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Biochemical properties of free and immobilized *Candida viswanathii* lipase on octyl-agarose support: Hydrolysis of triacylglycerol and soy lecithin

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ABSTRACT

Microbial lipases are important enzymes in food and pharmaceutical industries. In this work, a *Candida viswanathii* lipase was purified by hydrophobic interaction chromatography on octyl Sepharose. The purification presented 78.4% yield and the enzyme was 8.7-fold purified, with specific activity 700.4 U/mg protein and 69 kDa molecular weight. Immobilization of the enzyme on the same support presented 72.5% yield and a derivative with 101% expressed activity (109.2 U/g support), indicating hyperactivation of the enzyme. Optimal activity for both free and immobilized lipase was observed at pH 4.0 and 45 °C. The free and immobilized lipase showed broad-range stability from acid to neutral pH, and apparent activation and stability on organic solvents. The derivative was 60-fold thermostabilized in relation to the free enzyme and fully retained its activity after four cycles of *p*-nitrophenyl palmitate hydrolysis. Slight activation was observed with dithiothreitol and *β*-mercaptoethanol. The free and immobilized lipase efficiently hydrolyzed monoesters, simple and mixed long chain triacylglycerols, as well as soy lecithin. The activity and stability in acid pH, the organic solvent tolerance and the lecithin hydrolysis indicate high potential application of the enzyme and its derivative in textile, food and pharma industries and for chemical synthesis.

1. Introduction

Microbial lipases are excellent biocatalysts with important properties such as high specificity and selectivity, promoting reactions under mild conditions [1]. These enzymes are used in numerous industrial applications, including detergent formulation for the degradation of oils and fats, synthesis of pharmaceuticals and esters responsible for flavor and taste, as well as in cosmetics [2]. Lipases used in processes such as biodiesel production, oil and fats hydrolysis, effluent treatment and detergent production may be used as heterogeneous or partially purified mixtures, while food, pharmaceutical and cosmetic industries require purified preparations [3].

Lipases (triacylglycerol acyl hydrolases, E.C. 3.1.1.3) naturally catalyze the hydrolysis of ester bonds from long-chain triacylglycerols in organic-aqueous interfaces by a peculiar mechanism, the so-called interfacial activation. In homogenous media, most lipases have their active center covered by a polypeptide chain called lid, which isolate the molecule from the reaction medium (closed form); in the presence of hydrophobic surfaces, however, the enzyme becomes adsorbed on it, changing the structure to the open form in which the active center become fully exposed, allowing the lipases to hydrolyze drops of oils [4]. Their physiological role is to catalyze hydrolysis and sequential synthesis of triacylglycerols, providing diacylglycerol, monoacylglycerol, free fatty acids and glycerol [5]. The equilibrium displacement towards hydrolysis or synthesis is controlled by the amount of water in the reaction medium, *i.e.*, in water presence hydrolysis is the main reaction, while esterification occurs in the presence of organic solvents [6].

Apart from the inherent solubility of the proteins, lipases usually present low stability even at mild conditions, and the high cost for a single use limits therefore its industrial application. Enzyme immobilization may provide an effective method to circumvent these issues not only by improving catalytic properties and operational stability of enzymes, but also by facilitating multiple reuse and ease separation of the biocatalyst, enabling continuous industrial operation [7]. The choice of an immobilization technique for successful lipase

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immobilization should consider several concerns: the enzyme should be stable during the immobilization process; the procedure should be robust, reproducible, cost-effective and scalable; the materials and equipment should apply to food grade regulations if the immobilized lipase is intended to be used in food applications; the immobilization should preferably stabilize the enzyme in terms of temperature, pH, organic solvents and operational reusability; and the biocatalyst should be physically robust and rather applicable in both batch and fixed bed processes [8].

Immobilization of lipases using interfacial adsorption on hydrophobic supports has been proposed as a simple method to prepare robust derivatives [9,10]. This method take into account a simple adaptation of the Michaelis-Menten-Henri kinetic model to the interfacial hydrolysis of oil drops, in which the first step correspond to the fixation of a water-soluble enzyme to the lipid-water interface via a reversible adsorption-desorption mechanism. The penetration/adsorption step lead to a more favorable energy state of the enzyme present at the interface, which binds to substrate molecule, resulting in the formation and subsequent dissociation of the enzyme-substrate complex [11,12]. By using hydrophobic supports that resembles the surface of natural substrates and very low ionic strength, lipases can be selectively immobilized on the support. This mechanism proved to be advantageous allowing immobilization of lipases *via* an "affinity-like" strategy that can also significantly enhances enzyme activity [10]. Manoel et al. [4] immobilized lipases from *Thermomyces lanuginosus* and *Pseudomonas cepacea* on octyl and cyanogen bromide agarose, confirming that the octyl agarose derivatives present their open form stabilized while the covalent preparation maintains the closing/opening equilibrium.

Lipases from the *Candida* genus, including those from *Candida rugosa*, *Candida antarctica*, *Candida cylindracea*, present molecular weight ranging from 33 to 64 kDa and multiple isoforms [13–16]. These lipases normally show similar biochemical properties such as optimal activity in the pH range 7.0–8.0 and at 30–60 °C [13–16]. Lipases from *C. antarctica* (CALB), *C. rugosa* and *Mucor miehei* were immobilized by adsorption on hydrophobic supports as a quick and inexpensive alternative to obtain purified samples, rendering high yield and, in some cases, activation of the enzyme [17]. Adsorption technique allows the regeneration of the support, easing enzyme immobilization, and reducing costs and industrial wastes [18].

A *Candida viswanathii* strain grows and efficiently produces lipase when cultivated in liquid medium containing natural triacylglycerols, under agitation and temperature control [19,20]. Partial characterization showed that the crude enzyme is an acid and organic solvent tolerant lipase that could potentially be applied in bioprocesses. The aims of this study were to purify and immobilize this lipase on hydrophobic support. The purified free-enzyme and the derivative were biochemically characterized and its potential for hydrolyzing triacylglycerol and soy lecithin were also evaluated.

2. Materials and methods

2.1. Strain and maintenance

C. viswanathii strain is available in the Culture Collection of the Environmental Studies Center − CEA/UNESP, Brazil. *C. viswanathii* was routinely cultivated on malt extract agar (MEA) for 3 days at 28 °C and then stored at 4 °C. Cultures were also performed in MEA slants at the same conditions for inoculum preparation.

2.2. Liquid culture and protein extract preparation

Modified Vogel liquid medium [21] was prepared using 1.5% (w/v) olive oil and 0.2% (w/v) yeast extract as carbon and nitrogen sources, respectively. Erlenmeyer flasks (125 mL) containing 25 mL of the medium were inoculated with 1.0 mL of cells suspension (1×10^7 cells/mL). Cultivation was carried out at 27.5 °C, 210 rpm for 72 h [20].

Biomass was removed by filtration using 0.45 μm cut-off cellulose acetate membrane followed by centrifugation (8500 *g*, 20 min, 4 °C). The supernatant was used as source of lipase for purification and immobilization experiments.

2.3. Lipase activity

Lipase activity was assayed with *p*-nitrophenyl palmitate (p-NPP) (Sigma-Aldrich) as substrate [20]. *p*-NPP was initially dissolved in 0.5 mL of dimethyl sulfoxide (DMSO), then diluted to 0.5 mM with McIlvaine buffer pH 3.5 containing 0.5% (w/v) Triton X-100. The hydrolysis of *p*-NPP was determined discontinuously by measuring the released *p*-nitrophenolate (*p*-NP) at 40 °C. After 5 min pre-incubation of 0.9 mL of the substrate solution in a water bath, the reaction was started by adding 0.1 mL of appropriately diluted enzyme solution. The reaction was stopped after 1 and 2 min by heat shock (1 min, 90 °C), followed by the addition of 1 mL of saturated sodium tetraborate solution. The absorbance was read at 405 nm and the activity was determined using a *p*-NP standard curve ($\varepsilon = 1.8 \times 10^4\ M^{-1}\ cm^{-1}$). Controls were prepared without enzyme. One enzyme unit (U) was defined as the amount of enzyme that releases 1 μmol of product per min.

