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Optimization of Yield in Kinetically Controlled Synthesis of Ampicillin with Immobilized Penicillin Acylase in Organic Media

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Abstract

Immobilized penicillin acylase is a moderately priced versatile enzyme, that is able to catalyze the synthesis of derived penicillins and cephalosporins from the corresponding β-lactam nuclei and proper side-chain precursors. Kinetically controlled synthesis is a better strategy when product yield is a key issue. Yield should increase at reduced water activity by depressing the competing hydrolytic reactions in favor of synthesis; therefore, organic cosolvents can be a suitable reaction media for synthesis. Using response surface methodology and product yield as objective function, temperature and pH were optimized in the kinetically controlled synthesis of ampicillin using previously screened cosolvents and reaction conditions. Optimum pH was 6.0 for ethylene glycol (EG) and glycerol (GL) and 6.6 for 1-2 propanediol (PD); optimum temperature was 30°C for GL and for EG and PD was in the lower extreme of the range studied, optimum lying below 26°C. Maximum molar yields predicted by the model were 58, 51, and 46% for EG, GL, and PD, respectively, which were experimentally validated. Highest yield in aqueous buffer was always <40%. Molar yields about 60% compare favorably with values reported for the kinetically and thermodynamically controlled synthesis of ampicillin and other derived penicillins.

Index Entries: Penicillin acylase; ampicillin; β-lactam antibiotics; enzymatic synthesis; organic cosolvents.

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Introduction

Penicillin acylase (penicillin amidohydrolase; EC 3.5.1.11) is a moderately priced readily available enzyme *(1)* with remarkable versatility *(2)*. It is currently used for the industrial production of 6-aminopenicillanic acid (6-APA) by hydrolysis of penicillin G *(3)* or V *(4)* and 7-aminodesacetoxycephalosporanic acid (7-ADCA) by hydrolysis of cephalosporin G *(5)*. It can also be used for the synthesis of derived penicillins *(6)* and cephalosporins *(7)* from the corresponding β-lactam nuclei and suitable side-chain precursors. Several other reactions relevant in organic synthesis can be performed by penicillin acylase, such as the kinetic resolution of alcohols and amines by performing hydrolytic or synthetic reactions to produce pure enantiomers *(8–10)*, the nonspecific transfer of acyl moieties to nucleophiles *(11)*, and the protection of groups in peptide synthesis *(12)*.

Peptides *(13,14)* and β-lactam antibiotics *(15,16)* can be produced by thermodynamically or kinetically controlled synthesis using enzymes. Kinetically controlled synthesis of β-lactam antibiotics, although requiring activated acyl donors, is usually a better strategy when product yield is a main issue *(17)* as is the case for antibiotic production *(18)*, since it is not restricted by the equilibrium of the reaction. On the other hand, thermodynamically controlled synthesis does not require of activated acyl donor, but yield is entirely determined by the equilibrium of the reaction, and conditions hardly compatible with enzyme activity and stability are often required to displace it in favor of synthesis *(7,19,20)*. Yield is expected to increase at reduced water activity since this will depress the competing hydrolytic reactions in the case of kinetically controlled synthesis, as well as displace the equilibrium toward synthesis in the case of a thermodynamically controlled strategy *(21)*. A powerful tool to depress water activity is the use of organic solvents as reaction medium, and much effort has been devoted to the development of biocatalysis in nearly anhydrous hydrophobic organic solvents, in which hydrolytic reactions are almost completely avoided in favor of synthesis *(22)*. Penicillin acylase is barely active and substrates poorly soluble in such solvents *(6)*; the low activity exhibited seems to be related to a direct solvent effect rather than to reduced water activity, since the enzyme has proven to be active at very low water activity in solid-phase systems *(23)*. However, water-miscible organic cosolvents are a suitable medium to perform the synthesis of β-lactam antibiotics, because they will favor synthesis by reducing water activity and increasing the proportion of reactive nonionized species *(24)*. Water-miscible organic cosolvents have been discredited for being deleterious to enzyme activity and stability; however, this is by far not a general rule, and there are cosolvents in which the enzymes can be at least as active and stable and substrates as soluble as in water *(25–27)*.

A screening of cosolvents and variables for the synthesis of ampicillin with immobilized penicillin acylase has been previously reported: ethylene glycol (EG), glycerol (GL), and 1-2 propanediol (PD) were the best

cosolvents, while temperature and pH were the most relevant variables *(27)*. In this article, results are presented on the optimization of pH and temperature for the kinetically controlled synthesis of ampicillin in those three cosolvents using response surface methodology and product molar yield (referred to simply as yield hereafter) as evaluation parameter.

Materials and Methods

Materials

Immobilized penicillin acylase from *Escherichia coli* was a commercial product from Roche (Darmstadt, Germany) with a specific activity of 320 ± 20 U_H/g, corresponding to 78.5 ± 5 U_S/g. Penicillin G potassium salt and 6-APA were kindly supplied by Sinquisa (Lima, Perú). (*R*)-(–)-2 phenylglycine methyl ester hydrochloride (PGME) and D[–]-α-aminobenzylpenicillin (ampicillin) were from Sigma-Aldrich (Milwaukee, WI). Organic solvents and all other reagents were of analytical grade from either Sigma-Aldrich (St. Louis, MO) or Merck (Darmstadt, Germany).

