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Physical immobilization of *Rhizopus oryzae* lipase onto cellulose substrate: Activity and stability studies

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ABSTRACT

Rhizopus oryzae lipase (ROL) was immobilized by adsorption onto oxidized cellulose fibers and regenerated films. The maximum adsorption level increases with the raise in the amount of carboxylic groups on cellulose surface confirming that adsorption is being governed mainly by electrostatic interaction between the enzyme and the substrate. This hypothesis was further confirmed by ζ-potential measurements showing a decrease in the ζ-potential of the fibers after enzyme adsorption. XPS analysis showed an intensification of the N 1s peak attesting the presence of the enzyme on the surface. The effect of temperature, pH and solvent polarity on the immobilized enzyme activity and stability was investigated. The catalytic esterification of oleic acid with *n*-butanol has been carried on using hexane as an organic solvent. A high conversion yield was obtained (about 80%) at 37 °C with a molar ratio of oleic acid to butanol 1:1 and 150 IU immobilized lipase. The adsorption achieved two successive cycles with the same efficiency, and started to lose its activity during the third cycle.

1. Introduction

Lipases (triacylglycerol ester hydrolases, E.C.3.1.1.3) have been classified as enzymes that hydrolyze fats and oils with subsequent release of free fatty acids, diacylglycerols, monoacylglycerols and glycerol [1,2]. During the last decade, lipases have gained a great interest in biotechnology applications. This interest arises from the ability of these enzymes to catalyze synthetic reactions occurring in non-aqueous media [3]. Thanks to their commercial availability, low cost, high stereoselectivity and the possibility of use at large range of pH and temperature; lipases are among the most used biocatalysts in organic synthesis [4]. They have been employed for direct esterification and transesterification reactions in organic media to produce esters having potential applications in fine chemicals, pharmaceuticals and agrochemicals industries. Moreover, numerous works reported the aptitude of lipases to catalyze the synthesis of short chain fatty acids and alcohols used as additives for a variety of perfumes and flavours [5–7], biosurfactants [8,9] and biofuels [10,11].

To further expand the use of lipases in synthetic reactions, immobilization of lipases is needed as it may protect the enzyme from solvent denaturation, and facilitate a continuous process to be carried on without a need to proceed on further purification steps to isolate the enzyme [12]. Another advantage of immobilization is the enhancement of enzyme thermostability. Thus enzymatic reactions at higher temperatures have resulted in: higher conversion rates, higher substrate solubility and lower viscosity of the reaction medium, thereby favouring mass transfer [13]. Several approaches have been reported for the immobilization of lipases: they consisted either on physical adsorption of the enzyme on a carrier material [14,15], its entrapment or microencapsulation in a solid support [16,17] or on the covalent binding to a solid matrix [18,19]. The selection of an immobilization strategy is based on effectiveness of enzyme utilization, cost of the immobilization procedure, toxicity of immobilization reagents and the desired final properties of the immobilized biocatalyst [20]. Physical adsorption may have a high commercial potential as it is simple and economic to carry on and is reported to be suitable for large amounts of lipases allowing to work at large scale. Numerous studies have been reported describing the lipase immobilization by adsorption on various supports. Natural kaolin was used as support for the immobilization of *Candida rugosa* lipase [21]. Wilson et al. reported the improvement of the functional properties of a thermostable lipase from *alcaligenes* sp. by adsorption on octadecyl–sepabeads supports [22]. *Candida antarctica B* lipase was immobilized, by adsorption, onto polypropylene coated glass balls, and then it was used to synthesize ethyl oleate [23]. This same lipase was also immobilized by adsorption on polyethylene–agarose [24]. Magnin et al. reported

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the immobilization of *C. rugosa* lipase into porous chitoxan beads; they found that the immobilization enhanced the lipase lipolytic activity and its tolerance for organic solvents [25]. In a previous paper, we immobilized the *Rhizopus oryzae* lipase (ROL) on $CaCO_3$. This immobilized lipase has been used successfully to synthesize ethyl oleate [26]. In this study, we report the physical adsorption of ROL, produced and purified in our laboratory, on cellulose fibers previously oxidized to generate carboxylic groups. The stability and the activity of the immobilized ROL onto the cellulose fibers were investigated, and the ability of the immobilized enzyme to synthesize esters was also explored.

2. Materials and methods

2.1. Materials

The fibers used in this work were commercial microcrystalline cellulose (TECHNOCEL-150DM). Their average length was about 50 μm, and their specific surface, measured by the BET technique using nitrogen as the adsorbed gas, was found to be 2.5 m^2/g. In order to have a flat surface suitable for XPS analysis, commercial regenerated cellulose film was used. Chemicals used to oxidize cellulose fibers, namely, TEMPO (2,2,6,6-tetramethyl-1-piperidinyloxy radical), sodium bromide (NaBr) and 12% sodium hypochlorite solution, were laboratory grade and used without further purification.

2.2. Microorganisms

The strain was isolated from olive in decomposition and identified at the Pasteur Institute of Paris as corresponding to *R. oryzae* [27].

2.3. Oxidation procedure of cellulose fibers

Oxidation of cellulose fibers and regenerated cellulose film was carried on following the procedure well-detailed elsewhere [28]. This method of oxidation offers the advantage of being highly selective for primary hydroxyl groups, whereas secondary hydroxyl groups were in most cases insensitive toward oxidation. The oxidation reaction and the structure of the ensuing polymer are depicted in Fig. 1.

Conductimetric titration was performed by neutralisation of the carboxylic groups COO^- with 0.1 M HCl solution. The following procedure was adopted: 0.3 g of dry fibers were diluted in distilled water to a volume of 200 ml. NaCl solution (5 ml) of 0.1 M was added to minimize the uneven distribution of ions between the fiber phase and the surrounding water phase caused by Donnan equilibrium in the fiber/water system. The slurry was then titrated with 0.1 M HCl solution and the conductivity was followed.

Hereafter, the polymers are represented by the abbreviation Cell-*X*, in which *X* is a value representing the amount of carboxylic groups per gram of fibers.

2.4. Enzyme adsorption

ROL was produced and partially purified as described by Ben Salah et al. [27]. At the end of the cultivation period, the mycelium was removed by filtration. The supernatant was precipitated by addition of ammonium sulphate ($(NH_4)_2SO_4$) up to 65% saturation followed by centrifugation at 8000 rpm at 4 °C for 20 min. The pellet was dissolved in 50 mM sodium acetate buffer pH ~ 6 containing 100 mM NaCl and 2 mM benzamidine (protease inhibitor). Then, the enzyme solution was centrifuged at 8000 rpm for 10 min and the supernatant containing the lipase (crude lipase) was used for immobilization [27].

