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# Evaluation of the potential of polymeric carriers based on photo-crosslinkable chitosan in the formulation of lipase from *Candida rugosa* immobilization

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ABSTRACT

Using an EDC/NHS conjugation method, chitosan was chemically modified to incorporate a photosensitive α-cyano-4-hydroxycinnamic acid moiety with various degrees of substitutions. The chitosan α-cyano-4-hydroxycinnamate was fully characterized by FTIR, $^1$H NMR and UV–vis spectra. Lipase from *Candida rugosa* was entrapped in the modified photo-crosslinkable chitosan membranes. Crosslinking was carried out by irradiation in the ultraviolet region. The activities of free and immobilized lipase have been studied. The efficiency of the immobilization was evaluated by examining the relative enzymatic activity of free enzyme before and after the immobilization of lipase. The obtained values were found to reach 98.6%. The results showed that the optimum temperature of immobilized lipase was 40 °C, which was identical to that of the free enzyme, and the immobilized lipase exhibited a higher relative activity than that of free lipase over 40 °C. The optimal pH for immobilized lipase was 8.0, which was higher than that of the free lipase (pH 7.5), and the immobilization resulted in stabilization of enzyme over a broader pH range. The kinetic constant value ($K_m$) of immobilized lipase was 1.332 mg ml$^{-1}$ ($1.50 \times 10^{-3}$ M), which was higher than that of free lipase. On the other hand, the activity of immobilized lipase decreased slowly against time when compared to that of the free lipase, and could retain 75.5% residual activity after 6 consecutive cycles.

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## 1. Introduction

Enzymes are catalysts bearing some excellent properties (high activity, selectivity and specificity) that may permit to perform the most complex chemical processes under the most suitable experimental and environmental conditions [1–3]. Thus, the engineering of enzymes from biological entities to industrial reactors is a very exciting goal. Fortunately, there are many techniques available that may permit to improve the enzyme features, involving many areas of science that have suffered impressive developments in the last years: microbiology, protein engineering, protein chemistry, etc. However, some apparently older fashioned techniques, as immobilization, have been revealed in the last times as a very powerful tool to improve almost all enzyme properties, if properly designed: e.g., stability by protecting the active site from the deactivation, significant reduction in the operation cost, activity, specificity and selectivity, reduction of inhibition and recovery of the biocatalyst [3–5]. There are many methods available for immobilization which span from binding on prefabricated carrier materials to incorporation into in situ prepared carriers [6]. Operative binding forces vary between weak multiple adsorptive interactions and single attachments [7,8] through strong covalent binding [9,10]. Which of the methods is the most appropriate is usually a matter of the desired applications.

The immobilization via entrapment of enzyme in natural carrier polymers is of interest for a variety of scientific and industrial applications [11,12]. Enzyme entrapment is typically achieved using a polymer network such as an organic polymer or sol–gel and is usually performed in situ [12,13]. Entrapment protects enzymes by preventing direct contact with the environment, thereby minimizing the effects of gas bubbles, mechanical sheer and hydrophobic solvents [12].

Natural polymers used as carrier materials in entrapment technology, such as alginate, carrageenan, agarose, chitin, and chitosan, have the advantages of being nontoxic, biocompatible, and biodegradable [14]. Certain polysaccharides have played an important role in biotechnological processes including their use in recovery and delivery systems, as support materials, and as precursors or raw materials for further processing [15,16].

Chitosan is amine-bearing, linear polysaccharide produced by alkaline hydrolysis of naturally occuring chitin (i.e., poly-N-acetyl-

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glucosamine), a process that results in its N-deacetylation and depolymerization [17,18].

As enzyme immobilization supports chitin- and chitosan-based materials are used in the form of powders, flakes and gels of different geometrical configurations [19,20]. Preparation of chitosan gels is promoted by the fact that chitosan dissolves readily in dilute solutions of most organic acids, including formic, acetic, tartaric and citric acids, to form viscous solutions that precipitate upon an increase in pH and by formation of water-insoluble ionotropic complexes with anionic polyelectrolytes. In this way chitosan gels in the form of beads, membranes, coatings, capsules, fibres, hollow fibres and sponges can be manufactured. Commonly, different follow-up treatments and modifications are applied to improve gel stability and durability [20,21].

Lipases (triacylglycerol acylhydrolases, EC 3.1.1.3) are perhaps the most popular enzymes in biocatalysis because they couple a wide specificity to a high regio and enantioselectivity and specificity, therefore, may be used in many different reactions [22].

However, lipase catalysis implies dramatic conformational changes of the enzyme molecule. Lipases may be in two different structural forms. One of them, where the active center of the lipase is secluded from the reaction medium by a polypeptide chain called "lid", is considered an inactive (closed) form. The other one, which presented the lid displaced and the active center exposed to the reaction medium, is considered as the lipase in an active (open) form. The lipase molecule is in equilibrium between the open-active and the closed-inactive structures of the immobilized lipases. This equilibrium shifts towards the open form in the presence of hydrophobic interfaces by the adsorption of the open form [22–25]. Lipases have widely been used in many industrial fields such as organic synthesis, paper manufacture, oleochemistry, dairy industry and detergents. When used as a detergent additive, more than 1000 tons of lipases are needed each year for the worldwide market [26–29].

In this report, using an EDC/NHS conjugation method, chitosan was chemically modified to incorporate a photosensitive α-cyano-4-hydroxycinnamic acid moiety with various degrees of substitutions. The chitosan α-cyano-4-hydroxycinnamate was fully characterized by FTIR, $^1$H NMR and UV–vis spectra. Then, Lipase from *Candida rugosa* was entrapped in the modified photocrosslinkable chitosan membranes and crosslinking was carried out by irradiation in the ultraviolet region. The effect of significant parameters such as degree of substitution temperature and pH on the entrapment efficiency and retained activity was investigated.

