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Immobilization of *Candida antarctica* Lipase B by Adsorption to Green Coconut Fiber

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Abstract An agroindustrial residue, green coconut fiber, was evaluated as support for immobilization of *Candida antarctica* type B (CALB) lipase by physical adsorption. The influence of several parameters, such as contact time, amount of enzyme offered to immobilization, and pH of lipase solution was analyzed to select a suitable immobilization protocol. Kinetic constants of soluble and immobilized lipases were assayed. Thermal and operational stability of the immobilized enzyme, obtained after 2 h of contact between coconut fiber and enzyme solution, containing 40 U/ml in 25 mM sodium phosphate buffer pH 7, were determined. CALB immobilization by adsorption on coconut fiber promoted an increase in thermal stability at 50 and 60 °C, as half-lives ($t_{1/2}$) of the immobilized enzyme were, respectively, 2- and 92-fold higher than the ones for soluble enzyme. Furthermore, operational stabilities of methyl butyrate hydrolysis and butyl butyrate synthesis were evaluated. After the third cycle of methyl butyrate hydrolysis, it retained less than 50% of the initial activity, while Novozyme 435 retained more than 70% after the tenth cycle. However, in the synthesis of butyl butyrate, CALB immobilized on coconut fiber showed a good operational stability when compared to Novozyme 435, retaining 80% of its initial activity after the sixth cycle of reaction.

Keywords *Candida antarctica* lipase B · Enzyme immobilization · Coconut fiber · Physical adsorption · Methyl butyrate hydrolysis · Butyl butyrate synthesis

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Introduction

Most proteins, amongst them enzymes, are large amphiphatic molecules, and this characteristic makes them intrinsically surface-active molecules, which might lead them to adsorb in some interfaces [1]. Thus, enzyme immobilization by adsorption occurs through a binding between an enzyme and a solid support. Depending on the nature of the surface, enzyme binding may be the result of ionic interactions, physical adsorption, hydrophobic bonding, Van der Waals attractive forces, or a combination of these interactions. This technique, although simple and of low cost, possesses disadvantages, such as low linking energy between enzyme and support, which may cause enzyme desorption in presence of the substrate or when it is subjected to variations on temperature, pH, and ionic force. However, although immobilized enzymes are susceptible to desorption, immobilization by adsorption is one of the most used techniques in the attainment of insoluble biocatalysts [2].

In literature, a great number of publications [3–11] using immobilized lipases by physical adsorption is observed. Initially, porous glass beads, diatomaceous earth, silica, and alumina were the commonest support used for lipase immobilization. However, recently, ionic exchange resins, celite, and biopolymers are preferred [3]. Some factors that influenced in lipase immobilization were discussed, e.g., use of hydrophilic or hydrophobic supports [4, 5], enzyme loading and coupling time [6, 7], and electrostatic interactions [8]. An increase in its stability on organic solvents [7], as well as on thermal and operational stabilities [5, 9, 10], was obtained after immobilization of lipases by adsorption. Furthermore, in some cases, an improvement on specific activity of lipase is achieved by fixing it to the support in its open-form conformation [11].

Candida antarctica lipase B (CALB) is a triacylglycerol hydrolase (E.C. no. 3.1.1.3) and also an effective carboxylesterase that has a molecular weight of 33 kDa and an isoeletric point (p*I*) of 6.0 [12]. Because of its stereospecificity and regioselectivity, CALB has been used as biocatalyst in several applications such as cosmetics, food, and pharmaceutical industries [13–15]. Several supports have been proposed to CALB immobilization, e.g., silica functionalized with octyltriethoxysilane [9], duolite A568 [5], EP 100 [16], silica gel [17], CoFoam [18], activated carbon [19], octyl-agarose, octadecyl-Sepabeads, and hydrophobin-agarose [20]. When CALB was adsorbed on functionalized silica, for instance, interesting results were obtained on the *N*-acylation of ethanolamine with lauric acid. The catalyst remained fully active after 15 cycles of reaction, similar to the result obtained using Novozyme 435 [9], a commercial biocatalyst prepared by lipase immobilization on a macroporous polyacrylic resin.

The high cost of some available commercial support is promoting a search for cheaper substitutes. Low-cost supports can be organic (chitin, chitosan) or inorganic ($CaCO_3$) [21]. From organic group, it has been detached from lignocellulosic agroindustrial waste supports, such as saw dust, straw, wood chips/shavings, rice husk [22], spent grains [23], rice straw [21, 24], cellulignin [25], and coconut fiber [26, 27]. These studies showed that agroindustrial wastes are a suitable raw material source for immobilization matrixes. Previous studies [27] had shown that green coconut fiber is a suitable support for lipase immobilization by covalent attachment, as an increase in thermal and operational stabilities were obtained when compared with the soluble enzyme.

Thus, in order to produce a low-cost immobilized lipase, the objective of this work is to evaluate the immobilization of CALB by adsorption on green coconut fiber. For this purpose, the influence of contact time, lipase concentration, and pH of lipase solution during the adsorption step on the biocatalyst activity and stability was investigated.

