



## Research article

# Ethanol as additive enhance the performance of immobilized lipase LipA from *Pseudomonas aeruginosa* on polypropylene support

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## ABSTRACT

Immobilization is practical to upgrade enzymes, increasing their performance and expanding their applications. The recombinant, solvent tolerant lipase LipA PSA01 from *Pseudomonas aeruginosa* was immobilized on polypropylene Accurel® MP1004 to improve its performance. We investigated the effect of ethanol as an additive during the immobilization process at three concentrations (20%, 25%, and 30%) on the operational behavior of the enzyme. The immobilization efficiency was higher than 92%, and the immobilized enzymes showed hyperactivation and thermal resistance depending on the concentration of ethanol. For example, at 70 °C, the free enzyme lost the activity, while the prepared one with ethanol 25% conserved a residual activity of up to 73.3% ( $\Delta T_{50}^{15} = 27.1$  °C). LipA immobilized had an optimal pH value lower than that of the free enzyme, and the organic solvent tolerance of the immobilized enzymes depended on the ethanol used. Hence, the immobilized enzyme with ethanol 25% showed hyperactivation to more solvents than the soluble enzyme. Remarkable stability towards methanol (up to 8 folds) was evidenced in all the immobilized preparations. The immobilized enzyme changed their chemo preference, and it hydrolyzed oils preferentially with short-chain than those with long-chain. LipA had a notable shelf-life after one year, keeping its activity up to 87%. Ethanol facilitated the access of the enzyme to the hydrophobic support and increased its activity and stability according to the amount of ethanol added.

## 1. Introduction

Microbial enzymes are sustainable and environmentally friendly alternatives for developing biotechnological processes [1]. Among the enzymes, lipases are an outstanding group, recognized for their activities in a broad range of natural and artificial substrates and catalyzing reactions in hydrolytic, micro-aqueous, and solvent-free environments. The versatility of lipases has had applicability in different fields: oleochemicals, pharmaceuticals, cosmetics, foods, detergents, biomedical devices, and biofuels [2–9]. Thus, they can participate in reactions of esterification, interesterification, alcoholysis, acidolysis, and aminolysis [9–14].

Lipases are soluble in water, but usually, their substrates are water-insoluble. Therefore, in aqueous systems, the hydrolysis of substrates such as triacylglycerols occurs into the lipid-water interfaces [15]. In such interfaces, a structure covering the active site cavity suffers a displacement exposing the active site pocket to the substrate. The

movement of this structure is similar to a lid, which is formed by an amphiphilic subdomain composed of alpha-helices that triggers structural changes inside the enzyme deriving in a notable increase of the enzyme activity, a phenomenon known as interfacial activation [15,16]. The structural modifications associated with interfacial activation rely on the nature of the enzyme, the substrate concentration, and the ionic strength surrounding the enzyme [17].

Lipases are part of the diverse group of  $\alpha/\beta$  hydrolases, some of them with remarkable attributes for industrial application, such as tolerance to extreme pH, organic solvents and high temperatures, regio and enantioselectivity, and resistance to the high concentration of substrates and products [18–20]. However, these characteristics are not found in all lipases, and many of them are not suitable for industrial use. Enzymes are the most expensive input in industrial bioprocesses; thus, to reduce costs, enzymes should be resistant to extreme conditions and reusable [21]. Accordingly, enzymes are improved to maintain or even increase their properties and long-term stability to ensure cost-effective processes

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[22].

Enzyme immobilization has been used as an effective strategy to upgrade lipases, improving their stability at high temperatures, tolerance to solvents, and pH without affecting their catalytic activities [23–25]. Moreover, some of them, after immobilization, have acquired new traits that include enhanced activity, resistance to inhibitors, and changes in chemo, regioselectivity, and enantioselectivity [26–31]. Other benefits reported in immobilized lipases are their easier dispersion in the reaction medium, safe handling, storage, and recovery at the end of the process [32–34]. Further, immobilization gives long-lasting enzymes with resistance to harsh conditions and reusability for subsequent catalytic reactions, significantly reducing the cost of the process [35].

Nevertheless, immobilization is not always practical for enzyme performance. In some cases, the most noticeable impact is the decrease or even loss of enzyme activity due to a direct impact on the enzyme structure (distortion or increased stiffness of the active site); other effects include reduced affinity towards the substrate, mass transfer problems, and denaturation. Hence, the immobilization effects depend on the mechanism used for fixation, the type of carrier or support used, and the enzyme itself [36–40]. Although an immense variety of enzymes gain positive effects after immobilization, a universal and optimal method for immobilizing enzymes is still not available. Thus, each enzyme must be individually evaluated with each particular immobilization support to estimate the effects and changes on the enzyme traits by comparison with those in free conditions [41].

Currently, a wide variety of mechanisms are available to immobilize enzymes. These include adsorption, entrapment, covalent coupling, microencapsulation, crosslinking, and crosslinked aggregates formed with the same enzyme [5,32,37,42–46]. Among the available alternatives, physical adsorption on hydrophobic supports is one of the most straightforward techniques for immobilizing lipases [36,46,47]. In this strategy, lipases are adsorbed on the surface of hydrophobic supports, usually porous materials with small particle sizes and large surface area, such as natural water-insoluble carbon-chain polymers [46]. The adsorption of lipases in such materials occurs thanks to hydrophobic amino acid patches near the active site entrance, forming a non-polar region essential to the access of insoluble substrates such as lipids [48, 49].

Even though other proteins could be present in the medium simultaneously with the lipase, the hydrophobic nature of both lipase and support favors the selective fixation of the enzyme on the hydrophobic surfaces [36,50]. The attachment enzyme-support occurs through Van der Waals forces and hydrophobic interactions, which results in a reversible process in conditions such as aqueous media, detergents, co-solvents, substrates, and aggressive conditions [46,51,52].

Immobilization by adsorption produces conformational changes, ending in enzymes with activities often increased than the soluble enzyme [53]. The hyperactivation is usually a direct consequence of the opening of the lid, and the open conformation is fixed on the surface of the support, which exposes the cavity permanently to solvents and substrates, analogous to what occurs with interfacial activation [17,36, 54].

The binding of LipA on the surface of hydrophobic supports often requires pre-moistening of the support with ethanol, which decreases the hydrophobicity of the surface, facilitating the access of the enzyme solution [55]. Blanco et al. showed the effect of the addition of ethanol 10% during the immobilization process of the commercial and solvent tolerant *Candida antarctica* lipase fraction B (CalB) using silica functionalized with hydrophobic octyl groups as support [56]. Immobilized CalB did not show hyperactivation when it was immobilized without ethanol. However, using a low concentration of this solvent, the lipase was hyperactivated [56]. However, the authors did not evaluate higher ethanol concentrations because they achieved the maximum activity of the immobilized derivative at this concentration.

Recently, the use of additional reagents or molecules during the immobilization process has diversified and dynamized the application of

this strategy to improve the properties of the enzymes. These additives generally seek to protect the enzyme from inactivating reagents or substrates, to favor a microenvironment that eases the dispersion of the immobilized preparation or enhances the enzyme activity [57–59].

LipA from *P. aeruginosa* is an enantioselective and organic solvent tolerant valuable lipase, useful in biotechnological applications such as resolving racemates mixtures in hydrolytic and non-aqueous reactions [60–62]. Due to the tightly controlled expression of LipA and the opportunistic pathogenic nature of this bacterium, lipase production is preferred in organisms such as *Escherichia coli*. However, the low amounts of soluble and active LipA produced are the main drawbacks of recombinant production in this host [63–65]. Furthermore, LipA requires the protein foldase Lif (Lipase-specific foldase) for its appropriate folding. Thus, the expression of the enzyme in its active conformation requires the concomitant expression of Lif, which helps the correct folding of the enzyme [66,67]. Previously, we cloned and overexpressed the mature LipA PSA01 in the cytoplasm of *E. coli* BL21(DE3) along with its foldase chaperone, obtaining a soluble and active enzyme tolerant to high ethanol concentrations [68]. Despite recombinant production, the functionally active enzyme production is low. Hence, subsequent purification steps would further decrease the yields.

