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Wood Cellulignin as an Alternative Matrix for Enzyme Immobilization

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Abstract

The objective of this work was to select an efficient methodology for preparing active samples of *Candida rugosa* lipase immobilized in wood cellulignin, to be applied in hydrolysis and ester reactions. For this purpose, lipase was immobilized in the matrix by physical adsorption (pure cellulignin) and covalent binding (activated cellulignin with glutaraldeyde or carbonyldiimidazole [CDI]) in the presence or absence of polyethylene glycol (PEG) (Molecular mass of 1500 Daltons) as stabilizing agent. The activating agent and the presence of PEG-1500 in the immobilization procedure showed a strong influence on enzyme retention in the support. The values for enzyme retention ranged from 20 to 68%, and the highest yield was obtained when the enzyme was immobilized in cellulignin activated with CDI in the presence of PEG-1500. This immobilized derivative presented high hydrolytic (193.27 $\mu M/[mg \cdot min]$) and synthetic (522.92 $\mu M/[g \cdot min]$) activities when compared with those obtained by other techniques. The superiority of this immobilized system was confirmed by additional analyses, such as infrared spectroscopy and elemental analysis, which demonstrated an appropriate enzyme fixation and the highest level of protein incorporation in the support. Further information on the immobilized derivative was obtained by assessing the recycle potential in both aqueous and nonaqueous media.

Index Entries: Cellulignin; immobilizing support; lipase; *Candida rugosa*; polyethylene glycol; carbondiimidazole.

Introduction

The demand for industrial enzymes, particularly of microbial origin, is increasing owing to their applications in a large number of fields such

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as the food, dairy, pharmaceutical, detergent, textile, and cosmetic industries *(1,2)*. In this scenario, enzymes such as proteases and amylases have dominated the world market owing to their hydrolytic reactions for proteins and carbohydrates *(1)*. However, with the recognition of the biocatalyst potential of microbial lipases in both aqueous and nonaqueous media in the last decades, industrial fronts have shifted toward utilizing this enzyme for a wide spectrum of biotechnological processes *(3–6)*.

Currently, lipases have gained importance, to a certain extent, over proteases and amylases, especially in the area of organic synthesis *(5)*. The enantioselective and regioselective nature of lipases have been utilized for the resolution of chiral drugs; fat modification; and the synthesis of cocoa butter replacements, biofuels, personal care products, and flavor enhancers *(3–6)*. Thus, lipase-mediated reactions are attractive alternatives to bulk chemical routes. In addition, the fraction of unwanted intermediates and byproducts is reduced, giving products of high purity and improved quality *(4–6)*.

Lipase-catalyzed reactions, however, have a major inconvenience: mainly the conversions are relatively low when compared with chemical processes if crude commercial enzyme preparations are employed. These intrinsically low volumetric productivities may lead to products quantitatively less pure than those obtained via chemical synthesis, and such a drawback can be coupled with inhibition of the biocatalyst by products and/or substrates and deactivation of the biocatalyst by heat (thermal deactivation) or by several compounds (chemical deactivation). Such limitations can be overcome by using several techniques, including immobilization in solid supports in the presence or absence of stabilizing additives *(7–10)*.

In view of the current high cost of some available commercial support matrixes, the possibility of using cheap and/or alternative supports for lipase immobilization such as rice husk or rice straw *(11,12)*, $CaCO_3$ powder *(13)*, and chitin or chitosan *(14,15)* has been evaluated.

The present work relates to a method for immobilizing microbial lipase in wood cellulignin, aiming at its application in hydrolysis and ester reactions. The goal was to contribute toward the search for an inexpensive support for optimum lipase performance.

Cellulignin is the product obtained from biomass acidic prehydrolysis carried out in a steel reactor lined with titanium metal. It is a catalytic fuel with an intermediate heating power between wood and coals, with the aim of being used in single-or combined-cycle thermoelectric power generation *(16)*. Owing to its high degree of polymerization (35% lignin + 65% cellulose) and physical/chemical properties, such as porosity and surface area, this material can also be used as an alternative matrix for immobilizing catalysts. In this way, the present work contributes to the evaluation of methodologies for lipase immobilization in this novel support.

