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Low temperature effect on production of ampicillin and cephalexin in ethylene glycol medium with immobilized penicillin acylase

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Abstract

The effect of temperature was studied for the kinetically controlled synthesis of cephalexin and ampicillin with penicillin acylase immobilised in glyoxyl agarose. Yield increased at low temperatures in the absence and presence of ethylene glycol, while the initial ratio of synthesis to hydrolysis decreased. Arrhenius equations were used to describe the temperature dependency of the hydrolysis and synthesis rates. The effect of ethylene glycol was stronger over the yield of synthesis of cephalexin than ampicillin. In the case of cephalexin, yield increased from 82.8% in aqueous buffer to 97.6% in 50% (v/v) ethylene glycol medium at 0 °C, while at 20 °C an increase from 68.8% to 78.7% was obtained. The presence of ethylene glycol produced a greater increase in the energies of activation of the hydrolysis reactions than of the synthesis reactions, which explains the higher conversion yields obtained in the presence of the cosolvent, both for cephalexin and ampicillin. Cephalexin synthesis was optimized using an experimental design based on surface of response methodology.

Keywords: Penicillin acylase; Cephalexin; β-Lactam antibiotics; Enzymatic synthesis; Organic cosolvents; Energy of activation

1. Introduction

Biocatalysis is a valuable alternative to conventional, but cumbersome, chemical processes, not only for the well-established production of β-lactam nuclei from first-generation β-lactam antibiotics [1,2], but also for the production of semi-synthetic antibiotics derived from those leader molecules [3]. From the latter aspect, better enzymes and careful control of process conditions are required to compete with chemical synthesis in terms of product yield [4]. Penicillin acylase (penicillin amidohydrolase; E.C. 3.5.1.11) is a remarkably versatile enzyme which can conduct both hydrolytic and synthetic reactions over a wide range of compounds [5–7], playing a key role in the synthesis of β-lactam antibiotics [4]. Advances in the field of enzyme immobilization have been determinant for the industrial success in the production of β-lactam nuclei and, very recently, for the synthesis of β-lactam antibiotics as well [8–10].

New antibiotics have to be continuously developed to overcome the constant development of microbial resistance. Semi-synthetic cephalosporins (SSC) and penicillins (SSP) are important families of antibiotics, usually produced by chemical methods from precursor molecules, such as penicillin G (PG) and cephalosporin G [3,11]. Pharmaceutically relevant SSC can arise from 7-aminodesacetoxycephalosporanic acid (7-ADCA), such as cephalexin [12], cefadroxil [4] and cefachlor [13], or from 7-aminocephalosporanic acid (7-ACA), such as cephalotin [14] and cefamandole [15]. SSP, such as ampicillin [16] and amoxicillin [17], arise from 6-aminopenicillanic acid (6-APA). The enzymatic synthesis of SSC and SSP can be conducted either under thermodynamic or kinetic control. The latter, although requiring activated acyl donors, such as amides or esters, is usually a better strategy when product yield is the main issue, since it is not limited by the equilibrium of the reaction [18]. Kinetically controlled synthesis of β-lactam antibiotics has, however, some drawbacks since the synthesis reaction will occur simultaneously with the hydrolysis of both the activated acyl donor and the antibiotic product [19]. This can produce a rather sharp peak of antibiotic production [20]

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and require an excess of activated acyl donor that can increase costs and hamper product recovery [4]. Conditions that depress hydrolytic reactions can increase product yield and facilitate product recovery at maximum yield.

Organic cosolvents are suitable media for performing the synthesis of β-lactam antibiotics, because they can favor synthesis by reducing water activity and increasing the proportion of reactive non-ionized species [21]. Polyols are especially suitable, since they strongly depress water activity and increase enzyme stability, those effects being correlated with the number of hydroxyl groups in the polyol moiety [16,22,23]. In the enzymatic synthesis of ampicillin and cephalexin with immobilized penicillin acylase, ethylene glycol (EG) and 1,2-propanediol (PD) were selected as the best among several poloyols tested [20,24]. Temperature was reported also to be a key variable, with a remarkable increase in yield at low temperatures [25,26] even though no explanation was given for this phenomenon.

A study of the temperature effect on the conversion yield in the synthesis of cephalexin and ampicillin with immobilized penicillin acylase in glyoxyl agarose was conducted, using 7-ADCA and 6-APA as nucleophiles and (*R*)-(−)-2-phenylglycine methyl ester (PGME) as acyl donor in 50% (v/v) EG medium and in aqueous buffer. The objective was to explain the higher conversion yields obtained at low temperatures in terms of the activation energies of the individual reactions that take place in the kinetically controlled mechanism of synthesis.

