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Immobilization of lipase on aminopropyl-grafted mesoporous silica nanotubes for the resolution of (*R*, *S*)-1-phenylethanol

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ABSTRACT

Mesoporous silica nanotubes and aminopropyl-grafted mesoporous silica nanotubes were prepared as supports to immobilize lipase from *Candida* sp. 99-125(ClLip) by physical adsorption. The immobilization conditions were investigated. Moreover, immobilized lipases on both kinds of supports were employed to catalyze olive oil hydrolization and resolution of 1-phenylethanol by esterification. The results showed that the hydrolization activity of the lipase immobilized on aminopropyl-grafted mesoporous silica nanotubes was almost twice of that on mesoporous silica nanotubes. In addition, the resolution of 1-phenylethanol catalyzed by the former catalyst also increased 22%. Circular dichroism spectra revealed a reduction of α-helix and an increase of β-sheet when lipase was adsorbed on aminopropyl-grafted mesoporous silica nanotubes, which suggested that parts of α-helix were extended and reformed to be β-sheet.

1. Introduction

The extracellular lipase from *Candida* sp. 99-125(ClLip), an important industrial biocatalyst, has been widely used in esterification and transesterification reactions, resolution of various chiral compounds, and production of biodiesel because of its high reactive activity. These reactions are heterogeneous and take place exclusively at oil–water interfaces. The unique property of the activation of lipase at an oil–water interface draws special demands of supports for enzyme immobilization, which should offer not only a stable host but also the consistency with the catalytic environment.

Mesoporous silicates, synthesized using a surfactant templating method, have ordered porous structures with narrow pore size distributions and thick walls, enhancing their stability. The large regular repeating mesoporous structures of mesoporous silicates offer the possibility of adsorbing or entrapping large biomolecules within their pores as well as on the external surface area [1]. Since the first report by Díaz and Balkus [2] in 1996, this research area has grown rapidly [3,4]. To provide a strong interaction for immobilization, mesoporous silicates have been functionalized with a large variety of functional groups [5]. The method and extent of functionalization can influence the final loading and activity of the immobilized protein [6,7]. Covalent coupling with surface amine functional groups on the support using glutaraldehyde, which can bind to enzyme amine groups, can increase the stability of enzyme, significantly reduce the amount of leaching, and allow the immobilized enzyme to be reused. A range of enzymes, including penicillin G acylase [8], glucose oxidase [9], α-amylase [9], Mucor javaniscus lipase [10], glucose isomerase [11], trypsin [12], invertase and glucoamylase [13] has been covalently immobilized on mesoporous silicates in this manner. However, the increased rigidity in a covalently bound enzyme can cause a reduction in activity. On the other hand, although examples of enzyme immobilizations on amine functional mesoporous silicates by physical adsorption are relatively rare, these materials also proved promising supports without covalent bounding. Xu et al. reported lipase physically adsorbed on amino-functionalized ordered mesoporous SBA-15 shows excellent thermal stability and better recycle potential comparing with SBA-15-PPL [14]. Based on the above discussions, we conclude that mesoporous silicates with tailored particle size, morphology and surface functional groups are of great significance to the development of biocatalysis and enzyme engineering.

In this paper, we synthesized mesoporous silica nanotubes (MSNTs) via a sol–gel route using needle-like $CaCO_3$ nanoparticles and modified them with aminopropyl groups to obtain aminopropyl-grafted mesoporous silica nanotubes (NH_2-MSNTs). These two materials were employed as supports to immobilize lipase from *Candida* sp. 99-125. After that, we investigated the

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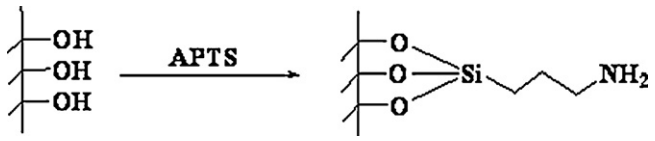


Scheme 1. The amine-functionalization of MSNTs.

preferences of immobilization conditions for the two different supports and applied these two immobilized lipases to the resolution of (*R*, *S*)-1-phenylethanol. Some possible reasons in terms of the secondary structure of the lipase were then discussed to explain the different results.

2. Experimental

2.1. Materials

Mono Q 5/50 GL column and ion exchange media were from Amersham Biosciences (Uppsala, Sweden). Coomassie blue G-250, Trishydroxymethylaminomethane (Tris), olive oil, and polyvinyl alcohol (PVA) were obtained from Sanbo Biotech (Beijing, China). Needle like $CaCO_3$ templates were prepared by a unique high gravity reactive precipitation (HGRP) technology as previously described [15]. 3-Aminopropyltriethoxysilane (APTS) was purchased from Tokyo Chemical Industry. 1-Phenylethanol was procured from Fluka (Sigma Aldrich, USA). All other chemicals used in the experiments were obtained from commercial sources as analytical reagents without further purification. Milli-pore water with a resistivity of 18.2 $M\Omega\,cm^{-1}$ was used throughout the study.

