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Immobilization of *Candida rugosa* lipase on electrospun cellulose nanofiber membrane

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ABSTRACT

A biocatalyst with high activity retention of lipase was fabricated by the covalent immobilization of *Candida rugosa* lipase on a cellulose nanofiber membrane. This nanofiber membrane was composed of nonwoven fibers with 200 nm nominal fiber diameter. It was prepared by electrospinning of cellulose acetate (CA) and then modified with alkaline hydrolysis to convert the nanofiber surface into regenerated cellulose (RC). The nanofiber membrane was further oxidized by $NaIO_4$. Aldehyde groups were simultaneously generated on the nanofiber surface for coupling with lipase. Response surface methodology (RSM) was applied to model and optimize the modification conditions, namely $NaIO_4$ content (2–10 mg/mL), reaction time (2–10 h), reaction temperature (25–35 °C) and reaction pH (5.5–6.5). Well-correlating models were established for the residual activity of the immobilized enzyme (R^2 = 0.9228 and 0.8950). We found an enzymatic activity of 29.6 U/g of the biocatalyst was obtained with optimum operational conditions. The immobilized lipase exhibited significantly higher thermal stability and durability than equivalent free enzyme.

1. Introduction

Enzymes are highly specific, efficient, and "green" catalysts in fine-chemical or pharmaceutical synthesis, food processing, biosensor fabrication and bioremediation [1–5]. In practical applications, enzymes are always attached or incorporated onto or into an inert, insoluble material to overcome the limitations of instability and non-reusability for the free enzymes [6–10]. This protocol can provide increased resistance to changes in conditions such as pH or temperature. It also allows enzymes to be held in place throughout the reaction, following which they are easily separated from the products and may be used again – a far more efficient process and so is widely used in industry for enzyme catalysed reactions [11–13]. However, these advantages strongly depend on the properties and microstructures of the supports for enzyme immobilization. It is thus important that the choice of support materials and enzyme immobilization processes should be well understood.

Since the immobilization process for enzymes will inevitably result in some loss of activity, improving the activity retention of the immobilized enzyme is critical. An effective support surface for enzyme immobilization should suppress the non-biospecific interactions of enzyme support and the denaturation of enzyme protein, and thus allow high enzyme activity to take place. Among various materials evaluated as effective supports, cellulose is inert and biocompatible under physiological conditions, and has a dual hydrophilic/hydrophobic character that can be "tuned" for enzyme immobilization, making cellulose-based supports for enzyme immobilization of great potential for both science and industry [9,10]. On the other hand, the microstructures of support also have great effect on the catalytic behaviors of immobilized enzyme. Nanoscale materials have large surface to volume ratios for high enzyme loading. We propose that electrospun polymer non-woven nanofiber membranes are promising support materials for enzyme immobilization due to the properties such as high specific surface area for high enzyme loading per unit mass, fine porous structure allowing ready accessibility to active sites and the low diffusion resistance, easy recoverability as well as potential applicability for continuous operations [13–19].

It is known that the enzyme immobilization is affected by many factors such as activated reagent content, reaction time, reaction temperature and reaction pH and thus it is important to optimize these factors in order to improve the performance of the system and to enhance the yield without increasing the cost. Most of the studies on enzyme immobilization used one-variable-at-a-time technique, meaning one parameter is changed during the process while keeping the others at a constant level [20,25]. This approach of optimization is not only time-consuming but also ignores interaction effects of multiple parameters. In order to overcome this problem, optimization can be applied using response surface methodology (RSM). RSM is a statistical and

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mathematical technique used for improving and optimizing processes in which a response of interest is influenced by several variables and the objective is to optimize this response [20]. The graphical presentation of RSM function is called response surface. Central composite design has been the most successful factorial design for the optimization of parameters with a limited number of experiments and estimates the response surface [21]. RSM has been popular for optimization studies in recent years and has been applied in biochemical and biotechnological processes [22–25]. Some examples of its successful optimization in enzyme immobilization are covalent immobilization of *C. rugosa* lipase on poly(γ-glutamic acid) [24] and cellulase on the smart polymer Eugragit L-100 [25]. The advantage of this statistical design of experiments compared to conventional method is that the number of experiments to optimize a number of factors is minimized and observations of interaction effects between individual components in the response are possible. Consequently, the method also offers a large amount of information from a small number of experiments [26].

