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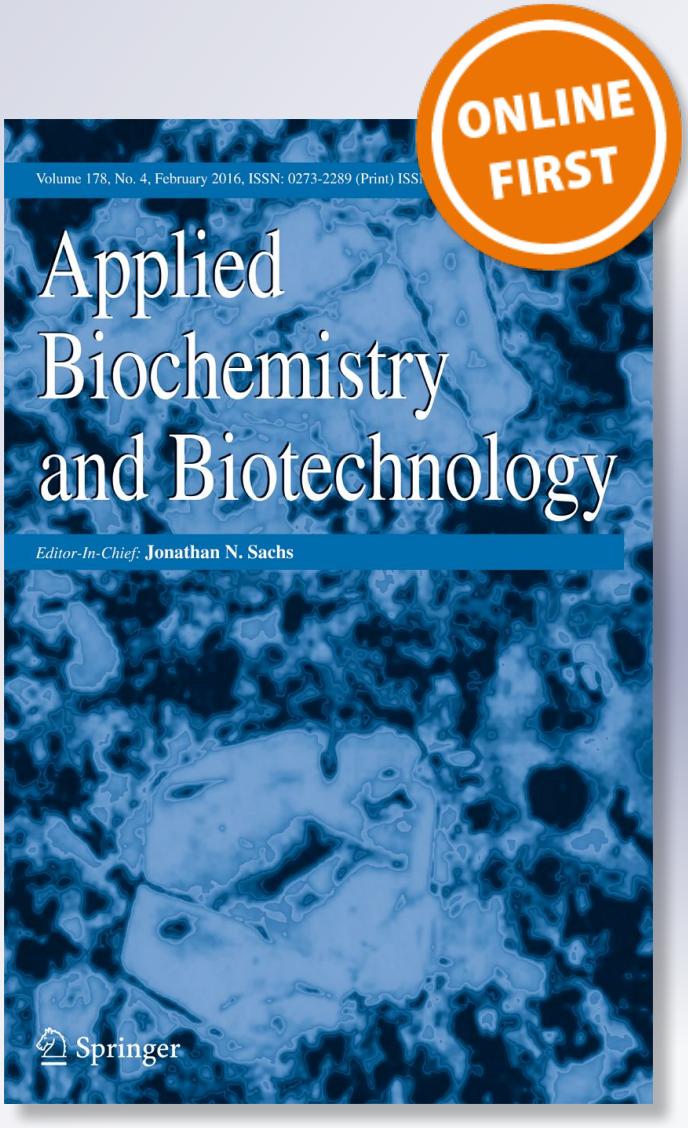

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Operational and Thermal Stability Analysis of *Thermomyces lanuginosus* Lipase Covalently Immobilized onto Modified Chitosan Supports

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Abstract The aim of this paper was to evaluate different strategies of chitosan activation using cross-linking reagent like glycidol, epichlorohydrin, and glutaraldehyde for *Thermomyces lanuginosus* lipase (TLL) immobilization. Operational activity and stability by esterification of oleic acid with ethanol and thermal inactivation using these derivatives were investigated. Derivative obtained by sequentially activation with glycidol, ethylenediamine, and glutaraldehyde and subsequent TLL immobilization showed the best performance, with high hydrolytic activity value. Its stability was 15-fold higher than solubilized TLL in the evaluated inactivation conditions (60 °C, 25 mM sodium phosphate buffer pH 7). After 5 cycles of oleic acid

Chemical compounds used in this article
Chitosan (PubChem CID: 71853)Epichlorohydrin (PubChem CID: 7835)Glycidol (PubChem CID: 11164)Ethylenediamine (PubChem CID: 3301)p-Nitrophenyl butyrate (PubChem CID: 75834)

Highlights
✓ *Thermomyces lanuginosus* lipase was immobilized on activated chitosan.
✓ The best activation strategy involved glycidol, ethylenediamine, and glutaraldehyde.
✓ The stability of the best derivative was 15-fold higher than solubilized TLL.
✓ More than 90% of oleic acid conversion was reached after 12 h.
✓ The best derivative lost only 18% activity after 5 cycles of oleic acid esterification.

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esterification, only a few percentage of its conversion has reduced. On the other hand, glycidol-activated chitosan derivative showed very low hydrolytic activity value. Epichlorohydrin-activated chitosan derivative showed regular hydrolytic activity value. Both derivatives showed low immobilization yields. Operational stability of this last derivative was very low, where after the first cycle of oleic acid esterification, only 56% of its initial conversion was obtained.

Keywords Lipase · *Thermomyces lanuginosus* · Immobilization · Chitosan · Esterification · Biodiesel

Introduction

Lipases (EC 3.1.1.3, triacylglycerol acylhydrolases) represent a group of water soluble enzymes that originally catalyze the hydrolysis of ester bonds of insoluble lipid substrates by means of water, acting at the interface between the aqueous and the organic phases. However, under appropriate experimental conditions, these enzymes are also very active biocatalysts for the esterification of fatty acids including alcoholysis, interesterification, transesterification, acidolysis, and amynolysis reactions and, in some cases, may possess enantioselective properties [1], which confer a wide spectrum of biotechnological applications. They are known to be strongly hydrophobic due to the presence of the alkyl groups on the surface of their structure [2]. Some amino acids of lipases form an amphiphilic peptide loop that acts like a lid that covers the active site in aqueous solution (in the absence of an interphase or organic solvent). However, at the lipid–water interface, this lid undergoes conformational change moving away from the active site and thus turning the "closed" form (inactive) of the enzyme into an "open" form (active) with the active site now accessible to the substrate. Thus, the conformational state of the lid and the hinge regions determines the enzyme in an active or inactive conformation [3].

