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Synthesis of calix[*n*]arene-based silica polymers for lipase immobilization

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ABSTRACT

The objective of this study was to prepare new calix[*n*]arene-based silica polymers for immobilization of *Candida rugosa* lipase. The amino functionalized calix[4]arene (C4P), calix[6]arene (C6P) and calix[8]arene (C8P)-based silica polymers were used for the covalent attachment of *C. rugosa* lipase using glutaraldehyde as a coupling agent. The characterization of synthesized CnP polymers and immobilized lipases were made by Fourier transform infrared spectroscopy (FTIR), thermal gravimetric analysis (TGA) and scanning electron microscope (SEM) techniques. The hydrolytic activities of immobilized lipases (CnP-L) were evaluated and compared with the free enzyme. The activity recovery of immobilized CRL (*C. rugosa* lipase) based on the carrier C4P, C6P and C8P reaches 74.6%, 68.5% and 51.4%, respectively. The optimal pH and temperature region of the immobilized lipases for the hydrolysis of *p*-NPP were 7.0 and 50 °C. Nevertheless, the immobilized lipase has good stability, adaptability and reusability in comparison with the free enzyme.

1. Introduction

During the last decades enzyme-catalyzed reactions, especially in organic solvents, have gained a lot of interest. Compared with conventional chemical catalysts, enzymes as biocatalysts exhibit a number of features that make their use advantageous. The specific characteristics presented by enzymes are high level of catalytic efficiency and high degree of specificities including substrate specificity, region-specificity and stereospecificity. These properties enable the enzymes to catalyze a series of biotransformation reactions which yield a wide range of useful biological and chemical compounds for pharmaceutical, food and agrochemical derivatives [1,2].

Lipases (E.C.3.1.1.3) are the most used enzymes in synthetic organic chemistry, catalyzing the hydrolysis of carboxylic acid esters in aqueous medium or the reverse reaction in organic solvents [3–12]. They are structurally characterized by a so-called lid. When hydrophobic substrates interact with the lipase, the lid opens and thus exposes the active site (serine) in a process called interfacial activation [6–12]. The catalytic triad, composed of aspartate, histidine and serine, then induces the formation of a short-lived acyl-enzyme intermediate which reacts with water or an alcohol liberating the acid or the ester, respectively.

Immobilization of enzymes is the key to expand the applications of these natural catalysts by enabling easy separation and purification of products from reaction mixtures and efficient recovery of enzyme proteins. Direct chemical cross-linking of enzyme proteins, can render enzymes insoluble, however, the extent of cross-linking is difficult to control and the enzymes tend to be denatured from the cross-linking reactions. The glutaraldehyde technique is very versatile and may be used in very different fashions [13,14]. However, in terms of stabilization, the treatment with glutaraldehyde of proteins previously adsorbed on supports bearing primary amino groups offers very good results in many cases, because it permits the cross-link between glutaraldehyde molecules bound to the enzyme and glutaraldehyde molecules bound to the support. Protocols for covalent enzyme immobilization often begin with a surface modification or activation step. The native amino groups or the active groups resulting from surface modification can be bound to aldehyde groups using glutaraldehyde [15,16]. Amine groups (terminal and in lysine residues) are very reactive, abundant on the enzyme surface and form Schiff bases with the aldehyde groups of the support. The number of covalent bonds between the support and the enzyme depends on the degree of activation of the support (concentration of aldehyde groups in the support surface) and on the concentration of amine groups in the enzyme molecule [17,18].

Thermal stability generally results from the molecular rigidity introduced by attachment to a rigid support and creation of a protected microenvironment. The four principal methods of immobilization are: (a) adsorption, (b) covalent binding, (c) entrapment and (d) membrane confinement. Among these, multipoint covalent attachment is the most effective in terms of thermal stabilization [19] although thermal stabilization has also been reported for gel-entrapped enzymes [20]. Multipoint immobilization of multimeric enzymes may prevent subunit dissociation by inter-subunit cross-linking while simultaneously reducing conformational inactivation by intra-subunit crosslinking [21].

