Biotechnology and Bioprocess Engineering 17: 711-721 (2012)
DOI 10.1007/s12257-011-0674-6

RESEARCH PAPER

Comparative Study of the Enzymatic Synthesis of Cephalexin at High Substrate Concentration in Aqueous and Organic Media Using Statistical Model

Carola Bahamondes, Lorena Wilson, Carolina Aguirre, and Andrés Illanes

Received: 21 December 2011 / Revised: 20 March 2012 / Accepted: 21 March 2012

Abstract Synthesis of cephalexin with immobilized penicillin acylase at high substrates concentration at an acyl donor to nucleophile molar ratio of 3 was comparatively evaluated in aqueous and ethylene glycol media using a statistical model. Variables under study were temperature, pH and enzyme to substrate ratio and their effects were evaluated on cephalexin yield, ratio of initial rates of cephalexin synthesis to phenylglycine methyl ester hydrolysis, volumetric and specific productivity of cephalexin synthesis, that were used as response parameters. Results obtained in both reaction media were modeled using surface of response methodology and optimal operation conditions were determined in terms of an objective function based on the above parameters. At very high substrates concentrations the use of organic co-solvents was not required to attain high yields and actually almost stoichiometric yields were obtained in a fully aqueous media with the advantages of higher productivities than in an organic co-solvent media and compliance with the principles of green chemistry.

Keywords: penicillin acylase, cephalexin, immobilized enzyme, β-lactam antibiotics

Carola Bahamondes, Lorena Wilson, Andrés Illanes*
School of Biochemical Engineering, Pontificia Universidad Católica de Valparaíso, Valparaíso, Chile
Tel: +56-32-2273-642; Fax: +56-32-2273-803
E-mail: aillanes@ucv.cl

Carolina Aguirre
Faculty of Sciences, Universidad Católica de la Ssma. Concepción Avenida Brasil 2147, Valparaíso, Chile

1. Introduction

The most important products of biotechnology are antibiotics, β-lactam antibiotics being the most outstanding [1,2]. In fact, a significant portion of the antibiotic market is shared by β-lactam antibiotics, particularly semi-synthetic penicillins and cephalosporins, with annual sales estimates of 15 billion US dollars and a production volume of 37,000 tons per year, representing more than 60% of total antibiotic sales [3]. Chemical synthesis requires many protection and deprotection steps and harsh conditions involving environmentally offensive chemicals [4]. Enzyme biocatalysis has slowly but steadily displaced chemical synthesis not only because of economic considerations but also for better compliance with the principles of green chemistry. In this way, the amount of waste produced per kilogram of product is severely reduced and effluent contaminants are far less hazardous [5]. Penicillin acylase (penicillin amidohydrolase, E.C.3.5.1.11) is massively used in the production of β-lactam nuclei by hydrolytic cleavage of the corresponding β-lactam antibiotics; however, the same enzyme, under appropriate conditions, can catalyze the reverse reaction of synthesis leading to the production of semi-synthetic β-lactam antibiotics [6,7] and enantiopure amino acids [8]. Penicillin acylases are produced by several bacteria, fungi and yeast, but the ones most used are from *Escherichia coli* and *Bacillus megaterium*. Efficient biocatalyst use is mandatory so the enzyme is used in an immobilized form. A plethora of strategies have been developed for penicillin acylase immobilization which reduces downstream operations and excludes allergenic macromolecules from the product [9]. New strategies for penicillin acylase immobilization are still emerging, aiming to produce robust and efficient biocatalysts [10-16]. Synthesis of β-lactam antibiotics with

penicillin acylase can be produced under thermodynamic [17] or kinetic control [18]. The latter requires activated acyl donors but has the advantage that yield is not constrained by the equilibrium conversion so that in practice very high yields, close to 100%, are attainable [19]. Kinetically controlled synthesis (KCS) is a more complex strategy since the enzyme catalyzes not only the formation of the product but also the hydrolysis of the product synthesized and also the hydrolysis of the activated acyl donor. As a consequence a maximum yield is obtained at a certain reaction time, with hydrolytic reactions prevailing beyond that point. Reaction conditions for KCS of β-lactam antibiotics have been optimized in terms of pH [20,21], ionic strength [22], temperature [23,24], acyl donor to nucleophile molar ratio [23] and medium composition with regard to organic cosolvents [25] that may improve synthesis by increasing the synthetase/amidase ratio (s/h1) [22]. This has been proven for the synthesis of cephaloglycine in methanol [18] and ampicillin and cephalexin in ethylene glycol [25]. It is believed that a structural change in the enzyme will have a stronger effect on amidase than on synthetase activity since the mechanism of amide hydrolysis is more complex [26,27]. An organic solvent may also increase substrate solubility, alter the pK values of the reacting species favoring the reactive non-ionized forms and favor synthesis by reducing water activity [4]. Synthesis at very high substrates concentrations has been proven beneficial in the case of ampicillin, amoxicillin and cephalexin [28-33]. However, synthesis at very high substrates concentrations is limited by solubility which varies significantly with pH and temperature, having a strong impact on the conversion of the limiting substrate (usually the nucleophile). Youshko *et al*. [31] studied the nucleophile reactivity with respect to the ratio of synthesis of product to hydrolysis of acyl donor rates (Vs/Vh) in the synthesis of cephalexin and observed higher yields in initially homogeneous than heterogeneous systems where the initial precipitation of the nucleophile reduced its availability for the acyl transfer reaction, so reducing Vs/Vh. Synthesis of β-lactam antibiotics has been also conducted in a one-pot system from the corresponding primary β-lactam antibiotics [34,35] and also in two-phase systems to increase yield and productivity [36].

A comparative study of the synthesis of cephalexin at very high substrates concentrations in fully aqueous and ethylene glycol containing media with a commercial *E. coli* immobilized penicillin acylase is presented. Variables under study are pH, temperature and enzyme to limiting substrate (nucleophile) ratio at an acyl donor to nucleophile molar ratio of 3, while response parameters are product yield, volumetric and specific productivity and Vs/Vh.

2. Materials and Methods

2.1. Materials

Polymethacrylate immobilized penicillin G acylase from *E.coli* covalently bound through epoxy groups with 232 IU/g was kindly supplied by Dalas Biotech Limited (India). (R)-(-)-2-phenylglycine methyl ester hydrochloride (97% pure) (PGME) and cephalexin hydrate were from Sigma (St. Louis, MO, USA); (R)-(-)-2-phenylglycine (PG) was from Aldrich (Milwaukee, WI, USA); 7-amino 3-desacetoxycephalosporanic acid (7-ADCA) was kindly donated by Antibióticos S.A. (León, Spain); penicillin G potassium salt was kindly donated by Natsus S.A. (Lima, Peru). All other reagents were analytical grade from Sigma-Aldrich or Merck (Darmstadt, Germany).