Different strategies have been used to improve enzyme performance: synthesis of enzyme-polymer conjugates [4–6], use of organic and another non-conventional media [7–11], enzyme immobilization [12], among others. In the literature, lipases have been widely used immobilized on different supports due to its high thermal stability as well as availability and specificity, but only a few were immobilized on chitosan [13, 14].

In this work, chitosan has been chosen as the support for lipase immobilization. It is a linear polymer being easily obtained by *N*-acetylation reaction of chitin. Hence, it is a highly available and cheap biomaterial mainly obtained as a waste from shellfish industries. Depending on the degree of deacetylation, chitosan contains from 5 to 8% (*w*/*v*) nitrogen in form of primary aliphatic amino groups that undergo the typical reactions of amines being *N*-acylation and Schiff base formation the most relevant reactions [15]. Furthermore, chitosan was used extensively in many fields due to their versatile biological activities and chemical applications [16].

Three strategies of activation were evaluated on chitosan matrix for the standard immobilization of the enzyme lipase from *Thermomyces lanuginosus* with potential applications in transesterification reactions for biodiesel production in free and organic solvent. The evaluated strategies of activation involved different activating reagents like glycidol (GLY), epichlorohydrin (EPI), ethylenediamine (EDA), and glutaraldehyde (GLU). Some strategies used a single reagent. It is the case of epoxy agents like GLY and EPI, which directly generate the

desired glyoxyl groups. In other strategy, EDA was used after glycidol activation for reaction of the terminal amine group of the enzyme for increasing the distance between the enzyme and support in order to avoid steric effects while increasing lipase immobilization yield [17] followed by GLU treatment to attach the aldehyde groups to the extended amino group present onto the chitosan matrix. Finally, in order to evaluate the potential applications of the prepared biocatalysts, thermal stability, activity through esterification of oleic acid, and operational activity by means of reusability assays were made.

Materials and Methods

Materials

Thermomyces lanuginosus lipase formulation (14.87 mg of protein/ml) was kindly donated by Novozymes (Araucária, Brazil). Powdered chitosan with 85.2% degree of deacetylation was purchased from Polymar Ind. Ltda (Ceará, Brazil). Glycidol 96% GC (GLY), epichlorohydrin (EPI), Triton X-100, *p*-nitrophenyl butirate (*p*-NPB), *p*-nitrophenol (pNP), and oleic acid were purchased from Sigma–Aldrich (St. Louis, USA). Ethylenediamine and 25% (*v*/*v*) glutaraldehyde grade II solution were purchased from Vetec (São Paulo, Brazil). All other reagents were of analytical grade.

Preparation of Chitosan Hydrogel Support

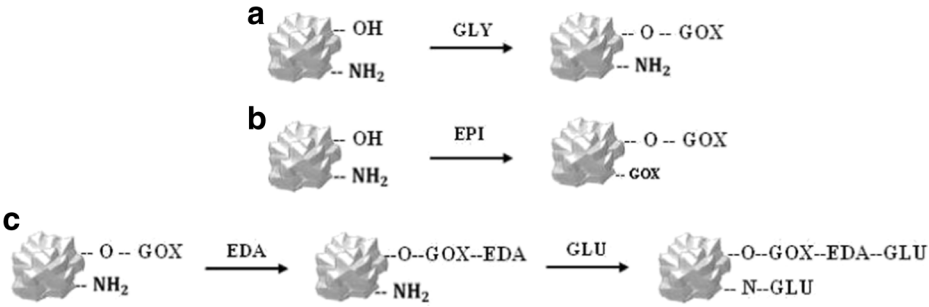
A mass of 5.02 g of powdered chitosan was dissolved into 200 ml of 5% (*v*/*v*) acetic acid solution by mixing the preparation until complete dissolution at room temperature. Then, 1.8 l of 0.1 M NaOH solution was slowly added to chitosan solution and mixed continuously under slow stirring during 4 h and left in repose for 24 h. The chitosan gel obtained was repeatedly washed with distilled water and filtered until the washing solution reached a neutral pH (checked with phenolphthalein indicator). Finally, the hydrogel stored at 4 °C until further use.

Activation of Chitosan Hydrogel with Different Chemical Reagents

Three strategies for activation were performed using chitosan hydrogels as support. Scheme 1 shows diagrams of the activation strategies studied in this work.

