Preparation of One-Pot Immobilized Lipase with Fe_3O_4 Nanoparticles Into Metal-Organic Framework For Enantioselective Hydrolysis of (*R,S*)-Naproxen Methyl Ester

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Immobilization of enzyme to magnetic metal-organic frameworks (MOF) can preserve biological functionality in harsh environments to increase enzymes activity, stability, and improve reusability. The magnetic Fe_3O_4 nanoparticles were treated with calix[4]arene tetracarboxylic acid (Calix) and *Candida rugosa* lipase (CRL), and then encapsulated into the zeolitic imidazole framework-8 (Fe_3O_4@Calix-ZIF-8@CRL). The lipase activity data of Fe_3O_4@Calix-ZIF-8@CRL was 2.88 times higher than that of the Fe_3O_4@ZIF-8@CRL (without Calix). The catalytic properties of immobilized lipases were studied on the enantioselective hydrolysis of R/S-naproxen methyl ester. It was also observed that Fe_3O_4@Calix-ZIF-8@CRL has excellent enantioselectivity (E = 371) compared to Fe_3O_4@ZIF-8@CRL (E = 131). Furthermore, Fe_3O_4@Calix-ZIF-8@CRL was seen to still retain 30% of the conversion rate after the fifth reuse. This work may also be useful for the pharmaceutical industry due to the increased reusability and stability of enzymes, the enantiomeric selectivity exhibited by MOF-enzyme biocomposites, and the significant differences in the biological activities of the enantiomers.

Introduction

Lipases are very important enzymes that catalyze hydrolysis, esterification, transesterification, regio and stereoselectivity reactions.[1–4] However, practical applications of enzymes are often hampered by poor thermal and storage stability, high cost, and difficulties in recovery and reusability.[5,6] Enzyme immobilization has proven to be a way to overcome these limitations in order to increase their stability, allow them to be used repeatedly, and facilitate continuous monitoring of the reaction process.[7–9]

Encapsulation method in porous metal-organic frameworks (MOF) use for preserving biomolecules.[10–12] The subclass of MOFs, the zeolitic imidazolate framework (ZIF), is known to exhibit high chemical and thermal stability, functionality and negligible cytotoxicity. Besides, it can be easily acquired under green synthesis conditions such as room temperature and aqueous solution.[13–15] The framework for ZIF-8, consisting of 2-methylimidazole (HmIm) and Zn^{2+}, spontaneously assembles and form a tetrahedral network.[10,16–19]

Calix[n]arene hosts synthesized from condensation reaction between para-substituted phenol derivatives and formaldehyde have attracted significant attention.[2,4] The calix[4]arenes, cone-shaped molecules with a hydrophobic cavity that can accommodate a variety of functional groups, have great potential as protein surface binders.[4] It is known that the CRL has an active site consisting of several amino acids. The carboxyl groups of calix[4]arene form complex with the guanidinyl group of arginine, the sulfhydryl group of cysteine, the imidazolyl group of histidine, the amino group of lysine, and the indolyl group of tryptophan. Our group has used calix[4]arene derivative for the first time in ZIF-lipase encapsulation synthesis.[20] The results indicate that immobilized lipase (CRL@Calix-ZIF-8) was higher activity and stability compared to the CRL@ZIF-8.

One of the biggest challenges in enzyme immobilization is the separation process. By applying an external magnetic field, the immobilized enzyme can be reused, quickly and easily separated from the product. Magnetizing MOF is one of the effective ways in this process. Recently, magnetic MOFs with specific functional properties have been designed for enzyme immobilization.[6,10,18–20] Moreover, the potential application of magnetic porous MOFs as the support for enzyme immobilization needs to be explored. To date, there are only a few reports on the preparation of magnetic ZIF biocomposites.[7,10,13,21,24–26] For example, Ricco co-workers have prepared the enzyme horseradish peroxidase and iron oxide magnetic nanoparticles in a one-pot synthesis and performed reusable biocatalysts.[10] In another study, Fe_3O_4@ZIF-8 nanoparticles were prepared and utilized for single-step purification and immobilization of recombinant laccase.[13] Lin and group have been immobilized the amidase onto the magnetic carrier and carried out high catalysis performance,[6] Cao and co-workers have been prepared ZIF-8-coated magnetic regenerated cellulose-coated nanoparticles and glucose oxidase was immobilized and enhanced catalytic activities.[21] Zou and group prepared the Fe_3O_4 embedded ZIF-8 composite with a solvent-free approach and indicated to be a suitable lipase carrier for Picking emulsion catalysis.[7] Feng and co-workers have been synthesized magnetic 3D ordered macroporous ZIF-8 and immobilized

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catalase.[26] To form CRL/MNP@ZIF-8, the encapsulate of lipase and Fe_3O_4 nanoparticle into ZIF-8, was reported by Ji and group.[27]

In our previous study,[20] *Candida rugosa* lipase was used for the first time in the enantioselective hydrolysis of racemic naproxen methyl ester, after immobilizing to metal organic framework. It was obtained that 38% conversion and enantioselectivity (E = 183) were found. However, there is no report on enantioselective catalyzed resolution of chiral enantiomers by using the magnetic-ZIF-8 based biocatalyst. Therefore, in this study magnetic Calix@ZIF-8 biocomposites were prepared by a simple method and were first utilized as a support for the immobilization of *Candida rugosa* lipase. Besides, the thermal stability and reusability of Fe_3O_4@Calix-ZIF-8@ CRL were studied. Immobilized enzymes were also used enantioselective hydrolysis reactions of (*R,S*)-naproxen methyl ester.

