

Optimization of Phospholipase A₁ Immobilization on Plasma Surface Modified Chitosan Nanofibrous Mat

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Abstract

Phospholipase A₁ is known as an effective catalyst for hydrolysis of various phospholipids in enzymatic vegetable oil degumming. Immobilization is one of the most efficient strategies to improve its activity, recovery and functional properties. In this study, chitosan-co-polyethylene oxide (90:10) nanofibrous mat was successfully fabricated and modified with atmospheric plasma at different times (2, 6 and 10 min) to interact with enzyme molecules. Scanning electron microscopy images revealed that the membranes retained uniform nanofibrous and open porous structures before and after the treatment. PLA₁ was successfully immobilized onto the membrane surfaces via covalent bonds with the functional groups of chitosan nanofibrous mat. Response surface methodology was used to optimize the immobilization conditions for reaching the maximum immobilization efficiency. Enzyme concentration, pH, and immobilization time were found to be significant key factors. Under optimum conditions (5.03 h, pH 5.63, and enzyme dosage 654.36 UI), the atmospheric plasma surface modified chitosan nanofibers reached the highest immobilization efficiency (78.50%). Fourier transform infrared spectroscopy of the control and plasma surface-modified chitosan nanofibers revealed the functional groups of nanofibers and their reaction with the enzyme. The results indicated that surface modification by atmospheric plasma induced an increase in PLA₁ loading on the membrane surfaces.

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1. Introduction

Phospholipase A₁ (PLA₁) hydrolyzes the acyl group of phospholipids at the sn-1 position, liberating the fatty acids, and producing 2-acyl-1-lyso-phospholipid [1]; this reduces phosphorus level to less than 10 mg kg⁻¹ on the degumming process of vegetable oils [2]. Most studies on the degumming of crude vegetable oils are based on free PLA₁ [3-5]. Unfortunately, free enzyme molecules, in most cases, are relatively expensive, chemically or thermally un-stable, and difficult for handling, purification and reuse, which limit the large-scale operations in industrial applications [6-7].

Therefore, immobilization of PLA₁ is a promising

technology, and has a large impact on the industrial scale degumming operations. Immobilization of enzymes onto solid support matrices including particles, fibers, porous films, and hydrogels is a hopeful way to circumvent the disadvantages mentioned above [8].

With the development of nanotechnologies, many efforts have been made to immobilize enzymes onto the surfaces of nano-scaled materials such as nanoparticles, nanotubes, mesoporous materials, and nanofibrous membranes [9].

The results of immobilization, including the performance of immobilized enzymes, strongly depend

on the properties of supports, which are usually referred to as material types, compositions, structures, etc. So far, different nanostructured materials have been used as supports, like mesoporous silica, nanotubes, nanoparticles, and nanofibers. They stand out of other supports because of their extremely high surface area-to-volume ratios, which can provide large specific surface areas for highly efficient immobilization and stabilizing enzymes. However, some of the nanostructured materials have disadvantages that are difficult to overcome. For example, mesoporous silica usually confines enzyme molecules on its inner surface, which limits the diffusion of substrate to/off the enzyme and results in lower enzyme activity. Nanoparticles and nanotubes are known to remarkably decrease mass transfer limitation, while their dispersion and recycling are more difficult. On the contrary, electrospun nanofibers have a great potential to overcome these problems, and may be promising supports for enzyme immobilization. Nanofibrous membranes exhibit intrinsic features including open porous structure and continuous nanofiber formation, enabling the easy accessibility of reactive sites toward enzyme molecules and preventing low mass diffusion in catalytic systems. So, substrate can easily diffuse into open porous nanofibers comparing to other nanostructures [10-12].

Chitosan is a natural cationic linear poly-saccharide composed essentially of β (1 \rightarrow 4) linked glucosamine units together with some proportions of N-acetylglucosamine units, and has excellent features due to its nontoxicity, biodegradability, biocompatibility, physiological inertness, hydrophilicity, remarkable affinity to proteins, and high mechanical strength. These significant chemical and biological characteristics make chitosan a desirable biomaterial for enzyme immobilization [13].

Surface treatment of biomaterials is performed to modify such characteristics as hydrophilicity and protein adsorption, and consequently, to improve cell attachment, proliferation and differentiation. Plasma treatment is a simple process to modify the physical and chemical characteristics of biomaterials without altering their bulk properties and without changing the mutagenic and toxic effects of chemical materials like glutaraldehyde [9]. Recently, plasma-based surface modifications of chitosan and chemically-modified chitosan have been widely studied and shown promising advantages [14]. Ni et al. found that, using open-air plasma, surface-modification could introduce chitosan with more efficient bioactive components [15].

Designing an efficient immobilized enzyme is a multivariate process involving many factors that could affect immobilization efficiency. Response surface methodology (RSM) is a statistically designed experimental protocol for developing, improving, and optimizing processes [16]. It helps to identify the effect of the interactions of different design variables on the response when they are varied simultaneously. Recently, RSM has been successfully used for the optimization of several immobilization processes, such as immobilization of β -galactosidase into chitosan beads

[17], and onto sephadex and chitosan beads [18].

In this research, PLA₁ was immobilized onto chitosan nanofibers for its application in food technology with focus on vegetable oil degumming. The conditions for immobilization of PLA₁ on plasma modified chitosan nanofibrous mats were determined by RSM for reaching the maximum activity recovery of enzyme. Various properties (including pH-activity curve, temperature-activity curve, storage stability, and reusability) of free and immobilized PLA₁ were investigated.

