IMMOBILIZATION OF PROTEASE EXTRACTED FROM *Bacillus* sp. P45 ON DIFFERENT SUPPORTS

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ABSTRACT

Immobilization is a direct tool which not only improves the activity and stability of enzymes, but also enables their reuse. Therefore, selecting methods and supports to obtain biocatalysts with high selectivity and thermal stability is an important step. This study aimed at evaluating the immobilization of partially purified protease from *Bacillus* sp. P45, by different methods with the use of the following carriers: Amberlite IR® 120, montmorillonite clay, chitosan, glutaraldehyde-activated chitosan, Eupergit® C, DEAE-Cellulose® and QAE-Sephadex®. It also aimed at determining the operational stability of the derivative. The best results were obtained with the use of glutaraldehyde-activated chitosan, Amberlite IR® 120 and montmorillonite clay, with loading capacities of 25.4, 6.2 and 2.3 U/g support, respectively. Regarding the operational stability of the derivative glutaraldehyde-activated chitosan, the enzyme was found to keep 53.5% of its residual activity after being reused four times.

KEY WORDS: ENZYME. CARRIERS. LOADING CAPACITY. REUSE.

IMOBILIZAÇÃO DE PROTEASE DE *Bacillus* sp. P45 EM DIFERENTES SUPORTES

RESUMO

Imobilização é uma ferramenta interessante para melhorar a atividade e a estabilidade de enzimas, permitindo sua reutilização. Desta forma, a seleção de métodos e suportes que permitam obter biocatalisadores, com elevada seletividade e estabilidade térmica é uma etapa importante. O objetivo desse estudo foi avaliar a imobilização de protease parcialmente purificada obtida a partir do cultivo submerso de *Bacillus* sp. P45, avaliando diferentes métodos através dos suportes: Amberlite IR® 120, argila montmorilonita, quitosana, quitosana ativada com glutaraldeído, Eupergit® C, DEAE-Cellulose® e QAE-Sephadex®, bem como determinar a estabilidade operacional do derivado. Os melhores resultados foram obtidos utilizando-se os suportes quitosana ativada com glutaraldeído, Amberlite IR® 120 e argila montmorilonita, com capacidades de adsorção de 25,4, 6,2 e 2,3 U/g de suporte, respectivamente. Já em relação a estabilidade operacional do derivado quitosana ativada com glutaraldeído, foi verificado que a enzima manteve 53,5% de sua atividade enzimática residual após quatro ciclos de reuso.

PALAVRAS-CHAVE: ENZIMA. MATRIZES. CAPACIDADE DE ADSORÇÃO. REUSO.

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1. INTRODUCTION

Enzymes have been successfully studied as catalysts in food [21,26], pharmaceuticals [3], agrochemicals and chemical synthesis of organic compounds [16,28]. They act under moderate conditions of temperature, pressure and pH, with reaction rates on the order of chemical catalysts under extreme conditions [34]. Although enzymes have been widely used in industrial processes, their application can be limited by certain factors, such as high cost, catalytic instability and difficult recovery at the end of industrial processes. Many restrictions can be avoided by using immobilized enzymes, since they make catalysis more advantageous by comparison with the use of free enzymes [25,29].

Immobilization processes enable enzymes to attach to the support by interactions through physical adsorption, ionic, covalent or inclusion bonds. Properties of the immobilized enzyme are determined by characteristics of the enzyme and the carrier material. Specific interaction between the enzyme and the support promotes immobilization with different chemical, biochemical, mechanical and kinetic properties [30].

Industrialized enzymes, such as proteases, represent a significant economic import, since they correspond to 60-65% of the global market [32]. Proteases have been used in food processing, such as meats [23], bread and dairy products [17,22], in detergent production [20], in the pharmaceutical industry [19], in leather [13], in the synthesis of protein hydrolysates and in the production of bioactive peptides [2,7,10].

Therefore, the aim of this study is to evaluate the immobilization of protease from *Bacillus* sp. (Amberlite IR® 120, montmorillonite clay, chitosan, glutaraldehyde-activated chitosan, Eupergit® C, DEAE-Cellulose® and QAE-Sephadex®) and the operational stability of the immobilized derivative, which showed the highest adsorption capacity.

2. MATERIAL AND METHODS

2.1 Microorganism, inoculum, cultivation and enzyme purification

Bacillus sp. P45 (GenBank accession number AY962474), maintained on brain-heart agar (BHA) at 4ºC, was used to produce the enzyme. For inoculum preparation, this strain was grown on BHA at 30ºC for 24 h. The cultures were scraped from the agar surface, added to a sterile 0.85% (w/v) NaCl solution, and mixed until a

homogeneous suspension with O.D.600 of 0.5 was obtained. The enzyme was produced by submerged cultivation using feather meal as substrate as described by DAROIT, CORRÊA and BRANDELLI [11]. After cultivation, the culture was then clarified by centrifugation (5000 × g for 20 min) and the supernatant containing the enzyme was used in the purification.

