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Immobilization and stabilization of microbial lipases by multipoint covalent attachment on aldehyde-resin affinity: Application of the biocatalysts in biodiesel synthesis

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ABSTRACT

Microbial lipase preparations from *Thermomyces lanuginosus* (TLL) and *Pseudomonas fluorescens* (PFL) were immobilized by multipoint covalent attachment on Toyopearl AF-amino-650M resin and the most active and thermal stable derivatives used to catalyze the transesterification reaction of babassu and palm oils with ethanol in solvent-free media. For this, different activating agents, mainly glutaraldehyde, glycidol and epichlorohydrin were used and immobilization parameters were estimated based on the hydrolysis of olive oil emulsion and butyl butyrate synthesis. TLL immobilized on glyoxyl-resin allowed obtaining derivatives with the highest hydrolytic activity (HA_{der}) and thermal stability, between 27 and 31 times more stable than the soluble lipase. Although PFL derivatives were found to be less active and thermally stables, similar formation of butyl butyrate concentrations were found for both TLL and PFL derivatives. The highest conversion into biodiesel was found in the transesterification of palm oil catalyzed by both TLL and PFL glyoxyl-derivatives.

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1. Introduction

Fatty acid alkyl esters (biodiesel) are important alternatives to the conventional diesel from petroleum. Since biodiesel comes from renewable sources, it does not contribute to new carbon dioxide emission – one of the factors responsible for the greenhouse effect. The other main characteristic of this fuel is the almost total absence of sulphur and the low production of soot particulate after combustion [1,2]. Biodiesel is industrially produced by homogeneous alkaline catalysis vegetable oil and methanol. However, several problems are associated with this process such as difficulty in glycerol recovery, excessive energy cost and the need for removal of the catalyst from the product. Due to these drawbacks, alternative, and more sustainable routes for biodiesel production are being sought. In this context, enzymatic process employing lipases is very promising because of its high selectivity and mild operative conditions [1–4].

Lipases (triacylglycerol acyl hydrolases – EC 3.1.1.3) are an important group of enzymes that catalyze the breakdown of oils and fats, with subsequent release of free fatty acids, acylglycerols and glycerol. In addition, lipases can also catalyze the transesterification, aminolysis and synthesis of a broad range of natural and unnatural esters, while retaining high enantio or regioselectivity [5,6].

For practical and economical reasons, it is advantageous to use lipase in its immobilized form. Lipase has been immobilized by several methods, namely adsorption, cross-linking, adsorption followed by cross-linking, multipoint covalent attachment and physical entrapment [7]. However, the selection of an immobilization strategy and support should be based on process specifications for the catalyst, including such parameters as overall enzymatic activity, inactivation and regeneration characteristics, cost of the immobilized procedure, toxicity of immobilization reactants and the desired final properties of the immobilized derivative [8].

Among the available immobilization methods, multipoint covalent attachment is the most effective in terms of thermal and operational stabilization of enzymes [9–16]. This stabilization is due to the formation of covalent linkages between primary amine groups of the enzyme and aldehyde groups of the support. Multipoint attachment may increase the rigidity of the immobilized enzyme, hence inducing higher resistance to small conformational changes caused by heat, organic solvents, denaturing agents, among others. Improvement of stability of the enzymes, nonetheless, is still one of the main issues for the implementation of enzymes as industrial biocatalysts [15].

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The objective of the present work was to immobilize and stabilize microbial lipases from *Thermomyces lanuginosus* (TLL) and *Pseudomonas fluorescens* (PFL) on a commercial matrix Toyopearl AF-amino-650M by multipoint covalent attachment to be used on biodiesel production by transesterification of palm and babassu oils with ethanol in solvent-free system. Toyopearl AF-amino-650M resin, commercially available from Tosoh Bioscience, can be easily functionalized with chemically active groups. This matrix is hydrophilic, dimensionally stable base resin with excellent pressure and flow characteristics, large pores (1000 Å) to accommodate the largest proteins and particle size of 40–90 μm. Toyopearl AF-amino-650M resin is ideal for coupling either proteins or low molecular weight ligands (Tosoh Bioscience technical information). Despite these satisfactory properties, its use as support matrix for immobilization of lipase has not been reported in the literature yet. In this work a screen of chemical agents was performed, mainly glycidol, epichlorohydrin and glutaraldehyde to active Toyoperal surface for optimizing the formation of immobilized derivatives with high activity and thermal stability. Immobilized parameters were estimated based on both aqueous (hydrolysis of olive oil emulsion) and non aqueous media (butyl butyrate synthesis) and the most active and thermal stable derivatives were used to carry out the transesterification reactions. The goal was to obtain full conversion of the selected vegetable oils so that the results could be applied to the production of ethyl esters that fulfill its use as biofuel. This is an exceptional option for Brazilian biodiesel production, since both vegetable oils and ethanol are readily available in the country.

