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Preparation of Robust Biocatalyst Based on Cross-Linked Enzyme Aggregates Entrapped in Three-Dimensionally Ordered Macroporous Silica

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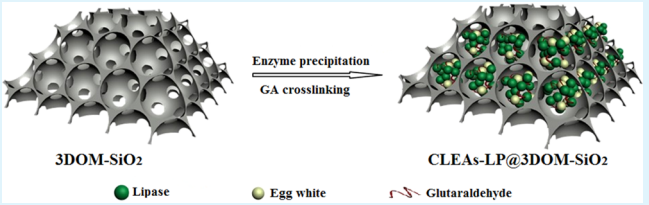
Supporting Information

ABSTRACT: With the aim to provide a highly stable and active biocatalyst, cross-linked enzyme aggregates (CLEAs) of lipase *Candida* sp. 99-125 were prepared in three-dimensionally ordered macroporous silica materials (CLEAs-LP@3DOM-SiO_2). Lipase *Candida* sp. 99-125 was first precipitated in the pores of 3DOM SiO_2 (named EAs-LP@3DOM-SiO_2), and further cross-linked by glutaraldehyde to form CLEAs-LP@3DOM-SiO_2. Saturated ammonium sulfate was used as a precipitant and glutaraldehyde with a concentration of 0.25% (w/w) was employed as a cross-linker. Compared with EAs-LP@3DOM-SiO_2 and native lipase, CLEAs-LP@3DOM-SiO_2 exhibited excellent thermal and mechanical stability, and could maintain more than 85% of initial activity after 16 days of shaking in organic and aqueous phase. When CLEAs-LP@3DOM-SiO_2 was applied in esterification and transesterification reactions, improved activity and reusability were achieved. This method can be used for the immobilization of other enzymes of interest.



KEYWORDS: *Three-dimensionally ordered macroporous materials, cross-linked enzyme aggregates, lipase, enzyme immobilization, biodiesel*

1. INTRODUCTION

Enzymes, which demonstrate high efficiency and high chemo-, regio-, and stereo-selectivities in catalyzing reactions, have attracted increasing attention for well over a century that continues to expand.[1,2] However, the industrial application of enzymes in many fields is often hampered by their low stability, poor reusability, and high cost.[3,4] Enzyme immobilization provides one of the most attractive concepts to overcome these drawbacks.[5] After immobilization, enzymes with improved stability can be isolated from the reaction mixture and the reusability is achieved; at the same time, the process cost can be dramatically reduced.[6]

Among various immobilization methods, the cross-linked enzyme aggregates (CLEAs) technique seems to be a promising method for enzyme immobilization.[7,8] This technique is exquisitely simple and amenable to rapid optimization, which translates to low costs and short time-to-market.[9] In recent years, the formation of CLEAs in the pores of mesoporous materials results in highly loaded, stable, and active heterogeneous biocatalysts, which are significantly more stable than conventional biocatalysts prepared by physical adsorption.[10−13] A ship-in-a-bottle approach for CLEAs shipped in hierarchically ordered mesocellular mesoporous silica was also developed and resulted in the improvement of enzymes' operational stability.[14,15] Until now, considerable research has been directed toward using this ship-in-a-bottle approach for enzyme immobilization.[16−19] Nevertheless, developing a novel support and immobilization method that can confer a substrate high transfer rate and endow enzymes' high activity and stability will have great potential to generate better biocatalysts in the future.

Three-dimensionally ordered macroporous (3DOM) materials, which possess an open, interconnected macropore structure, and nanosized wall components, are being explored for use in photonic devices, catalysis, sorption, and other potential applications.[20,21] In comparison to mesoporous materials, surface areas of 3DOM materials are lower, but pore interconnectivity and surface accessibility are high.[20] Moreover, in well-ordered 3DOM materials, each macropore has 12 windows to neighboring cages, facing in multiple directions. Thus, compared to diffusion through microporous or mesoporous materials, mass transport through 3DOM materials is quite efficient. Given these factors, 3DOM materials are well-suited to the challenges presented by heterogeneous catalysis. However, to the best of our knowledge, there is no report concerning enzyme immobilization in/on 3DOM materials. The special structure of 3DOM materials that possesses big chambers and small windows is very suitable for the ship-in-a-bottle approach. The superiorities of preparing CLEAs in 3DOM materials are probably as follows: (1) cross-

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linking in pores can prevent the formation of large CLEAs particles;[19] at the same time, the large interconnected cavities of 3DOM materials can enhance the accessibility of substrates to the enzyme active sites and the short diffusion path of the thin pore walls can accelerate the material exchange;[22] (2) the cross-linking step would inhibit the enzyme denaturation effectively because of the multipoint attachment between enzyme molecules; at the same time, CLEAs formed in macropores make enzymes' leaching through the small windows difficult and enhances the maximum loading of CLEAs to a certain degree; (3) the 3DOM materials can protect CLEAs from the damage of mechanical stirring.[19,23] Therefore, 3DOM material could be applied as a promising candidate to host CLEAs.

