



Easy and Fast Method of *Candida antarctica* B Lipase Immobilization in Polyurethane Foam

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ABSTRACT

In this study, we present a novel method to achieve *Candida antarctica* B lipase immobilization on polyurethane (PU) foam. The PU was an extremely simple support to be prepared and, the enzyme immobilization occurs simultaneously with the polymerization, leading <5 min. Thus, the enzyme distribution was of 100% by irreversible binding in the PU support matrix. The immobilization process provided a high yield of 250% in the enzymatic catalytic activity compared to the free process. The immobilized presented recycles 35 of reuse, with the higher residual activity of 80% in all cycles. In this way, this process provides an important technique associated with low-cost of the support, simple procedure, and possibility of being employed as a catalyst in reactions for the food industry.

Key words: Immobilization, Polyurethane, Lipase *Candida antarctica* B.

1. INTRODUCTION

Lipases (triacylglycerol ester hydrolase, EC 3.1.1.3) are between the enzymes the most commonly used in biocatalyst. Also present a wide variety of aminolysis reactions, acidolysis, alcoholysis, lactonization, hydrolysis, esterification, transesterification, interesterification and low environmental impact [1].

In addition, play an important role in industrial applications such as food industry (additives), fine chemistry (synthesis of esters), detergents (hydrolysis of fats), wastewater treatment (decomposition and removal of oily substances), cosmetics (removal lipids), pharmaceuticals (digestion of fats and oils in foods), leather processing (removal of lipids from animal skins), biomedical testing (triglycerides in the blood), bioenergy (especially in the biodiesel production) [2,3].

Thus, the enzyme immobilization techniques appear as alternative to solve the inconvenient mentioned above, allowing the enzyme recovery and reuse, with the displacement in pH value, adjustable porosity, low toxicity, favorable mechanical properties, temperature, and stability (thermal, operational and storage), allowing the biotechnology process becomes economically viable [3,4].

Furthermore, lipase B from *Candida antarctica* (CALB) has been recognized as an efficient biocatalyst with large capacity of catalyze reactions in aqueous and organic medium, where the water content is limited, resulting in a wide potential of application, presenting stability at temperature, pH and organic solvents and have chemo- and region-antiosseletivity [2,5]. Polyurethane (PU) polymer composed of a chain of organic units joined by urethane bonds, being used in many fields, as promising supports for enzymes, mainly in organic media reactions (resistance to oils, fats and solvents) [4]. Their properties are greatly influenced by the types of isocyanates and polyols, such as functional groups, thus, being affected by the polymerization process, resistance, density, cross-linking, flexibility, and high performance [6].

In the literature small number of studies reported *in situ* process (direct immobilize the enzyme onto support), or entrapment the enzyme in porous polymeric matrix structure (PU), which may occur interactions that promote the catalytic activity of the enzyme. Moreover, reduced enzyme escape, pore size control, amount maintenance of cells, application in a large scale at low cost, ease interaction between the enzyme and support in organic medium reactions and ease separation of reaction products [2,7]. In this sense, this study aims to evaluate *in situ* immobilization of

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lipase CALB using rigid PU foam as immobilization support.

2. EXPERIMENTAL

2.1 Materials

2.1.1. Enzyme

The enzyme used was lipase from CALB (Novozyme NZL-102-LYO-HQ) obtained in lyophilized form, from Novozymes Latin América LTDA.

2.1.2. Support

The commercial polyol and isocyanate monomers used in this work were produced for a specific formulation for mattresses and foam injected by the Company Flexible PUs - Mannes.

2.2. Methods

2.2.1. Monomers polymerization

The *in situ* polymerization procedure was carried out to obtain the support; this method was adapted by Silva *et al.*, [8]. For PU preparation was evaluated different proportions of polyol-isocyanate monomers (10/90; 20/80; 30/70; 40/60; 50/50; 60/40; 70/30; 80/20 and 90/10 v/v).

In situ polymerization was conducted with addition of monomers, using a graduate syringe. After they have been mixed and homogenized with a glass rod in an ice bath for 30 s. Subsequently, the reaction occurred within 5 min, by the foam expansion and complete solidification, observing the conformation flexibility, softness, strength, porosity, and internal resistance [7].

