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ORIGINAL ARTICLE

Improvement of catalytic activity of lipase in the presence of wide rim substituted calix[4]arene carboxylic acid-grafted magnetic nanoparticles

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Abstract *Candida rugosa* lipase immobilized on calix[4]arene carboxylic acid-grafted magnetic nanoparticles using a sol–gel encapsulation technique was tested for activity, which was assessed both in the enantioselective hydrolysis of racemic Naproxen methyl ester and that of *p*-nitrophenylpalmitate. It has also been noticed that, compared to the free enzyme ($E = 137$) with an ee value of >98 %, *S*-Naproxen calix[4]arene carboxylic acid-grafted magnetic nanoparticles based on encapsulated lipase (**Calix-1-MN** and **Calix-2-MN**) offer excellent enantioselectivity ($E = 373$ and $E = 381$). Moreover, the results indicated that after the fifth reuse in the enantioselective reaction, the encapsulated lipase (**Calix-2-MN**) still retained about 43 % of its conversion power.

Keywords Lipase · Enantioselectivity · Calix[4]arene · Magnetic nanoparticles

Introduction

In biotechnology, lipases are the class of enzymes most widely used in the kinetic resolution of racemic compounds and organic synthesis [1]. In particular, lipase from *Candida rugosa* has important industrial uses. It is well known that *Candida rugosa* is used in a wide variety of esterification reactions and hydrolysis [2]. The activity of *Candida rugosa* lipase (CRL) is high and it also has broad specificity in reaction medium, as compared to free lipase, which has low activity, and is usually unstable in organic medium or in a harsh conditions such as high temperature or excessive pH. The stability, catalytic activity, and reusability of immobilized lipase are improved in continuous operations by the immobilization of CRL on various supports, providing the separation of products [1–3]. Investigations of the immobilization of CRL on different carriers have been reported by a series of recent studies [3], and carriers have included chitosan, amberlite, cyclodextrin, and calixarene [4–8]. The calix[4]arene platform in supramolecular chemistry shows interesting organizational properties for the construction of ligating sites to recognize different species, which includes anions, cations, and neutral molecules [9]. As receptors in supramolecular chemistry, they have attracted much attention in the last 25 years. The increasing interest in these compounds is due to the simple large-scale synthesis of calixarenes, and the various methods by which they can be selectively functionalized either at the wide rim or the narrow rim. The nature and the number of donor groups and the conformation of the calix[4]arene moiety are highly responsible for the complexation properties of these molecules [9, 10].

Magnetic supports have been used in enzyme immobilization [11–13] and cell separation [14, 15]. Magnetically supported immobilized lipases can be recovered more easily from a reaction with the help of external magnetic field, in addition to offering the benefits of other solid support media [16–18]. Nanoparticles of paramagnetic iron oxides have been used in many applications in the last decade [19], including for bioseparation [20, 21], tumor hyperthermia [22], magnetic resonance imaging (MRI) diagnostic contrast agents [23], magnetically guided site-specific drug delivery agents [24], and the immobilization of biomolecules [25–27].

In our previous study [6], the use of calix[n]arenes and their lower rim substitute amine and carboxyl derivatives

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as additives for lipase sol–gel encapsulation and the effect of the derivatives of calix[n]arene on the hydrolysis and enantioselectivity of racemic Naproxen methyl ester was reported. Herein, we report about upper rim substitute calix[4]arene carboxylic acid derivative-grafted magnetic nanoparticles that were used as additives in the sol–gel encapsulation process, and explore the influence of the material on the hydrolysis and enantioselectivity of racemic Naproxen methyl ester.

Materials and methods

Materials

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. IR spectra were obtained on a Perkin-Elmer spectrum 100 FTIR spectrometer (ATR). ^{1}H NMR spectra were recorded on a Varian 400 MHz spectrometer. Reactions were monitored by TLC on pre-coated silica gel plates (SiO2, Merck, 60F254). UV–vis. spectra were obtained on a Shimadzu 160A UV–visible recording spectrophotometer. High-performance liquid chromatography (HPLC) Agilent 1200 Series were carried out using a 1200 model quaternary pump, a G1315Bmodel Diode Array and Multiple Wavelength 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. purchased from Sigma-chemical Co. (St. Louis, MO). Pure *S*-Naproxen was purchased from Sigma (USA). Racemic Naproxen was produced in the laboratory by the racemization of optically pure *S*-Naproxen as described by Wu and Liu [28]. Racemic naproxen methyl ester has been prepared according to published method [6, 29].

Synthesis

The syntheses of compounds **1**, **2**, Fe_3O_4 nanoparticles, and [3-(2,3-epoxypropoxy)propyl]-grafted Fe_3O_4 nanoparticles (EPPTMS-MN) were carried out according to published procedures [27, 30, 31]. The other materials (**3**, **Calix-1-MN** and **Calix-2-MN**) used in this work were prepared according to the methods given below, as illustrated in Scheme 1.

5,11,17,23-Tetrakis[[(isonipecoticacido)methyl]-25,26,27,28-tetrahydroxycalix[4]arene (3)