The crude lipase was immobilized by simple adsorption technique onto oxidized cellulosic fibers. Adsorption of lipase was carried out in batch by adding to a suspension containing 1% (w/w) of cellulose modified fibers, lipase dissolved in acetate buffers (pH 6). The proteins concentration was varied between 0.5 mg/ml and 5.0 mg/ml and the adsorption was conducted at 4 °C in a shaking water bath for 1 h. The lipase-adsorbed fibers were recovered by filtration and washed, at room temperature, with the same buffer and

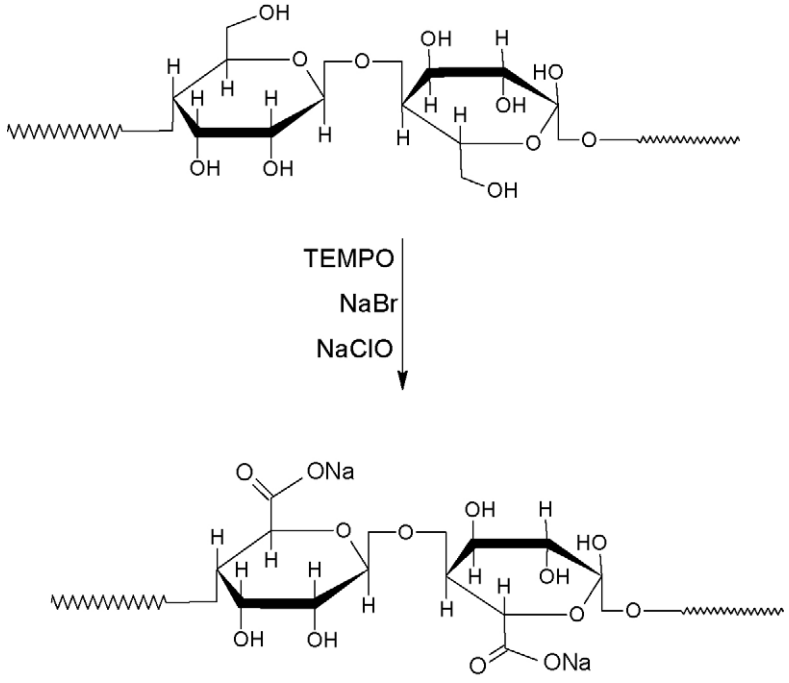

Fig. 1. TEMPO-mediated oxidation of cellulose primary hydroxyl groups to carboxyl groups.

deionised water, then dried by lyophilization at room temperature. Immobilization was estimated as following:

$$\text{immobilization yield}\,(\%) = \left(\frac{\text{initial enzyme activity} - \text{non-immobilized activity}}{\text{initial enzyme activity}}\right) \times 100$$

$$\text{activity yield}\,(\%) = \left(\frac{\text{immobilized activity}}{\text{initial enzyme activity} - \text{non-immobilized activity}}\right) \times 100.$$

2.5. Protein assay

The amount of residual proteins in the supernatant solution was determined according to Bradford's method [29] using BSA as a standard. The amount of adsorbed proteins was calculated by subtracting the initial amount of the protein from the residual concentration in the solution, after immobilization. The loading percentage of proteins was calculated as following:

$$\text{proteins loading}\,(\%) = \left(\frac{\text{amount of adsorbed proteins}}{\text{initial amount of proteins}}\right) \times 100$$

2.6. Lipase hydrolytic activity

The activities of the free and immobilized lipases were measured titrimetrically with a pH-Stat, under the standard assay conditions described previously using olive oil emulsion as substrate [30]. The reaction mixture contains 10 ml of olive oil emulsion (1 ml of olive oil and 9 ml of arabic gum at 10%), 20 ml of distilled water and 100 μl of bovine albumin serum 12.5%. The reaction was carried out at 37 °C and pH 8.3. The amount of free fatty acid released during hydrolysis was estimated by titration with 0.01N NaOH solution. Activity was expressed as units per milliliter of broth. One unit (IU) of lipase activity was defined as the amount of enzyme that catalyzes the liberation of 1 μmol of fatty acid from olive oil as substrate per minute at pH 8.3 and at 37 °C.

2.7. XPS analysis

The modification in chemical composition of the functionalized cellulose film as compared to native cellulose was analyzed by X-ray photoelectron spectroscopy (XPS) using an XSAM800 (KRATOS) X-Ray Spectrometer. Non-monochromatised Al Kα X-radiation ($h\nu$ = 1486.6 eV [31]) was generated with a power of 120 W (12 kV × 10 mA). Other experimental conditions were as in Ref. [32]. The reference used to compute charge shifts was the cellulosic carbon bound to a single oxygen (binding energy = 286.7 eV). The curve fitting for component peaks was carried out with a Shirley background and a non-linear least-squares algorithm using a product of Gaussian and Lorentzian peak shapes (XPSPeak 4.1). X-ray source satellites were previously subtracted. For quantification purposes, sensitivity factors were 0.66 for O 1s, 0.25 for C 1s, 0.42 for N 1s. Cell-50 and Cell-850 without any lipase and when treated with lipase (L) were analyzed.

2.8. ζ-Potential

A commercial ζ-potential analyzer (Nano-Zs from Malvern) was used to measure the electrophoretic mobility of cellulose fibers in the aqueous suspension. Measurements were conducted on the suspension fine fraction, which was obtained after filtration of the original suspension through a 45-μm screen. To avoid the fluctuation of ζ-potential due to ionic strength changes generated by the increase in the enzyme concentration, the aliquot sample was diluted with a concentrated solution of KCl to buffer the ionic strength to 10^{-2} mol/l. Four consecutive measurements were taken for each sample at room temperature and averaged. The ζ-potential was calculated from electrophoretic mobility data by using the Smoluchowski equation.

2.9. Characterisation of immobilized lipase

2.9.1. Effect of temperature on the free and the immobilized lipase activities and stabilities

The effect of temperature on the hydrolytic activities of both forms of ROL was determined at 25–50 °C, the activities were determined at pH 8.3 using olive oil emulsion as substrate (see Section 2.6). Relative activities were calculated as the ratio of the activity of enzyme measured at different temperatures to the activity of the enzyme at the standard conditions (pH 8.3, 37 °C) as described above.

The thermal stability assays were performed by incubation of the immobilized and free forms of ROL at different temperatures (25–60 °C) for 1 h, cooled down to room temperature and the activity of enzyme was measured by pH-Stat under standard conditions (pH 8.3, 37 °C) as described above. Residual activities were calculated as the ratio of the activity of enzyme measured after incubation to the activity of the enzyme at the standards conditions.

