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ORIGINAL ARTICLE

Catalytic effect of calix[n]arene based sol–gel encapsulated or covalent immobilized lipases on enantioselective hydrolysis of (R/S)-naproxen methyl ester

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Abstract In this paper the catalytic performance of the immobilized lipases was investigated in the presence of calixarene based polymers using different immobilization techniques. For this reason, *Candida rugosa* lipase was encapsulated in sol–gel matrices using alkoxysilane precursors and calix[n]arene based silica polymers as additives. The hydrolytic activities of encapsulated lipases were evaluated and compared with the free lipase and covalently immobilized lipases (CnP-L). These encapsulated lipases were also used in the kinetic resolution of the R/S-Naproxen methyl ester. The results indicated that the C6P encapsulated lipase has significantly higher conversion and enantioselectivity as compared to the free lipase; other encapsulated lipases and CnP-L. The optimal pH and temperature region of the C6P encapsulated lipase in the kinetic resolution of the R/S-naproxen methyl ester were 7.0 and 55 °C. Nevertheless, the encapsulated lipases have good stability, adaptability and reusability in comparison with the free enzyme.

Keywords Calixarenes · Silica · Naproxene · Enantioselectivity · Sol–gel

Introduction

Lipase (triacylglycerol ester hydrolase, EC 3.1.1.3) is an efficient enzyme which catalyses the hydrolysis of triacylglycerol to glycerol and fatty acids. Lipases have attracted considerable attention as biocatalysts for hydrolytic and esterification reactions [1]. In particular, due to their ability to catalyze conversions of low water-soluble substrates at the oil–water interface and to discriminate between enantiomers, they have been used as phase-transfer catalysts for enantioselective bioconversion in the production of optically pure enantiomers [2]. The need to use pure enantiomers for the preparation of drugs, foods, agrochemicals, and so on is well recognized, because of the well-known different recognition that enantiomers may have at the molecular level [3]. However, only one of the two isomers has a beneficial effect, whereas the other may have side effects; even when the unwanted isomer is not harmful, it represents a useless load to be metabolized by the liver.

The immobilization of enzymes is a key to expand the applications of these natural catalysts by enabling easy separation and purification of products from the reaction mixtures and efficient for the recovery of enzyme proteins. Enzymes can be immobilized on a support by adsorption, electrostatic attraction, covalent binding, or/and encapsulation [4]. They can also be self-reticulated to produce enzyme crystals [5]. Amongst the various immobilization techniques, sol–gel encapsulation is interesting because it tends to mimic living organisms such as cells, although in a very simple fashion in the present stage of development. A well-established sol–gel processing technique consists of 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 [6]. 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 also used in the mid-1980s to prepare modified silicates (Ormosils) for the encapsulation of antibodies and enzymes [7, 8]. Although, the final structure of the material is

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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; such as surfactants [9–11], room-temperature ionic liquids [12], crown ethers, β-cyclodextrins or porous solid supports like Celite [11]. Moreover, commonly used catalysts are weak acids or bases [6, 13]. Recent research describes the use of other species that include peptides like silaffins [14], polyamines [15] or enzymes such as hydrolases [16] and silicateins [17].

Calixarene skeleton represents one of the most important macrocyclic host molecule in supramolecular chemistry [18, 19], together with crown ethers [20] and cyclodextrins [21]. They are prepared by the base-catalyzed reaction of formaldehyde with phenol derivatives. Calixarene-based molecules have received intense attention in the last few years. The important features of these compounds are as selective binders and carriers, analytical sensors, catalysts and as model structures for biomimetic studies [22–25].

In our previous study, we have demonstrated the role of calix[n]arene binding site on the lipase activity and stability, and also reported the synthesis of calix[n]arene based silica polymers and covalently immobilization of lipase [26]. The present work is an extension of our previous study. In this study, we have used calix[n]arene based silica polymers on sol–gel encapsulation procedure as additive materials and observed the effects of calix[n]arene based polymers in the enantioselective hydrolysis reaction of (R/S)-naproxen methyl ester and compared with covalently immobilized lipases with the calix[n]arene based polymers (CnP-L). In particular, the influence of immobilization techniques and calix[n]arene based silica polymers with different cavity-size is also studied on lipase enantioselectivity and activity.