In this paper, an effective and convenient protocol to immobilize lipase using cellulose nanofiber membrane is described. Cellulose acetate (CA) was electrospun into a non-woven nanofiber membrane and then oxidized by $NaIO_4$ to generate aldehyde groups [27–29], to which enzyme molecules containing primary amino groups can attach by covalent bonding. Lipase from *Candida rugosa* was used as the model enzyme. RSM was used to optimize the extent of partial modification of the cellulose nanofiber membrane prior to enzyme immobilization. Four conditions, namely $NaIO_4$ content, reaction time, reaction temperature and reaction pH, were optimized. Furthermore, these factors can be related to produce performance metrics such as enzyme loading and activity, optimum processing pH and temperature, thermal stability, reusability and storage stability of the immobilized lipase.

2. Experimental

2.1. Materials

CA with an acetyl content of 29.6% was purchased from Sinopharm Chemical Reagent Co. Ltd. (Shanghai, China) and used without further purification. Lipase (from *C. rugosa*) powders (1150 units/mg solid), Bradford reagent, bovine serum albumin (BSA, molecular mass: 67,000 Da) and *p*-nitrophenyl palmitate (*p*-NPP) were commercial products of Sigma–Aldrich Chemical Co. (St. Louis, MO, USA) and used as received. All other chemicals were of analytical grade and used without further purification.

2.2. Membrane preparation

CA was stirred for 2 h in a 3:2 mass ratio mixture of acetone/*N*,*N*-dimethyl-acetamide (DMAc) to form a 15% (w/w) homogeneous solution. The solution was left to settle until free of air bubbles, and then injected into a 20 mL syringe with a 0.7 mm inner diameter metal needle connected to a high voltage power supply (GDW-a, Tianjin Dongwen High-voltage Power Supply Plant, China). Typically, electrospinning was performed at a potential of 17 kV, with 15 cm between the needle tip and the earthed aluminum collector. The solution feed rate was controlled by a micro-infusion pump (WZ-50C2, Zhejiang University Medical Instrument C., Ltd., China). It took 8 h to obtain a nanofiber membrane with sufficient thickness for further processing.

The method described by Liu and Hsieh [27] was used to convert the nanofiber membrane surface to regenerated cellulose (RC). The CA nanofiber membrane was immersed in 0.5 M KOH in ethanol and shaken at ambient temperature for 3 h [30], followed by washing with deionized water for several times to remove the residual KOH adsorbed on the membrane surface. The RC nanofiber membrane was then gently spread on a filter paper and dried at 60 °C under vacuum.

For oxidation of the cellulose membrane, the RC nanofiber membrane was submerged into deionized water overnight, and then oxidized by $NaIO_4$ solution at different contents ranging from 2 mg/mL to 10 mg/mL to obtain aldehyde groups capable for immobilizing lipase. The oxidation process was carried out in a shaking water bath. After this, the nanofiber membrane was taken out, thoroughly rinsed with deionized water and dried under vacuum at 60 °C.

Table 1
Central composite rotatable design for the oxidation of cellulose nanofiber membrane.

Exp. no.	Design 1		Design 2	
	t (h)	*c* (mg/mL)	*T* (°C)	pH
1	6.00	2.00	30.0	6.0
2	4.00	5.00	30.0	5.5
3	10.00	4.00	25.0	6.0
4	2.00	4.00	30.0	6.5
5	6.00	4.00	25.0	5.5
6	4.00	3.00	30.0	6.0
7	8.00	5.00	35.0	6.0
8	6.00	4.00	35.0	5.5
9	6.00	6.00	35.0	6.5
10	6.00	4.00	30.0	6.0
11	8.00	3.00	30.0	6.0
12	6.00	4.00	30.0	6.0
13	6.00	4.00	25.0	6.5

c, $NaIO_4$ content.