Alternatives to increase its activity and performance of lipases such as immobilization by physisorption have demonstrated the selective nature of hydrophobic supports preferentially adsorbing lipases such as LipA, fixing them in the open conformation triggering their hyperactivation, with which a previous purification process could be omitted [36]. Equally in lipases such as LipA, ethanol treatment has resulted in hyperactivation of these enzymes by a similar mechanism to immobilization [63,68]. Ethanol in immobilization has been implicated in increasing access to the aqueous solution where the enzyme is dissolved. However, another study with *Candida rugosa* lipase has shown that the concentration of ethanol in touch with the support is related to reducing the particle size of low molecular weight polypropylene supports, which in turn was implicated in lower adsorption of lipases and a lower activity [69]. Another study asserts that ethanol increases the capacity of hydrophobic supports to harbor more enzymes [56]. Alternatively, other approaches have evidenced that once the enzymes have been immobilized by adsorption, they resulted sensitive to the effects of different amounts of organic polar solvents, suffering hyperactivation previous their lyophilization step, or even a decreasing of the activity, evidencing that modifications of the enzyme instead of the support are responsible for the observed activity [32,70]. Therefore, it is worth evaluating the effect of hyperactivate enzymes with solvents, followed by immobilization, in the activity and stability of the immobilized derivative.

This paper examined the impact of different ethanol concentrations as an additive in the immobilization of LipA PSA01 by adsorption on hydrophobic polypropylene. Besides the effects in the activity, we evaluate the effects of using ethanol in some operational parameters such as resistance to temperature, polar and nonpolar solvents, different pH buffers, hydrolytic and synthetic activities, reusing, and stability under storage.

## 2. Materials and methods

### 2.1. Materials

Accurel MP1004, a porous polypropylene homopolymer powder with a particle size lesser than 0.4 mm, was purchased from Membrana GmbH (Obenburg, Germany). We purchased p-nitrophenyl palmitate (pNPP) and oleic acid (95%) from Sigma Aldrich (St Louis, Mo, USA). Tripalmitin was acquired from Spectrum Chemicals Mfg. Corp. (Gardena, CA, USA), and the coconut and Spanish extra virgin olive oils were bought from a local market. The reagents Bradford dye, acrylamide, and bisacrylamide were purchased from Biorad (Richmond, CA, USA).

We used the autoinducing medium for growth and expression of the recombinant lipase LipA PSA01: tryptone, yeast extract, lactose,

glucose, glycerol, lactose, potassium dihydrogen phosphate, disodium hydrogen phosphate, ammonium chloride, and magnesium sulfate were purchased from PanReac AppliChem (Barcelona, Spain). All solvents (hexane, ethanol, acetone, 2-propanol, ethyl acetate, and methanol) were acquired from JT Baker (Phillipsburg, NJ, USA). Gas chromatography standards and other chemicals used in this study (sodium, sodium hydroxide, TRIS, acetic acid, HEPES) were analytical reagents obtained from Sigma Aldrich (St Louis, Mo, USA).

## 2.2. Production of the recombinant LipA PSA01

We expressed LipA from *Pseudomonas aeruginosa* PSA01 (Genk bank: MK336958) in the strain *E. coli* BL21 (DE3) containing the plasmid pYLF6, which harbors the sequence of lipA (coding the mature lipase LipA) and the sequence of the truncated gene lif coding the chaperone Lif (Genk bank: MK336959) [67]. The lipase was produced in a medium for autoinduction: yeast extract 0.5%, tryptone 1.0%, Na<sub>2</sub>HPO<sub>4</sub> 25 mM, KH<sub>2</sub>PO<sub>4</sub> 25 mM, MgSO<sub>4</sub> 0.02%, NH<sub>4</sub>Cl 50 mM, glucose 0.05%, lactose 0.2% and glycerol 0.5% [70]. The broth was supplemented with chloramphenicol (100 µg/mL) and inoculated at 4% with an inoculum grown overnight in Luria Bertani with glucose 1%. The microorganism was incubated for 24 h at 16 °C, 100 rpm, and the cells were retrieved by centrifugation at 8000 rpm for 30 min. The pellets were weighed and stored at -20 °C until their lysis. The crude lysates were obtained using a home-made lysis buffer (Buffer Tris HCl pH 8.0 20 mM, Glycerol 5% v/v, Triton 0.3%, NaCl 400 mM, CaCl<sub>2</sub> 5 mM, and lysozyme 50 µg/mL). We added 7 mL of this buffer per gram of wet cells, and the suspensions were incubated with slow orbital agitation (70 rpm) at 37 °C for 30 min. We repeatedly pipetted the lysates to decrease their viscosity. The crude extracts were centrifuged at 8000 rpm for 30 min, and the supernatants were filtered through 0.45 µm and 0.22 µm membranes and stored at -80 °C. They were lyophilized and dissolved again using distilled water with half of the initial volume to concentrate the lysates.

## 2.3. Immobilization with ethanol

We followed the method described elsewhere with some modifications for the immobilization of LipA [35,54]. We prepared three lysates solutions with different amounts of ethanol in a final volume of 64 mL. Three different percentages of ethanol were used in the immobilization process, and they were added in two steps: in the first step, we poured 13 mL into 2 g of Accurel MP1004 to facilitate the wettability of the support. The ethanol was not removed from the powder. In a second step, we added more ethanol directly on 25 mL of two crude lysates solutions (3 mL and 6.5 mL), and they were poured on the suspensions with the polypropylene and ethanol (Final concentration of ethanol 25% and 30%). The third crude lysate was added without additional ethanol (final concentration 20%). The suspensions were completed to the desired volume with HCl 10 mM pH 7.0, pH closer to the isoelectric point of our lipase (5.87) ([http://web.expasy.org/compute\\_pi/](http://web.expasy.org/compute_pi/)), instead of using a buffer pH 8, the optimal for enzyme activity, assuming that fewer charged residues could favor the adsorption process [38]. The mixtures were continuing mixed at 37 °C, 150 rpm for 24 h. The immobilization described above was repeated by triplicate.

As an indirect indication of the immobilization process, we monitored the time course of the adsorption for 24 h by measuring the activity and protein contents of supernatants without support before the ethanol treatment, when the ethanol was added to the enzyme lysates, and at 2 h, 4 h, 6 h, and 24 h after pouring the lysates into the support suspensions with ethanol and buffer. The particles of support were vacuum filtered from supernatants and rinsed with 200 mL of distilled water. After 24 h, the supports were dried under vacuum and stored in a desiccator at room temperature. The activities of aliquots were monitored with the artificial substrate pNPP. We calculated the relative activities, taking initial activities as 100%.

To evaluate the adsorption process, we calculated the fixation level

(%), which corresponds to the amount of protein adsorbed for the carrier [32]:

$$\text{Fixation level (\%)} = \frac{C_i V_i - C_f V_f}{C_i V_i} \times 100\% \quad (1)$$

Where  $C_i$  represents the concentration of protein (mg/mL) of the lysate solution with the enzyme at the beginning of the experiment,  $V_i$  corresponds total volume (mL) of each solution,  $C_f$  is the residual concentration of protein after the immobilization process (mg/mL), and  $V_f$  corresponds to the final volume of the suspensions [32].

