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# IMPROVING SELECTIVITY AND PRODUCTIVITY OF THE ENZYMATIC SYNTHESIS OF AMPICILLIN WITH IMMOBILIZED PENICILLIN G ACYLASE

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**Abstract -** An experimental design was applied to improve the reaction conditions for enzymatic synthesis of ampicillin from phenylglycine methyl ester (PGME) and 6-aminopenicillanic acid (6-APA), catalyzed by penicillin G acylase from *E. coli* immobilized on an agarose-glyoxyl derivative. The presence and magnitude of interactions between reaction variables were estimated using a $2^5$ factorial design. A batch reactor was employed to assess the influence of the following variables: pH, temperature, initial 6-APA concentration, buffer concentration, and the presence of methanol. Response variables were productivity, selectivity, and yield (based on initial 6-APA concentration). The best synthesis yield (56.9%) was at T = 4ºC and pH 6.5. The highest productivity ($49.3 \times 10^{-3}$mM of antibiotic/min) was achieved at T = 25ºC and pH 6.5. Our results indicate that it is possible to achieve high productivity for this system while maintaining a high selectivity and yield.

*Keywords*: Ampicillin, immobilized enzyme, β-lactam antibiotic synthesis, factorial design.

## INTRODUCTION

Ampicillin (6- [2– amino– 2– phenyl acetamide] penicillanic acid) is one of the most widely used semi-synthetic β-lactam antibiotics (Ospina et al., 1996). It has an estimated market of 20,000 tons/year (Bruggink and Roy, 2001).

The conventional process for industrial production of ampicillin uses a complex series of chemical reactions, requiring protection and de-protection of reactive groups, toxic solvents, and low temperatures (app. -30°C). Indeed, solvents as methylene chloride and dimethylaniline are employed to protect the reactive groups (Ospina, et al., 1996, Croci and Cotti, 1980, Cowley and Martin, 1976). These solvents are undesirable as residues or impurities in the antibiotic produced, requiring rigid quality control of the downstream purification steps.

The enzymatic production of ampicillin (or "green chemistry" process) offers an attractive alternative to the chemical synthesis. It involves one single step (apart from the synthesis of ester/amide precursors) under mild conditions: an aqueous medium, ambient temperature and pressure, and a neutral pH. Its residues may easily meet strict environmental standards. However, the yield of enzymatic synthesis of ampicillin is lower than that of the chemical reaction.

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This work is an optimization study of the enzymatic synthesis of ampicillin, to identify process conditions under which this "green process" might become economically competitive. The kinetically controlled synthesis of ampicillin from phenylglycine methyl ester (PGME) and 6-amino penicillanic acid (6-APA), catalyzed by penicillin G acylase (PGA), was studied. A thermodynamically controlled synthesis of the antibiotic, where the free amino acid (phenylglicine, PG) would be the acyl donor, is not favored. PGA from *Escherichia coli* requires that the PG carboxyl group should be protonated while, at the same time, the amino group of the β-lactam nucleus should be neutral, available for nucleophilic interactions (Ospina et al., 1996). However, for the range of pHs where the enzyme is active (pH 6-8), the number of substrate molecules having the reactive groups with the proper charge is negligible.

The kinetically controlled synthesis of ampicillin is a strategy presented by several authors (Cole, 1969a; Kasche et al., 1987; Konecny et al., 1983). In this strategy, PG is replaced by an activated substrate, such as an amide, ester, or anhydride. Kasche (1986) and Fernandez-Lafuente et al. (1991a) reported that PGME provided higher yields than amides. Figure 1 shows the kinetically controlled synthesis of ampicillin from PGME and 6-APA. Two side reactions, also catalyzed by PGA, compete with the synthesis of ampicillin. Phenylglycine (PG) and methanol are the products of hydrolysis of PGME (a parallel reaction). PG and 6-APA are produced by the hydrolysis of ampicillin (in series with the synthesis). The yield of the kinetically controlled synthesis of ampicillin depends on the ratio between the rates of synthesis and hydrolysis of the antibiotic (*s/h2*) and between the synthesis and hydrolysis of the activated substrate (*s/h1*). According to Gonçalves et al. (2000a), the degree of saturation of the enzyme active site by 6-APA is a determining factor in increasing the synthesis yield. Kasche (1986) reported that the antibiotic synthesis requires adsorption of 6-APA on the enzyme active site before the substrate-enzyme complex is formed. Ferreira et al. (2000) studied the side reactions that compete with the enzymatic synthesis of ampicillin and concluded that the rate of hydrolysis of PGME decreased when this reaction was carried out in the presence of ampicillin. The rate of hydrolysis of ampicillin also decreased when PGME was present, but the inhibitory effects were very different in each case: the inhibitory effect of PGME on hydrolysis of ampicillin was much higher than that of ampicillin on hydrolysis of PGME. Consequently, productivity increased for higher concentrations of PGME, but the results of Ferreira et al. (2000) on the selectivity of the reaction were not conclusive.