2.3. Experimental design

Response surface methodology (RSM) was applied to optimize the reaction conditions to maximize residual enzyme activity. Four variables ($NaIO_4$ content, reaction time, reaction temperature, reaction pH), affecting the residual activity of immobilized enzyme significantly, were optimized. A 2-factor-5-level CCD and a 2-factor-3-level CCD were used as two central composite designs (CCD). Each design was constructed with Design Expert 7.0 software (Minneapolis, MN, USA), and generated a total of 13 unique experiments (see Table 1). All experiments were carried out as near simultaneously as possible to minimize the effect of experimental error in the observed response.

2.4. Determination of aldehyde contents

Aldehyde content on the oxidized cellulose membrane surface was determined by a titration method [27]. Accurately weighed 0.1 g oxidized cellulose nanofiber membrane was treated with 10 mL hydroxylamine hydrochloride in a 100 mL conical flask at 50 °C for 2 h. Then the mixture was cooled to room temperature and titrated with 0.05 M HCl solution to pH 3.2. A blank titration using 10 mL of hydroxylamine hydrochloride solution was conducted by the same method. The aldehyde content of the oxidized cellulose nanofiber membrane was calculated according to the following equation:

$$\text{aldehyde contents (wt./wt.\%)} = \frac{0.05 \times \text{MW}_{\text{CO}}(B - S)}{1000\ (\text{weight of sample})} \times 100$$

where B and S are volumes (milliliters) of 0.05 M HCl solution consumed in titrations for the blank and sample, respectively. MW_{CO} is the molecular weight of carbonyl group.

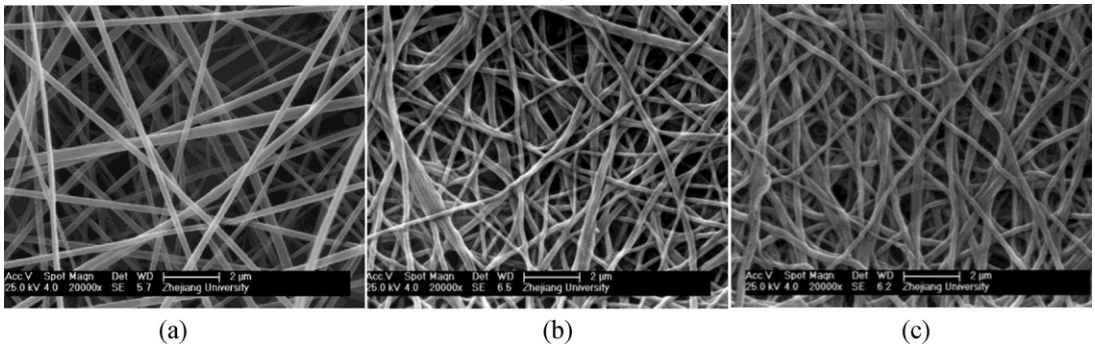

Fig. 1. SEM micrographs of CA (a), RC (b), and oxidized RC (c) nanofiber membranes.

2.5. Immobilization of lipase onto the oxidized cellulose nanofiber membrane

Lipase solution (8 mg/mL) was prepared by adding appropriate amount of lipase powder into phosphate buffer solution (PBS, 0.05 M, pH 7.0). A 5 mg sample of the oxidized cellulose nanofiber membrane was submerged into 3 mL lipase solution and shaken gently in water bath for 2 h at 25 °C. The enzyme-immobilized nanofiber membrane was then taken out and washed three times with 3.0 mL PBS (0.05 M, pH 7.0). The washings together with the reaction solution were collected for determination of protein concentration.

The amount of enzyme immobilized on the nanofiber membrane was measured by Bradford's method [31] with Coomassie brilliant blue reagent. The amount of protein immobilized on membrane was determined by the initial and final protein concentration difference in the enzyme solutions and the washings. BSA was used as standard to construct the calibration curve. The enzyme loading was defined as the amount of enzyme (mg) per gram of the nanofiber membrane. Each value was the mean of three parallel experiments at least, and the standard deviation was within ca. ±5%.