## 2. Materials and methods

### 2.1. Materials

Chitin was isolated from pink shrimp (Solenocera melantho) shell waste by treatment with 2.5N NaOH (12.5 ml per gram of shrimp shell powder at 75 °C for 6 h) and 1.7N HCl (9 ml per gram of shrimp shell powder at ambient temperature for 6 h). Chitosan (M.W. $1.79 \times 10^6$ amu) and degree of deacetylation 85% was prepared by N-heterogeneous deacetylation of chitin in aqueous 50% sodium hydroxide solution under solid–liquid–liquid phase transfer catalytic condition according to our previous study [30]. Briefly, 5 g chitin was soaked overnight in chloroform as swelling medium. After decantation of the solvent, the swollen polymer was treated with 500 ml 50% aqueous NaOH solution and $5.16 \times 10^{-3}$ mol/l of the PT-catalyst Benzyltriphenyl phosphonium chloride (BTPP) (BDH) at 50 °C. The reaction mixture was mechanically stirred for 6 h. After cooling the resulted deacetylated chitin was washed with distilled water until alkali free. α-cyano-4-hydroxycinnamic acid (ACHCA), N-(3-dimethylaminopropyl)-N′-ethylcarbodiimide hydrochloride (EDC, commercial grade), N-hydroxysuccinimide (NHS, 98%) and *Candida rugosa* lipase enzyme were purchased from Sigma–Aldrich. All chemicals were used as received.

**Table 1**
Synthesis of chitosan-ACHCA using EDC/NHS conjugation method[a].

| Formulation code | ACHCA (mol/l) | EDC (mol/l) | NHS (mol/l) | DS[b] (mol%) |
|---|---|---|---|---|
| CACN-1 | 0.0264 | 0.0528 | 0.0528 | 28% |
| CACN-2 | 0.0449 | 0.0898 | 0.0898 | 43% |
| CACN-3 | 0.0687 | 0.1374 | 0.1374 | 54% |
| CACN-4 | 0.0952 | 0.1904 | 0.1904 | 69% |
| CACN-5 | 0.1269 | 0.2538 | 0.2538 | 78% |

[a] Concentration of chitosan solution = 5 g/l; reaction time: 24 h at room temperature.
[b] Degree of ACHCA substitution (DS) determined by $^1$H NMR, DS (mol%) = $(I_8 + I_9 + I_{10} + I_{11})/(6 \times I_2)$, where $I_8$, $I_9$, $I_{10}$ and $I_{11}$ denote the integrals of the peaks of the introduced ACHCA moiety, and $I_2$ denotes the integral of the peak of the proton H$^2$ in C-2 of the chitosan [31,35].

### 2.2. Synthesis of chitosan-α-cyano-4-hydroxycinnamated (chitosan-ACHCA)

Chitosan-ACHCA was prepared by the reaction of chitosan with ACHCA in presence of EDC and NHS which were used as activating agents for the amide bond formation [31] as shown in Scheme 1. The formulation details used in synthetic procedure are summarized in Table 1. First, 0.5 g chitosan was dissolved in 50 ml mixture of 2% wt acetic acid in water:acetonitrile (1:1) (sol A). Specific amounts of EDC, NHS and ACHCA were then charged in another vial that contained a 50 ml mixture of water: acetonitrile (1:1) and stirred for 5 min until full dissolution (sol B). Sol B was then added drop wise into sol A and the obtained solution was stirred for 24 h in dark at room temperature. The polymer was recovered with the addition of a large amount of acetone. The gelatinous precipitate was then dried in oven at 60 °C for 24 h and kept in a dark place in a vacuum dessicator for further analysis and use. The quantitative determination of the degree of substitution (DS) of the final modified chitosan was calculated using $^1$H NMR spectra.

The modified chitosan-ACHCA with DS values 28%, 43%, 54%, 69% and 78% were named respectively, as CSCN-1, CSCN-2, CSCN-3, CSCN-4 and CSCN-5.

### 2.3. Characterization

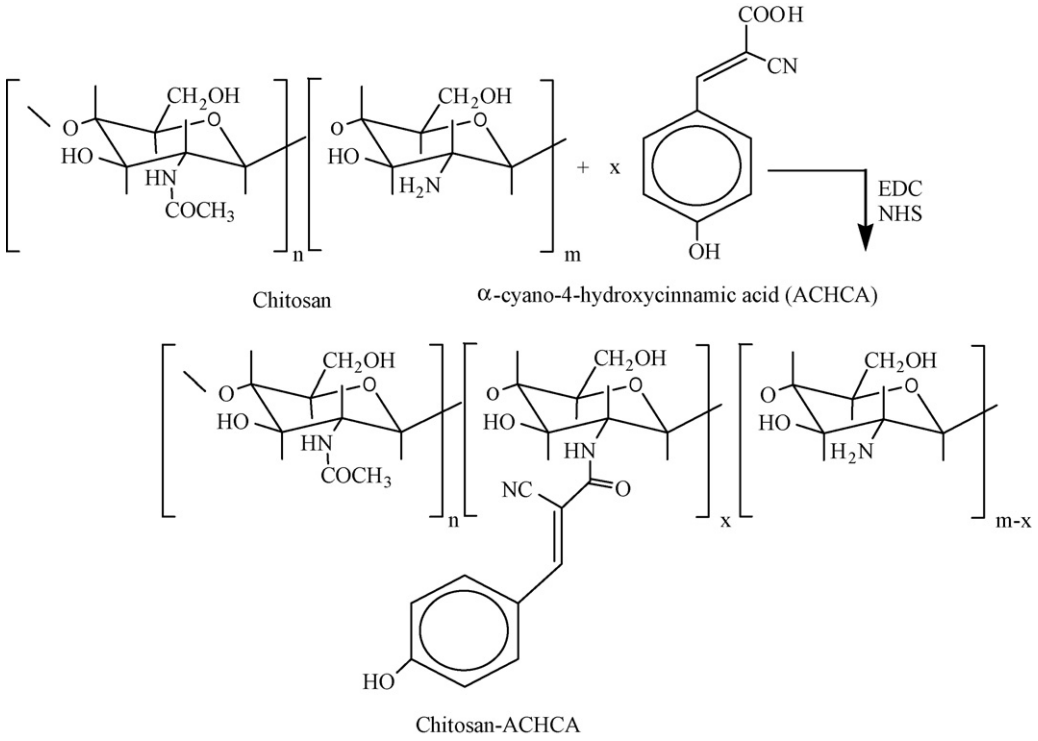
Infrared spectra (FTIR) were obtained with a PerkinElmer spectrophotometer. The chitosan and modified chitosan were dried overnight at 60 °C under reduced pressure and pressurized with a glass slide on top of the quartz window of the ATR instrument.

