ELSEVIER

Process Biochemistry 38 (2002) 351–360

PROCESS BIOCHEMISTRY

www.elsevier.com/locate/procbio

Effect of cosolvent and pH on the kinetically controlled synthesis of cephalexin with immobilised penicillin acylase

Carolina Aguirre [a,*], Mauricio Toledo [a], Valentina Medina [a], Andrés Illanes [b,1]

[a] *Faculty of Sciences, Universidad Católica Ssma. Concepción, P.O. Box 297, Concepción, Chile*
[b] *School of Biochemical Engineering, Universidad Católica Valparaíso, P.O. Box 4059, Valparaíso, Chile*

Received 14 February 2002; accepted 19 March 2002

Abstract

The effect of co-solvents was studied on the kinetically controlled synthesis of cephalexin with immobilised penicillin acylase (PA). Yield correlated well with the ratio of initial rates of cephalexin synthesis to acyl donor hydrolysis and water activity. Yield was substantially higher in EG than in aqueous medium and was selected to study the effect of (apparent) pH on initial rates of synthesis and hydrolysis. The ratio of initial rates of product synthesis to hydrolysis (r_2), adequate to assess the potential for synthesis in kinetically controlled systems under excess acyl donor, was maximal at pH 7.5 and correlated well with yield. The effect of ethylene glycol was very strong with an improvement of 230% in r_2, whose value was higher than 1, hardly attainable in aqueous medium. This reflected in a considerable improvement (13%) in product yield, which highlights the potential of EG as a medium for the production of cephalexin.

Keywords: Penicillin acylase; Cephalexin; β-Lactam antibiotics; Immobilised enzyme; Enzymatic synthesis; Organic cosolvents

1. Introduction

Second-generation cephalosporins are antibiotics of considerable commercial and pharmaceutical relevance [1]. Among them, 7-aminodesacetoxycephalosporanic acid (7-ADCA)-derived cephalexin is outstanding: most of the 5000 tons of 7-ADCA produced worldwide is used in its production [2]. The present process involves the chemical ring expansion of the leading molecule penicillin G to cephalosporin G, followed by enzyme cleavage of the side chain by immobilised penicillin acylase (PA) to produce 7-ADCA, from which cephalexin is chemically synthesised, i.e. via the Dane salt route [3]. The present trend is the replacement of chemical steps by biological conversions to avoid cumbersome reaction schemes, extreme conditions and hazardous chemicals [4,5]. Recent advances in the production of cephalexin include metabolic pathway engineering to produce 7-ADCA by fermentation with recombinant *Penicilliun chrysogenum* and enzymic synthesis of cephalexin from 7-ADCA and activated phenylglycine [2,4].

Penicillin amidohydrolase (PA; E.C. 3.5.1.11) is a key enzyme for the synthesis of β-lactam antibiotics. It is able, not only to hydrolyse penicillin G or cephalosporin G to produce 6-aminopenicillanic acid (6-APA) or 7-ADCA [3,6–8], but also to synthesise from them the corresponding semisynthetic penicillins [9] or cephalosporins [10]. Considerable waste reduction has been estimated by moving from chemical to enzymic synthesis of cephalexin and this might be on the baseline of its commercial success [3].

Synthesis of β-lactam antibiotics with PA can be conducted under thermodynamic [11–13] or kinetic control [11,14,15]. However, the former is not feasible for the synthesis of cephalexin because of the incompatibility of the zwitterionic phenylglycine with the enzyme [3,4]. Synthesis under kinetic control, despite the requirement of an activated acyl donor, is a viable option for cephalexin production [15,16] and conditions can be established that depress the hydrolytic reactions (of product and activated acyl donor) in favour of synthesis, increasing yield. One such condition is the presence of

* Corresponding author. Fax: +56-41-735-251
E-mail addresses: caguirre@ucsc.cl (C. Aguirre), aillanes@ucv.cl (A. Illanes).
[1] Fax: +56-32-273-642.

0032-9592/02/$ - see front matter
PII: S0032-9592(02)00092-4

organic cosolvents that may favour synthesis by reducing water activity [17,18] and by increasing the proportion of reactive non-ionised species [19]. PA instability in organic cosolvents has been a drawback [20,21]. However, this is not so in all cases, i.e. in polyols [22–24], and the advances in enzyme immobilisation techniques make now possible the construction of robust biocatalysts to perform in harsh environments [25–28]. In this scenario, the use of organic cosolvents as reaction medium for the synthesis of β-lactam antibiotics seems quite promising.

High product yield is a requisite for enzymic synthesis to become competitive with chemical synthesis [29]. In the synthesis of β-lactam antibiotics under kinetic control, yield will be determined by the balance between the rates of antibiotic production, antibiotic hydrolysis and acyl donor hydrolysis [30]. Therefore, the determination of such reaction rates is important since their ratios can be good estimates of the potential for antibiotic synthesis. These rates are to be critically affected by pH [31] making it a candidate variable for optimisation. In fact, pH was determined as the most critical variable in the kinetically controlled synthesis of ampicillin [32].

This work presents results on the effect of cosolvent and pH on such rates and in product yield for the synthesis of cephalexin. Solvents were first screened and ethylene glycol was then selected to study the effect of pH.

2. Materials and methods

2.1. Materials

Penicillin G and 6-aminopenicillanic acid (6-APA) were kindly supplied by Natsus S.A (Lima, Perú), D-α-phenylglycine (PG), cephalexin (Ceph), ampicillin (Amp), D-α-phenylglycine methyl ester (PGME), 7-amino desacetoxycephalosporanic acid (7-ADCA) and all other reagents and solvents were analytical grade and obtained from Sigma-Aldrich (St. Louis, MO, USA). PA from *Escherichia coli* immobilised in polyacrylamide carrier (PGA-450) with a specific activity of 340 UI_H per g was from Roche Molecular Biochemicals (Mannheim, Germany).

2.2. Enzyme assay

PA activity was determined from initial rates of 6-APA production by hydrolysis of penicillin G potassium salt (PenG). 6-APA was determined by HPLC. One International Unit of PA hydrolytic activity (IU_H) was defined as the amount of enzyme hydrolysing one μmol of PenG per min at pH 7.8 and 27 °C from 134 mM PenG in 0.1 M phosphate buffer.

