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Silanized palygorskite for lipase immobilization

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ABSTRACT

Lipase from *Candida lipolytica* has been immobilized on 3-aminopropyltriethoxysilane-modified palygorskite support. Scanning electron micrographs proved the covalently immobilization of *C. lipolytica* lipase on the palygorskite support through glutaraldehyde. Using an optimized immobilization protocol, a high activity of 3300 U/g immobilized lipase was obtained. Immobilized lipase retained activity over wider ranges of temperature and pH than those of the free enzyme. The optimum pH of the immobilized lipase was at pH 7.0–8.0, while the optimum pH of free lipase was at 7.0. The retained activity of the immobilized enzyme was improved both at lower and higher pH in comparison to the free enzyme. The immobilized enzyme retained more than 70% activity at 40 °C, while the free enzyme retained only 30% activity. The immobilization stabilized the enzyme with 81% retention of activity after 10 weeks at 30 °C whereas most of the free enzyme was inactive after a week. The immobilized enzyme retains high activity after eight cycles. The kinetic constants of the immobilized and free lipase were also determined. The K_m and V_{max} values of immobilized lipase were 0.0117 mg/ml and 4.51 μmol/(mg min), respectively.

1. Introduction

Attention has been paid to enzyme immobilization due to the limited recovery yield and the reusability of free enzymes as industrial catalysts. Enzyme immobilization facilitates the purification of reaction system and the recovery of enzyme and makes it possible to use the enzyme repetitively or continuously [1]. Immobilization is also accompanied by some changes in the enzymatic activity, affinity towards substrate, stability of the enzyme, etc. And the changes depend on the combination of enzyme and carrier and the immobilization conditions [2–6].

Clay minerals can be used as effective carriers for enzyme immobilization [7,8]. Palygorskite is a special class of clay mineral under the 2:1 layer composition with commonly a lath or fibrous morphology. Palygorskites are hydrated magnesium silicates. In such materials octahedral layers of magnesium with partial substitution with aluminium and/or iron is sandwiched between $(SiO)_4$ tetrahedral layer and $Al(OH)_3$ octahedral unit. The tetrahedral sheet is continuous across ribbons at the apical oxygen alternately pointing up and down in adjacent ribbons. The octahedral sheet is discontinuous with a variable charge imbalance [9]. The employ of palygorskite as lipase carriers presents numerous advantages, such as the high specific surface, the facility of water dispersion/recuperation, and the excellent mechanical resistance of these materials. Last, but not least, its natural origin and the low cost make it even more attractive from an applied point of view.

Functionalization of carriers is one of the frequently used methods to increase the immobilization of protein [10–13]. Differently modified palygorskites were used for lipase immobilization in our previous work. And the results showed the silanized palygorskite was a better carrier for lipase immobilization. The physico-chemical characterization for silanized palygorskite has been previously reported [14]. Owing to the presence of the $-NH_2$ functional groups in the silanized palygorskite, the enzyme can be immobilized on the support with glutaraldehyde through the amine–aldehyde Schiff linkage (Fig. 1). The glutaraldehyde covalent linkage technique is employed, as the binding is strong and probability of enzyme leakage is low. The present work is to characterize the immobilization process, enzyme loadings, and enzymatic properties of the immobilized *Candida lipolytica* lipase on silanized palygorskites. *C. lipolytica* lipase is a nonspecific lipase and can simultaneously or randomly catalyze the hydrolysis of 1,2,3-ester bonds. Due to the high lipase activity produced by *C. lipolytica*, it presents many potential applications for the food industry, pharmacology, and for the environment. For example, it has been widely used to produce unsaturated fatty acids and glycerides in oil industry [15].

2. Materials and methods

2.1. Materials

The palygorskite with the size between 10 and 25 μm used in this work was supplied by Oilbetter Co. (China). Lipase from

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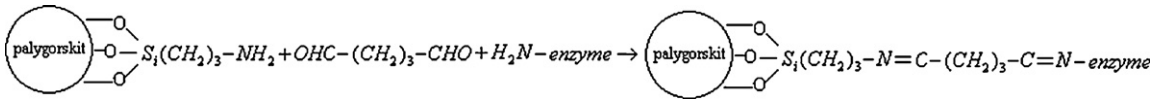

Fig. 1. Lipase covalently bonded on the palygorskite through glutaraldehyde.

