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Enantioselective hydrolysis of rasemic naproxen methyl ester with sol–gel encapsulated lipase in the presence of sporopollenin

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ABSTRACT

Sporopollenin is a natural polymer obtained from *Lycopodium clavatum*, which is highly stable with constant chemical structure and has high resistant capacity to chemical attack. In this 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 sporopollenin and activated sporopollenin as additive. The catalytic properties of the immobilized lipases were evaluated into model reactions, i.e. the hydrolysis of *p*-nitrophenylpalmitate (*p*-NPP), and the enantioselective hydrolysis of rasemic Naproxen methyl ester that was studied in aqueous buffer solution/isooctane reaction system. The results indicated that the sporopollenin based encapsulated lipase particularly had higher conversion and enantioselectivity compared to the sol–gel free lipase. In this study, excellent enantioselectivity ($E > 400$) has been noticed for most lipase preparations ($E = 166$ for the free enzyme) with an ee value ~98% for *S*-Naproxen. Moreover, (*S*)-Naproxen was recovered from the reaction mixture with 98% optical purity.

1. Introduction

Sporopollenin is a natural biopolymer which occurs in the outer membranes of moss and fern spores and most pollen grains. It has been shown that spore and pollen membranes have two layers, an inner one known as intine, and an outer one the exine containing a material to which the name sporopollenin was given. Sporopollenin is stable and highly resistant to chemicals. It has a constant chemical structure and exhibits very good stability even after a prolong exposure to mineral acids and alkalies [1]. The mechanism of its synthesis and its consolidation are not yet understood [2]. In the past two decades modified forms of sporopollenin have been utilized as anion, ligand [3], and as cation exchangers for removal of heavy metal ions from aqueous solutions [4].

Sporopollenin offers several advantages over man made and other naturally occurring materials (e.g. acrylic resins, chitosan). It is easily extracted from its natural source, spores or pollen, using cheap non-toxic reagents which are used in the food industry. It forms microcapsules which have a large internal cavity available for encapsulation with very high loadings. The particles are monodispersed and, from one species, uniform in size, morphology and chemical composition, i.e. sporopollenin contains only carbon, hydrogen and oxygen. It is thus free from any allergens. Such uniformity is difficult and expensive to achieve in man made products, especially possessing a large cavity capable of being filled with a wide range of polar and non-polar materials. Particular encapsulants previously used for macromolecules are relatively expensive with lower loadings than sporopollenin [5].

Sol–gel encapsulation has proven to be a particularly easy and effective way to immobilize enzymes [6]. Following isolated reports describe the specific examples; it was the seminal work of Avnir and co-workers that led to the generalization of this technique [6–8].

A well-established sol–gel processing technique consists in hydrolyzing adequate precursors in aqueous solutions to produce soluble hydroxylated monomers, followed by polymerization and phase separation to produce a hydrated metal or semi-metal oxide hydrogel [9]. Removal of water from the wet gel, which is usually accompanied by changes in the structure of the pores and of the gel's network, results in a porous xerogel. The most widely used precursors are alkyl-alkoxysilanes. These precursors were used already in the mid-1980s these precursors were used to prepare organically modified silicates for the successful encapsulation of antibodies and enzymes [10,11]. Although the final structure of the material is basically determined by the differences in chain length, functionality and hydrophobic character of the precursors, it can be tailored *via* the addition of a wide range of molecules. Examples include surfactants [12–14], room-temperature ionic liquids [15], crown ethers, β-cyclodextrins or porous solid supports like Celite [16]. The commonly used catalysts are weak acids or bases [9,16]. Recent research describes the use of other species that include

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peptides like silaffins [17], polyamines [18] or enzymes such as hydrolases [19] and silicateins [20].