Glycidol Activation

Activation of chitosan gel with GLY (Scheme 1a) was carried out according to the methodology described elsewhere [18]. In this way, in an ice bath, 10 g of chitosan gel was mixed with 112.5 ml of distilled water and 37.7 ml of 1.7 M NaOH solution containing 7.92 g of sodium borohydride ($NaBH_4$); pH was checked to be around 13–14. Immediately, 36 ml of GLY was slowly added (drop by drop) to the mixture keeping a constant slow stirring for 18 h at 25 °C. Next, the preparation was rinsed with distilled water, filter-dried and immediately treated with a 0.1 M sodium periodate ($NaIO_4$) solution, and stirred for 2 h at 25 °C. When the time was accomplished, non-consumed periodate of supernatant was measured by titration with KI in saturated bicarbonate [19].



Scheme 1 Schematic illustration of the different immobilization strategies. **a** Activation with Glycidol (Ch-GLY). **b** Activation with epichlorohydrin (Ch-EPI). **c** Activation of glycidol-activated chitosan with ethylenediamine and glutaraldehyde (Ch-GLY/EDA/GLU). *GLY* glycidol, *EPI* epichlorohydrin, *GLU* glutaraldehyde, *GOX* glyoxyl

Activated support was extensively washed with deionized water for removal of excess of the oxidant agent and stored until immobilization process.

Epichlorohydrin Activation

The methodology used for activation of chitosan gel with EPI (Scheme 1b) has been previously described [20]. A mass of 10 g chitosan hydrogel preparation was mixed with 100 ml of 2.0 M NaOH and 0.6 g of $NaBH_4$. pH was checked to be around 13–14. Immediately, 10 ml of EPI was carefully added to avoid an excessive increase of temperature reaction. Additionally, the mixture was kept under moderate stirring at 0 °C for the first 2 h and then at room temperature for an additional 12 h. Finally, the activated gel was washed with distilled water, filtered, and then oxidized with 0.1 M sodium periodate solution prior to the immobilization process.

EDA Cross-Linking and Glutaraldehyde Activation

EDA cross-linking (see Scheme 1c) was performed by treating 10 g of GLY-activated chitosan with 40 ml of a 2 M ethylenediamine solution (pH 10) for 2 h by moderate stirring at room temperature and subsequent reduction with sodium borohydride for 2 h [21]. Then, the gel was washed with distilled water and filtered. After treatment with EDA, chitosan gel was suspended (chitosan/buffer ratio of 1:10) in 100 ml of 100 mM sodium bicarbonate/carbonate buffer (pH 10) containing 5% (*v*/*v*) glutaraldehyde [22]. The mixture was kept under agitation for 18 h at 25 °C. Finally, the activated gel was washed with distilled water, filtered, and immediately used for enzyme immobilization.

TLL Immobilization onto Activated-Chitosan Matrices

TLL immobilization assays were carried out in a batch reactor under gentle orbital agitation at room temperature in 25 mM bicarbonate/carbonate buffer solution (pH 10.0) using a 1:10 ratio (g of chitosan/ml of total buffered solution) containing 0.15% (*v*/*v*) Triton X-100. The use of Triton X permitted to obtain the enzyme with the active center oriented towards the reaction medium. Moreover, immobilized enzyme presents the capacity of specifically adsorbing other TLL molecules that can be easily desorbed by the use of detergents [23]. From previous tests, 2 mg of TLL per gram of support was selected as the enzyme charge given to supports for their use in the thermal

stability studies. On the other hand, for esterification studies, an enzyme concentration of 20 mg of TLL per gram of support was chosen in order to achieve a maximum charge on chitosan support. Immobilization time for EPI- and GLY-activated chitosan supports was 24 h, whereas those supports activated with GLY/EDA/GLU, and the selected immobilization time was 6 h. Each derivative was produced by triplicate in three independent assays.

Reduction of Schiff's Bases

After enzyme immobilization process, Schiff's bases reduction was performed. For that reason, 1 mg of $NaBH_4$ per milliliter of solution was added to the reaction mixture and the suspension was kept under agitation during 30 min at room temperature. Then, the derivative was filtered, thoroughly rinsed with distilled water, and stored until enzyme assay was done.

Determination of TLL Hydrolytic Activity

The hydrolytic activity of soluble and immobilized TLL was determined using 50 mM *p*-nitrophenyl butirate (*p*-NPB) solubilized in acetonitrile as substrate following the methodology previously described [17] with few modifications.

Immobilization Parameters

Immobilization yield (IY) was evaluated through immobilized enzyme activity, according to Eq. 1, from the measure of the hydrolytic activities of the supernatant before (At_i) and after (At_f) enzyme immobilization. The difference of enzyme activity between the beginning and the end of the immobilization reaction was estimated to be the enzyme immobilized in the support. Furthermore, a blank assay using the soluble enzyme without support was also carried out to evaluate the possible enzyme deactivation under the same immobilization conditions. This deactivation was estimated by the ratio of final and initial activity measured in the blank assay ($f_{Bl} = At_{iBl}/At_{fBl}$).

$$IY(\%) = \left(\frac{At_i - \left(f_{Bl} At_f\right)}{At_i}\right) \times 100 \tag{1}$$

On the other hand, theoretical specific activity (At_t) obtained in all biocatalysts by immobilization process was calculated using Eq. 2. Furthermore, the activity of immobilized enzyme (At_d; U/g) allowed to estimate the recovered activity (At_r) considered as the biocatalyst effectiveness [22], employing Eq. 3.

$$At_t(U/g) = \frac{IY\% \times At_{off}}{100} \tag{2}$$

where At_{off} is the total enzyme activity offered per gram of chitosan.

$$At_r(\%) = \frac{At_d}{At_t} \times 100 \tag{3}$$

Protein Determination

Protein concentration was determined according to the Bradford methodology [24] employing bovine serum albumin (Sigma-Aldrich) as protein standard.