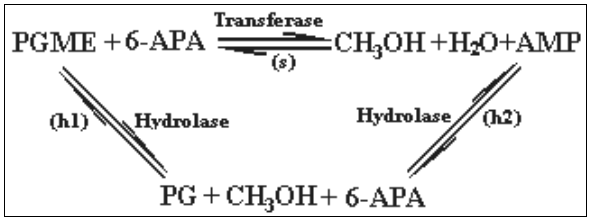


**Figure 1:** Enzymatic synthesis of ampicillin.

The catalyst used in this work had the enzyme immobilized on agarose gel. Experimental conditions that eliminate mass transfer limitations for these reactions were previously investigated (Ferreira et al., 1999): high-speed mechanical stirring and low enzymatic load (below 90IU/g of agarose gel).

The enzymatic synthesis of ampicillin with PGA from *E. coli* [EC 3.5.1.11] has been reported ever since 1969 (Cole, 1969a,b), though its low yield discouraged industrial implementation of the process. For enzymatic synthesis of antibiotics in industrial reactors, it is essential that operational variables be set so that some performance parameters are maximized. Each experimental variable of the reaction medium (concentration of reactants, pH, temperature, solvents, etc.) has different effects on these performance indices. If the reaction variables are changed one at a time, around a central point, only part of the experimental domain is explored. Factorial design is a useful procedure that systematically changes the variables, simultaneously and in a suitable manner (Barros Neto et al., 1995; Montgomery and Runger, 1999). The aim of this work was to find operational conditions that would simultaneously improve yield, selectivity, and productivity.

## MATERIALS AND METHODS

### Materials

#### a) Support

Agarose 10 BCL (10% w/w, crosslinked) was donated by Hispanagar S.A., Spain.

#### b) Enzyme

Penicillin G Acylase (PGA) [EC 3.5.1.11] from *Escherichia coli* was donated by Antibioticos S.A., Spain.

#### c) Substrates

Phenylglycine methyl ester (PGME) was purchased from Aldrich Chem. Co., USA; 6-aminopenicilanic acid (6-APA) and ampicillin from Winlab, U.K.; and D-Phenylglycine from Bachem, Switzerland. All other chemicals were of laboratory grade from different commercial suppliers.

### Methods

#### a) Immobilization of PGA

Agarose gel (Ag-O-$CH_2$-CHOH-$CH_2$OH) was activated by etherification with glycidol (2, 3 epoxypropanol) and oxidation with sodium periodate, resulting in glyoxyl -gels (Ag-O-$CH_2$-CHO). Further control of the PGA (amine)-agarose (aldehyde) multipoint attachment was achieved at pH 10 (bicarbonate buffer, 50mM), with 100mM of phenylacetic acid (PAA) during 3 hours at 20°C. Final reduction of the Schiff bases formed by the reaction of the PGA terminal and lysine amine groups with the aldehyde groups in the support was done with sodium borohydride (1mg/ml of solution) during 30 minutes at room temperature (Guisán, 1988). After each step, the gel was filtered and washed with distilled water. The enzymatic load of agarose-glyoxyl gel was 45 IU/g of gel.