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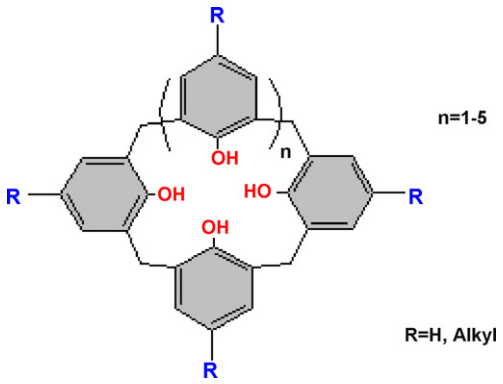


Fig. 1. The general molecular structures of calix[*n*]arenes.

Out of numerous strategies, covalent immobilization is undoubtedly considered to be one of the most efficient methods to enhance stability of enzymes, often at the cost of partial deactivation due to the conformational restrictions imposed by the bonding of enzyme residues to the support. Properties such as stability and kinetics of immobilized enzymes depend largely on the nature of supports, as well as immobilization strategy. Currently, a large number of organic and inorganic materials [22–24], synthetic polymers [25,26] and macromolecules [27,28] have been used as support materials.

Calix[*n*]arenes (Fig. 1) are a class of cyclooligomers formed via a phenol–formaldehyde condensation. They exist in a 'cup' like shape with a defined upper and lower rim and a central annulus. Their rigid conformation enables calixarenes to act as host molecules because of their preformed hydrophobic cavities. Due to this ability to form host–guest type complexes with a variety of organic or inorganic compounds the calixarenes have received increasing attention during the last two decades [29–31]. In addition, the simple introduction of various functional groups to either the upper or the lower rim of the 'cup' makes it easy to change the affinity of these cyclooligomers towards target molecules.

Over the last decade, many crown ether activation of enzymes in organic media have been reported [32]. Mainly, crown ethers form complexes with surface ammonium groups of lysine. Previously, we synthesized various calix[4]arene derivatives, immobilized them onto different supporting materials and tested for their molecular and enantiomeric recognition capabilities [33–36].

Cyclodextrins (CDs), a class of compounds with a macrocyclic structure, have been successfully used to improve enzymes activity and to increase the reaction rate and *E* in enzyme-catalyzed reactions in organic solvents [37,38]. In our previous work [39], *Candida rugosa* lipase was immobilized by covalent binding on beta-cyclodextrin-based polymer chemically modified with different activating agents as glutaraldehyde, and hexamethylene diisocyanate. The use of different activation agents allowed the immobilization of CRL with different properties. It was observed that the specific activity of the immobilized lipase with glutaraldehyde was 62.75 U/mg protein, which is 28.13 times higher than that of the immobilized lipase with hexamethylene diisocyanate.

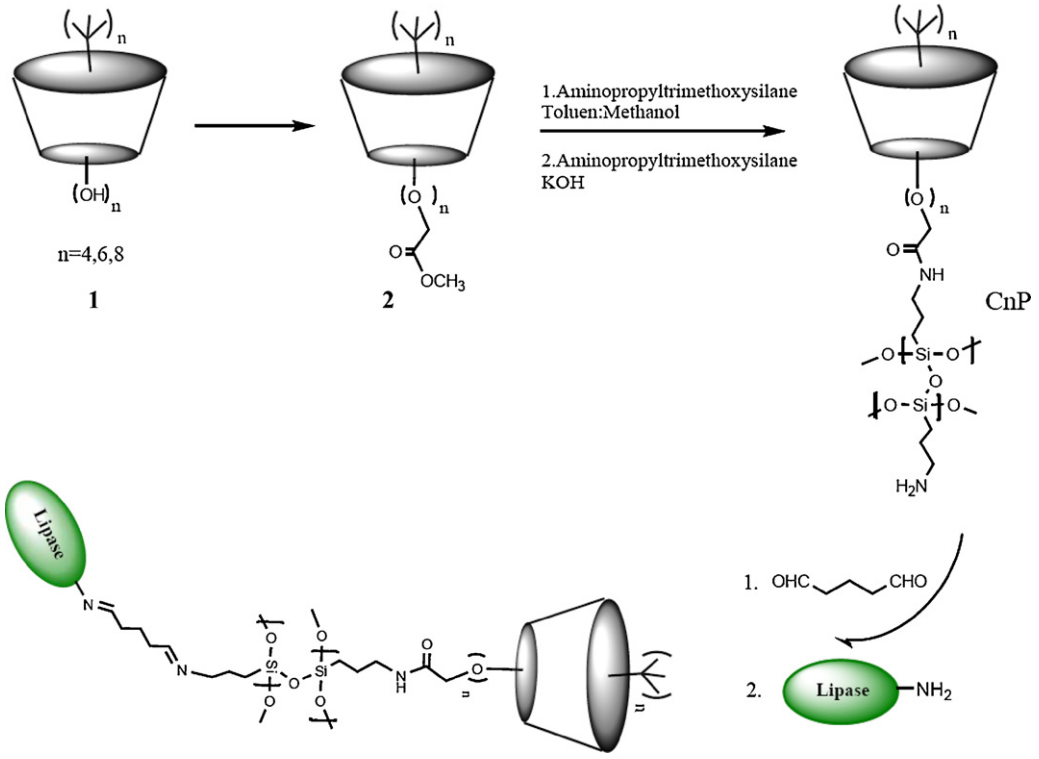
Recently, in order to see the role of calix[4]arene binding site on the lipase activitiy and stability, we have described the synthesis of a new calix[4]arene 1,3-distal glutaraldehyde derivative as a cross-linker-reagent for immobilization of *C. rugosa* lipase (CRL) [40].