2.2. Analyses

Enzyme activity was determined as the initial rate of penicillin G hydrolysis by measuring NaOH consumption in a pH-stat (Mettler Toledo, DL50), as previously described [32]. One international unit of penicillin acylase activity was defined as the amount of enzyme that hydrolyzes 1 μmol of penicillin G per minute in a reaction mixture containing 10 mM penicillin G solution in 0.1 M phosphate buffer at pH 7.8 and 30ºC. Substrates and products of synthesis were identified and analyzed by HPLC in a Shimadzu LC-10AS delivery system with SPD-10AV UV-Vis detector and a Shimadzu HPLC/PC integrator, equipped with a C18 μ-Bondapack column (300×3.9 mm) from Waters (Milford, MA, USA). Samples were gradient eluted with a sonicated mixture of methanol and 10 mM phosphate buffer pH 7.0 (from 20 to 55% v/v methanol during 17 minutes) at a flow-rate of 1 mL/min and the elution profile monitored at 220 nm. Elution times were 2.4, 3.3, 5.0, and 7.5 min for 7-ADCA, PG, cephalexin and PGME, respectively. Concentrations of substrates and products of reaction were calculated from calibration curves using stock solutions of pure compounds. HPLC samples were analyzed in triplicate and the residual standard deviation of monitored compounds never exceeded 3%.

2.3. Synthesis of cephalexin at high substrates concentrations

Cephalexin synthesis was conducted batch-wise under temperature and pH control in a 30 mL working volume mechanically stirred (flat blade impeller) reactor equipped with a bottom stainless steel filter for biocatalyst recovery. Reactor operated at 200 rpm to keep the biocatalyst particles in suspension. Eight samples were taken during the first 20 min of reaction to determine initial rates. Then samples were taken at five minute intervals during synthesis.

At very high substrates concentrations the system is heterogeneous and initially some of the substrate and later on the product are insoluble. In such cases, samples containing the suspension were diluted prior to analysis in order to solubilize the compounds and the biocatalyst particles filtered out (biocatalyst volume was insignificant).

Experiments of cephalexin synthesis were designed and analyzed using the MODDE 4.0 software package from Ůmetri AB (Uppsala, Sweden). Initial concentrations of substrates were 167 mM 7-ADCA and 500 mM PGME in all experiments. The ranges of variables were: temperature, 10 ~ 20ºC; pH, 6.5 ~ 7.5; enzyme to limiting substrate ratio (E/S), 31.25 ~ 125 IU/mmol of 7-ADCA, as suggested by previous studies [21,23,28,29,32,33,37]. In the case of the organic medium, 30% ethylene glycol (v/v) was used [38-40). For the experimental design three variables (temperature, pH and E/S) and four response parameters (product yield, volumetric and specific productivity and Vs/Vh) were considered. A composite centered face (CCF) experimental design was chosen considering 17 experiments including three central points. The central point for E/S was changed from 78.125 (design value) to 62.5 (experimental value) to compare those results with others obtained at such condition with other immobilized penicillin acylases [32,38]. Values obtained were modeled with a PLS polynomial (partial least squares) second order equation to yield a surface of response. Two additional variable combinations were included in the model: one at pH 7.0, 15ºC and 125 IU/mmol and other at pH 6.5, 15ºC and 62.5 IU/mmol 7-ADCA to further validate the model and reduce the experimental error. In the case of synthesis in a fully aqueous medium, a simplified design was used considering only two variables: temperature and E/S. pH was not considered a variable since the best value obtained in the ethylene glycol containing medium was always 7.5 regardless of the values of the other variables. However, to validate this as the best pH for synthesis in aqueous medium, three additional experiments were conducted at pH 6.5, 7.0, and 7.5 at 12ºC and 62.5 IU/mmol 7-ADCA. The same experimental design as in ethylene glycol medium was used in a total of 11 experiments (factorial design with three levels and two central point replicas). All experiments were duplicated and samples assayed in triplicate with errors lower than 5% in all cases.

3. Results and Discussion

3.1. Synthesis of cephalexin in organic medium

Experimental design and results obtained are shown in Table 1. To further analyze the nature of the effects on the response parameters, models were used to generate surfaces of response and make predictions for operating conditions not evaluated experimentally as will be analyzed ahead.

Table 1. Experimental design and results obtained in the synthesis of cephalexin with immobilized penicillin acylase at 30% v/v ethylene glycol, 167 mM 7-ADCA, and 500 mM PGME

Exp. Nr	pH	Temp (ºC)	E/S IU/mmol	Y %	Vs mM/min	Vh mM/min	Vs/Vh	VP mM/h	SP mM/h*g
1	7.5	20	125	93	11.8	2.60	4.54	206.17	115.83
2	7.5	10	125	88	6.08	0.80	7.60	146.96	82.56
3	7	15	125	96	5.15	0.65	7.92	87.34	49.07
4	7	15	125	91	5.27	0.63	8.37	91.43	51.37
5	7	10	125	90	3.70	0.50	7.40	52.83	29.68
6	6.5	10	125	90	2.01	0.27	7.44	37.43	21.03
7	7.5	15	62.5	95	3.80	0.68	5.59	126.70	142.36
8	7	20	62.5	97	4.29	1.37	3.13	58.91	66.19
9	7	15	62.5	97	3.86	0.61	6.33	74.59	83.81
10	7	15	62.5	95	3.60	0.58	6.2	79.3	89.1
11	7	15	62.5	97	3.75	-	-	75.8	85.2
12	7	10	62.5	99	1.53	0.54	2.83	58.20	65.40
13	6.5	15	62.5	92	0.91	0.25	3.64	30.73	34.53
14	6.5	15	62.5	92	0.91	0.27	3.52	30.73	34.62
15	7.5	20	31.25	82	0.53	0.16	3.31	22.90	51.46
16	7.5	10	31.25	88	1.26	0.35	3.5	57.8	130
17	7	15	31.25	87	0.85	0.21	4.01	16.9	38
18	6.5	20	31.25	80	0.44	0.15	2.93	16.36	36.76
19	6.5	10	31.25	83	0.40	0.15	2.6	10.35	23.2

Vs/Vh: ratio of initial rates of cephalexin synthesis and PGME hydrolysis; Y: cephalexin yield; VP: volumetric productivity of cephalexin synthesis; SP: specific productivity of cephalexin synthesis.

The statistical method PLS was used to estimate the regression coefficients corresponding to each term of the functions representing the models for cephalexin yield (Y), volumetric productivity (VP), specific productivity (SP) and Vs/Vh. Satisfactory models with a 95% confidence were obtained and are described by equations 1 to 4:

$$Vs/Vh = 6.488 - 0.280*T + 0.156*pH + 1.265*(E/S) - 1.206*T^2 - 0{,}163*pH^2 - 0.171\ (E/S)^2 + 0.063*T*pH - 0.460*T*E/S + 0.066*pH*E/S \quad (1)$$

$$Y = 99.382 - 0.939*T - 0.054*pH + 2.831*(E/S) - 0.034*T^2 - 0.987*pH^2 - 7.048\ (E/S)^2 + 0.517*(T*pH) + 0.810*(T*E/S) + 0.822*(pH*E/S) \quad (2)$$

$$VP = 98.943 + 4.901*T + 34.52*pH + 37.013*E/S - 6.668*T^2 + 13.871*pH^2 - 28.050*\ (E/S)^2 + 1.274*T*pH + 11.643*T*E/S + 16.442*pH*E/S \quad (3)$$

$$SP = 95.569 - 0.761*T + 29.854*pH + 3.031*E/S - 6.310*T^2 + 13.336*pH^2 - 31.680\ (E/S)^2 - 4.318*T*pH + 13.918*T*E/S - 1.005*pH*E/S \quad (4)$$

The most significant effects of each response parameter correspond to the variables with the higher coefficients (positive or negative) in the corresponding equation.

