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Binary immobilization of *Candida rugosa* lipase on chitosan

Tien-Chieh Hung, R. Giridhar, Shao-Hua Chiou, Wen-Teng Wu*

Department of Chemical Engineering, National Tsing Hua University, No. 101, Section 2, Guangfu Road, Hsinchu 30043, Taiwan

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Abstract

Industrial application of lipase requires efficient methods to immobilize the enzyme, yielding a biocatalyst with high activity and stability compared to free lipase. In the present study, lipase was immobilized to chitosan beads utilizing its amino and the hydroxyl groups, which is called "binary immobilization". Lipase was first immobilized to chitosan beads by activating its hydroxyl groups with carbodiimide followed by cross-linking more lipase to the amino groups with glutaraldehyde. Under optimum conditions, the binary method of immobilization yielded a higher protein loading of 287.2 μg/g-chitosan and an activity of 13.8 U/g-chitosan as compared to the immobilized lipase prepared by activation and by cross-linking. Broader pH tolerance and higher heat stability could be achieved by this method. Immobilized lipase retained 74% residual activity after ten hydrolysis cycles and 67% after 7 days of storage. Kinetic parameters V_{max} and K_m and the energy of activation (E_a) were determined for the immobilized lipase.

Keywords: Lipase; EDC; Chitosan; Binary immobilization; Activation; Cross-linking

1. Introduction

Lipases find a broad variety of industrial applications due to the multiplicity of reactions they catalyze. Owing to unique structural characteristics, lipases can catalyze reactions involving insoluble organic substrates at the interphase of organic and aqueous phases and are able to preserve their catalytic activity in organic solvents, biphasic systems and in micellar solutions [1]. Versatility of lipase leads to multiple industrial applications in food and flavor making, pharmaceuticals, synthesis of carbohydrate esters, amines and amides, biodetergents and recently cosmetics and perfumery [2]. In order to use them more economically and efficiently in aqueous as well as in non-aqueous solvents, their activity, selectivity, and operational stability can be modified by immobilization. Methods to immobilize lipases are numerous [3–10] and a lot of effort is still being devoted to the search for new support materials and novel techniques. The type of support as well as the method of immobilization influences the activity and operational stability of immobilized lipases. By an appropriate choice of the immobilization process, operational costs of industrial processes involving lipases can be significantly reduced.

One common method of protein immobilization is by cross-linking to polymeric materials like chitosan using cross-linking reagents such as glutaraldehyde, which establishes intermolecular cross-links with amino groups of the enzyme and those of the polymer. The binding yield may be enhanced if the hydroxyl groups of chitosan molecule are also used for immobilization. The method of immobilization involving the amino and hydroxyl groups of chitosan

* Corresponding author. Tel.: +886-3-5742509; fax: +886-3-5728212.
E-mail address: wtwu@che.nthu.edu.tw (W.-T. Wu).

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is named the "binary immobilization". Application of 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC) for carboxyl group activation for covalent immobilization of a lipase to a Eudragit S-100 polymer [11] has been attempted. EDC reacts preferentially with the carboxyl groups forming *O*-acylisourea that can be readily attached to the protein's amino groups [12]. Recently, we have demonstrated that EDC can be used for activating the hydroxyl groups of chitosan [13]. No literature reports exist indicating the hydrolytic activity as well as the functional stability of lipase immobilized by chemical activation of hydroxyl groups of chitosan and cross-linking with the amino groups of lipase (binary immobilization). The present study explores the use of the binary method for immobilizing lipase to chitosan and tests its suitability for hydrolytic reactions.

2. Materials and methods

2.1. Materials

Chitosan beads, used as the support for lipase immobilization, were obtained from Kiotek Corporation (Taiwan). *Candida rugosa* type VII lipase, 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride used as the activation reagent, *p*-nitrophenyl palmitate (*p*-NPP) used as the substrate to estimate the enzyme activity, 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) and protein assay dye from Bio-Rad laboratories (Hercules, CA). All other chemicals used in this study were of analytical grade. All the solutions were prepared in deionized water.

