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Esterification activity and stability of *Talaromyces thermophilus* lipase immobilized onto chitosan

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ABSTRACT

The *Talaromyces thermophilus* lipase (TTL) was immobilized by different methods namely adsorption, ionic binding and covalent coupling, using various carriers. Chitosan, pre-treated with glutaraldehyde, was selected as the most suitable support material preserving the catalytic activity almost intact and offering maximum immobilization capacity (76% and 91%, respectively). The chitosan-immobilized lipase could be reputably used for ten cycles with more than 80% of its initial hydrolytic activity. Shift in the optimal temperature from 50 to 60 °C and in the pH from 9.5 to 10, were observed for the immobilized lipase when compared to the free enzyme.

The catalytic esterification of oleic acid with 1-butanol has been carried out using hexane as organic solvent. A high performance synthesis of 1-butyl oleate was obtained (95% of conversion yield) at 60 °C with a molar ratio of 1:1 oleic acid to butanol and using 100 U (0.2 g) of immobilized lipase. The esterification product is analysed by GC/MS to confirm the conversion percentage calculated by titration.

1. Introduction

Lipases have gained a great interest in several biotechnology applications due to their broad specificity coupled with high enantio and regio selectivity. This interest arises from the ability of these enzymes to catalyze synthetic reactions occurring in non aqueous media. In fact, lipase have been employed for direct esterifications and transesterifications reactions in organic media to produce esters used as additives for a variety of perfumes and flavours [1], biosurfactants [2] and biofuels [3]. The main hurdle to the use of lipase for these applications is the cost of biocatalysts. One of the principal goals of immobilization procedures is to fix the maximum amount of enzymes by keeping maximum activity at the lowest cost. Lipase immobilization onto solid materials not only enhances the operational lifetime and stability of biocatalysts but also facilitates the recovery, reuse and continuous operation of lipases. It may also protect the enzyme from solvent denaturation and enhance the enzyme thermostability [4].

Several approaches have been reported for the immobilization of lipases, they consisted either on physical adsorption of the enzyme on a carrier material [5,6], its entrapment or microencapsulation in a solid support [7,8] or by covalent binding to a solid matrix [9,10]. Immobilization using covalent binding has been most widely studied. Covalent binding provides a powerful link between the lipase and its carrier matrix and allowed its reuse more often than other available immobilization methods such as adsorption and entrapment [11]. Inorganic and organic supports have been successfully used for the immobilization of enzymes. Although there is no universal support still suitable for all enzymes and all their applications, any material that is to be considered as enzyme support must fulfil some requirements: high affinity for proteins, availability of reactive functional groups, mechanical stability, rigidity, feasibility of regeneration, non toxicity and biodegrability [12]. However, the high cost of the materials that are commonly used for lipase immobilization (namely, silica-based carriers, acrylic resins, synthetic polymers, active membranes, exchange resins, etc.) as well as the technology necessary to apply fixation methods greatly increase the costs of biocatalysts. Some cheaper substitutes, exhibiting high activity and stability to the immobilized lipases, have been widely used, such as rice husk, rice straw [13], amberlite [14], chitin and chitosan [15].

Biotechnology production of esters from long chain fatty acids using immobilized lipases has recently received greater consideration over the traditional chemical synthetic methods. Among these esters, 1-butyl oleate is used as biodiesel, poly vinyl chloride plasticizer, water-resisting agent and hydraulic fluid [16,17]. The most desired characteristics of lipases used for this process, are its ability to utilize all mono, di, and triglycerides as well as the free fatty acids in transesterification, low product inhibition, high activity and yield in non-aqueous media, low reaction time, reusability of immobilized enzyme, temperature, solvent resistance and alcohol resistance [18,19]. Various lipases from different sources, immo-

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bilized on different carriers, have shown a range of yields with different oil substrates and different acyl acceptors. The most previously reported reactions of the 1-butyl oleate production have been performed by commercial lipases such as from *Candida rugosa* [20], *Rhizomucor miehei* [21,22] and *Pseudomonas fluorescens* [23,24].

A search for an ideal enzyme with more suitable properties remains important. In this present investigation, we are developed an appropriate method to immobilize a new purified lipase from a thermophilic fungus *Talaromyces thermophilus* (TTL). The stability of this immobilized lipase and its suitability for reuse were evaluated. Since the *T. thermophilus* fungus produces high stable xylanolytic activities, the production of fermentable sugar from lignocellulosic material waste was previously undertaken [25]. Further studies are currently under way to achieve the bioethanol production. This present contribution established another alternative exploring the lipolytic activity of this fungus for produce a biodiesel (butyl oleate) as a valuable starting material for further selective enzymatic synthesis of oleyl alkyl esters using different oil sources.

2. Materials and methods

2.1. Chemicals

Chitosan flaks form (having a deacetylation degree of 85% and a bulk density of 0.15–0.3 g/cm^3) was provided from Sigma, chitin (powder form) was obtained from Sigma, glutaraldehyde and Gelatin were from Amersham. Amberlite IRC-50 and Duolite C26 were provided from Rohm and Haas (France). DEAE-Sephadex was from Pharmacia. Arabic gum from Merck (D-6100 Darmstadt, Germany); oleic acid, hexane and 1-butanol were purchased from Prolabo (Paris, France); pH-stat (718 Stat Titrino) was from Metrohm (Herisau, Switzerland).

2.2. Microorganism and culture conditions

The present study reports on a newly isolated thermotolerant fungal strain isolated from a soil sample collected in the thermal station of El Hamma in the south of Tunisia. The fungal isolate was identified as *T. thermophilus* by CBS (Centraalbureau voor schimmelculturen, Holland Code reference: detail 274-2003). The *T. thermophilus* strain was also deposed in a national strain bank of Tunisia: Tunisian Collection of Microorganisms CTM10.103 (Centre of Biotechnology of Sfax, Tunisia) [25]. The *T. thermophilus* was cultivated in a modified liquid Mandels medium [26]. The basal medium contained KH_2PO_4 1 g/l, K_2HPO_4 2.5 g/l, $(NH_4)_2SO_4$ 1.4 g/l, $MgSO_4{\cdot}7H_2O$ 0.3 g/l, $CaCl_2$ 0.3 g/l, yeast extract 1 g/l, urea 0.7 g/l, Tween 80 1 ml/l and 1 ml/l of an oligoelement solution ($MnSO_4$: 1.6 g/l, $ZnSO_4$: 1.4 g/l, $FeSO_4$: 5 g/l, $CoCl_2$: 2 g/l). Wheat bran at 2% was added to one litre of this medium, as a carbon source.