From the statistical analysis of data, values of Q^2 and R^2 gave a confidence level of 95%, meaning that the models and predictions made from them are trustworthy. Q^2 values were always higher than 70%, meaning that the model adequately predicts responses for new experimental conditions within the range of values considered for the variables. R^2 values were in all cases close to 1, meaning a low variability in the responses explained by the model. According to the ANOVA tests for each response parameter (not shown), the Fischer regression of distribution was in all cases significantly higher than the F value of the lack of fit, which supports the confidence of the models. Cephalexin yields were in all cases higher than 80% and the most significant variable was E/S, lower values of Y being obtained at the lower E/S value of 31.25 IU/mmol 7-ADCA.

3.2. Synthesis of cephalexin in aqueous medium

Cephalexin yield in the ethylene glycol medium was always higher than 80% within the pH range studied and the optimum obtained at apparent pH 7.5. Then, pH was ruled out as a variable for the synthesis in aqueous medium, its value being fixed at 7.5. The variables under study were only temperature and E/S. The experimental design and results obtained are shown in Table 2. The data in Table 2 were modeled according to the statistical PLS polynomial method. Experiment Nr 1 was excluded to obtain a more representative model of the experimental data. Experiment Nr 10 was conducted at 12ºC instead of 15ºC in order to compare with the optimum obtained in organic medium (see Table 3). Values for regression coefficients Q^2 and R^2 close to 1 were obtained at a level of confidence of 95%, so that the model correlates very well with the experimental data. From the data on Y, VP, SP, and Vs/Vh, equations 5 to 8 were obtained for each response parameter:

$$Vs/Vh = 5.851 + 0.036T + 1.887*E/S - 0.09*T^2 - 1.828*(E/S)^2 - 0.205*T*E/S \quad (5)$$

$$Y = 99.098 - 0.263*T + 4.379*E/S - 1.556*T^2 - 3.057*(E/S)^2 + 0.208*T*E/S \quad (6)$$

$$VP = 201.09 + 8.377*T + 87.709*E/S - 7.654*T^2 - 23.783*(E/S)^2 - 4.348*T*E/S \quad (7)$$

Table 2. Experimental design and results obtained in the synthesis of cephalexin with immobilized penicillin acylase in aqueous medium at 167 mM 7-ADCA and 500 mM PGME

Experiment Nr	Temp ºC	E/S IU/mmol	Y %	Vs mM/min	Vh mM/min	Vs/Vh	VP mM/h	SP mM/h*g
1	20	125	0.98	18.4	5.8	3.17	327.32	183.89
2	20	62.5	0.94	7.65	1.4	5.46	188.38	211.66
3	20	31.25	0.88	3.95	1.65	2.39	92.82	208.58
4	15	125	0.98	11.09	3.62	3.06	280.56	157.62
5	15	62.5	0.97	6.95	1.22	5.70	194.39	218.41
6	15	31.25	0.91	3.5	1.7	2.06	86.84	195.15
7	10	125	0.99	6.96	1.8	3.87	283.42	159.23
8	10	62.5	0.98	5.57	1.03	5.41	178.54	200.60
9	10	31.25	0.9	3.01	1.68	1.79	78.42	176.22
10	12	62.5	1.0	6.29	1.09	5.77	200.4	225.2
11	15	62.5	0.99	7.2	1.28	5.63	180.36	202.65

Vs/Vh: ratio of initial rates of cephalexin synthesis and PGME hydrolysis; Y: cephalexin yield; VP: volumetric productivity of cephalexin synthesis; SP: specific productivity of cephalexin synthesis.

Table 3. Comparison of values of response parameters of cephalexin synthesis predicted by a model based on an objective function (W_{PS} = 0.7 in ethylene glycol medium and 0.8 in fully aqueous medium, and other W_i = 0.1) and experimental results obtained at such conditions

	Y %	Vs/Vh	VP mM/h	SP mM/ h*gr cat
Model (ethylene glycol)	95.6	5.95	127.9	148.2
Model (aqueous)	98.3	5.6	185.5	209.3
Experimental (ethylene glycol)	92	5.47	123.2	138.4
Experimental (aqueous)	100	5.77	211.5	237.9

Experiments were conducted at the optimum conditions: 12ºC, 62.5 IU/ mmol 7-ADCA, and pH 7.5.

$$SP = 213.95 + 1.266*T - 2.966*E/S - 3.246*T^2 - 24.554*(E/S)^2 - 15.181*T*\hat{E}/S \quad (8)$$

The most significant effect of each response parameter corresponds to the variables with the higher coefficients (positive or negative) in the corresponding equations.

ANOVA tests for each response parameter in aqueous medium showed that the Fischer distribution (F) of the regression was in all cases significantly higher than the F value of the lack of fit, which supports the confidence of the models.

3.3. Analysis of the statistical model and comparison between both reaction media for cephalexin synthesis

In order to separately analyze the effect of each variable on the response parameters, the magnitude of each variable was varied to the limiting condition maintaining the values of the other variables in their mean values and the responses were calculated from equations 1-8.

Fig. 1 shows the behavior of the response parameters with respect to pH in the ethylene glycol medium. Both Y and Vs/Vh slightly increased with pH, but the effect on VP and SP was much stronger. This may be attributed to the increase in the rate of cephalexin synthesis with pH at which the neutral reactive species of PGME is augmented so the rate of the limiting step of acyl-enzyme intermediate formation is increased [23,41].

Fig. 2 shows the behavior of each of the response parameters with respect to the other variables both in the ethylene glycol and the fully aqueous medium. Y decreased with temperature over 12ºC in the ethylene glycol medium and over 10ºC in the fully aqueous medium. It has been shown that in the synthesis under kinetic control the hydrolysis rate is more affected by temperature than the rate of synthesis [42], meaning that Vs/Vh is higher at low temperatures. Besides, solubility of cephalexin is severely reduced at low temperatures so that less antibiotic remains in solution to be hydrolyzed by the enzyme; therefore Y is increased.

Y and Vs/Vh are tightly related, the latter representing

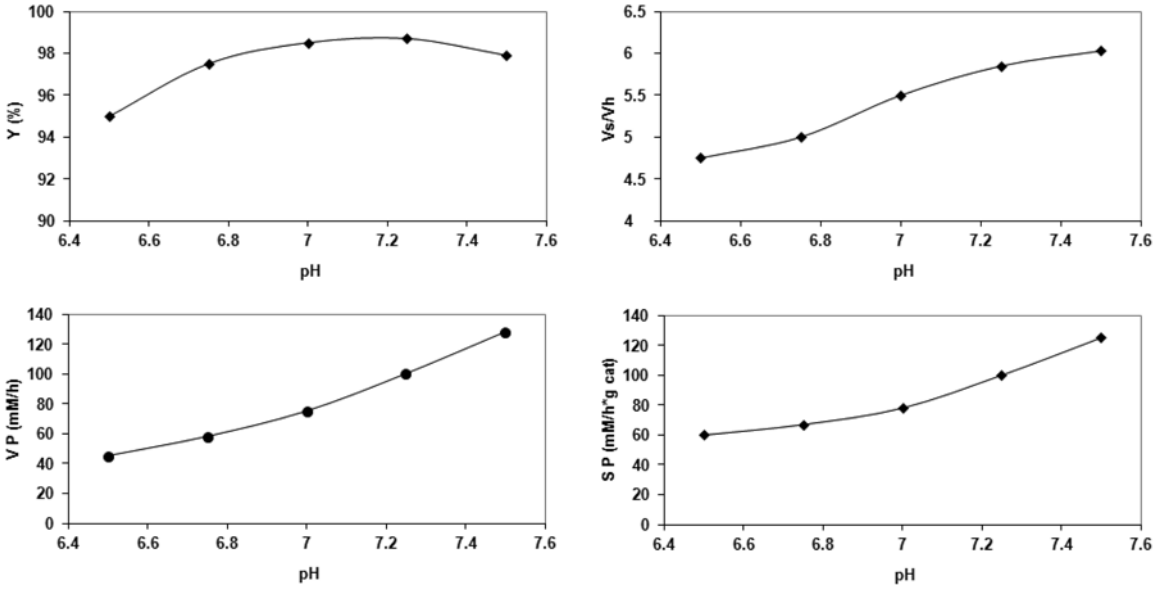

Fig. 1. Effect of response parameters on cephalexin synthesis at 15ºC, 62.5 IU/mmol 7-ADCA, and 30% (v/v) ethylene glycol. Y: cephalexin yield; Vs/Vh: ratio of initial rates of cephalexin synthesis and pH PGME hydrolysis; VP: volumetric productivity of cephalexin; synthesis SP: specific productivity of cephalexin synthesis.