2.3. Assays for substrates and products of synthesis

The amounts of Ceph, Amp, PGME, 7-ADCA, 6-APA and PG in the reaction mixtures (synthesis or hydrolysis) were analysed by high-performance liquid chromatography (HPLC). Samples (0.1 ml) appropriately diluted with eluant, were applied to Licrospher 100 RP-18 (Merck, Darmstadt, Germany) column (125 × 4 mm, 5 μm) and eluted isocratically with 30% methanol (M) in phosphate buffer 20 mM, pH 6.0 at a flow rate of 0.9 ml/min. In the case of PenG, M at 40% in phosphate buffer was used.

2.4. Initial rates

Initial rates of antibiotic hydrolysis $v_{H,Anbtibiotic}$ and PGME hydrolysis ($v_{H,PGME}$) were determined using phosphate buffer 100 mM over the pH range from 6.0 to 7.5 and borate buffer 100 mM over the pH range from 8.0 to 8.5. The former rates were determined at 30 mM Ceph or 30 mM Amp or 90 mM PenG and 52 IU_H per mmol Ceph or Amp, or 17.3 IU_H per mmol PenG; the later was determined at 90 mM PGME and 17.3 IU_H per mmol PGME. Experiments were conducted in fully aqueous medium and in EG 50% v/v, at 27 °C.

Initial rates of antibiotic synthesis $v_{S,Antibiotic}$ were determined for Amp and Ceph over the pH range under study. These experiments were conducted in fully aqueous medium and in EG 50% v/v at 27 °C, 30 mM 7-ADCA (6-APA), 90 mM PGME and 52 IU_H per mmol 7-ADCA (6-APA).

The cosolvents used were: acetonitrile (AN), 1,3-butanediol (BD), diglyme (DG), dimethyl sulfoxide (DMSO), ethanol (E), ethyleneglycol (EG), glycerol (G), methanol (M) and 1,2-propanediol (PD). Values for pH in EG–water mixtures are to be considered as apparent.

2.5. Synthesis

The reactions of synthesis of Ceph and Amp were conducted batch-wise, under nucleophile limitation, in a thermoregulated magnetically stirred reactor with pH control under the same conditions at which initial rates of synthesis were measured. Samples were removed periodically and analysed by HPLC. The molar yield of antibiotics synthesis was determined based on the initial concentration of the limiting substrate (7-ADCA or 6-APA) and expressed as percentage. Productivity was defined as the molar amount of antibiotic produced per unit time and unit reaction volume (mM/h) at maximum yield.

2.6. Water activity (a_W) calculation

Water activities for the reaction media were estimated theoretically from vapour–liquid activity coefficients calculated by the UNIFAC group contribution method [33].

3. Results and discussion

Fig. 1 represents the kinetically controlled syntheses of Ceph and Amp in fully aqueous media with phosphate buffer at pH 7.0, 27 °C, 30 mM nucleophile (7-ADCA, or 6-APA), 90 mM acyl donor (PGME) and 52 IU_H per mmol nucleophile (7-ADCA, or 6-APA). In both cases, product concentration reached a maximum and then gradually decreased due to hydrolysis, as expected from a kinetically controlled synthesis. Maximum molar yields of Ceph and Amp were 55.3 and 36.7%, while productivities at the points of maximum yield were 38.07 and 19.72 mM/h, respectively. These results show that 7-ADCA is a better nucleophile than 6-APA for this immobilised PA, which is consistent with previous results with soluble PA in aqueous buffer and also in organic cosolvents [34].

Nine organic cosolvents, mainly polyalcohols, were selected based on previous works [17,19,24] to examine the effect of their presence in the reaction medium on the kinetically controlled synthesis of Ceph. Time–course syntheses of Ceph in organic media at 50% v/v cosolvent concentrations are presented in Fig. 2 and results are summarised in Table 1, where the initial rates of Ceph synthesis ($v_{S,Cef}$), PGME hydrolysis ($v_{H,PGME}$), maximum molar yield (Y) and productivity at that yield (P) are presented. EG was the best cosolvent in terms of Y at the conditions tested, with a value substantially higher than in aqueous buffer. Y on PD and BD were similar than in aqueous buffer, while being substantially lower in the rest of the cosolvents; AN arrested the synthesis of Ceph almost completely. As expected, P was always reduced by the presence of organic cosolvents. However, among cosolvents, P in EG was the highest. The peak of Y was sharper in buffer than in most cosolvents. In the case of BD, EG, G and PD there is a good correlation between a_w and $v_{S,Ceph}/v_{H,PGME}$ with Ceph Y, as seen in Table 1. However, this is not so for AN, DMSO, E and M. The same pattern of synthesis is observed in these cosolvents (Fig. 2): after reached the product its highest concentration (comparatively small indeed) it remained constant, meaning that no product synthesis or hydrolysis occurred, which is indicative of enzyme inactivation and can explain the poor correlation obtained with these cosolvents between a_w or $v_{S,Ceph}/v_{H,PGME}$ and Ceph Y (see Table 1). This explanation is consistent with results reported on enzyme inactivation in organic cosolvents [22]. The ratio of initial rates of product synthesis and acyl donor hydrolysis ($v_{S,Ceph}/v_{H,PGME}$), sometimes referred to as the synthesis to hydrolysis ratio, has been considered a good parameter to assess the potential of a given enzyme for synthesis in a kinetically controlled system [35]. This parameter was 2.4 times higher in EG than in water (see Table 1) and this co-solvent was precisely the one that increased Y. Only in EG, DMSO and M were higher $v_{S,Ceph}/v_{H,PGME}$ were obtained than in water, but only in EG was this reflected by an increased Y, because of the strong enzyme inactivation in DMSO and M. Y was