2.2. Purification of crude lipase

The lipase of *Candida* sp. 99-125 was purified after ammonium sulphate–acetone precipitation [16] followed by anion exchange chromatography [17]. However, because of fermentation differences between batches, the crude lipase activities varied.

The culture broth was centrifuged (4000 rpm, 15 min) initially to remove culture medium and cells. Three volumes of ice-cold acetone were then slowly added to the supernatant under constant stirring during the addition of acetone and for 10 min afterwards. The precipitate was collected by filtration and dried at room temperature.

The anion exchange chromatography was run on ÄKTA basic 100 (Amersham biosciences). The crude lipase solution was loaded on a Q Sepharose Fast Flow column (1.5 cm × 11.3 cm) equilibrated with 20 mM Tris–HCl buffer (pH 8.0). The column was eluted with 0–400 mM NaCl linear gradient buffer at a flow rate of 1.0 mL/min. The active fractions were collected, concentrated and desalted by ultrafiltration with an Amicon cell using a PM 10 membranes. After gradient washing the proteins bound to the column were washed out with equilibration buffer containing 1 M NaCl.

2.3. Fabrication of supports

MSNTs were fabricated according to the methods published by Yang et al. [18], using needle-like $CaCO_3$ templates [26].

The amine-functionalization of MSNTs made the supports structurally different, which were named NH_2-MSNTs afterwards (Scheme 1). To obtain NH_2-MSNTs, some MSNTs powders were dispersed in toluene before the addition of APTS ($N_{Si}/N_{APTS}/N_{toluene}$ = 5:1:500). The suspension was subsequently heated under reflux at 125 °C in nitrogen, after 24 h the particles were filtrated and washed respectively with toluene and ethanol twice. At last, the particles were dried at 80 °C for 12 h to make them the final product of NH_2-MSNTs.

2.4. Immobilization

Lipase solution was prepared in 0.02 mol/L phosphate buffer with a protein concentration of 8.25 g/mL. The solution was then added with some support followed by 10 min ultrasonic dispersion to make it a visually homogeneous suspension.

The conventional way of physical immobilization was acquired in a shake flask with a suitable stirring speed at 20 °C, and after 12 h the immobilized lipase was separated by centrifugation (5000 rpm, 5 min). To cast free lipase off, the solid collection was then washed twice with the immobilizing buffer. The amount of adsorbed lipase was calculated indirectly by determining the protein concentration of supernatant and the wash buffers according to the Bradford method using bovine serum albumin as a standard [19].

The immobilized lipase was placed in a freezer at −20 °C for several hours until frozen, and the frozen sample was then lyophilized for 8 h to make it dry. The mass of buffer salts that may be contained in the final sample is negligible due to its low concentration. The immobilized lipase on MSNTs was named as MSNTs-lipase afterwards, similarly, immobilized lipase on NH_2-MSNTs was denoted as NH_2-MSNTs-lipase.

2.5. Characterization of supports and immobilized lipase

Contact angle measurements were performed in an OCA (Data Physics Co., Germany) apparatus equipped with a digital camera.

The morphology and structures of the samples were characterized by scanning electron microscopy (SEM, Hitachi S-4700) and transmission electron microscopy (TEM, Hitachi H-800).

2.6. Circular dichroism (CD) spectra

Circular dichroism spectra were recorded on a JASCO J-810 spectropolarimeter (JASCO) at 10 °C. The protein concentration and optical path length was 0.2 mg/mL and 5 mm respectively. The secondary structure was analyzed using four component model (Helix, Beta, Turn, and random coil) reference spectra.

To test free lipase, the sample was lipase solution in phosphate buffer at pH 7.5, while the blank sample is only buffer. To test the sample of immobilized lipase, the sample was dispersed ultrasonically previously in its optimized buffer. So was the blank sample, which is prepared by the supports of the same concentration instead.

2.7. Hydrolization

Lipase activity was determined according to an olive emulsion method [20]. The substrate solution consisting of olive oil (20 mL) and PVA (60 mL) was emulsified in a homogenizer for 6 min at maximum speed. Then the enzyme solution or powder was added to 5 mL of substrate emulsion and 4 mL of 20 mM phosphate buffer, pH 8.0 (K_2HPO_4–KH_2PO_4). Samples were incubated for 10 min at 40 °C. The reaction was stopped by adding 20 mL ethanol. Enzyme activity was determined by titration of the fatty acid released with 50 mmol/L sodium hydroxide. One activity unit of lipase was defined as the amount of enzyme required to release 1 μmol of fatty acid per min under assay conditions.