The protein loading on the surface of the support, corresponding with the mg of protein adsorbed per gram of polypropylene, was evaluated with the following formula:

$$\text{Protein loading in support} \left( \frac{\text{mg}}{\text{g support}} \right) = \frac{C_i V_i - C_f V_f}{\text{Support weight (g)}} \quad (2)$$

The support weight corresponds to the grams of support used for each immobilization, corresponding to two grams [71].

## 2.4. Activity of the immobilized enzymes

The activity of the lysates containing the lipase LipA PSA01 (U/mL) and the apparent activity of the immobilized enzyme (U/g) was evaluated using the substrate pNPP 1.0 mM. The release of p-nitrophenol was measured by the absorbance changes at 405 nm of 40 µL of soluble lysate or 5 mg of the immobilized enzyme to 960 µL or 1000 µL of reaction buffer, respectively [72]. The molar absorptivity coefficient of p-nitrophenol using buffer Tris HCl 50 mM pH 8.0, CaCl<sub>2</sub> 5 mM, and Triton X-100 0.3% (v/v) was  $1.793 \times 10^4 \text{ M}^{-1} \text{ cm}^{-1}$ . We defined one lipase unit as the amount of enzyme that produces one µmol of p-nitrophenol per min at 37 °C and pH 8.0 ( $U = \mu\text{mol} / \text{min}$ ). All the measurements were carried out in triplicate.

The specific activity of the free enzyme (U/mg) was determined with the concentration of protein in the sample:

$$\text{Specific activity} \left( \frac{\text{U}}{\text{mg}} \right) = \frac{(\Delta \text{AbsSample})}{\Delta t} \times \frac{V_T}{\varepsilon \times d \times V_E} \times \frac{1}{\text{mg/mL protein}} \quad (4)$$

Being  $\Delta \text{Abs}$  the change in the absorbance after 20 min ( $\Delta t$ ),  $\varepsilon$  is the coefficient of molar absorptivity,  $V_E$  is the volume of the enzyme used, and  $V_T$  the total volume of the reaction.

The apparent activity of the fixed enzyme was calculated as the activity of lipase (µmol of pNPP/min \* mL, per gram of support [35]. We stated the specific activity of the immobilized enzyme as the µmol of p-nitrophenol produced per minute, per mg of protein retained in the sample of support evaluated. We calculated both parameters as follows [33]:

$$\text{Apparent lipase activity} = \frac{U}{\text{weight support used (g)}} \quad (5)$$

$$\text{Specific activity} = \frac{U}{\text{protein in support used (mg)}} \quad (6)$$

The immobilization efficiency shows the amount of lipase adsorbed for the support, was calculated according to previously stated [73]:

$$\text{Immobilization efficiency (\%)} = \frac{U_i - U_o}{U_i} \times 100 \quad (7)$$

Where  $U_i$  is the enzyme activity in the solution before the immobilization and  $U_o$  is the activity remaining at the end of the immobilization, measured in the supernatant of the suspensions [73].

## 2.5. SDS-PAGE and protein quantification

We obtained the protein profiles of the crude lysates and the retained by the support by SDS polyacrylamide gel electrophoresis (12.5%). To

desorb the proteins retained on the support, we boiled for 30 min fifty mg of immobilized with 2% SDS [74]. The protein quantification of the initial lysates and the aliquots taken at different times as indicated was calculated using the modified Bradford method at 590 nm and 450 nm, using bovine serum albumin as reference for standard curve [75].

## 2.6. Temperature Stability of immobilized lipases

We evaluated the stability of the immobilized enzymes at different temperatures. Five mg of each immobilized enzyme and 40  $\mu$ L of the soluble LipA in lysis buffer were mixed with 100  $\mu$ L of buffer Tris HCl 20 mM pH 8.0. The samples were placed in a dry block heater at different temperatures (37 °C, 40 °C, 50 °C, 60 °C, 70 °C, and 80 °C) for 15 min. The tubes were then cooled down to 4 °C with ice-cold water for 10 min and warmed again to 37 °C for 5 min. Subsequently, 900  $\mu$ L of reaction buffer with 1.0 mM of pNPP were added, mixed, and the production of p-nitrophenol was measured as above. We estimated the residual activity by taking the immobilized enzymatic activity at 37 °C as 100%. The thermal stability of the immobilized enzymes was evaluated with the  $T^{15}_{50}$  (the temperature in which, after 15 min, the residual activity is reduced by half). The sigmoidal curve and inflection point were analyzed by four parameters logistic fit regression (SigmaPlot) [76].

We followed the stability of the immobilized enzymes at 80 °C for 80 min. Five mg of the support and 100  $\mu$ L of Tris HCl 20 mM pH 8.0 were subjected to this temperature, and aliquots were collected every 10 min up to 80 min and cooled down at 4 °C for 5 min. After we removed any aggregated protein by centrifugation, we calculated the residual activity of the immobilized enzymes (at 37 °C) [77]. The curves obtained were fitted to a first-order plot (Ln residual activity vs. time), and the constant of deactivation (kd) was calculated [77–79]. The half-life was obtained for each sample using the formula:

$$t \frac{1}{2} = \frac{\text{Ln}2}{\text{kd}} \quad (8)$$

## 2.7. Stability to pH and solvents

The stability to solvents and pH were examined using five mg of each immobilized enzyme and one mL of the following buffers 50 mM: sodium acetate pH 4.0, sodium acetate pH 5.0, MES 6.0, HEPES pH 7.0, HEPES pH 7.5, Tris HCl pH 8.0, Glycine pH 9.0, and Glycine pH 10 [71]. Likewise, 100  $\mu$ L of the crude LipA lysate were dissolved in the same buffer and incubated at room temperature for 96 h with a sporadic vortex. Then, the buffers were removed from the immobilized enzymes, and the activity was evaluated with pNPP as substrate. For evaluating the impact of organic solvents with different Log P, we put a known amount of immobilized enzymes (5.0 mg) in one mL of the following pure solvents: hexane, 2-propanol, ethyl acetate, acetone, ethanol, and methanol. The buffers and the supports were incubated at room temperature with an intermittent vortex. After 96 h of incubation, solvents were removed, and the immobilized lipases were air-dried. Afterward, the residual activity was estimated with pNPP as described before, and the residual activity calculated taking the activity of LipA incubated with the respective solvent as 100%. Equally, the residual activity of the free lipase was obtained, taking the activity calculated in Tris HCl pH 8.0 as 100%.

## 2.8. Hydrolytic activities on natural substrates

Homogeneous oil emulsions were elaborated as published by Bosley (coconut oil, olive oil, and tributyrin) [54]. The composition analysis of the fatty acids in these oils was done by derivatizing to the corresponding methyl esters with sodium methoxide and analysis through gas chromatography [7,80]. Each hydrolytic reaction was made with 10 mL of oil emulsion and 20 units of the immobilized enzyme and was incubated for 24 h at 37 °C and 120 rpm. Similarly, crude lysates with the

soluble enzyme and blank reactions without enzyme were prepared and placed at the same conditions. The enzymes immobilized were removed by vacuum filtration, and three drops of phenolphthalein 1% were added to each emulsion to be titrated with sodium hydroxide 0.05 N until a pale pink color was observed [81]. One unit was specified as the amount of enzyme able to release one  $\mu$ mol of free fatty acid per min at the conditions described above. The specific activity for the immobilized enzyme was calculated as follows (U/g of protein) [81].

$$\text{Specific activity} = \frac{50 (\text{VolF} - \text{VolB})}{\text{mg support used} \times 1440} \times 1000$$

Where VolF is the volume in milliliters of NaOH 0.05 N used to neutralize the free fatty acids present in the emulsions with enzyme after the hydrolysis, and VolB is the volume used to neutralize the fatty acids found in the blank reaction.