2.4. Protein

Protein was determined with bicinchoninic acid (BCA) [22], using bovine serum albumin as standard. Protein was followed by reading absorbance at 280 nm during purification chromatography.

2.5. Purification

The crude extract was previously dialyzed against 0.05 M ammonium acetate buffer pH 6.9 (8 h, 3 changes, 4 °C). The dialyzed extract was applied to a hydrophobic octyl column (Hiprep™ 16/10 Octyl Sepharose FF fast flow, GE Healthcare) previously equilibrated in the same buffer; at 2 mL/min flow rate. The column was washed with 50 mL of the same buffer and 3.0 mL fractions were collected. Elution of bounds proteins was performed with 100 mL of a 0.0–1.0% (w/v) Triton X-100 linear gradient prepared in the same buffer. Fractions with lipase activity were pooled and sample purity was evaluated by SDS-PAGE. All purification procedures were carried out at 4 °C.

2.6. Immobilization

The crude extract was previously dialyzed against 0.05 M ammonium acetate buffer pH 6.9 (8 h, 3 changes, 4 °C). The dialyzed extract was applied to 10 g of octyl Sepharose Fast Flow (GE Healthcare) previously packed in a column (1.5 × 12.0 cm) and equilibrated in the same buffer; at 2 mL/min flow rate. The column was washed with 50 mL of this buffer and the resin containing the immobilized lipase (derivative) was manually collected and stored at 10 °C.

Immobilization efficiency was presented by the efficiency factor (η):

$$\eta = \frac{V_{immobilized}}{V_{free}}$$

where, $V_{immobilezed}$ is maximum reaction velocity of the derivative and V_{free} is maximum reaction velocity of the free enzyme.

2.7. Derivative reuse

Successive cycles of 0.5 mM *p*-NPP hydrolysis were carried out in batch mode using McIlvaine buffer pH 3.5 at 40 °C. After each 1 min-cycle, the immobilized lipase was recovered from the reaction medium by centrifugation (8000 *g*, 10 min, 4 °C) and abundantly washed with 0.05 M ammonium acetate buffer pH 6.9. Then, fresh reaction medium

was added for the next run. Enzymatic activity in the derivative was determined at the end of each run and expressed in relation to the initial activity.

2.8. Enzyme characterization

2.8.1. Sample treatment and electrophoresis

The purified enzyme was previously treated with Calbiosorb™ adsorbent resin (Calbiochem®, San Diego, USA) to remove Triton X-100. The resin was equilibrated in 0.05 M ammonium acetate buffer pH 6.9 and loaded to the purified enzyme. Samples were incubated under slow stirring (45 min, 10 °C), centrifuged (8500 x *g*, 4 °C, 20 min) and the supernatant containing the enzyme was submitted to electrophoresis.

SDS-PAGE was performed using 10% (w/v) polyacrylamide gels according to Hames [23]. Samples were previously treated with 8 M urea, according to Lesuisse et al. [24]. Resolved protein bands were visualized after staining with 0.1% (w/v) coomassie brilliant blue R-250 in methanol, acetic acid and distilled water (4:1:5, v/v/v).

2.8.2. Optimum pH and stability to pH

The activity of the free and immobilized lipase was measured at 40 °C in different pH values using 0.05 M glycine-HCl buffer for pH from 2.0 to 3.0 and McIlvaine buffer from pH 3.0–8.0. Enzyme stability was carried out with the same buffers and also with 0.05 M glycine-NaOH from pH 8.0–10.0. Enzyme samples were 1:2 (v/v) diluted in each buffer and incubated at 10 °C. After 24 h, activity was assayed and expressed in relation to the initial activity.

2.8.3. Optimum temperature and thermal stability

The optimum temperature was determined by carrying out the enzymatic reactions at 25 − 60 °C, in McIlvaine buffer pH 4.0. For thermal stability, the free enzyme was incubated at 30, 40 and 45 °C, and the immobilized enzyme was incubated at 40, 45, 50 and 60 °C in the same buffer. Free enzyme was incubated up to 60 min and derivative was incubated up to 180 min. Samples were collected in several time intervals and the activity was analyzed in McIlvaine buffer pH 4.0 at 45 °C. Half-lives were calculated by the ratio ln 2/K_d. The constant of thermal inactivation (K_d) was determined by the equation: $\ln A = \ln A_0 – K_d \times t$, where A_0 and A are, respectively, the initial activity and the activity after time t (min). Stabilization was calculated as the ratio between half-lives of the immobilized and free lipase.

2.8.4. Effect of chemical compounds on enzyme activity and stability

The effect of metallic ions and other compounds on the free and immobilized lipase was evaluated at 2 and 10 mM. Samples were collected in several time intervals and the activity was assayed in McIlvaine buffer pH 4.0 at 45 °C. Half-lives were calculated by the ratio ln 2/K_d. The constant of inactivation (K_d) was determined by the equation: $\ln A = \ln A_0 – K_d \times t$, where A_0 and A are, respectively, the initial activity and the activity after time t (h).

2.8.5. Effect of organic solvents on enzyme activity and stability

The effect of organic solvents on the activity of the free and immobilized lipase was determined using 10% (v/v) glycerol, DMSO, propylene glycol, methanol, acetonitrile, ethanol, acetone, 1-propanol, 2-propanol, *n*-butanol, toluene, xylol, *n*-hexane and isooctane in a reaction medium containing 0.5 mM *p*-NPP dissolved in McIlvaine buffer pH 4.0 at 45 °C.

Stability of the free and immobilized lipase on organic solvents was evaluated using 10% (v/v) glycerol, DMSO, propylene glycol, methanol, acetonitrile, ethanol, acetone, 1-propanol, 2-propanol, *n*-butanol, toluene, xylol, *n*-hexane and isooctane. Experiments were carried out in sealed flasks at 200 rpm orbital agitation and 30 °C. Samples were withdrawn at several time intervals and the activity was analyzed in McIlvaine buffer pH 4.0 at 45 °C. Half-lives were calculated by the ratio ln 2/K_d. The constant of inactivation (K_d) was determined by the equation: $\ln A = \ln A_0 – K_d \times t$, where A_0 and A are, respectively, the initial activity and the activity after time t (h).

2.8.6. Specificity for substrate

Specificity for substrate of the free and immobilized lipase was verified by using 0.5 mM *p*-nitrophenyl acetate, *p*-nitrophenyl butirate, *p*-nitrophenyl octanoate, *p*-nitrophenyl decanoate, *p*-nitrophenyl laurate, *p*-nitrophenyl myristate, *p*-nitrophenyl palmitate and *p*-nitrophenyl stearate on enzyme assays performed in McIlvaine buffer pH 4.0 at 45 °C.

Hydrolysis of tributirin, triolein, canola oil, corn oil, olive oil, soybean oil and sunflower oil and soy lecithin was developed at 40 °C. Emulsion (10%, w/v) was carried out in McIlvaine buffer pH 4.0, containing 5% (w/v) Triton X-100. The reaction was started by adding 1 mL of enzyme sample to 5 mL of this emulsion, and then it was maintained at 300 rpm orbital agitation for 30 min. The reaction was interrupted by adding 16 mL of an acetone:ethanol solution (1:1, v/v) to the mixture. The released fatty acids were titrated to pH 11.0 with 0.05 M NaOH. One enzyme unit (U) was defined as the amount of enzyme that releases 1 μmol of fatty acid per min. The results were expressed as percentage of hydrolyzed triolein.

