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Immobilization of *Candida* sp.99-125 lipase onto silanized SBA-15 mesoporous materials by physical adsorption

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Abstract SBA-15-NH_2 and SBA-15-Cl Mesoporous Materials were prepared by modifying SBA-15 with silane coupling agent 3-aminopropyltriethoxysilane and 3-chloropropyltriethoxysilane using the post-synthesis method. The mesoporous samples were characterized by Fourier transform infrared spectra and nitrogen adsorption. Compared with SBA-15-NH_2, SBA-15-Cl has suitable pore opening for further utilization in the immobilization of Candida sp.99-125 lipase by physical adsorption. The influences of lipase concentration and immobilizing time on the immobilization efficiency were investigated. Meanwhile, the lipase immobilized on SBA-15-Cl showed higher thermal, pH and storage stability than that of free lipase. Further study demonstrated that lipase immobilized on SBA-15-Cl could be used eight times without significant decrease of enzyme activity. The phenomenon was associated with the shrinkage of the pore opening of SBA-15-Cl keeping lipase from leaping out.

Keywords: Silanization, SBA-15, Lipase, Immobilization, Stability, Reusability

INTRODUTION

Lipases (triacylglycerol ester hydrolases, EC 3.1.1.3) are ubiquitous enzymes which catalyze the hydrolysis of triacetin to give acetic acid and glycerol, and are thus widely used in dairy industries, chemical industries and enantiomerically pure pharmaceuticals [1,2]. Unfortunately, the practical application of lipases is often handicapped by their sensitivity to temperature and instability, as well as the difficulty in recovering activity for reuse [3-5]. Several methods have been proposed to overcome these limitations, one of the most successful being enzyme immobilization. Lipase can be immobilized on carrier by a variety of methods, which may be broadly classified as physical and chemical [6-9]. Compared with other methods, immobilization of lipase by physical adsorption is currently attracting an enormous attention given its simplicity. The stability of the immobilization often depends on the type of support. Therefore, it is important to choose the most suitable support to immobilize lipase.

Among numerous supports applied till now [10-16], ordered mesoporous materials (OMMs) have gathered particular interest. Lipase immobilized in OMMs exhibits higher stability than free lipase [17-22]. SBA-15, a mesoporous molecular sieve with large pore diameters, has also been used as a carrier for lipase [23,24]. Though SBA-15 support has been demonstrated to protect the lipase from aggregation, the insufficient strength of interaction between lipase and the mesoporous support causes significant lipase leaching from the support [25,26]. Thus, surface modification of mesoporous silicas with organofunctionalities was actively investigated to reduce the degree of enzyme leaching [27,28]. Through organic functionalization of the internal surface, the hydrophobic groups attached to the surface of the mesoporous material and the increase of the surface area can strengthen interactions between mesoporous material and lipase (i.e., hydrophobic, and electrostatic interactions) [29]. In addition, the surface functionalization also partially reduces the pore openings, especially at the external surface. This will 'trap' the lipase molecules within the pores while still allowing reactant and product molecules to diffuse in and out of the pores [30].

In the present work, NH_2-SBA-15 and Cl-SBA-15 mesoporous materials were prepared by modifying SBA-15 with 3-aminopropyltriethoxysilane (APTES) and 3-chloropropyltriethoxysilane (CPTES) using the post-synthesis method. The original SBA-15 mesoporous and its derivatives were then characterized by Fourier transform infrared spectra and nitrogen adsorption. Compared with SBA-15-NH_2, SBA-15-Cl has suitable pore opening for immobilization of Candida sp.99-125 lipase. The influences of lipase concentration and immobilizing time on lipase immobilization in terms of activity recovery were reported. The thermostable, pH and storage stability of lipase immobilized on SBA-15-Cl and free lipase as well as reusability of lipase immobilized on SBA-15-Cl were studied.

MATERIALS AND METHODS

1. Materials

Candida sp.99-125 lipase was purchased from Beijing CTA New Century Biotechnology Co. Ltd. (PR China). Poly (ethylene glycol)-block-poly (propylene glycol)-block-poly (ethylene glycol) (P123) was obtained from Sigma-Aldrich. 3-aminopropyltriethoxysilane (APTES) and 3-chloropropyltriethoxysilane (CPTES) were procured from Chemical Reagent Corp. in China. Other reagents were obtained from Sinopharm Chemical Reagent Co., Ltd. All reagents

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were of AR grade.