## 2.9. Acidolysis reactions on natural substrates

We evaluated the ability of the immobilized enzymes to carry out acidolysis reactions. We made two mixtures composed of tripalmitin (203 mg) and oleic acid or octanoic acid in a mole ratio of 1:3 in hexane (3 mL), an amount of enzyme equivalent to 20 units and water (3.5% based on the total quantity of reagents). The reactions were held at 50 °C in erlenmeyers with screw caps at 150 rpm for 24 h. We stopped the reactions by separating the enzyme by filtration. The remaining free acids were neutralized, pouring 1.5 equivalents of KOH 0.5 N dissolved in ethanol solution 30% into the reactions maintained at 50 °C [26]. We separated the upper layer and repeated a second extraction with 3 mL of hexane. The upper two layers were collected, and a stream of nitrogen gas was used to evaporate the solvent.

The acylglycerols were derivatized to methyl esters using the Bannon et al. methodology with some modifications [80]. We used fifty mg of the triacylglycerols, 3 mL of sodium methoxide (0.25 M) in anhydrous methanol: diethyl ether (1:1), and molecular sieves at 100 mg/mL, to adsorb water traces; all the mixture was shaken for 2 min at room temperature [7,80], and subsequently, 3 mL of hexane were added, followed by 15 mL of NaCl 36% in water, and vortexed for 15 min. Two extractions with hexane recovered the methyl esters produced. We calculated the amount of oleic acid and caprylic acid as published before, using heptanoate methyl ester as the internal standard [82]. The response factors were calculated by comparing the chromatographic areas from six different concentrations of the standards methyl palmitate, methyl octanoate, and methyl oleate, with those obtained from known quantities of methyl heptadecanoate [83]. The oleic and octanoic acids incorporated in the acidolysis reactions with the soluble lipase and the immobilized ones prepared with different ethanol concentrations were expressed as fraction mole (as mol%).

One  $\mu$ L of the sample was injected at a split ratio of 1/100 into a gas chromatograph with flame ionization detection (Perkin Elmer Clarus 580, Norwalk, CT). The column was a DB-23 capillary column of 60 m x 0.25 mm x 0.2  $\mu$ m (Agilent, Santa Clara, US) [7]. The oven was warmed at 50 °C for one min; after, two ramps were programmed: the first at 25 °C/min up to 200 °C. The second at 30 °C/min up to 230 °C for 3 min. The injector and detector temperature was set at 250 °C; helium was the carrier gas at a 2.0 mL/min rate.

## 2.10. Reusability and storage stability of the immobilized enzymes

The reusability of the immobilized lipase was established through the activity with pNPP, as described before [84,85]. Fifteen milligrams of the different immobilized experiments were used to evaluate the initial hydrolytic activity with this substrate. The powder was recovered and rinsed with buffer Tris HCl 50 mM pH 7.0 and used for a new hydrolysis cycle with a fresh substrate. The subsequent cycles were compared with the initial activity measured in the first cycle [85,86].

The stability of the immobilized lipases was established after one



year of storage. The powders with the enzymes fixed were stored in desiccators at room temperature (approx. 23 °C); the enzyme activities were calculated with pNPP and compared with those initially obtained on their freshly prepared immobilized counterparts.

### 2.11. Statistical analysis

We made pair-wise comparisons from the results expressed as means and standard deviation; the Post-hoc Tukey test was carried out after univariate analysis of variance to determine the statistically significant differences in means that were significant at  $p < 0.05$  (SPSS IBM, Armonk, NY, USA).

## 3. Results and discussion

### 3.1. Production of enzyme and immobilization at different ethanol concentrations

The recombinant LipA in *E. coli* BL21(DE3) was grown in auto-inducing broth to favor the slow production of the enzyme inside the bacteria. The crude lysates had a pH between 6.5 - 6.8, and after the concentration step, the activity calculated was  $0.65 \pm 0.02$  U/mL, and the specific activity was  $1.09 \pm 0.06$  U/mg. Despite the low production of the functional LipA, the immobilization in polypropylene as support was advantageous for this enzyme due to the preferred adsorption of this protein on the surface of the polymer, avoiding further purification steps and additional losses of the enzyme [35].

At the beginning of the process, residual activities at zero hours decreased due to the dilution effect of the enzyme into the mixture of ethanol, buffer, and support. During the first two hours, the adsorption process was fast for the three preparations, and at 24 h, the activity was reduced to over 96% compared with the initial activity measured before the addition of ethanol, indicating that the enzyme bound to the polypropylene (Fig. 1A). The rapid and easy adsorption of lipases on these hydrophobic surfaces has been shown before [35]. Moreover, the adsorption process in the outer cortex of the support particles does not seem to suffer any restriction nor any detriment in the activity related to the access of the substrate, considering the size of the enzyme (~50 Angstrom) and being the support predominantly a macroporous polymer (87% with pore diameter >500 Angstrom) with the bulk of particles ranging between 420 and 177  $\mu\text{m}$  [87,88].

We used the support previously wetted with 13 mL of ethanol to ease the moistening of the polypropylene. However, we did not remove this solvent or use it in lower concentration as it has been reported [89,90], since, in preliminary assays, we observed a reduction in the adsorption of the enzyme once the ethanol is removed, finding most of the enzyme in the supernatant reaction instead of the support (data not shown). In

the process we followed, the reduced contact between the proteins and the support may hinder the access of the enzyme through the support's pores, making necessary the use of a hydrophilic solvent during the immobilization process [54]. Ethanol produced hyperactivation of LipA PSA01, as we evidenced in the enzyme solutions with ethanol 25% and 30% (121% and 104%, respectively) concerning their initial activities (Fig. 1A).

The adsorption of proteins in the suspension was not complete during the immobilization, but it was similar in the three experiments (there were statistical differences for the preparations immobilized with ethanol 20% and 25% got a fixation of proteins between 65% and 79%) (Table 1). The fixation level resulted quite similar to the reported in another study using the same support [32]. On the contrary, the immobilized activity in terms of immobilization efficiency was greater than 93%, indicating that the enzyme could be selectively adsorbed (Table 1). However, the profiles of proteins adsorbed showed that besides the lipase PSA01 (29 KDa), other proteins present in the crude lysates were adsorbed on the support surface (Fig. 1B). The adsorption of these proteins on the support seems to benefit the low loading of protein present in the lysates. Otherwise, the enzyme could occupy places of difficult access for the substrates into the support pores. Bosley reported deactivation when the immobilization occurred a low loading and the effect of other proteins such as ovalbumin to prevent such deactivation [54,91]. Perhaps, the successful adsorption of LipA PSA01 and other proteins present in the lysates occurred because the latter favored the adsorption process by occupying sites in the support with high affinity and by reducing the excess of surface available that may increase the contact area between enzyme and support, which may distort the active site [32,54].

The most noticeable effect of the immobilization of LipA PSA01 in hydrophobic matrices is the hyperactivation of the enzyme. We observed specific activities from 41 to 62 U/mg of protein immobilized in the support, clearly, a huge difference compared with the initial specific activities obtained with the free enzyme ( $1.09 \pm 0.06$  U/mg). These results indicate hyperactivation of the lipase in this support in addition to a purification step. Hydrophobic polymers have been used as a step in lipase purification processes, and once fixed, they showed much higher activities than those observed with the free enzymes [53,90,92]. When the lipase is adsorbed on the polypropylene, the opening and exposure of the active-site cavity appear to be the currently accepted mechanism for hyperactivation [38,48]. Although it has been established that LipA does not undergo interfacial activation the enzyme activity increased significantly here once LipA was immobilized, compared with the soluble enzyme. The conformational changes that occurred due to the opening of the lid and their preservation after immobilization could explain what we observed with LipA PSA01.