2.6. Activity assay of free and immobilized lipases

The activity for the immobilized lipase in aqueous medium was determined using the method of Chiou and Wu [32] and Huang et al. [33]. Briefly, the reaction was started by immersing 1.0 mg immobilized lipase preparation in the reaction mixture composed of 1.0 mL ethanol containing 14.4 mM *p*-NPP and 1.0 mL PBS (0.05 M, pH 7.0). The mixture was then incubated at 37 °C under reciprocal agitation. After 5 min, the reaction was terminated by adding 2.0 mL 0.5 M Na_2CO_3, followed by centrifuging for 10 min (10,000 rpm). 0.50 mL supernatant was diluted 10 folds with de-ionized water, and measured at 410 nm in an UV–vis spectrophotometer (UV-2450, Shimadzu, Japan) against a blank without enzyme and treated in parallel. Molar extinction coefficient was adopted as $14.5 \times 10^3\ M^{-1}\ cm^{-1}$ for *p*-nitrophenol (*p*-NP), which was determined from the absorbance of standard solutions of *p*-NP in the reaction medium.

One enzyme unit is the amount of biocatalyst liberating 1.0 μmol *p*-NP min^{-1} in these conditions. The enzyme activity was the number of lipase unit per gram nanofiber membrane. Specific activity was defined as the number of lipase unit per milligram of protein. Activity retention value was the ratio of specific activity of immobilized lipase to that of free one. Each data was the average of at least three parallel experiments, and the standard deviation was within ca. ±5%.

2.7. Stability measurements

Activities of both free and immobilized lipases at different temperatures in the range of 20–60 °C were measured. The relative activities of free and immobilized lipase were normalized to the highest activity within this temperature range. Activity of both free and immobilized lipases under different pH values was tested by incubated in different reaction mixtures at pH of 4.5, 5.5, 6.1, 6.5, 7.0, 7.5, 8.0 and 9.2. The relative activities of free and immobilized lipase were normalized to their highest activity under the assay pH range.

For the measurement of thermal stability, both free and immobilized lipases were stored at 60 °C for 180 min. 0.1 mL free lipase solution or 5.0 mg immobilized enzyme was withdrawn at same timed intervals (30 min) during incubation with its residual activity measured. In the measurement of reusability, immobilized lipase was recovered from the reaction mixture before adding 2.0 mL 0.5 M Na_2CO_3, washed with PBS to remove any residual substrate on the fibrous membrane, and then used for the next assay. The storage stability was determined as follows. Free and immobilized lipases were respectively stored in PBS (0.05 M, pH 7.0) at 4 °C for 15 days. Parts of them were periodically withdrawn for activity assay. The residual activities were then determined as described above (Section 2.6).

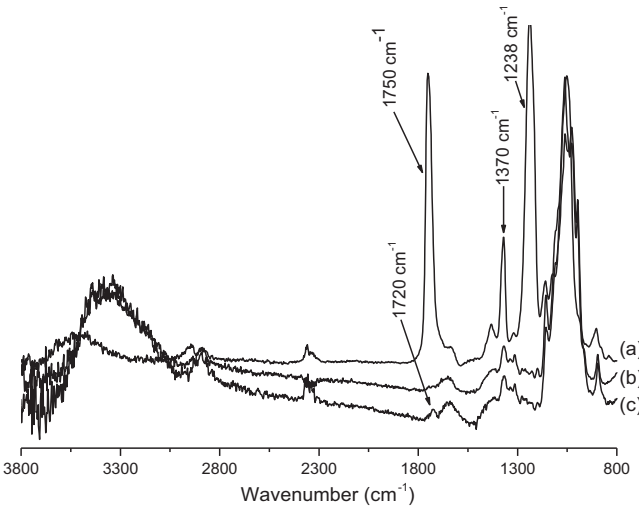

Fig. 2. FT-IR/ATR spectra of the CA (a), RC (b), and oxidized RC (c) nanofiber membranes.