2.9.2. Effect of pH on the free and the immobilized lipases activities and stabilities

The effect of pH on lipase (free and immobilized) activities was studied in the pH range 7.5–9.5 at 37 °C using olive oil as substrate. Relative activities were calculated as the ratio of the activity of enzyme measured at different pH to the activity of enzyme at the standard conditions (pH 8.3, 37 °C). The enzyme stability was determined by exposing immobilized and free lipase to different pH values ranging from 3 to 10 for 1 h at 4 °C, by using four different buffer conditions: glycine–HCl 50 mM (pH 3–4), sodium acetate 50 mM (pH 5–6), phosphate 50 mM (pH 7) and Tris–HCl 50 mM (pH 8–10). Then the hydrolytic lipase activity was measured at 37 °C by following the standard assay method. Residual activities were calculated as the ratio of the activity of enzyme measured after incubation to the activity of the enzyme at the standard conditions.

2.9.3. Effect of organic solvents on the stability of immobilized lipase

The effect of solvents on the stability of immobilized lipase (ROLi) was studied using solvents of polarity (log *P*) ranging from −0.23 to 3.5. The immobilized lipase was treated with the experimental solvent. The solvent was then separated by centrifugation. The immobilized lipase was dried under vacuum and then studied for lipase activity using oil olive emulsion as substrate at standard conditions. The residual activity of the solvent-treated lipase was expressed as the ratio of the activity of treated immobilized lipase to the activity of the untreated immobilized lipase at standard conditions.

Lipase activity and stability at different pHs, temperatures and solvents are the average of three experiments or more with a variation within 10%. For all cases, a blank experiment without enzyme was performed in parallel.

2.9.4. Effect of immobilization on the kinetic parameters

The effect of immobilization of lipase on the kinetic parameters was determined by measuring the initial reaction rates of the

hydrolytic reaction with varying concentration of olive oil. K_m and V_{max} values were determined from the Lineweaver–Burk graph.

2.9.5. Esterification assay

Unless otherwise stated, the esterification reactions were performed in screw-capped flasks containing 30 mg of butanol (0.1 M), 120 mg of oleic acid (0.1 M), 150 IU of immobilized lipase and 5% of water (w/w) dissolved in 4 ml of anhydrous *n*-hexane. The reaction mixture was shaken at 220 rpm and 37 °C in a shaking incubator.

2.9.6. Analysis of the samples

Aliquots of 200-μl volume were withdrawn periodically from the reaction mixture. The immobilized enzyme was removed by centrifugation at 2000 rpm for 5 min, then the supertnatant residual acids contents were assayed by titration with 0.8N sodium hydroxide, using phenolphthalein as an indicator and 3 ml of ethanol as a quenching agent. The yield of ester synthesis was calculated based on the conversion of the acid to ester.

3. Results and discussion

3.1. Adsorption isotherms of ROL on oxidized cellulose fibers

The adsorption isotherms of ROL on oxidized cellulose fibers bearing different amounts of carboxylic groups viz., 50, 250 and 450 μmol/g are depicted in Fig. 2. Results indicated that adsorption level is relatively low for initial concentration lower than 300 μg/g and started to go up once the proteins loading exceeds 400 μg/g. Then the proteins adsorption starts to level off for a concentration exceeding 950 μg/g. The adsorption capacity increased with the raise in the surface carboxylic groups' density and attained about 230, 600 and 800 μg/g for Cell-50, Cell-250 and Cell-450, respectively. One can note that virgin non-oxidized fibers displayed a low adsorption capacity being in the range of 200 μg/g. This result could be rationalized if we consider that electrostatic interaction between enzyme and the surface greatly contributes to the adsorption process. Indeed, under pH ∼ 6 where experiments are carried on, a high fraction of surface carboxylic groups are ionised and the structure of the enzyme involved inevitably some cationic sites or amino groups that are prone to establish electrostatic interaction with the surface carboxylic sites.

Analysis of the activity of immobilized lipase carried on Cell-450 has shown that the protein loading, immobilization yield and the activity yield were about 66%, 70% and 15%, respectively. This result indicated that lipase immobilization brings about a drop in the activity even though a high proportion of the enzyme is adsorbed, which may be either due to the change in spatial conformation induced by the immobilization or due to the substrates diffusion to the active sites of the immobilized lipase and the lower accessibility of enzyme active sites caused by random immobilization [33].

3.2. Study of electrokinetic proprieties of oxidized cellulose fibers before and after immobilization

To further support the high contribution of electrostatic interaction on adsorption process the evolution of ζ-potential as a function of pH of the cellulose fibers suspension saturated with lipase was investigated. The aim of this study is to access the surface charge density of the crude lipase, the cellulose matrix and the cellulose matrix treated with crude lipase, through ζ-potential measurement. In the colloidal chemistry, ζ-potential is the electric potential in the interfacial double layer at the location of the slipping plane. It corresponds to the potential difference between the dispersion medium and the stationary layer attached to the dispersed particle, and provides a useful indication to quantify the sign and the magnitude of the electrical charge at the colloidal surface particle.

In pH ranging from 3 to 4, Fig. 3 showed that the ζ-potential of the crude protein preparation is lower than −3 mV indicating that the macromolecule species of the crude lipase being globally neutral as a result of the presence of positive and negative charges in equal amounts. The isoelectric point (IEP) of the crude lipase is therefore close to 3. Above pH 6, the decrease in the ζ-potential with a plateau around −9 mV over pH 8 denotes a slight excess in the negative charge over positive one as a result of further dissociation of amino acid bearing carboxylic groups. The IEP of oxidized cellulose fibers appears to be close to 2, meaning that the surface of the oxidized cellulose matrix is negatively charged in pH range from 3 up to 12 as the result of the dissociation of the carboxylic groups. In the presence of enzyme, the shift in the ζ-potential of the fibers toward a lower value over the pH range from 3 up to 9 is the result of the enzyme adsorption and suggests that the adsorption involves both electrostatic and acid–base interaction between enzyme and carboxylic negative sites on the surface. Indeed, in the pH range between 4 and 8, the amino acid bearing lateral amino groups such as histidine, lysine and arginine are either protonated giving raise to ammonium species or in their basic form (–NH– or $–NH_2$). The former contributes to the adsorption through electrostatic interaction with ionized carboxylic site resulting in ion-pairing with the

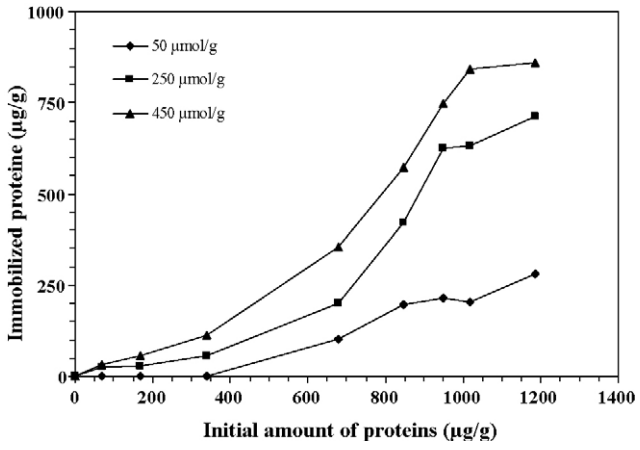

Fig. 2. Adsorption isotherm of ROL onto oxidized cellulose fibers as a function of initial protein loading at pH 6.