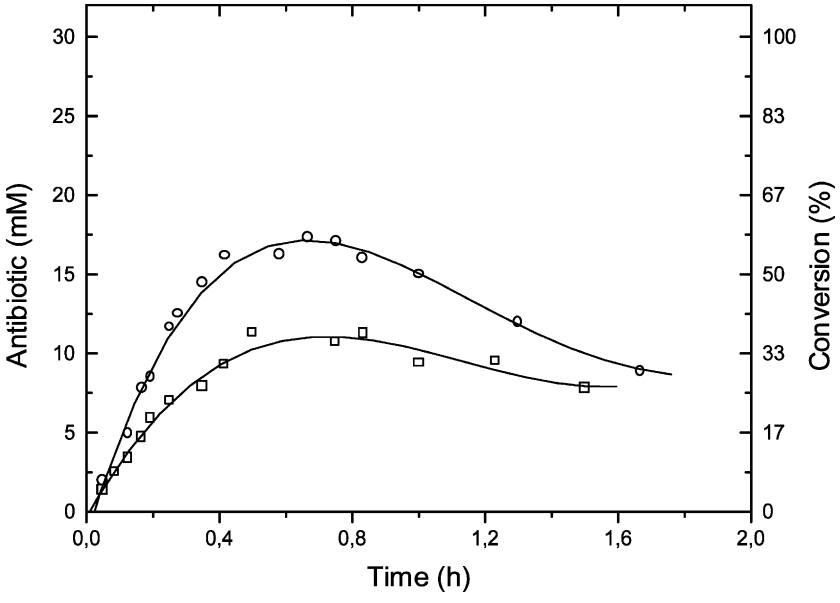

Fig. 1. Synthesis of (○) cephalexin and (□) ampicillin in fully aqueous medium in phosphate buffer pH 7.0 at 27 °C, 30 mM 7-ADCA (or 6-APA), 90 mM PGME and 52 IU_H per mmol 7-ADCA (or 6-APA).

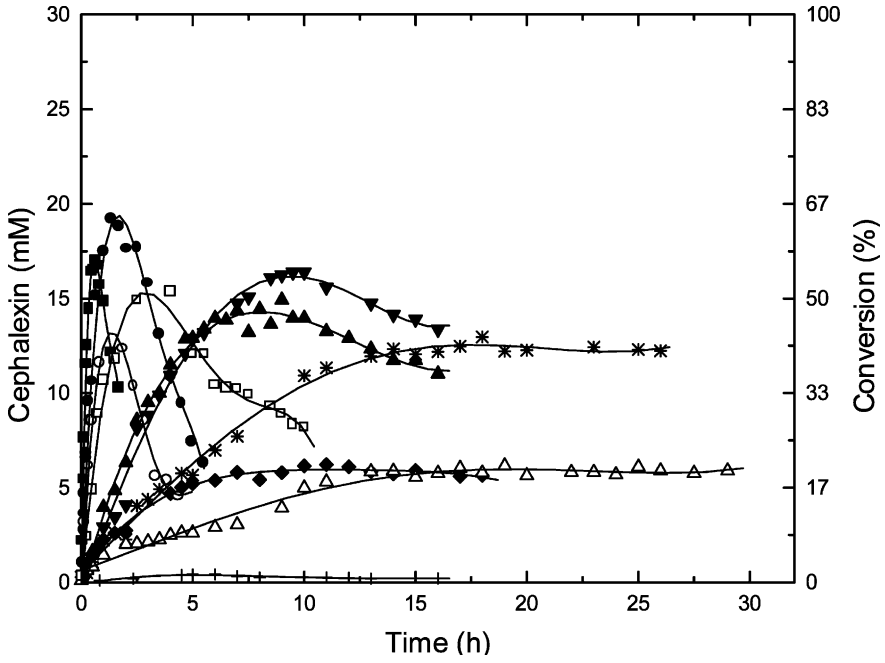

Fig. 2. Time–course of synthesis of cephalexin in organic cosolvents at 50% v/v. (■) control (fully aqueous medium); (+) AN; (▲) BD; (□) DG; (*) DMSO; (△) E; (●) EG; (○) G; (◆) M; (▼) PD. Conditions: pH 7.0, 27 °C, 30 mM 7-ADCA, 90 mM PGME and 52 IU_H per mmol 7-ADCA.

similar in water and in those cosolvents where the $v_{S,Ceph}/v_{H,PGME}$ was similar (BD, PD).

EG was the cosolvent in which Ceph *Y* was higher and less enzyme inactivation occurred. It has been previously determined that it is also the one in which substrates and products are more stable [24]. EG was then selected to study the effect of pH on the initial rates of Ceph synthesis and hydrolysis and acyl donor (PGME) hydrolysis. The same study was conducted on Amp, used for comparison, and rates on fully aqueous medium were also determined. EG has been determined previously as the best cosolvent for the synthesis of Amp as well [24].

The effect of pH on the initial rates of synthesis of Ceph and Amp in 50% v/v EG and in fully aqueous media (control) is shown in Fig. 3. The effect of pH on the initial rates of antibiotics and PGME hydrolysis is presented in Figs. 4 and 5, respectively. The effect of pH on initial rates of synthesis was very strong for both antibiotics, especially in aqueous medium, rates increasing with pH upto 8.0. This increase can be explained considering that the non-ionised reactive species of substrates will be favoured at higher pH [20] and is consistent with the proposed reaction mechanism for the kinetically controlled synthesis of β-lactam antibiotics [36]. The decrease over pH 8.0 might be a consequence of adverse configurations of the enzyme molecule at such extreme conditions, as suggested by the pH profile of antibiotics hydrolysis rate. This is further supported by the profile for PenG hydrolysis shown in Fig. 7. In the case of PenG, with an uncharged side-chain, the hydrolysis rate was maximum at a higher pH because of

Table 1
Cosolvent effect on initial rates of cephalexin synthesis ($v_{S,Cef}$) and PGME hydrolysis ($v_{H,PGME}$), water activity (a_w), maximum yield (*Y*) and productivity at that yield (*P*)

Cosolvent	$v_{S,Cef}$ (mM/min)	a_w	$v_{S,Cef}/v_{H,PGME}$	*Y* (%)	*P* (mM/h)
Control (buffer)	0.932	1	0.561	55.3	38.07
AN	0.011	0.896	0.456	1.4	0.42
BD	0.067	0.940	0.462	49.7	2.35
DG	0.181	–	0.390	51.2	6.30
DMSO	0.023	0.683	1.21	43.2	0.91
E	0.011	0.889	0.262	20.5	0.42
EG	0.350	0.810	1.351	64.1	15.15
G	0.181	0.826	0.263	41.2	6.30
M	0.023	0.780	0.846	20.7	0.91
PD	0.039	0.886	0.501	54.6	2.13

Conditions, pH 7.0, 27 °C, 52 IU_H per mmol 7-ADCA, 30 mM 7-ADCA and 90 mM PGME.