2.2.2. Lipase CALB immobilization on PU foam

The immobilization of lipase CALB on PU foam was performed using 60-40% v/v of monomers proportion, with 0.8 ml of enzymatic solution (0.8 g enzyme in 5 ml of solvent). Enzyme solutions were prepared using a different solvent (ethanol, acetone, and water). This immobilization method consists in entrapment, where the formation occurs on the porous structure, with the enzyme presence, entrapment in a three-dimensional structure, performing the protein "encapsulation" in insoluble polymer, resulting in immobilized biocatalyst [2,9].

To evaluate the enzyme distribution on the support, after polymerization, the structure formed was divided vertically into two parts (1 and 2) and horizontally in three portions (A, B, and C) (Figure 1). Which were submitted separately to quantify enzyme activity and to identify the distribution on the support (PU).

2.2.3. Immobilized enzyme stability in hydrogen peroxide

To evaluate the stability of the immobilized enzyme in hydrogen peroxide, immobilized enzyme on PU

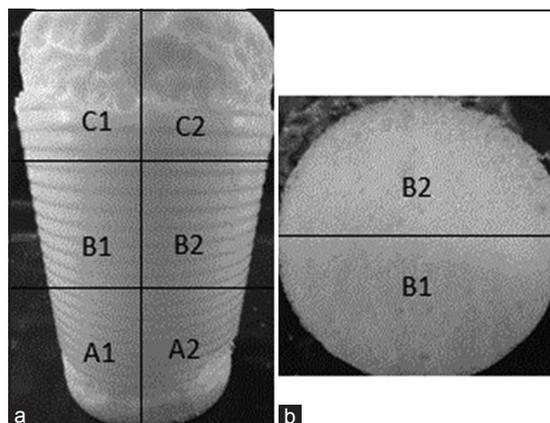


Figure 1: Polyurethane foam structure formed with immobilized *Candida antarctica* B, considering in, (a) External view of the division in three portions of the polymer (A, B, and C), and (b) horizontally division in two parts (B1 and B2).

(500 mg) was incubated in the presence of hydrogen peroxide with different molarities (1 M, 5 M and 10 M) at 10 and 25°C. The solution pH has not been adjusted; however, was observed a variation 3.8-4.3 of values, depending on the hydrogen peroxide concentration.

Samples were collected periodically, filtered, kept in an oven at 40°C for 30 min to evaporate the solvent and then carried out the measurement of esterification activity. The experiments were performed in triplicate and results are shown as means of residual activity [10,11,12].

2.2.4. Reuse of immobilized lipase

The main interest in immobilizing enzymes is to promote the reuse. Thus, the capacity of reactional recycles in the synthesis of ethyl oleate (oleic acid and ethanol) with immobilized lipase CALB try to simulate the reuse of the biocatalyst in reactions of interest were evaluated.

This study was conducted storing the immobilized (dry and reaction medium) at different temperatures to quantify the activity. For the dry condition, after each reaction the excess was withdraw of the reaction medium (oleic acid and ethanol) by centrifugation (2000 rpm for 5 min), where the supernatant was removed with a micropipette, and then stored for 24 h at different temperature (cooling - 2-8°C, room ambient - 10-25°C and 40°C), for later reuse. For the reaction medium, after each reaction the immobilized was left in the reaction medium (oleic acid and ethanol) for 24 h at different temperature (cooling - 2-8°C, room ambient - 10-25°C and 40°C). After 24 h the immobilized was centrifuged (2000 rpm for 5 min) and the supernatant removed with a micropipette and performed a new reaction to quantify the activity.

2.2.5. Determination of synthesis

2.2.5.1. Esterification activity

The esterification reactions were performed by preparing a reaction mixture of oleic acid and ethanol (1:1 molar ratio) as the reactants, according to the methodology described by Paroul *et al.*, [13]. The activity measurement was carried out with addition of free (0.1 g enzyme solution) and immobilized (0.1 g immobilized solution) CALB. The reaction was conducted under the same conditions described before.

