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STABILIZATION OF PENICILLIN G ACYLASE BY IMMOBILIZATION ON GLUTARALDEHYDE-ACTIVATED CHITOSAN

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Abstract - The objective of this work was to study enzyme immobilization on chitosan activated with glutaraldehyde, aiming to produce a cheap biocatalyst. Two different immobilization strategies were studied: one-point and multipoint covalent attachment to the solid matrix. The multipoint covalent attachment derivative had an 82% immobilization yield. It was 4.9-fold more stable than the free enzyme at 50°C and 4.5-fold more stable than soluble enzyme at pH 10.0. The one-point derivative had an 85% immobilization yield. It was 2.7-fold more stable than the free enzyme at 50°C and 3.8-fold more stable than soluble PGA at pH 10.0. Results indicated that chitosan can be loaded with PGA above 330 IU/g. Intraparticle diffusive effects, however, limited hydrolysis of penicillin G catalyzed by those derivatives at 37°C and 25°C. Operational stability assays were performed and the multipoint derivative exhibited a half-life of 40 hours.
Keywords: Stabilization of enzymes; Penicillin G acylase; Chitosan and immobilization of enzymes.

INTRODUCTION

The specificity of enzymes and their ability to catalyze reactions make them appealing for many applications in biochemical and industrial fields. One major advantage of this biocatalyst specificity is the achievement of products free of side products. However, the recovery and re-usability of free enzymes as catalysts are quite limited and this has resulted in the development of a wide variety of immobilization techniques. Immobilization also offers some other operational advantages over free enzymes, such as choice of batch or continuous process, rapid termination of reactions, controlled product formation, ease of removal from the reaction mixture and adaptability to a variety of engineering designs (Brahim et al., 2002). By careful selection of the matrix, it is also possible to vary the nature of the immobilized derivative in order to improve enzyme activity and stability and also to enable easier handling and storage (Mohy et al., 2000).

Penicillin G acylase is an important industrial enzyme used in the production of synthetic penicillins and cephalosporins, which remain the most widely used group of antibiotics. It catalyses the deacylation of penicillin G to produce 6-aminopenicillanic acid, an important intermediate in the manufacture of β-lactam antibiotics (Bianchi et al., 1996). Moreover, biotechnological processes for the large-scale production of synthetic antibiotics are focused on the condensation of the appropriate D-amino acid derivative with the β-lactam ring catalysed by penicillin G acylase (Arroyo et al., 2003).

Several supports used for PGA immobilization have been described. One of the most frequently

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used is agarose (Guisan et al., 1991; Guisan, 1987; Blanco et al., 1989). This support, however, is very expensive and its replacement by a cheaper one will result in a significant reduction in biocatalyst price and therefore in process costs. The support used in this work for PGA insolubilization and stabilization, chitosan, is one of the most abundant renewable polysaccharides, prepared from chitin by chemical N-deacetylation. Chitin, a polysaccharide containing 2-deoxy-2-amino glucose units linked by b-1,4 linkages, is obtained as a by-product of crustaceans, insects, marine crabs, etc. (Zeng and Zheng, 2002) and is much cheaper than agarose.

The various methods used for immobilization of penicillin acylase include adsorption, fibre entrapment, microencapsulation, cross-linking, copolymerization, and covalent attachment. In this work, a protocol to achieve stabilized derivatives by covalent immobilization of PGA on glutaraldehyde-activated chitosan is studied. Two covalent immobilization strategies were compared: one-point covalent attachment and multipoint covalent attachment. The derivatives formed by a one-point covalent bound have almost the same properties as the free enzyme and can be used as a standard to test activity/stability of the original enzyme tertiary structure. In this immobilization strategy, only one (or two) amine residue of the enzyme molecule is involved in the PGA-support covalent bound (Cardias et al., 1999).

It is generally accepted that a significant increase in enzyme stability will be achieved if the immobilization of each enzyme molecule occurs through several lysine residues, by multipoint attachment, mainly if the reactive groups in the support are secluded from its surface by short spacer arms. Therefore, all the residues of the enzyme molecule involved in immobilization have to preserve their relative positions almost completely unaffected during any conformational change produced by heat, organic solvents or any other distorting agent. Thus, these multipoint covalently immobilized enzymes should become much more stable than their soluble counterparts or than randomly immobilized derivatives (Guisan et al., 1991; Mateo et al., 2000). On the other hand, a high concentration of aldehyde groups on the support surface may resulted in several multipoint bounds between the enzyme and the matrix, distorting its three-dimensional structure and its active site. Therefore, control of the experimental conditions is critical to achieve a significant multipoint covalent attachment.