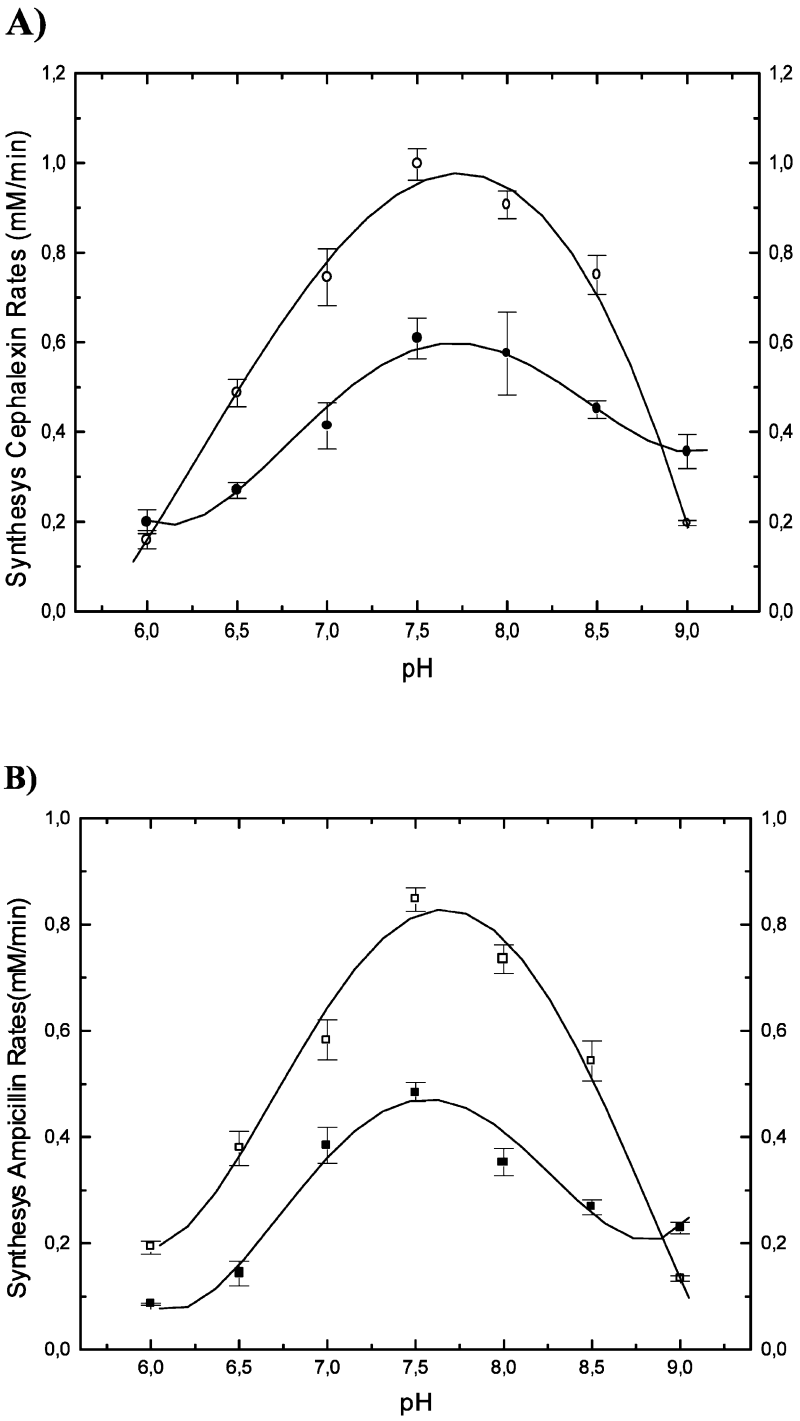

Fig. 3. Effect of pH on the initial rates of synthesis of (A) cephalexin (circles) and (B) ampicillin (squares). Open symbols, control (fully aqueous buffer); closed symbols: EG 50% v/v. Conditions, 27 °C, 30 mM 7-ADCA (or 6-APA), 90 mM PGME and 52 IU_H per mmol 7-ADCA (or 6-APA).

the compromise between the substrate charge and the enzymic ionic structure required for catalysis. The effect of pH on antibiotic hydrolysis rates was milder, especially in EG.

Initial rates of synthesis and hydrolysis of antibiotics were lower in EG than in the control but hydrolysis rates (Fig. 4) were much more affected than synthesis rates (Fig. 3) showing the beneficial effect of EG. No such differences were observed with respect to PGME hydrolysis rates. According to a kinetically controlled synthesis, Y will be the result of a balance between all those rates [37]. It is expected then, that Y will be higher in those cases where the ratio of antibiotic synthesis rate to the sum of antibiotic and acyl donor hydrolysis rates:

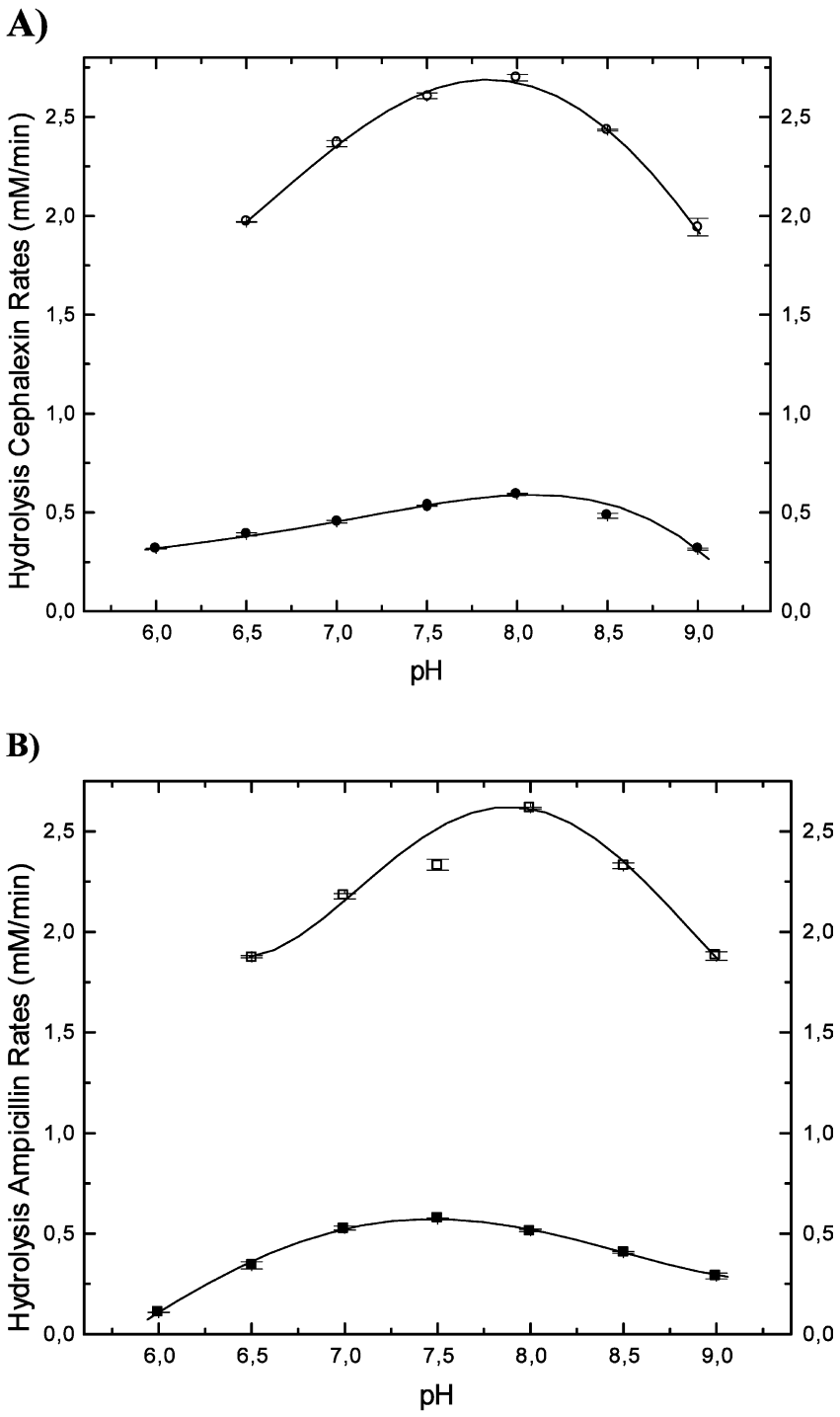

Fig. 4. Effect of pH on the initial rates of hydrolysis of (A) cephalexin (circles) and (B) ampicillin (squares). Open symbols, control (fully aqueous buffer); closed symbols: EG 50% v/v. Conditions, 27 °C, 30 mM 7-ADCA (or 6-APA), 90 mM PGME and 52 IU_H per mmol 7-ADCA (or 6-APA).

$v_{S,Antibiotic}/(v_{H,Antibiotic}+v_{H,PGME})$ is higher. Values for this ratio (r_1) at different pH values are presented in Table 2 both for Ceph and Amp in 50% v/v EG and in fully aqueous buffer. The ratio of antibiotic synthesis to hydrolysis rate: $v_{S,Antibiotic}/v_{H,Antibiotic}$ (r_2), is also presented in Table 2.

The effect of EG was quite beneficial for the synthesis of both antibiotics, increasing r_1 and r_2 in the whole range of pH studied, the effect being somewhat stronger for Ceph than for Amp. The kinetically controlled synthesis of both antibiotics was conducted under excess acyl donor (molar ratio acyl donor/nucleophile = 3), so

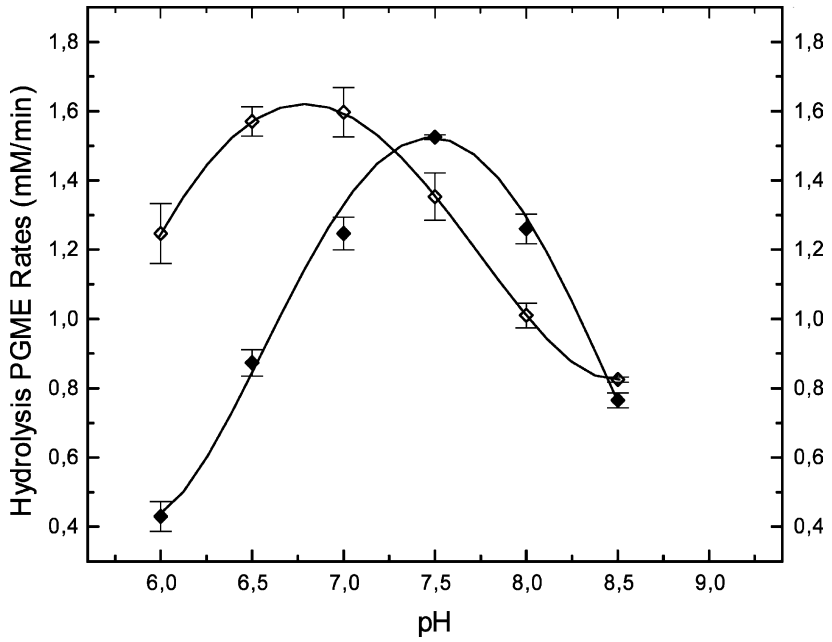

Fig. 5. Effect of pH on the initial rates of hydrolysis of PGME. Open symbols, control (fully aqueous buffer); closed symbols, EG 50% v/v. Conditions, 27 °C, 30 mM 7-ADCA (or 6-APA), 90 mM PGME and 52 IU_H per mmol 7-ADCA (or 6-APA).

that r_2 is more indicative than r_1 of the potential for synthesis. The increase in r_2 due to the presence of EG in the reaction media was in most cases well over 100% being, at pH 7.5, 230% for Ceph and 133% for and Amp and, at pH 7.0, 190 and 172%, respectively. This latter result is consistent with the values of Y obtained in the synthesis of Ceph in the absence and presence of EG (see Fig. 2) and strongly supports the use of EG as a suitable cosolvent for the enzymic synthesis of β-lactam antibiotics.