Lipase (triacylglycerol acylhydrolase, EC 3.1.1.3), one of the most important and versatile enzymes, has very broad substrate specificity and can be used in a wide range of reactions including hydrolysis, esterification, transesterification, etc.[24] There has been a noted increase in the number of studies using mesoporous silica for lipase immobilization with respect to the requirements of enzyme supports such as large surface area, high porosity, thermal and mechanical stability, and toxicological safety.[25−31] However, exploring the specific features of porous silica supports for lipase immobilization has not been disclosed. Besides, it is always highly desirable to develop high-performance materials to immobilize lipase. Thanks to the delicate structures, 3DOM materials could be applied as promising candidates to host lipase CLEAs. To the best of our knowledge, rare reports are related to immobilizing lipase CLEAs in 3DOM materials and studying their properties.

Thus, in this study, 3DOM silica (3DOM-SiO_2) was employed as a host material for lipase *Candida* sp. 99-125 CLEAs. Especially, lipase *Candida* sp. 99-125 was first precipitated in the pores of 3DOM-SiO_2 (named EAs-LP@3DOM-SiO_2), and further crosslinked by glutaraldehyde to form CLEAs-LP@3DOM-SiO_2. The effect of precipitant type and glutaraldehyde concentration on the enzyme activity was studied. The thermal and mechanical stability of EAs-LP@3DOM-SiO_2 and CLEAs-LP@3DOM-SiO_2 were also investigated in detail. In addition, the properties of CLEAs-LP@3DOM-SiO_2 that applied in hydrolysis, esterification, and transesterification reactions were also estimated.

2. EXPERIMENTAL SECTION

2.1. Materials. Lipase *Candida* sp. 99-125 (the protein concentration of the crude lipase is 112.6 mg protein g^{-1}, as determined by a UV detector at the wavelength of 280 nm) was purchased from Beijing CAT New Century Biotechnology CO, Ltd (China). 4-nitrophenyl palmitate (pNPP) was purchased from Sigma Chemical (St. Louis, MO). Ammonium sulfate, glutaraldehyde (GA), oleic acid, ethanol, isopropyl alcohol, acetone, *n*-butanol, cyclohexane, ethyl oleate and *Jatropha* oil were from Jiangtian Chemical Reagent Co., Ltd. (Tianjin, China). All other chemicals and reagents were analytical grade.

2.2. Preparation of 3DOM-SiO_2. The 3DOM-SiO_2 was prepared according to Stein group's work.[32] The difference of the procedure was that polystyrene latex spheres of 200 nm were used as colloidal crystal template and the template was removed by calcination.

2.3. Immobilization of Lipase in 3DOM-SiO_2. The process for immobilizing lipase in 3DOM-SiO_2 is as follows. (1) Five milligrams of 3DOM-SiO_2 was added into 100 μL of lipase solution (11.26 mg protein/mL, egg white was used as proteic feeder), and then the mixture was vacuumed for 5 min to force the enzymes to enter the pores of 3DOM-SiO_2. After that, the mixture was shaken for 2.5 h at 25 °C. The supernatant was withdrawn by a syringe and collected for next cycle of immobilization. The activity of lipase solution in the 3DOM-SiO_2 was measured and defined as starting activity; (2) one milliliter of precipitant was added and the mixture was shaken for 0.5 h at 0 °C, and then the supernatant was withdrawn by a syringe and the obtained product was named EAs-LP@3DOM-SiO_2; (3) GA solution was then added to EAs-LP@3DOM-SiO_2 and shaking was continued for 3 h at 0 °C. After cross-linking, the product was washed five times with phosphate buffer (50 mM, pH 8.0) and then CLEAs-LP@3DOM-SiO_2 was obtained. Both EAs-LP@3DOM-SiO_2 and CLEAs-LP@3DOM-SiO_2 were freeze-dried in a vacuum chamber and stored in refrigerator at −20 °C until use. The effect of precipitant type and GA concentration on the activity recovery of EAs-LP@3DOM-SiO_2 and CLEAs-LP@3DOM-SiO_2 was optimized through single factor experiment.