Candida rugosa is an important industrial lipase due to its wide application in oil hydrolysis, transesterification, esterification and enantioselective biotransformation [21,22]. Thus, lipase is becoming one of the most industrially used enzymes due to its high stereoselectivity, regioselectivity, and low price [23]. (*S*)-(+)-2-(6-Methoxy-2-naphthyl)propionic acid (Naproxen) is a non-steroidal anti-inflammatory drug that belongs to the family of 2-aryl propionic acid derivatives, it is widely used as a drug for human connective tissue diseases. The physiological activity of the *S* form of Naproxen is 28-fold that of the *R* form [24]. Hence, only the *S* form is used as a drug for humans [25–27]. Of the profen drugs, an important family of non-steroidal anti-inflammatory drugs, Naproxen is now the only member to be sold as a single enantiomer.

In the case of Naproxen, lipase has been used to prepare optically pure Naproxen by enantioselective hydrolysis of its racemic esters [28–30]. In addition, the preparation of Naproxen ester prodrugs has been accomplished by resolution of racemic Naproxen mixtures [31–33]. More recently, alcohol and buffer or acetone treatment method has been developed to improve the enantioselectivity of CRL towards the hydrolysis of racemic Naproxen methyl ester [34,35]. The changes in the enantioselectivity of CRL for *S*-Naproxen with the alterations in its microenvironment by changing the medium properties [36], by immobilization [37] and by introducing additives into the reaction medium [38] have also been reported.

To the best of our knowledge, there exists no report on the use of sporopollenin from *Lycopodium clavatum* as support for immobilization of lipase. It has been thought that the sol–gel procedure could be interesting for lipase immobilization. Therefore, we now report the use of sporopollenin (Spo) and activated sporopollenin (Spo_{act}) as additives on lipase immobilization made by sol–gel process and explore the effect of these materials in the enantioselective hydrolysis of (*RS*)-Naproxen methyl ester. The effect of temperature, pH and thermal/storage stability was also investigated.

2. Materials and methods

2.1. Materials

C. rugosa lipase (*CRL*) was a commercial enzyme 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 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

FT-IR spectra were recorded on a PerkinElmer 1605 FT-IR spectrometer as KBr pellets. UV–vis spectra were obtained on a Shimadzu 160A UV-vis recording spectrophotometer. High-performance liquid chromatography (HPLC) Agilent 1200 Series was carried out 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 of 1 mL/min; the UV detector was fixed at 254 nm.

The surface morphology of samples was examined by scanning electron microscope (SEM, Jeol, JSM 5310, Japan).

2.3. Preparation of activated sporopollenin

The sporopollenin exines (25 μm) were extracted from *L. clavatum* as follows: raw *L. clavatum* spores (powdered form, 50 g) were suspended in acetone (150 mL) and stirred under reflux for 6 h. The defatted spores were filtered and then treated with 8% (w/v) potassium hydroxide (150 mL) solution. After that the mixture was refluxed for 15 h. This base-hydrolyzed sporopollenin was filtered and washed with hot water and hot ethanol. It was finally suspended in 85% (w/v) *ortho*-phosphoric acid (150 mL) and stirred under reflux for 7 days. This acid-hydrolyzed sporopollenin was filtered, washed with water, acetone, 2 M hydrochloric acid solution, 2 M sodium hydroxide solution, water, acetone and ethanol and dried at 60 °C until constant weight [39].

2.4. General procedure for sol–gel encapsulation of lipases

Sol–gel encapsulated lipases were prepared according to a modified method of Reetz et al. [14]. 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 Spo or Spo_{act} (0.05 g) was included. Then 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 homogenized using a Vortex-Mixer. Then the alkylsilane (2.5 mmol) and TMOS (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 removal of the white solid material (filtration). The gel was successively washed with distilled water (10 mL), isopropyl alcohol (10 mL). The resulting encapsulated lipases were lyophilized and stored at 4 °C prior to use.

2.5. Determination of enzyme activity

Activity of the free and encapsulated lipases were assayed using 14.4 mM *p*-nitrophenyl palmitate in 2-propanol as substrate. The reaction mixture consisting of 1 mL of 50 mM phosphate buffer (pH 7.0 for immobilized lipase) containing 25 mg of immobilized lipase (or 0.1 mL free lipase) was initiated by adding 1 mL of substrate and mixed for 5 min at 30 °C. The reaction was terminated 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 at 410 nm produced by the release of *p*-nitrophenol in the enzymatic hydrolysis of *p*-NPP was measured in a Shimadzu UV-160A (Japan) spectrophotometer. A molar extinction coefficient (ε410) of 15,000 M^{-1} cm^{-1} for *p*-nitrophenol was used [40]. One unit (U) of lipase activity was defined as the amount of enzyme necessary to hydrolyze 1 μmol/min of *p*-NPP under the conditions of assay. All measurements were performed in triplicate and an average was taken as final result.