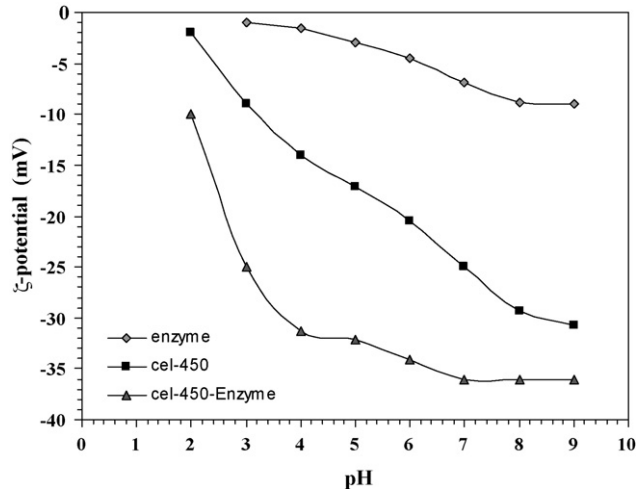

Fig. 3. Evolution of ζ-potential as a function of pH for the enzyme, the oxidized cellulose fibers (Cell-450) and Cell-450 treated with the enzyme.

Fig. 4. Schematic representation of physical adsorption of lipase onto cellulosic fibers.

surface, while the latter are likely to generate a complex with undissociated surface carboxylic group (–COOH). The involvement of the ammonium groups on the adsorption process will inevitably lead to the overcompensation of negative sites on the dangling chains of the enzyme, thus giving raise to a negatively charged surface which explains the decrease in the ζ-potential of the fibers after interaction with enzyme. One might suggest the contribution of other forces such as hydrogen bonding or van der Waals interactions to the adsorption process onto cellulose, however, their contribution is probably limited compared to the electrostatic and acid–base interaction. Fig. 4 schematically illustrates the adsorption mechanism on the substrate.

3.3. Characterisation of oxidized cellulose by XPS

Four samples were analyzed by XPS: Cell-50, Cell-850, Cell-50L and Cell-850L as described in Section 2. For all the samples, the C 1s, O 1s and N 1s were studied. Also minor amounts of other elements were detected such as sulphur, silicon and sodium. Fig. 5 shows that C 1s region displays four components for all the samples.

Three of them were centered at 286.7 eV (cellulosic C–O), 288.1 eV (cellulosic O–C–O) and 288.9 ± 0.3 eV assignable to carboxylic groups. The ratio between the intensity of this last component and the cellulosic carbon is maximum in sample Cell-850 followed by sample Cell-50, Cell-850L and Cell-50L. This shows that the relative amount of carboxylic groups decreases when the protein is grafted and it is more important in samples Cell-850,

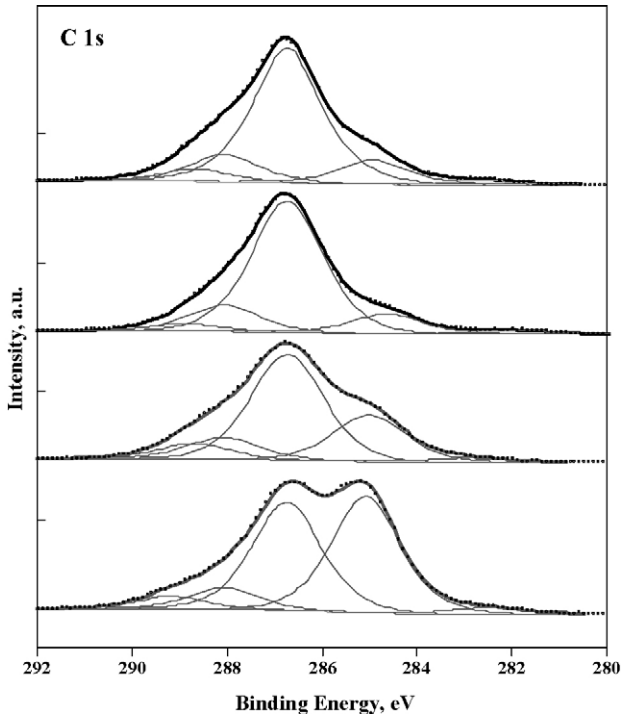

Fig. 5. XPS C 1s region for the four samples studied here: from bottom to top, CellR-50, CellR-850, CellR-50L and CellR-850L. Spectra were setoff for clarity sake.

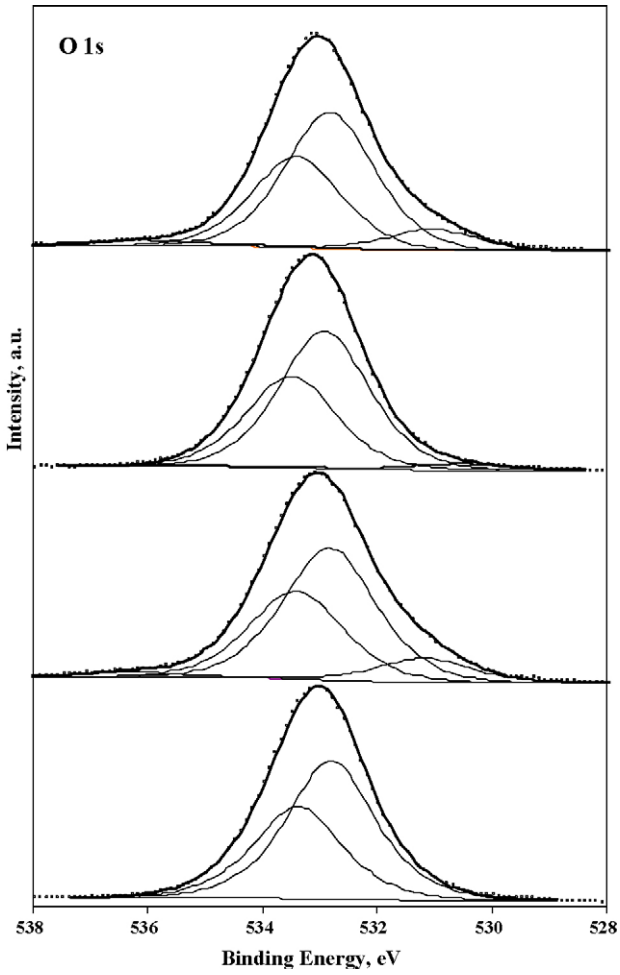

Fig. 6. XPS O 1s region for the four samples studied here: from bottom to top, samples CellR-50, CellR-850, CellR-50L and CellR-850L. Spectra were setoff for clarity sake.

the more oxidized ones. Finally, at 285.0 ± 0.1 eV an sp3 peak was found for all the samples with a single exception for sample Cell-50L, where a more sp2 carbon was found at 284.6 eV. This sp3/sp2 peak represents an important fraction of the whole carbon in sample Cell-50 (0.45), a moderate fraction in sample Cell-50L (0.25) and the smaller fractions (0.11–0.12) occur for samples Cell-850 and Cell-850L (the more oxidized ones).