Thermal Stability Assay of Supports

Soluble and immobilized TLL samples with similar enzyme charge were incubated in 25 mM sodium phosphate buffer (pH 7) at 60 °C for 24 h. At different established time intervals, samples were withdrawn and residual activity (At_{res}) was measured using *p*-NPB as substrate [25]. At_{res} was calculated as a ratio between final and initial enzymatic activity for each deactivation time. The half-life times ($t_{1/2}$) were calculated according to the model proposed by Sadana and Henley [26] through non-linear exponential regression using Microcal Origin version 8.0 (Northampton, USA).

The stabilization factor *(SF)* of each derivative was estimated through the ratio between the half-life times of the immobilized derivative ($t^d_{1/2}$) and the soluble enzyme ($t^S_{1/2}$) through Eq. 4. Each corresponding half-life time was defined as the time required to reduce in 50% the initial activity at a fixed process condition.

$$SF = \frac{t^d_{1/2}}{t^s_{1/2}} \tag{4}$$

Activity of Derivatives: Esterification of Oleic Acid

Assays for esterification of oleic acid on each derivative were made batchwise using a thermostatic bath with orbital stirring (120 rpm) at 37 °C. The offered quantity of each biocatalyst was 50 mg, the amount of oleic acid was 1000 mg, and the employed ethanol/oleic acid ratio was 1:1. In order to evaluate the percentage of conversion of oleic acid to ethyl oleate, samples were withdrawn at 1, 2, 4, 8, 24, 48, and 72 h. Then, the remaining oleic acid content was determined through the free fatty acid titration method proposed by Official Methods of Analysis of AOAC (Section 41.940.28) using 0.25 M NaOH in alcoholic medium with phenolphthalein as indicator. In this sense, the acidity index (AI) at each reaction time was established and the residual oleic acid (OA) was calculated employing Eq. 5.

$$OA(\%) = \left(\frac{AI_i - AI_f}{AI_i}\right) \cdot 100 \tag{5}$$

A blank assay without enzyme was also checked, and titration reactions were performed by triplicate.

Operational Stability Tests

The study of the reusability of the derivatives was done by submitting 50 mg of each biocatalyst to subsequent cycles of esterification of oleic acid with ethanol as described above. Each reaction cycle was carried out in a batch reactor for 12 h at 37 °C under constant agitation. After each cycle, the derivative was washed with distilled water for the removal of the unreacted substrates and dried through vacuum filtration using a sintered glass funnel before repeating the esterification cycle.

Scanning Electron Microscopy of Chitosan Derivatives

Scanning electron microscopy (SEM) was employed to observe the surface morphology of the supports following the methodology previously described [27].

Results and Discussion

TLL Immobilization onto Activated-Chitosan Hydrogel

Three activation strategies were used to immobilize TLL onto chitosan hydrogels. The first strategy consisted in the activation of chitosan hydroxyl groups with glycidol (Ch-GLY), and the second strategy employed epichlorohydrin (Ch-EPI). Subsequently, both strategies included the oxidation with sodium periodate to produce reactive aldehyde glyoxyl groups. A third strategy used glycidol-activated chitosan for reaction with ethylenediamine and finally with glutaraldehyde (Ch-GLY/EDA/GLU). When chitosan hydroxyl groups were activated with strategy Ch-GLY-EDA-GLU, GLU can react either with the amine of the EDA or directly with the amino groups of the chitosan, introducing aldehyde groups prone to react with L-lysine exposed amino groups of lipase to form Schiff bases. When this occurs, a binary immobilization takes place [3]. Thus, a higher immobilization yield would be expected due to the presence of a larger number of reactive groups.

The selected time for enzyme immobilization for each treatment was chosen as the time where the decrease in the residual activity in the immobilization supernatant was negligible. Little enzymatic deactivation (less than 5%) was observed in the blank test but was, nevertheless, taken into account in the activity calculations (see Eq. 1). For stability and activity studies, the enzyme loading for the immobilization assays was 2 mg of TLL per gram of support of wet chitosan (135 μl of commercial lipase solution). On the other hand, for assuring a maximum protein charge onto chitosan supports, an enzyme loading of 20 mg of TLL/g of support was used. Table 1 shows the results of the most relevant immobilization parameters (At_d, At_r, and IY) for the evaluated strategies for the two enzymatic charges.