2.6. Protein assay

Protein content was estimated by the method of Bradford [41] using Bio-Rad protein dye reagent concentrate. Bovine serum albumin was used as the standard.

2.7. Effect of pH and temperature on activity

The optimum pH and reaction temperature of free and encapsulated lipases were determined as the relative activity under the variety of pH (50 mM phosphate buffer for pH 4.0–10.0) and temperature (25–60 °C). Relative activities were calculated as the ratio of the activity of encapsulated enzyme after incubation to the activity at the optimum reaction pH and temperature.

2.8. Thermal stability and reusability of encapsulated lipases

Free and encapsulated lipase preparations were stored in 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.

The stability of encapsulated enzymes on repeated use was also examined by measuring the activity towards the hydrolysis of *p*-NPP assay. After each activity determination, the encapsulated enzyme was washed with buffer solution (PBS, 50 mM, pH 7.0) and reintroduced into a fresh medium, this procedure being repeated for up to 8 cycles.

2.9. Storage stability

The stabilities of the free and encapsulated lipase were measured by calculating the residual activity after long time storage. Free lipase was stored as a solution of 2 mg/ml in PBS (50 mM, pH 7.0) and the encapsulated lipase was stored in wet form at 4 °C. Enzyme activity was assayed at regular intervals.

2.10. Racemic Naproxen methyl ester production

Racemic Naproxen was prepared by the racemization of optically pure *S*-Naproxen as described by Wu and Liu [42]. The synthesized Naproxen methyl ester was identified with FT-IR, ^{1}H NMR and ^{13}C NMR. FT-IR (KBr); 1765 cm^{-1} (C=O, ester), ^{1}H NMR ($CDCl_3$); δ = 1.58 (s, 3H, CH$\underline{CH_3}$), 3.67 (s, 3H, O$\underline{CH_3}$), 3.86 (q, 1H, $\underline{CH}CH_3$), 3.91 (s, 3H, COO$\underline{CH_3}$), 7.09–7.17 (m, 2H, Ar–H), 7.37–7.43 (m, 1H, Ar–H), 7.64–7.73 (m, 3H, Ar–H). ^{13}C NMR ($CDCl_3$); δ = 18.81, 45.57, 52.27, 55.54, 105.81, 119.21, 126.15, 126.40, 127.39, 129.15, 129.49, 133.91, 135.89, 157.86, 175.36.

2.11. Hydrolysis of racemic Naproxen methyl ester

Hydrolysis reactions were performed in an aqueous phase–organic solvent batch reaction system. A solution of racemic Naproxen methyl ester (20 mM) in 2 mL of isooctane was added to 2 mL buffer solution (pH 7.0, 50 mM phosphate buffer solution) containing encapsulated lipases (5–50 mg depending on the activity), the mixture was treated in an 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.

Enantiomeric excess of the substrate (ee_s) and the product (ee_p) were analyzed by HPLC using Chiral column and the enantiomeric ratio (enantioselectivity, *E*) was calculated according to the equation of Chen et al. [43].

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

where

$$x = \frac{ee_s}{ee_s + ee_p} \quad ee_s = \frac{C_R - C_S}{C_R + C_S} \quad ee_p = \frac{C_S - C_R}{C_S - C_R}$$

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

3. Results and discussion

3.1. Sol–gel encapsulation procedure using sporopollenin as additives

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. Paunov et al. [39] showed that *alfa*-amylase, *beta*-lactase, anti-AIDS drug or oligonucleotides ranging in molecular mass from 1 to 116 kDa, are capable of being encapsulated efficiently into sporopollenin exine particles.

