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Kinetically controlled synthesis of ampicillin with immobilized penicillin acylase in the presence of organic cosolvents

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Abstract

Penicillin acylase (PA) is used in the industrial production of 6-amino penicillanic acid (6-APA). However, by proper control of reaction medium, the enzyme can be used in the reverse synthesis of β-lactam antibiotics from the corresponding β-lactam nuclei and suitable acyl donors. Under thermodynamically controlled strategy, the use of organic cosolvents can favor synthesis over hydrolysis by lowering water activity and favoring the non-ionic reactive species. Under kinetically controlled strategy using activated acyl donors, organic solvents can favor synthesis by depressing hydrolytic reactions. Results are presented on the synthesis of ampicillin from phenylglycine methyl ester and 6-APA with immobilized *Escherichia coli* PA in the presence of organic cosolvents. Several solvents were tested in terms of enzyme stability and solubility of substrates. Ethylene glycol, glycerol, 1–2 propanediol and 1–3 butanediol were selected accordingly and ampicillin synthesis was performed in all of them. Best results in terms of yield and productivity were obtained with ethylene glycol, with which further studies were conducted. Variables studied were enzyme to limiting substrate ratio, acyl acceptor to acyl donor ratio, organic solvent concentration, pH and temperature. Experimental design based on a two-level fractional factorial design was conducted. pH was determined as the most sensitive variable and was further optimized. The best conditions for ampicillin synthesis in terms of productivity, within the range of values studied for those variables, were pH 7.4, 28°C, 36 U_S PA/mmol 6-APA, 3 mol PGME/mol 6-APA and 45 % (v/v) ethylene glycol concentration. Productivity was 7.66 mM ampicillin/h, which corresponds to a specific productivity of 7.02 μmol ampicillin/h U_S at 55 % yield. Productivity was lower than in buffer but product yield was higher because of the much lower relative hydrolysis rates.

Keywords: Penicillin acylase; Ampicillin; Organic solvents; β-Lactam antibiotics; Enzymatic synthesis

1. Introduction

The use of enzyme biocatalysts in organic synthesis has been developed vigorously in the last decade [1]. Cheap, robust hydrolases can be used to perform such reactions if the medium is engineered to depress hydrolysis in favor of synthesis. Much effort has been devoted to the study of enzymes in nearly anhydrous hydrophobic organic solvents [2], exploiting the remarkable properties that enzymes exhibit in such media with respect to stability and specificity [3–5]. Despite the fact that enzymes are poorly active in such media, significant improvements have been reported recently [6–8], which certainly will pave the way from applied research to industrial

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development. Water-miscible organic cosolvents have also been studied as media for enzymatic syntheses where more hydrophobic solvents are inapplicable [9]. This is the case for the synthesis of β-lactam antibiotics with penicillin acylase (PA), where hydrophobic solvents, even at low concentrations, abate enzyme activity to a significant extent [10], and substrates are highly insoluble. PA is a very flexible enzyme [11–13] and several applications have been explored in organic synthesis [14–17], including the production of penicillins and cephalosporins in the presence of organic cosolvents under thermodynamically [9,10,18,19] and kinetically controlled synthesis [10,19–21]. The former, although simpler, is a rigid strategy, product yield being determined exclusively by the equilibrium constant. In this case, organic cosolvents might be advantageous by decreasing the activity of water, therefore pushing equilibrium to synthesis, and by increasing the pK_a of the carboxylic group, therefore favoring the non-ionized active form of the acyl donor [9]. However, conditions that favor equilibrium displacement towards synthesis (hard cosolvents at high concentrations, low pH) are hardly compatible with fair PA activity and stability [19]. Kinetically controlled synthesis requires an active form of the acyl donor (usually as an ester), but the system is more flexible and prone to optimization, with yields being determined by the balance between synthetase, esterase and amidase activities, which are differentially affected by reaction conditions. Synthesis can be performed under milder conditions, more favorable for PA activity and stability; therefore, higher transient yields and productivities can be expected. In this case, organic cosolvents might be advantageous by depressing hydrolytic reactions, improving the balance between activities and hence favoring synthesis. It has been proposed that changes in substrate solvation by the presence of the cosolvent can be responsible for affecting the kinetics of synthesis [22], but direct effects of the solvent on the enzyme molecule cannot be ruled out [23].

