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## *In situ* immobilization of commercial lipase in rigid polyurethane foam and application in the esterification of geraniol and oleic acid

### ABSTRACT

The aim of this work was to evaluate the immobilization of *Candida antarctica* lipase B (CaLB) using polyurethane (PU) foam. The stability of the enzymatic derivative against temperature (40, 60 and 80 °C) together with the recycle capacity and storage stability (4 and 25°C) were evaluated. The application of the enzymatic derivative of CaLB in esterification reactions was accomplished. Results indicated that the molar ratio between polyol and isocyanate monomers of 5:3 (v/v) was the best one for the immobilization. The immobilization process resulted in a 1363% increase in activity for the enzymatic derivative. The enzymatic derivative obtained kept its initial activity even after 21 h of exposure to high temperatures. The enzymatic activity of the complex was maintained during 30 days at low temperatures, and showed stability during 4 consecutive reuse cycles. The synthesis of geranyl oleate catalyzed by the immobilized derivative presented conversion of 87%. Taking into account the promising results obtained, the low cost of the immobilization support employed and the whole technique developed, this work comprise an innovative contribution.

**KEYWORDS:** Lipase; immobilization; Polyurethane; esterification; application.

## INTRODUCTION

The application of lipases as biocatalysts in industrial processes has been under continuous increase in food, textile, paper and cellulose, detergents, fat and oil industry. Thus, this class of enzymes is winning a growing range of industrial enzyme market with new biotechnological applications. It has established successfully in the synthesis of esters, just like the production of enantiopure pharmaceuticals, agrochemicals and foods (Dalla Vecchia et al., 2004; Brigida et al., 2007; Hasan et al., 2009; Kapoor and Gupta, 2012).

Polyurethanes are widely applied in different fields, such as the production of plastic foams, cushions, rubber articles, leather, stickers, paints and fibers. These foams can be considered promising to use as carriers for immobilizing enzymes for its application in organic medium reactions, considering their unique properties, like the exhibited high resistance to oils, solvents and fats (Guncheva et al., 2011). Furthermore, this technique is very simple, comprising simultaneous polymerization and enzyme immobilization in one step.

Correia et al. (2011) described the application of PU as enzymatic carriers applied in organic medium reactions. The authors considered its resistance to the organic solvents, which in this case lipase was secured in the porous matrix structure with covalent bonds.

The advantages of using immobilized lipases in esterification processes are well besides the specificity for different types of substrate; high yields, possibility of working under mild operating conditions, easy recovery and reusability of the catalyst, considering it would allow the enhancement of ester yield under mild operation conditions, hence avoiding later purification steps (Garcia et al., 2000). This tendency is envisioned for the reactions of enzymatic synthesis for the production of natural aromatic esters, with lipase as biocatalyst. Usually, large-scale synthesis of flavoring agents is performed with chemical catalysts that commonly lead to the formation of undesired byproducts and is high-energy consumption.

## MATERIALS AND METHODS

### MATERIALS

The immobilization was done using the soluble free lipase fraction B of *Candida antarctica* (Novozyme NZL-LIQUID, CalB), purchased in the liquid form from Novozymes Latin America Ltda. The esterification activity was measured using oleic acid method (Sigma Aldrich). The CalB liquid enzyme was previously diluted in a sodium phosphate buffer 25 mmol.L<sup>-1</sup>, pH 7, in a 1:10 (v/v) enzyme to buffer.

The commercial monomers polyol polyether and toluene isocyanate, used in this work at the polymerization step for producing the polyurethane foam, were produced with a particular formulation for mattresses and injected foams by Flexível Poliuretanos/Mannes Company (Brazil).

During the application of the enzyme derivative as catalyst of the esterification reactions, it was used geraniol (Sigma Aldrich, purity  $\geq 97\%$ ) and oleic acid (Sigma Aldrich, purity  $\geq 99\%$ ) as substrates.