2.8. Esterification

The racemic compound 1-phenylethanol was resolved by esterification when lipase showed highly enantioselectivity that the R-enantiomer was consumed preferably. The process was schemed as Scheme 2.

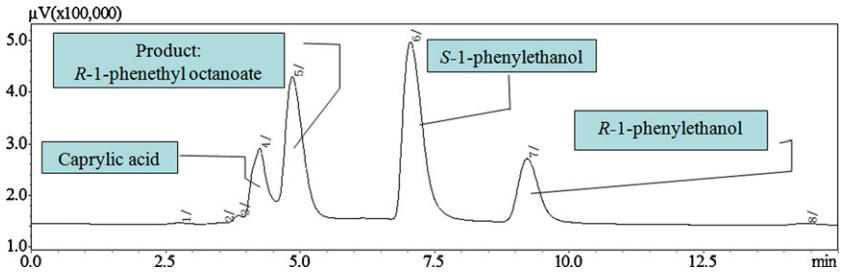
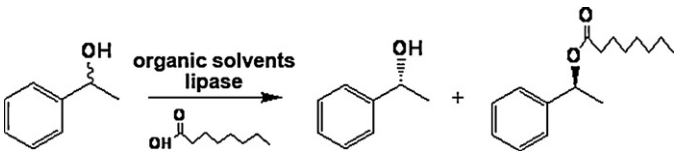


Fig. 1. HPLC figure of the kinetic resolution of (*R*, *S*)-1-phenylethanol.



Scheme 2. Enzyme catalyzed kinetic resolution of (*R*, *S*)-1-phenylethanol.

Using caprylic acid as the acyl donor and lipase, the kinetic resolution of 1-phenylethanol was achieved. In the presence of hexane, the reaction progress went along efficiently at 35 °C.

The reaction results were determined by HPLC equipped with a chiralcel OB-H column (0.46 cm × 15 cm) (DAICEL CHEMICAL). The mobile phase was a mixture of n-hexane and isopropyl alcohol (V/V = 9/1), with the velocity 0.5 mL/min. Detection wavelength was 211 nm (Fig. 1). Because of the high volatility of the solvent hexane, the enantiomer excess ratio of the substrate (e.e.$_s$%) was considered the effective parameter indicating the reaction process. The calculation of e.e.$_s$% is given below, in which *C* stands for concentration.

$$\text{e.e.}_s\% = \frac{C_{S\text{-1-phenylethanol}} - C_{R\text{-1-phenylethanol}}}{C_{S\text{-1-phenylethanol}} + C_{R\text{-1-phenylethanol}}} \times 100$$

3. Results and discussion

3.1. Characterization of supports

The SEM images of MSNTs and NH_2-MSNTs (Fig. 2) showed their outlooks were approximately the same. Using nano-sized needle like $CaCO_3$ as template, both kinds of supports possess mesoporous tubular structure with openings, an inner diameter of 200–400 nm, a length of 8–10 μm and a wall thickness of approximately 40 nm, which is consistent with the report by our group [21]. The surface area and pore distribution of the samples were determined by an ASAP 2010 surface area analyzer. From Fig. 3, it can be seen in the pore volume of small pores, 2–4 nm, decreases dramatically after APTS modification, but the larger pores, 8–30 nm, are hardly influenced, resulting in an increase in the average pore diameter and a decrease in the BET surface area and pore volume of the nanotubes as a whole (Table 1). The zeta potentials of MSNTs and NH_2-MSNTs were also listed in Table 1.

3.2. Properties of immobilizing conditions for two different supports

3.2.1. Effect of the ratio of carries to free lipase

If pore size and surface area could take the full responsibility of lipase loading, NH_2-MSNTs would be assumed an inferior kind of supports. However, in practical it is quite the contrary (Figs. 4–10). The lipase loading proportion on NH_2-MSNTs seemed always higher than that on MSNTs, and NH_2-MSNTs-lipase always showed higher catalytic activity in both hydrolization and