2. Materials and methods

2.1. Materials

Lipases from *T. lanuginosus* (TLL) and *P. fluorescens* (PFL) were from Novozymes (Araucária, PR, Brazil) and Amano Pharmaceutical (Nagoya, Japan), respectively, and used as received without further purification. The characteristics of these microbial lipase preparations are presented in Table 1. Toyopearl AF-Amino-650M resin was acquired from Tosoh Bioscience (Montgommery, USA). Glycidol (GLY) and epichlorohydrin (EPI) were purchased from Sigma Aldrich Co. (St. Louis, MO, USA). Glutaraldehyde (GLU), 25% (v/v) solution was obtained from Vetec (São Paulo, Brazil). Ethanol (minimum 99%) was supplied by Chromoline (SP, Brazil). Olive oil (low acidity) from Carbonell (Spain) was purchased at a local market. Refined bleached palm oil was a kind gift from Agropalma (Belém, PA, Brazil) having the following composition in fatty acids (% w/v): 0.1% lauric, 1.2% mirystic, 46.8% palmitic, 3.8% stearic, 37.6% oleic, and 10.5% linoleic, with 849.0 g mol^{-1} average molecular weight. Babassu oil was kindly supplied by Cognis (Jacareí, SP, Brazil) having the following composition in fatty acids: (w/v): 3.5% octanoic, 4.5% decanoic, 44.7% lauric, 17.5% myristic, 9.7% palmitic, 3.1% steriac, 15.2% oleic and 1.8% linoleic with average molecular weight 709.90 g mol^{-1}. All the other reagents used were of analytical grade.

2.2. Methods

2.2.1. Activation beads procedure

Toyopearl resin affinity was activated with different agents: glycidol, epichlorohydrin and glutaraldehyde. For glycidol activation, 10 g resin was added to a solution composed of 3 mL distilled water, 5 mL 1.7 M NaOH solution containing 0.15 g sodium borohydrate ($NaBH_4$). Following this, a suitable amount of glycidol (3.6 mL) was slowly added and the mixture kept at 0 °C for 15 h [22]. For epichlorohydrin activation, 10 g gel was suspended in 100 mL 2 M NaOH solution containing 0.6 g $NaBH_4$. Then, 10 mL of epichlorohydrin were slowly added and the suspension was kept at 0 °C for 15 h [23]. Glyceryl-resins (activated with glycidol and epichlorohydrin) were further suspended in 60 mL Milli-Q water and 30 mL 0.1 M sodium periodate to produce glyoxyl groups [22]. The suspension was kept under slight stirring for 2 h at room temperature. Glyoxyl-resins were washed with Milli-Q water and vacuum dried. For activation with glutaraldehyde, the resin was prepared from glyoxyl-resin by adding 10 g of this activated resin to a 40 mL of a 2 M ethylenediamine (EDA) solution at pH 10. Afterwards, 11.2 mL 0.2 M phosphate buffer solution pH 7.0 and 16.8 mL 25% glutaraldehyde solution were added to the glyoxyl-amine-resin and the system was kept under gentle stirring for 16 h at 25 °C to produce glyoxyl-amine-glutaraldehyde-resin (GAG-resin) [24]. Finally, the activated resin was washed with Milli-Q water and vacuum dried.

2.2.2. Immobilization procedure

Lipase preparations were added to 9 mL 0.1 M bicarbonate buffer (pH 10.05) containing 0.15 wt.% Triton X-100 and mixed with 1 g support under low stirring for 24 h at room temperature. The loading protein offered was 5 mg g^{-1} resin. After this, the derivative was filtered (Whatman filter paper 41) and thoroughly rinsed with Milli-Q water. Immobilization was followed by measuring the hydrolytic activities and protein concentration in the supernatant solution. TLL immobilized on Toyopearl AF-amino-650M resin was further tested by offering different loadings of protein (5.0, 10.0, 30.0, 60.0 and 80.0 mg g^{-1} resin) to determine the resin saturation enzymatic loading.

2.2.3. Schiff's bases reduction

After the enzyme immobilization step, 1.0 mg mL^{-1} sodium borohydride was added to the immobilization suspension and kept under agitation during 30 min at 25 °C. After this, the derivative was filtered (Whatman filter paper 41) and thoroughly rinsed with

Table 1
Some characteristics of the microbial lipases used in the present work.

