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Immobilization of *Candida rugosa* lipase on magnetic sol–gel composite supports for enzymatic resolution of (*R*,*S*)-Naproxen methyl ester

Elif Yilmaz, Mehmet Sezgin, Mustafa Yilmaz*

Department of Chemistry, Faculty of Science, Selcuk University, Konya 42075, Turkey

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ABSTRACT

In the present study, the *Candida rugosa* lipase (CRL) was encapsulated within a chemically inert sol–gel support prepared by polycondensation with tetraethoxysilane (TEOS) and octyltriethoxysilane (OTES) in the presence and absence of magnetic Fe_3O_4 nanoparticles or sporopollenin with Fe_3O_4 as additive. The catalytic properties of the immobilized lipases were evaluated through model reactions, i.e. the hydrolysis of *p*-nitrophenylpalmitate (*p*-NPP), and the enantioselective hydrolysis of racemic Naproxen methyl ester that was studied in aqueous buffer solution/isooctane reaction system. The results indicate that the sporopollenin based encapsulated lipase (Fe_3O_4-Spo-E) particularly has higher conversion and enantioselectivity compared to the encapsulated lipase without supports (lipase-enc). It has also been noticed that the sporopollenin based encapsulated lipase has excellent enantioselectivity (E = >400) as compared to the free enzyme (E = 166) with an ee value of ~98% for *S*-Naproxen.

1. Introduction

Lipases (triacylglycerol acylhydrolases, EC 3.1.1.3) are enzymes that are extensively used in organic chemistry and can catalyze a wide range of enantio- and regioselective reactions such as hydrolysis, esterifications, transesterifications, aminolysis and ammoniolysis [1–3]. Lipases have been used for the synthesis or resolution of chiral compounds such as (*R*,*S*)-profens by enantioselective esterification in organic media or enantioselective hydrolysis of their chemically synthesized racemic esters [4–7].

Due to the wide variety of environmental conditions, lipases are often easily inactivated and difficult to be separated from the reaction system for reuse. Consequently, the further industrial applications of lipases are limited [2]. The applications of immobilized enzymes may be challenged by difficulties that arise in enzyme recovery and recycling, which are the most important processes that can decrease the overall cost of the enzyme immobilization process [8–15]. Enzyme recycling using a magnetically separable carrier has proven to be an efficient means to recover coated enzymes [16]. In order to resolve all of the above questions, new immobilized methods have been designed and new supports have also been synthesized [17].

Magnetic supports have been used in enzyme immobilization [18–20] and cell separation [21], which were first applied to immobilize enzymes in 1973 [22]. 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 [23,24].

The sol–gel process is a method that has been found to be suitable for the immobilization of enzymes and other biological molecules. Compared to the other immobilization matrices, sol–gels have many advantages such as entrapment of large amount of enzymes, thermal and chemical stabilities, and simplicity of preparation without any covalent modification and flexibility of controlling pore size and geometry. The most widely used precursors are alkyl-alkoxysilanes. These precursors were used already in the mid-1980s to prepare organically modified silicates for the successful encapsulation of antibodies and enzymes [25–29]. Reetz et al. [30] reported that sol–gel encapsulation has proven to be a particularly easy and effective way to immobilize enzymes and the sol–gel lipase immobilizates were excellent catalysts in the kinetic resolution of chiral alcohols and amines, recycling without any substantial loss in enantioselectivity.

In our previous work [31], sporopollenin which is a natural biopolymer was utilized to encapsulate lipases, and prepared the enzyme by polycondensation with tetraethoxysilane (TEOS). The catalytic properties of the immobilized lipases were evaluated through model reactions, i.e. the hydrolysis of *p*-nitrophenylpalmitate (*p*-NPP), and the enantioselective hydrolysis of racemic Naproxen methyl ester. The results indicate that the sporopollenin based encapsulated lipase particularly has higher

* Corresponding author. Tel.: +90 332 2233873; fax: +90 332 2412499.
E-mail address: myilmaz42@yahoo.com (M. Yilmaz).

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conversion and enantioselectivity compared to the sol–gel free lipase.

In separation science, the application is generally based on the solid-phase magnetic feature which endows a rapid and easy separation with recovery of immobilized enzyme from the reaction mixture by applying an external magnetic field. In the present work, we wish to report the presence and absence of sporopollenin together with Fe_3O_4, as additives on lipase immobilization made by sol–gel process. This strategy provides an efficient way to improve the separation capability of the immobilized lipases as well as those can easily be separated from reaction mixture due to its magnetism.

In this study, we describe another facet of these lipase-immobilizates, namely the use of magnetite-containing analogs. Although we generally separate the catalyst from the reaction mixture by simple filtration, we envisioned an alternative method, specifically magnetic separation known to be efficient in other applications. Thus, in this work *Candida rugosa* lipase (CRL) was encapsulated within a chemically inert sol–gel support prepared by polycondensation with tetraethoxysilane (TEOS) and octyltriethoxysilane (OTES) in the presence and absence of magnetic Fe_3O_4 nanoparticles, or sporopollenin with Fe_3O_4 as additive and explores the effect of these materials in the enantioselective hydrolysis of racemic Naproxen methyl ester. The effect of temperature, pH and thermal/storage stability was also investigated.

