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Synthesis of Retinyl Palmitate Catalyzed by *Candida sp.*99-125 Lipase Immobilized on Fiber-Like SBA-15 Mesoporous Material

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*Candida sp.*99-125 lipase was suitable for transesterification of fats and oils to produce fatty acid methyl ester. The adsorption of *Candida sp.*99-125 lipase onto the fiber-like SBA-15 mesoporous material has been studied. The unaltered structural order of the fiber-like SBA-15 before and after the adsorption has been confirmed by FT-IR, SEM and N_2 adsorption. The amount of adsorbed *Candida sp.*99-125 lipase depends both on the solution pH and reaction time. Good adsorption capacity of *Candida sp.*99-125 lipase on fiber-like SBA-15 may be due to solution pH from 5.0 to 9.0 especially at 7.0 (93.99 mg enzyme per gram silica is obtained and the activity recovery is 281.05%). A high lipase loading (135.9 mg enzyme per gram silica) was obtained, but it did not produce a proportionate level of catalytic activity. The immobilized *Candida sp.*99-125 lipase showed increased adaptability in the hydrolysis of *p*-nitrophenyl acetate compared to free *Candida sp.*99-125 lipase at pH 5.0–9.0. Meanwhile, the immobilized *Candida sp.*99-125 lipase showed higher thermal stability than that of free *Candida sp.*99-125 lipase. And the synthesis of retinyl palmitate in organic solvent with the immobilized *Candida sp.*99-125 lipase was investigated. The influence factors, such as: the solvent used, the molar ratio and concentrations of substrates, the reaction time and the amount of lipase were studied and optimized. In the conditions of transesterificating 0.164 g retinyl acetate and 0.32 g palmitic acid, 10 mL of solvent hexane, 1:4 of mass ratio of lipase to retinyl acetate, and 6 hours of reaction time, 74.6% of retinyl acetate was converted into retinyl plamitate.

Keywords: Immobilization, *Candida sp.*99-125 Lipase, SBA-15, Hydrolysis Activity.

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1. INTRODUCTION

Lipases (triacylglycerol acylhydrolases, EC 3.1.1.3) were widely used as versatile biocatalysts in modern organic chemistry, especially for synthesis of enantioenriched compounds and polymerization reactions, esterification, and interesterification.[1–3] The lipase *Candida sp.*99-125 has good esterification ability without positional specificity for triglycerides and has potential in the synthesis of short chain esters as flavor compounds or biofuels such as biodiesel.[4–6] In practice, a free *Candida sp.*99-125 lipase has inherent problems, such as stability, recovery from the reaction system, and operational cost. However, immobilized enzymes have the advantages of stability and separation and thus can avoid these problems.[7]

The immobilization of biomolecules such as proteins and peptides onto solid supports has attracted much attention because of its scientific importance, and *Candida sp.*99-125 lipase was relevant in many areas, such as biology, medicine, biotechnology, and food processing.[1–4] Because of the interesting properties of lipases, several studies reported the immobilization of these enzymes using different protocols: adsorption on hydrophobic supports, entrapment in gels, and covalent attachment to solid supports. Immobilization of enzymes by physical procedures such as adsorption on a solid was very attractive because of its simplicity.[8–14] Mesoporous materials have been a hot topic in last 20 years. However, many papers about preparation and application of mesoporous materials have been published very recently.[15–31] In particular, use of mesoporous materials and nanoorganized materials

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as the supports leads to development of nanosize reactors and sensors. Among the mesoporous materials, nanoorganized materials have received considerable attention in the adsorption of large molecules such as enzymes and vitamins because of their well-ordered pore structure with high specific surface areas and pore volume. Recently, high enzyme loading has attracted much attention in the area of enzyme immobilization.[32–34]

Vitamin A plays an important role in the control of anti-aging skin and cancer, et al. Vitamin A and its derivatives have been widely used in food, cosmetics and drugs.[35–39] Vitamin A and retinyl acetate were the most commonly used in vitamin A series, but they were readily oxidized by light, air, oxidizing agents or heat.[40] Now a amount of researches have focused on the development of the stable synthesis of retinyl derivatives, in particular, retinyl palmitate. At present, retinyl ester was manufactured by chemical methods using inorganic alkaline catalysts at high temperature, and the unstable retinol and retinyl ester result in the formation of by-products easily. At the same time, there were high energy consumption, high requirements for equipment and environmental pollution.[41,42] Lipase-catalyzed reactions can avoid these problems. Lipase-catalyzed reactions were superior to conventional chemical methods owing to their mild reaction conditions, high catalytic efficiency and the inherent selectivity of natural catalysts that have been used in the synthesis of a variety of esters, in line with today's "green chemistry" requirement, which has become the development direction of the synthesis of retinyl esters in future.