The enzyme was partially purified from a strategy determined by SALA et al. [24], which consisted of an aqueous two-phase system integrated into the diafiltration process. The biphasic aqueous system was composed (w /w) by 3% polyethylene glycol (PEG) 1500 Da, 23% potassium phosphate (pH 7.0), 8% sodium chloride (NaCl), 20% enzymatic extract and 46% deionized water. The second biphasic aqueous system was formed by the addition (w /w) of the top phase from the first biphasic aqueous system (36%), 0.1 mol/L Tris-HCl buffer pH 7.0 (36%) and ammonium sulfate. Removal of PEG was performed by diafiltration using 10 kDa membrane of regenerated cellulose, 5 cycles of diafiltration, pressure of 1.5 kgf /cm^2 at 15ºC. The purified enzyme was lyophilized and stored at 4ºC.

2.2 Enzyme immobilization

The partially purified protease was immobilized by seven different methodologies so that the adsorption capacity of the enzyme on the support were evaluated.

2.2.1 Immobilization on Amberlite IR® 120

4 g cation resin was equilibrated with Tris-HCl buffer (0.1 mol/L, pH 7.0) and incubated with 20 mL enzyme extract prepared in the same buffer at 10ºC for 8 h. Unbound enzymes were removed by washing them with the same buffer (adapted from ABDEL-NABY et al. [1]).

2.2.2 Immobilization on montmorillonite clay

2 g carrier was added to 25 mL enzyme extract prepared with Tris-HCl buffer (0.1 mol/L, pH 7.0). The solution was kept under stirring at 10°C for a 2 h reaction period. The solution was then vacuum filtered (adapted from BRAGA et al. [6]).

2.2.3 Immobilization on chitosan

2 g carrier was added to 25 mL enzyme extract prepared with Tris-HCl buffer (0.1 mol/L, pH 7.0) and kept under stirring at 10°C for 12 h. Unbound enzymes were removed by washing them with the same buffer (adapted from ABDEL-NABY et al. [1]).

2.2.4 Immobilization on glutaraldehyde-activated chitosan beads

Preparation of chitosan beads: chitosan (2 g) was dissolved in 100 mL 1.5% (v/v) acetic acid and heated at 60ºC for 1 h at 150 rpm. The resulting viscous solution was submitted to ultrasound for 30 min, and then sprayed drop-wise through a syringe, at constant rate, into a 1 mol/L KOH solution. Resulting beads were washed with Milli Q water – to reach a neutral pH – and stored at 4ºC with Tris-HCl buffer (0.1 mol/L, pH 8.0) until activation with glutaraldehyde.

Activation of chitosan beads: chitosan beads were incubated at 30ºC and 100 rpm with 50 mL 40 g/L glutaraldehyde for 10 h. After activation, beads were washed with Tris-HCl buffer (0.1 mol/L, pH 8.0) to have the excess of glutaraldehyde removed and stored at 4ºC in the same buffer until further use. For immobilization of the enzyme, 1 g chitosan beads was incubated with 25 mL enzyme extract prepared with Tris-HCl buffer (0,1 mol/L, pH 8.0) for 12 h at 10°C. After incubation, beads were washed with the same buffer to remove unbound enzymes and stored at 4°C (adapted from SILVEIRA et al. [27]).

2.2.5 Immobilization on Eupergit® C

0.8 g carrier was added to 20 mL enzyme extract prepared with Tris-HCl buffer (2 mol/L, pH 7.0). The solution was kept under stirring at 10°C, for a 12 h reaction period. The solution was then vacuum filtered (adapted from BRAGA et al. [6]).

2.2.6 Immobilization on DEAE-Cellulose®:

4 g anionic resin was equilibrated with Tris-HCl buffer (0.1 mol/L, pH 8.0) and added to 20 mL enzyme extract prepared in the same buffer. The solution was kept under stirring at 4°C for a 12 h reaction period (adapted from FARAG and HASSAN [12]).

2.2.7 Immobilization on QAE-Sephadex®

4 g anionic resin was equilibrated with Tris-HCl buffer (0.1 mol/L, pH 8.0) and added to 20 mL enzyme extract prepared in the same buffer. The solution was kept under stirring at 4°C for a 12 h reaction period (adapted from FARAG and HASSAN [12]).

2.3 Proteolytic activity and loading capacity of immobilized enzymes

Azocasein was used as substrate for the determination of proteolytic activity, in agreement with DAROIT, CORREA and BRANDELLI [11]. A unit of proteolytic activity (U) is defined as the amount of enzyme required to cause an increase in 0.1 unit of absorbance under the conditions of the assay. Control assays, without the addition of supports, were performed under the same treatment conditions to quantify enzyme activity during the reaction.