Results are presented on the synthesis of ampicillin with immobilized PA under kinetically controlled strategy in the presence of organic cosolvents, with phenylglycine methyl ester (PGME) as acyl donor and 6-amino penicillanic acid (6-APA) as acyl acceptor. Organic cosolvents were tested, and four of them were selected in terms of enzyme stability and solubility of substrates. Synthesis of ampicillin was performed with them and one was selected, in terms of productivity and product yield, to be further studied. Experimental design based on a two-level fractional factorial design was conducted considering enzyme to limiting substrate and excess substrate to limiting substrate ratios, organic solvent concentration, pH and temperature as process variables, using ampicillin productivity and yield as evaluation parameters, under the hypothesis that organic cosolvents will improve synthesis. In the first stage, relevant variables were identified, as well as their direction of movement towards optimum, in terms of ampicillin productivity. In the second stage, optimum was approached according to the method of the steepest ascent.

2. Experimental

2.1. Materials

Immobilized penicillin acylase (IPA) from *Escherichia coli* was a commercial product from Roche (Darmstadt, Germany). Declared activity was 147 U/g, measured as initial rate of penicillin G hydrolysis (5% w/v, pH 8 and 28°C). IPA activity of synthesis was 78.5 U_S/g. Penicillin G and 6-APA were kindly supplied by Sinquisa (Lima, Perú); (*R*)-(−)-2 phenylglycine methyl ester hydrochloride and ampicillin were from Sigma-Aldrich (Milwaukee, WI, USA). Organic solvents and all other reagents were analytical grade either from Sigma-Aldrich (St. Louis, MO, USA) or Merck (Darmstadt, Germany).

2.2. Assays

Substrates and products of enzymatic synthesis were analyzed by HPLC using a Shimadzu delivery system LC-10AS with a Shimadzu UV SPD-10AV detector and a μ-Bondapack C_{18} column (300 × 3.9 mm) from Waters (Milford, MA, USA). Samples were eluted isocratically with 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 a UV detector at 214 nm. Amounts of reactants and products were calculated from calibration curves using stock solutions.

One unit of activity of synthesis (U_S) was defined as the amount of IPA that synthesizes 1 μmol of ampicillin/min at 25°C and pH 7 from 45 mM 6-APA and 135 mM PGME in 0.1 M phosphate buffer.

2.3. Selection of solvents

Solvents were selected in terms of solubility of substrates (6-APA and PGME) and IPA stability. Solubility tests were performed by dissolving increasing concentrations of 6-APA and PGME in a 1:3 molar ratio at increasing concentrations of solvents in phosphate buffer pH 7.8. The highest concentration of solvent, in which a mixture of substrates of given concentration was dissolved, was recorded. Stability of IPA was determined by measuring residual activity after incubating IPA for 12 and 24 h at 27°C in a non-reactive medium composed by 50% (v/v) of solvent in phosphate buffer pH 7.8. The time course of ampicillin synthesis was recorded for each solvent at 50% (v/v) in phosphate buffer pH 7 at 27°C with 30 mM 6-APA, 90 mM PGME and 37 U_S/mmol 6-APA. One solvent was finally selected in terms of ampicillin productivity and yield.

2.4. Synthesis of ampicillin with IPA

All reactions were carried out batchwise in 80-ml reactors containing 50 ml of reaction medium under pH and temperature control, using 6-APA as limiting substrate at 30 mM concentration. Agitation was kept to a minimum, just to maintain enzyme particles suspended. Samples were taken at intervals and assayed for product and residual substrates. Evaluation parameters were ampicillin molar yield and productivity.