In this study, we described the preparation of fiber-like SBA-15 and a method of immobilizing the *Candida sp.*99-125 lipase via physical adsorption in the channels of the fiber-like SBA-15 support. The hydrolytic activity of the *Candida sp.*99-125 lipase was determined by following the formation of the product, *p*-nitrophenol (pNP), from hydrolysis of *p*-nitrophenyl acetate. The influences of buffer pH and time on the amount of *Candida sp.*99-125 lipase immobilized onto fiber-like SBA-15 were reported. The thermal stability of immobilized and free *Candida sp.*99-125 lipase as well as reuse of immobilized *Candida sp.*99-125 lipase, the effects of various factors such as solvents, substrates, immobilized lipase lifetime and absorbent agent, on esterification were studied. In the investigation of the transesterification of retinyl actate and palmitic acid, the author discovered that the immobilized *Candida sp.*99-125 lipase enhance the yield of the transesterification reaction. This has demonstrated the possibility of the immobilized *Candida sp.*99-125 lipase accelerating enzyme-catalyzed reaction in organic phase. But so far the immobilized *Candida sp.*99-125 lipase adopted in Vitamin A esterification has not been reported. In this paper, we also have studied the immobilized *Candida sp.*99-125 lipase-catalyzed synthesis of retinyl ester in organic solvent.

2. MATERIALS AND METHODS

2.1. Materials

Candida sp.99-125 lipase (68.06 U/mg) was purchased from Beijing CTA New Century Biotechnology Co. Ltd. (PR China). The *p*-nitrophenyl acetate (*p*-NPB) was obtained from TCI (Shanghai). Poly(ethylene glycol)-block-poly(propylene glycol)-block-poly(ethylene glycol) (P123) was obtained from Sigma-Aldrich in the highest grade commercially available. Vitamin A acetate (96.5%) was purchased from Xin Hecheng Biochemical Corp. (China); palmitic acid was of analytical grade and obtained from Sinopharm Group Chemical Reagent Co., Ltd. (China); Vitamin A palmitate (99%) was obtained from SIGMA Co. Ltd.(USA); Methanol, ethanol, *n*-propanol, *iso*-propanol, butanol, hexane and other reagent were provided by Sinopharm Chemical Reagent Co., Ltd. All reagents and solvents were of AR grade.

2.2. Preparation of Fiber-Like SBA-15 Mesoporous Material

The SBA-15, which was prepared in our laboratory, was prepared using reported procedures.[6,12] The P123 was used as a surfactant template. The molar composition of the mixture was 1 TEOS: 0.017, P123:5.7, HCl:173 H_2O. First, 4 g P123 was dissolved in the mixture of 30 g water and 120 g 2 mol/L HCl aqueous solution, the resulting solution was stirred at room temperature until the solution became clear. Then, 8.5 g TEOS was added drop wise into the solution of P123 in acid media at 40 °C. After TEOS was added, the mixture was stirred at 40 °C for 24 h. This mixture was then transferred into a Teflon-lined autoclave and heated to and kept at 100 °C for 24 h. Finally, white precipitates were collected, filtered, and washed with water, air-dried at room temperature, and calcined at 550 °C for 5 h at a heating rate of 1 $°C \cdot min^{-1}$ in a tube furnace to remove the organic template.[43,44]

2.3. Immobilization of Lipase on SBA-15

SBA-15 is a kind of pure silica material consisting of SiO_2 units with free hydroxyl groups on its surface that can form hydrogen bonds with the functional groups of the side chains of amino acids of enzyme. Besides this force, a weak van der Waals interaction also assists in the enzyme adsorption on the surface of the support. Hydrophobic interactions contribute to adsorption by interactions of hydrophobic patches on the enzyme with the silica network of support. Moreover, *Candida sp.*99-125 lipase has a molecular dimension of 3 nm × 4 nm × 5 nm and hence the *Candida sp.*99-125 lipase may enter the pores of fiber-like SBA-15 (pore size 7.1 nm).

A total of 20 mg of activated SBA-15 was dispersed in 2 ml phosphate buffer solution (pH 7.0, 0.1 mol/L) containing approximately 8 to 24 mg *Candida sp.*99-125. The

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container with this mixture was put in an ice-water system, and then the suspensions were separated by centrifugation and washed with phosphate buffer (pH 7.0, 0.01 mol/mL) three times. The amount of *Candida sp*.99-125 immobilized on the SBA-15 was determined by measuring the concentration of protein in the supernatant using the Bradford protein assay method.[45]

$$P_a = \frac{(C_i - C_f)}{W} \times V \quad (1)$$

where P_a is the amount of immobilized enzyme onto carriers ($mg \cdot g^{-1}$, mg enzyme per gram silica); C_i and C_f were the concentration of the enzyme initial and final in the reaction medium (mg/mL), respectively; V is the volume of the reaction medium (mL); and W is the weight of the carriers (g).