The treatments with different concentrations of ethanol had no

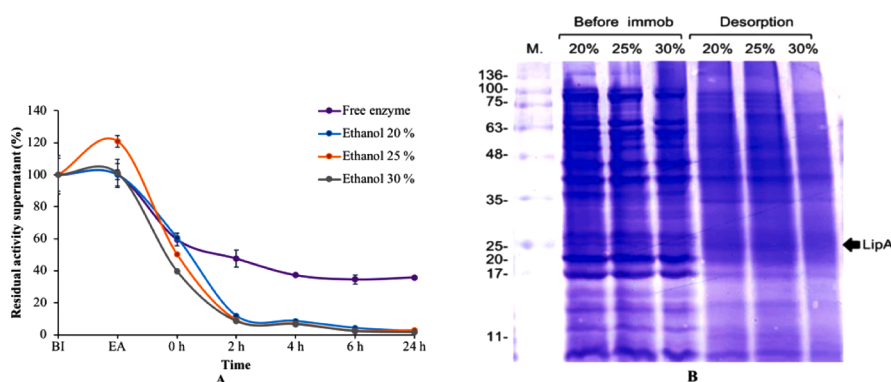


Fig. 1. (A) Time course of the immobilization of LipA in polypropylene MP1004 with three ethanol percentages. BI: Before immobilization. EA: relative activity of the lysates once added 3.0 mL (25%) and 6.25 mL (30%) and before they were added into the wetted support. (B) Besides the LipA, other proteins were adsorbed in the support.

**Table 1**

Performance of the LipA immobilization in polypropylene MP1004 with different ethanol concentrations.

Ethanol used (%)	Initial amount of protein (mg)	Protein loaded on support (mg/g)	Fixation level (%)	Immobilization efficiency (%)	Apparent activity (U/g support)	Specific activity immobilized (U/mg protein immobilized)
20	12.3 ± 0.5	4 ± 0.2	65.4 ± 1.8 <sup>a</sup>	94 ± 0.3	263 ± 5.5 <sup>a</sup>	62 ± 4.4 <sup>a</sup>
25	12.4 ± 0.5	4.8 ± 0.1	79 ± 12.1 <sup>b</sup>	92.1 ± 1.1	304.1 ± 9 <sup>b</sup>	60 ± 4.0 <sup>ab</sup>
30	12.1 ± 0.2	4.4 ± 0.3	73 ± 6 <sup>ab</sup>	95.2 ± 0.6	179 ± 3.4 <sup>c</sup>	41.4 ± 0.4 <sup>c</sup>

Means ± SD, are based on three replicates. Different letters in superscript (a, b, c) in the same column indicates means statistically different (Tukey test p value <0.05).

significant effect on the percentages of protein adsorbed. However, we observed differences in the apparent activity (U/g of support) with pNPP as substrate, being higher for the immobilization with ethanol 25% than the derivatives prepared with ethanol 20% and 30% (Table 1). Simulation approaches with the thermotolerant enzyme BTL2 in ethanol showed an increase in the lid's fluctuation and increased enzyme flexibility and the rearrangement of some polar groups that become buried in the core of the enzyme when this was exposed to less polar solvents than water [93]. Accordingly, we believe that ethanol caused these changes. At the same time, more hydrophobic residues from the enzyme likely resulted exposed, that are indeed compatible with the polypropylene, distorting the catalytic site and triggering slight changes that varied according to the amount of ethanol in the media, obtaining catalyzers with slight differences between them. Other approaches modifying some immobilization conditions, such as detergents, pH, and temperature variations, have been used to modulate the activity in the immobilization of lipases [30].

*Psa01 per se* can tolerate ethanol that could harm other enzymes and shows hyperactivation with this solvent. Few studies relate the effects of ethanol and the immobilization of enzymes in hydrophobic matrices. Blanco et al. used ethanol 10% to immobilize CalB, using silica support derivatized with octyl groups [55]. They indicated that such enzymes do not undergo hyperactivation with ethanol, but once immobilized at this ethanol concentration, their activities were increased three times more than that of the free enzyme [55]. The authors claim that the hyperactivation resulted from small changes into the microenvironment of the support, doubling the capacity on the surface of the support and allowing more attachment of lipase [55]. This assumption could explain the hyperactivation of an enzyme just as CalB, which has a small lid, so solvents such as ethanol may not provoke interfacial activation nor significant changes in the activity as LipA does.

In this study, LipA *Psa01* suffers hyperactivation, as we saw with the preparations at different ethanol concentrations in which the specific activities reach up to 63.5 U/mg of immobilized lipase (Table 1). Our results show that ethanol concentrations could influence the enzyme and its activity, as a load of protein on the support at different ethanol concentrations is almost the same (no significant differences), but the apparent activities obtained were different, being the immobilized prepared with ethanol 25% the one which exhibited the highest activity (304.1 ± 9 U/g) (Table 1). Therefore, we believe that this solvent probably accomplished two functions in the immobilization of LipA *PSA01*: The first is to allow the access of the enzyme on the porous surface of the hydrophobic support, and the second is to provoke changes in the enzyme, that include not just the opening of the lid and the further hyperactivation, but also conformational changes in the active site, that according to the amount of this solvent, altered its interaction with the substrate evaluated. This effect, known as bioimprinting, consists of adding some compounds before the immobilization that alter the enzyme structure to some extent, seeking to preserve once the additive is removed, such structure on the support. Bioimprinting has been used in other investigations to improve the enzyme before its fixing to supports [94,95].

### 3.2. Stability at the temperature

The results showed that the enzymes immobilized were much more

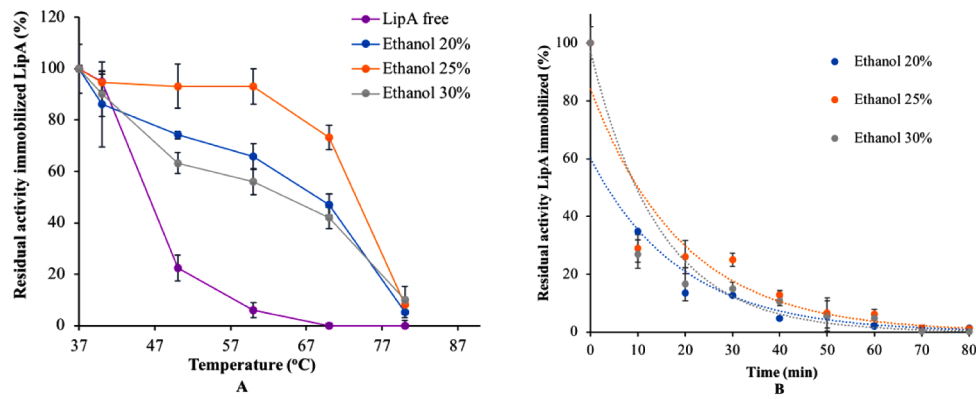
resistant to high temperatures than the free protein. The tolerance to temperature was more significant for the supports prepared with ethanol 25%, exhibiting resistance of up to four folds higher than the free enzyme at 50 °C, and almost 16 times at 60 °C (Fig. 2A). The  $T_{50}^{15}$  calculated were 69 °C, 73.2 °C, and 68 °C for the enzymes prepared with ethanol 20%, 25%, and 30%, respectively. In contrast, the free enzyme had a  $T_{50}^{15}$  of 46.1 °C. At 70 °C, the enzyme immobilized with ethanol 25% retained the 73.3% of activity, but at 80 °C, the activity declined rapidly, with  $t_{1/2}$  of 13 min for the enzymes exposed to 20% and 25% of ethanol, and 10 min for the support exposed to 30% ethanol (Fig. 2B).

