AMPICILLIN SYNTHESIS CATALYZED BY PENICILLIN ACYLASE: EFFECT OF pH AND TYPE OF CARRIERS ON SELECTIVITY AND YIELD

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Abstract. Penicillin G acylase (PGA) (EC 3.5.1.11) from *Escherichia coli* catalyzes the deacylation of penicillin G to yield 6-aminopenicillanic acid, which is an important intermediate in the manufacture of semisynthetic penicillins such as ampicillin. PGA has been immobilized onto chitosan and agarose and the catalytic efficiencies of the derivative for the enzymatic synthesis of ampicillin were compared. These biocatalysts were used to study the effect of pH on the synthesis of ampicillin from phenylglycine metyl ester (PGME) and 6-aminopenicillic acid (6-APA). Bacterial PGAs catalyze the synthesis/hydrolysis of acyl derivatives of phenylacetic acid by the formation of covalent intermediates (the acyl-enzyme complex). The use of phenylglycine derivatives (esters or amines) is necessary, since the direct, thermodynamically-controlled synthesis of ampicillin is not favored. Two other reactions compete with the synthesis: hydrolysis of the ester (a parallel reaction) and of the antibiotics (in series with the synthesis) both providing phenylglycine as end product. It is know that, by controlling the pH, it is possible to inhibit the two undesirable hydrolysis reactions and increase yield of synthesis. This study has investigated the effect of pH (6.0-7.0) on ampicillin synthesis. Assays have been performed using the two biocatalysts in order to improve the yield. The experimental results were obtained in a 20 mL, stirred-tank batch reactor, with suspended biocatalist particles at 25°C.

Keywords: Ampicillin, Enzymatic synthesis, Agarose, Chitosan.

1. Introduction

Semi-synthetic β-lactam antibiotics are the most important class of antibacterial agents. Their use in veterinary and human medicine is in continuous expansion. Some examples of semi-synthetic penicillins and cephalosporins are: amoxicillin, ampicillin, cephalexin, cefadroxil, cefazolin, among many others. They have in common the presence of the β-lactam ring, responsible for their anti-microbial activity, when irreversibly inhibiting the last step of the bacterial cell wall biosynthesis. β-lactam antibiotics can be described as a β-lactam nucleus with a side-chain (Figure 1). Many different nuclei and side-chains are found in the antibiotics that are in use today. Different combinations of side-chains and nuclei form antibiotics with distinctive properties. For example, replacing the phenylacetic acid side-chain of penicillin G with D-phenylglycine (PG) results in ampicillin which, in contrast to penicillin G, is stable for oral administration (Cole, 1969; Bruggink et al., 1998; Wegman et al., 2001; Ribeiro *et al.*, 2005).

Conventional industrial processes for manufacturing these drugs were established in the 60’s, and make use of chemical reactions for synthesis of side-chains and condensation, Elander (2003). Starting in the 90’s, intensive research efforts in academy and industry have been directed towards “clean”, enzymatic routes that would

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comply with the ever-growing environmental restrictions in the legislation. Chemical synthesis would be replaced by enzymatic reactions, working in aqueous medium and physiological conditions. These “clean”, environmental-friendly processes, based on enzymatic catalysis, have been called “green chemistry” (van Langen *et al*., 2001).

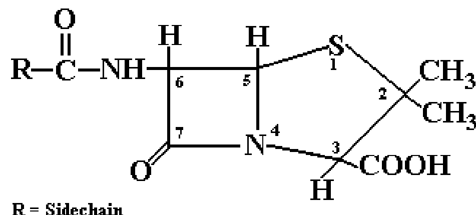

Figure 1. General structure of β-lactam antibiotics.

Ampicillin (6-[2-amino-2-phenylacetamide]penicillanic acid) is in the semi-synthetic penicillin that was first used in therapy (Pessina et al., 1988; Bruggink *et al*., 2001). It is produced by chemical synthesis in a complex process requiring protection of the α-amino group of phenylglicine (PG), highly reactive derivatives of PG, very low temperature (- 30 ºC), anhydrous conditions, and the use of highly toxic compounds (Ospina *et al*., 1996a).