2. Materials and methods

2.1. Materials

A commercial lipase powder (lyophilizate) such as *Candida rugosa* lipase (CRL) type VII was obtained from Sigma-Chemical Co., (St. Louis, MO) used in the immobilization. *Lycopodium clavatum* with a particle size of 25 μm was purchased from Fluka Chemicals. Bradford reagent, bovine serum albumin 99% (BSA), *p*-nitrophenylpalmitate (*p*-NPP), TEOS (tetraethoxysilane) and OTES (octyltriethoxysilane) were also purchased from Sigma-Chemical Co., (St. Louis, MO). Pure *S*-Naproxen was purchased from Sigma (USA). The solvents used in HPLC analyses were HPLC grade (Merck, Germany). All aqueous solutions were prepared with deionized water that had been passed through a Millipore Milli-Q Plus water purification system. All other chemicals (Merck, Darmstadt, Germany) were of analytical grade and used without further purification.

2.2. Instrumentation

UV–vis spectra were obtained on a Shimadzu 160A UV–visible recording spectrophotometer. High-performance liquid chromatography (HPLC) analyses were carried out on Agilent 1200 Series using a 1200 model quaternary pump, a G1315B model diode array and multiple wavelength UV–vis detector, a 1200 model standard and preparative auto sampler, a G1316A model thermostated column compartment, a 1200 model vacuum degasser, and an Agilent Chemstation B.02.01-SR2 Tatch data processor. The enantiomeric excess determination was performed with HPLC (Agilent 1200 Series) by using a Chiralcel OD-H column at the temperature of 25 °C with n-hexane/2-propanol/trifluoroacetic acid (100/1/0.1, v/v/v). The flow rate was 1 mL/min and the UV detector was fixed at 254 nm. The surface morphology of samples and particle size were examined by transmission electron microscopy (TEM, FEI Company-TecnaiTM G2 Spirit/Biotwin, USA).

2.3. Synthesis of Fe_3O_4 nanoparticles

The preparation of Fe_3O_4 nanoparticles was followed by a chemical co-precipitation of Fe(III) and Fe(II) ions described previously [32]. With some modifications, 50 mL of 1.0 M $FeCl_2$ and 1.75 M $FeCl_3$ solutions were prepared with deionized water in two beakers, and then transferred to a 250 mL three-necked flask together, stirred under nitrogen. When the solution was heated up to 60 °C NH_4OH (25 wt%) was added dropwise until pH = 10–11. After the addition of base, the solution immediately became dark brown, which indicates the formation of iron oxide in the system. Then solution was heated up to 80 °C for 1 h. The precipitates were isolated from the solvent by magnetic decantation and repeatedly washed with deionized water until neutral and finally dried at room temperature under vacuum for 12 h.

2.4. General procedure for sol–gel encapsulation of lipases (Scheme 1)

Sol–gel encapsulated lipases were prepared according to a modified method of Reetz et al. [30]. A commercial lipase powder (lyophilizate) such as CRL type VII (60 mg) was placed in a 50-mL Falcon tube (Corning) together with phosphate buffer (390 μL; 50 mM; pH 7.0) and the mixture was vigorously shaken with a Vortex-Mixer. The sporopollenin with Fe_3O_4 (0.05 g) or the magnetic Fe_3O_4 nanoparticles (0.05 g) was added. To this solution 100 μL of aqueous polyvinyl alcohol (PVA) (4% w/v), aqueous sodium fluoride (50 μL of 1 M solution) and isopropyl alcohol (100 μL) were added and the mixture was homogenized using a Vortex-Mixer. Then the alkylsilane (2.5 mmol) and TEOS (0.5 mmol; 74 μL; 76 mg) were added and the mixture agitated once more for 10–15 s. Gelation was usually observed within seconds or minutes while gently shaking the reaction vessel. Following drying overnight in the opened Falcon tube, isopropyl alcohol (10 ± 15 mL) was added in order to facilitate the removal of the white solid material (filtration). The gel was successively washed with distilled water (10 mL) and isopropyl alcohol (10 mL). The resulting encapsulated lipases were lyophilized and stored at 4 °C prior to use.