2. Silanization of SBA-15 Mesoporous Material

Mesoporous SBA-15 was prepared using triblock copolymer surfactant as the template in acid media as referenced to the previous work [31]. The functional group used to modify the SBA-15 was APTES or CPTES. The silanization procedure was performed using a post-synthesis method as reported [32]. In brief, 2 g SBA-15 mesoporous material was suspended in 50 mL of dry toluene containing 2 g of APTES or CPTES. The mixture was stirred for 48 h at 100 °C in a nitrogen atmosphere under reflux conditions. Then, the white solid was filtered out and washed with toluene (30 mL), ethanol (30 mL), and diethyl ether (30 mL), respectively. The resulting white powders, denoted as SBA-15-NH_2 or SBA-15-Cl, were dried under vacuum.

3. Characterization of SBA-15 and its Derivatives

The analyses of chemical functional groups in the SBA-15 and its derivatives were conducted by means of a Thermo Corporation Nexus FT-IR spectrophotometer (USA). For the analyses of surface area, pore characteristics, pore size distribution and adsorption-desorption isotherm, a Micromeritics ASAP 2000 surface analyzer was used.

4. Enzyme Immobilization

Direct immobilization method as reported was used to immobilize the Candida sp.99-125 lipase [33]. 1-10 mg of Candida sp.99-125 lipase was mixed with 40 mg of SBA-15-Cl in 10 ml phosphate buffer solution (pH 7.0, 0.1 mol·L^{-1}). The mixture was shaken at 37 °C for 1-8 h. The supernatant was separated from the solid materials by centrifugation. Retained immobilized lipase then was washed with a phosphate buffer (pH of 7.5) solution. The wet sample was then kept overnight in a vacuum oven for a complete drying.

5. Measurement of Protein Content and Enzymatic Activity

The protein content in the crude enzyme or immobilized enzyme preparations was determined by the Bradford method, using bovine serum albumin as the standard [34]. The activity of free and immobilized lipase was determined by monitoring the release of the acid moiety of the ester by titration with 0.02 M NaOH [35]. The lipase was added in the triacetin emulsification solution, which was a pH stabilized mixture of triacetin (2.0 g), deionized water (50 mL) and pH 7.0 phosphate buffer solutions (10 mL). After exactly 15 min of incubation at 40 °C, the reaction was stopped by adding 15.0 ml of 95% alcohol solution. Finally, the reaction solution was titrated with 0.02 M sodium hydroxide solution. The volume of consumed sodium hydroxide was recorded and the activity of lipase calculated in the standard way from which the unit of enzyme activity was denoted with U, and one lipase unit is defined as 1 mol of the fatty acid produced by the catalysis per min under the assay conditions. The activity recovery (%) remaining after immobilization was the ratio between the activity of immobilized lipase and the total activity of lipase added in the initial immobilization solution.

6. Stability Lipases

To investigate thermo- and pH stability, free lipase or lipase immobilized on SBA-15-Cl was incubated in buffers with different pH value, different temperatures for 8 h. The stability of lipases was determined by measuring residual activity. Storage stability of free lipase or lipase immobilized on SBA-15-Cl was investigated by measuring their activities after being stored at 20 °C for a week period and the residual activity was measured once a day. The relative activity of the lipase is calculated from the formula:

$$R(\%) = \frac{A}{A_0} \times 100\% \quad (1)$$

where, R is the relative activity (%), A is the residual activity of the immobilized lipase (U) and A_0 is the initial activity of the immobilized lipase (U).

7. Reusability

Lipase immobilized on SBA-15-Cl was recovered through centrifugation, washing and drying after each hydrolysis reaction of triacetin was finished. The reusability was also characterized by determining residual activity of lipase immobilized on SBA-15-Cl compared to that of their initial activity.