Materials and Methods

Materials

Commercial soluble and immobilized lipase B from *C. antarctica* (Lipozyme® CALB L and Novozyme 435, respectively) were kindly donated by Novozymes Latin America (Araucária, Brazil), with 1,780 U/ml and 1,039 U/g of hydrolytic activity for Lipozyme® CALB L and Novozyme 435, respectively. Methyl butyrate was from Sigma-Aldrich Chemical (St. Louis, MO, USA). Butyric acid and butanol were purchased from Merck (Rio de Janeiro, Brazil). Molecular sieve 4A ($Na_2O[Al_2O_3(5.0SiO_2)]12H_2O$) was from W. R. Grace (Massachusetts, MA, USA). The support used for enzyme immobilization was green coconut fiber that was kindly donated by Embrapa Agroindústria Tropical, Ceará State, Brazil. Other chemicals were of analytical grade.

Support

Green coconut fiber was obtained from green coconut husk through a process developed by Embrapa Agroindustria Tropical [28]. It was cut and sieved to obtain particles between 32 and 35 mesh. It was then washed with distilled water and dried at 60 °C before being used as immobilization matrix.

Protein Determination

Protein content was determined according to the Bradford method [29] using bovine serum albumin as a standard. Adsorbed protein was calculated by Eq. 1, where: P_{ads} is the adsorbed protein for gram of fiber (μg/g), P_o (μg/ml) and P_{sob} (μg/ml) are, respectively, protein concentration on the supernatant before and after adsorption, V is volume of lipase solution used during immobilization, and m_{fiber} is the mass of fiber used during immobilization.

$$P_{ads} = \frac{(P_o - P_{sob}) \times V}{m_{fiber}} \quad (1)$$

Preparation of Immobilized Enzyme

Immobilized enzyme was obtained by adsorption, at room temperature, by contacting enzyme solution and support in 5 ml syringes, stirred using the apparatus presented in a previous work [27]. For each gram of dry support, 10 ml of lipase solution in 25 mM sodium phosphate buffer was used. After immobilization, the biocatalyst was separated by filtration, rinsed with phosphate buffer (10 ml), and dried at vacuum for 10 min. In this study, recovered activity was defined as the ratio of enzymatic activity of the immobilized enzyme and the total units of soluble lipase that disappeared from the supernatant during immobilization [19]. Immobilization yield was defined as the difference between enzyme activity in the supernatant before and after immobilization divided by the enzyme activity in the supernatant before immobilization [29].

Assay of Hydrolytic Activity: Methyl Butyrate Hydrolysis

Hydrolytic activity of immobilized or soluble enzyme was determined by methyl butyrate hydrolysis [30]. Experiments were performed using an automatic titrator (pHstat) and

50 mM NaOH as titrating agent. The pH was set at 7.0. The reaction was initiated with the addition of 0.1 ml of soluble enzyme solution, 0.4 g of CALB immobilized on fiber, or 0.03 g of Novozyme 435 to a 30-ml methyl butyrate solution dissolved in 25 mM phosphate buffer pH 7.0. In this work, 1 U of enzymatic activity was defined as the amount of enzyme that hydrolyzes 1 μmol of methyl butyrate per minute at pH 7.0 and 28 °C.

Esterification Yield: Butyl Butyrate Synthesis

Stock solutions of butyric acid (150 mM) and butanol (150 mM) were prepared in *n*-heptane. Experiments were set up in 250-ml flasks containing 20 ml of stock solution, 1 g of molecular sieve 4A, and 0.3 g of the immobilized CALB or 0.012 g of Novozyme 435. The flasks were kept at 30 °C under agitation at 150 rpm for 24 h [31]. The consumption of butyric acid was measured by titration with 0.02 M NaOH and using phenolphthalein as indicator. The total acid content before reaction was determined by titration of a blank sample, without enzyme. The esterification yield was calculated from the decrease in butyric acid concentration after 24 h of reaction.

Operational Stability: Methyl Butyrate Hydrolysis

Immobilized enzyme stability was assayed by using 0.4 g of the immobilized CALB on fiber or 0.01 g of Novozyme 435 in successive batches of methyl butyrate hydrolysis. The operational conditions were the same as described for the assay of hydrolytic activity. At the end of each batch, the immobilized lipase was removed from the reaction medium, washed with phosphate buffer to remove any remaining substrate or product, dried under vacuum (10 min), and assayed again. The residual activity of the biocatalyst was calculated in terms of percentage of activity (U) of the immobilized enzyme measured after each cycle compared with the activity of the immobilized enzyme before the first cycle.

Operational Stability: Synthesis of Butyl Butyrate

Immobilized enzyme stability was assayed by using 0.3 g of the immobilized CALB on fiber or 0.012 g of Novozyme 435 in successive batches of butyl butyrate synthesis. Assay conditions were the same as described for the determination of esterification yield. At the end of each batch, the immobilized lipase was removed from the reaction medium and rinsed with hexane (20 ml) to extract any substrate or product eventually retained in the matrix. After 1 h at room temperature, the immobilized derivative was introduced into a fresh medium. The residual conversion is given as percentage of initial conversion of butyric acid (first cycle of synthesis) under standard conditions (described in "Esterification Yield: Butyl Butyrate Synthesis").