2.4. Activity Assays of Free and Immobilized Lipase

The activity of free and immobilized *Candida sp*.99-125 lipase was determined by using *p*-nitrophenyl acetate as a substrate according to previously reported assay method. The reaction mixture (3.0 mL) was composed of 2.7 ml phosphate buffer (pH 6.5, 0.01 mol/L), enzyme solution of appropriate dilution (0.15 mL) and appropriate immobilized *Candida sp*.99-125 (approximately 3 to 5 mg) where the buffer was used for the blank. The mixture was incubated for 2 min at 37 °C. Reactions were initiated by adding 0.15 ml of *p*-nitrophenyl acetate solution and stopped by addition of acetone (3 mL) after 10 min. The mixtures were clarified by filtration, and the absorbance of solution resulting from the release of *p*-nitrophenol was measured at 400 nm. One unit of lipase activity was defined as the amount of enzyme that liberates 1 μmol of *p*-nitro-phenol per min. The specific activity is the number of lipase units per mg of enzyme protein. The coupled yield and the relative activity and were defined as follows.[46]

$$\text{Coupled } yield\ (\%) = \frac{C_i - C_f}{C_i} \times 100 \quad (2)$$

$$R_a\ (\%) = \frac{A}{A_0} \times 100 \quad (3)$$

$$\text{Realtive } activity\ (\%) = \frac{B}{B_m} \times 100 \quad (4)$$

where R_a is the activity recovery of the immobilized lipase (%), A is the activity of the immobilized enzyme ($U \cdot mg^{-1}$), A_0 is the activity of the equal weight free enzyme ($U \cdot mg^{-1}$), B is the activity of the free/immobilized enzyme ($U \cdot mg^{-1}$), and B_m is the maximum activity of the free enzyme in solution ($U \cdot mg^{-1}$).

2.5. Thermal Stability of Free and Immobilized Lipase

Thermal stabilities of free and immobilized enzymes were determined by measuring their residual activities after they were incubated at 50 °C for 8 h.

2.6. Synthesis of Retinyl Palmitate

The transesterification reactions were conducted in a series of 25 ml screw-caped glass vials. 0.04 g lipase was added into 10 mL hexane containing 0.164 g retinyl acetate and 0.32 g palmitic acid. The reaction was carried out by agitation at 180 oscillations/min and 30 °C. These conditions were used unless otherwise stated. At 1–6 h intervals, the yield of samples was detemined by HPLC. The yield of retinyl palmitate was defined as:

$$\text{Transesterification } yield\ (\%) = \frac{M_p}{M_{a_0}} \times 100 \quad (5)$$

where M_p was the mole of retinyl palmitate in the product, and M_{a0} was the mole of retinyl acetate added.

2.7. Determination of Initial Rate

The transesterification reactions were conducted in a series of 25 ml screw-caped glass vials. 0.04 g lipase was added into 10 mL hexane containing 0.164 g vitamin A acetate and 0.32 g palmitic acid. After 30 min, the yield of samples was detemined by HPLC. The initial rate of transesterification reaction was defined as the increase of retinyl palmitate in unit time.

2.8. HPLC Analysis of Retinyl Palmitate

Quantitative analyses of the reactants and the products were obtained by a HPLC with SCL-10AVP system (Shimadzu, Japan). The detector was SPD-10AVP UV/V, which was operated at 364 nm. And a reversed-phase column (Alltech C18 250 mm × 4.6 mm, 5 μm) was used. A 20 μL sample of proper dilution of the reaction mixture was injected. 100% methanol was used as the mobile phase at room temperature at a flow rate of 1 $mL \cdot min^{-1}$.

3. RESULTS AND DISCUSSION

3.1. Structure of SBA-15 Mesoporous Material by SEM and TEM

Figures 1(a) and (b) shows scanning electron micrograph (SEM) images of calcined fiber-like SBA-15 material prepared at 100 °C. The SBA-15 has a fiber-like morphology, by stacking and coupling of rod-like particles of which respective length and width were approximately 6 μm and 0.5 μm. Typical TEM images of the fiber-like SBA-15 were shown in Figures 1(c) and (d). The presence