The enzyme production was carried out in 500 ml flasks containing 100 ml of culture medium that was incubated at 50 °C at an agitation rate of 160 rpm for 4 days.

2.3. Enzyme preparation protocol

The extracellular proteins were recovered by centrifugation and the supernatant was treated with ammonium sulfate (60% saturation). The precipitate was collected by centrifugation at 9000 rpm for 30 min, dissolved in buffer A (20 mM Tris–HCl, pH 8.5; 20 mM NaCl and 1 mM benzamidine). Insoluble material was removed by centrifugation at 9000 rpm during 10 min. The enzyme solution was loaded on a column (3 × 160 cm) of gel filtration Sephacryl S-200 equilibrated with buffer A.

The elution of lipase was performed with the same buffer at a rate of 45 ml h^{-1}. The fractions containing the lipase activity (eluted at 1.7 void volume) were pooled, concentrated and injected to the FPLC column Mono-Q Sepharose (trimethylammonium anion exchange) equilibrated in buffer A. The column (1 cm × 10 cm) was rinsed with 40 ml of the same buffer. Adsorbed material was eluted with a linear gradient of 100–400 mM NaCl in the same buffer at a rate of 120 ml h^{-1}. TTL activity was eluted at 220 mM NaCl. The highly active lipase fractions were pooled, concentrated and used as purified enzyme for subsequent studies. The purified enzyme has a high specific activity reached 9800 ± 139 U mg^{-1} using olive oil emulsion as substrate in the presence of 2 mM $CaCl_2$ at pH 9.5 and 50 °C [27].

2.4. Lipase immobilization

T. thermophilus lipase was immobilized by different methods on different supports. The immobilization steps and enzyme storage were carried out at 4 °C. The supernatants and washing volumes were pooled after each step and non immobilized activity was determined.

2.4.1. Adsorption

Adsorption followed by precipitation ($CaCO_3$): The enzyme immobilization was made onto $CaCO_3$ according to Rosu et al. [28] with a slight modification. A support powder (1 g) was added to 2 ml of enzymatic solution (285 U/ml, 29 μg/ml). The mixture was incubated 1 h at 4 °C under mild agitation. Afterwards, 10 ml of chilled acetone were added and the suspension was filtered through a Buchner funnel. The preparation of immobilized lipase was washed two times with 20 mM Tris–HCl buffer, centrifuged for 2 min at 4500 × *g*, dried at room temperature and stored at 4 °C until use.

Ionic binding (Amerlite IRC-50, Duolite, DEAE-Sephadex)

- The Amberlite IRC-50 or Duolite were swollen in distilled water, and equilibrated with 20 mM Tris–HCl buffer. One gram of each of the suction-filtered support material was mixed with 2 ml of enzyme solution (285 U/ml, 29 μg/ml). The mixture was intermittently stirred with a glass rod for 5 h at 4 °C. Then, it was washed twice with an equal volume of 20 mM Tris–HCl buffer, centrifuged for 2 min at 4500 × *g*, dried at room temperature and stored at 4 °C until use.
- 1 g resin DEAE-Sephadex was washed twice with 20 mM Tris–HCl buffer pH 9 and centrifuged for 2 min at 4500 × *g*. The resin was mixed with 2 ml of the enzyme preparation (285 U/ml, 29 μg/ml) and 2 ml Tris–HCl buffer for 20 min under agitation. The mixture was then washed twice with 20 mM Tris–HCl buffer and centrifuged for 2 min at 4500 × *g*.

2.4.2. Covalent coupling (chitosan, chitin and gelatin)

- The chitosan (0.5 g) was completely dissolved in 50 ml of 0.1 M HCl, after continuous agitation for 24 h at 30 °C. Then, a glutaraldehyde solution of 1.5% (v/v) was added for 2 h at the same temperature. The solubilized chitosan was precipitated by the addition of 1 ml NaOH (1 M). The precipitate was separated by centrifugation (10 min at 4500 × *g*) and washed with distilled water to remove excess of glutaraldehyde, which did not react with the active groups of the support, to ovoid the reticulation of the enzymes molecules; affecting therefore their activities [29].

The wet chitosan was mixed with 1 ml (285 U/ml) of the enzyme solution and stirred at 4 °C for 24 h. The unbound enzyme was removed by washing with Tris–HCl buffer 20 mM until no protein or activity was detected [30]. This protocol was repeated in the presence of different glutaraldehyde concentrations (0.5–3%) (v/v) to investigate the latter's effect on the immobilization and activity yields of the enzyme.

- Chitin (0.5 g) was shaken with 5 ml 2.5% (v/v) glutaraldehyde. It was then collected by centrifugation (10 min at 4500 × *g*) and washed with distilled water to remove excess of glutaraldehyde. The wet chitin was mixed with 2.0 ml of the enzyme solution at 4 °C for 24 h. The unbound enzyme was removed by washing with Tris–HCl buffer 20 mM as described earlier [30].
- The gelatin powder (5%, w/v) was swelled in 5 ml of Tris–HCl buffer (50 mM, pH 9.0) and heated at 50 °C for 10 min to ensure its complete solubilization. The mixture was then cooled and the enzyme solution was added. After the thorough mixing of the enzyme, the required amount of organic cross-linker (0.6%, w/v) glutaraldehyde was added. The mixture was constantly stirred and then poured on a (5 cm × 5 cm) glass plate to prepare a thin film of the enzyme. The film was stored at 4 °C for complete cross-linking. The immobilized enzyme film was thoroughly washed with 20 mM Tris–HCl buffer (pH 9.0) and cut into small blocks before being used in subsequent experiments.

The immobilization was estimated as following:

$$\text{Immobilization yield}\,(\%) = \frac{\mathbf{A}-\mathbf{B}}{\mathbf{A}} \times 100$$

$$\text{Activity yield}\,(\%) = \left(\frac{\mathbf{C}}{\mathbf{A}}\right) \times 100$$

with **A** is the total enzyme activity used for immobilization; **B** is the unbound enzyme activity; **A** − **B** is the theoretical immobilized enzyme activity; and **C** is the obtained immobilized enzyme activity. The total enzyme activity is the total number of units added to the support during the immobilization reaction; the non-immobilized activity is the number of units found in filtrates and washing volumes after immobilization; and the immobilized activity is the number of units detected in the support after immobilization and washing.