The results showed that the observed enantioselectivity and conversion effect of the C6P encapsulated lipase proved significantly high than that for the free lipase, other encapsulated lipases (C4P and C8P) and covalently immobilized lipases.

Materials and methods

Chemicals

Lipase from *C. rugosa* (E.C.3.1.1.3, Type VII), *p*-nitrophenyl palmitate (*p*-NPP) used as the substrate to estimate the enzyme activity, bovine serum albumin (BSA) used as the standard for protein assay, TEOS (tetraetoxysilane) and OTES (octyltrietoxysilane) were acquired from Sigma (St. Louis, MO). HPLC grade organic solvents were used as the mobile phase without further purification or drying. All other chemicals used in this work were of analytical or of reagent grade and became available from various commercial sources.

High-performance liquid chromatography (HPLC) Agilent 1200 Series were carried out using a 1200 model quaternary pump, a G1315Bmodel Diode Array and MultipleWavelength UV–vis detector, a 1200 model Standard and preparative autosampler, a G1316A model thermostated column compartment, a 1200 model vacuum degasser, and an Agilent Chemstation B.02.01-SR2 Tatch data processor. The concentrations of S- and R-enantiomers of Naproxen methyl ester were measured with HPLC (Agilent 1200 Series) by using Chiralcel OD-H column at the temperature of 25 °C. In the analyses, n-hexane/2-propanol/trifluoroacetic acid (100/1/0.1, v/v/v) was used as the mobile phase at the flow rate of 1 mL/min; and UV detection was done $\lambda = 254$ nm.

Synthesis of calix[n]arene based silica polymers

Calix[n]arene based silica polymers employed in this work as illustrated in Scheme 1 were synthesized previously by our group [26] and the characterization of synthesized calix[n]arene based silica polymers (CnP) were made by Fourier transform infrared spectroscopy (FTIR), thermal gravimetric analysis (TGA) and scanning electron microscope (SEM) techniques.

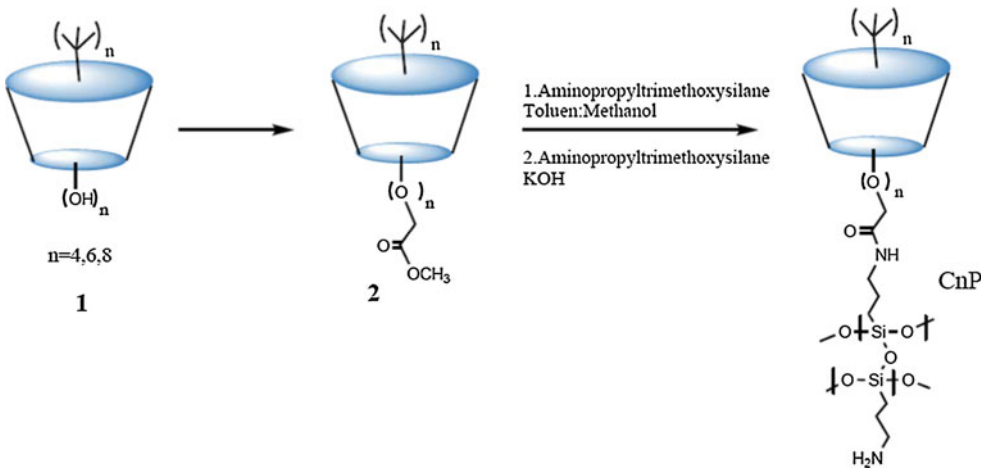
Synthesis of (R,S)-naproxen methyl ester

Racemic Naproxen was produced in the laboratory by the racemization of optically pure S-naproxen as described by Wu et.al. [27]. A complete racemization was confirmed as evidenced by two peaks of nearly the same area measured from the HPLC system. Racemic Naproxen methyl ester was produced by referring to the literature [28]. 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 ($CDCl_3$); $\delta = 1.58$ (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 ($CDCl_3$); $\delta = 18.81$, 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 Scheme 2.