### PU PRODUCTION

The polymerization reaction for producing polyurethane was carried out varying the volume ratio of the polyol polyether to toluene isocyanate monomers at 5:2, 5:3, 5:4, 1:1 and 3:5 (v/v). This variable was established based on the modified work of Silva et al. (2013) and Nyari et al (2015). The polymerization reaction was conducted with the help of a graduated syringe, through which the monomers were mixed and homogenized with the assistance of a glass rod, during 30 seconds. Afterwards, the polymerization step was accomplished (5 min), and then the polyurethane foam was expanded and finally completely solidified.

### LIPASE IMMOBILIZATION USING POLYURETHANE AS CARRIER

Immobilization of CalB in PU was performed after evaluation of the characteristics of the polymer formed with conditions of assay 2 in Table 1 chosen as being the most appropriated. The enzyme was previously solubilized in ethanol (1), acetone (2) and water (3), in a volume ratio of 1:10 (v/v), and the visual enzyme miscibility in the monomers was observed for later study of the immobilization and consequent formation of the polymer.

Accordingly, the enzyme, previously solubilized in water, ethanol or acetone, in a volume ratio of 1:10 (v/v), was added to the polyol. After the homogenization step, the isocyanate was added and then the mixture was stirred for 30 s and let polymerize for 5 min.

The immobilization was carried out adding 10% of the *Candida antarctica* lipase B diluted to the monomers. For the immobilization, the enzyme was homogenized in the monomer mixture of polyol and isocyanate, with the reaction vessel kept in an ice bath in order to avoid excessive temperature rise due to the exothermic reaction, according to the scheme presented in the Figure 1.

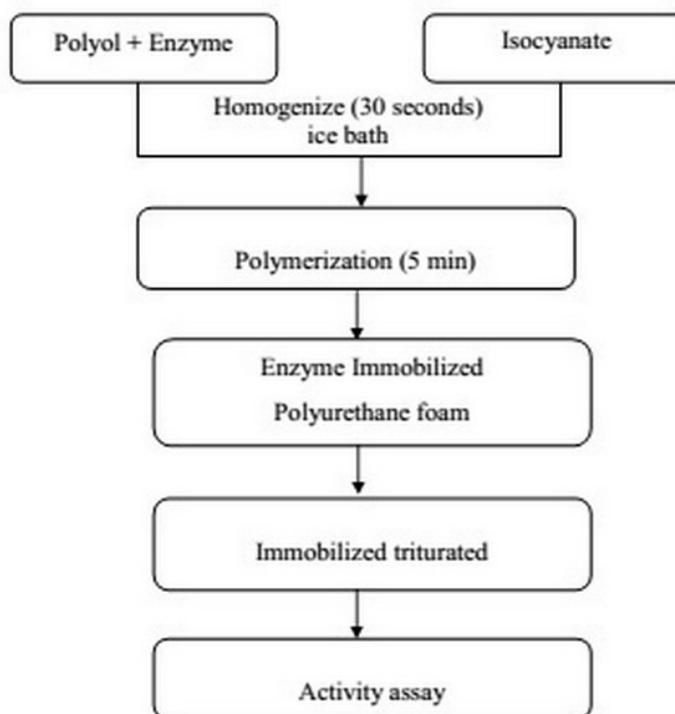


Figure 1 - Scheme of immobilization procedure of CalB lipase in PU.

Enzymatic activity was measured for all parts, and then they were mixed and the activity was determined again, being this considered the “whole immobilized enzyme material” (100%) with the parts having pertinent activity related in percent to the whole activity.

#### ESTERIFICATION ACTIVITY

Esterification activity of the lipases in different forms (free, immobilized, fractioned and crushed) presented in this work was quantified through the synthesis reaction of oleic acid and ethanol (molar ratio of 1:1 by volume). The reaction was performed at 40 °C, 160 rpm for 40 minutes. It was started by the addition of 0.1 g of enzyme to the reaction medium, in glass flasks with lids, kept in a shaker. Aliquots of 500 µL were taken out from the medium in triplicate at the beginning of the reaction. To each sample, 15 mL of an acetone-ethanol (1:1) (v/v) were added to extinguish the reaction and extraction of the oleic acid (Ferraz et al., 2012).

