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BIOCATALYSIS

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Immobilization of *Pseudomonas fluorescens* lipase on hydrophobic supports and application in biodiesel synthesis by transesterification of vegetable oils in solvent-free systems

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Abstract This work describes the preparation of biocatalysts for ethanolysis of soybean and babassu oils in solvent-free systems. Polystyrene, Amberlite (XAD-7HP), and octyl-silica were tested as supports for the immobilization of *Pseudomonas fluorescens* lipase (PFL). The use of octyl-silica resulted in a biocatalyst with high values of hydrolytic activity (650.0 ± 15.5 IU/g), immobilization yield (91.3 ± 0.3 %), and recovered activity (82.1 ± 1.5 %). PFL immobilized on octyl-silica was around 12-fold more stable than soluble PFL, at 45 °C and pH 8.0, in the presence of ethanol at 36 % (v/v). The biocatalyst provided high vegetable oil transesterification yields of around 97.5 % after 24 h of reaction using babassu oil and around 80 % after 48 h of reaction using soybean oil. The PFL-octyl-silica biocatalyst retained around 90 % of its initial activity after five cycles of transesterification of soybean oil. Octyl-silica is a promising support that can be used to immobilize PFL for subsequent application in biodiesel synthesis.

Keywords Lipase · Hydrophobic adsorption · Octyl-silica · Biodiesel · Soy and babassu oils

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Introduction

Fossil fuels are of great importance to the economies of most countries, but the consumption of a non-renewable and polluting source of energy results in significant environmental impacts [26, 28, 63]. In recent years, the production of biodiesel has become increasingly attractive, because of its renewability, biodegradability, and non-toxicity [22, 57]. Commercial processes for biodiesel production frequently use alkaline media for the transesterification of triglycerides. However, there are major drawbacks of such chemical processes, including problems during the steps of removal of catalyst from the product, recovery of glycerol, treatment of alkaline wastewater, interference of free fatty acids and water in the reaction, and high energy consumption [2, 51]. Alternatively, biodiesel can be produced using enzymatic catalysis. Besides eliminating the disadvantages mentioned above, in enzymatic processes the biocatalyst can be reused and the purification step is simplified because the catalyst can be easily removed by simple filtration, thus reducing the amount of effluent generated [1, 31, 57]. However, a disadvantage of this biotechnological process is the high cost of the enzymes. Consequently, enzyme reutilization is essential from an economic perspective. Biocatalysts with immobilized enzymes can be used in successive batches or in continuous processes. The choice of a suitable immobilization methodology to obtain a highly stable, active, selective, and reusable biocatalyst is one of the key issues in the industrial application of enzymes [31, 40–42, 57].

Lipases have been immobilized on different organic or inorganic supports by physical adsorption, encapsulation, covalent attachment, or cross-linking [13, 15, 16, 25, 29, 31, 32, 38, 50, 55]. For application in biodiesel synthesis, physical adsorption of lipases on hydrophobic supports

may be a suitable method of immobilization, because of the intrinsic insolubility of the enzymes in organic media. The immobilization of lipases by adsorption on hydrophobic supports can be explained by the mechanism of "interfacial activation" of some these enzymes in the presence of hydrophobic interfaces [48]. Lipases such as those from *Mucor miehei* [48], *Pseudomonas fluorescens* [36], and *Thermomyces lanuginosus* [16] have an amphiphilic peptidic loop (like a lid or flap) that covers their active site and makes them inaccessible to substrates. The absence of an interface protects the active site from the reaction medium, in a "closed conformation". However, in the presence of a hydrophobic interface (such as a lipid/water interface), important conformational rearrangements take place, resulting in an "open conformation" [48]. In this case, the lipases are strongly adsorbed on hydrophobic interfaces by means of the hydrophobic area surrounding the active center (the hydrophobic face of the lid). Hence, lipases recognize the hydrophobic surfaces as being similar to those of their natural substrates (oil droplets) [3, 16, 25, 31, 34].

Commercially available immobilized lipases are generally immobilized by physical adsorption on an organic support. An example is the lipase from *Candida antarctica* type B immobilized on Lewatit VPOC 1600 (Novozym 435, supplied by Novozymes), which is the biocatalyst most widely used in biodiesel synthesis [9, 10, 14, 20, 23, 33, 43, 46, 47, 52, 56, 59, 60, 62]. However, recent studies have shown that the hydrophilic nature of this support, which is composed of poly(methyl methacrylate-*co*-divinylbenzene), is a limitation in heterogeneous catalysis, particularly in biodiesel production. Glycerol is adsorbed on the support surface, resulting in a dramatic decrease in the catalytic activity of the biocatalyst. Glycerol forms a hydrophilic layer around the enzyme, resulting in strong diffusional limitation of the mass transfer of triglycerides to the enzyme molecules [50]. As reported by Séverac et al. [50], lipase immobilized on Accurel MP (hydrophobic macroporous particles of propylene) can be used in continuous transesterification because the hydrophobic nature of the support prevents the adsorption of the glycerol on its surface. Hence, the chemical hydrophobization of pre-existing matrices is an interesting strategy for the preparation of supports with good physical–chemical properties for the immobilization of lipases, in applications involving organic media.

In addition to the existing commercial hydrophobic supports (such as Accurel, Celite, and Duolite), hydrophilic silica particles have been hydrophobized using alkyldimethylchlorosilanes, improving the adsorption of lipases on the hydrophobized surface [6]. The activation of the silica surface with octyl groups to produce highly hydrophobic supports for immobilization of lipases has been described in some papers [6, 7]. This functionalization transforms the hydrophilic material into a hydrophobic one, providing suitable properties for the interaction of lipases and enabling monolayer immobilization with high enzymatic loading [7].

The choice of support for enzyme immobilization is a key step in the preparation of biocatalysts with high catalytic activity and minimal diffusional limitations of substrates and/or products [24, 32]. The key physical properties of the support that influence these parameters are the surface area and the pore size. The former should be high enough to allow high enzymatic loading within the internal structure of the support, while the latter should be large enough to permit free access of substrates to the active site and output of the products to the bulk medium [6, 21].

Meso- or macroporous inorganic materials with high internal surface areas and pore diameters of around 100 nm (such as macroporous silica) are suitable for the preparation of active biocatalysts with minimal diffusional delays [6].