The results with the enzyme immobilized with 25% of ethanol allow us to infer that this solvent can provoke slight changes in the enzyme structure, making it more tolerant to denaturant effects of the temperature. Therefore, a specific concentration of ethanol seems necessary to trigger the hyperactivation of the enzyme by allowing the open conformation and favor subtle changes in the structure, which improve its temperature resistance. The stability at higher temperatures has been shown in other studies using hydrophobic supports, and it has been attributed to an increased rigidity that restricts the movements of the enzyme, which usually occurs with the increasing of the temperature [53,92]. Other assumptions suggest that the support protects some regions of the enzyme that function as a nucleus of unfolding, preventing the denaturing effect of the temperature and maintaining stability [96].

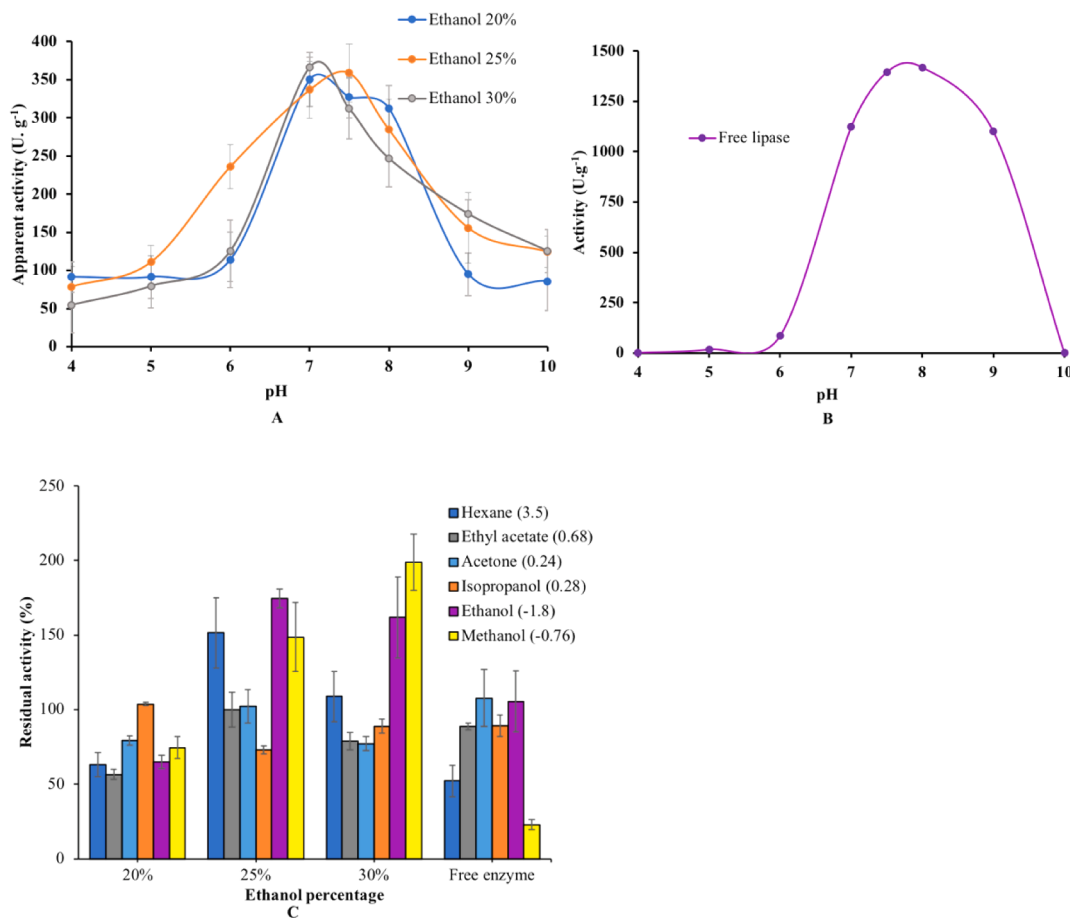
The use of ethanol in low concentrations could favor the stability of the enzyme as it has been stated that enzymes in solvents combined with water confer stability to the structure [97]. Another study also confirmed that mixtures of water and solvents (up to 20%) appear not to alter either the alpha-helices or beta-sheets and the changes in the enzyme activity could be more related to disturbances in the environment surrounding the active site [98]. Similarly, studies with molecular dynamics have shown that mixtures of ethanol stabilize some enzymes than pure water. This stability was seen as conserving the secondary structure domains, specifically the alpha-helices and beta-sheets [99].

### 3.3. Tolerance to pH variations and organics solvents of the immobilized enzymes

The enzymes fixed on the support showed activities in a broad range of pH, just as the free enzyme. The best activities for the enzymes prepared with ethanol, 20% and 30%, were observed at pH 7.0, while the enzyme fixed with ethanol 25% had a better activity at pH 7.5 (Fig. 3A). Although the soluble LipA performs better at pH 8.0 (Fig. 3B), we consider that structural changes that occurred at pH 7.0 were conserved when the enzyme was adsorbed onto the support resulting in high activity at pH closer to 7.0. This effect could be the same as "pH memory", which consists of retaining the last ionization state of the enzyme before the immobilization [100]. Since the free and immobilized enzyme activity was measured at pH 8.0 with pNPP, we assumed that the lipases suffered changes in their ionization pattern, which derived from irreversible alterations of the structure. Nevertheless, the immobilized enzyme responded better to the extreme pH than the free enzyme (pH 4 and pH 10), suggesting that immobilization can stabilize the enzyme against these harsh conditions. This effect was also reported with the *Candida rugosa* lipase immobilized in amberlite and Al<sub>2</sub>O<sub>3</sub>, especially in extreme pH, where the enzyme exhibited a stable behavior [71]. In other investigation, the effects of the pH used during immobilization of the



**Fig. 2.** Effect of the temperature in the activity of the immobilized enzyme prepared with different ethanol percentages. (A) Residual activity of the immobilized enzyme exposed at different temperatures (40 °C - 80 °C) for 15 min (the activity of the immobilized enzyme at 37 °C was established as 100%) (B) Deactivation profile after exposure of the three immobilized lipases at 80 °C for 80 min (dotted lines are a guide to the eye).

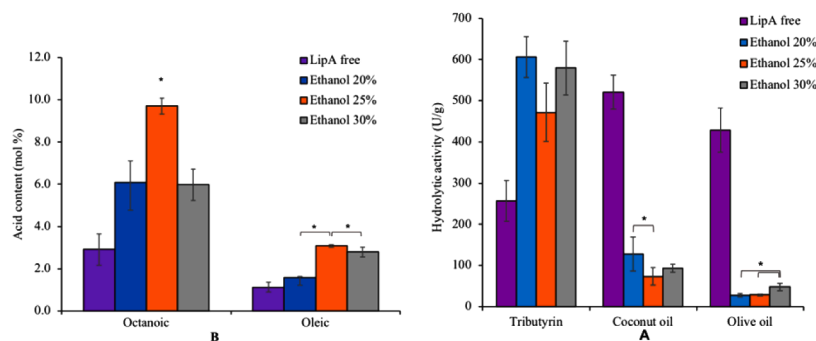


**Fig. 3.** Effects of ethanol amount used in the immobilization of LipA on the tolerance to pH and solvents. (A) Activities of LipA immobilized with three amounts of ethanol (20%, 25%, and 30%) incubated in different pH solutions from pH 4 to pH 10, for 96 h. (B) Activities of the free enzyme subjected at different pH buffers in the same conditions. (C) Residual activities of lipA immobilized using three ethanol percentages, after incubation with six pure solvents of different partition coefficients (Log P) for 96 h. The activity of the soluble enzyme in the respective buffer was taken as the 100%. The residual activity of the free enzyme was calculated taking the activity of LipA in buffer Tris HCl 50 mM pH 8.0 as 100%.

lipase of *P. fluorescens* were related to the high loading of the enzyme used, showing immobilization yields higher at pH 9.0 than at pH 7.0, but best activities with p-nitrophenyl butyrate when the buffer used for immobilization had the pH 7.0 [101]. Likewise, Arana et al. reported opposite effects when using low loadings during the immobilization process [101].

