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Immobilization of lipase onto micron-size magnetic beads

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Abstract

A novel and economical magnetic poly(methacrylate-divinylbenzene) microsphere (less than 8 μm in diameter) was synthesized by the modified suspension polymerization of methacrylate and cross-linker divinylbenzene in the presence of magnetic fluid. Then, surface aminolysis was employed to obtain a high content of surface amino groups (0.40–0.55 mmol g^{-1} supports). The morphology and properties of these magnetic supports were characterized with scanning electron microscopy, transmission electron microscopy, Fourier transform infrared spectroscopy and a vibrating sample magnetometer. These magnetic supports exhibited superparamagnetism with a high specific saturation magnetization (σ_s) of 14.6 emu g^{-1}. *Candida cylindracea* lipase was covalently immobilized on the amino-functionalized magnetic supports with the activity recovery up to 72.4% and enzyme loading of 34.0 mg g^{-1} support, remarkably higher than the previous studies. The factors involved in the activity recovery and enzymatic properties of the immobilized lipase prepared were studied in comparison with free lipase, for which olive oil was chosen as the substrate. The results show that the immobilized lipase has good stability and reusability after recovery by magnetic separation within 20 s.

Keywords: Magnetic microsphere; Synthesis; Suspension polymerization; *Candida cylindracea* lipase; Immobilization

1. Introduction

Lipases (E.C. 3.1.1.3) are ubiquitous enzymes with various biological activities, including triacylglycerols hydrolysis, esterification between fatty acid and alcohol, and other enzymatic reactions [1–3]. In practical applications the activity recovery and repeated use of lipases are very important for the process economy. Many immobilization techniques of enzymes have been employed and reviewed recently [4]. There are many factors affecting the activity recovery and reusability of enzymes in immobilization process. Some of the most important factors are the choice of a support and the selection of an immobilization strategy. Thus, exploiting good supports and immobilization strategy has been an attractive work for enzyme engineering. Magnetic supports have been used in enzyme immobilization [5–7] and cell separation [8], which were first applied to immobilize enzymes in 1973 [9]. Besides the merits of other solid supports, lipases immobilized by magnetic supports can be more easily recovered from a reaction system, and stabilized in a fluidized-bed reactor by applying an external magnetic field. The use of magnetic supports can also reduce the capital and operation costs. However, for these presently available magnetic supports, complex preparation process, insufficient enzyme loading capacity and high cost restrict their wider applicability in enzymatic engineering [10,11].

Magnetic polymeric supports are often prepared by the co-polymerization of monomers (one of them is the functional monomer) including suspension polymerization, emulsion polymerization, dispersion polymerization and two-step swelling [12]. Among them suspension polymerization is simple and easy to scale up, hence, more suitable for mass

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production. However, the magnetic polymeric supports made by conventional suspension polymerization were mostly in the size of several hundred micrometers with a very broad size distribution [13,14]. During polymerization process, moreover, a large amount of functional groups were buried in the polymer with only a small part localized on the surface [10]. The main disadvantages of those magnetic supports are their large size, low density of surface functional groups and weak magnetism. It is hardly reported in literature that micron-size (several microns) magnetic polymeric supports with high density of surface functional groups could be prepared by suspension polymerization.

Choosing a suitable method of lipase immobilization also enables an increase in the stability without negative influence on their catalytic activity. Covalent immobilization of enzymes onto spacer-arm attached magnetic supports can lead to a high activity yield and stability [5]. Since the interactions between lipases and magnetic supports are not sufficiently clear, the study of immobilization process is important for optimizing conditions for preparation and application of the immobilized lipases.