$^1$H NMR spectra were recorded by an Oxford NMR instrument at 500 MHz at room temperature using 1% deuterated acetic acid in $D_2O$ as a solvent. The experimental DS of the final modified chitosan was calculated from the integration of the appropriate peaks [31].

UV–vis spectroscopy was used to monitor the photocrosslinking process of chitosan α-cyano-4-hydroxycinnamate membrane irradiated with multiband UV lamp 254/365 nm (Mineralight lamp model UV GL-25). All UV–vis spectrophotometric measurements were executed at room temperature on a PerkinElmer Bio UV–vis spectrophotometer.

### 2.4. Entrapment of Candida rugosa lipase enzyme into the photo-crosslinked chitosan-ACHCA modified polymer

*Candida rugosa* lipase enzyme was immobilized into the photocrosslinked chitosan-ACHCA modified polymer via entrapment method [20]. 500 mg of the modified chitosan-ACHCA polymer




**Scheme 1.** Synthesis of chitosan-ACHCA.

of the desired degree of substitution was dissolved in 33 ml of distilled water. After *Candida rugosa* lipase enzyme solutions (2–10 mg ml$^{-1}$) were prepared by adding appropriate amounts of lipase powder to phosphate buffer (0.05 M, pH 7.0), 7 ml of enzyme solution of the desired concentration was mixed with the desired modified chitosan-ACHCA polymer solution and magnetically stirred for 1 h in ice bath. Then, the gelatinous solution was poured into a flat bottom Petri dish and dried in ice bath under vacuum for 14 h to obtain a uniform membrane on the Petri dish. The membrane was irradiated under a multiband UV lamp 254/365 nm (Mineralight lamp model UV GL-25) for 1 h. The resulted photocrosslinked hydrogel membranes were washed with phosphate buffer (0.05 M, pH 7.0) to remove the untrapped lipase enzyme, and then dried again in ice bath under vacuum for 14 h. Finally, the dry hydrogel membranes loaded with lipase were cut into equal size squares (5 cm$^2$) with a thickness of approximately 150 μm and stored at 4 °C.

The immobilization efficiency (*IE*%) was calculated using Eq. (1) [32]:

$$IE\% = \left(\frac{E_0V_0 - E_fV_f}{E_0V_0}\right) \times 100 \quad (1)$$

where $E_0$ is the initial lipase activity (U ml$^{-1}$); $V_0$ is the initial volume of lipase solution (ml); $E_f$ is the lipase activity in the washing buffer (U ml$^{-1}$); and $V_f$ is the washing buffer volume (ml).

The amount of entrapped lipase could be estimated from the *IE*% values multiplied by the initially added lipase amount [9].

### 2.5. Staining test

This staining test for the determination of proteins on polymeric carriers was a modification of the Coomassie protein assay (Bradford method) [33].

#### 2.5.1. Preparation of dye solution

100 mg Coomassie Brilliant Blue G-250 was dissolved in 50 ml of 95% ethanol plus 100 ml of 85% (w/v) phosphoric acid. Then the combined solution was diluted to 1000 ml with distilled water. Filtration prior to use was performed.

#### 2.5.2. Staining procedure

The samples of membranes with and without immobilized lipase were stained in Coomassie Brilliant Blue G-250 solution at room temperature for 5 min followed by washing with distilled water for two cycles.

### 2.6. Activity assays of free and immobilized lipase

The hydrolytic activities of free and immobilized lipase were assayed by the olive oil emulsion method, according to the modification proposed by Soares et al. [34]. The substrate was prepared by mixing 50 ml of olive oil with 50 ml of gum Arabic solution (7%, w/v). The assay mixture containing 5 ml of the emulsion, 4 ml of phosphate buffer (0.05 M, pH 7.0) and either free (1 ml, 2–10 mg ml$^{-1}$) or immobilized (5 cm$^2$ of the desired membrane) lipase was incubated for 5 min at 37 °C. The reaction was stopped by the addition of 10 ml of acetone–ethanol solution (1:1). The liberated fatty acids were titrated with 25 mM sodium hydroxide solution in the presence of phenolphthalein as an indicator. One lipase unit (U) corresponded to the release of 1 μmol of fatty acid per minute under the assay conditions.

### 2.7. Kinetic properties

The kinetic constant value ($K_m$) was determined by measuring the initial reaction rates of free and immobilized lipase using different concentrations of olive oil as substrate in phosphate buffer (0.05 M, pH 7.0) at 37 °C.

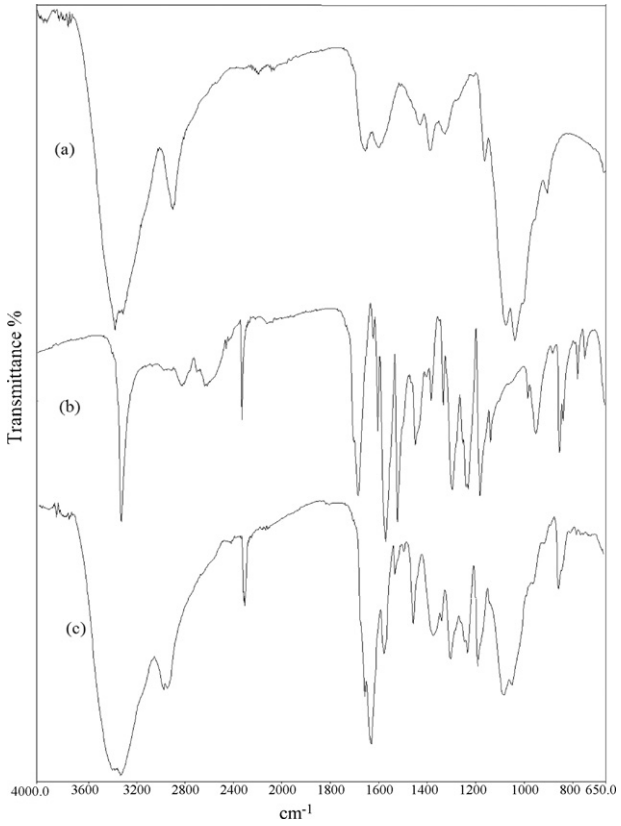



**Fig. 1.** FTIR spectra of (a) chitosan, (b) ACHCA and (c) chitosan-ACHCA (CACN-4).

### 2.8. Enzyme stability and reusability

Thermal stability was determined by incubating free and immobilized lipase at 70 °C in 0.05 M phosphate buffer at pH 7.0 for variable incubation periods. Samples were withdrawn every 60 min, immediately assayed for residual activity. Several consecutive operating cycles were performed by hydrolyzing olive oil in order to assess the operating stability of the immobilized lipase, at the end of each cycle, the immobilized enzyme was collected, and the procedure repeated with a fresh aliquot of substrate after the immobilized enzyme was stored for 24 h at 4 °C.