XPS O 1s region shown in Fig. 6 displays, as the main components, the cellulosic peaks centered at 532.8 ± 0.1 eV (C–O–H) and at 533.4 ± 0.1 eV (C–O–C). For the more oxidized samples (Cell-850), a small component at 536.2 ± 0.2 eV, assignable to water aggregates entrapped in the film was detected [34]. In the same pair of samples, also a peak at 531.1 ± 0.1 eV is seen and is tentatively assigned to the carbonyl oxygen in the carboxylic groups.

N 1s region is displayed in Fig. 7. For all the samples, it was fitted with peaks having fwhm = 1.6 ± 0.1 eV. Even samples without any immobilized enzyme present small amounts of nitrogen. This is probably due to the oxidizing agent used (2,2,6,6-tetramethylpiperidine-1-oxyl, TEMPO) that could have been adsorbed or reacted, by a condensation mechanism, putting a few nitrogen atoms on the cellulose chains. The more intense peaks arise for samples where the lipase was immobilized testifying the adsorption of the protein. In those samples, the more important peak is centered at 400.2 ± 0.1 eV: it is 57% of the total N 1s area in the Cell-50L and 66% in the Cell-850L. It is assignable both to

amine and to peptidic functionality [31]. Components with lower binding energy have low intensity and, hence the contribution of the noise for them is very important as can be seen in Fig. 7. The higher binding energy is due to positively charged nitrogen atoms [31].

3.4. Effect of the temperature on lipase activity and stability

The effect of temperature on hydrolytic enzyme activity was investigated at pH 8 in the range 25–50 °C. Results show that the activity of the free lipase is maintained roughly constant until temperature 37 °C, above which a continuous decrease in the activity is noted which results from the thermal denaturation of the enzyme. On the other hand, the thermal activity of the immobilized enzyme is slightly better as it is preserved until temperature 40 °C (Fig. 8A).

Regarding the temperature stability, Fig. 8B showed a significant enhancement in this property after immobilization of the enzyme. Indeed, at a time where the free enzyme lost its thermal stability above 37 °C the activity was preserved till 45 °C for the immobilized enzyme. Thus, after heat treatment for 1 h at 50 °C, 90% drop of the initial activity is observed for the free lipase whereas it attains only 50% for the immobilized one.

This result could be the consequence of conformational limitations on the enzyme movements because of multi-point attachment and it is in agreement with other reported works [35,36]. The better thermal stability of the enzyme after physical immobilization will extend the potential application as a biocatalyst.

3.5. Effect of pH on lipase activity and stability

The effect of pH on the hydrolytic activity of free and immobilized lipase in olive oil hydrolysis was investigated in the pH range 7.5–9.5. Results shown in Fig. 9A indicated an optimum activity around 8.3 for the free enzyme, whereas the activity is maintained constant till pH 8.3 and drops rapidly above this pH for the immobilized enzyme, so the immobilized lipase exhibits a wider pH range of hydrolysis activity than the free enzyme. Furthermore, immobilized lipase was stable within the pH range 3–8; whereas the free lipase was stable only at pH 6 as clearly illustrated in Fig. 9B. Similar trends have been reported regarding the hydrolysis activity versus pH for immobilized lipases [37,38].

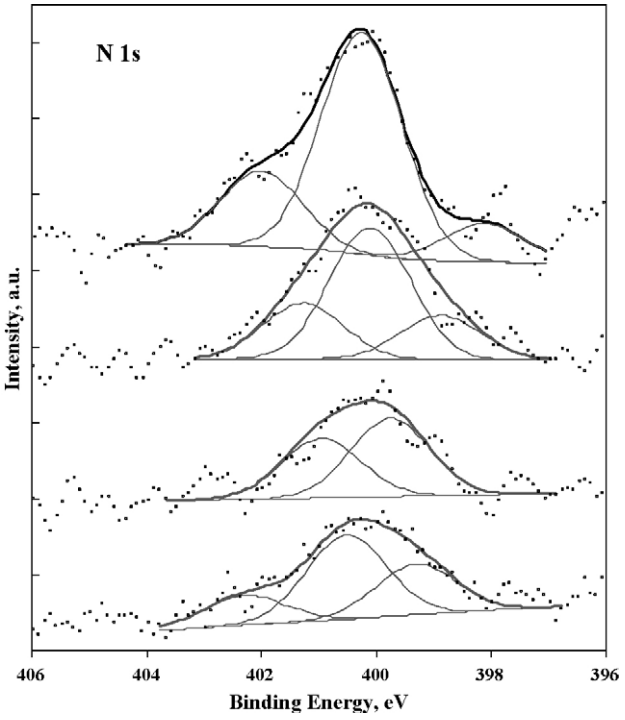

Fig. 7. XPS N 1s region for the four samples studied here: from bottom to top, samples CellR-50, CellR-850, CellR-50L and CellR-850L. Spectra were setoff for clarity sake.