In this study, we develop an economical, high loading capacity magnetic support, which could be chemically derivatized for covalent immobilization of lipase. First, micron-size magnetic polymeric spheres were synthesized by the modified suspension polymerization of methacrylate (MA) and cross-linker divinylbenzene (DVB) in the presence of oleic acid-coated magnetite nanoparticles. Then, surface aminolysis was employed to introduce functional groups (–NH_2). *Candida cylindracea* lipase (CCL) was covalently immobilized onto the amino-functionalized magnetic supports by the glutaraldehyde method. The magnetic supports were characterized with scanning electron microscopy (SEM), transmission electron microscopy (TEM), diffusive reflectance infrared spectroscopy (DR-IR) and a vibrating sample magnetometer (VSM). The factors affecting the activity recovery and properties of the immobilized lipase were investigated.

2. Experimental

2.1. Materials

Commercial lipase (E.C. 3.1.1.3) from *C. cylindracea* and bovine serum albumin (BSA) were obtained from Sigma. Other chemicals were generally of reagent grade and purchased from Beijing Chemical Reagent Company. Methacrylate and DVB were distilled under a reduced pressure to remove the inhibitor prior to use. All other materials were of analytical grade and used without any further purification, including ferric chloride hexahydrate ($FeCl_3{\cdot}6H_2O$), ferrous chloride tetrahydrate ($FeCl_2{\cdot}4H_2O$), poly(vinyl alcohol) (PVA-1788), aqueous ammonia (25% (w/w)), oleic acid, benzoyl peroxide (BPO), ethylenediamine, dimethylformamide (DMF), glutaraldehyde, and ethanol.

2.2. Synthesis of magnetic PMA-DVB microspheres

The oleic acid-coated Fe_3O_4 was obtained by the method described previously [15]. Magnetic PMA-DVB microsphere was prepared by modified suspension polymerization. Methacrylate (95 ml), DVB (5 ml), magnetic fluid (30 g) and BPO (4.0 g) were mixed to form the organic phase. PVA-1788 (25 g) was dissolved in 1000 ml deionized water to form the aqueous phase. They were mixed together and transferred to a 2-l beaker equipped with four vertical stainless steel baffle-plates, a condenser, a nitrogen inlet, and a four-paddle stirrer. The mixture temperature was maintained at 45 °C for 45 min and then increased to 60 °C within 10 min. Finally, the temperature was increased to 70 °C and the reaction was carried out for two more hours with the stirring speed of 1000 rpm. The resulting magnetic PMA-DVB microspheres were isolated by magnetic decantation and washed with deionized water and ethanol several times.

2.3. Surface functionalization and activation

Magnetic PMA-DVB (10.0 g) was washed with DMF twice and then mixed with DMF (100 ml) and ethylenediamine (100 ml). The mixture was shaking gently at 110 °C for 12 h. After washing with deionized water and ethanol two times, the amino-functionalized magnetic microspheres were obtained. To facilitate the covalent attachment of enzyme, the amino groups on the surface were transferred to aldehyde groups by the glutaraldehyde method [16]. After agitating at 30 °C overnight, the glutaraldehyde-activated magnetic supports were washed with deionized water three times and stored for future use.

2.4. Characterization of magnetic microspheres

The morphology and structure of the magnetic microspheres were observed by scanning electron microscopy (SEM; JSM-6700F, JEOL, Japan) and transmission electron microscopy (TEM, H-8100, Hitachi, Japan). The DR-IR spectra were recorded in KBr on a Fourier transform infrared spectrophotometer (FT-IR; Vecter 22, Bruker, Germany). The sample was placed in the sample port of an integrating sphere (diameter 110 mm), and the diffuse reflectance was measured at 610 nm with a Varian cary 5 spectrophotometer. The magnetization curves of samples were measured with a vibrating sample magnetometer (VSM; Model-155, Digital Measurement System, USA). The amount of amino group of on the surface of magnetic microspheres was determined from elemental analysis device (CHNS-932, Leco, USA).