In this paper, lipase from *Pseudomonas fluorescens* (denoted PFL) was immobilized on two commercially available hydrophobic matrices (polystyrene and Amberlite XAD 7HP) and on silica that had been chemically modified with octyl groups (octyl-silica). The latter was chosen because the immobilization of PFL on octyl-silica has not been reported in the literature. The three supports were characterized in terms of surface area, pore size, and hydrophobicity. The prepared biocatalysts were characterized with respect to their hydrolytic activity, immobilization yield, and recovered activity. The functionalization of silica particles with octyl groups and the immobilization of PFL molecules on the surface of octyl-silica were confirmed by thermogravimetric analysis. Furthermore, the adsorption of PFL on octyl-silica was fitted using the Langmuir isotherm, and evaluation was made of glycerol adsorption on the support surface. The biocatalysts prepared using octyl-silica (PFL-octyl-silica), polystyrene (PFL-polystyrene), and Amberlite (PFL-XAD 7HP) were used in the ethanolysis of babassu (*Orbignya* sp.) oil. PFL-octyl-silica was also used in ethanolysis of soybean (*Glycine max*) oil, and its performance was compared to that obtained with commercial immobilized lipases from *Candida antarctica* type B (CALB IM), and *Thermomyces lanuginosus* (TLL IM). Reuse assays with PFL-octyl-silica were performed using ethanolysis of soybean oil, in order to determine the operational stability of the biocatalyst.

Materials and methods

Materials

Lipase from *Pseudomonas fluorescens* (PFL, ≥20,000 IU/g at 55 °C, pH 8.0) was acquired from Sigma-Aldrich Co.

(St. Louis, USA). Commercial immobilized lipases from *Candida antarctica* type B (CALB IM—T2-350, 2,500 TBU/g at 40 °C, pH 7.5) and *Thermomyces lanuginosus* (TLL IM—T2-150, 10,000 TBU/g at 40 °C, pH 7.5) were acquired from Chiral Vision (Leiden, The Netherlands). These commercial biocatalysts are prepared by covalent attachment on acrylic resin activated with epoxy groups (Immobead™ 150/350-P). Amberlite XAD 7HP acrylic resin was acquired from Sigma–Aldrich Co. (St. Louis, USA), and macroporous silica (Immobead S60S) and macroporous polystyrene particles (Immobead S861) were purchased from Chiral Vision (Leiden, The Netherlands). Octyltriethoxysilane (OTES), Rose Bengal, bovine serum albumin (BSA), ethyl heptadecanoate, and molecular sieve UOP type 3 Å (rod, size 1/16 in.) were acquired from Sigma–Aldrich Co. (St. Louis, USA). Anhydrous ethanol (purity ≥99.7 %) was acquired from J. T. Baker (New Jersey, USA). Gum Arabic was acquired from Synth (Sao Paulo, Brazil). Olive oil (low acidity) from Carbonell (Spain) was purchased at a local market. Babassu oil was kindly supplied by Pulcra Chemical (Jacareí, SP, Brazil) and had the following fatty acid composition (% m/m): 3.5 % octanoic, 4.5 % decanoic, 44.7 % lauric, 17.5 % myristic, 9.7 % palmitic, 3.1 % stearic, 15.2 % oleic, and 1.8 % linoleic. Refined soybean oil from Liza (Brazil) was purchased at a local market and had the following fatty acid composition (% m/m): 10.7 % palmitic, 3.0 % stearic, 24.0 % oleic, 56.7 % linoleic, and 5.5 % linolenic.

Determination of hydrolytic activity

The hydrolytic activities (HA) of the crude PFL extract and the immobilized lipase were determined by the hydrolysis of olive oil emulsion [54]. The substrate was prepared by mixing 100 g of olive oil with 100 g of gum Arabic solution (7 % m/v). The reaction mixture containing 5 mL of the emulsion, 4 mL of 100 mM sodium phosphate buffer at pH 7.0, and lipase (100 mg of immobilized PFL, or 1 mL of PFL extract) was incubated for 5 min at 37 °C, under continuous agitation in a shaker (250 rpm). The reaction was stopped by adding 10 mL of an ethanol-acetone (1:1) solution. The fatty acids released were titrated with 25 mM potassium hydroxide solution in the presence of phenolphthalein as indicator. Reaction blanks were prepared by adding immobilized lipases after the ethanol-acetone solution. One international unit (IU) of activity was defined as the amount of enzyme required to release 1 µmol of free fatty acid per minute under the conditions described above.

Preparation of the octyl-silica support

In order to increase the hydrophobicity of the silica, the support was chemically modified with octyl groups, according to the methodology described by Tani and Suzuki [58]. The silica particles were first treated with 0.1 M hydrochloric acid, followed by thorough washing with distilled water until neutral pH, and drying at 200 °C for 5 h. A suspension was prepared containing 1 g of dried silica and 20 mL of a mixture of octyltriethoxysilane and toluene in a volumetric ratio of 1:10. This suspension was vigorously stirred at 85 °C for 3 h under reflux condenser. After the activation period, the suspension was filtered and the support was thoroughly washed with toluene, methanol, and distilled water. The process was then completed by drying the support at room temperature for a maximum period of 24 h.

PFL immobilization on hydrophobic supports

The immobilization consisted of the preparation of a suspension containing the enzymatic solution, previously prepared in sodium phosphate buffer solution (5 mM, pH 7.0), and the support (octyl-silica, polystyrene, or Amberlite XAD 7HP), in a proportion of 1:19 (m/v) (support:enzymatic solution), with provision of 5 mg protein/g of support in order to avoid diffusional delay. The pH and salt concentration were set at 7.0 and 5 mM, respectively, because under such conditions PFL is stable and is preferentially adsorbed on hydrophobic supports [3, 25]. The suspension was kept under mild agitation in an orbital shaker at 25 °C for 24 h, according to the procedure described by Bastida et al. [3]. The prepared biocatalysts were then filtered (using Whatman no. 41 filter paper) and washed with Milli-Q water. The immobilization was monitored by measuring the activity of the supernatant solution in the hydrolysis of emulsified olive oil, compared to an enzyme solution (control) incubated under the same conditions.