Herein, we report for the first time immobilization of *C. rugosa lipase* onto calix[*n*]arene-based supports and studied the effect of calix[*n* = 4,6,8]arene molecules on lipase activity and the other parameters.

2. Materials and methods

2.1. 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,



Scheme 1. Synthesis of CnP polymers and covalent immobilization of *Candida rugosa* lipase.

and bovine serum albumin (BSA) used as the standard for protein assay were acquired from Sigma (St. Louis, MO). Glutaraldehyde was purchased from Fluka (Milwaukee, WI). All aqueous solutions were prepared with deionized water passed through a Millipore Milli-Q Plus water purification system.

FTIR spectra were recorded on a PerkinElmer 1605 FTIR spectrometer as KBr pellets. Reactions were monitored by TLC on pre-coated silica gel plates (SiO_2, Merck, $60F_{254}$). UV–vis spectra were obtained on a Shimadzu 160A UV–vis recording spectrophotometer. TGA was carried out with Seteram thermogravimetric analyzer. Analysis was performed from room temperature to 900 °C at heating rate of 10 °C/min. in argon atmosphere with a gas flow rate of 20 mL/min. Morphology of the polymers before and after lipase immobilization was investigated using a scanning electronic microscope (SEM, Jeol, JSM 5310, Japan) at 10 kV without gold sputtering.

2.2. General procedure for the synthesis of calix[n]arene-based silica polymers (CnP)

The *p-tert*-butylcalix[4,6,8]arenes **1** and their ester derivatives **2** were synthesized according to the literature procedures [41–43].

The CnP polymers employed in this work as illustrated in Scheme 1 were synthesized by adapting known procedures [44]. The ester derivative of *p-tert*-butylcalix[*n*]arene (1 mmol) and 3-aminopropyl trimethoxysilane (APTES) (10 mmol) were refluxed in a 1:1 mixture of methanol:toluene for 5 days under a nitrogen atmosphere. Reaction was monitored by TLC and FTIR. The formation of amide derivatives of calixarene CnP was confirmed by the appearance of the characteristic amide bands at about 1674–1684 cm^{-1} in their IR spectra, and by the disappearance of ester carbonyl band at 1742–1760 cm^{-1} in the IR spectra.

After the amide derivative of *p-tert*-butylcalix[*n*]arene was formed, APTES (5 mmol) and KOH (0.5 mL, 0.1 mol/L) were added in reaction medium and stirred at room temperature for 5 h under nitrogen atmosphere, and then refluxed for 5 h. After that 2 mL distilled water was dropped into the flask, the reactants were stirred continuously at reflux for 3 h. The viscosity of the reactants increases slowly and turned into light yellow solid after cooling. The solid was washed with distilled water until the filtrate remained neutral and dried at 110 °C under reduced pressure. Then resin was grounded and extracted by CH_3CN for 12 h, dried at 110 °C under reduced pressure till constant weight. Calix[*n*]arene-based silica polymers were obtained as light yellow powder; yields for C4P, C6P and C8P were 80%, 82% and 85%, respectively. FTIR (KBr); 1674, 1684 and 1677 cm^{-1} (NHCO), 3450–3200 cm^{-1} (NH and Si–OH) and 1146–1053 cm^{-1} (Si–O–Si).

