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Preparation of poly(glycidylmethacrylate–methylmethacrylate) magnetic beads: Application in lipase immobilization

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Abstract

Magnetic bead was prepared from the monomers glycidylmethacrylate (GMA) and methylmethacrylate (MMA) via suspension copolymerization in the presence of ferric ions. The magnetic beads were characterized with scanning electron microscope (SEM), FT-IR and ESR spectrophotometers. The beads were sieved and 100–150 μm size of fraction was used in enzyme immobilization. The specific surface area of the magnetic beads was measured by the BET method and was found to be 16.2 m^2/g beads. The reactive character of the epoxy groups allowed the attachment of the amino groups during thermal precipitation reaction. The resulting magnetic beads were used for the covalent immobilization of *Candida rugosa* lipase via glutaraldehyde activation and glutaraldehyde was also acted a 5-carbon spacer arm. The maximum lipase immobilization on magnetic poly(GMA–MMA) was 23.4 mg g^{-1}. The activity yield of the lipase immobilized on the spacer-arm attached magnetic beads was up to 81%. Kinetic analysis shows that the dependence of lipolytic activity of both free and immobilized lipase on trybutyrin substrate concentration can be described by Michaelis-Menten model with good agreement. The estimated Michaelis constants (K_m) for the free and immobilized lipase are 2.6 and 12.3 mM, respectively. The V_{max} values of free and immobilized enzymes were calculated as 984 and 773 U/mg enzymes, respectively. Employment of immobilization seemed to result in an increase in K_m and a decrease in V_{max}. Optimal operational temperature was 5 °C higher for immobilized enzyme than that of the free counterpart. Thermal and storage stabilities were found to be increase with immobilization.

Keywords: Lipase; Immobilized enzyme; Enzyme stability; Enzyme kinetic; Magnetic beads

1. Introduction

A number of methods for immobilization of enzymes have been reported in the literatures such as adsorption onto an insoluble material, and covalent linking to an insoluble carrier [1–7]. 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 [5,8–12]. A variety of support materials have been used for lipase immobilization [13–18]. After immobilization of lipase, changes were observed in enzyme activity, optimum pH, affinity to substrate and stability [14–16]. The extent of these changes depended on the source of enzyme, the type of support and the method of immobilization.

Several kinds of magnetic particles are produced from various polymers with different functional groups. These magnetic particles have wide range of applications in the immobilization of cells and enzymes [19–22], bio-separation systems [19] immunoassays [21], biosensors and so on [23]. Permanent magnetization could cause the particle to aggregate even if the supports have been removed from the magnetic field. To solve this problem, super-paramagnetic particles have to be developed. Fe_3O_4 is one of the super-paramagnetic materials and it is commonly used in the biotechnological and biomedical areas [10,19]. Besides the merits of other supports, magnetic ones can be more easily recovered from the reaction medium, cultivation media and waste from food or fermentation industries [19,20]. In the magnetic separation techniques, there is no need for expensive liquid chromatography systems, centrifuges, filters or other equipment. The separation process can be also carried out in crude sample containing suspended solid materials [19–22]. As a result of the magnetic properties of the adsorbents, they can be selectively removed from the medium under the applied magnetic force.

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Lipases (E.C. 3.1.1.3) from different sources are currently used in various biochemical reactions including triacylglycerols hydrolysis, esterification between fatty acid and alcohol, and other enzymatic reactions [10,14]. The rising interest in lipase mainly lies on its wide industrial applications, including detergent formulation, oils/fats degradation, pharmaceuticals synthesis, and cosmetics production [24]. Lipases display a peculiar mechanism of action, "interfacial activation" [25]. Lipases may exist in two different forms. One of them, where the active centre of the lipase is secluded from the reaction medium by a polypeptide chain called "lid", is considered to be inactive (closed form). The other one, presenting the lid displaced and the active centre exposed to the reaction medium, is considered to be active (open form) [25,26].

In this study, magnetic poly(GMA–MMA) beads were synthesized from the monomers glycidylmethacrylate and methylmethacrylate and cross-linked with ethyleneglycol dimethacrylate. The epoxy groups of the magnetic poly(GMA–MMA) beads were converted into amino groups in the presence of ammonia during thermal precipitation iron oxide crystal in the bead structures. The magnetic supports were characterized with scanning electron microscopy (SEM), FT-IR spectroscopy and a vibrating sample magnetometer (VSM). The resulting magnetic beads were used for the covalent immobilization of *Candida rugosa* lipase after activation with glutaraldehyde [27,28]. The immobilization of lipase via covalent attachment onto the magnetic poly(GMA–MMA) beads from solutions containing different amounts of enzyme was investigated in a batch system. The factors affecting the activity recovery and properties of the immobilized lipase were investigated.