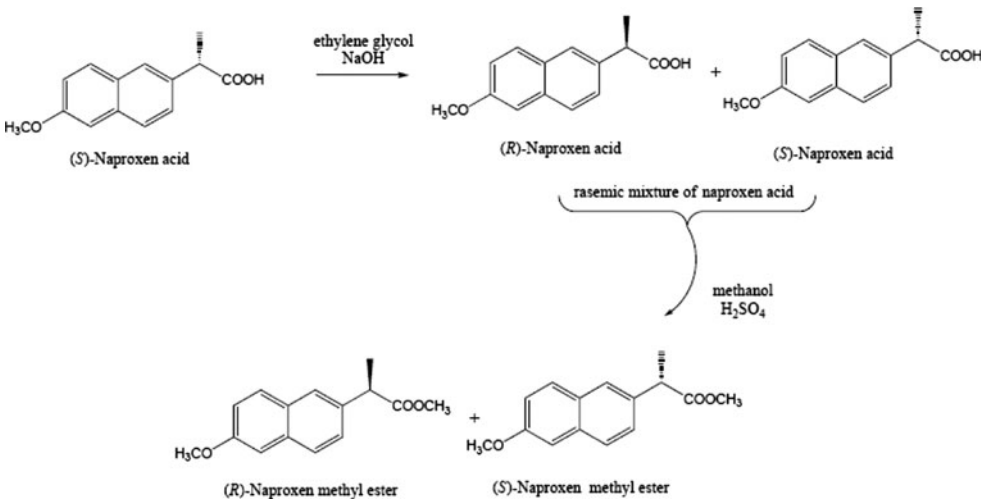
Sol–gel encapsulation procedure

Calixarene based sol–gel encapsulated lipases were prepared accordingly [11]. Candida rugosa lipase (60 mg) was placed in a 50 mL Erlenmeyer flask mixed with phosphate buffer solution (390 μL; 0.05 M; pH 7.0) and the mixture was vigorously stirred on a horizontal shaker. The calix[n]arene

Scheme 1 Calix[n]arene based silica polymers using in sol–gel encapsulation



Scheme 2 Racemization of (S)-naproxen acid and synthesis of (R,S)-naproxen methyl ester



based silica polymer (0.05 g) was included followed by addition 100 μL of aqueous PVA (4% W/V), aqueous sodium fluoride (50 μL of a 1 M solution) and isopropyl alcohol (100 μL) and the mixture homogenized using a shaker. Then the OTES (2.5 mmol) and TEOS (0.5 mmol; 120 μL) were added and the mixture agitated once again for 10–15 s. Gelation was usually observed within seconds or minutes while gently shaking the reaction vessel. The gel was lyophilizated and successively washed with distilled water (10 mL), isopropyl alcohol (10 mL). The resulting encapsulated lipases were held at 4 °C prior to use. The amount of protein in the enzyme solution and the elution solutions were determined by the Bradford method [29] using bovine serum albumin as a standard.