2.8.7. Kinetic parameters

The activity of free and immobilized lipase was assayed with *p*-nitrophenyl palmitate at concentrations from 0.0 to 1.0 mM. The Michaelis-Menten constant (K_m) and maximum reaction velocity (V_{max}) were estimated from the Lineweaver-Burk plot [25].

3. Results and discussion

3.1. Purification, immobilization and reuse of C. viswanathii lipase

The dialyzed extract was directly used as initial sample for purification of the *C. viswanathii* lipase by hydrophobic interaction chromatography. The adopted strategy did not request previous salt addition to the sample and under these conditions most of proteins did not bind to the resin. Accordingly, most of non-target proteins were washed, while no lipase was eluted from the column. Elution of bound proteins was performed with a Triton X-100 gradient (Fig. 1). Adsorbed proteins eluted with about 0.88% Triton X-100, which also absorbs at 280 nm. Thus, fractions were also assayed for lipase activity and those with high activity were pooled. Part of the sample was treated to remove the detergent and applied to SDS-PAGE, which showed electrophoretic homogeneity with only one 69 kDa band (Fig. 2). Samples of the crude and dialyzed filtrates were detergent free, while samples of the purified enzyme still contained Triton X-100, since Calbiosorb could not completely remove this detergent. The purified lipase presented slightly higher MW than the crude enzyme probably due to the presence of residual Triton X-100. Hughey et al. [26] also observed differences in MW of ϒ-glutamyltranspeptidase samples treated with Triton X-100. According to these authors, estimation of the enzyme MW may be complicated by the presence of carbohydrates and binding sites, which interact with large amounts of charged detergents and therefore cause the abnormal protein migration in electrophoresis gels.

Following the proposed protocol, *C. viswanathii* lipase could be purified by a practical, fast and relatively inexpensive one-step procedure, which is very interesting for further process scale-up. The enzyme was 8.7-fold purified, presenting specific activity of 700.4 U/mg of protein and the process presented 78.4% yield (Table 1). Attempts to determine native molecular mass by size exclusion chromatography on Sephadex G-100 and G-200 columns, even in the presence of urea or Triton-X100, were not successful because the enzyme was always eluted in the void volume (not shown), indicating the formation of soluble and catalytically active aggregates with MW higher than 600 kDa. Aggregation is commonly verified among lipase molecules and its occurrence is due to surface hydrophobicity. This phenomenon was

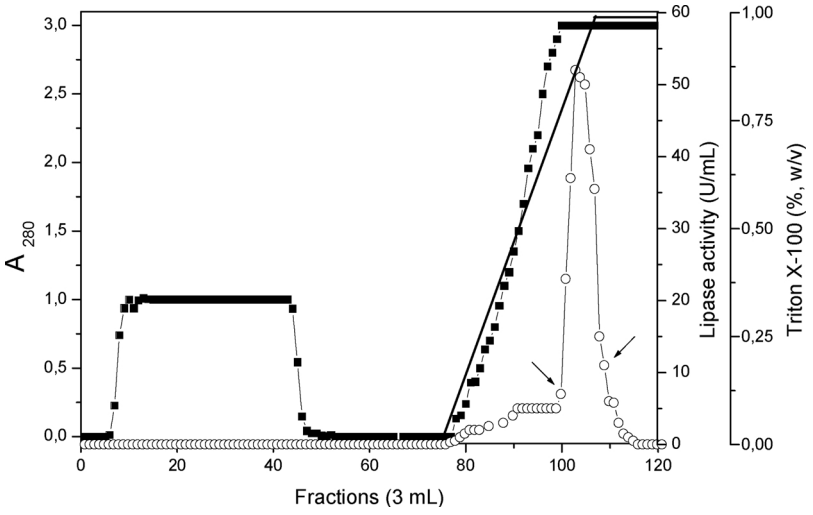

Fig. 1. Hydrophobic interaction chromatography profile of *C. viswanathii* lipase on octyl Sepharose. The column was equilibrated with 0.05 M ammonium acetate buffer pH 6.9 and eluted with Triton X-100 gradient using 2 mL/min flow rate. (■) Absorbance 280 nm; (○) lipase activity (U/mL); (−) Triton X-100 gradient; Arrows indicate the pooled fractions.

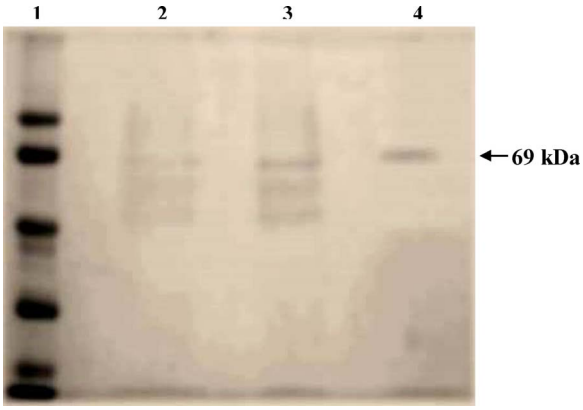

Fig. 2. Electrophoretic profile SDS-PAGE of *C.viswanathii* lipase. Column 1–standards: phosphorilase b (97.0 kDa), albumin bovine serum (66.0 kDa), ovoalbumin (45.0 kDa), carbonic anhydrase (30.0 kDa), trypsin inhibitor (20.1 kDa), and α-lactalbumin (14.4 kDa); Column 2–crude filtrate; Column 3–dialyzed filtrate; Column 4–purified lipase.

previously reported for the lipases from *C. rugosa*, *Mucor miehei*, *Humicola lanuginosa* [17], and *Pseudomonas fluorescens* [27]. Aggregates of the *C. rugosa* lipase with different MW was clearly demonstrated by Liou et al. [28] that verified four proteins peaks of 440, 240, 130 and 60 kDa using a Sephadex G-200 column and only one 60 kDa band by SDS-PAGE.

Considering that lipases immobilized on hydrophobic supports involve their open form, the chromatographic process was repeated in the same conditions but without elution of bound proteins. The process presented 72.5% yield and the derivative was obtained with 109.2 U/g support. The immobilization efficiency factor ($\eta = V_{immobilized}/V_{free}$) was 1.01 for the *C. viswanathii* lipase. Guo et al. [29] immobilized *C. cylindracea* lipase on magnetic hydrophobic microspheres, and the efficiency factor was 1.50 when olive oil was used as substrate and 0.32 for *p*-nitrophenyl acetate as substrate.

Selective adsorption of lipases involves the hydrophobic areas surrounding the active center and the internal face of the lid, while other water soluble proteins usually do not adsorb on these supports under these mild conditions [10]. Bastida et al. [9] previously observed that the lipase from *Rhizopus niveus* is much faster immobilized on octyl agarose by decreasing ammonium sulfate concentration from 1 M to 10 mM. This behavior was quite different from standard hydrophobic adsorption of proteins in which adsorption rate and yield strongly increase by increasing salt concentration. Manoel et al. [4] demonstrated that the hydrophobic adsorption of *Pseudomonas cepacia* and *Thermomyces lanuginosus* lipases in different ionic strength resulted in an immobilization via surface affinity adsorption involving the open form of the enzymes.

The stability of the immobilized lipase was evaluated during successive batches of p-NPP hydrolysis (Fig. 3). The derivative retained 100% of activity after 4 reuse cycles. After 6 and 7 cycles, the activity corresponded to 50 and 20% of the initial activity, respectively. The main reason for the loss of activity probably was the enzyme desorption from the support, since the reaction medium contained 0.5% Triton X-100. Palomo et al. [10] demonstrated that lipases from *C. antarctica*, *C. rugosa* and *M. miehei* were desorbed from octyl agarose supports by the addition of 1.0, 0.5 and 0.07% Triton X-100, respectively.