Best results in terms of r_1 and r_2 were obtained for both antibiotics at pH 7.5. It is remarkable that r_2 is over 1 in the case of Ceph, a result that will not be easily attainable in fully aqueous medium. Results in EG were

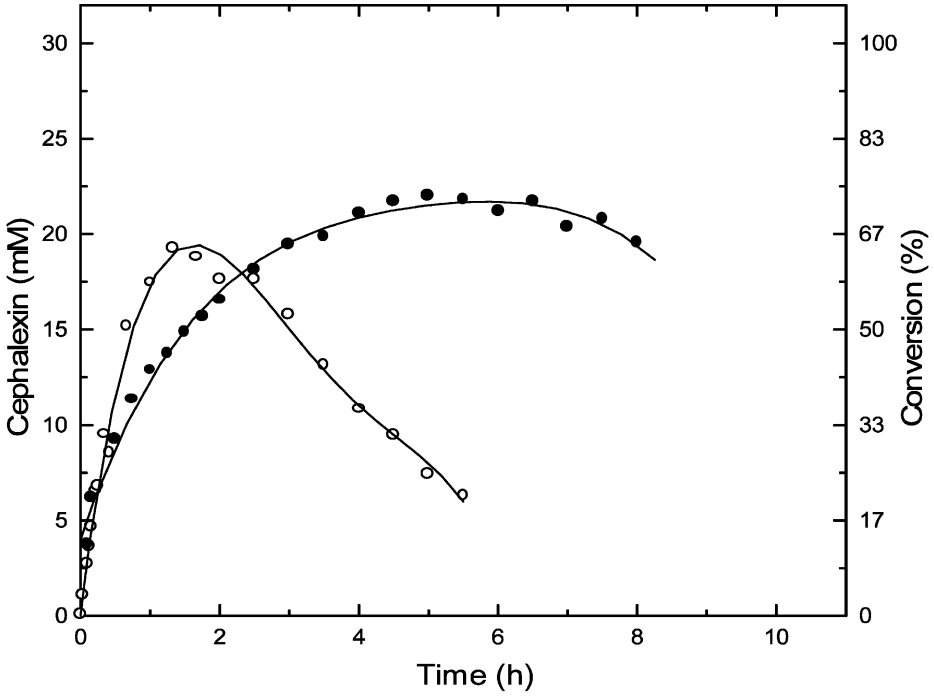

Fig. 6. Time–course of synthesis of cephalexin in EG 50% v/v (○) pH 7.0; (●) pH 7.5. Conditions, 27 °C, 30 mM 7-ADCA (or 6-APA), 90 mM PGME and 52 IU_H per mmol 7-ADCA (or 6-APA).

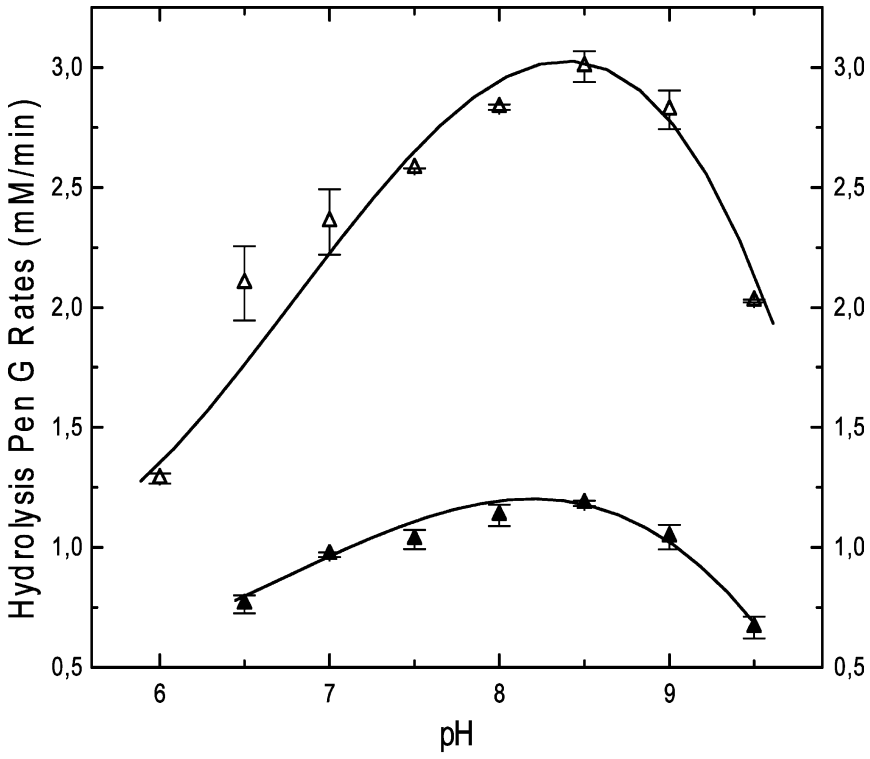

Fig. 7. Effect of pH on the initial rates of hydrolysis of penicillin G. Open symbols, control (fully aqueous buffer); closed symbols, EG 50% v/v. Conditions, 27 °C, 90 mM Pen G 17.3 IU_H per mmol PenG.

always better for the synthesis of Ceph than Amp, which is consistent with the fact that 7-ADCA is a better nucleophile than 6-APA for most PAs. An optimum pH of 6.0 was reported for the synthesis of Amp with PA immobilised on agar [31], but this optimum was determined based on $v_{S,Amp}/v_{H,PGME}$, not considering the effect on antibiotic hydrolysis rate.