The amount of acid consumed was determined by titration with 0.05 M NaOH. One unit of enzyme activity was defined as the amount of enzyme which consumes 1 μ mol of fatty acid per minute under the assay conditions. The enzyme activity was calculated by Equation 1.

$$AE = \frac{(V_b - V_a) \times M \times 1000 \times V_f}{t \cdot M_{EL} \times V_c} \quad (1)$$

Where, AE = Esterification activity ($U \text{ g}^{-1}$); V_a = Volume of NaOH spent in titration taken after 40 min (ml); V_b = Volume of NaOH spent in titration taken after 1 min (ml); M = Molarity of the NaOH solution; V_f = Final volume of the reaction medium (ml); t = time (min); M_{EL} = Weight of the free enzyme (enzyme solution)/immobilized; V_c = Volume of the aliquot from the reactional medium necessary for titration (ml).

2.2.5.2. Efficiency of immobilization

The immobilized efficiency was calculated considering total activity of free lipase solution (amount of enzyme extract used in the immobilization test) and the total activity of the immobilized (total mass produced by immobilized) according to Equation 2.

$$IE(\%) = \frac{UT_{\text{immobilized}}}{UT_0} \times 100 \quad (2)$$

Where, IE (%) = Immobilization efficiency; $UT_{\text{immobilized}}$ = Total activity in the immobilized synthesized; UT_0 = Total activity of the enzymatic solution for the immobilization.

2.2.5.3. Residual activity

The residual activity used to evaluate thermal stability (free and immobilized lipase) and operating/recycles (immobilized lipase) was calculated using as reference the initial activity for each system, according to Equation 3.

$$RA(\%) = \frac{U_x}{U_{\text{initial}}} \times 1000 \quad (3)$$

Where, RA (%) = Residual activity; U_x = Enzymatic activity after recycles or storage time; U_{initial} = Enzymatic activity of reference (initial).

2.3. Statistical Analysis

Each experiment was done in triplicate. Data were expressed as means \pm standard deviation, and subjected to one-way analysis of variance (Tukey) using Statistica 8.0 (StatSof) software. A significance level of 95% ($p=0.05$) was used.

3. RESULTS AND DISCUSSION

3.1. Polymerization Reaction: Synthesis of PU

For the PU synthesis were evaluated different proportions of isocyanate and polyol monomers, since the preparation and selection of PU foam is related to the different compositions of polyol and isocyanate in the formulation. As assessment parameters was evaluated the visually consistency, stability, conformation defects, flexibility and conformation of the foam porous [8,14].

Figure 2 shows the PU foam conformational aspect, which were synthesized employing different proportions of polyol and isocyanate. According to the (Figure 2c), we ratio 60/40 polyol/isocyanate, showed the better physical characteristics (pore distribution, flexibility and uniform cell), corresponding to the requirements specified by Garcia-Galan *et al.*, [15]. As the formation of a uniform polymer, with a lower shrinkage, without structure defects, porosity, and uniform pore size. This result corroborates with those found by Silva *et al.*, [8] who found the best ratio of monomers 60/40 (polyol/isocyanate).

The results of this study present an extremely simple support to be prepared leading <5 min. It is important to note that the enzyme immobilization occurs simultaneously, not generating any waste, contamination, and other interperere. Therefore, the tendency toward a ratio of the monomers, where it was found that with higher concentration polyol, the shrinkage effect is lower on PU structure, forming a soft and ductil structure with compact cells.

The PU can present a variety of densities and hardness, which change according to the monomer used and with the addition of modifying substances properties. The PU developed with aromatic diisocyanates shows yellow color with the light exposure, whereas those made with aliphatic diisocyanates are stable. Several studies in the literature report the use matrices of commercial PU foam.

Martins *et al.*, [16] evaluated the immobilization of *Pseudomonas aeruginosa* ATCC 25619 in PU foam and compare the effect of phenol on the free and immobilized cells. The foam (10 mm \times 10 mm \times 5 mm)

was cleaned and immersed in 70% ethanol for 1 h. Nest, washed with distilled water and dried at 70°C for 4 h, sterilized at 121°C, inoculated and incubated at 26°C for 24 h. The results obtained in the experimental conditions suggest that cell immobilization on PU foam can be used as a tool in the biological treatment of polluted environments with phenol.