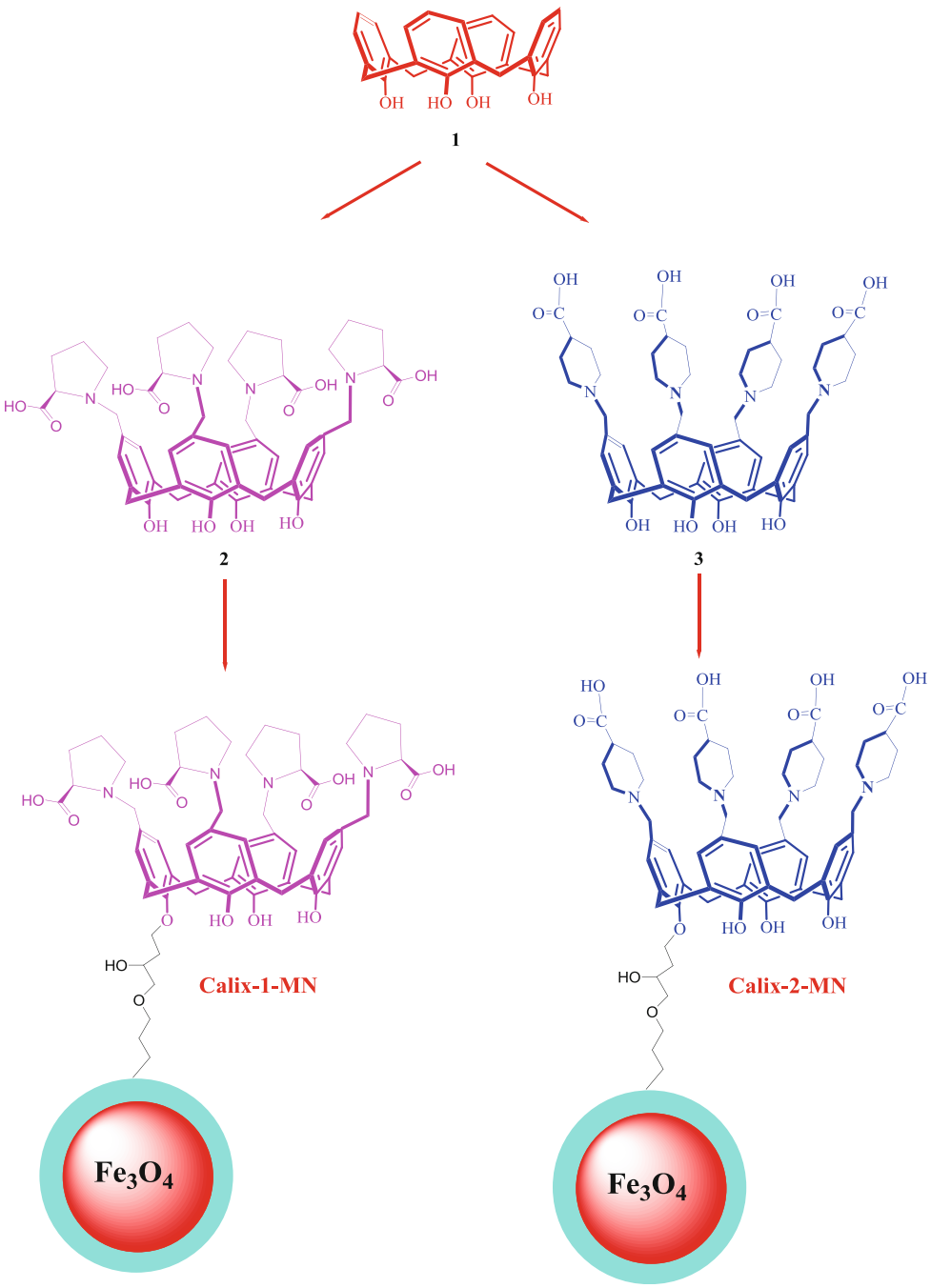
To a solution of calix[4]arene (**1**) (10 mmol) in 90 mL of THF-DMF were added 11 mL of acetic acid, isonipecotic acid (50 or 100 mmol), and 37 % aqueous formaldehyde (50 mmol) and the reaction mixture was stirred for 24 h at room temperature. The precipitate that formed was removed by suction filtration. Received product was washed with water and acetone and dried under vacuum. Compound **3** was obtained in 79 % yield, m.p. 328–330 °C. The IR (ATR) spectral data is as cm^{-1}: 1,655 (COO). ^{1}H NMR (400 MHz, $CHCl_3$): $\delta = 1.55$ (q, 8H, $J = 11.1$ Hz, NCH_2CH_2), 1.77 (m, 8H, NCH_2CH_2), 2.02–2.3 (m, 10H, NCH_2CH_2, CH_2CH), 2.78 (m, 8H, NCH_2CH_2), 3.15 (d, 4H, $J = 14$ Hz, $ArCH_2Ar$), 3.32 (s, 8H, $ArCH_2N$), 4.25 (d, 4H, $J = 14$ Hz, $ArCH_2Ar$), 6.85 (s, 8H, Ar). Anal. Calcd for $C_{56}H_{68}O_{12}N_4$: C, 68.0; H, 6.93; N, 5.66 %. Found: C, 68.31; H, 6.99; N, 5.71 %.

Preparation of magnetic calix[4]arene derivative (Calix-1-MN and Calix-2-MN)

A mixture of the compound **2** or **3** (0.6 g), potassium carbonate (0.5 g) in acetonitrile (30 mL) was stirred for 30 min before adding 0.9 g of EPPTMS-MN and heated under reflux for 3 days. After magnetic separation, the resulted compound was washed with DMF (three times) to remove excess compound **3**, then washed with water and dried under vacuum. The IR (ATR) spectral data of the **Calix-1-MN** is as cm^{-1}: 1,624 (C = O), 1,341 (aromatic C = C), 1,031, 948 and 788 (Si–O) and **Calix-2-MN** is as cm^{-1}: 1,651(C = O), 1,578, 1,451 and 1,410 (aromatic C = C), 1,035, 939 and 783 (Si–O).