Materials and Methods

Lipase and Chemicals

Commercial *Candida rugosa* lipase (Type VII) and bovine serum albumin (BSA) were from Sigma (St. Louis, MO). The lipase was a crude preparation with a lipolytic activity of 1400 U/mg of solid. Glutaraldehyde (GA) (25% solution), carbonyldiimidazole (CDI), and dimethylsulfoxide (DMSO) were from Aldrich (Milwaukee, WI). Polyethylene glycol (PEG)-1500 (Reagen, SP, Brazil) was used as stabilizing agent. Arabic gum was from Synth (SP, Brazil). Olive oil (low acidity) was purchased at a local market. Alcohol (*n*-butanol) and butyric acid were purchased from Merck (Darmstadt, Germany). Solvents were standard laboratory grade (Synth, São Paulo, SP, Brazil). Heptane was dried with metallic sodium and used as solvent for all experiments. Substrates for esterification reactions were dehydrated with 0.32-cm molecular sieves (aluminum sodium silicate, type 13X-BHD, Chemicals, Toronto, Canada) previously activated in an oven at 350°C for 6 h.

Support

Wood cellulignin from *Eucalyptus grandis* with 3.5% (w/w) moisture content was kindly supplied by RM Materiais Refratários Ltda. (São Paulo, Brazil) in the form of a dark brown powder, with a density of 0.35 g/cm^3. The sample was sieved to obtain particle sizes between 80 and 100 mesh. Because of its acid 1C characteristic, a limiting factor to be used as a support for immobilizing lipases *(9)*, cellulignin was initially neutralized with 0.1 *M* NaOH solution at a 1:15 (solid:liquid) ratio, under agitation for 30 min. The material was then washed with distilled water and dried at 60ºC before being used as the support matrix.

Support Activation Procedures

Cellulignin activation with GA was based on the methodology described by Castro et al. *(12).* Initially, the support was submitted to vacuum for 10 min. Under vacuum, buffered GA solution (2.5% [v/v], 0.1 *M*, phosphate buffer pH 8.0) was slowly added in order to reach a complete solid immersion. Then, the material was transferred to a 100-mL beaker and 4.6 mL of glutaraldehyde solution (2.5% [v/v]) was added. The reaction was carried out at room temperature for 1 h. The activated support was filtered and washed with distilled water to eliminate excess GA.

The methodology described by Carneiro-da-Cunha et al. *(17)* was used for cellulignin activation with CDI. Support sample (4 g, dry wt) was immersed in a solution containing CDI in DMSO (20 mg/mL) in closed flasks for 2 h at room temperature. Afterward, the support was thoroughly washed with a water:DMSO (1:1) solution and then with water to eliminate excess CDI.

Immobilization Procedure

Lipase was immobilized by physical adsorption (pure cellulignin) and covalent binding in either cellulignin activated with GA or with CDI. Cellulignin samples (4 g, dry wt) were previously soaked in hexane under agitation (100 rpm) for 1 h. The excess solvent was discharged, and the amount of enzyme necessary to give a lipase loading of 700 U/g of dry support was dissolved in 20 mL of distilled water and added to the support under low stirring for 2 h at room temperature. When appropriate, PEG (Molecular mass of 1500 Daltons) was added together with the enzyme solution at a fixed amount (5 mg/g of support). Next, 10 mL of hexane was added to the enzyme-support mixtures and coupling took place overnight at 4ºC. Immobilized derivatives were filtered (Whatman filter paper 41) and thoroughly rinsed with hexane.

Protein Assay

Protein was determined according to Bradford's method *(18)* using BSA as a standard. The amount of bound protein was determined indirectly from the difference between the amount of protein introduced into the coupling reaction mixture and the amount of protein present in the filtrate and in the washing solutions.

Coupling Yield

Hydrolytic activities of free and immobilized lipase derivatives were assayed by the olive oil emulsion method according to the modification proposed by Soares et al. (*19).* One unit of enzyme activity was defined as the amount of enzyme that liberated 1 μmol of free fatty acid/min under the assay conditions (37°C, pH 7.0, 150 rpm). Analyses of hydrolytic activities carried out on the lipase loading solution and immobilized preparations were used to determine the coupling yield (η%) according to Eq. 1:

$$\eta(\%) = \frac{U_s}{U_o} \times 100 \quad (1)$$

in which: U_s is the total enzyme activity recovered in the support, and U_o is the enzyme units offered for immobilization.

Esterification Reactions

Reaction systems consisted of heptane (20 mL), *n*-butanol (250 m*M*), butyric acid (280 m*M*), and immobilized lipase derivatives (0.50 g, dry wt). The mixture was incubated at 37°C for 48 h with continuous shaking at 150 rpm. The consumed butanol and the formed product were determined by gas chromatography using a 6-ft 5% DEGS on Chromosorb WHP,

80/10 mesh column (Hewlett Packard, Palo Alto, CA) and hexanol as an internal standard. Esterification activity was expressed as micromoles of formed butyl butyrate per minute per gram of dry support.