2.3. Preparation of GA activated CnP polymers

CnP (1 g) was suspended in ethanol (6 mL). Upon this solution was added 20 mL of phosphate buffer (PBS) solution (0.05 M, pH 7.0) and 10 mL of 25% glutaraldehyde, and stirred for 12 h in a shaking condition (200 rpm). After the reaction, residue was filtered; excess GA was removed by washing twice with 0.05 M phosphate buffer (pH 7.0) and stored at 4 °C.

2.4. Immobilization of lipase on GA activated CnP polymers (CnP-L)

Immobilization of lipase on GA-activated CnP polymers was carried out at room temperature in a shaking condition. The GA-activated CnP polymer (0.5 g) was mixed with 20 mL of CRL solution (5 mg/mL in PBS, 0.05 M, and pH 7.0). Reaction was performed at room temperature for 7 h with continuous stirring of the reaction medium. After that, the immobilized lipases were washed with phosphate buffer (2 × 20 mL). The elution solutions containing residual enzyme were collected. The resulting immobilized lipases were held at 4 °C prior to use. The amount of protein in the enzyme solution and the elution solutions was determined by the Bradford method [45] using bovine serum albumin as a standard.

2.5. Characterization of immobilized lipases

Thermal properties of immobilized lipases (CnP-L) and GA-activated CnP polymers were analyzed by thermogravimetric analysis under Ar purge at a 10 °C/min heating rate. Morphology of immobilized lipases was investigated using a scanning electronic microscope at 10 kV without gold sputtering.

2.6. Activity of free and immobilized lipases

The activity of free and immobilized lipases was measured by dissolving 0.5 g of *p*-nitrophenyl palmitate (*p*-NPP) in 100 mL of ethanol as substrate at room temperature [46]. Free lipase of 0.1 mL (or 25 mg immobilized lipase) 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 0.5N Na_2CO_3 to the mixture followed by centrifugation (4000 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–vis recording spectrophotometer. One unit of enzyme activity was defined as the amount of enzyme, which liberates 1 μmol *p*-nitrophenol min^{-1} [47]. Specific activity was calculated by dividing total activity (U) by amount of lipase bound to CnP-GA polymers and expressed as U/mg protein. Activity yield (%) was calculated by dividing specific activity of lipase in the immobilized preparation by specific activity of soluble lipase. All measurements were performed in triplicate and an average was taken as final result.

2.7. Optimum conditions of immobilized lipases on enzymatic activity

The optimum pH and reaction temperature of free and immobilized lipases (CnP-L) were determined as the relative activity under the variety of pH (0.05 M phosphate buffer for pH 4.0–10.0) and temperature (25–60 °C). Relative activities were calculated as the ratio of the activity of immobilized enzyme after incubation to the activity at the optimum reaction pH and temperature.

2.8. Thermal stability and reusability of immobilized lipases

The thermal stability was determined by incubating the free and immobilized lipase at 60 °C in phosphate buffer pH 7.0 for 120 min. To evaluate the reusability of the immobilized lipase, after each reaction run, the immobilized lipase preparation was removed from the reaction medium 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-introduced into fresh reaction medium. This process was repeated up to 9 cycles. The activity of freshly prepared immobilized lipase in the first run was defined as 100%.

3. Results and discussion

3.1. Preparation of CnP polymers and lipase immobilization

The main focus of this work is the design of the first calixarene-based polymeric resin that is easily accessible, and the effect of

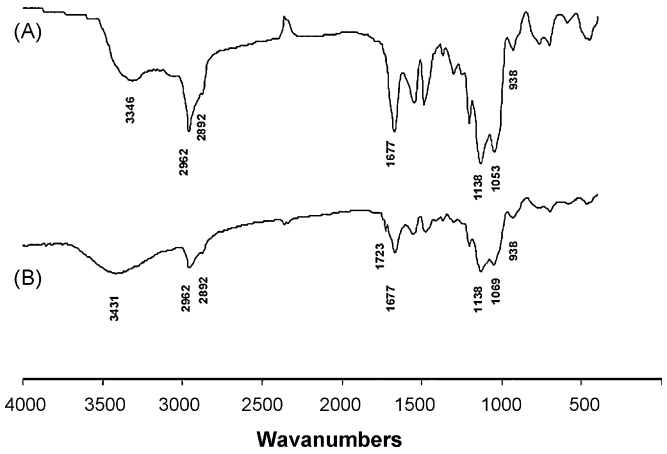


Fig. 2. The FT-IR spectrum of C6P (A) and C6P-GA (B) compounds.

calix[*n* = 4,6,8]arene molecules on lipase activity and the other parameters.

