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Synthesis of cephalexin in ethylene glycol with glyoxyl-agarose immobilised penicillin acylase: temperature and pH optimisation

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Abstract

The synthesis of cephalexin with glyoxyl-agarose immobilised penicillin acylase (GAPA) in 50% (v/v) ethylene glycol at 90 mM acyl donor was optimised in terms of pH and temperature, using molar yield as objective function. Yield was substantially increased by the presence of the cosolvent and stability was also higher than in aqueous medium. pH and temperature optima were 7.0 and 28 °C and maximum yield was 96%, substantially higher than previously obtained with a commercial biocatalyst. The operational stability of the biocatalyst was determined as a half-life of 135 h at 28 °C. Synthesis of cephalexin at low temperatures is being studied to determine if the increase in biocatalyst stability will outweigh the longer times and higher enzyme loads required. Optimisation is still to be conducted at higher substrate concentrations. Reduction in acyl donor excess is advisable but it will reduce yield.

Keywords: Penicillin acylase; Cephalexin; β-Lactam antibiotics; Enzymatic synthesis; Organic cosolvent

1. Introduction

Semi-synthetic β-lactam antibiotics are produced mostly by chemical synthesis, despite the drawbacks of low specificity and heavy environmental burden [1]. Replacement of the chemical route for a more specific and environmentally sound biocatalytic process have met with limited success mainly because lower yields are obtained that increase downstream processing cost significantly. Penicillin acylase (penicillin amidohydrolase; E.C. 3.5.1.11) has been used for more than three decades now to produce 6 aminopenicillanic acid (6APA) from the hydrolysis of penicillin G or V [2,3]. It can catalyse also the synthesis of derivatives from the corresponding β-lactam nuclei and suitable acyl donors [4] using both kinetic and thermodynamic approaches [5]. Limited stability of penicillin acylase biocatalysts under process conditions and low product yields have precluded the widespread success of biocatalysis in the synthesis of β-lactam antibiotics. Therefore, a definite input is required in this field to produce more robust biocatalysts and to engineer the reaction medium for improved yields. Novel type biocatalysts have emerged and very promising robust penicillin acylases have been recently produced in tailor-made carriers [6] and as protein aggregates [7,8]. Product yield is a key technological issue in antibiotic production [9]; however, yield in conventional (aqueous) media is limited because reverse or competing hydrolytic reactions will be favoured in a thermodynamically [10] or kinetically [11] controlled synthesis, respectively. Several attempts have been made to increase yield. Precipitation-driven synthesis in predominantly solid systems has been claimed as a good option to attain high yields and has proven to be so in peptide synthesis [12], but not in the case of β-lactam antibiotics [13]. Notwithstanding, higher yields of ampicillin and cephalexin have been obtained with soluble penicillin acylase in frozen aqueous medium [14] and very good yield of ampicillin synthesis was obtained

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keeping saturated substrate concentrations in a fed-batch reactor [15].

Reduced water activity is beneficial since it depresses the competing hydrolytic reactions in the case of kinetically controlled synthesis, and displaces the equilibrium towards synthesis in the case of a thermodynamically controlled strategy. Water-miscible organic cosolvents are suitable media to perform the synthesis of β-lactam antibiotics [16], because they effectively reduce water activity and increase the proportion of reactive non-ionised species [17]. Polyols, and among them ethylene glycol, have proven to be particularly suited [18], being penicillin acylase at least as active and stable and substrates as soluble as in water [19,20]. In fact, significant increase in ampicillin yield has been obtained in ethylene glycol medium with a commercial immobilised penicillin acylase [21].

Multi-point covalent attachment of enzymes to glyoxyl-agarose gels is an efficient system to produce robust biocatalysts that has been used for penicillin acylase [22]. The system can be further improved by creating nano-environments that preclude the contact of the enzyme with harsh bulk environmental conditions [23]. High yields have been obtained in the synthesis of derived cephalosporins at moderate concentrations of cosolvents, like methanol and dimethyl formamide, under high excess of acyl donor [24]. However, yields were significantly lower for derived cephalosporins synthesised at limiting or equimolar acyl donor concentrations and even under moderate excess of acyl donor, both in cosolvent and in fully aqueous media [4,25,26].

This work presents the optimisation of temperature and pH for the synthesis of cephalexin in ethylene glycol medium with glyoxyl-agarose immobilised penicillin acylase (GAPA), using product yield as objective function.