Cadena *et al.*, [9] evaluated the catalytic performance of invertase covalently immobilized on PU, inox plate covered with plast-film layer and ferromagnetic azide-dacron for invert sugars production. The preliminary studies for selection of the support showed that the best activity was obtained for PU treated with HCl, polyethylenimine and glutaraldehyde (156.7±4.9 U/g support). All plast-film-invertase derivatives did not show activity and the dacron-invertase derivative showed an activity of 105.39 U/g support. The invertase immobilized in the presence of substrate (10% w/v sucrose) was the most efficient (832.74 ± 1.48 U/g support). The optimal pH was shifted from 4.5 (free enzyme) to 5.0 (immobilized derivative) and optimal temperature was not affected. The immobilized samples obtained were stable and could be used over a period of 2 months without considerable activity loss (68.5% activity retention), and retained 12.6% (287.97±27.9 U/g support) of the activity after five cycles of batch reactions.

Second Correia *et al.*, [17] and Pitt *et al.*, [18] increasing the isocyanate proportion results in more rigid structure with deformed porosity and reduced apparent volume. The increased stiffness of the structure is related to an increase of the intermolecular forces. In General, PU with high isocyanate content becomes more rigid due to the increased crosslinking, providing greater adhesion and pores compacted.

The PU chemical structure is very complex and dependent on the diisocyanate component, can be diol or polyol, resulting in completely different PU. The isocyanate may react with different chemical groups and the final properties will vary according with the reaction route [9].

3.2. Evaluation of the Immobilized CALB Conformation

After the immobilization process, the PU with the enzyme was fractionated in three parts (lower, middle, and upper) as shown in Figure 1, which were individually submitted to enzymatic activity assays. The esterification activity results of different regions of the foam containing the immobilized CALB lipase are shown in Table 1.

In Figure 1 (Fraction A) only the bottom fraction of the foam showed high activity, the middle and upper fractions (Fraction B and C) showed low enzyme activity. Thus, indicating that the enzyme is not presented a uniform distribution over the PU. This heterogeneous distribution of the enzyme in the foam is bound to the absence of the expansive agent, such as water, which assists in the foam formation [6].

3.3. Evaluation of Immobilized CALB Conformation Solubilized in Different Solvents

The enzyme previously solubilized in water, ethanol or acetone at 0.16% (w/v) was added to the polyol, as shown in Section 2.3. After the polymerization stage, the visual analyzed was performed to evaluate the activity of polymeric structures generated. The test using acetone as a solvent (Figure 3a) produced a rigid polymer structure, compact and biphasic, with two distinct phases in terms of densities. The lower phase showed characteristics of compaction and yellow color, indicating be enriched with isocyanate.

The solvents ethanol (Figure 3b) and water (Figure 3c) resulted in a homogeneous polymeric foam structures with good flexibility and pore distribution.

Table 1: Results of the esterification activity obtained in different regions of the foam with immobilized CALB lipase.

Sample (foam fraction)	Enzymatic activity (U g ⁻¹)
Upper	31.394±3.4
Middle	63.782±6.5
Bottom	472.740±50.2

CALB=Candida antarctica B

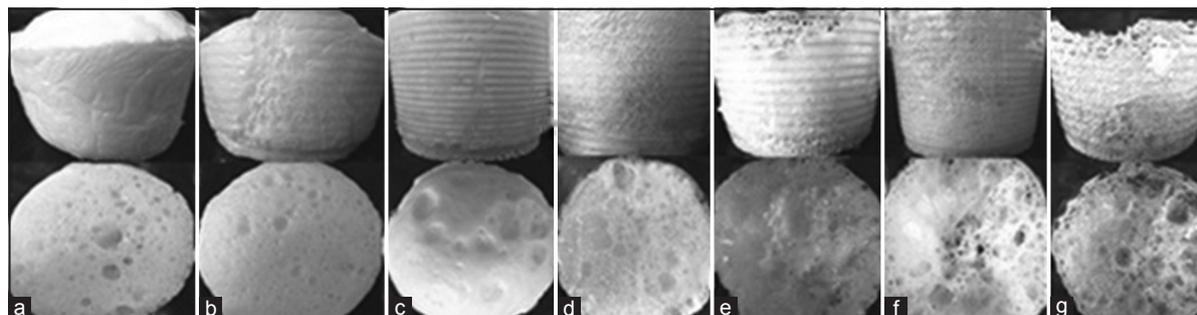


Figure 2: Conformational aspect of polyurethane foam with different proportions of polyol-isocyanate monomers, (a) 10/90, (b) 70/30, (c) 60/40, (d) 40/60, (e) 30/70, (f) 20/80, and (g) 70/30 v/v, respectively.