C. lipolytica (300 U/mg) was purchased by the Xueyan Enzyme Co. (China). 3-Aminopropyltriethoxysilane (SAPTES) was a gift by Shuguang Co. (China). Other chemicals and solvents were obtained from Chinese Chemical Company and were of A.R. grade.

2.2. Preparation of silanized palygorskite

The silanize palygorskite was prepared and characterized according to our previous report [14]. Palygorskite was acid treated firstly. The specimens were acid treated with dilute HCl. Excess acid was removed by repeated water rinsing and the acid treated palygorskites were dried. The acid treated palygorskite was further modified by 3-aminopropyltriethoxysilane. Ten percent by volume of 3-aminopropyltriethoxysilane was dissolved in water, after which the pH of the solution was adjusted to 3–4 with concentrated HCl. Samples with the ratio of 2:5 (w/v) were stirred slowly overnight at room temperature, removal from the solution over a filter, rinsed with water and dried in vacuo. The resulting modified palygorskite had a surface area of 182.09 m^2/g, larger than that of natural palygorskite (167.24 m^2/g).

2.3. Immobilization of lipase on glutaraldehyde-activated silanized palygorskite

To activate silanized palygorskite with glutaraldehyde, the palygorskite support was suspended in glutaraldehyde solution at a ratio of 25 ml solution/g silanized palygorskite. The suspension was magnetically stirred at room temperature for 30 min, vacuum filtered to remove glutaraldehyde from the solution, and then washed extensively with deionized water. The final product was dried in vacuo.

Glutaraldehyde-activated silanized palygorskite supports were added to 40 ml enzyme solution in 0.1 M phosphate buffer pH 7.5, with a protein content of 1 mg/ml. The covalently bond process was performed over 12 h at 25 °C with shaking at 110–120 rpm shaking frequency.

2.4. Determination of protein content

The protein content in the crude enzyme or immobilized enzyme preparations was determined by the Bradford method, using bovine serum albumin (BSA) as the standard (the protein is, therefore, expressed in BSA equivalents) [16]. The amount of protein bounded onto the palygorskite support was determined indirectly from the difference between the initial total protein exposed to the supports and the amount of protein recovered in the wash.

2.5. Determination of enzyme activity

The activity of free and immobilized enzyme was assayed by titrating the fatty acid with 0.05 M NaOH. The fatty acid was liberated from the hydrolysis of olive oil under the catalysis of enzymes in phosphate buffer at 37 °C. Activities were assayed by adding lipase in the phosphate buffer, using 5 ml 20% (v/v) olive oil emulsification solution as the substrate, which was obtained after pure olive oil dispersed in water solution containing polyvinyl alcohol (4%, w/v). After exact 15 min of incubation at 37 °C, the reaction was stopped by adding 15.0 ml of alcohol solution (95%, w/w). Finally, the reaction solution was titrated with 0.05 M of NaOH. The blank hydrolysis of olive oil was a same process, except that the alcohol solution was added at the beginning of the hydrolysis. The fatty acid content was calculated from the difference between the blank and acid equation of the titration. One unit of the activity was defined as 1 μmol of the fatty acid produced by the catalysis in 1 h under the assay conditions.

2.6. Effect of pH

The effect of pH on enzyme activity was determined by incubating free and immobilized lipase separately at different pHs ranging from 6.0 to 8.0 at 30 °C for 30 min. At the end of incubation time the residual lipase activity of each sample was determined.

2.7. Effect of temperature

The effect of temperature on enzyme activity was determined by incubating free and immobilized lipase separately at different temperature in the range of 20–90 °C at pH 7.5 for 30 min. At the end of incubation time samples were analyzed for residual lipase activity.

2.8. Storage stability

Both free and immobilized lipases were stored at two different temperatures (4 and 30 °C). The storage stability was evaluated by determining the enzyme activity of them at regular time intervals up to 7 weeks.