Synthesis of ampicillin on the selected solvents was studied considering the following variables: concentration of organic solvent in percent v/v (C_S); enzyme to limiting substrate ratio in units of activity of synthesis per millimole 6-APA (r_{ES}), limiting substrate to excess substrate ratio in millimoles 6-APA per millimole PGME (r_{SS}), pH and temperature in degrees centigrade (T). Experimental design based on a two-level fractional factorial design was conducted. First, variables were screened for significance using a 2^{5-2}_{III} design, considering productivity as the evaluation parameter. Conditions for experiments, numbered 1 to 11, including three center point repeats are in Table 1. From the screening stage, most significant variables and their direction of movement towards the optimum were determined. Then, based on the results from the screening stage, experimental design towards the optimum was performed according to the method of steepest ascent. Conditions for such experiments, numbered 12 to 15, are also in Table 1.

The rate of approach to maximum yield during ampicillin synthesis is quite different for each solvent and each condition, so, to avoid bias, volumetric productivity (Pr) was defined as the value obtained (mM ampicillin/h) at 95% of maximum molar yield (Y).

Table 1
Experimental design and results for screening of variables (experiments 1–11) and for the optimum approach stage according to the method of the steepest ascent (experiments 12–15). Nomenclature is in the text

Experiment no.	Variable					Result	
	pH	T (°C)	r_{ES} (U_S/mmol)	C_S (% v/v)	r_{SS}	Pr (mM/h)	Y (%)
1	6.6	25	27.5	40	1/3	4.25	45.2
2	5.5	25	27.5	50	1/5	0.25	21.6
3	5.5	30	27.5	40	1/3	0.45	26.7
4	6.6	30	27.5	50	1/5	4.48	48.6
5	6.6	25	46	40	1/5	5.43	48.6
6	5.5	25	46	50	1/3	0.25	19.1
7	5.5	30	46	40	1/5	1.08	32.8
8	6.6	30	46	50	1/3	7.20	50.6
9	6.05	27	36.7	45	1/3.75	1.72	49.6
10	6.05	27	36.7	45	1/3.75	1.90	48.8
11	6.05	27	36.7	45	1/3.75	1.98	48.7
12	6.6	27.5	36.3	44.5	1/3.78	3.87	56.7
13	7.15	28	35.9	43.9	1/3.81	7.56	54.7
14	7.7	28.5	35.5	43.4	1/3.84	7.18	49.4
15	8.25	29	35.1	42.8	1/3.87	6.04	43.2

3. Results and discussion

3.1. Selection of solvents

Organic cosolvents were considered according to a preliminary literature screening and solubility of substrates was determined in all of them. Table 2 shows the maximum concentration of each solvent, in which a mixture of substrates of given concentration was dissolved. Substrates were insoluble on water-immiscible hydrophobic substrates (ethyl acetate and toluene) with no added water. Solubility was higher in polyols and acetonitrile, and lower in dimethyl formamide (DMF) and dimethyl sulfoxide (DMSO). Among alcohols, solubility of substrates correlated with the number of hydroxyl groups.

Stability of IPA was determined in all solvents, except polyethylene glycol 400 and sorbitol where substrates were chemically altered. Results are summarized in Table 2. Stability was high in polyols, as expected since they are well-known enzyme stabilizers [24], rather low in glymes and extremely low in primary and secondary alcohols and the rest of the solvents. Almost no IPA activity was recovered after 24 h in methanol, DMF and DMS. Kim and Lee [25] reported almost full recovery of enzyme activity after exposure to such solvents, but at significantly lower concentrations. The deleterious effect of hard cosolvents like DMSO and DMF at high concentrations is in agreement with previous reports on IPA, even at lower temperatures [18]. Although polyols can be considered ideal cosolvents with respect to enzyme stability, they have been questioned because of the increase in viscosity that may hinder the reaction. However, it was shown that increase in viscosity of the reaction medium up to 26.5 cP did not affect the enzymatic synthesis of cephalexin [26], whose similarity with the case under study is apparent.