Analysis

Substrates and products of enzymatic synthesis were analyzed by high-performance liquid chromatography 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). Samples were eluted isocratically with 70% (v/v) 20 m*M* 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 hydrolysis (U_H) was defined as the amount of immobilized penicillin acylase that hydrolyzes 1 µmol of penicillin G potassium salt/min at 30°C and pH 7.8 from 134 m*M* penicillin G potassium salt in 0.1 *M* phosphate buffer. One unit of activity of synthesis (U_S) was defined as the amount of immobilized penicillin acylase that synthesizes 1 µmol of ampicillin/min at 25°C and pH 7.0 from 45 m*M* 6-APA and 135 m*M* PGME in 0.1 *M* phosphate buffer.

Synthesis of Ampicillin with Immobilized Penicillin Acylase

Reactions were performed batchwise under pH and temperature control in 80-mL reactors containing 50 mL of reaction medium, using 6-APA as limiting substrate at 30 m*M*. Agitation was kept to a minimum, just to maintain enzyme particles suspended. Samples were taken at intervals and assayed for product and residual substrates and yield and volumetric productivity (referred to hereafter as productivity) were calculated from these data. Yield was defined as the maximum molar conversion of 6-APA into ampicillin (%), and productivity was evaluated as the amount of ampicillin produced per unit time and unit reaction volume at that yield (m*M*/h).

Table 1
Experimental Design and Results for Kinetically Controlled Synthesis of Ampicillin in Organic Cosolvents and Control (phosphate buffer) at Varying pH and Temperature at C_S = 50% (v/v), r_{SS} = 1:3 mmol of 6-APA/mmol of PGME, and r_{ES} = 125 U_H/mmol of 6-APA

		Yield (%)				Productivity (m*M*/h)			
T (°C)	pH	EG	GL	PD	Buffer	EG	GL	PD	Buffer
25	6.0	55	49	33	40	2.1	5.0	1.2	13.3
25	7.5	52	32	38	39	7.8	12.8	2.5	14.6
35	6.0	53	47	34	38	3.3	7.4	1.4	12.0
35	7.5	42	26	28	28	10.6	17.5	1.9	9.8
30	6.75	56	47	46	35	8.4	15.8	2.8	17.5
30	6.75	56	46	43	36	8.5	15.6	2.7	17.7
30	6.75	56	47	44	35	8.5	15.7	2.9	17.5
23	6.75	52	37	30	30	6.1	8.6	1.2	7.2
30	5.7	45	48	35	33	0.9	4.7	0.6	3.5
30	7.8	40	28	29	32	6.0	14.1	2.2	31.2
37	6.75	42	34	24	29	6.9	16.1	1.6	30.5

Syntheses of ampicillin on EG, GL, and PD were performed at varying pH and temperature at previously selected conditions for other variables: solvent concentration (C_S), 50% (v/v); 6-APA to PGME ratio (r_{SS}), 1:3; immobilized penicillin acylase to 6-APA ratio (r_{ES}), 125 U_H/mmol *(27)*. Yield was optimized with respect to temperature and pH of synthesis using response surface methodology *(28)*, with a two-factor central composite design, which is presented in Table 1. A software package (Modde 4.0 for Windows; Umetri, Umeå, Sweden) was used to fit the second-order models with respect to temperature and pH.

Operational Stability of Immobilized Penicillin Acylase

The stability of immobilized penicillin acylase was tested under sequential batch operation, in which the enzyme was recovered after each batch, washed and fresh medium was added again. Reactors were of the same type as described previously but were provided with a bottom filter to recover the enzyme and remove the product. Each batch was run until the designed yield (that of the first batch) was obtained and the increase in time (decrease in productivity) was recorded.

Results

Temperature and pH were previously determined as the most relevant variables for the synthesis of ampicillin in organic cosolvents *(27)*. To study the effect of such variables on yield, a temperature range from 25 to 35°C and a pH range from 6.0 to 7.5 were chosen.

The time courses of the syntheses of ampicillin in EG, GL, and PD media, according to the experimental design in Table 1, are presented in

