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0273-2289/1559–0291 (Online)/07/136–140/67–80/$30.00

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

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Abstract

The objective of this study was to covalently immobilize *Candida antarctica* type B lipase (CALB) onto silanized green coconut fibers. Variables known to control the number of bonds between enzyme and support were evaluated including contact time, pH, and final reduction with sodium borohydride. Optimal conditions for lipase immobilization were found to be 2 h incubation at both pH 7.0 and 10.0. Thermal stability studies at 60°C showed that the immobilized lipase prepared at pH 10.0 (CALB-10) was 363-fold more stable than the soluble enzyme and 5.4-fold more stable than the biocatalyst prepared at pH 7.0 (CALB-7). CALB-7 was found to have higher specific activity and better stability when stored at 5°C. When sodium borohydride was used as reducing agent on CALB-10 there were no improvement in storage stability and at 60°C stability was reduced for both CALB-7 and CALB-10.

Index Entries: Coconut fiber; covalent attachment; enzyme immobilization; lipase; hydrolysis; esterification.

Introduction

Lipases (triacylglycerol ester hydrolases, E.C. 3.1.1.3) catalyze both the hydrolysis and synthesis of esters from glycerol and long-chain fatty acids *(1)*. They catalyze interesterification, aminolysis, and thiotransesterification reactions *(2)*. Industrial applications of lipases include food processing, detergent formulations, and the synthesis of fine chemicals *(3)*. The use of immobilized enzymes provides for several advantages such as enzyme recycle or reuse and reduced cost. Therefore, many methods have been used to immobilize lipases, such as adsorption *(4)*, covalent attachment *(5)*, and chelation *(4)*. The multipoint attachment of proteins involves

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several covalent attachments between one molecule of enzyme and the activated support. Attachment on a solid support should increase the rigidity of the immobilized enzyme molecules, making them more resistant to small conformational changes induced by heat, organic solvents, denaturing agents, and so on. To be successful the support surface should be complementary to that of the enzyme molecule. Not all supports or methods of activation offer the same possibilities to force an intense covalent multipoint attachment and in some conditions the immobilized enzyme can be less stable than the soluble one *(6)*.

Covalent immobilization is based on the retention of enzymes to support surfaces by covalent bonds between functional groups of the enzymes and reactive groups on the support *(7)*. The majority of supports must be activated before immobilization making the technique more expensive than simple adsorption *(4,7)*. Biocatalysts that can be covalently attached are usually more stable and resistant to extreme conditions (pH range and temperature). This stability and resistance are very important characteristics for industrial biocatalysts *(8)*. Lipase immobilization on supports activated with 3-glycidoxypropyltrimethoxysilane ([GPTMS] Sigma-Aldrich Chemical Co, St. Louis) (a silane coupling agent) occurs through a reaction between glyoxyl (aldehyde) groups, present on the support, and amines groups from the enzyme. As a consequence of the amine–aldehyde reaction, Schiff's bases between the enzyme and support are formed. At neutral pH, the reactivity of enzyme lysine residues is very low because of their pK_a (around 10.5). Thus, the covalent attachment under these conditions is said to be one-point. However, at pH 10.0, the multipoint covalent attachment is possible because of the increase in the lysine residues reactivity, and consequently, a larger number of Schiff's bases are formed *(9,10)*.

Considering the high cost of some available commercial support matrixes, studies have been intensified in order to obtain cheaper supports. Some articles have reported the use of agroindustrial wastes as an immobilization matrix for α-amylase *(11)*, invertase *(12)*, and lipase *(13)*. These studies showed that agroindustrial wastes are a suitable raw material source for an immobilization matrix. In Brazil, an increase in the green coconut water market had a direct impact on the increase of coconut husk production, an agroindustrial waste. This waste takes up to seven years to decompose, contributes to spreading tropical diseases, and is responsible for the overfilling of sanitary landfills when not disposed properly *(14)*.

In this study, we examine the immobilization of *Candida antarctica* lipase B by covalent attachment to green coconut fiber. Green coconut fiber was first silanized using GPTMS followed by immobilization of the enzyme. Two covalent immobilization strategies were compared: one-point covalent attachment (pH 7.0) and multipoint covalent attachment (pH 10.0). The derivatives formed by a one-point covalent bound have almost the same properties as the free enzyme and can be used as a standard to test activity/stability of the original enzyme. In this immobilization strategy,

only one (or two) amine residue of the enzyme molecule is involved in the CALB-support covalent bound *(15)*. In general, enzyme stability can be improved if the immobilization occurs through multipoint attachment at multiple lysine residues. Because attachment to the support stabilizes the enzyme structure they should become much more stable than their soluble counterparts or their randomly immobilized derivatives *(9,16)*. High concentrations of aldehyde groups on the support surface may also result in several multipoint bounds between the enzyme and the matrix, which can distort its three-dimensional structure and its active site. Different factors may influence the immobilization process, such as: activating agent, nature of the support, and interaction with enzyme *(17)*. The objective of this work was to study the immobilization of CALB on green coconut fiber by one-point (pH) and multipoint covalent binding (pH 10.0), and investigating the effects of immobilization on the activity and stability of the enzyme.