2.5. Lipase activity determination

Lipase activity was assayed potentiometrically by automatically titrating the free fatty acids released from mechanically stirred triglyceride emulsions at pH 9.5 and 50 °C, using 0.1 N NaOH and a pH-stat device (718 Stat Titrino, Metrohm, Switzerland). The triglycerides were added directly to the pH-stat vessel containing the assay solution and were emulsified by mechanical stirring. Long-chain triglycerides (e.g. olive oil) had first to be pre-emulsified with gum arabic (GA) by mixing 5 ml of olive oil with 45 ml of a 10% (w/v) GA solution prepared as previously described [31]. Ten ml of this olive oil-GA emulsion were then mixed in the pH-stat vessel with 30 ml of a solution containing 20 mM Tris–HCl, 2 mM $CaCl_2$ [32].

The determination of immobilized lipase activity was initiated by the addition of 0.1 g of support containing the immobilized enzyme to the substrate. One unit of lipase activity corresponds to 1 μmol of fatty acid released per minute under the assay conditions used.

2.6. Protein assay

The protein content in the free enzyme or immobilized enzyme preparations was determined by the Bradford method, using bovine serum albumin (BSA) as the standard (the protein is, therefore, expressed in BSA equivalents) [33]. The amount of protein bounded onto the support was determined indirectly from the difference between the initial total protein exposed to the supports and the amount of protein recovered in the wash. The loading percentage of proteins was calculated as following:

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

2.7. Scanning electron microscopy (SEM)

A Baltec Critical Point Dryer 30 field emission scanning electron microscope (Balzers Union, Germany) equipped with a secondary electron detector and energy dispersive X-ray microanalysis (EDX) was used to examine the surface morphology of the carrier before and after the immobilization of TTL on chitosan. The samples were sputter-coated with gold prior to analysis.

2.8. Characterization of immobilized lipase

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

The effect of pH on free and immobilized lipase activities was studied in the pH range 7.5–11 at 50 °C using olive oil as substrate. Each measurement was performed three times and standard error was included.

The enzyme stability was determined by incubation of the immobilized and free lipase at different pH values ranging from 3 to 11 for 24 h at 4 °C using the following 50 mM buffers systems: Na_2HPO_4/citrate, pH 3.0–5.0; KH_2PO_4/K_2HPO_4, pH 6.0–7.0; 3-N-(α,α-dimethylhydroxyethyl)-amino-2-hydroxypropanesulfonic acid (AMPSO), pH 8–9; and glycine, pH 10–11 and their residual activities were determined at the optimum pH (9.5 for the free enzyme and 10.0 for the immobilized enzyme). Each measurement was performed three times and standard error was included.

2.8.2. 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 TTL was determined at various temperature values ranging from 30 to 70 °C. Their relative activities were determined at pH 9.5 for the free lipase and at pH 10.0 for the immobilized lipase, using olive oil emulsion as substrate. Each measurement was performed three times and standard error was included.

The thermal stability assays were performed by incubation of the immobilized and free forms of TTL at different temperatures (30–70 °C) for 1 h, cooled down to room temperature and their residual activities were measured under optimal conditions (pH 9.5 and 50 °C for the free lipase; pH 10.0 and 60 °C for the immobilized lipase). Each measurement was performed three times and standard error was included.

2.9. Reusability of immobilized lipase

For the reusability, after each reaction run, the immobilized lipase preparation was removed and washed with 20 mM Tris–HCl buffer to remove any residual substrate. It was then reintroduced into fresh reaction medium to determine enzyme activity. The experiment was performed three times and standard error was included.

2.10. Esterification assay

The reactions were carried out in screw-capped flasks containing 1 g of oleic acid and butanol taken at various molar ratios, in the presence of 4 ml of anhydrous hexane. Different amounts of chitosan immobilized lipase (50 U in 0.1 g); (100 U in 0.2 g); (200 U in 0.4 g) and (300 U in 0.6 g) and different levels of added water (0%, 2%, 5% and 7%) were tested. The reaction mixture was incubated at

Table 1
Variation in immobilization and activity yields of lipase as function of resin used.

Type of resin	$CaCO_3$	Amerlite	Duolite	DEAE-Sephadex	Chitin	Chitosan	Gelatin
Activity yield (%)	15.0	24.0	20.0	48.0	65.0	76.0	56.0
Activity (U/g carrier)	85.5	137	114	274	371	433	319
Immobilization yield (%)	ND	32.0	30.0	50.4	88.5	91.0	62.6
Immobilized proteins (%)	ND	31.0	29.6	46.0	66.2	70.0	51.0

60 °C in a shaking incubator at 200 rpm. Each experiment was twice repeated.

Aliquots of 200 μl were withdrawn periodically and centrifuged at 2000 rpm for 5 min. The supernatant residual acids contents were assayed by titration with 0.1 N sodium hydroxide, using phenolphthalein as indicator and 3 ml of ethanol as quenching agent. The yield of ester synthesis was calculated based on the amount of acid consumed.

Analyses were confirmed by GC/MS using a GC system equipped with a series 5975 B Insert MSD mass-selective detector (Agilent technologies, France). 2 μl portion of the organic phase was analysed after splitless injection employing a HP-5MS Phenyl Methyl Siloxane capillary column (30 m × 250 μm × 0.25 μm nominal, Agilent Technologies, France). Helium (constant flow, 1 ml min^{-1}) was used as a carrier gas. The temperatures of the injector and detector were 250 °C and 240 °C, respectively. The following temperature program was applied: 120 °C for 5 min, increase of 3 °C min^{-1} to 180 °C, increase of 10 °C min^{-1} to 220 °C, and 220 °C for 31 min. Data were evaluated using the NIST Mass Spectral Search program [34]. The conversion percentage calculated by both GC/MS analysis (which showed product formation) and titrimetry (which showed acid consumption) were found to be in good agreement.

3. Results and discussion

3.1. Study of immobilization support

The lipase from *T. thermophilus* (TTL) was immobilized on different matrix with various methods. Table 1 shows the immobilization and activity yields of the used matrix and the percentages of bound proteins.