2. Materials and methods

2.1. Materials

Chitosan (CS, low molecular weight, degree of deacetylation 91.2%), polyethylene oxide (PEO, MW 900 KDa), PLA₁ (Lecitase™ Ultra from *Thermomyces lanuginosus*), and soy-phospholipid (PL) were purchased from Easter Groups (Dong Chen Co., Ltd, China), Acros Organics Co. (New Jersey, USA), Novozymes A/S (Bagsvaerd, Denmark) and Behpak Co. (Behshar, Iran), respectively. All other chemicals were obtained from Merck Chemical (Darmstadt, Germany).

2.2. Preparation of CS/PEO nanofibers and plasma treatment

Chitosan and PEO solutions were prepared separately by dissolving chitosan and PEO powders in aqueous acetic acid (80% v/v) under magnetic stirring at 37°C for 24 h. The obtained solutions were then mixed together in a weight ratio of CS/PEO, 90:10, as the required electrospinning solution. The electrospinning processes were carried out at injection rate of 3.0 ml h⁻¹, 20 kV, drum speed of 200 rpm, and electrospinning time of 1 h, using Electroris (FNM, Tehran, Iran). For every run, the polymer solution was placed into a plastic syringe with a stainless steel needle (18 G). An aluminum sheet was wrapped on the Electroris rotating drum as collector, and was located at the distance of 12 cm from the needle [19]. Electrospun fibers were treated by homemade air-dielectric barrier discharge (DBD) plasma consisting of two parallel copper-electrodes where the upper electrode was covered by quartz-dielectric. A DC-pulsed high voltage power supply with a power of 30W and a frequency of 6 kHz was applied to the DBD reactor. Electrospun fiber mats, each measuring 2 cm \times 2 cm (~200 μ m thick), were placed in the chamber, and plasma treatment was performed for 2, 6 and 10 min [14, 20].

2.3. Characterization

To characterize the morphology of electrospun nanofibers before and after plasma treatment, a scanning electron microscope (SEM; Philips XL30, USA) was used. To test the chemical structure changes, the fourier transform infrared (FTIR) spectra of original and atmospheric plasma surface modified CS/PEO nanofibrous mats (APSM CS/PEO NM)

were measured from 4000 to 500 cm^{-1} at a resolution of 4 cm^{-1} by a TENSOR 27 spectrometer (Bruker Optics, USA).

2.4. Enzyme assay

PLA₁ assay was performed with soy-phospholipid emulsion using the method of Yang *et al.* [21]. One unit of PLA (UI) is the amount of enzyme, which releases 1 μmol of titratable free fatty acids per minute. Four ml of substrate emulsion (25% PL and 4% polyvinyl alcohol solution with a volume ratio of 1:4), 5 of 0.01M citric acid buffer (pH 5.0), and 1 ml of enzyme solution were mixed and incubated at 37°C for 10 min. The reaction was terminated with the addition of ethanol 95% (15 ml) after incubation, and the liberated free fatty acids were titrated with 0.05 M NaOH. Blanks were measured with heat-inactivated PLA samples (95°C, 10 min). All experiments were carried out in triplicate

2.5. Immobilization of PLA₁

PLA₁ solution was prepared by solving appropriate amount of PLA₁ enzyme in phosphate-citrate buffer (0.05 M, pH 4.5-6.5). Varying amounts of PLA₁ enzyme (200-1000 U) were added to the APSM CS/PEO NM and the control nanofibers (before plasma treatment), and incubated overnight at 4°C for different times (1.5-7.5 h). The efficiency of immobilization was evaluated for chitosan nanofiber using Eq. 1:

$$Y = (A_1 / A_2) \times 100 \quad \text{Eq.1}$$

Where, Y is immobilization efficiency; A₁ is Specific activity of immobilized PLA₁ (U mg^{-1}); and A₂ is the specific activity of soluble free PLA₁ (U mg^{-1}).

Specific activity of the immobilized enzyme was calculated by subtracting the specific activity of washed fractions from the specific activity of the added enzyme [18]. Protein concentration in the solution was determined with Coomassie Brilliant Blue reagent following the Bradford's method with Bovin Serum Albumin as standard [22].

2.6. Effect of pH and temperature on enzyme activity

The effect of pH on the activity of free and immobilized PLA₁ was studied by immersing the enzymes onto 50 mM phosphate-citrate buffer with pH ranging from 4.5 to 7.5 at 25°C (ambient temperature). The effect of temperature on enzyme activity was determined by immersing the enzymes into phosphate-citrate buffer (pH 5.5) with temperature ranging from 30 to 70 C.

The initial activity was designated as 100%, and the activities at all the remaining pH and temperature values were proportional to it [2, 23].

2.7. Storage stability and Reusability tests

Storage stability of the free and immobilized PLA₁ was measured by keeping them in phosphate-citrate buffer (50 mM, pH 5.5) and storing at 4°C for up to 30 days. The remaining activity of the enzymes was measured periodically [31, 36]. To evaluate the reusability, the tested immobilized PLA₁ membranes were washed with phosphate-citrate buffer to remove any residual chemicals, followed by immersing into fresh catalytic reaction medium under the same experimental conditions. This procedure was repeated up to 10 times. The residual enzyme activity after each cycle was defined as the value proportional to the initial activity (100%) [23].