2.5. Enzyme activity assay

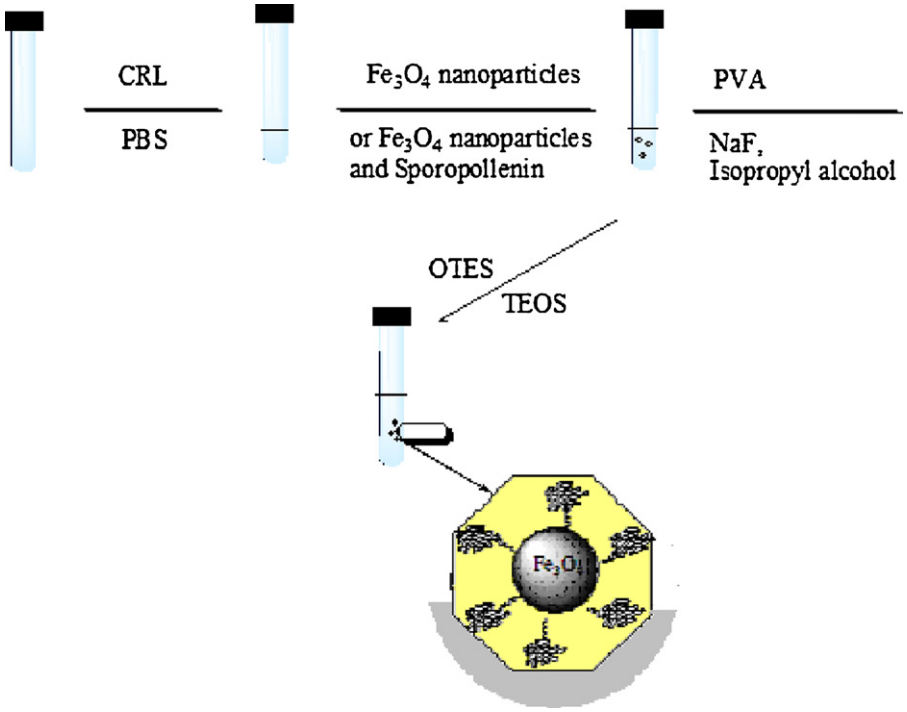
Activity of the free or immobilized lipase was assayed using 0.5% (w/v) *p*-nitrophenyl palmitate in 2-propanol as substrate. To the reaction mixture consisting 1 mL of 0.05 M phosphate buffer (desired pH) and 25 mg of immobilized lipase (or 0.1 mL free lipase) was added 1 mL of substrate that was mixed for 5 min at room temperature [33] for initiation of the reaction. The termination of the reaction has been achieved by adding 2 mL of 0.5 N Na_2CO_3 followed by centrifuging at 4000 rpm for 10 min. The increase in the absorbance measured at 410 nm by a Shimadzu UV-160A (Japan) spectrophotometer is caused by releasing *p*-nitrophenol in the enzymatic hydrolysis of *p*-NPP. A molar extinction coefficient (ε = 410) of 15.000 M^{-1} cm^{-1} for *p*-nitrophenol was used in Beer's law. One unit (U) of lipase activity was defined as the amount of enzyme necessary to hydrolyze 1 μmole/min of *p*-NPP under the conditions of assay [34]. The efficiency of immobilization was evaluated in terms of lipase activity, specific activity and activity yields as follows:

$$\text{lipase activity (U/g-support)} = \frac{\text{activity of immobilized lipase}}{\text{amount of immoblized lipase}}$$

$$\text{specific activity (U/mg-protein)} = \frac{\text{activity of immobilized lipase}}{\text{amount of protein loading}}$$

$$\text{activity yield (\%)} = \frac{\text{specific activity of immobilized lipase}}{\text{specific activity of free lipase}} \times 100$$

The amount of protein in the enzyme solution and the elution solutions was determined by the Brad-



Scheme 1. Schematic illustration of the sol–gel encapsulation protocol.

ford's method [35] using bovine serum albumin as a standard.

2.6. Effect of pH and temperature on activity

The effect of pH on activity of free and immobilized lipases were assayed in the phosphate buffer (50 mM) of pH ranging from 4 to 9 by using the standard activity assay procedure mentioned above.

The rates of thermal inactivation of the free and immobilized lipases were studied in the temperature range 25–60 °C. Both forms of enzyme were incubated in PBS (50 mM, pH 7.0) for 20 min at different temperatures and, after cooling, the remaining activity was assayed under the standard conditions and measured.

2.7. Thermal and storage stabilities

Free and immobilized lipase preparations were stored in the phosphate buffer solutions (50 mM, pH 7.0) at 60 °C for 2 h, respectively. Samples were periodically withdrawn for activity assay. The residual activities were determined as above.

Free and immobilized enzymes were stored at 4 °C in 50 mM phosphate buffer (pH 7.0). The storage stability of enzymes was determined by measurement of the activity of samples taken at regular time intervals and compared.

2.8. Hydrolysis of racemic Naproxen methyl ester

Racemic Naproxen was produced in the laboratory by the racemization of optically pure *S*-Naproxen as described by Wu and Liu [36].

Hydrolysis reactions were carried out in an aqueous phase–organic solvent batch reaction system consisted of 2 mL isooctane as solvent dissolving racemic Naproxen methyl ester (20 mM) and 2 mL buffer solution (pH 7, 50 mM phosphate buffer solution) including encapsulated lipases (5–50 mg depending on the activity). The reactions were carried out in a horizontal shaker at 150 rpm at 30 °C and samples drawn from isooctane phase at 24 h were analyzed by HPLC to calculate the conversion and enantioselectivity.