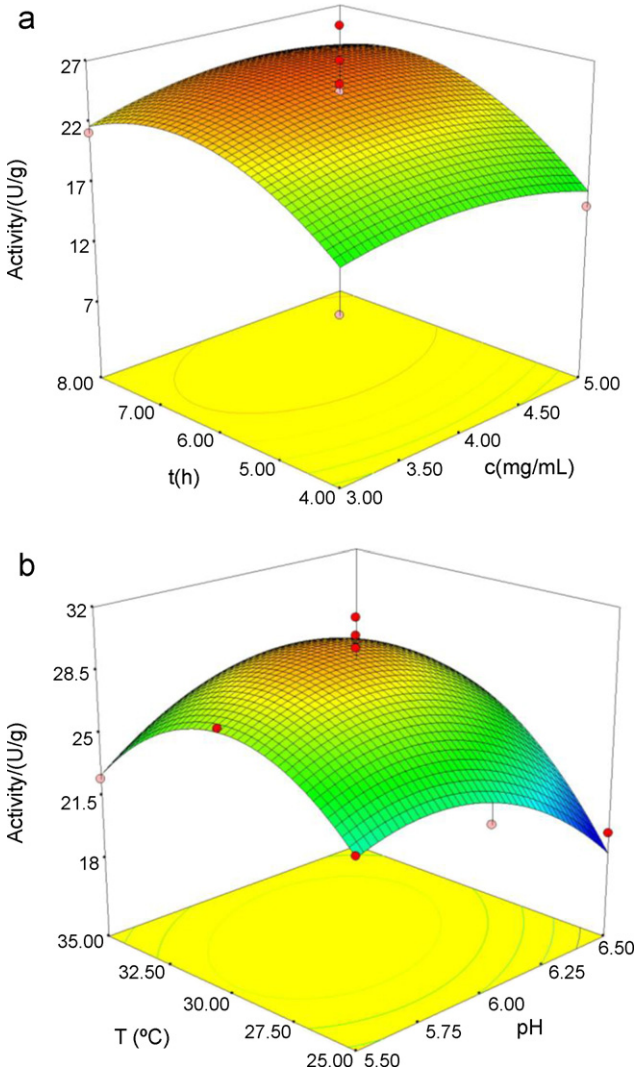

Fig. 3. Response surface plots of the interactions between t and c (a) and between T and pH (b) on the residual activity of immobilized lipase.

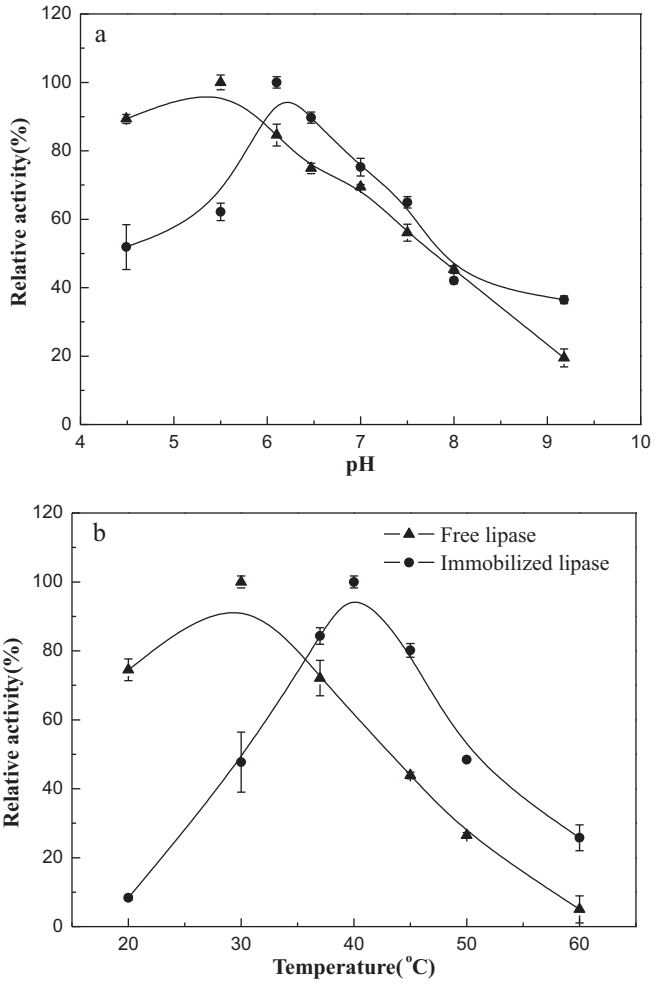

Fig. 4. Effect of pH (a) and temperature (b) on the lipase activity of free (▲) and immobilized (●) preparations.

