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Separation and Immobilization of Lipase from *Penicillium simplicissimum* by Selective Adsorption on Hydrophobic Supports

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Abstract Lipases are an enzyme class of a great importance as biocatalysts applied to organic chemistry. However, it is still necessary to search for new enzymes with special characteristics such as good stability towards high temperatures, organic solvents, and high stereoselectivity presence. The present work's aim was to immobilize the lipases pool produced by *Penicillium simplissicimum*, a filamentous fungi strain isolated from Brazilian babassu cake residue. *P. simplissicimum* lipases were separated into three different fractions using selective adsorption method on different hydrophobic supports (butyl-, phenyl-, and octyl-agarose) at low ionic strength. After immobilization, it was observed that these fractions' hyperactivation is in the range of 131% to 1133%. This phenomenon probably occurs due to enzyme open form stabilization when immobilized onto hydrophobic supports. Those fractions showed different thermal stability, specificity, and enantioselectivity towards some substrates. Enantiomeric ratio for the hydrolysis of (R,S) 2-*O*-butyryl-2-phenylacetic acid ranged from 1 to 7.9 for different immobilized *P. simplissicimum* lipase fractions. Asymmetry factor for diethyl 2-phenylmalonate hydrolysis ranged from 11.8 to 16.4 according to the immobilized *P. simplissicimum* lipase fractions. Those results showed

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that sequential adsorption methodology was an efficient strategy to obtain new biocatalysts with different enantioselectivity degrees, thermostability, and specificity prepared with a crude extract produced by a simple and low-cost technology.

Keywords Fraction separation · Immobilization · Enantioselective hydrolysis

Introduction

Lipases (glycerol ester hydrolases E.C. 3.1.1.3) have triglycerides as their natural substrate, being able to catalyze a wide range hydrolysis or synthesis of soluble and insoluble esters in water. In the last two decades, the interest for lipases increased due to their industrial applications in oil and fats hydrolysis [1], fatty acid esters synthesis as cosmetic ingredients or surfactants [2], and kinetic resolution of racemic mixtures with high regio- and stereospecificities producing intermediates for pharmaceuticals, pesticides, and agro-chemistry synthesis [3].

A great number of bacterial and fungi lipases have been commercially produced and used under the crude extract form as biocatalyst in some reactions. However, crude extract utilization without purification frequently takes a great time demand and decreases the enantioselectivity due to contaminant presence, including enzymes with different catalytic activities. The traditional microbial lipases purification methods require, in most cases, at least four or five stages including hydrophobic interaction chromatographic methods and ionic exchange, increasing these biocatalysts' production cost. Currently, new enzyme purification strategies with low cost, fast, and high yield have been developed, for example the selective adsorption on hydrophobic supports at low ionic strength. This method has been used for lipases' crude extract purification, immobilization, and hyperactivation from different sources via a single adsorption step [4, 5].

Mandelic acid enantiomers [2] and their derivatives are valuable chemicals that have been utilized extensively for synthetic purposes as well as stereochemical investigations. Mandelic acid enantiomers are employed for racemic alcohols and amines resolution. R(−)-mandelic acid is used as semi-synthetic cephalosporin's and penicillin's manufacture precursor. It is also used as a chiral resolving agent and chiral synthon for anti-tumor and anti-obesity agents' synthesis [6].

Recently, our laboratory isolated and identified one wild fungal strain of *Penicillium simplicissimum* that produces large amounts of lipase in solid-state fermentation in babassu cake, a Brazilian agro-industrial residue, consisting of a low-cost production system. The medium composition and culture conditions for lipases production were previously optimized [7].

In the present work, lipases contained in crude extract from *P. simplicissicimum* were separated in three different fractions via sequential adsorption on supports with different hydrophobic groups at low ionic strength. The fractions were characterized and showed different stability and catalytic activity. Furthermore, the enantioselective hydrolysis of 2-*O*-butyryl-2-phenylacetic acid and diethyl 2-phenylmalonate were carried out using different biocatalysts.

Materials and Methods

Materials

The *P. simplicissimum* strain was isolated and selected as described by Gutarra et al. [7]. Butyl-, phenyl-, and octyl-agarose were obtained from Hispanagar S.A. (Burgos, Spain). *p*-Nitrophenyl-butyrate (*p*NPB), Triton X-100, Cetyl-trimethyl-ammonium bromide (CTAB),

and diethyl 2-phenylmalonate were obtained from Sigma Chemical (St. Louis, USA). 2-*O*-Butyryl-2-phenylacetic acid was synthesized as previously described [8]. All other chemicals were of analytical or chromatographic grade.