2.8. Experimental designs and statistical analyses

Experimental designs and statistical analyses reported in this paper were generated using DX7 software (Trial version, Stat-Ease, Inc., USA). Effect of various parameters was optimized using the response surface method (RSM). A 20 run central composite rotatable design (CCRD) was performed in order to determine optimum conditions for enzyme immobilization. This design assesses the influence of the main factors, as well as their interaction. Three main factors namely pH (4.5, 4.9, 5.5, 6.1, and 6.5), enzyme concentration (200.0, 362.1, 600.0, 837.8, and 1000 UI), and immobilization time (1.5, 2.72, 4.5, 6.28, and 7.5 h) were studied in five levels. The response was immobilization efficiency (%) in each case. Analysis of variance (ANOVA) was used to compare the other parameters, and Duncan's test was used as post hoc. All experiments were carried out in triplicate, and the data were represented as mean \pm SD (SPSS, ver. 16). The significance was considered as $p < 0.05$.

3. Results and Discussion

3.1. Surface morphologies of CS/PEO and APSM CS/PEO nanofibrous mat

To prepare a support medium for enzyme immobilization, CS/PEO polymer containing 90% chitosan and 10% polyethylene oxide was fabricated into nanofibrous mat. The morphology of the electrospun mats was investigated through the SEM micrographs. The original CS/PEO NM presented homogeneous and uniform network of continuous nanofibers with a diameter range of 70–240 nm. (Figure 1a).

After surface activation by atmospheric plasma at different times, total fibrous structures of the APSM CS/PEO NM remained intact; however, there were a few structural deformations, especially at 10 min plasma treatment, as shown in Figure 1 (b, c and d).

The surface modification by atmospheric plasma caused remarkable change in the stability of CS/PEO nanofibers (Figures 1b', c' and d') in comparison with

the non-treated CS/PEO nanofibers (Figure 1a') after immersing in distilled deionized water (DDW).

The FTIR spectra verified that CS/PEO nanofibers are a combination of chitosan and polyethylene oxide (Figure 2).

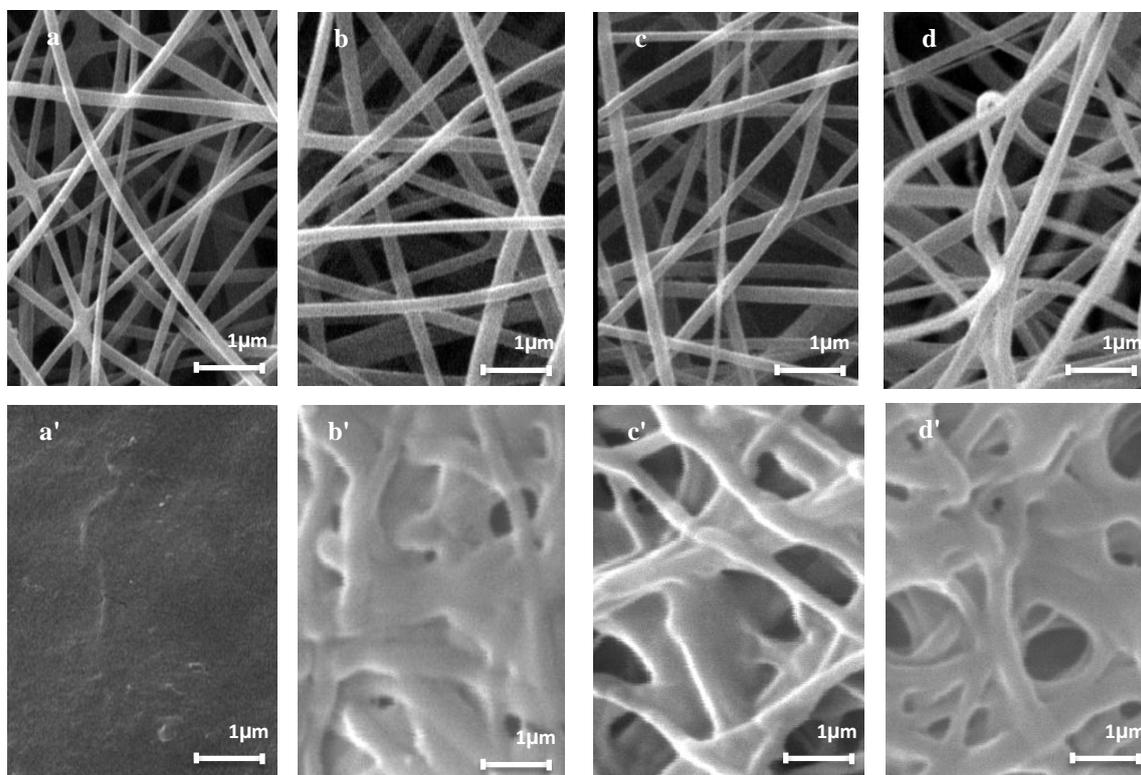


Figure 1. SEM images of control CS/PEO NM(a), and 2 (b), 6 (c) 10 (d) minute APSM-CS/PEO NM before immersing in DDW, and control CS/PEO NM(a'), and 2 (b'), 6 (c') 10 (d') minute APSM-CS/PEO NM after immersing in DDW

Major characteristic peaks of chitosan were observed at the wavenumbers of 3450 cm^{-1} (stretching N–H and O–H), 1650 cm^{-1} (stretching C=O of carbonyl group), 1375 cm^{-1} (stretching C–H of methyl group), and 1092 cm^{-1} (stretching C–O) [24].