The kinetically controlled synthesis of cephalexin was optimized in terms of molar product yield using quadratic response surface methodology (RSM), considering pH, temperature and cosolvent concentration as the most relevant variables. Using this methodology, valuable information was obtained on the relevance of each variable and the interactions among them employing a limited number of experiments [27–29].

2. Materials and methods

2.1. Materials

Free penicillin G acylase (PA) from recombinant *Escherichia coli* was a product from Antibióticos SA (Spain), 7-aminodesacetoxycephalosporanic acid, (*R*)-(−)-2-phenylglycine methyl ester hydrochloride (97% pure) (PGME), cephalexin (Cep) hydrate, ampicillin (Amp), penicillin G potassium salt (Pen G) and phenylglycine (PG) were from Sigma Chemical Company Inc. (St. Louis, MO, USA). Ethylene glycol (EG), and all other reagents were analytical grade either from Sigma–Aldrich (St. Louis, MO, USA) or Merck (Darmstadt, Germany).

2.2. Immobilization of penicillin G acylase

IPA was obtained by immobilization of recombinant *E. coli* penicillin acylase (PA) through covalent multipoint attachment to activated agarose [30] with a specific activity of 256 IU_H/g.

2.3. Analysis

7-ADCA, Cep and PGME were identified and analyzed by HPLC using a system with a Lichrospher 100 RP-18 125 mm × 4 mm 5-μm column, a Bruker UV-detector at 220 nm, a Rheodyne 7125i 20-μL injector, and a Knauer 64 pump with a flow-rate of 1 mL/min. The eluant was composed of 30% (v/v) methanol in 20 mM phosphate buffer pH 6.0. Product concentrations were calculated from calibration curves using stock solutions. In the case of Pen G, methanol at 40% (v/v) in phosphate buffer was used.

One international unit of activity of hydrolysis (IU_H) was defined as the amount of IPA that hydrolyzes 1 μmol of Pen G per minute at 30 °C and pH 7.8 from 134 mM Pen G in 0.1 M phosphate buffer.

2.4. Hydrolysis of β-lactam antibiotics with IPA

Batch-wise hydrolysis reactions were performed at 30 mM Cep (or Amp), 125 IU_H/mmol 7-ADCA (or 6-APA) at pH 7.0 in a temperature range from 0 to 20 °C in 50% (v/v) ethylene glycol and aqueous buffer. In all cases initial rates of antibiotic hydrolysis (v_h) were measured.

2.5. Synthesis of cephalexin with IPA

Batch-wise synthesis reactions were performed at 30 mM 7-ADCA (6-APA), 90 mM PGME and 125 IU_H/mmol 7-ADCA (6-APA). The temperature effect on conversion yield (Y) and initial rate of antibiotics synthesis (v_s) were studied in the range from 0 to 20 °C, both in the presence (50%, v/v) and the absence of EG at pH 7.0. Yield was defined as the maximum molar conversion of 7-ADCA into Cep (%) and productivity (P) as the amount of antibiotic produced per unit time and unit reaction volume at maximum yield (mM/h).

For Cep optimization, temperature (T), pH and cosolvent concentration (Cs) varied according to the experimental design below. During synthesis, the pH and temperature were monitored and samples were taken to analyze product and substrates to determine yield and productivity. The amount of enzyme added at each temperature tested was corrected by a factor equivalent to the variation in initial reaction rate of penicillin G hydrolysis, using 30 °C and pH 7.8 as control. This is why the reaction of synthesis was optimized in terms of conversion yield, since the productivity will be affected by the introduction of the enzyme load as a new variable.

2.6. Energy of activation

Considering that the effect of the kinetic constants of hydrolysis k'_H and synthesis k'_S are proportional to the initial reaction rates (expressed by unit mass of IPA), when Arrhenius equations (Eqs. (1) and (2)) are applied, the apparent energies of activation of hydrolysis and synthesis (Ea'_H; Ea'_S) are obtained:

$$k'_H = A_H e^{-Ea'_H/RT} \quad (1)$$

$$k'_S = A_S e^{-Ea'_S/RT} \quad (2)$$

where A is the preexponential term, R the universal gas constant and T is the absolute temperature.