Materials and Methods

Materials

Commercial *C. antarctica* lipase type B (1780 U/mL) was kindly donated by Novozymes Latin America Ltd. and was used as received. Methyl butyrate and GPTMS was obtained from Sigma-Aldrich Chemical Co. Butyric acid and butanol were purchased from Merck S. A. (Rio de Janeiro, Brazil). Molecular sieve 4A ($Na_2O[Al_2O_3(5.0SiO_2)]12H_2O$) was from W. R. Grace & Co (Massachusetts, MA). Polyethyleneglycol 6000 was from Vetec (Rio de Janeiro, Brazil). All chemicals were of analytical grade.

Support Activation

Green coconut fiber was obtained from green coconut husks through a process developed by Embrapa Agroindustria Tropical, Ceará State, Brazil *(14)*. It was cut and sieved to obtain particles between 32 and 35 mesh, washed with distilled water, and dried at 60°C before being used as an immobilization matrix. The support was activated using a four-step process. The support was first protonated with nitric acid (10%, [v/v]) under low stirring for 30 min at 30°C. It was then rinsed with nitric acid (10%, [v/v]) and acetone–water solutions (20, 50, and 100%, [v/v]) and dried at 60°C for 1 h. The support was then silanized using GPTMS (1%, [v/v]) at pH 8.5 under low stirring for 5 h at 60°C. The fiber was rinsed with water, acetone–water solutions, and dried. In the third step, the hydrolysis of epoxy groups was done with 0.1 *M* sulphuric acid at 85°C under low stirring for 2 h. Again, the fiber was rinsed with water and acetone–water and dried. For each gram of dry support used in the previous stages, 30 mL of each solution was used. Finally, oxidation was achieved by reaction with 0.04 *M* sodium periodate solution (5 mL/g fiber) under low stirring at room temperature for 1 h. After oxidation, the activated fiber was thoroughly

rinsed with water then with 5 m*M* sodium phosphate buffer pH 7.0. Before immobilization, activated fiber was dried under vacuum *(18)*.

Preparation of Immobilized Enzyme

Lipase was immobilized by covalent attachment by contact at room temperature. The enzyme and support were mixed by repeated inversion using the apparatus shown in Fig. 1. For each gram of dry support, 10 mL of lipase solution (80 U/mL) in 25 m*M* sodium phosphate buffer pH 7.0 or in 200 m*M* sodium bicarbonate buffer pH 10.0, were used. After immobilization, the biocatalyst was separated by filtration, rinsed with phosphate buffer (10 mL) and dried at vacuum for 10 min.

Assay of Hydrolytic Activity: Methyl Butyrate Hydrolysis

Methyl butyrate hydrolysis was used to determine the hydrolytic activity of the immobilized or soluble enzyme *(19)*. Experiments were performed using an automatic titrator (pHstat Netrohm Titrino 751, Switzerland) and 50 m*M* NaOH as titrating agent. The pH was set at 7.0 and the reaction initiated by the addition of 0.1 mL of free enzyme solution or 0.4 g of immobilized enzyme to 30 mL methyl butyrate solution dissolved in 25 m*M* phosphate buffer pH 7.0. One unit (U) of enzymatic activity is defined as the amount of enzyme that releases 1 µmol of methyl butyrate per min at pH 7.0 and 28°C.

Esterification Yield: Butyl Butyrate Synthesis

Stock solutions of butyric acid (150 m*M*) and butanol (150 m*M*) were prepared in *n*-heptane. Experiments were set up in 250-mL flasks containing 20 mL of stock solution, 1.0 g of molecular sieve 4A, and 0.3 g of the biocatalyst *(20)*. The flasks were kept at 30°C under vigorous agitation for 24 h. The consumption of butyric acid was measured by titration with 20 m*M* NaOH using phenolphthalein as an indicator. The total acid content before the 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 biocatalyst in successive batches of methyl butyrate hydrolysis. Assay 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.