Sol–gel encapsulation of lipase

Sol–gel encapsulated lipase with and without the calix[4]arene grafted magnetic nanoparticles (**Calix-1-MN** or **Calix-2-MN**) were prepared according to a modified method of Reetz et al. [32]. A mixture of CRL (60 mg) placed in a 50 mL erlenmeyer together with phosphate buffer solution (PBS) (390 µL; 0.05 M; pH 7.0), which was vigorously stirred on a horizontal shaker and a mixture of **Calix-1-MN** or **Calix-2-MN** (50 mg), 100 µL of aqueous polyvinyl alcohol (PVA) (4 % w/v), aq. NaF (50 µL of a 1 M solution) and *i*-PrOH (100 µL) was homogenized using a shaker. Then, OTES (2.5 mmol) and TEOS (0.5 mmol; 120 µL) were added and the mixture was agitated once more for 10–15 s. Gelatin was usually observed within seconds or minutes while gently shaking the



Scheme 1 A schematic representation of the synthesis of calix[4]arene carboxylic acid-grafted magnetic nanoparticles

reaction vessel. The gel was lyophilized and successively washed with distilled water (10 mL) and isopropyl alcohol (10 mL). The resulting encapsulated lipase was held at 4 °C prior to use.

Activity of the sol–gel encapsulated lipases

The catalytic activity, specific activity and the effect of pH and temperature on activity of the sol–gel encapsulated lipases were measured according to method in literature [6, 33, 34]. All measurements were performed in triplicate and an average was taken as final result.

Determination of protein assay

Protein content was determined by the dye-binding method of Bradford, using bovine serum albumin as a standard [35]. The amount of bound protein was determined indirectly from the difference between the amount of protein introduced into the coupling reaction mixture and the

amount of protein in the filtrates and also in washings after immobilization. The amount of immobilised enzyme was calculated by subtracting the amount of unimmobilised enzyme from the total amount of the lipase used for the immobilisation.

General procedures for encapsulated lipase-catalyzed enantioselective hydrolysis of esters

Hydrolysis reactions were carried out in an aqueous phase/organic solvent batch reaction system. To a solution of 2 mL buffer solution (pH = 7.0, 50 mM PBS) containing encapsulated lipases (5–50 mg depending on the activity) was added a solution of racemic methyl ester (20 mM) in 2 mL of isooctane. The reactions were performed in a shaker at 150 rpm at 35 °C and drawn samples from isooctane phase after 24 h.

HPLC were used to calculate the conversion and enantioselectivity being expressed as the enantiomeric ratio (E) calculated from the conversion (x) and the enantiomeric excess of the substrat (ee_s) and the product (ee_p) using the equation of Chen et al. [36].

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

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.

Results and discussion

Calix[n]arenes are a very important class of macrocycles extensively used in supramolecular chemistry. They are composed mainly of cyclic oligomers built with phenol units through methylene bridges [9]. It has been suggested that calixarenes could be regarded as the third generation of supramolecules, after crowns and cyclodextrins [10].

In this study, we report the synthesis of two calix[4]-arene carboxylic acid derivatives immobilized onto magnetic nanoparticles used as an additive in the enanatioselective hydrolysis reaction of Naproxen methyl ester. To achieve the desired goal, the synthesis of the L-proline derivative of calix[4] arene **2** was carried out according to the published method [31] and the calix[4]-arene derivative **3** was conducted in the presence of AcOH in THF with secondary amine (piperidyl-4-carboxylic acid) and formaldehyde to afford the cone conformer a 77 % yield of **3**. The structure of **3** was confirmed by spectroscopic and analytical data. The IR spectra of compound **3** shows a carbonyl band at 1,655 cm^{-1}. In general, the ^1H NMR spectra of compound **3** showed singlet of δ 3.32 ppm (8H each) for ArCH_2N. The conformational characteristics of calix[4]arenes were conveniently estimated by the splitting pattern of the $ArCH_2Ar$ methylene protons in the ^1H NMR spectrum. The cone conformation of compound **3** was confirmed from ^1H NMR spectroscopic data. The methylene bridge of $ArCH_2Ar$ protons was observed by a typical AB pattern at δ 3.15 and 4.25 ppm (J = 14 Hz) for **3**, in ^1H NMR. The high field doublets at δ 3.15 ppm were assigned to the equatorial protons of methylene groups, whereas the low field signals at δ 4.25 ppm were assigned to the axial protons in the ^1H NMR. According to the described method, the magnetic nanoparticles of Fe_3O_4 were prepared by the chemical co-precipitation of Fe^{2+} and Fe^{3+} ions, with the ratio of their concentration selected in the stoichiometric ratio of 2:1. By this method the Fe_3O_4 nanoparticles have a number of hydroxyl groups on the surface to interact with the aqueous phase. EPPTMS-modified Fe_3O_4 nanoparticles (EPPTMS-MN) were formed by reaction between the hydroxyl groups on the surface of the magnetite and the EPPTMS. As a result, the nanoparticles were directly modified by [3-(2,3-epoxypropoxy)propyl]-trimethoxysilane (EPPTMS) to introduce reactive groups on the particle surfaces. Finally, calix[4]-arene derivatives (**2** or **3**) were immobilized in the presence of K_2CO_3 in acetonitrile to nanoparticles modified by that surface [27]. The new compounds were characterized by a combination of FTIR, TGA, elemental analyses, and SEM.

In the FTIR spectra given in Figs. 1 and 2, the appearance of carbonyl bands at 1,624 cm^{-1} for **Calix-1-MN** and at 1,578 cm^{-1} for **Calix-2-MN** also offer evidence for the existence of carboxylate moieties on the nanoparticles. In addition, the characteristic bands of the silane group at 1,031, 788 cm^{-1} for **Calix-1-MN** and at 1,035, 783 cm^{-1} for **Calix-2-MN** (Si–O) placed on the FTIR spectra have confirmed the structure of **Calix-1-MN** and **Calix-2-MN**.

Thermal properties of **Calix-1-MN** and **Calix-2-MN** were analyzed by thermogravimetric methods (Figs. 3 and 4). The indication of the coating formation on the magnetic nanoparticles' surface can be obtained from TGA measurement. The main weight loss temperature ranges were 250–600 °C (32 %) for **Calix-1-MN** and 310–570 °C (39 %), for **Calix-1-MN**. Thermogravimetric results showed a direct relationship between the loss of mass and the number of calixarene molecules bound to the nanoparticle surfaces.