As seen in Table 1, IY and At_d for Ch-GLY obtained after immobilization of a low charge of TLL were very low, mainly because a low amount of aldehyde group was generated with glycidol activation. This fact was evidenced by the high amount of periodate that had not been consumed, and therefore, it was present in the supernatant. These results were also observed in [28, 29] publications. Moreover, the preferential reaction of glycidol with hydroxyl groups at alkaline pH and the presence of the intact amino chitosan groups converted the matrix into a highly hydrophilic material. In that way, the generated microenvironment was not hydrophobic enough for a successful lipase immobilization with high activity.

From the results shown in Table 1, it can be observed, for Ch-EPI-TLL derivative, that moderate IY (50.9%) and At_d (42.4 U/g) values were obtained (for the lower charge of TLL) mainly due to the large amount of aldehyde groups produced by EPI activation evidenced by the low amount of non-consumed periodate present in the supernatant and due to the more hydrophobic microenvironment on the support surface which possibly improved the TLL immobilization and enzyme activity. This result is in consonance with [29] report. When the support previously activated with EPI was immobilized with a maximum charge of TLL, the At_d was increased (68.1 U/g of support) and the IY was very low (15.6%).

Table 1 Immobilization results of chitosan-TLL derivatives

Strategy	At_i (U/ml)	At_f (U/ml)	At_d (U/g)	At_r (%)	IY(%)
Ch-GLY[a]	133.5 ± 5.3	99.2 ± 4.9	7.6 ± 0.9	1.9	28.3
Ch-EPI[a]	121.2 ± 4.0	62.3 ± 4.6	42.4 ± 6.0	6.9	50.9
Ch-GLY/EDA/GLU[a]	126.7 ± 3.2	3.6 ± 3.1	190.6 ± 14.2	15.5	97.3
Ch-EPI[b]	1273.8 ± 30.9	1120.2 ± 28.1	68.1 ± 5.6	3.4	15.6
Ch-GLY/EDA/GLU[b]	1214 ± 29.1	570.6 ± 18.5	283.2 ± 21.6	4.2	54.9

Enzyme activities of the supernatant (soluble enzyme) before (At_i) and after (At_f) immobilization, derivative activity (At_d), recovered activity (At_r), and immobilization yield (IY). Immobilization conditions: 10 ml of enzymatic solution in 25 mM bicarbonate buffer (pH 10.0), contacted with 1 g of support at 25 °C. The immobilization time was 6 h (Ch-GLY/EDA/GLU) and 24 h (Ch-GLY and Ch-EPI). In all of cases, the data represents mean ± SD from three independent experiments

[a] Enzyme offered: 2 mg protein/g support (135 μl of commercial lipase solution)

[b] Enzyme offered: 20 mg protein/g support (1350 μl of commercial lipase solution)

Finally, the best result was obtained with Ch-GLY/EPI/GLU-TLL derivative where a high IY (97.3%) and a good At_d (190.6 U/g) values were achieved with a low charge of TLL. This might be explained mainly due to binary immobilization evidenced in this strategy, where the two functional groups (amino and hydroxyl groups) of chitosan were activated to generate aldehyde groups at alkaline pH. In addition, the use of EDA generated a spacer arm between the enzyme and the chitosan support improving the interaction of the enzyme active site with the substrate possibly due to a reduction of steric hindrance [30], leading to an increase of the derivative activity. When a maximum charge of TLL was used for Ch-GLY/EPI/GLU derivative, At_d was significantly improved (283.2 U/g of support) and an IY of 55% was obtained.

Thermal Stability of Derivatives

Enzyme activity at different physicochemical conditions is directly related to the enzyme stability. In that way, the stability of the enzyme both soluble and immobilized is influenced on how the enzyme active site undergoes conformational changes which cause loss of activity. Thermal inactivation profiles of soluble and carrier-bound TLL are shown in Fig. 1.

As seen in Table 2, the half-life times of all assayed derivatives were much higher than soluble TLL, due to the conformational rigidity that covalent immobilization to chitosan matrices conferred to the enzyme lipase. In that way, active site conformation was better preserved in comparison with soluble enzyme at the same reaction conditions.

The Ch-GLY/EDA/GLU-TLL derivative was the most stable showing a stabilization factor 15.26-fold higher than soluble enzyme under the evaluated inactivation conditions. This is due to that the Ch-GLY/EDA/GLU-TLL derivative allowed the formation of a greater number of covalent linkages between the enzyme units and the support due to binary immobilization which increased TLL rigidity and preserved enzyme activity for a higher time (the derivative kept more than 20% of its initial activity after 24 h).

Ch-GLY-TLL derivative showed a moderate half-life time and stabilization factor values (Table 2) losing almost all of its activity after 24 h. In addition, the Ch-EPI-TLL derivative showed the lowest half-life time and stabilization factor values losing all its activity after 10 h incubation time.