2.2. Binary immobilization

Before establishing the suitable method of binary immobilization of lipase, the conditions for EDC activation and glutaraldehyde cross-linking were optimized as follows. A total of 2 g of chitosan beads were activated with 0.25% EDC for 20 min and immobilized with 6 ml of 0.5% (w/v) lipase for 1 h at 25 °C and pH 7.0. Similarly, 2 g of chitosan beads were cross-linked with 0.0025% (v/v) glutaraldehyde for 40 min and immobilized with 6 ml of 0.5% (w/v) lipase for 30 min at 25 °C and pH 7.0. The activation and cross-linking in the binary immobilization were carried out under the optimized conditions. To identify the best method for the binary immobilization of lipase, three different protocols were tested as follows.

A schematic illustration of the three different methods is shown in Fig. 1(a) to (c). The method that gave the highest yield of protein coupling and activity was implemented for further immobilization studies.

2.2.1. Method I

In the first method, activation and cross-linking reactions were performed simultaneously. A total of 2 g of chitosan beads were activated and cross-linked simultaneously in 6 ml of a solution containing 0.25% (w/v) EDC and 0.0025% (v/v) glutaraldehyde. After 30 min, beads were transferred to 12 ml of 0.5% (w/v) lipase for immobilization at room temperature, at pH 7. After 45 min, beads were washed thrice in deionized water, resuspended in it and stored at 4 °C.

2.2.2. Method II

In the second method, lipase was first immobilized to the amino groups of chitosan by cross-linking with glutaraldehyde followed by the immobilization of more lipase molecules through its hydroxyl groups which were activated with EDC. A total of 2 g of chitosan beads were mixed with 6 ml of 0.0025% (v/v) glutaraldehyde. After 40 min, beads were transferred to 6 ml of 0.5% (w/v) lipase for immobilization at room temperature, at pH 7. Supernatant was removed after 30 min and 6 ml of 0.25% EDC was added to the beads. After 20 min of activation, supernatant was removed and 6 ml of 0.5% (w/v) lipase was added to the beads and allowed to react. After 1 h, beads were washed thrice in deionized water, resuspended in it and stored at 4 °C.

2.2.3. Method III

In the third method, lipase was first immobilized to the hydroxyl groups of chitosan by activation with EDC, followed by the immobilization of more lipase molecules through its amino groups by cross-linking with glutaraldehyde. A total of 2 g of chitosan beads were mixed with 6 ml of 0.25% (v/v) EDC. After 20 min, beads were transferred to 6 ml of 0.5% (w/v) lipase at room temperature, at pH 7. After 1 h, the supernatant was decanted and 6 ml of 0.0025% (v/v)