Assay of free and encapsulated lipases activity

The hydrolytic activities of the sol–gel encapsulated and free lipases were measured by dissolving 0.5 g of *p*-nitrophenyl palmitate (*p*-NPP) in 100 mL of ethanol as substrate at room temperature [30]. The sol–gel encapsulated lipase (25 mg) was added to the mixture of 1 mL 0.5% (w/v) *p*-NPP solution and 1 mL 0.05 M PBS buffer (pH 7.0) and incubated for 5 min at 25 °C. The reaction was terminated by adding 2 mL of 0.5 N Na_2CO_3 to the mixture followed by centrifugation (4,000 rpm for 5 min). The increase in the absorbance at 400 nm produced by the release of *p*-nitrophenol in the enzymatic hydrolysis of *p*-NPP was measured in a Shimadzu 160A UV–visible recording spectrophotometer. One unit of enzyme activity was defined as the amount of enzyme, which liberates 1 μmol *p*-nitrophenol min^{-1} [31]. Specific activity was calculated by dividing total activity (U) by amount of lipase bound to sol–gel polymers and expressed as U/mg-protein. Activity yield (%) was calculated by dividing specific activity of lipase in the encapsulated preparation by specific activity of encapsulated free lipase without the calixarene derivatives. All measurements were performed in triplicate and an average was taken as final result. The hydrolytic activities of covalently immobilized lipases (CnP-L) on calixarene based silica polymers were presented previously in our study [26]. Effect of these covalently immobilized lipases on the enantioselective

hydrolysis reaction was observed and compared with encapsulated lipases in this paper.

Kinetic resolution of (R,S)-naproxen methyl ester

Hydrolysis reactions of the (R,S)-naproxen methyl ester 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 7.0, 0.05 M phosphate buffer solution) including encapsulated lipases or covalently immobilized lipases (5–50 mg depending on the activity) (Scheme 3). The reactions were carried out in an horizontal shaker at 200 rpm at 35 °C and samples drawn from isooctane phase at 24 h were analyzed by HPLC to calculate the conversion and enantioselectivity [32].

The enantioselectivity (E) was calculated from the conversion (x), the enantiomeric excess of the substrate (ee_s) and the product (ee_p) using the equation of Chen et al. [33];

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

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_R and C_S denote enantioselectivity for irreversible reactions, enantiomeric excess of substrate, enantiomeric excess of product, racemate conversion, concentration of R-enantiomer and concentration of S-enantiomer, respectively.

Optimum conditions of encapsulated lipases on enantioselective hydrolysis

The optimum pH and reaction temperature of encapsulated lipases were determined as the conversion (x) and enantioselectivity (E) at different pH (0.05 M phosphate buffer for pH 4.0–10.0) and temperature (25–65 °C).

Reusability of encapsulated lipases on enantioselective hydrolysis

To evaluate the reusability of the encapsulated lipases, after each reaction run, the encapsulated lipase preparation was removed from the water phase by centrifugation (4000 rpm, 5 min, at room temperature) and washed with phosphate buffer (0.05 M, pH 7.0) to remove any residual substrate. It was then re-treated with racemic naproxen methyl ester in batch reaction system. This process was repeated up to five cycles.