3.2. Biochemical properties of free and immobilized C. viswanathii lipase

3.2.1. Effect of pH on activity and stability

When the activity of the free and immobilized lipase was evaluated in the pH range from 2.0 to 8.0 (Fig. 4a), both forms presented similar profiles with the highest activity at pH 4.0. At pH from 2.0 to 3.0 the immobilized enzyme was more active presenting 55% of activity while the free enzyme presented 15–40% of activity. Free and immobilized enzyme showed 80 and 90% of activity at pH 3.5 and 4.5, respectively; and in the pH range 5.0–6.5, the activity corresponded to 65% of the maximum. Above pH 6.5 there was a decrease in the activity up to pH 8.0. The crude *C. viswanathii* lipase shows optimum activity in slightly more acid condition (pH 3.5) compared to the free purified and immobilized lipase; and also, presents higher activity at pH from 2.0 to 3.0

Table 1
Purification of lipase from *C. viswanathii*.

Step	Total activity (U)	Total protein (mg)	Specific activity (U mg protein^{-1})	Yield (%)	Purification (fold)
Dialyzed filtrate	1250.4 ± 15.4	15.6 ± 1.2	80.2 ± 1.0	100.0 ± 1.8	1.0
Octyl Sepharose	980.6 ± 7.4	1.4 ± 0.2	700.4 ± 4.2	78.4 ± 0.9	8.7

Experimental conditions: the crude filtrate was dialyzed against 0.05 M ammonium acetate buffer pH 6.9 (8 h, 3 changes, 4 °C). Assay conditions: lipase activity was carried out in McIlvaine buffer pH 3.5 at 40 °C.

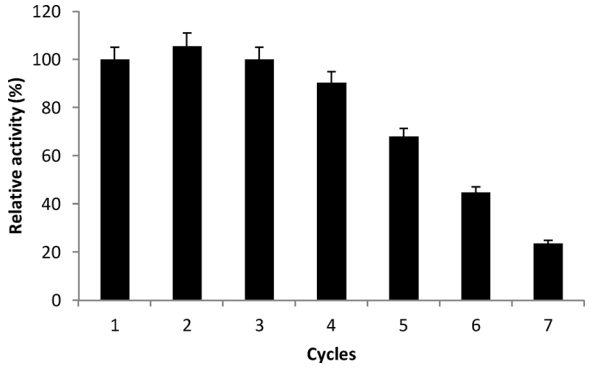

Fig. 3. Reusability of *C. viswanathii* lipase immobilized on octyl agarose. Lipase activity was determined with McIlvaine buffer pH 3.5 at 40 °C.

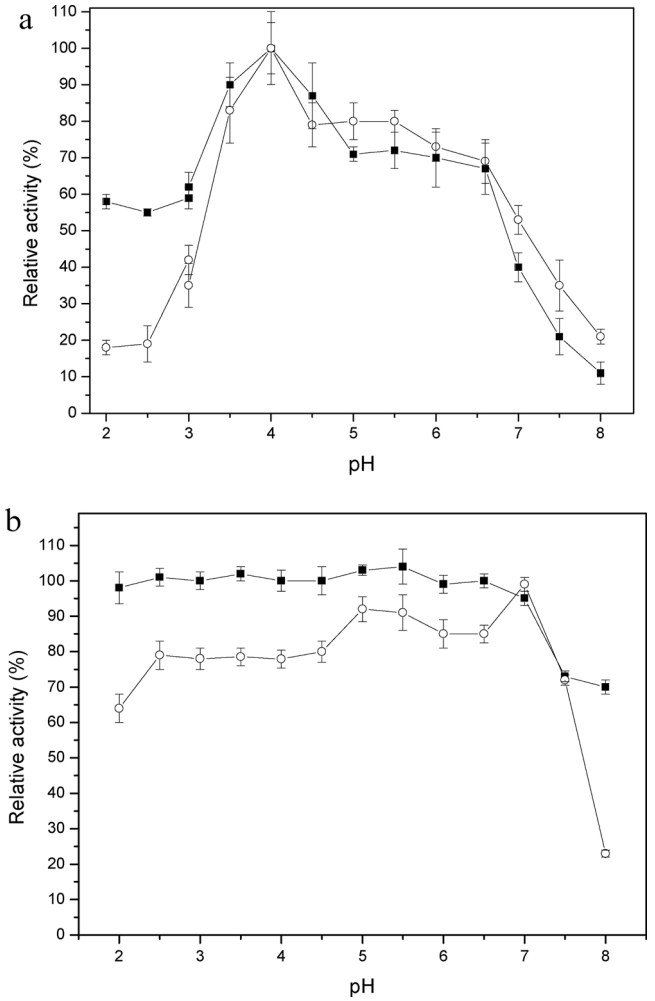

Fig. 4. Effect of pH on activity (a) and stability (b) of free and immobilized *C. viswanathii* lipase. (a) Activity was determined in 0.05 M glycine-HCl buffer pH from 2.0 to 3.0 and McIlvaine buffer for pH from 3.0 to 8.0. (b) Lipase activity was determined in McIlvaine buffer pH 4.0 at 40 °C. (○) free lipase, (■) immobilized lipase. Free lipase activity corresponded to 36.0 U/mg prot. and immobilized lipase corresponded to 38.0 U/mg prot.

(45–53%, respectively) [20]. Besides, the present lipase differs from those of *C. antarctica*, *C. rugosa*, and *C. cylindracea* which are neutral or alkaline enzymes [13,14].

During incubation at different pH (Fig. 4b), both lipase forms showed long-term stability in the pH range from 2.0 to 7.0. Immobilized lipase retained more than 90% of activity in this pH range, while the free lipase maintained its activity between 60 and 90% from

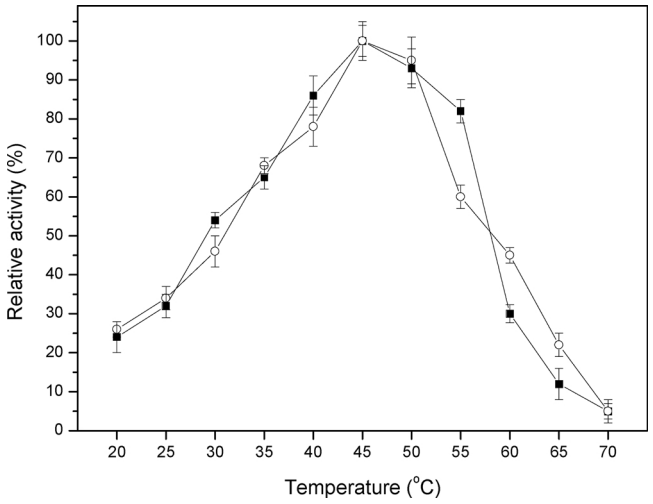

Fig. 5. Optimal temperature of free and immobilized *C. viswanathii* lipase. Activity was determined in McIlvaine buffer pH 4.0. (○) free lipase, (■) immobilized lipase. Free lipase activity corresponded to 36.0 U/mg prot. and immobilized lipase corresponded to 38.0 U/mg prot.

pH 2.0–7.5. Crude lipase from *C. viswanathii* presents different stability at pH 2.0 and 2.5, in which the enzyme is completely inactivated [20]. The immobilized lipase retained 100% of its activity in this pH range; at pH 7.5 and 8.0 more than 70% of activity was retained, indicating that the hydrophobic immobilization increased its stability to a wider pH range.