Enzymatic synthesis of ampicillin can be carried out by thermodynamically or kinetically controlled routes. However, thermodynamic studies have demonstrated that synthesis is highly unfavorable for ampicillin (Margolin *et al*., 1980) due to the low chemical energy of zwitterionic phenylglycine (Diender *et al*., 1998; Wegman *et al*., 2001). Penicillin G acylase (PGA) from *Escherichia coli* requires that the PG carboxyl group should be protonated while, at the same time, the amino group of 6-amino penicillanic acid (6-APA) should be neutral, available for nucleophilic interactions. 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 (Blinkovsky and Markaryan, 1993; Youshko *et al*., 2000). The kinetically controlled synthesis of ampicillin can be reached by the use of activated substrates such as phenylglycine methyl ester (PGME) at pH values that are optimal for the enzyme.

In this work, we have focused on the optimization of the enzymatic synthesis of ampicillin from PGME and 6-APA (Figure 2) catalyzed by PGA, to verify the experimental range of pHs to improve the yield of synthesis. Despite the great number of publications involving pH, its optimal value is still controversial. Some authors suggest that pH is not important to improve yield in the range between 6.0 and 8.0 (Ospina *et al*., 1996b; Boccu *et al*., 1991), others conclude that optimal conditions of synthesis could be in the pH range 6.0-7.0 (Youshko *et al*., 2000; Ferreira *et al*., 2004). This question still remains open because there is not accord with respect to some important experimental conditions (organics solvents, temperature, substrate concentrations, etc, Boccu *et al*., 1991; Ospina *et al*., 1996a; Illanes *et al*., 2002; Ferreira *et al*., 2004). But it is known that, by controlling pH, it is possible to inhibit the two undesirable hydrolysis reactions and increase the yield of synthesis (Figure 2). Therefore, this study has investigated the effect of pH (between 6.0 and 7.0) on the yield and selectivity of the penicillin acylase-catalyzed synthesis of ampicillin.

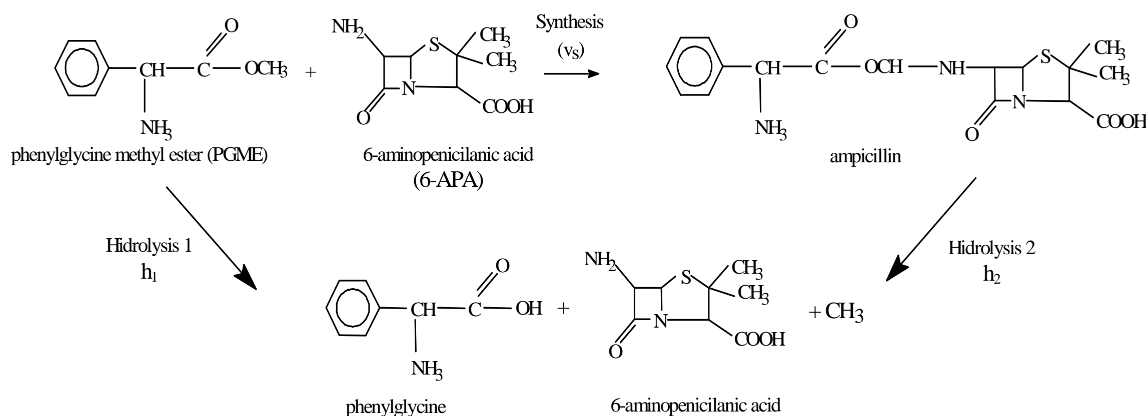

Figure 2. Enzymatic synthesis of ampicillin.

The use of enzymes to catalyze the reaction allows the production of β-lactam antibiotics in a single step. However, enzymes are quite complex and very sensitive to any change in their environment. These features make them have a low operational stability. Nevertheless, the structure of enzymes can become more rigid through immobilization on different supports. Here, we also presented results of immobilization of PGA on chitosan and agarose, viewing the enhancement of the enzyme catalytic properties.

Methods to immobilize PGA are reported by Hernandez-Justiz *et al*., 1998; Fernández-Lafuente *et al*., 2001; Cao *et al*., 2001, but great effort is still being dedicated to the search for new support materials and novel techniques. The type of support as well as the method of immobilization influences the activity and operational stability of immobilized enzyme. By an appropriate choice of the immobilization process, operational costs of industrial processes can be significantly reduced.