Results and Discussion

The Fe_3O_4 nanoparticles[28–30] and tetracarboxylic acid derivative of calix[4]arene[27,28] were synthesized according to published procedures. The Calix was reacted to CRL solutions at room temperature, then the reaction mixture was added Fe_3O_4 nanoparticles and stirred for 10 minutes at room temperature (Scheme 1). Following, treated with imidazole and Zn^{2+} by co-precipitation. The encapsulated Fe_3O_4@Calix-ZIF-8@CRL biocomposite structure was designed. Our approach allows the zeolitic imidazolate frameworks to be encapsulated with CRL-Calix complex and Fe_3O_4 on the inside.[20,33]

The structures of ZIF-8, Fe_3O_4@Calix-ZIF-8, Fe_3O_4@ZIF-8@CRL and Fe_3O_4@Calix-ZIF-8@CRL were displayed, respectively (Figure 1A). The strong bands at 421 cm^{-1} and 1567 cm^{-1} of ZIF-8 belong to the out-of-plane bending of the imidazole ring and the Zn–N tension band, respectively. For Fe_3O_4 nanoparticles, a well-defined band at 550 cm^{-1} showed the presence of Fe–O bonds. Moreover, Fe_3O_4@Calix-ZIF-8, Fe_3O_4@ZIF-8@CRL and Fe_3O_4@Calix-ZIF-8@CRL FTIR spectra were independently observed in Fe–O stretching mode with a 551 cm^{-1} peak.[34,35]

The crystal structures of ZIF-8, Calix@ZIF-8, Calix@ZIF-8@CRL and Fe_3O_4@Calix-ZIF-8@CRL were confirmed by XRD patterns (Figure 1B). The characteristic peaks at $2\theta = 7.2°$, 10.5°, 12.8°, 14.8°, 16.5°, 18.1°, 19.7°, 22.4°, 24.6°, 25.3°, 26.8° were assigned for pure ZIF-8. It was observed that the peaks did not change much with the addition of calixarene to the structure of ZIF-8. The characteristic peaks of Fe_3O_4 crystal are $2\theta = 27°$, 30.7°, 33.3°, 35.6° and 37.4°. In addition, it was suggested that the density of the crystal peaks decreased by immobilizing the CRL to the Calix@ZIF-8 structure.[35,36]

SEM photos confirm the morphological differences between Fe_3O_4@Calix-ZIF-8 and Fe_3O_4@Calix-ZIF-8@CRL (Figure 2a-b). The diameters of the Fe_3O_4@Calix-ZIF-8 were approximately 200 nm. As the enzyme is loaded, the morphology changes and larger and more irregularly shaped particles are formed. Moreover, the EDX of Fe_3O_4@Calix-ZIF-8 was carried out and exhibits the presence of Zn and Fe (Figure 2c–d). Figure 2e-f showed the TEM images of Fe_3O_4@Calix-ZIF-8 and Fe_3O_4@Calix-ZIF-8@CRL. Further, TEM and SEM images exhibited that Fe_3O_4 nanoparticles and lipase were encapsulated into ZIF-8 successfully.[37]

The TGA analysis of the Fe_3O_4@Calix-ZIF-8 and Fe_3O_4@Calix-ZIF-8@CRL was given in Figure 3. The TGA results showed that Fe_3O_4@Calix-ZIF-8, 56% weight loss at 200 to 520 °C is due to the decomposition of the calixarene molecule and ZIF-8. Fe_3O_4@Calix-ZIF-8@CRL was observed as 75% weight loss in the same temperature range. Compared with the remaining amount of both Fe_3O_4@Calix-ZIF-8 and Fe_3O_4@Calix-ZIF-8@CRL, the content of CRL in the magnetic ZIF-8 was estimated to be about 19%.

Table 1 displays the activity of immobilized lipases and the enantioselective hydrolysis of the racemic Naproxen methyl ester. The results showed that lipase activity of Fe_3O_4@Calix-ZIF-8@CRL and Fe_3O_4@ZIF-8@CRL were obtained to be 207 Units/g and 72 Units/g, respectively. Besides, enzyme loading values of Fe_3O_4@Calix-ZIF-8@CRL and Fe_3O_4@ZIF-8@CRL were found 40 mg/g and 20 mg/g, respectively. Fe_3O_4@Calix-ZIF-8@CRL was 2.88 times higher lipase activity and 2 times lipase loaded. It is known that lipase has two different conformations, open and closed-form and the outer surface of the lipase lid is hydrophobic and the inner surface is hydrophilic. The carboxyl groups