3.2.2. Effect of temperature on activity and stability

The effect of temperature on free and immobilized lipase activities is presented in Fig. 5. Maximal activities for both free and immobilized lipase were observed at 45 °C. The crude *C. viswanathii* lipase presents optimum activity at 40 °C, and high activity is also observed at 45 and 50 °C with a sharp decrease at 60 °C [20]. The similarity in activity profiles for the free and immobilized enzyme indicated there were no significant changes in the conformation of the immobilized enzyme promoted by interactions with the support. Similar behavior is also found for *C. rugosa* lipase adsorbed on calcium carbonate matrix [30].

When thermal stability of the free and immobilized lipase was assayed in absence of substrate, the free enzyme maintained about 65% of activity up to 20 min at 30 °C, and the half-life was 27 min. The half-lives of the free enzyme at 40 and 45 °C were 5.0 and 4.5 min, respectively (Fig. 6a). Immobilized lipase showed higher thermal stability than the free enzyme, maintaining more than 70% of activity even after 180 min both at 40 and 45 °C. At 50 °C, the enzyme retained above 80% after 60 min and more than 70% of activity up to 120 min, presenting half-life of 175 min. At 60 °C, the immobilized lipase presented half-life of 7.5 min (Fig. 6b). Immobilization by interfacial activation on hydrophobic supports was very suitable to stabilize the *C. viswanathii* lipase, *i.e.* the immobilized enzyme was 60- and 62.2-fold stabilized in relation to the free enzyme at 40 and 45 °C, respectively. The increased thermostability of the immobilized lipase suggests that the hydrophobic immobilization helps preserving enzyme structure from conformational changes caused by temperature, *i.e.* immobilization fixes the enzyme in such a way that reduces the susceptibility to denaturation by heat. Ours results is consistent with others dealing with lipase immobilization on hydrophobic supports. Lipases from *C. rugosa* and *M. miehei* hydrophobically immobilized on octadecyl-Sepabeads retain, respectively, 100 and 60% of activity after 2 h [10]. Wilson et al. [31] reported a 23-fold increase on thermal stability of the lipase from *Alcaligenes* sp. immobilized on octadecyl-Sepabeads. Fernandez-Lorente et al. [32] evaluated the thermal stability of *Bacillus thermocatenulatus* lipase immobilized on octyl Sepharose, butyl Sepharose, butyl Toyopearl or hexyl Toyopearl. The octyl Sepharose derivative was the most stable

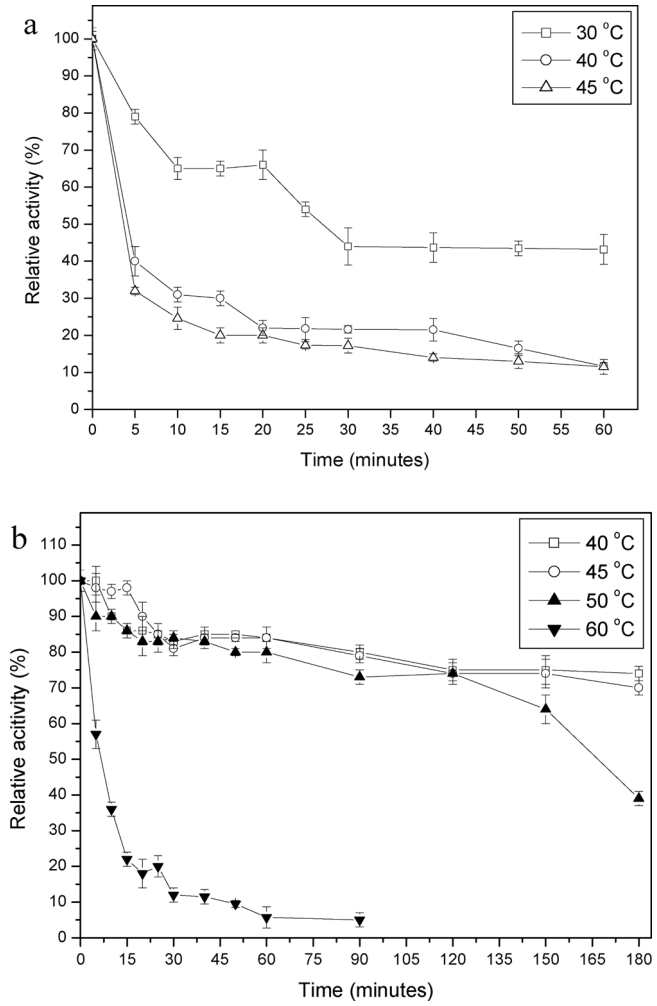

Fig. 6. Thermostability of free (a) and immobilized (b) *C. viswanathii* lipase. Lipase activity was determined in McIlvaine buffer pH 4.0 at 45 °C. Free enzyme was incubated up to 60 min and derivative was incubated up to 180 min. Free lipase activity corresponded to 36.0 U/mg prot. and immobilized lipase corresponded to 38.0 U/mg prot.

preparation, followed by butyl Sepharose, while either butyl or hexyl Toyopearl preparations presented low stabilities.

3.2.3. Effect of ions and different chemical compounds

The effect of ions and substances on free and immobilized lipase is shown in Table 2. Among metal ions, Mn^{+2}, Mg^{+2} and Zn^{+2} slightly activated the free enzyme, but only Mn^{+2} activated the immobilized lipase. Mg^{+2} and Ca^{+2} are well known activators of lipases because these metal ions form complexes with ionized fatty acids which may facilitate their removal in reactions at the water-oil interface, changing their stability and behavior [33].

Co^{+2} had inhibitory effect at 10 mM for both free and immobilized lipase. The free lipase activity was strongly inhibited by Cu^{+2} at 10 mM, but the immobilized lipase was not affected by this ion, whereas, Hg^{+2} moderately and strongly inhibited the free and the immobilized enzyme, respectively. Possibly, the active site of the immobilized enzyme is more exposed and therefore more sensitive to Hg^{+2}. Inhibition by Hg^{+2} was also observed for lipases from *Mucor* sp. [34] and *Penicillium* sp. DS-39 [35] at 1–20 mM. These results suggest the presence of thiol groups of cysteine close or in the active site. The active site of several lipases consists of the catalytic triad Ser (nucleophile)-Asp/Glu (acid)-His, similar to those from serine proteases; the triad residues are variable and Cys was identified as the nucleophile, which can modulate conformational changes of lipase interfaces and may be involved in substrate recognition [36].

1,4-dithiothreitol (DTT) and β-mercaptoethanol slightly increased the activity of both free and immobilized lipase at 10 mM. These results confirm the structural or functional importance of cysteine for the enzyme, what may be explained by preventing oxidation of thiol groups [37]. These reducing thiol agents also act decreasing the activity of enzymes that have disulfide bonds, disassembling the three-dimensional protein structure. Usually, these agents did not influence the activity of lipases, suggesting that there are small quantities of cysteine or cystine in these enzymes, or even that these amino acids have little relationship to their activities [33,38–40]. Exceptionally, activation with β-mercaptoethanol is also observed for the lipases from *Burkholderia* sp. HY-10 [41] and *Metarhizium anisopliae* [42], whereas activating effect by DTT was observed for the *Candida deformans* lipase [43].

Ethylenediaminetetraacetic acid (EDTA) moderately inhibited the free enzyme at 2 and 10 mM, however, this effect was not observed for the immobilized enzyme. Chelation of divalent ions by EDTA has important influence in the formation of the enzyme-substrate complex for metalloenzymes that require these ions for activity. These results suggest that the conformation of the free *C. viswanathii* lipase may be modulated by cations and the immobilization could stabilize the active conformation, preventing loss of activity or of the ion when incubated with EDTA. This effect is also observed for the *Pseudozyma hubeiensis* lipase, in which EDTA reduces the activity of the free enzyme by 45% and had no effect on the immobilized form [44]. Some studies also report inhibition by EDTA for the lipases from *Burkholderia multivorans* [45] and *Pseudomonas gessardii* [40], suggesting that they are metalloenzymes.