Moreover, in another study Kazlauskas demonstrated that the presence of a small amount of isopropyl alcohol is beneficial [44–46]. Usually interactions of the additive and the lipase on the basis of hydrogen bonds were postulated, although in some cases ambiguities as to the origin of the activating effect remain. In preliminary experiments it was shown that the presence of isopropyl alcohol during the sol–gel process does indeed exert a beneficial effect [14]. Therefore, isopropyl alcohol and PVA were included in all experiments. Besides this, considerably higher loadings were strived for.

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 [47]. Therefore, in this study the sporopollenin and activated sporopollenin were chosen as the suitable adsorbents for *C. rugosa* lipase. Sporopollenin was activated according to published procedure [39]. This is evidenced by the aggressive extraction procedure using strong alkali and acid. After extraction, exines retain the same shape as the parent spores and as such constitute an empty shell that can be used for encapsulation.

Most importantly, the encapsulated lipases exhibited enzymatic activity against the *p*-NPP substrate. These results demonstrate that the *C. 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 sporopollenin and activated sporopollenin as additive.

Scanning electron microscopy (SEM) allowed the verification of morphological differences between the sporopollenin, activated sporopollenin and lipase encapsulated on sporopollenin (Spo-E) (Fig. 1). For the pure support, microcapsule has a large internal cavity. After the activated process, pore structure of the sporopollenin (Spo_{act}) was decomposed (Fig. 1(b)). After encapsulation, the surface cavity of the sporopollenin was filled with lipase and the other materials. Fig. 1(c) shows the rounded structure, which is presumably protein aggregate.

Table 1 shows the activity of the encapsulated lipases. However, the encapsulated lipase with sporopollenin (Spo-E) was found to be more efficient compared to activated sporopollenin (Spo_{act}-E) with respect to expression of encapsulated lipase activity. The Spo-E was found to give 71 U/g of support with 88.8% activity yield, while Spo_{act}-E was found to give 68.4 U/g of support with 61.74% activity yield.

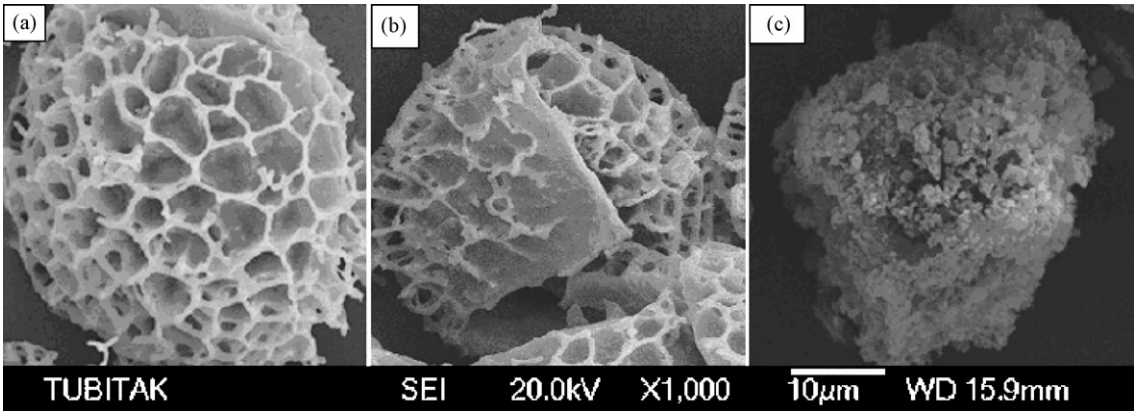


Fig. 1. SEM photographs: sporopollenin (a), activated sporopollenin (b) and encapsulated lipase (c).

Table 1
Activity of the free and encapsulated lipases under optimum reactions conditions.

	Encap. protein (mg/g)	Encap. protein yield (%)	Lipase activity (U/g support)	Spesific activity (U/mg protein)	*Activity yield (%)*
Free lipase[a]	28.56	58.4	95.1	3.32	*100*[b]
Encapsulated lipase (Spo-E)	24.0	57.14	71.0	2.95	*88.8*
Encapsulated lipase (Spo_{act}-E)	*33.3*	*79.33*	*68.4*	*2.05*	*61.74*

[a] Encapsulated free lipase without sporopollenin.
[b] Activity yield for free lipase was defined as 100%.

