Improved Catalytic Performance of Lipase Supported on Clay/Chitosan Composite Beads

Ni Tu [1], Jianxin Shou [2], Huaping Dong [1,*], Jin Huang [1] and Yimin Li [1,*]

[1] College of Chemistry and Chemical Engineering, Shaoxing University, Huancheng West Road 508, Shaoxing 312000, China; tuni1118@163.com (N.T.); cathyhuang_92@163.com (J.H.)

[2] College of Life Science, Shaoxing University, Huancheng West Road 508, Shaoxing 312000, China; jxshou@126.com

* Correspondence: olive180@163.com (H.D.); liym@usx.edu.cn (Y.L.); Tel.: +86-575-8834-2592 (H.D.); +86-575-8834-2386 (Y.L.); Fax: +86-575-8834-2383 (H.D.)

Abstract: Clay/chitosan composite beads were prepared and used as the carrier to support lipase by adsorption, to improve the activity and stability of lipase in the hydrolysis of olive oil. Under conditions of pH 6.0, 25 °C and adsorption for 10 h, immobilized lipases on chitosan bead (CB–lipase) and three clay/chitosan composite beads, at different clay to chitosan proportions of 1:8 (CCB-8-lipase), 1:5 (CCB-5-lipase) and 1:3 (CCB-3-lipase), were prepared. By comparing the activity of these immobilized lipases, CCB-5-lipase showed the highest activity, followed by CCB-8-lipase > CCB-3-lipase > CB-lipase; this improvement was attributed to the synergetic effect of enrichment of olive oil by clay at the reaction surface and better biocompatibility of chitosan with lipase molecules. The optimum pH and temperature in the reaction respectively changed from 7.0 and 30 °C for free lipase to 7.5 and 35 °C for immobilized forms. Furthermore, the thermal stability and repeated usability of these immobilized lipases were sequenced as CCB-3-lipase > CCB-5-lipase > CCB-8-lipase > CB–lipase, due to greater rigidity of immobilized lipase with the addition of clay, which was further confirmed by SEM. The study shows that the incorporation of clay with chitosan creates a good synergetic effect to improve the catalytic performance of immobilized lipase on clay/chitosan composite.

Keywords: lipase; immobilization; clay/chitosan composite; hydrolysis; catalytic performance

1. Introduction

Lipase is a versatile and important enzyme which can catalyze various reactions, such as hydrolysis, esterification, alcoholysis and transesterification, producing many kinds of important intermediates and products in pharmaceutical and chemical industries, such as chiral alcohol, ester, carboxylic acids, amines, and so on [1–3]. However, lipase used in its free form is very sensitive to heat and pH variation, and it is hard to separate free lipase from the reaction medium to be reused for continuous cycles [4,5]. To overcome these problems, lipase has been immobilized onto various natural and synthetic carriers to maintain its activity and stability [6–9]. Compared with synthetic matrix [10,11], natural materials are more eco-friendly and compatible with lipase molecules, contributing to good catalytic performance of immobilized lipase [12,13].

Chitosan is one of the most abundant natural polysaccharides and has the advantages of non-toxicity, biocompatibility and biodegradation, leading to its low steric hindrance and good affinity to enzymes [14]. Therefore, chitosan has been frequently used as the support for several forms including gel, fiber and membrane for enzyme immobilization [15,16]. For example, Alarcón-Payán et al. prepared chitosan particles to encapsulate peroxidase for pollutant removal; except for its lower

activity relative to free peroxidase, the thermal stability of peroxidase was enhanced [17]. Gilani et al. supported lipase on mesoporous chitosan beads to improve its thermal and storage stabilities in catalytic reactions [18]. However, chitosan matrix is not rigid enough and can easily be dissociated by vigorous stirring during continuous use, hampering the application of immobilized enzymes on chitosan carriers in industrial manufacture [14]. To improve the strength and stability of chitosan support, some inorganic mechanical materials with high strengths have been mixed with chitosan to prepare composite materials, achieving obvious progresses in the stability and reusability of immobilized enzymes, compared with those on chitosan support [19–22]. Multiwalled carbon nanotube was mixed with chitosan to prepare an ideal composite carrier for β-glucosidase immobilization, improving its pH and storage stability and allowing its continuous use [20]. Chang et al. used chitosan–clay composite materials to immobilize α-amylase, β-glucosidase, and glucoamylase; these immobilized enzymes showed higher activities than free enzymes over broader pH and temperature ranges, and they still kept high residual activity after repeated use for many times [21]. In addition, clay is also very rich in nature, and it has a high adsorption capacity, to enrich substrate compounds at the reaction interface [9,23]. Therefore, clay is an ideal candidate to prepare a composite carrier with chitosan for enzyme immobilization [14,21]. However, the use of clay/chitosan composite supports for lipase immobilization, and an investigation of how the content of clay affects the catalytic performance of immobilized lipase, have never been studied.

In this study, Na–bentonite, which is a typical clay was mixed with chitosan to prepare a clay/chitosan composite bead for lipase immobilization, in order to improve the activity and stability of lipase in hydrolysis of olive oil. The structure and morphology of the prepared beads were investigated by X-ray powder diffraction (XRD) and scanning electron microscopy (SEM). The conditions for the preparation of immobilized lipase and catalyzing hydrolysis of olive oil were optimized. The effect of clay content in the composite carrier on the activity and stability of immobilized lipase was also elucidated. In addition, the thermal and operational stability of immobilized lipases were also studied.