3. Results and discussion

3.1. Preparation and characterization of cellulose nanofiber membrane

Several authors [26–29] have shown that CA can be electrospun into nonwoven nanofiber membranes and further deacetylated to form RC on the surfaces of the nanofiber membranes. The diameter and morphology of the electrospun nanofibers are mainly influenced by the solution properties, processing parameters, and environmental conditions. The effects of solution properties and processing parameters on nanofiber membranes were focused in previous work [26–29]. However, environmental conditions such as temperature, humidity and atmospheric pressure also have a significant impact on the electrospun nanofibers. We particularly concentrated upon the effect of humidity on the nanofiber diameter. In general, the spinning process is highly sensitive to atmospheric humidity. A critical relative humidity value of $60 \pm 10\%$ was found to be most suitable for continuous electrospinning nanofibers with an average diameter of 200 nm. Furthermore, the average diameter of nanofibers increased with the relative humidity, as was presented in Supporting Information. To speculate on the origin of this diameter increase, a small amount of water was added to a sample of cellulose solution. A white precipitate formed immediately. It can be inferred from this observation that the spun CA jet can absorb a significant proportion of water vapor from a humid environment, thus prompting the precipitation to occur during CA in-flight fiber formation. This precipitation restricts further drawing or splitting of the jet and hence leads to thicker fiber.

The resulting CA nanofiber membrane was then deacetylated with KOH in ethanol for 3 h. Fig. 1(a and b) shows the SEM images of the electrospun CA nanofiber membrane and its RC analogues. It can be seen that part of the fabric shrank after deacetylation and the surface of the fibers got rough, but the total membrane structure and the average diameter were maintained.

3.2. Optimization of oxidation on the surface of cellulose nanofiber membrane for lipase immobilization

Treating the RC nanofiber membrane with $NaIO_4$ is a well described approach to the oxidation of membrane surface and the generation of aldehyde groups [34,35], which can be further employed as binding sites for enzyme immobilization. Only a slight difference was observed between the RC nanofiber membrane (Fig. 1(b)) and the oxidized analogue (Fig. 1(c)). The oxidation was also quantified by FTIR/ATR spectroscopy (Fig. 2). As can be seen from the spectra, the CA nanofiber membrane shows three characteristic peaks at 1750 cm^{-1} ($\upsilon_{C=O}$), 1370 cm^{-1} (υ_{C-CH_3}) and 1238 cm^{-1} (υ_{C-O-C}), which were attributed to the vibrations of the acetate group. These peaks disappeared and the peak at ~3500 cm^{-1} (υ_{O-H}) had an obvious increase for the RC nanofiber