The SEM image of 3DOM-SiO_2 was obtained on a scanning electron microscope (SEM, Seron Technology S-4800). Fourier-transform infrared (FT-IR) spectra of 3DOM-SiO_2, CLEAs-LP@3DOM-SiO_2 and, native lipase were recorded on a Bruker Vector 22 FT-IR spectrophotometer using KBr pellets.

2.4. Enzymatic Activity Assay. Activities of the native, EAs-LP@3DOM-SiO_2, and CLEAs-LP@3DOM-SiO_2 were measured by the hydrolysis of 4-nitrophenyl palmitate (pNPP). For native lipase, 100 μL of lipase solution (2.25 mg protein/mL) was added to a mixture of 4 mL of PBS solution and 100 μL of pNPP (5 mg/mL in ethanol) at room temperature. After reaction for 1 min, 2 mL of Na_2CO_3 (0.25 M) was added to terminate the reaction and the reaction mixture was filtered and the spectrophotometric absorbance of the supernatant was measured at 405 nm. For the immobilized lipase, 5 mg of immobilized lipase was added to a mixture of 4 mL of PBS solution and 100 μL of pNPP (5 mg/mL in ethanol) at room temperature. After reaction for 1.5 min, the reaction mixture was filtered and the spectrophotometric absorbance of the supernatant was measured at 405 nm. One unit of enzyme activity was defined as the amount of enzyme, which liberated 1 μM of pNP per minute under the assay conditions. The activity recovery was calculated as follows:[4]

$$\text{activity recovery}(\%) = \text{observed activity after immobilization} / \text{starting activity}$$

2.5. Thermal Stability. Thermal stabilities of the native lipase, EAs-LP@3DOM-SiO_2, and CLEAs-LP@3DOM-SiO_2 in organic media were determined by incubating enzyme samples in isooctane at 70 °C for a certain time. The enzyme samples were taken out at each time point, and isooctane was carefully removed with a micropipette after centrifugation. Then the samples were dried under vacuum to remove isooctane completely. Thermal stabilities of the native lipase, EAs-LP@3DOM-SiO_2, and CLEAs-LP@3DOM-SiO_2 were also determined by incubating enzyme samples in aqueous solution at 60 °C, and the enzyme samples were taken out at each time point. The residual activity of each enzyme sample was measured. The relative activity was calculated from the ratio of the residual activity to the initial activity of each sample.

2.6. Mechanical Stability of Native and Immobilized Lipase in Aqueous Solution and Organic Solvent under Shaking Conditions. The native lipase, EAs-LP@3DOM-SiO_2, and CLEAs-LP@3DOM-SiO_2 were immersed in phosphate buffer (50 mM, pH 8.0) or isooctane at room temperature and shaken at 200 r/min for a certain time. Then the enzyme samples were taken out at each time point and the residual activities were measured.

2.7. Applications. The hydrolytic application of lipase *Candida* sp. 99-125 was tested with ethyl oleate emulsion that contains 4% (w/v) polyvinyl alcohol (PVA) as the substrate. The native lipase, EAs-LP@3DOM-SiO_2, and CLEAs-LP@3DOM-SiO_2 with equal activity (10 U) were added to the mixture of 4 mL of emulsion and 2 mL of the phosphate buffer (50 mM, pH 8.0). The reactions were conducted at 40 °C for 12 h, and the amount of the released oleic acid was determined by titration with a 50 mM NaOH solution

For applications in esterification and transesterification, the native lipase, EAs-LP@3DOM-SiO_2, and CLEAs-LP@3DOM-SiO_2 with equal activity (30 U) were added to the mixture of 0.21 g of oleic acid or *Jatropha* oil, 0.175 mL of ethanol, and 1.5 mL of cyclohexane.

The reactions were carried out at 40 °C for 24 h. After the reactions, the lipases were separated from the previous reaction mixture by centrifugation and washed three times with cyclohexane, and then the lipases were reused for another cycle.

The amount of product, fatty acid ethyl esters (FAEEs, the main components of biodiesel), was determined by using a gas chromatograph (Beifen-Ruili Analytical Instrument Co., Beijing, China) with internal standard method. The GC oven was primarily kept at 160 °C for 2 min, and then heated to 220 °C at a rate of 15 °C/min, where it was kept for 4 min, then increased to 260 °C at a rate of 3 °C/min and then maintained at this temperature for 10 min. The injector and detector temperatures were set at 240 and 280 °C, respectively.[33] All the experiments in this study were performed in triplicate.