2.7. Experimental design

The effects of pH, T and ethylene glycol concentration (Cs) on the synthesis of Cep under the conditions defined above were determined by modulating the variables 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, requiring a total of 17 experiments for this experimental design. A software package (Modde 4.0 for Windows, Umetri, Umeå, Sweden) was employed to fit the second order models using multiple linear regression, for the response (Y) with respect to T, pH and Cs, according to Eq. (3):

$$Y = \beta_o + \Sigma \beta_i X_i + \Sigma \beta_{ii} X_i^2 + \Sigma\Sigma \beta_{ij} X_i X_j \quad (3)$$

where Y is the molar conversion yield, β_o the offset term, β_i the linear effect, β_{ii} the squared effect, β_{ij} the interaction effect and X_i and X_j are the independent variables or experimental factors (T, pH and Cs).

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.

Ranges for the selected variables in the optimization of Cep synthesis in ethylene glycol medium were: pH, 6.5–7.5; *T*, 0–20 °C and Cs, 40–60% (v/v).

Optimum reaction conditions predicted from such models, in terms of *T*, pH and Cs, were experimentally validated.

3. Results and discussion

3.1. Effect of temperature and ethylene glycol in the kinetically controlled synthesis of cephalexin and ampicillin

As seen in Fig. 1, the initial rates of hydrolysis of Amp and Cep decreased markedly with ethylene glycol. Reaction rates were similar for both antibiotics, although slightly lower for Cep. As expected, reaction rates for Cep and Amp, both in the presence and in the absence of EG, decreased at lower temperatures.

The same effect was observed for the initial reaction rates of synthesis (Fig. 2). However, in this case initial reaction rates for Cep were much higher than for Amp even though the effect of temperature is slightly lower in the former case. Higher initial reaction rates for Cep synthesis will explain the higher conversion yields obtained with respect to Amp.

In summary, v_s and v_h decreased with Cs, v_s and v_h increased with temperature, v_s for Cep was much higher than for Amp and v_h for Amp was slightly higher than for Cep.

The effect of temperature on the time course of Cep synthesis in the absence of ethylene glycol (fully aqueous buffer) is shown in Fig. 3. As expected for a kinetically controlled reaction, Cep concentration increased up to a maximum, after which it decreased as product hydrolysis outweighed synthesis. During synthesis, the acyl donor PGME was also hydrolyzed as revealed by the continuous increase in PG concentration. The same pattern of synthesis was observed in all experimental conditions tested. It can be seen that the lower the temperature, the higher the synthesis yield, reaching a value close to 83% at 0 °C.

The effect of EG on the conversion yield at different temperatures is shown in Fig. 4. Yields always increased with Cs due to the decreasing effect on hydrolysis reaction rates. The effect is stronger for Cep, where the yield increased from 83% to 98% at 0 °C, while Amp yield increased only from 77% to 83% at the same temperature.