The time–course of Ceph synthesis in 50% v/v EG was conducted at pH 7.5 to verify the best value predicted in Table 2. Results at pH 7.5 and 7.0 are presented in Fig. 6 at comparable conditions. Results are fully consistent with those predicted from Table 2. Ceph Y at pH 7.5 was 72.3%, which is 13% higher than at pH 7.0. Y decreased at higher pHs which confirms pH 7.5 as the best for Ceph synthesis with this immobilised PA.

4. Conclusions

- EG was selected as the best cosolvent for the kinetically controlled synthesis of cephalexin with immobilised PA, considering product yield and stability of enzyme, substrates and product.

Table 2
Effect of pH on the ratios of antibiotic synthesis to antibiotic hydrolysis plus acyl donor hydrolysis rates $v_{S,Antibiotic}/(v_{H,antibiotic}+v_{H,PGME})$ (r_1) and antibiotic synthesis to antibiotic hydrolysis rates $v_{S,Antibiotic}/v_{H,antibiotic}$ (r_2) for ampicillin and cephalexin

	Cephalexin				Ampicillin			
	r_1		r_2		r_1		r_2	
	Control (buffer)	EG (50% v/v)	Control (buffer)	EG (50% v/v)	Control (buffer)	EG (50% v/v)	Control (buffer)	EG (50% v/v)
6.0	–	0.267	–	0.625	–	0.167	–	0.818
6.5	0.138	0.214	0.249	0.692	0.110	0.116	0.202	0.412
7.0	0.189	0.241	0.316	0.911	0.153	0.213	0.266	0.717
7.5	0.253	0.325	0.383	1.264	0.231	0.229	0.365	0.842
8.0	0.245	0.314	0.337	0.983	0.201	0.197	0.279	0.673
8.5	0.230	0.360	0.309	0.938	0.171	0.229	0.232	0.659
9.0	–	–	0.103	1.194	–	–	0.069	0.793

Conditions, 27 °C, 52 IU_H per mmol 7-ADCA (6-APA), 30 mM 7-ADCA (6-APA) and 90 mM PGME.

- Initial rates of antibiotic production and hydrolysis and acyl donor hydrolysis were determined in the presence and absence of EG at different pHs both for cephalexin and ampicillin to calculate the ratios of synthesis to hydrolysis rates (r_2). This parameter is proposed as a good estimate for the potential of antibiotic synthesis.
- The effect of pH on initial rates was very strong, especially for antibiotic synthesis. Rates of synthesis increased with pH upto 8.0, which is consistent with the proposed mechanism for the kinetically controlled synthesis of ampicillin.
- Maximum r_2 was obtained at pH 7.5 and results correlated well with cephalexin Y, which was also maximum at that pH.
- Results were substantially better for the synthesis of cephalexin than ampicillin, which gives further evidence that 7-ADCA is a better nucelophile than 6-APA in the synthesis of β-lactam antibiotics with PA.
- EG improved antibiotic synthesis strongly by increasing the ratio of synthesis to hydrolysis significantly. At the best pH, this ratio was over 1, 230% higher than in fully aqueous medium, where a value close to 1 is hardly attainable.
- The use of EG as co-solvent is very promising for the kinetically controlled synthesis of cephalexin with immobilised PA. Optimisation of cephalexin synthesis in EG is underway and yields well over 90% are predicted.

Acknowledgements

This work was granted by chilean Fondecyt project 1990793, project DIN 01/98 of the Universidad Católica de la Ssma. Concepción and project DI 203-709/99 of the Universidad Católica de Valparaíso.

References

[1] Jústiz O, Fernández-Lafuente R, Guisán J, Negri P, Pagani G, Pregnolato M, Terreni M. Chemoenzymatic synthesis of 3′ functionalized cephalosporines (cefazolin) by three consecutive biotransformations in fully aqueous medium. J Org Chem 1997;62:9099–106.

[2] Wegman M, Janssen M, van Rantwijk F, Sheldon R. Towards biocatalytic synthesis of β-lactam antibiotics. Adv Synth Catal 2001;343:559–76.

[3] Bruggink A, Roy P. Industrial synthesis of semisynthetic antibiotics. In: Bruggink A, editor. Synthesis of β-lactam antibiotics. Dordrecht: Kluwer Acad Publishers, 2001:13–56.

[4] Bruggink A, Roos E, de Vroom E. Penicillin acylase in the industrial production of β-lactam antibiotics. Org Proc Res Dev 1998;2:128–33.

[5] Tramper J, Beeftink H, Janssen A, Ooijkaas L, van Roon J, Strubel M, Schroën C. Biocatalytic production of semi-synthetic cephalosporins: process technology and integration. In: Bruggink A, editor. Synthesis of β-lactam antibiotics. Dordrecht: Kluwer Acad Publishers, 2001:207–50.

[6] Shewale J, Desphande B, Sudhakaran V, Ambedkar S. Penicillin acylases: applications and potentials. Proc Biochem 1990;25:97–103.

[7] Shewale J, Sudhakaran K. Penicillin V acylase: its potential in the production of 6-aminopenicillanic acid. Enzyme Microb Technol 1997;20:402–10.

[8] Parmar A, Kumar H, Marwaha S, Kennedy J. Advances in enzymatic transformation of penicillins to 6-aminopenicillanic acid (6-APA). Biotechnol Adv 2000;18:289–301.

[9] Youshko M, van Langen L, de Vroom E, van Rantwijk F, Sheldon R, Švedas V. Highly efficient synthesis of ampicillin in an aqueous solution precipitate system: repetitive addition of substrates in a semi-continuous process. Biotechnol Bioeng 2001;73:426–30.

[10] Schroën C, Mohy Eldin M, Janssen A, Mita G, Tramper J. Cephalexin synthesis by immobilised penicillin G acylase under non-isothermal conditions: reduction of diffusion limitation. J Mol Catal B: Enzymatic 2001;15:163–72.