According to the FTIR spectra, the functional group and wavenumbers observed for the CS/PEO nanofibers were the same as those of chitosan and PEO alone. The new peaks observed in the $1550\text{--}1650\text{ cm}^{-1}$ were assumed to be related to the functional groups of asymmetric C=O in the CS/PEO nanofibers and contingency resonant. This means that double bond leads to the creation of C–O and C=O structures to link the carbon and oxygen. Despite the fingerprint, carbon-oxygen double bond and the hydroxyl group of chitosan and polyethylene oxide showed the successful synthesis of CS/PEO nanofiber [24].

In this study, the NH_2 groups existing on the membrane surface were converted into more reactive NH^{3+} groups via atmospheric plasma activation, and the surface NH^{3+} groups could further form covalent bonds with the enzyme molecules, leading to the immobilization of the enzymes [25]. Covalent immobilization provides strong bonding between the enzyme and support, and thus reduces enzyme leakage [26].

A series of atmospheric plasma with different initial times ranging from 2 to 10 minutes were applied to investigate the effect of time on surface functionalization and enzyme immobilization. The chemical structure changes were characterized via FTIR. Figure 3 shows the FTIR spectra for the unmodified and APSM CS/PEO nanofibers with different treatment times. The original nanofibers exhibited an stretching C–O bond (C from the carboxyl and O from the hydroxyl groups) at approximately 1100 cm^{-1} , and then an stretching amino bond at approximately 1600 cm^{-1} [24]. The C = O carbonyl stretch of a carboxylic acid bond appears at approximately 1650 cm^{-1} [27]. The observed bond at approximately $3000\text{--}3500\text{ cm}^{-1}$ is contributed by the stretching vibrations of –NH and –OH groups [28]. Furthermore, the FTIR spectra for the APSM CS/PEO NM are similar to those of the control CS/PEO nanofibers; however, during the atmospheric plasma treatment, the intensity of some links such as hydroxyl and carboxylic acid at 1100 cm^{-1} and 1650 cm^{-1} were increased on 6 minutes but they were decreased on 2 and 10 minutes.

Also the intensity of –NH group on 6 minutes was more than that of other samples. This phenomenon

represents that the functional groups of CS/PEO NM were involved after treatment with plasma.

In addition, the peak related to C–C bond from both the combined CS/PEO and CH₂ in ethylene oxide group from PEO in 1360-1460 cm⁻¹ after plasma treatment was observed sharper in all samples than in

the control CS/PEO nanofibers ; however, on 6 min of treatment, it was increased significantly.

As shown in Figure 3, after 6 min atmospheric plasma treatment, the binding strength was increased approximately for all peaks.

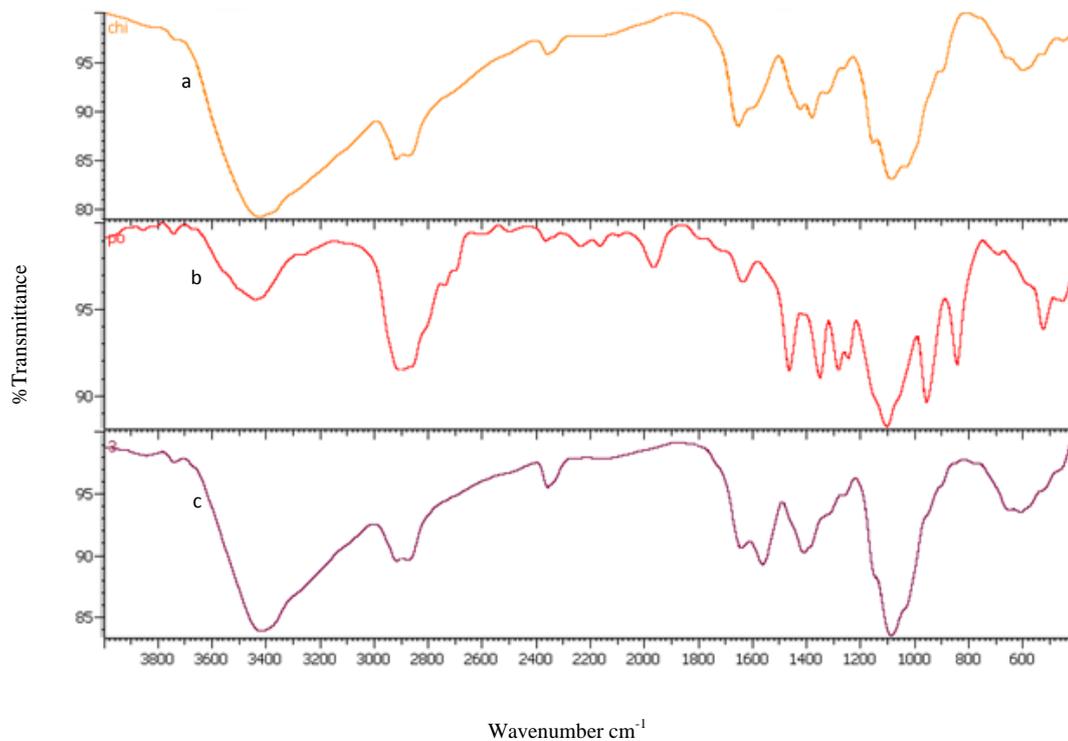


Figure 2. FTIR spectra of chitosan (a), polyethylene oxide (b), and CS/PEO NM (c)