The **Calix-1-MN** and **Calix-2-MN** gave also satisfactory analytical data, consistent with the proposed formulas

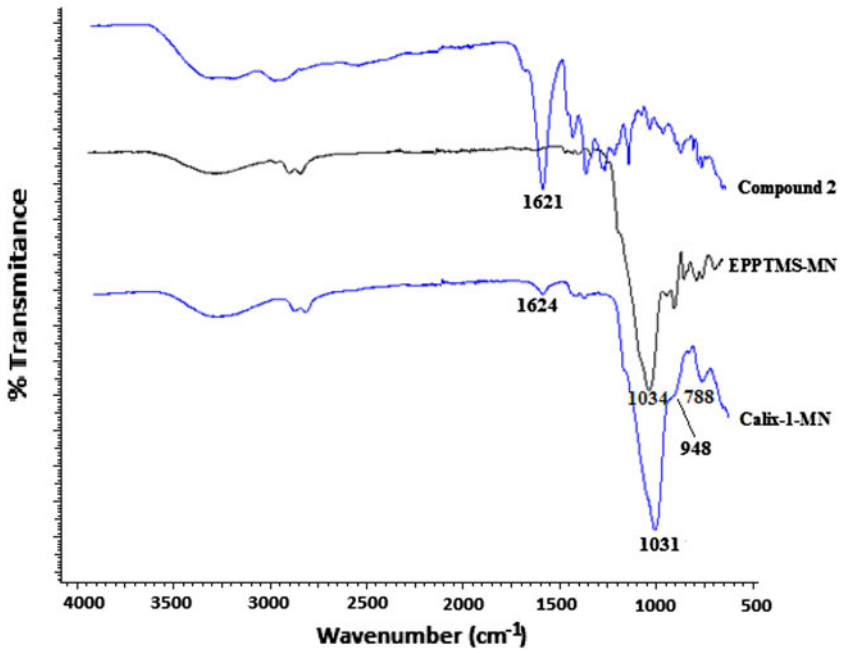


Fig. 1 The IR spectrum of compound **2**, magnetic nanoparticle (**EPPTMS-MN**) and **Calix-1-MN**

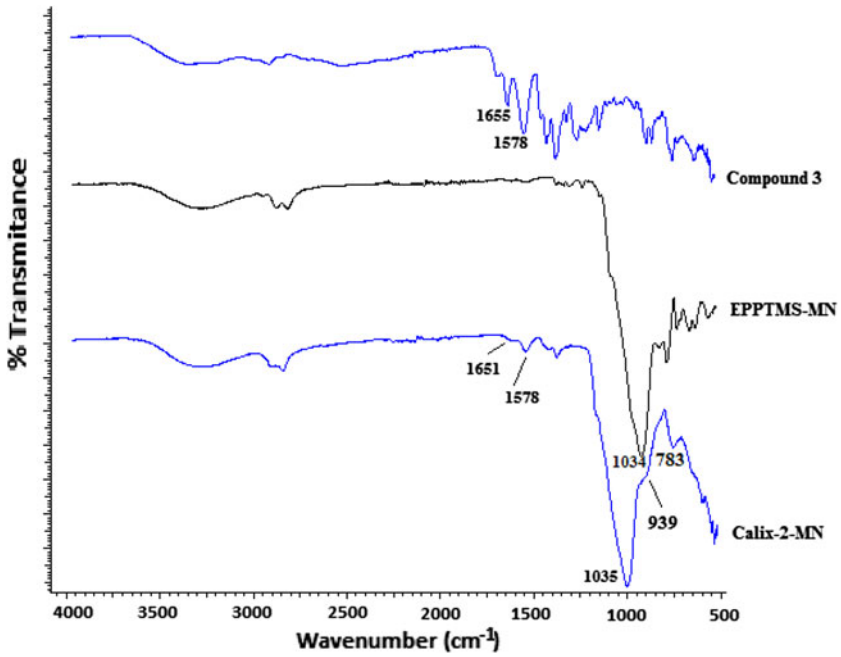


Fig. 2 The IR spectrum of compound **3**, magnetic nanoparticle (**EPPTMS-MN**) and **Calix-2-MN**

integrating residual molecules. According to the elemental analysis, (Table 1) the resulting **Calix-1-MN** contains 1.71 %, corresponding to 0.30 mmol, of **2**/g of support and **Calix-2-MN** contains 1.89 % nitrogen, corresponding to 0.34 mmol of **3**/g of support.

Scanning electron micrographs comparing the sol–gel encapsulated lipases (**Calix-1-MN-E** and **Calix-2-MN-E**) with the calixarene immobilized nanoparticles (**Calix-1-MN** and **Calix-2-MN**) showed that the sol–gel encapsulated nanoparticles had a porous surface compared with the irregular surface cavities of the **Calix-1-MN** and **Calix-2-MN** (Fig. 5).