In this paper, we have addressed the possibilities of using chitosan to achieve PGA stabilization via covalent attachment, focusing our attention on the variables that determine the enzyme-support interaction.

MATERIALS AND METHODS

Materials

Powdered chitosan, 85.2 % deacetylation degree, was purchased from Polymar Ind Ltda, Fortaleza, Ceará. Penicillin G acylase from *E. coli* [E.C.3.5.1.11] with 489 IU/mL of enzyme activity, was the kind gift of Antibióticos S.A, Burgos, Spain. Penicillin G (Megapacilina) was purchased from Laboratório Itafarma, Brazil and p-dimethylaminobenzaldehyde (PDAB) was purchased from Sigma. All other reagents were of analytical grade.

Methods

- **Preparation and Activation of Chitosan Beads**

Chitosan beads were prepared by dissolving powdered chitosan in a solution of 5% acetic acid and glyoxal (40% v/v). The obtained solution was dropped into a gently stirred NaOH 1M solution. Next, the formed beads were treated with different glutaraldehyde concentrations, ranging from 1% to 9%, in 0.1M phosphate buffer at pH 8.0 and 28ºC for 30 minutes and washed with distilled water to remove the excess of activating agent.

- **Activity Assays of Penicillin G Acylase**

Enzyme activity was assessed via colorimetric analysis using p-dimethylaminobenzaldehyde (PDAB). The product, 6-aminopenicillanic acid (6-APA), released by hydrolysis of 5% penicillin G in 0.1M phosphate buffer at pH 8.0 and 37ºC, reacts with PDAB to yield a colored product, measured at 415nm. One IU corresponds to the amount of enzyme that hydrolyzes 1μmol of penicillin G (5% mass/volume) per minute at pH 8.0 and 37°C.

- **One-Point Covalent Immobilization of PGA on Glutaraldehyde-Activated Chitosan:**

PGA was immobilized on glutaraldehyde-activated chitosan following a previously developed methodology (Braun et al., 1989) that had been slightly modified. The enzyme was dissolved in 0.1M phosphate buffer at pH 8.0 and added to the

activated support (1g of beads to 10 mL of solution). The preparation was kept under gentle stirring at 28°C for 22 h.

- **Multipoint Covalent Immobilization of PGA on Glutaraldehyde-Activated Chitosan**

The multipoint covalent immobilization methodology was developed and has been studied in detail by different authors (Guisan et al., 1991; Guisan, 1987; Blanco et al., 1989; Cardias et al., 1999). The favourable conditions for multipoint covalent immobilization of PGA are established in the literature (Adriano et al., 2005). A solution of PGA in 100 mM bicarbonate buffer at pH 10, containing 100 mM phenyl acetic acid, was added to the activated support (2g of beads to 20 mL of solution). The preparation was kept under gentle stirring at 20°C for 8 h.

- **Enzyme Loading**

The enzyme loading of the derivatives was calculated by measuring the difference between enzyme activities of the supernatant (free enzyme) before (At_0) and after (At_t) immobilization. The immobilization yield (*IY*) was calculated with equation 1.

$$IY\% = \frac{At_0 - At_t}{At_0} \times 100 \quad (1)$$

- **Assays of Thermal Stability**

Free enzyme and immobilized derivatives were incubated in 0.1M phosphate buffer at pH 8.0 and 50ºC. Periodically, samples were taken and their residual activities were assayed as described above.

- **Alkaline pH Stability**

Free and immobilized PGA were incubated in 0.1M bicarbonate buffer solution at pH 10.0 and 25ºC. Residual activity was periodically measured.

- **Residual Activity and Stabilization Factor**

The residual activity is the percentage activity of the enzyme immobilized under standard conditions, taken as 100%. The stabilization factor is given as the ratio between the half-life of the immobilized derivative divided by the half-life of the free enzyme under the same conditions.