2. Results and Discussion

2.1. Characterization of Clay/Chitosan Composite Beads and Chitosan Bead

Figure 1 showed the XRD patterns of pristine clay and chitosan, as well as chitosan bead (CB), three clay/chitosan beads at clay to chitosan proportion of 1:8 (CCB-8), 1:5 (CCB-5) and 1:3 (CCB-3); the pristine chitosan and CB had the same diffraction peaks at $2\theta = 10.5°$ and $19.8°$ (Figure 1a) which were assigned to the crystal structure of chitosan, showing that the formation of gel beads did not change the crystal structure of chitosan; while the former peak ($2\theta = 10.5°$) disappeared on the curves of three composite beads (Figure 1b), and one new peak appeared at $2\theta = 5.5°$, which corresponded to an extended basal spacing of 1.61 nm for these composite materials, compared with that (1.25 nm) of pristine clay (Figure 1a). The results confirmed the following mutual interaction between chitosan and clay in the formation of composite beads: some chitosan molecules intercalated into the interlayer space of clay, and clay also successfully broke and replaced some crystal positions of chitosan, leading to the unique properties of composite beads. The increased intensity of the peak at $2\theta = 5.5°$ on the curves of CCB-8, CCB-5 and CCB-3 resulted from the orderly increased content of clay in the three composite beads. The morphology of CB and CCB were observed in Figure 2 by SEM; the sphere shape of CB was shrunk and depressed (Figure 2a) due to the loss of water after drying. In contrast, the spherical shapes of CCB-8, CCB-5 and CCB-3 were all complete and not changed; as a typical picture of CCB-5 shown in Figure 2b. Figure 2c showed that the surface of CB was unstable and partly broken under rather high voltages of SEM (10 kV), while the surface of CCB-5 was very smooth and complete (Figure 2d). This improvement in the rigidity of the bead, which was composed of clay/chitosan compound materials, helped to enhance the stability of immobilized lipase. Figure 2e,f showed the

surfaces of immobilized lipases on CB (CB-lipase) and CCB-5 (CCB-5-lipase), confirming some of lipases were immobilized onto the micro-porous surfaces of CB and CCB-5 as aggregates.
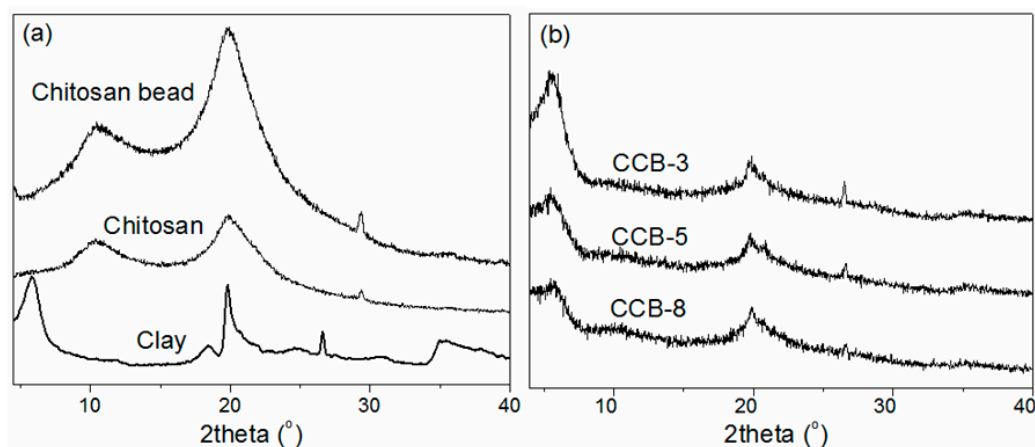

Figure 1. XRD patterns of various materials, chitosan, clay and chitosan bead (**a**); clay/chitosan composite materials (**b**).
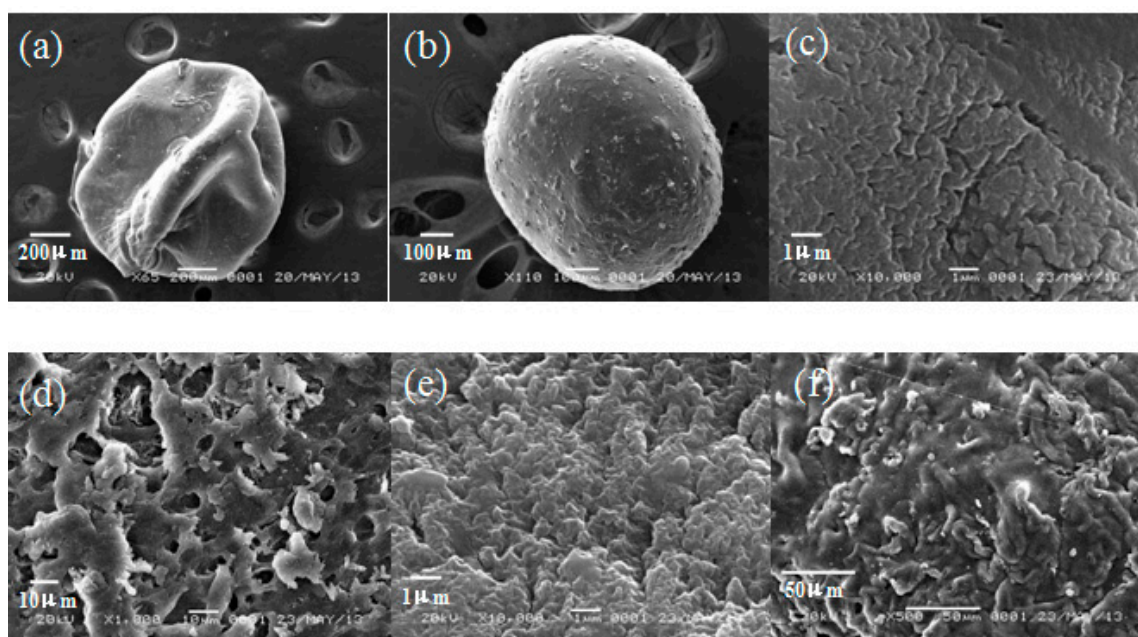

Figure 2. SEM of chitosan bead, clay/chitosan composite beads and immobilized lipases, spherical shapes of CB (**a**) and CCB-5 (**b**) surfaces of CB (**c**); CCB-5 (**d**); CB-lipase (**e**) and CCB-5-lipase (**f**).