## 3. Results and discussion

### 3.1. Synthesis of chitosan-ACHCA

Chitosan-ACHCA was synthesized in a controllable manner using an EDC/NHS conjugation method as presented in Scheme 1. The formulation details used in synthetic procedure are summarized in Table 1. FTIR spectra of chitosan, ACHCA and chitosan-ACHCA (CACN-4) modified polymer were shown in Fig. 1. The principal spectra character of chitosan could be seen in the figure: 1076 $cm^{-1}$ (O—C strech), 1159 $cm^{-1}$ (bridge—O strech), 1313–1481 $cm^{-1}$ (—CH bend), 1627 $cm^{-1}$ (C=O strech), 2927 $cm^{-1}$ (C—H strech) and 3431 $cm^{-1}$ (O—H strech) which was in accordance with a previous report [35]. The spectrum of ACHCA shows the characteristic carbonyl peak of the —COOH group at 1700 $cm^{-1}$ and sharp intense peak at 2350 $cm^{-1}$ which correspond to the —CN group, whereas in the spectrum of the modified chitosan-ACHCA CACN-4 there is no peak corresponding to the carbonyl —COOH group of the ACHCA due to the amide bond formation between ACHCA and the amino group of chitosan. The prominent peaks at 1640 and 1550 $cm^{-1}$ which are observed in the IR spectrum of the modified chitosan are assigned to the carbonyl stretching vibration of amides (amide I) and bending vibration of amides (amide II). Also, the appearance of the sharp peak at 2350 $cm^{-1}$ corresponding to the —CN group confirm the incorporation of the ACHCA on the chitosan backbone.

The $^1H$ NMR spectra of chitosan in $D_3C$ COOD/$D_2O$ and chitosan-ACHCA in $D_2O$ were shown in Fig. 2. The spectrum of chitosan (Fig. 2a) shows a small peak at about $\delta$ 2.03 ppm assigned to the presence of —$CH_3$ of the N-acetylated glucosamine residue. The peak at $\delta$ 3.08 ppm was assigned to $H^2$ of glucosamine and N-acetylated glucosamine, and the multiplet peaks from $\delta$ 3.6 to 3.9 ppm were attributed to $H^3$, $H^4$, $H^5$, and $H^6$ of glucosamine and N-acetylated glucosamine. There existed a peak at $\delta$ about 4.78 ppm because of the presence of $H^1$ of glucosamine and N-acetylated glucosamine [35].

As a representative example, the $^1H$ NMR spectra is presented of a chitosan-ACHCA (CACN-5) (Fig. 2b). The spectrum confirms incorporation of the α-cyano-4-hydroxycinnamate group by the presence of phenolic proton peaks at $\delta$ 2.475 ppm, a doublet phenyl proton peaks at $\delta$ 6.630–6.647 ppm and at $\delta$ 8.208–8.225 ppm, which are characteristic for para disubstituted benzene derivatives, and vinyl proton peak at $\delta$ 8.332 ppm. Conveniently, the DS can be determined by comparing the integrated intensity of the phenyl, vinyl or phenolic peaks of the α-cyano-4-hydroxycinnamate group to the integral intensity of the $H^2$ of glucosamine and N-acetylated glucosamine.

### 3.2. Photo-induced crosslinking of chitosan-ACHCA

The photo-induced crosslinking chitosan-ACHCA was followed by UV–vis spectroscopy (Fig. 3). Absorbance at 326 nm decreased with exposure time without appearance of an isosbestic point, which is characteristic of a cis–trans isomerization mechanism for cinnamate crosslinking [31,36]. Within 60 min, crosslinking efficiency was 70%. This implies that photo-crosslinking of modified chitosan occurs mainly through a [2π + 2π] electron cycloaddition reaction (Scheme 2). Similar results were reported [31].

### 3.3. Immobilization of lipase

On the basis of the calculation from Eq. (1), we obtained the lipase immobilization efficiency and immobilized amount into photo-crosslinked CACN-4 membrane vs. the lipase added amount. As can be seen from Fig. 4, the immobilized lipase amount on CACN-4 membrane was increased (the membrane-enzyme composite was later crosslinked), while the immobilization efficiency decreased slowly with the increase of the amount of lipase added. Increasing the amount of the added lipase, would increase the immobilization of lipase as a result of trapping in the vacant spaces inside the crosslinked network or due to the electrostatic attraction with the cationic chitosan derivative until the maximum loading on the gel [20], then after the saturation, any excess lipase will be lost in the filtrate and washing buffer solutions, which may explain the low immobilization efficiency in case of higher amounts of lipase addition. The maximum quantity of lipase immobilized on 1 $cm^2$ membrane was 1.04 mg.