2.9. Reusability of immobilized lipase

For the reusability, after each reaction run, the immobilized lipase preparation was removed and washed with phosphate buffer solution to remove any residual substrate. It was then reintroduced into fresh reaction medium to determine enzyme activity.

2.10. Kinetic parameters

The kinetic parameters, K_m and V_{max}, of free and immobilized lipase were calculated from the Lineweaver–Burk and Michaelis–Menten models using varying concentrations of olive oil emulsification solution as the substrate (as described in Section 2.5) [17].

2.11. Sample preparation for SEM (scanning electron microscopy)

SEM analysis of the support before and after lipase immobilization was performed with an XL-30 scanning electron microscope (Philips, The Netherlands). Samples were prepared for SEM by mounting on aluminium stubs using a carbon double-sided tape. Either to make specimens adhere better or to increase the conductivity. All samples were sputter coated with 2 mm thin layer of gold prior to viewing for enhancing conductivity.

3. Results and discussion

3.1. Optimization of glutaraldehyde concentration

Glutaraldehyde is a bifunctional crosslinker commonly used to couple components with amino functional groups. Results showed a glutaraldehyde concentration of 0.5% (v/v) is optimal for activity of the immobilized lipase preparation, producing immobilized lipase samples with activity of 2800 U/g, which corresponded to a lipase recovery of 42% on the support. Similar result was observed for the immobilization of lipase on the polysulfone membrane surface [18]. At higher glutaraldehyde concentrations, more protein is bond, however, the activity of enzyme bound to the palygorskite support decreases. This may be due to the reticulation among enzyme molecules favored by the use of a bifunctional molecule and therefore affecting the activity [19]. Another reason for the low activity may be due to a partial denaturation of enzyme as a result of "rigidification" of the enzyme molecule or steric hindrance which prevents the substrate from reaching the active site [20].

3.2. Optimization of pH for lipase immobilization

The effect of a lipase pH value on the lipase immobilization process was studied (Fig. 2). No significant differences were observed in the immobilization yield obtained for pH values of 6.4–8.0. However, the activity of enzyme bound to the palygorskite support was changed with the increase of the immobilization pH value, and lipase immobilized at pH 7.0 showed a maximum activity of 3300 U/g. It was reported the optimal pH for the immobilization of porcine pancreas lipase was also pH 7.0 [21].

3.3. pH stability and optimum pH

The effect of pH on the stability of both free and immobilized lipase on silanized palygorskite in olive oil hydrolysis was determined in the pH range of 6.0–8.0 and the results were presented in Fig. 3. The pH profiles of the immobilized lipases were broader than that of the free enzyme. Furthermore, the optimum pH of the immobilized lipase was at pH 7.0–8.0, while the optimum pH of free lipase was at 7.0. The retained activity of the immobilized enzyme was improved both at lower and higher pH in comparison to the free enzyme. The results meant that the immobilization methods preserved the enzyme activity over a wider pH range. Lipase of *Bacillus coagulans* BTS-3 immobilized on Nylon-6 by covalent bonding was fairly stable within a pH range of 7.5–9.5 with optimum pH 7.5 [22]. Lipase from *Candida rugosa* covalently immobilized onto polyacrylonitrile nanofibers was less sensitive to pH changes with unchanged optimum pH when compared to the free lipase [23]. Lipase from *Arthobacter* sp. immobilized on porous polypropylene hollow fiber membrane also remained the unchanged optimum pH with a broader pH profiles than that of the free enzyme which loses activity at pH 4.0 [24].

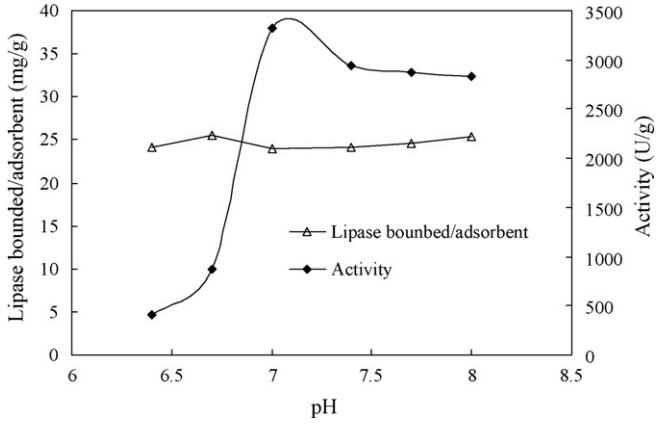

Fig. 2. Effect of pH on lipase immobilization. Immobilization process was achieved by mixing 400 mg of glutaraldehyde-activated silanized palygorskite with 40 ml of lipase (in 0.1 M phosphate buffer), at 25 °C for 6 h.