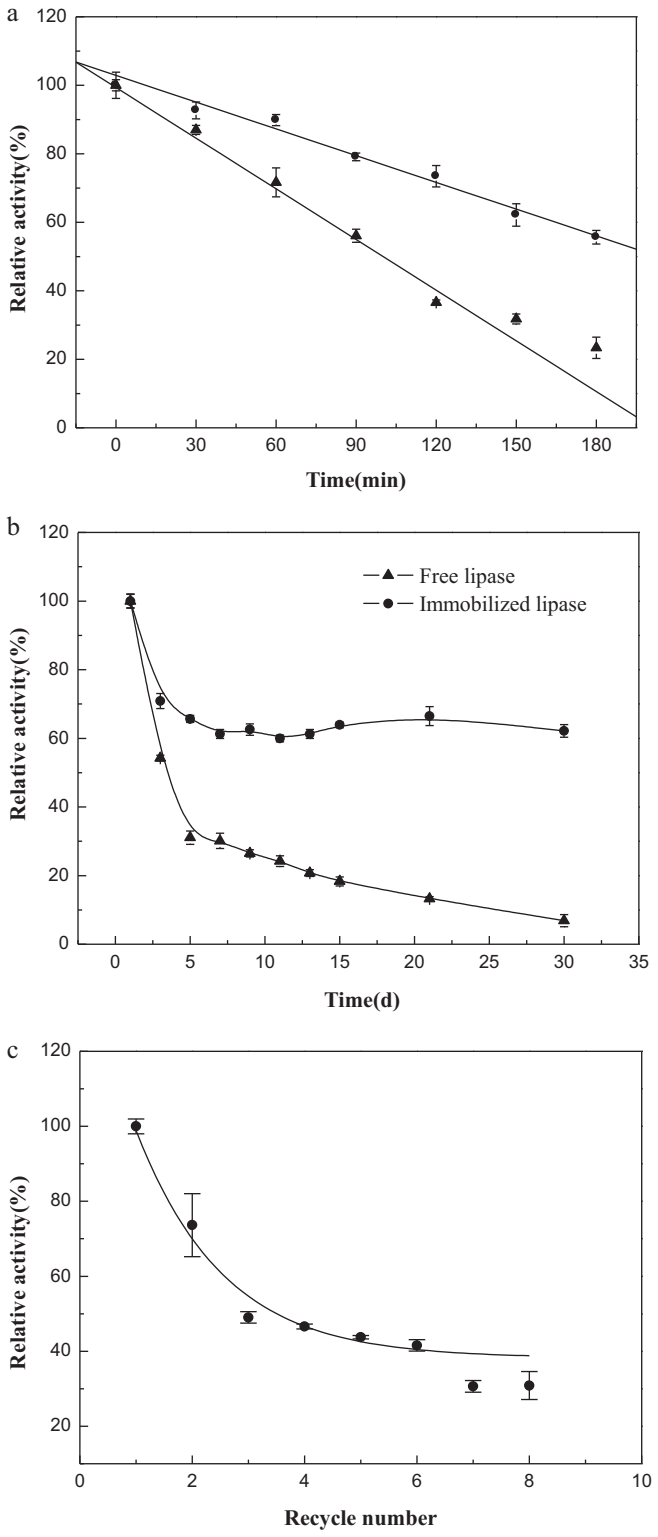

Fig. 5. Thermal (a), storage (b) and operational (c) stabilities of free (▲) and immobilized (●) lipases.

membrane. The changes in these peaks indicate the removal of acetyl groups from the membrane surface. Furthermore, a small peak at ~1720 cm^{-1} was observed for the oxidized cellulose nanofiber membrane. It was attributed to the characteristic absorption of aldehyde groups on the membrane surface [35].

To optimize the oxidation of the RC nanofiber membrane for enzyme immobilization, RSM was applied to model the residual activity of immobilized lipase using four parameters: $NaIO_4$ content (c), reaction pH (pH), reaction temperature (T) and reaction time (t). These four variables were optimized using two central composite designs (CCD), a 2-factor-5-level CCD, and a 2-factor-3-level CCD. Table 1 shows the levels of both designs at each of the 13 experimental sets generated. The accuracy of the models was evaluated by the coefficient of determination (R^2) and absolute average deviation (AAD) [19]. The AAD was calculated using the following equation:

$$\mathrm{AAD} = \left\{ \sum_{i=1}^{p} (|y_{i,\mathrm{exp}} - y_{i,cal}|/y_{i,\mathrm{exp}})/p \right\} \times 100$$

Value of R^2 and AAD for design 1 and design 2 was 0.9228, 8.17% and 0.8950, 3.56%, respectively. ANOVA (Table 2) shows that both models for design 1 and design 2 were statistically good with a significance level of $P < 0.0500$ and the models had no significant ($P > 0.05$) lack of fit. This demonstrates the precision and reliability of the design 1 and 2 models.

To evaluate the interaction between t and c, a response surface plot was constructed at medium levels of T and pH (Fig. 3). Based on the response surface plot, it was found that the residual activity of immobilized lipase increased with t and c. Sufficient oxidation time and amount of oxidizer need to be applied to maximize the yield of aldehyde groups and hence to maximize the enzyme binding capacity on the cellulose nanofiber membrane. However, when c and t were increased beyond 4.2 mg/mL and 6.8 h, the residual activity decreased. Two speculations can be used to explain this phenomenon. First, the aldehyde groups will be inevitably oxidized into carboxyl groups with the increase of $NaIO_4$ concentration [34], reducing the number of aldehyde groups which can directly react with amino groups contained by enzyme molecules. Thus, the lipase loading decreases, and in turn reduces the residual activity of immobilized enzyme. Second, the oxidation will also break the polymer chains, resulting in a gradual degradation of the cellulose nanofibers [35]. This effect is prone to happen for nanofiber membrane with large surface area to mass ratio. It is therefore losing the immobilized enzyme and decreasing the residual activity.