- **Recovered Activity and Coupling Yield**

In this work, recovered activity is defined as the enzyme activity of the immobilized derivative, measured by the hydrolysis of penicillin G with the immobilized enzyme. The coupling yield (η) was calculated as the ratio of the recovered activity (At_{deriv}) to the difference between enzyme activities of the supernatant (free enzyme) before (At_0) and after (At_t) immobilization (see equation 2).

$$\eta(\%) = \frac{At_{deriv}}{At_0 - At_t} \times 100 \quad (2)$$

- **Operational Stability of the Immobilized Enzyme**

The operational stability of the multipoint covalent immobilized PGA was assayed by using 3g of beads, containing 2.08IU/g in penicillin G hydrolysis in successive batches. The operational conditions were pH 6.5 and 25ºC, similar to the ones for amoxicillin synthesis. At the end of each batch, the immobilized PGA was removed from the reaction medium and washed with phosphate buffer to remove any remaining substrate or product and activities were assayed as described above. Then, the derivative was introduced into a fresh medium.

- **Physical and Chemical Characterization of Chitosan Beads**

Diameter and density of the chitosan beads were determined by picnometry and bead porosity was determined using a gravimetric method (Gonçalves et al., 1997). The chitosan degree of deacetylation was obtained by potentiometric titration (Raymond et al., 1993). Using the infrared method, the chemical characterization of activated chitosan has been done with a Nicolet Protege 460 FTIR spectrometer.

RESULTS AND DISCUSSION

Physical and Cchemical Characterization of Chitosan Beads

Glutaraldehyde is a very reactive substance and the use of increasing concentrations of it to activate a support may result in matrices with different internal structures. In this work, chitosan beads were prepared and treated with glutaraldehyde concentrations ranging from 1% to 9%, in order to study the effect of this

activating agent on the immobilized derivative. Table 1 shows the results of the physical characterization of the chitosan beads activated with different concentration of glutaraldehyde. It can observed that the increase in glutaraldehyde concentration appeared not to affect particle porosity, although activated beads showed a decrease in particle porosity when compared to non activated particles. This decrease was probably due to the cross-linking of the polymeric chitosan chains with glutaraldehyde that approached the chains and therefore decreased the pore sizes of the matrix. On the other hand, particle density (1.0023 ± 0.006 g/mL) and diameter (0.2084 ± 0.004 cm) did not change after activation, which means that 1% was sufficient to produce all the possible cross-linking.

Figure 1 depicts the ATR-FTIR spectrum of natural chitosan and 5% glutaraldehyde- activated chitosan. It can be observed that the intensity of the amine aliphatic band ($1100cm^{-1}$) decreased, which indicates the blocking of those groups by bounding to glutaraldehyde. Moreover, a band appearing at 1562 cm^{-1} indicates a C=C bound, which was probably a result of the resonance of the Schiffs basis formed between chitosan and glutaraldehyde. A band which can be observed at $1720cm^{-1}$ corresponds to a free aldehydic bound and water.

Chitosan beads were activated with different glutaraldehyde concentrations in order to obtain distinct quantities of reactive arm chains on the support surface. Figure 2 pictures the effect of glutaraldehyde concentration on the immobilization yield of penicillin G acylase on chitosan under conditions of one-point immobilization.

Table 1: Physical characterization of chitosan beads activated with different glutaraldehyde concentrations

$C_{glutaraldehyde}$ (%)	Density (g/ml)	Bead diameter (cm)	Porosity
1	1.0043±0.006	0.2042±0.004	0.809±0.001
3	1.0085±0.006	0.2050±0.004	0.805±0.001
5	1.0052±0.006	0.2116±0.004	0.816±0.001
7	1.0073±0.006	0.2084±0.004	0.800±0.001
9	1.0065±0.006	0.2128±0.004	0.815±0.001
Non activated beads	1.0023±0.006	0.2084±0.004	0.929±0.001