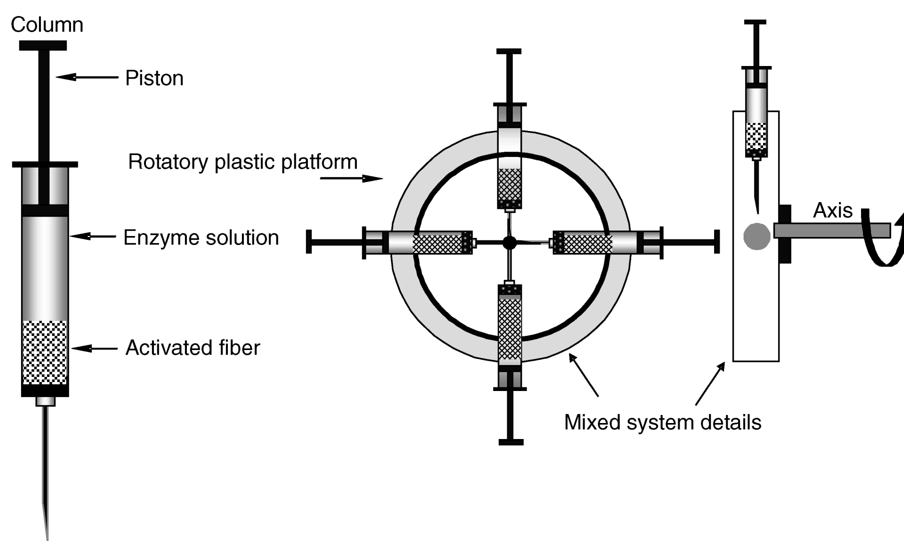

Fig.1. Details of the apparatus used for enzyme immobilization *(15)*.

Operational Stability: Synthesis of Butyl Butyrate

Operational stability of the immobilized enzyme was also assayed by using 0.3 g of the biocatalyst in successive batches of butyl butyrate synthesis. The operational 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), in order to extract any substrate or product retained in the matrix. After 1 h at room temperature, the immobilized enzyme was introduced to fresh medium. 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.

Thermal Stability

The thermal stability of soluble or immobilized enzyme was determined by incubating them in 100 m*M* sodium phosphate buffer at 60°C and pH 7.0. Periodically, samples were withdrawn and their residual activities were assayed by the hydrolysis of methyl butyrate. Residual activity is given as percentage of activity taken as 100% of the hydrolytic activity of the enzyme immobilized before incubation. Thermal deactivation curves follow the first-order deactivation model *(21)*, *see* Eq. 1. First-order deactivation rate coefficients (K_d) were estimated from experimental data.

$$\ln(A) = \ln(A_0) - K_d t \quad (1)$$

where A_0 is the initial residual activity, A is the residual activity in time t, and K_d are first-order deactivation rate coefficients. The biocatalyst half-life

($t_{1/2}$) was estimated by Eq. 2 using the estimated parameter k_d. In this article, stabilization factor (F) was considered as the ratio between soluble and immobilized enzymes half-lives.

$$t_{1/2} = \frac{\ln(0.5)}{-k_d} \quad (2)$$

Storage Stability

To evaluate the storage stability of the immobilized enzyme, derivatives produced by covalent attachment were stored dry packed in aluminum paper at 5°C and their residual hydrolytic activities were determined every 24 h.

Effect of Sodium Borohydride Reduction

The effect of sodium borohydride ($NaBH_4$), used as reducing agent, was studied. $NaBH_4$ concentration ranged from 0.5 to 6.0 mg/mL. After immobilization, solid $NaBH_4$ was added directly to the system and the reaction occurred for 30 min at room temperature. At the end of the reaction, the immobilized enzyme was separated by filtration, thoroughly rinsed with phosphate buffer and dried at vacuum for 10 min. The concentration of sodium borohydride that promoted minor loss on hydrolytic activity was selected based on results of thermal and operational stabilities.

Scanning Electron Microscopy

In order to evaluate changes in the surface provoked by the activation process, natural fibers and activated fibers were analyzed by scanning electron microscopy (SEM) using a Zeiss DSM 940A SEM (Zeiss, Germany) operating at 10 kV. All samples were glued onto special stubs and gold-coated with a Sputter Emitech K550 (Emitech Ltd., Kent, UK) to avoid electrostatic charge and to improve image resolution.