Characteristics	Lipase preparations		References
	TLL	PFL	
Supplier	Novozymes	Amano	
Formulation	Liquid	Powder	
PDB file	1TIB	2LIP	
Molecular weight (kDa)	33	33	[13,17]
Lysine residues	7	7	[13,18]
Substrate specificity	1,3-Specific	Non-specific	[19,20]
pH optimum	8.0	8.3	[17,21]
T optimum (°C)	60	58	[17,21]
Protein content (mg g^{-1})	18.7	20.0	[17]
Specific hydrolytic activity (IU mg^{-1} protein)	195.6	217.5	[17]
Half-life ($t_{1/2}$) at 70 °C (min)	4.8	2.5	[17]

0.2 M buffer phosphate pH 7.0 and finally washed thoroughly with distilled and Milli-Q water.

2.2.4. Thermal stability

Soluble and immobilized lipase were incubated in the presence of 1 mL buffer phosphate pH 8.0 (0.1 M) at 70 °C for different time intervals. The half-life times ($t_{1/2}$) were determined by applying an exponential nonlinear decay model [25].

2.2.5. Determination of hydrolytic activity

Hydrolytic activities of soluble and immobilized lipase were assayed using olive oil emulsion as substrate, according to the methodology described by Soares et al. [26] with slight modifications. The substrate was prepared by mixing 50 g olive oil with 150 g Arabic gum solution (3 wt.%). The reaction mixture containing 5 mL emulsion, 5 mL 0.1 M phosphate buffer (pH 8.0), and immobilized (0.1 g) or soluble (0.2 mL, 0.5 mg mL^{-1}) lipase was incubated for 5 min at 37 °C. The reaction was stopped by addition of 10 mL commercial ethanol. The fatty acids formed were titrated with 0.02 M sodium hydroxide solution in the presence of phenolphthalein as indicator. One international unit of activity was defined as the amount of enzyme that liberates 1 μmol free fatty acid per minute (1 IU) under the assay conditions.

2.2.6. Determination of esterification activity

Reaction systems consisted of heptane (20 mL), *n*-butanol (0.1 M), butyric acid (0.1 M) and immobilized lipase (1.0 g, dry weight). The mixtures were incubated at 37 °C for 24 h with continuous shaking at 150 rpm, according to methodology described by Soares et al. [26]. The butyl butyrate conversion percentage was quantified by measuring the concentration of residual butyric acid in the reaction mixture after 24 h. For these, samples withdrawn (150–200 mg) were diluted in 10 mL of an ethanol/acetone 50:50 (v/v) mixture and titrated with KOH solution (0.02 M) in ethanol using phenolphthalein as the end-point indicator.

2.2.7. Determination of protein

Protein was determined according to the methodology described by Bradford [27] using bovine serum albumin (BSA) as standard.

2.2.8. Immobilization parameters

Immobilized protein (IP) was calculated after determining the amount of protein and enzyme units that disappeared in the supernatant, and comparing with the initial protein and enzyme concentration that were offered (U g^{-1} of gel). Recovered activity (RA) was calculated after determining the activity of the immobilized enzyme (apparent hydrolytic activity) and comparing with the number of enzyme units that disappeared from the supernatant (theoretically immobilized). Stability factor (SF) is the ratio between the half-life of the immobilized derivate and the half-life of the soluble enzyme.

2.2.9. Biodiesel synthesis

The reactions were performed in closed 25 mL-flasks containing 20 g substrate consisting of babassu or palm oils and anhydrous ethanol, without addition of solvents, at fixed molar ratio oil to alcohol 1:9 [28] and 1:18 [29], respectively. The mixtures were incubated with immobilized lipase at proportions of 2 mg immobilized enzyme per gram of oil. The experiments were carried out at 45 °C. Reactions were performed for a maximum period of 48 h under constant magnetic agitation at 180 rpm. For the time course studies, an aliquot of reaction medium was taken at different time intervals and diluted in *n*-hexane for determining fatty acid ethyl esters (FAEE) by gas chromatograph analysis.

2.2.10. Gas chromatograph analysis

Samples prepared as described above were analyzed by injecting 1 μL hexane solution and internal standard hexanol into a FID gas chromatograph (Varian CP 3800), using a 6-ft 5% DEGS on Chromosorb WHP, 80/10 mesh column (Hewlett Packard, Palo Alto, CA, USA) following previous established conditions [30]. The yield was defined as the concentration ratio of transformed oil to initial oil × 100.