2.5. Lipase immobilization

All lipase immobilization experiments were carried out batchwise in 5 ml of 0.1 M phosphate buffer pH 7.0 at continuous shaking of 150 rpm at room temperature. In a typical experiment, 50 mg magnetic support was dispersed in 5 ml of 0.1 M buffer and a predetermined amount of lipase powder

was added to the suspension. The mixture was placed in a shaking incubator at 150 rpm and the immobilization was carried out at room temperature. After completion of the reaction, the lipase-immobilized support was recovered by magnetic separation. The amount of lipase protein in the supernatant was determined by Bradford method using BSA as a standard [17]. The amount of lipase adsorbed onto the magnetic supports was calculated as

$$q = (C_i - C_f)\frac{V}{W} \ (\mathrm{mg/g})$$

where q is the total binding quantity (mg g^{-1}), C_i and C_f the concentrations of the initial soluble enzyme and final in the supernatant after immobilization, respectively (mg ml^{-1}), V the reaction volume (ml), W the weight of the magnetic supports (g). All data used in this formula are averages of at least duplicated experiments.

2.6. Enzymatic reaction and activity assay

The specific activities of the free and immobilized lipase were determined by measuring the fatty acid content in the medium according to a method described previously [2,18]. The hydrolytic activity of both forms of the lipase were tested with 5% (w/v) olive oil emulsion (pH 7.0) containing 4% (w/v) PVA. To 20 ml of the emulsion, a predetermined amount of the free or immobilized lipase was added and the hydrolysis reaction was carried out in a shaker (150 rpm) at 37 °C for 30 min. The quantity of fatty acid liberated was measured by titration with 5 mM NaOH. One unit of enzyme activity was defined as the amount of lipase which liberates 1 μmol fatty acids per minute under the assay condition. The activity recovery (%) remaining after immobilization was the effective activity after immobilization referred to the activity which the bound protein amount would have in solution.

2.7. Stability and recycled use of the immobilized lipase

To investigate the thermal stability of the enzyme, both free and immobilized CCL preparations were incubated in 0.1 M phosphate buffer pH 7.0 at 50 °C with continuous shaking at 150 rpm. Aliquots of 0.5 ml free CCL solution or immobilized lipase suspension were withdrawn at 10 min intervals and the remaining activities were measured as described above. In addition, the durability of the immobilized lipase was determined by the hydrolysis of olive oil by the recovered immobilized CCL with magnetic separation and compared with the first run (activity defined as 100%).

3. Results and discussion

3.1. Characteristics of magnetic supports

In suspension polymerization, the size and size distribution of droplets are controlled by the stirring condition and the concentration of stabilizer. Therefore, the drawback of suspension polymerization (large size and broad size distribution) can be overcome to a certain extent by improving the stirring conditions and modifying the reaction process [19]. Since poly(methacrylate-divinylbenzene) microspheres are much easier to be functionalized with surface chemical reaction, methacrylate was selected as monomer for polymerization. In the present work, three modifications were adopted for modified suspension polymerization. First, the polymerization was conducted in a 2-l beaker equipped with four vertical stainless steel baffleplates and a four-paddle stirrer, so that the droplets dispersed were very uniform. Second, the viscosity of droplets decreases with the increase of reaction temperature. It is critical to control the process of

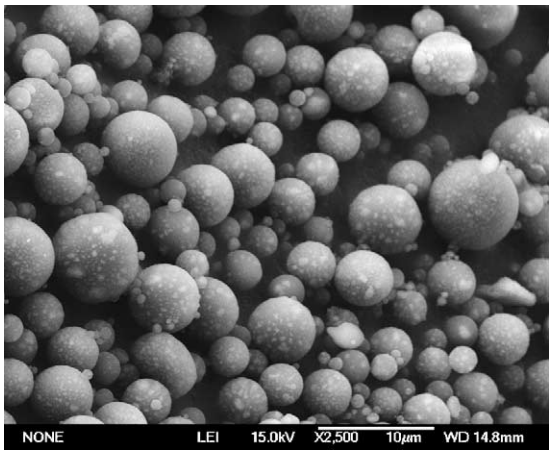


Fig. 1. SEM of magnetic poly(MA-DVB) microspheres.