Results and Discussion

Green Coconut Fiber Activation With GPTMS

Lignocellulosic materials, such as coconut fiber, can be activated by inserting functional groups owing to the presence of hydroxyl and carbonyl groups *(22)* on its surface. In the specific case of GPTMS, functional groups are added by reaction with hydroxyl groups *(18)* present in the support. Some techniques allow observing the axial deformation of hydroxyl groups promoted by the addition of functional groups *(23)*. Scanning electron micrographs shown in Fig. 2 demonstrates the presence of this activating agent on the surface of the support. The enzyme was successfully coupled to the activated support, as hydrolytic activity could be measured after immobilization. Other authors have also studied enzyme

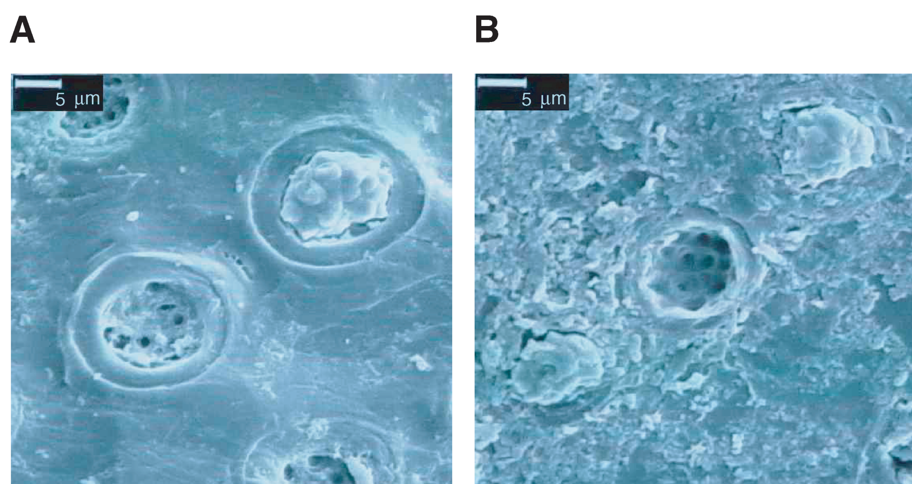

Fig. 2. Scanning electron micrographs of **(A)** natural green coconut fiber and **(B)** activated fiber with GPTMS (×2000).

immobilization on nonporous supports *(11,23)*, mainly by covalent attachment *(23)*, obtaining good results.

Enzyme Immobilization and Effect of Contact Time on Biocatalyst Hydrolytic Activity

The influence of lipase contact on fiber activated with GPTMS was investigated for different time intervals. As shown in Fig. 3, when the immobilization was performed at pH 7.0, after 1 h of contact between enzyme and support, no improvement or decrease on immobilized enzyme hydrolytic activity was observed. Nevertheless, when immobilization was performed at pH 10.0, a decrease on immobilized enzyme hydrolytic activity was observed after 2 h of contact. According to the literature *(24)*, this behavior suggests that more than one Schiff's base was formed between enzyme and support, promoting a larger rigidity on the enzyme molecule, and consequently, losses on enzyme activity. Based on these results, the contact time of 2 h was selected for further immobilization studies.

Effect of pH of Lipase Solution on Biocatalyst Thermal and Operational Stabilities

The effect of pH of lipase solution during the binding step of *C. antarctica* lipase B on coconut fiber activated with GPTMS was investigated. The objective of this study was to obtain one-point and multipoint covalent immobilized enzymes. Derivatives prepared at pH 7.0 are expected to be one-point attached. On the other hand, owing to the pK_a of lysine residues on the enzyme surface (pK_a = 10.5), the immobilization at

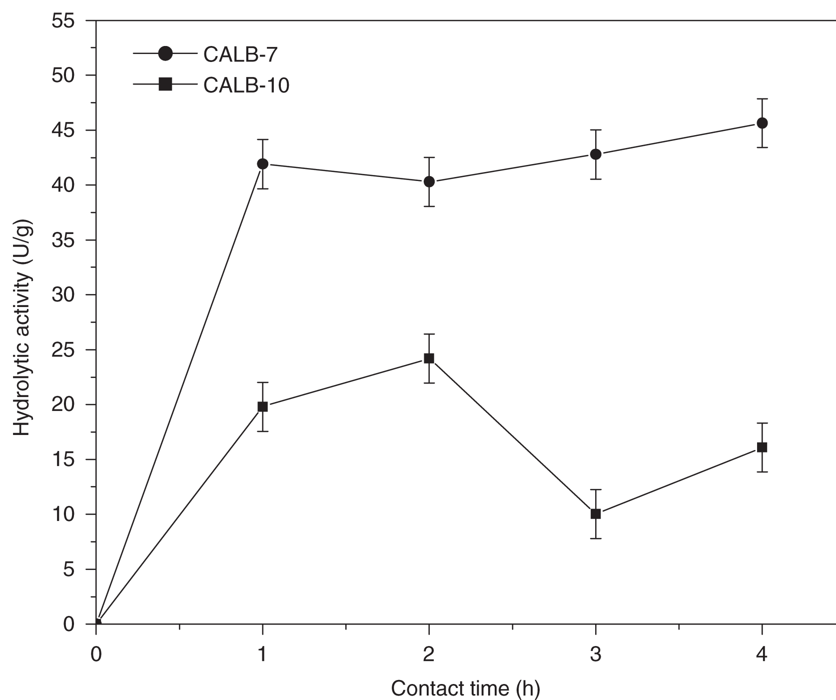

Fig. 3. Effect of contact time on the hydrolytic activity of the derivative obtained through lipase immobilization in coconut fiber by covalent attachment at pH 7.0 (CALB-7) or pH 10.0 (CALB-10).