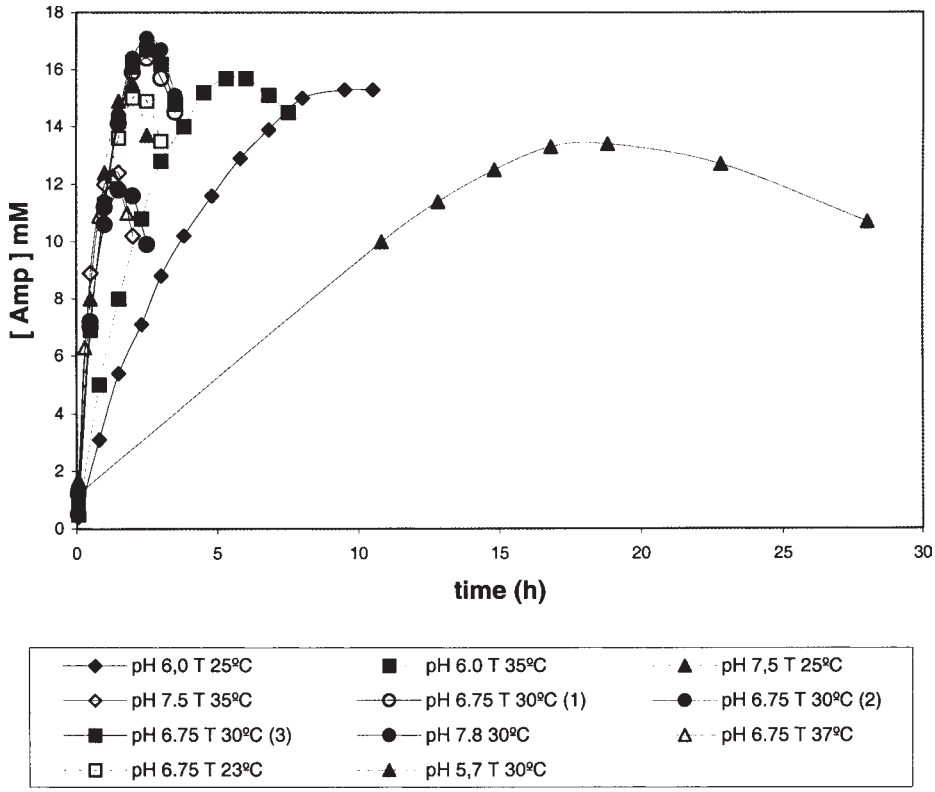

Fig. 1. Time course of ampicillin synthesis in EG, according to experimental design in Table 1. C_S = 50% (v/v); r_{ES} = 125 UI_H/mmol of 6-APA; r_{SS} = 1:3 mmol of 6-APA/mmol of PGME.

Figs. 1, 2, and 3, respectively. Results under the same experimental design are presented in the aqueous buffer control in Fig. 4, and a comparison of the synthesis in organic cosolvents and aqueous buffer is shown in Fig. 5. A 2^2 experimental design with three central repeats was used in the first stage, and curvature was obtained, meaning that optimum pH and temperature are within the ranges considered. In the second stage, four additional experiences were added to the previous seven. The whole experimental design and results are presented in Table 1.

A quadratic model was fitted from the experimental data and the best fit is represented by Eqs. 1, 2, and 3 for EG, GL, and PD, respectively. Regression coefficients were >0.95 in all cases:

$$Y_{EG} = 56 - 3.091\ pH - 2.311T - 1.563\ pH \cdot pH - 1.313T \cdot T - pH \cdot T \quad (1)$$

$$Y_{GL} = 46 - 8.442\ pH - 0.479T - 3.375\ pH \cdot pH - 5.626T \cdot T - 1.75\ pH \cdot T \quad (2)$$

$$Y_{PD} = 44 - 1.707\ pH - 5.579T - 6.876\ pH \cdot pH - 3.875T \cdot T - pH \cdot T \quad (3)$$

in which Y is the yield, T is the temperature (°C), and variables are in their coded form.

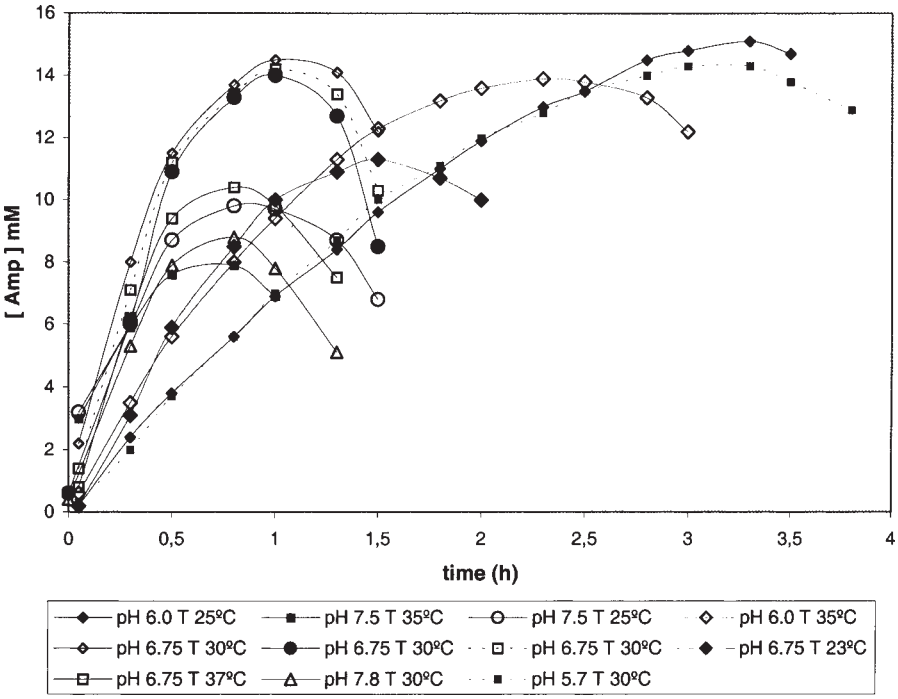

Fig. 2. Time course of ampicillin synthesis in GL, according to experimental design in Table 1. C_S = 50% (v/v); r_{ES} = 125 UI_H/mmol of 6-APA; r_{SS} = 1:3 mmol of 6-APA/mmol of PGME.