In addition, a staining test derived from the Coomassie protein assay (Bradford method) was used for the determination of proteins on the polymeric support in this work. In a previous report, it was demonstrated that the enzymatic proteins on the supports can be qualitatively characterized by using this staining test [37]. This modified Bradford method is based on the fact that the dye–protein complex with a characteristic blue would present due to the bonding between the anionic Coomassie Brilliant Blue G-250 in an

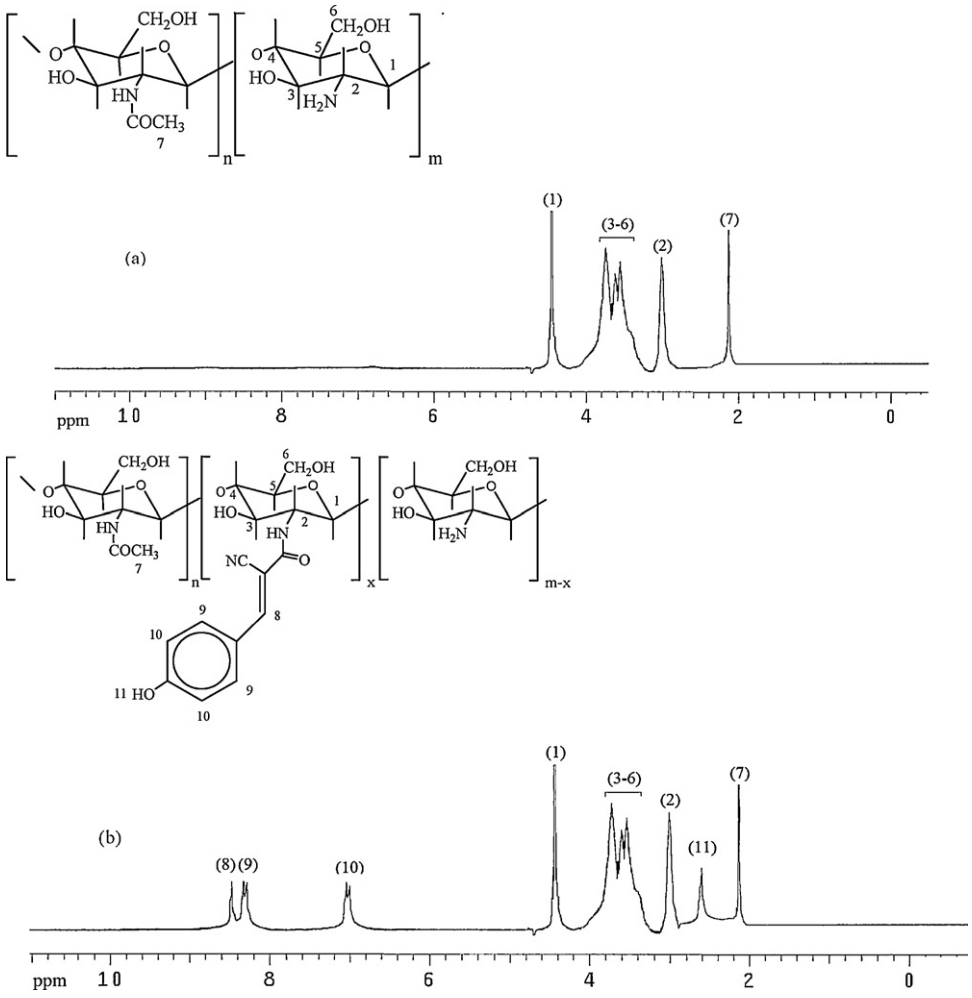



**Fig. 2.** $^1$H NMR spectra of (a) chitosan and (b) modified chitosan-ACHCA (CACN-5) with $CD_3COOD/D_2O$ as solvent.

acidic solution and proteins on the membranes. The Coomassie blue reagent has been shown to interact chiefly with arginine residues, but weakly with histidine, lysine, tyrosine, tryptophan and phenylalanine residues. Vander Waals forces and hydrophobic interactions might also participate in the binding mechanism [37]. As shown in Fig. 5, by immersing modified photo-crosslinkable chitosan membranes in Coomassie Brilliant Blue solution, the membranes with immobilized lipase changed to greenish blue color after 5 min with observed increasing in the color depth by increasing DS value (Fig. 5b and c), which is different from that of sample without immobilized lipase (Fig. 5a). The blue color indicates the formation of protein/Coomassie Brilliant Blue G-250 dye complex and consequently suggests the immobilization of lipase on the modified chitosan membranes.

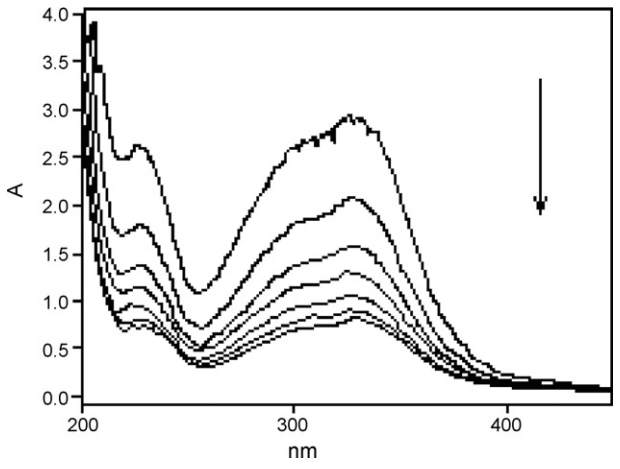



**Fig. 3.** Changes in the UV spectral patterns of CACN-4 in thin film upon after UV irradiation time $t$ = 0, 10, 20, 30, 40 and 60 min.