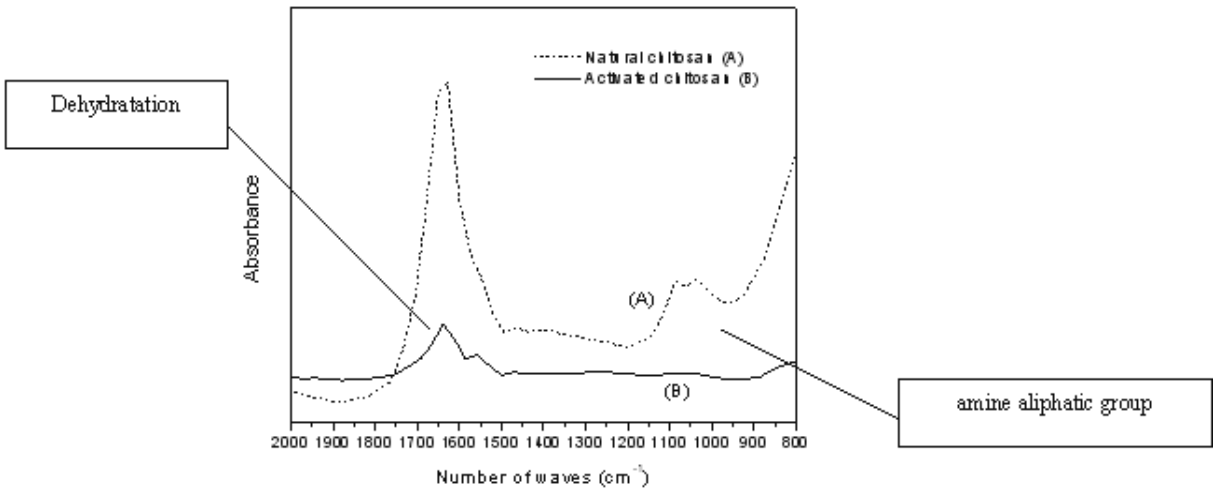


Figure 1: Natural and 5% glutaraldehyde-activated chitosan FTIR-ATR spectrum.

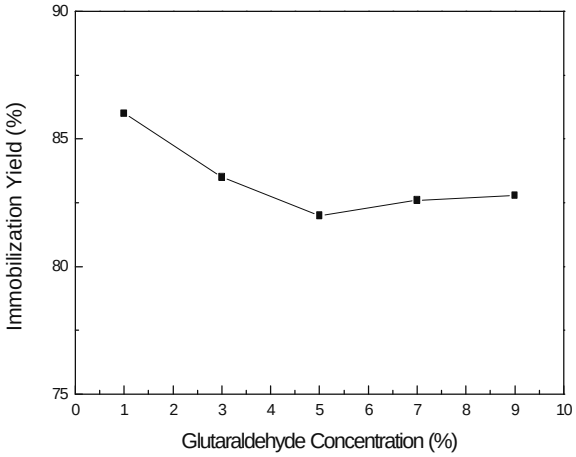


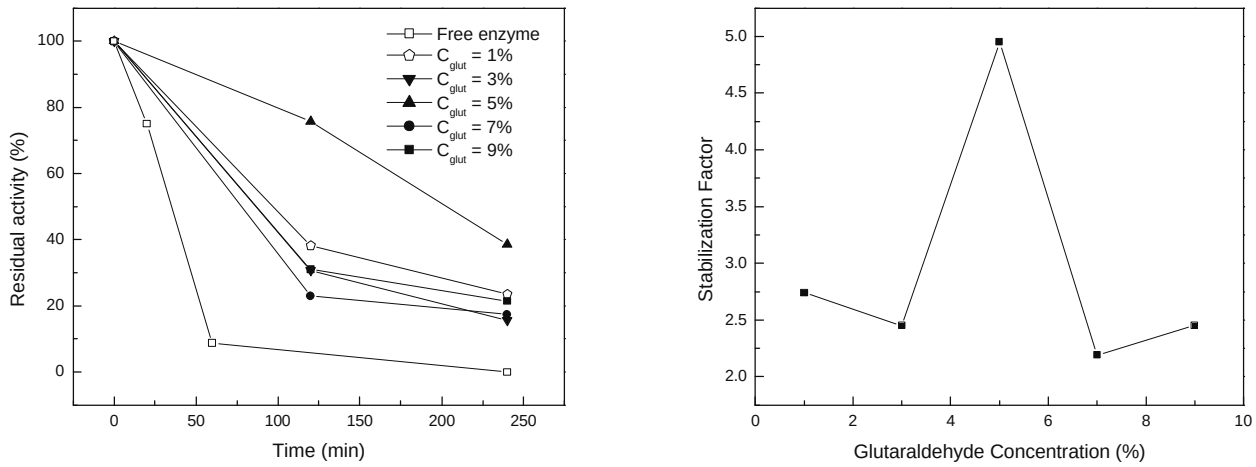
Figure 2: Immobilization yield of PGA on chitosan activated with different concentrations of glutaraldehyde with 10IU/g at 20°C and pH 10.0 during a period of 8 hours.