2.2. Lipase Immobilization and Enzyme Leakage from Supports

2.2.1. Effect of pH and Adsorption Time on Lipase Immobilization

The effect of pH on the immobilization of lipase on chitosan beads and clay/chitosan composite beads is shown in Figure 3a. As pH increased from 5.0 to 8.0, the immobilization yields of lipases on these beads all increased firstly and peaked at pH 6.0; then they all dropped obviously, as pH increased over 6.0, indicating that pH 6.0 was the best for lipase immobilization on each kind of bead. To elucidate the reason for this trend, the zeta potentials of lipase and these various supports at pH 6.0 were determined. Lipase had a charge of −11.6 mV, and the supports of CB, CCB-8, CCB-5 and CCB-3 had charges of −0.58 mV, −1.69 mV, −2.75 mV and −4.86 mV, respectively. Thus, lipase

molecules disperse well in solution at pH 6.0 due to electrostatic repulsion between them; this reduces the nonspecific adsorption between lipase molecules and promotes the attachment of lipase molecules onto the supports. Otherwise, these far less negatively charged beads hardly showed any electrostatic repulsion to lipase molecules and did not prevent the lipase molecules from adsorption onto the supports. The lipase molecules adsorbed onto the supports through Van der Walls forces, electrostatic attraction from cationic amino groups of chitosan, and hydrogen bonds between lipase molecules and supports [14,15,18]. As the pH reduced to 5.0, the lipase molecules tended to aggregate by nonspecific adsorption, due to the weak electrostatic repulsion and stronger hydrogen bonds between them; these aggregated lipases were easily washed off during immobilization. In addition, the CB and composite beads were a little unstable and tended to dissociate in a weak acid solution during immobilization. Thus, the immobilization yields of lipases at pH 5.0 were lower than that at pH 6.0. When the pH increased to be above 6.0, lipase and these beads were charged more negatively; this would increase the electrostatic repulsion between them, so the immobilization yields also decreased obviously. Therefore, pH 6.0 was the best for lipase immobilization on these beads. With the increase of clay content, the supports were charged more negatively and prevented more lipase molecules from adsorption onto their surface, so the immobilization yields decreased accordingly (Figure 3a). In addition, the porous surface of CB (Figure 2c) could also help to adsorb more lipase protein, compared with the compact surface of clay/chitosan composite bead (Figure 2d).

The immobilization yields of lipase on these beads vs. their adsorption times were also investigated. As Figure 3b shows, the amounts of lipase immobilized on these beads increased with time until 10 h; then these immobilization yields all dropped a little, indicating the adsorption of lipase on these beads had reached equilibrium after 10 h. Thus, the best immobilization time for lipase was 10 h for each immobilized lipase.
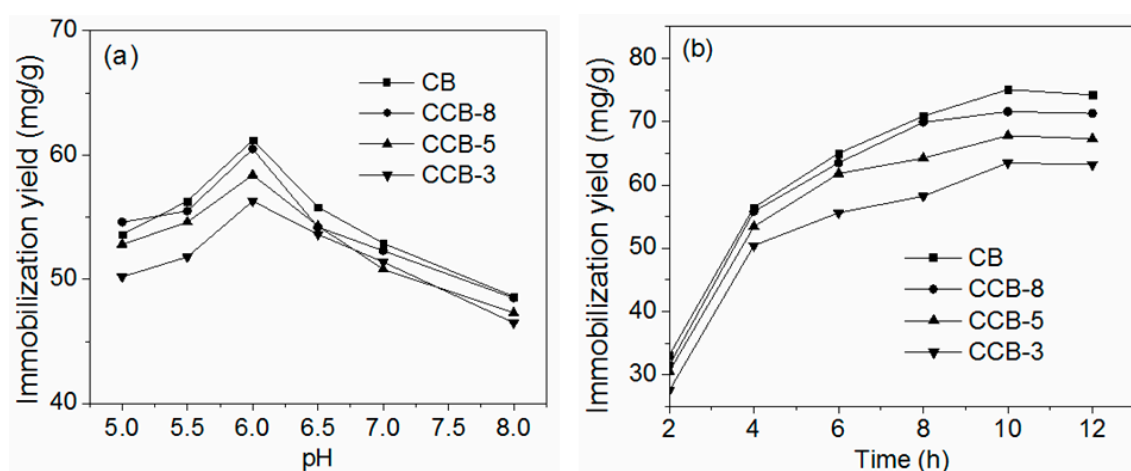

Figure 3. Immobilization yield of lipase on chitosan bead and clay/chitosan composite beads vs. pH (**a**) and contact time (**b**). Initial enzyme concentration: 10 mg/mL, agitation speed: 180 rpm, temperature: 25 °C.

2.2.2. Enzyme Leakage from Supports

The immobilized enzymes leaking from corresponding supports after 24 h were monitored, as shown in Table 1. The maximum amount of enzyme leaked from CB (23.5%), and the enzyme leakage decreased as the clay content in the support increased; the lowest leakage was observed with CCB-3 (14.3%). This result showed that the addition of clay in the composite beads improved the adsorption capacity of the support for enzyme molecules, helping to enhance the rigidity and stability of immobilized lipases.