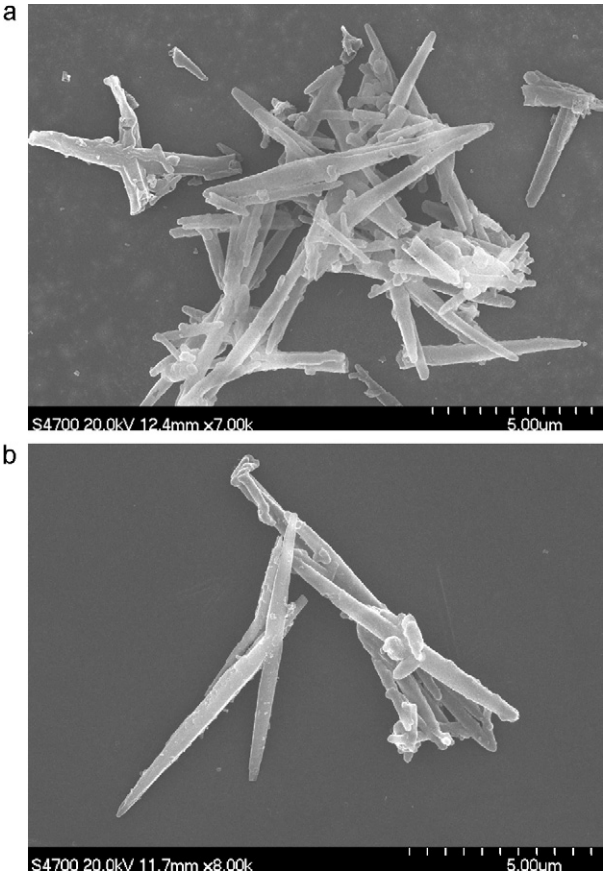


Fig. 2. SEM images of MSNTs (a) and NH_2-MSNTs (b).

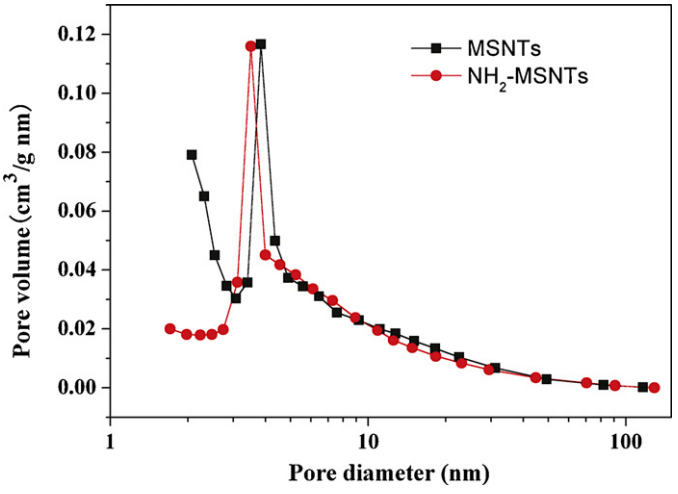


Fig. 3. The distribution of pore diameter of two different supports.

Table 1
Characterization of MSNTs and NH_2-MSNTs.

	Surface area (m^2/g)	Pore volume (cm^3/g)	Pore size (nm) (d-BJH)	Zeta potential[a] (mV)	Contact angle (°)
MSNTs	638.45	0.78	8.2	−33.4	26
NH_2-MSNTs	285.43	0.72	8.67	31.4	44

[a] Zeta potential values given here are in pure water.

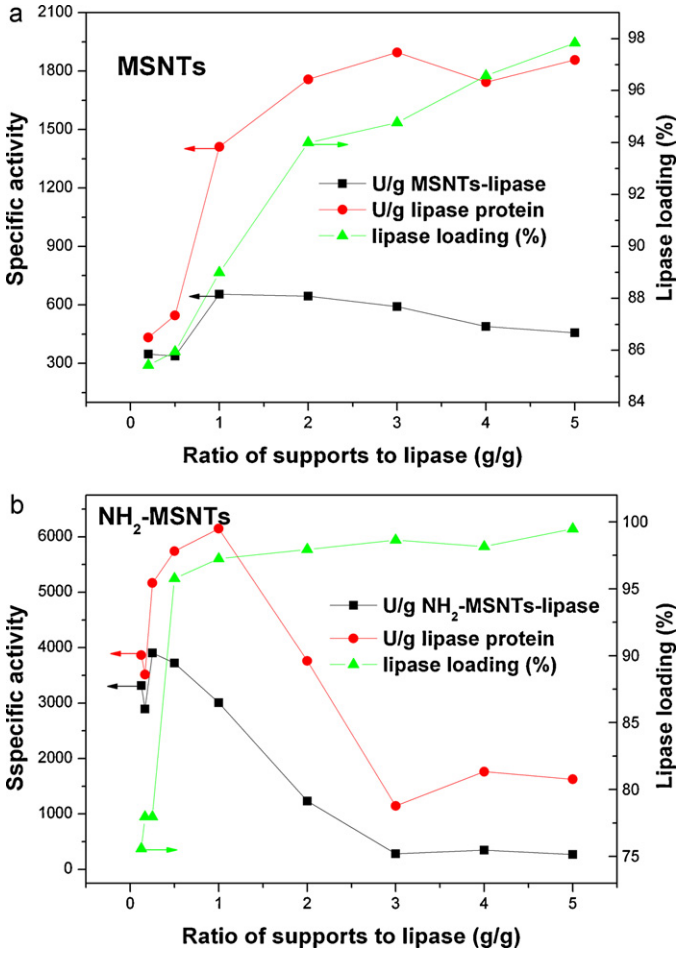


Fig. 4. The effect on hydrolization activity of ratios of two different supports to lipase (the initial lipase contents were same for different samples).