#### b) Enzyme Activity

Colorimetric analysis of the 6-APA released during hydrolysis of penicillin G provided the basis for evaluation of enzyme activity. 6-APA reacted with p-dimethyl-amino-benzaldehyde (PDAB) in 10mM phosphate buffer, pH 8 (Balasingham et al., 1972). The difference between enzymatic activities of the supernatant (free enzyme) before and after immobilization was used to assess the enzymatic load of the gel. One IU (international unit) of enzyme was defined as the amount of enzyme that hydrolyzes 1μmol of penicillin G (5% mass/volume) per minute at pH 8.0 and 37°C.

#### c) Analysis

Concentrations of PGME, ampicillin, 6-APA, and PG were determined using HPLC. A C18 column (Waters Nova-Pack, USA, C18, 60Å, 4μm, 3.9 × 150mm) with 1ml/min of eluent (mobile phase) composed of 35% acetonitrile, 2‰ SDS (lauryl sodium sulphate) and 10mM $H_3PO_4$, 5mM $K_2H_2PO_4$ was used at 25°C and $\lambda$ = 225nm.

#### d) Ampicillin Synthesis Experiments

A jacketed batch reactor with mechanical stirring was used in all the experiments. The amount of biocatalyst was constant in all assays (1.0 g of agarose with 45 IU of immobilized PGA/$g_{gel}$). The pH of the solutions during the enzymatic synthesis reactions was controlled adding concentrated NaOH. Stirring rate was 800rpm and the total reactor volume was $50 \times 10^{-6}$ $m^3$. Samples of 10μl were taken from the reaction mixture, diluted in the mobile phase (990μl), and analyzed using HPLC.

The variables studied were ones that could be easily used to define a cost- or profit-objective function for the process. These responses were the overall reaction yield with respect to 6-APA ($Y_{6\text{-APA}}$), productivity (P), and global selectivity (S). Their definitions for a batch system follow: here $N_{amp}$ is the number of moles of ampicillin; $N_{6-APA}^{initial}$ is the number of moles of 6-APA at the beginning of the batch; $C_{amp}^{max}$ is the maximum concentration of ampicillin, which is reached at time $t_{max}$ for each run; and $N_{PG}$ is the number of moles of undesired product (phenylglycine).

$$Y_{6-APA} = \frac{N_{amp}}{N_{6-APA}^{initial}} \quad (1)$$

$$\text{Productivity} = \frac{C_{amp}^{max}}{t_{max}} \quad (2)$$

$$\text{Selectivity} = \frac{N_{amp}}{N_{PG}} \quad (3)$$

**e) Experimental Design**

Enzymatic synthesis assays were performed using a $2^5$ factorial analysis. The experimental variables were temperature, pH, ionic strength, presence of organic solvent (methanol), and substrate concentrations. The response variables are defined by equations (1) to (3). These variables and their ranges were selected based on several sources (Boccu et al., 1991; Ospina, et al., 1996; Rosell, et al., 1998; Fernandez-Lafuente, et al., 1991a, 1998; Langen, et al., 1999) and on preliminary studies in our laboratory.

A number of experimental variables may influence the enzymatic synthesis of β-lactam antibiotics. However, not all these variables exert a strong influence; thus, a screening was carried out using factorial analysis. The factorial planning is reported in Table 1. Assays were carried out on two levels: (–1) and (+1): at temperatures of 4ºC and 25ºC, pHs of 6.5 and 7.5, phosphate buffer concentrations of 0 (zero) and 50mM, concentrations of methanol, 0 (zero) and 500mM, and concentrations of 6-APA 10 and 50mM. The experiments were carried out in a random sequence.

The experimental results were treated according to Barros Neto et al. (1995) and Montgomery and Runger (1999).

In all assays, initial PGME concentration was 50mM, based on the conclusions of Ferreira et al. (2000): high concentrations of PGME inhibited the hydrolysis of ampicillin, but decreased the selectivity of the reaction. PGME concentration was not changed in the experimental planning because the most important factor in selectivity is the 6-APA/PGME ratio, which was modified when the initial 6-APA concentration was changed.

Coefficients of the responses (yield, productivity, and selectivity) and their cross interactions were estimated. The significance of these coefficients was evaluated using a standard deviation analysis. Normal probability plots were used to discern significant effects, in accordance with Barros Neto et al. (1995), p.94-96 and Montgomery and Runger (1999), p.318-320. Figure 2 shows normal probability plots of the three responses (yield, selectivity, and productivity). Effects in the middle of plots were "noise" with Gaussian distribution and zero mean. These coefficients reflected random fluctuations in the process. The assays were not replicated. The significant effects were assumed to represent all responses satisfactorily, and the other ones were eventually used to estimate the standard deviation of the effects.