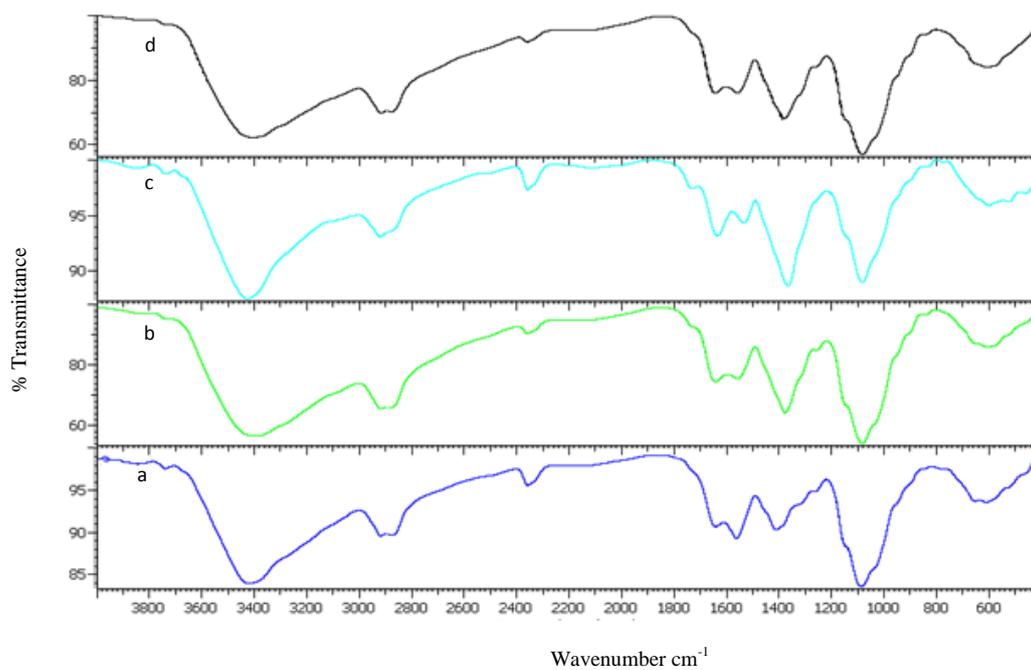


Figure 3. FTIR spectra of CS/PEO NM before (a), and after 2 (b), 6 (c), and 10 (d) minute atmospheric plasma treatment

So, 6 min atmospheric plasma treatment was chosen as the suitable plasma reaction time for CS/PEO NM mats for enzyme immobilization. The results showed that plasma treatment caused to changes in the surface structure of the functional groups and increased the ion exchange property of the membranes without using chemicals.

3.2. Optimization of immobilization conditions

To optimize the balance between enzyme loading and the activity of the immobilized enzyme, the properties of the immobilized PLA₁ on 6 min APSM CS/PEO NM were tested. Studies have shown that enzyme concentration, pH and immobilization time significantly affected the immobilization of enzyme into nanofibers [2,18,24]. Thus, these three factors were further optimized using the RSM to obtain the maximum activity recovery of immobilized PLA₁.

Table 1. Levels of design variables ($\alpha=1.68$)

Variables	Range of levels				
	$-\alpha$	-1	0	+1	$+\alpha$
Time (h)	1.50	2.72	4.50	6.28	7.50
PLA ₁ (U)	200	362.16	600	837.84	1000
pH	4.50	4.91	5.5	6.09	6.50

To assess the influence of each factor and their interactions, a CCRD of 6 central points with 14 experiments was performed. The levels of each factor are given in Table 1, and the experimental data are recorded in Table 2. The summary of ANOVA for the model of immobilization efficiency is given in Table 3. By regression analysis, a quadratic polynomial equation was established to explain the relationship between the activity recovery and the independent variables as follows:

Eq.2

$$Y = 75.85 + 6.83 X_1 + 5.57 X_2 + 50.90 X_3 + 3.62 X_1 X_3 - 13.17 X_1^2 - 13.04 X_2^2 - 16.41 X_3^2$$

Where, Y, X₁, X₂, and X₃ represent efficiency, immobilization time, enzyme dosage, and pH, respectively. The calculated F ratio was 112.50 and P value was less than 0.0001, implying that the model is extremely significant and satisfying.

A verification experiment was performed under the optimal conditions to validate the reliability and accuracy of the model. The experimental activity recovery was determined to be 78.50%, which is in excellent agreement with the value predicted by the model (Table 4).

Accordingly, this model could be considered reliable and accurate for predicting the immobilization efficiency and activity recovery of immobilization of PLA₁. The coefficients of the terms along with their p-values (P<0.01) show which terms contributed significantly to the responses. A smaller P-value indicates a higher level of significance for the corresponding coefficient [17].

According to the ANOVA, which was used to check the significant of the effects and assess the

goodness of fit (Table 3), a predicted response surface model was statistically significant ($p<0.0001$).

The non-significant value of lack-of-fit ($p>0.2781$), and high values of R² (0.9902), adjusted R² (0.9814) and predicted R² (0.9476) revealed that the model is statistically significant for the response to predict and explain the variations in immobilization efficiency formulation. Central point of CCRD design (enzyme concentration, 600 UI; pH, 5.50; and immobilization time, 4.50 h) leads to the maximum immobilization efficiency of PLA₁ on APSM CS/PEO NM with 72.5-80.06%.