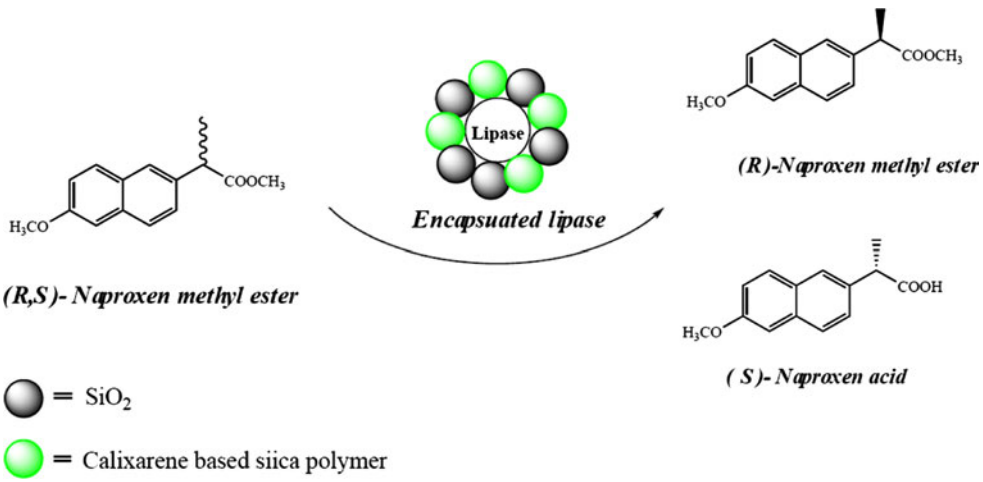
Results and discussion

Calixarene based silica polymers

To examine the effect of calix[n]arene based silica polymers using as additives on the enantioselective hydrolysis reaction of racemic naproxen methyl ester is the main focus of this work. For this reason, we used the calixarene based silica polymers synthesized previously our study on sol–gel encapsulation procedure and compared with covalently immobilized lipases and free lipase in point of the enantioselectivity and conversion.

Sol–gel encapsulation of Candida rugosa lipase (CRL) using calixarene based silica polymers

The studies carried out by Reinhoudt et al., Griebenow et al., Dordick et al., and Xu et al. [34–37], respectively, have been observed that lipases show higher activities and occasionally enhanced stereoselectivities when used in the presence of 18-crown-6, or cyclodextrine derivatives. Reinhoult and



Scheme 3 Enantioselective hydrolysis of (R,S)-naproxen methyl ester

Table 1 Initial specific activities and protein amounts of encapsulated lipases toward p-NPP

Additives used in sol–gel process	Protein loading (mg/g-sol gel)	Protein loading yield (%)	Lipase activity (U/g-sol–gel)	Specific activity (U/mg-protein)	Activity yield (%)
C4P	25.6	58.3	53.7	2.10	63.3
C6P	10.5	37.5	39.5	3.00	90.4
C8P	27.4	50.2	40.1	1.47	44.3
Free lipase[a]	28.6	58.4	95.1	3.32	100[b]

[a] Encapsulated free lipase without calixarene derivative

[b] The activity of encapsulated free lipase was defined as 100%

coworker described a study on the elucidation of the mechanism of crownether-introduced activation of enzymes in organic solvents. Because 18-crown-6 is a highly effective complexing agent of ammonium groups [20], it may form complexes with cationic lysine residue of enzymes. After complexation, the charge of lysine residues is screened, and therefore becomes less available for salt bridge formation. Hence, the formation of ether-lysine complexes might reduce the formation of inter- and intramolecular salt bridges. Moreover, they observed that the activation is clearly a macrocyclic effect. Calixarenes represent one of the most important macrocyclic host molecules in supramolecular chemistry [22, 38], together with crown ethers [39]. In literature calix[n]arene derivatives form complexes with cationic lysine [40, 41].

Most importantly, the encapsulated lipases exhibited enzymatic activity against the *p*-NPP substrate. 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 the calix[n]arene based silica polymers (n = 4,6,8) as additives.

Table 1 shows the activity of the encapsulated lipases. It can be seen that the C6P encapsulated lipase was found to be more efficient compared to the others (C4P and C8P encapsulated lipases) with respect to expression of encapsulated lipase activity. The encapsulated lipase retains 63.3% of the activity on C4P, 90.4% on the C6P and 44.3% on the C8P. Calixarenes have different cavity sizes and the cavity-size of calix[6]arene is more suitable for complexation with ammonium groups [32] In addition, all of the encapsulated lipases have better activity than immobilized lipases as covalently [26]. This result was not surprising due to the conformational restrictions imposed by the covalent bonding of enzyme residues to the calixarene based silica polymers. Many physical and chemical effects contribute to the activity changes, including changes in molecular structure during coupling, steric hindrance of access to the catalytic sites, and physical denaturation caused by adsorption or proximity to the solid–liquid interface [42].