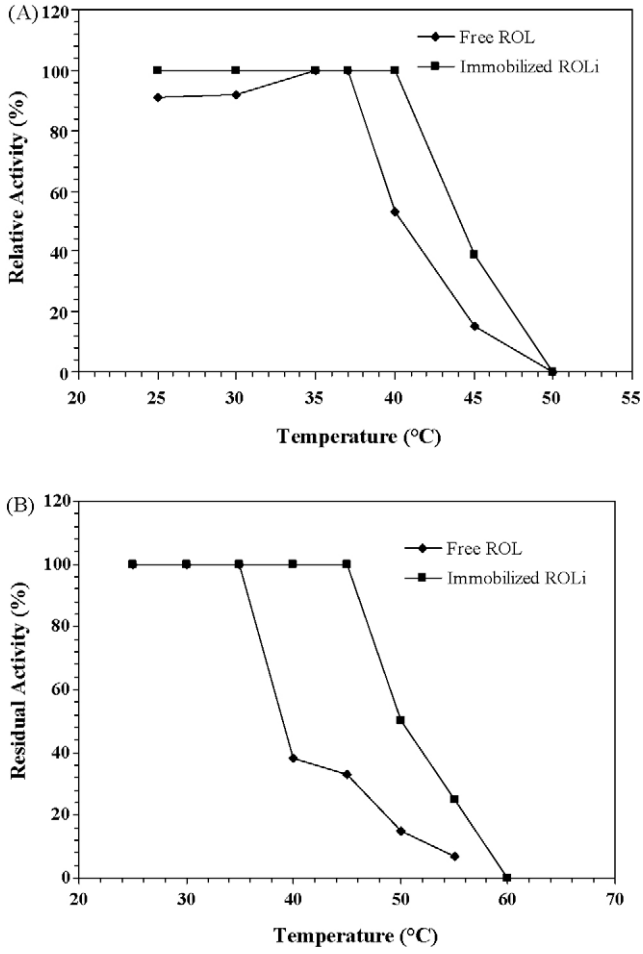

Fig. 8. Effect of temperature on the (A) hydrolytic activity and (B) stability of the free and immobilized ROL. Enzyme activities were assayed with olive oil as substrate at pH 8.3.

3.6. Effect of solvent on lipase stability

The use of enzymes in organic medium is of great interest as it permits to carry on esterification reactions that are difficult to occur in presence of a high amount of water.

However, it is well known that enzyme activity is strongly affected by the choice of the organic solvent which may bring about the denaturation of the enzyme thus leading to the loss of the catalytic activity. In order to study tolerance of immobilized enzyme to organic solvent the activity of the immobilized lipase versus time was monitored in different organic solvents varying from polar to non-polar with polarity index ($\log P$) ranging from −0.23 to 3.5 (Fig. 10). The $\log P$ value of the solvents is the widely used parameter to describe solvent polarity and their possible effects on enzyme activity, where P is the partition coefficient of a given solvent between water and octanol in a two-phase system [39].

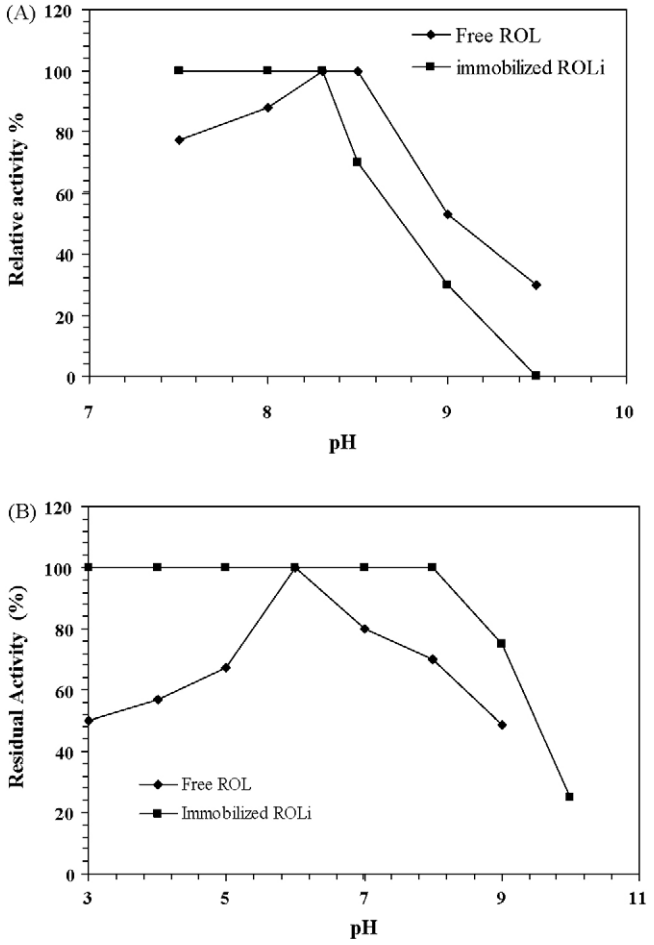

Fig. 9. Effect of pH on the (A) hydrolytic activity and (B) stability of the free and immobilized ROL. Enzyme activities were assayed with olive oil emulsion as substrate at 37 °C.

Among four tested solvents, the tolerance of the immobilized lipase is higher for hexane and *ter*-butanol for which the activity is maintained during long time incubation, whereas, the lipase lost 50% of hydrolytic activity by its exposure to acetonitrile for 60 min. Finally, it lost all of its activity in the presence of the acetone after 120 min of incubation. The lower lipase activities in acetone and

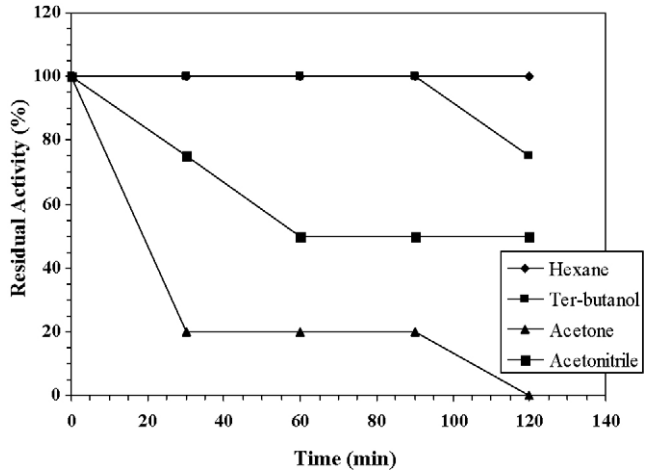

Fig. 10. Effect of organic solvents on the stability of immobilized ROL. Enzyme activities were assayed with olive oil as substrate at pH 8.3 and 37 °C.

Table 1
Michaelis parameters K_m and V_{max} at pH 8.3 and 37 °C

Enzyme	K_m (mmol/l)	V_{max} (μmol/(l min μg))
Free ROL	15.67	2.93
Immobilized ROL	23.36	0.80

acetonitrile could be due to the fact that the more polar solvents on which water is highly miscible could strip off the essential water layer around the enzyme present as microaqueous layer and thus distort the catalytic conformation of the enzyme [40]. This result is in agreement with the literature reports showing low ester conversions in solvents with log $P < 2.5$ [41].

3.7. Kinetic parameters

The effect of immobilization of ROL on the kinetic parameters for the hydrolytic activity (K_m and V_{max}), was investigated. V_{max}, which defines the highest possible velocity when all the enzyme sites were saturated with substrate, reflects the intrinsic characteristics of the immobilized enzyme, and may be affected by diffusion limitations. K_m, or apparent K_m, which is defined as the substrate concentration that gives a reaction velocity of $1/2V_{max}$, reflects the effective characteristics of the enzyme and depends upon both partition and diffusion effects [42]. These parameters were determined from the Lineweaver–Burk graph obtained by plotting inverse values of substrate concentration against inverse values of initial reaction rates. The values of apparent K_m and V_{max} of the free and immobilized ROL as obtained from the Lineweaver–Burk graph are presented in Table 1.