pH 10.0 should be multipoint *(17)*. It can be observed (Fig. 3) that CALB-7 exhibited higher hydrolytic activity than CALB-10, around 42 U/g and 20 U/g, respectively. This result may be an indication of a multiinteraction between enzyme support at pH 10.0. Because a low density of active groups was present on the activated fiber, enzyme immobilization by multipoint attachment would allow a small number of enzyme molecules to bind on the support. However, activity is not the only important parameter when industrial biocatalysts are designed. Therefore, thermal and operational stabilities of the immobilized enzyme were determined.

A direct correlation between the number of attachment points and enzyme thermal stability has been previously reported *(25,26)*. Moreover, multipoint binding between the enzyme and the support, which is responsible for enhancing thermal stability, would provide better stability against deleterious effects of organic solvents *(25)*, which are usually used as reactional media when lipases are used to catalyze the synthesis of esters. Therefore, inactivation profiles at 60°C of free and immobilized lipases (Fig. 4) were studied in this work.

It can be seen in Fig. 4 that the immobilized enzyme is more stable than free enzyme suggesting that immobilization protects the enzyme from

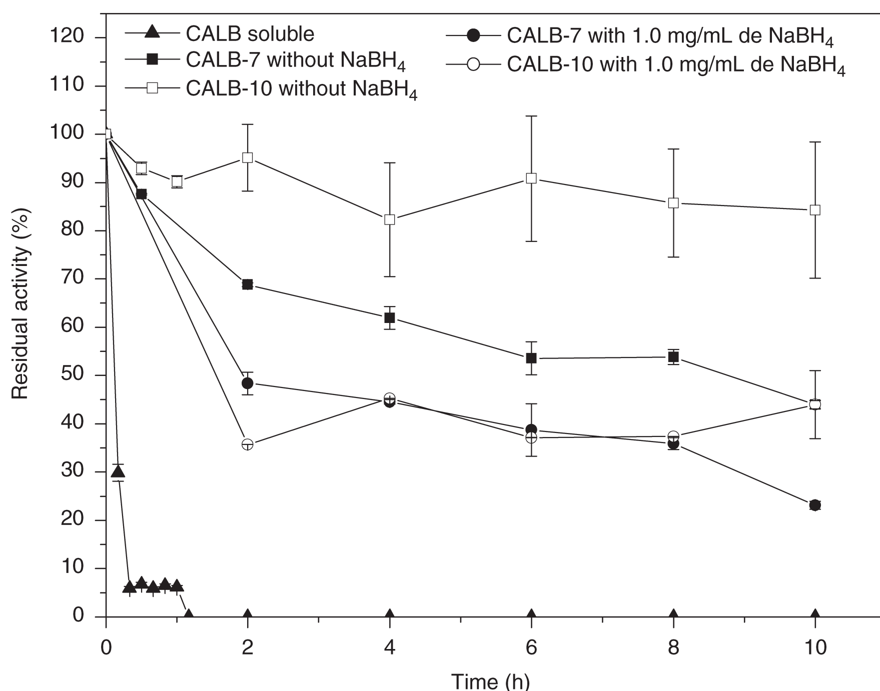

Fig. 4. Thermal stability of derivatives from lipase B from *C. antarctica* obtained by covalent attachment at pH 7.0 (CALB-7) or pH 10.0 (CALB-10) with or without reduction with sodium borohydride ($NaBH_4$), incubated in 0.1 *M* sodium phosphate buffer, pH 7.0, at 60°C. Initial hydrolytic activity of the immobilized enzyme (before thermal treatment): (a) Soluble CALB 160 U/mL; (b) CALB-7 without reduction with $NaBH_4$ 42.0 U/g; (c) CALB-7 treated with $NaBH_4$ 32.3 U/g, (d) CALB-10 without reduction with $NaBH_4$ 20.0 U/g; and (e) CALB-10 treated with $NaBH_4$ 12.0 U/g.

thermal inactivity. The plots of residual activity vs incubation time (at pH 7.0 and 60°C) adjust quite well with the proposed model (*see* Eq. 1) and allow calculating the half-lives of inactivation (*see* Table 1). The most stable derivative was prepared at pH 10.0, with stabilization factor (F) equal to 363.71, which is 5.4-fold more stable than the derivative prepared at pH 7.0. Hence, thermal stability is very sensitive to the process of enzyme-support multiinteractions *(25)*, indicating that a multipoint interaction might have occurred when immobilization was conducted at pH 10.0.