In our previous work [48], *C. rugosa lipase* (CRL) on sporopollenin by adsorption method is reported. It has been observed that under the optimum conditions (enzyme/support: 0.3/1 (w/w)), the activity yield (%) of the immobilized lipase was 45.8, which is approximately 2.0 times less than that of the encapsulated lipase. Besides the enantioselective hydrolysis of rasemic Naproxen methyl ester that was studied in aqueous buffer solution/isooctane reaction system. It was observed that non-encapsulated lipase shows poor enantioselectivity (*E*: 24.2).

3.2. pH effect on the activity of encapsulated lipases

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 (4.0–10.0). Upon immobilization on sporopollenin, the optimum pH for reactions catalyzed by free lipase was slightly shifted towards acidic values. Generally, an acidic shift in the pH optimum is expected when enzymes are immobilized onto polycationic supports [47].

As shown in Fig. 2 the optimum pH of the encapsulated lipases was 5.0. Immobilized lipase showed better pH stability and resistance to acidic environments than free lipase. Pereira et al. reported the optimum pH values as 6.0 and 7.0, respectively, for immobilized and free lipase [49]. The pH shift depends mainly on the method of immobilization and the interaction of enzyme and support. A change of the optimum pH for *C. rugosa* lipase immobilized on poly(vinyl alcohol) microspheres was reported by Oh et al. [50].

3.3. Temperature effect on the activity of encapsulated lipases

The effect of temperature on free and encapsulated lipases is given in Fig. 3. The effect of temperature on the activity of free and encapsulated lipases for *p*-NPP hydrolysis at pH 7.0 in the temperature range of 25–60 °C is shown in Fig. 3. It was found that the optimum temperature for the free enzyme was approximately

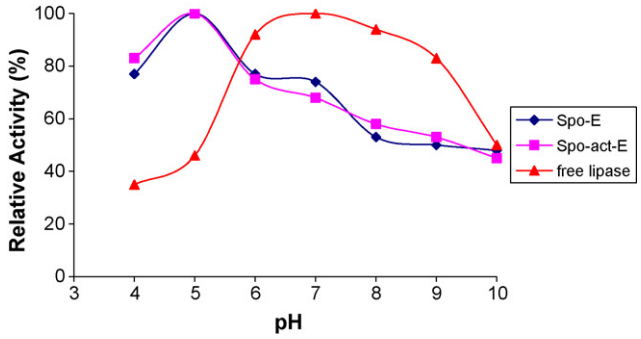


Fig. 2. Effect of substrate pH on residual activity of free (▲), encapsulated lipase. The activities of the enzymes were monitored with *p*-NPP assay at different pH values.

35 °C, while it shifted nearly to 40 °C for encapsulated lipase (Spo) and 45 °C for encapsulated lipase (Spo_{act}).

Furthermore, the temperature profiles of the immobilized lipases are broader than that of the free enzyme, which means that the immobilization methods preserved the enzyme activity over a wider temperature range. One of the main reasons for enzyme

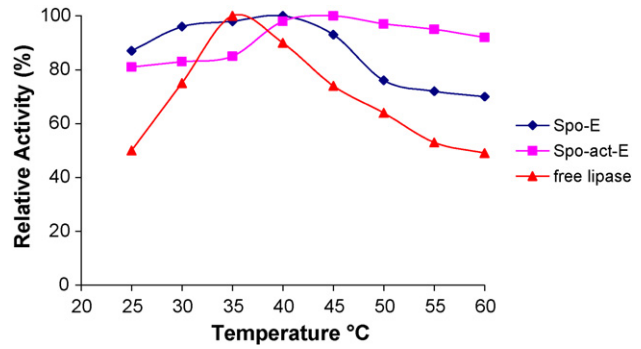


Fig. 3. Effect of reaction temperature on the residual activity of free (▲), encapsulated lipase (Spo-E) (♦) and encapsulated lipase (Spo_{act}-E) (■). The activities of the enzymes were monitored with *p*-NPP assay at pH 7.0.