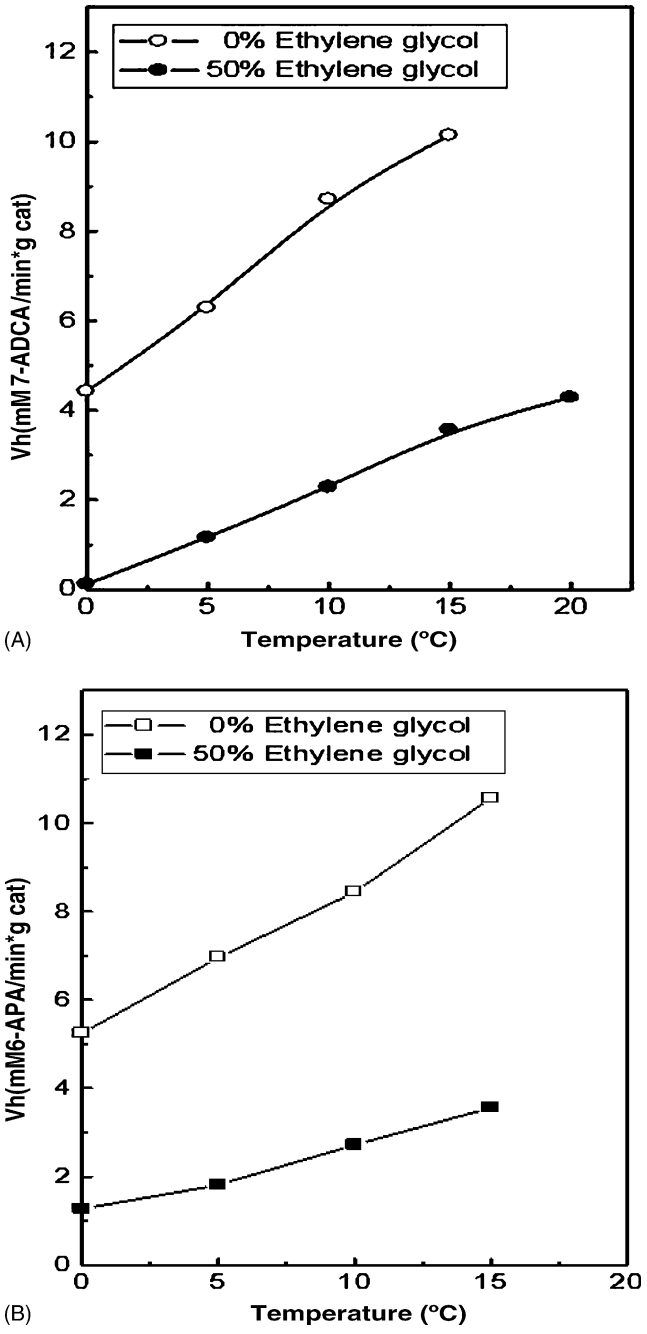

Fig. 1. Effect of temperature on the initial rates of hydrolysis of (A) cephalexin (○) and (B) ampicillin (□). Open symbols, control (fully aqueous buffer); closed symbols, EG 50% (v/v). Conditions: pH 7.0, 30 mM antibiotics, 125 IU_H per mmol antibiotics.

3.2. Activation energies in the kinetically controlled synthesis of cephalexin and ampicillin

A summary of the values of activation energies (kJ) for the synthesis and hydrolysis of Amp, Cep and PGME with and without EG is presented in Table 1. r_{EG} is the ratio of energies of activation (Ea) with and without EG.

Both in the synthesis and hydrolysis reactions, Ea was higher in EG than in aqueous buffer, with a stronger effect for the antibiotics hydrolysis reactions. Ea for the hydrolysis of PGME varied only slightly with the presence of EG. Ea for the hydrolysis of Cep is 31% higher than for Amp in EG medium (60.55 and 46.13 kJ, respectively). In the absence of EG (fully aqueous buffer) the difference is only 23% (37.19 and 30.25 kJ, respectively). The increase in Ea in the presence of EG is rather small for the synthesis reactions as compared to the hydrolysis reactions, being somewhat higher for Amp than for Cep.

Conversion yield of both antibiotics increased by the presence of EG, which can be explained satisfactorily in terms of the Ea values obtained. For instance, in the absence of EG, the ratio of $E a'_H$ to $E a'_S$ (r_{HS}) was 1.2 and 1.9 for Amp and Cep,