Sol–gel encapsulation of *Candida rugosa* lipase (CRL) using calix[4]arene based magnetic nanoparticles

The original procedure for the encapsulation of lipases in sol–gel materials produced by the fluoride-catalyzed hydrolysis of mixtures of $RSi(OCH_3)_3$ and $Si(OCH_3)_4$ was

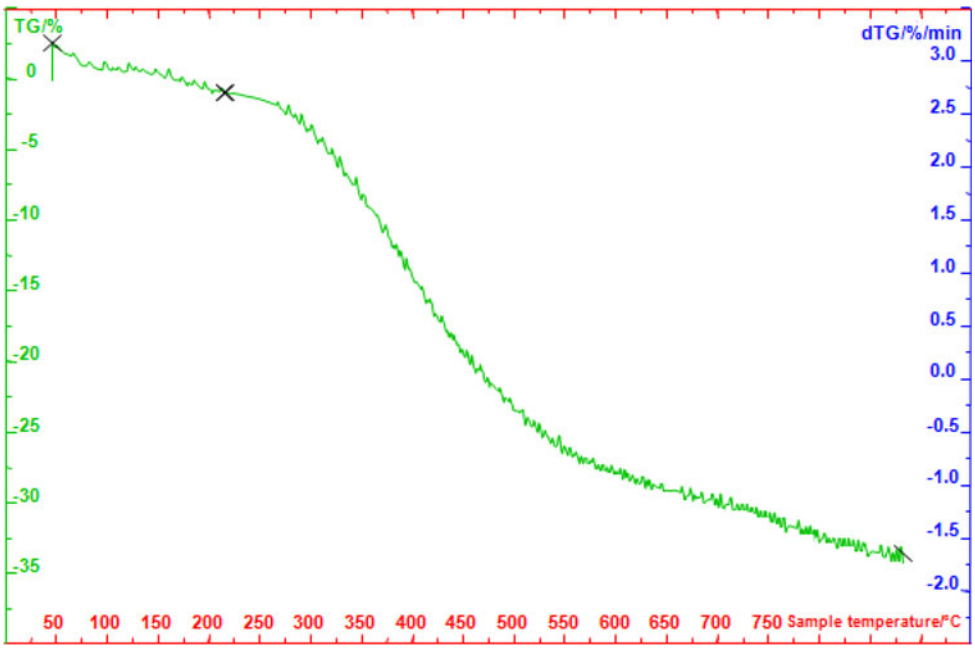


Fig. 3 The TGA spectrum of **Calix-1-MN**

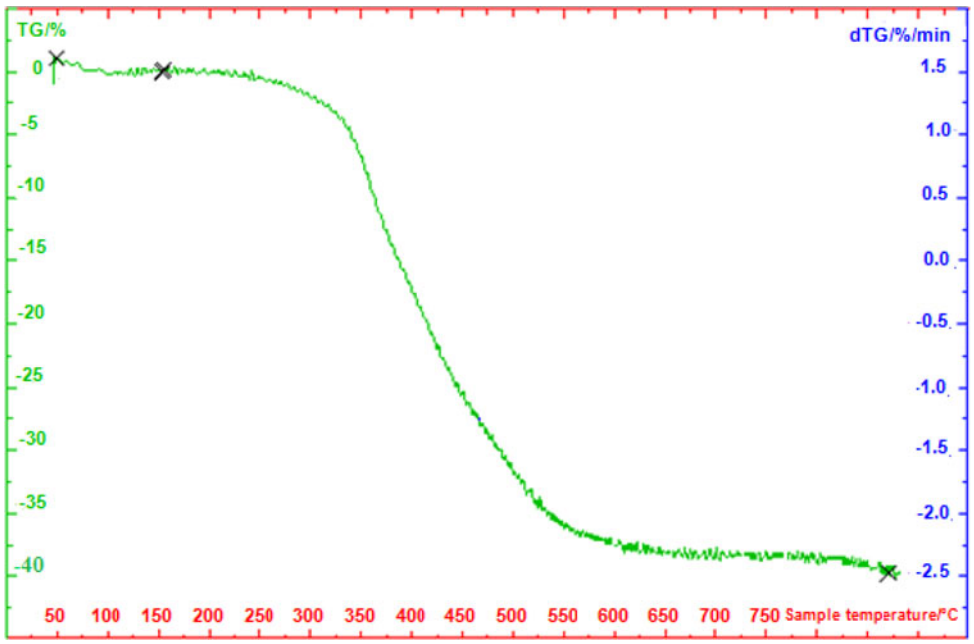


Fig. 4 The TGA spectrum of **Calix-2-MN**

Table 1 Results of elemental analysis for **EPPTMS**, **Calix-1-MN** and **Calix-2-MN**

	C (%)	H (%)	N (%)
EPPTNS-MN	13.20	2.61	–
Calix-1-MN	23.06	4.44	1.71
Calix-2-MN	29.12	5.12	1.87

described by Reetz et al. [32]. Furthermore, this study showed that in the presence of different additives such as derivatives of cyclodextrin and 18-crown-6, encapsulated lipases show higher activity and enhanced enantioselectivity. The mechanistic evidence for organic solvents in the crown-ether-introduced activation of enzymes was described by Reinhoudt et al. [37]. The 18-crown-6 may also form complexes with cationic lysine group of enzymes, as it is already known that it has a high affinity for forming complexes with ammonium groups. The charge of the lysine groups was screened after complexation, and showed lower availability for salt bridge formation. Therefore, the formation of intra- and inter-molecular salt bridges of ether-lysine complexes might be reduced [1, 37].