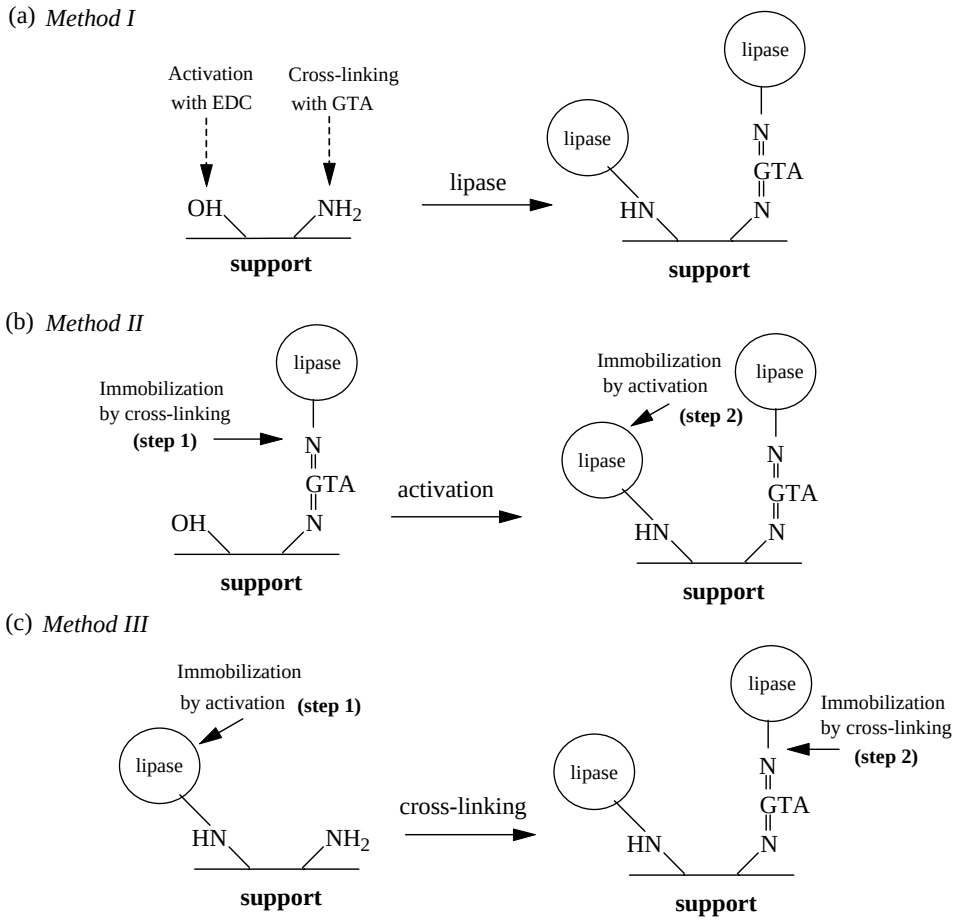

Fig. 1. Schematic illustration of three different methods of binary immobilization of lipase (GTA: glutaraldehyde).

glutaraldehyde was added to the beads. After 40 min, the supernatant was removed and 6 ml of 0.5% (v/v) lipase was added to the beads and allowed to react for 30 min. Finally, the beads were washed thrice in deionized water, resuspended in it and stored at 4 °C.

2.3. Enzyme activity assay

Activity of the free and immobilized lipase was assayed using 0.5% (w/v) *p*-nitrophenyl palmitate in ethanol as substrate. The reaction mixture consisting of 1 ml of 0.05 M phosphate buffer (pH 8 for free and 9 for immobilized lipase) containing 0.2 g of immobilized lipase (or 0.1 ml free lipase) was initiated by adding 1 ml of substrate and mixed for 5 min at 30 °C. The reaction was terminated by adding 2 ml of 0.5N Na_2CO_3 followed by centrifuging at 10,000 rpm for 10 min. The increase in the absorbance at 410 nm produced by the release of *p*-nitrophenol in the enzymatic hydrolysis of *p*-NPP was measured in a Beckman DU 530 (Fullerton, CA) spectrophotometer. A molar extinction coefficient (ε_{410}) of 15,000 $M^{-1}\,cm^{-1}$ for *p*-nitrophenol was used [14]. One unit (U) of lipase activity was defined as the amount of enzyme necessary to hydrolyze 1 μmol/min of *p*-NPP under the conditions of assay.

2.4. Protein assay

Protein concentration was estimated by the Bradford protein assay method [15] using bovine serum albumin as a standard.

2.5. Immobilization efficiency

The efficiency of immobilization was evaluated in terms of protein coupling and activity yields as follows.

$$\text{protein coupling yield (\%)} = \frac{\text{amount of protein coupled}}{\text{amount of protein introduced}} \times 100$$

$$\text{activity yield (\%)} = \frac{\text{specific activity of immobilized lipase}}{\text{specific activity of free lipase}} \times 100$$

2.6. pH and thermal stability of immobilized lipase

The pH stability of lipase immobilized by the binary method was studied by incubating the immobilized enzyme at 25 °C in buffers of varying pH (3–13) for 1 h and then determining the hydrolytic activity at the optimum pH and temperature. Residual activities were calculated as the ratio of the activity of immobilized enzyme after incubation to the activity at the optimum reaction pH.

The thermal stability of lipase immobilized by the binary method was tested by incubating the immobilized enzyme at varying temperatures (25–60 °C) and determining the activity at its optimum reaction temperature. Relative activities were calculated as mentioned above and plotted against temperature.