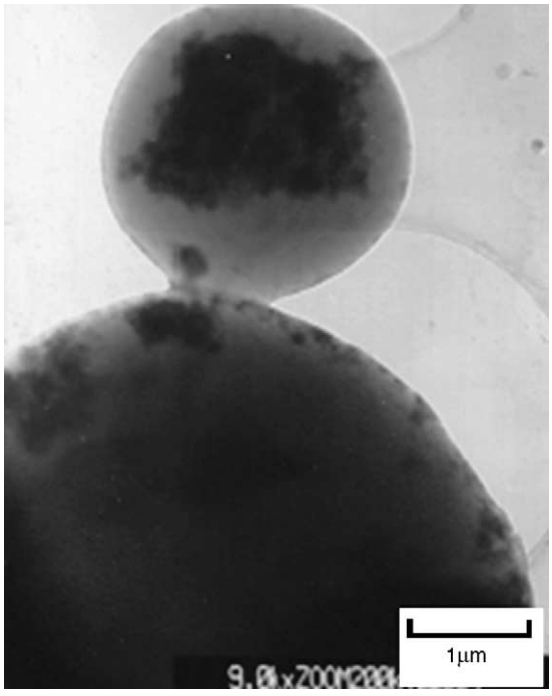
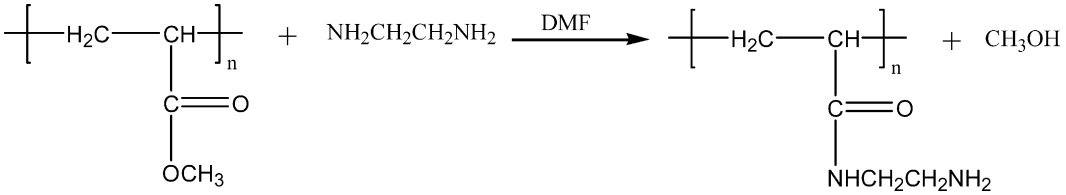


Fig. 2. TEM of magnetic PMA-DVB microspheres.

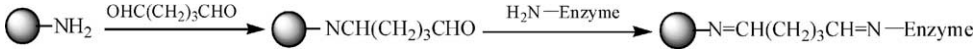
temperature increase to ensure a period of droplet break-up and the formation of small droplets (otherwise large droplets will form). Third, a large amount of stabilizer PVA (above 20 wt.% of monomer) was added to obtain stable microspheres with narrow size distribution. As expected, micron-size (less than 8 μm in diameter) magnetic PMA-DVB spheres were obtained by this modified suspension polymerization. The morphology and structure of the resulting microspheres were observed by SEM as shown in Fig. 1 and with TEM in Fig. 2.

In addition, surface aminolysis was employed to introduce surface functional groups instead of conventional co-polymerization of monomers (one of them is the functional monomer). The $-OCH_3$ groups on the surface of magnetic microspheres were replaced with ethylenediamine in the presence of DMF at the temperature of 110 °C as follows.



The fact was confirmed by the comparison of DR-IR spectra of magnetic PMA-DVB before (A) and after (B) aminolysis as shown in Fig. 3. Before aminolysis, the strong band at 1735 cm^{-1} indicates the presence of extensive carboxylic ether and the $-OCH_3$ characteristic band at 1373 cm^{-1}. After aminolysis, the intensity of the carboxyl band at 1735 cm^{-1} decreased greatly and the $-OCH_3$ characteristic band at 1373 cm^{-1} disappeared due to the aminolysis that took place between $CH_3O-C=O$ groups and amino groups. The C–O–C characteristic bands at 1160 and 1270 cm^{-1} decreased accordingly. Compared with the unmodified magnetic microspheres, the amino-modified microspheres possess characteristic bands at 1650 and 1545 cm^{-1} due to the stretching vibration of amide I (mainly due to C=O stretching) and amide II (mainly due to N–H bending), respectively. Elemental analysis of the amino-modified magnetic supports were performed, and the amounts of the surface amino group were found to be 0.40–0.55 mmol g^{-1} supports from the nitrogen stoichiometry, much higher than those produced by co-polymerization [20,21].