The lipase adsorption followed by precipitation was carried out with a non-ionic carrier $CaCO_3$. This support carrier was previously described as a suitable adsorbent leading to high dispersion of the crude *Rhizopus oryzae* lipase and preserving the catalytic activity [17]. The same support was found to be appropriate for the *Pseudomonas SP KWI 56* [28]. In contrast to these results, the purified TTL adsorbed on $CaCO_3$ show a very low yield of the immobilized lipase activity. It has been reported that the presence of ammonium sulfate was needed to help the enzyme immobilization on $CaCO_3$ [17,35].

The enzyme adsorption by ionic binding was occurred by three binding resins: two carboxylic cation exchangers (Amberlite IRC-50 and Duolite C26) and an anionic exchanger (DEAE-Sephadex). The adsorption on Amberlite IRC-50 and Duolite C26 led to a poor yield of adsorbed lipase. It is known that the ionic adsorption is based on electrostatic interactions between differently charged ionic groups of the matrix and of the enzyme. Also, a weakly hydrogen bonds and Van Der Waals forces are formed during the adsorption. A repulsion force between the negative surface charge of the Amberlite IRC-50 and Duolite C26 carriers and the negative charged of the TTL occurred at the pH of the immobilization (pH 9), can lead to the leaching of enzyme weakly adsorbed, which explains the poor yield of the immobilized enzyme. This minimally adsorption was happened only due to the hydrophobic properties of these two carriers. The anionic DEAE-Sephadex resin performed a moderate enzyme adsorption. Further investigations to optimize the enzyme loadings and the contact time between enzyme solution and these supports need to be carried out.

Low yields of immobilized enzyme activity were obtained for all this binding resins, essentially for Amerlite IRC-50 and Duolite C26 carriers. This could imply that their surfaces are not physically inert towards lipase. Thus, interaction of lipases with these supports often leads to structural deformations and to reduced catalytic activity [36]. An additional factor would be a weak interaction between the lipase and the support. The adsorbed lipase was sensitive to the pH and the ionic strength variations and the protein might be stripped off from the carrier.

It has been found in many examples that the immobilization of lipases on hydrophobic supports permits to get a higher activity recovery of the immobilized preparations [37]. In fact, these supports involving the adsorption of the hydrophobic areas surrounding the active site and leaving stabilized the lid-opened form of the lipase [37]. However, if the substrate is very large (e.g. olive oil), the near presence of the hydrophobic support surface may generate steric hindrances, reducing largely the activity of the lipase [38].

Covalent coupling was more advantageous than other, since diffusional restrictions of substrates and products are decreased considerably. On the other hand, multipoint covalent liaison via short spacer arms promotes the stabilization of the immobilized structure enzyme. In fact, the relative distances among all residues involved in the covalent immobilization have to be unaltered during any conformation change induced by any distorting agent (heat, extreme pH values, organic solvents, ...) [38]. This also should reduce the steric hindrance which restraining the access of the substrate to the lipase active site [39]. TTL was therefore covalently coupled to chitin, chitosan and gelatin, through cross-linking mediated by glutaraldehyde. Compared to chitin and gelatin, chitosan provided the highest activity (433 U/g chitosan) and immobilization yields (76%). Chitosan is a cheap support material since it was obtained from prawn's shells. Moreover, this polyaminosaccharide was recognized by excellent properties for lipase support, such as biocompatibility, biodegradability, physiological inertness, hydrophilic character and great affinity for proteins [15].

Furthermore, this support was selected as a suitable adsorbent leading to high dispersion of the TTL in the support and preserving the catalytic activity. Magnin et al. (2001), reported the immobilization of *C. rugosa* lipase into porous chitosan beads; they found that immobilization enhanced the lipase activity and its tolerance to organic solvents [40].

A possible explanation for the low loss of TTL activity is the potential for glutaraldehyde reactive group to link protein through amino groups of lysines existing in the lid (data not shown). This interaction may generate some steric hindrances, avoid the displacement of the lid and then reducing the catalytic action of the lipase.

3.2. Influence of glutaraldehyde concentration on the immobilization and activity yields of lipase

Glutaraldehyde is a bifunctional crosslinker commonly used to couple components with amino functional groups. However, glutaraldehyde is toxic and causes the denaturation of immobilized

Table 2
Effect of glutaraldehyde concentration on immobilization and activity yields of lipase.

Glutaraldehyde (GA) concentration (%)	0.5	1	1.5	2	2.5	3
Activity yield (%)	57	70	77	65	43	37.5
Immobilization yield (%)	68	84	90	87	82	70.6

enzymes [41]. Therefore, the effects of glutaraldehyde concentration on the enzyme's immobilization and activity yields were investigated (Table 2). The chitosan support was prepared in the presence of different glutaraldehyde concentrations (0.5–3%) (v/v) and the immobilization and activity yields were measured. Results showed that a glutaraldehyde concentration of 1.5% (v/v) is optimal for lipase recovery reaching 77% of the yield activity. Similar result was observed for the immobilization of lipase on the polysulfone membrane surface [42]. At higher glutaraldehyde concentrations, more protein is bond; but with lower activity. This may be due to the steric hindrance occasioned by the increased level of polymerization in the enzyme-support matrix which impedes the access of the substrate to the active site of the immobilized enzyme [43].

3.3. Morphology by scanning electron micrograph (SEM)

The morphology of the chitosan pre-treated with glutaraldehyde before and after the immobilization was examined. We observed that the chitosan activated by glutaraldehyde has a large surface area (Fig. 1A). After lipase immobilization, the support surface area was filled with rounded structures, which were likely to be protein aggregates (Fig. 1B). The flat chitosan surface seems to provide a large contact area for a multipoint attachment with the enzyme. This observation may offer an explanation for the high yield of the *T. thermophilus* lipase immobilization on this support. Same results were also observed by Foresti and Ferreira after immobilization of *C. rugosa* lipase onto chitosan [12].

3.4. pH stability and optimum pH of immobilized lipase

The effect of pH on the activity of both free and chitosan immobilized lipase on olive oil hydrolysis was determined in the pH range of 7.5–11 and the results were presented in Fig. 2A. The pH profile seems to be identical for the free and the immobilized enzyme. Furthermore, the optimum pH shifted from pH 9.5, which was the optimum for the free enzyme, to more alkaline range (pH 10).