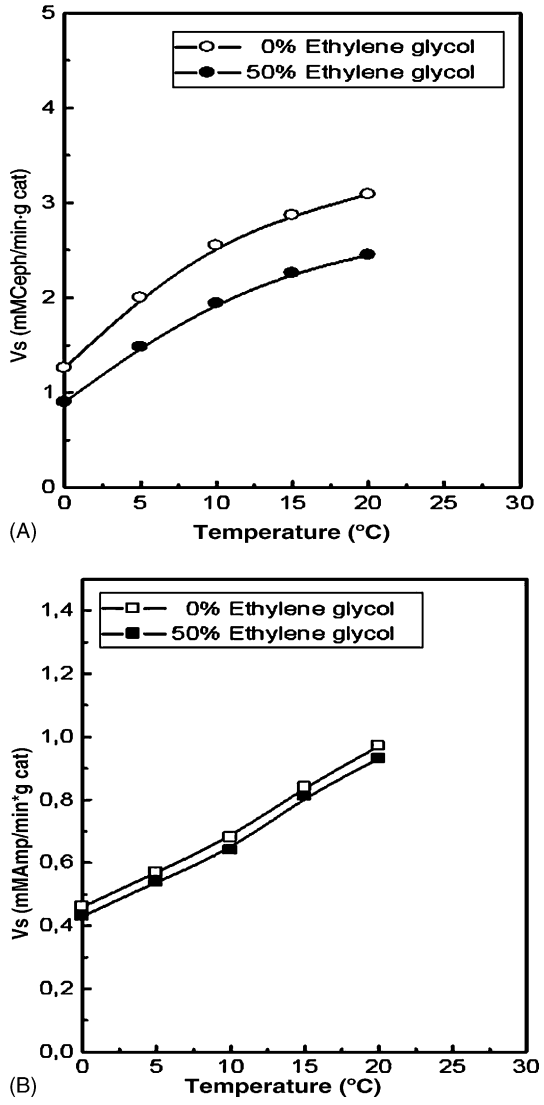

Fig. 2. Effect of temperature on the initial rates of synthesis of (A) cephalexin (○) and (B) ampicillin (□). Open symbols, control (fully aqueous buffer); closed symbols, EG 50% (v/v). Conditions: pH 7.0, 30 mM nucleophile, 90 mM PGME, 125 IU_H per mmol nucleophile.

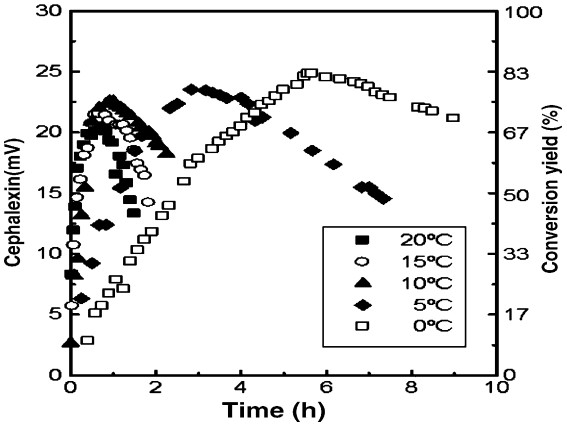

Fig. 3. Time course of the enzymatic synthesis of cephalexin with IPA at pH 7.0, 125 IU_H/mmol nucleophile and 0% (v/v) ethylene glycol.

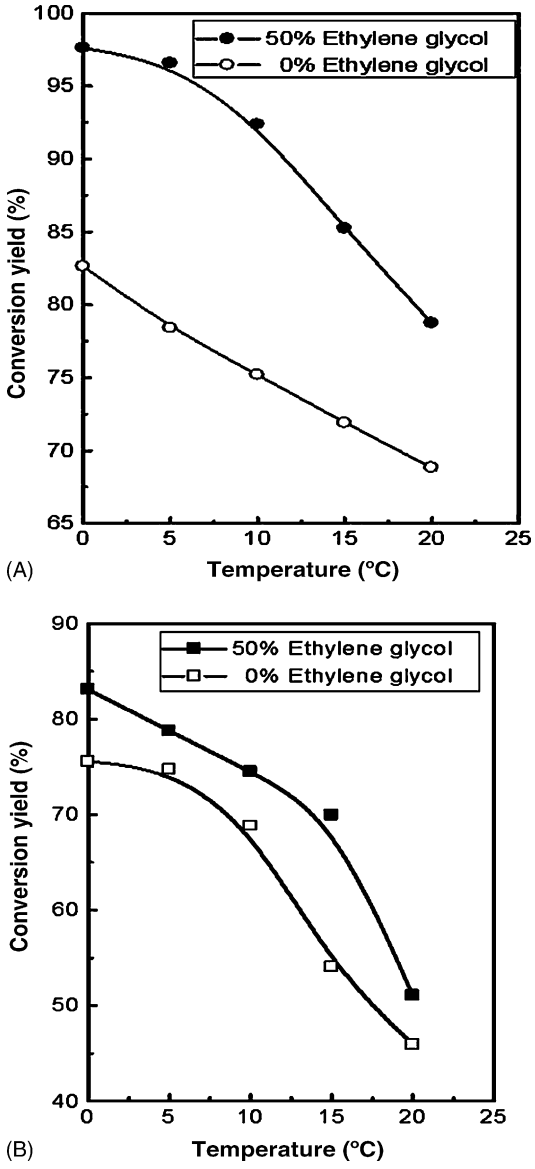

Fig. 4. Effect of temperature on conversion yields of synthesis of (A) cephalexin (○) and (B) ampicillin (□). Open symbols, control (fully aqueous buffer); closed symbols, EG 50% (v/v). Conditions: pH 7.0, 30 mM nucleophile, 90 mM PGME, 125 IU_H per mmol nucleophile.