The enantioselectivity was expressed as the enantiomeric ratio (E) calculated from the conversion (x) and the enantiomeric excess of the substrate (ee_s) and the product (ee_p) using equation of Chen et al. [37].

$$E = \frac{\ln[(1-x)(1-ee_s)]}{\ln[(1-x)(1+ee_s)]}$$

where

$$x = \frac{ee_s}{ee_s + ee_p}, \qquad ee_s = \frac{C_R - C_S}{C_R + C_S}, \qquad ee_P = \frac{C_S - C_R}{C_S + C_R}$$

where E, ee_s, ee_p, x, C_R and C_S denote enantiomeric ratio for irreversible reactions, enantiomeric excess of substrate, enantiomeric excess of product, racemate conversion, concentration of *R*-enantiomer and concentration of *S*-enantiomer, respectively.

3. Results and discussion

3.1. Sol–gel encapsulation procedure using magnetic Fe_3O_4 nanoparticles as additives

In our previous studies [31,38] it has shown that lipases show higher activities and occasionally enhanced stereoselectivities when used in the presence of sporopollenin, or calix[n]arene, calix[n]-NH_2 and calix[n]-COOH (n = 4, 6, 8) compounds.

Sporopollenin forms microcapsules, which have a large internal cavity available for encapsulation with very high loadings. Particular encapsulants previously used for macromolecules are relatively expensive with lower loadings than sporopollenin.

Infrared and ^{13}C NMR spectroscopic studies on sporopollenin derived from pteridophyta and spermatophyta have shown that sporopollenin has aliphatic, aromatic, hydroxyl, carbonyl/carboxyl and ether functions in various portions in its polymeric structure [39]. Therefore, in this study the sporopol-

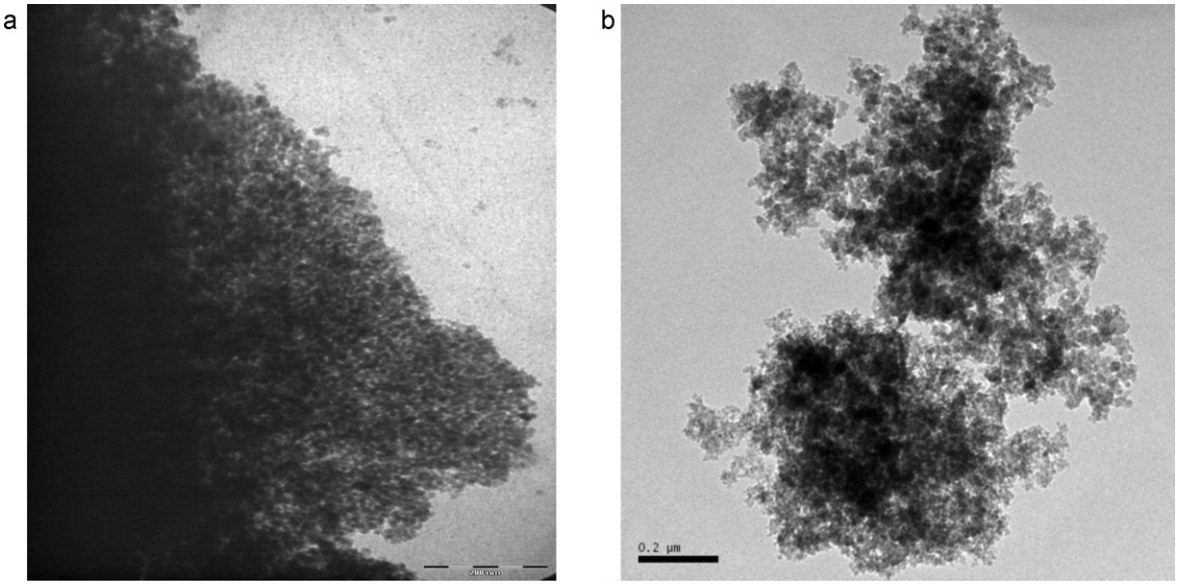


Fig. 1. TEM micrographs of (a) pure Fe_3O_4 nanoparticles and (b) Fe_3O_4-Spo-E nanoparticles.

lenin was chosen as a suitable adsorbent for *Candida rugosa* lipase.

In the literature, magnetic nanoparticles can be easily separated from the reaction medium, stored, and reused with consistent results. This system offers a relatively simple technique for separating and reusing enzymes over a longer period than that for free enzymes alone and for enzymes which are immobilized by physisorption [40,41]. To achieve this goal, we have attempted to synthesize Fe_3O_4 nanoparticles and APTES-modified Fe_3O_4 nanoparticles based on previously published procedures [40]. Most importantly, the encapsulated lipases exhibited enzymatic activity against the *p*-NPP substrate or on the enantioselective hydrolysis reaction of racemic Naproxen methyl ester. These results demonstrate that the *Candida rugosa* lipase was encapsulated within a chemically inert sol–gel support prepared by polycondensation by tetraethoxysilane (TEOS) and octyltriethoxysilane (OTES) in the presence and absence of Fe_3O_4 nanoparticles, or sporopollenin with Fe_3O_4 that was used as an additive.