The retained activity of the immobilized enzyme was improved both at lower and higher pH in comparison to the free enzyme, showing that the immobilization methods preserved the enzyme activity over a wider pH range. Lipase of *Candida lipolytica* immobilized on silanized palygorskite support by covalent bonding retained nearly 100% of activity within a pH range of 6.5–8 whereas it loses more than 87% of its activity at pH 6.5 in its free state [44]. Similarly, the lipase from *C. rugosa* covalently immobilized onto polyacrylonitrile nanofibers was less sensitive to pH changes exhibiting the same optimum pH than the free lipase [44].

The stability of the free and immobilized TTL was compared at pH ranging between 3.0 and 11.0 at 4 °C during 24 h of incubation. At different pH conditions, the immobilized lipase was more stable than the free enzyme (Fig. 2B). At acid pH (3.0 and 5.0) the immobilized lipase lost 45% of its residual activity, whereas in its free state this loss was twice higher. This could be attributed to the ability of the micro-environment, created between the support and the immobilized enzyme, to protect the latter from the denaturation caused by the change in pH. This stabilizing effect in acidic and alkaline media has been previously reported for *C. lipolytica* immobilized lipase on silanized palygorskite [45]. Then, we can conclude that the chitosan support has a special importance of this immobilization technique in the lipase stabilization at different ranges of pH.

3.5. Thermal stability and optimal temperature of immobilized lipase

The temperature dependence of the activity of the soluble and immobilized lipase was studied (at pH 9.5 for the free enzyme and pH 10.0 for the immobilized enzyme). The free and the immobilized enzyme exhibited different temperature profiles with a shift of optimal reaction temperature from 50 to 60 °C (Fig. 2C). A similar shift of 10 °C was also reported for *C. rugosa* lipase immobilized on styrene divinylbenzene copolymer [46].

Thermal stability of the free and the immobilized lipase was followed after their incubation at different temperatures ranging from 30 to 70 °C for 60 min. As seen in Fig. 2D, the immobilized lipase was much more stable than the free enzyme particularly when the temperature exceeded 50 °C. Thus, after heat treatment for 60 min at 70 °C, more than 50% drop of the initial activity is observed for the free lipase whereas it attains only 22% for the immobilized enzyme. This result clearly demonstrates the efficiency of the immobilization method in the enzyme protecting against heat inactivation. It could be the consequence of conformational limitations of the enzyme movements due to multi-point attachment and it is in agreement with other reported works [47,48]. The better thermal stability of the immobilized will extend the potential application of the lipase as a biocatalyst.

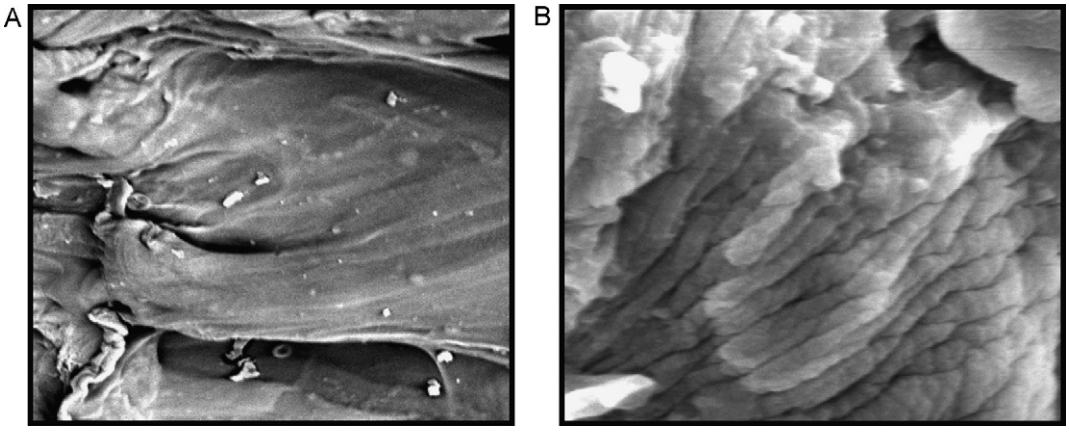

Fig. 1. Scanning electron micrographs of the chitosan (A) before (950×) and (B) after the immobilization of TTL (1200×).