3. Results and discussion

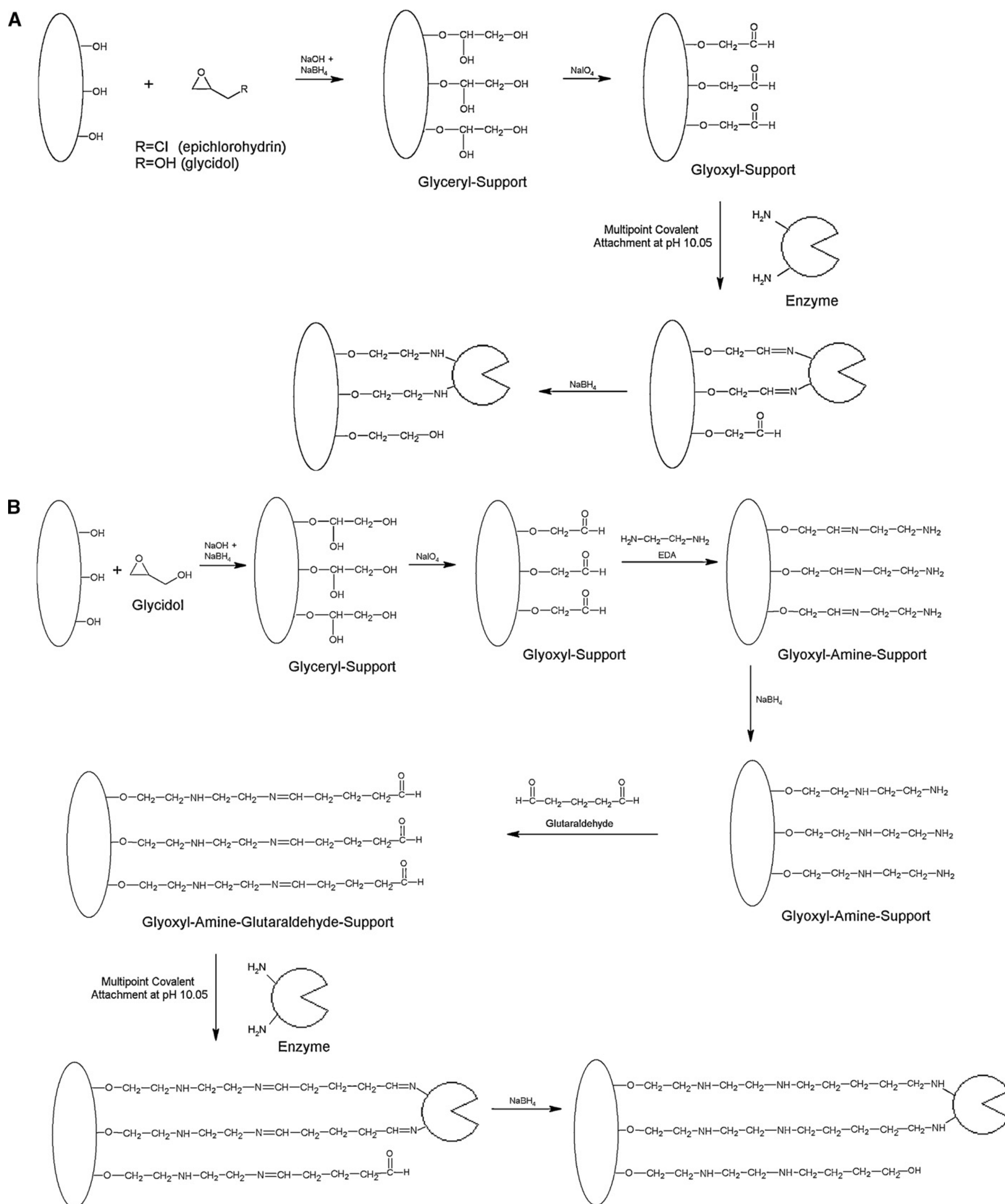
3.1. Determination of the immobilization parameters of TLL and PFL derivatives

Lipases from *P. fluorescens* (PFL) and *T. lanuginosus* (TLL) were immobilized on Toyopearl AF-amino-650M resin, a commercially available resin with excellent chemical and mechanical properties, aiming at obtaining thermo-stable biocatalysts to be used on the transesterification of vegetable oils with ethanol. Lipases were immobilized via multipoint covalent attachment on glyoxyl-resin, activated with glycidol and epichlorohydrin (Scheme 1A), and glyoxyl-amine-glutaraldehyde-resin (Scheme 1B) at pH 10.05. Support activation with these agents generates high concentration of aldehyde groups on the support surface [9,16]. Aldehyde groups in the support and amine groups in the enzyme from lysine residues are a good choice to make multipoint attachment and, therefore, to obtain highly thermo-stable enzyme derivatives [9–16].