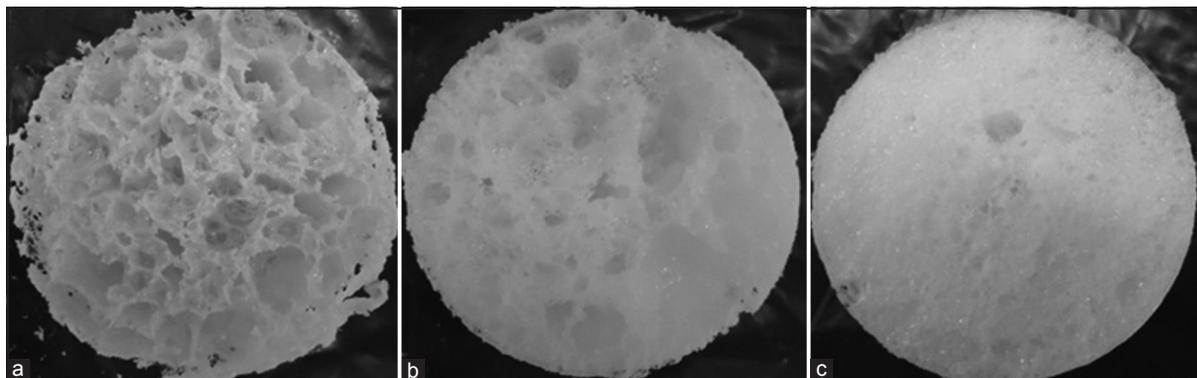


Figure 3: Conformational aspect of the immobilized polyurethane foam with the enzyme diluted in, (a) Acetone, (b) ethyl alcohol, and (c) water.

According Cadena *et al.*, [9] water can help viscosity, increasing the mass and decreasing the organic solvent use between the polymer chains. The water contributes to maintain the structural integrity for the active site polarity and protein stability, may also limit the solubility of hydrophobic substrates around the enzyme.

After evaluating the immobilized CALB lipase conformation in water, ethanol and acetone, the PU foam was divided in two parts as shown in Figure 1. Then, all parcels were submitted to enzyme activity determination.

The enzyme activity results are shown in Table 2. In the enzyme dilution with water was observed a similarity activity for all parts of the PU foam, on the other hand, the enzyme diluted with ethanol and acetone no showed enzymatic activity.

Second García-Arrazola *et al.*, [19] noted that PU immobilization preferably occur by the confinement method (matrix including), who the enzyme molecules is entrapment between the polymer meshes, creating an artificial cell delimited by a porous membrane, where the large molecules, such as enzymes, not are able to diffuse, whereas small molecules such as substrates and products, diffuse. In Additional, occur physical bonds between the support and enzyme, where the enzyme does not interact chemically with the polymer, thus avoiding denaturation.

The results presented in Table 3, for the enzyme solubilized in water before the immobilization demonstrated that the polymerization process kept the enzyme activity, indicating that protein not was denatured or caused undesirable changes conformation and allow the permeability of the substrate between the support matrix a product output.

It was observed high levels of hydration on the material surface, which may have an important role in maintaining the enzyme conformation when adsorbed. This solubility is selected according to the

Table 2: Enzymatic activity obtained in the many regions of the foam with immobilized lipase CALB, diluted in water.

Fraction	Activity (U g ⁻¹)
1A	958.55±149.25 ^a
1B	895.55±163.05 ^a
2A	917.90±101.40 ^a
2B	947.68±69.00 ^a
3A	911.17±115.37 ^a
3B	900.37±106.12 ^a

*Same letters in the same column (a, b, c) no indicate a significant difference (p<0.05). CALB=*Candida antarctica* B

Table 3: Enzymatic activity obtained in different regions of the foam with immobilized CALB lipase, previously diluted in water.