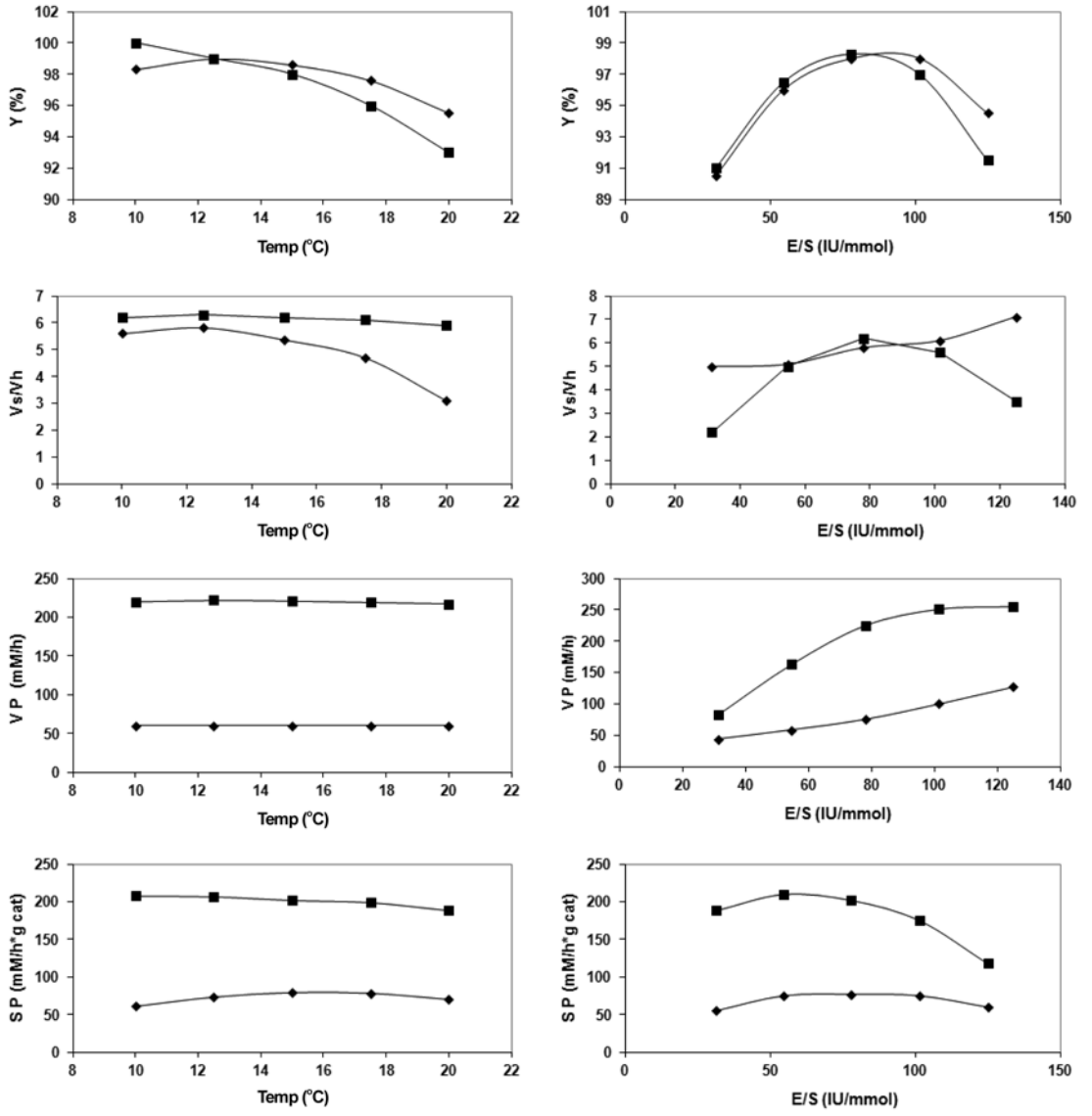

Fig. 2. Effect of temperature and E/S on response parameters of cephalexin synthesis at pH 7.5 in 30% (v/v) ethylene glycol medium (■) and in fully aqueous medium (◆). Y: cephalexin yield; Vs/Vh: ratio of initial rates of cephalexin synthesis and PGME hydrolysis; VP: volumetric productivity of cephalexin; synthesis; SP: specific productivity of cephalexin synthesis.

the amount of β-lactam nucleus available to be attacked by the acyl-enzyme complex to yield the antibiotic [22]. Therefore, Y should increase as Vs/Vh increases. According to Figs. 1 and 2, both response parameters decreased with temperature and increased with pH and E/S within the ranges studied. Vs/Vh increased at low temperatures for the same reason that Y increased: the rate of hydrolysis was more sensitive to temperature than the rate of synthesis. E/S however, produced different effects according to the reaction medium: in the case of ethylene glycol medium, Vs/Vh increased steadily with E/S while in a fully aqueous medium a maximum was observed at an E/S value of about 80 IU/mmol. The difference may be due to the different water activity of the reaction medium and the different solubilities of the compounds in both media. When the amount of enzyme activity is high, at high water activity water acting as nucleophile will produce substantial hydrolysis of the enzyme-acyl complex and also of the activated acyl donor. This effect can be observed by analyzing initial rates at increasing enzyme concentrations in an aqueous medium at a constant temperature (Table 2). Vh increased more than Vs as E/S increased meaning that the enzyme becomes more reactive towards PGME to hydrolyze it than to 7-ADCA to react with the acyl-enzyme complex to yield cephalexin.

VP is determined by the accumulation of cephalexin

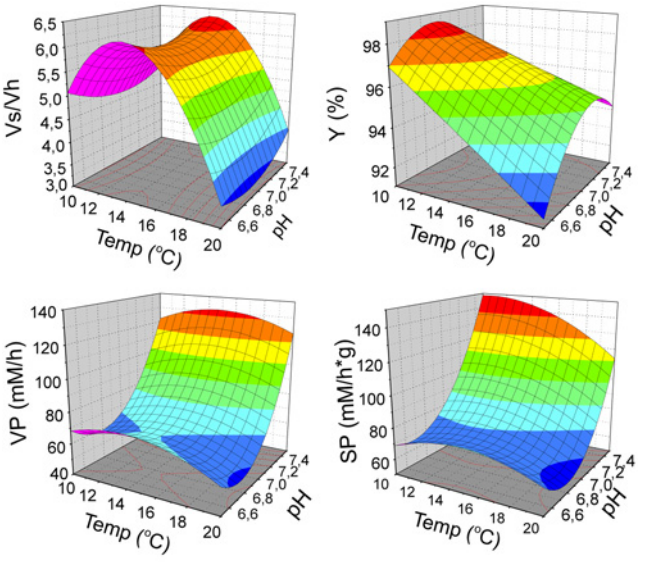

Fig. 3. Surface of response for the synthesis of cephalexin in ethylene glycol medium at varying pH and temperatures at 62.5 IU/mmol of 7-ADCA.