2. Experimental

2.1. Materials

Lipase (from *Candida rugosa*, lyophilised powder) was supplied by the Sigma Chemical Co. (St. Louis, MO, USA) and used as received. Glycidylmethacrylate (GMA), methylmethacrylate (MMA) and ethyleneglycoldimethacrylate (EGDMA) and α,α′-azoisobisbutyronitrile (AIBN) were obtained from Fluka AG (Switzerland), and the monomers distilled under reduced pressure in the presence of hydroquinone and stored at 4 °C until use. Glutaraldhyde, polyvinyl alcohol (PVA; MW: 50,000), tributyrin, gum Arabic and sodium cholate were obtained from Sigma Chem. Co. All other chemicals were of analytical grade and were purchased from Merck AG (Darmstadt, Germany). The water used in the present work was purified using a Barnstead system (Dubuque, IA, USA).

2.2. Preparation of magnetic poly(GMA–MMA) beads

The magnetic poly(GMA–MMA) beads were prepared via suspension polymerization. The organic phase contained GMA (7.5 ml), MMA (7.5 ml), EGDMA (7.5 ml; as cross-linker) and 5.0% polyvinyl alcohol (20 ml, as stabilizer) were mixed together with 0.2 g of AIBN as initiator in 20 ml of toluene. The aqueous dispersion medium comprised from $FeCl_3$ solution (0.3 M, 400 ml) which was used as a precursor for the thermal iron-oxide precipitation in the beads. The polymerization reactor was equipped with a mechanical stirrer, nitrogen inlet and reflux condenser. The polymerization reaction was maintained at 70 °C for 2.0 h and then at 80 °C for 1.0 h. After the reaction, the resultant beads were filtered and washed with distilled water and ethanol. For the thermal magnetization of the beads, 5.0 g $FeCl_2$ was dissolved in purified water (100 ml) and then was transferred into a reaction vessel containing ferric-poly(GMA–MMA) beads (15 g) in $NH_3{\cdot}H_2O$ (50 ml, 25%, w/v). The thermal precipitation reaction was carried out under nitrogen atmosphere and at three different temperatures (i.e., at 40, 50 and 90 °C for 2 h). After reaction, the magnetic poly(GMA–MMA) beads were washed in ethanol solution (50%; 250 ml), and then with purified water. The magnetic poly(GMA–MMA) beads (∼5.0 g) were transferred in phosphate buffer (pH 8.0; 25 ml), containing glutaraldehyde (5%, w/w). The reaction was carried out at 25 °C for 6 h. The activated beads (poly(GMA–MMA)–GA), were washed with distilled water, acetic acid solution (0.1 M, 100 ml) and phosphate buffer (50 mM, pH 7.0). The magnetic beads were used for the covalent immobilization of lipase [26,27].

2.3. Immobilization of lipase on the magnetic beads

Immobilization of lipase on the magnetic poly(GMA–MMA)–GA beads was carried out at 22 °C in a shaking water bath for 1.0–8.0 h using poly(GMA–MMA) beads as a control blank support. The magnetic beads (2.0 g) equilibrated in phosphate buffer (50 mM, pH 7.5) for 2 h, were then transferred to 10 ml enzyme solution containing of 5.0 mg/mL lipase. After this reaction period, the enzyme immobilized magnetic beads were removed from the medium by a magnetic separation device. Schematic representation of chemical route for the enzyme immobilization is presented in Fig. 1.

Physically bound enzyme from control blank poly(GMA–MMA) and poly(GMA–MMA)–GA beads were removed by washing with a solution containing ionic detergent (sodium cholate, 50 mM) and salt (NaCl, 1.0 M) at pH 8.0 for 2 h. It should be noted that all the physically bound enzyme were removed from the control blank poly(GMA–MMA) beads after washing with detergent and salt solution. After this washing, the control blank poly(GMA–MMA) beads did not yield any lipase activity in the assay medium. The enzyme immobilized on the activated poly(GMA–MMA)–GA beads were washed with phosphate buffer (50 mM, pH 7.0) and stored in the same fresh buffer (50 mM, pH 7.0) at 4 °C until use. After predetermined period, the enzyme-loaded magnetic beads were immediately transferred ethylene diamine solution (5.0 mg/ml ethylene diamine) in same buffer solution to block the free reactive groups on the beads. The amount of immobilized lipase on the magnetic beads was determined by measuring the initial and final concentration of protein in the immobilization medium using the Bradford protein assay method [29]. A calibration curve constructed with lipase solution of known concentration (0.05–0.50 mg/ml) was used in the calculation of protein in the enzyme and wash solutions.