In order to obtain more direct information on particle size and morphology, TEM micrographs of pure Fe_3O_4 and Fe_3O_4-Spo-E were obtained (see Fig. 1). Observing the photographs, nanoparticles are seen as dense aggregates due to the lack of any repulsive force between the magnetite nanoparticles, which is due to the nano size of the pure Fe_3O_4 nanoparticles. After immobilization, the size of the particles was changed and the dispersion of particles was improved greatly, which can be explained by the electrostatic repulsions and steric hindrance between the lipase and sporopollenin on the surface of Fe_3O_4 nanoparticles.

Table 1 shows the activity of the encapsulated lipases. However, the encapsulated lipase with Fe_3O_4-Spo-E was found to be more efficient as compared to the Fe_3O_4-E with respect to expression of immobilized lipase activity. The Fe_3O_4-Spo-E was performed to give 212.9 U/g of support with 200% activity yield. The encapsulated lipase retains 93%, and 200% of the magnetic nanoparticles activity yield on the Fe_3O_4-E and Fe_3O_4-Spo-E encapsulated lipases, respectively.According to the results obtained, however, the conformational structure of the encapsulated lipase Fe_3O_4-Spo-E does not suffer too much as compared to the encapsulated lipase (Fe_3O_4-E) as their activity was decreased too much.

In our previous work [31], immobilization *of Candida rugosa* lipase was made by sol–gel process and used the sporopollenin (Spo) as additive. It has been observed that under the optimum conditions, the activity yield (%) of the immobilized lipase was 88.8, which is approximately 2.3 times less than that of the encapsulated lipase with sporopollenin with Fe_3O_4 used as additive (Fe_3O_4-Spo-E).

3.2. Effect of pH and temperature on the activity of immobilized enzyme

Fig. 2 illustrates the effect of pH on the activity of the encapsulated lipases. pH is one of the most influential parameters altering enzymatic activity in an aqueous medium. Immobilization of enzyme is likely to result in conformational changes of enzyme resulting in a variation of optimum pH. It is well known that the procedure of enzyme immobilization on insoluble supports has a variety of effects on the state of ionization and dissociation of the enzyme and its environment. Immobilization is likely to result in a conformational change of the enzyme, which leads to inactivity of the enzyme. The catalytic activity of the free and encapsulated lipases in the hydrolysis of *p*-NPP was investigated at different pH values (4.0–9.0).

Upon immobilization on Fe_3O_4-E or Fe_3O_4-Spo-E, the optimum pH for reactions catalyzed by free lipase was slightly shifted toward

Table 1
Activity of the encapsulated lipases under optimum reaction conditions.

	Encap. protein (mg/g)	Encap. protein yield (%)	Lipase activity (U/g support)	Specific activity (U/mg protein)	Activity yield (%)
Encapsulated lipase[a]	28.6	58.4	95.1	3.30	100[b]
Fe_3O_4-E	33.0	78.6	101.8	3,10	93
Fe_3O_4-Spo-E	32.0	76.2	212.9	6.65	200

[a] Encapsulated lipase without Fe_3O_4 or Fe_3O_4-Spo.
[b] Activity yield for encapsulated lipase was defined as 100%.

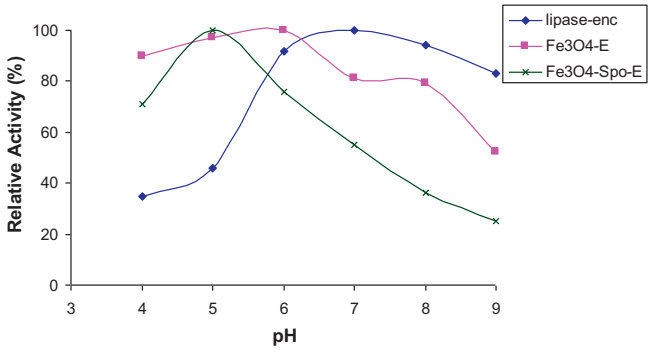


Fig. 2. Effect of substrate pH on residual activity of encapsulated lipases.

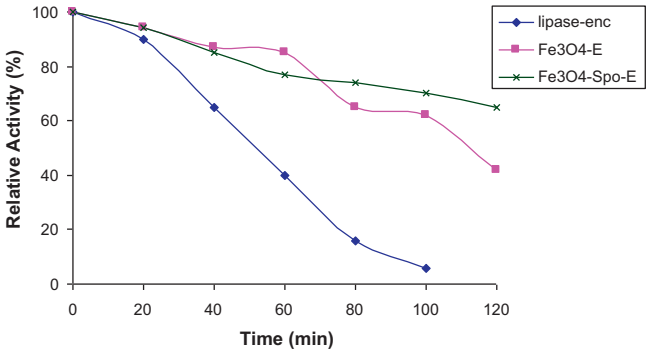


Fig. 4. Thermal stability of immobilized lipases.

acidic values. Generally, an acidic shift in the pH optimum is expected when enzymes are immobilized onto polycationic supports [39]. As shown in Fig. 2, the optimum pH of the encapsulated lipase of magnetic Fe_3O_4 nanoparticles, or Fe_3O_4-Spo-E as additive was 6.0 and 5.0, respectively. Immobilized lipase shows better pH stability and resistance to acidic environment than free lipase. The pH shift depends mainly on the method of immobilization and the interaction of enzyme and support. A change of the optimum pH for *Candida rugosa* lipase immobilized on poly(vinyl alcohol) microspheres was reported by Oh et al. [42]. In our recent study, it has been noticed that lipase (*Candida rugosa*) could be stabilized when encapsulated in the presence of sporopollenin in the pH range of 4.0–10.0 with optimum pH 5.0 [31].