P. simplicissimum Lipases

The crude extract containing extracellular lipases from *P. simplicissimum* was produced by solid-state fermentation using a residue from babassu oil industry kindly provided by TOCANTINS BABAÇU S.A. (Tocantinópolis, Brazil). Ten grams of babassu cake medium was supplemented with sugar cane molasses (6.25%, *w*/*w*) and moistened to 70% (*w*/*w*) and inoculated with 10^7 spores per gram of dry babassu cake. After 72 h of fermentation (maximum production), the enzyme was extracted with 45 mL of 100 mM sodium phosphate buffer at pH 7.0 [7]. The protein solution was precipitated with ammonium sulfate (60% saturation) and centrifuged at 10,000 rpm for 30 min. The crude extract containing *P. simplicissimum* lipase dissolved in 5 mM sodium phosphate buffer at pH 7.0 and dialyzed against the same buffer for 2 h at 4°C.

Lipase Activity Assay for *p*NPB Hydrolysis

This assay was carried out by the absorbance increase (348 nm) over time produced by *p*-nitrophenol release in the 0.4 mM *p*NPB hydrolysis in 25 mM sodium phosphate buffer at pH 7.0 and 28 °C. The reaction was initialized by the addition of 0.2 mL diluted lipase suspension to 2.3 mL of substrate solution. One international unit (IU) of *p*NPB was defined as the amount of enzyme necessary to hydrolyze 1 μmol of *p*NPB per minute under assay conditions [5].

Immobilization on Hydrophobic Supports

The supports butyl-, phenyl-, and octyl-agarose were exhaustively washed with distilled water. One gram of the support was suspended in 10 mL of enzyme solution (1.6 mg/ml of protein) in 5 mM sodium phosphate buffer at pH 7.0, and the mixture (support and enzyme solution) was shaken at 25 °C and 250 rpm for 2 h. Thereafter, the immobilized enzyme was washed with distilled water and stored at 4 °C. Suspensions samples and the supernatants were withdrawn periodically, and the hydrolytic activity was measured using *p*-NPB as substrate. Figure 1 presents the flow chart of separation and immobilization of the different lipase fractions from *P. simplicissimum*.

Immobilization Parameters

Enzyme immobilization effect on activity (hyperactivation) was evaluated by retention activity parameter (R, %) calculated according to Eq. 1.

$$R(\%) = \frac{U_H}{U_A - U_E} \times 100 \quad (1)$$

where U_A—added units or units of activity offered for immobilization, U_E—output units or units of activity in the solution after immobilized procedure, and U_H—immobilized units or units of immobilized activity measured experimentally.

Thermal Inactivation Kinetic Parameters

The thermal inactivation assays of soluble lipase and immobilized preparations on butyl-agarose and octyl-agarose were carried out by incubating the same lipase amount in three