One common method of enzyme immobilization is cross-linking to polymeric materials like chitosan, using linking molecules such as glutaraldehyde, which establishes intermolecular cross-links between amino groups of the enzyme and those of the polymer (Hung *et al*., 2003). Chitosan has recently been appointed as a biomaterial with significant potential for use in many fields: medical science, biotechnology, food science etc. Chitosan has received much attention recently because it is a renewable, biodegradable and biocompatible material (Beppu and Santana, 2002). Composed mainly of 2-amino-2-deoxi-D-glucose, chitosan is a linear polysaccharide obtained from the deacetylation of chitin. Chitin is second only to cellulose as the most plentiful natural polymer. Together with its deacetylation product chitosan, chitin is manufactured commercially from the outer shell of crustaceans, particularly crabs and shrimp. The vast quantities of available shellfish wastes easily supply the chitosan needed for many applications (Bullock *et al*., 2000).

2. Materials and Methods

2.1. Materials

Phenylglycine methyl ester (PGME) purchased from Aldrich Chem. Co., USA; 6-aminopenicilanic acid (6-APA), from Winlab, U.K.; Penicillin G Acylase from recombinant *Escherichia coli* was donated by Antibioticos S.A., Spain; Agarose 8 BCL was donated by Hispanagar S. A.; Spain; Powder Chitosan, 85.2 % of deacetylation

degree, was purchased from Polymar Ind LTDA, Fortaleza, Brazil. All other chemicals were of laboratory grade from commercial suppliers.

2.2. Methods

Preparation of agarose supports (Fernandez-Lafuente, 1992). Activation of agarose gel was performed by etherification with glycidol and oxidation with sodium periodate. Further control of the PGA (amine)-agarose (aldehyde) multi-point attachment was achieved by reaction at pH 10 (bicarbonate buffer, 50 mM), in the presence of phenylacetic acid. Final reduction of the amino double bonds was performed with sodium borohydride.

Preparation of chitosan supports. Activation of chitosan beads was performed by dissolving powder chitosan in a solution of acetic acid 5 % and glyoxal (40 % v/v). The obtained solution was dropped into a gently stirred NaOH 1 M solution. Afterwards, the formed beads were treated with glutaraldehyde concentration of 5 %, in phosphate buffer 0.1 M and pH 8.0 for 30 minutes at 28ºC and washed with distilled water to remove the excess of the activating agent.

Enzyme activity. Enzyme activity was evaluated by colorimetric analysis of 6-APA released during hydrolysis of penicillin G. 6-APA reacted with p-dimethylaminobenzaldehyde (PDAB) in 10 mM phosphate buffer, pH 8 (Balasingham *et al.*, 1972). The difference between enzymatic activities of the supernatant (soluble enzyme) before and after immobilization was used to assess the enzymatic load of the gel. 1 IU (international unit) of enzyme was defined as the amount of enzyme that hydrolyses 1 μmol of penicillin G (5 % mass/volume) per minute at pH 8.0 and 37 °C.

Analysis. Concentrations of phenylglycine methyl ester, ampicillin, 6-APA and phenylglycine were determined using HPLC: C18 column (Waters Nova-Pack, USA, C18, 60Å, 3.9 × 150mm) and mobile phase with 35 % acetonitrile, 2 ‰ SDS (Lauryl sodium sulphate); 5 mM $K_2H_2PO_4$ and 10 mM H_3PO_4 at 25 °C. In order to analyze compounds concentrations in the reaction mixture, samples of 50 μL were taken and diluted in the mobile phase (950 μL).

Experiments. A jacketed, batch reactor with mechanic stirring was used in all the experiments described in this paper to eliminate the extra-particle mass transport resistance. Mechanical agitation was preferred rather than magnetic stirring, to avoid gel disruption and loss of enzyme activity. The pH of the solutions during the enzymatic synthesis reactions was kept constant by an automatic titrator. The same amount of biocatalyst was used in all assays (0.5×10^6 IU/m^3 of reactor), 1.0 g of derivative with 10 IU of immobilized PGA. This enzymatic load was low enough to avoid intra-particle diffusion effects. All experiments were performed in a batch reactor at 25 ºC. Stirring speed was 600 rpm and the total reactor volume was 20 mL. Initial substrate concentrations were 50 mM in aqueous solution. The choice of these reaction parameters were selected from results of previous studies of our group (Ferreira *et al*., 2000 and 2004; Ribeiro et al., 2005) where the effect of pH were more relevant.