Thermal Stability

Thermal stability of soluble or immobilized enzyme was determined by incubating the biocatalyst in 100 mM sodium phosphate buffer pH 7.0 at 50 or 60 °C. Periodically, samples were withdrawn, and their residual activities were assayed by the hydrolysis of methyl butyrate. Residual activity is given as percentage of initial activity (hydrolytic activity before incubation). Thermal deactivation curves have been described following the

deactivation model proposed by Henley and Sadana (1985) referenced by Arroyo et al. [10]; see Eq. 2.

$$A = \left(100 + \frac{\alpha_1 k_1}{k_2 - k_1} - \frac{\alpha_2 k_2}{k_2 - k_1}\right) e^{-k_1 t} + \left(\frac{\alpha_2 k_1}{k_2 - k_1} - \frac{\alpha_1 k_1}{k_2 - k_1}\right) e^{-k_2 t} + \alpha_2 \quad (2)$$

where A is the residual activity in time t, k_1 and k_2 are first-order deactivation rate coefficients, and α_1 and α_2 ratios of specific activities (E_1/E and E_2/E, respectively) to the different states, see Eq. 3.

$$E \xrightarrow{k_1} E_1 \xrightarrow{k_2} E_2 \quad (3)$$

First-order deactivation rate coefficients (k_1 and k_2) and ratios of specific activities to the different states (α_1 and α_2) were estimated from experimental data. Biocatalyst half-life ($t_{1/2}$) was estimated by Eq. 2, using the estimated parameters (k_1, k_2, α_1, and α_2) and making A equal to 50%. In this work, stabilization factor (F) was considered as the ratio of immobilized enzymes' half-lives to soluble enzyme half-life [27].

Results and Discussion

Immobilization of *C. antarctica* Lipase B on Coconut Fiber by Adsorption

Influence of Contact Time and Enzyme Concentration on the Immobilized Enzyme Properties

Adsorption kinetic was investigated, and the influence of contact time between coconut fiber and lipase, at different enzyme concentrations from 0 U/ml (control without enzyme) to 90 U/ml, were evaluated. No hydrolytic activity was detected when the fiber without immobilized enzyme (control) was used as catalyst. Figure 1 pictures the influence of different initial concentrations of lipase in the supernatant (E_0 equal to 30, 60, or 90 U/ml) on the hydrolytic activity of immobilized CALB. The experimental data were subjected to statistical analysis (analysis of variance). At the probability level of $p<0.05$ (data not shown), it was observed that immobilized amount increases as time increased until 2 h (in

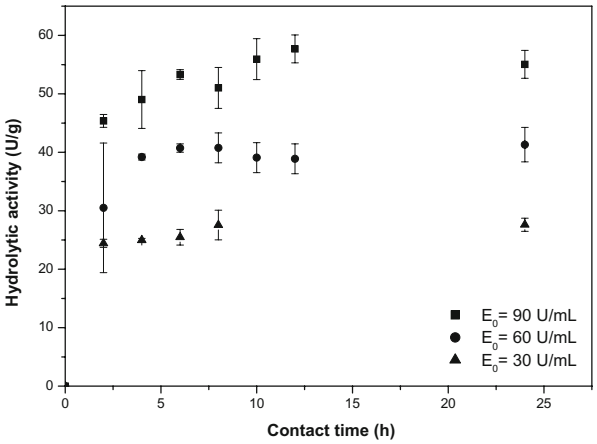

Fig. 1 Effect of contact time on the hydrolytic activity of lipase immobilized on coconut fiber by adsorption using initial enzyme concentrations of 30 U/ml (*closed triangle*), 60 U/ml (*closed circle*), or 90 U/ml (*closed square*)

case of E_0=30 U/ml and E_0=60 U/ml) or 6 h (E_0=90 U/ml), which is probably the point of dynamic balance between adsorption and desorption [32]. Furthermore, after 2 h of contact between enzyme and support, no expressive changes in hydrolytic activity of immobilized CALB was observed, as, after this time, loading amounts were almost 70–80% of the maximum loading achieved for the three initial concentrations studied. These results indicate that immobilization was achieved in a short time.

In subsequent experiments, to evaluate the influence of enzyme loading on the properties of the immobilized enzyme, contact time was set in 2 h, and enzyme concentration in the supernatant, during incubation, was changed from 30 to 500 U/ml (Fig. 2). It can be seen that hydrolytic activity of the immobilized enzyme enhances with increasing lipase concentration in the supernatant. However, the higher value of hydrolytic activity achieved by adsorption of CALB on coconut fiber (135.14 U/g) was still lower than the hydrolytic activity of Novozyme 435 (1,039 U/g, determined according to the methodology described in "Assay of Hydrolytic Activity: Methyl Butyrate Hydrolysis"), a commercial derivative.

Recovered activity and immobilization yield were calculated, and results are listed in Table 1. It can be observed that recovered activity increased when the initial enzyme concentration in the supernatant (E_0) was increased to 60 and 90 U/ml, but it remained almost constant for the other concentrations studied. Immobilization yield, on the other hand, decreased when high concentrations of enzyme were used (60 to 150 U/ml, compared to 30–40 U/ml). When E_0=90 U/ml, the highest value of recovered activity was obtained, which suggests that protein molecules are probably immobilized at close proximity to each other, which may prevent deactivation caused by enzyme unfolding by covering the support surface. In other words, when enzyme load was increased, more enzyme molecules were immobilized and less area of the support is available for lipase to spread itself, which may prevent loss in activity [31].