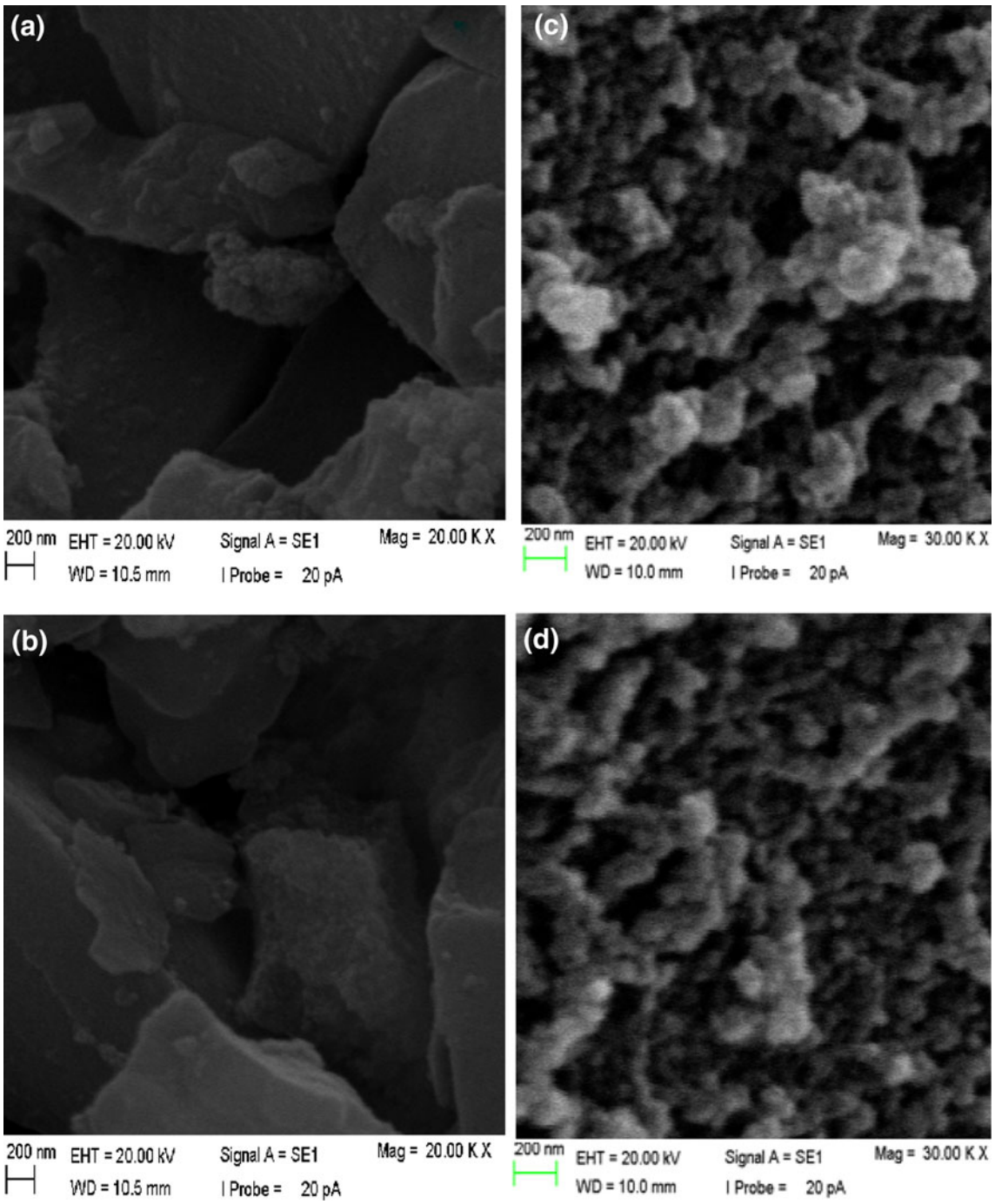


Fig. 5 SEM micrographs of **a Calix-1-MN**, **b Calix-2-MN**, **c Calix-1-MN-E** and **d Calix-2-MN-E**

Itoh et al. [38] also demonstrated that the crown ether derivatives have the potential to enhance both the reaction rate and enantioselectivity of the lipase-catalyzed hydrolysis of 2-cyano-1-methylethyl acetate. These crown compounds cannot change the original enantioselectivity of the enzyme, but enhance its potential ability to a level at which the reaction can be used practically [38]. Moreover, they observed that the macrocyclic effect causes the activation. Like crown ethers, calix[n]arenes are also the most important macrocyclic host molecules. The calixarene derivatives are known to form complexes with cationic lysine [39].

We have successfully used carboxylic acid groups on lower rim calixarene as additives for lipase immobilization by sol–gel methods in our previous work [6]. The results showed that, as compared to sol–gel free lipase, in the

hydrolysis reaction of (*R*,*S*)-Naproxen methyl ester, calixarene-based encapsulated lipases offered distinctly higher conversion rates and enantioselectivity. The purpose of this study was to use the sol–gel encapsulation in the presence of a chiral proline derivative of calix[4]arene (**Calix-1-MN**) and a piperidyl-4-carboxylic acid derivative of calix[4]arene-grafted magnetic nanoparticles (**Calix-2-MN**) as the new additives to produce immobilized lipase. These immobilized derivatives were used as a catalyst in both the enantioselective hydrolysis reaction of (*R*,*S*)-Naproxen methyl ester and hydrolysis of *p*-nitrophenylpalmitate.

Table 2 shows the specific activities and protein amounts of encapsulated lipases toward *p*-NPP. However, the encapsulated lipase with **Calix-2-MN** was found to be more efficient than **Calix-1-MN** with respect to the expression of immobilized lipase activity. This result was not surprising, because **Calix-1-MN** contains proline groups, which ease intramolecular hydrogen bonding. In this case, **Calix-1-MN** shows weaker interactions with the enzyme than does **Calix-2-MN**. However, **Calix-1-MN** was found to be less efficient compared to **Calix-2-MN**.

pH and temperature effect on the activity of encapsulated lipases

Because environmental conditions also affect enzymatic activity, it was observed that when altering enzymatic activity in an aqueous solution, pH is also one of the most efficient parameters. In this study, the activity and the pH effect on the free and immobilized lipase in the hydrolysis of *p*-NPP were determined by altering the reaction medium pH from 3 to 9.

As shown in Fig. 6, the optimum pHs of encapsulated free lipase (**enc lipase**), **Calix-1-MN-E** and **Calix-2-MN-E** as additives were 7.0, 6.0, and 5.0 respectively. The immobilized lipase offered better pH stability and hence resistance to acidic environments than did free lipase.