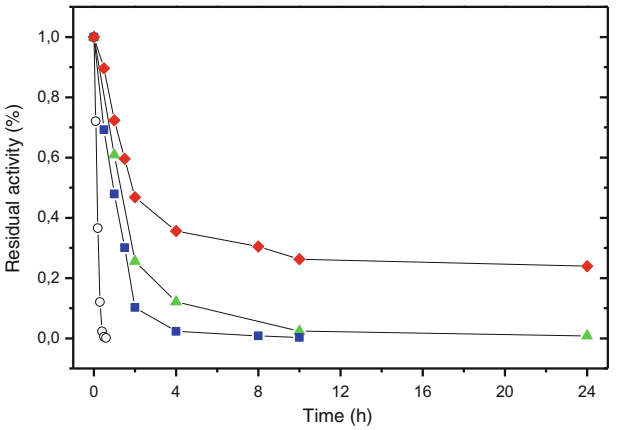


Fig. 1 Thermal inactivation profile at 60 °C in 25 mM sodium phosphate buffer (pH 7) of soluble (○) and TLL-chitosan derivatives, obtained after 24 h: Ch-EPI (■); Ch-GLY (▲); Ch-GLY/EDA/GLU (♦). Each profile (experimental curve of At_r vs *time*) was analyzed with Sadana-Henley model [26]

Esterification Activity of Derivatives Employing Oleic Acid

Ch-EPI-TLL and Ch-GLY/EPI/GLU-TLL derivatives were used in esterification of oleic acid with ethanol assay due to their good hydrolytic activity obtained with low loading of enzyme as previously seen in Table 1. On the other hand, Ch-GLY-TLL derivative showed low hydrolytic activity and therefore was not used in this study.

The percentages of oleic acid conversion to ethyl oleate versus time for the two best derivatives are shown in Fig. 2. The acidity index (AI) was measured by triplicate for each evaluated time (Table 3). As mentioned in "TLL Immobilization onto Activated-Chitosan Matrices", the enzymatic immobilization loading was high (20 mg/g of support) in order to achieve a maximum charge on chitosan support.

The best insoluble biocatalyst turned out to be Ch-GLY/EDA/GLU-TLL with an oleic acid conversion up to 90% after 12 h of incubation time not reaching at 100% of conversion even after 72 h. In this sense, it has been reported [31] that high water content significantly decreases the ester production through increase in hydrolysis. Furthermore, the enzyme conformation in reaction media with high water content may be more flexible, which may also decrease the transesterification activity of enzyme [32]. Hence, to avoid excess water in reaction media, Zong and co-workers [33] analyzed the effect of several adsorbents, where both 3, 4, and 5 Å molecular sieve and blue silica gel had the best performance. Another

Table 2 Thermal inactivation at 60 °C and pH 7.0 of soluble TLL and derivatives obtained in "TLL Immobilization onto Activated-Chitosan Hydrogel" section

Biocatalyst	k_d (h^{-1})	$t_{1/2}$ (h)	SF
Soluble TLL	4.26 ± 1.02	0.13	1.00
Ch-GLY	0.60 ± 0.07	1.17	8.69
Ch-EPI	0.84 ± 0.07	0.84	6.25
Ch-GLY/EDA/GLU	0.55 ± 0.06	2.06	15.26

In all of cases, the data represents mean ± SD from three independent experiments

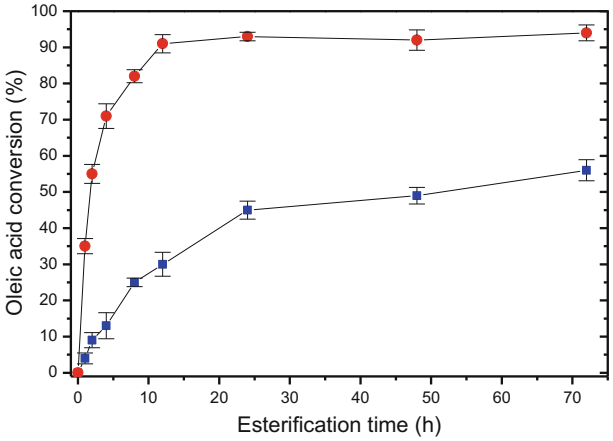


Fig. 2 Kinetics of esterification of oleic acid to ethyl oleate using the TLL-chitosan derivatives at 37 °C for 72 h with orbital stirring. Ch-EPI $At_d = 68.12 \pm 4.56$ U/g (■); Ch-GLY/EDA/GLU $At_d = 283.23 \pm 21.57$ U/g (●). The Derivative mass employed was 50 mg

possible way to perform that is by using zeolites [34]. Furthermore, and as a technological relevant alternative, the employment of enzyme-inorganic crystal nanoflowers complexes, such as copper hydroxysulfate nanocrystals, could be advantageous for increasing ester synthesis due to the high activity and stability of these supports attributed to mainly both their innovative and complex skeleton structures [35, 36]. On the other hand, Ch-EPI-TLL derivative showed a moderate to low oleic acid conversion being of 45% after 24 h and 50% after 72 h reaction time. The low conversion could be a result of both enzyme inactivation due to esterification conditions (a low thermal stability was achieved for this derivative) and the low TLL charge provided to chitosan support.

Operational Stability of Derivatives

The enzyme reutilization was evaluated to study the potential use of biocatalysts in batch and continuous enzymatic processes. In this sense, operational stability of Ch-GLY/EDA/GLU-TLL and Ch-EPI-TLL derivatives was established. The esterification process of each derivative was repeated five times where each cycle lasted 12 h.