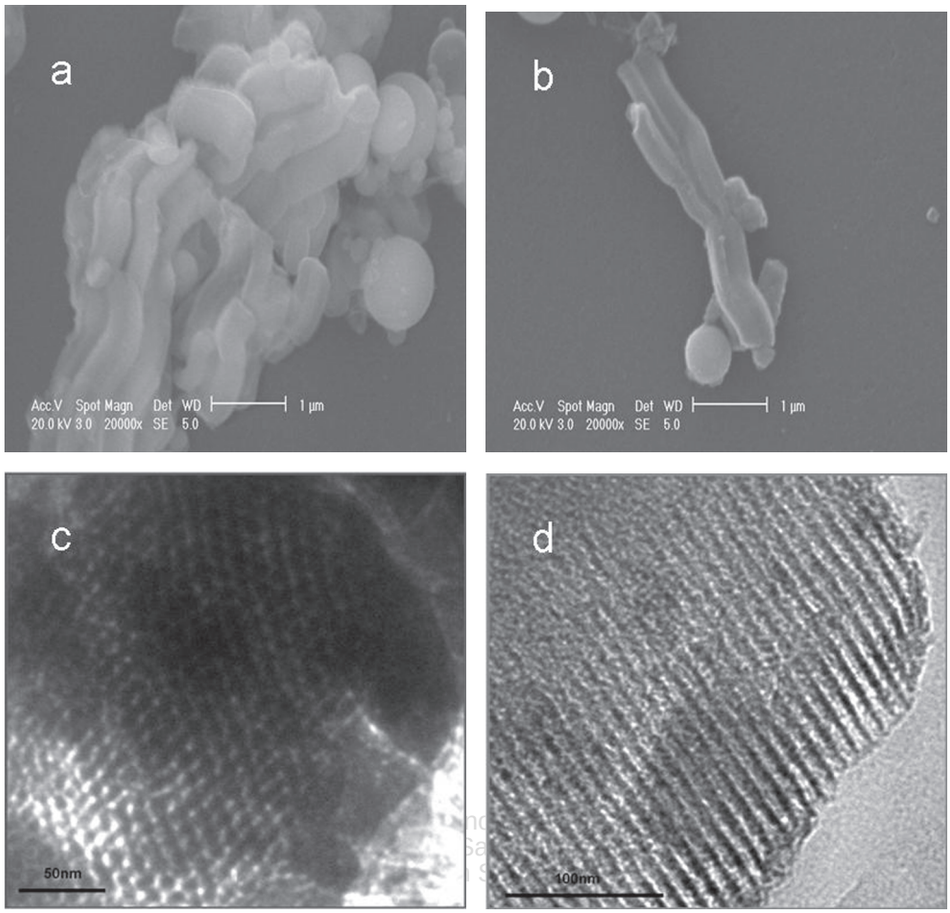

Fig. 1. Scanning electron micrographs of SBA-15 (a) and (b) and transmission electron micrographs of SBA-15 (c) and (d).

of ordered arrays of silica channels with wall thickness (approximately 3.4 nm) and uniform diameter (approximately 7.1 nm) were clearly observed, which was fitting for the entrapment of *Candida sp.*99-125 lipase molecules sized 3 nm × 4 nm × 5 nm.

3.2. FTIR Spectra of Immobilization of *Candida sp.*99-125 lipase

To confirm that *Candida sp.*99-125 lipase was immobilized on SBA-15, the Fourier transform infrared (FTIR) spectra of SBA-15, *Candida sp.*99-125 lipase, and immobilized *Candida sp.*99-125 lipase were studied. As shown in Figure 2, the typical Si–O–Si bonds around 1082, 798 and 462 cm^{-1} were associated with the formation of a condensed silica network. The weak band in the range of 940–960 cm^{-1} was assigned to non-condensed Si–OH groups. After *Candida sp.*99-125 lipase was adsorbed, the intensity of this band dramatically decreased. The result indicates that a hydrogen bonding interaction occurred between free Si–OH groups and the N–H groups of *Candida sp.*99-125 lipase. Furthermore, absorption bands associated with C–H

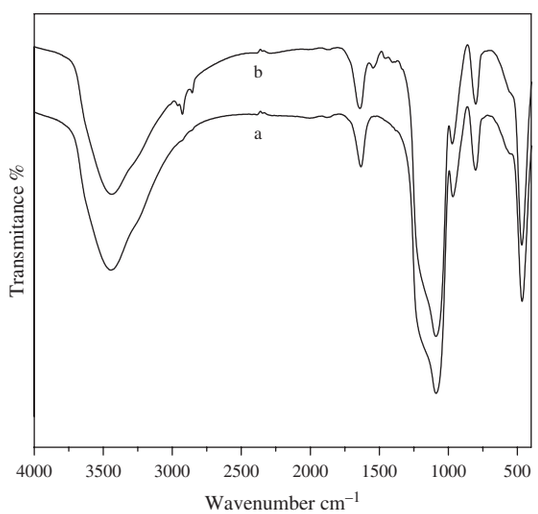

Fig. 2. FT-IR spectra of silica sphere (a) and silica spheres adsorpted with *Candida sp.*99-125 Lipase (b).

stretching (between 2885 and 2995 cm^{-1}), C–O stretching (1577 cm^{-1}), and C–H deformations (around 1484 cm^{-1}) were observed in the spectra of *Candida sp.*99-125 lipase and immobilized *Candida sp.*99-125 lipase but not in the spectra of SBA-15. These results confirmed that the *Candida sp.*99-125 lipase had been successfully immobilized on the SBA-15.[9]

3.3. N_2 Adsorption–Desorption Isotherm

Figure 3 reports the 77 K nitrogen adsorption–desorption isotherms of the fiber-like SBA-15 material before and after *Candida sp.*99-125 lipase was adsorbed. The isotherms can be classified as type IV and exhibit an H1-type hysteresis loop at high relative pressure. These were typical features of hexagonal cylindrical channel mesoporous materials. Compared to SBA-15, SBA-15 after *Candida sp.*99-125 lipase adsorption shows a marked decrease in the specific surface area (from 575.49 m$^2\cdot$g^{-1} to 440.72 m$^2\cdot$g^{-1}) and pore volume (from 0.966 m$^3\cdot$g^{-1} to 0.874 m$^3\cdot$g^{-1}). The pore size of the SBA-15 before and after *Candida sp.*99-125 lipase was adsorbed was around 7.10 nm. Similar results have been reported for the adsorption of lysozyme, cytochrome *c*, porcine pancreas lipase, or CRL (lipase from *Candida* rugosa) onto MCM-41 and SBA-15.[47–49] The decrease in surface area and pore volume distribution suggests that the immobilization of *Candida sp.*99-125 lipase in the channels of SBA-15 has occurred.