The amount of acid consumed was determined by titration with NaOH 0.05 mol.L<sup>-1</sup>. One unit of enzymatic activity (U) was defined as the amount of enzyme necessary to consume 1 µmol of fatty acid per minute, under the described assay conditions. The enzymatic activity was calculated according to (Equation 1) (Paroul et al., 2011).

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

Where: *EA* denotes the esterification activity (U.g<sup>-1</sup>), *V<sub>a</sub>* is the volume of NaOH required on titration of the sample taken after 40 min (mL), *V<sub>b</sub>* is the volume of NaOH required on titration of the sample taken at the time 0 (mL), *M* the molarity of the NaOH solution, *V<sub>f</sub>* the final volume of the reaction medium (mL), *t* is the time (min), *M<sub>EL</sub>* represents the mass of the enzymatic preparation lyophilized used in the reaction (g) and *V<sub>c</sub>* the volume of the aliquot of the reaction medium taken for titration (mL).

### IMMOBILIZATION YIELD

The immobilized yield was calculated considering the total activity of the free lipase in solution given on the immobilization process (that considers the volume of the enzymatic extract employed on the immobilization test and its activity (U/mL)) and the immobilized total activity (that considers the total mass of the produced immobilized and its activity (U/g)) according to Equation 2 (Nyari et al., 2016).

$$RI(\%) = \frac{UT_{immobilized}}{UT_o} \times 100 \quad (2)$$

Where: *RI (%)* = Immobilization yield; *UT<sub>immobilized</sub>* = total activity on synthesized immobilized; *UT<sub>o</sub>* = total activity of the enzymatic solution offered for immobilization.

The esterification reaction was conducted with 0.1 g of biocatalyst, at 40 °C, 40 min reaction time and 160 rpm.

### STABILITY OF FREE AND IMMOBILIZED ENZYME AT HIGH AND LOW TEMPERATURES

The free enzyme was immobilized in PU and kept at vacuum oven under 40, 60 and 80 °C for 21 h, following the methodology described by Silva et al. (2012). Periodically, a sample was withdrawn and the enzyme esterification activity was measured.

To evaluate the stability of CalB lipase immobilized in PU, the subject was fractioned and crushed in a blender, in order to identify if the shape of the immobilized would interfere the enzyme activity during the storage. Afterwards, the immobilized CalB, the fractioned and crushed one, was stored in a refrigerator

(4 °C) in glass flasks (without buffer). The exposure to room temperature (25 °C) was also evaluated for the fractioned and crushed CalB/PU. The dosage of the esterification activity with oleic acid and ethanol was done in a regular basis.

#### OPERATIONAL STABILITY AND RESIDUAL ACTIVITY (%) OF THE IMMOBILIZED ENZYME

0.1 g of the immobilized enzyme was added to a beaker of 50 mL containing 5 mL of the oleic acid-ethanol reaction medium (1:1) (v/v). The reaction was held at 40 °C, 160 rpm for 40 minutes. Aliquots of 500 µL were taken out in triplicate at the end of the reaction. To each sample, 15 mL of an acetone-ethanol (1:1) (v/v) were added to extinguish the reaction and extract the oleic acid. The amount of consumed acid was determined by titration with NaOH 0.05 mol.L<sup>-1</sup>. Later, the dosage of enzyme esterification activity was measured using oleic acids as substrate. After each cycle, the immobilized was washed with 50 mL of ethanol and the moisture equalized in a vacuum pump during 30 min (Nyari et al., 2016).