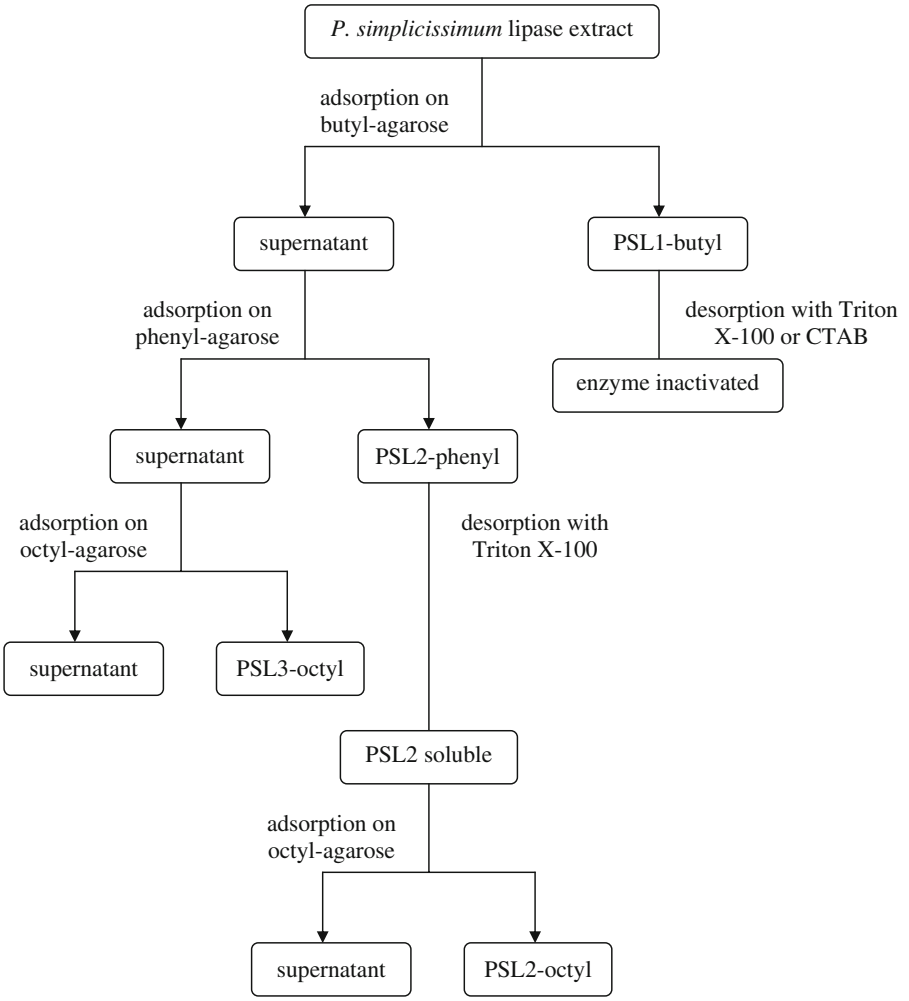

Fig. 1 Flow chart of the separation and immobilization of the different fractions of lipases from *P. simplicissimum*

different buffers at three different temperatures, 30, 40, and 50 °C: 25 mM sodium acetate at pH 5.0; 25 mM sodium phosphate at pH 7.0; and 25 mM sodium carbonate at pH 9.0. Periodically, residual enzyme activity was measured by *p*NPB hydrolysis. The soluble enzyme was submitted to the same conditions.

The half-life ($t_{1/2}$) time was calculated according to Eqs. 2 and 3:

$$E = E_0 \exp(-kt) \tag{2}$$

$$t_{1/2} = \frac{\ln 2}{k} \tag{3}$$

where E is the enzyme-specific activity for a reaction in time t (U/g), E_0 is the enzyme-specific activity for t=0 (U/g), and k is the observed deactivation rate constant.

Sodium Dodecyl Sulfate–Polyacrylamide Gel Electrophoresis

One milliliter of enzyme solution or 0.7 g of immobilized enzyme preparations was suspended in 1 mL of 0.125 mM Tris containing bromophenol, 10% (*v*/*v*) mercaptoethanol, 40% glycerol, and 4% (*w*/*v*) sodium dodecyl sulfate (SDS) and incubated at 100 °C for 15 min. This treatment desorbed any protein physically adsorbed on the support [9]. SDS–polyacrylamide gel electrophoresis (PAGE) electrophoresis was performed according to the Laemlli's method [10] in a SE 250-Mighty Small II electrophoretic unit (Hoefer Co.) The gels were stained following the silver staining method [11]. Molecular weight markers were the LMW kit (14,400–94,000 Da) from Pharmacia.

Esters Enzymatic Hydrolysis

Enzymatic activities of *P. simplicissimum* lipase preparations were analyzed during the different esters hydrolysis reaction.

2-*O*-butyryl-2-phenylacetic acid was dissolved in a 3 mL solution of 25 mM sodium phosphate buffer at pH 7.0 and 25 °C to a substrate concentration of 0.5 mM, and 0.5 g of immobilized preparation was added.

Diethyl 2-phenylmalonate was dissolved in 5 mL of 25 mM sodium phosphate buffer at pH 7.0 to a final substrate concentration of 1 mM, and 0.5 g of immobilized preparation was added.

Enzymatic activity (micromole of substrate hydrolyzed per minute per gram of immobilized preparation) was evaluated via reverse-phase high-performance liquid chromatography (HPLC; Spectra Physic SP 100 coupled with an UV detector Spectra Physic SP 8450) on a Kromasil C18 (15×0.4 cm) supplied by Analisis Vinicos (Spain). The mobile phase was acetonitrile (35%) and 10 mM ammonium phosphate buffer (65%) at pH 2.3 at a flow rate of 1.5 ml/min. Detection was carried out at 225 nm.

Enantiomeric Excess and Enantioselectivity Determination

Enantiomeric excess (ee) of the released acid after 2-*O*-butyryl-2-phenylacetic acid hydrolysis catalyzed by lipase was analyzed by the chiral reverse-phase HPLC. The column was a Chiracel OD-R and the mobile phase was an isocratic mixture of acetonitrile (20%) and 10 mM ammonium phosphate buffer (80%) with a final pH of 2.3 at a flow of 0.45 mL/min and UV detection performed at 225 nm. The ee and enantiomeric ratio (*E*) in all cases were calculated using the equations reported by Chen et al. [12].

$$ee(P) = \frac{(P_1 - P_2)}{(P_1 + P_2)} \tag{4}$$

$$E = \frac{\ln[1 - c(1 + ee(P))]}{\ln[1 - c(1 - ee(P))]} \tag{5}$$

where P_1 is the concentration of enantiomer 1, P_2 is the concentration of enantiomer 2, and c is the conversion.