The Langmuir isotherm model (Eq. 1) was employed in order to understand the immobilization of PFL by adsorption on octyl-silica particles. In this set of experiments, the activity loading was varied from 4,710 to 23,550 IU/g of support. The experimental data were analyzed using Origin Pro v. 8.0.

$$q_e = \frac{K_L q_m C_e}{1 + K_L C_e}, \quad (1)$$

where q_e is the activity adsorbed on the solid (IU/g), K_L is the binding affinity (mL/IU), which reflects the binding energy between the enzyme molecules and the solid surface (the greater the K_L value, the higher the affinity), q_m is the maximum theoretical adsorption capacity for complete monolayer coverage (IU/g), and C_e is the activity (IU/mL) in the solution at equilibrium [61].

Immobilization parameters

The immobilization yield and the recovered activity, expressed as percentages, were the parameters used to

compare the efficiency of immobilization on the hydrophobic supports.

The immobilization yield (IY), in terms of hydrolytic activity (with olive oil as substrate), was calculated by Eq. (2):

$$\text{IY}\,(\%) = \frac{U_0 - U_\text{f}}{U_0} \times 100, \tag{2}$$

where U_0 is the initial activity in the immobilization supernatant, and U_f is the final activity in the immobilization supernatant (expressed in U/g of support).

The recovered activity (RA) was calculated by Eq. (3):

$$\text{RA}\,(\%) = \frac{U_\text{EI}}{U_\text{I} \times \text{IY}} \times 100, \tag{3}$$

where U_EI is the apparent activity of the immobilized enzyme (expressed in U/g of support), and U_I is the activity provided for immobilization (expressed in U/g of support), calculated by Eq. (4):

$$U_\text{I} = \frac{U_\text{ES} \times V_\text{ES}}{M_\text{S}}, \tag{4}$$

where U_ES is the activity of the soluble enzyme (U/mL), V_ES is the volume (mL) of enzyme provided for immobilization, and M_S is the mass of support (g) used in the immobilization.

Surface area determination

The measurements of the surface areas of the supports were performed by adsorption using nitrogen as adsorbate. The samples were previously degassed to below 50 mm Hg and the analyses were performed at 77 K using a surface area analyzer (NOVA 1200, Quantachrome Instruments). The surface areas of the hydrophobic supports were calculated using the BET (Brunauer, Emmett, and Teller) method. Pore diameter, based on the BJH calculation, was determined using the BET apparatus software (NovaWin2 v. 2.2, Quantachrome Instruments).

Hydrophobicity determination

The relative hydrophobicity of the supports was determined by adsorption of Rose Bengal dye, according to the procedure described by Mendes et al. [29]. A fixed mass of support (0.15 g) was added to flasks containing 20 mL of a 20 μg/mL solution of dye. The flasks were kept under agitation for 1 h at room temperature. Samples of the supernatant solution were removed in order to quantify the dye concentration by measuring the initial and final absorbances at 549 nm, and comparing them with those of Rose Bengal standard solutions. The adsorption efficiency (E) was calculated as the amount of Rose Bengal dye adsorbed per unit of area of the supports, described by Eq. 5.

$$E(\mu g/m^2) = \left(\frac{C_\text{Csol} V_\text{sol} - C_\text{Csob} V_\text{sol}}{m_S} \right) \div \text{SA}, \tag{5}$$

where C_Csol is the initial dye concentration, V_sol is the volume of dye solution, C_Csob is the dye concentration in the final supernatant solution, m_S is the mass of the support, and SA is the surface area of the support determined by the BET method.

Thermogravimetric analysis

Thermogravimetric (TGA) and differential thermogravimetry (DTG) analyses of octyl-silica and the immobilized lipase (PFL-octyl-silica) were carried out using a Model SDT 2960 Simultaneous DSC-TGA analyzer (TA Instruments). The samples (10 mg) were examined in a synthetic air atmosphere, with heating from 20 to 1,000 °C at a rate of 10 °C/min.

Thermal stability tests

Stabilization of the PFL due to immobilization on the octyl-silica support was evaluated by thermal inactivation in the presence of ethanol. This test was conducted by incubation of PFL, either in the soluble form or immobilized on octyl-silica, at 45 °C in sodium phosphate buffer solution (100 mM, pH 8.0), in the presence of ethanol solution (36 %, v/v). Samples were periodically removed and immediately cooled in an ice bath to interrupt the inactivation reaction. The thermal inactivation constant was calculated according to the nonlinear decay model proposed by Sadana and Henley [44], described by Eq. 6. The experimental data were analyzed using Origin Pro v. 8.0.

$$\underset{\approx}{a} = (1 - \alpha_1)\ \exp(-k_1 t) + \alpha_1, \tag{6}$$

where $\underset{\approx}{a}$ is the activity (dimensionless), α_1 is the ratio of the specific activity of the final state to that of the initial state, k_1 is the first order deactivation rate constant (h^{-1}), and t is the incubation time (h). The parameter k_1 describes the unfolding (inactivation) process, and the parameter α_1 describes the long-term level of activity [44].

Glycerol adsorption tests

PFL-octyl-silica (0.5 g) was suspended in a glycerol solution (25 %, m/m) prepared in anhydrous ethanol, and kept under 500 rpm agitation at 40 °C for 72 h. The initial and final concentrations of glycerol were quantified by HPLC

Table 1 Morphological characteristics and hydrophobicity of the supports tested in this work

Supports	Surface area (m^2/g)	Pore diameter (Å)	Hydrophobicity[a] (μg dye/m^2)
Polystyrene	647.80	91.80	0.93
XAD 7HP	330.00	64.00	7.43
Silica	74.80	193.00	5.56
Octyl-silica	73.20	159.50	16.55

[a] Hydrophobicity was defined in this work as the amount of Rose Bengal adsorbed per unit area of the support

analysis, using a Shimadzu SCL-10A instrument equipped with a refractive index detector (Model RID 10-A). The compounds were separated on an Aminex HPX-87H column (300 × 7.8 mm, Bio-Rad), using 5 mM sulfuric acid as the mobile phase, at a flow rate of 0.6 mL/min, and an oven temperature of 45 °C.