Table 1
Activation energies (Ea) in kJ for the synthesis and hydrolysis of ampicillin, cephalexin and PGME with and without EG, at pH 7.0

Reaction	Ea (kJ)		
	0% EG	50% EG	r_{EG}
Amp hydrolysis	30.25	46.13	1.52
Cep hydrolysis	37.19	60.55	1.63
PGME hydrolysis	32.68	37.35	1.14
Amp synthesis	24.25	25.63	1.06
Cep synthesis	19.53	23.00	1.18

r_{EG} is the ratio of Ea with and without EG.

Table 2
Operating variables and experimental data for the optimization of cephalexin synthesis in ethylene glycol medium, using RSM

No.	pH	T (°C)	Cs (%)	Y (%)	P (mM/h)
1	6.5	0	40	65.06	3.09
2	7.5	0	40	72.43	22.53
3	6.5	20	40	58.37	19.54
4	7.5	20	40	65.00	116.07
5	6.5	0	60	64.96	1.99
6	7.5	0	60	65.30	8.60
7	6.5	20	60	41.06	1.76
8	7.5	20	60	50.66	41.75
9	6.5	10	50	60.56	12.04
10	7.5	10	50	64.87	44.56
11	7.0	0	50	84.37	12.03
12	7.0	20	50	69.87	74.86
13	7.0	10	40	82.20	62.11
14	7.0	10	60	73.53	18.52
15	7.0	10	50	76.63	28.95
16	7.0	10	50	76.02	29.25
17	7.0	10	50	75.32	29.87

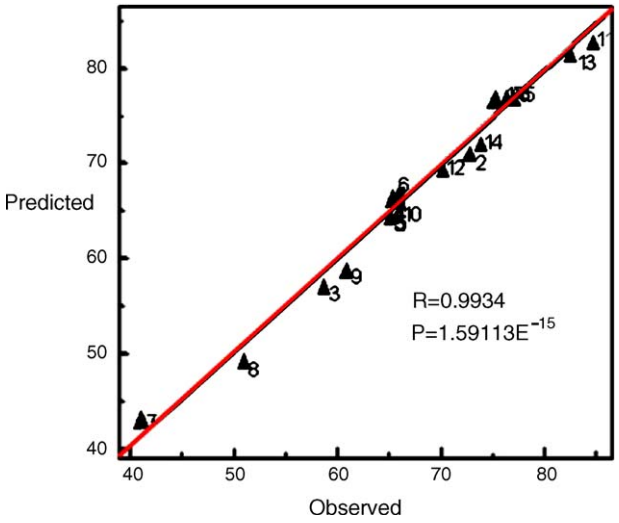

Fig. 5. Correlation between the observed responses and the predicted values by SRM for the conversion yield of cephalexin synthesis with ethylene glycol.

while in the presence of EG these values increased to 1.8 and 2.6, respectively. This means that the reactions of hydrolysis are comparatively less favoured because of a higher energetic barrier, which is reflected in higher conversion yields for both antibiotics. Thus, the highest the value of r_{HS}, the higher the conversion yield. Based on the values of these ratios, it is also possible to explain the higher yields at lower temperatures. If the value of r_{HS} is 1, this means that temperature affects hydrolysis and synthesis rates to the same extent; values higher than 1 means that temperature decrease will reduce the rate of hydrolysis to a higher extent than the rate of synthesis, thus increasing the conversion yield.

3.3. Optimization of cephalexin synthesis in ethylene glycol

Enzyme load was modified to compensate for the lower reaction rates at lower temperatures. Our objective was the optimization of conversion yield, considering that the correction by temperature will only affect productivity, but not yield. The experimental design was intended to determine the influence of each independent variable (pH, T and Cs) on conversion yield.

The experimental design and its results in terms of yield and productivity are summarized in Table 2 for the optimization of the kinetically controlled synthesis of Cep in EG, considering productivity as a control parameter. As shown in Table 3, the ANOVA test indicates (F_{ratio} versus $F_{T,95\%}$) that quadratic models derived from RSM are adequate for description of the yield from Cep synthesis in EG under the range of operating conditions established. No lack of fit was obtained in any case and values of R^2 and Q^2 validate the model for the synthesis. Fig. 5 shows the correlation between experimental results and model-predicted values of conversion yield in the synthesis of Cep in EG. After eliminating non-significant coefficients ($P > 0.05$), the model for Y in EG is reduced to Eq. (4):

$$Y = 77.2 + 2.8\text{pH} - 6.7T - 4.8\text{Cs} - 15.4\text{pH}^2 - 3.1T^*\text{Cs} \quad (4)$$

As suggested by the model coefficients, temperature was the most significant variable, and the effect of pH was the least significant among the variables studied within the range considered. This variable represents, however, the apparent pH of the reaction medium, since it was measured conventionally using a fully aqueous buffer to calibrate the instrument, not considering the effect of the cosolvent in the liquid phase.