Synthesis of ampicillin was conducted on polyols, to select one for further studies. The time course of such reactions is shown in Fig. 1 and exhibited the expected pattern for a kinetically controlled reaction [27]. The best results in terms of productivity and product yield were obtained with ethylene glycol, being therefore selected for further studies. Even though initial rate was lower than that in buffer, as expected, yield was 30% higher and, in the absence of cosolvents, synthesized ampicillin was hydrolyzed extremely fast. This is consistent with results previously obtained with other IPAs at moderate concen-

Table 2
Maximum concentration of solvent (% v/v in 0.1 M phosphate buffer pH 7.8) in which mixtures of 6-APA and PGME of different concentrations were completely dissolved at 27°C; r_{SS} was always 1/3. Residual activity of IPA in 50% (v/v) concentration of solvents in 0.1 M phosphate buffer pH 7.8 at 27°C

Solvent	Maximum solvent concentration (% v/v)			Residual activity (% of initial)	
	6-APA-PGME (mM)				
	20–60	30–90	40–120	24 h	12
Acetonitrile	70	60	50	0	n.d
1–3 Butanediol	50	40	40	76	95
Dimethyl formamide	30	30	30	0	n.d.
Dimethyl sulfoxide	40	40	40	0	n.d.
Diglyme	50	40	30	43	60
Ethanol	50	50	40	0	n.d.
Ethylene glycol	70	60	50	100	100
Glycerol	70	60	50	100	100
Glyme	60	50	40	41	59
Methanol	60	50	50	0	n.d.
Polyethylene glycol 400	60	60	50	–	–
1–2 Propanediol	60	50	50	71	90
2 Propanol	50	50	50	0	n.d.
Sorbitol[a]	80	80	80		

[a] Grams per 100 ml of phosphate buffer.

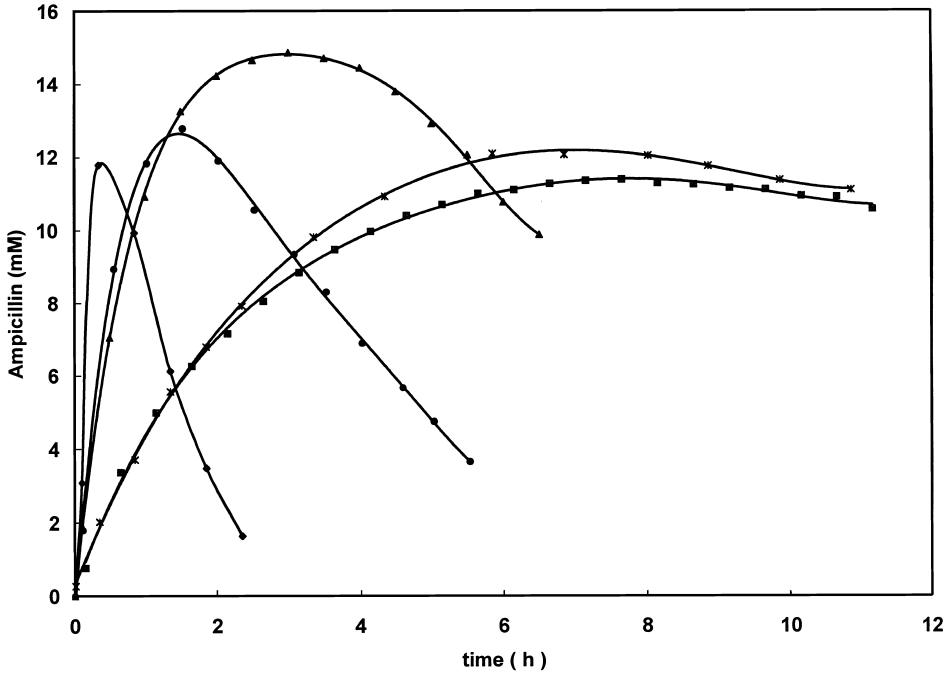

Fig. 1. Time course of ampicillin synthesis for each solvent at 50% (v/v) in phosphate buffer 0.1 M pH 7 at 27°C with 20 mM 6-APA, 60 mM PGME and 37 U_S/mmol 6-APA. ♦: buffer; ●: glycerol; ▲: ethylene glycol; ×: 1–2 propanediol; ■: 1–3 butanediol

trations of methanol (20–50% v/v) ethanol (25% v/v) and butanol (10% v/v), where yields were improved but initial rates of synthesis of ampicillin were severely reduced [10,28].