To facilitate the covalent attachment of lipase, the amino group on the magnetic PMA-DVB was transferred to the aldehyde group. Lipase was then covalently bound via the amino group to the activated magnetic support. The amino group of the lipase is involved in the formation of a Schiff base linkage between the lipase and support as follows.



A spacer-arm comprising aliphatic chains of five carbon atoms has been used to separate the immobilized lipase from the support. The attachment of five-carbon atom hydrophobic spacer-arms on the magnetic microspheres surface could prevent undesirable side interactions between the enzyme molecule and the support.

Fig. 4 shows the magnetization curves of the amino-modified and unmodified magnetic PMA-DVB at room temperature. The specific saturation magnetization (σ_s) of amino-modified microspheres, which was found to be 14.6 emu g^{-1}, is comparable to the unmodified microspheres of 15.9 emu g^{-1}. The results show that the surface modification has little impact on the magnetism of microspheres. It has been clear that, for ultrafine magnetically ordered particles, there exists a critical size below which the granules can acquire only single magnetic domains even in zero magnetic fields. It has been estimated that the oleic acid-coated Fe_3O_4 has superparamagnetism [22,23]. Thus,

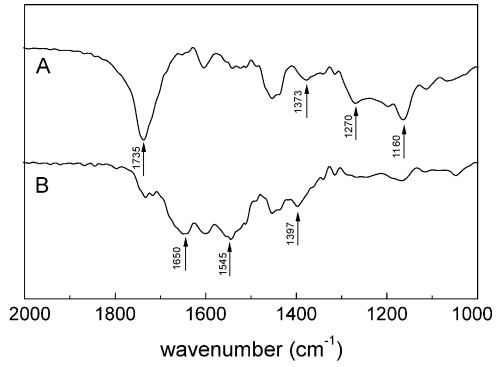


Fig. 3. Comparison of DR-IR spectra of magnetic PMA-DVB microspheres before (A) and after (B) aminolysis.

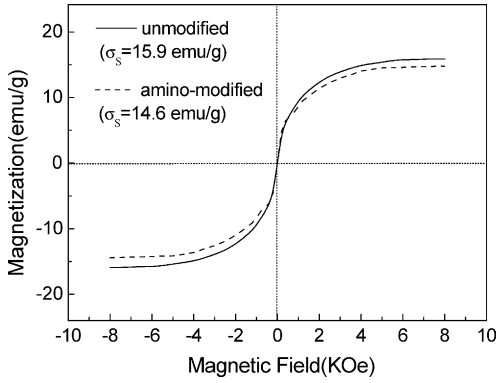


Fig. 4. VSM of amino-modified and unmodified magnetic PMA-DVB microspheres.

these samples prepared are also expected to be superparamagnetic. The coercive force, $H_c = 2.12$ Oe, and remanence, $M_r = 0.06$ emu g^{-1} are so small that the hysteresis could hardly be observed. Both the amino-modified and unmodified magnetic microspheres can be easily separated within 20 s by a conventional permanent magnet (2000 Oe). When the external magnet was removed, the magnetic microspheres could be well dispersed by gentle shaking. This indicates the advantage of the magnetic supports in ease of recovery and recycling.