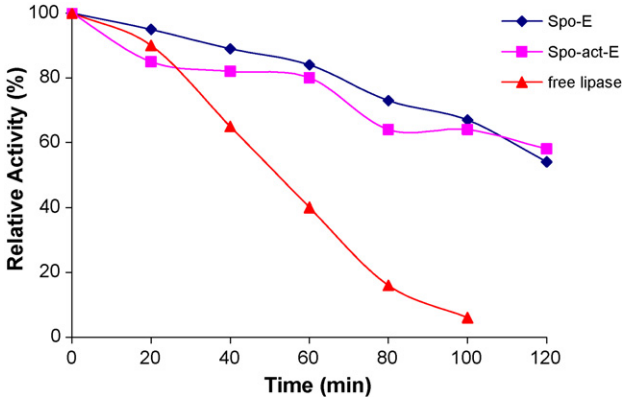


Fig. 4. Thermal stability of free (▲), encapsulated lipase (Spo-E) (♦) and encapsulated lipase (Spo_{act}-E) (■). The activities of the enzymes were monitored with *p*-NPP assay at pH 7.0.

immobilization is the anticipated increase in stability towards various deactivating forces, due to restricted conformational mobility of the molecules following immobilization [51–53]. 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 [54]. In literature, similar changes in the optimum temperature have also been reported [55]. In our previous work it was found that the optimum temperature for immobilized CRL on cyclodextrin based polymer or calix[n]arenes based polymers was 45 and 50 °C respectively [56,57].

3.4. Thermal stability and reusability on the activity of encapsulated lipase

In this work, Fig. 4 shows the thermal stabilities of the free and encapsulated lipases. Free and 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 free lipase loses its initial activity within around 80 min at 60 °C, while the encapsulated lipases retain their initial activities of about 54% by Spo and 58% by Spo_{act} 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 [58,59]. 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 [45]. These results indicate that the thermal stability of the immobilized lipases is much better than that of the free one since the interaction between the enzyme and the support, which could prevent the conformation transition of the enzyme at high temperature.

Reusability for the immobilized enzyme is very important in economics, and an increased stability can make the immobilized enzyme more advantageous than its free counterpart. To investigate the reusability, the enzymes immobilized were washed with PBS (50 mM, pH 7.0) after one catalysis run and reintroduced into a fresh *p*-NPP solution for another hydrolysis at 30 °C. Fig. 5 shows that the encapsulated lipase activities were maintained at levels exceeding 28% and 26% of their original activities for encapsulated lipase Spo and Spo_{act} after 8 reuses, respectively. This shows that immobilized lipase using this technique can be used successfully for industrial applications requiring long-term reaction stability.

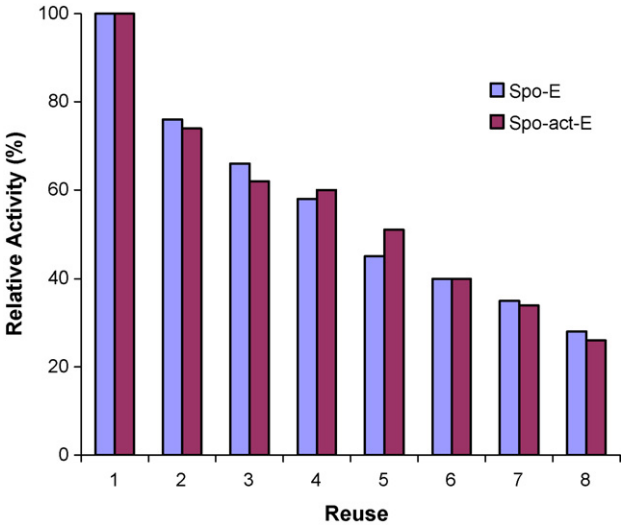


Fig. 5. Reusability of the encapsulated lipase (Spo-E) and encapsulated lipase (Spo_{act}-E). The activities of the enzymes were monitored with *p*-NPP assay at pH 7.0.

The activity of immobilized enzymes decreases with the increase in reuse number.