RESULTS AND DISCUSSION

1. Characterization of SBA-15 and Its Derivatives

The Fourier transform infrared spectra of the SBA-15 and its derivatives are shown in Fig. 1. To the spectrum of SBA-15 (Fig. 1(a)), the broad and strong peak centered peak at 1,085 cm^{-1} and those relatively weak peaks near at 800 cm^{-1} and 470 cm^{-1} correspond to the asymmetric stretching vibration of typical Si-O-Si bands [36]. A peak near 1,637 cm^{-1} is observed in the spectrum of parent SBA-15, mainly resulting from the bending vibration of absorbed H_2O. To the spectrum of SBA-15-Cl and SBA-15-NH_2 (Fig. 1(b) and 1(c)), the absorption band at 2,930 cm^{-1} is associated with the C-H stretching vibration of CH_2 groups of CPTES and APTES. The new broad band at 698 cm^{-1} can be assigned to the C-Cl bond in Fig. 1(b), and the new broad bands at 1,566 cm^{-1} and 1,495 cm^{-1} can be assigned to the N-H bond of amino in Fig. 1(c), suggesting that the silane coupling agent reacted with the -OH groups of SBA-15 [37,38].

Fig. 2 exhibits the nitrogen adsorption-desorption isotherms and the corresponding BJH pore size distributions of SBA-15 and its derivatives at 77 K. The curves are Langmuir IV-type adsorptions, which are the typical adsorption curves of SBA-15. For the SBA-15 sample (Fig. 2(a)), the relative pressure was 0.7, so, the nitrogen adsorption suddenly increased, corresponding to a suddenly rising

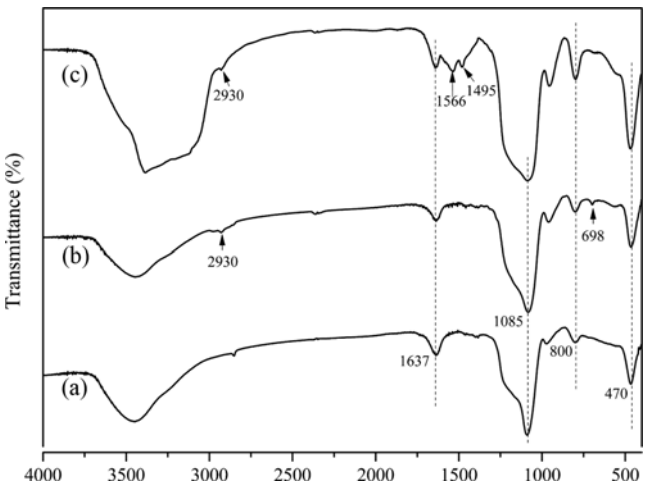

Fig. 1. FTIR spectra of SBA-15 (a), SBA-15-Cl (b) and SBA-15-NH_2 (c).

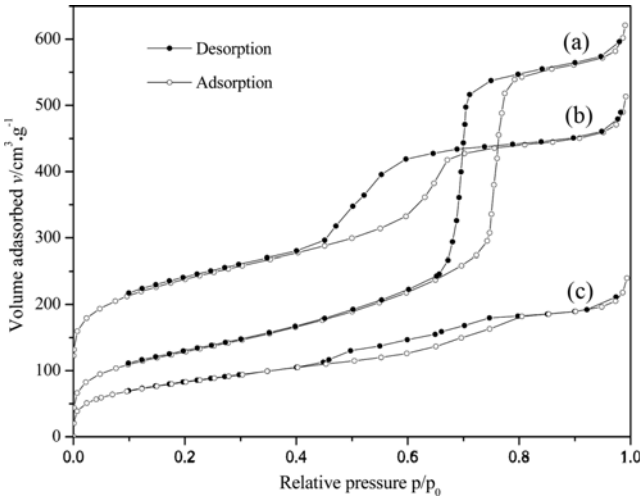

Fig. 2. N_2 adsorption-desorption isotherms of SBA-15 (a), SBA-15-Cl (b) and SBA-15-NH_2 (c).

Table 1. BET parameters of SBA-15 and Its derivatives

Samples	Surface area/ $m^2 \cdot g^{-1}$	Average pore diameter/nm	Pore volume/ $cm^3 \cdot g^{-1}$
SBA-15	460.9	7.05	0.9571
SBA-15-Cl	512.0	4.71	0.6282
SBA-15-NH_2	303.1	2.63	0.3550

curve. For SBA-15-Cl and SBA-15-NH_2 (Fig. 2(b) and 2(c)), the mutation relative pressure dropped to about 0.5, while its adsorption amount of nitrogen gas obviously decreased compared with that of SBA-15. This shows that the silanizations of SBA-15 lead to the decrease of pore size and pore volume. Also as shown in Table 1, compared with SBA-15, the pore size and volume of surface modified SBA-15 materials were significantly decreased, while there was an obvious increase in surface area. It showed that the material used for modification of zeolite had been successfully introduced and was grafted in the channels. Meanwhile, Table 1 shows that the average pore diameter of SBA-15-NH_2 and SBA-15-Cl was 2.63 nm and 4.71 nm respectively. Therefore, SBA-15-Cl was suitable for utilization as supports for the immobilization of lipase by physical adsorption.