Since these lipase preparations have great tendency to yield bimolecular aggregates [13,31], immobilization procedures were carried out in the presence of Triton X-100 (0.15 wt.%). In addition, it has been shown that immobilized lipase activity in aqueous and anhydrous media can be improved in the presence of detergents probably due to the breakage of lipase aggregates and/or to the shift on the closed-open equilibrium of the individual lipase molecules [31]. In this sense, the application of immobilizing procedure under dissociation conditions (e.g., in the presence of detergents) may allow to obtain fully dispersed immobilized lipase molecules oriented towards the medium [13,31].

After immobilization, the prepared derivatives were incubated in sodium borohydrate solution for reduction Schiff's bases (C=N double bond), which were formed between the aldehyde group of the support and amine groups of the enzyme (Scheme 1A and B). The reduction of these Schiff's bases transform them in stable covalent bonds, as well as change the reactive aldehyde groups to the inert hydroxyl groups which is an important step in the immobilization process [9]. In this set of experiments, a fixed protein loading of 5 mg g^{-1} of resin was used and the catalytic properties of the resulting derivatives are shown in Table 2.

Among the tested activating agents, glutaraldehyde gave the highest percentage of immobilized protein. For both lipase preparations, all protein initially offered was immobilized. For TLL immobilized on resin activated with glycidol or epichlorohydrin, values for percentage of immobilized protein were 32.4 and 36.4%, respectively. For PFL, the percentage of immobilized protein ranged from 37.8 to 44% after 24 h incubation. According to these results, it can be verified that the percentage of immobilized protein was proportional to the length of the spacer arm from the activating agents. This may be explained by steric hindrance on lipase molecules that may occur when short spacer arm as glyoxyl groups (2 carbon atoms) was used (Scheme 1A). Under this condition, lipase molecules are very close to the support surface. Consequently, lipase immobilization on glyoxyl-amine-glutaraldehyde-resin (9 carbon atoms) allowed obtaining derivatives with higher immobilized protein amount (Scheme 1B). Similar results were reported



Scheme 1. Mechanism of multipoint covalent attachment of enzymes on glyoxyl (A) and glyoxyl-amine-glutaraldehyde-supports (B).

by Bulmus et al. [32] for experiments dealing with the immobilization of glucose oxidase on PMMA via spacer arms at different length, in which the amount of immobilized enzyme was found to increase as a function of the spacer arm length.

Although, high protein amounts can be immobilized on GAG-resin, immobilized lipase activities and enzyme recovered were higher for derivatives obtained using glyoxyl-resin. The highest hydrolytic activities were obtained for TLL immobilized on glyoxyl-

Table 2
Immobilization parameters for TLL and PFL immobilized on Toyopearl AF-amino-650M resin activated by different protocols offering protein loading of 5 mg g^{-1} of resin.

Lipase preparations	Activating agent	HA_{der} (IU g^{-1})	IP (mg g^{-1})	RA (%)
TLL	Glycidol	188.4	1.62	60.9
	Epichlorohydrin	244.9	1.84	69.7
	Glutaraldehyde	169.6	5.00	17.7
PFL	Glycidol	44.0	1.89	10.7
	Epichlorohydrin	58.7	2.20	12.3
	Glutaraldehyde	33.4	5.00	3.07

$HA_{der.}$: hydrolytic activity (IU g^{-1} of resin); IP: immobilized protein concentration (mg g^{-1} of resin) RA: recovered activity (%).

resin activated with epichlorohydrin (244.9 IU g^{-1} resin), followed by glycidol (188.4 IU g^{-1} resin) and glutaraldehyde (169.6 IU g^{-1} resin). According to Table 1, soluble PFL preparation exhibited higher specific hydrolytic activity (217.5 IU mg^{-1} protein) than soluble TLL (195.6 IU mg^{-1} protein). On the other hand, when PFL was immobilized on resin activated with epichlorohydrin, maximum achieved hydrolytic activity (58.7 IU g^{-1} resin) was 4 times lower than the value attained for TLL-epichlorohydrin derivative (244.9 IU g^{-1} resin). This may be related to the formation of bonds between the enzyme and support that cause distortion in the enzyme three dimensional structure, leading to an inactive conformation of many enzyme molecules, or to poor orientation of the immobilized protein, preventing substrate access to the catalytic site.

Recovered activity values were also influenced by the activating agent type. For both lipase preparations, the recovered activity percentage of the derivatives prepared by activation via glyoxyl was 3–4 times higher than those obtained with glyoxyl-amine-glutaraldehyde derivatives. The recovered activity for TLL immobilized on resin activated via glyoxyl varied from 60.9 to 69.7%, while for glyoxyl-amine-glutaraldehyde derivative the recovered activity was as low as 18%. Such strong reduction on the recovered activity may be due to high glutaraldehyde reactivity, which distorts the enzyme three-dimensional structure.