The Residual Activity used to evaluate thermal stability (immobilized lipase) and operating was calculated using as reference the initial activity for each system, according Equation 3 (Nyari et al., 2016).

$$AR(\%) = \frac{U_x}{U_{inicial}} \times 100 \quad (3)$$

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

#### ESTERIFICATION OF GERANIOL AND OLEIC ACID USING THE ENZYMATIC DERIVATIVE AS CATALYST

The enzymatic esterification was carried under the optimized conditions in previous works (Paroul et al., 2011), where geraniol alcohol and oleic acid were mixed in the molar ratio of 3:1. The substrates were added to Erlenmeyer flasks of 50 mL with an average reaction medium volume of 5 mL. 0.5 g (10% by weight, based on the substrates) of CalB lipase immobilized in PU and the reaction time was counted from the addition of it to the flasks. All the experiments were carried out in shaker, with a constant agitation of 160 rpm and temperature of 40 °C. After the end of the reaction, fixed in 6 hours, the biocatalyst was filtered with filter paper. The conversion to esters was determined by titration with sodium hydroxide (NaOH) 0.05 mol.L<sup>-1</sup> until a pH.

## SYNTHESES REACTIONS CONVERSION

The syntheses reaction conversions were assessed using the Equation 4 by methodology Nyari et al., 2016.

$$EC = 100 - \left[ \frac{\left( \frac{M \times V_a}{V_c} \right) \times V_f}{MR} \right] \times 100 \quad (4)$$

Where: *EC* = Esters Conversion (%); *V<sub>a</sub>* = NaOH volume spent in the sample titration removed after 40 min (mL); *M* = NaOH molarity of the solution; *V<sub>f</sub>* = final volume of reaction medium (mL); *MR* = moles react (initial 100%); *V<sub>c</sub>* = volume of reaction medium removed for titration (mL).

## STRUCTURAL CHARACTERIZATION OF PU AND CALB ENZYME IMMOBILIZED IN PU BY X-RAY DIFFRACTION

The XRD patterns of the samples of polyurethane and CalB immobilized in PU were obtained through an X-ray diffractometer brand Rigaku, Miniflex II, operated in the conventional 2 theta geometry. The angle of examination was extended from 1.35 to 70 degrees and conditions of analysis: scan speed: 5.00 °/min counting step: 0.05 ° with an emitting tube of Cu-1.54.

## STATISTICAL ANALYSIS

Each experiment was done in triplicate. Data were expressed as means ± 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.

## RESULTS AND DISCUSSION

### DEFINITION OF POLYMERIZATION CONDITIONS AND LIPASE IMMOBILIZATION IN PU

As summarized in Table 1, different polyol to isocyanate ratios tested led to distinct characteristics of formed foams.

**Table 1.** Characteristics of the polymers formed according to the ratio of the monomers.

Polyol to isocyanate ratio (v/v)	Polymer characteristic
5:2	No polymerization observed
5:3	Flexible uniform foam
5:4	Foam with big pores
5:5	Hollow foam
3:5	Damp foam

The choice of the monomers isocyanate and polyol employed for foam formation afford distinct characteristics and functionalities that can interfere on the quality of the foams produced (Pinto et al., 2005; Meng et al., 2008).

After evaluation of the foams produced, it was defined the ratio of 5:3 (v/v) polyol to isocyanate as the most appropriate, since it led to a flexible and uniform material by Silva et al. (2013) and Nyari et al. (2016) with modifications, hence convenient to be applied to the immobilization process of CalB.

After the polymerization step using the three solvent media (water, ethanol or acetone), visual inspection of the polymeric structure generated was performed. The assay conducted applying acetone as enzyme solvent generated a strict polymeric structure, compact and biphasic, with two different phases in terms of density, a lower compressed phase with a yellowish color, indicating it was enriched with isocyanate. However, no enzymatic activity was verified in neither of the phases. The other solvents (water and ethanol) led to the formation of foams with homogeneous polymeric structures, with good malleability and pore distribution. Nevertheless, only the PU incorporated with the enzyme previously diluted in water showed enzymatic activity.