[11] Fernandez-Lafuente R, Rossell C, Piatkowska B, Guisán J. Synthesis of antibiotics catalyzed by penicillin G acylase: evaluation and optimization of different synthetic approaches. Enzyme Microb Technol 1996;19:9–14.

[12] Fernandez-Lafuente R, Rossell C, Guisán J. Dynamic reaction design of enzymic biotransformations in organic media. Biotechnol Appl Biochem 1996;24:139–43.

[13] Schroën C, Nierstrasz V, Kroon P, Bosma R, Janssen A, Beeftink H, Tramper J. Thermodynamically controlled synthesis of β-lactam antibiotics. Equilibrium concentration and side-chain properties. Enzyme Microb Technol 1999;24:489–506.

[14] Hernández-Jústiz O, Fernández-Lafuente R, Terreni M, Guisán J. Use of aqueous two-phase systems for in situ extraction of water soluble antibiotics during their synthesis by enzymes immobilized on porous supports. Biotechnol Bioeng 1998;59:73–9.

[15] Hernández-Jústiz O, Terreni M, Pagani G, García J, Guisán J, Fernández-Lafuente R. Evaluation of different enzymes as catalyst for the production of β-lactam antibiotics following a kinetically controlled strategy. Enzyme Microb Technol 1999;25:336–43.

[16] Schroën C, Nierstrasz V, Moody H, Hoogschagen M, Kroon P, Bosma R, Beeftink H, Janssen A, Tramper J. Modelling of enzymatic kinetic synthesis of cephalexin-influence of substrate concentration and temperature. Biotechnol Bioeng 2001;73:171–8.

[17] Hyun C, Kim J, Ryu D. Enhancement effect of water activity on enzymatic synthesis of cephalexin. Biotechnol Bioeng 1993;42:800–6.

[18] Kim M, Lee S. Effect of organic solvents on penicillin acylase-catalyzed reactions: interaction of organic solvents with enzymes. J Mol Catal B: Enzymatic 1996;1:181–90.

[19] Rosell C, Terreni M, Fernández-Lafuente R, Guisán J. A criterion for the selection of monophasic solvents for enzymatic synthesis. Enzyme Microb Technol 1998;23:64–9.

[20] Kim M, Lee S. Penicillin acylase-catalyzed synthesis of β-lactam antibiotics in water-methanol mixtures: effect of cosolvent content and chemical nature of substrate on reaction yields. J Mol Catal B: Enzymatic 1996;1:201–11.

[21] Youshko M, Sinev A, Švedas V. Stability and catalytic properties of penicillin acylase in systems with low water content. Biochemistry (Moscow) 1999;64:1186–95.

[22] Arroyo M, Torres-Guzmán R, de la Mata I, Castillón M, Acebal C. Prediction of penicillin V acylase stability in water-organic cosolvent monophasic systems as a function of solvent composition. Enzyme Microb Technol 2000;27:122–6.

[23] Arroyo M, Torres-Guzmán R, de la Mata I, Castillón M, Acebal C. Activation and stabilization of penicillin V acylase from

Streptomyces lavendulae in the presence of glycerol and glycols. Biotechnol Prog 2000;16:368–71.
[24] Illanes A, Fajardo A. Kinetically controlled synthesis of ampicillin with immobilized penicillin acylase in the presence of organic cosolvents. J Mol Catal B: Enzymatic 2001;11:605–13.
[25] Margolin A. Novel crystalline catalysts. Trends Biotechnol 1996;14:223–30.
[26] Fernandez-Lafuente R, Rosell C, Caanan-Haden L, Rodes L, Guisan J. Facile synthesis of artificial enzyme nano-environments via solid-phase chemistry of immobilized derivatives: dramatic stabilization of penicillin acylase versus organic solvents. Enzyme Microb Technol 1999;24:96–103.
[27] Tischer W, Kasche V. Immobilized enzymes: crystals or carriers. Trends Biotechnol 1999;17:326–35.
[28] Cao L, van Langen F, van Rantwijk F, Sheldon R. Cross-linked aggregates of penicillin acylase: robust catalysts for the synthesis of B-lactam antibiotics. J Mol Catal B: Enzymatic 2001;11:665–70.
[29] Baldaro E. Effect of temperature on enzymatic synthesis of cephalosporins. In: Pandit U, Alderweireldt F, editors. Bioorganic chemistry in healthcare and technology. New York: Plenum Press, 1991:237–40.
[30] Fernandez-Lafuente R, Rosell C, Guisan J. The use of stabilised penicillin acylase improves the design of kinetically controlled synthesis. J Mol Catal A: Chem 1995;101:91–7.
[31] Ospina S, Barzana E, Ramírez O, López-Munguía A. Effect of pH in the synthesis of ampicillin by penicillin acylase. Enzyme Microb Technol 1996;19:462–9.
[32] Illanes A, Anjarí S, Arrieta R, Aguirre C. Optimization of yield in the kinetically controlled synthesis of ampicillin with immobilized penicillin acylase in organic media. Appl Biochem Biotechnol 2002;97(3):165–180.
[33] Sandler S. Chemical and engineering thermodynamics, vol. 7. New York: Wiley, 1989.
[34] Aguirre C, Baeza J, Illanes A. Cosolvent effect on the synthesis of ampicillin and cephalexin with penicillin acylase. In: Ballesteros A, Plou F, Iborra J, Halling P, editors. Stability and stabilization of biocatalysts. Amsterdam: Elsevier, 1998:95–100.
[35] Terreni M, Pagani G, Ubiali D, Fernández-Lafuente R, Mateo C, Guisán J. Modulation of penicillin acylase properties via immobilization techniques: one pot chemoenzymatic synthesis of cephamandole from cephalosporin C. Bioorg Med Chem Lett 2001;1:2429–32.
[36] Kasche V. Mechanism and yields in enzyme catalysed equilibrium and kinetically controlled synthesis of β-lactam antibiotics, pèptides and other condensation products. Enzyme Microb Technol 1986;8:4–16.
[37] Kasche V. Ampicillin and cephalexin synthesis catalyzed by *E. coli* penicillin amidase. Yield increase do to substrate recycling. Biotechnol Lett 1985;12:877–82.