The effect of temperature on encapsulated lipases is given in Fig. 3. The effect of temperature on the activity of encapsulated lipases for *p*-NPP hydrolysis at pH 7.0 in the temperature range of 30–60 °C is shown in Fig. 3. It was found that the optimum temperature for the encap-lipase without supports and Fe_3O_4-E was approximately 35 °C, while it shifted nearly to 40 °C for Fe_3O_4-Spo-E. Furthermore, the temperature profiles of the immobilized lipases are broader than those of the encap-lipase without supports, which means that the immobilization methods preserved the enzyme activity over a wider temperature range. One of the main reasons for enzyme immobilization is the anticipated increase in stability toward various deactivating forces, due to restricted conformational mobility of the molecules following immobilization [43–45]. This was either due to the creation of conformational limitation on the enzyme movement as a result of electrostatic interaction and hydrogen bond formation between the enzyme and the support or a low restriction in the diffusion of the substrate at high temperature. Thus, the immobilized enzymes showed their catalytic activities at a higher reaction temperature [46]. In the literature, similar changes in the optimum temperature have also been reported [47]. In our previous work, it was found that the optimum temperature for encapsulated CRL in the presence of sporopollenin was 40 °C [31].

3.3. Thermal stability and storage on the activity of immobilized lipase

Fig. 4 shows the thermal stabilities of the encap-lipase without supports (lipase-enc) and encapsulated lipases (Fe_3O_4-E and Fe_3O_4-Spo-E). These encapsulated lipases were incubated for 2 h at 60 °C and the enzyme activity was measured at various time intervals. It can be observed that the lipase-enc loses its initial activity within around 100 min at 60 °C, while the encapsulated lipases retain their initial activities of about 42% by Fe_3O_4-E and 65% by Fe_3O_4-Spo-E after 120 min of heat treatment at 60 °C.

Utilization of enzymes in processes often encounters the problem of thermal inactivation of enzyme. At high temperature, enzyme undergoes partial unfolding by heat-induced destruction of non-covalent interaction [48,49]. The resistance of immobilized lipase to temperature at a certain time is an important potential advantage for practical applications of this enzyme. Thermal stability of the immobilized enzyme was greatly improved. Thermal stability of lipase is obviously related with its structure [50]. These results indicate that the thermal stability of the immobilized lipases are much better than that of the free one may be due to the interaction between the enzyme and the support, which could prevent the conformation transition of the enzyme at high temperature.

The storage stability of the encapsulated enzymes was clearly better than the lipase-enc (Fig. 5). Thus, the support and the technique of immobilization provided a longer shelf life than those of free counter part [51]. The retention in activity is usually observed after enzyme immobilization. This could be explained by the modification in three-dimensional structure of the enzyme, which leads to conformation change of the active center. The presence of matrix hinders the accessibility of substrate to the enzyme active site, and

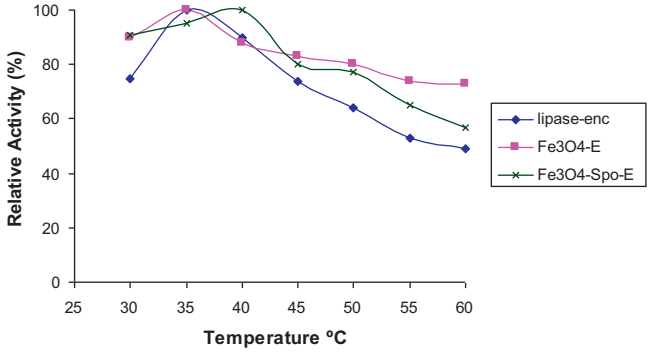


Fig. 3. Effect of reaction temperature on the residual activity of immobilized lipases.

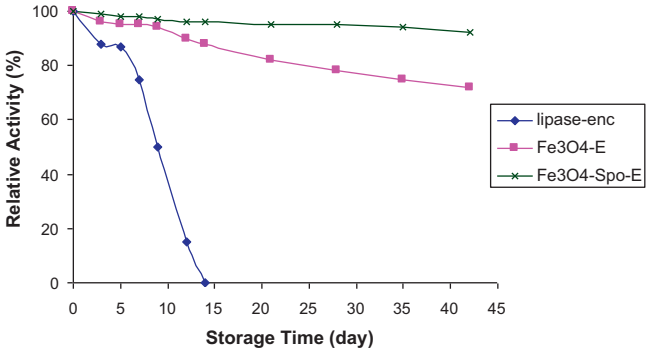


Fig. 5. Storage stability of immobilized lipases.

