

Short communication

Partition and substrate concentration effect in the enzymatic synthesis of cephalixin in aqueous two-phase systems

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ABSTRACT

The kinetically controlled synthesis of cephalixin in aqueous two-phase systems was studied, using immobilized penicillin acylase, 7-amino 3-desacetoxycephalosporanic acid as nucleophile and phenylglycine methyl ester as acyl donor. The organic phases used were 80% (v/v) polyethyleneglycol 400 and 600 and the aqueous phase was 2.5 M $(\text{NH}_4)_2\text{SO}_4$. 7-amino 3-desacetoxycephalosporanic acid and cephalixin partition coefficients were determined at pH 7.4 and 7.8, at 14 °C and 20 °C. Highest partition coefficient for cephalixin was obtained for polyethyleneglycol 400– $(\text{NH}_4)_2\text{SO}_4$ at pH 7.4 and 20 °C, while the lowest partition coefficient for 7-amino desacetoxycephalosporanic acid was obtained in the same system at pH 7.8 and 14 °C. No significant effect of pH was observed on conversion yield and productivity of cephalixin synthesis; however, higher values were obtained with polyethyleneglycol 400 as organic phase. Higher conversion yields with both biphasic systems were obtained at the lowest temperature, where product hydrolysis was lower; volumetric productivity was higher for the fully aqueous medium (control), being higher at 20 °C. All parameters of synthesis were improved at higher substrates concentrations, obtaining conversion yields of 78.2% and 65.4%, with 60 mM 7-amino desacetoxycephalosporanic acid for the polyethyleneglycol 400– $(\text{NH}_4)_2\text{SO}_4$ system and the control, respectively.

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1. Introduction

Substrates and products of enzymatic reactions are usually similar in terms of structure and chemical behavior, which makes their separation after reaction cumbersome. On the other hand, product accumulation may inhibit the enzyme which is a distinctive disadvantage of biocatalysts. To overcome it, extractive bioconversion is a good option since it may reduce product inhibition by in situ removal from the vicinity of the biocatalyst while keeping the substrate in contact with it. Liquid–liquid extraction is a unit operation based on the difference in partition coefficients among the substances to be separated. Aqueous two-phase systems (ATPS) is a liquid–liquid extraction in which two phases are formed, being both aqueous but containing dissolved incompatible polymers or a polymer and a salt. ATPS have a low interfacial tension between phases and mass transfer can be easily attained by agitation. ATPS have been applied to the enzymatic synthesis of β -lactam antibiotics [1–5] and oligosaccharides [6–8], and in protein purification [9–14].

Enzymatic synthesis of cephalixin under kinetic control has some drawbacks, like the requirement of a significant excess of acyl donor [5,15,16], phenylglycine methyl ester (PGME) or phenylglycine amide [17], and product decrease after reaching maximum conversion because of the prevalence of hydrolysis over synthesis [18–22]. On the other hand, separation of cephalixin is cumbersome because it has similar properties than 7-ADCA and copious amounts of poorly soluble phenylglycine are produced that contaminate the product. The purpose of this work is the evaluation of ATPS systems composed by polyethyleneglycol of low molecular weight (PEG 400 and 600) and $(\text{NH}_4)_2\text{SO}_4$ at different pHs and temperatures as reaction media for the synthesis of cephalixin with immobilized penicillin acylase as a way of improving conversion yield and/or volumetric productivity and specific productivity with respect to conventional one-phase systems.

2. Materials and methods

2.1. Materials

Spherical particles of around 0.1 mm in diameter of polyacrylamide gel surface bound penicillin acylase (PGA-450) from *Escherichia coli* with $380 \pm 20 \text{ IU}_H/\text{g}$ were obtained from Roche Molecular Biochemicals (Mannheim, Germany). The biocatalyst was stored wet at 5 °C with no loss of activity during the whole working period. Penicillin G potassium salt (PGK) was kindly provided by Sinqusa S.A. (Lima, Perú). 7-Amino 3-desacetoxycephalosporanic acid (7-ADCA), (R)-(–)-2-Phenylglycine methyl ester hydrochloride 97% (PGME), cephalixin hydrate and polyethylene gly-

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col (PEG) 400 and 600 were purchased from Sigma Aldrich Inc. (St. Louis, MO, USA). All other chemicals were either from Sigma Aldrich or Merck (Darmstadt, Germany).

2.2. PEG–salt aqueous two-phase systems

Biphasic systems were formed by PEG 600 or 400 (80%, v/v in water) and aqueous ammonium sulfate 2.5 M, at pH 7.4 and 7.8, and 14 °C and 20 °C.