The stability of the enzyme three-dimensional structure exposed to high temperature can be considered as an indirect methodology to confirm the formation of multipoint covalent attachment [9,16]. Thermal stabilities for both soluble and immobilized lipases were evaluated by measuring the residual activity of the lipase preparations exposed to 70 °C in buffer phosphate 0.1 M at pH 8.0, as shown in Fig. 1A and B. Data for thermal inactivation of TLL and PFL derivatives was adjusted to an exponential nonlinear decay model to estimate the half-life ($t_{1/2}$) and stability factor (SF).

The length of spacer arm from activating agents was found to have also influence on the thermal-stability of TLL-derivatives. The best results for thermal stabilities were obtained for TLL immobilized derivatives on glyoxyl-resin, activated with either glycidol or epichlorohydrin. These derivatives were 27 and 31 times more thermally stable than soluble lipase, respectively, while the lipase immobilized on GAG-resin was slightly less thermo-stable (SF = 21). These results suggest that multipoint covalent attachment occurred for these derivatives. Half-life ($t_{1/2}$) values for TLL derivatives varied from 1.77 to 1.93 h (Fig. 1A). The stabilization factors for PFL derivatives were less than 5 times in relation to the soluble lipase ($t_{1/2} \approx 15$ min) at 70 °C (Fig. 1B). These results indicated that PFL immobilization on Toyopearl AF-amino-650M resin was not successfully achieved by multipoint covalent attachment. It is possible that under these conditions, the PFL immobilization on resin affinity may distort the enzyme three-dimensional structure (random immobilization), diminishing the protein structure rigidity and, consequently, reducing its thermal-stability. Similar results were found for *Candida antarctica* lipase (CALB) covalently immobilized on chitosan hydrogel activated with epichlorohydrin [9].

3.2. *Performance of the lipase derivatives on the butyl butyrate synthesis*

The catalytic activity of immobilized derivatives prepared by multipoint covalent attachment on Toyopearl AF-amino-650M activated by different protocols was also investigated in anhydrous medium reaction on the butyl butyrate synthesis and values for ester concentrations are presented in Table 3. The highest butyl butyrate concentrations (>60 mM) were found for reactions mediated by immobilized derivatives on glyoxyl-resin. In this set of experiments, similar butyl butyrate concentrations were found for TLL and PFL derivatives. However, in aqueous medium (olive oil

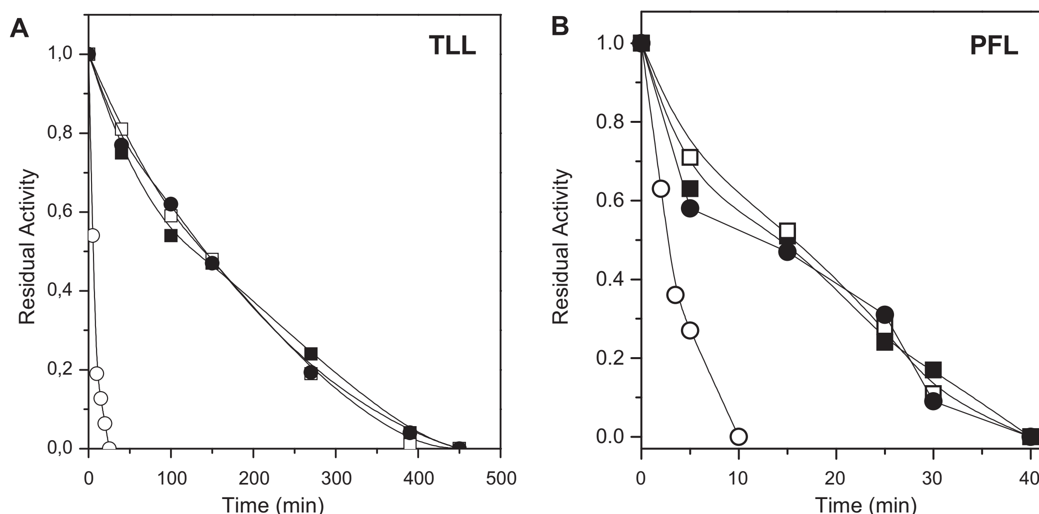


Fig. 1. Thermal stability of TLL (A) and PFL (B) soluble (○) and immobilized by multipoint covalent attachment on Toyopearl AF-amino-650M resin activated with glycidol (■), epichlorohydrin (□) and glutaraldehyde (●). Inactivation tests were carried out at 70 °C in 0.1 M sodium phosphate buffer pH 8.0.

Table 3
Butyl butyrate synthesis catalyzed by TLL and PFL immobilized on Toyopearl AF-amino-650M activated by different protocols.