3.4. Effect of Reaction Time on Amount Adsorbed and Activity Recovery of Immobilized *Candida sp.*99-125 Lipase

The effect of reaction time on activity recovery (*Ra*) of the immobilized *Candida sp.*99-125 lipase is shown in Table I. According to Table I, a maximal enzyme activity recovery is about 2 times higher than that of the free lipase. The activity recovery of the immobilized *Candida sp.*99-125 lipase increases with enhancement of reaction time and the maximum activity recovery is 221.05% obtained under immobilization proceeding for 60 min, however, it decreases when the reaction time is further prolonged. *Candida sp.*99-125 lipase molecules form gradually a monolayer adsorption in 60 mins, and the increase of activity recovery with an increment of adsorption time can be explained in terms of available space for the adsorbed protein molecules to optimize their interactions with the adsorbent surface. However, if the adsorption time of *Candida sp.*99-125 lipase is more than 60 mins, *Candida sp.*99-125 lipase molecules form a multilayer adsorption and the overlay and aggregation of the adsorbed enzyme molecules would increase accordingly, which cause that some of enzymatic activities cannot be expressed. The adsorbed amount and immobilized *Candida sp.*99-125 lipase activity at different time are shown in Table I. The adsorbed amount of the *Candida sp.*99-125 lipase increases with enhancement of immobilization time, and the maximum immobilization percent of *Candida sp.*99-125 lipase on fiber-like SBA-15 is up to 90.6%. Moreover, there is no obviously increasing amount adsorbed of the *Candida sp.*99-125 lipase after 360 mins. At this time, electrostatic repulsion and attraction between adsorbed proteins reach to the equilibrium. In this case, lipase immobilization reaches the adsorption equilibrium. However, only 109.2% of the activity recovery is retained on the support at 360 mins. The results from Table I show that higher loadings do not result in significant increase in activity recovery on support. This may be due to longer incubation time would imply more enzyme-support links as a result of multilayer adsorption of enzyme and formation of enzyme aggregates which may decrease the enzyme activity, as it may prevent the interaction of the lid with external hydrophobic surfaces. Moreover, the enzyme immobilized may become a barrier to products and substrates diffusion.

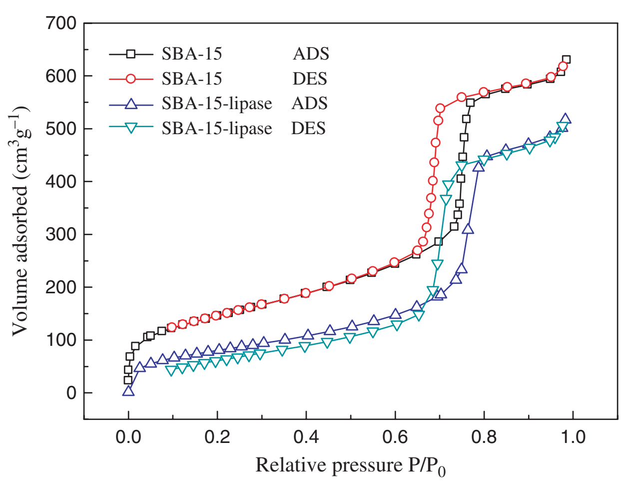

Fig. 3. N_2 adsorption–desorption isotherms of SBA-15 before *Candida sp.*99-125 lipase was adsorbed.

Table I. The amount adsorbed and immobilized *Candida sp.*99-125 lipase activity at different time (pH 7.0 phosphate buffer solution).

Time (min)	P_a (mg·(g·carrier)$^{-1}$)	IY (%)	activity[a] (U·(g·carrier)$^{-1}$)	R_a (%)
0	0.0	0.0	0.0	0.0
15	59.1	39.4	6093.5	151.5
30	92.7	61.8	10188.7	161.5
60	121.1	80.7	18211.6	221.1
90	116.9	77.9	13959.6	175.5
150	128.7	85.8	11333.7	129.4
360	135.9	90.6	10100.3	109.2

[a]The activity of free *Candida sp.*99-125 lipase: 68.08 U·mg^{-1}.

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3.5. Effect of Reaction pH on Amount Adsorbed and Activity Recovery of Immobilized *Candida sp.*99-125 lipase

Table II shows the amount adsorbed of *Candida sp.*99-125 and immobilized enzyme activity at different pH. It can be clearly seen from Table II that the amount adsorbed is significantly affected by the solution pH. The maximum adsorption amount of *Candida sp.*99-125 is 96.88 mgg^{-1} at pH 5.0 while only the activity recovery is 121.3%. Although the amount adsorbed is 93.29 mg/g at pH 7.0 which is lower than that at pH 5.0, the activity recovery is 281.05%. The activity recovery is also clearly a function of solution pH. It has been reported that the maximum amount adsorbed of enzyme occurs at a pH near the pI of the protein–substrate complex and not at the pI of the protein. The candidates for the dominant driving forces for the adsorption of *Candida sp.*99-125 include hydrophobic interactions (a kind of van der Waals attraction), electrostatic repulsion and attraction between the amino acid residues on the surface of *Candida sp.*99-125 and silanol groups on the surface of the silica walls of the mesoporous materials, the intramolecular cohesive attraction and repulsion. Hydrophobic interactions are more dominant near the pI than electrostatic interactions. These hydrophobic interactions originate from

(1) the interaction between the nonpolar side chains of the amino acids residues on the surface of *Candida sp.*99-125 and surface siloxane bridges or

(2) from the lipase–lipase interactions between the hydrophobic side chains of neighboring *Candida sp.*99-125 molecules adsorbed on the surface of SBA-15.