Phenylmethylsulfonyl fluoride (PMSF) showed moderate and pronounced inhibitory effect at 2 and 10 mM, respectively, for both free and immobilized enzyme. PMSF inhibits enzymes containing serine and cysteine residues in the active site, such as some proteases [46], suggesting that *C. viswanathii* lipase can be a serine or cysteine hydrolase. Inhibition by PMSF is also observed for several other microbial lipases [37,45,47–49]. Nam et al. [39] studied the inhibitory effect of PMSF on microbial lipases using X-ray crystallography. These authors found that the serine residue of the catalytic triad and the sulfonyl center of PMSF form a covalent bond, being the structural configuration of this complex similar to that from serine proteases.

The anionic SDS detergent resulted in complete activity loss of both free and immobilized lipase, because SDS binds and denature hydrophobic regions. Non-ionic detergents such as Tween 80 and Tween 20 and anionic detergent such as sodium deoxycholate decreased the activity of both free and immobilized lipase. In many cases, anionic detergents negatively affect lipase activity; and this effect can be associated with repulsion of protein molecules from the substrate interface [50]. According to Guncheva and Zhiryakova [49] surfactants at high concentrations have negative effect on lipase activity probably because they block substrate access to the active site. Exceptionally, the purified lipase from *B. multivorans* is activated by SDS, Triton X-100, Tween 80 at 0.1% and sodium deoxycholate, what can be attributed to a change in enzyme structure resulting in increased substrate accessibility [45].

Stability of free and immobilized lipase were also evaluated in these same chemical compounds at 2 and 10 mM. High stabilities of free enzyme were observed with Co^{+2}, DTT, Na^{+}, PMSF, Mg^{+2}, EDTA, Hg^{+2}, Ba^{+2} and Ca^{+2} at 2 mM. At 10 mM, high stabilities were observed with Mn^{+2}, NH_4^{+}, Na^{+} and DTT, respectively. For immobilized lipase, it was observed high stability with NH_4^{+}, PMSF, Co^{+2}, EDTA, β-mercaptoethanol, Tween 20 and DTT, at 2 mM, respectively; and at 10 mM, with Ca^{+2}, Mn^{+}, Hg^{+2}, Co^{+2}, Cu^{+2}, Tween 20, respectively. The immobilization of lipase improved the stability in the presence of the majority of ions. These results can be used to improve the operational conditions in enzymatic process.

3.2.4. Effect of organic solvents

Table 3 shows the effect of organic solvents on the stability of free

Table 2
Effect of ions and other chemical compounds on free and immobilized lipase from *C. viswanathii*.

Substances	2 mM				10 mM			
	Free Enzyme		Immobilized enzyme		Free Enzyme		Immobilized enzyme	
	Relative activity (%)	$t_{1/2}$ (h)	Relative activity (%)	$t_{1/2}$ (h)	Relative activity (%)	$t_{1/2}$ (h)	Relative activity (%)	$t_{1/2}$ (h)
Control	100.0 ± 4.5	43.3 ± 1.2	100.0 ± 7.7	43.6 ± 2.0	100.0 ± 7.4	45.6 ± 2.1	100.0 ± 4.1	46.4 ± 1.9
$HgCl_2$	94.5 ± 9.4	68.4 ± 2.1	93.5 ± 8.7	32.4 ± 1.8	60.1 ± 5.6	47.6 ± 2.5	14.7 ± 3.9	71.8 ± 3.3
$ZnSO_4$	96.8 ± 8.5	32.6 ± 2.3	99.4 ± 7.9	40.8 ± 2.4	112.4 ± 3.2	8.5 ± 0.8	100.4 ± 7.8	15.8 ± 1.2
$CuCl_2$	91.8 ± 10.2	26.4 ± 1.3	97.6 ± 5.2	37.4 ± 2.0	3.9 ± 5.1	28.6 ± 1.3	97.6 ± 6.9	60.0 ± 2.5
$CoCl_2$	96.1 ± 11.9	72.0 ± 3.3	92.4 ± 4.7	47.5 ± 1.9	50.1 ± 5.1	66.8 ± 2.3	56.3 ± 5.2	70.8 ± 1.5
$CaCl_2$	101.9 ± 11.9	64.0 ± 3.5	100.0 ± 8.9	44.8 ± 1,3	101.0 ± 9.9	44.8 ± 1.4	104.4 ± 12.2	72.0 ± 2.1
$BaCl_2$	107.9 ± 3.5	66.8 ± 3.3	96.2 ± 6.3	35.2 ± 1.2	100.3 ± 6.1	54.0 ± 2.5	100.3 ± 7.7	40.0 ± 3.2
$MnSO_4$	113.0 ± 5.2	13.2 ± 1.3	120.9 ± 9.4	72.0 ± 3.5	108.8 ± 8.3	12.8 ± 1.0	107.2 ± 2.8	72.0 ± 2.4
NaCl	103.0 ± 8.6	70.8 ± 2.7	106.4 ± 8.0	71.2 ± 2.9	102.4 ± 7.7	15.8 ± 1.3	105.3 ± 6.3	51.4 ± 1.8
NH_4Cl	103.2 ± 5.5	60.0 ± 2.8	104.9 ± 7.0	67.6 ± 3.7	102.0 ± 8.4	70.0 ± 3.4	105.6 ± 9.7	22.0 ± 1.2
$MgSO_4$	117.4 ± 8.5	68.0 ± 3.7	108.2 ± 6.2	43.6 ± 2.7	101.7 ± 7.5	42.8 ± 2.2	106.9 ± 8.9	17.0 ± 0.7
$Pb(CH_3COO)_2$	104.2 ± 6.4	14.4 ± 1.1	107.1 ± 5.9	39.5 ± 2.1	107.3 ± 10.6	66.0 ± 1.3	104.9 ± 10.3	62.0 ± 3.1
EDTA	71.0 ± 7.6	71.6 ± 3.4	93.6 ± 6.4	36.0 ± 1.3	61.1 ± 2.8	67.2 ± 2.1	106.0 ± 7.8	44.8 ± 2.1
DTT	104.9 ± 10.7	72.0 ± 2.8	99.0 ± 2.7	66.5 ± 3.3	119.3 ± 9.5	54.4 ± 1.8	124.3 ± 3.6	29.4 ± 1.1
β-mercaptoethanol	97.0 ± 8.7	56.0 ± 1.4	98.62 ± 4.7	38.0 ± 1.7	113.1 ± 5.8	66.2 ± 3.6	134.1 ± 10.8	43.2 ± 2.1
PMSF	89.8 ± 7.7	70.0 ± 2.2	89.0 ± 0.89	41.2 ± 1.2	20.5 ± 9.2	66.8 ± 2.7	23.4 ± 2.8	10.0 ± 0.8
Tween 20	77.6 ± 9.4	56.8 ± 2.9	68.1 ± 5.1	14.0 ± 1.0	45.5 ± 5.2	65.8 ± 2.1	34.3 ± 7.8	59.2 ± 1.3
Tween 80	59.5 ± 6.6	45.6 ± 2.0	52.6 ± 2.4	24.0 ± 1.2	32.4 ± 8.8	20.2 ± 0.9	22.4 ± 2.8	21.8 ± 1.1
SDS	ND	ND	ND	ND	ND	ND	ND	ND
Sodium deoxycholate	80.3 ± 4.7	46.8 ± 1.9	89.5 ± 5.5	28.8 ± 1.1	81.8 ± 8.6	13.4 ± 1.2	93.7 ± 1.8	36.0 ± 2.1