3.2. Synthesis of ampicillin with IPA

Results for the screening stage are presented in Fig. 2. Values for productivity and product yield are presented in Table 1 (experiments 1–11). Using multiple linear regression and productivity as the evaluation parameter, a linear model was validated from such results, with $R^2 = 0.99$ and $Q^2 = 0.87$ [29]. At a 95% confidence level, pH was the most significant variable, with much smaller effects for T and r_{ES}, all three effects being positive. The magnitude of the effects of C_S and r_{SS} was below the confidence interval, meaning a negligible effect within the ranges considered.

Results for the optimum approach stage according to the method of the steepest ascent are presented in Fig. 3. Values for productivity and product yield are presented in Table 1 (experiments 12–15). The magnitude of the effects is reflected in the size of the path for each variable, being much smaller for T, r_{ES}, C_S and r_{SS} than for pH, which means that productivity is being optimized in terms of pH. Optimum was 7.4, but yield decreased with pH, meaning that optimum pH for yield is outside the range considered in this stage.

Values for productivity and yield in all experiments from both stages are plotted as a function of pH in Fig. 4. A compromise exists for the pH of synthesis, with 7.4 being the optimal for productivity, but 6.5 being the best for yield. Productivity decreased more sharply below 7.4 than did yield over 6.5. The highest value for yield obtained should not be considered as an optimum, since a linear model was not validated for yield in the screening stage. In this case, a surface of response methodology (SRM) has to be used, which will expand the ranges of values originally considered for the variables and, eventually, alter the significance of vari-

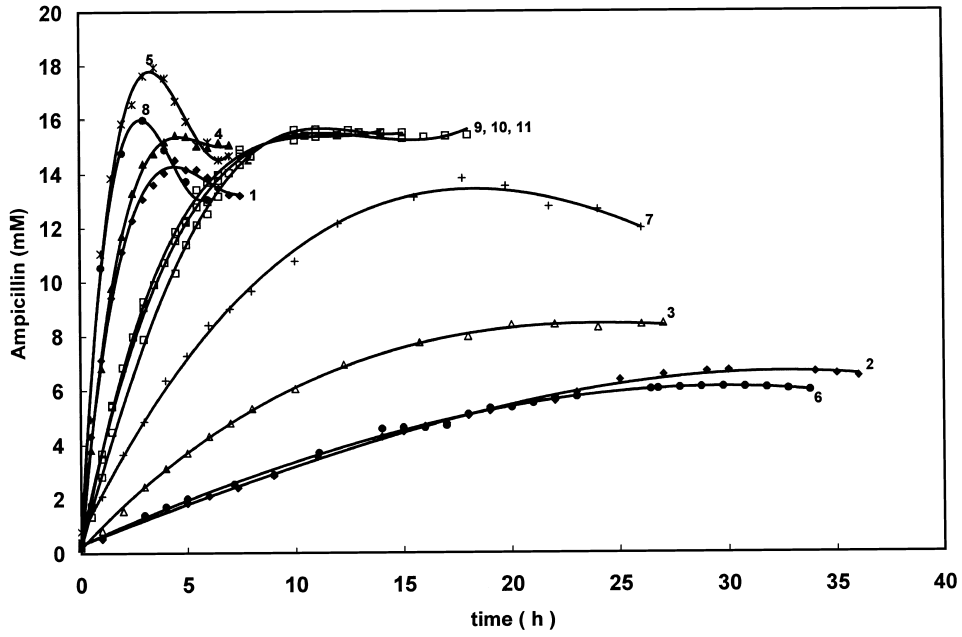

Fig. 2. Time course of ampicillin synthesis on ethylene glycol-buffer mixtures in the screening stage. Conditions of experiments (1 to 11) are those in Table 1.