The biphasic system was prepared in a funnel with each phase saturated in the other, mixing 70 mL of (NH₄)₂SO₄ aqueous solution and 30 mL of organic phase. The system was then agitated and then the phases let to separate. Equal volumes of both saturated solutions, previously incubated at the desired pH and temperature, were then taken to perform the partition experiments and the reactions of synthesis. For the PEG 400–(NH₄)₂SO₄ biphasic system at 25 °C and pH 7.8, salt concentrations at equilibrium were 2.3% (w/w) and 47.1% (w/w) in the organic and aqueous phase, respectively, while the corresponding values for the polymer were 59.7% (w/w) and 6.9% (w/w). (NH₄)₂SO₄ concentration was determined by gravimetry of the sulfate ion, while the polymer concentration was determined by mass balance using the experimental values of density, volume and percentage water (dry basis).

2.3. Determination of the partition coefficients of 7-ADCA and cephalixin

Partition coefficients of 7-amino desacetoxycephalosporanic acid (7-ADCA) and cephalixin in the PEG 600 (PEG 400)/(NH₄)₂SO₄ biphasic systems were determined at the maximum solubility of the compounds at pH 7.4 and 7.8, and at 14 °C and 20 °C. The antibiotic and 7-ADCA are distributed between both phases according to the partition coefficient (K_p), defined as the ratio of compound concentration in the organic phase to compound concentration in the aqueous phase. K_p was determined by adding the amounts corresponding to the maximum solubility of the compound (Table 1) in the biphasic system conformed by equal volumes of both phases saturated in each other. The system was agitated at 250 rpm in a reactor provided with a paddle impeller at constant temperature and pH for 30 min, after which agitation was stopped to allow the complete separation of phases, aliquots being taken to quantify the amount of compounds in each phase. K_p for PGME in 3:1 (mole:mole) mixtures with 7-ADCA was determined under the same conditions described above.

2.4. Analyses

The concentrations of cephalixin, 7-ADCA and PGME in the top and bottom phases were measured in an Agilent HPLC system (USA) with a UV detector (set at 220 nm) and a Kromasil KR 100-5 C18 150 mm × 4.6 mm column. Elution was performed by a gradient of methanol and phosphate buffer (20 mM, pH 7.0) at a flow rate of 1 mL/min at 20 °C. Amounts of reactants and products were calculated from calibration curves using stock solutions of pure compounds.

One international unit of activity (IU_H) was defined as the amount of PGA-450 that hydrolyzes 1 μmole of PGK per minute at 30 °C and pH 7.8 from 134 mM PGK in 0.1 M phosphate buffer.

2.5. Syntheses in aqueous two-phase systems at different substrate concentrations

Cephalixin synthesis was studied in the 80% (v/v) PEG 600 (PEG 400)/2.5 M (NH₄)₂SO₄ biphasic systems, at the same pH and temperatures than the ones used for the determination of partition coefficients. The substrates concentrations were 30 mM and 90 mM for 7-ADCA and PGME, respectively, with an enzyme load of 125 IU_H/mmole 7-ADCA. Control syntheses were run in parallel where a homogeneous aqueous system (phosphate buffer 0.1 M) was used as reaction medium under the same experimental conditions. For the syntheses at different substrate concentrations, PEG 400 was selected and the range of 7-ADCA concentration in the whole system was from 30 mM to 75 mM. Nucleophile to acyl donor molar ratio of 1:3, previously determined as optimum for cephalixin synthesis [23], was employed throughout and 7-ADCA concentrations over and below its solubility limit were studied, as indicated in Table 1.

2.6. Reaction kinetics

Time course of cephalixin synthesis was followed by sampling at intervals. Samples were conveniently diluted in 20 mM phosphate buffer pH 7.0:methanol 70:30 (v/v) mixture prior to assay by HPLC. Synthesis in biphasic systems was performed using equal volumes (15 mL) of each phase previously saturated in one another;

samples from each phase and the whole mixture were taken for analysis. Concentration of substrates and products in the whole system (30 mL) were determined by adding the amounts in each phase and dividing them for the total volume.

Maximum conversion yield (%Y) was determined as the molar percentage of 7-ADCA (the limiting substrate) converted into cephalixin at the point of its highest concentration. Volumetric productivity (P) was calculated as the amount of cephalixin produced per unit time and unit of reaction volume at the point of maximum conversion. Specific productivity (P_{sp}) was calculated as the ratio of volumetric productivity to biocatalyst mass. In biphasic systems these parameters were determined considering the amounts of product in both phases.