The adsorption capacity of the immobilized enzyme, defined by the active enzyme (U) effectively bound to the support, was determined by measuring enzyme activity in a reaction medium composed of azocasein with Tris-HCl buffer (0.1 mol/L, pH 8.0) and immobilized derivative (in g). The adsorption capacity was expressed as the amount of enzyme immobilized on the U/g support.

2.4 Operational stability of immobilized enzymes

Reuse of the selected derivative was monitored by checking changes in the activity of the immobilized protease. It was repeatedly used in the enzyme reaction at 30°C; azocasein was the substrate. After each cycle, the immobilized enzyme was filtered, washed in the same buffer and reintroduced into a fresh reaction medium.

2.5 Statistical analysis

Data were subjected to analysis of variance to detect significant differences between treatments by the Tukey's test. Differences were considered significant when $p < 0.05$.

3. RESULTS AND DISCUSSION

Figure 1 shows the derivatives immobilized on the following supports: Amberlite IR® 120, montmorillonite clay and glutaraldehyde-activated chitosan.

The supports Eupergit® C, DEAE-Cellulose® and QAE-Sephadex® showed no capacity to adsorb on the enzyme. The results of the other supports under evaluation are shown in Table 1. Regarding protease immobilization, the most promising result was obtained with the use of chitosan activated with glutaraldehyde, whose enzyme activity was 25.4 U/g support. This value is four-fold higher than the second-best result, i.e., Amberlite IR® 120.

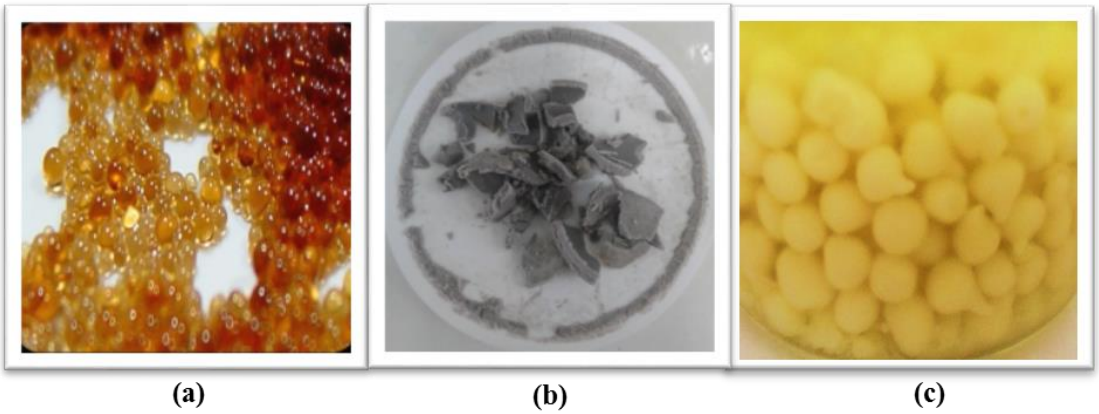

FIGURE 1. Immobilization of protease extracted from *Bacillus* sp. P45 on (a) Amberlite IR® 120, (b) montmorillonite clay and (c) glutaraldehyde-activated chitosan

TABLE 1. Immobilization of partially purified protease from *Bacillus* sp. P45

Carrier	Loading capacity of immobilized enzyme (U/g support)
Amberlite IR® 120	6.2 ± 0,13[b]
Montmorillonite clay	2.3 ± 0,29[c]
Chitosan	1.6 ± 0.07[d]
Glutaraldehyde-activated chitosan	25.4 ± 0,24[a]
Eupergit® C	-
DEAE-Cellulose®	-
QAE-Sephadex®	-

*The same letters in the columns represent no significant differences at 95% significance level.

The technique of immobilization by physical adsorption is considered a simple technique, which involves ionic interactions, hydrogen bonds and hydrophobic interactions between the support and the enzyme [15,18]. However, the results of both supports, montmorillonite clay and chitosan, showed low capacities of adsorption (2.3 and 1.6 U/g support, respectively). This may have occurred due to the fact that these

interactions are reversible and superficial, leading to desorption and causing significant losses in the enzyme activity of the immobilized derivative [15].