Operational Stability of Immobilized Lipase

The operational stability of the immobilized system was assayed using 0.5 g (dry wt) of immobilized lipase in either olive oil hydrolysis or butyl butyrate synthesis in successive batches. At the end of each batch, the immobilized lipase was removed from the reaction medium and washed with hexane to remove any substrate or product retained in the matrix. One hour later (the length of time required for evaporation of the solvent), the immobilized lipase was introduced into a fresh medium. Activities were estimated at the end of each cycle and expressed as micromoles per minute per milligram of catalyst. The biocatalyst half-life time ($t_{1/2}$) was determined by applying the inverted linear decay model *(20)*.

Elemental Analyses

Analyses of carbon, hydrogen, and nitrogen were carried out to determine the enzyme fixation on the support by mass balance using data obtained from an elemental analyzer (Perkin Elmer CHN 2400, Norwalk; CT).

Fourier Transform Infrared Spectroscopy

The samples of free lipase, pure cellulignin, activated cellulignin, and immobilized derivatives were submitted to Fourier Transform Infrared the (FTIR) analysis (spectrophotometer FTIR BOMEM MB-100). The spectra were obtained in the 400 to 4000-cm^{-1} wavelength range for evaluation of the immobilization procedures.

Scanning Electron Microscopy

Structural integrity and conformational changes, such as surface cavities in the support, set in by the lipase procedure were observed by scanning electron microscopy (SEM) on Leica, LEO 440i.

Results and Discussion

Wood cellulignin was used to immobilize *C. rugosa* lipase following two procedures. The first one consisted of immobilizing the lipase on pure cellulignin by physical adsorption (ADS). In the second procedure, the enzyme was covalently bonded to the support previously activated with either GA or CDI. When PEG was employed as stabilizing agent for the enzyme, the following nomenclature was used: ADS-PEG, GA-PEG, and CDI-PEG.

Table 1
Elemental Analysis for Pure and Activated Cellulignin Samples

Material	Carbon (%)	Hydrogen (%)	Nitrogen (%)
Pure cellulignin	49.20	6.86	0
Activated cellulignin with GA	39.58	5.19	0.31
Activated cellulignin with CDI	45.16	5.60	0.51

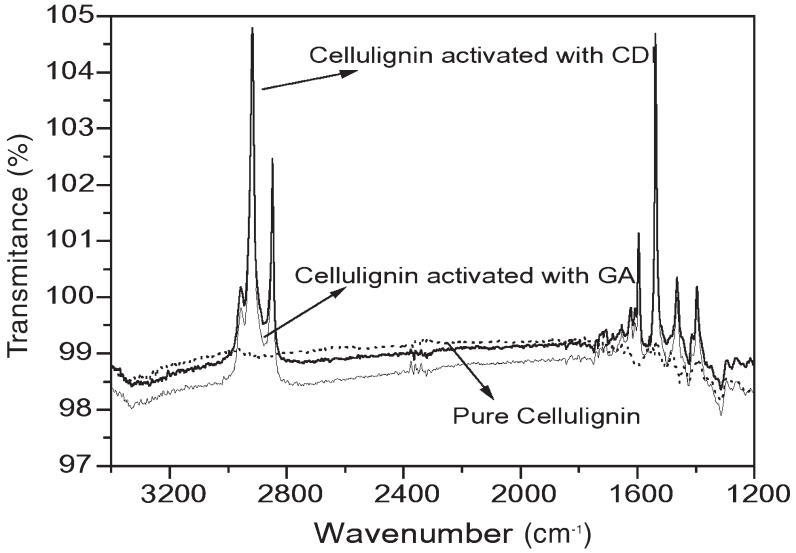

Fig. 1. FTIR spectra for pure cellulignin and cellulignin activated with GA and CDI samples.