Table 2 Catalytic performance of encapsulated (a) and covalently immobilized lipases (b) towards the enantioselective hydrolysis of racemic naproxen methyl ester

Additives	x (%)	ee_s (%)	ee_p (%)	E
(a)				
C4P	24.6	32.0	>98	142
C6P	39.5	64.0	>98	195
C8P	27.5	37.1	>98	131
Free lipase (encapsulated)[a]	37.9	60.2	>98	166
(b)				
C4P-L	10.7	11.7	>98	94
C6P-L	28.0	30.2	>98	119
C8P-L	21.2	26.3	>98	115
Free lipase (powder)	39.1	62.9	>98	187

[a] Encapsulated free lipase without calixarene derivatives

Kinetic resolution of racemic Naproxen methyl ester with the encapsulated lipases

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 (R,S)-naproxene 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. [33].

Table 2 shows that the conversion (x), enantiomeric excess (ee) and enantioselectivity (E) results in the course of (R,S)-naproxen methyl ester hydrolysis by the sol–gel encapsulated lipases and immobilized lipases as covalently. The enantioselective hydrolysis reactions of racemic Naproxen methyl ester were studied in aqueous buffer solution/isooctane reaction system. From the results, it has been revealed that the sol–gel encapsulated lipases have a highly enantioselectivity (*E*) and conversion (*x*) compared with the covalently immobilized lipases. Particularly, the resolution reaction with C6P based encapsulated lipase was

terminated after 24 h, obtaining Naproxen methylate (unreacted R-ester) and corresponding acid (ee_p) 98% at conversion 39.5% and the enantioselectivity being very high ($E = 195$). While the resolution reactions with encapsulated free lipase was obtaining unreacted Naproxen methylate (R-ester) and corresponding acid (ee_p) 98% at conversion 37.9% and the enantioselectivity (E) being 166. Consequently, whereas the sol–gel encapsulation of lipase led to high enantioselectivity, high conversion and fast recovery of product compared to encapsulated free enzyme, the covalent immobilization of lipase led to low enantioselectivity and conversion compared to free lipase due to the conformational limitation on the enzyme as a result of covalent bond formation between the enzyme and the support.

The reason for the increased coversion (x) and enantioselectivity (E) with CnP additives is not yet clearly understood. Possible explanations include protection of the enzyme from aggregation or from denaturing effects due to the silanes used during the formation of the silica matrix, changes in gel properties, for example, porosity and pore diameter, or stabilization of the enzyme during the reaction in organic solvent [13].

The effect of pH on enantioselectivity of encapsulated lipase was determined by incubating encapsulated lipase in the presence of C6P at different pH from 4.0, 5.0 and so on up to 9.0 at 35 °C for 24 h. The pH plays an important role in affecting enzyme activity because the charge density of enzyme surface and enzyme conformation change with pH [43, 44]. At the end of incubation time the rate of enzyme reaction and enantiomeric excess (ee) were determined using HPLC (Agilent 1200 Series) by using Chiralcel OD-H column at the temperature of 25 °C. The optimum pH value was determined from the graph of pH plotted against the percentage of conversion (x) and enantioselectivity (E) (Fig. 1). The optimum pH values were found to be 7.0 for C6P based encapsulated lipase.

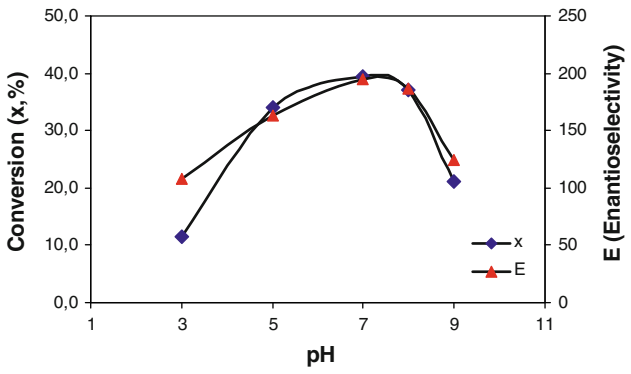


Fig. 1 Effect of pH on the conversion (x) and enantioselectivity (E) in the hydrolysis of racemic naproxen methyl ester with C6P based sol–gel encapsulated lipase