**Table 1: Levels of the $2^5$factorial design (–1) and (+1): T, 4ºC and 25ºC; pH, 6.5 and 7.5; phosphate buffer, 0 (zero) and 50mM; methanol, 0 (zero) and 500mM; 6-APA, 10 and 50mM.**

| Run | T (ºC) | pH | Methanol (mM) | Buffer (mM) | $C_{6\text{-APA}}$ (mM) |
|---|---|---|---|---|---|
| 01 | + | + | + | + | + |
| 02 | + | – | – | + | + |
| 03 | + | – | + | – | + |
| 04 | + | + | – | – | + |
| 05 | – | – | – | – | + |
| 06 | – | + | + | – | + |
| 07 | – | + | – | + | + |
| 08 | – | – | + | + | + |
| 09 | – | + | + | + | – |
| 10 | – | – | – | + | – |
| 11 | – | – | + | – | – |
| 12 | – | + | – | – | – |
| 13 | + | – | – | – | – |
| 14 | + | + | + | – | – |
| 15 | + | + | – | + | – |
| 16 | + | – | + | + | – |
| 17 | – | + | – | – | + |
| 18 | + | – | – | + | – |
| 19 | + | – | + | + | + |

**Continuation Table 2**

| Run | T (°C) | pH | Methanol (mM) | Buffer (mM) | $C_{6\text{-APA}}$ (mM) |
|---|---|---|---|---|---|
| 20 | – | + | – | + | – |
| 21 | + | – | + | – | – |
| 22 | – | + | + | – | – |
| 23 | – | + | + | + | + |
| 24 | + | – | – | – | + |
| 25 | + | + | + | – | + |
| 26 | – | – | – | – | – |
| 27 | – | – | – | + | + |
| 28 | + | + | – | + | + |
| 29 | – | – | + | – | + |
| 30 | + | + | – | – | – |
| 31 | + | + | + | + | – |
| 32 | – | – | + | + | – |