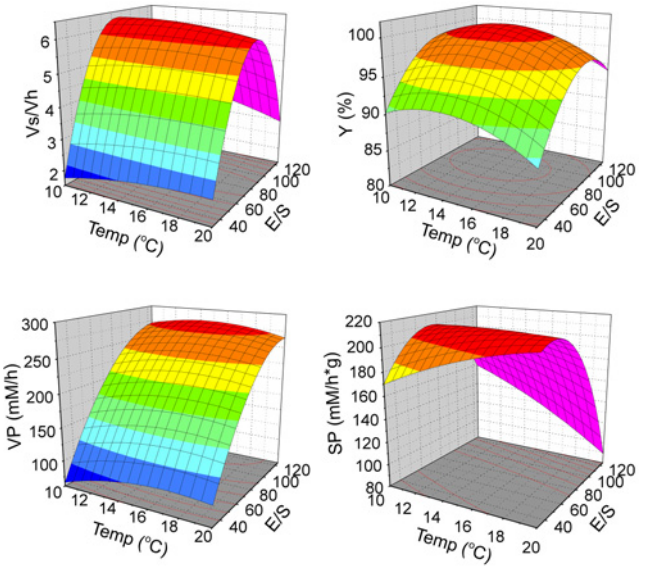

Fig. 4. Surface of response for the synthesis of cephalexin in fully aqueous medium at pH 7.5 and varying temperatures and enzyme to substrate ratio.

over time which results from the balance between its rate of synthesis and hydrolysis. Therefore, a high initial rate of cephalexin synthesis (initially, hydrolysis of cephalexin is insignificant) will produce a higher VP for the same amount of enzyme. VP is significantly affected by pH and E/S. Increasing E/S the initial rate of cephalexin synthesis obviously increased. Temperature had no significant effect on VP presumably due to its opposing effects on the initial reaction rate of cephalexin synthesis and on product solubility.

The effect of the variables on VP and SP is shown in Fig. 2. Both response parameters decreased with temperature. However, VP increased with E/S and the opposite effect was observed with respect to SP. Both VP and SP were higher in the aqueous than in the organic medium as a consequence of the lower initial reaction rates of cephalexin synthesis in ethylene glycol medium.

Each prediction model (Eqs. 1-8) was used to generate a surface of response showing the optimum values for each response parameter. Figs. 3 and 4 show the surface of responses in ethylene glycol and fully aqueous medium respectively at an E/S value of 62.5 IU/mmol 7-ADCA in the whole range of temperature and pH values. However, there is a compromise among the conditions that maximize each response parameter. For instance, the maximum value of 6.4 for Vs/Vh in fully aqueous medium (see Fig. 4) was obtained at 12.5ºC and 84 IU/mmol 7-ADCA, while maximum Y of 100% was obtained at 13.9ºC and 86.4 IU/mmol 7-ADCA; maximum VP of 270.6 mM/h was obtained at 14.4ºC and 105.1 IU/mmol 7-ADCA and maximum SP of 215.2 mM/h*g cat was obtained at 18.8ºC and 50.5 IU/mmol 7-ADCA. Therefore, an objective function weighing these responses should be developed to determine optimum conditions for cephalexin synthesis. Such an objective function is represented by equation 9:

$$Y = W_Y{*}Y + W_{Vs/Vh}{*}Vs/Vh + W_{VP}{*}VP + W_{SP}{*}SP \qquad (9)$$

Where Y, Vs/Vh, VP and SP are the response parameters represented by Eqs. 1-8 and W_i is the relative weight (importance) of each response parameter i, so that:

$$\sum W_i = 1$$
$$0 \le W_i \le 1$$

In order to obtain optimum conditions based on this objective function a linear programming strategy (Nerder Mead Simplex Method) was used and solved using the statistical software MODDE (Ůmetri AB, Sweden). The optimization method proceeds with one criterion at a time and optimal values maximizing the objective function are obtained for each variable according to such criterion. For example, if equal relative weights are assigned to each response variable in the synthesis of cephalexin in ethylene glycol medium (W_i = 0.25), optimal conditions would be 13ºC, pH 7.5 and E/S 102.7 IU/mmol 7-ADCA, at which conditions Y is 97.8%, Vs/Vh is 7.7, VP is 180.7 mM/h, and SP is 139.6 mM/h*g cat. However, not all variables have the same impact so, in order to determine the best reaction conditions in high co-solvent concentration and fully aqueous media, different weights should be assigned to each response parameter or introduce certain restrictions. Enzyme cost is a most important factor in the synthesis of β-lactam antibiotics [33]; therefore, a higher weight was

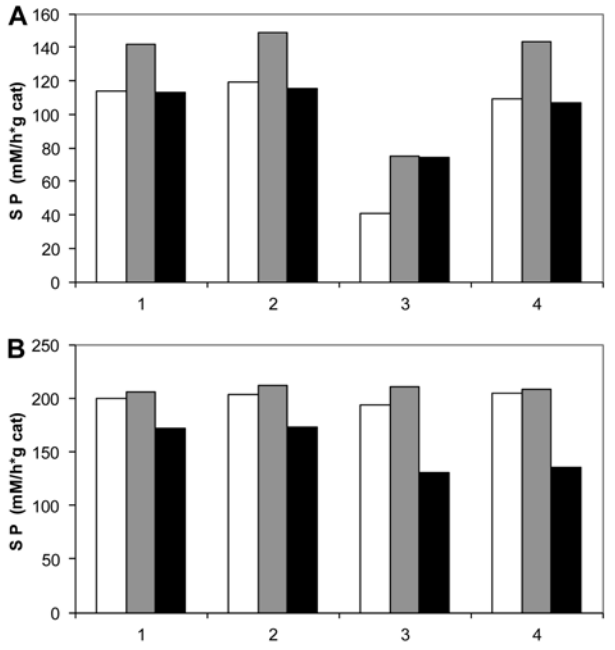

Fig. 5. Optimization of cephalexin synthesis in terms of SP at different E/S values in ethylene glycol medium (A) and in fully aqueous medium (B). 1 corresponds to $W_{VP} = 1$ ($W_i = 0$ for the other variables), 2 corresponds to $W_{SP} = 1$ ($W_i = 0$ for the other variables), 3 corresponds to $W_Y = 1$ ($W_i = 0$ for the other variables), and 4 corresponds to $W_{Vs/Vh} = 1$ ($W_i = 0$ for the other variables).

assigned to SP.