The remaining ester ee after the diethyl 2-phenylmalonate hydrolysis catalyzed by lipase was analyzed by the chiral reverse-phase HPLC. The column was a Chiralcel OD-R with an isocratic mixture of acetonitrile (25%) and 10 mM ammonium phosphate buffer (75%) as mobile phase and with a final pH of 2.3 at a flow of 0.45 mL/min and

UV detection performed at 225 nm. Asymmetry factor (A) was calculated in all cases using the equations:

$$A = \frac{(1 + ee(P))}{(1 - ee(P))}. \tag{6}$$

Results and Discussion

Lipases Adsorption on Different Hydrophobic Supports

Lipases contained in crude extract from *P. simplicissimum* were separated in different fractions via sequential adsorption on hydrophobic supports at low ionic strength in 5 mM sodium phosphate buffer at pH 7.0 and room temperature. The supports were used increasing the hydrophobicity degree: butyl-agarose (low hydrophobicity), phenyl-agarose (medium hydrophobicity), and octyl-agarose (high hydrophobicity) [9]. Lipase crude extract from *P. simplicissimum* was incubated with butyl-agarose, and 64% of the hydrolytic activity against *p*-NPB was adsorbed on the support after 30 min (Fig. 2a). The support and the supernatant were separated by simple filtration. Then, the supernatant was incubated in phenyl-agarose presence and 60% of the hydrolytic activity was adsorbed on the support (20% of the crude extract activity; Fig. 2b). After separation, the second supernatant was incubated with octyl-agarose and the support adsorbed 85% of hydrolytic activity in the solution (12% of the crude extract activity; Fig. 2c). The methodology here in described and designed selective adsorption method allowed separation of contained in crude extract in three active fractions due to their selectivity adsorption differences on the hydrophobic surface. PSL1 was able to be immobilized even on butyl-agarose, PSL2 on phenyl-agarose but not on butyl-agarose, and PSL3 on the most hydrophobic support octyl-agarose.

The lipase crude extract SDS-PAGE from *P. simplissicimum* and three immobilized active fractions, after desorption, showed that the enzyme hydrophobic adsorption from *P. simplicissimum* lipases were partially selective as the fractions presented different protein profiles (Fig. 3). A great protein amount was restrained on butyl-agarose support. The phenyl-agarose and octyl-agarose supports presented a reduced amount of proteins in comparison with butyl-agarose. Sabuquillo et al. [4] observed similar behavior when studying different fractions separation of commercial lipases from *Rhizopus niveus* and *Candida antarctica* by selective adsorption on activated supports with hydrophobic groups (butyl-, phenyl-, octyl-agarose) at low ionic strength. The different lipase fractions from these microorganisms showed different thermal stabilities, different esterase activities, and enantioselectivities in the (R,S) 2-hidroxy-4-phenylbutanoic acid ethyl ester hydrolysis.

The fractions PSL1, PSL2, and PSL3 showed a retention activity of 131%, 240%, and 1,133%, respectively, as a hydrophobic adsorption consequence. The hydrophobic supports used, which somehow resembles the lipases natural substrates drops surface, and the low ionic strength force lipases adsorption through hydrophobic areas surrounding the active center and the internal flap face (other water-soluble proteins are not adsorbed on the support under these mild conditions). These adsorbed lipases present an open form, with the active center accessible to small substrates; in fact, immobilized enzymes usually exhibit a significantly enhanced enzyme activity (by the "interfacial activation mechanism"). In this way, the lipase open form has been "fixed" onto hydrophobic supports and has lost its dependence on the external hydrophobic substrate interfaces [13, 14]. The lipases' hyperactivation by hydrophobic