3. RESULTS AND DISCUSSION

3.1. Characterizations of 3DOM-SiO_2 and Immobilized Lipase. The morphology of 3DOM-SiO_2 is shown in Figures 1

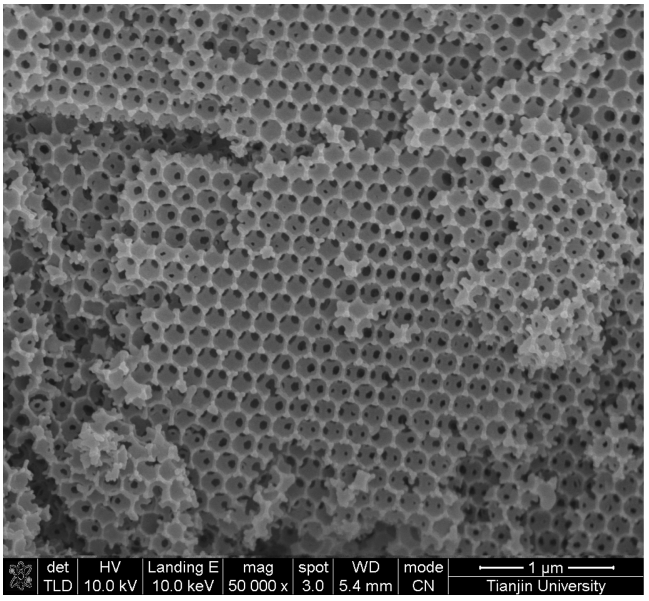

Figure 1. SEM image of the 3DOM SiO_2.

and S1 (Supporting Information), proving the three-dimensionally ordering structure of 3DOM-SiO_2. The macropore size of 3DOM-SiO_2 was around 180 nm, and each macropore was connected to its twelve neighbors by small "windows", which were about 50 nm. The specific surface area was estimated to be 94.84 m^2/g, using the BET model. Compared with mesoporous materials, the surface area of 3DOM-SiO_2 was lower, but the pore interconnectivity and surface accessibility were high.[20] For enzyme immobilization, larger pore size in the supports was of great importance. The ordered and interconnected pore distribution in 3DOM-SiO_2 may be more beneficial to the mass transfer of reactants and products. At the same time, the special structure of 3DOM materials that possessed big chambers and small windows was crucial for preventing enzyme leakage.

To confirm that lipase *Candida* sp. 99-125 was immobilized on 3DOM-SiO_2, the FT-IR spectra of 3DOM-SiO_2, CLEAs-LP@3DOM-SiO_2, and native lipase were studied here (Figure 2). For 3DOM-SiO_2, the bands at 798 and 1099 cm^{-1} can be assigned to the symmetric stretching vibration peak of Si−O and antisymmetric stretching vibration peak of Si−O−Si.[34] For native lipase, the peaks observed in the regions 1407 and 1545 cm^{-1} corresponded to the bending vibration peaks of −C−H

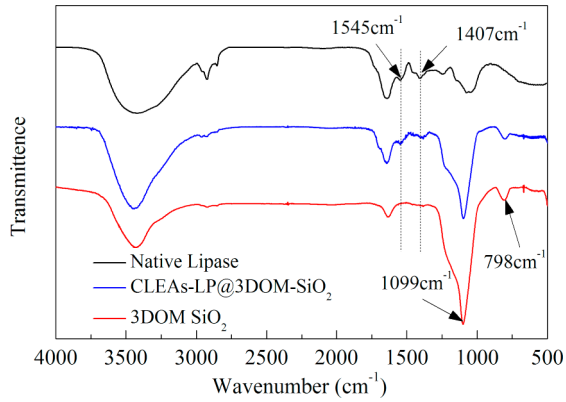

Figure 2. FT-IR spectra of 3DOM SiO_2, CLEAs-LP@3DOM-SiO_2, and native lipase.

and −N−H.[35] The −C−H and −N−H absorption peaks observed in the FT-IR spectrum of CLEAs-LP@3DOM-SiO_2 confirmed that lipase *Candida* sp. 99-125 had been successfully immobilized on 3DOM-SiO_2.