Table 1. Enzyme leakage from various supports.

Support	CB	CCB-8	CCB-5	CCB-3
Enzyme leakage (%)	23.5	19.1	16.5	14.3

2.3. Activities of Immobilized Lipases in Hydrolysis of Olive Oil under Different pHs and Temperatures

The activities of the immobilized lipases during the hydrolysis of olive oil under different pHs and temperatures were investigated and are shown in Figure 4a,b. The immobilized lipases, including CCB-8-lipase, CCB-5-lipase, CCB-3-lipase and CB-lipase, exhibited their highest activity at pH 7.5, which was higher than the optimum pH (7.0) for free lipase. This change of optimum pH for lipase after immobilization probably resulted from the carrier effects of chitosan and clay/chitosan on lipase's active structure. Except for the lower activity of CB–lipase than that of free lipase, the other three immobilized lipases on clay/chitosan composite beads including CCB-8-lipase, CCB-5-lipase and CCB-3-lipase showed higher activities than free lipase, and the highest activity was observed with CCB-5-lipase. The enrichment of olive oil onto these beads under reaction conditions was compared by carbon content analysis; the results showed that the relative adsorption yield of olive oil on CCB-5 was the highest (100%), followed by CCB-8 (93.6%) > CCB-3 (84.5%) > CB (63.7%). This confirmed that the addition of clay could enrich more substrates at reaction interfaces to improve the activity of immobilized lipases. Furthermore, the enriched olive oil on the beads was able to provide a suitable hydrophobic/hydrophilic interface to open a lid on the active center of lipase, to activate lipase for catalysis, and greater amounts of hydrophobic olive oil adsorbed on the beads activated lipase better [4,9]. Thus, the activities of immobilized lipases were listed as CCB-5-lipase > CCB-8-lipase > CCB-3-lipase > CB-lipase.

In addition, previous studies have always used chitosan beads and membranes as ideal supports to make the immobilized enzymes more active because of the better biocompatibilities of these carriers to the enzyme molecules [12,14,18,21,24]. Therefore, the improved activity of immobilized lipases on clay/chitosan beads was due to the synergetic effects of the enrichment of olive oil by clay at reaction surfaces and the better biocompatibility of chitosan to lipase molecules, and the timely consumption of fatty acid product by amino groups of chitosan. An excessive content of clay decreased the biocompatibility of the carrier to lipase, so CCB-3-lipase showed lower activity than CCB-5-lipase. Likewise, as shown in Figure 4b, the optimum temperature for immobilized lipase (35 °C) during the hydrolysis of olive oil was also higher than that for free lipase (30 °C). Furthermore, the immobilized lipases showed higher activities than free lipases at higher reaction temperatures ($\geq$35 °C), and such advantages in the activities of CCB-8-lipase, CCB-5-lipase, CCB-3-lipase over that of free lipase were expanded with the temperature increase, showing that the resistance of lipase towards heating was much improved after immobilization on clay/chitosan composite beads. In contrast, CCB-5-lipase showed the highest activity at the optimum temperature, and CCB-3-lipase showed the strongest resistance to heating inactivation among these immobilized lipases, which was contributed to by the increased content of rigid clay in clay/chitosan composite bead.
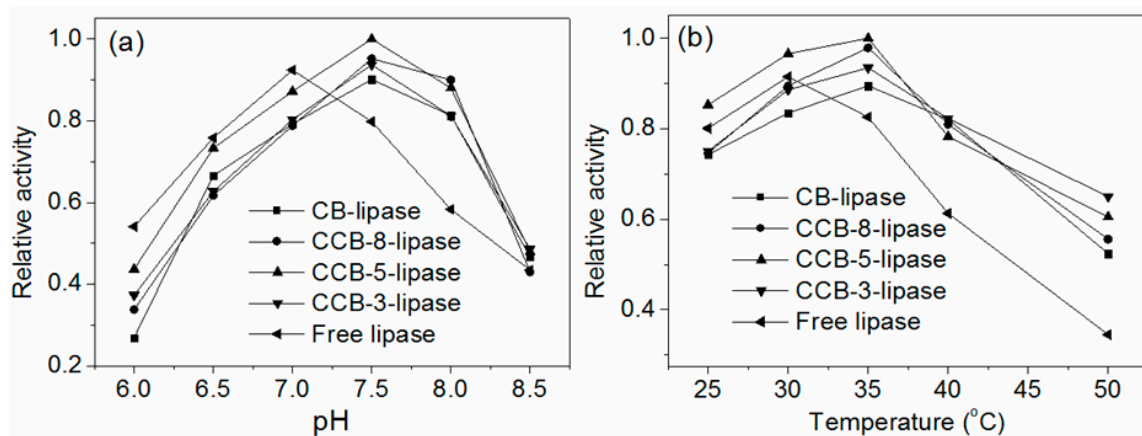
Figure 4. Effect of pH (**a**) and temperature (**b**) on the activity of immobilized lipase. Substrate concentration: 0.18 g/mL, 0.2 g immobilized beads.