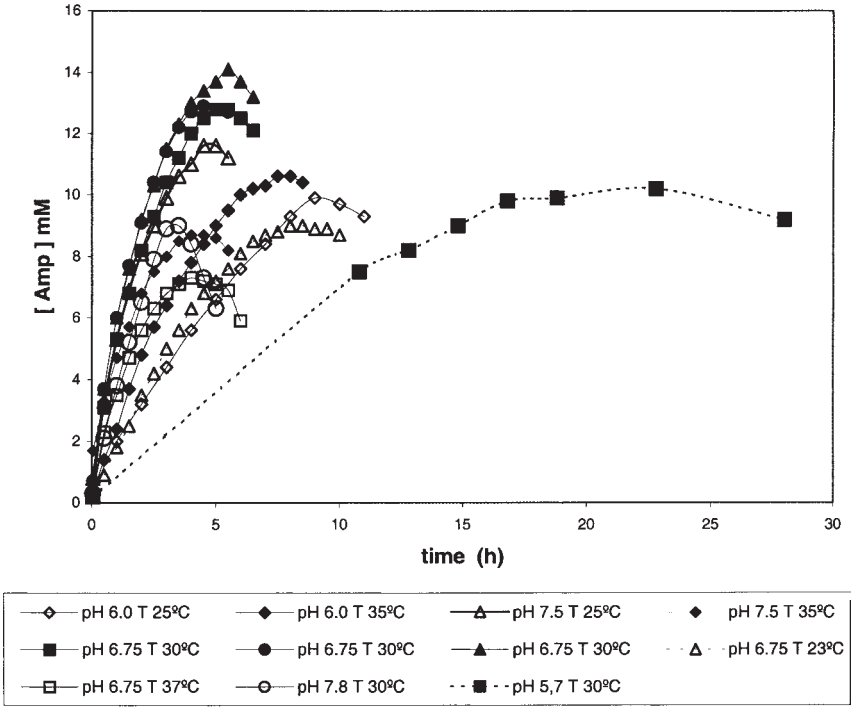

Fig. 3. Time course of ampicillin synthesis in PD, according to experimental design in Table 1. C_S = 50% (v/v); r_{ES} = 125 UI_H/mmol of 6-APA; r_{SS} = 1:3 mmol of 6-APA/mmol of PGME.

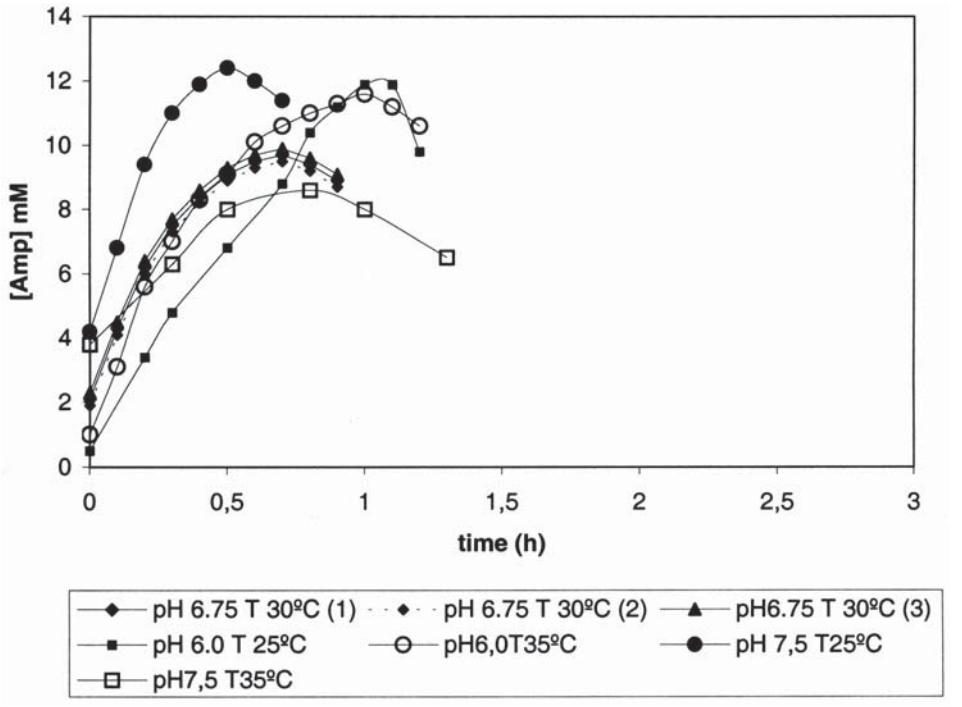

Fig. 4. Time course of ampicillin synthesis in phosphate buffer, according to experimental design in Table 1. r_{ES} = 125 UI_H/mmol of 6-APA; r_{SS} = 1:3 mmol of 6-APA/mmol of PGME.

