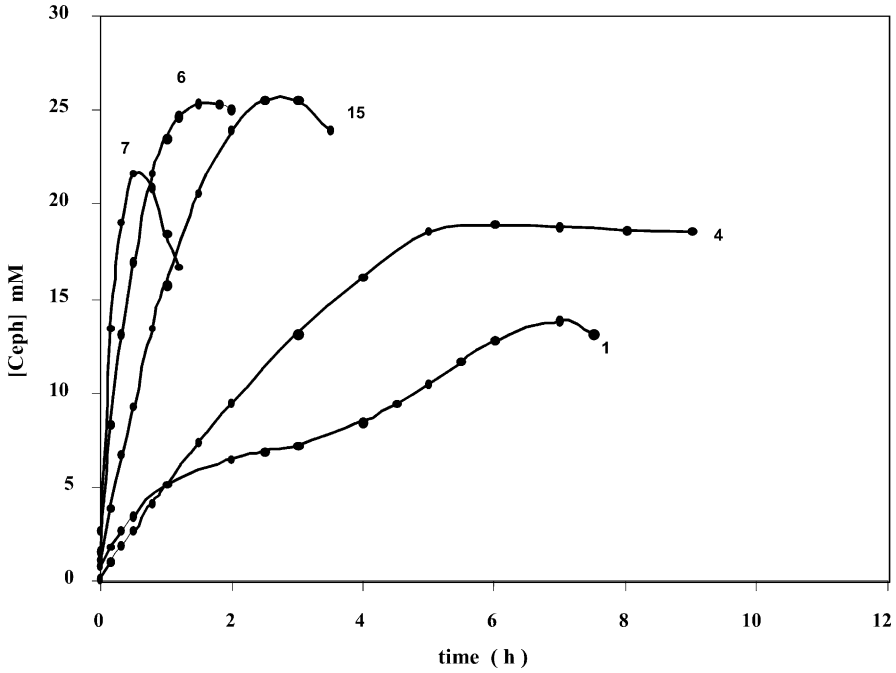

Fig. 1. Time course of cephalexin synthesis with PGA-450 at selected conditions, according to experimental design. Numbers denote experiments in Table 1.

non-significant coefficients ($P > 0.05$), is represented by the second order polynomial in Eq. (2):

$$Y = 91 + X_T + 8.6X_{\mathrm{pH}} + 3.6X_{C_{\mathrm{EG}}} - 10.5X_T^2 - 18X_{\mathrm{pH}}^2 + 2X_{C_{\mathrm{EG}}}^2 - 9.5X_T X_{\mathrm{pH}} - 1.5X_T X_{C_{\mathrm{EG}}} - 5.6 \times 10^{-6} X_{\mathrm{pH}} X_{C_{\mathrm{EG}}} \quad (2)$$

ANOVA test indicates that the quadratic model adequately described yields for cephalexin synthesis, with values for R^2 of 0.99 and for Q^2 of 0.61, which are statistically sound. Fig. 2 shows the correlation between experimentally determined and model predicted values of yield. A straight line is obtained, which highlights the predictive value of the model. Maximum yields predicted at the values of experimental conditions considered in the experimental design are summarized in Table 2.

Optimum conditions for cephalexin synthesis predicted by the model were 12 °C, pH 6.8, and C_{EG} 60% (v/v), at which predicted yield was 99%. Surfaces of response at the optimum conditions for each variable are presented in Fig. 3. Experimental yields obtained in three different runs at such optimum conditions were 98, 99, and 99%, validating the prediction. Synthesis at such optimum conditions is shown in Fig. 4, both in EG medium and in phosphate buffer. As seen, such high yield was unattainable in aqueous medium, where only 50% was obtained and decay after maximum was sharper because of the higher hydrolytic rates. Even though conditions were not optimized for aqueous medium, synthesis in such medium has been performed in a wide range of conditions, yields never exceeding 50%, which validates the hypothesis of this work. Productivity at optimum conditions was 36 mM/h, while at the same pH and temperature in aqueous medium, productivity was only 14 mM/h (Fig. 4).