To achieve the desired goal, we have prepared *p-tert*-butylcalix[*n* = 4,6,8]arenes **1** as a starting materials through the base catalyzed condensation reaction. A synthetic scheme has been developed to enable their derivatization. This synthetic route is depicted in Scheme 1.

The synthesis of the ester derivatives of *p-tert*-butylcalix[*n* = 4,6,8]arenes **2** are based on previously published procedures while the synthesis for the other compounds CnP and lipase immobilization reported here is for the first time. Therefore, the following strategy was outlined in Scheme 1, the ester derivatives of *p-tert*-butylcalix[*n* = 4,6,8]arenes were synthesized referring to the literature by the reaction of *p-tert*-butylcalix[*n* = 4,6,8]arenes with methylbromoacetate in dry acetone in the presence of anhydrous potassium carbonate. These ester derivatives were converted to the CnP polymers using 3-aminopropyl trimethoxysilane in toluene/methanol. Completion of the reaction has been followed by IR spectroscopy, which shows the disappearance of ester carbonyl band at 1742–1760 cm^{-1} and appearance of new band at 1674–1677 cm^{-1} due to the amide carbonyl group (Fig. 2A).

Treatment of silanized calix[*n*]arene amide derivatives with 3-aminopropyltriethoxysilane in dry toluene in the presence of KOH under nitrogen atmosphere gave the polymerization product of calix[*n* = 4,6,8]arene with the amino groups. The calixarene-based structures are suitable for immobilization of lipase because the reactive groups on the long space-arms are easily approachable for enzyme coupling covalently.

To obtain the aldehyde functional group onto the support CnP, the amino groups of CnP were treated with glutaraldehyde (GA) solution in water. The proof of aldehyde groups onto the support CnP was confirmed by FT-IR spectrum measurement. The FT-IR spectrum of the C6P shows the appearance of a weak absorption band at 1723 cm^{-1} which is the characteristic of the carbonyl (aldehyde) groups (Fig. 2B).

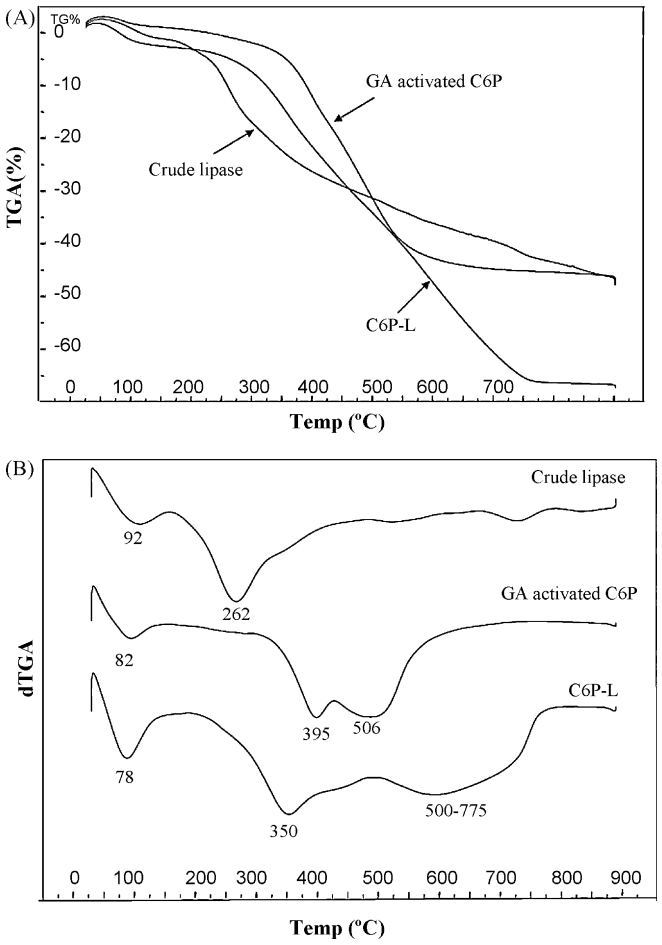


Fig. 3. TGA (A) and their 1st derivatives (B) of crude lipase, GA activated C6P and C6P-L.