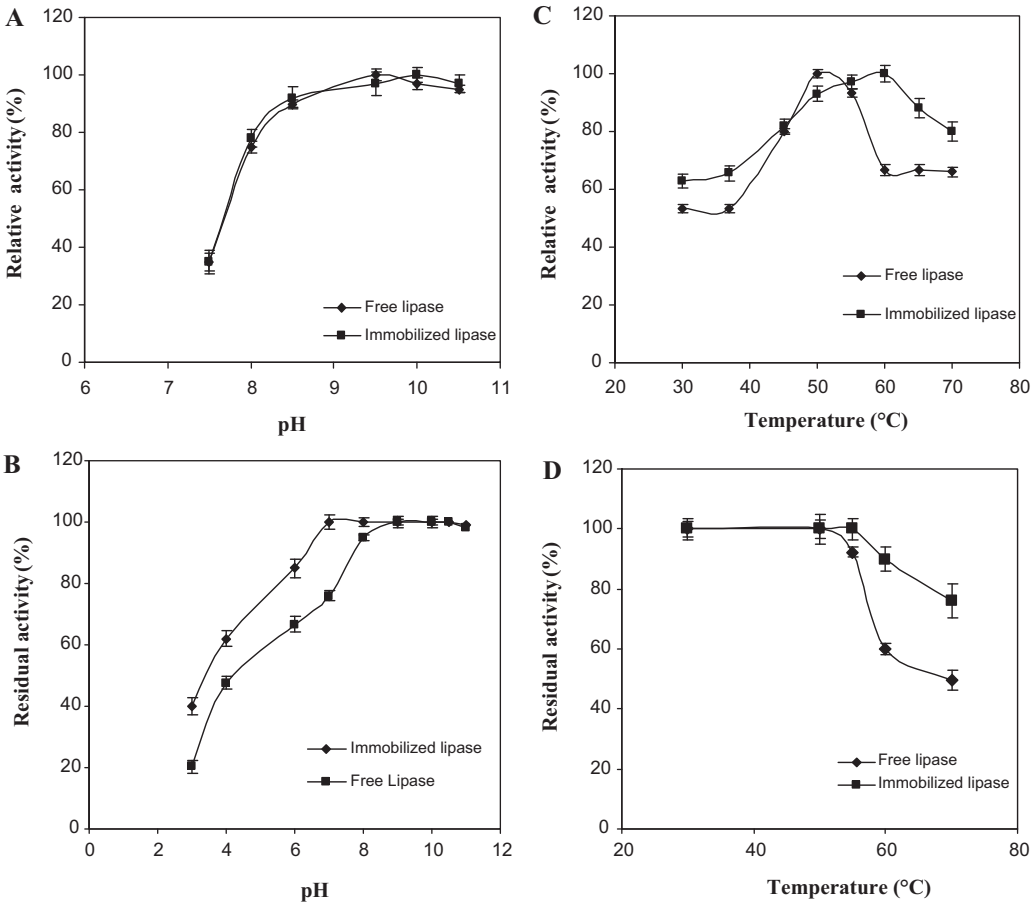

Fig. 2. (A) Effect of pH on free (♦) and immobilized (■) *T. thermophilus* lipase activities. Enzymes were assayed with olive oil emulsion as substrate at 50 °C. (B) The effect of pH on the stability of free (♦) and immobilized (■) *T. thermophilus* lipase. Enzymes were assayed with olive oil emulsion as substrate at 50 °C. Bars indicate standard deviation. (C) Effect of temperature on free (♦) and immobilized (■) lipase activities. Enzymes were assayed with olive oil emulsion as substrate at pH 9.5 and pH 10, respectively. (D) Thermal stability of free (♦) and immobilized (■) TTL. Enzymes were assayed, after incubation for 1 h at different temperatures, with olive oil emulsion as substrate at optima conditions. Bars indicate standard deviation.

3.6. Reusability

Chitosan immobilized lipase was tested repeatedly to hydrolyze olive oil at 60 °C and the reusability was examined because of its importance in batch and in continuous reactor for bioconversion processes.

As shown in Fig. 4, the immobilized enzyme displayed a good reusability, since it retained more than 80% of its initial hydrolytic activity in reuse up to 10 cycles. This finding is of a great interest, as it would allow the set-up of a bioreactor for continuous application.

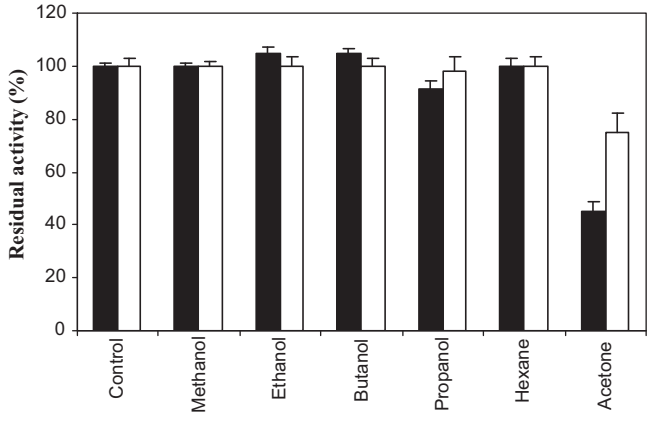

Fig. 3. Effect of organic solvents on the activity of immobilized TTL. Enzyme activities were assayed, after 2 h of incubation with different solvents, using olive oil as substrate at pH 10.0 and 60 °C. The experiments were conducted three times and standard errors are reported.

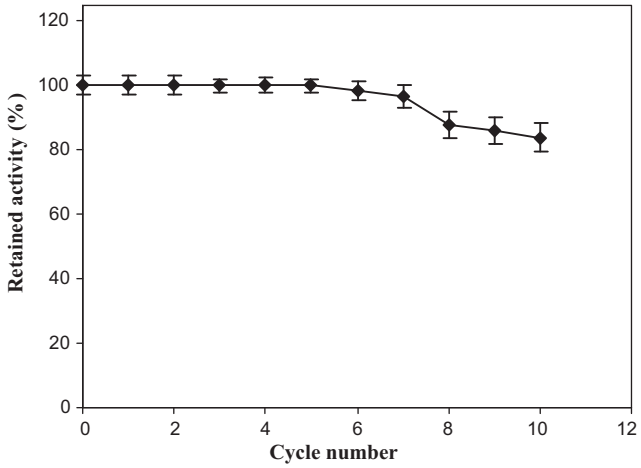

Fig. 4. Effect of repeated use on the olive oil hydrolysis by the immobilized TTL at 60 °C and pH 10.0. The experiments were conducted three times and standard errors are reported.