This strategy is based on linear programming using numerical methods like Runge-Kutta, so the result will depend on the initial value selected for iteration. To improve the quality of the result, optimal values of the E/S studied (31.25, 62.5, and 125 IU/mmol 7-ADCA) were first determined by assigning a value of W = 1 to each response parameter separately and considering $W_i = 0$ for the other parameters. For example, assigning $W_{VP} = 1$ and W=0 for all others, optimal conditions are obtained at 31.25 IU/mmol 7-ADCA, 12ºC and pH 7.5, being Y = 84.4%, Vs/Vh = 4.53, and VP = 113.87 mM/h obtained at such condition. Results of this study on the response parameter SP at different E/S values are presented in Fig. 5 for ethylene glycol (A) and fully aqueous medium (B) respectively. As seen, highest SP was obtained at 62.5 IU/mmol 7-ADCA in both media; however in aqueous

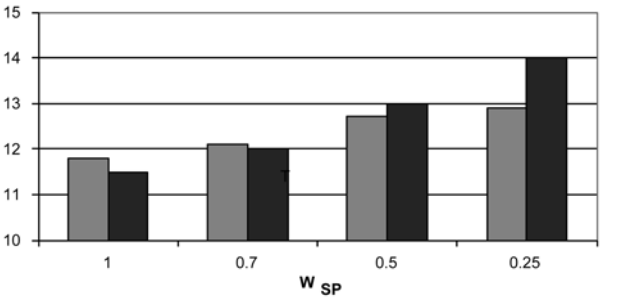

Fig. 6. Determination of the effect of W_{SP} on the optimum temperature for cephalexin synthesis, determined according to objective function (equation 9), at pH 7.5 and 62.5 IU/mmol 7-ADCA in ethylene glycol (E.G.) and fully aqueous media.

medium SP obtained at 31.25 and at 62.5 UI/mmol differed only slightly but the other response parameters were significantly reduced at 31.25 UI/mmol (values not shown). Therefore, at E/S= 62.5 IU/mmol 7-ADCA and $W_{SP} = 0.7$ ($W_i = 0.1$ for each of the other three response parameters) pH and temperature were varied in organic medium and only temperature was varied in aqueous medium. According to this an optimum condition was obtained in both media at 62.5 IU/mmol 7-ADCA, 12ºC and pH 7.5. In all scenarios, best results for SP were obtained at 62.5 IU/mmol 7-ADCA and pH 7.5. However, best temperature is strongly dependent on the weights assigned to W_{SP}. Fig. 6 shows the optimum values obtained at each temperature when different weights were assigned to SP. As seen, optimum temperature decreased with the increase in W_{SP}.

The experimental values obtained at optimal conditions are presented in Table 3, to validate the models. Reported results correspond to the average of two runs, with differences below 3% between replicas. Experimental results obtained in ethylene glycol medium were slightly lower (4-7%) than predicted by the model, except for Vs/Vh where they closely matched. For the synthesis in aqueous medium, experimental results correspond to the average of two reactions (those reported in Table 4 and in experiment Nr 10 in Table 2). Prediction was not so accurate in this case for VP and SP, but it was quite accurate in the case of Y and Vs/Vh (experimental values were only 2-3% higher than prediction). Higher error in the case of VP and SP can be attributed to the fact that these response parameters are

Table 4. Summary of results obtained in the synthesis of cephalexin at different pHs in aqueous medium at 12ºC, 62.5 IU/mmol 7-ADCA, initial substrates concentrations 167 mM 7-ADCA and 500 mM PGME.

pH	Temp ºC	E/S UI/mmol	Y %	Time for Y_{max} min	Vs mM/min	Vh mM/min	Vs/Vh	VP mM/h	SP mM/h*g
6.5	12	62.5	96	125	3.1	0.8	3.88	76.95	86.47
7.0	12	62.5	98	90	3.9	0.95	4.11	109.11	122.60
7.5	12	62.5	100	45	6.29	1.09	5.77	222.67	250.19

considered independent functions, but both are in fact time dependent. Since Y values are very close to 100% and the approach is smooth to that value, there is some uncertainty on the time considered for maximum Y, so that by adjusting this time acceptable predictions can be obtained. The pH was not considered a variable in the model for cephalexin synthesis in aqueous medium, but in order to validate pH 7.5 as the optimum value, three additional experiments were conducted at 12ºC, 62.5 IU/mmol 7-ADCA, at pH 6.5, 7.0, and 7.5. Values higher than pH 7.5 were not considered because of the sharp decrease in FGME solubility. Results are presented in Fig. 7 and summarized in Table 4, validating pH 7.5 as optimum. SP and VP at pH 7.5 were three times higher than at pH 6.5; however, Y was close to 100% for all pHs with only a 4% decrease at pH 6.5 with respect to 7.5. Higher productivities at pH 7.5 are the consequence of the much smaller times required to attain maximum yield.

Synthesis at optimum conditions (12ºC, pH 7.5 and E/S 62.5) in ethylene glycol and aqueous medium are compared in Fig. 8. As can be seen, time to attain maximum yield was lower than in ethylene glycol medium and yield was about 10% higher. PV and PS in aqueous medium almost doubled those obtained in ethylene glycol medium.

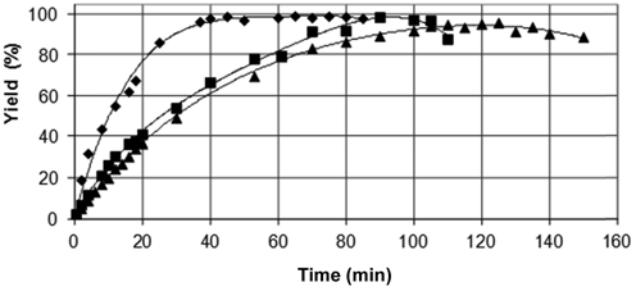

Fig. 7. Synthesis of cephalexin at 12ºC, E/S 62.5 IU/mmol 7-ADCA, initial substrates concentrations 167 mM 7-ADCA and 500 mM PGME. ◆: pH 7.5; ■: pH 7.0; ▲: pH 6.5.

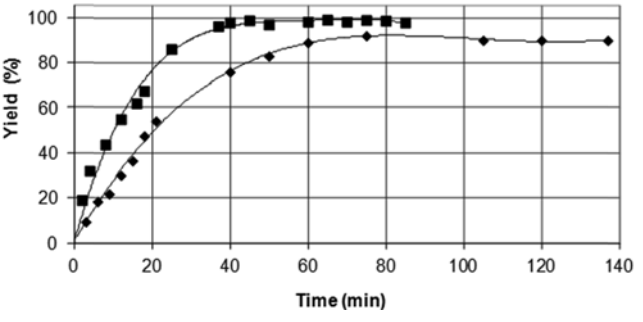

Fig. 8. Comparison of cephalexin synthesis in 30% v/v ethylene glycol (◆) and aqueous medium (■) at optimum conditions (12ºC, pH 7.5, and E/S 62.5 IU/mmol 7-ADCA). Substrates concentrations were 167 and 500 mM for 7-ADCA and PGME respectively.

Experimental results obtained in the synthesis of cephalexin in organic and aqueous media at high substrates concentrations agreed well with those reported by other authors with different penicillin acylases. Schroën *et al.* [43] observed that increasing the temperature of synthesis from 2 to 32ºC in aqueous medium at pH 8 and 100 mM 7-ADCA and FGME, both Y and Vs/Vh decreased. Youshko *et al*. [11,21] studied the effect of pH on Vs/Vh in the synthesis of ampicillin obtaining a maximum at pH 7.0 and a Vs/Vh value close to 10 at 25ºC, 100 mM phenylglycine amide and 2.3 M 6-APA. Yields were in all cases over 80% and close to 100% when working at pH 7.5, being the highest reported for ampicillin synthesis. With respect to VP, values varied between 10 and 327 mM/h in ethylene glycol and in aqueous medium [32,33,38]. Values of PS obtained cannot be properly compared with those reported by other authors because of the different specific activities of the enzyme catalysts used, which are not always reported; however, this is a cost effective process parameter that is mostly important. Li *et al.* [37] studied the synthesis of cephalexin at different E/S values going from 10 to 90 IU/mmol 7-ADCA, working at 150 mM 7-ADCA and 300 mM PGME; best results were obtained at 65 IU/mmol 7-ADCA with a Y of 81% and Vs/Vh of 2.9.