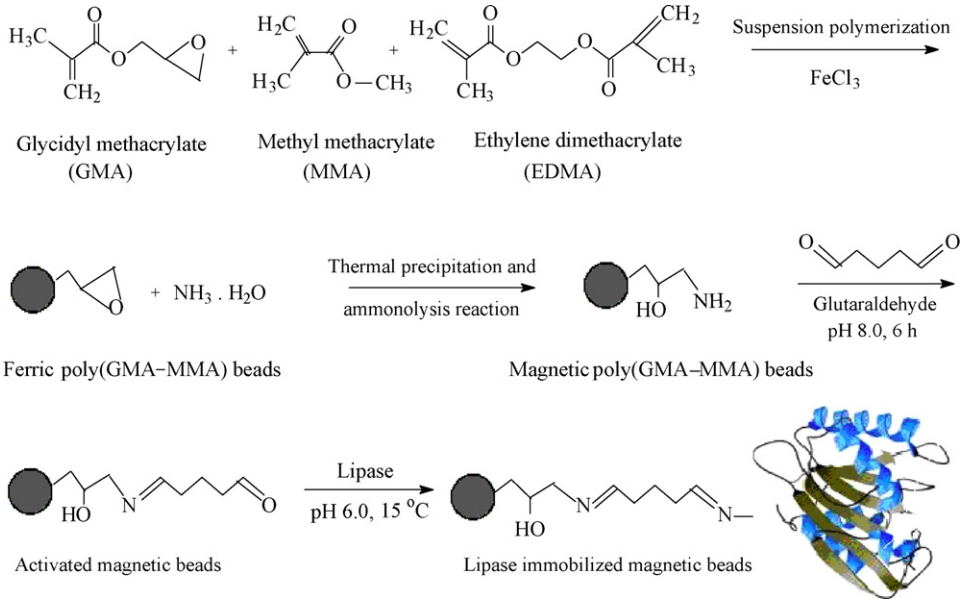

Fig. 1. The reaction schemes for immobilization of lipase onto magnetic beads.

2.4. Activity assays of lipase

The activity of free and immobilized lipase was determined by olive oil hydrolysis. A 100 ml olive oil emulsion was prepared by mixing olive oil (50 ml) and gum Arabic solution (50 ml, 7%, w/v). The assay mixture consisted of emulsion (5 ml), phosphate buffer (2.0 ml, 100 mM, pH 7.0) and free enzyme (0.5 ml, 1.0 mg/ml) or immobilized enzyme (0.5 g magnetic beads). Oil hydrolysis was carried out at 35 °C for 30 min in a shaking water-bath at 150 rpm. The reaction was stopped by the addition of 10 ml of acetone–ethanol solution (1:1, v/v). The liberated fatty acid in the medium was determined by titration with 50 mM NaOH solution. These activity assays were carried out over the pH range 4.0–9.0 and temperature range 20–60 °C to determine the pH and temperature profiles for the free and the immobilized enzymes.

2.5. Determination of the kinetic parameters of the free and immobilized enzyme

The kinetic constants were determined using tributyrin as substrate (in the concentrations range (5–50 mM) using free and immobilized lipase and titrating the butyric acids produced with 50 mM NaOH as described above. The experiments were conducted under the optimized assay conditions. The apparent K_m and V_{max} values for the free and immobilized lipase were calculated from Lineweaver–Burk plots by using the initial rate of the enzymatic reaction data:

$$v^{-1} = \left\{ \frac{K_m}{V_{max}[S]^{-1}} \right\} + V_{max}^{-1} \quad (1)$$

where [S] was the concentration of substrate, v and V_{max} represented the initial and maximum rate of reaction, respectively. K_m was the Michaels constant. One lipase unit corresponded to release of 1 μmol fatty acid per minute under assay conditions. The specific activity is the number of lipase units per mg protein.

2.6. Thermal stability and repeated use of the immobilized lipase

The thermal stabilities of the free and immobilized lipase were determined by incubation in substrate-free phosphate buffer solution (pH 7.0, 50 mM) at two different temperatures (55 and 65 °C) under continuous shaking at 150 rpm. At 15 min time intervals, the remaining activities of the free and immobilized lipase were measured as described above. In addition, the repeated usability of the immobilized lipase was determined by the hydrolysis of olive oil after removal of immobilized lipase with magnetic separation device from reaction medium and compared with the first run (activity defined as 100%).

2.7. Storage stability

The storage stability of free and immobilized lipase were determined after storage in phosphate buffer (50 mM, pH 7.0) at 4 °C during 8 weeks. The residual activities were then determined as described above and activity of the each enzyme was expressed as percentages of its residual activity compared to the initial activity.