Foam fraction	Activity (U g ⁻¹)
1A	958.55±149.25 ^a
1B	895.55±163.05 ^a
2A	917.90±101.40 ^a
2B	947.68±69.00 ^a
3A	911.17±115.37 ^a
3B	900.37±106.12 ^a

*Same letters in the same column (a, b, c) no indicate a significant difference (p<0.05). Activity=840.04 g U⁻¹ of support activity blended, CALB=*Candida antarctica* B

reaction, where the amount of water and the solubility of the substrates must be evaluated and optimized, contributing for structural integrity, active site polarity and protein stability.

Moreover, can act as expansive agent resulting in hard segments of polyuria, and increasing the foam hardness or the free radicals in the pre-polymer releasing carbon dioxide during the polymerization process.

3.4. Reaction Yield

The reaction yield is the relation between the total activities (U_T), calculated considering the amount of free enzyme (12.8 mg) used in the incorporation step and experimental total activity (U_{TExp}), and calculated taking account the total activity of the support (PU) incorporated with the enzyme (10.0 g). The U_T and U_{TExp} values obtained for the free and immobilized enzyme, respectively, were shown in Table 4.

There was an increase in immobilized enzyme activity up to 2,250.0% in relation to the other enzyme. It was observed 1,140.162 $U\ g^{-1}$ enzymatic activity of the immobilized support, while the free enzyme shows activity of $U\ 489.20\ g^{-1}$. These results suggest a beneficial effect of immobilization on the enzyme activity. These values are much higher than those found in the literature, where Souza [20] evaluated the immobilization of CALB lipase in magnetic nanoparticles of iron toward the implementation in the esters synthesis and obtained a yield of 41.8%. This trend can be associated with several factors such as the availability of easy access of new active sites, the possibility of reuse the immobilized biocatalyst, ease separation from the reaction medium, without contamination of the final product by the enzyme and improved the process control [11].

Silva [21] after studies immobilization CALB on chitosan activated with glycidol and chitosan-alginate both activated with glutaraldehyde, specific activities was obtained $422.44 \pm 50.4\ U\ g^{-1}$ and $378.30 \pm 34.7\ U\ g^{-1}$, respectively for retinyl palmitate synthesis, with free initial activity of $945.69\ U\ ml^{-1}$, being around 37% lower than the immobilized with PU which was $1,140.162\ U\ g^{-1}$.

3.5. Stability of Immobilized Enzyme in Hydrogen Peroxide

Immobilized CALB in PU present initial activity of $1,666.73\ U\ g^{-1}$ (0.16 g enzyme added on support). The stability analysis in relation to values of relative activity at different hydrogen peroxide concentration (1 M, 5 M and 10 M), was show in Figure 4.

It was possible to observe that the immobilized CALB in PU foam submitted at 1 M hydrogen peroxide presents 36.5% residual activity, for 80 days. This

Table 4: Initial activity of the free and immobilized CALB lipase in PU. Synthesis conversion defined as the ratio between the concentration.

Sample	Sample mass (g)	Activity ($U\ g^{-1}$)
Free	0.1	489.2 ± 40.0
Immobilized	0.1015	$1,140.16 \pm 76.4$
Yield	10 g of PU	$10,707.0 \pm 98.0$

CALB=Candida antarctica B, PU=Polyurethane

suggests a beneficial effect of the immobilization on enzyme activity, due to several factors such as ease access of new active sites, possibility of reuse, low contamination of the final product and better process control. Thus, it demonstrates that the CALB in PU has atypical characteristics and offers multiple possibilities for industrial use [17]. The enzyme behavior at high concentrations of hydrogen peroxide corroborate with literature data [11,12,22].

Severiano [23] studied the stability of the Novozym 435 enzyme in 5% (w/w) hydrogen peroxide and verified the complete deactivation after 8 h of reaction Chung *et al.*, [24] evaluated the stability of the *Rhizomucor miehei* enzyme at 5 M hydrogen peroxide and observed in 4 h the inactivation.