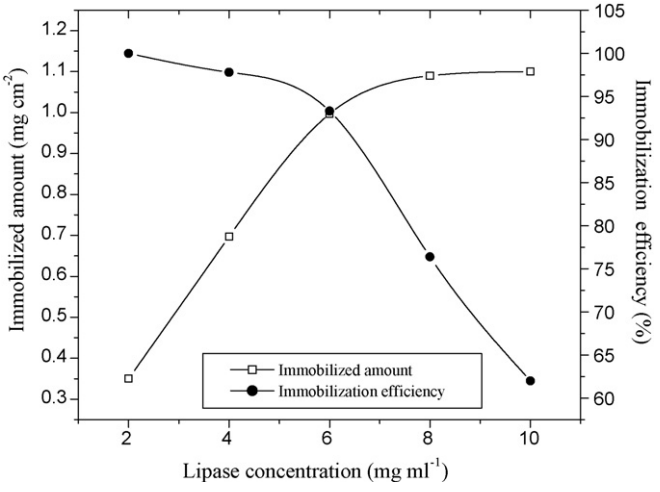
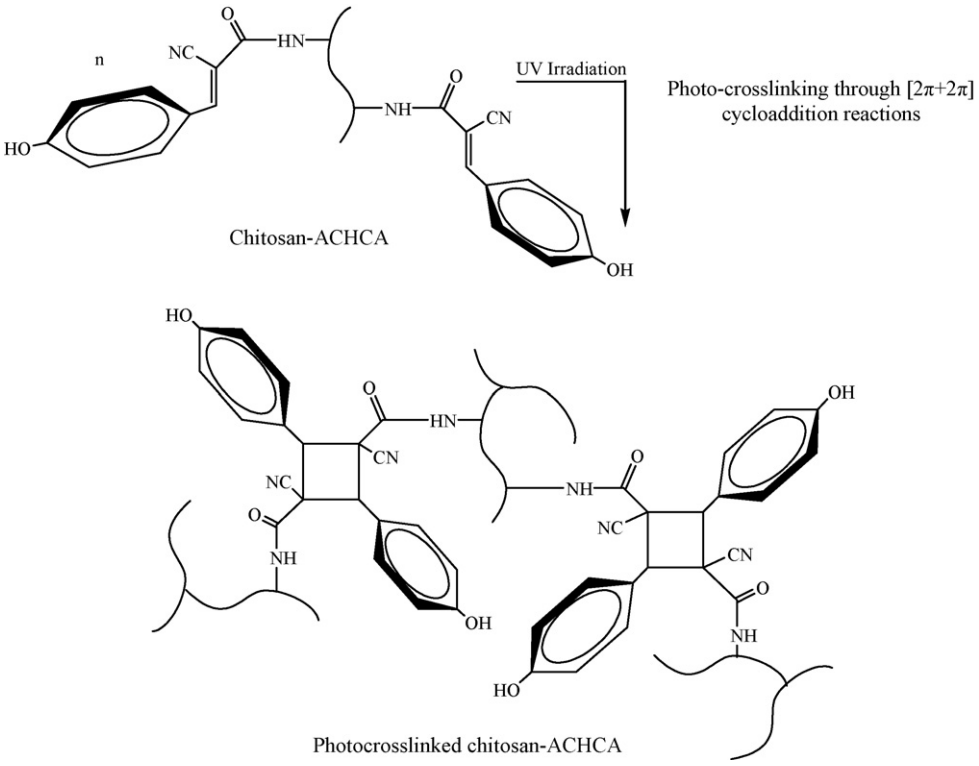



**Fig. 4.** Lipase immobilization in photo-crosslinked chitosan-ACHCA (CACN-4).




**Scheme 2.** Photo-crosslinking of chitosan-ACHCA.

### *3.4. Effect of degree of substitution on the immobilization efficiency and enzyme activity*

Immobilization of enzymes inside crosslinked chitosan-based membrames via entrapment is based on both the encapsulation of enzyme proteins on the crosslinked network of the polymeric carrier membrane support, and the electrostatic attraction between the enzyme and the chitosan-based polymeric carrier which has a cationic nature [20]. The effects of degree of substitution on the immobilization efficiency and on the enzyme activity are presented in Table 2. An increase in the degree of substitution led to an increase in the immobilization efficiency from 78% to 98.8%. The photo-crosslinked membrane CACN-5 has the maximum degree of substitution (78%) and resulted in higher enzyme immobilization efficiency which could be attributed to the corresponding increase in the degree of crosslinking of the polymeric carrier network containing the enzyme and hence, decreases the loss of the enzyme in the washing buffer solution. On the other hand, the retained activity of the immobilized enzyme shows a decrease from 85% to 77% with increasing the degree of substitution. This observation can be explained as being due either to overcrowding of the entrapped lipase in the photo-crosslinked network of the membrane, as a result of which relatively large substrate (olive oil) diffusion limitations occur, or to the presence of protein–protein interactions becoming more important and thus hindering the substrate conversions.

### *3.5. Effect of pH and temperature on the catalytic activity*

The effect of pH on the activity of free and immobilized lipase was also studied at 37 °C (Fig. 6a). The immobilized lipase by CACN-5 exhibited maximal activity at pH 8.0, which was higher than that of the free lipase (pH 7.5). The microenvironment of immobilized lipase and the bulk solution usually has unequal partitioning of $H^+$ and $OH^-$ concentrations due to electrostatic interactions with the photo-crosslinked matrix of the modified chitosan network, which can lead to the displacements in the pH activity profile or probably due to the $H^+$ diffusion problems that significantly displace the pH [38–40]. In this study, the optimal pH of the immobilized lipase was displaced towards more alkaline values. Fig. 6a shows that immobilization resulted a broader pH range where the enzyme showed significant activity, this may be due to the protection function of the modified photo-crosslinked CACN-5 membranes microenvironment or probably due to the fact that the produced fatty acid, accumulating on the membrane surfaces, would reduce the mesh size of the porous network and hence limit solute diffusion [40–42].

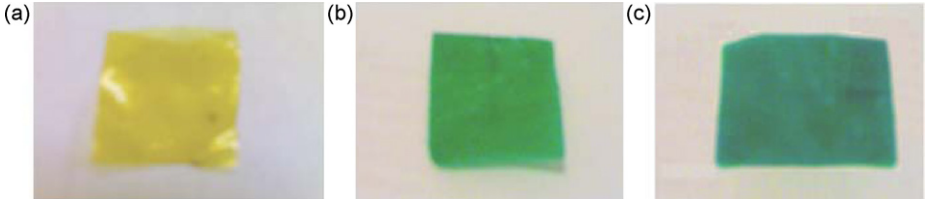



**Fig. 5.** Digital photograph of chitosan-ACHCA membranes after dipped into Coomassie Brilliant Blue solution of 5 ml for 5 min: (a) CACN-5 without lipase, (b) CACN-2 with lipase and (c) CACN-5 with lipase.

**Table 2**
Comparison of entrapment capacity and activity of lipases in modified chitosan-ACHCA membranes.

| Membrane | DS% | Entrapped enzyme (mg cm$^{-2}$) | IE% | Enzyme activity U cm$^{-2}$ membrane | Activity retention % |
|---|---|---|---|---|---|
| CACN-1 | 28 | 0.836 | 78 | 17.765 | 85 |
| CACN-2 | 43 | 0.909 | 85 | 19.089 | 84 |
| CACN-3 | 54 | 0.995 | 93 | 20.143 | 81 |
| CACN-4 | 69 | 1.040 | 98 | 20.436 | 78 |
| CACN-5 | 78 | 1.055 | 98.6 | 20.309 | 77 |

The activity of free and immobilized lipase was determined in phosphate buffer (0.05 M, pH = 7.0) by measuring residual enzyme activity as function of temperature in the range of 20–55 °C. The activity profiles of free and immobilized enzyme by CACN-5 at different temperatures are represented in Fig. 6b. The optimal temperature for immobilized lipase was 40 °C, which was identical to that of free enzyme, but immobilized lipase exhibited a higher residual activity than that of free lipase over 40 °C. This would be due to the encapsulation in modified chitosan membrane increased the stabilization of lipase molecule and even at a higher temperature the immobilized lipase could retain its active structure compared to free enzymes. This is consistent with the results of Zang et al. [38].