Table 2
Enantioselective hydrolysis of racemic Naproxen methyl ester using immobilized lipases[a].

	x (%)	ee$_s$ (%)	ee$_p$ (%)	E
Lipase encap.[b]	37.9	60	>98	166.0
Fe_3O_4-E	30.7	44	>98	170.5
Fe_3O_4-Spo-E	47.0	88	>98	>400

[a] Enantiomeric excess (ee) as determined by Chiral HPLC, Agilent 1200 Series-chiral column (Chiralcel OD-H); n-hexane/2-propanol/trifluoroacetic acid (100/1/0.1, v/v/v) as mobile phase; time, 24 h; concentration of substrate, 20 mM; temperature, 35 °C.
[b] Encapsulated free lipase without Fe_3O_4 or Fe_3O_4-Spo.

limitation of mass transfer of substrate and product to or from the active site of the enzyme may also be responsible. This explanation is in agreement with the results reported [52]. The ability to be stored for a period of time at a certain temperature is one of the key factors to be considered when using immobilized lipases. Both lipases obtained show their full activity (100%) when stored at 4 °C. Generally, enzymes are still active when kept at low temperature probably because lipases tend to lock to its original conformation, which is catalytically active. The encapsulated lipase without Fe_3O_4-E, Fe_3O_4-Spo-E rapidly loses its activity with a residual value of 15% after 12 days, the decrease in activity occurs more slowly with the encapsulated lipases, and about 90% (Fe_3O_4-E) and 96% (Fe_3O_4-Spo-E) of their initial activity were recovered after the same period. The stability achieved was referred to multiple attachment of the enzyme to the support, preventing any intermolecular process such as proteolysis and aggregation, therefore creating a more rigid enzyme molecule [53].

3.4. Enantioselective hydrolysis of racemic Naproxen methyl ester with the immobilized lipases

From an industrial point of view, the quality of a given kinetic resolution depends not only on the degree of enantioselectivity, but also on the activity and the possibility of recycling and reusing the lipase. We therefore studied all of these factors in a test reaction involving the hydrolysis reaction-kinetic resolution of (*R*,*S*)-Naproxen methyl ester, although complete optimization was not strived for. All reactions were carried out on a small scale and stereoselectivity was ascertained by measuring the selectivity factor E on the basis of the formula of Chen et al. [37].

Table 2 shows the results obtained in the resolution of racemic Naproxen methyl ester catalyzed by encapsulated lipases. It shows the conversion (x), enantiomeric excess (ee) and enantiomeric ratio (E) in the course of (*R*,*S*)-Naproxen methyl ester hydrolysis by the encapsulated lipases. The enantioselective hydrolysis of racemic Naproxen methyl ester by sol–gel encapsulated lipases was studied in aqueous buffer solution/isooctane reaction system.

The resolution reaction with encapsulated lipases (Fe_3O_4-E, Fe_3O_4-Spo-E) was terminated after 24 h, obtaining Naproxen methylate (unreacted *R*-ester) and corresponding acid (ee$_p$) 98% at conversion of 47% and the enantioselectivity being very high (E > 400). Whereas the resolution reactions with encapsulated lipase without Fe_3O_4-E, Fe_3O_4-Spo-E (lipase-enc) gave an unreacted Naproxen methylate (*R*)-ester and corresponding acid (ee$_p$) 98% at conversion of 37.9% and the enantioselectivity (E) being 166. Immobilization led to high enantioselectivity, high conversion and fast recovery of product compared to free enzyme.

The result was not surprising because in the literature Tsai et al. [54] has used lipase MY from *Candida rugosa* and catalyzed hydrolysis of (*R*,*S*)-Naproxen esters in water-saturated isooctane as the model system. They found E value as 510. In our recent study [38] we reported to use of the calix[n]arenes and their derivatives (carboxyl and amine) as additives for the sol–gel encapsulation

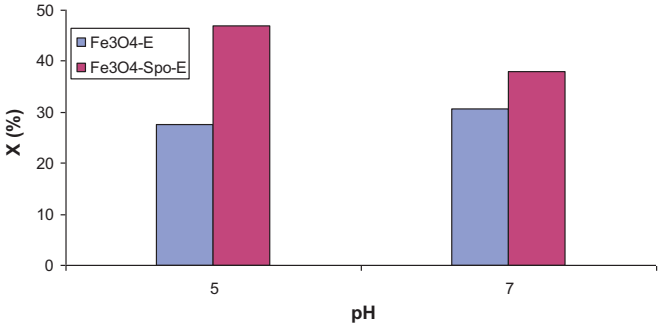


Fig. 6. Effect of pH on the conversion (x) in the hydrolysis of racemic Naproxen methyl ester.

of lipase and effect of the calix[n]arene derivatives in the enantioselective hydrolysis reaction of (*R*/*S*)-Naproxen methyl ester. The resolution reaction with encapsulated lipase was terminated after 24 h, obtaining Naproxen methylate (unreacted R-ester) and corresponding acid (ee$_p$) 98% at conversion 46.3% and the enantioselectivity being very high (E > 200).

In our previous work [31] it was found that excellent enantioselectivity (E > 400) has been noticed for most lipase preparations with an ee value ~98% for *S*-Naproxen. The results indicate that in particular sporopollenin based encapsulated lipases have higher conversion and enantioselectivity compared to the sol–gel free lipase.