One of the most important parameters that must be known to understand how the procedure of immobilization affects enzymatic activity is the temperature profile of immobilized lipase. Generally, compared to free enzymes, the optimum temperatures of immobilized enzymes are shifted towards higher temperatures. At pH 7.0, the temperature dependence of the *p*-NPP hydrolysis reaction catalyzed by immobilized and free lipases was studied from 30 to 60 °C, and these results are given in Fig. 7. The observed optimum temperature for the encapsulated lipase (**enc-lipase**) was approximately 35 °C, while it shifted to nearly 40 °C for the **Calix-1-MN** and **Calix-2-MN**.

Table 2 Initial specific activities and protein amounts of encapsulated lipases toward *p*-NPP

Additives used in sol–gel process	Protein loading (mg/g-sol–gel)	Lipase activity (U/g-sol–gel)	Specific activity (U/mg-protein)
Calix-1-MN	27.3	42.4	1.60
Calix-2-MN	19.1	47.5	2.48
Free lipase[a]	28.5	95.1	3.32

[a] Encapsulated lipase without support

Enantioselective hydrolysis 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 depends on the activity and the possibility of recycling and reusing the lipase. The crystal structure of *Candida rugosa* lipase exhibits two known conformations [40, 41]. In one, called the "closed form," the helical surface loop partially covers the hydrophobic crevice containing the active site, while in the other conformation, called the "open form," the surface loop moves and the space is uncovered [42].

It was recently reported by Kazlauskas et al. [43] that 2-propanol treatment of *Candida rugosa* lipase caused modification of enantioselectivity. 2-Propanol treatment was proposed to convert the closed form of this lipase to the open form, thereby enhancing the enantioselectivity [38, 43]. The present study demonstrated that the calixarene carboxylic acid derivatives have the potential to enhance both the enantioselectivity and reaction rate of the lipase-catalyzed hydrolysis of rasemic-Naproxen methylester. These calixarene-based compounds cannot change the basic enzymatic enantioselectivity, but increase its potential ability to a level at which the reaction can be used practically [6].

In this work, to encapsulate CRL within a chemically inert sol–gel support prepared by polycondensation by (TEOS) and (OTES), derivatives of calix[4]arene carboxylic acid were used as additives. The reactions were carried out on a small scale, and on the basis of the formula of Chen et al. [36] the enantioselectivity was determined by measuring the selectivity factor E. A high catalytic activity on the hydrolysis of (*R*,*S*)-Naproxen methyl ester was exhibited by the immobilized CRL. Using the **Calix-1-MN** and **Calix-2-MN,** high enantioselectivity $E > 300$ was achieved (Table 3).

It was also found that calix[4]arene carboxylic acid based additives have important effect son the stability of CRL. All the results showed that the catalytic activity of the CRL might have improved due to the sol–gel processes with the calix[4]arene carboxylic acid derivatives used as

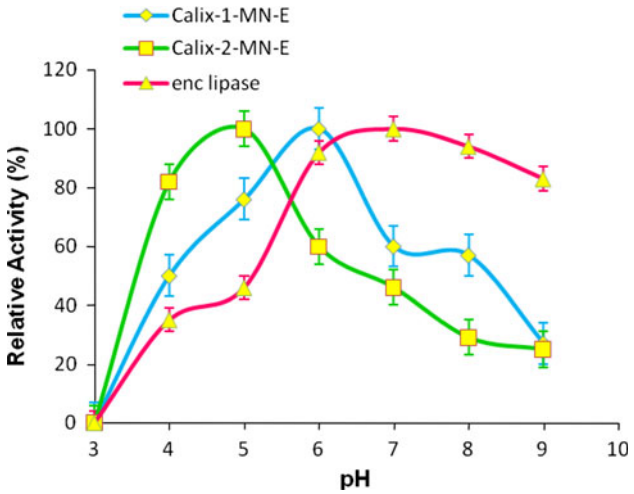


Fig. 6 Effect of substrate pH on residual activity of encapsulated lipases

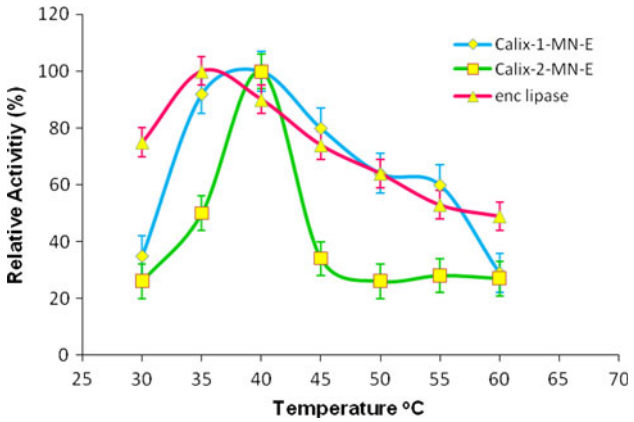


Fig. 7 Effect of substrate temperature on residual activity of encapsulated lipases

additives. The calix[4]arene-based nanoparticles employed may interact with certain sites of the lipase as proposed in some proteins, thereby activating the lipase and changing its enantioselectivity.

In order to investigate whether or not variations in pH affect the chiral selectivity of the enzyme as a catalyst depending on the ionization state of the lipase, the effects of pH on the enantioselectivity of lipase encapsulated **Calix-1-MN** and **Calix-2-MN** was determined by incubating at 35 °C at pH 7.0 and at optimum pH. The results given in Fig. 8 show the optimum pH value was 6.0 for lipase encapsulated **Calix-1-MN** and the optimum pH value was 5.0 for lipase encapsulated **Calix-2-MN**.