Assay conditions: lipase activity was performed in McIlvaine buffer pH 4.0 at 45 °C. Activities were carried out using 15 U/mg of protein for the free lipase and 13 U/mg of protein for the immobilized lipase. Relative activity was expressed in relation to the control (absence of any substance in the reaction medium). Half-lives were determined by incubating the enzyme in each substance without substrate. ND activity not detected under assays conditions; EDTA: ethylenediaminetetraacetic acid; DTT: dithiothreitol; PMSF: phenylmethylsufonyl fluoride; SDS: sodium dodecyl sulfate.

and immobilized *C. viswanathii* lipase. Solvents are listed according to their hydrophobicity (Log *P*) ranging from −1.67 to 4.51 and according to its polarity $E_T(30)$. Negative hydrophobicity (Log *P*) values indicate that the solvent is water soluble, whereas positive values indicate they are insoluble, occurring separation of the aqueous from the organic phase [51]. The $E_T(30)$ is defined as the molar transition energies (kcal mol^{-1}) of the standard betaine dye [52]. High $E_T(30)$ values correspond to high solvent polarity. Lipases have different sensitivity to solvents, but in general, water-miscible polar solvents are more destabilizing than water-immiscible solvents [48]. Non-polar solvents probably promote changes in the equilibrium between the open and closed conformation of lipases and also modify solubility of substrates and reaction products; while polar solvents are more destabilizing to the protein structure by removing the solvation water [38,49]. Under these conditions, activation and inactivation of lipases was observed using non-polar or polar organic solvents. Enzyme inactivation might be due to the relatively high viscosity of the solvents, which hindered efficient interaction between the enzymes and substrates; on the other hand, the activation of lipase in the presence of some water-miscible organic solvents can be explained possibly by the disruption of aggregates formed by enzyme molecules [53].

Nevertheless, no correlation between Log *P* and $E_T(30)$ values of solvents with relative stability was observed for the *C. viswanathii* lipase. The enzyme was very stable in most of them, retaining more than 80% of activity for both free and immobilized enzyme, except for the free enzyme in xylol, for the immobilized form in methanol and

Table 3
Effect of organic solvents on free and immobilized lipase from *C. viswanathii*.

Organic solvents	Log P	Et(30) (kcal mol^{-1})	Free lipase		Immobilized lipase	
			Relative activity (%)	$t_{1/2}$ (h)	Relative activity (%)	$t_{1/2}$ (h)
Control			100.0 ± 4.1	16.8 ± 0.3	100.0 ± 4.6	46.4 ± 2.1
Glycerol	−1.67	57.9	110.4 ± 5.7	36.0 ± 1.2	139.3 ± 2.4	45.2 ± 1.9
DMSO	−1.38	45.1	108.5 ± 2.5	31.2 ± 0.9	159.3 ± 2.9	42.0 ± 2.5
Propylene glycol	−0.92	–	90.5 ± 7.7	23.2 ± 1.1	176.1 ± 6.8	40.0 ± 1.8
Methanol	−0.76	55.4	104.1 ± 7.5	16.4 ± 0.4	63.0 ± 7.0	37.2 ± 2.1
Acetonitrile	−0.40	45.6	86.6 ± 8.2	14.2 ± 0.3	118.4 ± 10.5	42.4 ± 1.6
Ethanol	−0.24	51.9	116.7 ± 6.1	17.6 ± 0.7	82.2 ± 1.9	36.4 ± 1.4
Acetone	−0.23	42.2	81.8 ± 6.8	17.2 ± 0.2	73.4 ± 10.9	38.1 ± 0.9
2-propanol	0.07	48.6	101.5 ± 9.8	18.0 ± 0,9	126.8 ± 1.0	39.4 ± 1.3
1-propanol	0.25	50.7	87.0 ± 4.3	15.2 ± 0.8	167.5 ± 7.3	22.0 ± 0.3
n-butanol	0.80	49.7	88.0 ± 9.9	30.4 ± 1.3	107.8 ± 5.0	14.3 ± 0.8
Toluene	2.50	33.9	72.2 ± 5.3	15.0 ± 0.7	62.0 ± 4.0	30.8 ± 1.6
Xylol	3.15	34.7	78.0 ± 7.4	49.2 ± 2.1	139.9 ± 1.5	27.0 ± 0.9
n-Hexane	3.50	30.9	86.5 ± 8.5	40.4 ± 2.3	120.0 ± 10.7	30.4 ± 1.9
Iso-octane	4.51	30.9	98.4 ± 7.8	24.8 ± 0.2	135.9 ± 7.4	42.1 ± 3.1

Assay conditions: experiments were conducted in sealed flasks using 10% (v/v) organic solvent in McIlvaine buffer pH 4.0, 30 °C, 200 rpm. Lipase activity was determined using the same buffer at 45 °C. Activities were carried out using 15 U/mg of protein for the free lipase and 13 U/mg of protein for the immobilized lipase. Relative activity was expressed in relation to the control (absence of organic solvent in the reaction medium). Half-lives were determined by incubating the enzyme in each organic solvent without substrate. Log *P* logarithm of the partition coefficient of a particular solvent *n*-octanol and water (Sangster, 1989).

acetone, and for both forms in toluene. This important characteristic enables various industrial applications as transesterification reactions, synthesis and resolution of racemic esters [45]. Half-lives of the immobilized lipase were more than 40 h using glycerol, DMSO, propylene glycol, acetonitrile, and isooctane. Intermediate half-lives were observed with methanol, ethanol, acetone, 2-propanol, 1-propanol, toluene, xylol, and *n*-hexane, while the shortest half-life was observed with *n*-butanol. On the other hand, the free lipase presented shorter half-lives in most solvents. Prolonged half-lives were observed only with xylol and *n*-hexane; intermediate values were verified with glycerol, DMSO, propylene glycol, *n*-butanol, and isooctane; and the shorter values with methanol, acetonitrile, ethanol, acetone, 2-propanol, 1-propanol and toluene.

The activity of the free enzyme was slightly stimulated after 1 h incubation with ethanol, glycerol and DMSO (117, 110 and 108%, respectively). Methanol, 2-propanol and isooctane did not affect enzyme activity. The results obtained with the free lipase from *C. viswanathii* were similar to the lipases from *Rhizopus homothallicus* var. *rhizopodiformis*, *Aspergillus niger* MYA 135 [54] in organic solvents, which also show no correlation between stability and Log *P* values [55].

The immobilized enzyme was moderately stimulated in the presence of xylol, glycerol, isooctane, 2-propanol, *n*-hexane and acetonitrile, and stronger activations were found with propylene glycol (176.1%), 1-propanol (167.7%), and DMSO (159.3%). Butanol did not affect enzyme activity, while in methanol and ethanol stability was lower for the immobilized form than for the free enzyme.

3.2.5. Specificity for substrate

Hydrolytic activity of free and immobilized lipase was evaluated on *p*-nitrophenyl ester substrates (Fig. 7a). The activity increased by increasing ester chains of fatty acids up to *p*-nitrophenyl palmitate. Lower levels of activity were observed from acetate to myristate and stearate for both free and immobilized enzyme. The lipase specificity for long-chain hydrophobic substrates can be attributed to the geometry, size and structure of the enzyme active site [56]. Specificity for long-chain fatty acids esters has also been reported for lipases from *Pseudomonas aeruginosa* BN-1 [57] and *Yarrowia lipolytica* [58]. Other lipases present higher activity on intermediate-chain fatty acids esters such as those from *Mucor* sp. [34], *Burkholderia* sp. HY-10 [41] and *Amycolatopsis mediterranei* DSM 43304 [59]. On the other hand, lipases from *Metarhizium anisopliae* [42] and *C. rugosa* [60] show higher specificity for short-chain fatty acids esters (C2-C8), being classified as esterase or lipases with esterase activity [61].

