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Micron-sized flower-like Fe_3O_4@GMA magnetic porous microspheres for lipase immobilization

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Abstract:

Flower-like Fe_3O_4 microspheres prepared by a fast solvothermal method were selected to fabricate micron-sized Fe_3O_4@glycidyl methacrylate (GMA) magnetic porous microspheres. The magnetic porous microspheres were endowed with appropriate specific surface area (39.54 m^2/g) and appropriate pore diameter (16 nm), which could be served as a carrier for immobilized lipase. Meanwhile, the prepared magnetic microspheres with high saturation magnetization could realize rapid separations. After those microspheres were aminated and activated, the obtained immobilized lipase possessed high efficiency of immobilization and catalytic activity,

with the optimum pH and temperature 8.0 and 40℃,respectively. The thermal stability of immobilized lipase was obviously exceed free lipase.

Keywords: micron-sized, flower-like, magnetic, porous, lipase immobilization

1. Introduction

For the past few years, more and more attention has been paid to biocatalyst, especially enzymes [1-4]. Compared with the issues such as low yield, severe pollution in the traditional pharmaceutical intermediates and manufacturing technology of fine chemical products, enzymatic synthesis has been widely used in medicine, food processing, biomass energy and other fields because of its simple process, high product purity, low cost, no poisonous reagents and environmentally friendly [5, 6].

Lipase (EC 3.1.1.3) is a significant biocatalyst which can be available for catalyzing a variety of reactions, such as esterification, transesterification, esterolysis, especially has an important role in the preparation of chiral drugs [7-9]. However, it's sensitive to the environment for the free lipase when directly used in the catalytic process. The free lipase would also be unstable and easily deactivated in the alkali, acid, high temperature and organic solvents, and contaminates the products. Immobilized lipase, which was prepared by immobilizing free lipase on the carrier by a certain physical and chemical methods, not only overcomes the deficiency of the free lipase, but also maintains the inherent biological catalytic activity. As a result, immobilized lipase has been extensively researched and extensively used in biological

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medicine, food, et al [2, 10-12].

Among the numerous carriers of immobilized lipase, magnetic composite microsphere with mangnetic responsiveness is the most common choice. Firstly, magnetic microsphere is easy to be modified (like silane coupling agent) due to abundant functional groups on the surface, and good biocompatibility [13-15]; for another, because of the property of rapid separation, it improves the reusability of immobilized enzyme and reduces production costs. Tan's group [16] prepared submicron hollow Fe_3O_4 particles via solvothermal method for immobilized lipase after amino functionalization. Further, Zhu et al [6] utilized succinic anhydride modified co-precipitation Fe_3O_4 nanoparticles to immobilize porcine pancreatic lipase. Li's group immobilized candida rugosa lipase (CRL) using hollow Fe_3O_4 nanoparticles [17] and amine or acid modified Fe_3O_4 nanoparticles [18], the immobilized lipase revealed excellent catalytic activity and reusability. Nevertheless, the particle size of Fe_3O_4 particles above mentioned is popularly nanometer or submicron range. Although the relatively high specific surface area is achieved, the directly contact area with lipase is low, and binding sites are limited by the steric hindrance. Therefore, it is crucial to fabricated micron-sized hierarchical Fe_3O_4 porous microspheres for immobilized lipase.

In this study, micron-sized flower-like Fe_3O_4 porous microspheres were produced by solvothermal method with the aftertreatment of calcination under nitrogen atmosphere. Then, the Fe_3O_4 porous microspheres modified with methacryloxy propyl trimethoxyl silane (MPS) were polymerizated with GMA to fabricate flower-like

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Fe_3O_4@GMA magnetic porous microspheres (MPMs) for the immobilized lipase CRL. The structure and character of the MPMs were studied in detail, and the performance of the immobilized lipase was also investigated.