Nevertheless, when higher concentrations of lipase in the supernatant were used, E_0= 150 U/ml, recovered activity decreased, and immobilization yield was enhanced (Table 1). According to the literature [4, 6, 9, 10], protein adsorption is not restricted to a monolayer on the support, and adsorption of secondary layers has been reported. Therefore, when E_0= 150 U/ml, probably a second layer of lipase was adsorbed on the first layer, leading to an improvement on immobilization yield, as more enzyme molecules were adsorbed. However, although more molecules were immobilized on coconut fiber, not all of them

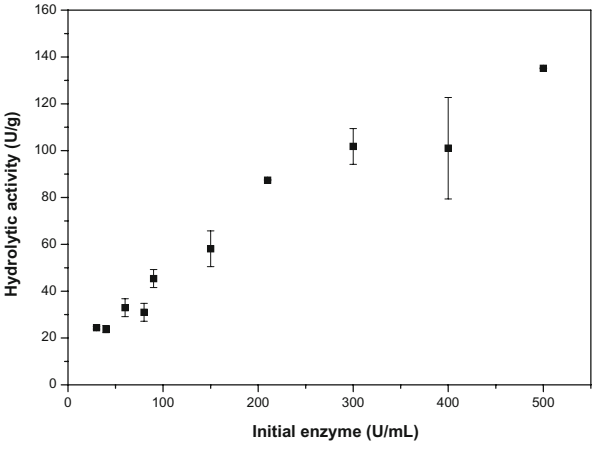

Fig. 2 Hydrolytic activity of immobilized enzyme as a function of initial enzyme activity in the supernatant. Lipase was immobilized on coconut fiber by adsorption after 2 h of contact time at room temperature

Table 1 Influence of enzyme concentration on recovered activity and immobilization yield.

Concentration of enzyme (U/ml)	Recovered activity (%)	Immobilization yield (%)
30	32.9	33.4
40	32.9	33.4
60	42.1	9.0
80	33.3	14.0
90	86.9	5.2
150	39.6	9.5
210	0	0
300	0	0
400	0	0
500	0	0

CALB immobilized by adsorption on coconut fiber at pH 7 and room temperature

were available to the substrate, causing a decrease in recovered activity. Coconut fiber surface does not have a porous structure, and it has a low surface area [27]. This poor surface area limits the number of enzyme molecules to be immobilized, facilitating the multilayer adsorption of proteins.

In order to evaluate the formation of enzyme multilayers [10], thermal stability studies of lipase immobilized in coconut fiber, obtained by using E_0=40 U/ml or E_0=280 U/ml, were performed at 60 °C. The thermal deactivation model (Eq. 2) was fitted to experimental data (Fig. 5), and the model parameters are listed in Table 2.

At 60 °C, the deactivation of soluble CALB and the immobilized CALB, obtained at pH 7.0 and E_0=280 U/ml, followed a first-order pattern. The other biocatalyst tested followed a double exponential decay. In all cases, $k_1 > k_2$, $\alpha_1 < 100\%$, and $\alpha_2 = 0$ (Table 2 and Eq. 2). According to the literature [10], the small values of parameter k_2 mean that there is a good stabilization of the enzymatic state E_1.

By comparing the remaining activity of the intermediate state E_1, expressed by α_1, for the two biocatalysts obtained at pH 7.0 and different initial enzyme activities in the supernatant, it is possible to evaluate the formation of lipase multilayers during adsorption. The intermediate of CALB-7A, CALB immobilized at pH 7 using E_0= 40 U/ml, has higher activity (α_1=59.2%) than the intermediate of CALB-7B (α_1=14.3%), CALB immobilized at pH 7 using E_0=280 U/ml, probably because of the formation of multilayers on CALB-7B. Due to this enzyme aggregation, lipase is weakly linked, and it is quickly deactivated [10]. As it can be seen, CALB-7A is 4.6-fold more stable than CALB-7B.

Effect of pH on the Adsorption of C. antarctica Lipase B on Coconut Fiber

According to the literature, hydrophobic interactions should not be affected by changes in the pH of adsorption. On the other hand, if electrostatic forces are important, changes over the isoelectric point of lipase will have a large impact on the binding constants [6]. Therefore, in this work, the effect of pH in the adsorption of *C. antarctica* lipase B on coconut fiber was investigated. The results of lipase adsorption, after 2 h of contact between coconut fiber and enzyme solution (E_0=40 U/ml), are presented in Fig. 3. Similar profiles for hydrolytic activity of immobilized lipase and adsorbed protein on fiber can be observed, with two plateau regions. This behavior is typical of ionic supports [6], and adsorption is governed by electrostatic forces. The same enzyme (CALB), when immobilized on a

Table 2 Kinetics parameters of thermal deactivation, at 50 and 60 °C, of soluble CALB, CALB immobilized by adsorption on coconut fiber and Novozyme 435.

Enzyme	k_1 (h^{-1})	k_2 (h^{-1})	α_1	α_2	$t_{1/2}$ (h)	F
Soluble CALB (T=50 °C)	0.0791	–	4.53	–	7.813	1
CALB-7A (T=50 °C)	0.0309	–	4.412	–	16.180	2
Soluble CALB (T=60 °C)	7.153	0.0000	99.908	0	0.0968	1
CALB-7A (T=60 °C)	1.642	0.0204	59.236	0	8.92	92
CALB-7B (T=60 °C)	0.451	0.0000	14.25	0	1.94	20
CALB-4A (T=60 °C)	0.503	0.0215	36.436	0	2.92	30
CALB-5A (T=60 °C)	0.521	0.0096	29.885	0	2.37	24
Novozyme 435 (T=60 °C)	0.955	0.0043	81.280	0	114.04	1178

CALB-7A CALB immobilized by adsorption on coconut fiber at pH 7, using E_0=40 U/ml; *CALB-7B* CALB immobilized by adsorption on coconut fiber at pH 7, by using E_0=280 U/ml; *CALB-4A* CALB immobilized by adsorption on coconut fiber at pH 4, by using E_0=40 U/ml; *CALB-5A* CALB immobilized by adsorption on coconut fiber at pH 5, by using E_0=40 U/ml.

hydrophobic support (activated carbon), did not show important differences on the amount of bound protein for the different values of pH studied [19].