For the control CS/PEO NM, immobilization efficiency was detected as 59.2-63.50% in the same conditions. The results confirmed that in this system, plasma activation is an important factor that affects the performance of the immobilized PLA₁. As shown in Figure 4, under optimum conditions, the enzyme was successfully loaded on APSM CS/PEO NM.

The ATR-FTIR spectra showed that the intensity of some links such as C=O and C-N in 1500-1650 cm⁻¹, O-H and N-H in 3000-3500 cm⁻¹ significantly increased after PLA₁ immobilization. Additionally, the efficiency of PLA₁ immobilization in this study is much higher than that of the immobilized PLA₁ on other different supports, such as magnetic nanoparticles (64.7%) [2], calcium alginate (56.2%), calcium alginate-chitosan (65.5%) and calcium alginate-gelatin (60.2%) [29].

The change in pH value can influence the electrical charges on the surface of the nanofibers, and limit the interactions between the functional groups on the surface of nanofibers and those of PLA₁. Protein denaturation can occur under conditions of excess acid or alkali [30-31].

Therefore, at pH values under and above 5.3-5.7, the efficiency of PLA₁ immobilization significantly decreases.

Table 2. Central composite design of factors and efficiency for PLA₁ immobilization on 6 min APSM-CS/PEO NM

Run	Time	PLA ₁ dosage	pH	Efficiency
1	0	0	0	72.50
2	0	0	0	74.60
3	+1	+1	+1	56.08
4	0	$-\alpha$	0	26.60
5	-1	+1	-1	26.05
6	+1	-1	+1	44.56
7	0	0	$+\alpha$	36.56
8	0	0	0	80.06
9	$-\alpha$	0	0	25.86
10	$+\alpha$	0	0	51.06
11	0	0	0	76.00
12	+1	+1	-1	33.56
13	0	0	0	77.00
14	-1	-1	-1	21.05
15	0	0	$-\alpha$	22.02
16	0	$+\alpha$	0	51.06
17	-1	+1	+1	35.05
18	+1	-1	-1	24.55
19	0	0	0	75.00
20	-1	-1	+1	25.65

Table 3. ANOVA for the results of the central composite design

Source	DF	SS	MS	Coefficient	F-value	P-value
Model	9	9052.24	1005.80	75.85	112.50	<0.0001 ^a
X ₁	1	637.83	637.83	6.83	71.34	<0.0001 ^a
X ₂	1	423.68	423.68	5.57	47.39	<0.0001 ^a
X ₃	1	475.49	475.49	5.90	53.18	<0.0001 ^a
X ₁ × X ₂	1	4.70	4.70	0.77	0.53	0.4852
X ₁ × X ₃	1	104.62	104.62	3.62	11.70	0.0065 ^a
X ₂ × X ₃	1	5.97	5.97	0.86	0.67	0.4329
X ₁ ²	1	2500.58	2500.58	-13.17	279.69	<0.0001 ^a
X ₂ ²	1	2451.16	2451.16	-13.04	274.16	<0.0001 ^a
X ₃ ²	1	3882.97	3882.97	-16.41	434.31	<0.0001 ^a
Residual error	10	89.40	8.94	-	-	-
Lack of fit	5	56.83	11.37	-	1.74	0.2781
Pure error	5	32.58	6.52	-	-	-
Corrected total	19	9141.64	-	-	-	-
R ² = 0.9902		R ² (adj) = 0.9814		R ² (pre) = 0.9476		

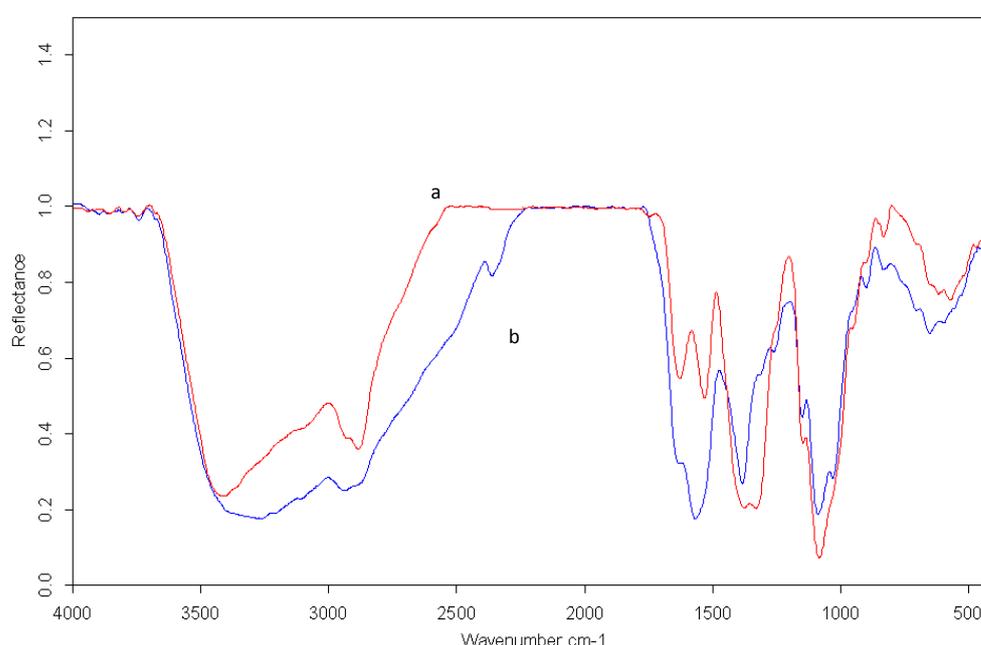


Figure 4. ATR-FTIR spectrum of 6 minutes APSM-CS/PEO NM before (a) and after (b) PLA₁ immobilization in optimum conditions