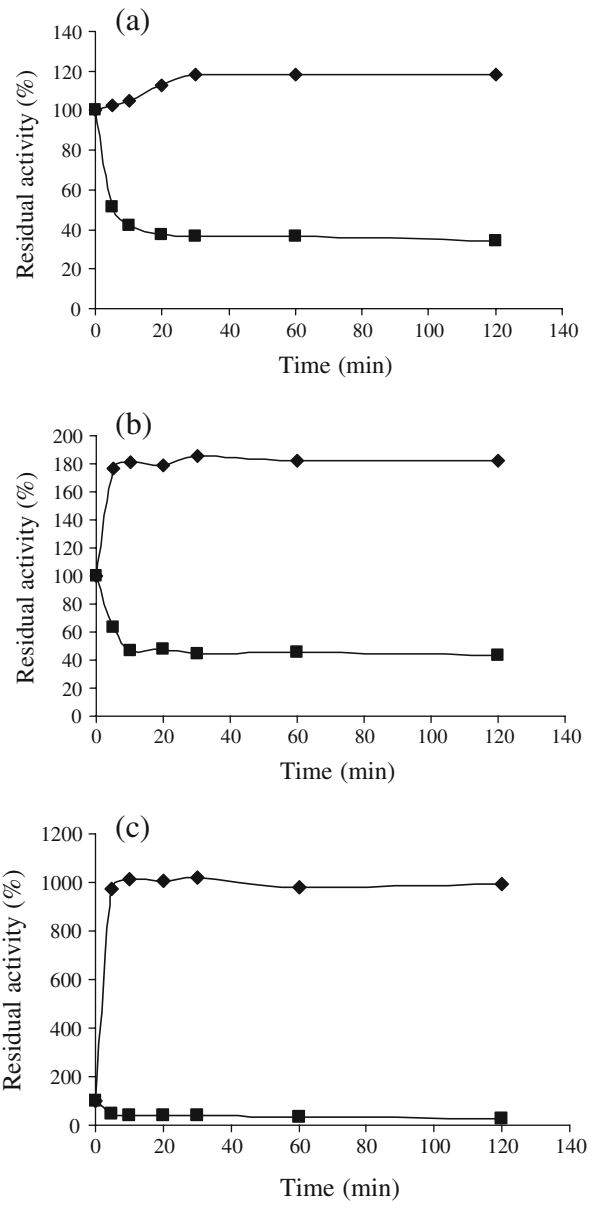

Fig. 2 Adsorption of the lipases from *P. simplicissimum* on hydrophobic supports at low ionic strength: **a** butyl-agarose, **b** phenyl-agarose, **c** octyl-agarose. Immobilization was performed as described in "Immobilization on Hydrophobic Supports". Supernatant (*square*) and suspension (*diamond*)

adsorption is usually reported in literature. For example, lipases from *Humicula lanuginosa*, *R. niveus*, and *Rhizomucor miehei* immobilized on octyl-agarose were 2,000%, 700%, and 600% more active than the corresponding soluble enzyme when catalyzing fully soluble substrate hydrolysis [9]. Palomo et al. [15] reported a lipase hyperactivation from *Bacillus thermocatenulatus* adsorbed on octyl-agarose (300%) and octadecyl-sepabeads (120%). Wilson et al. [16] described that immobilized lipase from *Alcaligenes* sp. onto octadecyl-sepabeads showed a hyperactivation of 135%.

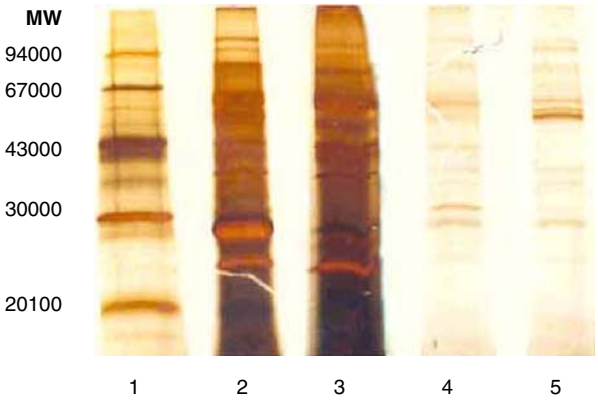

Fig. 3 Analysis of lipase fractions from *P. simplicissimum* by SDS-PAGE. Experiments were performed as described in "Sodium Dodecyl Sulfate–Polyacrylamide Gel Electrophoresis". *Lane 1*: Molecular weight standards. *Lane 2*: Soluble crude extract of lipases from *P. simplicissimum*. *Lane 3*: *P. simplissicimum* lipase immobilized on butyl-agarose. *Lane 4*: *P. simplissicimum* lipase immobilized on phenyl-agarose. *Lane 5*: *P. simplissicimum* lipase immobilized on octyl-agarose using silver staining

Preparation of Octyl-Agarose Derivatives of the Fractions PSL1 and PSL2

Some characterization studies of the different fractions were carried out using the immobilized fractions on the same support type preventing possible interferences caused by enzyme–support interactions and microenvironment formed after immobilization [14]. Thus, the lipases desorption from butyl- and phenyl-agarose were attempted in order to immobilize all the lipase fractions on octyl-agarose, the unique support able to immobilize the three lipase fractions from *P. simplicissimum* due to its high hydrophobicity. Fractions PSL1 and PSL2 were desorbed using different concentrations of Triton X-100 and CTAB in 5 mM sodium phosphate buffer at pH 7.0. The detergents used in PSL1 desorption from butyl-agarose inactivated the enzyme probably due to the enzyme denaturation by disrupting its tertiary structure. The response to the detergents presence by reported lipases has variable extent. Helistoa and Korpela [17] reported that lipases from *Candida cylindracea*, *Penicillium* sp., and *Aspergillus carneus* were inhibited by Triton X-100, whereas lipases from *Chromobacterium viscosum*, *Pseudomonas fluorescens*, and *Bacillus* sp. were activated by Triton X-100. Mateos Diaz et al. [18] found a total loss of lipase activity from *Rhizopus homothallicus* in the Triton X-100 presence.