2.8. Characterization of magnetic poly(GMA–MMA) beads

The free amino group's content of the magnetic poly(GMA–MMA) beads was determined by potentiometric titration. The aminated magnetic poly(GMA–MMA) beads about 1.0 g were transferred in HCl solution (0.1 M, 20 ml) and it was then incubated in a shaking water-bath at 35 °C for 6 h. After this reaction period, the final HCl concentration

in the solution was determined by a potentiometric titration with 50 mM NaOH solution. The surface morphology of the magnetic beads was observed by scanning electron microscopy (SEM). The dried magnetic poly(GMA–MMA) beads were coated with gold under reduced pressure and their scanning electron micrographs were obtained using a JEOL (Model JSM 5600; Japan). The average size and size distribution of the poly(GMA–MMA) beads were determined by screen analysis performed by using molecular sieves. The surface area of the magnetic poly(GMA–MMA) beads was measured with a surface area apparatus (BET method). The magnetization curves of the dried magnetic poly(GMA–MMA) beads were determined with a vibrating-sample magnetometer (VSM, model 155, Digital Measurement System Inc., Westwood, MA, USA).

3. Results and discussion

3.1. Properties of magnetic poly(GMA–MMA) beads

The ferric ions containing poly(GMA–MMA) beads were prepared from GMA and MMA via suspension polymerization in the presence of $FeCl_3$ as a precursor for the thermal precipitation of iron oxide. In the next step, the classical thermal precipitation reaction was carried out in $NH_3{\cdot}H_2O$ aqueous solution containing Fe(II) ions for the formation of iron oxide crystal within the structure of the beads. The formation of iron oxide crystal within the poly(GMA–MMA) bead structure has been evidenced by gravimetric analysis, FTIR and vibrating-sample magnetometer. During the thermal precipitation reaction, the epoxy groups of the magnetic beads (size between 100 and 150 μm) were converted into amino groups via ammonolysis reaction due to the presence of ammonia in the medium. The enzyme "lipase" was immobilized on the magnetic beads after glutaric dialdhyde activation of the amino groups. The specific surface area of the magnetic poly(GMA–MMA) beads was measured by BET method and was found to be 16.2 m^2/g beads.

The formation of iron oxide crystals within the magnetic beads have been also evidenced by VSM. The plots of magnetization versus magnetic field curve for the bare and enzyme immobilized magnetic beads at room temperature are presented in Fig. 2. The specific saturation magnetization (σ_s) was found to be 19.8 emu/g for magnetic beads. In the literature, the reported (σ_s) values for magnetic poly(methacrylate–divinylbenzene) beads and magnetic nanoparticles were 14.6 emu g^{-1} [30] and 92.0 emu/g [31], respectively. As seen in this figure, the very weak hysteresis indicated the super-paramagnetic nature of the beads. This could be due to the formed magnetic nanoparticles within the beads structure should be smaller than 20 nm, and they might be considered to have a single magnetic domain. Finally, the magnetic beads developed in this study were not needs high magnetic intensity. So, the magnetic beads can be easily separated from reaction medium within a few second by a conventional permanent magnet. When the applied magnetic force is removed, the magnetic beads can easily be dispersed by simple shaking. Thus, the magnetic beads can be removed or recycled in the medium with a simple magnetic device [8,20,21].

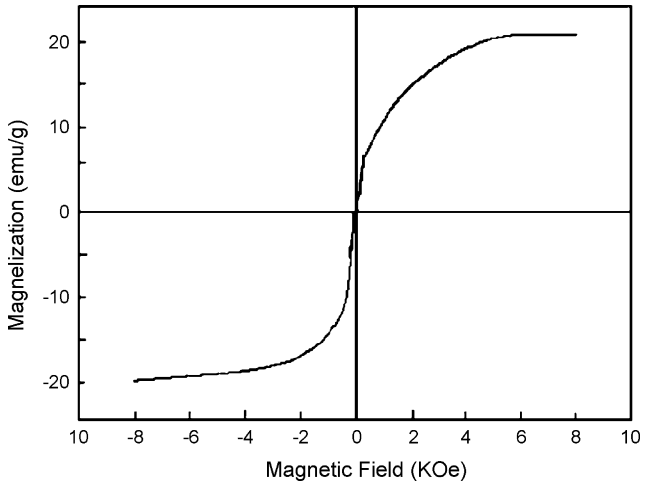

Fig. 2. Magnetization vs. magnetic field for the magnetic poly(GMA–MMA) beads at room temperature.

The amount of formed of iron oxide crystal in the beads structure was by gravimetric analysis and was found to be 134 mg/g beads.