Treatment time in the range of 4.7-5.3 h causes to reach a maximum immobilization efficiency and then steadily decline over the next 5.3 h. It shows that longer immobilization times could result in a greater loading of PLA₁ molecules on the surface of the support, and limit the substrate diffusion due to steric inhibition. In order to find the optimal concentration of PLA₁, the immobilization of various concentrations of PLA₁ was investigated. The highest efficiency was obtained at the range of 590-690 UI. Further increase in PLA₁ concentration caused a decrease in its activity and thus immobilization. It means that enzyme

activity does not increase constantly after a distinct enzyme concentration because of saturation of the surface of the support, and intra-interactions of the extra enzymes' functional groups, especially the amino and carboxylic groups.

Moreover, the saturation and multi-layering of the enzyme on the surface could cause intermolecular steric hindrance to substrate diffusion at higher enzyme/support ratios. The enzymatic aggregation on the surface of APSM CS/PEO NM could block the active sites of the enzyme [31].

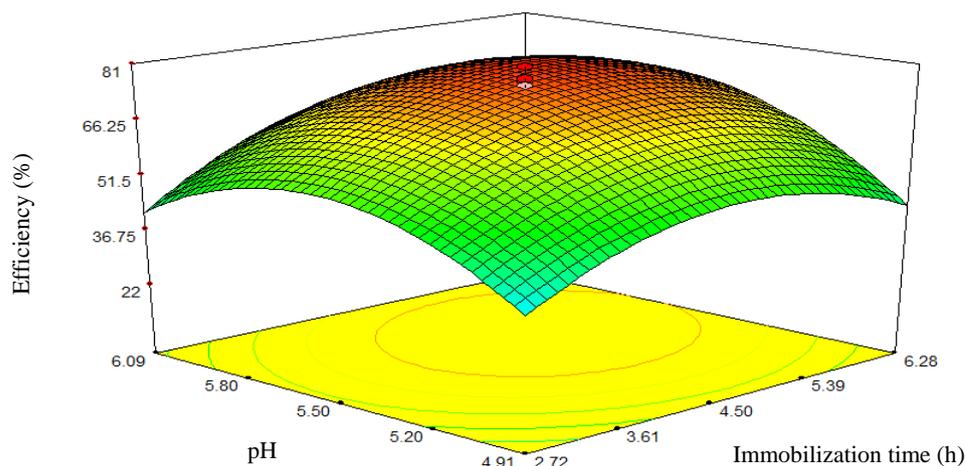


Figure 5. Response surface for (A) Immobilization time (B) Efficiency and (C) pH

3.3. PLA₁ activity

3.3.1. Effect of pH and temperature

The effect of media pH on the activity of free and immobilized PLA₁ was studied in the pH range of 4.5–7.5, and the results are shown in Figure 6a. The optimum pH for the free and immobilized PLA₁ on APSM CS/PEO NM was 5.5 and 6.0, with 64% and 78% relative activity, respectively. Compared to the free PLA₁, the pH-activity profile of the immobilized enzyme showed an upward shift in pH with considerable broadening due to greater pH stability. This phenomenon might be due to the immobilized enzyme molecules preserved from the conformational alterations induced by pH changes and the enzyme can be more active in the wide range of pH values [2].

This shift was attributed to the partitioning effects of the supports. Since the reaction medium and the insoluble support are two different phases, the depletion in one of the phases is possible. When this occurs, the immediate microenvironment of the enzyme on the surface of the support material will be different from that of the bulk solution. Similar results have been reported by other researchers [2,31]. The broadened pH/activity profile of immobilized PLA₁ may have important benefits when developing the industrial applications. The optimum temperature for the free PLA₁ was 50°C, while the maximum activity of the immobilized PLA₁ was at 60°C (Figure 6b).

The shift in optimal temperature toward higher values could be due to the immobilization of the enzyme and resulted in the formation of the enzyme–substrate complex, hindering the access of substrates to the active sites and covalent bond between the enzyme and support medium. There are many advantages of running bioprocesses at elevated temperatures such as higher diffusion rates, lower substrate viscosities, increased reactant solubility, and reduced risk of microbial contamination [23,31].

The PLA₁ immobilized on the control CS/PEO NM had intermediate state between the free and the

PLA₁ immobilized on APSM CS/PEO NM in the effect of pH and temperature. Indeed, it shows that unmodified CS/PEO NM has functional groups too. These groups can make a covalent attachment between the nanofibers and enzyme; however, this ability can be extremely improved by treating with plasma.

3.3.2. Reusability and storage stability

Rapid decrease of catalytic activity during the storage time and difficult recovery after catalytic reactions limit broader applications of free enzymes. Thus, a variety of solid support media have been developed for enzyme immobilization to overcome these drawbacks. Figure 7a shows that the activity of the immobilized PLA₁ on APSM CS/PEO NM decreases gradually during the repeated tests, and 80% of its initial activity is still retained after 10 cycles, while the PLA₁ immobilized on control CS/PEO NM shows a greater reduction of its initial activity after 10 cycles.