Considering that green coconut fiber is a lignocellulosic material, interactions between charged groups and/or dipoles of lipase and cellulose were expected. Furthermore, when electrostatic interactions are the driven force of adsorption, the amount of adsorbed protein is pH-dependent, i.e., dependent on the charge of the protein and, possibly, the sorbent [8]. For better understanding immobilization of CALB on coconut fiber, it is important to imagine the distribution of charges of CALB, based on its isoelectric point of 6 [12], and coconut fiber, supposing an isoelectric point of 2 [33]. Based on these suppositions, a maximum plateau value would be expected for pH values smaller than 6, as enzyme is positively charged and fiber, negatively charged. This behavior was experimentally confirmed; higher values of hydrolytic activities were obtained when enzyme was immobilized between pH 3 and 6. Moreover, as hydrolytic activity and adsorbed protein profiles are similar, only lipase molecules must be adsorbed. It is known [19] that the crude extract used has contaminant proteins, but the obtained results show that they are not adsorbed on coconut fiber and that lipase is preferentially adsorbed from the crude extract.

The two plateaus on Fig. 3, constant specific activity values, indicate that there is a stability of the enzyme structure on the range of pH studied. For some enzymes, there may be partial destruction of cystine residues (in alkaline solutions) or hydrolysis of the labile peptide bonds (in acid solutions), as observed by Akova and Ustun [34] during adsorption of *Nigella sativa* lipase on Celite. Other authors [8] have also found similar values of specific activity for the different pH values studied for the adsorption of lipase from *Candida rugosa*.

Immobilization parameters (recovered activity and immobilization yield) were calculated for the biocatalyst with higher hydrolytic activities (adsorptions performed between pH 3 and 6), and results are presented in Fig. 4. It can be observed that best results for recovered activity and immobilization yield were obtained at pH 4 and at 5 or 6, respectively.

Although the amount of *C. antarctica* lipase type B adsorbed to coconut fiber was nearly independent on the pH of adsorption (between pH 3 and 6), immobilization yield and recovered activity were dependent on the pH of adsorption because interactions between the molecule and its environment influence the structure of a protein molecule, and these interactions are pH-dependent [8].

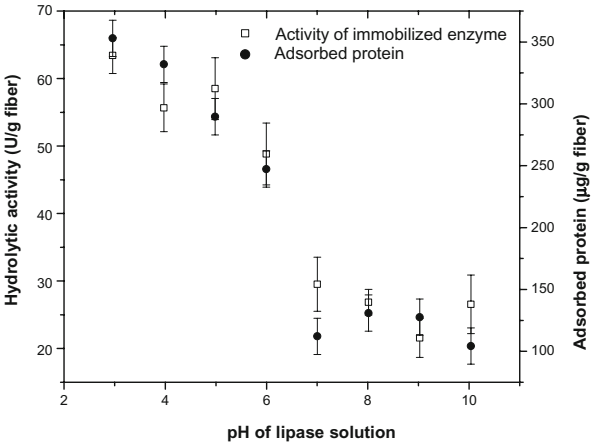

Fig. 3 Effect of the pH of immobilization on the amount of adsorbed protein (*closed circle*) and on the hydrolytic activity (*open square*). Lipase was immobilized on coconut fiber by adsorption after 2 h of contact time at room temperature

Based on the obtained results, immobilized enzyme prepared at pH 4 (CALB-4) and 5 (CALB-5) were selected for thermal stability studies at 60 °C. Table 2 shows the kinetic parameters of thermal deactivation at 60 °C estimated by fitting deactivation model, Eq. 2, to experimental data. It can be observed that the deactivation profiles of CALB-4 and CALB-5 were similar, as k_1 and k_2 are almost the same. However, by comparing the half-lives of CALB-4 and CALB-5 to CALB-7A, it can be observed that the biocatalyst prepared at pH 7 is 3- and 3.8-fold more stable than the ones prepared at pH 4 and 5, respectively. Considering that CALB has an optimum pH between 7 and 8 [35], when the enzyme is adsorbed on coconut fiber at pH 7, a favorable molecule conformation is preserved, being, therefore, more thermal stable than CALB-4 and CALB-5. Therefore, lipase immobilized at pH 7 was selected for further kinetic, operational, and thermal stability studies.