Both free and immobilized lipases were evaluated on the hydrolysis of triacylglycerols and phospholipid (soy lecithin) by titration of free fatty acids (Fig. 7b). Both lipase forms presented a significant preference for the pure triacylglycerol triolein that presents long-chain monounsaturated fatty acids (oleic acid) esterified in the glycerol compared to tributyrin. Among natural triacylglycerols, free and immobilized lipase presented high activity on olive oil (85 and 90% hydrolysis, respectively), followed by soybean oil (~80% hydrolysis) and canola oil (74 and 79% hydrolysis, respectively). Intermediate hydrolytic activity was found with sunflower oil (~65% hydrolysis) and corn oil (50 and 60% hydrolysis, respectively). Remarkably, both free and immobilized lipases were highly active on soy lecithin (~87%) in comparison to triolein hydrolysis. Microbial lipases naturally hydrolyze plant or animal triacylglycerols, but hydrolysis of soy lecithin, a phospholipid, has been rarely verified [62]. Free and immobilized *C. viswanathii* lipase hydrolyzed olive oil and soy lecithin with the same efficiency, resulting in 85 and 87% hydrolysis, respectively, compared to triolein hydrolysis. High triacylglycerol hydrolysis was also observed on soybean, canola and sunflower oils. Intermediate activity was verified on corn oil, and the lowest activity was verified on tributyrin, that has the smallest chain.

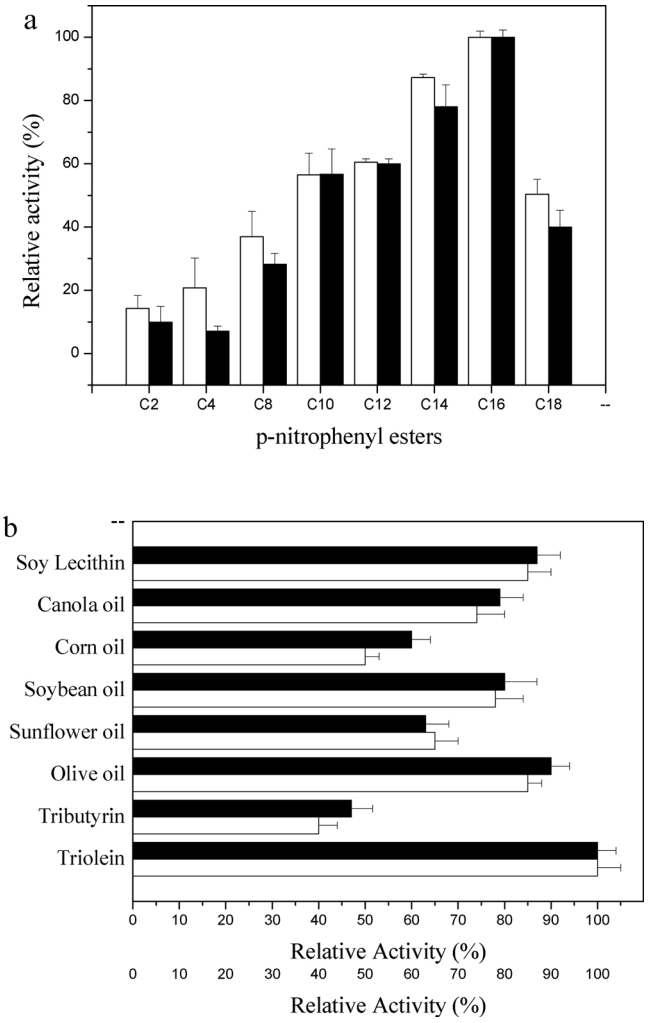

Fig. 7. Activity on *p*-nitrophenyl esters (a) and triacylglycerol and lecithin hydrolysis (b) of free and immobilized *C. viswanathii* lipase. Activity was determined in McIlvaine buffer pH 4.0 at 45 °C. (□) Free lipase, (■) immobilized lipase. Free lipase activity corresponded to 36.0 U/mg prot. and immobilized lipase corresponded to 38.0 U/mg prot. C2 acetate, C4 butirate, C8 caproate, C10 decanoate, C12 laurate, C14 myristate, C16 palmitate, C18 stearate.

3.2.6. Enzyme kinetics

Substrate hydrolysis reactions were performed for free and immobilized lipase with *p*-NPP (0.0–1.0 mM) to determine K_m and V_{max}. From these values, the turnover number (k_{cat}) and the catalytic efficiency (k_{cat}/K_m) for the free enzyme were calculated (Table 4). Free lipase showed K_m 0.16 mM and V_{max} 889.6 μmol min^{-1} mg $protein^{-1}$. K_m value for the immobilized enzyme was 0.08 mM and V_{max} of 900.1 μmol.min^{-1} mg $protein^{-1}$. The hydrophobic immobilization of *C. viswanathii* lipase improved the catalytic properties of the immobilized enzyme, allowing increased access of the substrate to the

Table 4
Kinetic parameters of free and immobilized lipase from *C. viswanathii* for *p*-nitrophenyl palmitate hydrolysis.

Enzyme form	Parameter			
	K_m (mM)	V_{max} (μmol min^{-1} mg $prot^{-1}$)	k_{cat} (s^{-1})	k_{cat}/K_m (M^{-1} s^{-1})
Free lipase	0.16	889.6	868	5.4×10^6
Immobilized lipase	0.08	900.1	–	–

Assay conditions: lipase activity was determined using McIlvaine buffer pH 4.0 at 45 °C.

catalytic site, as verified by the lower K_m.

These results indicate that the hydrophobic adsorption on octyl agarose resulted in a conformational change that exposed the catalytic residues towards the solvent, making the binding site more accessible to the substrate (lower apparent K_m). Rodrigues et al. [63] reported that in some instances, immobilization might greatly alter the physicochemical properties of the enzyme surroundings, which can produce some partition of different compounds away or towards the enzyme. If a partition of the substrates or products is achieved after immobilization, this may affect enzyme activity depending on the different possibilities of the enzymatic kinetics.

Changes in kinetic parameters obtained for the immobilized lipase from *C. viswanathii* in relation to the free enzyme were similar to those from other lipases immobilized on the same support. According to Fernandez-Lafuente et al. [64] and Palomo et al. [10], the large pocket around the active site of the open structure of lipases is strongly adsorbed on octyl-agarose even at low ionic strength, suggesting that this adsorption promotes hyperactivation of lipases. k_{cat} values and catalytic efficiency for the free *C. viswanathii* lipase were $868\ s^{-1}$ and $5.4 \times 10^6\ M^{-1}\ s^{-1}$, respectively. Purified lipases A and B from *C. cylindracea* present k_{cat} values of 1010 and $1600\ s^{-1}$ and k_{cat}/K_m 2.5×10^7 and $0.42 \times 10^7\ M^{-1}\ s^{-1}$, respectively, using *p*-nitrophenyl butyrate as substrate [13].

4. Concluding remarks

This work relates the purification, immobilization and a broad biochemical characterization of the *C. viswanathii* lipase produced under submerged culture conditions and its immobilized derivative. The high enzyme hydrophobicity allowed the purification to be carried out with octyl agarose using low ionic strength, resulting in a fast and relatively inexpensive one-step purification strategy. This property also allowed the lipase immobilization on the same support, giving rise to a hyperactivated derivative, which was stabilized in relation to pH, temperature and organic solvents. Immobilization also improved the catalytic properties of the immobilized enzyme, shown by the K_m decrease.

In contrast to other *Candida* lipases, the free *C. viswanathii* lipase and its derivative present high activity and stability in more acid pH, what can be considered interesting for application in process such as acid bating of fur and wool, and also in the composition of digestive aids for medical treatment. Besides, the tolerance to organic solvents, the stability during several re-use cycles and the ability to hydrolyze triacylglycerols and phospholipid indicate high potential application in food and pharma industries, as well as for chemical synthesis.

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