2.4. Catalytic Kinetics of Free and Immobilized Lipase

The kinetic parameters of free and immobilized lipases in olive oil hydrolysis, determined by Lineweaver–Burk plots (Figure 5), are shown in Table 2. The Michaelis–Menten constant (K_m) value of free lipase (0.040 g/mL) was lower than those of CB–lipase (0.060 g/mL) and CCB-5-lipase (0.052 g/mL), due to the diffusion resistance of carriers for olive oil during the reaction. The CCB-5-lipase had a lower Michaelis–Menten constant (K_m) than CB–lipase, showing that the affinity of immobilized lipase towards olive oil was improved by addition of clay, due to the higher enrichment of olive oil by clay. The higher maximum reaction rate (V_{max}) of CCB-5-lipase than those of free lipase and CB–lipase also confirmed the improved activity of lipase by immobilization on composite beads.
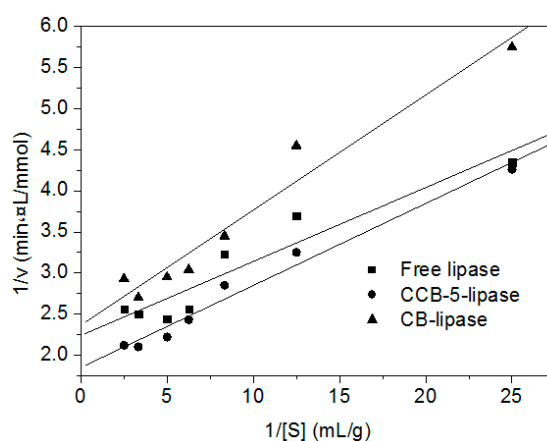

Figure 5. Lineweaver–Burk plots for free and immobilized lipases.

Table 2. Kinetic parameters of free and immobilized lipases.

Biocatalyst	Michaelis–Menten Constant (K_m) (g/mL)	Maximum Reaction Rate (V_{max}) (mmol/min·L)	r *
Free lipase	0.040	0.447	0.950
CCB-5-lipase	0.052	0.541	0.988
CB-lipase	0.060	0.423	0.978

* Correlation coefficient.

2.5. Thermal Stability of Immobilized Lipases

Generally, an improvement in thermal stability can expand the practical fields of enzymes. Figure 6a,b show the residual activities of free and immobilized lipases after heating at 30 °C and 60 °C for certain periods of time. Free lipase lost 75% of its initial activity after heating at 30 °C for 6 h; in contrast, CB–lipase, CCB-8-lipase, CCB-5-lipase and CCB-3-lipase kept 30%, 34.5%, 40% and 45%, respectively, of their initial activities, after heating at 30 °C for 10 h. Through heating treatment of these lipases at 60 °C, free lipase quickly lost activity after 1 h; CB–lipase and CCB-8-lipase, CCB-5-lipase and CCB-3-lipase prolonged their inactivation times from 1 h to 2 h, 3 h, 4 h and 4 h, respectively, and the residual activity of immobilized lipase was improved as the clay content increased, within the period of heating treatment. The results indicated that the thermal stability of lipase was notably enhanced by immobilization on clay/chitosan composite beads, and the enhancing range was proportional to the content of clay in composite beads.
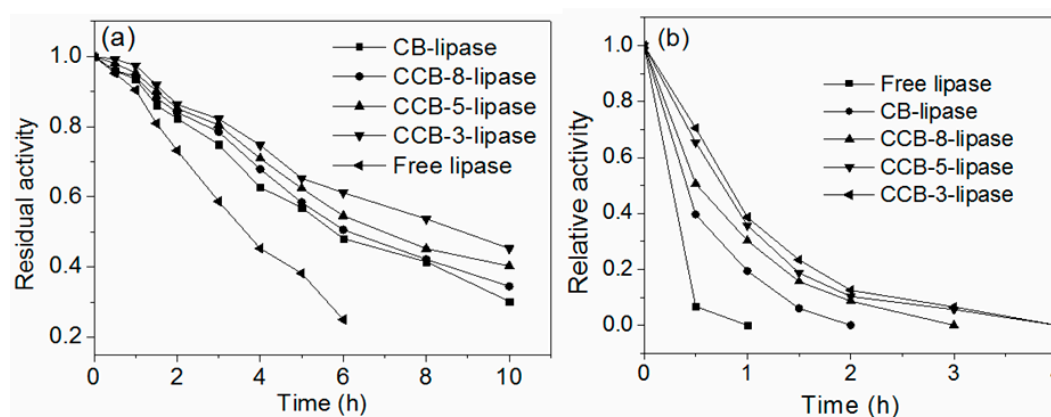

Figure 6. Thermal stability of free and immobilized lipases after heating treatment at 30 °C (**a**) and 60 °C (**b**). Substrate concentration: 0.18 g/mL, 0.2 g immobilized beads, pH 7.0 for free lipase and pH 7.5 for immobilized lipase.