Pure and activated cellulignin samples were first characterized by the standard techniques *(21)* elemental analysis (Table 1) and infrared (IR) spectroscopy (Fig. 1). Cellulignin activation renders chemical modifications on its original structure in terms of carbon, hydrogen, and nitrogen contents, which is in agreement with data obtained by IR spectroscopy. As can be seen in Fig. 1, spectra for activated cellulignin samples exhibit two bands at 1500 and 2800 cm^{-1}, which were not present in the pure cellulignin sample. These bands can be attributed to the axial deformation of the carbonyl and hydroxyl groups, suggesting that the functional groups of the activating agents (GA or CDI) were successfully inserted within the support *(21).*

Table 2 summarizes the results for the immobilization of lipase in cellulignin regarding coupling yield (η%). As expected, the lowest coupling yield (η = 20.87%) was verified for the sample prepared by physical adsorption. Experiments performed by covalent binding gave much higher coupling yields (>30%). These data suggested that the activation of cellulignin might produce chemical modifications, which provide

better conditions for enzyme binding in the support. Between the tested activation agents, CDI showed the best performance, producing immobilized lipase samples with an average activity of 113.77 U/mg, which corresponded to a coupling yield of 38.6%. The use of PEG-1500 as stabilizing additive showed a positive effect for all derivatives. The highest coupling yield (62.58%) was verified for the immobilized derivative obtained by using the cellulignin activated with CDI in the presence of PEG-1500, confirming the efficiency of this kind of additive *(22,23).*

The CHN contents for the immobilized derivatives were also analyzed to verify nitrogen incorporation into the supports, with typical results as shown in Table 2. These values can be directly correlated with the amount of protein incorporated in the support because pure and activated cellulignin samples have extremely low nitrogen levels (Table 1). Therefore, the nitrogen levels quantified for the immobilized derivatives were originated from the enzyme immobilized on the support. The highest percentage of nitrogen fixation (79.35%) was achieved for the immobilized derivative obtained by covalent bonding on the cellulignin activated with CDI (immobilized sample CDI-PEG).

Additional information on the catalytic activity was obtained by testing the derivatives prepared in synthetic applications, i.e., is, in esterification reactions with *n*-butanol and butyric acid. These reactions were selected because they gave measurable results with the greatest accuracy in a short span of time and with a minimum amount of lipase. In addition, our grow has used this reaction system as a standard reaction system for lipases immobilized in several supports *(12,15,19).* The data plots are shown in Fig. 2A–E.

In the case of the lipase immobilized on pure cellulignin (ADS), maximum conversion of *n*-butanol into butyl butyrate was found to be 150 m*M* after 48 h of reaction (Fig. 2A). This value was significantly higher (>200 m*M*) when lipase was immobilized in cellulignin activated with either GA or CDI (Fig. 2C,E). The highest esterification activity (317.58 m$M/g \cdot$ min]) was found for the derivative produced with cellulignin activated with CDI (Fig. 2E).

In Fig. 2B,D,F, the curve profiles for the synthesis of butyl butyrate using the immobilized derivatives obtained in the presence of PEG-1500 are shown. By analyzing these data, we observed that the influence of PEG-1500 on the immobilized derivative was quite positive, promoting an increase of 1.5 to 2 times in esterification activity for all immobilized derivatives. It appears that PEG might have improved the esterification activity of the immobilized lipase by better preserving the native structure of the enzyme in the organic medium. It is also worth noting that the use of PEG-1500 protected the enzyme from denaturation effects, without interfering with the reaction rate. However, it is probable that the solubility of PEG in heptane favored the substrate partition to the solid phase, reducing eventual diffusion limitations of the immobilized enzyme.

Table 2
Hydrolytic Activities and Activity Coupling Yield of the Immobilized Derivatives Obtained with Cellulignin in the Presence and Absence of Additive[a]

Experiment	Sample designation	Activation agent	Additive[b]	Hydrolytic activity (U/mg)	Coupling yield (%)[c]	Incorporation of nitrogen (%)[d]
1	ADS	None	A	94.88	20.87	22.28
2	GA	GA	A	75.78	24.36	41.01
3	CDI	CDI	A	113.77	38.60	46.65
4	ADS-PEG	None	P	108.33	23.91	40.90
5	GA-PEG	GA	P	154.67	50.49	72.10
6	CDI-PEG	CAI	P	193.27	62.58	79.35

[b]A, absence of PEG-1500; P, presence of PEG-1500.

[a]The total amount of protein offered to the immobilization was 28 mg/g of support. Lipase loading was 700 U/g of support.

[c]Calculated using Eq. 1.

[d]Calculated from results of elemental analysis.