The solvent impact over LipA preparations, measured as their

residual activities, revealed contrasting results depending on the solvent nature (Fig. 4). In general, we observed high stabilities even though we used solvents in high concentrations (90% for the free enzymes and neat for the immobilized ones). Accordingly, the tested solvents produced the hyperactivation of either the free enzyme or any of the three immobilized preparations produced. Five out six solvents produced hyperactivation in the immobilized prepared using 25% ethanol (Fig. 3C), and



**Fig. 4.** Hydrolytic and synthetic activities of the free enzyme and their immobilized preparations with different ethanol percentages. (A) hydrolytic activities with three natural substrates. (B) Mole Fraction (mol%) of the octanoic and oleic acid incorporated in acidolysis reactions with tripalmitin. \*Means with P values < 0.05 considered statistically significant.

the enzyme fixed with ethanol 20% showed the lowest tolerance to the solvents evaluated.

Free LipA showed tolerances higher towards hydrophilic solvents such as ethanol and acetone (105.5% and 107.8%, respectively). As stated, hydrophilic solvents present deleterious effects for many enzymes by removing the stabilizing water layers essential for the enzyme's flexibility and activity [102]. However, with acetone, we evidenced hyperactivity of both the free and the fixed enzyme with ethanol 25%. Concomitantly, there was a slight decrease in the activity with the preparations made with 20% and 30% (77% and 79% respectively). It is possible that acetone, a polar solvent with a large dielectric constant (21), can influence the conformational structure but with a different mechanism, as other solvents do, resulting in a hyperactivated enzyme. Other lipases such as CALB lost their activity after incubation with ketones, contrary to what we observed, and it seems that a possible competitive inhibition mechanism is the one that affects the activity [103]. Another possible explanation is the partitioning of water molecules within the active site between the enzyme and the bulk solvent, replacing the removed water with solvent molecules affecting the catalysis [104].

Opposite to the above, our lipase underwent a significant impact after incubation with methanol. This loss of activity, perhaps, is related to the penetration of methanol into the catalytic cavity, disrupting the hydrophobic interactions and hydrogen bonds present, causing the unfolding and deactivation of the enzyme [105,106]. These results indicate that incubation with different alcohols exerts different outcomes on the stability of LipA, and as it has been reported, the tolerance depends on the enzyme itself [107]. Immobilization improved LipA tolerance to methanol, especially to those prepared with ethanol 25% and 30%, with residual activities reaching up to approximately eight folds for the immobilized prepared with ethanol 30%, concerning the activity of the free lipase. Our findings suggest that denaturing effects of methanol can be mitigated because the structure is stable enough through its fixation to the support to prevent the loss of activity. The resistance to this solvent could expand the potential of LipA PSA01 for applications in biofuel synthesis.

We used a hydrophobic solvent such as hexane (log P 3.5) to determine its effect in the enzymes immobilized with polypropylene. The activity on pNPP after the exposure to this solvent showed a beneficial effect on the enzyme's activity than with the free enzyme (151.5% and 108% for immobilized prepared with ethanol 25% and 30%, respectively). However, the enzyme activity fixed with 20% of ethanol was lower than the measured with the free counterpart (63%). Kamal et al. reported an increase in the stiffness in solvents with high Log P and the rearrangement of the side chains of some hydrophobic residues placed in the vicinity of the active site cavity, affecting its affinity for the substrate and therefore the activity [98]. On the contrary, studies with CalB showed that non-polar solvents change the orientation of the amino acids inside the active site cavity (measured as root mean square

deviation or RMSD) more than the polar solvents, and there was better conservation of the structure of alpha-helices in solvents with more polarity than those with lower polarity, and therefore lower impact in the activity of the enzyme [105]. These contrasting reports emphasize the need to carry out more studies that explain the mechanisms behind the hyperactivation or denaturation of enzymes like LipA PSA01.

Thus, the enzyme is tolerant to the effects of both polar and non-polar solvents. In this way, immobilization with specific ethanol concentrations gave LipA PSA01 operational stabilization towards solvents with different partition coefficients. Our results are in correspondence with the reports published by Peng et al. examining the organic solvents resistance for 24 h of LipA from *P. aeruginosa* (they used a concentration of 50%), showing that LipA is a solvent-resistant enzyme tolerating different organic protic and non-protic solvents [62]. Moreover, in the present study, the incubation of the free and immobilized LipA with pure and highly concentrated solvents resulted in higher stabilities that depended on the immobilized assayed and the solvent used.

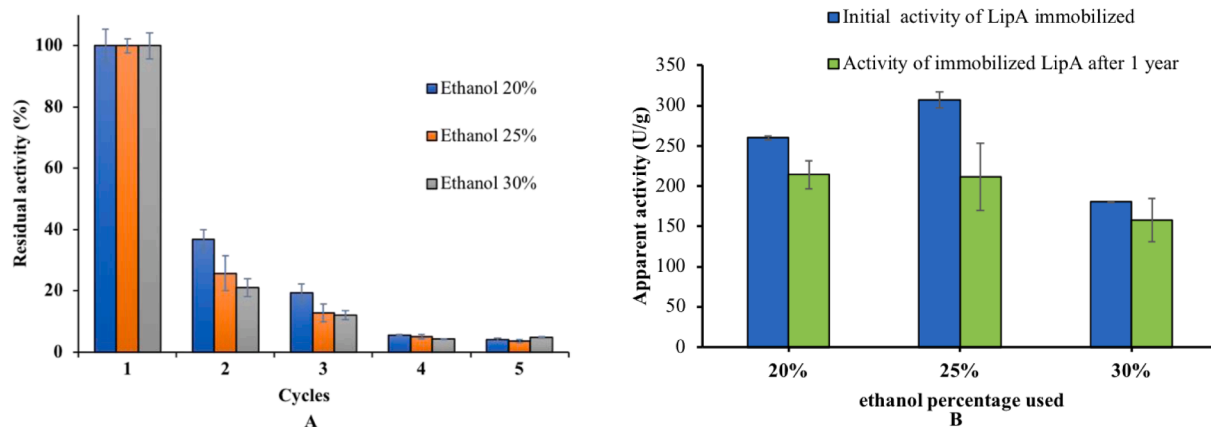
#### 3.4. Hydrolytic and synthetic activities on natural substrates

LipA free and immobilized counterparts were subjected to hydrolytic reactions using oils with different chain lengths as natural substrates (Fig. 4A). After 24 h at 37 °C, the LipA free showed activity for the three oils, but preferentially over those present in coconut oil, compared with the hydrolysis of olive oil and tributyrin (Fig. 5B). The triacylglycerols in olive oil were mainly composed of oleic acid (18:1) and palmitic acid (16:0), while coconut oil had more abundant lauric and myristic fatty acids (Table 2). In contrast, the hydrolytic activity with olive and coconut oil was severely affected with minor differences in the three preparations. Conversely, the activity of immobilized enzymes with tributyrin was higher than the observed with the free lipase. In this way, we evidenced a change in the activity with the immobilization resulting in an enzyme with preferential activity for short fatty acids instead of medium and large fatty acids.