3.2. Optimization of the Preparation Process of CLEAs-LP@3DOM-SiO_2. *3.2.1. Selection of Optimum Precipitant.* Precipitation, by adding salts, organic solvents, or nonionic polymers to aqueous solutions of enzyme, can cause physical aggregation of enzyme molecules into supramolecular structures.[36] In this study, when precipitants were added, free enzymes that embedded in the pores of 3DOM-SiO_2 would aggregate. Activity recoveries were calculated after precipitation and after cross-linking with GA. Table 1 shows the activity

Table 1. Effect of Precipitant on Activity Recoveries of EAs-LP@3DOM-SiO_2 and CLEAs-LP@3DOM-SiO_2

precipitant type	activity recoveries of EAs-LP@3DOM-SiO_2 (%)	activity recoveries of CLEAs-LP@3DOM-SiO_2 (%)
saturated ammonium sulfate	97	19
isopropyl alcohol	87	12
acetone	68	11
ethanol	79	14
n-butanol	90	16

Conditions: the concentration of glutaraldehyde was 1% (w/w) and the cross-linking time was 3 h.

recoveries of EAs-LP@3DOM-SiO_2 and CLEAs-LP@3DOM-SiO_2. When saturated ammonium sulfate was used as a precipitant, the activity recoveries of EAs-LP@3DOM-SiO_2 and CLEAs-LP@3DOM-SiO_2 were 97% and 19%, which were better than that of the other precipitants. Thus, in further experiments, saturated ammonium sulfate was used as a precipitant. Additionally, the activity recoveries of CLEAs-LP@3DOM-SiO_2 were lower than that of EAs-LP@3DOM-SiO_2. These can be explained as follows: (1) the enzyme aggregates formed in precipitation procedure may be in an unfavorable conformation, which was not conducive for reaction. When they were cross-linked, this unfavorable conformation would be remained and displayed lower activity;[7] (2) the active conformation might be ruined if cross-linking reaction happened in the active sites of enzyme, inducing the lower activity recovery of CLEAs-LP@3DOM-SiO_2.[37]

3.2.2. Effect of GA Concentration. After cross-linking, CLEAs were formed in the macropores of 3DOM-SiO_2. GA concentration is one of the key parameters in the preparation of CLEAs. Insufficient cross-linking may occur when lower GA concentration was used, and un-cross-linked enzyme molecules may leach out during the cross-linking process.[9] On the contrary, excessive cross-linking occurred when too high of a concentration of GA was used, resulting in a loss of enzyme's activity.[38] Table 2 showed the effect of GA concentration (w/w) on the activity of CLEAs-LP@3DOM-SiO_2. It can be observed that the activity recovery increased with the increase of GA concentration and reached a maximum value when GA concentration was 0.25%. Under this condition, the highest activity (104 U/g) and activity recovery (33%) was obtained. With further increases of GA concentration, the activity recovery decreased. Thus, in the subsequent experiments, 0.25% of GA concentration was adopted.

Table 2. Effect of Cross-Linker Concentration on Activities of CLEAs-LP@3DOM-SiO_2

cross-linker (%)	CLEAs-LP@3DOM-SiO_2 activity (U/g)	activity recovery (%)
0.10	65	20
0.25	104	33
0.50	80	25
1.00	61	19
2.50	49	15

Condition: the cross-linking time was 3 h.

3.3. Properties of CLEAs-LP@3DOM-SiO_2. The thermal stabilities of native and immobilized lipases were determined by incubating them in isooctane at 70 °C for different times. As can be seen in Figure 3, the native and immobilized lipases were activated when they were incubated in isooctane for a short time. This can be ascribed to the hydrophobic interaction between the hydrophobic phase and part of the lipase molecule, which can cause the opening of the "lid" to make the active site accessible, and thus result in a dramatic increase in catalytic activity.[39] The native lipase, EAs-LP@3DOM-SiO_2, and CLEAs-LP@3DOM-SiO_2 showed the highest activity after 4h (117%), 4h (121%), and 12h (127%) incubation, and their half-lives were 12, 15, and 60 h, respectively. However, as can be seen in Figure S2 (Supporting Information), the activities of native lipase, EAs-LP@3DOM-SiO_2, and CLEAs-LP@3DOM-SiO_2 did not decrease after incubation for 70 h in isooctane at room temperature. These results indicated that thermal effect may be the major reason of the inactivation of lipase in isooctane at 70 °C. The stabilities of native and immobilized lipase in aqueous solution at 60 °C were also investigated. As shown in Figure S3 (Supporting Information), CLEAs-LP@3DOM-SiO_2 exhibited an improved stability compared with native lipase and EAs-LP@3DOM-SiO_2. All these results indicated that the thermal stability of CLEAs-LP@3DOM-SiO_2 was better than that of both native lipase and EAs-LP@3DOM-SiO_2. The increased stability of CLEAs-LP@3DOM-SiO_2 can be ascribed to the following reasons: (1) the multiple covalent bonds between the lipase can enhance enzyme rigidification and reduce the heat extensional deformation of enzyme active site;[17,40] (2) the silica shell may provide a suitable microenvironment to prevent enzymes from thermal damage.[19] Additionally, the lipases displayed markedly enhanced thermostability in isooctane compared to that in aqueous solution at high temperature. These phenomena were due to the conformational rigidity of the lipases in the dehydrated state and their resistance to the covalent reactions causing irreversible thermoinactivation of lipases in aqueous solution.[41,42]