2. Optimization of Parameters for Lipase Immobilization

2-1. Lipase Concentration

The effect of initial Candida sp.99-125 lipase concentration on the immobilization was studied at different initial lipase concentrations in the range of 0.1-1.0 mg/mL. The immobilization experiments were conducted at 37 °C for 4 h with continuous shaking. As shown in Fig. 3, the amounts of lipase immobilized increased significantly with the initial lipase concentration. While the activity recovery first increased with the increased amount of lipase; a maximum was reached at approximately 0.6 mg/mL of lipase concentration, and decreased thereafter. It is considered that the higher lipase loading makes the lipase form an intermolecular steric hindrance, which restrains the diffusion of the substrate and product. Similar phenomena were also observed in the previous reports.

2-2. Immobilizing Time

The effects of the immobilizing time on the immobilization capacity and on the enzyme activity were studied at different reaction time. The initial lipase concentration was 0.6 mg/mL. Fig. 4 shows that an increase in the immobilizing duration time led to an increase in the lipase immobilized in the present study. Meanwhile, the activity recovery of the immobilized lipase kept pace with the immobilizing time and the highest relative activity was obtained under immobilization allowed to proceed for 4 h. However, it decreased when the reaction time was further prolonged. It can be explained that the immobilizing time of lipase was more than 4 h, so Candida sp.99-125 lipase molecules formed a multilayer adsorption and the overlay of the adsorbed lipase molecules increased accordingly, which caused that some of enzymatic activities could not be expressed [39]. Given these results, it can be inferred that the optimum immobilizing time for the immobilization process was 4 h.

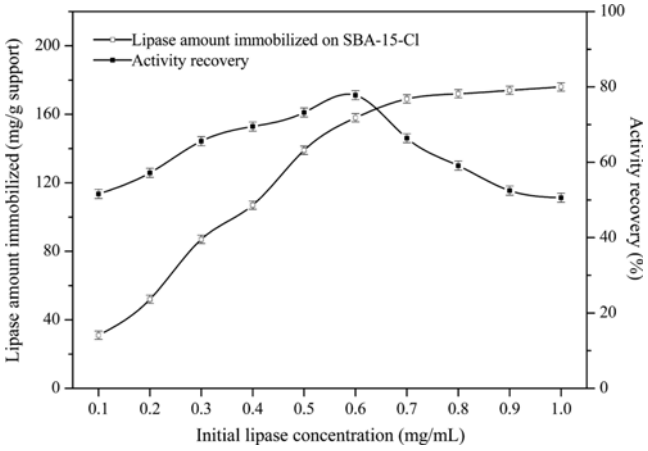

Fig. 3. Effect of lipase concentration on immobilization.

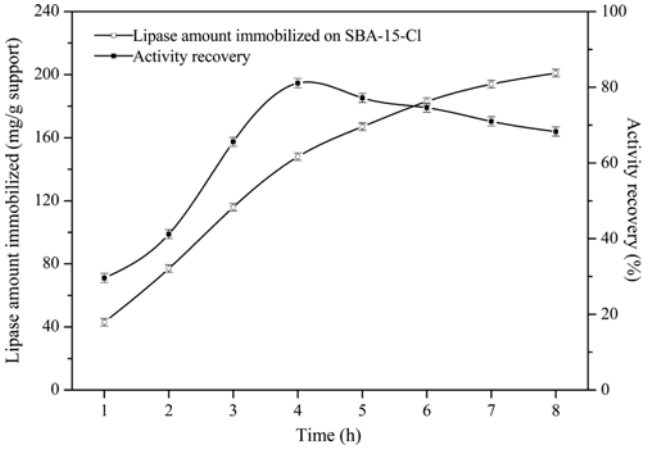

Fig. 4. Effect of immobilizing time on lipase immobilization.