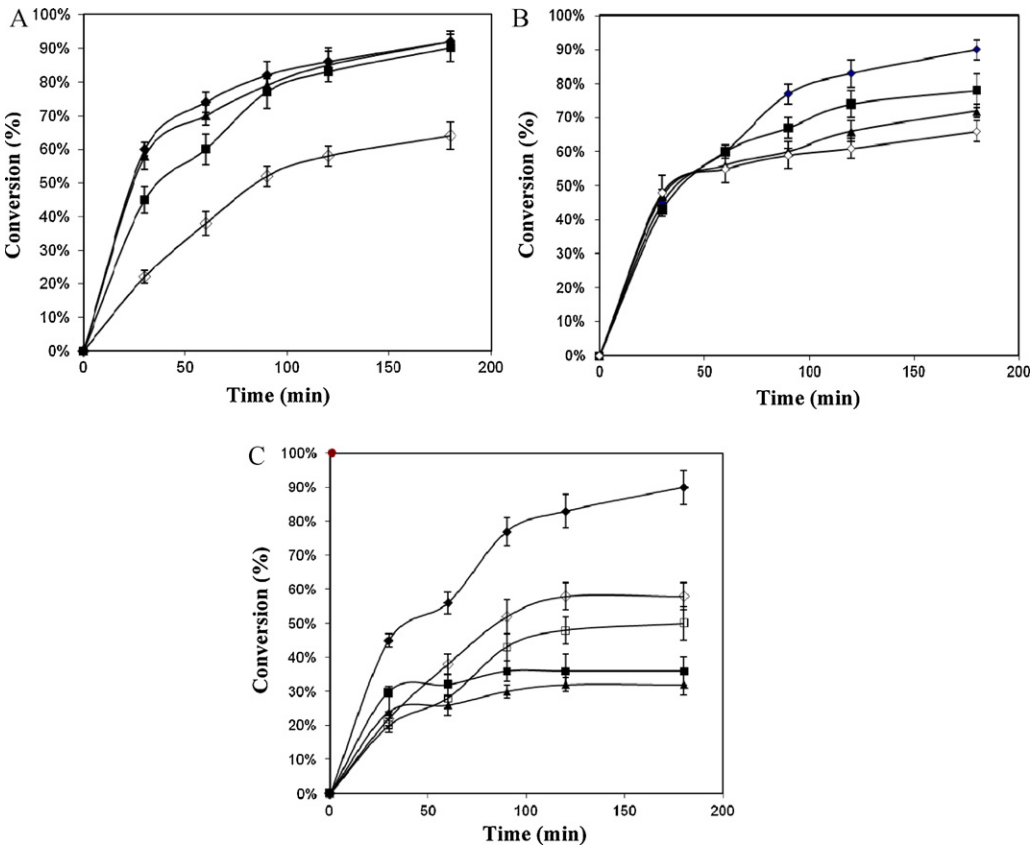

Fig. 5. (A) Effect of different amounts of immobilized TTL: (◊) 50 U, (■) 100 U, (▲) 200 U and (♦) 300 U, on the conversion yield during butyl oleate synthesis. Reaction conditions were oleic acid/butanol molar ratio of 1/1 at 60 °C and stirred at 250 rpm. Bars indicate standard deviation. (B) Effect of different initial added water: (♦) 0%, (■) 2%, (▲) 5% and (◊) 7%, on the conversion yield during butyl oleate synthesis. Reaction conditions were 100 U of lipase, an oleic acid/butanol molar ratio of 1/1 at 60 °C and stirred at 250 rpm. Bars indicate standard deviation. (C) Effect of different oleic acid/butanol molar ratios (*R*): (♦) 1/1, (■) 1/2, (▲) 1/3, (◊) 2/1 and (□) 3/1, on the conversion yield during butyl oleate synthesis. Reaction conditions were 100 U of lipase; 0% added water at 60 °C and stirred at 250 rpm. Bars indicate standard deviation.

Similar trends have been reported with *C. rugosa* lipase covalently immobilized on poly(glycidylmethacrylate-methylmethacrylate) magnetic beads [49].

3.7. Effect of solvent on lipase stability

The use of enzymes in organic medium is of great importance as it allows the occurrence of esterification reactions which are difficult to occur in presence of a high amount of water.

However, it is well known that enzyme activity could be strongly affected by the organic solvent which may bring about the denaturation of the enzyme thus leading to the loss of the catalytic activity. The activity of the immobilized lipase was monitored in different organic solvents varying from polar to non-polar. Among the six tested solvents, immobilized TTL has a high tolerance for water-immiscible organic solvents such as hexane, for which the activity is maintained during long time incubation. Interestingly, the immobilized TTL showed a high stability in the presence of water-miscible organic solvents, since it retained almost 100% activity after exposure, for 2 h at room temperature, to 40% of butanol, methanol, ethanol, 2-propanol or acetone (Fig. 3). The loss of 25% of immobilized TTL activity after its exposure to acetone for 120 min could be due to the fact that this more polar solvent could strip off the essential water layer around the enzyme and thus distort the catalytic conformation of the enzyme [50].

3.8. Esterification activity of the immobilized TTL

The ability of the chitosan-immobilized TTL to catalyze the synthesis of esters was investigated by taking the synthesis of butyl oleate, carried out in hexane at 60 °C, as reaction model. The influence of three main esterification parameters was tested.

3.8.1. Effect of amount of lipase

The influence of enzyme amounts on the esterification reaction of oleic acid and butanol was revealed in Fig. 5A. The percentage conversion increased with the increase in lipase amount and remains constant with an enzyme load of 100 U (0.2 g). The excess in enzyme did not contribute to the increase of the conversion yield. At saturation point, all the substrates are bound to the enzyme and added enzyme molecule could not find any substrate to serve as a reactant. This was reported for the esterification of oleic acid catalyzed by *R. oryzae* lipase [51]. According to Bloomer et al. [52] the amount of enzyme would influence the total reaction time, which is required to achieve desired conversion in esterification reaction.

3.8.2. Effect of initial addition of water

Water plays an essential role in the lipase-catalyzed esterifications. It is a well-known fact that water content affects the equilibrium of esterification/hydrolysis reactions as well as the distribution of products in the media as the result of water acting as a substrate [53]. Although the accurate amount of water for a given enzymatic reaction depends on system components (enzyme, solid support and solvent). Generally, it is reported that the optimum level of added water is within the range of 0.2–3% based on dry enzyme [54].

The effect of initial water content on enzymatic activity was examined through the addition of water ranging from 0% to 10% (w/w) of the total amount of reaction mixture. The synthesis in