3. Results and discussion

3.1. Solubility of 7-ADCA and cephalixin in polymer–salt biphasic systems

The formation of a two-phase system was checked before determining the partition coefficients of substrates and products. The ATPS selected was composed by 80% (v/v) PEG as organic phase and 2.5 M (NH₄)₂SO₄ as aqueous phase. Temperatures higher than 20 °C were not considered based on previous results in homogeneous systems where a sharp decrease in Y was observed over that temperature [24,25].

Maximum solubility for 7-ADCA was obtained for the 80% (v/v) PEG 600–2.5 M (NH₄)₂SO₄ biphasic system at pH 7.8 and 20 °C, while maximum solubility for cephalixin was obtained in the 80% (v/v) PEG 400–2.5 M (NH₄)₂SO₄ biphasic system at pH 7.8 and 20 °C. Solubility of both compounds decreased at lower temperatures in the latter system, while the effect of temperature on solubility was not significant for the former. Increase in pH produced a significant increase in the solubility of 7-ADCA (between 10% and 36%) and cephalixin (between 12% and 40%). However, when using PEG 600, temperature had no significant effect on the solubility of 7-ADCA and cephalixin in the range studied. There was no definite trend of solubility with respect to the type of PEG used: under certain conditions solubility was higher with PEG 600 and the opposite occurred in others, as can be appreciated in Table 1. Solubility of 7-ADCA in both systems increased in the presence of PGME, reaching a maximum value of 60 mM at 14 °C for the 80% (v/v) PEG 400–2.5 M (NH₄)₂SO₄ system, when using a 7-ADCA–PGME molar ratio of 1:3 (60 mM and 180 mM, respectively).

The synthesis of cephalixin should be conducted at the condition of higher solubility of both 7-ADCA and cephalixin, which corresponds to 20 °C. However, it was not possible to distinguish which was the best system between PEG 600–(NH₄)₂SO₄ and PEG 400–(NH₄)₂SO₄. Another aspect to be considered is that 7-ADCA should be preferentially in the aqueous phase to be acted upon by the enzyme, while cephalixin should be preferentially in the organic phase to avoid its hydrolysis.

3.2. Partition of 7-ADCA and cephalixin in biphasic systems

The partition of 7-ADCA and cephalixin was evaluated in biphasic systems conformed by PEG 400 and PEG 600 (80%, v/v) organic phase (top) and 2.5 M (NH₄)₂SO₄ aqueous salt phase (bottom). Experiments were done in triplicate with variations below 3% among them in all cases; reported results are the average of these triplicates. Table 2 shows that the lowest value of K_p for

Table 1
Solubility of 7-ADCA and cephalixin in 80% (v/v) PEG 400 and PEG 600–2.5 M aqueous (NH₄)₂SO₄ systems. All standard deviations of solubility are below 0.5%.

Temperature (°C)	PEG 400				PEG 600			
	7-ADCA (mM)		Cephalixin (mM)		7-ADCA (mM)		Cephalixin (mM)	
	pH 7.4	pH 7.8	pH 7.4	pH 7.8	pH 7.4	pH 7.8	pH 7.4	pH 7.8
14	35	45	35	40	35	50	30	45
20	45	50	40	60	35	55	30	50

Table 2

Partition coefficients (K_p) in biphasic systems composed by 80% (v/v) PEG 400 (or PEG 600)–2.5 M aqueous $(\text{NH}_4)_2\text{SO}_4$. All standard deviations of K_p are below 3%.

Temperature ($^{\circ}\text{C}$)	PEG 400				PEG 600			
	7-ADCA (K_p)		Cephalexin (K_p)		7-ADCA (K_p)		Cephalexin (K_p)	
	pH 7.4	pH 7.8	pH 7.4	pH 7.8	pH 7.4	pH 7.8	pH 7.4	pH 7.8
14	1.34	0.94	6.94	8.80	3.24	3.46	3.10	7.80
20	2.91	1.40	11.56	3.22	1.01	1.19	10.45	3.59