2. Materials and methods

2.1. Materials

Penicillin acylase from *Escherichia coli*, with approximately 300 IU/ml and 25 mg/ml protein, was a product from Antibióticos S.A. (León, Spain) kindly provided by Dr José Manuel Guisán (Instituto de Catálisis, CSIC, Madrid, Spain). The enzyme was centrifuged and dialysed prior to use and has remained fully stable for more than a year stored at 5 °C. Agarose 10 BCL was from Hispanagar (Burgos, Spain). Penicillin G potassium salt (PGK) was kindly provided by Sinquisa S.A. (Lima, Perú); 7amino 3-desacetoxicephalosporanic acid (7ADCA), (*R*)-(−)-2-phenylglycine methyl ester hydrochloride (97% pure) and cephalexin hydrate were from Sigma Chemical Company Inc. (St. Louis, MO, USA). Ethylene glycol, glycidol and all other reagents were analytical grade either from Sigma–Aldrich (St. Louis, MO, USA) or Merck (Darmstadt, Germany).

2.2. Analysis

Substrates and products of enzymic synthesis were analysed by HPLC using a Shimadzu delivery system LC-10AS with a Shimadzu UV SPD-10AV detector and a μ-Bondapack C_{18} column (300 × 3.9 mm) from Waters (Milford, MA, USA). Samples were eluted isocratically with 70% (v/v) 20 mM phosphate buffer pH 6.0 and 30% (v/v) methanol at a flow rate of 1 ml/min, and analysed in a UV detector at 214 nm. Amounts of reactants and products were calculated from calibration curves using stock solutions of pure compounds.

One international unit of activity (IU) was defined as the amount of penicillin acylase that hydrolyses 1 μmol of PGK per minute at 30 °C and pH 7.8 from 134 mM PGK in 0.1 M phosphate buffer.

2.3. Enzyme immobilisation

Glyoxyl-agarose gel was prepared as reported by Guisan [27]. Penicillin acylase was immobilised in glyoxyl-agarose gel, based on the procedure described by Alvaro et al. [28], using phenylacetic acid (PAA) instead of penicillin G sulphoxide as protecting agent during immobilisation. The concentration of PAA was determined to give maximum protection to the enzyme during immobilisation. The glyoxyl agarose immobilised penicillin acylase (GAPA) was stored as a wet gel at 5 °C. No enzyme inactivation or leakage was detected during prolonged storage.

2.4. Synthesis of cephalexin with GAPA

Synthesis was conducted under kinetic control using the methyl ester of phenylglycine (PGME) as acyl donor. Synthesis reactions were performed batch-wise with temperature and pH control at 30 mM 7ADCA, 90 mM PGME, 125 IU/mmol 7ADCA and 50% (v/v) ethylene glycol in 0.1 M phosphate buffer, as suggested from previous studies [18,21]. The amount of GAPA used at different temperatures and pHs was corrected according to the temperature-activity and pH-activity profiles to provide a comparable catalytic potential in each experiment, not to bias productivity. Temperature and (apparent) pH were varied according to the experimental design below. During synthesis, the pH and temperature were monitored and controlled, and samples were taken to analyse product and substrates. Molar yield (Y) was defined as the maximum molar conversion of 7-ADCA into cephalexin. Productivity (P) was determined as the amounts of cephalexin produced per unit time and unit reaction volume at maximum yield (mM/h).

2.5. Experimental design for optimisation

The effects of temperature and pH on the synthesis of cephalexin at the above determined conditions were studied according to a full 2^k factorial design (two factors at two levels) considering also three central points to evaluate the experimental error. The design was further expanded to a circumscribed central composite design, introducing $2k$ additional experiments. A total of eleven experiments were then required for this experimental design, which is shown in Table 1. A software package (MODDE 4.0 for WINDOWS, Umetri, Umeå, Sweden) was employed to fit the second order models for the response (Y) with respect to temperature and pH using multiple linear regression, according to:

$$Y = \beta_0 + \sum \beta_i X_i + \sum \beta_{ii} X_i^2 + \sum \beta_{ij} X_i X_j \quad (1)$$

where β_0 is the offset term, β_i the linear effect, β_{ii} the squared effect, β_{ij} the interaction effect, and X_i and X_j the independent variables or experimental factors (temperature and pH).

The determination coefficient (R^2) is the fraction of variation of the response explained by the model. The prediction coefficient (Q^2) is the fraction of variation of the response that can be predicted by the model. The range of temperature was from 18 to 28 °C and the range of pH was from 6.0 to 7.0. Optimum temperature and pH were experimentally validated.