2. Materials and methods

2.1 Materials

Tetraethoxysilane (TEOS), methacryloxy propyl trimethoxyl silane (MPS), candida rugosa lipase (CRL) were purchased from Sigma-Aldrich Co. LLC. Anhydrous $FeCl_3$, potassium persulfate (KPS), ethylene glycol dimethacrylate (EGDA), glutaraldehyde and ethylene glycol (EG) were provided by Tianjin Fuchen Chemical Reagent Factory. Glycidyl methacrylate (GMA) was obtained from Sartomer Company. Deionized water was ultrapure produced by an apparatus for pharmaceutical purified water (Aquapro Co. Ltd.).

2.2 The preparation of flower-like Fe_3O_4 porous microspheres

The preparation of flower-like Fe_3O_4 porous microspheres was referred to the literature [19-21] with a certain improvement. A typical procedure was as follows: First, 0.1 g $FeCl_3$ and 3 g urea were dissolved in 40 ml EG. Then, the mixture was moved to a Teflon-lined stainless-steel autoclave at 200℃ for a period of time after stirring for 30 min at 50℃. The product was washed with ethanol and water for three times. The flower-like Fe_3O_4 porous microspheres were produced after calcination under nitrogen atmosphere for 1 h.

2.3 Synthesis of flower-like Fe_3O_4@GMA magnetic porous microspheres

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0.5 g prepared Fe_3O_4 porous microspheres were dispersed in 320 ml ethanol/100 ml water. Subsequently, 5 ml MPS was added. The mixture was immersed in a water bath at 40℃ for 12 h. The produce was washed with ethanol and water for three times, marked as Fe_3O_4-MPS. Then, 0.2 g Fe_3O_4-MPS microspheres above-mentioned were dispersed into the aqueous solution contained right amount of GMA and 5% EGDA. When the temperature of mixed solution was reached to 70℃, 10 ml 0.5% KPS solution was added. The polymerization reaction was lasted 6 h. The flower-like Fe_3O_4@GMA MPMs were formed after washing and drying.

2.4 Lipase immobilization

The lipase solution was prepared by PBS buffer solution with a certain concentration. 0.2 g carrier microspheres were dispersed in the solution, then incubated for 4 h at room temperature. Finally, the residual lipase was washed, and the supernatant was collected to determine the efficiency of immobilized lipase by Bradford method [22]. Bovine serum albumin (BSA) was used as a standard.

The activity of immobilized lipase was monitored by titrating the fatty acid hydrolysed from olive oil referred to the literature [23]. The relative activity (%) was calculated as the ratio of the activity of every sample and the maximum activity of the sample.

3. Results and discussion

3.1 Synthesis and characterization of flower-like Fe_3O_4@GMA magnetic porous microspheres

To identify the composition of the products, the typical FTIR spectrum of

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Fe_3O_4@GMA MPMs were firstly determined as shown in figure 1. In figure 1A, the absorption peak at 3433 cm^{-1} was referred to stretching vibration absorption peak of hydroxyl on the surface of Fe_3O_4 microspheres. 557 cm^{-1} showed the characteristic absorption peak of Fe-O. It revealed flower-like magnetic porous microspheres were Fe_3O_4. The absorption peak at 1627 cm^{-1} in the figure 1B was assigned to stretching vibration of C=C. The in-plane deformation vibration of C-H was at 1390 cm^{-1}, while 1178 cm^{-1} and 1060 cm^{-1} were referred to Si-O-Si bond. It illustrated MPS has been modified on the Fe_3O_4@GMA MPMs. 1730 cm^{-1} belonged to the absorption peak of carbonyl group (C=O) as shown in figure 1C and 1D. The peaks at 1149 cm^{-1} and 906 cm^{-1} were attributed to C-O-C and epoxy group, respectively. Therefore, we could confirm Fe_3O_4@GMA MPMs were formed with the polymerization of GMA.