3.2. Factors affecting the activity recovery for lipase immobilization

3.2.1. Effect of glutaraldehyde concentration

To extend the spacer and facilitate the covalent attachment of enzymes, the amino groups on the surface of magnetic microspheres were transferred to aldehyde groups by the glutaraldehyde method. Fig. 5 shows the effect of the glutaraldehyde concentration on the activity recovery of immobilized lipase. The highest activity recovery was obtained by using 3% (v/v) glutaraldehyde as a cross-linking agent. Glutaraldehyde was used to link the amino-functional groups on the magnetic support and the amino group of the lipase through a coupling reaction. Any glutaraldehyde concentration less than 3% (v/v) resulted in insufficient activation of the surface of magnetic support, while higher concentrations caused excessive self-crosslinking of glutaraldehyde, which might have a steric effect for lipase. Owing to the steric hindrance, the activity of the immobilized lipase decline. Thus, an initial glutaraldehyde concentration of 3% (v/v) was applied for the surface activation of the magnetic supports.

3.2.2. Effect of time

In order to effectively facilitate the covalent coupling and prevent enzyme deactivation at longer reaction time, it is important to choose the optimum coupling time. Fig. 6 illustrates the activity recovery of the immobilized lipase prepared at different reaction time. The activity recovery of the immobilized lipase increased with prolonged coupling time and the highest activity recovery was obtained under immobilization allowed to proceed for 6 h. However, the activity recovery decreased if the reaction time was longer. The immobilization process would decrease the enzyme activity because of the long time coupling reaction.

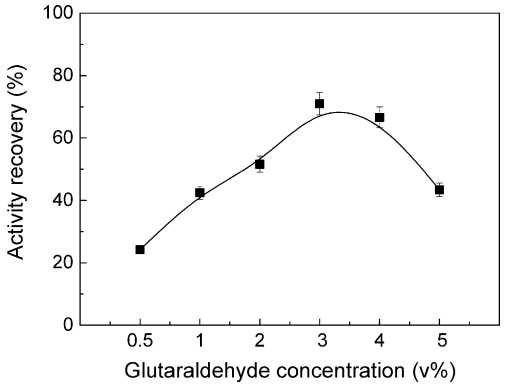


Fig. 5. Effect of glutaraldehyde concentration (vol.%) on the activity recovery of immobilized lipase at pH 7.0 and room temperature for 6 h.

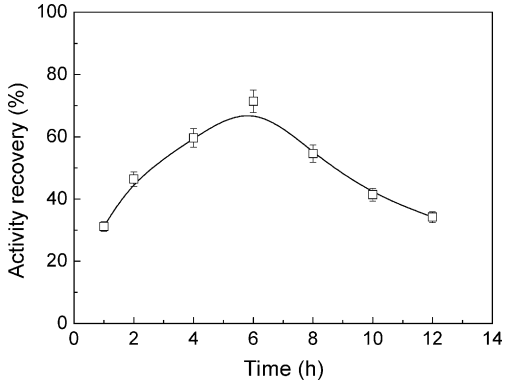


Fig. 6. Effect of the coupling time on the activity recovery of immobilized lipase incubated at pH 7.0 and room temperature.

3.2.3. Effect of lipase concentration added

CCL was immobilized to the magnetic supports at different initial lipase concentrations (from 0.2 to 1.4 mg ml^{-1}). Table 1 indicates the effect of initial lipase concentration on the enzyme loading and the activity recovery when 50 mg of supports was used in the immobilization. At this concentration range, the amounts of the lipase loading increased greatly with the initial lipase concentration and the activity recovery reached a maximum value at an initial lipase concentration of 1.0 mg ml^{-1}. It is considered that the higher enzyme loading makes the enzyme form an intermolecular steric hindrance, which restrains the diffusion of the substrate and product. Therefore the activity recovery decreased slowly above 1.0 mg ml^{-1} of lipase. The highest activity recovery was 72.4%, obtained with a protein load of 34.0 mg g^{-1} support, which is remarkably higher than previous reports [5,24]. In the following experiments, the enzyme-immobilizing support with a protein loading of 34.0 mg g^{-1} support was used due to its higher activity recovery.