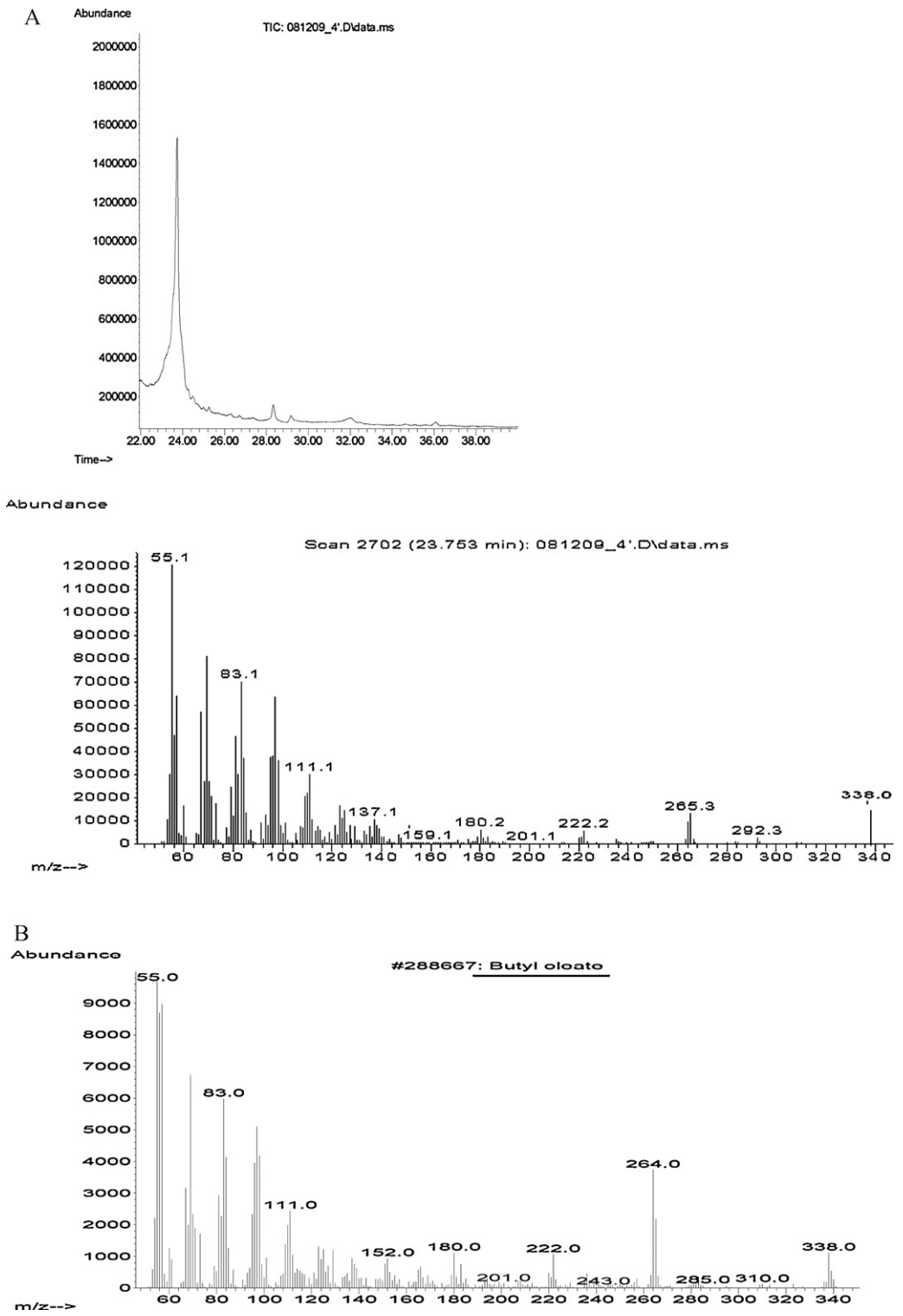

Fig. 6. GC/MS analyses of butyl ester obtained by esterification of oleic acid using *T. thermophilus* lipase. (A) Chromatographic profile of butyl oleate after 180 min of esterification reaction. (B) Mass spectrum of butyl oleate compared to the GC/MS library.

hexane was carried out with 100 U (0.2 g) of immobilized lipase. As shown in Fig. 5B, the conversion percentage was relatively higher without initial added water. This may be due to the fact that water already exist in substrates and enzyme preparation, as well as the water produced during the esterification reaction was sufficient to moist the enzyme and to render it active. The high hydrophilicity of chitosan may explain why the conversion percentage is high without addition of water. In fact, when water is added, the support becomes more hydrated and so was the microenvironment of the immobilized lipase. As a consequence, the esterification reaction was thermodynamically disfavoured with respect to the hydrolysis reaction.

3.8.3. *Effect of molar ratio of substrates*

The last important variables affecting the conversion percentage in esterification reactions is the relative proportions of the

substrates. The esterification was carried out with various molar ratio oleic acid/butanol (1:1, 1:2, 1:3, 2:1 and 3:1) at the conditions described above. Results depicted in Fig. 5C show that the highest conversion yield was obtained at a stoichiometric ratio close to 1:1 between oleic acid and butanol after 180 min of reaction. The conversion yield decrease gradually with the increase in acid or alcohol. On the one hand, this observation may reflect the ability of the alcohol excess to distort the essential water layer from lipase, which disrupts the conformation of the protein structure. On the other hand, the presence of an excess of substrates can lead to the competitive binding of either acid or alcohol to the immobilized lipase due to the hydrophilic character of the chitosan matrix that favours the accumulation of substrate molecules with their terminal polar group pointed toward the surface. Thus, the excess of one of the reagents will hinder the interaction frequency between substrates and the immobilized lipase [55].

With respect to the immobilized lipases previously employed for biodiesel production, the performance of the chitosan-immobilized TTL in this contribution is promising, since it showed a high activity toward butyl oleate production with a yield of 95% obtained at a short esterification time (180 min) and with a low cost. Most workers have needed much longer times in order to obtain high ester yields. For instance, 350 min were required for an 80% yield of butyl-oleate, using an immobilized lipase of *R. oryzae* [17]. Even longer times have been reported using the *Thermomyces lanuginosus* lipase immobilized by covalent attachment onto glutaraldehyde activated styrene-divinylbenzene copolymer for a 97% yield of biodiesel at 24 h [56]. There are two kinds of lipase used most frequently, especially for large scale industrialization. One is the *Candida antartica* lipase immobilized on acrylic resin, known by its commercial name Novozym 435, which can catalyze the esterification of vegetable oils with a yield higher than 90% at 500 h [57]. The other is the *Candida* sp. 99–125 lipase immobilized on cheap textile membrane, which can catalyze the esterification of various vegetable oils with yield higher than 87% at 210 h [58].

3.9. GC/MS analysis of reaction products

To deep furthermore investigate the finding obtained by titration assay, the esterification product is analysed by GC/MS (Fig. 6A). The mass spectrum is in agreement with a butyl oleate molecule ($C_{22}H_{42}O_2$) (Fig. 6B).

4. Conclusion

We have shown that the covalent binding to chitosan through glutaraldehyde was the best method for *T. thermophilus* lipase immobilization. This strategy combines the excellent properties of chitosan as carrier with an advantageous low cost source for industrial use. Immobilization of *T. thermophilus* lipase on chitosan enhanced its tolerance to the temperature and pH and offers therefore an interesting operational stability.

The catalytic activity for the esterification reaction was evaluated using oleic acid and butanol as reagents in hexane solvent. The highest yield of conversion was obtained with a molar ratio of oleic acid to butanol 1:1 and 100 U (0.2 g) of immobilized lipase. This certainly prompts further investigations on continuous bioreactor for esterifications reaction on a large scale.

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