Biodiesel synthesis

Biodiesel was produced by transesterification of vegetable oils (babassu or soybean) with ethanol in solvent-free systems at 40 °C, catalyzed by immobilized PFL (PFL-octyl-silica, PFL-polystyrene, and PFL-XAD 7HP biocatalysts) or commercial immobilized lipases (CALB IM and TLL IM). All the reaction media were composed of 20 g of oil (soybean or babassu oil) and anhydrous ethanol, at an oil:alcohol molar ratio of 1:7 [18]. The reactions were conducted in duplicate for a maximum period of 72 h, under agitation at 500 rpm in a mechanically stirred reactor equipped with a reflux condenser, using 130 units of hydrolytic activity per g of oil. For the time course studies, an aliquot of reaction medium was removed after different time intervals for the determination of fatty acid ethyl esters (FAEE) by gas chromatography (GC).

The samples (250 mg) were diluted in 5 mL of ethyl heptadecanoate solution (10 mg/mL) as internal standard. Aliquots (1 μL) were injected into an HP5890 GC equipped with a Restek Crossbond® Carbowax® polyethylene glycol column (15 m, 0.32 mm ID, 0.45 μm), kept at 230 °C, and a flame ionization detector operating at 250 °C was employed. The analysis was performed for 30 min using nitrogen as carrier gas.

The ester content (C), expressed in terms of percentage mass, was calculated using Eq. 7 [53].

$$C = \frac{\left(\sum A\right) - A_{EI}}{A_{EI}} \times \frac{C_{EI} \times V_{EI}}{m} \times 100\,\%, \quad (7)$$

where ΣA is the total peak area for the $C_{14:0}$ to $C_{24:1}$ FAEEs, A_{EI} is the peak area of ethyl heptadecanoate (C_{17}), C_{EI} is the ethyl heptadecanoate concentration (10 mg/mL), V_{EI} is the ethyl heptadecanoate volume (5 mL), and m is the sample mass (250 mg).

Operational stability

The operational stability of PFL immobilized on octyl-silica (high loading) was evaluated using the transesterification of soybean oil with ethanol (molar ratio 1:7, and 0.2 g of biocatalyst/g of oil) in a batch reactor equipped with a reflux condenser. The reaction was performed for 24 h, with a thermostatically controlled temperature of 40 °C and stirring at 500 rpm. In this set of experiments, soybean oil was selected as feedstock because it is readily available in Brazil and is an attractive option for use in the country's oleochemical industry. At the end of each batch (five cycles, each of 24 h), the biocatalyst was removed from the reaction medium and washed with hexane to remove compounds adsorbed on the octyl-silica surface (such as mono-, di-, and triglycerides, and glycerol). The FAEEs were analyzed by gas chromatography for calculation of the transesterification yield.

Results and discussion

Morphological characteristics and hydrophobicity of the supports

The essential requirement for the application of supports to immobilize enzymes is the need for a large surface area. Porous materials have therefore attracted attention due to their obvious advantage of high internal surface area [24]. Brunauer–Emmett–Teller (BET) analysis was carried out in order to determine the surface areas and the pore diameters of the four different supports tested to immobilize *Pseudomonas fluorescens* lipase (Table 1). Among the supports, polystyrene particles presented the highest surface area (647.8 m^2/g), followed by XAD 7HP (330.0 m^2/g), silica (74.8 m^2/g), and octyl-silica (73.2 m^2/g). Conversely, the silica-based supports exhibited greater pore diameters than the organic supports (polystyrene and XAD 7HP). There is usually an inverse correlation between the pore diameter and surface area of supports [24]. The silica particles presented a pore diameter of 193.0 Å, which decreased to 159.5 Å after chemical modification with the silane agent to produce octyl-silica. The pore diameters of the polystyrene and XAD 7HP particles were 91.8 and 64.0 Å, respectively, classifying these materials as mesoporous [6, 24]. *Pseudomonas fluorescens* lipase is a small globular protein with a molecular volume of 3 × 4 × 5 nm [45] and an average molecular diameter of about 5 nm (50 Å) [49]. The pore sizes of the four different supports tested were therefore sufficiently large to accommodate the PFL molecules.

The hydrophobicity of a support is an important property to be considered in the immobilization of lipases. Due to their catalytic mechanism (interfacial adsorption with modulation of open/closed lid), lipases can be preferentially adsorbed on hydrophobic surfaces in their active form (open structures), which in many cases results in a dramatic activation of the immobilized enzyme [3, 25, 34]. Adsorption assays using Rose Bengal dye were carried out in order to determine the hydrophobicity of the supports tested in this work (Table 1). Among the commercial supports, polystyrene particles presented the lowest hydrophobicity (0.93 μg dye/m^2), followed by silica (5.56 μg dye/m^2) and XAD 7HP (7.43 μg dye/m^2). However, chemical modification of the silica particles with octyltriethoxysilane (producing octyl-silica) greatly increased their hydrophobicity, with dye adsorption of 16.55 μg/m^2.

Thermogravimetric analysis (TGA)

Thermal analyses are used to characterize compounds by measuring changes in physicochemical properties as a function of increasing temperature. In the case of TGA, changes in weight are measured as a function of increasing temperature. The results obtained from a TGA run can be presented in the form of curves, plotting weight against temperature (thermogravimetric curve, TG), or the rate of loss of weight against temperature (differential thermogravimetric curve, DTG). This technique is commonly used to determine characteristics of materials that exhibit mass loss due to decomposition, oxidation, or loss of volatiles (such as moisture). A common application of TGA is determination of the organic content of a sample, which can be useful for corroborating predicted material structures [11].

Experimental confirmation of the introduction of octyl groups on the surface of the silica was obtained by TGA analysis. In the presence of O_2, the majority of organic compounds decompose between 300 and 600 °C. There is evidence in the literature that silica is thermally stable up to approximately 1,200 °C [27]. In the present work, loss of weight until this temperature was reached could therefore be explained by removal of the octyl groups (in the case of the support) or octyl groups and adsorbed proteins (in the case of the immobilized enzymes).

Figure 1a shows the TG/DTG curves obtained for the octyl-silica support heated in a synthetic air atmosphere. A peak corresponding to a weight loss of about 1.5 % was observed between 0 and 200 °C, which could be attributed to the removal of water present in the structure of the support. Between 300 and 600 °C, the sample displayed a weight loss of around 6.2 %, due to loss of the octyl groups of the organic modifier (this was confirmed by the fact that the unmodified silica showed no loss of weight in this temperature range). No significant losses of weight were observed between 600 and 1,000 °C. For the immobilized lipase (Fig. 1b), the TG/DTG analysis indicated a maximum rate of weight loss (around 17.0 %) in the range 300–600 °C, probably due to degradation of both the enzyme molecules and the octyl groups. A part of this weight loss could also have been due to the removal of water molecules that were tightly bound to the microenvironment of the biocatalyst.