2.6. Operational stability

The operational stability of the biocatalyst at the optimum conditions for synthesis was determined in repeated production batches. The experiments were carried in 50 ml reactors provided with a filter at the bottom to retain the GAPA. After each batch, the product was removed from the reactor, the catalyst thoroughly washed and the reactor loaded again. The reaction in each batch was conducted until the maximum yield of the first batch was reached.

All experiments were done in duplicate and samples assayed in triplicate. Differences among experiments and samples were determined by statistical analysis, with errors in the range from 1 to 3% at a 95% level of significance.

3. Results

Temperature and pH have been previously determined as the most relevant variables in the synthesis of ampicillin with commercial immobilised penicillin acylase PGA-450 in organic media [18,21]. The effect of cosolvent on Y can be seen in Fig. 1. Y was substantially higher and its decay after maximum was slower in organic than in aqueous medium. The time-course of the syntheses of cephalexin, according to experimental design in Table 1, are in Fig. 2. Results in terms of Y and P are summarised in Table 1. A quadratic model was fitted from the experimental data, considering Y as objective function. After eliminating the non-significant coefficients, the best fit is represented by Eq. (2):

$$Y = 86.99 + 15.62\text{pH} + 5.83T - 16.48\text{pH}^2 - 2.72T^2 - 6.77\text{pH}T \quad (2)$$

where T stands for temperature and variables are in their coded form. ANOVA test indicates that the quadratic model adequately describe yields for cephalexin synthesis, with values for R^2 and Q^2 of 0.999 and 0.994, respectively. A three-dimensional plot corresponding to this model is presented in Fig. 3. Optimum conditions for the synthesis of cephalexin, as predicted from Eq. (2), are shown in Table 2. Model prediction was experimentally validated, as also shown in Table 2. The most significant variable was pH with Y sharply

Table 1
Experimental design and results for the kinetically controlled synthesis of cephalexin in organic cosolvent at varying pH and temperature, at 30 mM 7ADCA, 90 mM PGME, 125 IU GAPA per mmol 7ADCA and 50% (v/v) ethylene glycol

Experiment number	pH	T (°C)	Y (%)	P (mM/h)
1	6.0	18	54.0	2.09
2	7.0	18	71.7	12.3
3	6.0	28	51.1	2.89
4	7.0	28	95.8	14.48
5	6.5	23	86.7	8.73
6	6.5	23	87.2	8.77
7	6.5	23	87.0	8.75
8	5.8	23	31.6	0.88
9	7.2	23	75.8	15.39
10	6.5	15	72.2	3.01
11	6.5	30	90.2	9.66

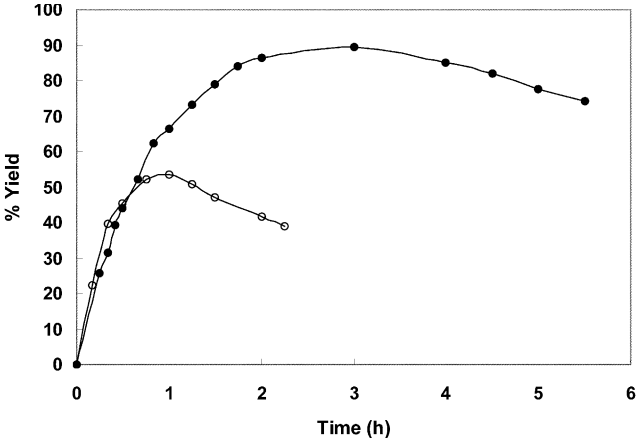

Fig. 1. Time-course of synthesis of cephalexin at pH 6.5 and 28° at 50% (v/v) ethylene glycol (closed symbols) and in phosphate buffer (open symbols).

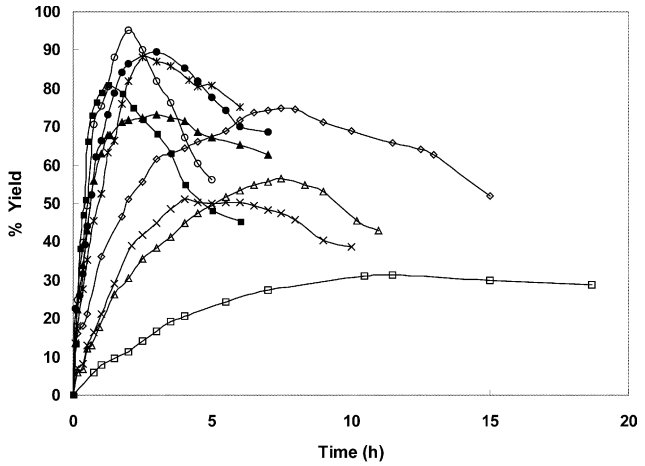

Fig. 2. Time-course of synthesis of cephalexin according to experimental design in Table 1, at 30 mM 7ADCA, 90 mM PGME, 125 IU/mmol 7ADCA and 50%(v/v) ethylene glycol. □, pH 5.8, 23 °C; △, pH 6.0, 18 °C; ×, pH 6.0, 28 °C; ◇, pH 6.5, 15 °C; *, pH 6.5, 23 °C; ●, pH 6.5, 30 °C; ▲, pH 7.0, 18 °C; ○, pH 7.0, 28 °C; •, pH 7.2, 23 °C.