It is noteworthy the reduced hydrolytic activity of LipA immobilized with these natural substrates with medium and long chains, considering that the preparations exhibited good activity towards the substrate pNPP. Our findings show that immobilization modifies the enzyme activity presumably by a distortion of regions in the catalytic site related to accommodating triacylglycerols of long fatty acids. Thus, the alterations in the activities could result from the increased stiffness of the enzyme on the support that inhibits the accurate induce-fit required to hydrolyze triacylglycerols. Equally, the emulsions are heterogeneous mixtures, and probably there were transfer mass issues that could affect the activity. The improvements in the resistance to operational conditions by placing enzymes in porous surfaces often go with changes in some attributes of the enzyme, adjustments that can be modulated for specific purposes as reported [30,108].

These results indicate that activities on the artificial substrate pNPP





**Fig. 5.** Reusability and shelf storage of the immobilized enzymes (A) : Residual activities of the immobilized preparations subjected to five cycles of reuse in hydrolytic reactions with p-nitrophenyl palmitate as substrate. The residual activities were estimated considering the activities of the first cycle as 100%. (B) Comparisons of the initial activities and those obtained after one year, of the three immobilized preparations, using three ethanol percentages.

**Table 2**

Fatty acids compositions of oils used in this study.

Fatty acids	Composition (%)	
	Coconut oil	Virgin olive oil
Octanoic acid (8:0)	8.3 ± 0.4	ND
Decanoic acid (10:0)	8.6 ± 0.9	ND
Lauric acid (12:0)	35.5 ± 4.1	ND
Myristic acid (14:0)	19.4 ± 0.1	ND
Palmitic acid (C16:0)	12.4 ± 1.4	15.0 ± 0.1
Palmitoleic acid (16:1)	ND	1.4 ± 0.01
Stearic acid (C18:0)	5.5 ± 0.9	4.2 ± 0.04
Oleic acid (C18:1)	9.1 ± 0.5	70.0 ± 0.6
Linoleic acid (C18:2)	1.1 ± 0.2	8.6 ± 0.05
Linolenic acid (C18:3)	ND	0.9 ± 0.03
Eicosenoic acid (C20:1)	ND	0.3 ± 0.1

Means ± SD; ND: Not detected.

cannot indicate the behavior of the enzyme on natural substrates. The former displays a three-dimensional structure, and each chain interacts with the three pockets integrating the catalytic cavity of this enzyme. Hence, the alcohol binding pocket hosts the *sn*-2 chain, the acyl pocket the *sn*-3, and the solvent pocket accommodates the *sn*-1 chain of the triglyceride [109]. On the other hand, the substrate pNPP is composed of a single 16-carbon chain linked with p-nitrophenol alcohol that forms an ester bond and feasibly occupies the solvent pocket; hence, fewer interactions would be required to fit this molecule to the enzyme, and the hydrolysis reaction could occur more easily than with the triglyceride.

We examined the performance of both the free enzyme and the lipase immobilized with different ethanol concentrations on acidolysis reactions at 50 °C using tripalmitin, octanoic acid, and oleic acid as substrates and hexane as solvent. The resistance to solvents of our lipase allowed it to carry out reactions of synthesis in where a minimum amount of water is necessary to favor the catalysis. Equally, with an enzyme more tolerant to temperature, we could synthesize methyl esters using tripalmitin as a substrate (melting point: 44.7 - 67.4 °C). Methyl ester response factors were calculated with six concentrations of the methyl esters: 0.93, 1.3, and 1.4 for methyl palmitate, methyl oleate, and methyl caproate, respectively. The experiments that used the free enzyme showed little incorporation of the fatty acids used, probably due to the denaturing effect of temperature and the effects of hexane on the activity (Fig. 4B). Hydrolysis rather than synthesis could also be favored in the aqueous microenvironment in which the enzyme was dissolved. Contrary to what we observe in hydrolytic reactions, there was a better performance in acidolysis experiments of the immobilized enzymes than those of the free lipase. Perhaps the hydrolytic reactions in a biphasic system such as emulsions can create mass transfer problems, as micelles

can be much larger than the pore diameters of the support and do not reach the enzymes fixed, resulting in a decrease of the measured activity [54].

We evidenced a better preference of LipA towards the fatty acids found in coconut oil than those contained in olive oil, and this trend was also observed with the immobilized enzymes. However, the enzyme immobilized with ethanol 25% presented greater incorporation of both fatty acids than the other immobilized preparations and the free lipase. Though LipA exhibits activity with a wide range of substrates, the enzyme shows a preference for medium-chain fatty acids, as reported by Bofil et al. using p-nitrophenyl derivatives substrates [110].

### 3.5. Reusability of the enzyme and storage

Reusing the enzyme is an advantage of immobilization, deriving in cost savings from using the enzyme in more than one cycle. The reuse of the immobilized enzyme showed that they could be used up to 5 cycles, but the grade of hydrolysis decreased profoundly in the second cycle and continued in the same way until the fifth cycle (Fig. 5A). LipA leaching occurred with not many differences in the three conditions examined. Although immobilization by adsorption is considered a straightforward process with many advantages, the main drawback of this methodology is the desorption of the enzyme, which occurs easily, mainly in the aqueous medium, because of the breakdown of the weak forces attaching the enzyme to the support [71,111,112]. However, in methodologies such as covalent bonding, the attachment to the support happen in the closed conformation, and the covalent bonds can trigger changes in the topology of the active site, resulting in a decrease or loss of activity [113]. Despite the disadvantages, the adsorption compared with other methodologies for immobilizing results in immobilized enzymes with the best activities [37,96]. The selection of polypropylene has demonstrated promising results in terms of activity. However, additional strategies must be considered to avoid desorption, such as the adsorption followed by crosslinking or precipitation [71] or the coating of the immobilized particles with silicone polymers, that allows the access of the substrate, avoiding the leaching of the enzyme and contributing with the mechanical strength of the support [114].

Concerning the stability under storage, the comparisons of the initial specific activities and the measured after one year revealed a reduction of this parameter, the highest being 31% for the one immobilized with ethanol 25% (Fig. 5B). The enzyme fixed with ethanol 30% showed upper stability (12.7% lower than the initial activity). These results highlight the lipase immobilized with higher ethanol concentration as the most stable, despite its activity was not the most outstanding. As explained above, besides the anhydrous conditions in which the enzyme was kept, probably the increased rigidity of this protein due to the

ethanol used and its fixation on the polypropylene seems to benefit the tolerance of the enzyme to the eventual changes in environmental conditions. The shelf stability is an essential characteristic for the possible use of lipases in industrial settings, and LipA acquired good stability with the immobilization without needing refrigeration. Other reports show that lipases in porous supports have stabilities even lower than found in this investigation [115].

#### 4. Conclusions

In this investigation, the immobilization using ethanol as an additive was a valuable strategy to enhance the performance of enzymes. The enzyme activity and stability were increased significantly concerning the free enzyme, and this increase was related to the amount of ethanol used. Ethanol in this study had two roles: To ease the access of the enzyme on the porous surface of the hydrophobic support and trigger modifications in the enzyme structure, including the opening of the lid and other changes that depended on the amount of ethanol. The enzyme immobilized increased its tolerance temperature and the denaturing effects of hydrophobic solvents and hydrophilic, especially methanol. The immobilized lipases preferably hydrolyzed triglycerides with short-chain fatty acids than those with long-chain fatty acids, while in acidolysis reactions, the behavior was similar to the exhibited by the free enzyme. Desorption of the enzyme is a disadvantage of the immobilization by adsorption, and further improvements must be undertaken to obtain an enzyme more competitive and potentially valuable for biotechnology processes where harsh conditions are frequently used.

#### Author contribution statement

All the authors listed have made substantial, direct, and intellectual contributions to the manuscript and approved it for publication.

#### Declaration of Competing Interest

The authors state that there are no declarations of interest.

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