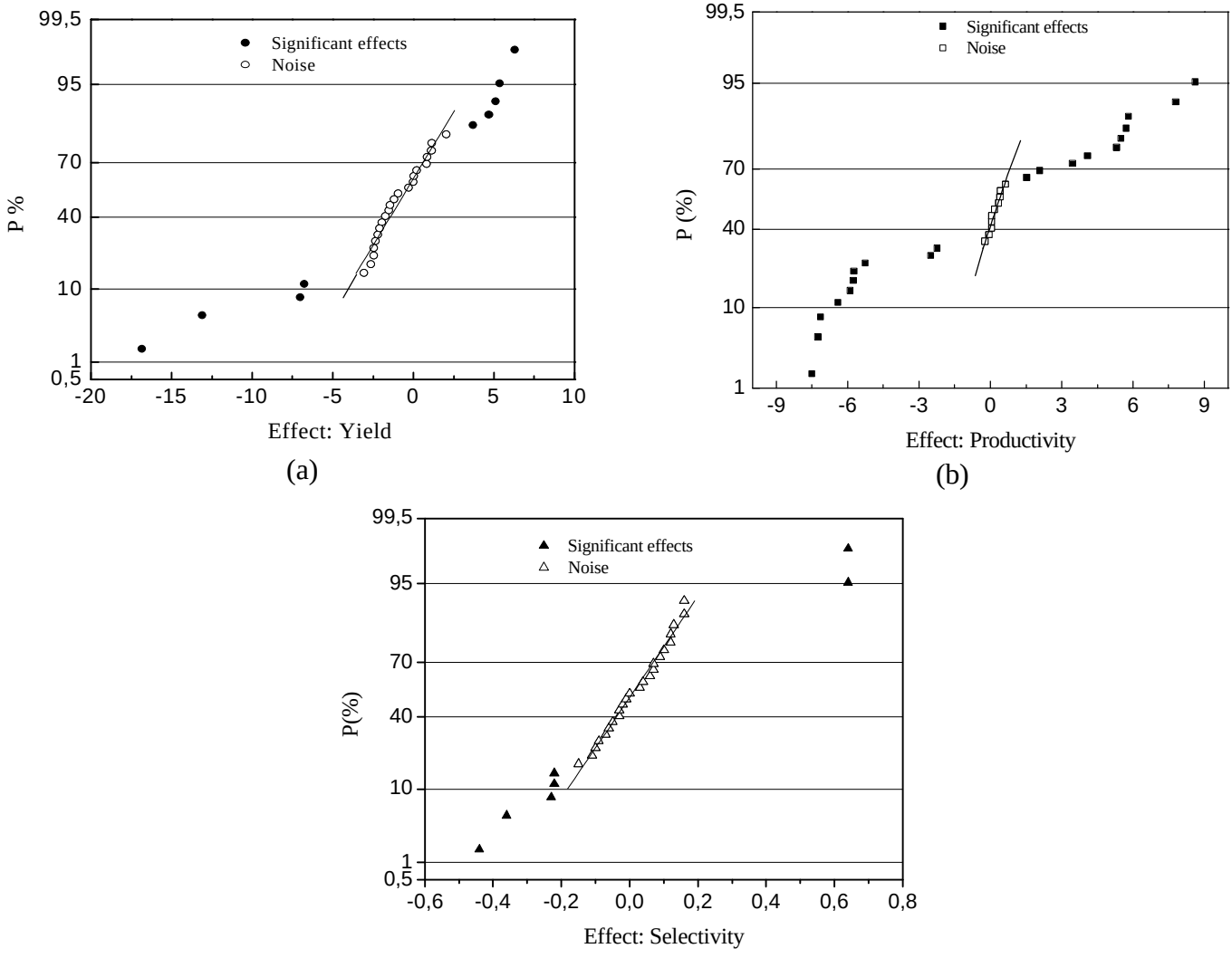


**Figure 2:** Normal probability plots for (a) yield with significant effects (●); (b) productivity with significant effects (■); and (c) selectivity with significant effects (▲).

## RESULTS AND DISCUSSION

Table 2 shows all effects on yield, productivity, and selectivity. The response surfaces had thirty-two terms, including five-variable cross-interactions, but only the most significant effects were selected. Analysis of the normal probability plots indicated that the effects of methanol and ionic strength were not significant; these less significant effects for the three responses were used to estimate the standard error of the effects. The yield had s.d. = 1.7%, the selectivity had s.d. = 0.09, and the productivity had s.d. = $1.2 \times 10^{-3}$ mM/min. The effect of pH, B in Table 2, is the largest of all responses. It is positive for productivity and negative for yield and selectivity, so low values of pH will enhance yield and selectivity of ampicillin synthesis at the price of decreasing productivity – a typical optimization problem. The effect of methanol, C in Table 2, is insignificant for all responses, but it must be considered when coupled with pH, BC effect in Table 2. BC is positive for productivity and yield and negative for selectivity. Once again, a contradictory overall effect is present. 6-APA concentration, E in Table 2, is significant for all responses. When 6-APA concentration is low, high yields are achieved, but selectivity and productivity of the reaction decrease. It is important to stress that, in order to optimize the production of ampicillin economically all three responses must be taken into account. High productivity and yield are important, but selectivity must also be considered because PGME is hydrolyzed (see Figure 1).

**Table 2: Estimates of principal and crossed effects upon yield, productivity, and selectivity. A = Temperature, B = pH, C = Methanol, D = Buffer, E = 6-APA concentration. Significant effects are highlighted.**