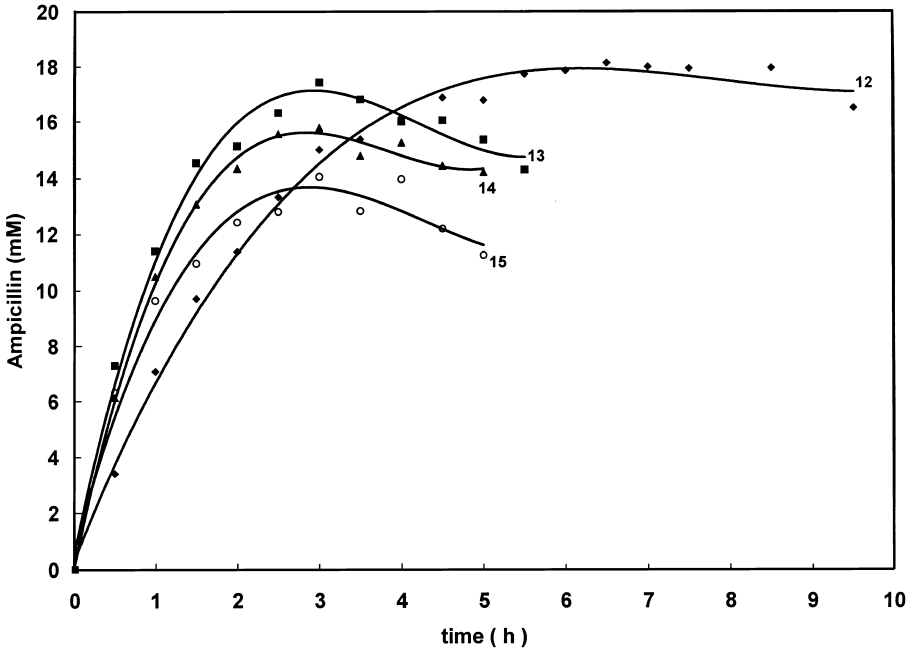

Fig. 3. Time course of ampicillin synthesis on ethylene glycol-buffer mixtures in the optimum approach stage according to the method of the steepest ascent. Conditions of experiments (12–15) are those in Table 1.

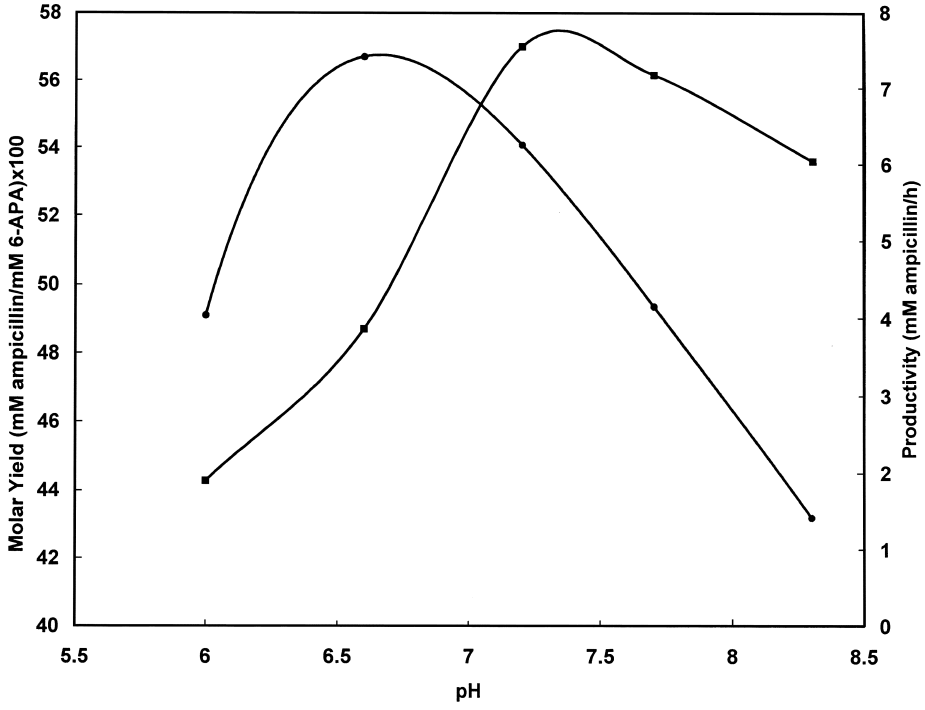

Fig. 4. Productivity (■) and molar yield (●) for ampicillin synthesis as a function of pH. Conditions of experiments are those in Table 1.