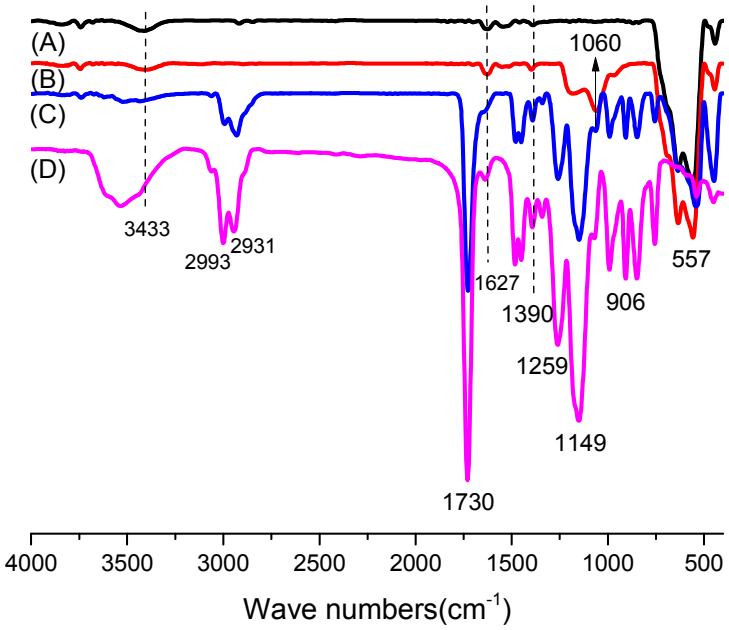

Fig. 1 FTIR spectra of flower-like Fe_3O_4 microspheres (A), Fe_3O_4-MPS microspheres (B), Fe_3O_4@GMA magnetic microspheres (C) and PGMA (D).

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The crystal structure of the obtained magnetic porous microspheres was determined, and the XRD pattern was shown in Figure 2. From the different intensity of crystal diffraction peaks, it indicated the inorganic particles in the prepared Fe_3O_4@GMA MPMs have fine crystallinity. Compared to the standard Fe_3O_4 XRD card, its peak positions were basically the same, which showed the inorganic particles were Fe_3O_4. Therefore, combined with the FTIR analysis results, the produced magnetic porous microspheres were Fe_3O_4@GMA MPMs. The magnetic responsiveness of Fe_3O_4@GMA MPMs were shown as the VSM curves in the figure 3. In figure 3A, the maximum saturated magnetization of flower-like Fe_3O_4 microsphere was 61 emu/g. The fairly low residual magnetization, coercivity, and the coincidence of the hysteresis loop reflected that the prepared flower-like Fe_3O_4 microspheres possessed superparamagnetic property. After polymerized with GMA, the saturation magnetization of Fe_3O_4@GMA MPM was reduce to 58 emu/g (as shown in figure 3B). It revealed the prepared Fe_3O_4@GMA MPMs still had strong magnetic responsiveness which could play a significant role in rapid separation and reutilization.

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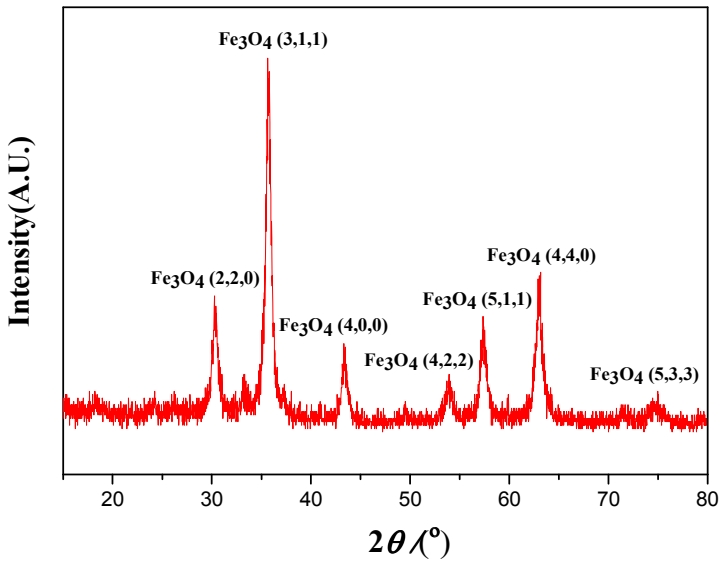

Fig. 2 XRD of flower-like Fe_3O_4@GMA magnetic microspheres.