3.6. Reuse of Immobilized CALB

The reuse of lipase from immobilized CALB using PU foam was evaluated for successive cycles in the ethyl oleate synthesis. Figures 5 and 6 present the recycle number of the immobilized CALB, stored at dry ambient and immersed in reactional medium, for 24 h at different temperature (2-8, 10-25 and 40°C), respectively. The immobilized present up to 35 cycles of reuse with residual activity higher of 80%. These results are considered excellent when compared

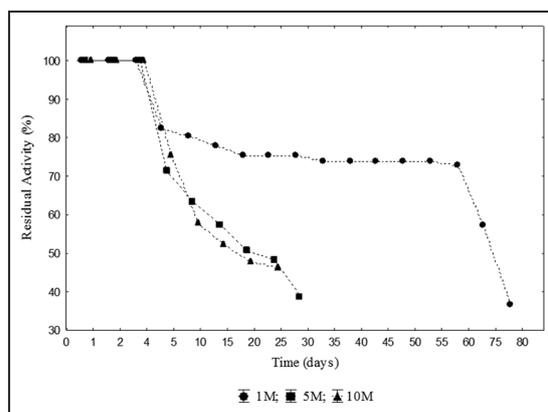


Figure 4: Residual activity of *Candida antarctica* B in polyurethane foam submitted at different hydrogen peroxide concentrations.

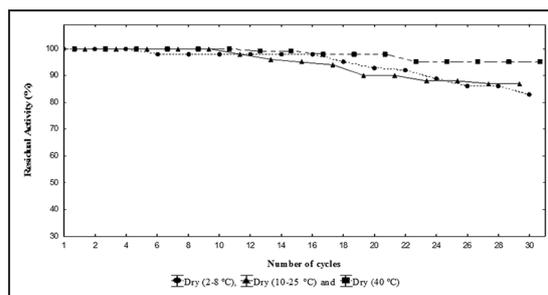


Figure 5: Recycles of the immobilized *Candida antarctica* B stored in dry ambient at different temperature (2-8, 10-25, and 40°C).

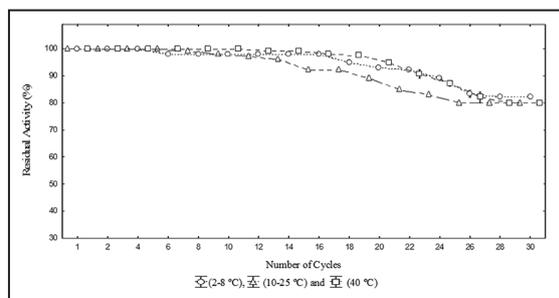


Figure 6: Recycles of the immobilized *Candida antarctica* B stored in reactional medium at different temperatures (2-8, 10-25 and 40°C).

with the literature. Barbosa *et al.*, [25] studied the immobilized *Bacillus* sp. ITP 001 lipase in a sol-gel, which retained only 20% activity after the second cycle.

Guncheva *et al.*, [4], observed that the immobilized lipase *Candida rugosa* in PU maintained 80% of the initial activity, after 15 cycles. Whereas, immobilized inulinase in PU maintained 49.7 and 49.4% of its activity after 24 cycles of reuse in sucrose and inulin, respectively.

Sebrão *et al.*, [26] immobilized lipases *Pseudomonas* sp. and *Rhizopus oryzae* in sodium caseinate/glycerol film for use in the esters synthesis and obtained eight cycles of reuse. While Fernandes [27] applied commercial CALB using poly(3-hydroxybutyrate-co-3-hydroxyvalerate) nanoparticles as support and observed 100% of residual activity for four cycles, in the esterification geraniol and oleic acid. Rodrigues *et al.*, [28] using the same immobilized enzyme in activated carbon, for butyl butyrate synthesis, after six cycles of use showed 15% activity in relation to the initial.

4. CONCLUSION

Through this study, it was possible to develop a new method of immobilization by entrapment of enzymes in PU support, which is extremely simple, easy, fast and inexpensive. The results of enzyme solubilization in water before the immobilization demonstrated that the polymerization process kept the enzyme activity. The immobilization process provided a high yield of 250% in the enzymatic catalytic activity compared to the free process. Immobilized CAL B on PU showed 36.5% residual activity for 80 days and 35 cycles of reuse, with residual activity higher than 80% for all cycles, when submitted to 1 M hydrogen peroxide. In this way, this process provides an important technique associated with low-cost of the support, simple procedure and possibility of being employed as a catalyst in reactions for the food industry.

5. ACKNOWLEDGMENTS

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