Properties of CALB Immobilized on Coconut Fiber by Adsorption

Based on the results obtained so far, CALB-7A, CALB immobilized on coconut fiber after 2 h of contact between the support and an enzyme solution containing E_0=40 U/ml, in

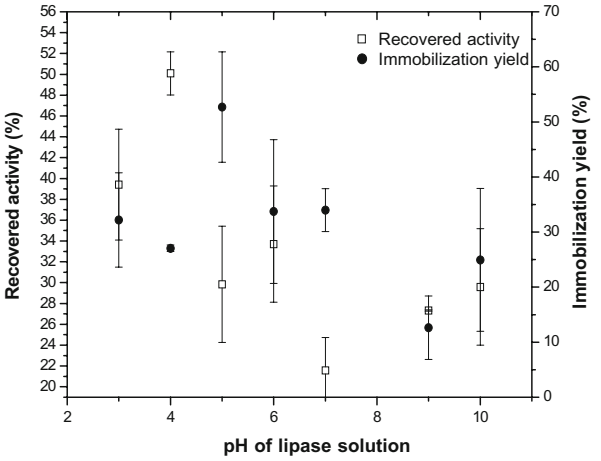

Fig. 4 Effect of pH enzyme solution on the recovered activity (*open square*) and immobilization yield (*closed circle*) of lipase immobilization on coconut fiber by adsorption after 2 h of contact time at room temperature

25 mM sodium phosphate buffer pH 7, was selected for comparative studies with a commercial immobilized CALB, Novozyme 435, and soluble CALB. A low enzymatic loading was selected to ensure lipases were immobilized in monolayers.

Thermal Stability

Thermal stabilities of CALB immobilized on coconut fiber were investigated at 50 and 60 °C, and results were compared to the stability of soluble CALB and Novozyme 435 (Fig. 5 and Table 2). It can be observed, by analyzing stabilization factors (F) on Table 2, that immobilization of CALB on coconut fiber promoted an improvement on thermal stabilities, as CALB-7A is 2- and 92-fold more stable than soluble CALB at 50 and 60 °C, respectively. Other authors [19] obtained similar results when immobilizing the same enzyme on activated carbon by adsorption; immobilized CALB was 2-fold more stable than soluble enzyme with $t_{1/2}$=8 h to thermal stability studies at 50 °C.

The thermal stability at 60 °C of CALB-7A was significantly higher than that of the soluble enzyme, with a stabilization factor of 92.15 (Table 2). In a previous study [27], when CALB was immobilized on coconut fiber by covalent attachment at pH 7 and 10, the immobilized enzyme was, respectively, 67- or 364-fold more stable than the soluble enzyme. Making a comparison between the thermal stabilization achieved by adsorption and covalent immobilization, it can be observed that the forces involved in immobilization of CALB on coconut fiber by adsorption are stronger than the bind between enzyme and support, formed during immobilization by covalent attachment at pH 7. Nevertheless, it is not stronger than the interaction that occurred at pH 10. Other authors [9], when immobilizing CALB by adsorption in octyl silica, obtained a biocatalyst with half-life of around 2 h, which is less stable than CALB-7A prepared in this work ($t_{1/2}$= 8.92 h).

The thermal stability at 60 °C of Novozyme 435, however, is higher than that of CALB-7A (Fig. 5). After 10 h of incubation at 60 °C, Novozyme 435 retained more than 70% of its initial activity, whereas CALB-7A retained only 50%. The higher stability of Novozyme 435 may be due to the hydrophobic nature of the support used for immobilization. When hydrophobic supports are used, the hydrophobic areas surrounding the enzyme active center are involved in adsorption, which stabilizes the active form of lipase [11].

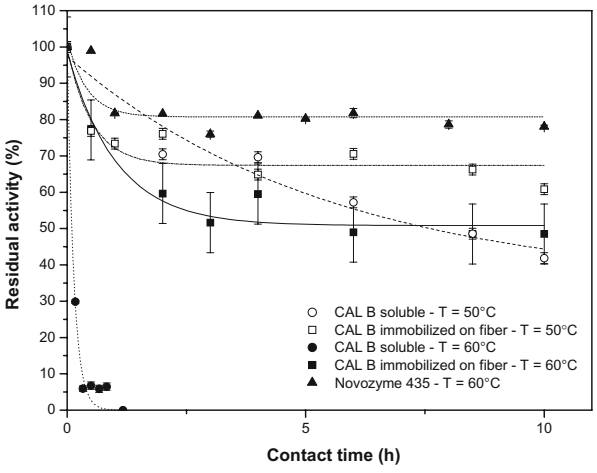

Fig. 5 Thermal stability of derivatives of lipase B from *Candida antarctica* obtained by adsorption, incubated in 0.1 M sodium phosphate buffer, pH 7, at 50 or 60 °C

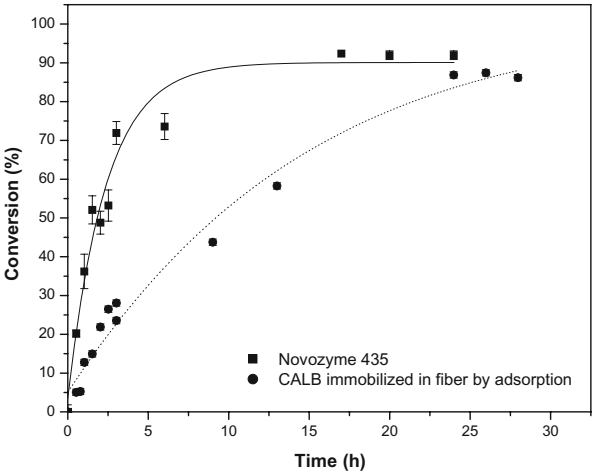

Fig. 6 Conversion of butyric acid in butyl butyrate synthesis catalyzed by immobilized lipase B of *Candida antarctica* on coconut green fiber (*closed circle*) and Novozyme 435 (*closed square*)

Time Course of Butyl Butyrate Synthesis Catalyzed by Immobilized CALB

Figure 6 shows the time course of butyl butyrate synthesis catalyzed by CALB-7A and Novozyme 435. It can be observed that at the beginning, the reaction catalyzed by Novozyme 435 achieved higher conversions quickly. It is known that the enzyme load in Novozyme 435 is higher than in CALB-7A, which explains the fast reaction rate of this well-characterized commercial biocatalyst. However, when equilibrium was reached, conversion values were very similar for both, 87.7% when using CALB-7A and 92.1% when using Novozyme 435. Rodrigues et al. [19] achieved equilibrium conversion values of 92.1 and 84.8% for Novozyme 435 and CALB immobilized on activated carbon, respectively, for the same reaction. Brigida et al. [27] achieved equilibrium conversion of 91.7% using CALB immobilized on coconut fiber by covalent attachment.