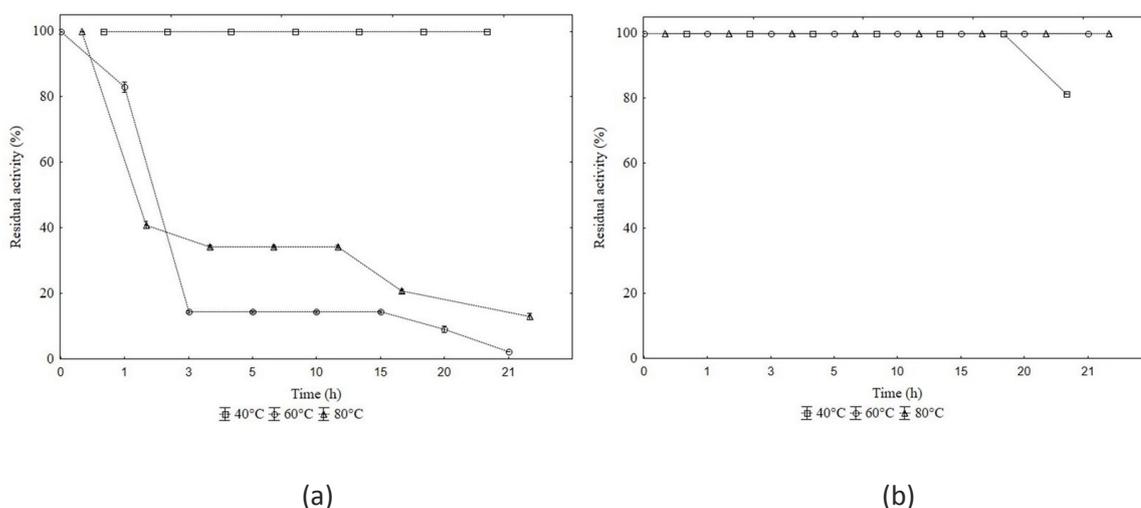
After immobilization step, PU incorporated with enzyme, the resulting material was divided into three parts (lower, middle and upper), which, individually, were submitted to enzymatic activity assays. Activity measurements were accomplished 24 h after the immobilization, enough for the sample completely dry. Each fraction was then crushed and activity measured and recorded for each one. Afterwards, all fractions of the immobilized enzyme were mixed and homogenized. The esterification activity of the free and whole PU immobilized CalB were of 104.00 U.mL<sup>-1</sup> and 172.91 U.g<sup>-1</sup>, respectively. The enzyme did not suffer desnaturation, even being verified an increase on immobilized enzyme activity in this support. It is worth mentioning that this process is fast and maintains the enzymatic activity, as also reported by Silva et al. (2013) which evaluated the immobilization of *Aspergillus niger* inulinase.

## IMMOBILIZATION YIELD

In order to evaluate the efficiency of the immobilization process (enzyme on PU support (8.0g), the immobilized derivative was submitted to washing with buffer solution (sodium phosphate pH 7). It was verified that the washing solution no showed esterification activity, indicating that all enzyme added to the support was irreversibly incorporated by confined method and/or covalently bound to PU. There was an increase in the activity of the immobilized enzyme (up to 1363.2%) in relation to the free enzyme. These results suggest a beneficial effect of immobilization on enzyme activity (Nyari et al., 2016).

## THERMAL STABILITY OF FREE CALB AND ITS IMMOBILIZED DERIVATIVE

After determining the best condition for the immobilization of lipase in PU (5:3 v/v of polyol and isocyanate), study of stability was performed for the free and immobilized enzyme as a function of exposure time at different temperatures, as shown in Figures 2a (free CalB at high temperatures) and 3 (immobilized CalB in PU at high temperatures).