Lipase preparations	Activating agent	Butyl butyrate concentration (mM)$^{24\,h}$
TLL	Glycidol	66.6
	Epichlorohydrin	60.0
	Glutaraldehyde	46.2
PFL	Glycidol	62.2
	Epichlorohydrin	65.5
	Glutaraldehyde	49.7

emulsion hydrolysis), TLL derivatives were more active than PFL ones. This lipase preparation showed to be more active in organic than aqueous medium. According to data reported [33], TLL is very efficient in emulsified triglycerides hydrolysis, but a fraction of this activity could be inactivated in organic medium due to an increase in the rigidification of the lid that covers its active site in anhydrous media. Synthetic activity is highly dependent on the degree of hydration and a minimum amount of water is necessary for the enzyme to maintain its optimal conformation [1]. For TLL and PFL derivatives immobilized on GAG-resin, butyl butyrate concentrations varied from 46.2 to 49.7 mM. These results corroborated with the hypothesis that immobilized derivatives on resin activated with glutaraldehyde undergo strong distortion of the enzyme three-dimensional structure.

3.3. Investigating the maximum protein loading of TLL on Toyopearl AF-amino-650M resin

Since TLL derivatives were more thermal-stable than *P. fluorescens* (PFL) the maximum protein loading that could be used in the immobilization of this lipase preparation on Toyopearl AF-amino-650M resin was established using different protocols. In this set of experiments, multipoint covalent attachment of TLL also was carried out in the presence of Triton X-100 (0.15 wt.%) and the amount of protein offered to the support varied from 5 to 80 mg g^{-1} resin. The immobilization parameters are summarized in Table 4.

As can be observed the high reactivity of glutaraldehyde led to TLL derivatives with the highest immobilized protein value (21.9 mg immobilized protein g^{-1} resin) for the maximum protein loading (80 mg g^{-1} resin). For glyoxyl-resin, the maximum protein immobilized concentration was 16.6 and 15.8 mg g^{-1} resin activated with glycidol and epichlorohydrin, respectively. However, the highest hydrolytic activities were obtained by activating the support with epichlorohydrin (437.8 IU g^{-1} resin) and glycidol (421.4 IU g^{-1} resin). It is possible that under these conditions, glutaraldehyde caused strong distortion of the enzyme three-dimensional structure, leading to an inactive conformation of many enzyme molecules.

For all derivatives the recovered activity decreased when protein loading increased, being more severe for glutaraldehyde derivatives. The recovered activity values displayed in Table 4 also indicate that the derivatives were subject to mass transfer limitations. The substrate diffusion towards the enzyme molecules decreases the biocatalyst activity biocatalysts [9]. For glyoxyl-derivatives, using the lowest lipase loading (5 mg g^{-1} resin) the recovered activities decreased, respectively, from 60.9 (support activated with glycidol) and 69.7% (support activated with epichlorohydrin) to less than 15% for the highest lipase loading (80 mg g^{-1} resin). The intrinsic enzyme rate increases with an increase on the lipase loading and the rate of substrate diffusion in the gel may decrease as the presence of more molecules of immobilized protein decrease the porous diameter [9,33].

3.4. Biodiesel synthesis catalyzed by glyoxyl-derivatives

Biodiesel synthesis was carried out as described in the material and methods using immobilized microbial lipases from *T. lanuginosus* (TLL) and *P. fluorescens* (PFL) by multipoint covalent attachment on glyoxyl-resin activated with glycidol and epichlorohydrin. Babassu and palm oils were employed as feedstocks under conditions previously established, i.e., ethanol to babassu oil molar ratio of 9:1 and ethanol to palm molar ratio of 18:1 [3,28,29]. All reactions were incubated with 2 mg of immobilized protein g^{-1} of oil. Tables 5 and 6 display the profile for ethyl esters formation in the transesterification reactions as well as corresponding transesterification yields at 48 h reaction. In this set of experiments, derivatives from GAG-resin were not investigated.

The observation of these tables indicate that, all tested TLL and PFL derivatives were able to form the main esters of fatty acids in the babassu and palm oils. Similar transesterification yields were found for TLL and PFL derivatives confirming the satisfactory performance of these derivatives to mediate synthetic reactions (butyl butyrate synthesis). As expected, the highest ethyl ester concentrations were in regard to palmitate and oleate esters for palm oil since such oil shows high concentration by palmitic and oleic acids in their composition. Similarity laurate, myristic and oleate ethyl esters were found for babassu oil.