The use of cationic resin Amberlite IR® 120 showed immobilization with enzyme activity of 6.2 U/g support. ABDEL-NABY et al. [1] found similar behavior in the immobilization of protease from *Bacillus mycoides* on DEAE-Cellulose, Dowex 1x4 and Eceola-Cellulose resins (5.9, 8.7 and 8.8 U/g support, respectively). The ionic adsorption technique involves parameters that depend on the competition among the ions of the bioproduct and the groups loaded of the carrier. When these parameters are analyzed, enzyme immobilization may be favored. In addition, the isoelectric point (pI) of the enzyme, as well as the pH of the buffer used for immobilization, must be observed since it must be adjusted to favor the enzyme adsorption on support. The result found by this immobilization technique can be explained by the fact that the pH of the reaction medium buffer (7.0) is above the pI estimated for protease produced by *Bacillus* (6.3), i.e., the enzyme may have had negative liquid charge throughout the immobilization process [33]. The use of a cationic support may have resulted in the release of the enzyme on the carrier during the process.

Furthermore, when ion-exchange resins are used in immobilization processes, the reaction time may influence enzyme kinetics. Long periods of immobilization may result in the displacement of the enzyme on the support and lead to low performance in the process.

Finally, immobilization by covalent bonding through a spacer group (glutaraldehyde and chitosan) showed the highest adsorption capacity of the enzyme (25.4 U/g). The introduction of a spacer contributes to the hydrophilization of the microenvironment of the enzyme and causes increase in the stability of the catalyst. Since the enzyme activity depends on the maintenance of the flexible conformation of the molecule, the introduction of a spacer between the enzyme and the surface of the charger is used for reducing the loss of activity due to immobilization [8,14].

Several authors have reported that the use of the covalent bond with the spacer group in enzyme immobilization processes is a successful technique [5,9]. However, most data found in the literature refer only to the immobilization of other enzymes, whereas studies of protease are still scarce. BHANDARI, GUPTA and SINGH [4] observed improved enzyme stability of a purified protease immobilized on chitosan and glutaraldehyde, which produced a biocatalyst with better characteristics and operational stability than the one produced by a soluble enzyme.

Based on the results of the adsorption capacity of enzymes on support, the operational stability of the best immobilization technique (chitosan activated with glutaraldehyde) was evaluated. The results of the reuse are shown in Figure 2.

The possibility of reusing immobilized protease is an important feature for industrial applications. Thus, to demonstrate the operational stability of the immobilized enzyme, repeated cycles of hydrolysis were performed with the same immobilized beads. The immobilized derivative kept 53.5% of its residual activity after four cycles of use. This result can confirm that the immobilized enzyme has stability and can be used consecutively in industrial processes. The immobilization technique is promising, even though further studies involving kinetic parameters should be carried out in order to maximize and improve the immobilization process.

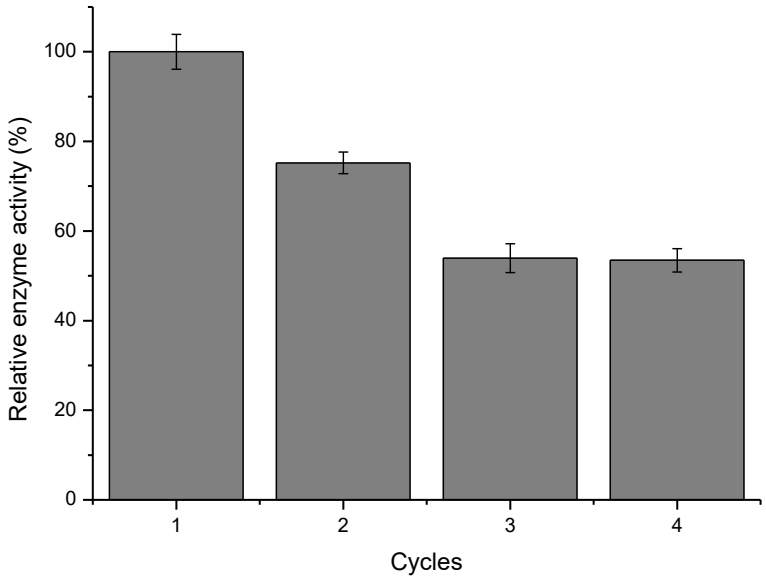

FIGURE 2. Operational stability of *Bacillus* sp. P45 immobilized on glutaraldehyde-activated chitosan

VIEIRA [31] found distinct behavior when immobilizing the β-galactosidase enzyme in glutaraldehyde-activated chitosan and observed the loss of 17% in the enzyme activity at the end of the fourth cycle. SILVEIRA et al. [27] reported similar results to the ones found by this study, i.e., the immobilization of a protease from

Chryseobacterium sp. Kr6 on chitosan and glutaraldehyde had 5-cycle operational stability, remained with 63.4% in its initial activity.

4. CONCLUSION

Among the different techniques and supports used for promoting the immobilization of *Bacillus* sp. P45, the best results were obtained by the support chitosan activated with glutaraldehyde, whose adsorption capacity was 25.4 U/g support, had 4-cycle operational stability and kept 53.5% of its residual enzyme activity, thus showing that the immobilized protease may be an alternative to industrial applications.

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