FTIR spectra of magnetic poly(GMA–MMA) beads are presented in Fig. 3. The FTIR spectra of magnetic poly(GMA–MMA) have the characteristic stretching vibration band of hydrogen-bounded alcohol at ~3480 cm^{-1}. Among the characteristic vibrations of both GMA and MMA is the methylene vibration at ~2960 cm^{-1}. The vibration at 1736 cm^{-1} represents the ester configuration of both MMA and GMA. The vibration peak is the carbonyl configuration at 1152 cm^{-1} of the polymer. On the other hand, several bands appear in the finger print region for magnetic beads between 1600 and 1200 cm^{-1}. These peaks are assigned to the $–CH_2$ scissoring band of both GMA and MMA at 1459 cm^{-1}, anti-symmetric and symmetric stretching band of carbonyl groups of GMA and MMA at 1395 and 1267 cm^{-1}, respectively. The epoxide group gives the band between at 971 cm^{-1} (epoxy ring vibrations). Fe_3O_4 has the characteristic band at 617 cm^{-1} and also this indicates that Fe_3O_4 molecules are successfully formed within the structure of poly(GMA–MMA) beads.

Scanning electron microscopy (SEM) micrographs presented in Fig. 4. The SEM micrograph shows that the magnetic beads

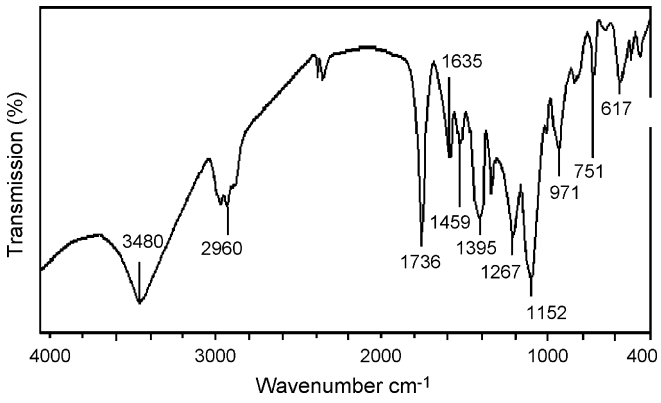

Fig. 3. FTIR spectrum of the magnetic poly(GMA–MMA) beads.

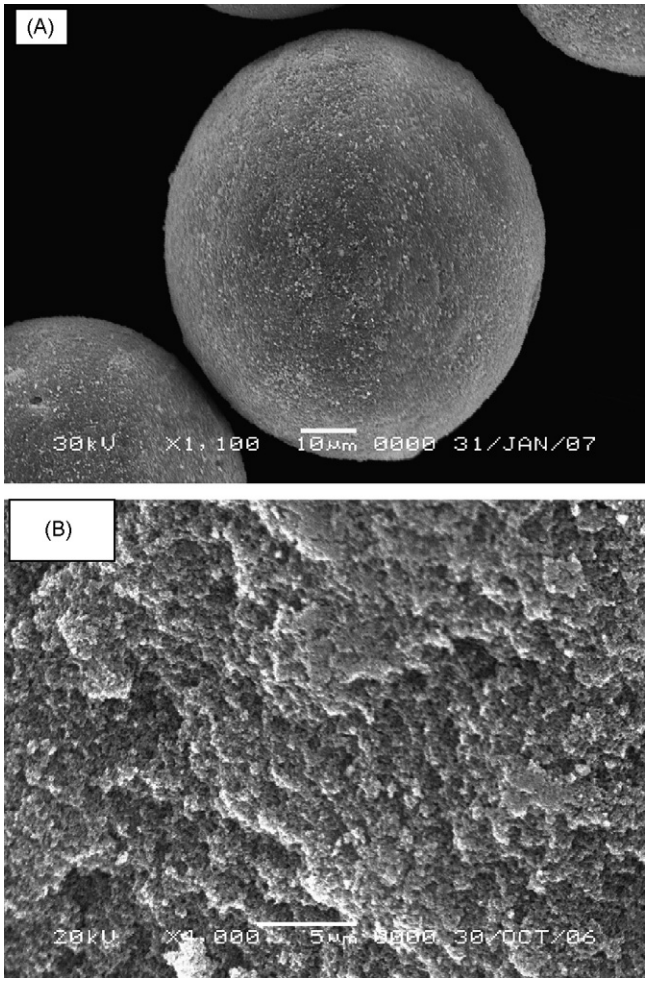

Fig. 4. SEM micrographs of the magnetic poly(GMA–MMA) beads: (A) 1100× magnification and (B) surface of magnetic beads at 4000× magnification.

have a porous surface structure. The porous surface properties of the magnetic poly(GMA–MMA) beads would favour higher immobilization capacity for the enzyme due to increase in the surface area.