These results could be explained by the inactivation of the enzyme by the denaturation of the protein and the leakage of protein from the support upon use [60].

3.5. Storage stability

The storage stability of the encapsulated enzymes was clearly better than the free lipase (Fig. 6).

Thus, the support and the technique of immobilization provided a longer shelf life than that of free counter part [61]. 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 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 [62].

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

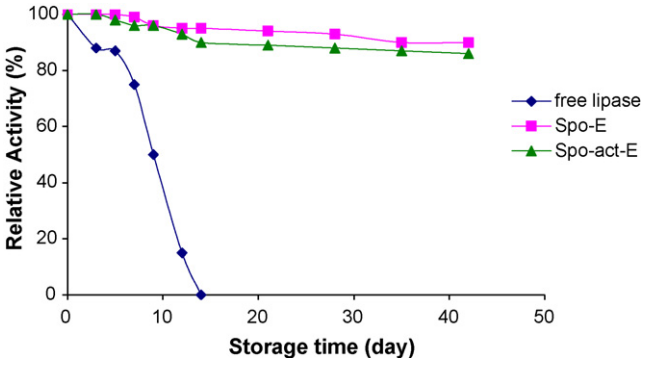


Fig. 6. Storage stability of free (♦) and encapsulated lipase (Spo-E) (■) and encapsulated lipase (Spo_{act}-E) (▲). They were stored at 4 °C in 50 mM phosphate buffer at pH value 7.0. The activities of the enzymes were monitored periodically with *p*-NPP assay at pH 7.0.

Table 2
Kinetic resolution of racemic Naproxen methyl ester using free and encapsulated lipases.[a].

	X (%)	ee_s (%)	ee_p (%)	E
Free lipase[b]	37.9	60.2	>98	166.0
Encapsulated lipase (Spo-E)	44.3	78.71	>98	>400
Encapsulated lipase (Spo_{act}-E)	23.0	29.0	>98	89.7
Non-encapsulated lipase	10.8	24.2	>98	24.2

[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; pH 7.0; temperature, 35 °C.
[b] Encapsulated free lipase without sporopollenin.

when kept at low temperature probably because lipases tend to lock to its original conformation, which is catalytically active. The free enzyme rapidly loses its activity with a residual value of 15% after12 days, the decrease in activity occurs more slowly with the encapsulated lipases, and about 95% (Spo-E) and 93% (Spo_{act}-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 [63].

3.6. Enantioselectivity of the encapsulated Candida rugosa lipase with sporopollenin and activated sporopollenin in the kinetic resolution of racemic Naproxen methyl ester

From an industrial point of view, the quality of a given kinetic resolution not only depends upon 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 (*RS*)-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. [43].

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 (*RS*)-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 (Scheme 1).

The resolution reaction with encapculated lipase (Spo) was terminated after 24 h, obtaining Naproxen methylate (unreacted *R*-ester) and corresponding acid (ee_p) 98% at conversion of 44.3%

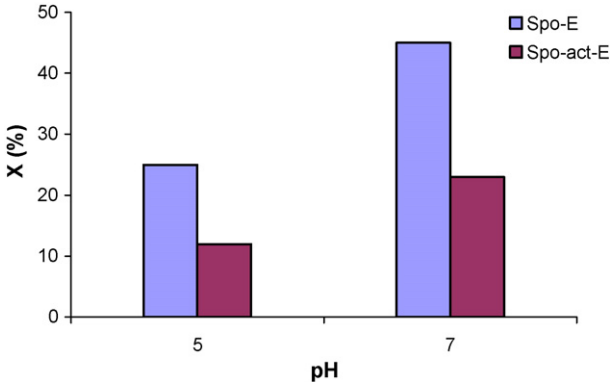
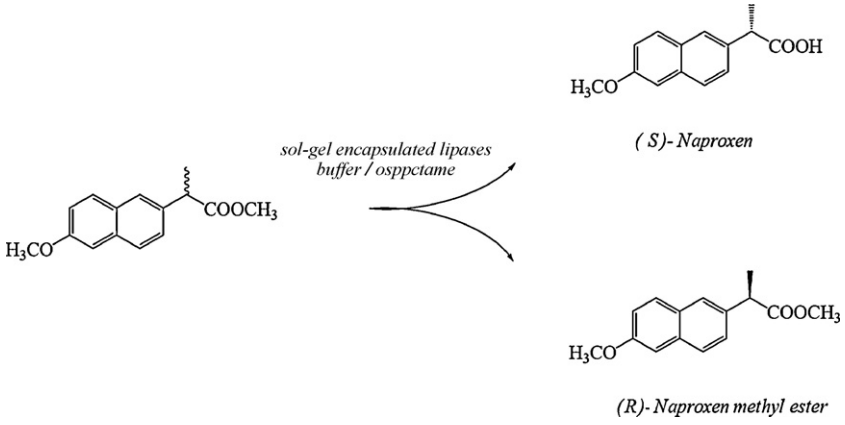