Yields of cephalexin synthesis are significantly higher than those obtained for ampicillin with the same biocatalyst, where maximum was 59% in a two-variable optimization at 50% (v/v) ethylene glycol [28]. This is the usual behavior, both in cosolvent [39,44] and in aqueous media [33,34,45,46], which highlights that 7ADCA is a better nucleophile than 6APA for antibiotic synthesis with penicillin

Table 2
Predicted maximum yields in the in the kinetically controlled synthesis of cephalexin with PGA-450 and values of temperature (T), ethylene glycol concentration (C_{EG}), and pH at which they are obtained

	Yield (%)
C_{EG} (% v/v)	
40	90 (pH 6.9 $T = 11$)
50	92 (pH 6.9 $T = 12$)
60	98 (pH 6.8 $T = 12$)
T (°C)	
0	88 (pH 7.0 $C_{\mathrm{EG}} = 60$)
12.5	98 (pH 6.8 $C_{\mathrm{EG}} = 60$)
2.5	88 (pH 6.7 $C_{\mathrm{EG}} = 60$)
pH	
6.0	73 ($T = 21$ $C_{\mathrm{EG}} = 60$)
6.75	97 ($T = 13$ $C_{\mathrm{EG}} = 60$)
7.5	88 ($T = 8$ $C_{\mathrm{EG}} = 60$)

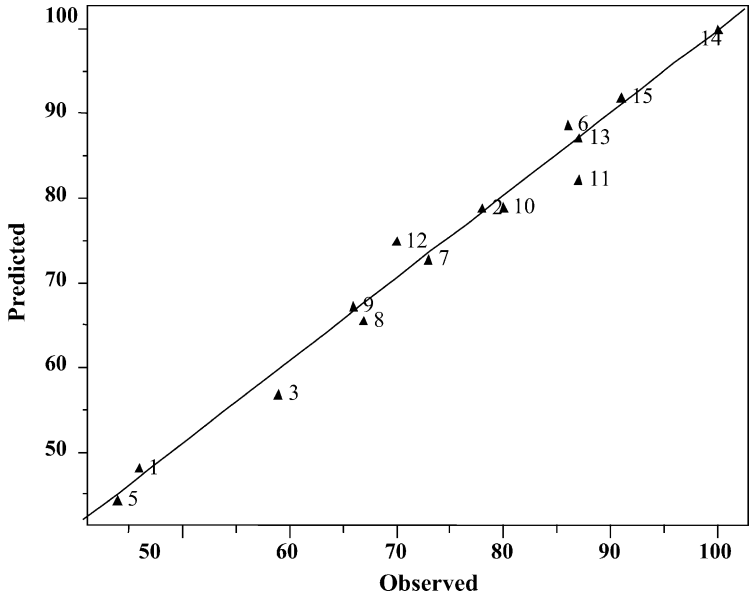

Fig. 2. Correlation between observed and predicted values of cephalexin yield. Numbers refer to experiments in Table 1.

acylase. Synthesis of cephalexin has been previously optimized in terms of pH and temperature at a constant C_{EG} (50% v/v) using a recombinant penicillin acylase immobilized in glyoxyl–agarose (GAPA). In that case, temperature range above ambient was considered; optimum values were 27 °C and pH 7.0 and maximum yield obtained was 96%, slightly lower than the one obtained here. Productivity at optimum conditions was reported as 15 mM/h for GAPA [40]. A sound straightforward comparison with the value obtained in this work (36 mM/h) is not possible because of the different activity of the biocatalysts. However, they can be compared in terms of specific productivity (per gram of biocatalyst): in that case, a value of 27.7 mM/h g is obtained with PGA-450, higher than 16.0 mM/h g obtained with GAPA [40].

Yield was the highest at the highest C_{EG} tested, which is consistent with the reduced water activity and the consequent reduction of hydrolytic rates in a kinetically controlled synthesis [39,47]. However, there is not much room for further yield increase at higher C_{EG}, which in turn will only reduce productivity and yield since C_{EG} values higher than 65% (v/v) produce considerable enzyme inactivation. At optimum conditions (pH 6.8, C_{EG} 60% (v/v), and 12 °C) the initial rate of synthesis (v_s) was 1.1 mM/min, while the initial rate of PGME (v_h) was 1.0 mM/min with a v_s/v_h ratio of 1.1. At the same conditions in fully aqueous medium the v_s/v_h ratio dropped to 0.7; this was a consequence of higher v_h since v_s was about the same as in EG medium. A similar effect was observed at temperatures above ambient, where yield increase in EG was the consequence of the increasing ratios of synthesis to hydrolysis of cephalexin and synthesis of cephalexin to hydrolysis of PGME [39]. The increase in yield with C_{EG} can be explained because EG is a polar, water soluble and protic solvent, that will solvate the already formed antibiotic, and will displace water from the active site of the enzyme thus impairing its hydrolysis. This is supported by the data in Fig. 1 where yield decay after maximum is steeper at the lower C_{EG}.