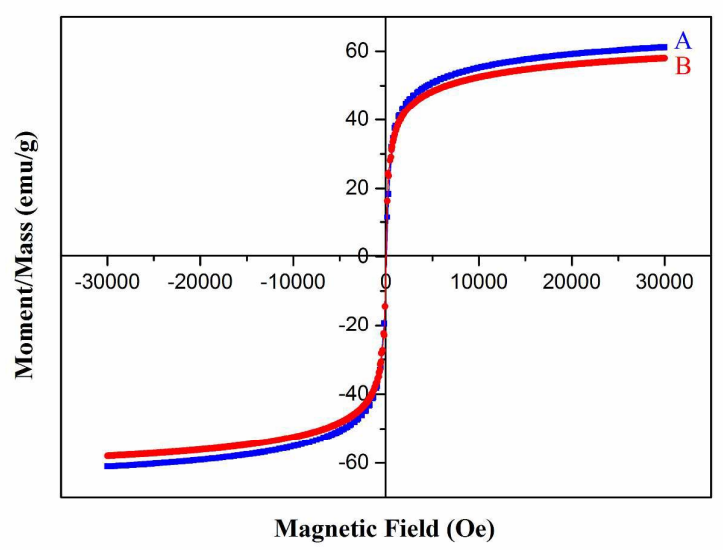

Fig. 3 The magnetization curves of flower-like Fe_3O_4 microspheres (A) and Fe_3O_4@GMA magnetic microspheres (B).

Figure 4 showed the flower-like Fe_3O_4 microspheres fabricated with different reaction time. From figure 4A, we could see that the flower-like Fe_3O_4 microspheres were assembled by multitudinous crystal plates. When the reaction time was 3 h, the

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petals of the prepared magnetic microspheres were small and dense. Then, flower morphology preliminarily revealed. With the further reaction to 4 h, the petals gradually grew up, and stretched to form the flower-like Fe_3O_4 microspheres. After reacting 6 h, flower morphology was disappeared. Spherical Fe_3O_4 particles was obtained, and their particle size was significantly reduced. So, the flower-like Fe_3O_4 microspheres prepared at 4 h were chose to be the carriers of immobilized lipase. The TEM image in figure 4D further proved the flower morphology of Fe_3O_4 microspheres which were attribute to petals aggregation.