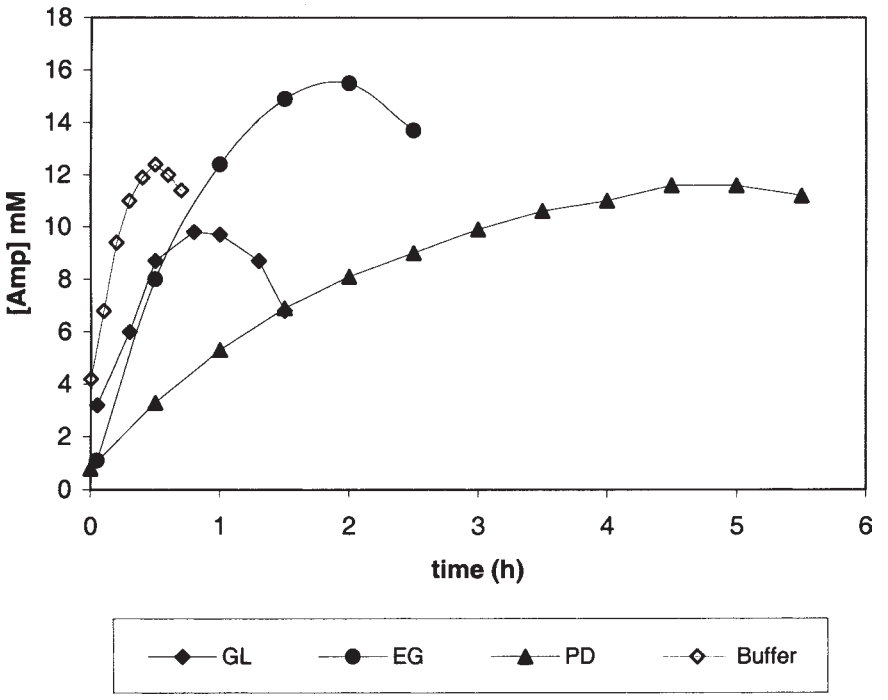

Fig. 5. Comparison of synthesis of ampicillin in organic cosolvents and aqueous buffer at pH 7.5 and 25°C. C_S = 50% (v/v); r_{ES} = 125 UI_H/mmol of 6-APA; r_{SS} = 1:3 mmol of 6-APA/mmol of PGME.

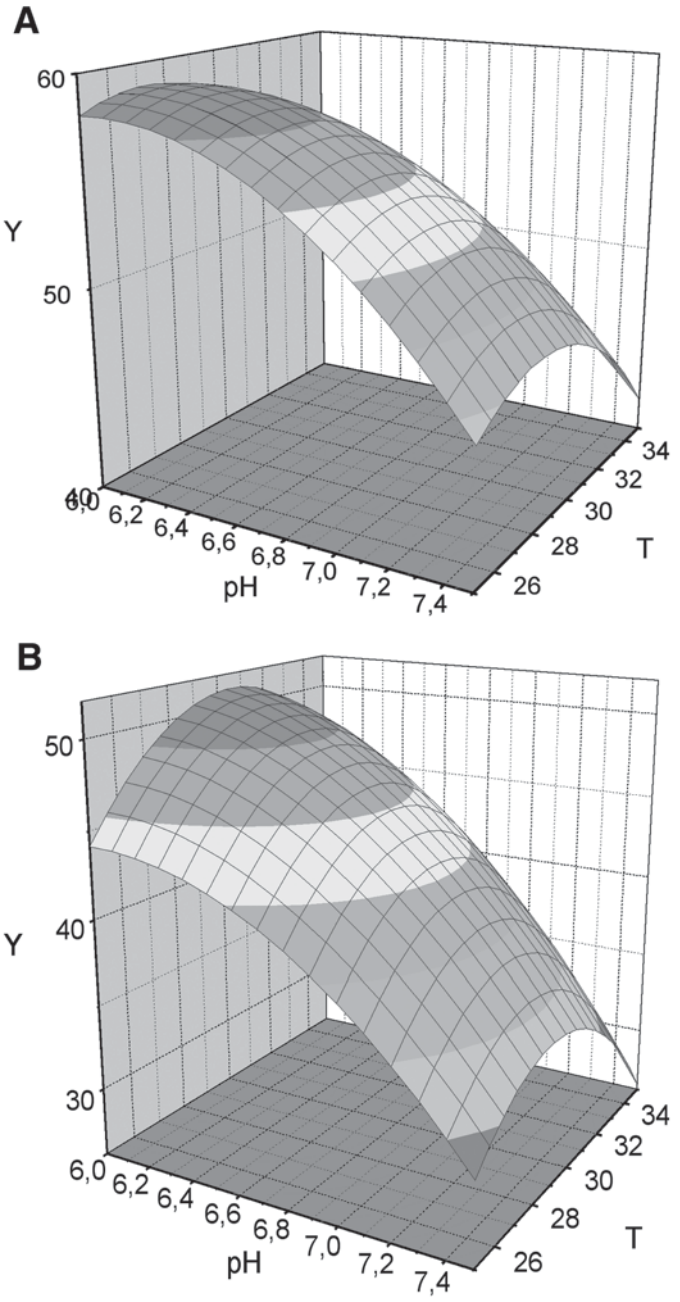

Fig. 6. Three-dimensional plot of effect of pH and temperature on ampicillin yield. **(A)** In EG, according to Eq. 1 and **(B)** in GL, according to Eq. 2. C_S = 50% (v/v); r_{ES} = 125 UI_H/mmol of 6-APA; r_{SS} = 1:3 mmol of 6-APA/mmol of PGME.