In chiral resolution using enzyme as a catalyst, it has been reported that variation of pH might influence chiral selectivity since the conformation of an enzyme depends on its ionization state [55]. The effects of pH on enantioselectivity of encapsulated lipase were determined by incubating encapsulated lipase in the presence of encapsulated lipase (Fe_3O_4-E or Fe_3O_4-Spo-E) at different pH values (i.e. pH 5.0 and 7.0) and, at 35 °C for 24 h. At the end of the incubation time the rate of enzyme reaction and enantiomeric excess (ee) were determined using HPLC (Agilent 1200 Series) equipped with Chiralcel OD-H column at the temperature of 25 °C. The optimum pH values were determined from the graph of pH plotted against the percentage of conversion (x) (Fig. 6). The optimum pH value was found to be 5.0 for Fe_3O_4-Spo-E.

The temperature dependence of the percentage of conversion (x) of the hydrolysis reaction catalyzed by immobilized lipases was studied in the interval from 35 °C to 50 °C and the results are shown in Fig. 7. The results showed that the conversion degree of the optimum temperature for both Fe_3O_4-E and Fe_3O_4-Spo-E was approximately 35 °C and decreased with increase in temperature from 35 °C to 50 °C.

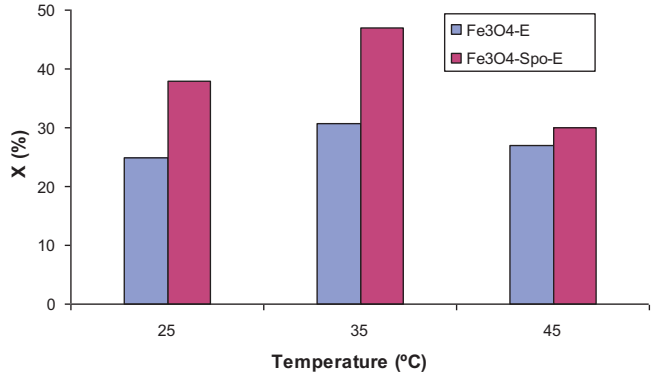


Fig. 7. Effect of temperature on the conversion (x) in the hydrolysis of racemic Naproxen methyl ester.

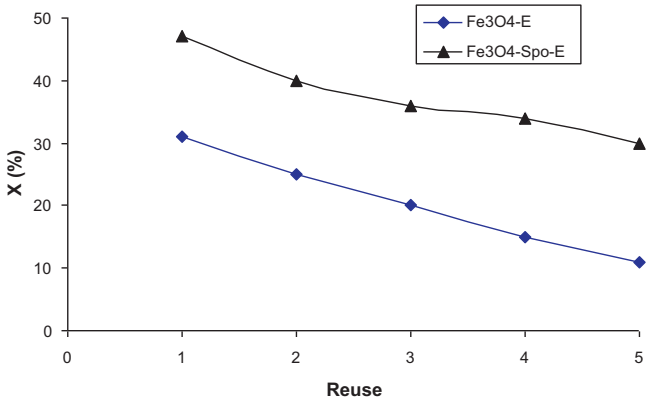


Fig. 8. Reusability on the conversion (x) in the hydrolysis of racemic Naproxen methyl ester.

After the encapsulation of CRL in the presence of Fe_3O_4 and Fe_3O_4-Spo, the encapsulated lipases were not soluble in water due to an increase in the cross-linked bond between the enzymes. Thus, it was used in the reusability studies, where after each run; the encapsulated lipases were washed with PBS. It was found that the percent conversion (x) of the encapsulated lipase with Fe_3O_4 or Fe_3O_4-Spo decreases after the fourth usage. Fig. 8 shows that the encapsulated lipases were still retained 11% and 30% of their conversion ratios for Fe_3O_4-E or Fe_3O_4-Spo-E after the 5th reuse, respectively. These results are due to the inactivation of the enzyme denaturation of protein and the leakage of protein from the supports upon use.

4. Conclusions

In this work, *Candida rugosa* lipase (CRL) was immobilized by sol–gel encapsulation technique within a chemically inert sol–gel support prepared by polycondensation with tetraethoxysilane (TEOS) and octyltriethoxysilane (OTES) in the presence and absence of magnetic Fe_3O_4 nanoparticles, or sporopollenin with Fe_3O_4 as additive. The catalytic activity of the encapsulated lipases was evaluated into model reactions, i.e. the hydrolysis of *p*-nitrophenylpalmitate (*p*-NPP) and the enantioselective hydrolysis of racemic Naproxen methyl ester. It was observed that the encapsulated sporopollenin Fe_3O_4 (Fe_3O_4-Spo-E) was found to give 212.9 U/g of support with 200% activity yield, while Fe_3O_4-E was found to give 101.8 U/g of support with 92.9% activity yield. It was observed that excellent enantioselectivity for the encapsulated sporopollenin ($E > 400$) was obtained ($E = 166$ for the lipase-enc) with an ee value of *S*-Naproxen about 98%. On the basis of these results, we recommend immobilized lipases as a prospective preparation for continuous industrial applications.

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