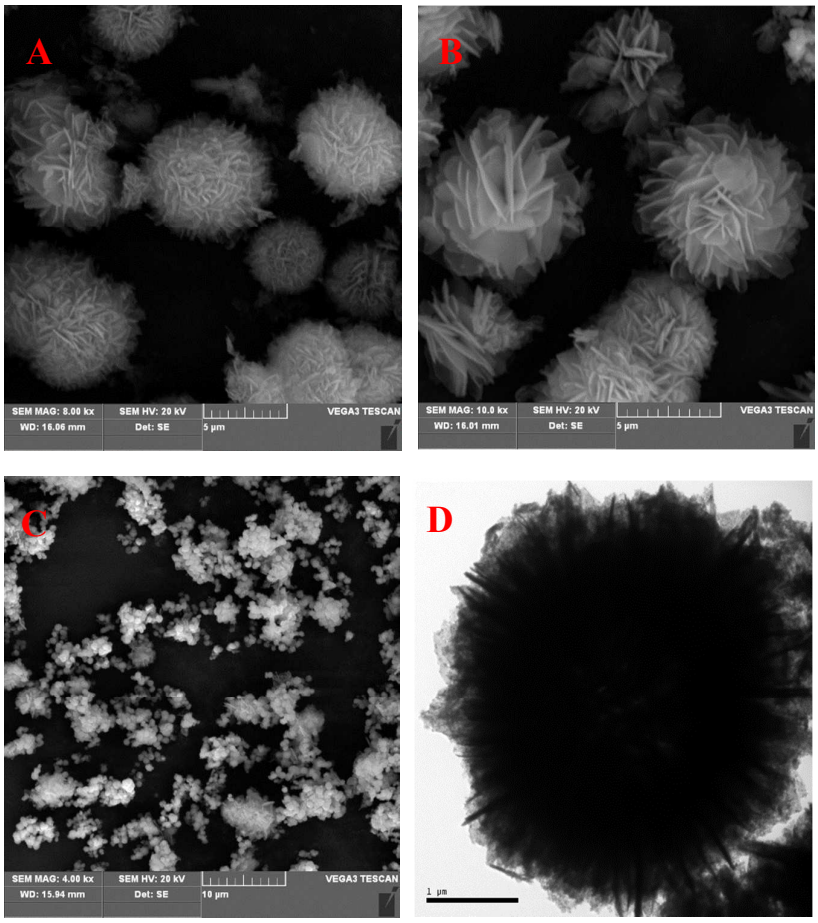

Fig. 4 SEM images of flower-like porous Fe_3O_4 nanoparticles prepared at 3h (A), 4h (B) and 6h (C), and TEM image prepared at 4 h (D).

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Figure 5 was the SEM and TEM images of prepared flower-like Fe_3O_4@GMA MPMs. From figure 5A, when the flower-like Fe_3O_4 microspheres were polymerized with GMA, Fe_3O_4@GMA MPMs still has the fine flower morphology, just the surface was more cluttered and rough. It illustrated the prepared magnetic porous microspheres possessed shape retention, and were suitable for immobilized lipase. In figure 5B, Fe_3O_4 microspheres were successfully coated by GMA. Poly(GMA) was a thin layer which did not destroy pore performance of the flower-like Fe_3O_4 microspheres.

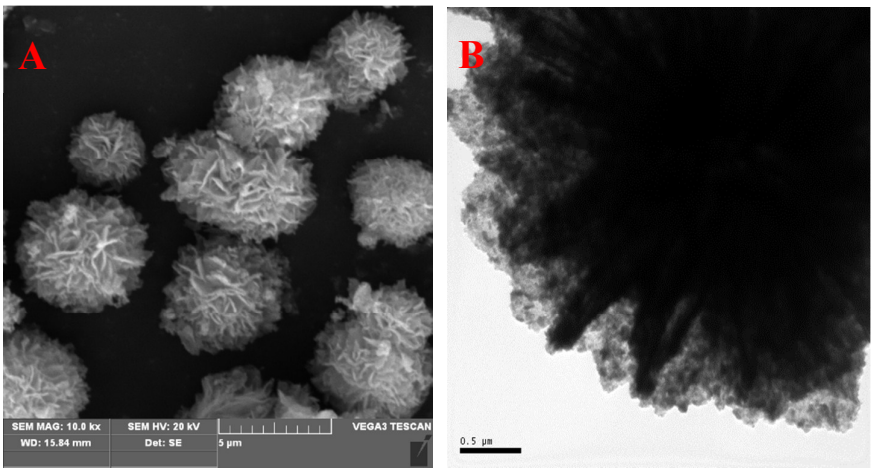

Fig. 5 SEM (A) and TEM (B) images of Fe_3O_4@GMA magnetic microspheres.

To analyze the pore performance and specific surface area of magnetic microspheres, Figure 6 showed the N_2 adsorption-desorption curves of the flower-like Fe_3O_4 microspheres before and after polymerization. From figure 6A and 6B, both flower-like Fe_3O_4 and Fe_3O_4@GMA presented the hysteresis loop which belonged to H3 type. It revealed that the pore of prepared microspheres was the slit pore piled up by plate-like particles, which were consistent with the results observed in the SEM images. Figure 6C showed the pore size distributions of two kinds of microspheres

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were relatively wide. This was beneficial to debase mass transfer resistance of substrate after immobilized lipase. From the adsorption-desorption isotherm, the BET specific surface areas of prepared flower-like Fe_3O_4 and Fe_3O_4@GMA MPMs were 45.17 m^2/g and 39.54 m^2/g, respectively. And average pore sizes were 16.08 nm and 15.99 nm. The above results indicated flower-like Fe_3O_4@GMA MPMs not only possessed appropriate specific surface area, but also appropriate pore diameter, which were an excellent carrier for immobilized lipase.