The effect of pH on yield was strong (see Eq. (2)) and yield was reduced at values below the optimum of 6.8. One possible explanation may be that the carbonyl group of the acyl donor will be progressively ionized at lower pH, so that the fraction of its reactive non-ionized form will be reduced [48]. However, pH effect is quite complex promoting also conformation changes of the enzyme and affecting substrate adsorption.

Yield increased when reducing temperature below ambient, which has been previously reported for the case of ampicillin [27], cephalexin [46], and cephalothin [8] and is consistent with the mechanistic prediction done by Kasche [49]. This behavior can be explained in terms of the values of the energies of activation of the reaction of cephalexin synthesis and hydrolysis. The former has been determined to be higher, which means that the rate of synthesis will be less affected by temperature than the rate of hydrolysis, favoring the process at low temperature [50]. A similar pattern has been determined with Assemblase® in which the energy of activation of synthesis was low, indicating a very moderate effect of temperature on reaction rate, while much higher values were obtained for the hydrolysis of cephalexin and phenylglycine amide [34]. A sharp decrease was observed in the K_m value for 7ADCA in the synthesis of cephalexin when the temperature was reduced from 25 to 15 °C, but the K_m for the hydrolysis of cephalexin was hardly affected by temperature in that range [50]. This is also consistent with increased yields at lower temperatures. Yields slightly decreased at temperatures below optimum; there is no clear explanation for this behavior, but diffusional restrictions may