It can be noticed that by increasing glutaraldehyde concentration, a slight decrease in the yield was achieved. This indicates that 1% glutaraldehyde provided enough aldehyde groups on the surface of the support that were capable of bounding to PGA. Therefore, no improvement in immobilization yield (IY) was achieved by increasing activating agent concentration. However, IY is not the only parameter to be considered when developing immobilization protocols. Thermal and alkaline pH stability must also be addressed.

Although glutaraldehyde concentration did not affect immobilization yield, thermal and alkaline stability of the derivatives were affected by this parameter, as can be observed in Figures 3 and 4.

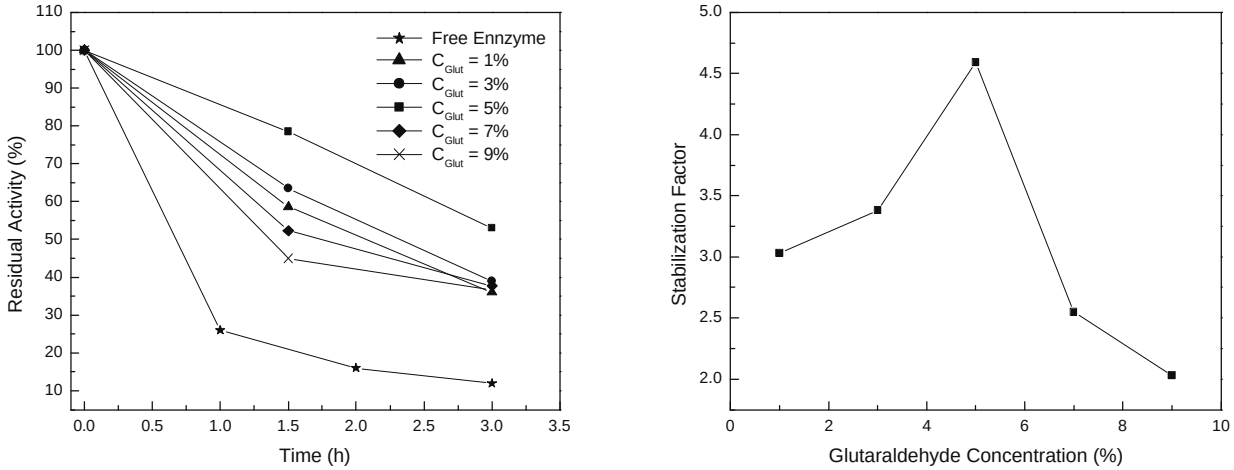
It can be seen that the stability of the immobilized enzyme increased with degree of activation of the support (Figures 3 and 4) up to a 5% glutaraldehyde concentration. For higher concentrations, the derivatives lost a very large percentage of their catalytic activity as a consequence of multi-point interaction with the activated support, which appears to threaten the preservation of the active enzyme conformation. The side reactions that occur between the support and the enzyme may form hydrophobic by-products, which make it more difficult to achieve the correct alignment of the reactive groups (Pereira, 1996).

Within the experimental range tested, the best results were achieved when 5% glutaraldehyde was used to activate the chitosan beads. Similar results were obtained by other authors (Cardias et al., 1999), who studied PGA immobilization on silica. The residual activity obtained by those authors after 2h was around 70%, which is close to the residual activity observed in the present work (Figure 3).



(a) (b)

Figure 3: Thermal stability of PGA at 50°C: a) residual activity over time for free and immobilized enzyme on chitosan treated with different glutaraldehyde concentrations (C_{glut}) at 20°C and pH 10 and b) stabilization factor as a function of glutaraldehyde concentration.



(a) (b)

Figure 4: Alkaline pH stability of PGA (at pH 10): a) residual activity over time for free and immobilized enzyme on chitosan treated with different glutaraldehyde concentrations (C_{glut}) at 20°C and pH 10 and b) stabilization factor as a function of glutaraldehyde concentration.