Three-dimensional plots corresponding to these models are presented in Fig. 6. Optimum pH and temperature, maximum yield, and productivity at maximum yield are presented in Table 2. Model optima were verified

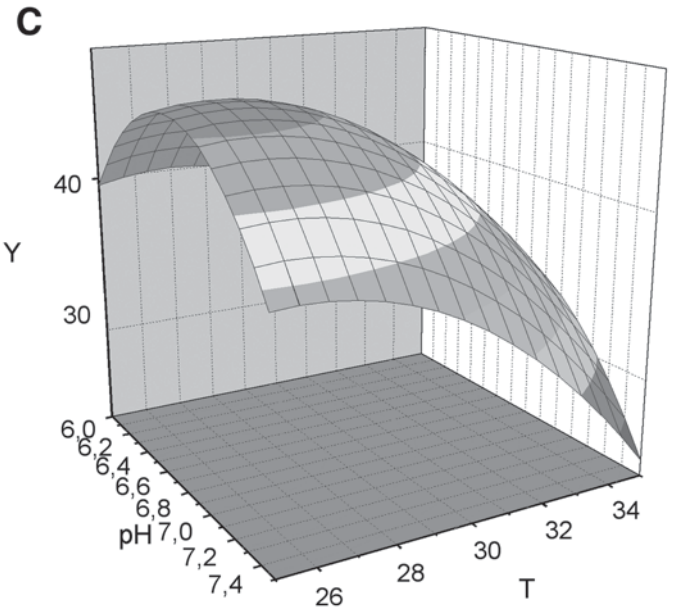

Fig. 6. *(continued)* Three-dimensional plot of effect of pH and temperature on ampicillin yield. **(C)** In PD, according to Eq. 3. C_S = 50% (v/v); r_{ES} = 125 UI_H/mmol of 6-APA; r_{SS} = 1:3 mmol of 6-APA/mmol of PGME.

Table 2
Optimum Temperature (T^*_Y) and pH (pH^*_Y) in Terms of Yield, Maximum Predicted Yield (Y^*), Experimental Yield at Optimum Conditions (Y^*_{exp}), and Productivity at Maximum Yield (P_{Y^*}) and Optimum Temperature (T^*_P) and pH (pH^*_P) in Terms of Productivity, Maximum Productivity (P^*), and Yield at Maximum Productivity (Y_{P^*}) in EG, GL, and PD at 50% (v/v)

	EG	GL	PD
T^*_Y	26.0	30.0	26.0
pH^*_Y	6.0	6.0	6.6
Y^*	58.0	51.0	46.0
Y^*_{exp}	59.0	53.0	—
P_{Y^*}	2.3	8.9	2.0
T^*_P	33.0	33.0	33.0
pH^*_P	7.8	7.3	7.1
P^*	12.6	19.7	3.3
Y_{P^*}	42.0	40.0	33.0

experimentally for the case of EG and GL obtaining yields of 59 and 53%, slightly higher than the model prediction, as shown in Table 2.

According to previous results *(27)*, C_S had a positive effect on yield, although not so strong as the variables under study. Therefore, the effect of