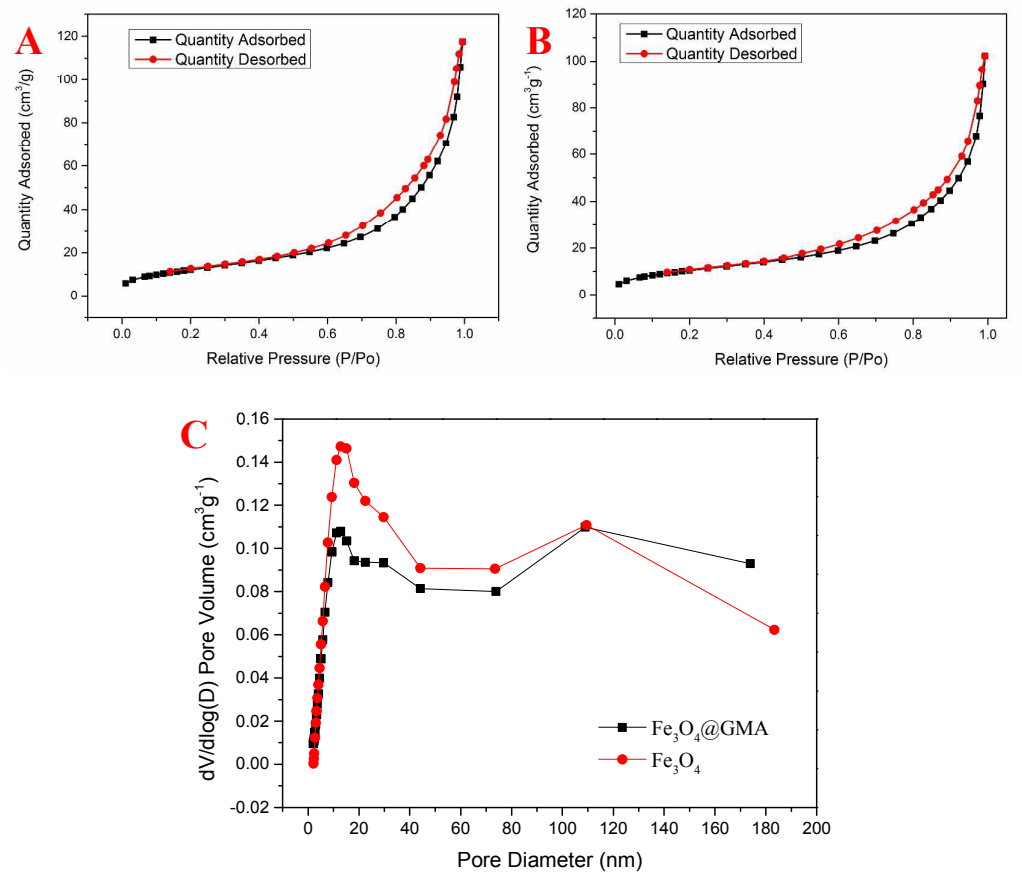

Fig. 6 N_2 adsorption/desorption isotherm curves of Fe_3O_4 (A), Fe_3O_4@GMA magnetic microspheres (B) and their pore size distributions (C).

3.2 Lipase immobilization

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GMA monomer endowed flower-like Fe_3O_4 microspheres with a large number of epoxide groups which were serviceable to immobilize biomacromolecules using glutaraldehyde as a linker. The immobilized lipase was achieved via covalent immobilization of carriers and lipase CRL. So, the dosage of GMA is an important factor to influence the content of epoxy groups on prepared carriers. Table 1 was the corresponding relation between the dosage of GMA and immobilization efficiency of immobilized lipase. From table 1, although the immobilization efficiency was the highest when GMA was used 1.45 g, there would appear white emulsion after washing the product under an external magnetic field, which revealed GMA was excessive. Thus, 1 g of GMA was the optimal dosage.