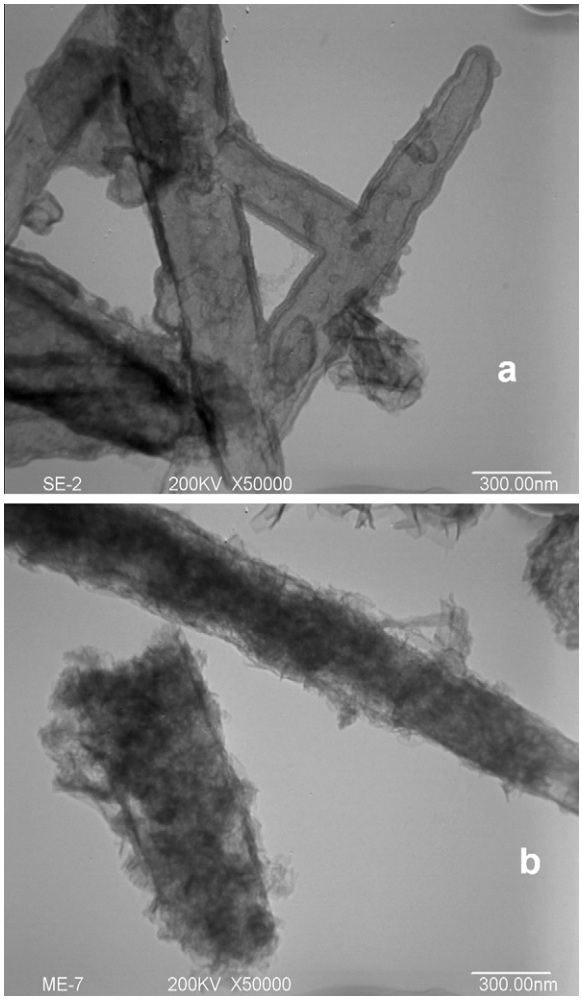


Fig. 5. TEM images of immobilized lipase on MSNTs (a) and NH_2-MSNTs (b).

esterification. Xu et al. [22] found that when lipase was immobilized by a PVA/PTFE composite membrane, the maximum reaction rate per unit of membrane area was much higher than that immobilized by a hydrophobic membrane. The accessibility of substrate to active sites was improved by a hydrophobic support, which is widely used in organic synthesis. The incompatibility of hydrophobic support and substrate with water-soluble lipase unavoidably leads to contradiction between hydrolytic rate and stability of lipase activity however. In order to take advantage of the virtues of hydrophobic and hydrophilic support, amphiphilic support, obtained by surface modification, was encouraged [25].

Besides catalysis performance, different supports also result in different preferences on immobilizing conditions. Fig. 4 shows the effect of ratio of carries to enzyme on the lipase loading process. It was found that the superior ratio of MSNTs to lipase turned to 3:1 for the activity recovery of free lipase, while for NH_2-MSNTs the superior ratio decreased to 1:1, which indicated that, to immobilize a certain amount of lipase, the NH_2-MSNTs would be consumed less vs. MSNTs. Furthermore, higher activity was retained on NH_2-MSNTs with specific activity of lipase rose up to 6000 U/g. Adopting

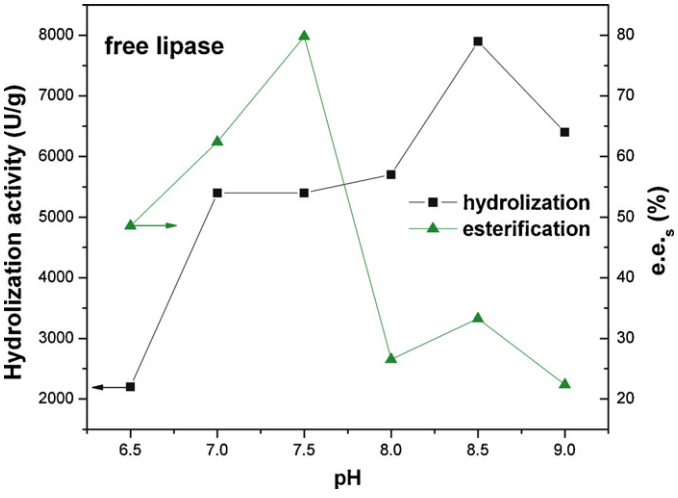


Fig. 6. The effect of pH value towards free lipase.