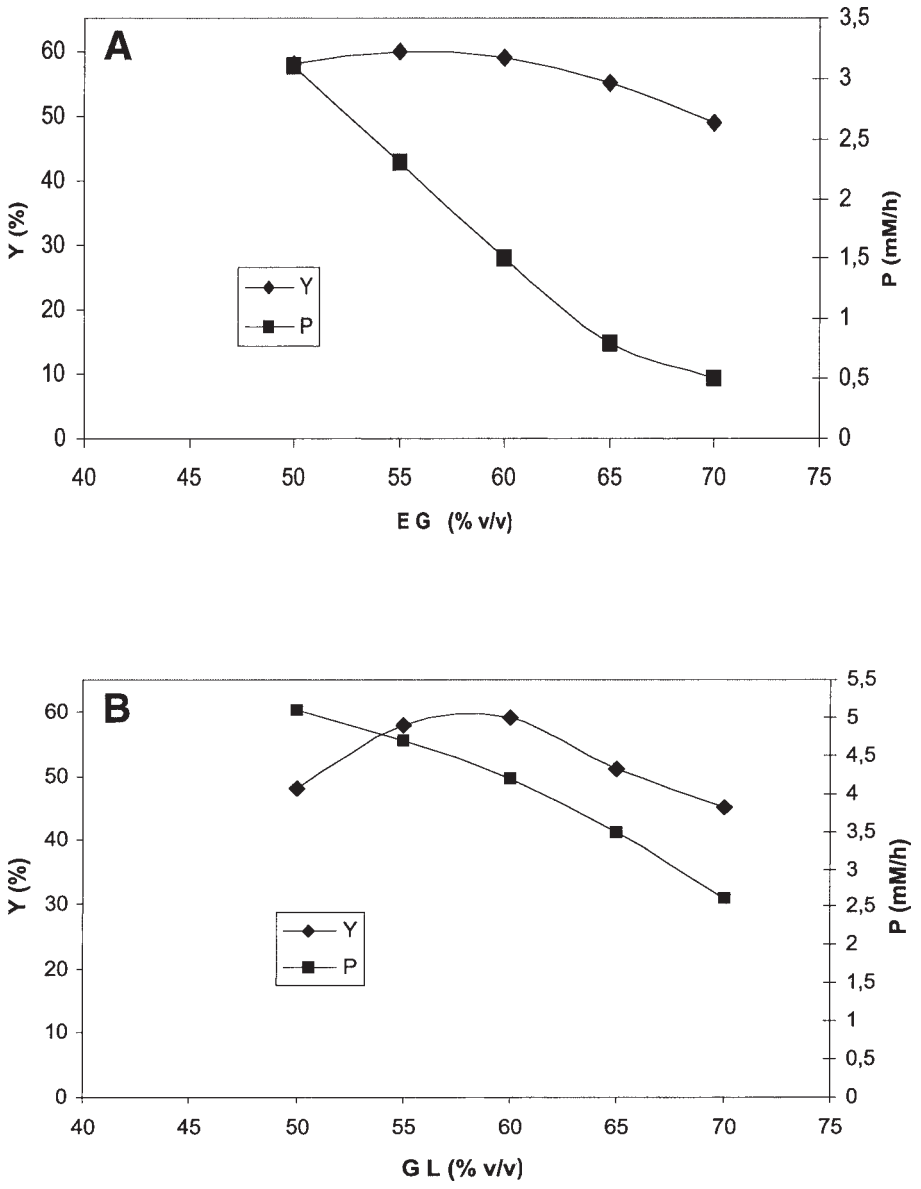

Fig. 7. Effect of cosolvent concentration on ampicillin yield (Y) and productivity (P) at optimized pH and temperature, according to Table 2. **(A)** In EG; **(B)** in GL. r_{ES} = 125 UI_H/mmol of 6-APA; r_{SS} = 1:3 mmol of 6-APA/mmol of PGME.

C_S on yield was evaluated at the already optimized pH and temperature in EG and GL media; results are presented in Fig. 7.

The stability of immobilized penicillin acylase was determined by the increase in time required to obtain the specified yield in a sequential batch operation. Results are summarized in Table 3 for EG and GL media under previously optimized conditions.

Table 3
Operational Stability of Immobilized Penicillin Acylase in Sequential Batch Operation for Synthesis of Ampicillin in EG (pH 6.0, 26°C) and GL (pH 6.0, 30°C) Media[a]

	EG		PD	
Batch no.	Reaction time (h)	Productivity of batch (m*M*/h)	Reaction time (h)	Productivity of batch (m*M*/h)
1	5.4	2.9	3.9	8.5
2	5.4	2.9	4.0	8.0
3	7.0	2.5	4.2	7.6
4	7.6	2.3	4.4	7.2
5	7.9	2.2	4.6	7.0

[a]Yield at the end of each batch was 58 ± 1% for EG and 52 ± 1% for GL.

Discussion

The time course of ampicillin production exhibited the typical pattern of a kinetically controlled synthesis in which a transient maximum product concentration is obtained, after which product hydrolysis prevails, as shown in Figs. 1–3. As seen in Figs. 4 and 5, the peak is much sharper in buffer than in organic cosolvents, where product decay rate is much lower because of the depressing effect of cosolvents on the hydrolytic reaction; this represents a definite advantage of organic over aqueous media. Yield was always lower in buffer than in EG and GL, but in a few conditions higher than in PD, as shown in Table 1. On the other hand, productivity was always higher in buffer. These results are consistent with those previously reported with other immobilized penicillin acylases in 10–50% (v/v) alcohol:buffer mixtures, in which yields were higher but initial rates of ampicillin synthesis (and productivity as a consequence) were drastically reduced *(6,29)*. With more hydrophobic cosolvents (at the same 50% [v/v] concentration) than the ones used in this work, an increase in initial rates has been observed in the thermodynamically controlled synthesis of ampicillin, which has been attributed to the favorable reduction in the ionization of the substrates *(24)*. Yields increased at lower pH for the case of EG and GL, which is consistent with the mechanism of reaction proposed *(30)*. Optimum was 6.0 in both cases; below pH 6.0 a decrease in yield can be the consequence of a direct adverse effect on enzyme catalytic structure. In the case of PD, the pH optimum was somewhat higher (6.6) but definitely lower than optimum for hydrolysis, which for this immobilized penicillin acylase is 7.8 *(31)*. Yield increased at lower temperatures in the case of EG and PD and the optimum was close to the lower limit of the range studied; this is consistent with preliminary results on EG that suggested a significant increase in yield at lower temperatures *(27)*. This effect has been previously reported for the enzymatic synthesis of β-lactam antibiotics *(32)* and peptides *(33)*.

EG and GL are clearly superior to PD as reaction medium for synthesis. PD has been discarded for future work because no clear advantage exists over a completely aqueous medium in which yields are comparable and productivity is much higher. Yields are higher in EG than in GL; furthermore, viscosity is much lower in EG (even lower than in buffer) than in GL, which is relevant in terms of mass transfer in the case of immobilized biocatalysts. It is tempting to correlate yield with water activity in the reaction medium. Water activity has been determined at 50% (v/v) cosolvent concentration, being 0.81, 0.83, and 0.89 for EG, GL, and PD, respectively *(34)*, so that the activity of water decreases from water to EG in the same order that yield increases.

A quadratic model for *Y* was validated for the three cosolvents using response surface methodology. Three-dimensional plots in Fig. 6 show the pH and temperature optima for each of the cosolvents. These optima were experimentally validated for EG and GL, with yield slightly higher than the values predicted by the model.