Table. 1 The immobilization efficiency of flower-like Fe_3O_4@GMA immobilized lipase synthetized with different amount of GMA.

Dosage of GMA	0.5 g	1.0 g	1.45 g
Immobilization efficiency	27.99 mg/g	133.32 mg/g	157.52 mg/g

Considering immobilized lipase required suitable environment to develop its optimal catalytic performance, we examined the pH and temperature of the system on the influence of the activity of immobilized lipase, as shown in figure 7 and 8. pH value directly decided the surface charge distribution of lipase which affected the activity [24]. Figure 7 was the relative activity of immobilized lipase at various pH conditions compared to that at the pH of 6.0. From figure 7, the optimal pH was 8.0, which was far higher than that at other pH.

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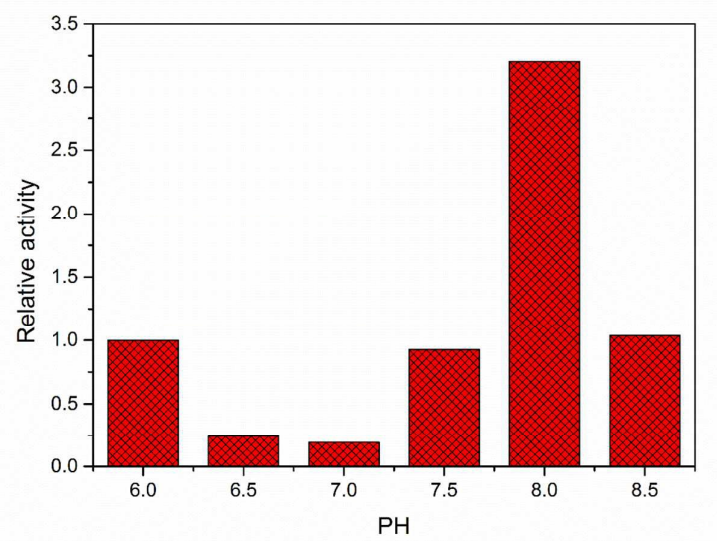

Fig. 7 Relative activity of immobilized lipase at various pH conditions compared to that at pH 6.0.

Figure 8A was the relative activity of different lipases at various temperatures. From figure 8A, the optimal temperature of immobilized lipase was 40℃, about 65% of the free lipase. In addition, it was higher than Novozym-435 in the determination of temperature. Figure 8B showed the thermal inactivation analysis of free lipase and immobilized lipase at 50℃. From figure 8B, the activity of both free lipase and immobilized lipase were trend to decline. However, the decline extent of free lipase was even bigger compared with each test point. When incubated 180 min, the activity of free lipase was dropped to 6.3%, almost deactivated. From the thermal inactivation fitting curve, it was accord with the two-step series-type enzyme deactivation model: $E_0 \xrightarrow{k_1} E_1 \xrightarrow{k_2} E_2$. The residual enzyme activity could be expressed as

$$\text{Residual activity}\ (\%) = \alpha \exp(-k_1 t) + \beta \exp(-k_2 t)$$

B

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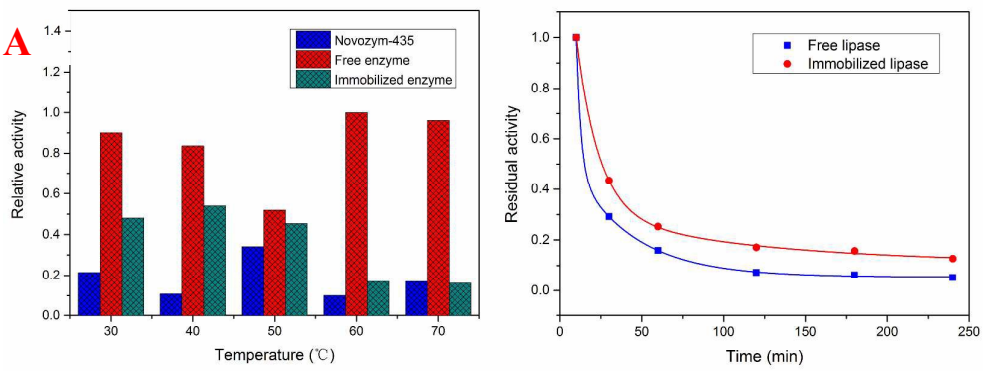

Fig. 8 Relative activity of different lipases at various temperature conditions compared to the activity of free enzyme at 60℃ (A). Thermal stability of free and immobilized lipase (B).