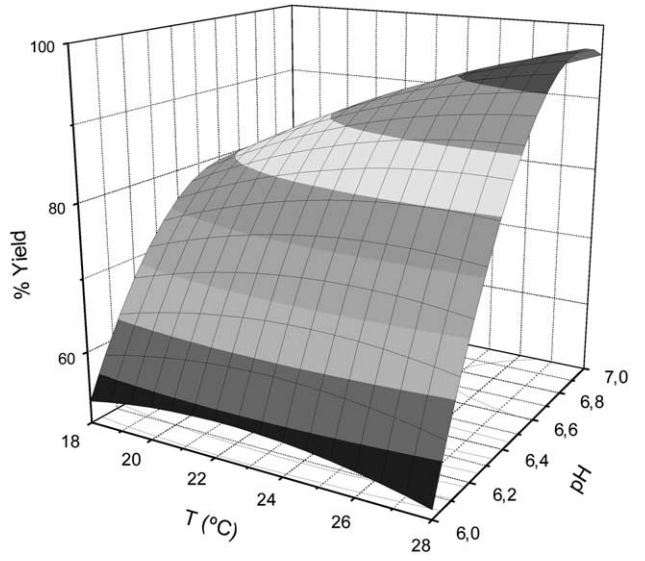

Fig. 3. Three-dimensional plots of the effect of pH and temperature on cephalexin yield according to Eq. 2, at 30 mM 7ADCA, 90 mM PGME, 125 IU/mmol 7ADCA and 50%(v/v) ethylene glycol.

decreasing below pH 7.0. Optimum temperature was near the high level of the range; however, Y decreased significantly at temperatures over 30 °C (data not shown). Excess acyl donor at the already optimised temperature and pH was reduced to evaluate its effect on Y. Y was about the same at 2.5 PGME/7ADCA molar ratio, but was reduced below 90% at a ratio of 2 and below 80% at equimolar conditions. At ratios higher than 3, yield was 100%.

The operational stability of the biocatalyst was determined in repeated production batches at the corresponding optimum conditions (pH 7.0 and 28 °C). The reaction in each batch was conducted until the value for Y in the first batch (96%) was obtained. Specific activity of the biocatalyst at the end of each batch was determined to assess its operational stability. Results are presented in Table 3 for a series of seven consecutive batches.

Table 2
Optimisation of the GAPA catalysed synthesis of cephalexin in 50% (v/v) ethylene glycol

$T^{*\,Y}$ (°C)	28
$pH^{*\,Y}$	7.0
Y^* (%)	97.7
$Y^{*\,exp}$ (%)	95.8
P_{Y*} (mM/h)	13,0
P_{Y*exp} (mM/h)	14.5
$T^{*\,P}$ (°C)	27
$pH^{*\,P}$	7.0
P^* (mM/h)	14.8
Y_{P*} (%)	95.1

Optimum temperature ($T^{*\,Y}$) and pH ($pH^{*\,Y}$) in terms of yield, maximum predicted yield (Y^*), experimental yield at optimum conditions ($Y^{*\,exp}$), and productivity at maximum yield (P_{Y*}). Optimum temperature ($T^{*\,P}$) and pH ($pH^{*\,P}$) in terms of productivity, maximum productivity (P^*) and yield at maximum productivity (Y_{P*}).