With repeated use of the immobilized PLA₁, the strength of binding between the matrix and the enzyme get weakened, leading to loss in the enzyme activity. Frequent encountering of the substrate into the active site causes its distortion, and thus its catalytic efficiency is reduced. In addition, the free and immobilized PLA₁ were kept in phosphate-citrate buffer at 4°C for 30 days, and their retained activities were tested periodically to examine their storage stability. As shown in Figure 7b, the free PLA₁ has lost more than 50% of its initial activity after 30 days, while the retained activity of the immobilized PLA₁ on APSM and original CS/PEO NM were 76% and 59% of their initial activity, respectively, during the same period.

These results indicate that the enzyme is bound to the plasma-modified chitosan with higher strength; hence, it is not leached out with repeated washings [18]. These attractive results demonstrate the great potential of using CS/PEO nanofibers for enzyme immobilization and related biocatalytic applications.

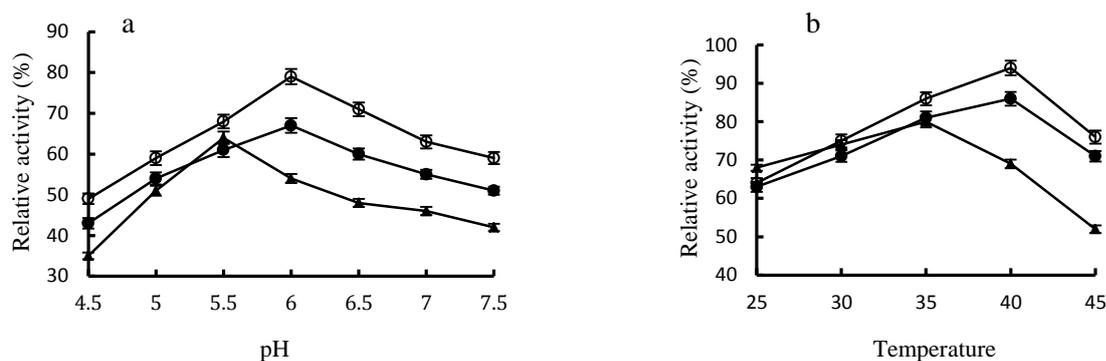


Figure 6. The effect of pH (a) and temperature (b) the on activity of free PLA₁ (▲), immobilized PLA₁ on CS/PEO NM (●), and APSM CS/PEO NM (○).

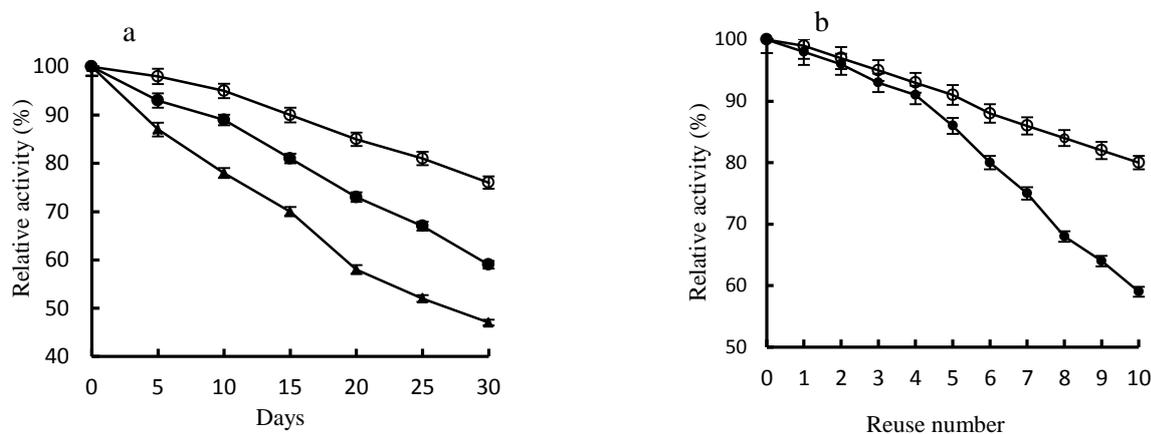


Figure 7. Reusability of immobilized PLA₁ (a) on CS/PEO NM (●) and APSM-CS/PEO NM (○); storage stability (b) of free PLA₁ (▲), immobilized PLA₁ on CS/PEO NM (●), and APSM CS/PEO NM (○).

4. Conclusions

In the present work, APSM CS/PEO NM was successfully prepared as solid support medium for PLA₁ immobilization. Media optimization for immobilization was developed by RSM.

Immobilization time, enzyme concentrations and pH were determined as effective factors on immobilization efficiency. The PLA₁ immobilized APSM CS/PEO NM not only provides a simple pathway to reach high enzyme loading, effective catalytic activity, easy recovery, and reusability, but also remarkably improves the enzyme stability under common pH, thermal, and storage conditions. This work demonstrates the great potential of APSM CS/PEO NM with good functional groups with covalent bonds for enzyme immobilization, and that the immobilized PLA₁ could have wide applications in biocatalytic fields such as enzymatic degumming of edible oils.

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6. Conflict of interest

The Authors declare that there is no conflict of interest.

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