Table 3 Acidity index results of oleic acid esterification of the two evaluated biocatalysts

Biocatalyst	Reaction time (h)								
	0	1	2	4	8	12	24	48	72
Ch-EPI	184.7	174.1	168.8	159.3	139.0	129.3	101.0	92.3	81.5
	183.1	174.9	167.4	160.9	138.8	130.5	102.3	91.5	80.9
	185.2	175.4	169.0	162.2	137.7	128.6	101.7	90.9	80.3
Ch-GLY/EDA/GLU	182.6	118.4	80.3	51.1	33.0	14.8	12.4	15.3	10.6
	183.2	117.3	79.7	51.6	32.5	15.7	12.6	13.9	9.8
	181.7	117.9	79.0	52.5	33.7	14.9	13.2	14.3	10.0

In all cases, the data represent measures acidity of remaining oleic acid in esterification reaction media, expressed as milligrams of KOH per gram of sample

Ch-GLY/EDA/GLU-TLL derivative showed a great operational stability along 5 cycles, maintaining in great measure its initial enzyme activity. As seen in Fig. 3, the derivative reached about 75% conversion of oleic acid to ethyl oleate after 5 cycles being only lost 18% of its initial activity. In addition, after 3 cycles of esterification, only a 6% of its initial activity was lost. Similar results related to reusability of derivatives were obtained when compared with the extremely innovative and highly stable metal-organic frameworks (MOFs), such as glucose oxidase (GOx) and horseradish peroxidase (HRP)/zeolitic imidazolate framework (ZIF-8) and polydopamine (PDA)@GOx /ZIF-8 derivatives, among others [37, 38]. These derivatives, which were obtained by encapsulation techniques, were used for immobilization of *Candida antartica* lipase B allowing a 400–1000 times increase in stability compared to free enzyme while maintaining same enantioselectivity [39].

On the other hand, Ch-EPI-TLL derivative lost almost half of its initial activity (44% conversion) after the first cycle was performed, as seen in the decrease in the conversion yield of oleic acid. Then, conversion continued to decrease until reached a 10% of oleic acid production after 5 cycles. The main reason that explains this behavior is that EPI derivative produces a physical change leading to some non-specific adsorption of the lipase on support. Hence, as esterification reaction was progressing, a continuous TLL desorption was seen. This phenomenon was detected by measuring protein concentration in reaction media (data not shown) and was not evidenced in the derivatives produced through Ch-GLY and Ch-GLY/EDA/GLU.

Scanning Electron Microscopy Analysis

Figure 4 shows the results of the surface texture and porosity of chitosan supports both before and after activation and immobilization treatments. It was possible to see the irregular surface, the size particles, and a low porosity of native chitosan support (Fig. 4a). In Ch-EPI (Fig. 4b), Ch-GLY (Fig. 4c), and Ch-GLY/EDA/GLU (Fig. 4d) supports, different surfaces were observed in comparison with non-activated chitosan support, although the difference was irrelevant between each other except for the glutaraldehyde-treated support. Micrographs of Ch-GLY/EDA/GLU-TLL derivative showed that the biocatalyst surface was partially covered with the enzyme as seen as white small beads. In addition, as shown in Fig. 4e, f respectively, a difference between low (2 mg/g) and high (20 mg/g) enzyme charge was demonstrated.

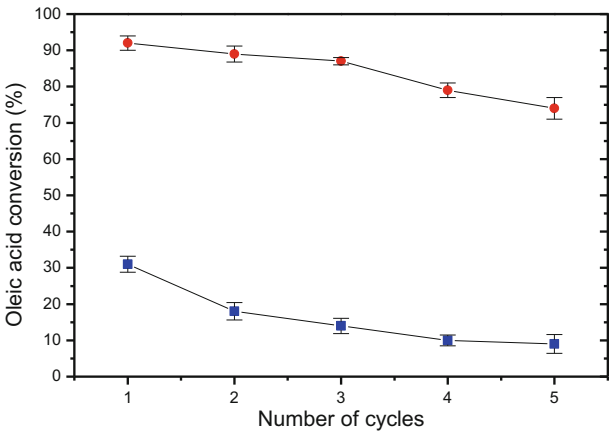


Fig. 3 Reuse TLL chitosan derivatives for 5 cycles of esterification of oleic acid and ethanol at 37 °C in orbital stirring: Ch-EPI (■) and Ch-GLY/EDA/GLU (●). Duration of each cycle is 12 h. Derivative mass was 50 mg