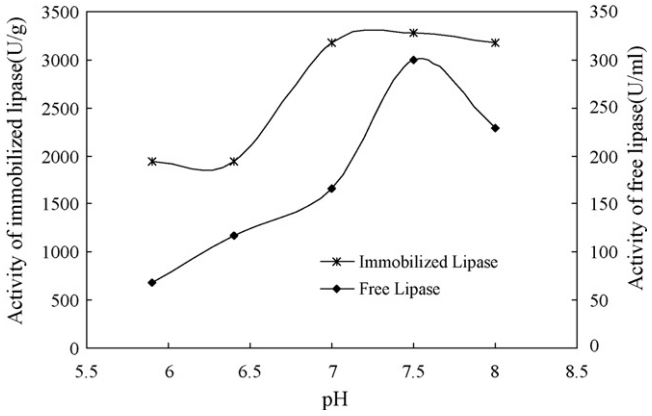

Fig. 3. pH stability of free lipase and immobilized lipase.

3.4. Thermal stability

Both of the thermal stability of the free and the immobilized lipase on silanized palygorskite was determined by measuring the hydrolysis of olive oil at 37 °C after the enzyme exposed to temperatures ranging from 20 to 60 °C in phosphate buffer (0.1 M, pH 7.5) for 30 min. As seen in Fig. 4, the free and the immobilized enzyme exhibited different temperature profiles. The immobilized lipase was stable at 30 °C, while the activity of the free enzyme was decreased drastically. The results also showed that the immobilized enzyme had more than 70% activity at 40 °C, while the free enzyme had only 30% activity. Thus, the immobilized lipase was much more stable than the free enzymes at higher temperatures. Lipase of *B. coagulans* BTS-3 immobilized on Nylon-6 by covalent bonding maintained 88% of the activity at 55 °C for 2 h [22]. Lipase from *Burkholderia* sp. C20 when immobilized onto cellulose nitrate membrane retained 80% activity after incubation at 55 °C for 2 h [25]. *Candida antarctica* lipase type B immobilized on agarose-glutaraldehyde retained around 30% of the initial activity after 24 h of incubation at 50 °C [26].

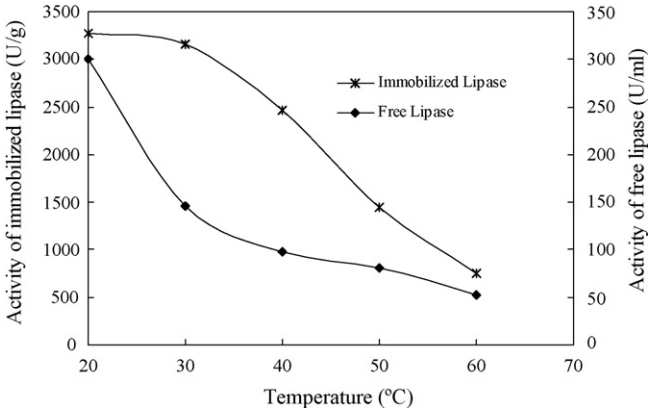

Fig. 4. Thermal stability of free lipase and immobilized lipase.