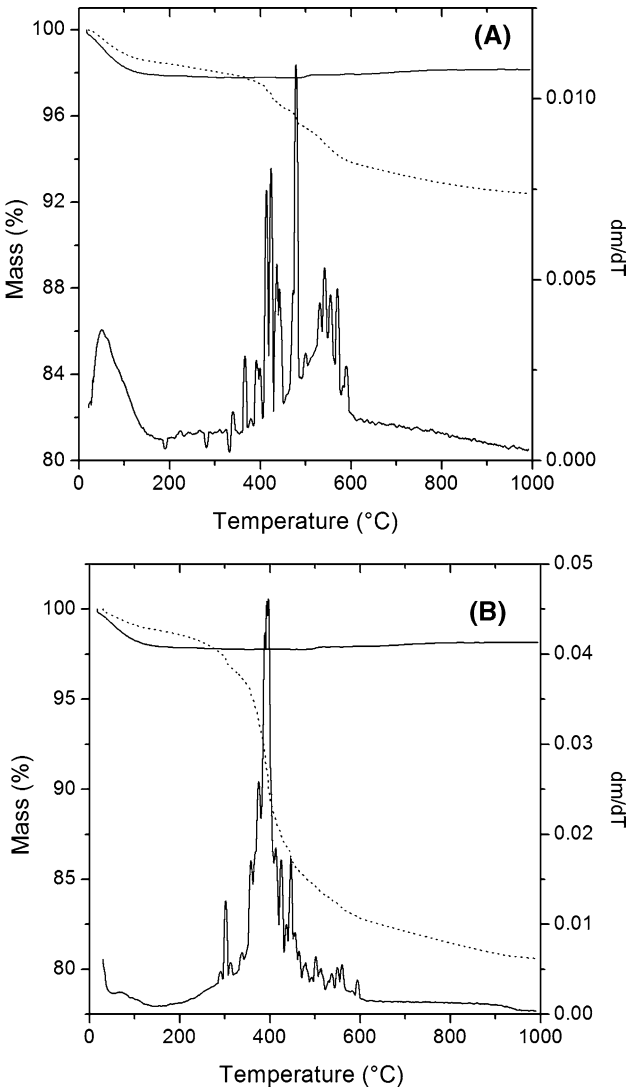

Fig. 1 Thermogravimetric analysis of the octyl-silica support (**a**) and PFL-octyl-silica biocatalyst (**b**). *Dotted lines* (TG curves) indicate the thermogravimetry results and *solid lines* (DTG) represent the derived lines

PFL immobilization

Pseudomonas fluorescens lipase (PFL) was immobilized on octyl-silica, polystyrene, and XAD 7HP by hydrophobic adsorption at 25 °C, pH 7.0, and low ionic strength.

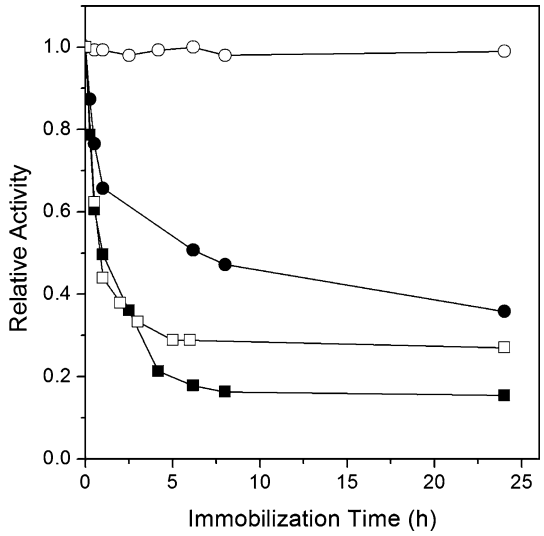

Fig. 2 Hydrolytic activity in the supernatant of the PFL immobilized at 25 °C, pH 7.0 (5 mM sodium phosphate buffer), on octyl-silica (■), polystyrene (●), and XAD 7HP (□), and in the control solution (○)

Immobilization resulted in a decrease of hydrolytic activity in the supernatant (Fig. 2), whereas the activity in the control (soluble enzyme solution) remained unchanged. The decrease of activity in the supernatant could therefore be attributed to the immobilization of the enzyme molecules by physical adsorption on the support surface.

After 24 h of incubation, the immobilization on octyl-silica and XAD 7 HP reached equilibrium, with immobilization percentages of approximately 91 and 76 %, respectively. However, the immobilization on polystyrene reached around 61 % after the same period. Although the polystyrene had the highest surface area and a pore size large enough to accommodate PFL molecules, its capacity to adsorb hydrophobic molecules was the lowest among the supports tested (0.93 μg dye/m^2, Table 1). As the immobilization was conducted at low ionic strength, absorption should have been favored between hydrophobic areas of the enzyme surface and highly hydrophobic areas of the support.

The results obtained for the immobilization yields (IY), recovered activities (RA), and hydrolytic activities (HA) of the immobilized biocatalysts are shown in Table 2. Immobilization on octyl-silica produced the most active biocatalyst, with a hydrolytic activity of 650 IU/g, corresponding to around 82 % of recovered activity. The decrease in recovered activity after immobilization on octyl-silica particles (with highly hydrophobic surfaces) could be attributed to small conformational changes in the tertiary structure of the lipase molecules, due to hydrophobic interactions with the support. These interactions can affect the tetrahedral intermediate and negatively influence the catalytic activity of the enzyme [6, 8].