7-ADCA is 0.94, for the PEG 400– $(\text{NH}_4)_2\text{SO}_4$ 2.5 M biphasic system at pH 7.8 and 14°C , which means that its concentration is slightly higher in the aqueous salt phase. On the contrary, the highest K_p for cephalaxin was 11.56 for the same system but at 20°C and pH 7.4, meaning that the product is strongly partitioned to the organic phase. Higher K_p values for cephalaxin are due to its more hydrophobic nature, conferred by the side-chain containing an additional phenyl group linked to the 7-ADCA moiety. For the reactions of synthesis, higher %Y are to be expected since 7-ADCA will be partitioned to the aqueous salt phase where the reaction occurs, while cephalaxin will do it to the organic phase where its hydrolysis can be avoided. Experimental conditions to fulfill that goal are in compromise so that the cephalaxin to 7-ADCA K_p ratio was considered as the objective function to determine the best reaction conditions. The best experimental conditions according to such function were pH 7.8 and 14°C for the PEG 400 biphasic system, where a K_p ratio of 9.36 was obtained, and pH 7.4 and 20°C for the PEG 600 biphasic system, where a K_p ratio of 10.35 was obtained.

Increase in pH had a slight effect on the K_p of 7-ADCA in both systems, but its effect was quite strong for cephalaxin, especially at 20°C . Highest K_p for cephalaxin were obtained in both systems at 20°C and pH 7.4. Higher pH and lower temperature slightly decreased K_p for 7-ADCA in the PEG 400–salt biphasic system, so favoring its partition to the aqueous salt phase. At such conditions a K_p of 8.8 was obtained for cephalaxin meaning a strong partition to the organic phase, being quite adequate for performing the synthesis. K_p values for PGME (when present at a 3:1 molar ratio with respect to 7-ADCA) at the pH, temperature and concentrations studied (from 90 mM to 225 mM) varied between 1.1 and 3.3 for PEG 400 and PEG 600 systems, which means a slightly higher partition to the organic phase which in principle may produce a reduced level of hydrolysis than in a one-phase fully aqueous system.

3.3. Synthesis of cephalaxin

Syntheses were performed in three different reaction media: 80% (v/v) PEG 400–2.5 M $(\text{NH}_4)_2\text{SO}_4$; 80% (v/v) PEG 600–2.5 M $(\text{NH}_4)_2\text{SO}_4$ and 0.1 M phosphate buffer used as control. Experiments were conducted at varying pHs and temperatures with 30 mM 7-ADCA and 90 mM PGME. Experiments were done in duplicate with variations below 5% between them; reported results are the average of these duplicates. Results are summarized in Table 3. Significantly higher values of %Y were obtained with the PEG 400 biphasic system when compared to the PEG 600 system and to the control (except at 20°C). However, productivity in biphasic system was significantly lower than in the control. %Y was not affected significantly by temperature and pH within the range studied. As expected, both P and P_{sp} increased dramatically with temperature in the control experiment, but this effect was not observed in the biphasic systems where actually a small decrease was observed. This different behavior may be the consequence of the higher values of the activation energy observed for the reactions of synthesis and hydrolysis when organic solvents are used [22], producing a decrease in the net reaction rate of synthesis and therefore a decrease in P and P_{sp} . van Langen et al. [25], reported a %Y of 22 in the synthesis of cephalaxin in aqueous medium at 20°C at a reac-

tion time of 0.8 h, while at -20°C in a frozen state %Y of 80 was attained after 46 h; even though reaction rate was severely reduced at such low temperature, %Y was significantly higher, which can be attributed to the selective depression of hydrolysis at lower temperatures [24,26,27].

Despite the reduction in productivity, biphasic systems provided a reaction medium in which product hydrolysis was severely reduced. This is illustrated in Fig. 1 that records the time course of cephalaxin synthesis at 14°C and pH 7.4 and 7.8 for the control and the two biphasic systems. In the aqueous systems, cephalaxin concentration decreased dramatically after reaching %Y maximum, while in biphasic systems decrease was mild to nil. This is clearly attributed to the partition of the product to the organic phase where its hydrolysis is strongly arrested. Additionally, enzyme is likely to be in a more protective environment as a consequence of the lower water activity than in a fully aqueous medium [18,28]. Best results in terms of %Y, P and P_{sp} were obtained with PEG 400 biphasic system so that further experiments were conducted in such reaction medium.