More than 95% of the lipase immobilized onto the magnetic support by simple adsorption could be desorbed by treatment of 1% sodium dodecylsulfate solution [25]. However, no protein was desorbed from the covalently immobilized lipase. Therefore, it can be concluded that all the immobilized lipase was not simply adsorbed but covalently bound.

3.3. Properties of the immobilized lipase

By taking the activity of the immobilized and free lipase under optimal conditions as 100%, respectively, the activity values obtained from different enzyme reactions were defined as residual activities.

Table 1
Effect of initial lipase concentration on the enzyme loading and the activity recovery of immobilized lipase

Lipase concentration (mg ml^{-1})	0.2	0.4	0.6	0.8	1.0	1.2	1.4
Protein loaded (mg g^{-1} support)	8.0	16.0	22.0	29.5	34.0	37.0	36.5
Activity recovery (%)	31.1	50.2	58.4	68.6	72.4	67.8	68.0

3.3.1. Effect of pH value on immobilized lipase activity

By studying the variation of the residual activity of the immobilized and free lipase at different medium pH values as shown in Fig. 7, it shows that their optimum medium pH values are 8.0 and 7.0, respectively. The optimum pH value of free enzyme shifted 1 unit to the alkaline region after covalent immobilization. The shift depends on the method of immobilization as well as the structure and charge of the matrix. With changing of medium pH values, the immobilized CCL was more sensitive to pH around 7.0. A similar observation with the lipase immobilized on supports was reported [7,26]. It might be a result of the conformation change (exposure of the catalytic site) of the lipase molecules after immobilization, making the catalytic site more easily accessible to H^+ or OH^- ions. This might change the dissociation state of the catalytic site, making the enzyme inhibited greatly.

3.3.2. Effect of temperature on immobilized lipase activity

The effect of temperature on the free and immobilized lipase activities were investigated by using olive oil as substrate as shown in Fig. 8. The maximum activity of the free lipase appeared at 37 °C, but the optimum temperature of the immobilized lipase was obtained at 50 °C, higher than that of the free lipase. The immobilized lipase was not inactivated at the temperature above 37 °C. As the temperature increases, the residual activity of the free lipase decreases greater than that of the immobilized lipase. The optimum enzymatic reaction temperature of the immobilized lipase was up to 50 °C, higher than its soluble counterpart. So the conclusion was drawn that the immobilized lipase showed good heat resistance. This implies that the immobilized lipase may be employed at higher temperature to achieve higher activity.

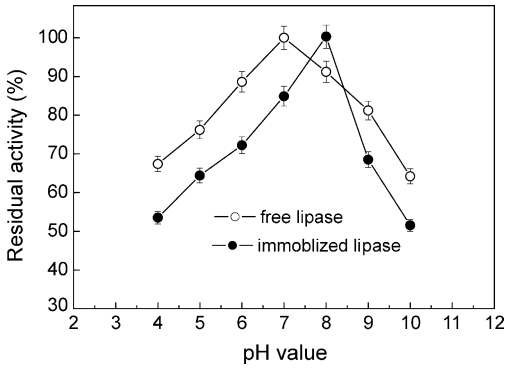


Fig. 7. Effect of pH value on the residual activity of *Candida cylindracea* lipase during olive oil hydrolysis at 37 °C after 30 min (●, immobilized enzyme; ◯, free enzyme).

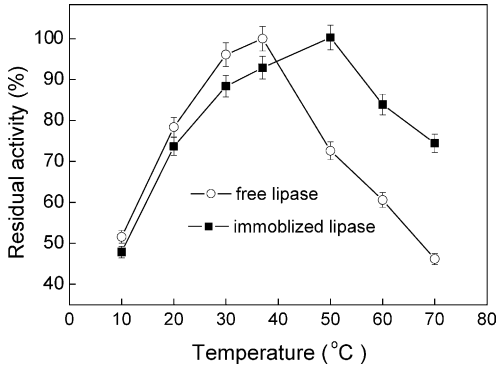


Fig. 8. Effect of temperature on residual activity of *Candida cylindracea* lipase during olive oil hydrolysis at pH 7.0 after 30 min (■, mmobilized enzyme; ◯, free enzyme).