The experimental results also suggest that PEG modifies the interactions between lipase and cellulignin, producing alterations in the coupling yield and esterification rate. It seems that PEG coating compromises a balance between attractive linking forces to the support and repulsive forces owing to the steric effect produced by the interaction of the polymeric chains in the solution *(23).* It is known that in its free form, PEG has a tendency to join proteins in solution, which possibly promotes enzyme occlusion and binding of the polymer protective layer onto the support. Hydrocarbon chain length influences directly the repulsive forces within polymeric matrixes and increases media viscosity, which has a negative effect on the chain flexibility in the matrix formation. On the other hand, according to the literature, PEG is soluble in water, toluene, and other solvents, but is insoluble in hexane, ether, and ethylene glycol *(24).* Based on the immobilization procedure adopted in our work, the removal of unbound enzyme from the immobilized system by hexane-washing steps attained its objective without promoting enzyme leakage from the support owing to the additive protection. The solubility of PEG in another solvent also favored the reaction system in which heptane was used as an organic medium. This may help to improve biocatalyst activities and suggests that internal diffusion limitations are less pronounced when the immobilization of lipase on cellulignin is performed in the presence of PEG-1500. Additionally, PEG treatment promoted a shift in chemical equilibrium toward synthesis. These results can be explained by the fact that PEG is highly hygroscopic and that the water present in the system binds to the hydroxyl alcohol. In this way, nucleophilic attack by water was minimized, resulting in optimization of butyl butyrate production.