Table 2 Catalytic properties of the biocatalysts prepared by immobilizing PFL on hydrophobic supports (protein loading: 5 mg/g of support)

Supports	IY (%)	RA (%)	HA (IU/g support)
XAD 7HP	75.8 ± 1.9	26.8 ± 3.2	221.1 ± 20.6
Octyl-silica	91.3 ± 0.3	82.1 ± 1.5	650.0 ± 15.5
Polystyrene	60.5 ± 5.1	15.5 ± 0.2	79.6 ± 7.8

Values are expressed as means of triplicates ± standard deviations

IY immobilization yield, *RA* recovered activity and *HA* hydrolytic activity

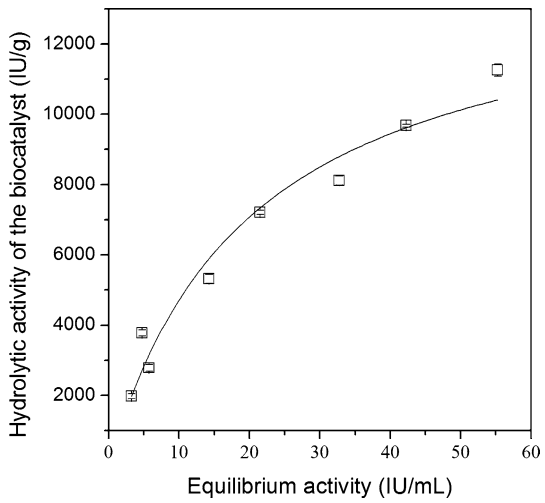

Fig. 3 Langmuir isotherm fitted to the experimental data for PFL adsorption on octyl-silica at 25 °C in sodium phosphate buffer solution (5 mM, pH 7.0)

The low recovered activities of the biocatalysts prepared by immobilizing PFL on polystyrene (15.5 %) and XAD 7 HP (26.8 %) might not only have been due to small conformational changes in the tertiary structure of the enzyme, as described above, but also to diffusional delays, because the pore sizes of these supports were 2–2.5 times smaller than that of octyl-silica.

Enzymatic loading of octyl-silica

Experimentally determined adsorption isotherms were used to evaluate the capacity of the support. In this study, enzymatic loadings varied from 4,710 to 23,550 IU/g of support, corresponding to 50–270 mg of protein/g of octyl-silica. The experimental conditions were 25 °C, pH 7.0, low ionic strength (5 mM), and 24 h (the time necessary to reach adsorption equilibrium in all assays).

Figure 3 shows a good fit of the Langmuir isotherm to the experimental data, with the theoretical hydrolytic activity of the biocatalyst plotted against the activity in the supernatant at equilibrium. From the parameters of the Langmuir model fitted to the experimental data, the maximum adsorption capacity of the support (q_m) was 15,222 ± 1,676 IU/g of support, corresponding to approximately 168 mg of protein/g of support, and the binding affinity (K_L) was 0.0421 ± 0.0105 mL/IU.

The surface area and pore diameter for PFL immobilized on octyl-silica (high loading) were determined using the BET method. The surface area decreased from 73.20 m^2/g (Table 1) to 46.10 m^2/g, which could be attributed to the adsorption of PFL molecules on the support surface. The pore diameter also decreased, from 159.50 Å (Table 1) to 115.50 Å. Even with this reduction, the porosity remained suitable for the access of macromolecules (substrates and products).

The loading assay showed that it was possible to prepare a biocatalyst with high activity (approximately 15,000 IU/g of support). Highly active derivatives are desirable for industrial purposes because they provide high catalytic activity per reaction volume, hence increasing the reaction rate and decreasing the time required to achieve the desired conversion [5, 19].

Thermal stability of PFL in the presence of ethanol

Immobilization is a technique used not only to render enzymes insoluble, but also to stabilize them. Depending on the bond strength, a greater rigidity of the tertiary structure can be achieved. As a result, the immobilized enzyme can show greater stability against heat, organic solvents, and other denaturing agents [25].

The thermal stability of PFL adsorbed on octyl-silica at 45 °C, pH 8.0 (100 mM sodium phosphate buffer solution), in the presence of 36 % (v/v) ethanol (a known protein denaturing agent), was 12-fold higher than that of soluble PFL. The nonlinear inactivation model proposed by Sadana and Henley [44] was fitted to the experimental data (Fig. 4) and used to estimate the half-life times ($t_{1/2}$). The half-life of soluble PFL was 0.66 h, while for PFL adsorbed on octyl-silica, $t_{1/2}$ increased to 7.76 h. For soluble PFL, ethanol exerted a negative effect on the tertiary structure of the enzyme, even in the presence of the bimolecular aggregates that are formed by the interaction of hydrophobic pockets surrounding the active sites of lipases in open conformation, which are more stable than the monomeric forms [17, 25, 35]. For PFL adsorbed on octyl-silica, strong interaction between the enzyme molecules and the support surface may have hindered inactivation of the enzyme, due to the stabilization of its three-dimensional structure. The higher stability of PFL adsorbed on octyl-silica in the presence of an organic co-solvent is indicative of the potential of this biocatalyst for use in biotransformations where ethanol acts as a co-solvent or substrate, such as the synthesis of fatty acid ethyl esters (for example biodiesel) by transesterification of oils and fats.

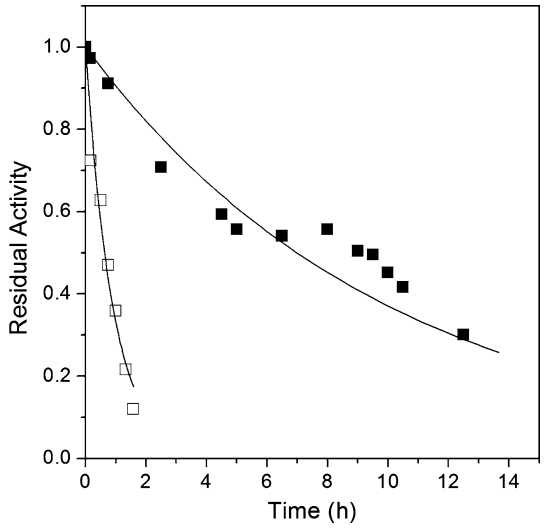

Fig. 4 Thermal inactivation profiles of soluble PFL (□) and the PFL-octyl-silica biocatalyst (■), in the presence of ethanol (36 % v/v) at 45 °C and pH 8.0