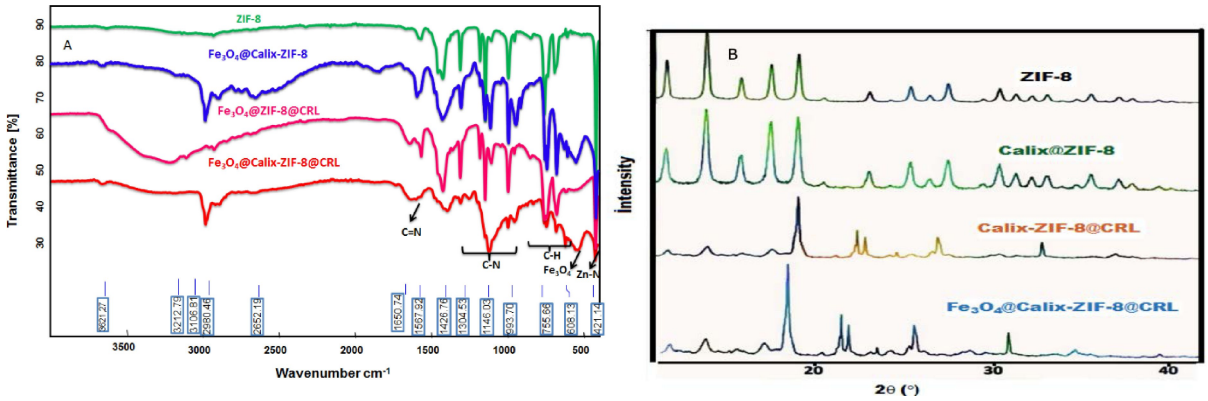
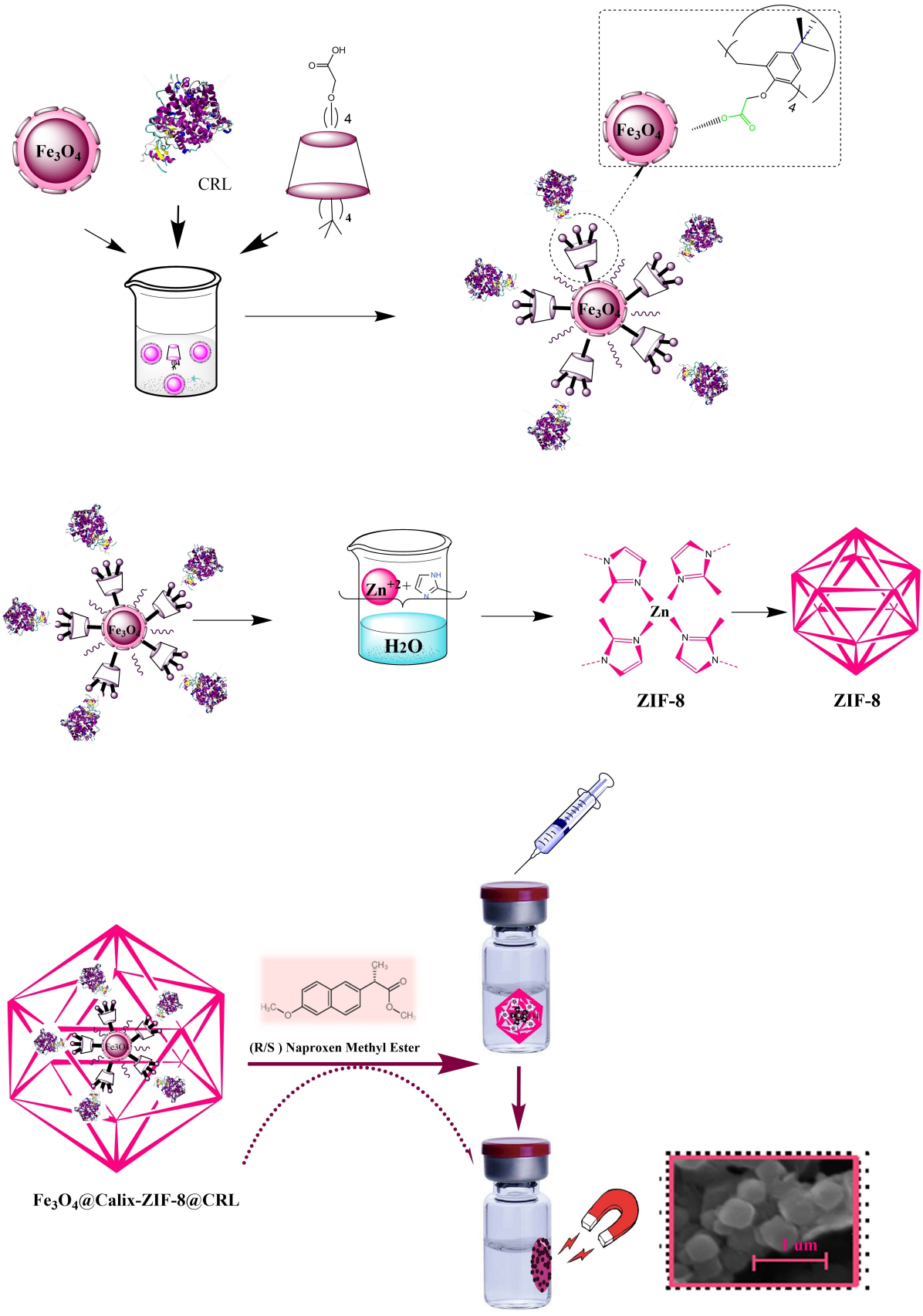


Figure 1. A) FTIR spectra of ZIF-8, Fe_3O_4@Calix-ZIF-8, Fe_3O_4@ZIF-8@CRL and Fe_3O_4@Calix-ZIF-8@CRL, **B)** XRD patterns of ZIF-8, Calix@ZIF-8, Calix-ZIF-8@CRL and Fe_3O_4@Calix-ZIF-8@CRL.