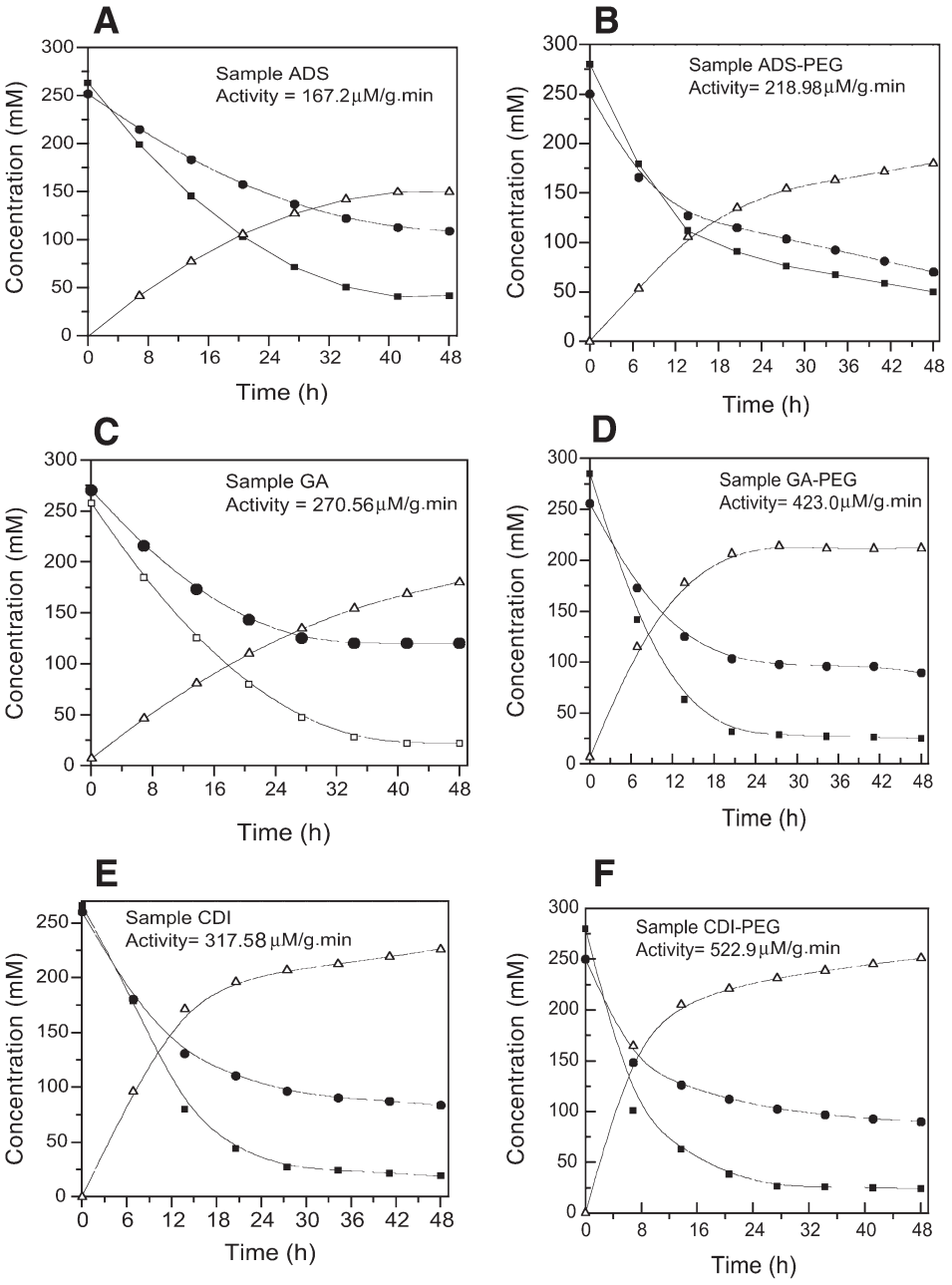

Fig. 2. Synthesis of (Δ) butyl butyrate from substrates containing (●) 250 m*M* butanol and (■) 280 m*M* butyric acid in heptane using lipase immobilized on pure (A,B) cellulignin, (C,D) cellulignin activated with GA, (E,F) and, cellulignin activated with CDI without (A,C,E) and with (B,D,F). PEG-1500 Reactions were carried out at 37°C for 48 h with shaking at 150 rpm.

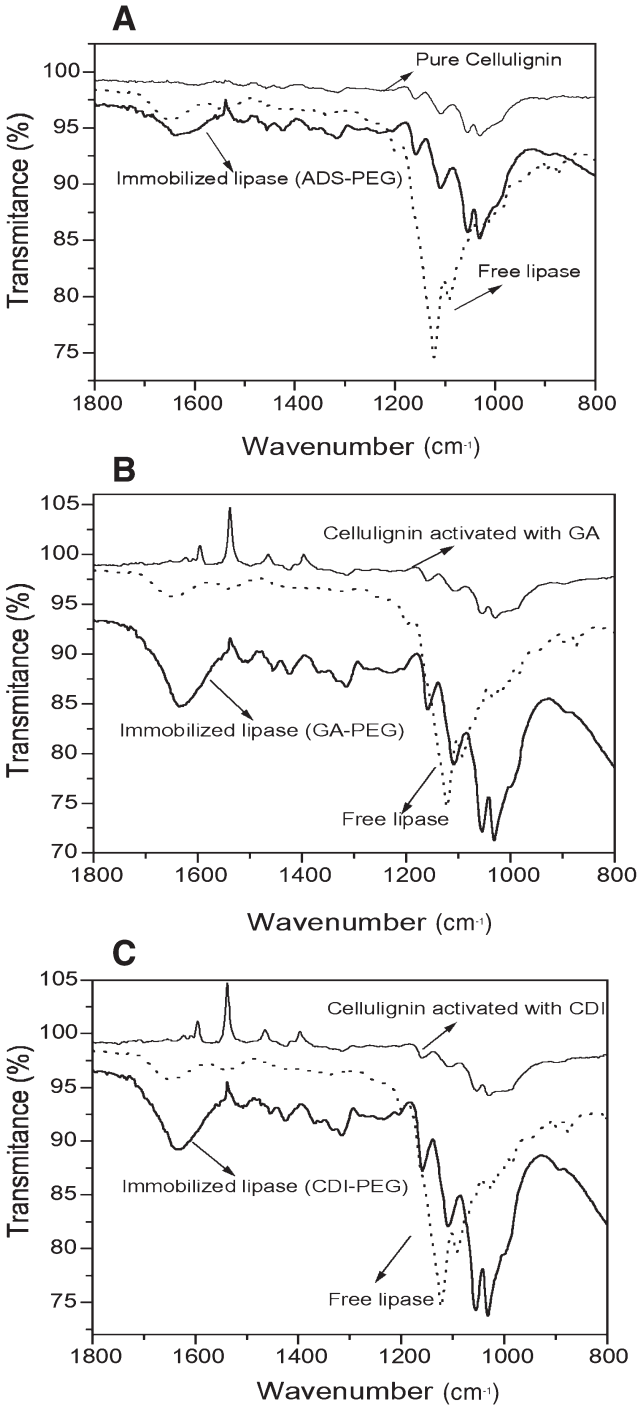

Fig. 3. FTIR spectra for free lipase, pure cellulignin, and cellulignin activated with GA or CDI and immobilized derivatives in presence of PEG: **(A)** cellulignin-lipase by physical adsorption; **(B)** cellulignin activated with GA with lipase covalently bonded; **(C)** cellulignin activated with CDI with lipase covalently bonded.