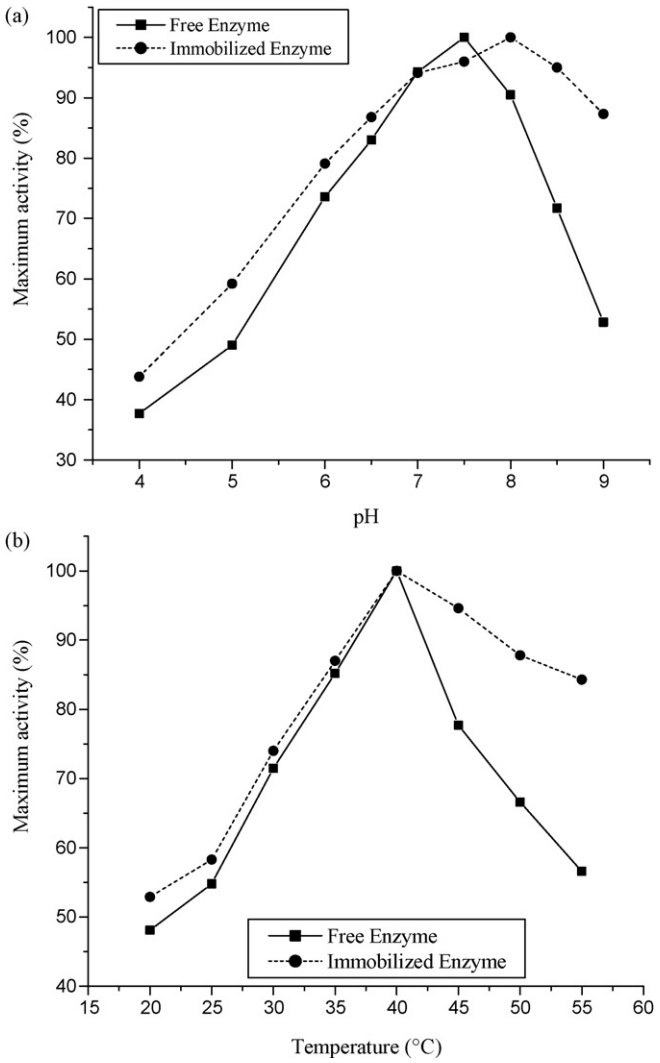



**Fig. 6.** Effect of (a) pH and (b) temperature on the activity of both free and immobilized lipase by CACN-5 membranes.

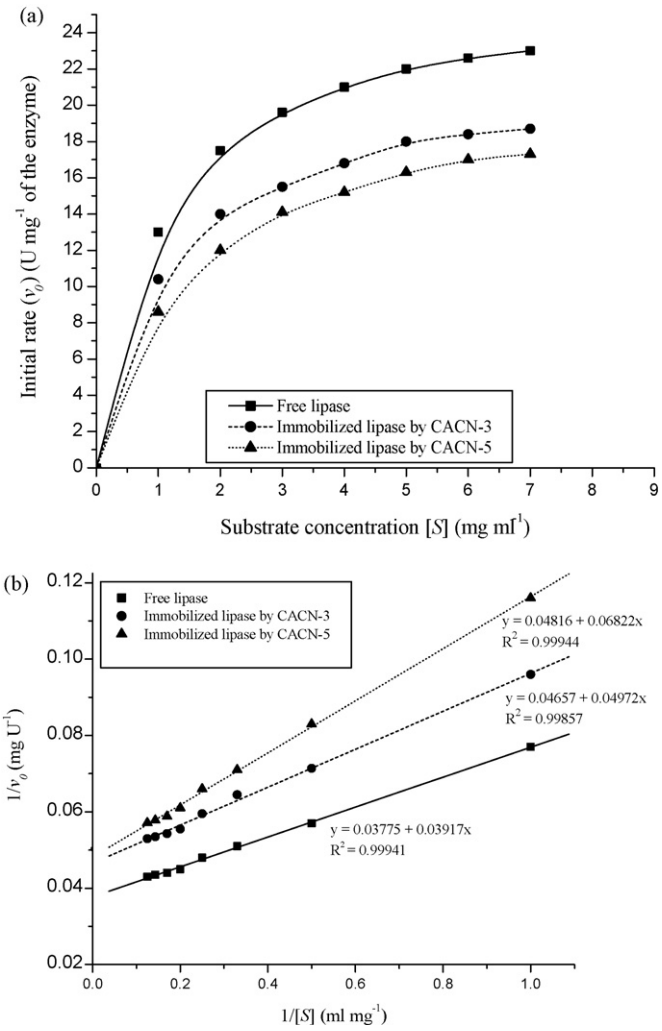



**Fig. 7.** (a) Variation of initial hydrolysis rate of olive oil per with substrate concentration. (b) Lineweaver–Burk plot of free and immobilized lipase by CACN-3 and CACN-5 membranes.

### 3.6. Kinetic properties

Fig. 7a shows the effect of substrate concentration on the initial hydrolysis rate. Increasing the substrate concentration resulted in an enhancement of enzyme activity. The Michaelis–Menten kinetics of free and immobilized lipase was investigated using a series of olive oil concentrations: 1.0, 2.0, 3.0, 4.0, 5.0, 6.0, 7.0 and 8.0 mg/ml according to Eq. (2) [9,38]:

$$\frac{1}{v_0} = \frac{K_m}{v_{\max}} \frac{1}{[S]} + \frac{1}{v_{\max}} \quad (2)$$

where $v_0$ (U mg$^{-1}$ of the enzyme) is the initial rate, [S] is the olive oil concentration (mg ml$^{-1}$), $K_m$ (mg ml$^{-1}$) is Michaelis constant and $v_{\max}$ (U mg$^{-1}$ of the enzyme) is the maximum rate.