3.2. Immobilization of lipase onto magnetic poly(GMA–MMA)beads

In order to optimize the immobilized lipase onto magnetic poly(GMA–MMA)–GA beads; the coupling reaction time was varied between 2.0 and 12.0 h. As seen in Fig. 5, an increase in the coupling duration time led to an increase in the immobilization efficiency (from 9.87 to 23.44 mg/g magnetic beads) but this relation levelled off at around 8.0 h. Further increase in the coupling duration time (up to 12.0 h) did not lead to a significant change in the immobilization capacity. As presented in Fig. 5, the amount of immobilized lipase was found to be 23.44 mg/g magnetic beads with a recovered activity yield of 81%. The protein content in the washed buffer solution was negligible. The measured specific activity of the free and immobilized lipase was about 935 U mg/protein and 757 U mg/immobilized protein, respectively.

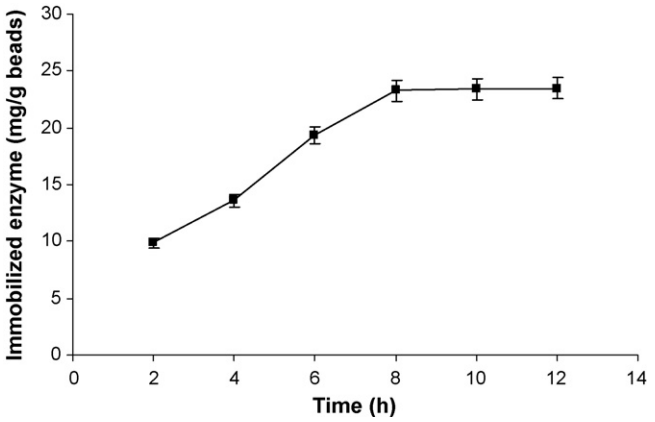

Fig. 5. Effect of coupling reaction time on the immobilization efficiency of lipase on the magnetic poly(GMA–MMA) beads: the experimental conditions are: pH 8.0, reaction coupling time varied between 1.0 and 8.0 h.

3.3. Properties of free and immobilized lipase

The effect of pH on the activity of free and immobilized lipase in olive-oil hydrolysis was carried out in the pH range 4.0–9.0 and the results are presented in Fig. 6. The maximum activity for free enzyme was observed at pH 7.0. The optimum pH value of the immobilized lipase was shifted 1.0 U to the alkaline region. In other similar research, the optimum pH value of the immobilized lipase was generally slightly shifted toward alkaline region [10,13]. The shift depends on the method of immobilization as well as the structure and charge of the matrix. It might be a result of the basic nature of the amino functionalized surface of the magnetic beads, the amino groups on the beads surface can prevent the uniform distribution of hydrogen ions between the surface and the bulk solution. In addition, an improved stability upon covalent immobilization may explain this shift in the optimal pH. It should be noted that the pH profiles of the immobilized lipases are broader than that of the free enzyme, which means that the immobilization methods preserved the enzyme activity in a wider pH range. These results could probably be attributed to the stabilization of lipase molecules resulting from multipoint attachment of the enzyme molecules on the surface

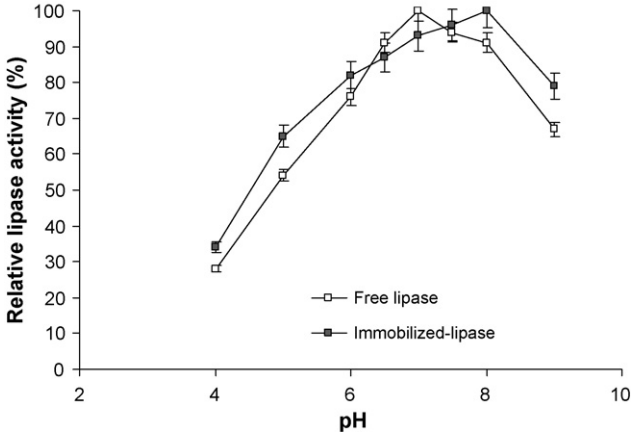

Fig. 6. pH profiles of the free and immobilized lipase.

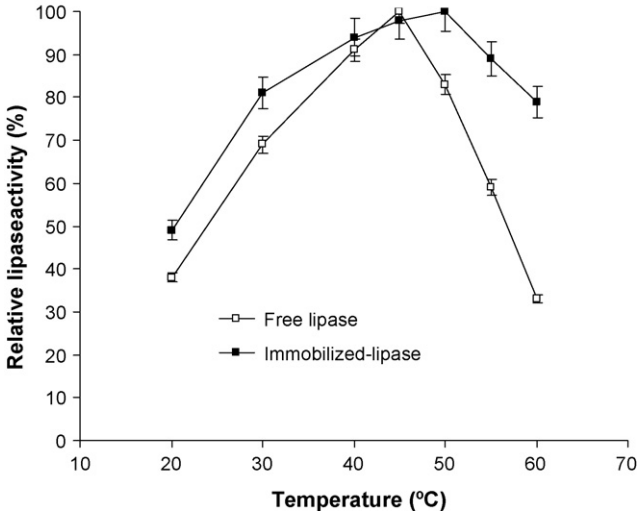

Fig. 7. Temperature profiles of the free and immobilized lipase.

of the magnetic poly(GMA–MMA)–GA beads.