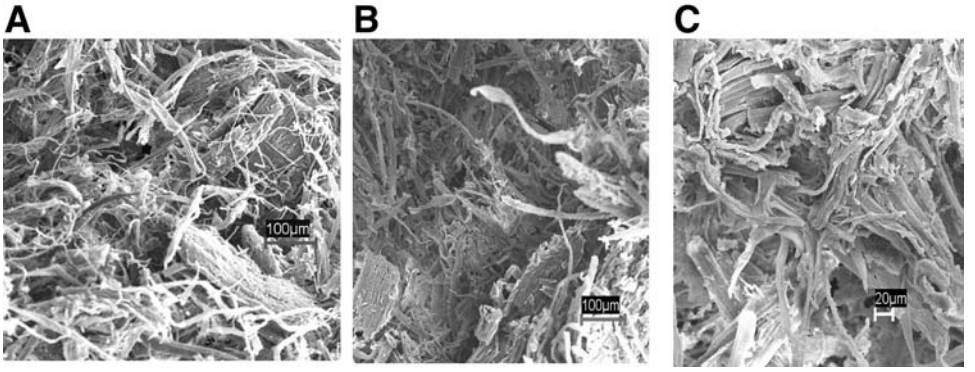

Fig. 4. Scanning electron micrographs of **(A)** cellulignin activated with CDI in absence of enzyme and **(B, C)** immobilized enzyme preparation obtained by immobilizing *C. rugosa* lipase in cellulignin activated with CDI in presence of PEG-1500.

From all the tested procedures, the immobilized derivative obtained in cellulignin activated with CDI was the most active sample to perform the synthesis of butyl butyrate, facilitating maximum product formation at a shorter reaction time (16 h). A similar performance was attained by the other immobilized systems (Fig. 2B,D) only after 36 h of reaction.

The efficiency of the methodology in relation to the incorporation of lipase on cellulignin was also assessed by IR spectroscopy *(25)*. Figure 3A–C shows a set of spectra for free lipase, support, and immobilized derivatives prepared in the presence of PEG (ADS-PEG, GA-PEG, CDI-PEG).

The characteristic bands of free lipase clearly appeared at 1600–1650 cm^{-1} (amine I) and 1120 cm^{-1} (amine II). The greater relative absorption owing to immobilized lipase are shown in Fig. 3B,C (samples GA-PEG and CDI-PEG) compared with Fig. 3A (ADS-PEG), suggesting higher fixation of lipase in cellulignin after derivatization. This agrees with the enzymatic activity. No information can be extracted from the spectra concerning formation of chemical bonds between lipases and the support.

SEM images for the support activated with CDI and the resultant immobilized derivative in the presence of PEG are shown in Fig. 4A–C. Although the surface of the support is highly fibrous or practically does not have a porous structure (Fig. 4A), it seems that the enzyme was able to couple with the support efficiently (Fig. 4B,C). The micrographs in Fig. 4B,C also show that the enzyme not only was well spread on the support surface but also penetrated inside the fibers. This may explain the high esterification activity found for this particularly immobilized derivative (CDI-PEG). It is important to note that SEM studies only provide information regarding the general morphology of the particles, and not the actual conformation of the immobilized enzyme. Further investigations to better characterize the immobilized derivative need to be carried out using specific techniques.