2.6. Reusability of Immobilized Lipases

Reusability is generally one of the important factors in determining the practical values of enzymes. The reusability of immobilized lipase on chitosan bead and clay/chitosan composite beads during the hydrolysis of olive oil, under each optimum condition, were investigated and shown in Figure 7. The CB–lipase kept 30% of its initial activity after five continuous runs, CCB-8-lipase improved its residual activity from 30 to 45% after the same continuous runs, and CCB-5-lipase and CCB-3-lipase could keep about 40% and 50% of their initial activity, respectively, after seven continuous cycles. The reusability of immobilized lipase on clay/chitosan composite beads was obviously better than that of CB–lipase, and the increase in the clay content of composite bead was able to improve the reusability of immobilized lipase, due to the improved rigidity of clay/chitosan composite beads. As shown in Figure 8, the hollow cross section of CB–lipase was observed after the second run of reaction (Figure 8a), and such erosion was more serious after the fifth run of reaction (Figure 8b); in contrast, the cross section of CCB-3-lipase was still complete and smooth even after the fifth run of reaction (Figure 8d), further confirming that the stability of immobilized beads was much enhanced by the addition of clay.
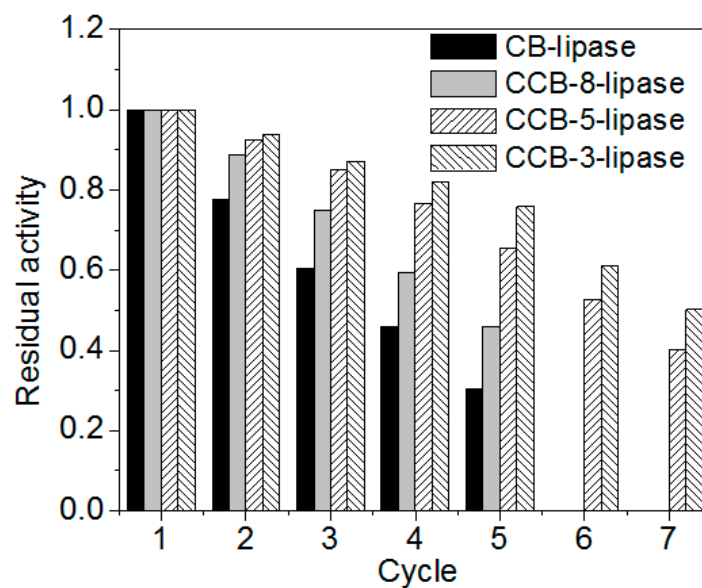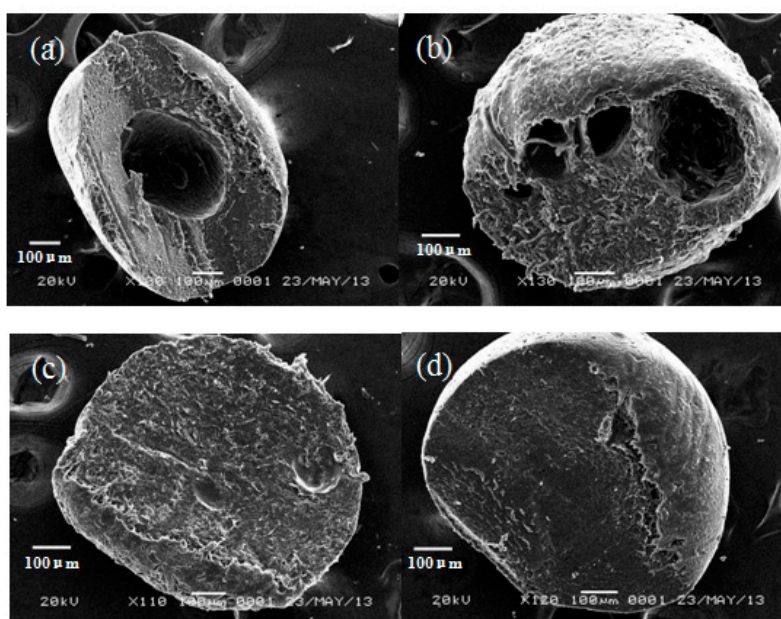
Figure 7. Reusability of immobilized lipases.

Figure 8. The cross sections of immobilized beads after the second (**a**,**c**) and fifth runs (**b**,**d**) of reactions, CB–lipase (**a**,**b**); CCB-3-lipase (**c**,**d**).

3. Materials and Methods

3.1. Materials

Clay (Na–bentonite) was purchased from Zhejiang Sanding Technology Company (Shaoxing, China), chitosan (95% of deacetylation) was purchased from Shanghai Aladdin Chemicals (Shanghai, China). *Yarrawia lipolytica* lipase, bovine serum albumin, olive oil, and polyvinyl alcohol were purchased from Sigma-Aldrich (St. Louis, MO, USA). All other chemicals were of an analytical grade.

3.2. Preparation of Clay/Chitosan Composite Beads

1 g chitosan was dissolved in 50 mL of acetic acid buffer (5%, v/v) at a concentration of 2% by stirring. Then, clay was added into the viscous chitosan solution with different mass proportions of clay to chitosan (1:8, 1:5 and 1:3) to prepare three different clay/chitosan suspensions. These clay/chitosan mixtures as well as chitosan solution were respectively dropped into 1 mol/L of NaOH solution to obtain various clay/chitosan composite beads and chitosan bead by gentle agitation. Each kind of bead was left in solution for 1 h; then the bead was washed with deionized water until the solution was neutral. After that, each kind of bead was orderly immersed with 20% ethanol, 50% ethanol and ethanol solution for 15 min, and the residual ethanol in the beads was evaporated after isolation of beads from the ethanol solution. Finally, these beads were dried at 40 °C and ready for use. These clay/chitosan composite beads were respectively named as CCB-8, CCB-5 and CCB-3, according to their corresponding proportions of clay to chitosan, and the chitosan bead was named CB.

3.3. Immobilization of Lipase on Chitosan Bead and Clay/Chitosan Composite Beads

A quantity of 0.2 g of each prepared bead was added into 10 mL lipase solution (10 mg/mL) within the pH range of 5.0 to 7.5, and the mixture was agitated at 180 rpm in a shaking bath at 25 °C. To avoid nonspecific adsorption of enzymes onto the flask wall, the stock enzyme solution was newly prepared and its initial concentration determined by the Bradford method, using bovine serum albumin as the standard before every immobilization [25]. At the end of immobilization, the supernatant solution was removed, the flask wall was rinsed twice with buffer solution, and the immobilized beads were washed with deionized water three times. The mixture of supernatant and washing solution was collected, to determine the protein un-immobilized onto the beads by the Bradford method [25]. The amount of lipase protein immobilized onto the beads was determined by comparing the difference between the total amount of protein and the amount of protein in the mixture of supernatant and washing solution, as done in previous studies [5,8,13,18]. In addition, the immobilization yield of lipase on each kind of bead vs. time was also investigated at each optimum pH. After optimization, these prepared immobilized beads were dried under vacuum at room temperature, and they were respectively named CCB-8-lipase, CCB-5-lipase, CCB-3-lipase and CB–lipase.