Scheme 1. Schematic illustration of the preparation of Fe_3O_4@Calix-ZIF-8@CRL and the enantioselective hydrolysis of (R,S)-Naproxen methyl ester

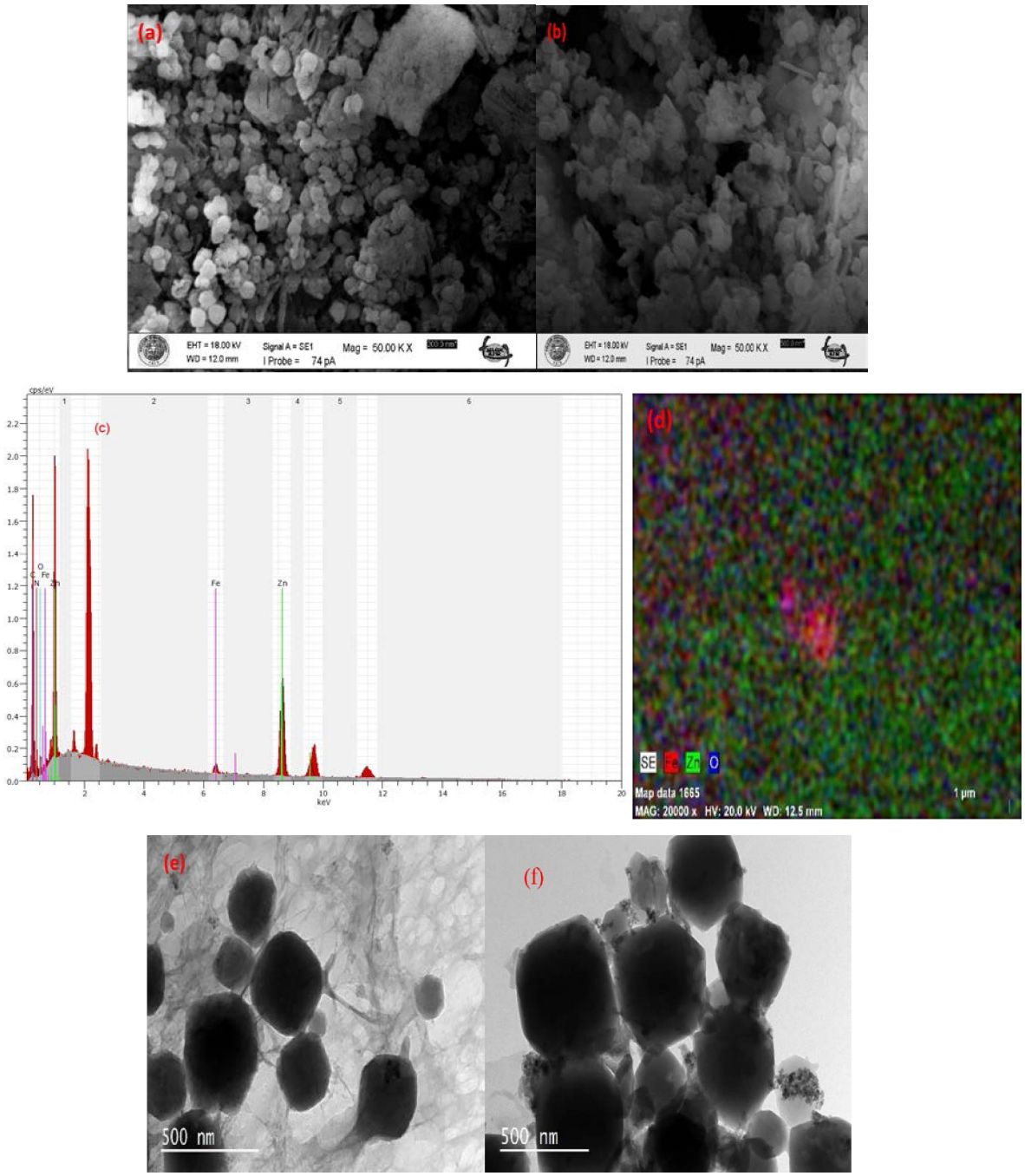


Figure 2. SEM (a,b) and TEM (e,f) micrographs for Fe_3O_4@Calix-ZIF-8 (a,e) and Fe_3O_4@Calix-ZIF-8@CRL (b,f); EDX analysis of Fe_3O_4@Calix-ZIF-8 (c,d).

Table 1. Enantioselective hydrolysis of racemic Naproxen methyl ester and activity of the immobilized lipases.[a]

Lipase	Activity [U/g]	x [%]	ee_s [%]	E
Fe_3O_4@Calix-ZIF-8@CRL (2)	207	49	95	371
Fe_3O_4@ZIF-8@CRL (1)	72	22	29	131

[a] HPLC mobile phase: n-hexane/2 propanol/trifluoroacetic acid, (100/1/0.1, v/v/v), $t = 24$ h, flow rate: 1 mL/min; UV detector wavelength: 254 nm.

of calix[4]arene is a highly effective complexing agent and can form complexes with the abundant cationic lysine residue on the enzyme surface and keep the lid open.[17,4,38,2] By the interaction of lipase with Calix, it enters the rigid structure of MOF, becomes immobilized to the enzyme, and thus the conformation of the enzyme is preserved for a long time.