Reactor Performance. Any optimization of the enzymatic reactor must consider the yield with respect to the β-lactam nucleus ($Y_{6\text{-}APA}$) and the reaction selectivity (S), defined as follows:

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

$$S = \frac{N_{AMP}}{N_{PG}} \quad (2)$$

where N_{AMP} is the number of moles of produced ampicillin, $N_{6-APA}^{initial}$ is the number of moles of 6-APA at the beginning of the batch, and N_{PG} is the number of moles of undesired product (phenylglycine).

3. Results and Discussion

The equilibrium yield of enzymatic synthesis of ampicillin can be overcome by the kinetically controlled reaction, depending on the reaction conditions and the proprieties of the biocatalyst. The reaction must be interrupted when ampicillin concentration passes through a maximum; otherwise the antibiotic is hydrolyzed (Figure 2) resulting in PG and 6-APA. It is known that pH, temperature, enzyme and substrate concentrations are the main reaction parameters that change the kinetics of the reaction (Kasche, 1985; Boccu *et al*., 1991; Illanes and Fajardo, 2001; Illanes *et al*., 2002; Ferreira *et al*., 2004). Hence, we show here results of a study of pH effect and changes on the catalytic properties of PGA from *E. coli* immobilized on chitosan and agarose.

3.1. Effect of pH on the Yields and Selectivity of ampicillin synthesis

In order to study in more details the effect of pH on enzymatic synthesis of ampicillin; yield (Y_{6-APA}) and selectivity (S) were chosen as the variables of interest. These variables are expected to have a strong impact on processing costs of antibiotics production. In Figures 3 and 4, the effect of pH on the ampicillin synthesis can be seen, using chitosan and agarose supports, respectively. It is evident that the larger ampicillin concentration is achieved at pH 6.5 (yield of 42.0 %). Here, the yield of synthesis is defined with respect to the initial 6-APA concentration. These results are consistent with previous work of our group (Ferreira *et al*., 2004). The yield was also seriously affected by other factors (temperature, substrate concentration and, substrate ratio). It was observed that pH should not be analysed separately, there were two- and/or three- significant interaction factors among variables. Here, we have concentrated the investigation within a narrow range of pH [6.0 – 7.0], maintaining the other variables at the optimal operational conditions described by Ferreira *et al*., 2004.

At pH 7.0, the synthesis yield only reached 6.0 % for chitosan after three hours of reaction. The hydrolysis reaction is larger than the synthesis at this pH (selectivity < 1). PMGE is readily hydrolyzed at higher pHs, thus Y_{6-APA} and S decrease. On the other hand, at pH 6.0, the yield reached 11.6 % (Figure 3). A lower pH means slower PGME hydrolysis, hence improving the yield. Here, we assume that PGME hydrolysis follows the same mechanism published for penicillin G hydrolysis (Duggleby *et al*., 1995), the amine group of serine B1 should be uncharged to bind the undissociated form of the PGME. When more PGME is in this form, greater the probability that hydrolysis happens, unless 6-APA directs the reaction to synthesis of antibiotic.

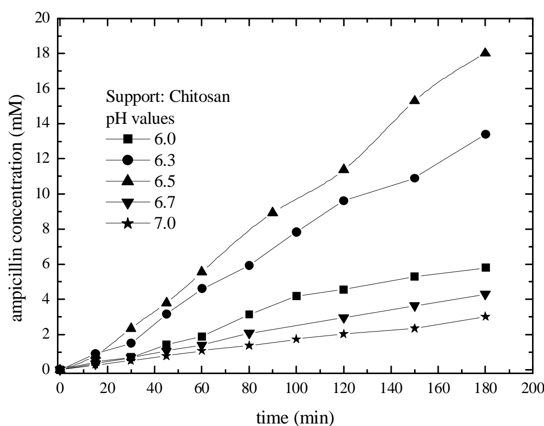

Figure 3. Effect of pH on enzymatic synthesis of ampicillin. Product concentration *versus* time at 25ºC, initial concentration of substrates: 6-APA, 50 mM and PGME, 50 mM. Support: chitosan.