The ability to reuse the biocatalyst is of practical importance; the operational stability of lipase preparations was determined. In this work, two model reactions were selected: methyl butyrate hydrolysis (aqueous media) and butyl butyrate synthesis (organic media). The results are summarized in Fig. 5. It can be observed that the biocatalyst prepared at pH 7.0 (CALB-7) retained an activity of about 55% in aqueous media (hydrolysis) and 75% in organic media (synthesis), respectively, after five reuses. The biocatalyst prepared at pH 10.0 (CALB-10) had a similar

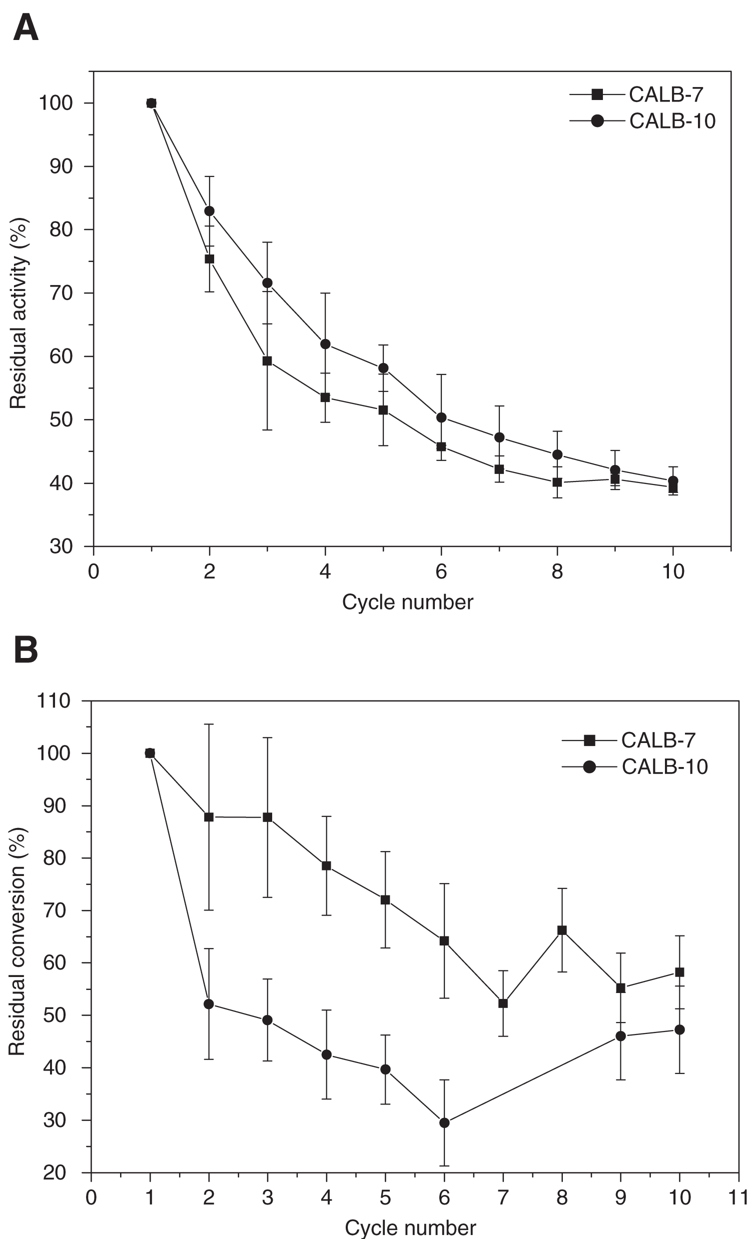

Fig. 5. Batch operational stability of covalent immobilized lipase B of *C. antarctica* on coconut green fiber activated with GPTMS: **(A)** methyl butyrate hydrolysis and **(B)** butyl butyrate synthesis. Initial hydrolytic activity of the immobilized enzyme (before operational stability tests): (a) CALB-7 42.0 U/g and (b) CALB-10 20.0 U/g.

behavior in aqueous media, around 55% of residual hydrolytic activity, but presented a poor result of synthetic activity, around 45% of residual activity. After 10 reuses, the residual activities of CALB-7 and CALB-10 were, respectively, around 40% and 45% in aqueous media and 55% and 45% in