These speculations can also be used to explain the following results. We found that the residual activity increased at medium levels of T and pH when the response surface plot was constructed at the optimum levels of t and c. It means that the residual activity increases with the decrease of pH value. Nevertheless, the residual activity does not correspondingly increase with T higher than 30.8 °C and pH lower than 6.1. This is because the oxidation of cellulose by $NaIO_4$ is much more severe in acidic medium and high temperature. Therefore, both pH and T should be controlled to limit the further oxidation of the aldehyde groups to carboxyl groups and the undesirable degradation of the cellulose chains.

From the above results, it is clear that high levels of t and c (about 6.8 h and 4.2 mg/mL) together with a center level of T and pH (about 30.8 °C and pH 6.1) are required for the oxidation of cellulose nanofiber membrane. This oxidized nanofiber membrane contains 13.7% (w/w) of aldehyde groups, which is comparable to the theoretical maximum value of 18.1% (w/w), indicating the possibility of sufficient sites for enzyme immobilization. Under these optimal conditions, a residual activity of 29.6 U/g was obtained for the immobilized lipase.

3.3. Stabilities of the immobilized lipase

The optimal pH and temperature for the activity were evaluated after optimizing the immobilization of lipase on the cellulose

Table 2
ANOVA table for design 1 and design 2.

	SS		Df		MS		*F*-values		*P*-values	
	1	2	1	2	1	2	1	2	1	2
Model	382.11	5.47	5	5	76.42	1.09	16.74	15.07	0.0009	0.0024
Residual	31.97	0.44	7	6	4.57	0.073				
Lack of fit	26.46	0.056	3	2	8.82	0.028	6.41	0.29	0.0523	0.7609
Pure Error	5.51	0.38	4	4	1.38	0.095				

SS, sum of squares; Df, degree of freedom; MS, mean square.

nanofiber membrane. According to Fig. 4(a), the optimum pH for free lipase was at pH 5.5. It may result from the ionization states of several groups on amino acid chains that result into the suitable conformation of lipase molecules [36]. While after immobilization procedure, the maximum activity was at pH 6.1 for the immobilized lipase, higher than that for the free lipase. This alkaline shift in the optimum pH could be attributed to the characteristics of oxidized cellulose. As mentioned above, the inevitable carboxyl groups on the membrane surface can absorb hydrogen ions from reactive solution and thus result in a more acidic microenvironment for the immobilized lipase than that for free lipase. A similar phenomenon has been reported previously [37]. Fig. 4(b) shows a higher optimum temperature and higher temperature stability for immobilized lipase activity than that for the free enzyme.

Thermal stability, storage life and reusability are of considerable practical significance for the commercial application of biocatalysts. Fig. 5(a) shows the relative thermal stabilities of the free and the immobilized enzymes at 60 °C. The linear trend lines were drawn for activity profiles of free and immobilized lipases as to determine their respective thermal half-lives. From this figure, we can see the immobilized lipase gave extended half-life of approximately 203 min as compared to free lipase (100 min), indicating that the high temperature resistance and enhanced thermal stability of immobilized lipase is induced by the presence of covalent bonds between the enzyme and the fibrous mesh preventing the conformational denaturation of the enzyme at higher temperature. According to Fig. 5(b), a highly significant difference was observed in the retained activity between the two lipases, indicating a much better storage ability for the immobilized lipase than that for the free one. Fig. 5(c) shows the effect of repeated uses on the activity of the immobilized enzymes. The activity of the immobilized enzyme did decay with repeated uses. The remaining activity of lipase immobilized on the cellulose fibrous membrane was about 30% after 8 cycles of batch operation. It is possible that this activity loss is related to the inactivation of enzyme by continuous use and the loss of the membrane fabric.

4. Conclusion

In this paper, we have comprehensively quantified the properties of immobilized lipase on oxidized cellulose electrospun nanofiber membrane. Response surface methodology is useful to optimize the oxidation of the membrane for enzyme immobilization. Optimal reaction conditions are: 4.2 mg/mL $NaIO_4$ content, a reaction time 6.8 h, and a reaction temperature 30.8 °C at pH 6.1. The immobilized lipase showed an activity of 29.6 U/g at these optimum conditions. The stabilities of the enzyme were significantly improved also.

Acknowledgements

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.molcatb.2011.02.010.

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