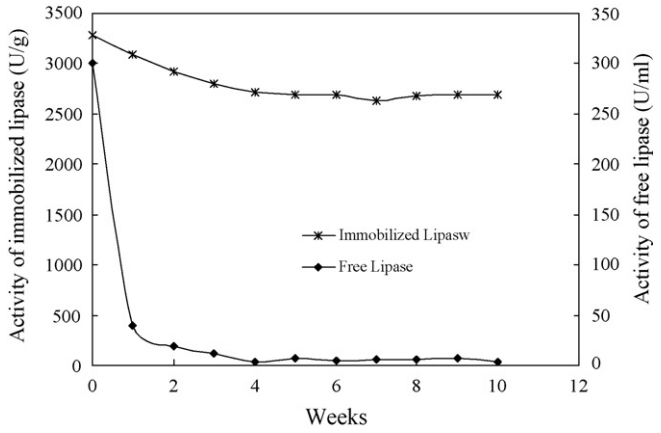

Fig. 5. Storage stability of free lipase and immobilized lipase.

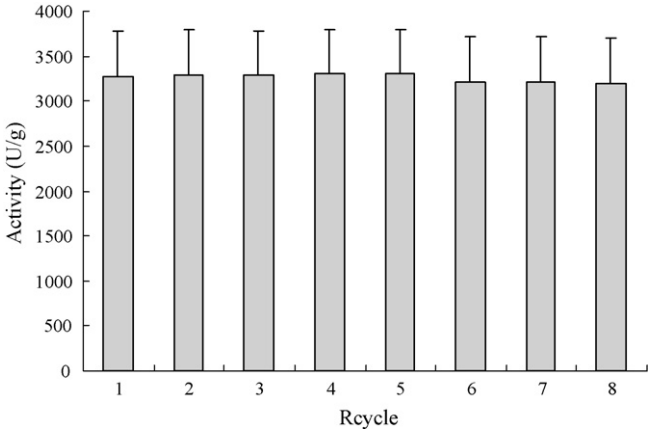

Fig. 6. Effect of recycling on immobilized enzyme activity.

3.5. Storage stability

The experimental results indicate that the immobilization holds the enzyme in a stable position in comparison to the free counterpart. The free and immobilized lipase was stored at 4 °C. The activity of the immobilized lipase decreased more slowly than that of the free lipase; after 10 weeks, the activity of the free enzyme was 67% of its original activity while that of the immobilized lipase was 92%. At 30 °C, the activity of free lipase decreased significantly and almost had no activity after 1 week. However, the immobilized enzyme retained 81% of its activity after 10 weeks (Fig. 5). It was reported the *C. rugosa* lipase covalently immobilized on poly(glycidylmethacrylate-methylmethacrylate) magnetic beads via glutaraldehyde lost 37% of its initial activity after 8 weeks storage at 4 °C [27]. Lipase from *C. rugosa* immobilized on the poly(acrylonitrile-co-2-hydroxyethyl methacrylate) fibrous membranes retained over 60% of its initial activity after 30 days storage at 4 °C [28]. The relative activity of the immobilized *C. rugosa* lipase stored at 4 °C did not change significantly after 30 days, however, dropped to 30% of its initial activity stored at room temperature for 30 days [29].

3.6. Reusability

Immobilized lipase was used repeatedly to hydrolyze olive oil at 37 °C for 15 min and the reusability was examined because of its importance for repeated applications in a batch or a continuous reactor. As shown in Fig. 6, lipase immobilized on palygorskite displayed a good reusability. The immobilized enzyme retains 3200 U/g of activity after eight cycles, which meant the *C. lipolytica* lipase immobilized on the palygorskite showed nearly complete retention of activity in reuse up to eight cycles. Lipase immobilized on cellulose nitrate membrane retained 60% of its original activity after repeated uses for nine times [25]. *C. rugosa* lipase covalently immobilized on poly(glycidylmethacrylate-methylmethacrylate) magnetic beads retained 62% of its initial activity after 10 cycles of usage [27]. It was reported lipase immobilized on glutaraldehyde-activated polymer Nylon-6 retained 85% of its original hydrolytic activity after eight cycles [22].

3.7. Kinetic parameters

The effect of substrate concentration on the initial rate catalyzed by free and immobilized lipases was studied using olive oil as substrate. From Lineweaver–Burk and Michaelis–Menten models (Fig. 7), Michaelis constant (K_m) and the maximum reaction velocity (V_{max}) of the free and immobilized enzymes were calculated and results were given in Table 1. The K_m value of immobilized lipase was 2.22 times higher than that of free enzyme and the V_{max} value of immobilized lipase was 1.67 times lower than that of free lipase. The change of kinetic constants may be a consequence of either the structural changes in the enzyme introduced by the immobilization procedure or lower accessibility of substrate to the active sites of the immobilized enzyme [30].