Conversion and enantioselectivity were calculated to find the hydrolysis capability of immobilized lipases against naproxen methyl ester (Table 1). However, there has been no report about the magnetic MOF platform so far. The mixture was

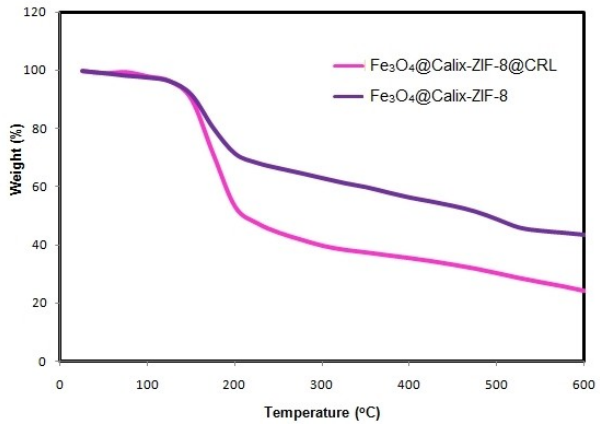


Figure 3. Thermogravimetric analysis (TGA) of Fe_3O_4@Calix-ZIF-8 and Fe_3O_4@Calix-ZIF-8@CRL.

stirred at 150 rpm, and after 24 hours, samples to the isooctane phase were measured by HPLC to calculate conversion and enantioselectivity. The conversion (x) and the values expressed as the enantiomeric ratio (E) were calculated using the equation of the literature procedure.[39] At optimum pH and temperature, Fe_3O_4@Calix-ZIF-8@CRL exhibited the highest enantioselectivity (E = 371) with conversion (X = 49%) (Table 1).

The pH is a critical factor in enzymatic hydrolysis. Free lipase, Fe_3O_4@ZIF-8@CRL and Fe_3O_4@Calix-ZIF-8@CRL were evaluated by incubating at various pH values (pH 4–9). As shown in Figure 4A, the optimum pH of the free enzyme was about 7.0, while both Fe_3O_4@ZIF-8@CRL and Fe_3O_4@Calix-ZIF-8@CRL were pH 8.0. The impact of temperature on the activity of free enzyme, Fe_3O_4@ZIF-8@CRL, and Fe_3O_4@Calix-ZIF-8@CRL in the temperature range of 30–60 °C is given in Fig. 4B. It was found that the optimum temperature for the free lipase was approximately 35 °C while it shifted nearly to 40 °C and 45 °C, for Fe_3O_4@ZIF-8@CRL and Fe_3O_4@Calix-ZIF-8@CRL, respectively.