For biodiesel from babassu oil, ethyl esters concentrations varied between 60.5 wt.% using TLL-glycidol to 66.3 wt.% for PFL-epichlorohydrin, corresponding to transesterification yields ranging from 86.6 to 94.9% (Table 5). For palm oil, ethyl esters concentrations varied between 52.4 wt.% using TLL-glycidol to 59.0 wt.% for TLL-epichlorohydrin, corresponding to transesterification yields ranging from 93.5 to 100% (Table 6).

Although, the difference among the transesterification yields was not considerable, slight higher yields and reaction rates were found for reactions performed with palm oil. TLL showed higher specificity on the transesterification of the lauric acid into ethyl laurate than PFL. However, such lipase preparation showed higher specificity for transesterification on the oleic acid than TLL, employing babassu oil as feedstock. In all reaction systems, a good dispersion of the biocatalyst was verified in the reaction medium

Table 4
Immobilization parameters for different protein loadings of TLL offered to Toyopearl AF-amino-650M activated by different protocols.

PL (mg g^{-1})	Glycidol			Epichlorohydrin			Glutaraldehyde		
	HA_{der} (IU g^{-1})	IP (mg g^{-1})	RA (%)	HA_{der} (IU g^{-1})	IP (mg g^{-1})	RA (%)	HA_{der} (IU g^{-1})	IP (mg g^{-1})	RA (%)
5	188.4	1.62	60.9	244.9	1.84	69.7	169.6	5.00	18.0
10	247.5	3.81	34.0	279.8	4.76	30.8	207.8	8.44	12.8
30	345.8	8.46	21.4	378.4	7.11	27.8	245.6	11.7	11.0
60	397.5	15.7	13.2	405.7	12.7	16.7	294.1	19.3	7.90
80	421.4	16.6	13.3	437.8	15.8	14.5	321.2	21.9	7.50

PL: protein loading (mg g^{-1} of resin).
HA_{der}: hydrolytic activity (IU g^{-1} of resin).
IP: immobilized protein concentration (mg g^{-1} of resin).
RA: recovered activity (%).

Table 5
Ethyl esters profile and yields attained in the transesterification reaction of babassu oil using glyoxyl-derivatives.

Lipase preparation	Activating agent	Ethyl esters (wt.%)							Total esters (wt.%)	Yield (%)
		C_8	C_{10}	C_{12}	C_{14}	C_{16}	C_{18}	$C_{18:1}$		
TLL	GLY	2.66	2.20	25.0	8.44	3.18	1.80	17.2	60.5	86.6
TLL	EPI	2.51	2.29	25.7	8.97	3.45	1.91	19.3	64.1	91.9
PFL	GLY	2.65	2.24	22.9	9.24	3.43	1.95	18.3	61.0	87.3
PFL	EPI	2.58	2.15	22.6	9.90	3.74	1.79	23.5	66.3	94.9

Table 6
Ethyl esters profile and yields attained in the transesterification reaction of palm oil using glyoxyl-derivatives.

Lipase preparation	Activating agent	Ethyl esters (wt.%)					Total esters (wt.%)	Yield (%)
		C_{14}	C_{16}	C_{18}	$C_{18:1}$	$C_{18:2}$		
TLL	GLY	1.37	24.9	1.90	18.9	5.30	52.4	93.5
TLL	EPI	1.78	25.9	2.30	22.9	6.40	59.0	100
PFL	GLY	1.62	26.5	2.40	22.5	6.50	58.9	100
PFL	EPI	1.58	26.2	2.27	22.4	6.30	58.8	100

as well as eases of the product separation (biodiesel) at the end of the reaction.

In agreement with these results, among the tested lipases, TLL showed the highest activity towards the transesterification of vegetable oils with ethanol and their derivatives were found to be more feasible than PFL derivatives due to the highest thermal-stability. Therefore, TLL immobilized by multipoint covalent attachment on glyoxyl-Toyopearl AF-amino-650M resin can be considered an attractive alternative to mediate transesterification of vegetable oils for biodiesel synthesis.

4. Conclusion

Toyopearl AF-amino-650M resin seems to be a very promising support for lipase immobilization. This support allows obtaining immobilized derivatives from *T. lanuginosus* (TLL) and *P. fluorescens* (PFL) with high hydrolytic activity and thermal stability. Results suggested that multipoint covalent attachment has occurred only for TLL immobilized on Toyopearl AF-amino-650M resin activated by glycidol, epichorohydrin and glutaraldehyde. For both lipase sources better catalytic properties were found when the support was activated with glycidol and epichlorohydrin, which allows for their successful application in biodiesel synthesis by transesterification of babassu and palm oils with ethanol. Although, the difference among the transesterification yields was not considerable, slightly higher yields were found for reactions performed with palm oil using both glyoxyl-derivatives of TLL and PFL.

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