3. Properties of Immobilized Lipase

3-1. Thermal Stability

It was well known that enzymes in solution are not stable and their activities also decrease gradually. To investigate the thermal stability of free lipase and lipase immobilized on SBA-15-Cl, their residual activities were measured and compared in Fig. 5 when they were incubated at 50 °C. All activities of lipases decreased with time. The free lipase almost lost its all activity after 7 h with high temperature preservation. However, the activity of lipase immobilized on

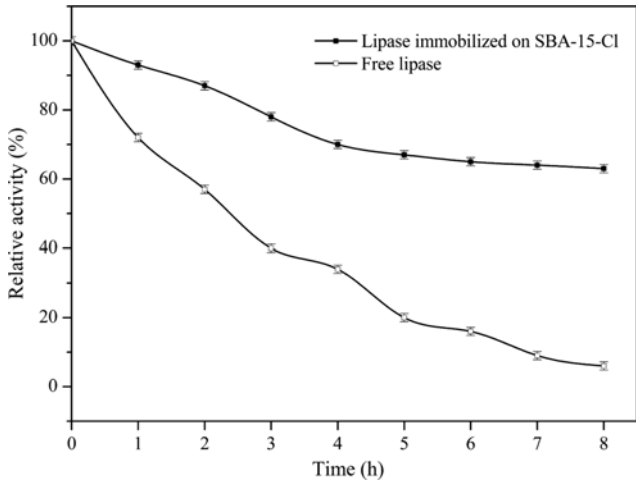

Fig. 5. Thermal stability of free lipase and lipase immobilized on SBA-15-Cl.

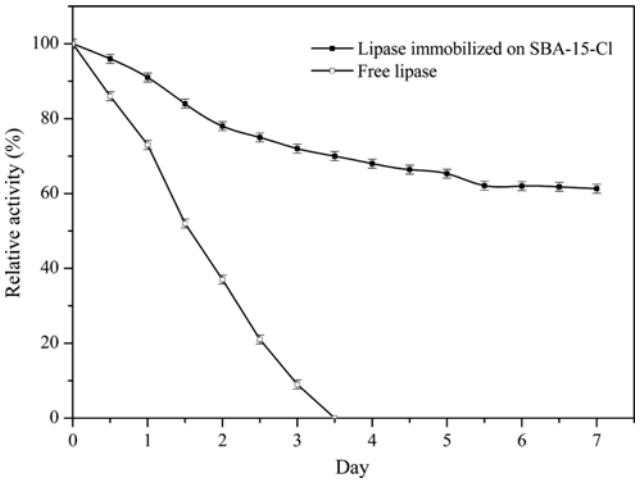

Fig. 7. Storage stability of free lipase and lipase immobilized on SBA-15-Cl.

SBA-15-Cl was always higher than the former. This result can be attributed to the increased protection from the stronger interaction between the Candida sp.99-125 lipase molecule and functionalized surface of the supports either by physical forces [26], resulting in the prevention of lipase heat denaturation and enhanced adaptability of immobilized lipase [40]. In addition, the excellent activity retention by the lipase immobilized on SBA-15-Cl could be explained on the basis of the smaller mesoporous pores of supports keeping lipase from leaping out of the channel.

3-2. pH Stability

Effect of medium pH on the enzyme activity of free lipase and lipase immobilized on SBA-15-Cl was determined after incubating in the pH range of 5.0-9.0 for 4 h at 50 °C and the results are presented in Fig. 6. The maximum value of relative activity was observed at pH of 8.0 for all lipases. However, the pH profile of the lipase was broader than that of the free enzyme. In other words, lipase immobilized on SBA-15-Cl holds excellent adaptability both at lower and higher pH in comparison to the free lipase. As for the lipase immobilized on SBA-15-Cl, the additional interaction of the support with lipase due to the presence of chloropropyl functional group might have reduced the sensitivity of lipase to low pH [30]. The reason for the lowest enzyme activity at pH 9.0 may be the change of lipase conformation, due to an unfavorable charge distribution on the amino acid residues.