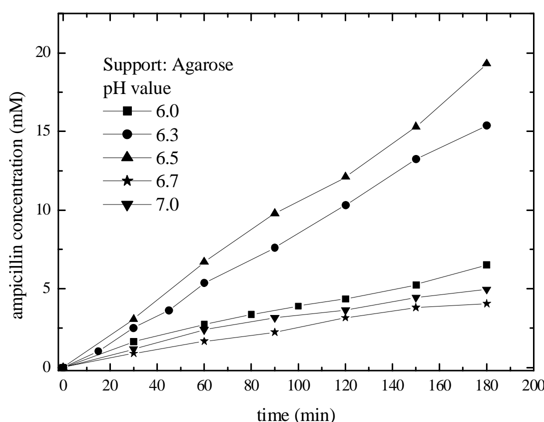

Figure 4. Effect of pH on enzymatic synthesis of ampicillin. Product concentration *versus* time at 25ºC, initial concentration of substrates: 6-APA, 50 mM and PGME, 50 mM. Support: agarose.

Another point is that at lower values of pH a greater availability of nuclei would facilitate its adsorption and favors its competition with water to promote the nucleophilic attack on the acyl-enzyme complex. At pH 6.5, almost all the 6-APA amine groups are still deprotonated ($pK_a = 2.5$ and $pK_b = 4.9$) thus improving adsorption of 6-APA on the active site of PGA, achieving the highest yield (38.2 % at 3 hours of reaction). However, only a yield of 26.4 % is reached when pH decreases to 6.3, probably due to the pH affects on the formation of acyl-enzyme complex. When the reaction takes place at pH 6.0, the yield decreases to 11.6 %, because less serine B1 with deprotonated amine groups are available (the pK of free α-amine groups is between 6.8 and 7.9) and the velocities of all reactions (hydrolyses and synthesis) decrease. For pH around 6.0, the formation of acyl-enzyme complex becomes very difficult. Hence, the dissociation of substrates is an important factor to increase the antibiotic production.

The selectivity (synthesis/hydrolysis ratio, mol product per mol PG) is an index of the viability of the process, since *S* rapidly decreases in the course of the reaction. The effect of pH on selectivity is shown in Figure 5 for two both supports. At pH 6.3, *S* is 2.2 for chitosan and 1.9 for agarose. On the other hand, at pH 7.0, selectivity is only 0.38 for chitosan and 0.43 for agarose.

To sum up, defining the best pH is an optimization problem: decreasing the pH would improve adsorption of 6-APA, but would also decrease the uncharged form of PGME and the number of neutral Ser B1-amine groups (undesirable effects). Hence, the pH for the enzymatic synthesis of ampicillin should be chosen for a maximum nucleophile reactivity of 6-APA, but below the value at which the hydrolysis of PGME starts to quench any effective synthesis. Therefore, considering all these effects, pH 6.5 was selected as the most convenient choice to improve the ampicillin production. Even though it does not provide the best selectivity, it is still an acceptable value. A compromise exists for the pH of synthesis: 6.5 being the optimal for yield, but 6.3 being the best for selectivity. It is important to stress that, in order to economically optimize the production of ampicillin, yield and selectivity must be taken into account. High yield is important, but selectivity must also be considered.

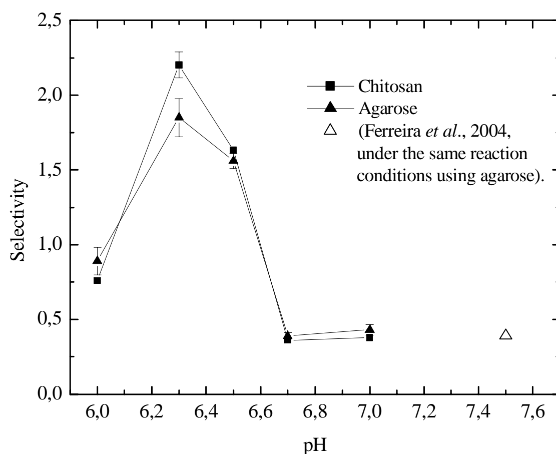

Figure 5. Selectivity as function of pH on the enzymatic synthesis of ampicillin (chitosan and agarose).