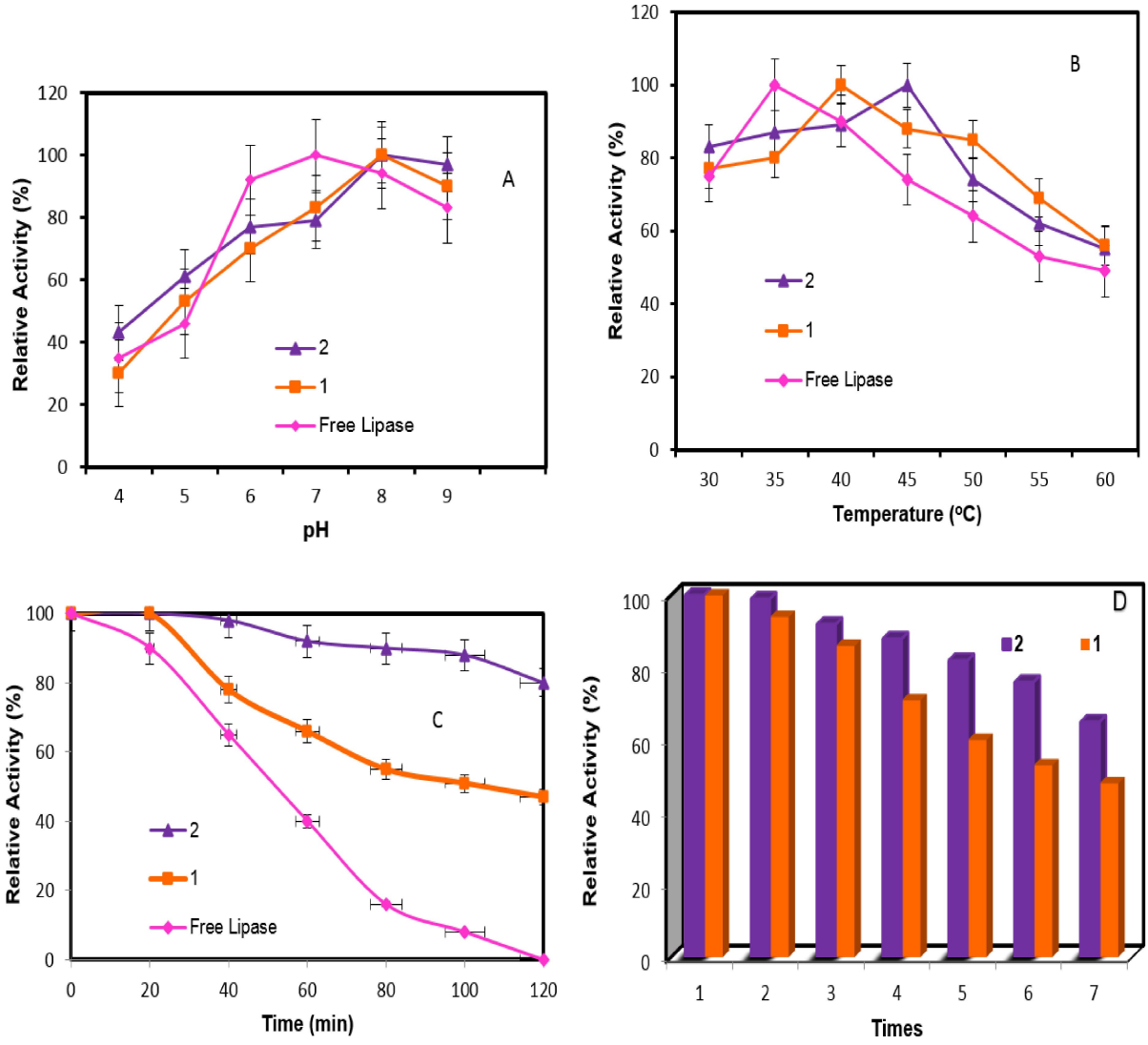


Figure 4. Effect of **(A)** pH and **(B)** temperature on enzyme activities, thermal stability **(C)** and reusability **(D)** of the free lipase, (**1**) Fe_3O_4@ZIF-8@CRL and (**2**) Fe_3O_4@Calix-ZIF-8@CRL.

This effect may arise due to conformational limits of enzyme movement and from electrostatic interactions and hydrogen bonding between Calix and lipase or improved substrate diffusion at a high temperature.[5,17,34]

The thermal stability is one of the important parameters to be considered in the enzyme catalysis reaction. The free and immobilized lipases were incubated at 60 °C for 120 min and the enzyme activity was assayed at different times as given in Figure 4C. Free lipase completely lost its hydrolytic activity in 100 minutes, Fe_3O_4@ZIF-8@CRL and Fe_3O_4@Calix-ZIF-8@CRL retained about 47% and 80% after 120 min, respectively. It can be clarified that the interactions between the lipase and Calix by the fact that the lipase preserves its tertiary structure during the immobilization. One of the main advantages of enzyme immobilization is the ability to reuse the biocatalyst. After each cycle, the Fe_3O_4@ZIF-8@CRL and Fe_3O_4@Calix-ZIF-8@ CRL were washed with deionized water and can be easily separated from the reaction medium by magnetic decantation. After 7th cycle, the Fe_3O_4@ZIF-8@CRL and Fe_3O_4@Calix-ZIF-8@CRL possessed around 48% and 65% of its initial activity, respectively (Figure 4D). The enhanced recyclability of Fe_3O_4@Calix-ZIF-8@CRL probably resulted from the inhibition of enzyme desorption.[6,26]

The kinetic properties of Fe_3O_4@ZIF-8@CRL and Fe_3O_4@Calix-ZIF-8@CRL were studied. The V_{max} value of Fe_3O_4@ZIF-8@CRL is 66.66 U/mg and Fe_3O_4@Calix-ZIF-8@CRL 119.04 U/mg. Furthermore, the Fe_3O_4@Calix-ZIF-8@CRL (0.4 mM) K_m value is lower than the Fe_3O_4@ZIF-8@CRL (0.6 mM), which demonstrates that the affinity of the enzyme to the substrate is higher after the CRL is immobilized.[40]

Optimum pH values were examined from the plot of pH plotted against percent conversion (x) (Figure 5A). The effect of immobilized lipases on enantioselectivity was determined by incubating at different pHs (7.0 and 8.0).[3,41] Optimum pH of both immobilized enzymes was observed as 8.0. To investigate the temperature of the percentage of conversion (X) of the hydrolysis reaction that is catalyzed by Fe_3O_4@ZIF-8@CRL and Fe_3O_4@Calix-ZIF-8@CRL, the experiments were carried out at three different temperatures, i.e., 25, 35 and 45 °C (Figure 5B). The optimum temperature of Fe_3O_4@ZIF-8@CRL (35 °C), Fe_3O_4@Calix-ZIF-8@CRL (45 °C) was found. These results may be due to the formation of electrostatic interaction and hydrogen bonding between the enzyme and the support, which can reduce conformational flexibility and result in higher enzyme stability.[42]

Reusability of immobilized enzymes is very easy and important due to their magnetic properties. Figure 5C displays that the Fe_3O_4@Calix-ZIF-8@CRL still kept 30% of their conversion ratios after the fifth reuse. It was found that the percent conversion (x) of the encapsulated lipase with Fe_3O_4@ZIF-8@CRL decreases after the third usage. These results show that