Effect of Enzyme Concentration on Immobilization Yield

Chitosan was activated with 5% glutaraldehyde and the glutaraldehyde-activated chitosan matrix was added to different amounts of enzyme, ranging from 9.78IU to 300IU at pH 10.0 and 20°C during 30 hours. Figure 5 shows the immobilization yield during the process of attachment of the enzyme to the support. It can be observed that for all enzyme loadings tested, the same immobilization yield of around 82% was achieved. However, results shown in Figure 6 indicate that the support was not saturated and therefore more enzyme molecules could have been immobilized if longer immobilization times had been used. As the immobilization reaction rate depended on the enzyme concentration, the concentration of enzyme remaining in the supernatant after 82% immobilization was lower. This implies the need for immobilization times longer than 30 hours to achieve yields higher than 82%. In addition, when larger of enzyme amounts were used another phenomenon occurred. For a fixed time, for instance 8h, a significant decrease in immobilization yield was obtained when the enzyme concentration in solution was increased. This behaviour may be credited to a combination of diffusion and exclusion effects in the vicinity of the pore entrance. Enzyme molecules diffusing from the bulk solution into the pores reacted primarily with the outer aldehydic chains, simply because those were the first chains available to the enzyme, making the other aldehydic groups in the inner pore space less accessible for linkage. When highly activated chitosan remained in contact with a large amount of enzyme, immobilization was very fast, causing a partial or total blockage of the pore entrance, which would affect the immobilization yield and kinetics. This phenomenon was enhanced when the support was activated with glutaraldehyde because of the long arm chains formed between the enzyme and the matrix (Cardias et al., 1999).

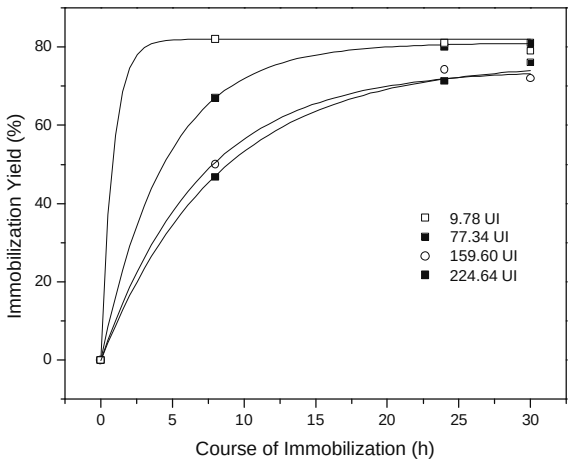


Figure 5: The effect of initial enzyme concentration on immobilization yield.

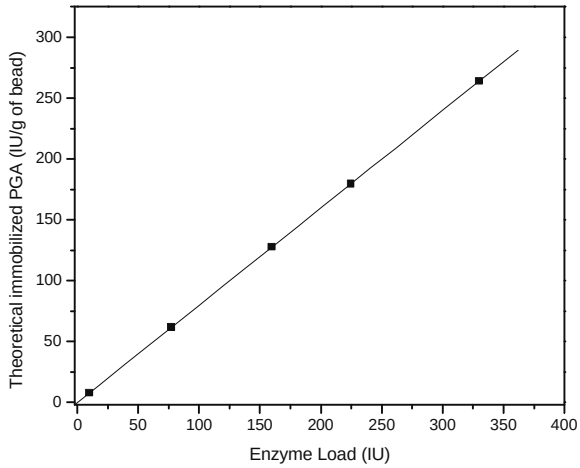


Figure 6: Theoretical immobilized PGA (IU/g bead) as a function of enzyme load. Immobilization time 30 hours, support glutaraldehyde- activated chitosan.