3.2. Stabilization of PGA via immobilization onto agarose and chitosan

The optimal conditions for the selectivity, yield, and stability of an enzyme may not happen at the same operational conditions. The immobilization of the enzyme may alter its catalytic properties and, consequently, enlarge the operational range of the biocatalysts. As mentioned previously, the synthesis of ampicillin is affected by simultaneous reactions, the hydrolyses of PGME and of the product itself. In order to inhibit the hydrolysis reactions, and consequently improve the ampicillin, two different supports were employed. PGA covalently immobilized displayed higher stability with respect to variations in pH than the soluble enzyme. Figure 6 shows the activity of immobilized PGA at different pHs. It is known that the highest activity of soluble PGA from *E. coli* occurs at pH 8.0 (Margolin *et al*., 1980; Böck *et al*., 1983; Pessina *et al*., 1988), and this activity decreases as pH decreases. Figure 6a shows that the stability of immobilized enzyme does not depend on the pH of solution. The immobilization of the enzyme onto agarose promoted only a slight decrease in its selectivity (see

Figure 5) while increasing the operational stability of the soluble enzyme. These results suggest that dissociation of the enzyme subunits may be responsible for the low stability of the soluble enzyme. Advantages of chitosan and agarose used like supports to immobilize enzymes have already been demonstrated (Pereira *et al.*, 2003; Fernandez-Lafuente *et al.*, 2001). Both supports have good stability, and their reaction courses have almost been identical. Moreover, they have promoted a good yield, suggesting that the immobilization has not altered PGA properties as a catalyst of kinetically controlled synthesis of ampicillin. The stabilized derivatives could be reused for 8 reaction cycles in the different conditions described in this paper without significant changes in their activities (See Figure 6b) or on the reaction yields.

The chitosan derivative presented a slight decrease in activity due to disruption of the beads during the last runs. Reactor agitation should be milder, and/or the preparation of chitosan should be changed to obtain a more resistant support. Furthermore, PGA immobilized on both matrices has long storage stability, keeping its activity after 30 days.

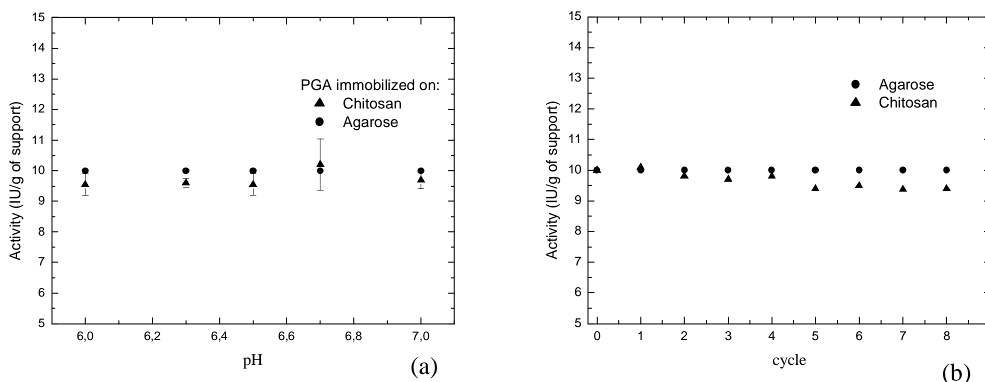

Figure 6. (a) Enzymatic activity of PGA from *E. coli* immobilized on chitosan and agarose *versus* pH. (b) Stability of PGA immobilized on chitosan and agarose at pH 6.5.

Conclusions

The main goals to make competitive the enzymatic synthesis of ampicillin are: to increase selectivity (lower losses of PGME caused by hydrolysis), and yield (better use of the β-lactam nucleus, 6-APA). In this context, we have obtained the best selectivity at pH 6.3, and the best yield at pH 6.5 for both supports. Among the pHs that were tested, we conclude that the best choice for the kinetically-controlled enzymatic synthesis of ampicillin is pH 6.5: maximum yield with an acceptable selectivity.

The use of appropriate biocatalysts certainly reduces impacts on process cost. With this aim, we combined immobilized enzyme with low pH to improve the yield of ampicillin synthesis: from 13 % (pH 6.0) to 42 % (pH 6.5), and selectivity from 0.4 to 1.85 for agarose, and from 12 % (pH 6.0) to 38 % (pH 6.5) and selectivity from 0.4 to 2.2 for chitosan.

It is noteworthy that the loading of 10 IU/g of support showed to be effective, resulting in a uniform enzyme fixation, with catalytic activity. The operational stability tests indicated that no enzyme deactivation occurred after eight batches and 30 days of storage, showing the success of the immobilization on chitosan and on agarose.

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