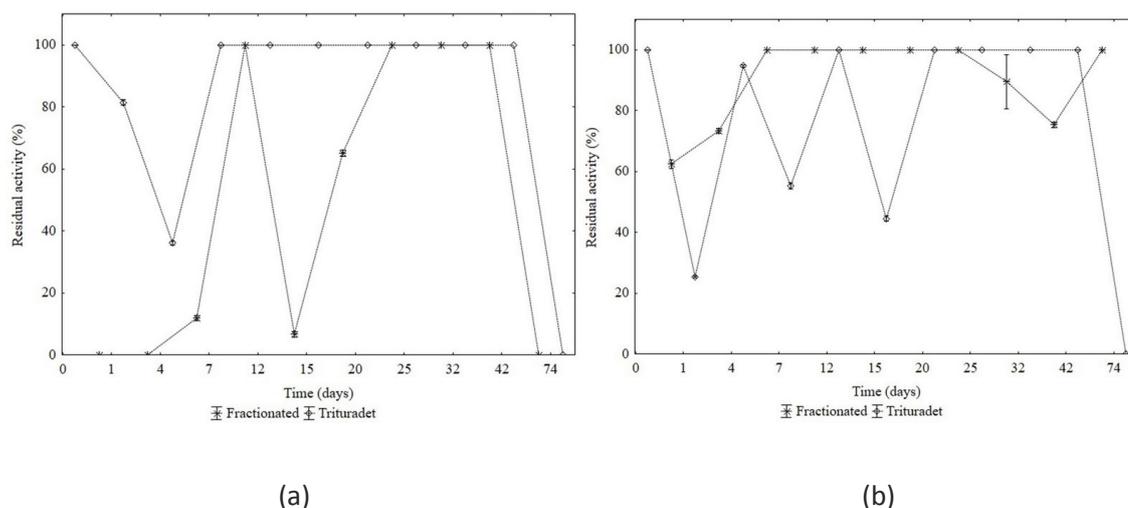
**Figure 2** - Stability evaluation of free (a) and immobilized (b) CalB lipase as a function of exposure time to high temperatures (40, 60 and 80 °C).

After 21 h of exposure, it was observed that the activity of the free enzyme submitted to the temperature of 40 °C did not decrease, keeping a residual activity of 100 %. At 60 °C, however, an activity loss was noted, leading to a residual activity of 6.74 %, as shown in Figure 2a. The high temperature degraded quickly the free enzyme, this fact also confirmed at 80 °C, when the residual activity after 21 h of exposure was only 2.14%.

Regarding the stability of the immobilized derivative exposed to 40 °C, during the first 3 h activity remained constant, not presenting any decrease. Besides, after

21 h of exposure, it was observed that the enzymatic activity of the immobilized complex submitted to the temperatures of 60 and 80 °C did not also show any decrease, keeping the residual activity of 100%, as shown in Figure 2b.

The stability at high temperatures was monitored for several days. After 15 days of exposure to 40, 60 and 80 °C, the residual activity was null, as the immobilized CalB was inactivated for all temperatures tested. Evaluation of the storage stability of the immobilized enzyme at low and room temperatures was performed in two ways: fractionated and crushed, as shown in Figure 3a (immobilized CalB storage at room temperature) and 3b (immobilized CalB storage in refrigerator).



**Figure 3** - Stability evaluation of immobilized CalB in PU as a function of temperature: room temperature (a) and 4°C (b).

The PU immobilized CalB, both in crushed and fractionated forms, showed similar behavior. The behavior of CalB against the PU carrier is unstable, presenting lots of oscillations. This observed behavior requires a deeper study, because the occurrence of reactions during the storage is possible. During the polymerization of the polyurethane, immobilization through clustering or chemical reaction may take place, which probably changes the different forms and fractions of the immobilized. The stability of the PU immobilized CalB was kept up to 74 days.

These findings show that the use of polyurethane foam as immobilization carrier may provide the enzyme to keep the activity for several days at room temperature. Ferraz et al. (2012) obtained a similar result when evaluating the stability of enzymatic extracts obtained from rice bran, soybean bran and sugarcane bagasse at room temperature for 60 days. In that case, results showed the same trend of activity conservation for longer times at this temperature condition.

The PU immobilized CalB in its crushed form presented a more linear behavior if compared to the fractionated form. The oscillations observed during the refrigeration storage are similar to those at room temperature. Based on these results, it can be concluded that the storage, both in fractionated and crushed forms, does not interfere on PU immobilized CalB activity.

## CHARACTERIZATION OF THE FOAMS OF PU AND ENZYMATIC DERIVATIVE

The XRD patterns of pure polyurethane and PU immobilized CalB are presented in Figure 4. The diffractograms of both samples exhibit an enlarged peak, i.e., the material possesses a more amorphous structure. Silva et al. (2012), identified a similar behavior, which characterized the pure polyurethane with clay.