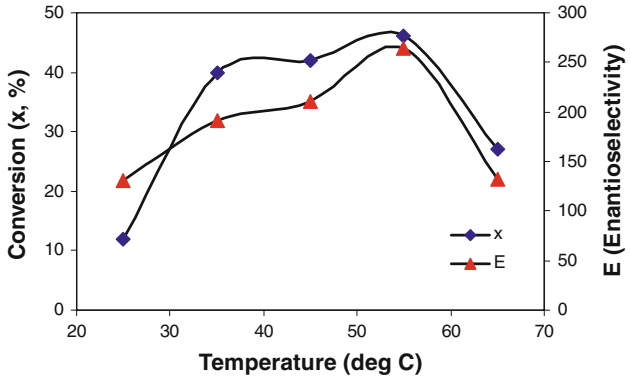


Fig. 2 Effect of temperature on the conversion (x) and enantioselectivity (E) in the hydrolysis of racemic naproxen methyl ester with C6P-based sol–gel encapsulated lipase

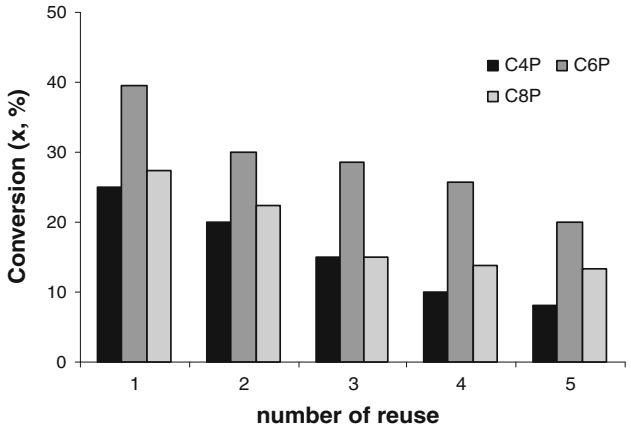


Fig. 3 Reuse of the sol–gel encapsulated lipases (CnP) in the hydrolysis of racemic naproxen methyl ester

The temperature dependence of the hydrolysis reaction catalyzed by C6P based encapsulated lipase was studied in the interval from 25 to 65 °C and the results are shown in Fig. 2. It was observed that the optimum temperature value was found to be 55.0 °C for C6P based encapsulated lipase. This behavior is considered to be advantageous and can be attributed to a more rigid structure of the encapsulated lipases, which prevent the split breaking of the interactions responsible for the proper globular, catalytically active structure of the lipase [45, 46]. It is likely that the lipase is not only physically encapsulated, but that additional multipoint interaction through hydrogen bonding, ionic, and hydrophobic interactions.

The reusability of encapsulated lipases is also important for economical use of the enzyme. After the encapsulation of CRL in the presence of CnP silica polymers based encapsulated lipases were not soluble in water due to increase cross-linked bond between the enzyme. In the reusability studies, where after each run, the encapsulated

lipases were washed with PBS. It was found that the percentage of conversion (x) and enantioselectivity (*E*) of the encapsulated lipase decreased after the second usage. Figure 3 shows that the encapsulated lipases were still retained 8, 20, and 14% of their conversion ratios for C4P, C6P and C8P based encapsulated lipases after the 5th reuse, respectively. These results are due to inactivation of the enzyme caused by the denaturation of protein and the leakage of protein from the supports upon use.

Conclusion

In the present study, 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 calix[n]arene based silica polymers as additive. The prepared encapsulated lipases were used in the enantioselective hydrolysis reaction of racemic Naproxen methyl ester and compared with the covalently immobilized lipases (CnP-L). The results indicated that the particularly C6P encapsulated lipase had higher conversion and enantioselectivity compared to the free lipase, other encapsulated lipases and immobilized lipases as covalently (CnP-L). The encapsulated lipases also showed good properties and stabilities, which are important factors when selecting an appropriate enzymic system for different biotechnological applications.

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