Figure 7 shows the effects of enzyme loading on the apparent coupling yield (η). It can be observed that η decreased with increasing concentration of enzyme added to the support during immobilization. When recovered activity of the immobilized derivative is analyzed (Figure 7), it can be seen that it initially increased with increasing enzyme loading added to the support. Nevertheless, for higher enzyme concentration it remained constant. This behaviour occurred due to diffusional limitations in the chitosan pores, when substrate was prevented from reaching PGA active sites. Figure 8 shows effectiveness of the reaction used to measure enzyme activity (relation between the apparent immobilized activity and the theoretical immobilized PGA) as a function of the theoretical immobilized PGA (disappearance of the supernant) at two temperatures. It can be observed that the effectiveness of the reaction decreased as the theoretical immobilized enzyme increased for the two temperatures studied. This can be explained by diffusion effects: if the apparent enzyme activity is limited by the rate of mass transfer, the higher the theoretical immobilized enzyme, the higher the intrinsic reaction velocity and, consequently, the more severe the diffusion effect for both temperatures. However, this effect was more pronounced at the highest temperature. This can be explained by the different effects of temperature on the molecular diffusivity of substances and on the reaction velocity. The former has a linear (Guisan et al., 1991) and the latter has an exponential dependence on temperature, in accordance with the Arrhenius law. Therefore, although a high enzyme load could not be measured under reaction conditions used in this work, in industrial operation it is important to use a support with a high loading. A high theoretical load would imply an apparently high operational stability of the biocatalyst: the theoretical immobilized enzyme deactivates, but the apparent activity remains constant, allowing steady reactor operation.

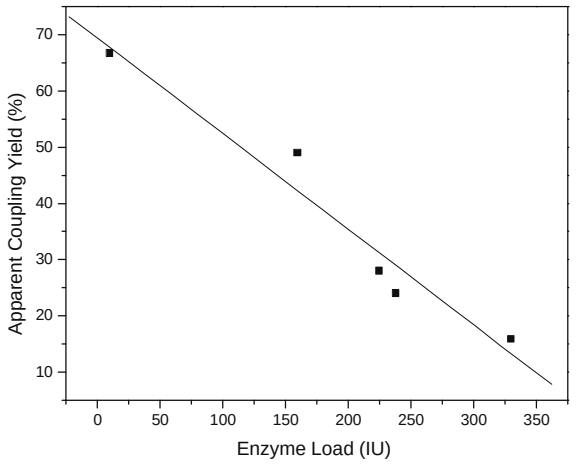


Figure 7: The effect of enzyme concentration during immobilization on the apparent coupling yield (η).

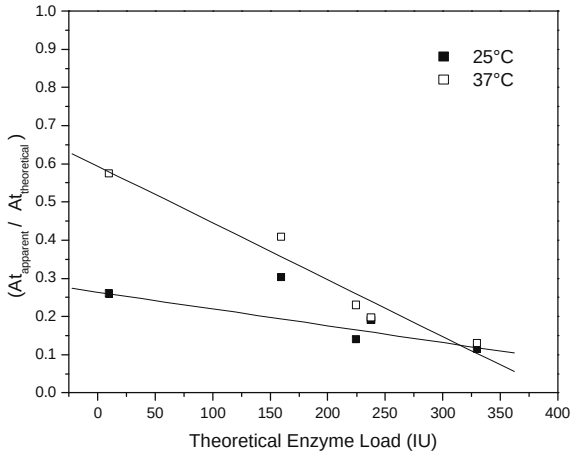


Figure 8: Relationship between apparent and theoretical immobilized PGA as a function of the theoretical enzyme load at two temperatures.

In this work, two immobilization strategies were compared: one-point and multipoint covalent attachments of the enzyme to the matrix. Table 2 shows the obtained results for immobilization yield (IY), recovered activity and stabilization factor at 50ºC and pH 10. Stability studies were carried out using derivatives containing small amount of enzyme. This condition resulted in slow reaction rates, minimizing the effects of diffusion. Figures 9 and 10 show the obtained results for thermal stability at 50ºC and stability of the free enzyme and the one-point and the multipoint covalent derivatives at an alkaline of pH 10.0.

Table 2: Immobilization parameters for penicillin G acylase: one-point and multipoint covalent attachment on glutaraldehyde-activated chitosan.

Immobilization Strategy	Immobilization Yield (%)	Coupling Yield (%)	FE (50°C)	FE (pH 10)
One-point	85	85	2.7	3.8
Multipoint	82	70	4.9	4.5

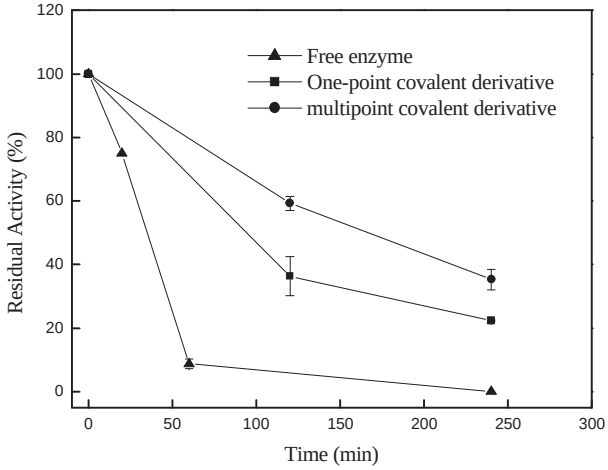


Figure 9: Thermal stability at 50ºC for free and immobilized penicillin G acylase on glutaraldehyde-activated chitosan by one-point and multipoint covalent attachment.