The fitting results were shown in table 2. From table 2, for immobilized lipase, $k_1 < k_2$. It illustrated similarly that the stability of the immobilized lipase was better than free lipase.

Table 2. Deactivation Kinetic Parameters of free lipase and immobilized lipase at 50 ℃.

	α	β	k_1/min^{-1}	k_2/min^{-1}	R^2
Free enzyme	19.2918	0.5385	0.3581	0.0273	0.999
Immobilized lipase	0.2188	1.4325	0.0097	0.0727	0.998

Kinetics of free lipase and immobilized lipase were explored via hydrolyzing olive oil with different initial concentrations. The Lineweaver-Burk plots were shown in Figure 9. From figure 9, it was shown that the K_m of free lipase and immobilized lipase were 15.4 and 3.1, respectively. And the V_{max} of immobilized lipase was 2.4

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U/mg, lower than that of free lipase (16.3 U/mg). The low K_m and V_{max} of immobilized lipase revealed that the affinity of immobilized lipase for the substrate was higher than free lipase. It could be attribute to the formation of oil-water by the exposed of Fe_3O_4 and poly(GMA). The mass transfer limitation caused by the carries expressed insignificance.

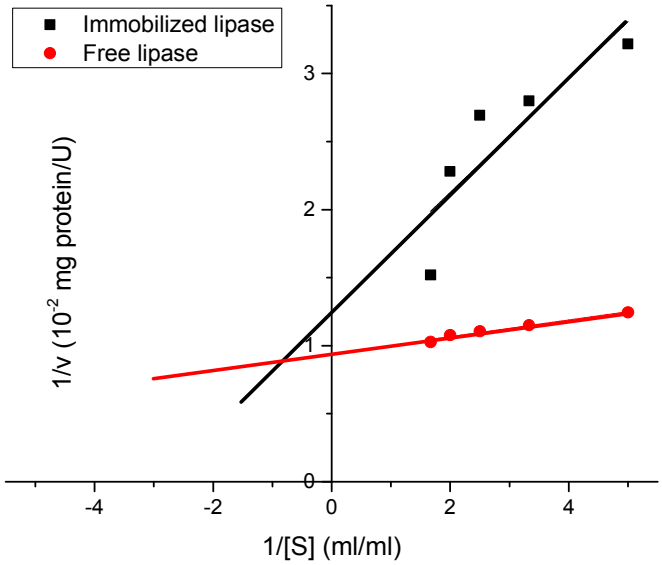

Fig. 9 Lineweaver–Burk plots for the immobilized lipase.

4. Conclusions

In conclusion, micron-sized flower-like Fe_3O_4@GMA magnetic porous microspheres were fabricated by one-step emulsion polymerization of flower-like Fe_3O_4 microspheres. The magnetic porous microspheres possessed high specific surface area (39.54 m^2/g) and appropriate pore diameter (16 nm). In addition, the magnetic responsibility of prepared Fe_3O_4@GMA magnetic porous microspheres was still up to 58 emu/g, which can be achieved rapid separation. It is doubtlessly an excellent carrier for immobilized lipase. The optimum pH and temperature of

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immobilized lipase were 8.0 and 40℃, respectively. Through the analysis of the thermal inactivation, the thermal stability of immobilized lipase was obvious higher than free lipase. The micron-sized flower-like Fe_3O_4@GMA magnetic porous microspheres greatly enriched the magnetic carrier of immobilized lipase, and expanded the application of magnetic composite microspheres.

Acknowledgements

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