In the process of industrial application, vigorous shaking is an effective method to reduce mass transfer limitation. Thus, the stability of native lipase, EAs-LP@3DOM-SiO_2, and CLEAs-LP@3DOM-SiO_2 was evaluated by incubating them in aqueous solution and organic solvent under shaking condition (200 r/min). In aqueous solution, the activity of native lipase decreased sharply and lost the total activity in 2 days (Figure 4). EAs-LP@3DOM-SiO_2 lost its initial activity dramatically in first 4 days, and retained 17% of its initial activity after 16 days. The residual activity for EAs-LP@3DOM-SiO_2 may be contributed by the lipase that firmly adsorbed on the surface of 3DOM-SiO_2. For CLEAs-LP@3DOM-SiO_2, no significant decrease of the activity was observed after 16 days of shaking. In organic solvent, the native lipase, EAs-LP@3DOM-SiO_2, and CLEAs-LP@3DOM-SiO_2 retained about 34%, 42%, and 87% of their initial activity after 16 days, respectively (Figure 5). The stabilization of lipase activity in CLEAs-LP@3DOM-SiO_2 can be explained by the multiple-point attachment of the lipase molecules in the form of cross-linked clusters, which effectively prevents the denaturation of the enzyme molecules.[43] Additionally, the protection role of 3DOM-SiO_2 may also contribute to the stability of CLEAs-LP@3DOM-SiO_2. Thus, this approach of CLEAs entrapped in 3DOM materials may be potentially useful in biocatalysis industries for it can protect

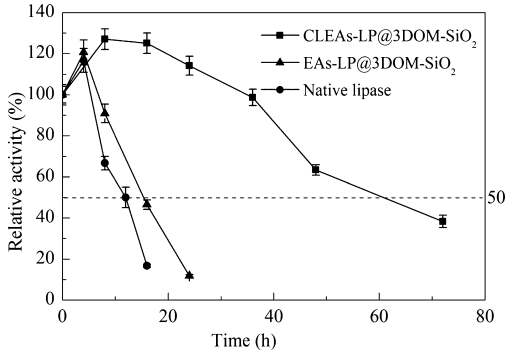

Figure 3. Thermal stability of native and immobilized lipases in isooctane at 70 °C. Reaction conditions: 2 mL of isooctane, 70 °C.

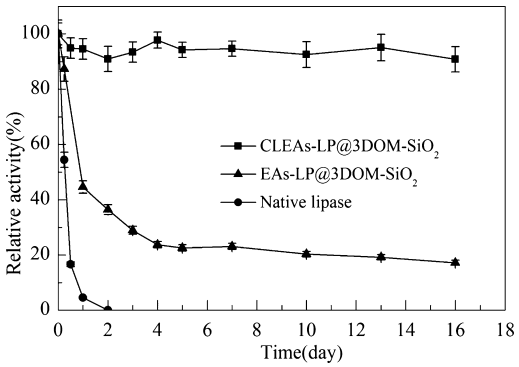

Figure 4. Mechanical stability of native and immobilized lipases in aqueous solution under vigorous shaking. Reaction conditions: 2 mL of phosphate buffer (50 mM, pH 8.0), 200 r/min, room temperature.

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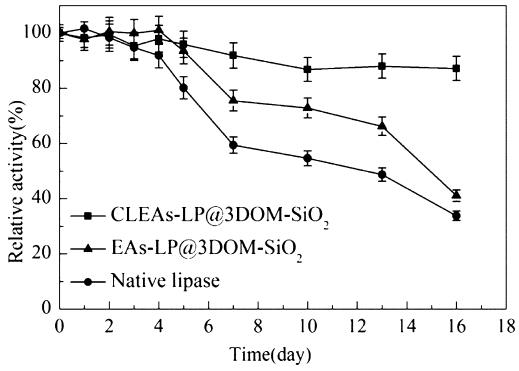

Figure 5. Mechanical stability of native and immobilized lipases in organic solvent under vigorous shaking. Reaction conditions: 2 mL of isooctane, 200 r/min, room temperature.

enzyme from mechanical damage caused by shaking or stirring during industry production.