Illanes *et al*. working at very high substrates concentration with other penicillin acylases, concluded that at such conditions results obtained in ethylene glycol and fully aqueous medium are similar [32,38] which is not the case at moderate substrates concentrations where much higher Y are obtained in ethylene glycol medium. The authors concluded that at very high substrates concentrations an organic co-solvent is no longer required to depress the hydrolytic reactions and then aqueous catalysis is a better option both in economic and environmental terms. In the present study with Dalenz PGA higher yields and productivities were obtained at the corresponding optimum conditions for the synthesis in fully aqueous medium as clearly shown in Fig. 5B; time for obtaining maximum yield was reduced from 75 min in ethylene glycol medium to 45 min in fully aqueous medium so productivities were correspondingly higher. Results reaffirm the idea that at very high substrates concentrations the presence of organic co-solvents becomes unnecessary. It is therefore possible to develop a fully aqueous environmentally friendly system for the production of cephalexin with immobilized penicillin acylase obtaining stoichiometric yields and high productivities. Optimum conditions should be determined based on a cost objective function where the response parameters are weighed accordingly. This implies a complete evaluation of the economic impact of these response parameters not only in enzyme reactor operation but also in downstream processing. This study is currently underway.

4. Conclusion

Experimental results of the synthesis of cephalexin at very high substrates concentrations, both in ethylene glycol and fully aqueous media, were satisfactorily fitted to multiple linear regression models describing the effect of each process variable on each of the response parameters considered.

Cephalexin yields were always high, over 85%, both in ethylene glycol and fully aqueous media. The most significant variable with respect to yield was E/S, its increase over 62.5 IU/mmol 7-ADCA being detrimental.

Yield and Vs/Vh responded in the same manner upon changes in the variables under study, while pH was the most significant variable with respect to volumetric and specific productivities, both increasing with the increase in pH within the range studied.

It is not possible to simultaneously optimize yield, productivity and Vs/Vh, so that a cost effective objective function is required for optimization that adequately weighs the impact of these response parameters. As an exercise, optimum conditions were determined to be 12ºC, pH 7.5 and 62.5 UI/mmol for a criterion in which specific productivity was given a higher weight than the other response parameters.

Statistical experimental design proved to be a sound method for determining optimum conditions for cephalexin synthesis at high substrates concentrations generating models that can be evaluated under different criteria of parameter weighing for optimization.

The results of cephalexin synthesis with immobilized penicillin acylase at high substrates concentrations were similar in terms of Y and Vs/Vh in both reaction media, but volumetric productivity and specific productivity were higher in fully aqueous than in ethylene glycol medium, being the former clearly advantageous in economic terms with the additional bonus of complying with the principles of green chemistry.

Acknowledgements

Work funded by Fondecyt Grant 1080122 from Conicyt, Chile.

References

1. Kardos, N. and A. L. Demain (2011) Penicillin: The medicine with the greatest impact on therapeutic outcomes. *Appl. Microbiol. Biotechnol*. 92: 677-687.
2. Deaguero, A. L., J. K. Blum, and A. S. Bommarius (2010) Biocatalytic synthesis of β-lactam antibiotics pp. 1-32. In: M. C. Flickinger (ed.). *Encyclopedia of Industrial Biotechnology: Bioprocess, Bioseparation, and Cell Technology*. John Wiley & Sons Inc. NY, USA.
3. Chandel, A. K., L. V. Rao, M. L. Narasu, and O. V. Singh (2008) The realm of penicillin G acylase in β-lactam antibiotics. *Enz. Microb.Tech.* 42: 199-207.
4. Shaw, S., J. Shyu, Y. Hsieh, and H. Yeh (2000) Enzymatic synthesis of cephalotin by penicillin G acylase. *Enz. Microb.Tech.* 25: 142-151.
5. Wegman, M., M. Janssen, F. van Rantwijk, and R. Sheldon (2001) Towards biocatalytic synthesis of β-lactam antibiotics. *Adv. Synth. Catal.* 343: 558-576.
6. Illanes, A. and L. Wilson (2006) Kinetically-controlled synthesis of β-lactam antibiotics. *Chim. Oggi* 24: 27-30.
7. Shewale, J. V. and K. Sudhakaran (1997) Penicillin V acylase: Its potential the production of 6-APA. *Enz. Microb. Tech.* 20: 402-410.
8. Gong, X., E. Su, P. Wang, and D. Wei (2011) *Alcaligenes faecalis* penicillin G acylase-catalyzed enantioslective acylation of DL-phenylalanine and derivatives in aqueous medium. *J. Mol. Catal. B-Enzym*. 71: 152-158.
9. Shewale, J. and H. Sivaraman (1989) Penicillin acylase: Enzyme production and its application in the manufacture of 6-APA. *Proc. Biochem*. 24: 146-154.
10. Žuža, M. G., B. M. Obradović, and Z. D. Knežević-Jugović (2011) Hydrolysis of penicillin G by penicillin G acylase immobilized on chiotosan microbeads in different reactor systems. *Chem. Eng. Technol.*34: 1706-1714.
11. Bernardino, S., N. Estrela, V. Ochoa-Mendes, P. Fernandes, and L. P. Fonseca (2011) Optimization of the immobilization of penicillin G acylase by entrapment in xerogel particles with magnetic properties. *J. Sol-Gel Sci. Technol.* 58: 545-556.
12. Zhou, C., S. Zhyu, X. Wu, B. Jiang, T. Cen, and S. Shen (2010) Post-immobilizations of modified macromolecular reagent using assembled penicillin acylase for microenvironmental regulation of nanopores and enhancement of enzyme stability. *Biotechnol. Bioproc. Eng.* 15: 376-382.
13. Grazú, V., F. López-Gallego, T. Montes, O. Abián, R. González, J. A. Hermoso, J. L. García, C. Mateo, and J. M. Guisán (2010) Promotion of multipoint covalent immobilization through different regions of genetically modified penicillin G acylase from *E. coli*. *Proc. Biochem*. 45: 390-398.
14. Bernardino, S. M. S. A., P. Fernandes, and L. P. Fonseca (2009) A new biocatalyst: Penicillin G acylase immobilized in sol-gel micro-particles with magnetic properties. *Biotechnol. J.* 4: 695-702.
15. Sun, H., X. Y. Bao, and X. S. Zhao (2009) Immobilization of penicillin G acylase on oxirane-modified mesoporous silicas. *Langmuir* 25: 1807-1812.
16. Pchelintsev, N. A., M. I. Youshko, and V. K. Švedas (2009) Quantitative characteristic of the catalytic properties and microstructure of cross-linked enzyme aggregates of penicillin acylase. *J. Mol. Catal. B-Enzym*. 56: 202-207.
17. Schroën, C. G. P., V. A. Nierstrasz, P. J. Kroon, R. Bosma, A. E. Jenssen, H. H. Beeftink, and J. Tramper (1999) Thermodynamically controlled synthesis of [beta]-lactam antibiotics. Equilibrium concentrations and side-chain properties. *Enz. Microb. Tech.* 24: 498-506.
18. Fernandez-Lafuente, R., C. Rosell, B. Piatkowska, and J. Guisan (1996) Synthesis of antibiotics (cephaloglycin) catalyzed by penicillin G acylase: Evaluation and optimization of different synthetic approaches. *Enz. Microb. Tech.* 19: 9-14.
19. Bruggink, A. (2001) *Synthesis of Beta-Lactam Antibiotics*. pp. 335. Kluwer Academic Publ. Dordrecht, The Netherlands.
20. Ospina, S., E. Barzana, O. T. Ramirez, and A. Lopez-Munguia (1996) Effect of pH in the síntesis of ampicillin by penicillin acylase. *Enz. Microb. Tech.* 19: 462-469.