| Effect | $Y_{6\text{-APA}}$ (%) | P x $10^3$ (mM/min) | S |
|---|---|---|---|
| **Mean** | 29.68 | 14.36 | 0.47 |
| **Standard error** | 1.7 | 1.2 | 0.09 |
| **A** | -13.10 | -7.75 | 0.16 |
| **B** | -16.85 | 7.81 | -0.44 |
| **C** | 0.03 | 4.09 | -0.06 |
| **D** | -1.52 | -5.50 | -0.02 |
| **E** | -7.02 | 5.49 | 0.64 |
| **AB** | 1.16 | 0.41 | 0.09 |
| **AD** | -2.62 | 0.19 | 0.06 |
| **BC** | 5.34 | 5.72 | -0.22 |
| **BE** | $-5.0 \times 10^{-3}$ | 0.06 | -0.22 |
| **CE** | -6.76 | 0.66 | 0.12 |
| **AC** | -2.46 | -6.40 | 0.10 |
| **AE** | 6.30 | -2.23 | -0.05 |
| **BD** | 0.86 | 0.43 | -0.01 |
| **CD** | -1.73 | 2.09 | -0.07 |
| **DE** | -1.43 | -5.25 | 0.00 |
| **ABC** | -0.28 | -5.88 | 0.64 |
| **ABE** | 1.14 | 1.54 | 0.16 |
| **ACE** | 5.12 | 0.35 | -0.36 |
| **BCD** | -2.34 | -2.49 | -0.15 |
| **BDE** | -1.19 | -7.24 | -0.10 |
| **ABD** | -2.18 | 0.05 | 0.04 |
| **ACD** | 0.22 | 0.05 | 0.07 |
| **ADE** | -2.07 | 5.82 | 0.07 |
| **BCE** | -0.93 | -0.05 | -0.23 |
| **CDE** | -3.05 | -0.22 | -0.11 |
| **ABCD** | 2.04 | 5.31 | 0.13 |
| **ABDE** | 0.84 | 3.47 | 0.12 |
| **BCDE** | -1.94 | -5.72 | -0.03 |
| **ABCE** | 4.70 | -7.12 | 0.03 |
| **ACDE** | 3.71 | 8.63 | -0.09 |
| **ABCDE** | -2.44 | 4.96 | -0.03 |

Table 3 shows the experimental results obtained according to the conditions reported in Table 1. It can be observed that the twenty-fourth run provided the best combination of $Y_{6\text{-APA}}$, S, and P. Higher yields were obtained in experiments with a low concentration of 6-APA (10mM) (runs 9, 10, 11, 22, 26, and 32), and hence with an excess of PGME (50mM). Actually, a low concentration of β-lactam nucleus means a higher probability of consuming this reactant, thereby improving the yield. Nevertheless, PGME is hydrolyzed and selectivity decreases in this region. All experiments with high values of selectivity were run with a high concentration of 6-APA (50mM) (runs 2, 3, 5, 8, 19, 24 25, 27, and 29). The greater availability of nuclei facilitated its adsorption and favored its competition with water to promote the nucleophilic attack on the acyl-enzyme complex. Thus, the s/h1 ratio increased. On the other hand, high selectivity resulted in higher maximum ampicillin concentrations and thus improved the yield. This combination of effects explains the results of assays 5, 24, and 27, which achieved high yields despite their high concentration of 6-APA, 50mM.

The effect of 6-APA on productivity is more complex. The highest productivities took place with low 6-APA concentrations (13, 15, and 16) as well as with high 6-APA concentrations (24 and 25). This is an indication that the role of the nucleus cannot be analyzed separately from other effects. Gonçalves et al. (2000b) describe the mixed inhibition/activation effect of 6-APA on the enzymatic synthesis of amoxicillin. The formation of the acyl-enzyme complex was inhibited by 6-APA when the concentration of para-hydroxyphenylglycine methyl ester was low and activated when the concentration of the ester was high. Here another antibiotic is under study, but the role of 6-APA seems to be equally complex.

**Table 3: Results of factorial design $2^5$ for enzymatic ampicillin synthesis. Responses: yield ($Y_{6\text{-APA}}$), productivity (P), and selectivity (S).**