ables. Yield increased at lower pH, which is consistent with the mechanism proposed for the reaction of synthesis [30]. Initial rates (and productivities), on the other hand, were higher at higher pH, which can also be explained from the mechanism of synthesis [27], considering that the non-ionized reactive species of substrates will be favored at higher pH [10].

Best conditions for ampicillin synthesis in terms of productivity, within the range of values studied for the variables, were pH 7.4, 28°C, 36 U_S PA/mmol 6-APA, 3 mol PGME/mol 6-APA and 45% (v/v) ethylene glycol concentration. Productivity was 7.66 mM/h, which corresponds to a specific productivity of 7.02 $\mu mol/h \cdot U_S$, at 55% yield. These figures compare favorably with previously reported data on ampicillin synthesis with other IPA. Working with varying concentrations of methanol, Kim and Lee [10] reported molar yields varying from 12% at 0% cosolvent to 26% at 40% (v/v) cosolvent; corresponding values for specific productivity, recalculated in terms of U_s, were 0.495 and 0.107 $\mu mol/ h \cdot U_S$. These figures are quite lower, which may be due, at least in part, to the fact that they used PGME as the limiting substrate. Ospina et al. [31], working with an IPA in buffer with 6-APA as limiting substrate reported, under comparable conditions than the present work, a specific productivity of 1.67 $\mu mol/h \cdot U_S$ at 60 % yield. They could increase yield up to 75% and specific productivity to 5.1 $\mu mol/h \cdot U_S$ when substantially higher substrate concentrations (200 mM 6-APA; 600 mM PGME) were used.

4. Conclusions

Kinetically controlled synthesis with IPA in the presence of organic solvents was studied, as a valid

alternative for the production of ampicillin. Water-miscible solvents were screened in terms of solubility of substrates and enzyme stability considering productivity and yield as evaluation parameters. Polyols were superior and, among them, ethylene glycol was the best. Operational variables were then screened for significance, using productivity as parameter for evaluation. Among those variables and within the ranges studied, pH was the most relevant and was further optimized. A compromise is shown for pH optimum between productivity and yield, being higher for the former. The best results in terms of productivity were obtained at pH 7.4, 28°C and 45% (v/v) ethylene glycol concentration under 3/1 PGME molar excess. Specific productivity of 7.02 $\mu mol/h \cdot U_S$ was obtained, which is significantly higher than previously reported values with similar systems at comparable yields.

Since the presence of organic solvent will increase yield at the expense of productivity, it is advisable to consider yield as parameter for optimization of ampicillin synthesis in the presence of organic solvents. When this was done using the same experimental data from the screening stage it was not possible to validate a linear model, and an experimental design using SRM is to be used. This is underway and preliminary results indicate that, beyond the limits in Table 1, yield will be improved by increasing solvent concentration and decreasing pH and temperature, but again, this will be obtained at the expense of productivity, whose behavior is exactly the opposite. This information will be a valuable guideline for process optimization when relative impacts of yield and productivity in processing costs can be incorporated into a cost-objective function for the enzymatic production of ampicillin.

Productivity has been considered within the framework of one batch. However, IPA enzymes will be used repeatedly (in fact, IPA activity was almost fully recovered after the first batch in all conditions tested), so global productivity will have to be assessed by considering enzyme inactivation through time. Information is still lacking on IPA inactivation under prolonged operation, but certainly it will have to be taken into consideration for process optimization. The problem has been addressed for the synthesis of cephalexin with IPA [20] and, more in depth, in less-related enzymatic processes [32,33].

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