The effect of temperature on the free and immobilized lipase activities was investigated between 20 and 60 °C by using olive oil as substrate. As seen in Fig. 7, maximum activity for the free and immobilized lipase was observed at 45 and 50 °C, respectively. This could be explained by creation of conformational limitations on the enzyme movements as a result of formation of covalent bonds between the enzyme and the support. In general, the effect of changes in temperature on the rates of enzyme-catalysed reactions does not provide much information on the mechanism of biocatalysts. However, these effects can be important in indicating structural changes in enzyme [31–34].

3.4. Kinetic parameters of free and immobilized lipase

The initial reaction rates of the hydrolysis of the substrate (tributyrin) were measured at different substrate concentrations (i.e. 5–50 mM) with the free and the immobilized lipase. The kinetic data for hydrolysis of tributyrin was fitted to the Michaelis-Menten equation. The Lineweaver–Burk plot of $1/v$ versus 1/S, Michaelis constant (apparent K_m) and the maximum reaction velocity (V_{max}) of the free and the immobilized enzymes were calculated. The kinetic parameters of free and immobilized enzymes are presented in Table 1. For the free lipase the apparent K_m value was found to be 2.6 mM, and the V_{max} was calculated to be 987 U/mg enzymes. K_m value was found to be 12.3 mM for the immobilized enzyme. The apparent K_m for the immobilized enzyme was increased by about 4.73-fold compared to the free enzyme. This indicates an alteration in the affinity of the enzyme towards to the substrate upon covalent immobilization on the magnetic beads and/or the higher apparent K_m value for the immobilized lipase might be due to mass transfer limitations [8,35]. The V_{max} value of immobilized enzyme was calculated as 773 U/mg enzymes. The V_{max} value of the immobilized enzyme decreased about 1.7-fold compared to the free enzyme (Table 1).

Several reasons can account for the variations of the K_m and V_{max} values of the enzyme upon immobilization [8,36]. These variations are attributed to several factors such as the non-covalent interactions of the immobilized enzyme molecule with the magnetic beads surface might have induced an inactive conformation to the enzyme molecules. It should be noted that the immobilization process does not also control the proper orientation of the immobilized enzyme on the support. This improper fixation and/or the change in the property of the active sites might hinder the active site for binding of substrates (i.e., tributyrin) to the immobilized lipase molecules. It should be noted that the solubility of the substrate could also affect the kinetic parameters of the immobilized enzyme. The efficiency factor η can be calculated from the maximum reaction rates of the immobilized enzyme over that of the free counterpart:

$$\eta = \frac{v_{\text{immobilized}}}{v_{\text{free}}} \quad (2)$$

where $v_{\text{immobilized}}$ was the reaction rate of the immobilized enzyme and v_{free} that of the free enzyme. From this calculation, magnetic beads enzyme system provided an efficiency factor of 0.783 for the immobilized lipase. The ratio A_{max}/K_m defines a measure of the catalytic efficiency of an enzyme–substrate pair. In this study, the catalytic efficiencies (A_{max}/K_m) of the free and immobilized lipase were found to be 379.6 and 62.8, respectively (Table 2). The catalytic efficiency of lipase was decreased about 6.0-fold upon immobilization.

Table 2
Half-lives ($t_{1/2}$) and inactivation rate constant (k_i) of the free and immobilized lipase at two different temperatures

Temperature (°C)	Free enzyme		Immobilized enzyme	
	$t_{1/2}$ (min)	k_i (min)	$t_{1/2}$ (min)	k_i (min)
55	162	3.84×10^{-3}	429	1.41×10^{-3}
65	77	1.06×10^{-2}	140	4.66×10^{-3}

3.5. Thermal stability and repeated use

The effect of temperature on the thermal stability of free and both immobilized enzymes are presented in Fig. 8. The free lipase retained about 63% and 28% of its initial activity at 55 and 65 °C after a 120 min incubation period, respectively. After a 120 min heat treatment at 55 °C, immobilized lipase retained about 84% its initial activity. At 65 °C, this was 57%. The half-lives and the thermal inactivation rate constant at 55 and 65 °C

Table 1
Properties of the free and immobilized lipase on the magnetic beads

Form of enzyme	K_m (mM)	V_{max} (U/mg enzyme)	Enzyme loading (mg enzyme/g beads)	Efficiency factor	Catalytic efficiency (V_{max}/K_m)
Free lipase	2.6	984	–	–	379.6
Immobilized lipase	12.3	773	23.44	0.783	68.2