3.3.3. Thermal stability and repeated use of the immobilized lipase

Fig. 9 is a comparison of thermal stability of both free and immobilized lipase at 50 °C in phosphate buffer pH 7.0. Both preparations exhibited a similar trend, however, the immobilized lipase is more stable than the free one. The half-life of the immobilized CCL is much longer than that of the free lipase. The stability and reusability of the immobilized lipase is very important for commercial application. The thermal stability and duration was determined by hydrolysis of olive oil with the recovered immobilized lipase at 50 °C and compared with the first run (activity defined as 100%). The used immobilized lipase was recovered by magnetic separation and washed three times with 0.1 M phosphate buffer (pH 7.0), and then supplied again to the fresh reaction solution

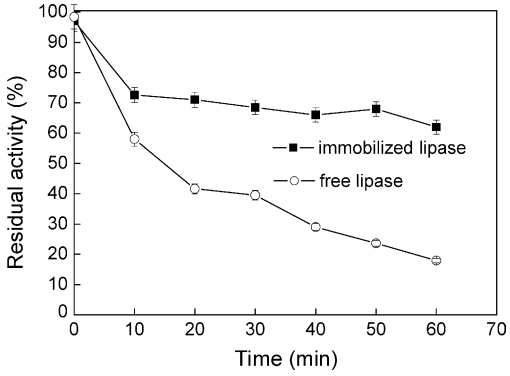


Fig. 9. Stability of (◯) free and (■) immobilized CCL in phosphate buffer pH 7.0 at 50 °C (initial free enzyme activity was defined as 100%).

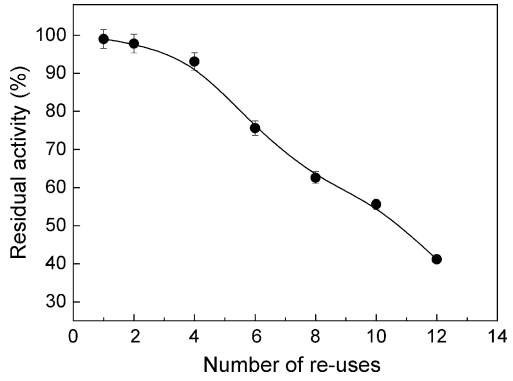


Fig. 10. Effect of repeated use on the activity of immobilized lipase during olive oil hydrolysis at pH 8.0 and 50 °C after 30 min.

to determine the enzymatic activity. This cycle was repeated. The activity of the immobilized lipase during the repeated use decreased significantly after four cycles as shown in Fig. 10. However, the immobilized enzyme has good stability and the residual activity remained about 75.6% of the first use after six cycles.

4. Conclusions

A novel and economical protocol for lipase covalent immobilization on magnetic supports has been developed. The magnetic enzyme particles prepared in this study present some advantages. First, the magnetic supports have reduced particle size (<8 μm) compared with those made by conventional suspension polymerization, together with a high density of surface amino groups (0.40–0.55 mmol g^{-1} supports), which allow high protein loading. Second, the magnetic enzyme particles, which having high magnetic properties (14.6 emu g^{-1}) can be easily and gently separated by using a magnetic field. Third, the magnetic supports have active groups for the enzyme immobilization without need for any particle activation process. On the other hand, the immobilized lipase shows high activity recovery (72.4%), enzyme loading (34.0 mg g^{-1} support) and good stability during the repeated use. It can be concluded that this micron-size magnetic polymeric support provides an economical, efficient and selective system for enzyme immobilization.

Acknowledgement

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