Fig. 7. Effect of pH on the conversion (x) in the hydrolysis of racemic Naproxen methyl ester.

and the enantioselectivity being very high ($E > 400$). Whereas the resolution reactions with free encapsulated lipase (free $lipase_{enc}$) 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. [64] used lipase MY from *C. rugosa* and catalyzed hydrolysis of (*R*, *S*)-Naproxen esters in water-saturated isooctane as the model system. They found E value was 510. In addition [65], racemic fluoxetine was catalyzed with sol–gel immobilized ABL and sol–gel/PVA immobilized ABL and found E values from 43 to 127–473.

The results indicate that in particular sporopollenin and activated sporopollenin based encapsulated lipases had higher conversion and enantioselectivity compared to the sol–gel free lipase. However during the activated process, because pore structure of activated sporopollenin was decomposed (Fig. 1). Thus its activity and enantioselectivity are lower than the non-activated sporopollenin.

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 [49]. The effects of pH on enantioselectivity of encapsulated lipase were determined by incubating encapsulated lipase in the presence of sporopollenin at different pH (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. 7).



Scheme 1. Enantioselective hydrolysis of (*R*, *S*)-Naproxen methyl ester.

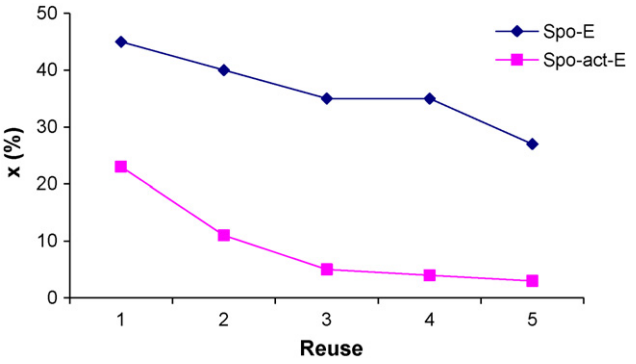


Fig. 8. Reusability on the conversion (x) in the hydrolysis of racemic Naproxen methyl ester.

After the encapsulation of CRL in the presence of sporopollenin and activated sporopollenin, 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 decreases after the fourth usage. Fig. 8 shows that the encapsulated lipases were still retained 27% and 3% of their conversion ratios for sporopollenin and activated sporopollenin 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

Sporopollenin was for the first time utilized to encapsulate lipases, and the prepared enzyme by polycondensation with tetraethoxysilane (TEOS) exhibited the highest activity in both the hydrolysis of *p*-nitrophenyl palmitate and the enantioselective hydrolysis of rasemic Naproxen. It was observed that the encapsulated sporopollenin (Spo-E) was found to give 71 U/g of support with 88.8% activity yield, while Spo_{act}-E was found to give 68.4 U/g of support with 61.74% activity yield. Furthermore, the immobilized lipase retained 95% (Spo-E) and 93% (Spo_{act}-E) of their initial activity after 50-d application, while the free enzyme left 15% after only 12 d. It was observed that excellent enantioselectivity ($E > 400$) was obtained for most lipase preparations in this work ($E = 166$ for the free enzyme) 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. And the sol–gel method was worthy of further investigations to achieve higher activity and stability of enzymes compared to conventional immobilization method.

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