Fraction PSL2 immobilized on phenyl-agarose was desorbed using 0.5% Triton X-100 with a yield of 70% of the hydrolytic activity. The supernatant was dialyzed and incubated in the octyl-agarose presence. This support adsorbed 80% of the hydrolytic activity and presented a hyperactivation of 216%.

Effect of pH on Thermal Stability of the Different Fractions

Thermal stability study was performed for soluble *P. simplissicimum* lipases and immobilized fractions on butyl-agarose or octyl-agarose. Initially, stability curves were carried out at 30 °C at three different pH values: 5.0, 7.0, and 9.0 as described in "Material and Methods". All the lipase fractions had presented a thermal stability increase with the pH reduction, showing the best thermal stability at 30 °C and pH 5.0, being able to retain 78% to 100% of residual activity for 48 h. The fractions' thermal stability at pH 7.0 was moderate, and at pH 9.0, all the fractions had been unstable, retaining up to 30% of their activity after 5 h of thermal inactivation. The same results were found by Gutarra [19] when working with lipase crude extract from *P. simplicissimum* also produced by solid-state

fermentation. These authors observed the increase of lipase activity as well as thermal stability with pH reduction.

Soluble and immobilized fractions were stable in pH 5.0 under elevated temperatures, retaining 60% to 95% of activity after 48 h at 40 °C. When stability curves were carried out at 50 °C, the lipases contained in the crude extract showed a half-life of 23 h, while immobilized fractions PSL1, PSL2, and PSL3 showed a half-life of 65, 14, and 41 h, respectively (Fig. 4). In general, lipases of mesophilic fungus are not stable at temperatures above 40 °C. The lipase from *Penicillium aurantiogriseum* shows low thermal stability at temperatures higher than 28 °C, presenting a residual activity of only 32% after 30 min of incubation at 50 °C [20]. The thermal stability of *Penicillium wortmanii* lipase, which is described as a moderate thermostable enzyme, retains only 55% of the initial activity after 1-h incubation at 50 °C [21].

The three immobilized fractions presented different stability probably due to the fact that those strains produced more than one lipase coded by different genes. Nawani and Kaur [22] have found that the isolated thermophilic *Bacillus* sp. produced two extracellular lipases with differences in thermal stability in which LIP1 and LIP2 showed a half-life of 65 and 45 min at 70 °C and pH 8.0, respectively. These authors suggest that the two lipases expression were influenced by the culture age.

Different Derivatives Specificity

Three different substrates were used to measure the fractions catalytic activity in order to demonstrate the possible supports selectivity towards *P. simplissicimum* lipase isoforms. The selected reactions were the hydrolysis of *p*-NPB, (R,S) 2-*O*-butyryl-2-phenylacetic acid (**rac-1)**, and diethyl 2-phenylmalonate (**pro-3**), which has different chain lengths and molecular sizes.

Table 1 shows the catalytic activity of the biocatalysts towards the three substrates. Although all fractions showed higher activity against *p*-NPB, their relative specificities with other substrates were very different. With regard to the relative activity, PSL1-butyl presented 100% of the esterase activity (with **rac-1** as substrate), while PSL2-phenyl presented 29% and PSL3-octyl only 24%. These different immobilized activity percentages indicate that among the *P. simplissicimum* lipase isoforms, those that recognize **rac-1** and **pro-3** as substrates are preferentially contained in PSL1-butyl. We may conclude that different isoforms have different enzymatic activity in the substrates' hydrolysis chosen,

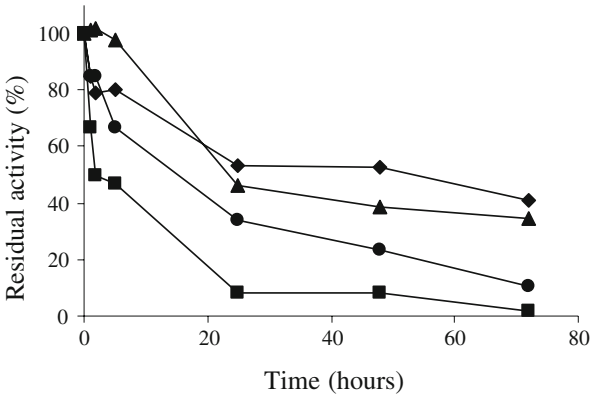

Fig. 4 Stability of the different fractions of immobilized lipase from *P. simplicissimum*. Inactivation was carried out in 25 mM sodium acetate buffer at pH 5.0 and 50 °C. PSL1-butyl (*diamond*), PSL2-octyl (*square*), PSL3-octyl (*triangle*) and soluble (*circle*)

Table 1 Specificity of the different lipase fractions from *P. simplicissimum*.