For comparison with mesoporous materials for lipase immobilization using the ship-in-a-bottle approach, SBA-15 was chosen as a host material. The lipase was first immobilized in SBA-15 by adsorption (this immobilized lipase was named as ADS-LP@SBA-15), then chemical crosslinking was conducted and CLEAs of lipase *Candida* sp. 99-125 in SBA-15 was obtained (this immobilized lipase was named as CLEAs-LP@SBA-15). The apparent K_m values of the immobilized lipases were obtained and summarized in Table S1 (Supporting Information). Lipase immobilized in 3DOM-SiO_2 showed lower K_m values than that of ADS-LP@SBA-15 and CLEAs-LP@SBA-15, which demonstrated that the mass transport through 3DOM materials was quite efficient. Thus, 3DOM materials may be well-suited to be applied in enzyme immobilization by the ship-in-a-bottle approach.

3.4. Catalytic Performance of the Lipases in Hydrolysis, Esterification and Transesterification Reactions. In the hydrolysis of ethyl oleate, the yields of oleic acid were 94%, 90%, and 70% when native lipase, EAs-LP@3DOM-SiO_2, and CLEAs-LP@3DOM-SiO_2 were used as catalysts, respectively (Figure 6). The yield of oleic acid catalyzed by EAs-LP@3DOM-SiO_2 was close to that of native lipase, which can be explained as follows: the lipase in EAs-LP@3DOM-SiO_2 would be redissolved in aqueous solution and the activity would be similar with native lipase.[44] CLEAs-LP@3DOM-SiO_2 exhibited lower catalytic efficiency in this reaction than that of native

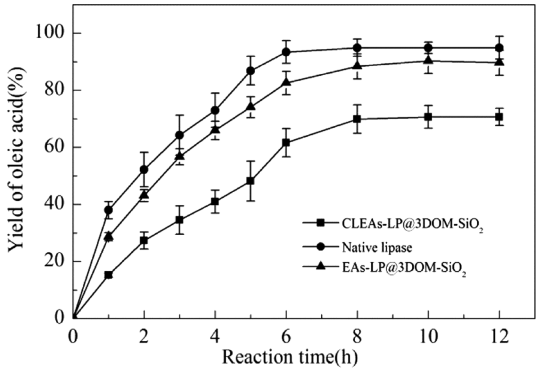

Figure 6. Time course of the hydrolysis of ethyl oleate. Reaction conditions: enzyme activity of 10 U, 4 mL of emulsion, 2 mL of phosphate buffer (50 mM, pH 8.0), 40 °C.

lipase and EAs-LP@3DOM-SiO_2, which could be due to the internal mass-transport limitations. Despite this drawback, lipase's stability has been significantly improved by immobilization, as shown in Figures 3−5. Additionally, CLEAs-LP@3DOM-SiO_2 could recover from the reactants for another cycle, which was very important in the industrial production, whereas free enzyme is unrecoverable due to its homogeneity.

The native lipase, EAs-LP@3DOM-SiO_2 and CLEAs-LP@3DOM-SiO_2 were also used to catalyze the esterification of oleic acid with ethanol. As can be seen in Figure 7a, in the first