It was observed from Table 1 that the values of apparent kinetic parameters get changed by the immobilization. The K_m value was 15.67 and 23.36 mmol/l for the free and the immobilized enzyme, respectively. Likewise, the higher value of V_{max} relative to free lipase (2.93 μmol/(l min μg) against 0.8 μmol/(l min μg)) was obtained. This change in apparent kinetic parameters after immobilization can be explained by several phenomena: (i) a lower substrates concentration in the microenvironment of the immobilized lipase caused by limitations diffusion; (ii) inaccessibility of enzyme active sites and (iii) change of the enzyme conformation induced by the immobilization [43]. Similar change in apparent hydrolytic kinetic parameters of *C. rugosa* lipase immobilized on chitosan nanofibrous membrane was reported [38].

3.8. Esterification activity of the immobilized ROL

The ability of the cellulose-immobilized ROL to catalyze the synthesis of esters was investigated by taking the synthesis of butyl oleate, carried out in hexane at 37 °C, as reaction model. The effect of different parameters was tested.

3.8.1. Effect of initial addition of water

It is a well-known fact that water plays an essential role on the lipase-catalyzed esterifications. On the one hand, water participates, directly or indirectly, in all non-covalent interactions that maintain the conformation of the catalytic site of enzymes, and on the other hand, in esterification/hydrolysis reactions, water content affects the equilibrium conversion of the reactions as well as the distribution of products in the media as a result of water acting as a substrate[44]. Although the proper amount of water for a given enzymatic reaction depends on many factors (the selected enzyme, support, solvent and substrates concentration), generally, it is reported that the optimum level of added water is within the range of 0.2–3% based on dry enzyme [45].

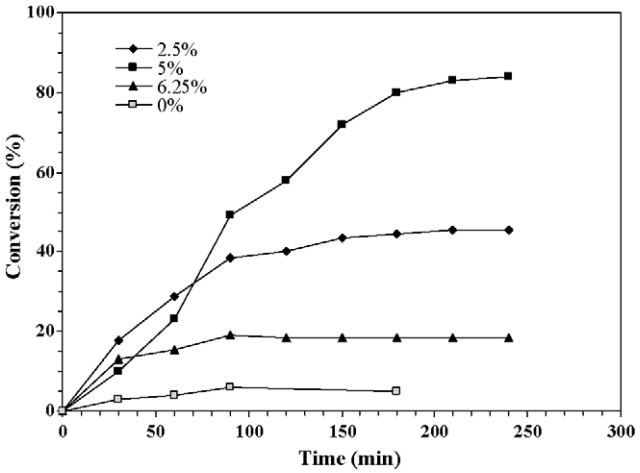

Fig. 11. Effect of different initial added water on the conversion yield during butyl oleate synthesis. Reaction conditions were 150 IU of lipase, an oleic acid/butanol molar ratio of 1 at 37 °C and stirred at 220 rpm.

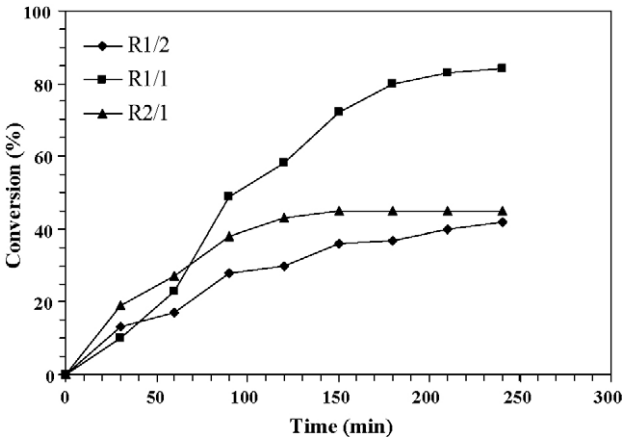

Fig. 12. Effect of different oleic acid/butanol molar ratios (*R*) on the conversion yield during butyl oleate synthesis. Reaction conditions were 150 IU of lipase, 37 °C and stirred at 220 rpm.

The effect of initial water content on the esterification reaction was investigated. The reaction was carried out at the conditions described in Section 2 with various amounts of added water ranging from 0% to 6.25% (w/w). As shown in Fig. 11, in the absence of any added water the biocatalytic activity seems to be inhibited as it does not exceed 5% after 3 h. The esterification reaction is triggered with the addition of water and the conversion limit fluctuates according to the initial water level. The high conversion yield was obtained by the addition of 5% of water.

At 0–6.25% with respect to the solvent and substrate, the amount of the added water (which is introduced just after the addition of the different reagent) ranged from 0 to 250 mg. If we consider that about 150 mg of immobilized cellulose matrix was used to carry on esterification reaction and the adsorbed amount of the enzyme is about 900 μg/g (according to Fig. 2), then the ratio water/enzyme (w/w) exceeds 650. This value is too high and will lead to a complete inhibition of the lipase synthetic activity if the esterification was conducted under homogenous condition, i.e., the lipase is added directly to the substrate solution [46,47]. This discrepancy could be rationalized if we take account of the high hydrophilic character of cellulose matrix and of its microporous structure, which brings about a high absorption capacity through capillary effect. These effects bring about fibers swelling that make the surface pore more expanded and the enzyme conformation more flexible. This hypothesis is corroborated by the high swelling capacity of the cellulose fibers matrix used when they are immersed into water. Indeed, after contact with water during 10 min, the fibers increased in volume and gained more than 200% with respect to its initial weight. Above 5% of added water it is likely that water will start to accumulate on the external surface and cover the enzyme molecule which becomes unavailable for biocatalysis as the substrate molecules dissolved in hexane will no longer reach the surface bound enzyme by the physical separation between the reactants and the lipase active sites.

3.8.2. Effect of molar ratio acid/alcohol

The esterification was carried out with various molar ratio oleic acid/butanol (1:2, 1:1 and 2:1) at the conditions described above. By varying the molar ratio between the acid and the alcohol, we noticed that the highest conversion yield was obtained at a stoichiometric ratio close to 1:1 between oleic acid and butanol after a 250 min reaction. The conversion is stabilized at roughly the same level (close to 40%) either in the presence of an excess acid or excess alcohol (Fig. 12). This result may be imputed, on the one hand, to the competitive binding of acid and alcohol to the immobilized enzyme due to the hydrophilic character of the cellulose matrix that favours the accumulation of the substrate molecule with their terminal polar group pointed toward the surface. Thus, the increase of the ratio of one of the reagents will disfavour the molecular contact between the acid and the alcohol with the immobilized enzyme. One can not only exclude the effect of the raise in the polarity of the medium causing more interaction with the aqueous boundary layer on the lipase surface, which disrupts the conformation of the protein structure. Similar results were reported in the synthesis of *n*-butyl oleate by lipozyme [48].