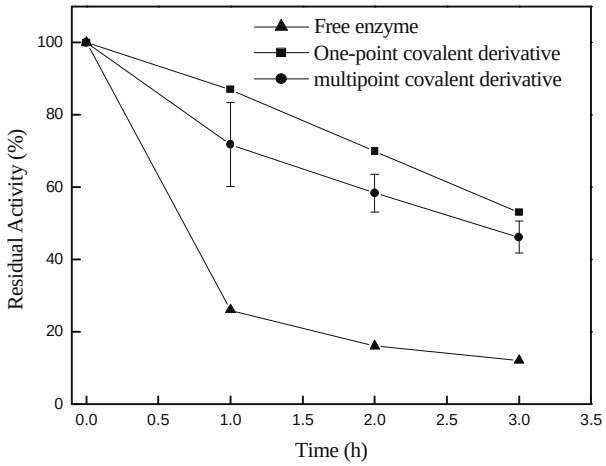


Figure 10: Stability at alkaline pH (pH 10) for free and immobilized penicillin G acylase on glutaraldehyde-activated chitosan by one-point and multipoint covalent attachment.

Similar results for coupling yield were obtained by other authors when studying the immobilization of PGA on chitosan by one-point covalent attachment, η = 86% (Braun et al., 1989). When thermal stability was concerned (Figure 8), it can be seen that immobilization strategy had an effect on the obtained results, since multipoint derivatives were more stable. This was caused by additional bounds between the enzyme and the matrix during the immobilization process, resulting in a more stable derivative (Cardias et al., 1999). On the other hand, when stability at an alkaline pH was considered (Figure 9), the immobilization strategy had little effect on biocatalyst stability. Nevertheless, both derivatives (one-point and multipoint) were much more stable than the soluble PGA, which was completely inactivated in less than 3h (Figures 8 and 9).

Biocatalyst Operational Stability

The operational stability of the multipoint covalent derivative was studied in a batch reactor and results are shown in Figure 10. Under the studied conditions, activity dropped to 50% of its initial value after 20 cycles. Therefore, the half-life of the catalyst can be determined to be 20 cycles or 40h of operation.

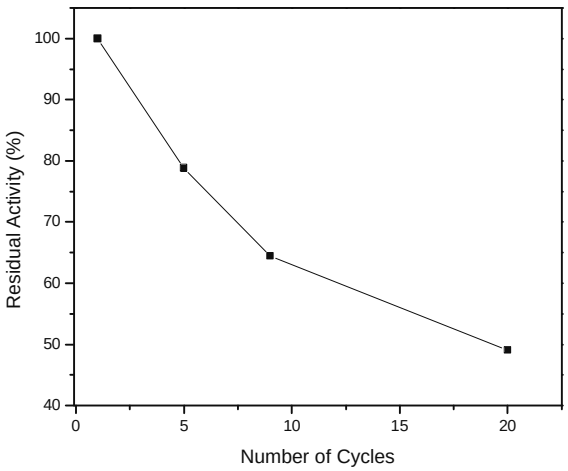


Figure 11: Operational stability of PGA immobilized on glutaraldehyde-activated chitosan during penicillin G hydrolysis at 25°C and pH 6.5.

CONCLUSIONS

In general the PGA immobilized on glutaraldehyde-activated chitosan (one-point and multipoint covalent attached to the matrix) showed significant advantages over free enzyme. The temperature, pH and operational stability of the immobilized enzyme were much better than those of the free enzyme. The obtained results allow the conclusion to be drawn that the multipoint derivative, activated with a 5% glutaraldehyde concentration, provided the best results when thermal and alkaline pH stabilities were analyzed. Moreover, the carrier is very cheap and the process is very simple, which makes it good for large-scale industrial applications.

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