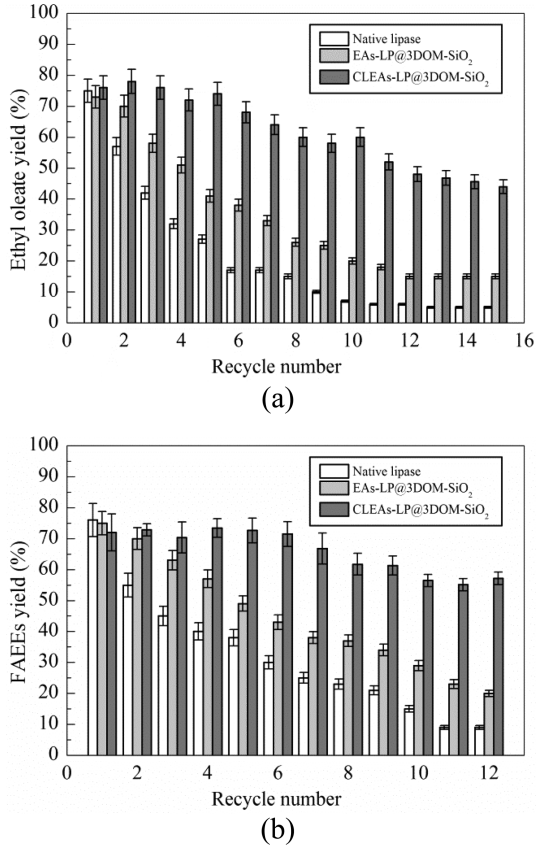

Figure 7. Catalytic performances of the native lipase, EAs-LP@3DOM-SiO_2, and CLEAs-LP@3DOM-SiO_2 in esterification (a) and transesterification (b) reactions. Reaction conditions: enzyme activity of 30 U, 0.21 g of oleic acid or *Jatropha* oil, 0.175 mL of ethanol, 1.5 mL of cyclohexane, 40°C, 24 h.

run, the yields of ethyl oleate that catalyzed by native lipase, EAs-LP@3DOM-SiO_2, and CLEAs-LP@3DOM-SiO_2 were 75%, 73%, and 76%, respectively. The yield of ethyl oleate that catalyzed by CLEAs-LP@3DOM-SiO_2 was maintained about 80% of the initial conversion after using 10 times but decreased to 59% when used for 15 times. However, only 6% and 15% of initial conversion was retained after using 15 times that catalyzed by native lipase and EAs-LP@3DOM-SiO_2. These results indicated that immobilized lipase through CLEAs-LP@3DOM-SiO_2 method was effective in improving the enzyme's catalytic performance and achieving an eventual success in a long-term stabilization of enzyme activity under continuous recycling.

Then the native lipase, EAs-LP@3DOM-SiO_2 and CLEAs-LP@3DOM-SiO_2 were evaluated in the transesterification of *Jatropha* oil with ethanol for the production of FAEEs at 40 °C (Figure 7b). The FAEEs yields that catalyzed by native lipase,

EAs-LP@3DOM-SiO_2, and CLEAs-LP@3DOM-SiO_2 were 76%, 75%, and 72% and declined to 9%, 20%, and 56% after using 12 times. CLEAs-LP@3DOM-SiO_2 exhibited wonderful reusability, which was impossible for native lipase to achieve. These results can be mainly due to: (1) the 3DOM-SiO_2 can protect lipase from the mechanical inactivation caused by shaking; (2) the multipoint covalent cross-linking can inhibit enzyme leakage and denaturation effectively;[45] (3) CLEAs-LP@3DOM-SiO_2 can be recovered easily compared with native lipase, which can reduce the loss of enzyme during recycling process. All of these results suggested that CLEAs-LP@3DOM-SiO_2 can be utilized efficiently as a biocatalyst in the biodiesel industry. As the lipases were recovered by centrifugation, it was expected that some lipases may have been lost during each cycle, and thus the true loss of enzyme activity should be lower than that shown in Figure 7.

4. CONCLUSIONS

In conclusion, 3DOM silica materials were employed as host for lipase *Candida* sp. 99-125 CLEAs. The cross-linking of lipase *Candida* sp. 99-125 precipitated in the pores of 3DOM-SiO_2 resulted in a highly stable and active ship-in-a-bottle enzyme system. Compared with EAs-LP@3DOM-SiO_2 and native lipase, CLEAs-LP@3DOM-SiO_2 exhibited excellent thermal stability and mechanical stability in aqueous and organic phases. When CLEAs-LP@3DOM-SiO_2 was used as a biocatalyst for the esterification and transesterification reactions, improved activity and reusability were achieved. With these desired characteristics, this approach of CLEAs entrapped in 3DOM materials can be employed to the stabilization of any other enzymes, and may have potential applications in various enzyme-based industrial processes. Additionally, although significant enhanced enzyme stability is achieved by this approach, many aspects such as optimizing pore size, decreasing mass transfer resistance, and improving the mechanical stability of 3DOM SiO_2 deserve further studies.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

Photograph and SEM micrograph of 3DOM-SiO_2 particles; stability of native and immobilized lipase in isooctane at room temperature; stability of native and immobilized lipase in aqueous solution at 60 °C; kinetic value of immobilized lipase in 3DOM-SiO_2 and SBA-15. This material is available free of charge via the Internet at http://pubs.acs.org.

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The paper was written through contributions of all authors. All authors have given approval to the final version of the paper.

Notes

The authors declare no competing financial interest.

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