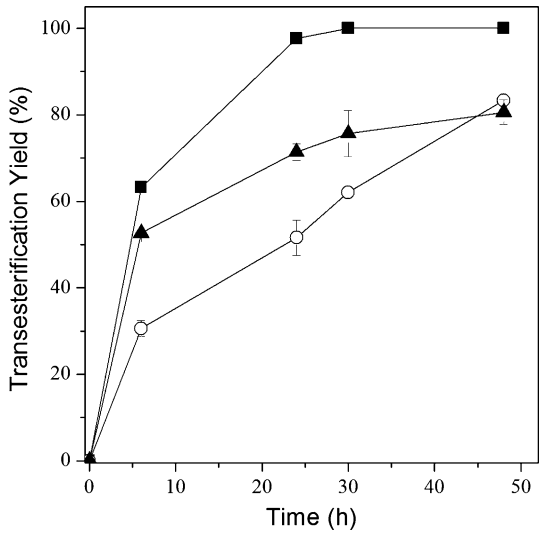

Fig. 5 Transesterification yields for babassu oil in solvent-free systems catalyzed by PFL immobilized on hydrophobic supports: (■) octyl-silica, (○) XAD 7HP, and polystyrene (▲) particles. The reactions were performed at 40 °C in a mechanically stirred (500 rpm) reactor equipped with reflux condenser, using an oil:ethanol molar ratio of 1:7 and 130 units of hydrolytic activity per g of oil

Table 3 Transesterification of vegetable oils with ethanol, catalyzed by immobilized lipases

Oil	Alcohol	Reaction conditions	Oil:alcohol molar ratio	Biocatalyst	Yield	References
Soy	Ethanol	57 °C, 4 h[a]	1:3	*Rhizomucor miehei* lipase adsorbed on anion exchange macroporous resin (Lipozyme RM IM)	86	[4]
		63 °C, 4 h[a]		*Candida antarctica* B lipase adsorbed on anion exchange macroporous resin (Novozym 435)	57	
Soy	Ethanol	30 °C, 10 h	1:10.2	Mix of *Thermomyces lanuginosus* lipase covalently immobilized on Lewatit VP OC 1600 (80 %) and *Rhizomucor miehei* lipase adsorbed on anion exchange macroporous resin (Lipozyme RM IM) (20 %)	78	[39]
Soy	Ethanol[b]	30 °C, 10 h	1:3	*Thermomyces lanuginosus* lipase covalently immobilized on Lewatit VP OC 1600	100	[42]
	Ethanol				60	
Soy	Ethanol	32 °C, 24 h	1:45	*Candida antarctica* B lipase adsorbed on anion exchange macroporous resin (Novozym 435)	87	[43]
Soy	Ethanol	65 °C, 6 h	1:6	Novozym 435	100	[13]
Soy[c]	Ethanol	40 °C, 24 h	1:4.5	Novozym 435	85.4	[47]
Babassu	Ethanol	45 °C, 48 h	1:9	*Pseudomonas fluorescens* lipase covalently immobilized on Toyopearl AF-amino-650 M activated with glycidol	87	[30]
				Pseudomonas fluorescens lipase covalently immobilized on Toyopearl AF-amino-650 M activated with epichlorohydrin	95	
Babassu	Ethanol	45 °C, 72 h	1:9	*Pseudomonas fluorescens* adsorbed on polyhydroxybutyrate pearls	100	[32]
Babassu	Ethanol	39 °C, 48 h	1:7	*Burkholderia cepacia* lipase immobilized on silica-PVA	98	[18]
Babassu	Ethanol	50 °C, 10 h[d] or 50 °C, 48 h	1:12	*Burkholderia cepacia* lipase immobilized on silica-PVA activated with epichlorohydrin	100	[12]
Babassu	Ethanol	40 °C, 48 h	1:10	Porcine pancreas lipase immobilized on polysiloxane–polyvinyl alcohol hybrid matrix activated with glutaraldehyde	75	[37]

[a] Transesterification in the presence of hexane as co-solvent and assisted by ultrasound; [b]Ethanol added in two steps; [c]oil refining by-product, micelle with 90 % oil; [d]Transesterification performed in a microwave reactor

Biodiesel synthesis

PFL immobilized on octyl-silica, XAD 7HP, and polystyrene were used as biocatalysts for the transesterification reaction of babassu oil with ethanol. However, for the transesterification reaction with soybean oil, PFL immobilized on octyl-silica and the commercial immobilized lipases (CALB IM and TLL IM) were used as biocatalysts.

The transesterification of babassu oil catalyzed by PFL-octyl-silica reached almost full conversion (≥97.5 %) after 24 h of reaction, while use of the biocatalysts prepared by immobilizing the lipase on XAD 7HP and polystyrene particles resulted in maximum transesterification yields of 83 and 80 %, respectively, after 48 h (Fig. 5). According to the specifications recommended by the Brazilian National Agency of Petroleum, Natural Gas, and Biofuels (ANP), a minimum transesterification yield of 96.5 % is required for use as biofuel (Method EN 14103). The present results indicated that a reaction time of 24 h was sufficient for PFL immobilized on octyl-silica to produce fatty acid ethyl esters (biodiesel) in accordance with the ANP specifications.

Yields varying from 75 to 100 % have been reported previously (see Table 3) for the transesterification of babassu oil with ethanol, catalyzed by different immobilized lipases. However, the experimental conditions differed, with temperatures varying from 40 to 50 °C, reaction times of between 48 and 72 h, and babassu oil:ethanol molar ratios ranging from 1:9 to 1:12. From an industrial point of view, the conditions adopted in this work (40 °C, 24 h, and 1:7) could have certain advantages, such as lower consumption of energy and raw materials. Da Rós et al. [12] reported a 100 % yield for transesterification of babassu oil with ethanol, within only 10 h at 50 °C, using immobilized *B. cepacia* lipase.