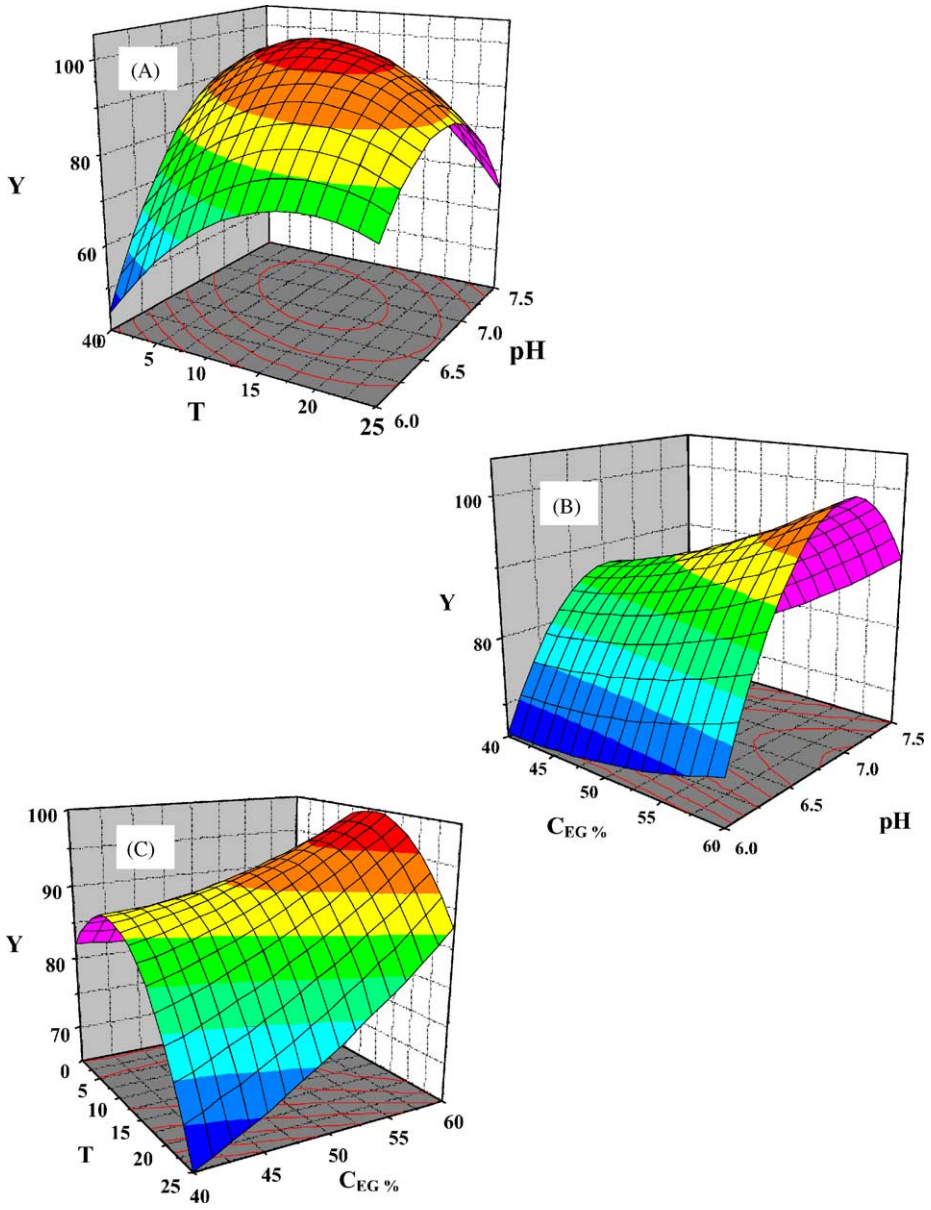

Fig. 3. Surfaces of response at the optimum conditions for each variable. (A) at 60% (v/v) ethylene glycol concentration; (B) at 12 °C; (C) at pH 6.8.

play a role in severely reducing substrate and product transport rates at very low temperatures.

3.2. Effect of acyl donor to nucleophile ratio on the synthesis of cephalexin at optimum pH, temperature and cosolvent concentration

The use of excess acyl donor is common practice in the kinetically controlled synthesis of β-lactam antibiotics to compensate for acyl donor hydrolysis [44]. Reported yields are significantly lower for derived cephalosporins synthesized at limiting or equimolar acyl donor concentrations and even under moderate excess of acyl donor, both in cosolvent and in fully aqueous media [32,33,51,52]. The effect of PGME/7ADCA molar ratio over and below 3 on cephalexin yield was studied and results are summarized in Table 3. Yield was severely reduced below a ratio of 3 and increased only slightly above it, so that a ratio of 3 can be considered optimum in terms of *Y*, since the very little increase in yield at ratios higher than 3 is outweighed by the higher level of phenylglycine produced. This in turn will produce precipi-

Table 3
Effect of PGME/7ADCA ratio on cephalexin yield at optimum conditions of pH, temperature, and ethylene glycol concentration

PGME/7ADCA molar ratio	Maximum yield (%)
1	47
2	86
2.5	87
3	98
4	100

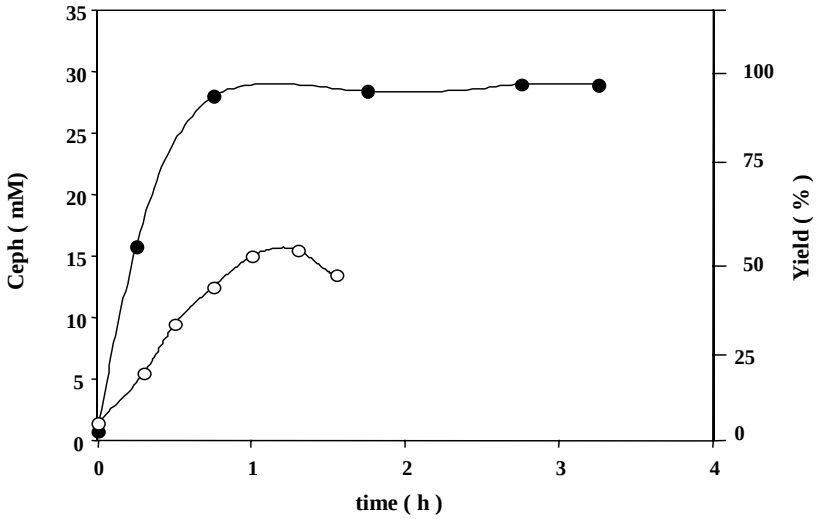

Fig. 4. Time course of cephalexin synthesis: (●) at predicted optimum conditions of pH (6.8), temperature (12 °C), and 60% (v/v) ethylene glycol concentration; (○) in phosphate buffer at the same pH and temperature.

tation, which may hinder the interaction of 7ADCA with the active site of the enzyme [53]. This is rather unfortunate because excess acyl donor is clearly undesirable for large-scale production. It could be possible to obtain higher yields at lower PGME/7ADCA molar ratio by conducting the synthesis at higher C_{EG}, but as said before C_{EG} cannot be increased significantly over 60% because of enzyme inactivation. Possible solutions like the use of better acyl donors and more robust biocatalysts are beyond the scope of this work.