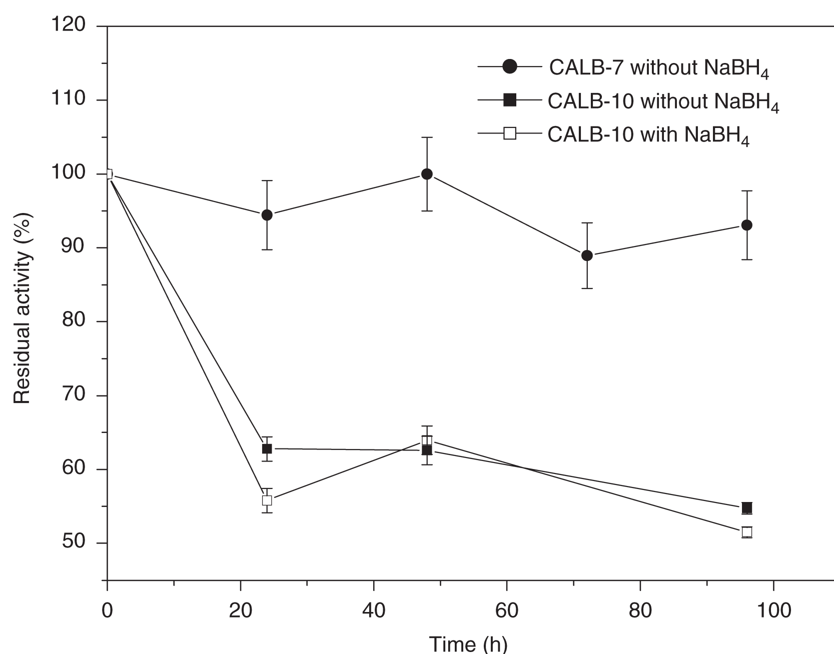

Fig. 6. Storage stability at 5°C of derivatives from lipase B from *C. antarctica* obtained by covalent attachment at pH 7.0 (CALB-7) and pH 10.0 (CALB-10), with or without reduction with sodium borohydride. Initial hydrolytic activity of the immobilized enzyme (before storage): (a) CALB-7 without reduction with $NaBH_4$ 42.0 U/g; (b) CALB-10 without reduction with $NaBH_4$ 20.0 U/g; and (c) CALB-10 treated with $NaBH_4$ 12.0 U/g.

Table 1
Kinetics Parameters of Thermal Desativation, at 60°C, of Soluble and Immobilized Lipase B From *C. antarctica* Obtained by Covalent Attachment at pH 7.0 (CALB-7) or pH 10.0 (CALB-10)

Enzymes	kd (h^{-1})	$t_{1/2}$(h)	R
Soluble enzyme	7.14	0.097	1.00
Immobilized enzyme at pH 7.0	0.106	6.51	67.11
Immobilized enzyme at pH 10.0	0.020	35.28	363.71

organic media. One possible explanation for the poor operational stability of CALB-10 in organic media is the lower enzyme loading in the support (20 U/g), because one protein molecule is coupled to more than one functional group on the support.

The effect of storage of the immobilized lipases at 5°C was determined and results are illustrated in Fig. 6. It can be observed that CALB-7 is very stable and maintained 100% of activity after 96 h of storage. On the other hand, CALB-10 lost more than 30% of its activity

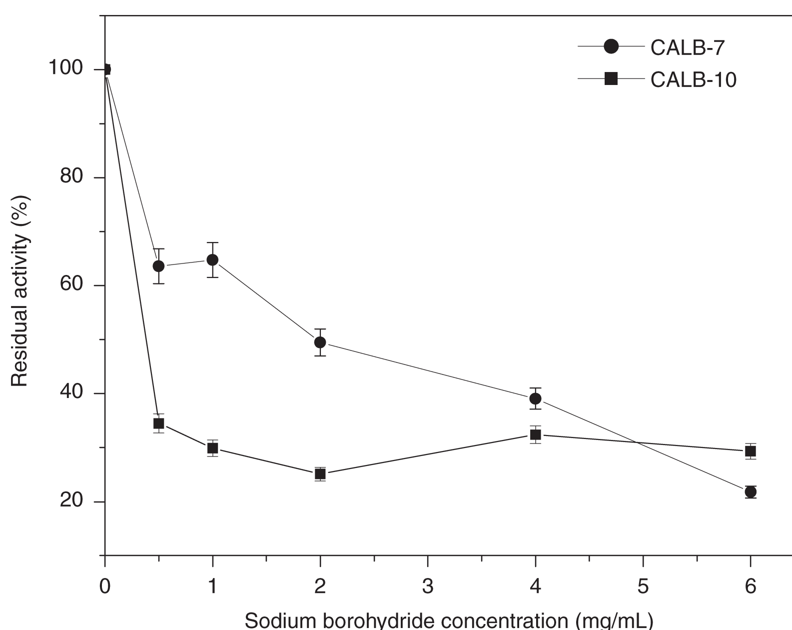

Fig. 7. Effect of sodium borohydride on the hydrolytic activity of derivatives obtained by covalent attachment of lipase B from *C. antarctica* in coconut fiber. Initial hydrolytic activity of the immobilized enzyme (before to $NaBH_4$ reduction): (a) CALB-7 42.0 U/g and (b) CALB-10 20.0 U/g.