3.8.3. Effect of enzyme amount

The effect of the amount of the immobilized enzyme on the course of the esterification reaction was also analyzed by varying the lipase loading from 150 to 200 IU (based on the hydrolytic activity). Results depicted in Fig. 13 showed that a jump in the conversion yield is observed as the lipase concentration exceeds 50 IU. For 100, 175 and 200 IU the conversion limit seems to be stabilized around 60% conversion. On the other hand, at 150 IU the highest conversion yield (around 80%) is obtained. It is worth noting that the kinetic conversion yield of the esterification reaction grows up continuously with the increase in the amount of the lipase for the

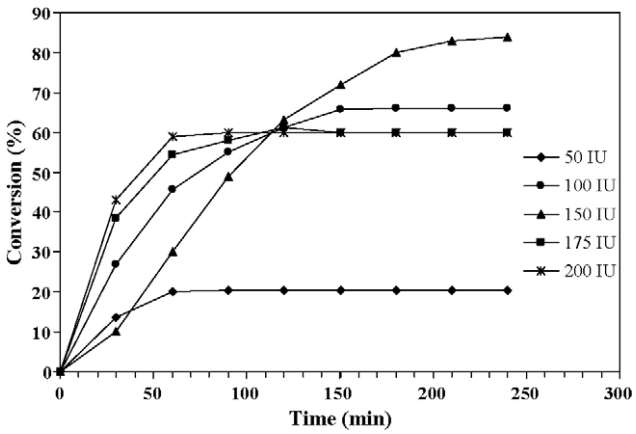

Fig. 13. Effect of different amounts of immobilized ROL on the conversion yield during butyl oleate synthesis. Reaction conditions were oleic acid/butanol molar ratio of 1 at 37 °C and stirred at 220 rpm.

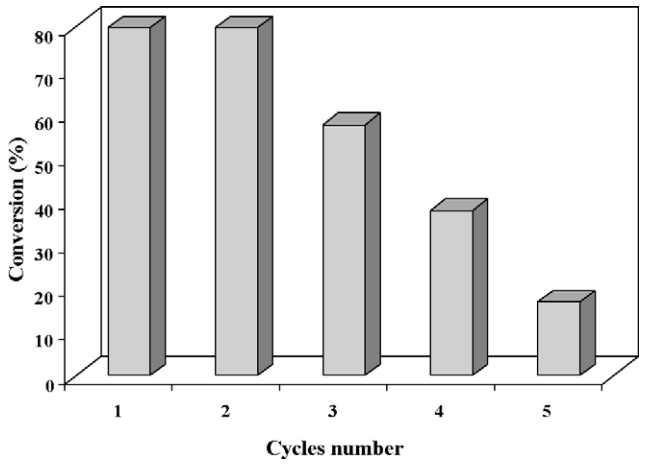

Fig. 14. Effect of repeated use on the conversion percentage for the synthesis of 1-butyl oleate in *n*-hexane with the immobilized ROL. Reaction conditions were 150 IU of lipase, oleic acid/butanol molar ratio of 1 at 37 °C and stirred at 220 rpm.

first 100 min, and then the conversion yield seems to be stabilized at 60% conversion for 175 and 200 IU. In fact, we should expect an increase in both the esterification kinetic and the conversion yield with the raise in the immobilized amount of enzyme. Thus, at 175 and 200 IU we ought to observe a higher conversion yield than for 100 or 150 IU. This discrepancy may be explained by the fundamental role of water in the biocatalytic activity of immobilized lipase. Indeed, in this part of the work where the amount of immobilized lipase was varied by changing the weight of cellulose fibers, we have maintained the same level of added water, the substrate and the solvent. Other factors could intervene in addition to the water level. Further research regarding the effect of immobilized enzyme is under investigation taking into account both the enzyme and the level of added water.

3.8.4. Repeated use of immobilized ROL

From an economic point of view, the reuse of the enzyme constitutes the main advantage of the process of biocatalysts immobilization. To check this parameter, cellulose-adsorbed lipase was used in five subsequent cycles in the esterification reaction of oleic acid with *n*-butanol under the same experimental conditions as described above at a lipase amount of 150 IU. At the end of each batch, the immobilized lipase was removed from the reaction medium and washed with *n*-hexane in order to remove any substrate or product retained in the support. Then, the immobilized lipase was used again for another reaction cycle. Results are shown in Fig. 14.

On the contrary, native lipase could not be recovered easily, and thus could not be used repeatedly since it adsorbed water produced during the reaction and formed a gelatinous mud as a result of hydration.

We noted that the enzyme achieved two successive cycles with the same efficiency, and started to lose its activity during the third cycle. Within the fifth cycle only 25% of the initial catalytic activity is preserved. The reduction of the enzymatic activity is probably related to lipase desorption from the support or to the denaturation of lipase during the repeated use. The reuse of this same lipase immobilized on the carbonate of calcium has been made with success during the synthesis of butyl oleate [49].

4. Conclusion

This work focuses on the physical immobilization of ROL on oxidized cellulose fibers prepared by chemical oxidation of cellulose. The cellulose oxidation brings about carboxylic groups on the surface with an amount that could be controlled through the dosage of the oxidant reagent.

Study of the adsorption isotherms reveals that the lipase adsorption level increases with the raise in the amount of carboxylic groups on cellulose indicating that electrostatic interactions between the enzyme and the surface is the main driving force for the adsorption process. This hypothesis was further confirmed by electrokinetic study where the absolute values of the fibers ζ-potential increase significantly after the contact with the enzyme as a consequence of the charge neutralisation between enzyme and the substrate. Comparison between the XPS spectra of the cellulose substrate before and after the contact with the enzyme revealed an intensification of an N 1s peak attesting the presence of the enzyme on the surface.

Immobilization of the enzyme on cellulose substrate enhanced the tolerance of the enzyme to the temperature and pH. Moreover, the activity of the enzyme is preserved when the immobilized enzyme is kept in contact with apolar solvent-like hexane.

The catalytic activity for the esterification reaction was evaluated using oleic acid and butanol as reagents in hexane solvent. Results showed that the highest yield was obtained at 37 °C with a molar ratio of oleic acid to butanol 1:1 and 150 IU immobilized lipase. The system could be reused three times without a significant loss of the catalytic activity. Study is under progress to immobilize the lipase onto ultrafiltration membranes made of regenerated cellulose having previously submitted the lipase to smooth oxidation treatment to test it as a membrane bioreactor for esterification reaction.

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