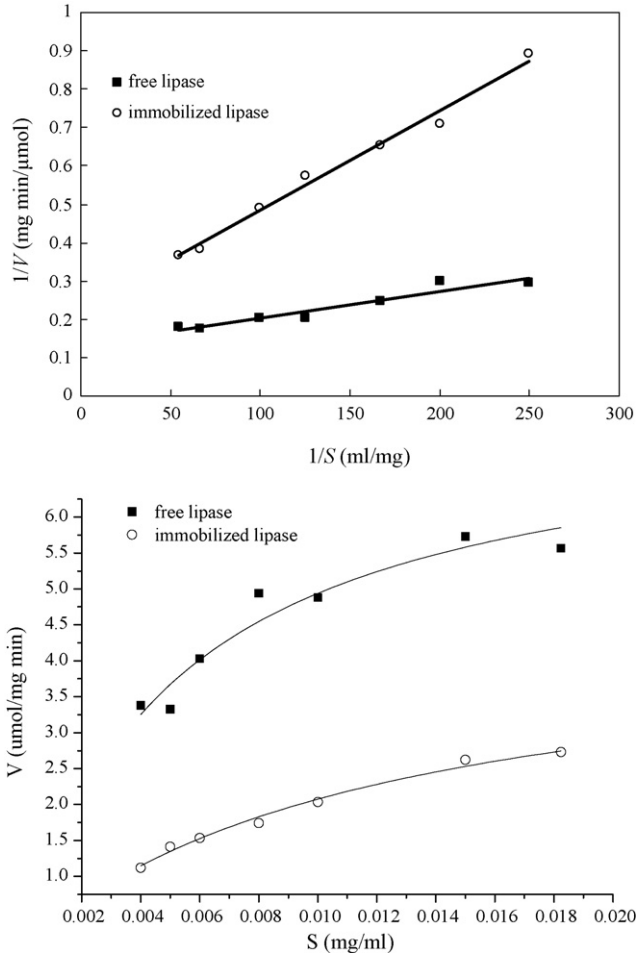

Fig. 7. Lineweaver–Burk and Michaelis–Menten plots of the free and immobilized lipase.