There is a decrease of the amount adsorbed of *Candida sp.*99-125 from pH 5.0 to pH 9.0. This can be interpreted as follows: charged amino acid residues on the protein surface change from positive charges to negative charges gradually with the increase of pH. Both the surface of the protein molecule and the silica surface are negatively charged, which creates a strong electrostatic repulsion between the enzyme and the absorbent and leads to a low amount of adsorption.

Table II. The amount adsorbed of *Candida sp.*99-125 lipase and immobilized enzyme activity at different pH levels (incubation time 1 h).

pH	P_a (mg·(g·carrier)$^{-1}$)	IY (%)	activity[a] (U (g·carrier)$^{-1}$)	R_a (%)
5	96.88	96.88	8000.45	121.30
6	93.99	93.99	9501.63	148.49
7	93.29	93.29	17850.00	281.05
8	56.28	56.28	7100.99	185.33
9	36.79	36.79	4196.06	167.53

[a]The activity of free *Candida sp.*99-125 lipase: 68.08 U·mg^{-1}.

3.6. Effect of pH on Activity of Free and Immobilized *Candida sp.*99-125 lipase

Effects of pH on the enzymatic activity of the free and immobilized *Candida sp.*99-125 lipase for hydrolysis of *p*-nitrophenyl acetate was determined with pH ranging from 5.0 to 9.0, and the results were shown in Figure 4. Although the maximum values of relative activity were observed at pH 8.0 and 7.0 for free and immobilized *Candida sp.*99-125 lipase, the immobilized *Candida sp.*99-125 lipase holds excellent adaptability in a wider pH range (5.0 to 9.0) compared to free *Candida sp.*99-125 lipase, and the shape of the enzymatic activity isotherm of the immobilized *Candida sp.*99-125 lipase was normally bell-shaped. It was known that polyionic matrices cause the partitioning of protons between the bulk phase and enzyme microenvironment causing a shift in the optimum pH value.[50]The adaptability should depend on the method of immobilization, as well as the structure and charge of the carrier; furthermore, the screen function of the structure would play an important role in enhancing the adaptability.[51]

3.7. Effect of Temperature on Activity of the Free and Immobilized Enzymes

The effect of the reaction temperature on the enzymatic activity of the free and immobilized *Candida sp.*99-125 lipase was shown in Figure 5. The optimum reaction temperatures of free and immobilized *Candida sp.*99-125 lipase were 40 °C and 50 °C, respectively. At other temperatures, the activity of the immobilized *Candida sp.*99-125 lipase was higher than that of the free *Candida sp.*99-125 lipase. In other words, the immobilized *Candida sp.*99-125 lipase holds more heat resistance than that of free *Candida sp.*99-125 lipase between 45 °C and 60 °C. The increasing temperature will be suitable for adjusting the conformational integrity of the

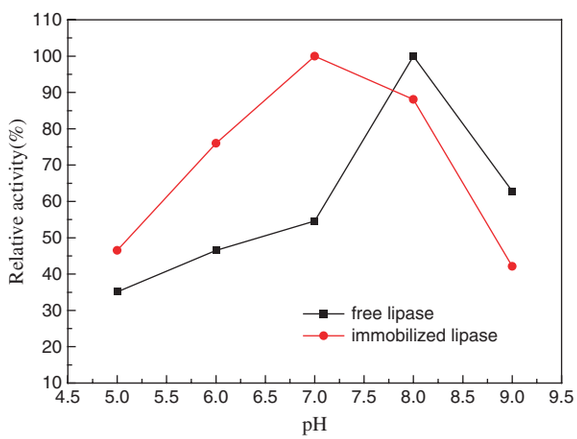

Fig. 4. Variation of enzymatic activity versus medium pH immobilized lipase and free lipase.

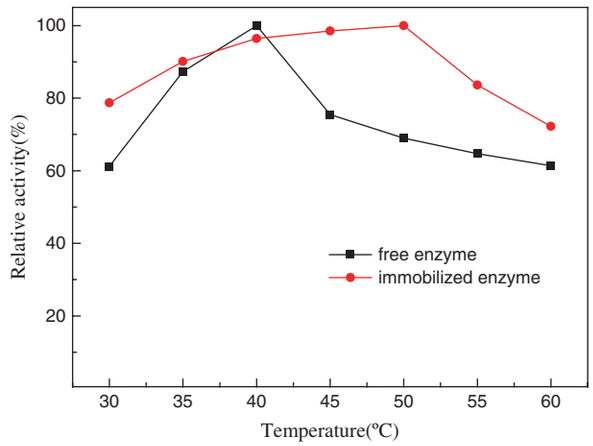

Fig. 5. Effect of temperature on activity on the free and immobilized *Candida sp.*99-125 lipase.

enzyme immobilization by adsorbing to the mesoporous materials.[52, 53]