3-3. Storage Stability

In industrial applications, lipase is generally stored to external environment and loses activity rapidly. So the storage stability of lipase immobilized on SBA-15-Cl has always been of interest. The immobilized and free lipase were stored in phosphate buffer (50 mM, pH 7.0) at 20 °C and the activity measurements were carried out for a period of a week. As shown in Fig. 7, lipase immobilized on SBA-15-Cl exhibits clearly better storage stability than that of free lipase. In the storage condition, the free lipase lost its all-initial activity within three days. Under the same storage conditions, a 38.7% decrease in activity was detected during a seven day storage period for the lipase immobilized on SBA-15-Cl. The relative severe decrease in activity of the free lipase might be due to its susceptibility to autolysis during the storage time [41,42]. It should be noted that the immobilization of lipase on SBA-15-Cl beads could decrease

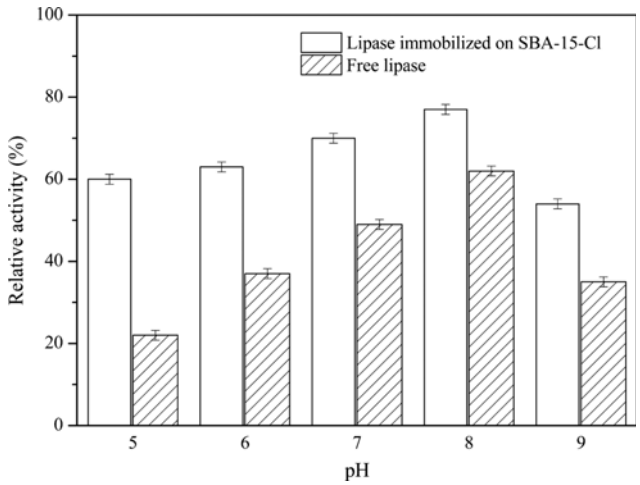

Fig. 6. pH stability of free lipase and lipase immobilized on SBA-15-Cl.

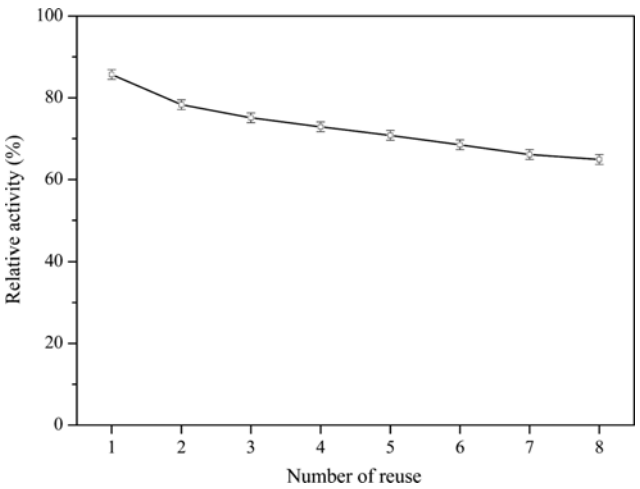

Fig. 8. The reusability of lipase immobilized on SBA-15-Cl.

the autolysis effect.

3-4. Reusability

Reusability of immobilized lipase is very important for practical applications, especially in the industrial fields. In this study, lipase immobilized on SBA-15-Cl was repeatedly used as the biocatalyst for the hydrolysis reaction and subsequently recovered and reused. As shown in Fig. 8, lipase immobilized on SBA-15-Cl could be reused for eight cycles without dramatic activity loss and still retained 64.9% of its initial activity, which indicates its robust operational stability. The excellent activity retention of lipase immobilized on SBA-15-Cl could be explained on the basis of the pore structure and the degree of leaching. Directly, the pore size of SBA-15-Cl is relatively small; thus enzyme molecules are difficult to leach out of the pore channel, keeping high enzyme activity. Also, the finding made in this study indicated that the immobilization mostly occurred within the mesopores rather than the external surface.

CONCLUSION

Mesoporous material SBA-15-Cl with a suitable pore diameter of 4.71 nm has been synthesized and used for Candida sp.99-125 lipase immobilization. The results showed that lipase could be successfully immobilized on SBA-15-Cl by physical adsorption. The relative activity was 81.1% when 0.6 mg/ml of lipase concentration and 4 h of immobilized time were the best immobilized conditions. More attention was paid to the stability and reusability of lipase immobilized on SBA-15-Cl. Lipase immobilized on SBA-15-Cl holds much more excellent adaptability compared with free lipase during pH 6.0-9.0. At 50 ºC, the immobilized lipase shows higher thermal stability than that of free lipase. Also, a high storage stability obtained with the immobilized lipase indicates that the stability of Candida sp.99-125 lipase was increased upon immobilization on SBA-15-Cl mesoporous material. In addition, lipase immobilized on SBA-15-Cl maintained 64.9% of its residual activity even after eight cycles of use.

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