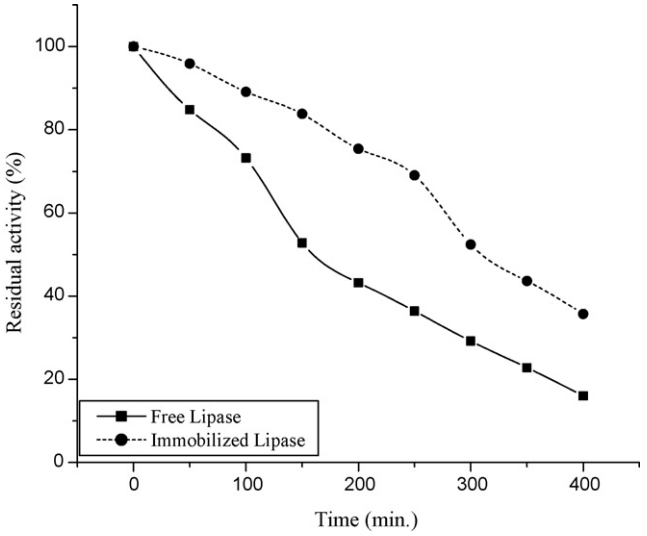



**Fig. 8.** Thermal stability of free and immobilized lipase by CACN-5.

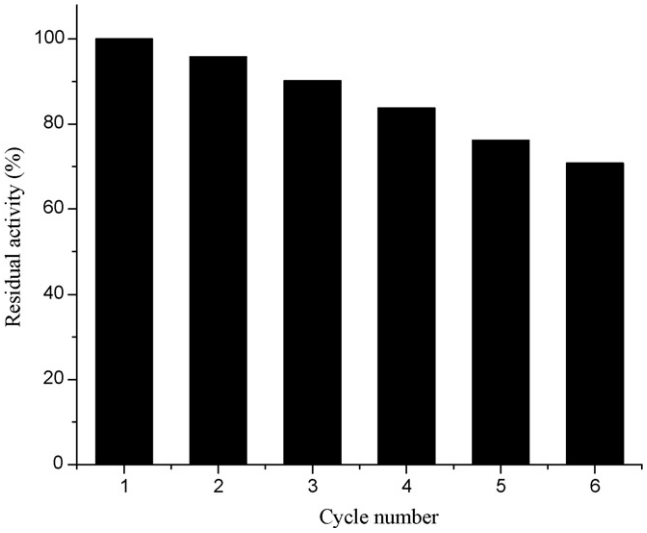



**Fig. 9.** Operating stability of immobilized lipase by CACN-5.

The Michaelis constant, $K_m$ and $v_{max}$ were evaluated from double-reciprocal plots (Fig. 7b). The calculated values of the $K_m$ of immobilized lipase by CACN-3 and CACN-5 were 0.987 and 1.332 mg ml$^{-1}$ ($1.12 \times 10^{-3}$ and $1.50 \times 10^{-3}$ M), respectively, which were higher than that of free lipase (0.932 mg ml$^{-1}$ or $1.05 \times 10^{-3}$ M). Also, the calculated values of $v_{max}$ of the immobilized lipase by CACN-3 and CACN-5 were 21.473 and 20.764 U mg$^{-1}$ of the immobilized enzyme respectively, which were lower than that of free lipase (26.490 U mg$^{-1}$). The increase in $K_m$ and decrease in $v_{max}$ values suggested that the lipase encapsulated in modified chitosan membrane had a lower affinity for binding substrate. This may be due to mass transfer limitations of the water-insoluble olive oil emulsion substrate through the crosslinked network of modified chitosan hydrogel membrane matrix. In other words, the drops of the emulsion may found a difficulty to diffuse inside the hydrogel polymeric support. In addition, the lower affinity for binding the substrate could also attributed to the steric effect arising from the structural rigidity of the entire enzyme structure, which may distorted after entrapment.

### 3.7. Stability properties

The immobilization of enzyme in a carrier often limits its freedom to undergo drastic conformational changes, thus resulting in increased stability towards denaturation. The thermal stabilities of free and immobilized lipase by CACN-5 were determined at 70 °C. As can be seen from Fig. 8, the activity of immobilized enzyme decreased slowly against the incubation time. 6 h later, the residual activities of free and immobilized enzyme were 16% and 35.7% of initial activity, respectively. Lipase immobilization in photo-crosslinked membranes led to a significant stabilizing effect towards heat denaturation. This property is an advantage for continuous use of immobilized lipase. The operational stability of immobilized lipase by CACN-5 was shown in Fig. 9. After 6 consecutive operations, the immobilized lipase could retain 75.5% residual activity.

## 4. Conclusion

In summary, using an EDC/NHS conjugation method, chitosan was chemically modified to incorporate a photosensitive α-cyano-4-hydroxycinnamic acid moiety with various degrees of substitutions. The chitosan α-cyano-4-hydroxycinnamate was fully characterized by FTIR, $^1$H NMR and UV–vis spectra. Lipase from *Candida rugosa* was entrapped into the modified photo-crosslinkable chitosan membranes and crosslinking was carried out by irradiation in the ultraviolet region. The maximum quantity of lipase immobilized on 1 cm$^2$ membrane was 1.04 mg. The optimum temperature for immobilized lipase was 40 °C, which was identical to that of free enzyme. The optimal pH for immobilized lipase was 8.0, which was slighty higher than that of the free lipase (pH 7.5). The apparent $K_m$ value of immobilized lipase was 1.332 mg ml$^{-1}$ ($1.50 \times 10^{-3}$ M), which was higher than that of free lipase. Therefore, the immobilization process slightly decreased the affinity of lipase to substrate. On the other hand, the activity of immobilized lipase decreased slowly against time as compared with that of free lipase, and could retain 75.5% residual activity after 6 consecutive cycles. This immobilization remarkably improved temperature and operational stability, which made it more attractive in the application aspects. On the bases of the obtained results, immobilization of lipase by CACN-3 is recommended as the optimal carrier due to the relatively higher IE% (93%) with 81% retained activity.

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