| Run | $Y_{6\text{-APA}}$ (%) | P x $10^3$ (mM/min) | S |
|---|---|---|---|
| 01 | 10.5 | 17.4 | 0.20 |
| 02 | 31.6 | 21.6 | 0.92 |
| 03 | 24.7 | 22.8 | 1.92 |
| 04 | 15.0 | 25.0 | 0.39 |
| 05 | 41.1 | 2.4 | 1.09 |
| 06 | 20.2 | 2.4 | 0.38 |
| 07 | 32.4 | 3.7 | 0.61 |
| 08 | 32.4 | 1.9 | 0.76 |
| 09 | 50.0 | 1.2 | 0.15 |
| 10 | 42.6 | 1.0 | 0.19 |
| 11 | 50.3 | 1.8 | 0.18 |
| 12 | 18.9 | 0.6 | 0.07 |
| 13 | 29.7 | 48.2 | 0.09 |
| 14 | 15.2 | 16.0 | 0.18 |
| 15 | 13.1 | 43.7 | 0.09 |
| 16 | 27.9 | 30.4 | 0.17 |
| 17 | 17.0 | 1.2 | 0.36 |
| 18 | 36.2 | 20.1 | 0.23 |
| 19 | 22.5 | 20.8 | 0.71 |
| 20 | 24.5 | 0.8 | 0.09 |
| 21 | 33.8 | 28.2 | 0.25 |
| 22 | 44.5 | 1.0 | 0.11 |
| 23 | 10.4 | 1.6 | 0.17 |
| 24 | 41.4 | 49.3 | 2.40 |
| 25 | 25.3 | 42.1 | 0.85 |
| 26 | 56.9 | 4.0 | 0.22 |
| 27 | 46.6 | 2.0 | 0.99 |
| 28 | 11.2 | 18.7 | 0.22 |
| 29 | 36.5 | 2.6 | 0.76 |
| 30 | 16.6 | 9.2 | 0.09 |
| 31 | 15.5 | 25.8 | 0.14 |
| 32 | 55.4 | 1.9 | 0.23 |

Considering all these effects, a high 6-APA concentration was selected as the most convenient choice for operating the reactor, because lowering the PGME/6-APA ratio would decrease losses of ester by hydrolysis 1 (see Figure 1).

According to Kato et al. (1980), Van der Wielen *et al.* (1997), and Hernández-Jústiz, (1996), the ionic strength of the medium might interfere with the catalytic behavior of the enzyme. A high ionic strength would inhibit 6-APA adsorption and therefore the rate of antibiotic synthesis would decrease. However, for the range studied here (0-50 mM of phosphate buffer), this effect was not significant.

Organic solvents may have two roles: a thermodynamic one, changing dissociation constants, i.e., increasing pK values of carboxylic groups, and a kinetic one, decreasing the concentration of water. Both effects disfavor hydrolysis reactions (Sadvige, 1984; Svedas et al., 1980; Fernández-Lafuente et al., 1991a,b, 1998; Kim and Lee, 1996b; Klibanov, 1997). Hence, a higher selectivity could be expected in the presence methanol, which is a product of reactions *s* and *h1* (see Figure 1). High concentrations of this alcohol would therefore increase the yield of synthesis (Fernandez-Lafuente et al., 1998; Kim et al., 1996a,b; Illanes and Fajardo, 2001). However, for the range of substrate concentrations studied here, no significant improvements in the response variables were observed when the solvent was present.

For an enzymatic synthesis under thermodynamic control, the effects of temperature on yield can be anticipated. However, in an enzymatic synthesis under kinetic control, the variable selectivity is essential. The kinetically controlled synthesis of ampicillin is a series-parallel system of reactions, and therefore forecasting the effect of temperature is not straightforward. When hydrolysis 1 is favored (see Figure 1), substrate is lost and the overall process costs increase. On the other hand, hydrolysis 2 can be favored when inadequate reactor residence times are used, once again increasing process costs.

Temperature was tested as a variable to enhance synthesis for both hydrolyses. The highest synthesis yields occurred at 4ºC (see Table 2, runs 9, 11, 26, and 32). The highest productivities occurred at 25ºC ($49.3 \times 10^{-3}$ mM/min). Maximum selectivity, 79.6%, was also obtained at 25ºC (see Table 2, run 24).

Higher selectivity (except for run 25), yield (except for runs 2 and 29), and productivity (except for runs 15 and 25) all occurred at pH 6.5. Gonçalves et al. (1998), Ospina et al. (1996), Boccu et al. (1991), and Kasche (1986) also reported that higher yields were observed for slightly acid pHs. At pH 6.5, 6-APA adsorption on the active site is stronger, favoring synthesis over hydrolysis.