3.3. Operational stability of PGA-450 in the synthesis of cephalexin

The operational stability of PGA-450 at the optimum conditions was tested through 10 consecutive production batches. Each batch was conducted until the maximum conversion was obtained and then the reaction medium was removed, the biocatalyst was recovered and washed to perform the following batch and its residual activity was determined. Results are presented in Table 4. As indicated in Section 2.5, enzyme concentration was reduced from 26 to 3.3 mg/mL to increase the time of each batch (see results in Fig. 4 and Table 4), so reducing the number of batches required to reveal enzyme inactivation. No carrier attrition or enzyme leakage was detected throughout the entire reactor operation. Very little enzyme inactivation was observed at the end of the ten batches and the time required to attain maximum yield increased only slightly. Enzyme inactivation under operation could be described by a series type mechanism with a grace period [54,55] and the projected half-life calculated according to that behavior is 1500 h. The stability of PGA-450 was lower under non-reactive conditions, with a projected half-life of 825 h at the same conditions of pH, temperature, and C_{EG}. This difference can be a consequence of modulation of enzyme inactivation by the substrates and or products of the reaction. In fact, 7ADCA protection of PGA-450 inactivation has been already reported [56]. The stability was even lower in fully aqueous medium with a projected half-life under non-reactive conditions of 95 h. The protective effect of polyols on enzyme stability at low temperatures has also been reported for other penicillin acylase biocatalysts [24,57]. Polyols, and among them, ethyleneglycol, are good hydrogen bonding compounds that can replace water without distorting the enzyme configuration, and help in creating a stable active center configuration [58]. The effect of organic cosolvents on penicillin acylase stability has been thoroughly studied and analyzed in terms of the $\log P$ and the ΔG_d values [24,26]. According to it, EG should destabilize the enzyme at temperatures higher than 40 °C and stabilize it at temperatures below ambient, which is exactly the case for PGA-450 (inactivation in the range from 40 to 60 °C is higher in EG than in aqueous medium, data not shown). A significant difference between biocatalyst stability exists between PGA-450 and GAPA. At optimum conditions for cephalexin yield, half-life was 135 h for GAPA [40] while an estimate of 1500 h was obtained for PGA-450, though this value was obtained by extrapolation.

Table 4
Operational stability of PGA-450 in sequential batch production of cephalexin at optimum conditions (12 °C, 60% (v/v) EG, and pH 6.8) and 3.3 mg PGA-450/mL

Batch number	Time of batch (h)	Total process time (h)	Residual specific activity (IU/g)	Y (%)
1	7	7	353	98
2	7	14	353	98
3	7.3	21.3	353	97
4	7.4	28.7	348	97
5	7.4	36.1	349	96
6	7.5	43.6	347	97
7	7.7	51.3	345	97
8	7.6	58.9	347	96
9	7.6	66.5	344	97
10	7.9	74.4	342	96

This is only in part a consequence of the higher temperature of synthesis used with GAPA, so that PGA-450 can be considered intrinsically more stable than GAPA under operating conditions in the synthesis of cephalexin.

4. Conclusions

The kinetically controlled synthesis of cephalexin was optimized in terms of yield, considering pH, temperature, and ethylene glycol concentration as key variables. Yields close to stoichiometric with respect to the limiting substrate 7ADCA were obtained at the optimum conditions: 12 °C, pH 6.8, and 60% (v/v) ethylene glycol. Yields were considerably higher than those obtained at higher temperatures and lower cosolvent concentrations, validating the hypothesis. Yields were much higher than those previously obtained with the same biocatalysts in the synthesis of ampicillin, which confirms that 7ADCA is a better nucleophile than 6APA for the reactions of synthesis with penicillin acylase. Results compare quite favorably with those already reported for the thermodynamically and kinetically controlled synthesis of cephalexin, both in organic and aqueous media.

Excess acyl donor had a strong effect on cephalexin yield. The PGME/7ADCA ratio of 3 was considered optimum, with negligible increase in yield above it and significant decrease below it. This is a limitation for large-scale production, so the problem of excess PGME needs to be addressed; better acyl donors, like phenylglycine amide [3,34,59], and more robust biocatalysts [15,60] are promising.

Stability of PGA-450 at optimum conditions for cephalexin synthesis was very high, with a projected half-life of 1500 h. This operational stability was much higher than in non-reactive conditions both in EG and in aqueous media, and much higher than previously reported with another immobilized penicillin acylase, which highlights the potential of this biocatalyst for the large-scale production of cephalexin. However, substrates concentrations used are still low to be industrially attractive, so that synthesis at conditions close or even over substrate solubility need to be studied. Yield and specific productivity are expected to increase, but new reaction strategies need to be envisaged to cope with the heterogeneity of the reaction. This study is underway.

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