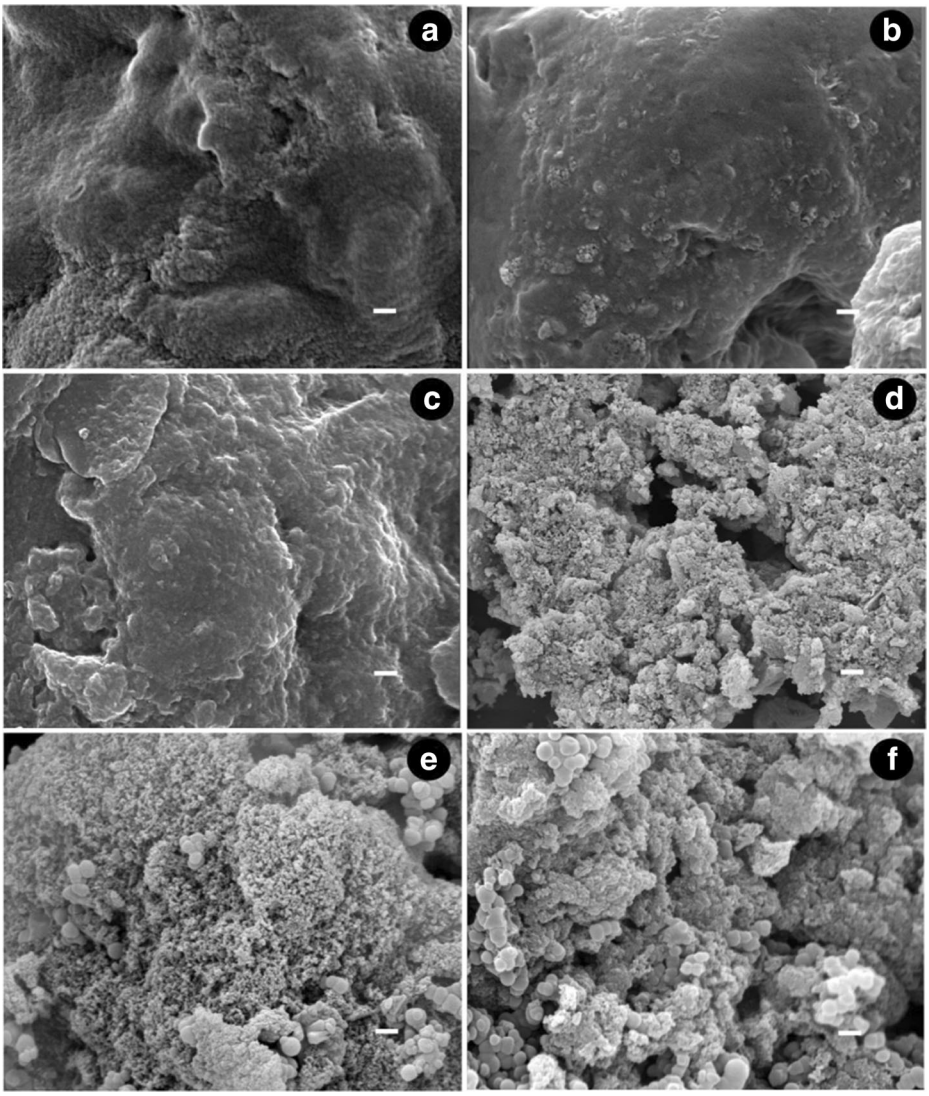


Fig. 4 Scanning electron microscopy (SEM) micrographs of supports of chitosan. No treatment (**a**). Activated with epichlorohydrin (**b**). Activated with glycidol (**c**). Sequentially activated with glycidol/ethylenediamine/glutaraldehyde (**d**). Derivative of Ch-GLY/EDA/GLU obtained with enzyme loading of 2 mg/g of support (**e**). Derivative of Ch-GLY/EDA/GLU obtained with 20 mg/g of support (**f**). All micrographies are magnified ×6000. *Bar* 1 μm

Conclusions

Different activation–immobilization strategies were performed, and chitosan-TLL derivatives were successfully obtained. The activation strategy that involved glycidol, ethylenediamine, and glutaraldehyde (Ch-GLY/EDA/GLU) was the best for TLL immobilization, where the highest hydrolytic activity value of 283.2 ± 21.6 U/g was obtained when maximum loading of enzyme (20 mg/g of support) was employed. Derivative of chitosan activated with

epichlorohydrin (Ch-EPI-TLL) showed moderate hydrolytic activity (68.1 ± 5.6 U/g) with maximum loading of enzyme (20 mg/g of support). However, when chitosan was activated with glycidol and immobilized with TLL (Ch-GLY-TLL), a low hydrolytic activity was obtained.

Thermal inactivation studies of derivatives showed an acceptable half-life time of 2 h for Ch-GLY/EDA/GLU-TLL. The stability of this derivative was 15-fold higher than soluble TLL at the evaluated inactivation conditions (60 °C, 25 mM sodium phosphate buffer, pH 7). The other derivatives showed a poor stability and half-life times due to the low number of covalent linkages between enzyme and support and the hydrophobic nature of the enzyme which caused a weak physical adsorption to chitosan matrix leading to progressive enzyme leakage to reaction media. Consequently, this behavior produced a rapid desorption and inactivation of the enzyme contained in chitosan supports when the derivatives were employed.

Finally, a great operational activity was obtained for Ch-GLY/EDA/GLU-TLL, which converted more than 90% of oleic acid after a period of 12 h. Stability study of this derivative showed 18% less conversion compared to the initial cycle after 5 cycles of esterification. Actual studies are focused on improving the activity and stability of the synthetized enzyme derivatives for their use in biodiesel production employing already used vegetable edible oils discarded as wastes from several food industries.

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