after 24 h of storage and almost 50% after 96 h. This deactivation profile observed in CALB-10 may be caused by the reaction of amine groups from the bound enzyme with aldehyde groups from the support, because no reduction steps to transform the Schiff's bases into stable secondary amino bonds and the remnant aldehydes into inert hydroxyl groups *(6)* were performed after immobilization. Other possible explanation would be enzyme leakage from the support. Although covalent bound formation is favored, the condensation reaction between amino groups and aldehydes is reversible, which can lead to loss of bound enzyme *(27)*.

Effect of the Sodium Borohydride Reduction on the Biocatalyst Activity and Stability

Sodium borohydride is a double-functional agent, which seems to be a very suitable reducing agent, and also, able to inactivate the remaining aldehydes groups present in the support after immobilization *(10)*. The final reduction of the immobilized enzyme with sodium borohydride transforms weak Shiff's bases into stable secondary amino bounds and remaining aldehyde on the solid support into inert hydroxyl groups. Undesired enzyme-support interactions may be caused by noninert support surfaces, promoting the decrease on enzyme activity and stability *(28,29)*. Therefore, the influence of

sodium borohydride ($NaBH_4$) reduction after the immobilization was evaluated (Fig. 7). All $NaBH_4$ concentrations studied promoted a decrease on the immobilized enzyme hydrolytic activity. For CALB-10, the significant loss in activity might be caused by the low enzyme loading, which is also the probable reason for the false absence of influence of $NaBH_4$ concentration on the biocatalyst activity. For CALB-7, 0.5 mg/mL and 1.0 mg/mL of $NaBH_4$ promoted the smallest reduction in activity values. In spite of the inactivation of bound enzyme, owing to the deleterious effect of $NaBH_4$, the derivatives treated with 1.0 mg/mL of $NaBH_4$ were selected for thermal and storage at 5°C stability studies, as this treatment would prevent further uncontrolled reaction between support and the enzyme that could decrease its stability *(28)*.

Figure 4 shows the thermal stability at 60°C of the immobilized derivatives obtained with and without sodium borohydride reduction. The thermal stability was reduced after the reduction step with sodium borohydride for both CALB-7 and CALB-10. It has been reported that sodium borohydride can have deleterious effects on protein structures: for example, disulfide bond splitting or reductive cleavage of peptide bonds *(30)*, which could justify this thermal stability results. Moreover, the reduction step was not able to improve the storage stability at 5°C (Fig. 6), and therefore, the use of $NaBH_4$ was unsatisfactory for this system. Other authors *(6,17)* have also observed that $NaBH_4$ was not an appropriate reducing agent and found different solutions to solve this problem, such as the use of competitive inhibitor to protect the active center of the enzyme during the reduction step with $NaBH_4$ *(6)* or use another reducing agent, for instance, and amino acids *(27)*.

Conclusions

The results obtained in this work show that green coconut fiber is a suitable support for lipase immobilization, enabling the preparation of highly stabilized immobilized enzymes, for example, around 363.71-fold (CALB-10) or 67.11-fold (CALB-7) more stable than the soluble enzyme. The higher thermal stabilization observed when immobilized enzyme was prepared at pH 10.0 might be owing to the generation of a multipoint covalent attachment between the aldehyde groups of the support and the lysine residues of the enzyme. Best results of operational stability of synthesis and storage at 5°C were obtained when lipase was covalently immobilized at pH 7.0. The reduction step using sodium borohydride had no influence on CALB-10 storage stability at 5°C. Moreover, it promoted a decrease in thermal stability of both CALB-7 and CALB-10, which shows that it's not an appropriated reducing agent for this system. Other strategies to improve CALB-10 storage and operational stabilities are currently being investigated that include enhancing superficial area of the support. However, we have demonstrated that coconut fiber, a cheap support, is compatible with *C. antarctica* lipase, rendering a biocatalyst with interesting properties that can be used in aqueous or organic media.

Acknowledgments

The authors would like to thank the Brazilian research-funding agencies FUNCAP (State of Ceará), FINEP, and CNPq (Federal). We are also grateful to Embrapa Agroindustria Tropical for the SEM analysis.

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