The effect of the initial concentration of 7-ADCA in the synthesis of cephalaxin was then studied. As seen in Table 4, best results in terms of %Y were obtained in the biphasic system at 60 mM 7-ADCA (78.2%) and decreased at higher concentrations. P followed the same pattern than %Y so that a clear optimum was obtained at a nucleophile concentration of 60 mM. At higher concentrations substrates are initially partly insoluble, then, being the enzyme immobilized onto a solid support, mass transfer limitations are likely to be significant, which will explain the decrease in %Y and P observed. On the other hand, the by-product phenylglycine is quite insoluble so that during reaction copious amounts are precipitated which may augment that effect. Higher %Y have been reported in biphasic systems at 100 mM 7-ADCA, but working with free enzymes or at lower PGME:7-ADCA ratios, which further support the explanation above [2,4]. No decrease in %Y was observed in

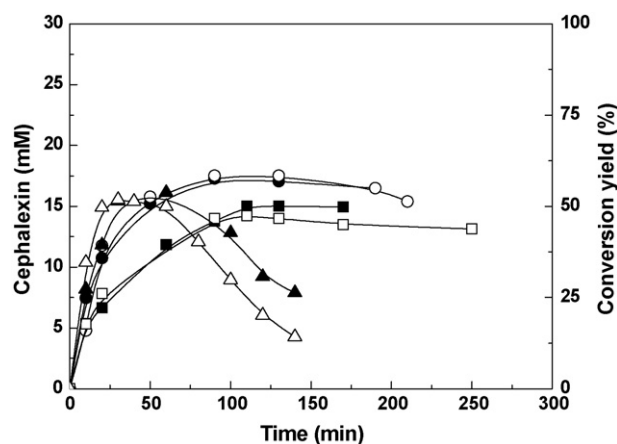


Fig. 1. Synthesis of cephalaxin in biphasic systems and control. PEG 400 (80%, v/v)–2.5 M aqueous $(\text{NH}_4)_2\text{SO}_4$ (circles); PEG 600 (80%, v/v)–2.5 M aqueous $(\text{NH}_4)_2\text{SO}_4$ (squares); control (fully aqueous buffer) (triangles). Open symbols, pH 7.8; closed symbols, pH 7.4. Conditions: 30 mM 7-ADCA; 90 mM PGME; 14°C ; 125 IU_H/mmole 7-ADCA.

Table 3
Effect of pH and temperature on the synthesis of cephalixin in biphasic system 80% (v/v) PEG 400 (or PEG 600)–2.5 M aqueous (NH₄)₂SO₄ and one-phase (control) at 30 mM 7-ADCA, 90 mM PGME and 125 IU_H/mmole 7-ADCA. All standard deviations of P are below 5%.

System	Temperature (°C)	pH	Y (%)	P (mM/h)	P _{sp} (mM/h g)
PEG 400	14	7.4	57.6	11.5	37.2
		7.8	58.4	11.7	37.6
	20	7.4	53.0	9.5	30.7
PEG 600	14	7.4	50.1	8.2	26.5
		7.8	47.3	7.8	25.0
	20	7.4	48.6	7.3	23.5
Control	14	7.4	53.8	16.1	52.0
		7.8	53.9	19.5	62.8
	20	7.4	55.1	50.1	161.4

Table 4
Effect of initial nucleophile concentration in the synthesis of cephalixin in biphasic systems 80% (v/v) PEG 400–2.5 M aqueous (NH₄)₂SO₄ and control at pH 7.8, 14 °C and 125 IU_H/mmole 7-ADCA. All standard deviations of P are below 5%.

System	7-ADCA:PGME (mM:mM)	Y (%)	P (mM/h)	P _{sp} (mM/h g)
PEG 400	30:90	58.37	11.67	37.63
	45:135	66.16	35.87	77.07
	60:180	78.22	46.93	75.62
	75:225	51.85	38.89	50.12
Control	30:90	53.90	19.48	62.80
	45:135	59.62	53.66	115.30
	60:180	66.60	79.92	128.80

the control experiment beyond 60 mM as in the case of the biphasic system; in fact, conversions over 90% have been obtained in one-phase aqueous systems at higher nucleophile concentrations [23,29]. The higher P_{sp} in the biphasic system was also obtained at 60 mM 7-ADCA; however, such value is lower than the one obtained in the control at the same nucleophile concentration.

4. Conclusions

The advantages of biphasic systems are the higher stability of the product synthesized as a consequence of the selective depression of hydrolysis and the more easy recovery of the product from the organic phase without requiring a time-dependent recovery. When compared with conventional one-phase aqueous reaction medium, higher %Y are attainable in a biphasic system at higher soluble concentration substrates. Temperature had no significant effect in the range studied and higher pH is convenient since it increases the solubility of the nucleophile substrate and favors the partition of the product into the organic phase. While productivity in two-phase systems are lower than those obtained with conventional one-phase system, %Y are higher at least at moderate substrate concentrations, but the limitation exists that lower initial substrate concentration can be dissolved in the biphasic systems which precludes from taking advantage of the benefits of working at very high substrates concentrations in one-phase aqueous systems [23].

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