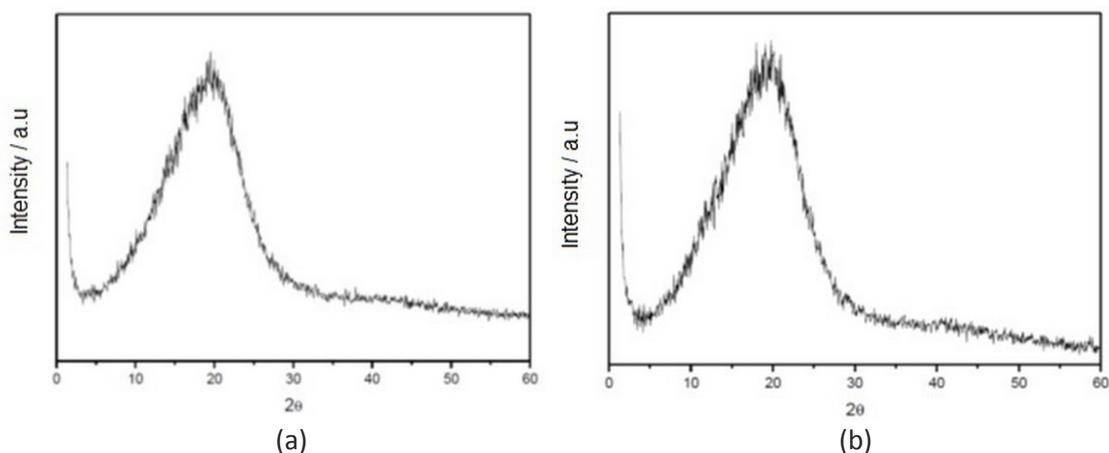


Figure 4. X-rays diffractograms (XRD) of pure PU (a) and enzymatic derivative (b) samples.

The characterization of polyurethane was also studied in the work of Fiorio et al. (2012) where the presence of an enlarged peak was identified in  $2\theta - 20^\circ$ .

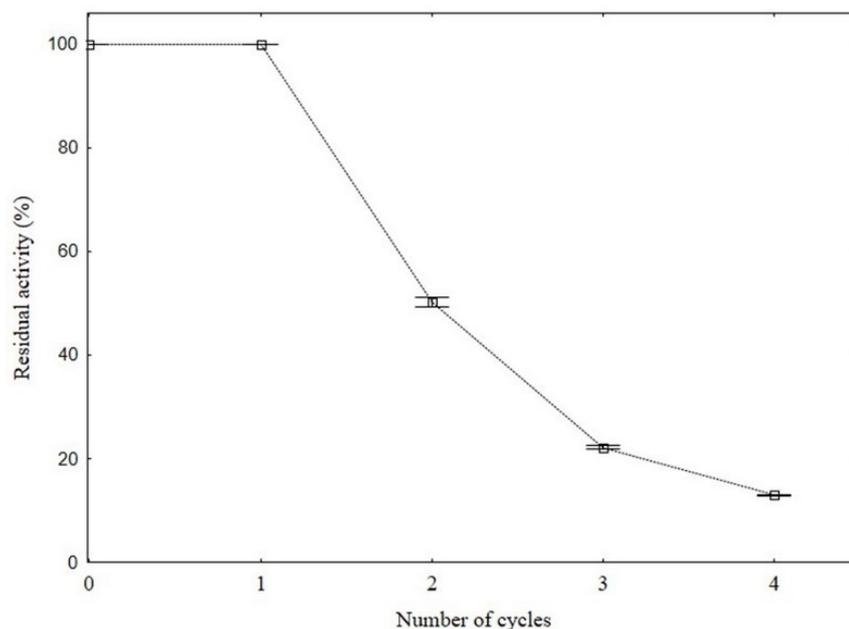
## OPERATIONAL STABILITY

The operational stability of the enzymatic derivate was evaluated in the reaction of esterification using geraniol and oleic acid as substrates. Results are shown in Figure 5, where it can be observed that the PU immobilized CalB presents a decrease in its activity after each cycle and after four reaction cycles the residual activity falls down to 12.9%.