	Relative activity (%)[a]		
	p-NPB	rac-1	pro-3
PSL1-butyl	39	100	100
PSL2-octyl	34	29	69
PSL3-octyl	100	24	48

[a] Percent of relative activity taken account the values obtained for fraction more active as 100%. **rac-1**: 2-*O*-butyryl-2-phenylacetic acid. **Pro-3**: diethyl 2-phenylmalonate. Experiments were performed as described in "Esters Enzymatic Hydrolysis".

and therefore, those reactions are suitable for demonstrating selective adsorption. However, the overall results indicate that this kind of immobilization allows obtaining biocatalysts where their catalytic properties depend on the used support hydrophobicity degree.

2-*O*-Butyryl-2-Phenylacetic Acid (**rac-1**) Hydrolysis

Three different *P. simplissicimum* lipase immobilized preparations were employed in the **rac-1** hydrolysis (Fig. 5). Table 2 presents **rac-1** enantioselective hydrolysis results using the fractions PSL1-butyl, PSL2-octyl, and PSL3-octyl as biocatalysts. PSL2-octyl presented ee value (64.5%) higher than the PSL3-octyl (50%). The biocatalyst PSL1-butyl did not show enantioselectivity. On the other hand, PSL2-octyl biocatalyst presented low conversion (23%) than PSL3-octyl (63%), although the ee values achieved in this work were low when compared to the ones in literature. It is worth noting that this work was done with a non-commercial lipase obtained by solid-state fermentation and the hydrolysis reaction conditions had not been optimized. *P. simplicissimum* lipases, as far we know, were never reported as biocatalyst in enantioselective hydrolysis. Torres et al. [23] reported that *C. antarctica* lipase (fraction B) adsorbed on PEI-agarose was used as catalysts in the enantioselective hydrolysis of R,S-mandelic acid methyl ester showing enantiomeric ratio value of 25. Palomo et al. [24] described 2-*O*-butyryl-2-phenylacetic acid hydrolysis by lipase from *B. thermocatenulatus* immobilized on octadecyl-Sepabeads. The enzyme resolved 2-*O*-butyryl-2-phenylacetic acid with enantiomeric ratio value of 100 of the release product.

Diethyl 2-Phenylmalonate (**pro-3**) Hydrolysis

Three different *P. simplissicimum* lipase immobilized preparations were employed in the diethyl 2-phenylmalonate (*pro*-**3**) hydrolysis (Fig. 6). Table 2 presents the results obtained by

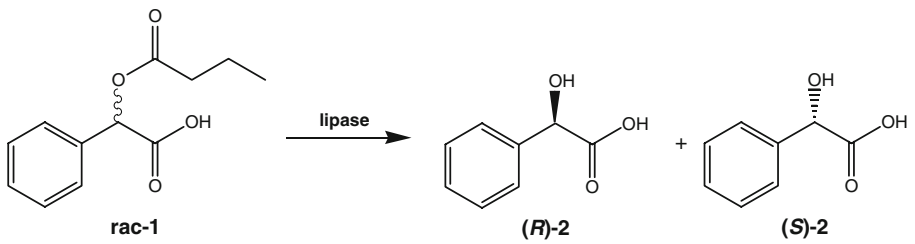

Fig. 5 Enantioselective hydrolysis of 2-*O*-butyryl-2-phenylacetic acid (**rac-1**)

Table 2 Activity and enantioselectivity of the different lipase fractions from *P. simplicissimum* in the hydrolysis of 2-*O*-butyryl-2-phenylacetic acid (**rac-1**) and diethyl 2-phenylmalonate (**pro-3**).

	2-*O*-Butyryl-2-phenylacetic acid				Diethyl 2-phenylmalonate				
	Enzyme activity (U/g_{sup})	2 (%)	ee (%)	*E*	Enzyme activity (U/g_{sup})	4 (%)	5 (%)	ee (%)	*A*
PSL 1-BUTYL	0.0210	100	0	1.0	0.0041	73	n.d.	88.5	16.4
PSL 2-OCTYL	0.0054	23	64.5	5.6	0.0024	49	n.d.	84.3	11.8
PSL 3-OCTYL	0.0130	63	50.0	7.9	0.0053	78	n.d.	85.1	12.5

2: mandelic acid. **4**: 2-(ethoxycarbonyl)-2-phenylacetic acid. **5**: 2-phenymalonic acid. . Experiments were performed as described in "Esters Enzymatic Hydrolysis"

n.d. not detected, *ee* enantiomeric excess, *E* enantioselective ratio, *A* asymmetry factor

hydrolysis reaction results of the prochiral substrate **pro-3** using PSL1-butyl, PSL2-octyl, and PSL3-octyl as biocatalysts. In all the cases, the reaction proceeded towards formation of the 2-(ethoxycarbonyl)-2-phenylacetic acid [4], yielding quantitatively the chiral monoester. The product 2-phenylmalonic acid [5] was not detected in the reaction carried out with three enzymes. Therefore, the *P. simplissicimum* lipase fractions seem to be highly selective, hydrolyzing only one ester group of the prochiral diethyl 2-phenylmalonate (**pro-3**). With respect to the asymmetry factor, the PSL1-butyl was more enantioselective, 16.4. However, PSL2-octyl and PSL3-octyl presented asymmetry factor values of 11.8 and 12.5, respectively.