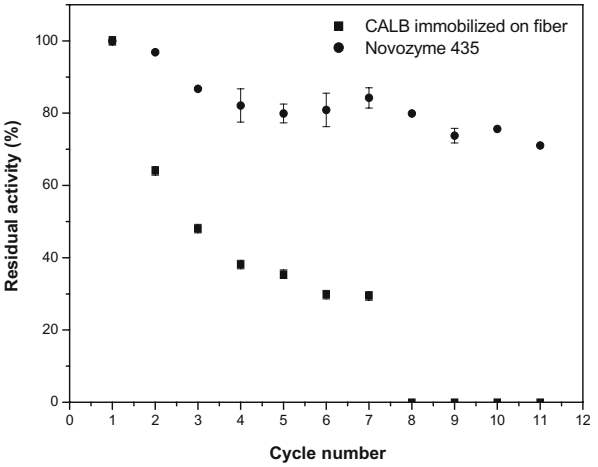

Fig. 7 Operational stability of (*closed square*) lipase type B of *Candida antarctica* adsorbed on green coconut fiber and (*closed circle*) Novozyme 435 in subsequent batches of methyl butyrate hydrolysis

Operational Stability

Reusability of immobilized CALB was tested in subsequent cycles of methyl butyrate hydrolysis. It can be observed in Fig. 7 that CALB-7A retained less than 50% of its initial hydrolytic activity after the third cycle of reaction whereas Novozyme 435 retained almost 70% after the tenth cycle (Fig. 7). Other authors [36] observed that CALB immobilized on activated carbon retained more than 55% of its initial activity after the sixth cycle of methyl butyrate hydrolysis. The worse operational stability of CALB immobilized on coconut fiber, when compared to CALB immobilized on activated carbon and to Novozyme 435, may be due to enzyme desorption during reaction, induced by the hydrophobic substrate, and by the low enzyme load adsorbed. As discussed before, the driven forces of CALB adsorption on coconut fiber are electrostatic interactions that are weaker than hydrophobic interactions, which predominate on Novozyme 435 and CALB adsorbed on activated carbon. Furthermore, both activated carbon and the resin used in the preparation of Novozyme 435 are porous support with high superficial area available for enzyme immobilization, allowing obtaining of high enzyme load. Coconut fiber, on the other hand, does not have a porous structure, and it has a low surface area [27], making it difficult to achieve high enzyme loads.

The effect of repeated use on immobilized lipase activity was also investigated in the synthesis of butyl butyrate, and results are pictured in Fig. 8. CALB-7A showed a good operational stability, retaining 80% of its initial activity after the sixth cycle of reaction, when compared to Novozyme 435, which remained fully active. Other authors [19, 37], when they studied the immobilization of *C. antarctica* type B and *C. rugosa* lipase by adsorption, obtained poor results of operational stability of synthesis. When *C. antarctica* type B lipase was immobilized on activated carbon, the immobilized enzyme retained, after six cycles, only 10% of its initial stability [19]. Both niobium oxide (crystalline and amorphous) supports, used for the immobilization of *C. rugosa*, showed poor operational stability resulting in high activity lost (over 75%) after five recycles [37]. Brigida et al. [27] found that CALB immobilized on coconut fiber by covalent attachment at pH 7 and 10 retained, respectively, only 65 and 30% of residual conversion after six cycles of reaction. Therefore, CALB-7A is a suitable biocatalyst to be used in organic synthesis rather than in hydrolysis reactions.

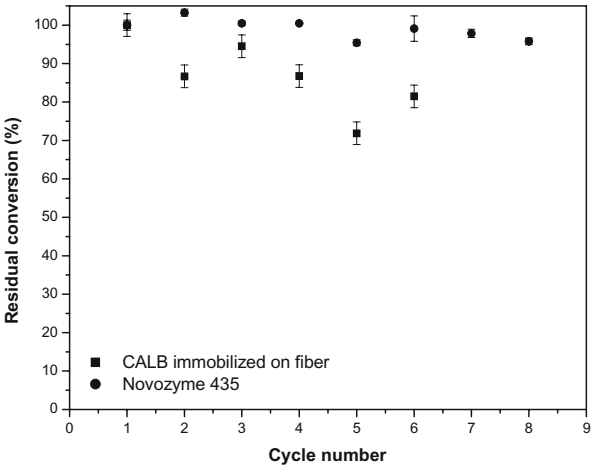

Fig. 8 Operational stability of (*closed square*) lipase type B of *Candida antarctica* adsorbed on green coconut fiber and (*closed circle*) Novozyme 435 in subsequent batches of butyl butyrate synthesis