According to Rodrigues et al. (2008) the soluble CalB lipase immobilized in activated charcoal for the synthesis of butyl butyrate, after six cycles, kept 10 to 15% of its initial activity.

Guncheva et al. (2011) reported that the lipase of *Candida rugosa* immobilized in polyurethane kept 8% of its initial activity after 15 cycles, being applied in the esterification of palmitic acid with ethanol.

The inulinase from *Aspergillus niger* immobilized in polyurethane kept 49.7 and 49.4% of its initial activity after 24 cycles of reuse in sucrose and inulin, respectively (Cui et al., 2013; Silva et al., 2013). Studied the operational stability of the lipase from *Yarrowia lipolytica* immobilized in polyurethane foams for the synthesis of lauryl laurate and the biocatalyst kept, after 15 cycles of reaction, a yield of 92%.



**Figure 5.** Operational stability of the esterification reaction of the oleic acid for PU immobilized CalB.

#### APPLICATION OF THE ENZYMATIC DERIVATIVE TO ESTERIFICATION OF GERANIOL AND OLEIC ACID

The application of the lipase to catalyze the esterification reaction of geraniol and oleic acid (3:1) presented 87.6% of conversion of geranyl oleate, after 6 hr de reaction. The acid/alcohol molar ratio is one of the most important parameters in enzymatic esterification. Since the reaction is reversible, an increase in the alcohol concentration should result in higher ester yields and shift the chemical equilibrium toward ester synthesis (Barbosa et al., 2012; Xu et al., 2013).

The results of the current study demonstrate the potential application of this immobilized enzyme in synthesis reactions, especially for geranyl oleate. Similar results were found by Paroul et al. (2011) which obtained 93% of conversion using a commercial immobilized lipase (Novozym 435). Experimental data on enzymatic esterification of geraniol and oleic acid production are reported in this work, showing a promising perspective of using low cost biocatalysts to overcome the well-known drawbacks of the chemical-catalyzed route.

The ethyl oleate is used as solvent for preparation of pharmaceutical drugs involving lipophilic substance as a steroid, it is also used as a lubricant, plasticizer, and biodiesel in the chemical industry as a stabilizer and emulsifier in the food industry. Being considered is regulated as a permitted food additive according Food and Drug Administration for human food consumption (Ferraz et al. 2015).

Despite the fact that most of these compounds are obtained by chemical synthesis or by extraction from plants, implementation of new biotechnological

processes has increased recently (Tan et al., 2006). Similar results were found by Paroul et al. (2011) and Nicoletti et al. (2015). Considering the high conversion and low cost of citronella oil, it can be confirmed by results obtained in this work that the substitution of commercial alcohols by essential oils can contribute to make the process economically viable.

## CONCLUSIONS

The key step in the enzymatic process consists in the successful immobilization of the enzyme allowing its recovery and reuse and the effectiveness of an immobilization process depends on the support used. It is worth mentioning that in this work the enzyme immobilization was accomplished in a single step, i.e., immobilization process occurred together with the formation of polyurethane foam, which clearly differs from the works presented in the current literature and may imply in an innovative. Indeed, the approach employed in this work results in a low-cost, much faster process with high activity retention, which mean important advantages over current methods. The obtained results indicated that the molar ratio between the monomers polyol to isocyanate at 5:3 (v/v) was the best one for the immobilization of lipase from *Candida antarctica* B in PU. The enzymatic derivative kept its initial activity after 21 h of exposure at high temperatures. The storage at low temperatures allowed the verification of the maintenance of the enzymatic activity of the derivative during 30 days, both in fractioned and crushed forms. Besides, the immobilized derivative showed stability during 4 consecutive cycles of reuse. Experimental data on enzymatic esterification of geraniol and oleic acid production are reported in this work, showing a promising perspective of using low cost biocatalysts to overcome the well-known drawbacks of the chemical-catalyzed route

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