These values were comparable to some reactions reported in the literature. Fernández-Lorente et al. [25], studying the hydrolysis of prochiral diethyl phenylmalonate by lipases from *Candida rugosa* and *Alcaligenes* sp. immobilized on octyl-agarose, obtained asymmetry factors of 7 and 12, respectively. The substrates were partially hydrolyzed to the corresponding chiral monoesters without the final achiral dicarboxylic acid production. Cabrera et al. [26] described the prochiral phenylmalonic acid diethyl ester hydrolysis by lipase from *Thermomyces lanuginosa* adsorbed on octyl-agarose showing asymmetry factor of 8. After the reaction media optimization, lipase from *T. lanuginosa* was covalently

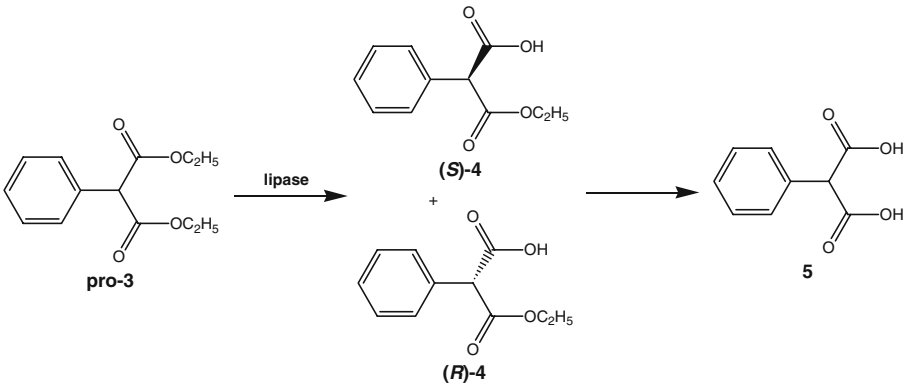

Fig. 6 Enantioselective hydrolysis of diethyl 2-phenylmalonate (**pro-3**)

immobilized on CNBr-activated agarose, and 0.03% CTAB was used as additive in the reaction, obtaining the (+)-1-(ethoxy-carbonyl)-phenylmalonic acid with an asymmetry factor of 20. Again, it is worth highlighting that this preparation is non-commercial and this is the first time that it were used in enantioselective hydrolysis.

The difference in the fractions' catalytic properties can be explained considering that the sequential adsorption method on hydrophobic supports at low ionic strength was able to separate three different fractions as a different intensity function of adsorption on activated supports with different hydrophobic groups. Bellezza et al. [27] reported that the lipase crude extract from *C. rugosa* was used as a biomaterial source for its adsorption on the layered micro-crystal surface of α-zirconium phosphate and phosphonates, which possess groups with different hydrophobic character anchored to the inorganic matrix. The biocomposites have shown different esterase activities and enantioselectivities in the *p*-nitrophenyl-acetate hydrolysis, ethyl butyrate and (±)-methyl-2-(4-chlorophenoxy) propionate as a support nature function. These results have been interpreted on selective adsorption basis of different enzyme isoforms.

On the other hand, the variation on catalytic characteristics can be explained by chemical and superficial differences of the supports used in immobilization. Sabbani et al. [28] observed the important role of particle size and pore size in enantiomeric ratio, which demonstrates the significance of this kind of variables.

Conclusion

The Brazilian *P. simplicissimum* strain can produce lipase by solid-state fermentation of babassu cake, low-cost agro-industrial residues. Lipases contained in the crude extract from *P. simplicissimum* were, simultaneously, separated and immobilized using the sequential hydrophobic adsorption method at low ionic strength getting three active fractions, PSL1, PSL2, and PSL3. The fractions characterization shows that the lipase crude extract is compounded by different enzymes with interesting catalytic properties and high thermal stability when compared with lipases of other mesophilic fungus. Enantioselective hydrolysis result of the diethyl 2-phenylmalonate was satisfactory when compared with the results reported in the literature. It is important to stress that the biocatalyst employed here was obtained from a wild fungal strain using a low-cost production process and mainly that the hydrolysis reactions have not been optimized. Besides that, these characteristics give this new biocatalyst obtained in this work a great potential for enantioselective reactions. It seems also that the non-commercial enzymes used may be feasible alternatives for the development of cost-effective enzymatically catalyzed processes.

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