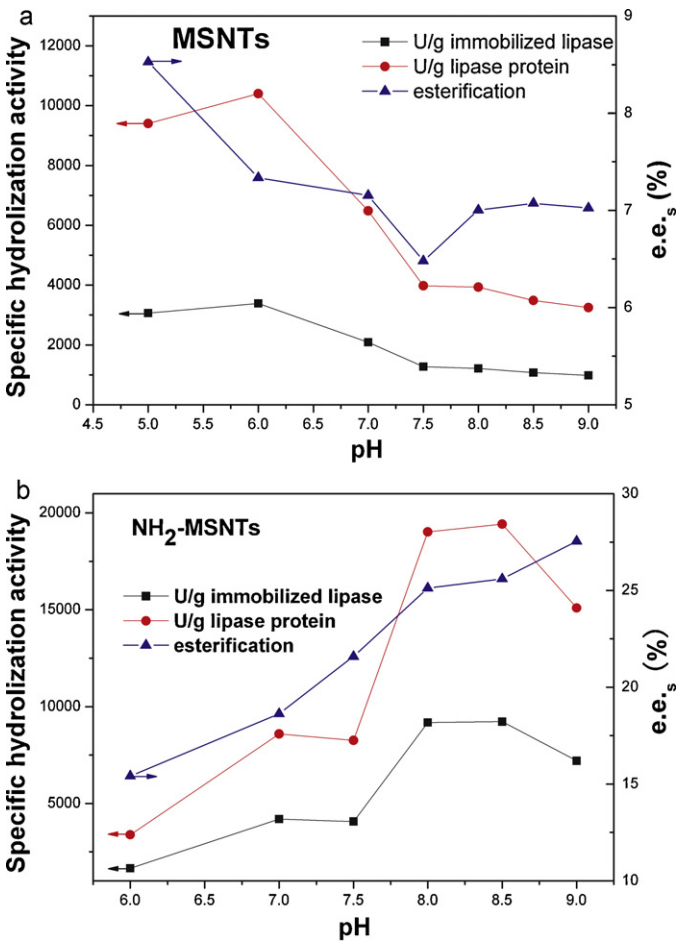


Fig. 7. The effect of pH towards immobilized lipase on MSNTs (a) and NH_2-MSNTs (b) (the lipase protein content were same for different samples).

the optimized ratio respectively in each case, the loading situations were shown in TEM images (Fig. 5). Obviously, the immobilized lipase on MSNTs was clearer, while the one on NH_2-MSNTs presented darker.

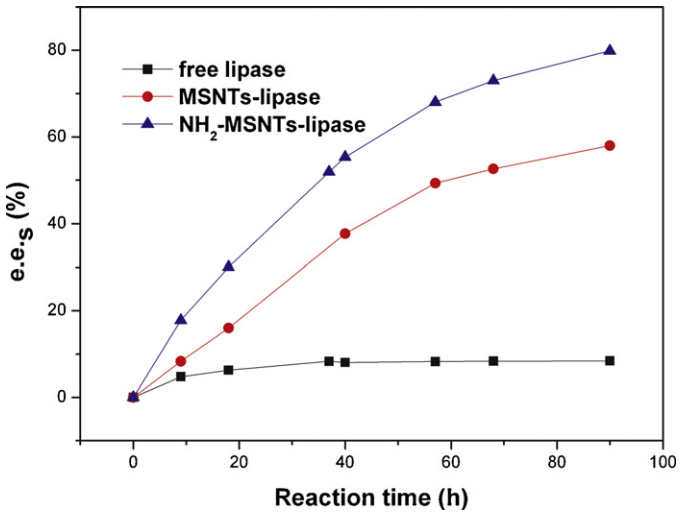


Fig. 8. The resolution process catalyzed by free and immobilized lipase (10 mmol/L 1-phenylethanol × 30 mL, lipase content 0.05 g, 35 °C, stirring speed 120 rpm).