The amino group of the enzyme is coupled to the carriers via free aldehyde group of the supports at room temperature for 7 h with low stirring in the standard buffer (50 mM phosphate buffer pH 7.0). Protein loading, activity and specific activity of the lipase immobilized by the immobilization protocol are shown in Table 1.

The thermal stability of the glutaraldehyde activated polymer (C6P) and the lipase immobilized polymer (C6P-L) was evaluated by thermal gravimetric analysis (TGA) and its 1st derivative (dTGA) (Fig. 3A and B). Upon heating in TGA, the crude lipase lost a few percent of mass at 92 °C, mainly to due the loss of physically adsorbed water on the material, followed by the major weight loss beginning at 262 °C. Glutaraldehyde activated polymer (C6P) was more thermally stable than the lipase immobilized polymer and showed two decomposing transitions at 395 and 506 °C. The lipase immobilized polymer (C6P-L) showed also two decomposing transitions, the major weight loss beginning at 350 °C and a broad temperature range, at 500-775 °C. The enhanced thermal stability of glutaralde-

Table 1
Yields of protein loading and activity of the immobilized lipases CnP-L prepared.

Immobilized lipase	Protein loading (μg/g CnP)	Protein loading yield (%)	Lipase activity (U/g CnP)	Specific activity (U/mg protein)	Activity yield (%)
C4P-L	536.0 ± 12	7.6 ± 0.2	14.1 ± 0.5	26.5 ± 1.4	74.6 ± 4.2
C6P-L	606.9 ± 17	8.0 ± 0.2	14.8 ± 0.6	24.4 ± 2.1	68.5 ± 3.0
C8P-L	616.4 ± 23	8.7 ± 0.4	11.0 ± 0.3	18.2 ± 0.8	51.4 ± 2.4
Free lipase	–[a]	–	2.49[b] ± 0.1	35.6 ± 2.1	100 ± 1.2

[a] Protein content of free lipase solution was 70 μg/mL.
[b] Activity of the free lipase is expressed for 1 mL (0.2%).

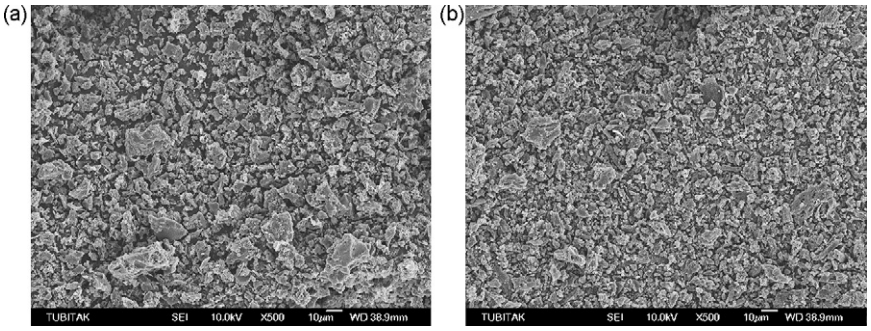


Fig. 4. SEM micrographs of the GA activated C6P (a) and C6P-L (b) before and after lipase immobilization.

hyde activated polymer is attributed to its cross-linked nature, since it is well known that cross-linking leads to increased thermal stability.

To verify changes in the immobilized enzymatic preparation during this reaction, a SEM image obtained after GA treated support (C6P) (Fig. 4a) was compared with image obtained for the immobilized lipase (C6P-L) (Fig. 4b). After immobilization, the surface cavity of the GA treated polymer was filled by the rounded structure, which is presumably protein aggregate.

Most importantly, the immobilized lipases exhibited enzymatic activity against the *p*-NPP substrate. These results demonstrate that lipase was covalently immobilized on the GA treated polymers successfully.

Table 1 shows the activity of the free and immobilized lipases. It can be seen that the amounts of bound protein are 536.0 μg/g on the C4P polymer, 606.9 μg/g on the C6P polymer, and 616.4 μg/g on the C8P polymer. The C6P and C8P polymers have more hydrophobic surfaces than the C4P polymer and in this case, lipase seem to become strongly adsorbed to hydrophobic interfaces through a large hydrophobic surface [48–51].