3.4. Enzyme Leaking Test

The immobilized enzyme proteins leaking from various supports into aqueous solution were determined with the following method: 0.2 g of various immobilized lipases (CCB-8-lipase, CCB-5-lipase, CCB-3-lipase and CB–lipase) were respectively added into a 10 mL phosphate buffer solution (pH 7.0), and each suspension was incubated at 25 °C. After 24 h, the leaked enzyme protein in the aqueous solution was determined by the Bradford method [25], and the leaking rate of enzymes at each support was determined as the ratio of leaked protein to the total protein immobilized on the support.

3.5. Characterizations

The beads were powdered and XRD patterns of these powders were performed on a Panalytical diffractometer (EMPYREAN, PANalytical, Almelo, The Netherlands) with CuKα radiation (40 kV, 40 mA), over a 2θ range from 2.5° to 40°. Scanning electron microscopy (SEM, JSM-6360LV, JEOL, Tokyo, Japan) was used to observe the surface appearance of these beads at a 10 kV accelerating voltage.

3.6. Activity Assay of Immobilized Lipases

Olive oil was mixed with 4% of polyvinyl alcohol (1:3, v/v) to prepare the olive oil emulsion; then a phosphate buffer at pH 7.5 was added into the emulsion at a ratio of 1:5 (v/v) to obtain the substrate solution. A quantity of 0.2 g of immobilized bead was added into 5 mL of prepared substrate solution to initiate the reaction at 30 °C. After 30 min, the reaction was ended by adding 10 mL of

95% ethanol solution, and the released fatty acid was measured with 0.02 mol/L NaOH titration. The blank hydrolysis of olive oil was conducted through the same method, except for the addition of ethanol solution at the beginning of the reaction. The produced fatty acid was determined by the difference between the blank and the acid equation of the titration. One unit of the activity of lipase was defined as one μmol of fatty acid produced by catalysis of per gram of protein in one min under the assay conditions.

3.7. Determination of Kinetic Parameters

According to the following Michaelis–Menten equation (Equation (1)), maximum reaction rate (V_{max}) and Michaelis–Menten constant (K_m) of free and immobilized lipases (CCB-5-lipase and CB-lipase) were determined by measuring the initial rates of olive oil hydrolysis at various concentrations within the range of (0.04–0.40 g/mL).

$$v = \frac{V_{max}[S]}{K_m + [S]} \tag{1}$$

where [S] is the concentration of olive oil (g/mL) and v is the initial reaction rate of olive oil hydrolysis by lipase (mmol/min·L).

3.8. Thermal Stability of Free and Immobilized Lipases

To determine the thermal stability, free and immobilized lipases were respectively incubated in the phosphate buffer solution at their corresponding optimum pHs, at 30 and 60 °C, for a certain period, and aliquots were taken periodically for enzymatic activity measurement under their optimum condition. The initial activity without heating treatment was defined as 100%.

3.9. Reusability of Immobilized Lipases

The immobilized lipases were respectively added into the substrate solution for the first run under the optimum condition; at the end of the reaction, the reaction mixture was filtrated to remove the supernatant and the beads were washed with buffer solutions. Then, the obtained beads were also added into a fresh substrate solution for next run. They were reused for several times following this method, and the residual activity of immobilized lipase was determined after each run. The initial activity of lipase in the first run was defined to be 100%.

4. Conclusions

In this study, lipase was immobilized on chitosan bead and clay/chitosan composite beads in order to improve the catalytic performance of lipase in the hydrolysis of olive oil. The immobilized lipases on the composite beads including CCB-8-lipase, CCB-5-lipase and CCB-3-lipase showed higher activity than free lipase and CB–lipase, and the highest activity was observed on CCB-5-lipase. This improvement was mainly due to the synergetic effect of clay adsorption and biocompatibility of chitosan on lipase catalysis. Furthermore, CCB-8-lipase, CCB-5-lipase and CCB-3-lipase exhibited better thermal stability and reusability than CB–lipase, and the enhancements were proportional to the content of clay in composite beads, showing that the addition of clay into the support can improve the catalytic stability of immobilized lipase, contributing to the expansion of the practical range of immobilized lipase. Therefore, good cooperation between clay and chitosan makes clay/chitosan composite bead an ideal support for enzymes in practical applications.

References

1. Sharma, R.; Chisti, Y.; Banerjee, U.C. Production, purification, characterization, and applications of lipases. *Biotechnol. Adv.* **2001**, *19*, 627–662. [CrossRef]

2. Acosta, A.; Filice, M.; Fernandez-lorente, G.; Palomo, J.M.; Guisan, J.M. Kinetically controlled synthesis of monoglyceryl esters from chiral and prochiral acids methyl esters catalyzed by immobilized *Rhizomucor miehei* lipase. *Bioresour. Technol.* **2011**, *102*, 507–512. [CrossRef] [PubMed]

3. Ziegler-Borowska, M.; Chelminiak-Dudkiewicz, D.; Siódmiak, T.; Sikora, A.; Wegrzynowska-Drzymalska, K.; Skopinska-Wisniewska, J.; Kaczmarek, H.; Marszałł, M.P. Chitosan-collagen coated magnetic nanoparticles for lipase immobilization-new type of "enzyme friendly" polymer shell crosslinking with squaric acid. *Catalysts* **2017**, *7*, 26. [CrossRef]

4. Sheldon, R.A.; van Pelt, S. Enzyme immobilisation in biocatalysis: Why, what and how. *Chem. Soc. Rev.* **2013**, *42*, 6223–6235. [CrossRef] [PubMed]