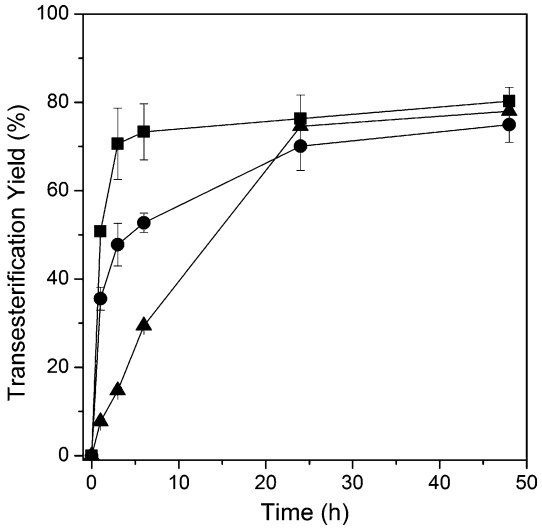

Fig. 6 Transesterification yields for soybean oil in solvent-free systems catalyzed by PFL-octyl-silica (■) and the commercial biocatalysts CALB IM (▲) and TLL IM (●). The reactions were performed at 40 °C in a mechanically stirred (500 rpm) reactor equipped with reflux condenser, using an oil:ethanol molar ratio of 1:7 and 130 units of hydrolytic activity per g of oil

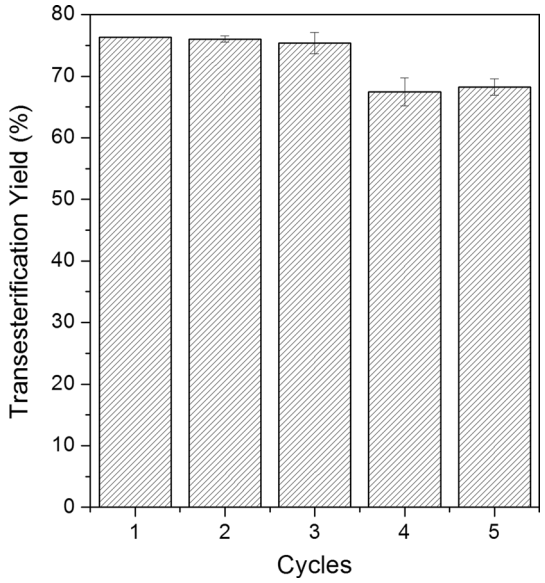

Fig. 7 Operational stability tests of PFL immobilized on octyl-silica particles, using successive 24-h cycles of transesterification of soybean oil in solvent-free systems. The reactions were performed under continuous agitation (500 rpm) at 40 °C, using an oil:ethanol molar ratio of 1:7 and 0.2 g of biocatalyst/g of oil

Interestingly, the authors used microwave-assisted heating to accelerate the transesterification reaction. Although promising, this technology remains in an early stage for enzyme-catalyzed reactions.

Figure 6 shows the transesterification yields of soybean oil with ethanol. Similar yields of around 80 % were obtained for all the biocatalysts (PFL-octyl-silica and commercial CALB and TLL) after 48 h of reaction. Some of the previously published studies (see Table 3) reported transesterification yields of soybean oil with ethanol in the range from 57 to 100 %. Technological strategies that have been used to increase transesterification yields and reduce reaction times include the use of a co-solvent, sonication to assist the enzyme-catalyzed reaction, combination of different lipases in the same reactor, addition of ethanol in two steps as a way of improving enzymatic biodiesel synthesis, and a high soybean oil:ethanol molar ratio. Under the experimental conditions adopted in the present work (conventional heating, addition of ethanol in a single step, and low oil:alcohol molar ratio), PFL hydrophobically adsorbed on octyl-silica is a promising biocatalyst for use in the transesterification of vegetable oils that contain high concentrations of medium-chain saturated fatty acids or high concentrations of mono- and polyunsaturated long-chain fatty acids. An example of the former is babassu oil, which is rich in lauric and myristic acids, while the latter include soybean oil, which is rich in oleic, linoleic, and linolenic acids.

Operational stability (reuse) of PFL immobilized on octyl-silica in biodiesel production

The PFL-octyl-silica biocatalyst remained fully active for three cycles of 24 h (Fig. 7), after which a decrease of around 10 % of its initial activity was observed. The fact that there was no decrease in activity for the first three batches suggests that the enzyme was not eluted from the support under the operational conditions employed. In general, lipases immobilized on hydrophobic supports (in this case, octyl-silica) that resemble their natural substrates remain strongly adsorbed on them in open form, with hardly any elution from the surfaces, because lipases are insoluble in organic media [15]. The decrease in activity therefore probably resulted from inactivation of the enzyme due to the detrimental effects of temperature and ethanol.

For continuous systems employing fixed-bed reactors, the adsorption of glycerol on the support surface should be avoided in order to prevent poisoning of the biocatalyst and the need to interrupt the process in order to remove the contaminant. PFL adsorbed on octyl-silica was incubated for 72 h in a solution of glycerol in ethanol at 40 °C, with stirring at 500 rpm. The initial and final concentrations of glycerol were determined by RI-HPLC. It was found that glycerol was not adsorbed on the octyl-silica surface (data not shown). The use of PFL adsorbed on octyl-silica is therefore advantageous for the continuous production of biodiesel, because washing steps are not required for biocatalyst regeneration.

Conclusions

Pseudomonas fluorescens lipase was strongly adsorbed on octyl-silica, increasing the thermal and operational stability of the enzyme. In aqueous ethanol solution, this biocatalyst was about 12-fold more stable than the soluble enzyme. PFL-octyl-silica was shown to be a good biocatalyst for the production of biodiesel from babassu and soybean oils. In the transesterification of babassu oil, almost full conversion into fatty acid ethyl esters (biodiesel) was achieved within 24 h of reaction, while for the reaction with soybean oil, a yield of 80 % was obtained within 48 h. Use of commercial lipase B from *Candida antarctica* resulted in a similar yield for the transesterification of soybean oil. Octyl-silica did not adsorb glycerol, which is an important consideration when the biocatalyst is reused successively in batch processes or employed in continuous processes for biodiesel production. The PFL-octyl-silica biocatalyst could be reused in three successive batches for the transesterification of soybean oil, without any significant reduction in activity. The findings demonstrated that silica particles functionalized with octyltriethoxysilane provided a support with excellent physicochemical properties for the immobilization of lipases, and could be used to prepare active and stable biocatalysts for the catalysis of reactions of industrial interest, such as the transesterification (in an organic medium) of vegetable oils. In this work, PFL-octyl-silica was shown to be suitable for use with non-aqueous media homogeneously suspended in a stirred tank reactor. However, the prepared biocatalyst could also be used in the design of continuous column reactors (fixed and fluidized bed reactors), because it offers the mechanical resistance to pressure of silica, combined with the presence of particles of a suitable size and density for fluidization.

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