The reusability of immobilized lipase is also important for economical use of the enzyme. Figure 9 shows that the ratios of conversion for **Calix-1-MN** after the fifth reuse of the encapsulated lipases were 23 % for pH 6 and 13 % for pH 7. For **Calix-2-MN**, these ratios were 43 % for pH 5, and 29 % for pH 7 (Fig. 10). These results are due to the

Table 3 The enantioselective hydrolysis of racemic Naproxen methyl ester of using sol–gel encapsulated lipases as catalysts

Additives used in sol–gel process	x (%)	ee_s (%)	ee_p (%)	E
Free-E[a]	20.3	25	>98	137
Calix-1-MN-E (pH 7)	43.4	75	>98	224
Calix-1-MN-E (pH 6)	49.2	95	>98	373
Calix-2-MN-E (pH 7)	49.5	96	>98	381
Calix-2-MN-E (pH 5)	48.7	93	>98	341

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) for **1** as mobile phase; time, 24 h; concentration of substrate, 20 mM; temperature, 35 °C

[a] Encapsulated *Candida rugosa* lipase without supports

inactivation of the enzyme's denaturation of protein and the leakage of protein from the supports upon use. This indicates that lipase encapsulated **Calix-1-MN** or **Calix-2-MN** could be used in industrial applications.

Conclusions

In this work, *C. rugosa* lipase was immobilized on calix[4]arene carboxylic acid-grafted magnetic

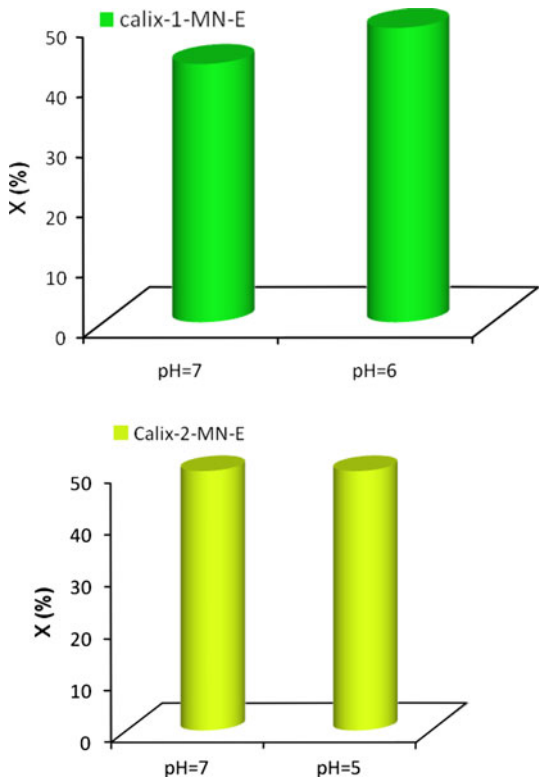


Fig. 8 Effect of pH on the conversion (x) in the hydrolysis of racemic naproxen methyl ester with **Calix-1-MN-E** and **Calix-2-MN-E**

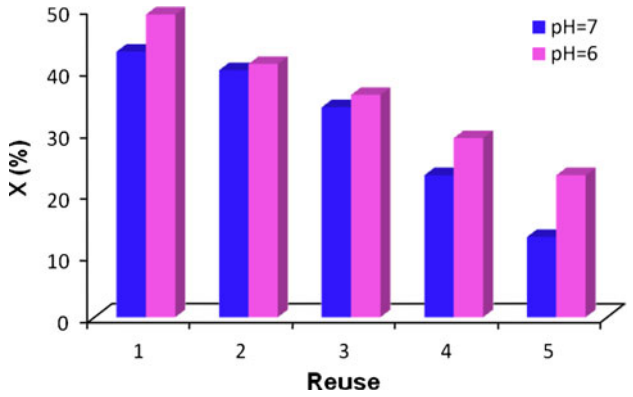


Fig. 9 Reusability on the conversion (x) in the hydrolysis of racemic Naproxen methyl ester with **Calix-1-MN-E** for different pH

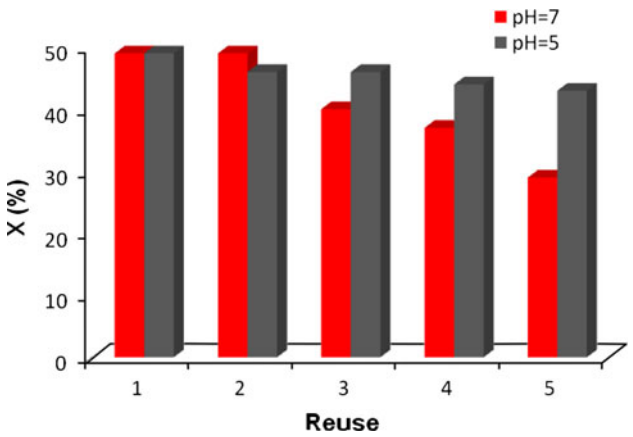


Fig. 10 Reusability on the conversion (x) in the hydrolysis of racemic Naproxen methyl ester with **Calix-2-MN-E** for different pH

nanoparticles using a sol–gel encapsulation technique. The prepared encapsulated lipases were used in the enantioselective hydrolysis reaction of racemic Naproxen methyl ester. The lipases demonstrated improved enantioselectivity, with an *E* value of 224 for **Calix-1-MN** and 381 for **Calix-2-MN,** whereas encapsulated free lipase has a lower *E* value of 137. Calix[4]arene carboxylic acid-grafted magnetic nanoparticle immobilization allows for high enantioselectivity, high conversion, and fast recovery of product as compared with unsupported encapsulated lipase. This work represents not only a significant advance in the improvement of lipase-catalyzed organic synthesis, but also provides an interesting combined use of calix[4]arene with an enzyme. In addition, the recovery and reusability of encapsulated lipase is also important for economical use of the enzyme, and this very easy due to its magnetic properties. These are all important factors when selecting an appropriate enzymic system for biotechnological applications.

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