Temperature and pH were also optimized in terms of productivity (data not shown), and a pattern opposite to yield was observed, with maximum productivity obtained at higher pHs and temperatures, as shown in Table 2. This is relevant for process optimization since an increase in yield will always be obtained at the expense of productivity. In other words, an increase in yield will demand a higher consumption of biocatalyst. However, yield is a key issue in the production of fine chemicals and biochemicals and, therefore, the use of robust cheap hydrolases for organic synthesis will certainly reduce the impact of productivity on processing cost. In addition, productivity, as defined here, refers merely to the first batch, which has relatively little meaning in the case of an immobilized biocatalyst that will be used for a considerable number of batches, given that global productivity is the correct parameter to assess (this comment is also valid for the initial rates of synthesis that are reportedly higher in fully aqueous media). This, in turn, will require stability data under process conditions, which are still to be obtained.

As a first approach, the synthesis of ampicillin was studied in EG and GL media in a repeated-batch mode of operation under the optimum conditions for yield. Productivity decreased at an average of 6 and 5% per batch in EG and GL, respectively (see Table 3). This decrease is rather high for an immobilized penicillin acylase, and it is yet to be elucidated if this is real inactivation or an artifact derived from the filtering and washing of the immobilized penicillin acylase. Stability of soluble penicillin V acylase was reported higher in GL and slightly higher in EG than in water and was correlated to the log *P* value of the cosolvent; log *P* for GL is –3.0 and –1.8 for EG, with –1.8 the breakpoint between higher and lower stability than in water *(25)*. We have obtained similar results with immobilized penicillin acylase (unpublished data). However these results were gathered under nonreactive conditions that do not represent actual behavior under reactive conditions *(31,35)*. Biocatalyst stability should not be a major concern

in this process *(36)* since optimum temperature is likely to be low *(18)* and very good immobilized penicillin acylases have been developed with extremely high stability in organic cosolvents by using artificial nanoenvironments to protect enzyme structure and function *(37)*.

The effect of C_S at already optimized pH and temperature was studied in EG and GL beyond 50% (v/v) since preliminary studies with EG suggested a moderate increase in yield at higher C_S *(27)*. As shown in Fig. 7, the increase in EG was modest from 50 to 60% (v/v) C_S and decreased thereafter. This slight increase was accompanied by a severe reduction in productivity, so that 50% (v/v) can be considered as optimum C_S in the case of EG. The increase in yield was substantial in GL, and values close to 60% were obtained in the 55–60% (v/v) range, with a smaller reduction in productivity. However, increase in viscosity of GL solutions over 50% made the system barely tractable.

Maximized values for yield compare well with those reported for the kinetically controlled synthesis of ampicillin in the absence *(17,38,39)* and presence of organic cosolvents *(6,29,40–42)* and also for those reported for other β-lactam antibiotics *(16–18,40)*. There are a few cases in which higher yields of ampicillin have been reported. Ospina et al. *(38)* reported 75% yield with an immobilized penicillin acylase from recombinant *E. coli* at high substrate concentration (100 m*M* 6-APA, 300 m*M* PGME) well over solubility of FGME; at lower substrate concentrations, yields were well below 60%. This heterogeneous system is attractive even though it hampers enzyme recovery from the precipitated phenylglycine. Hernández-Justiz et al. *(17)* reported 80% yield with a soluble penicillin acylase from *Acetobacter turbidans*, but this result was obtained at much lower substrate concentrations; reported yields with penicillin acylases from *Kluyvera citrophila*, *E. coli*, and *Bacillus megaterium* were only 40, 33, and 20%, respectively. Higher yields have been reported for the synthesis of derived cephalosporins with penicillin acylase *(7,18,43)*, which stems from the fact that 7-ADCA (or 7-ACA) is a better nucleophile for penicillin acylase than 6-APA *(44)*. Our strategy is being used for the synthesis of derived cephalosporins, in which very high yields are predicted for processes that have a much higher added value.

Conclusion

Product yield was chosen as the objective function to optimize the kinetically controlled synthesis of ampicillin. Yield is expected to have a strong relative impact on processing cost in the production of pharmaceuticals, even more so if the biocatalyst is relatively cheap and robust.

Yield in aqueous buffer was below 40% in all conditions tested and peak for maximum yield was very sharp, while values >60% were obtained in organic cosolvents with much lower decay after maximum, verifying the hypothesis that organic solvents are favorable for the kinetically controlled synthesis of ampicillin by hampering competing hydrolytic reactions in favor of synthesis.

The statistical model developed adequately predicted temperature and pH optima for ampicillin yield with immobilized penicillin acylase in organic media.

EG was the best cosolvent for the synthesis of ampicillin, with substantially higher yields obtained than in aqueous buffer and other cosolvents. Optimum conditions were pH 6.0 and 26°C. Temperature optimum was close to the lower extreme of the range studied, which makes interesting to study the synthesis at low temperatures. In addition, EG has very good properties in terms of substrate solubilization, enzyme protection, water activity reduction, low viscosity, and very low freezing point.

Yield was always in compromise with productivity, the latter higher in aqueous buffer, somewhat lower in GL, even lower in EG, and much lower in PD. However, productivity in one batch is not a very relevant parameter for an immobilized biocatalyst; global productivity is necessary to assess under prolonged reactor operation. Preliminary results in repeated-batch operation showed an acceptable stability in EG and GL, but inactivation data under operation conditions are required to optimize synthesis further.

Maximum yield obtained compares very favorably with those reported in the literature for the kinetically and thermodynamically controlled synthesis of ampicillin and other derived penicillins.

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