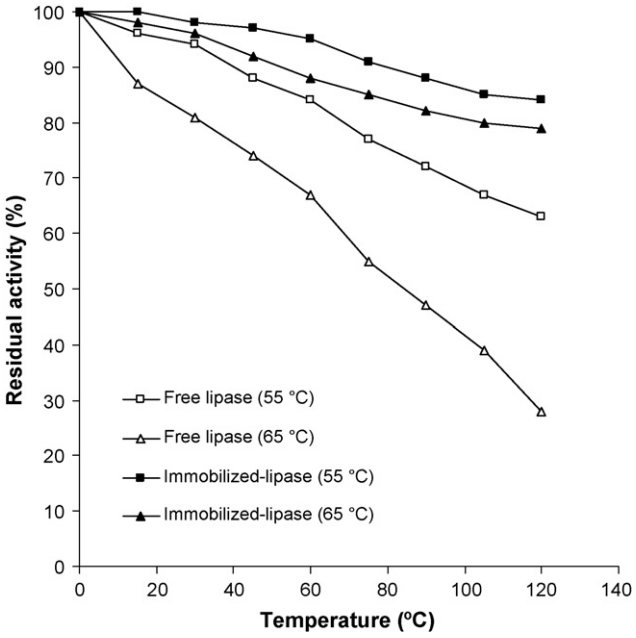

Fig. 8. Thermal stabilities of the free and immobilized lipase at two different temperatures.

were determined ($\ln A = \ln A_0 - k_i t$, where A_0 and A are the initial activity and the activity after time t, min) and presented in Table 1. These results suggest that the thermostability of immobilized lipase becomes significantly higher than that of the free enzyme at high temperature. This is to the covalently immobilized enzyme being protected from conformational changes causing effect of the environment. Similar results have been previously reported for various covalently immobilized enzymes [36–40].

The repeated usability of the covalently immobilized lipase was studied under batch operation mode. After first run, the immobilized lipase was recovered from reaction medium by magnetic separation device and washed extensively with phosphate buffer solution (50 mm, pH 7.0), and then fresh reaction medium was added for next run to determine the enzymatic activity. The data shows that the immobilized lipase retained 62% of its initial activity after 10 cycles of usage. This result suggested that the covalently immobilized lipase was stable on the magnetic beads surface. It should be noted that this high operational stability could significantly reduce the operation cost in practical applications.

3.6. Storage stability

The free and immobilized lipase were stored in phosphate buffer (50 mM, pH 7.0) at 4 °C and the activity measurements were carried out for a period of 56 days (Fig. 9). The free enzyme lost its all-initial activity within 28 days. Immobilized lipase lost 37% of its initial activity during 56 days storage period. The experimental results indicate that the immobilization definitely holds the enzyme in a stable position in comparison to the free counterpart. The storage stability of immobilized lipase was more than 80% higher than that of the free enzyme. Thus,

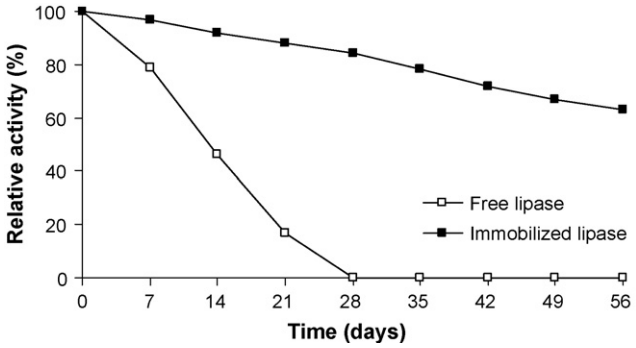

Fig. 9. Storage stability of the free and immobilized lipase.

the immobilized lipase exhibits higher storage stability than that of the free form. As previously reported, the immobilization of enzymes via glutaraldehyde coupling on the supports resulted in a significant storage stability compared to the free counterpart [27].

4. Conclusion

One of the most important aims of enzyme technology is to enhance the conformational stability of the enzyme. The extent of stabilization depends on the enzyme structure, the immobilization methods, and type of support. In this study, the magnetic poly(GMA–MMA) beads (diameter between 100 and 150 μm) were prepared via suspension polymerization. This poly(GMA–MMA)–GA beads has high protein binding capacity and hence can be used for enzyme immobilization. The magnetic beads were used for immobilization of *C. rugosa* lipase. The immobilized enzyme expressed high hydrolysis activity to both olive oil and tributyrin. It also showed a good thermal stability, storage stability and reusability. A high operational stability in repeated use obtained with this method indicates that this immobilized lipase can successfully be used for various biotechnological applications.

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