However, the support C4P was found to be more efficient compared to the others (C6P and C8P) with respect to expression of immobilized lipase activity. The immobilized lipase retains 74.6% of the activity on C4P, 68.5% on the C6P and 51.4% on the C8P. This result was not surprising because in the literature direct linkage of enzymes to surfaces is often reported to cause significant loss of activity when compared to the free enzyme [52]. 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 [53]. Calix[*n*]arene have various conformation and C6P and C8P have more efficient steric hindrance compared to the C4P.

3.2. Temperature and pH effect on the activity of free and immobilized lipases

The effect of pH of the medium on the enzymatic activity of free and immobilized lipases for the hydrolysis of *p*-NPP was determined in 0.05 M phosphate buffer at the pH range 6.0–8.0, and the results are shown in Fig. 5. The maximum value of relative activity was observed at pH 7.0 for free and all immobilized lipases. The buffer pH plays an important role in affecting enzyme activity because, the charge density of enzyme surface and enzyme conformation change with pH [54,55]. In addition, the pH shift depends mainly on the method of immobilization and the interaction of enzyme and support [56]. In the present study, the optimum pH of the free lipase did not shift when it was immobilized on the calixarene-based silica polymers (CnP).

The temperature dependence of the *p*-NPP hydrolysis reaction catalyzed by free and immobilized lipases was studied in the interval from 25 to 60 °C and the results are shown in Fig. 6. The

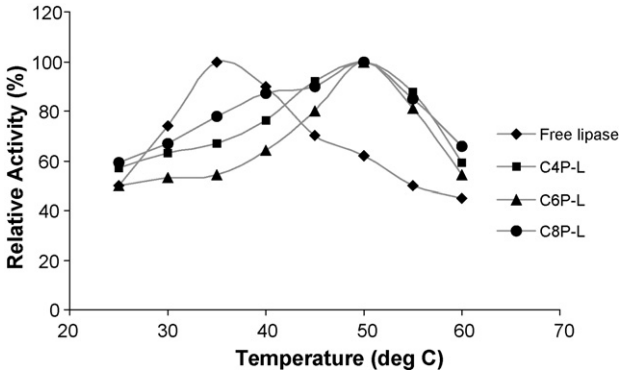


Fig. 6. Effect of temperature on the activity of the free and immobilized lipases.

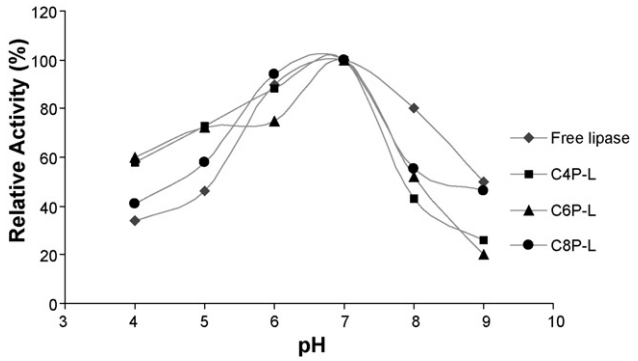


Fig. 5. Effect of pH on the activity of the free and immobilized lipases.

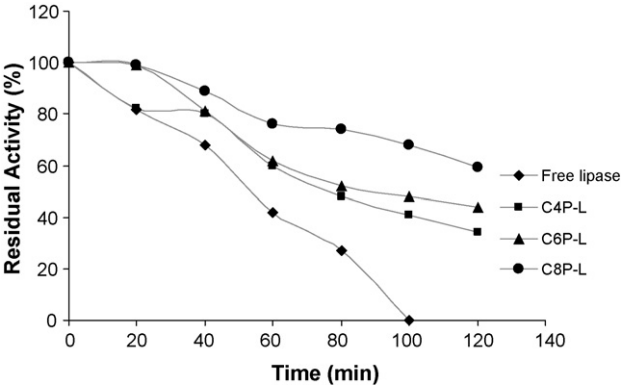


Fig. 7. Thermal stabilities of free and immobilized lipases at 60 °C.

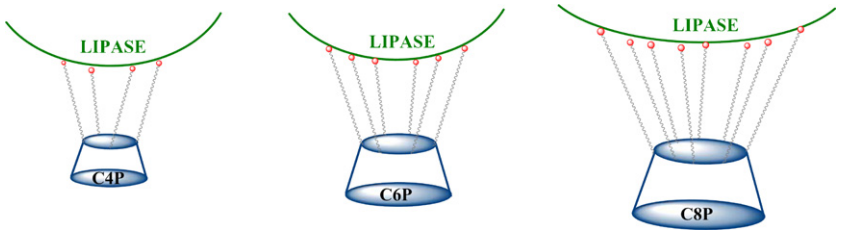


Fig. 8. Stabilization of lipase by multipoint immobilization.