4. Discussion

The positive effect of ethylene glycol on Y was clearly appreciated. Not only Y was substantially higher than in fully aqueous medium, but also Y decrease after maximum was much smoother, which makes production more stable and amenable for product recovery. These results are consistent with the mechanism of enzymic synthesis of β-lactam antibiotics under kinetic control [29,30] and are in agreement with previous results obtained in the synthesis of ampicillin with PGA-450 [18]. The positive effect of the cosolvent on Y has been explained by the reduction of water activity that ethylene glycol produces [21], which selectively depresses the hydrolysis of product and acyl donor (PGME). Productivity is usually decreased by the presence of

Table 3
Operational stability of GAPA in repeated production batches of cephalexin at the corresponding optimum conditions (pH 7.0 and 28 °C), at 30 mM nucleophiles, 90 mM PGME, 125 IU/mmol nucleophile and 50%(v/v) ethylene glycol

Batch number	Specific activity after each batch (% of initial in the first batch)	Time to obtain $Y = 96\%$ (h)
1	95	2
2	82	2.3
3	80	2.3
4	82	2.5
5	77	2.7
6	74	2.7
7	72	2.8

organic cosolvents [18,31,32]. However, in this case productivity was about the same in ethylene glycol and in aqueous medium. It is interesting to point out that the initial rates of synthesis are less affected by ethylene glycol than the initial rates of hydrolysis of the antibiotic and the acyl donor, which are higher in aqueous medium [33]. This is the reason underlying the net positive effect of ethylene glycol on cephalexin Y.

Using response surface methodology, a quadratic model was validated for cephalexin synthesis using Y as objective function. Y decreased at low pHs, which has been predicted for the kinetically controlled synthesis of β-lactam antibiotics in aqueous medium [34] and has been proven experimentally for the synthesis of ampicillin in the presence of cosolvents [18]. This behaviour has been explained in terms of unfavourable equilibrium for the reactive non-ionised substrate species at low pHs [35]. Y increased with temperature within the range studied, optimum being close to the upper extreme of the range. This was unexpected, based on the predictions done by Kasche [34] and results reported on the synthesis of cephalexin [14] and cephalothin [11], where Y was higher at lower temperatures. We have observed this behaviour in the synthesis of ampicillin with GAPA (data not shown). The positive effect of temperature on Y has been observed in kinetically controlled enzymic synthesis of peptides [36], whose mechanism of synthesis is similar to β-lactams [34], but not in the case of derived cephalosporins. The optimum predicted by the model was validated, experimental yield being only 1.9% below the predicted value. Optimum Y was significantly higher than that previously obtained with PGA-450 [33]. Y and P were much higher for cephalexin than for ampicillin (data not shown), which further supports that 7ADCA is a better nucleophile than 6APA for penicillin acylase [37]. Optimum Y for cephalexin was close to 100% and P at that optimum (14.5 mM/h) is significant and compare quite favourably with values reported in the literature for the production of semi-synthetic cephalosporins [4,31,38,39]. Conditions that maximise yield were almost the same at which productivity was maximum (see Table 3), so that no compromise occurred between these two operational parameters, as is usually the case. GAPA can then be considered as a suitable biocatalyst for the synthesis of cephalexin in organic medium.

Substrate concentrations used are still below those recommended for industrial operation [40]. Yield is expected to increase when working at higher 7ADCA and PGME concentrations [41], but the problem of substrate solubility is still to be worked out. A possible solution is the use of a fed-batch strategy as the one used for the synthesis of ampicillin [15]. A 200% excess in acyl donor can be considered too high and will increase the amount of phenylglycine to be removed, but excess below 150% will reduce Y.

To assess the potential of GAPA further for the synthesis of cephalexin, operational stability was determined under the corresponding optimum conditions obtained previously. From the results in Table 3, considering the time required in each batch to attain maximum Y, the time-course of enzyme inactivation was determined. GAPA inactivation under operation conditions could be modelled by a two-stage series type mechanism [42]. From this model, the projected half-life was calculated as 135 h. Synthesis of cephalexin at low temperatures is well-worth studying to determine if the expected increase in enzyme and antibiotic stability will outweigh the longer times and/or higher enzyme loads that will be required. Once this information is gathered, a cost-base objective function can be developed to optimise reactor operation conditions in terms of processing costs.

5. Conclusions

GAPA was a suitable biocatalyst for the kinetically controlled synthesis of cephalexin. Results are significantly better than those previously obtained with a commercial biocatalyst and compare quite favourably with those previously reported for the kinetically controlled synthesis of β-lactam antibiotics. The biocatalyst was robust, withstanding high concentrations of ethylene glycol and having a very high storage stability and fair operational stability. Yield was substantially increased by the presence of the cosolvent and values close to 100% could be obtained at optimised pH and temperature. This was not attainable in aqueous medium and, moreover, the stability of GAPA in ethylene glycol medium was somewhat higher than in water at optimum temperature for synthesis. Conditions for synthesis are still to be optimised at higher substrate concentrations. Yield is expected to increase, but solubility of 7ADCA will be exceeded so that a fed-batch strategy or pH profiling during operation might be required.

Acknowledgements

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