Optimum values for each of the variables under study were obtained within the ranges considered (Table 4), determining

Table 3
ANOVA test used to study the response Y for cephalexin synthesis in EG using RSM

Response		Y (%)	
Source	$F_{T,95\%}$	F-ratio	P-value
Model	3.68	58.358	$9.37E^{-6}$
Lack of fit	19.30	11.406	0.083
R^2; Q^2		0.987; 0.817	

$F_{T,95\%}$: F crit 95% confidence level.

Table 4
Optimum values of temperature (T^*), pH (pH^*) and ethylene glycol concentration (Cs^*) considering Y as objective function

Parameter	Value
T^*_Y (°C)	0
pH^*_Y	7.0
Cs^*_Y (vol%)	40
Y^* (%)	**84.5**
Y^*_{exp} (%)	**87.5**
P^*_Y (mM/h)	3.29
$P_{Y^*_{exp}}$ (mM/h)	16.0

r_{ES} = 125 IU_H/mmol 7-ADCA; 7ADCA: 30 mM; PGME: 90 mM.

the maximum yield of Cep synthesis. The effect of temperature on yield was negative for EG. Negative effects of temperature have been previously reported for the synthesis of Amp in EG [20], cephalotin in aqueous medium [14] and Cep in frozen medium [26]. Actually, low temperature in EG medium has been proposed as a very promising system for the synthesis of Amp [20]. This is in agreement with the results reported in the previous section and can be explained in terms of energies of activation. However, the positive effect of temperature on yield has also been reported for a kinetically controlled synthesis, in the case of peptide bond formation with protease in a reversed micelle system [31].

The effect of Cs on yield was significant, which demonstrates that the range of values considered contained the optimum and curvature occurred. The addition of cosolvents that depress a_w [32,33] such as polyalcohols should increase yield, and this has been shown in the synthesis of β-lactam antibiotics [20,34], peptides [35] and β-D-glucosides [36]. In the present case, a negative effect of Cs in yield was observed in the range under study. However, yields were much higher than those obtained in a fully aqueous medium used as a control (Fig. 4). The effect of pH on the synthesis of Cep, although milder, was also significant. Optimum pH is the result of a delicate balance between its effect on the reactions of synthesis and hydrolysis of the Cep produced and hydrolysis of the acyl donor substrate PGME. The net effect of pH on the yield is a consequence of this balance, which is different in cosolvent than in aqueous medium [12]. Crossed interaction effects between variables were always negative. Surface of response with respect to pH and T is presented in Fig. 6 for the yield of Cep synthesis in EG, where operation optima can be clearly appreciated within the range of values considered for the variables. Optimum values for pH (pH_Y^*), T (T_Y^*) and Cs (Cs_Y^*) and maximum predicted yields (Y^*) are presented in Table 4 for the synthesis of Cep in EG. Y^* was over 85% in EG. Values as high as 90%, have been reported for the kinetically controlled synthesis of cephaloglycin at 15 °C [19], and Cep at 4 °C [13].

Optimum conditions determined by the models were experimentally validated and the results (Y_{exp}^*) are shown in Fig. 7 and summarized in Table 4. Y_{exp}^* was 87.5% in EG; this is, 3.5% higher than predicted, which can be considered acceptable. Y_{exp}^* is high and compares quite well with values reported in the literature for the production of semi-synthetic cephalosporins [12–15,35,37,38]. Y^* of Cep in EG was much higher than that previously obtained for Amp [39]. In general, yields of semi-synthetic cephalosporins tend to be higher than for semisynthetic penicillins [7,12,38,40], because it is known that 7-ADCA is a better nucleophile than 6-APA [41]; besides, according to the results reported in the previous section, the energies of activation of hydrolysis were higher for Cep than for Amp.