5. Dong, H.; Li, Y.; Li, J.; Sheng, G.; Chen, H. Comparative study on lipases immobilized onto bentonite and modified bentonites and their catalytic properties. *Ind. Eng. Chem. Res.* **2013**, *52*, 9030–9037. [CrossRef]

6. Zhao, D.; Peng, C.; Zhou, J. Lipase adsorption on different nanomaterials: A multi-scale simulation study. *Phys. Chem. Chem. Phys.* **2015**, *17*, 840–850. [CrossRef] [PubMed]

7. Kobayashi, J.; Mori, Y.; Kobayashi, S. Novel immobilization method of enzymes using a hydrophilic polymer support. *Chem. Commun.* **2006**, 4227–4229. [CrossRef] [PubMed]

8. Xu, L.; Ke, C.; Huang, Y.; Yan, Y. Immobilized *Aspergillus niger* lipase with SiO_2 nanoparticles in sol-gel materials. *Catalysts* **2016**, *6*, 149. [CrossRef]

9. Dong, H.; Li, J.; Li, Y.; Hu, L.; Luo, D. Improvement of catalytic activity and stability of lipase by immobilization on organobentonite. *Chem. Eng. J.* **2012**, *181–182*, 590–596. [CrossRef]

10. Kang, Y.; He, J.; Guo, X.D.; Guo, X.; Song, Z.H. Influence of pore diameters on the immobilization of lipase in SBA-15. *Ind. Eng. Chem. Res.* **2007**, *46*, 4474–4479. [CrossRef]

11. Zdarta, J.; Klapiszewski, L.; Jedrzak, A.; Nowicki, M.; Moszynski, D.; Jesionowski, T. Lipase B from *Candida antarctica* immobilized on a silica-lignin matrix as a stable and reusable biocatalytic system. *Catalysts* **2017**, *7*, 14. [CrossRef]

12. Kim, H.J.; Jin, J.N.; Kan, E.; Kim, K.J.; Lee, S.H. Bacterial cellulose-chitosan composite hydrogel beads for enzyme immobilization. *Biotechnol. Bioprocess Eng.* **2017**, *22*, 89–94. [CrossRef]

13. Cabana, H.; Ahamed, A.; Leduc, R. Conjugation of laccase from the white rot fungus *Trametes versicolor* to chitosan and its utilization for the elimination of triclosan. *Bioresour. Technol.* **2011**, *102*, 1656–1662. [CrossRef] [PubMed]

14. Chang, M.-Y.; Juang, R.S. Use of chitosan-clay composite as immobilization support for improved activity and stability of β-glucosidase. *Biochem. Eng. J.* **2007**, *35*, 93–98. [CrossRef]

15. De Mello, M.D.; Cordeiro, D.; Costa, L.T.; Follmer, C. Catalytic properties of lipases immobilized onto ultrasound-treated chitosan supports. *Biotechnol. Bioprocess Eng.* **2013**, *18*, 1090–1100. [CrossRef]

16. Stefanescu, C.; Daly, H.W.; Negulescu, I.I. Biocomposite films prepared from ionic liquid solutions of chitosan and cellulose. *Carbohydr. Polym.* **2012**, *87*, 435–443. [CrossRef]

17. Alarcón-Payán, D.A.; Koyani, R.D.; Vazquez-Duhalt, R. Chitosan-based biocatalytic nanoparticles for pollutant removal from wastewater. *Enzyme Microb. Technol.* **2017**, *100*, 71–78. [CrossRef] [PubMed]

18. Gilani, S.L.; Najafpour, G.D.; Moghadamnia, A.; Kamaruddin, A.H. Stability of immobilized porcine pancreas lipase on mesoporous chitosan beads: A comparative study. *J. Mol. Catal. B Enzym.* **2016**, *133*, 144–153. [CrossRef]

19. Metin, A.Ü.; Alver, E. Fibrous polymer-grafted chitosan/clay composite beads as a carrier for immobilization of papain and its usability for mercury elimination. *Bioprocess Biosyst. Eng.* **2016**, *39*, 1137–1149. [CrossRef] [PubMed]

20. Çeklik, A.; Dinçer, A.; Aydemir, T. Characterization of β-glucosidase immobilized on chitosan-multiwalled carbon nanotubes (MWCNTS) and their application on tea extracts for aroma enhancement. *Int. J. Biol. Macromol.* **2016**, *89*, 406–414. [CrossRef] [PubMed]

21. Chang, M.Y.; Juang, R.S. Activities, stabilities, and reaction kinetics of three free and chitosan-clay composite immobilized enzymes. *Enzyme Microb. Technol.* **2005**, *36*, 75–82. [CrossRef]

22. Dinçer, A.; Becerik, S.; Aydemir, T. Immobilization of tyrosinase on chitosan-clay composite beads. *Int. J. Biol. Macromol.* **2012**, *50*, 815–820. [CrossRef] [PubMed]

23. Li, J.F.; Li, Y.M.; Meng, Q.L. Removal of nitrate by zero-valent iron and pillared bentonite. *J. Hazard. Mater.* **2010**, *174*, 188–193. [CrossRef] [PubMed]

24. Zhang, L.H.; Bai, C.H.; Wang, Y.S.; Jiang, Y.C.; Hu, M.C.; Li, S.N.; Zhai, Q.G. Improvement of chloroperoxidase stability by covalent immobilization on chitosan membranes. *Biotechnol. Lett.* **2009**, *31*, 1269–1272. [CrossRef] [PubMed]

25. Bradford, M.M. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* **1976**, *72*, 248–254. [CrossRef]