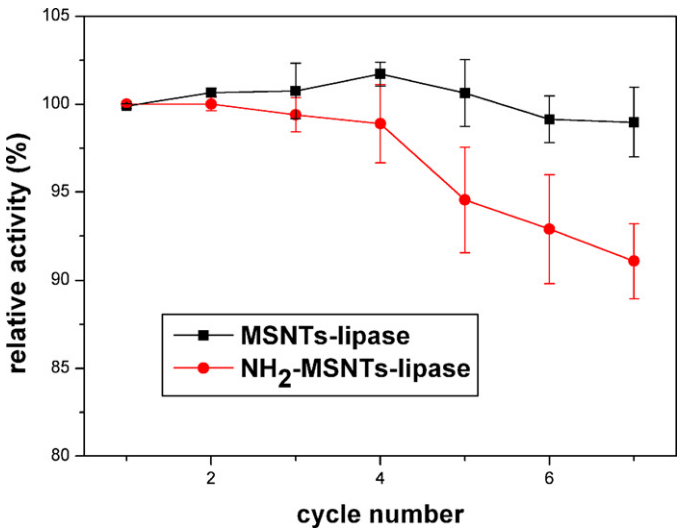


Fig. 9. Changes in the esterification activity of lipase along repeated use.

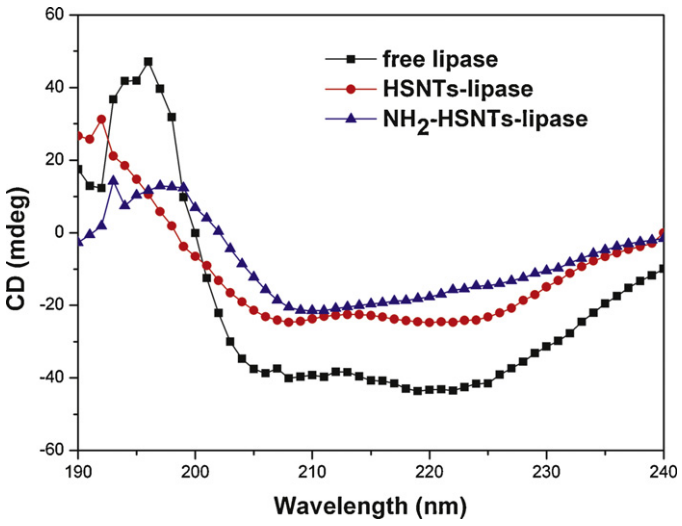


Fig. 10. Far-UV circular dichroism spectra of free and immobilized lipase.

3.2.2. Effect of pH value during the process of immobilization

The activities of free lipase catalyzing both hydrolization and esterification were measured with pH ranging from 6.5 to 9 (Fig. 6). For free lipase, the hydrolization activity reached the peak at 8.5 while its esterification activity reached the peak at 7.5 (the lipase powder acquired pH memory when the lipase solution was lyophilized). It indicates that the lipase molecular conformation at pH 8.5 suits hydrolization of olive oil whereas the esterification of 1-phenylethanol will be accelerated if the enzyme shows itself the conformation at pH 7.5.

However, the situation completely changed during the immobilizing process. Due to the surface charges of the supports, the optimal pH value for the immobilization system shifted (Fig. 7). For the adsorption of lipase on MSNTs, the immobilized lipase would show a higher activity for both hydrolization and esterification under the condition that the buffer solution is acidic and hold at a low pH. On the other hand, the immobilized lipase will show higher activity if the buffer solution for adsorption of lipase on NH_2-MSNTs is alkaline. When the NH_2-MSNTs-lipase obtained at different pH value was adopted to catalyze the hydrolization of olive oil and the resolution of 1-phenylethanol, it could be clearly observed that the optimal pH value for hydrolization is 8.5 and 9.0 for esterification.

Table 2
Secondary structure estimation from CD spectrometry.

	Lipase	MSNTs-lipase	NH_2-MSNTs-lipase
α-Helix	25.4%	23.7%	18.7%
β-Sheet	24.6%	25.6%	40.4%
β-Turn	19.2%	11.5%	14.8%
Other	30.8%	39.2%	26.2%
Total	100.0%	100.0%	100.0%

Another notable phenomenon was that the highest hydrolization activity of immobilized lipase on MSNTs was 10,400.0 U/g lipase, whereas the value almost doubled for that on NH_2-MSNTs with 19,429.5 U/g lipase. Moreover, with respect to esterification MSNTs-lipase also performed 2/3 less. These data obviously proved amino-modified support to be a superior support.

3.3. The catalyzed kinetic resolution of 1-phenylethanol

To achieve bio-catalyzed kinetic resolution of 1-phenylethanol, lipase with high enantioselectivity should be applied. Therefore, free lipase, immobilized lipase on MSNTs and NH_2-MSNTs were added respectively into three stirring tubes. In each tube, the substrate, solvent, lipase protein contents and other reaction conditions were controlled to be same. The reaction process is shown in Fig. 8. Because of the conglomeration in the presence of hexane, free lipase did not show high catalyzing activity in the beginning and e.e. value of 1-phenylethanol remained low within 90 h. On the other hand, immobilized lipase showed significantly higher activity, with e.e. value of 1-phenylethanol as 80% when the reaction was catalyzed by NH_2-MSNTs-lipase, while the data reached 58% using MSNTs-lipase.