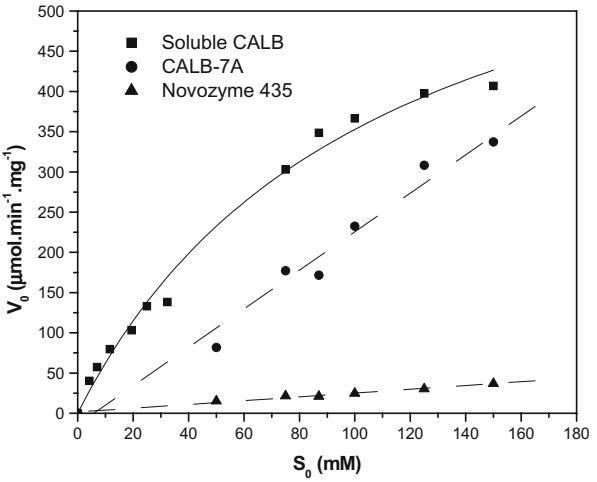

Fig. 9 Initial rate of hydrolysis of methyl butyrate by soluble lipase (*closed square*), lipase immobilized on green coconut fiber (*closed circle*) and Novozyme 435 (*closed triangle*) in fully aqueous medium. The *solid line* represents the fit of Michaelis–Menten model to experimental data. The *dashed lines* represent a linear regression of the experimental data. The amount of adsorbed protein (50 mg/g of catalyser) in Novozyme 435 was determined by Secundo et al. [42]

Hydrolysis of Methyl Butyrate: Kinetic Parameters

Initial reaction rates were determined at different initial methyl butyrate concentrations ranging from 4 to 150 mM. Higher concentrations could not be used because the substrate is not fully soluble in the aqueous medium upon this limit [38].

Figure 9 shows the initial hydrolysis rate of increasing concentrations of methyl butyrate, and Table 3 shows the estimated parameters. It can be seen that hydrolysis rate increases with substrate concentration for the three biocatalysts investigated. However, soluble enzyme follows a Michaelis–Menten-type kinetics with a value of K_M and V_{max} of around 80.65±39.7 mM and 625.01±168.5 μmol min^{-1} mg^{-1}, respectively, while immobilized enzymes, both CALB-7A and Novozyme 435, follow first-order kinetics. There are some reasons to explain the different behavior of the immobilized enzyme compared to the soluble enzyme. The immobilized enzyme residues are in an environment that is different from that of the soluble enzyme in the bulk solution, which may lead to apparent enzyme kinetics. Furthermore, immobilized enzyme might be influenced by mass transfer effects, i.e., substrate and products diffusion through a stagnant film that may surround the support (external diffusion) and through the pores of the support (internal diffusion) [39].

Enzyme activity can be reduced by mass-transfer effects as immobilization means the deliberate restriction of enzyme mobility, which can also affect the mobility of solutes [40].

Table 3 Kinetic parameters of free, immobilized lipase B from *Candida antarctica* obtained by adsorption and Novozyme 435.

Enzyme	Hydrolytic activity	K_m (mM)	V_{max} (μmol.min^{-1}.mg^{-1})
CALB soluble	40 U/mL	33.57	444.44
Immobilized CALB by adsorption at pH 7	27 U/g	49.37	341.30
Novozyme 435*	1,047 U/g	178.11	70.68

* The amount of adsorbed protein (50 mg/g of catalyser) in Novozyme 435 was determined by Secundo et al. [42].

These phenomena can lead to reduced reaction rate and to decrease efficiency compared to soluble enzyme, which can be observed in Fig. 9.

Although Novozyme 435 possesses high activity per gram of support, it has a low specific activity (20.94 U/mg protein) when compared to CALB-7A (245.45 U/mg protein). Higher values of specific activity at low degree of adsorption in cellulose have been reported [8] when compared to hydrophobic materials. Moreover, diffusional limitations are less significant in coconut fiber, as immobilization occurs on the surface due to the absence of porous. The support used to prepare the biocatalyst Novozyme 435, a macroporous resin, may be affect by both internal and external diffusional resistances. The influence of different particle sizes and different specific surface areas on the rate of enzymatic reaction has been investigated [41], and higher values of specific activity were also obtained for low specific surface area.

Conclusions

In the present work, green coconut fiber was successfully used to immobilize lipase B from *C. antarctica* by adsorption. During adsorption studies, it was observed that adsorption equilibrium was achieved after a contact time of 2 h (in case of E_0=30 U/ml and E_0=60 U/ml) or 6 h (E_0=90 U/ml). Moreover, an improvement of hydrolytic activity of immobilized CALB is also observed with increasing concentrations of lipase offered to immobilization. This increase in activity is due to formation of multilayers, confirmed by thermal stability essays. Two plateaus of enzyme activity were observed when the pH of lipase solution during adsorption was varied in the range studied. This behavior is typical of an ionic support. At 50 and 60 °C, the adsorbed enzyme was, respectively, 2- and 92-fold more stable than the soluble enzyme. At 60 °C, however, Novozyme 435's stability was higher than that of CALB-7A. After 10 h of incubation at 60 °C, Novozyme 435 retained more than 70% of its initial activity, whereas CALB-7A retained only 50%. Last but not least, operational stabilities studies of butyl butyrate synthesis, compared to a commercial derivative, showed that CALB-7A is a suitable biocatalyst to be used in the synthesis of flavors.

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