Nevertheless, pH affects the formation of acyl-enzyme complex in two opposite directions. The amine group of serine B1 (the active site of PGA, Duggleby et al., 1995) should be uncharged, ready to accept protons when directing the nucleophilic attack on the ester binding of the substrate (PGME); thus, decreasing pH would hinder the formation of the acyl-enzyme complex. Besides that, at a slightly acid pH almost all the 6-APA amine groups are still deprotonated ($pK_a$ = 2.5 and $pK_b$ = 4.9), which facilitates the nucleophilic attack on the acyl-enzyme complex by 6-APA. To summarize, defining the best pH is clearly an optimization problem: decreasing the pH would improve adsorption of 6-APA (a desirable effect), but would also decrease the number of uncharged serine B1 α-amine groups (undesirable). In addition, the need for deprotonated 6-APA amine groups places a lower limit on the range of feasible pHs. At pH 6.5, the rate of formation of the acyl-enzyme complex and the nucleophilic attack by the amine group of 6-APA (mostly still uncharged) are still not seriously quenched. The result is a higher selectivity (s/h2 ratio).

It should be observed that at pH 6.5 the value for catalytic activity of PGA is no longer the maximum; nevertheless, a multipoint, immobilized enzyme, which displays higher stability with variations in pH than the soluble enzyme was used in this study. The best combination of temperature and pH is 25ºC and 6.5. At this temperature, the yield decreases slightly but is still acceptable (41.4% against the overall maximum of 56.9% at 4ºC).

Of the combinations of experimental conditions that were tested, those in run 24 (Table 3) provided the best response: maximum productivity and selectivity with an acceptable yield. The experimental conditions were 25ºC, pH 6.5, no methanol (only the amount naturally produced by PGME hydrolysis and by ampicillin synthesis), no phosphate buffer, and initial concentrations of 6-APA and PGME of 50mM. A plot of a batch synthesis assay using these conditions is shown in Figure 3. Note that the operational conditions chosen took into consideration yield, productivity, and selectivity, which are important in defining the operational cost of production of the antibiotic on an industrial scale.

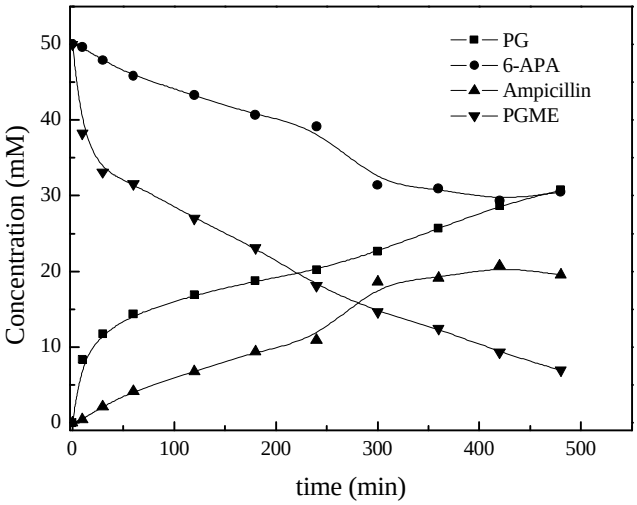


**Figure 3:** Ampicillin synthesis catalyzed by PGA immobilized on agarose at 25ºC, pH 6.5, $C_{initial}$ of 6-APA, and PGME = 50mM, no methanol and no phosphate buffer.

## CONCLUSIONS

Three performance indices for enzymatic synthesis of ampicillin were defined as the responses of a $2^5$ factorial design: yield, selectivity, and productivity. Five variables were studied. Temperature, pH, and the PGME/6-APA ratio significantly influenced the responses. The effects of ionic strength and of the presence of a solvent (methanol) were negligible within the ranges tested.

The highest yield was achieved at 4ºC; however, the best productivity and selectivity were obtained at 25ºC.

Optimum work conditions were selected: 25ºC, pH 6.5, and equimolar initial concentrations of 6-APA and PGME (50mM). Under these conditions, productivity and selectivity were maximized ($49.3 \times 10^{-3}$ mM/min and 2.40, respectively) and the yield was satisfactory (41.4%).

## ACKNOWLEDGMENTS

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