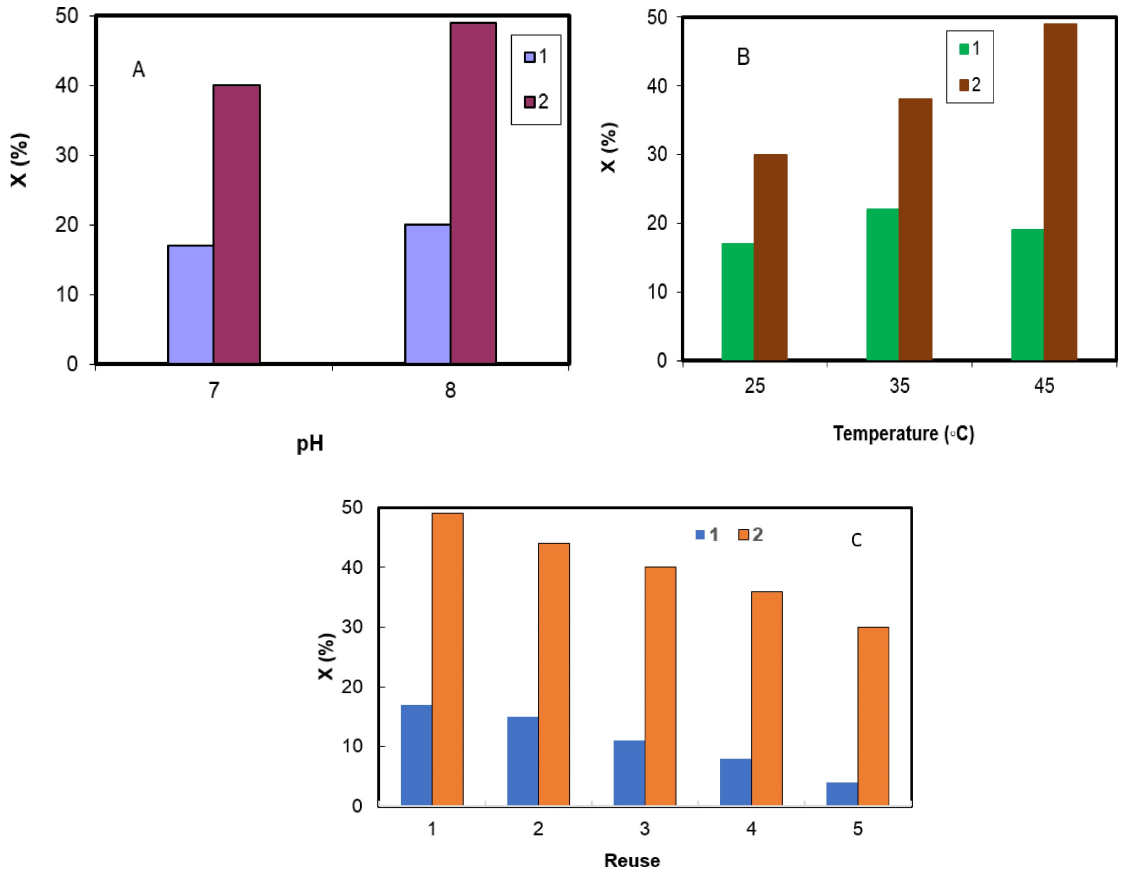


Figure 5. Influence of pH (**A**), temperature (**B**) and reusability (**C**) on the conversion (X) in the hydrolysis of racemic naproxen methyl ester with (**1**) Fe_3O_4@ZIF-8@CRL and (**2**) Fe_3O_4@Calix-ZIF-8@CRL.

with the calix-enzyme complex in the structure of Fe_3O_4@Calix-ZIF-8@CRL and its conformation is well preserved, and consequently, denaturation and enzyme leakage might be very low.[43,44] Significantly improved MOF-biocatalyst performance, as well as increased recyclability, shows that it can be utilized in very various industrial applications.[45]

Conclusions

The new magnetic MOF biocatalyst was successfully fabricated, immobilized lipase and utilized for enantioselective hydrolysis of (*R,S*)-naproxen methyl ester. The magnetic MOF biocatalyst was prepared by interacting with Calix, CRL, and Fe_3O_4 nanoparticles and then reacted with imidazole and Zn^{+2} by co-precipitate. It exhibited approximately 2.88-fold enhancement in hydrolytic activity of Fe_3O_4@Calix-ZIF-8@CRL. Additionally, the reusability of Fe_3O_4@Calix-ZIF-8@CRL is also important for economical use of the lipase, which is very simple due to its magnetic feature. It was observed that the immobilized enzyme (Fe_3O_4@Calix-ZIF-8@CRL) retained its initial activity by 65% after the 7th repeated use. In enantioselective enzymatic hydrolysis of (*R,S*)-naproxen methyl ester, the Fe_3O_4@Calix-ZIF-8@CRL was utilized as an efficient biocatalyst. It was observed that Fe_3O_4@Calix-ZIF-8@CRL has excellent enantioselectivity and conversion (X = 49%; E = 371) compared to immobilized lipase without calixarene derivative (Fe_3O_4@ZIF-8@CRL) (X = 22%; E = 131). Therefore, the immobilization of lipase on magnetic MOF suggestions a simple and inexpensive way for the pharmaceutical industry of significant differences in the biological activities of the enantiomers.

Experimental Section

Chemicals

Zinc nitrate hexahydrate ($Zn(NO_3)_2 \cdot 6H_2O$), 2- methylimidazole, *Candida rugosa* lipase (CRL, type VII), *p*-nitrophenyl palmitate (*p*-NPP), Iron(II) chloride tetrahydrate ($FeCl_2 \cdot 4H_2O$), Iron(III) chloride hexahydrate ($FeCl_3 \cdot 6H_2O$), Bradford reagent, S-naproxen were supplied from Sigma and Merck.