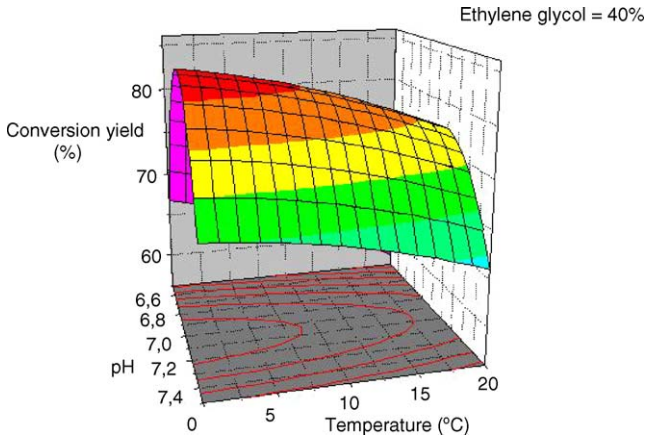

Fig. 6. Correlation between the observed responses and the predicted values by SRM for the conversion yield of cephalexin synthesis with ethylene glycol.

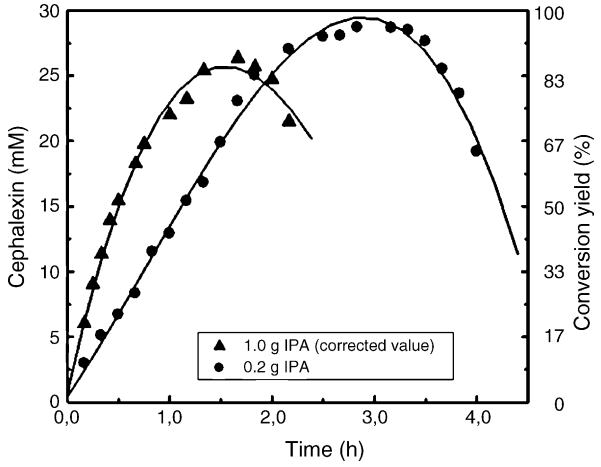

Fig. 7. Effect of enzymatic load on the conversion yield and productivity, under optimal conditions (pH 7.0, 40% EG, 0 °C, 125 IU_H per mmol nucleophile).

Yield of Cep in EG can be increased at the expense of productivity by lowering the temperature. In the experimental design presented, enzyme load was corrected by the effect of temperature on enzyme activity. There is an apparent contradiction in the yield obtained under the optimal conditions generated by the model (87.5% at 0 °C and 40% EG) and the higher value obtained experimentally as shown in Fig. 4 (98% at 0 °C and 50% EG) where only the effect of temperature on yield was studied at a fixed 50% cosolvent concentration. This difference is not attributable to the difference in EG concentration, since in that range (from 40 to 50%), yield is not affected. So, the explanation of this difference should be attributable to the higher enzyme load used when corrected by temperature (about five times higher). Some authors have considered that yield is not affected by the enzyme load [25,26]; however this will be so only within a certain range. Previous results not presented here show a reduction in yield when enzyme load is increased over a certain value. This reduction would be the consequence of the higher rate of hydrolysis of the antibiotic at a higher enzyme load, since the enzyme not only exhibits synthetic activity but also hydrolytic activity over the antibiotic product and the activated acyl donor. As shown in Fig. 7, yield and productivity without correction of enzyme load by temperature were 98% and 10.3 mM/h and with temperature correction 87.5% and 16.0 mM/h respectively, under the optimal conditions determined by the model.

4. Conclusions

The effect of EG and temperature on the conversion yield in the kinetically controlled synthesis of Cep and Amp can be explained satisfactorily in terms of activation energies of the corresponding reactions of synthesis and hydrolysis.

With higher values of r_{HS}, higher conversion yields were obtained, because temperature reduction produced a greater reduction of hydrolysis rates when compared with synthesis rates. These r_{HS} values satisfactorily explain the observed effect of cosolvent in conversion yield for both antibiotics and the higher conversions obtained for Cep than for Amp.

The kinetically controlled synthesis of Cep has been optimized in terms of conversion yield, considering pH, temperature and cosolvent concentration as key variables. The most relevant variable was *T*, followed by Cs, with the effect of pH being smaller within the range considered. Model predictions were accurate and their optima were experimentally validated. There is a compromise between yield and productivity, but the system was optimized in terms of yield, for being a key issue in antibiotic production. Optimum conditions were pH 7.0, 0 °C and 40% (v/v) of EG with a yield of 84.5% which is certainly a very promising value. The optimum temperature was close to the lower extreme of the range studied, which makes the work on synthesis of Cep in EG at low temperatures interesting. The negative effect of temperature and the crossed interaction effects between Cs and temperature give ample space for further optimization. The effect of enzyme loading on yield deserves more attention since the increase in productivity obtained at higher loads can be accompanied by a reduction in yield. This study is underway.

Acknowledgement

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