3.8. Thermal Stability of Free and Immobilized Lipase

It was well known that enzymes in solution were not stable and their activities also decrease gradually in the usage. To investigate the thermal stability of immobilized *Candida sp.*99-125 lipase on fiber-like SBA-15 materials, the residual activities of immobilized *Candida sp.*99-125 lipase and free *Candida sp.*99-125 lipase were measured and compared in Figure 6, when they were incubated at 50 °C. The residual activity of immobilized *Candida sp.*99-125 lipase maintained comparative stability and held over 42% of the hydrolysis activity at 6 h, whereas, that of the free *Candida sp.*99-125 lipase decreased quickly with increasing thermal treatment time and maintained only 25% of the activity at 4 h. Comparing the immobilized to the free *Candida sp.*99-125 lipase, the thermal stability of the immobilized sample was better than that of the free sample. Thus the mesoporous pores of supports can keep enzymes from injury resulting from direct exposure environmental changes.

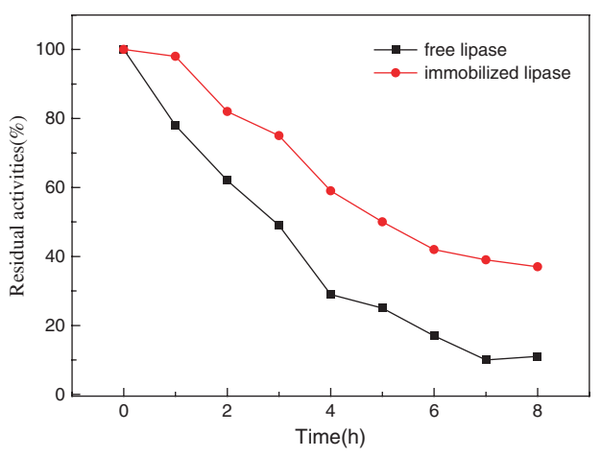

Fig. 6. Thermal stability of free and immobilized *Candida sp.*99-125 lipase.

3.9. Effect of Different Lipases in Synthesis of Retinyl Palmitate

Six lipases from different sources and different specificities were assayed for synthesizing the vitamin A palmitate, which was shown in Table III. Novozym 435 showed good activity for the synthesis of retinyl palmitate and the yield of retinyl palmitate reached 81.7%. Moreover the researches showed that Novozym 435 was the ideally lipase of ester synthesis. And the immobilized *Candida sp.*99-125 lipase on fiber-like SBA-15 materials was almost as good as Novozym 435.[54–59]

3.10. Effect of Organic Solvent in Synthesis of Retinyl Palmitate

As the reaction media of enzyme, organic solvents have a huge effect on enzyme-catalyzed reaction. It can directly affect the catalytic activity, stability, substrate specificity of enzyme, the balance and rate of reaction. Laane used polar parameters (logP) of organic solvents to describe effect of organic solvents on the enzyme reaction (logP was the partition coefficient of organic solvent in the octanol/water system).[59] We tested six kinds of commonly used organic solvents, whose relative polarity and yield show in Table IV catalyzed by immobilization of *Candida sp.*99-125 lipase on SBA-15.

As seen from Table IV, the yield increased while logP of organic solvents increased under the same other conditions. This further confirmed the Laane's conclusion,[60] hydrophobic solvent was in favor of esterification reaction. As was seen from the table, hexane was the best solvent, so we choose hexane as the reaction media in the following trials.

3.11. Effect of Substrates Molar Ratio on Reaction of Synthesis of Retinyl Palmitate

Lipase-catalyzed transesterification reaction was a reversible reaction. To make a thorough reaction, the

Table III. Effects of different lipases on reaction.

Lipase	Maximum yield/%
Porcine pancreatic lipase	0
Novozym 435	81.7
Lipozyme RM IM	34.1
Lipozyme TL IM	28.3
Free *Candida sp.* lipase	50.3
Candida sp. lipase on SBA-15	75.2

Note: Reaction condition: solvent hexane up to 10 mL, lipase 0.04 g, Time 6 h, $C_A = 0.05$ mol/L, $C_B = 0.125$ mol/L.

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Table IV. Effect of solvent on reaction.

Solvent	logP	yield/%
Butanone	0.29	0
Tetrahydrofuran	0.5	0
Tert-amyl alcohol	1.4	10.7
Toluene	2.5	54.8
N-hexane	3.5	74.2
N-octane	4.5	72.2

Note: Reaction condition: solvent up to 10 mL, lipase 0.04 g, Time 6 h, C_A = 0.05 mol/L, C_B = 0.125 mol/L.

reactant must be excess, or removed a certain product during reaction. In order to improve yield of the retinyl palmitate, we fixed the amount of retinyl acetate and altered the amount of palmitic acid, then several different substrate molar ratios were compared to determine the optimal molar ratio. The results were shown in Figure 7.

From Figure 7, we can see the yield increase with the increased molar ratio of palmitic acid and retinyl acetate. When the molar ratio of palmitic acid and retinyl acetate was 2.5:1, the yield was the highest of 74.6%. The yield of esterification got balance when acid continued to increase, which showed that the substrate concentration around enzyme molecules had reached a balance, and it had no effect on the reaction balance to continue to increase the amount of palmitic acid, and adding too much acid also caused a waste of raw materials. Therefore, the use of substrate molar ratio of 2.5:1 was appropriate.