Preparation of Fe_3O_4@ZIF-8@CRL and Fe_3O_4@Calix-ZIF-8@CRL

Fe_3O_4 nanoparticles and tetracarboxylic acid derivatives of calix[4] arene were synthesized according to our previous studies [29–31,33,46]. $Zn(NO_3)_2.6H_2O$ (92.5 mg/mL) and 2-methylimidazole (4.1 g) was mixed into an aqueous solution at room temperature for 10 minutes. Then, Calix (20 mg) were added to CRL solution (100 mg) and after 10 minutes incubation and Fe_3O_4 nanoparticles (50 mg) were added and mixed for 10 minutes. The mixture was added to the 2-methylimidazole solution and incubated at room temperature for 30 minutes, after which the mixture turned a milky brown color. The solution was then stored overnight at 4 °C, and after 24 hours it was washed three times with deionized water and centrifuged (2028 × g, 5 minutes). The immobilized lipases were dried by lyophilization.

Characterization

The vibrational spectra of biocomposites were recorded on a Bruker Fourier Transform Infrared FTIR. Transmission electron microscopy (TEM, FEI Company- Tecnai TMG2 Spirit / Biotwin, USA) and scanning electron microscopy (SEM, Jeol, JSM5310, Japan) was identified for particle size and surface morphology of the samples. The spectroscopic measurements were performed by Shimadzu UV-1700 Pharma spectrophotometer. X-ray diffractions (XRD, Bruker D8 Advance) were used to investigate crystal structures of the biocomposite. Surface distribution of Energy Dispersive X-Ray Analysis (EDX) elements was examined. Thermogravimetric analysis (TGA) was carried out on a TGA Perkin-Elmer Puris thermogravimetric analyzer in the 0–600 °C range under nitrogen atmosphere (heating rate 10 °C/min). The samples were analyzed by High Performance Liquid Chromatography (HPLC, Agilent 1200 Series) and enantiomeric excess (ee) was determined by using a Chiralcel OD–H column.

Determination of lipase activity and stability

The catalytic activities of free lipase, Fe_3O_4@ZIF-8@CRL and Fe_3O_4@Calix-ZIF-8@CRL were determined by hydrolysis of *p*-NPP.[1–4] It is found from the 405 nm absorbance of *p*-nitrophenol (p-NP) released using UV-visible spectrophotometer. 1 Unit (U) of lipase is the amount of lipase required to hydrolyze 1 μmol p-NPP per minute. Bradford method was used to calculate the protein amount of immobilized lipases.[47]

The activities of free and immobilized enzymes were measured at various pH (4.0–9.0) and temperature (30–60 °C). In order to examine their thermal stability, enzyme activities were determined by keeping immobilized lipase solutions at 60 °C and different time intervals (20–120 minutes). The immobilized lipases were washed with deionized water and easily separated by magnetic filtration, and the enzyme activity was measured after each use. All experiments were repeated three times.

Kinetic constants determination

Catalytic activities Fe_3O_4@ZIF-8@CRL and Fe_3O_4@Calix-ZIF-8@CRL were exanimated at various *p*-nitrophenyl palmitate (*p*-NPP) concentrations. Kinetic parameters (V_{max} and K_m) were determined.

Enantioselective hydrolysis

The preparation of racemic naproxen methyl ester was carried out according to previously described method.[48] Enantioselective hydrolysis reactions were carried out in aqueous buffer solution/isooctane reaction system. A solution of racemic Naproxen methyl ester (20 mM) in 2 mL isooctane was added to 2 mL of buffer containing immobilized lipases, the mixture was stirred at 150 rpm in the incubator. Samples taken from the isooctane phase after 24 hours were analyzed by HPLC to calculate conversion and enantioselectivity. Besides, the effect of immobilized lipases on enantioselectivity was determined by incubation at different pH and temperatures. It was also performed reusability of immobilized lipases.

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Conflict of Interest

The authors declare no conflict of interest.

Keywords: biocatalysis · enantioselectivity · lipase · magnetic nanoparticles · metal organic frameworks

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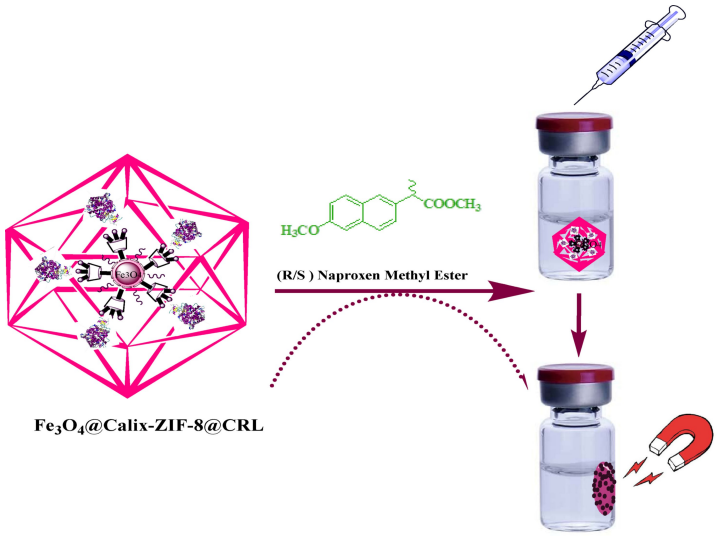

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MOF biocatalysis: Magnetic Calix@ZIF-8 biocomposites were prepared by a simple method and were utilized as a support for the immobilization of *Candida rugosa* lipase. Immobilized enzymes were also used enantioselective hydrolysis reactions of (*R,S*)-naproxen methyl ester. This work may also be useful for the pharmaceutical industry due to the increased reusability and stability of enzymes, the enantiomeric selectivity exhibited by MOF-enzyme biocomposites, and the significant differences in the biological activities of the enantiomers.