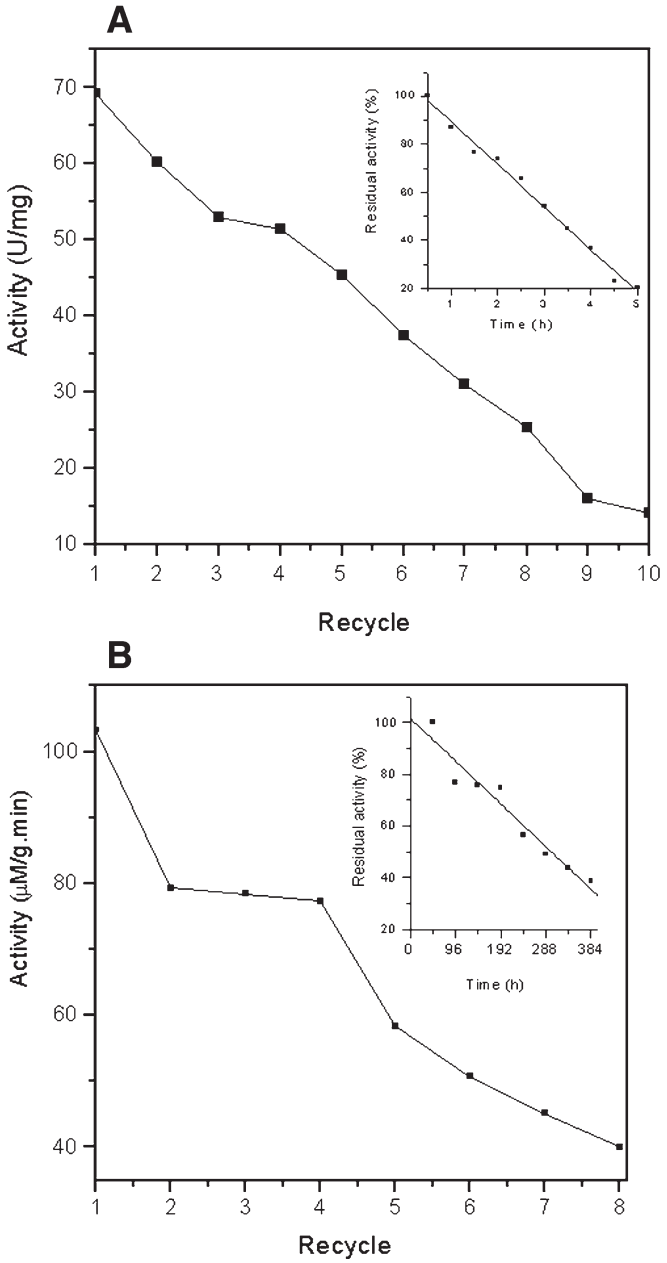

Fig. 5. Batch operational stability tests for lipase immobilized in cellulignin activated with CDI in presence of PEG in **(A)** aqueous and **(B)** organic media. Initial activities for (A) (68.4 (μM/[mg · min]) and (B) (261.5 (μM/[g · min]) were defined as 100%.

Considering that operational stability is a parameter of fundamental importance in developing processes with immobilized enzymes, complementary tests were performed to determine the biocatalyst half-life ($t_{1/2}$) for immobilized lipase in aqueous (hydrolysis of olive oil) and organic

Table 3
Comparison of Recycle Potential for *C. rugosa* Lipase Immobilized on Different Supports

Support	Immobilizing procedure	Reaction type	Biocatalyst half-life (h)	Reference
Chitin (crab shell)	Covalent binding	Synthesis	427	14
Chitosan porous beads	Physical adsorption	Synthesis	86	15
		Hydrolysis	4.3	
Poly (styrene-divinylbenzene)	Physical adsorption	Synthesis	620	26
Cellulignin	Covalent binding	Synthesis	364	This work

(synthesis of esters) media (Fig. 5A,B). In the olive oil hydrolysis (30 min at 37°C), a $t_{1/2}$ of 2.7 h was observed (Fig. 5A). For the esterification reaction of butanol with butyric acid (24 h at 37°C), a slow decrease in esterification activity was verified with a total reduction of 34% at the end of the seventh recycle, which corresponds to a $t_{1/2}$ of 364 h (Fig. 5B).

Data reported in the literature on the operational stability of immobilized lipases are scarce; however, for the purpose of comparison, the operational stability of *C. rugosa* lipase immobilized in different supports of polymeric nature according to the methodology previously established in our laboratory is provided in Table 3. The recycling potential of the lipase immobilized in cellulignin activated with CDI in the presence of PEG-1500 is favorable compared to data reported for lipase immobilized on chitin and chitosan *(14,15),* but it had lower stability than the derivatives obtained from the immobilization of lipases by adsorption on styrene-divinylbenzene copolymer *(26).*

Conclusion

Over the last decades significant advances have been made in the use of enzymes in nonconventional media. These advances are ushering in a new age in the application of enzymes to organic syntheses. Our efforts in this area have focused on the development of suitable support materials for biotransformations in nonconventional media. In this work, we demonstrated that a cheap support is biocompatible with lipases, rendering immobilized derivatives with similar or better characteristics than those previously obtained with natural and synthetic polymers, such as chitin and styrene-divinylbenzene copolymer. Various techniques were evaluated taking into consideration the information obtained from mass balance (CHN), chemical composition (FTIR), coupling yield (hydrolysis of olive oil), and catalytic activity in nonaqueous medium (synthesis of butyl butyrate). These were useful for validating the selection of the most active

biocatalyst, which was the covalent binding on cellulignin activated with CDI in the presence of PEG. It is likely that cellulignin will be applicable to the immobilization of other enzymes of industrial interest.

Acknowledgment

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