3.12. Effect of Reaction Time in Synthesis of Retinyl Palmitate

We have studied the effect of reaction time on esterification of retinyl palmitate. The results were shown in Figure 8, with the increase in reaction time the yield continued to increase, after 6 h the variation of yield was

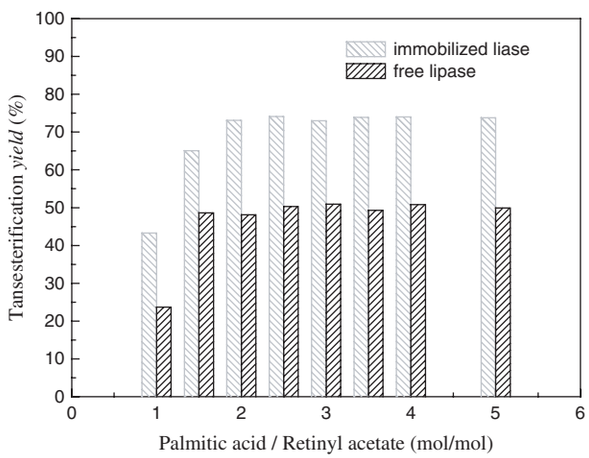

Fig. 7. Effect of substrates molar ratio on yield.
Note: (Reaction condition: solvent hexane up to 10 mL, lipase 0.04 g, Time 6 h, C_A = 0.05 mol/L).

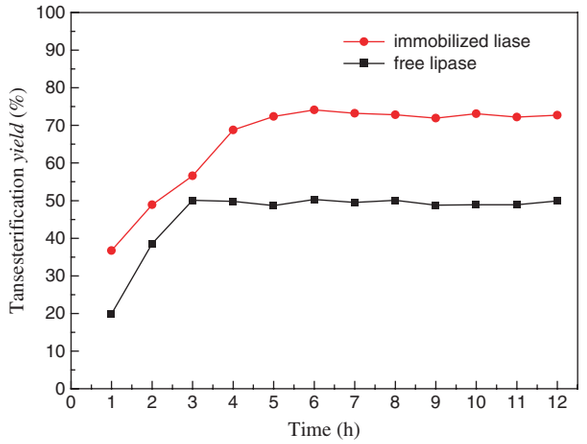

Fig. 8. Effect of reaction time on yield.
Note: Reaction condition: solvent hexane up to 10 mL, lipase 0.04 g, C_A = 0.05 mol/L, C_B = 0.125 mol/L.

little, and reaction was basically balanced. However, when the reaction time was more than 10 h, the yield decreased slightly. It may be that the long time reaction has a certain negative impact on the structure of retinyl palmitate. That was the structure of enzyme molecules tended to unreasonable with long reaction time, so impeded enzyme molecule itself exercise biological function and seemingly showed their activity decreased and even inactivated which lead to the descending the yield.

4. CONCLUSIONS

The adsorption of *Candida sp.*99-125 lipase on the mesoporous fiber-like SBA-15 from buffer solutions with different pH values and times were studied as a model protein adsorption system. The adsorbed amount depends on the solution pH and time.[61] The optimal pH and temperature regions of the immobilized *Candida sp.*99-125 lipase for the hydrolysis of *p*-nitrophenyl acetate were 5 and 35 °C, respectively. Meanwhile, the immobilized *Candida sp.*99-125 lipase exhibits much better adaptability compared to free enzyme at pH 5.0 to 9.0 and at a temperature of 50 °C. The immobilized *Candida sp.*99-125 lipase showed higher thermal stability than that of free *Candida sp.*99-125 lipase. Screening the enzyme and media, we found that the immobilized *Candida sp.*99-125 lipase was suitable for the reaction of synthesis of retinyl palmitate, while the best medium was hexane. We also determine the optimal reaction conditions: 0.164 g retinyl acetate and 0.32 g palmitic acid, 10 mL solvent hexane, 1:4 of mass ratio of lipase to retinyl acetate, and 6 hours of reaction time, 74.6% of retinyl acetate was converted into retinyl plamitate.

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Nomenclature

R_a	activity recovery of the immobilized lipase, %
A	activity of the immobilized enzyme, $U \cdot mg^{-1}$
A_0	activity of the equal weight free enzyme, $U \cdot mg^{-1}$
B	activity of the free/immobilized enzyme, $U \cdot mg^{-1}$
B_m	maximum activity of the free enzyme in solution, $U \cdot mg^{-1}$
P_a	amount of immobilized enzyme onto carriers, $mg \cdot g^{-1}$
C_i	concentration of the enzyme initial in the reaction medium, mg/mL
C_f	concentration of the enzyme final in the reaction medium, mg/mL
V	volume of the reaction medium, mL
W	weight of the carriers, g
M	the mole of retinyl palmitate in the product, mol
Ma_0	the mole of retinyl acetate added, mol
C_A	the mole concentration of retinyl acetate, mol/L
C_B	the mole concentration of palmitic acid, mol/L

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