One important reason for immobilization is the convenience of recovery. Although the porous structure enables an efficient and rather stable retain of the enzyme, it is unfortunately a shortcoming and also a challenge for nano-materials, because of its light weight and nearly invisible size.

As was showed in Fig. 9, the immobilized lipase was recovered by centrifugation and reused at 35 °C for several cycles, and both of lipase-MSNTs and lipase-NH_2-MSNTs exhibited above 90% retained activities after the 7th cycle, of which lipase-MSNTs performed better. Due to the hydrophobic property of aminopropyl modified supports, lipase-NH_2-HSNTs presented a cloudy disperse in organic solvent and much more easily to cling to the reactor wall, which, on the other hand, was not the case for lipase-MSNTs. After uses some of the wispy powders were detected adhered to the plastic wall of the stirring tube, and because of the solvent volatility the adhering catalysts were being exposed outside the solution gradually, which probably explained the activity loss.

3.4. Reformation of secondary structure of lipase through immobilization

The enhancement of lipase activity for resolution of 1-phenylethanol may be mainly due to the hydrophobic activation of NH_2-MSNTs. He et al. found that the hydrolytic activity for the hydrolysis of insoluble or partly soluble substrates increases with enhanced support surface hydrophobicity [23]. It is not surprising that similar effect was also found for the resolution by esterification in hexane. In addition, another effect was discovered when free and immobilized lipase were subjected to CD in the far UV region of 190–240 nm (Fig. 10). The secondary structure estimation of the samples was performed by the JASCO Secondary Structure Estimation Program. The distributions of secondary structure of free and immobilized lipase on MSNTs were slightly different (Table 2), with β-structure of MSNTs-lipase a little less than that of the free lipase,

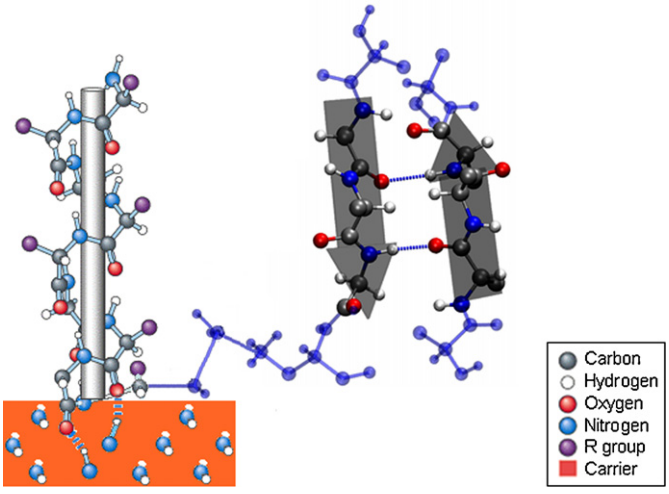


Fig. 11. The reformation of secondary structure when lipase was adsorbed to NH_2-MSNTs.

which, on the other hand, increased obviously when lipase was adsorbed to NH_2-MSNTs. Such contrast must be the result of the intermolecular force between the abundant $-NH_2$ on the supports surface and the lipase protein.

Hydrogen bond and the cumulative effect of many hydrogen bonds within α-helix stabilize this conformation. β-Sheet and β-turn structure can be considered as special structures of α-helix reformed by the extending of two amino acid residues [24]. In this case, $-NH_2$ on the supports surface competed for the hydrogen bonding, and obviously the inter-group forces between $-NH_2$ and –OH/–C=O would be stronger than that between –NH– of the amino acid residues and –OH/–C=O. Therefore, α-helix was extended and turned out to be β-sheet and β-turn (Fig. 11). The reformation of secondary structure of lipase indicates another explanation about the superiority of NH_2-MSNTs-lipase. Besides the hydrophobicity effect, the three-dimensional reformation of lipase molecule geometry uncovered catalyzing sites and subsequently enhanced its activity.

4. Conclusion

Mesoporous silica nanotubes and aminopropyl-grafted mesoporous silica nanotubes were prepared and used for the immobilization of lipase as new biocatalysts for the resolution of (*R*, *S*)-1-phenylethanol. The results showed that NH_2-MSNTs-lipase acquired higher activity than MSNTs-lipase for both esterification of 1-phenylethanol and hydrolization of olive oil. The reason is the reformation of secondary structure of lipase after the immobilization on aminopropyl-grafted mesoporous silica nanotubes.

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