21. Youshko, M. I., L. M. van Langen, E. de Vroom, F. van Rantwijk, R. A. Sheldon, and V. K Švedas (2002) Penicillin acylase-catalyzed ampicillin synthesis using a pH gradient: A new approach to optimization. *Biotechnol. Bioeng.* 78: 589-593.
22. Kim, M. G. and S. B. Lee (1996) Effect of organic solvents on penicillin acylase-catalyzed reactions: Interaction of organic solvents with enzymes. *J. Mol. Catal. B-Enzym*. 1: 181-190.
23. Illanes, A., Z. Cabrera, L. Wilson, and C. Aguirre (2003) Synthesis of cephalexin in ethylene glycol with glyoxyl-agarose immobilised penicillin acylase: Temperature and pH optimisation. *Proc. Biochem.* 39: 111-117.
24. van Langen, L. M., E. de Vroom, F. van Rantwijk, and R. A. Sheldon (1999) Enzymatic synthesis of beta-lactam antibiotics using penicillin-G acylase in frozen media. *FEBS Lett.* 456: 89-92.
25. Illanes, A., S. Anjarí, R. Arrieta, and C. Aguirre (2002) Optimization of yield in kinetically controlled synthesis of ampicillin with immobilized penicillin acylase in organic media. *Appl. Biochem. Biotechnol*. 97: 165-179.
26. Kim, M. G. and S. Bok (1996) Penicillin acylase-catalyzed synthesis of beta-lactam antibiotics in water-metanol mixtures: Effect of cosolvent content and chemical nature of substrate on reaction rates and yields. *J. Mol. Catal. B-Enzym*. 1: 201-211.
27. Kasche, V. V. (1985) Ampicillin and cephalexin synthesis catalyzed by *E. coli* penicillin amidase. Yield increase due to substrate recycling. *Biotechnol. Lett.* 7: 877-882.
28. Youshko M. I., H. M. Moody, A. L. Bukhanov, W. H. Boosten, and V. K. Švedas (2004) Penicillin acylase-catalyzed synthesis of beta-lactam antibiotics in highly condensed aqueous systems: Beneficial impact of kinetic substrate supersaturation. *Biotechnol. Bioeng*. 85: 323-329.
29. Schröen, C. G., V. A Nierstrasz., H. M. Moody, M. J. Hoogschagen, R. Bosma, H. H. Beeftink, A. E. Janssen, and J. Tramper (2001) Modeling of enzymatic kinetic synthesis of cephalexin-influence of substrate concentration and temperature. *Biotechnol. Bioeng.* 73: 171-178.
30. Youshko, M. I., L. M. van Langen, E. de Vroom, van Rantwijk, R. A. Sheldon, and V. K. Švedas (2001) Highly efficient synthesis of ampicillin in an "aqueous solution-precipitate" system: Repetitive addition of substrates in a semicontinuous process. *Biotechnol. Bioeng*. 73: 426-430
31. Youshko, M. I., L. M. van Langen, E. de Vroom, H. M. Moody, F. van Rantwijk, R. A. Sheldon, and V. K. Švedas (2000) Penicillin acylase-catalyzed synthesis of ampicillin in "aqueous solution–precipitate" systems. High substrate concentration and supersaturation effect. *J. Mol. Catal. B-Enzym*. 10: 509-515.
32. Illanes, A., L. Wilson, O. Corrotea, L. Tavernini, F. Zamorano, and C. Aguirre (2007) Synthesis of cephalexin with immobilized penicillin acylase at very high substrate concentrations in fully aqueous medium. *J. Mol. Catal. B-Enzym*. 47: 72-78.
33. Illanes, A., L. Wilson, and C. Aguirre (2008) Synthesis of cephalexin in aqueous medium with carrier-bound and carrier-free penicillin acylase biocatalysts. *Appl. Biochem. Biotechnol.* 157: 98-110.
34. Zhang, Y. W., R. J. Liu, and X. M. Xu (2010) One-pot two-step enzymatic synthesis of amoxicillin by complexing with Zn^{2+}. *Appl. Microbiol. Biotechnol.* 88: 49-55.
35. Du, L. L., Q. Wu, C. X. Chen, B. K. Liu, and X. F. Lin (2009) A two-step, one-pot enzymatic synthesis of ampicillin from penicillin G potassium salt. *J. Mol. Catal. B-Enzym*. 58: 208-211.
36. Aguirre, C., I. Concha, J. Vergara, R. Riveros, and A. Illanes (2010) Partition and substrate concentration effect in the enzymatic synthesis of cephalexin in aqueous two-phase systems. *Proc. Biochem.* 45: 1163-1167.
37. Li, D., Y. Zhang, S. Cheng, Q. Gao, and D. Wei (2008) Enhanced enzymatic production of cephalexin at high substrate concentration with *in situ* product removal by complexation. *Food Technol. Biotech*. 4: 461-466.
38. Illanes, A., L. Wilson, C. Altamirano, Z. Cabrera, L. Alvarez, and C. Aguirre (2007) Production of cephalexin in organic medium at high substrate concentrations with CLEA of penicillin acylase and PGA-450. *Enz. Microb.Tech.* 40: 195-203.
39. Illanes, A., C. Altamirano, M. Fuentes, F. Zamorano, and C. Aguirre (2005) Synthesis of cephalexin in organic medium at high substrate concentrations and low enzyme to substrate ratio. *J. Mol. Catal. B-Enzym*. 35: 45-51.
40. Illanes, A., S. Anjarí, C. Altamirano, and C. Aguirre (2004) Optimization of cephalexin synthesis with immobilized penicillin acylase in ethylene glycol medium at low temperatures. *J. Mol. Catal. B-Enzym*. 30: 95-103.
41. Aguirre, C., M. Toledo, V. Medina, and A. Illanes (2002) Effect of cosolvent and pH on the kinetically controlled synthesis of cephalexin with immobilized penicillin acylase. *Proc. Biochem.* 38: 351-360.
42. Aguirre, C., P. Opazo, M. Venegas, R. Riveros, and A. Illanes (2006) Low temperature effect on production of ampicillin and cephalexin in ethylene glycol medium with immobilized penicillin acylase. *Proc. Biochem.* 41: 1924-1931.
43. Schroën, C. G. P. H., V. A. Nierstraz, R. Bosma, P. J. Kroon, P. S. Tjeerdsma, E. de Vroom, J. M. van der Laan, H. M. Moody, H. H. Beeftink, A. E. M. Janssen, and J. Tramper (2002) Integrated reactor concepts for the enzymatic kinetic synthesis of cephalexin. *Biotechnol. Bioeng*. 80: 144-155.