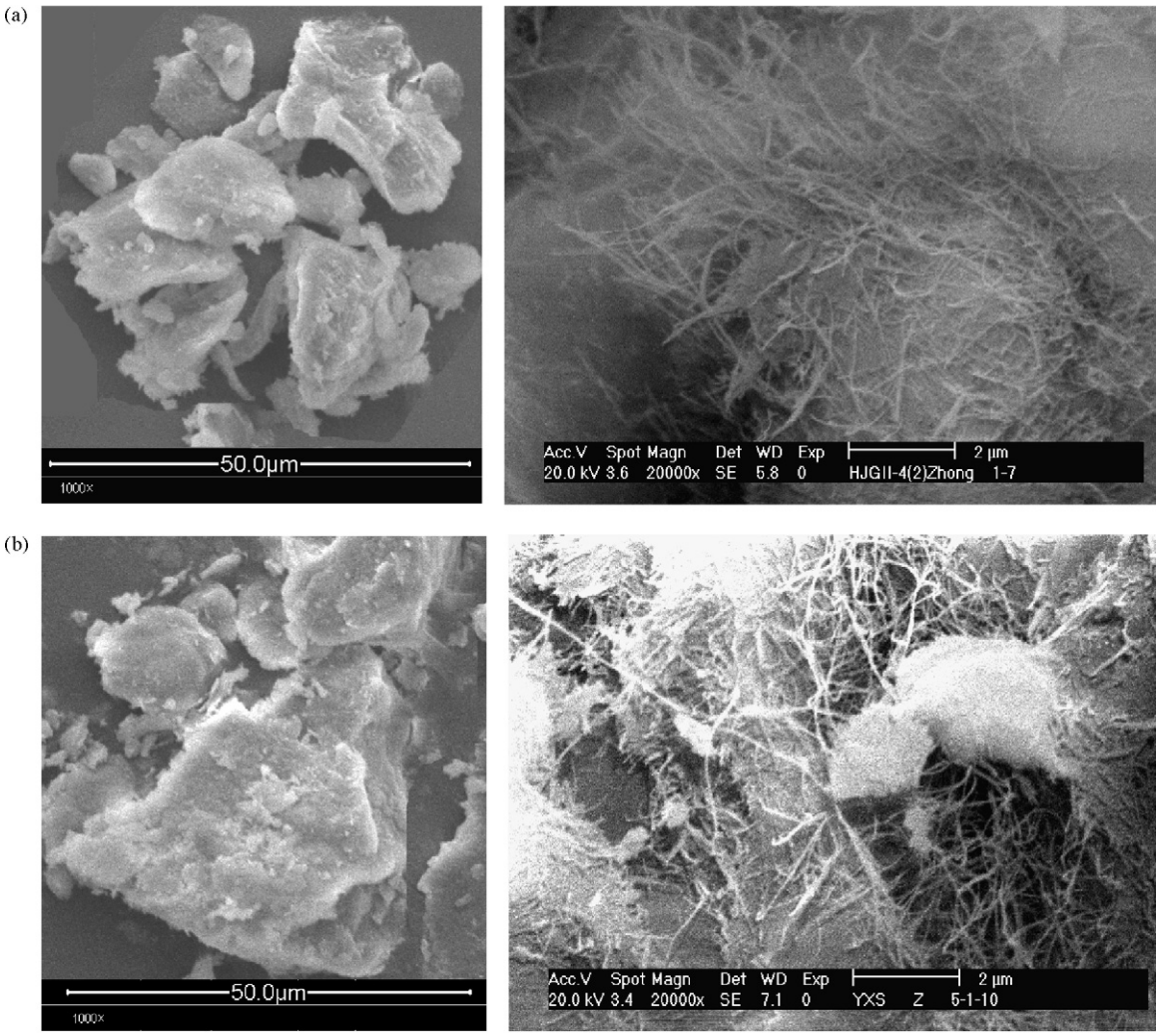

Fig. 8. Scanning electron micrographs of palygorskite without (a) and with (b) bound lipase. (a) Before lipase immobilization and (b) after lipase immobilization.

Table 1
Kinetic parameter of free and immobilized enzyme (K_m and V_{max})

	K_m (mg/ml)	V_{max} (μmol/(mg min))
Free lipase	0.0053	7.6
Immobilized lipase	0.012	4.5

3.8. Scanning electron microscopy

Scanning electron microscopy provided surface morphology of palygorskite supports and particle size. The support had a size in the range of 10–25 μm. After binding lipase, the palygorskites remained dispersed and the size was similar to that of unbound supports, which meant that the immobilization process did not significantly change the size of the supports. Scanning electron micrographs are the evidence of immobilization over the palygorskite (Fig. 8). The distinct dollops related to aggregates of proteins bonding on the surface of the support are visible in the photographs.

4. Conclusion

The silanized palygorskite could be used as a support for lipase immobilization. Palygorskite is mechanically stable and chemically inert, and is therefore environmental- and solvent-friendly for industrial manufacturing and processing. Scanning electron micrographs proved the immobilization of *C. lipolytica* lipase on the amino chemically surface modified palygorskite support. The K_m (0.0117 mg/ml) value of immobilized lipase was 2.22 times higher than that of free enzyme and the V_{max} (4.51 μmol/(mg min)) value of immobilized lipase was 1.67 times lower than that of free lipase. Lipase immobilized on modified palygorskite, retained activity over wider ranges of temperature and pH than those of the free form. The activity of the immobilized enzyme became less sensitive to reaction conditions (temperature and pH) than that of the free counterpart. The immobilized enzyme retains high activity after eight cycles. A high operational stability, obtained with the immobilized lipase, indicated that the immobilized lipase could successfully be used in a continuous system for the hydrolysis of lipids.

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