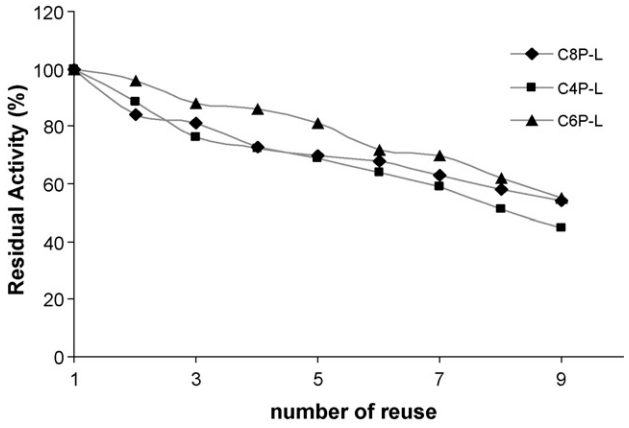


Fig. 9. Effect of repeated use on the activity of immobilized lipases.

maximum activity of the free lipase appeared at 35 °C, but the optimum temperature of the immobilized lipases (C4P-L, C6P-L and C8P-L) was obtained at 50 °C, higher than that of the free lipase. This behavior is considered to be advantageous and can be attributed to a more rigid structure of the immobilized lipases, which prevent the split breaking of the interactions responsible for the proper globular, catalytically active structure of the lipase [57,58]. However, it has been concluded that the immobilized lipases show good heat resistance due to the conformational limitation on the enzyme as a result of covalent bond formation between the enzyme and the support at high temperature [59]. Alternatively, a restriction in the diffusion of the substrate at high temperature may also be responsible [60].

3.3. Thermal stability of immobilized lipases (CnP-L)

The thermal stabilities of free and immobilized lipases onto GA activated CnP polymers were evaluated by incubating them in aqueous buffer at 60 °C. The thermal stabilities of the free and immobilized lipases are given in Fig. 7. According to Fig. 7, it can be seen that the free lipase loses its initial activity within around 100 min while the immobilized lipases retain their initial activity of about 34% for the C4P-L, 44% for the C6P-L and 59% for the C8P-L after a 120 min of heat treatment. These results indicate that the thermal stability of immobilized lipases is much better than that of the free one owing to the formation of multipoint covalent bond between the enzyme and the supports (Fig. 8), which prevents the conformation transition of the enzyme at high temperature. Multipoint immobilization of multimeric enzymes may prevent subunit dissociation by inter-subunit cross-linking while simultaneously reducing conformational inactivation by intra-subunit crosslinking [20,21]. This approach leads to a generation of multiple covalent links between the enzyme and matrix, both stabilizing the quaternary structure of the protein and increasing rigidity of the subunit structures [61]. Consequently, thermal stability of the enzyme was improved upon immobilization.

3.4. Reuse stability of the immobilized lipases (CnP-L)

The reusability of immobilized enzymes is very important for their application. In the reusability studies, it was observed that the immobilized lipases were still retained 45%, 56% and 55% of their original activities for C4P-L, C6P-L and C8P-L after the 9th reuse, respectively. Residual activity of the free and the immobilized lipases at repeated use is shown in Fig. 9. These results are due to the inactivation of the enzyme caused by the denaturation of protein and the leakage of protein from the supports upon use.

4. Conclusion

One of the most important aims of enzyme technology is to enhance the conformational stability of the enzyme. The extent of stabilization depends on the enzyme structure, the immobilization methods, and type of support. In this study, immobilization of *C. rugosa* lipase on the calix[*n* = 4,6,8]arenes-based silica polymers was first studied by the covalent immobilization methods. The activity recovery of immobilized CRL (*C. rugosa* lipase) based on the carrier C4P, C6P and C8P reaches to 74.6%, 68.5% and 51.4%, respectively. The optimum temperature for the enzymatic reaction increased from 40 to 50 °C by immobilization. The activity ratio between the initial and intermediate stages of the enzyme was reduced by immobilization, suggesting that there was a conformational change to the enzyme molecule due to the immobilization.

Acknowledgments

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