3. Results and discussion

3.1. Binary immobilization studies

An illustrative scheme of the chitosan–lipase complex formed by the immobilization of lipase to the amino groups of chitosan by cross-linking with glutaraldehyde and through the EDC-activated hydroxyl

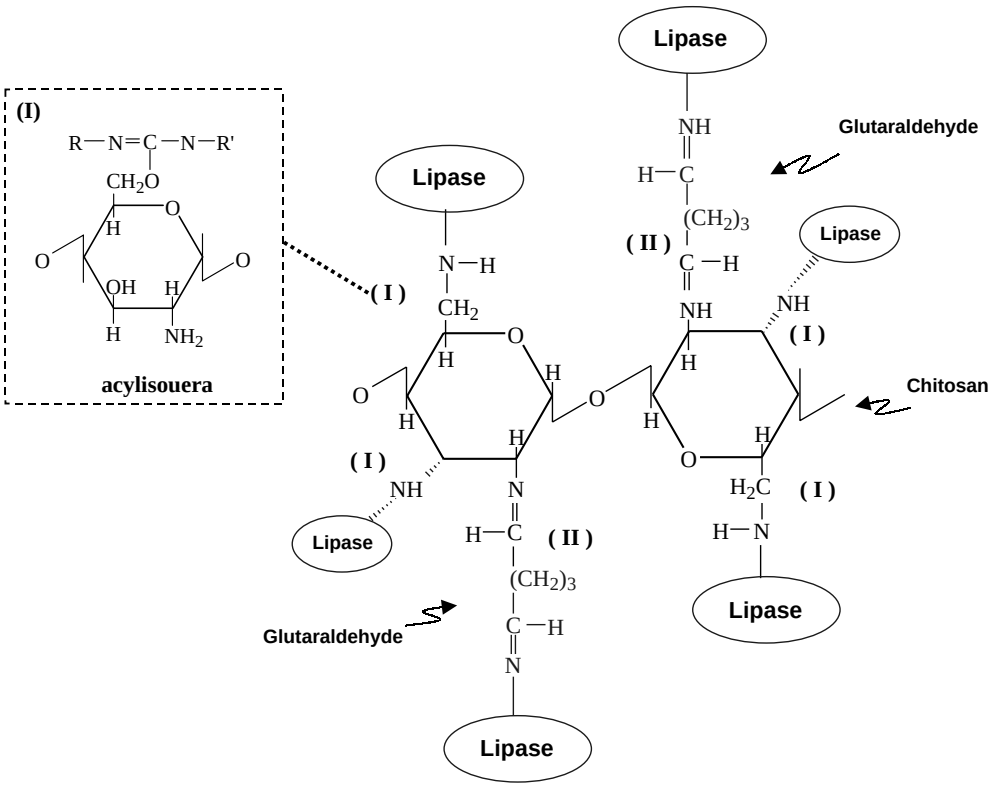

Fig. 2. Schematic illustration of lipase immobilization by activation with EDC and cross-linking with glutaraldehyde.

Table 1
Yields of protein loading and activity of the immobilizied lipase prepared by three different binary immobilization protocols

Method of binary immobilization	Total protein loading (μg/g-chitosan)	Total protein loading yield (%)	Lipase activity (U/g-chitosan)	Specific activity (U/mg-protein)	Activity yield (%)
Method I	123.2	10.8	2.7	21.9	43.4
Method II	281.6	24.7	10.1	35.9	71.0
Method III	287.2	25.2	13.8	46.2	91.5

Method I: Simultaneous activation and cross-linking; Method II: Cross-linking followed by activation; Method III: Activation followed by cross-linking.

groups is shown in Fig. 2. During activation, hydrogen ions are removed from the hydroxyl groups of chitosan and the resulting molecule reacts with EDC to form an unstable complex consisting of chitosan and acylisourea (I). Then, the alkyl group of chitosan which is formed by the release of acylisourea in presence of the enzyme binds to the amino group of the enzyme to form the immobilized lipase [13]. Whereas, during cross-linking, the aldehyde group of glutaraldehyde reacted with the amino group of chitosan or the enzyme to form imino group (–CH=N–) [16]. The imide bond is unstable in acid media (pH < 7.0) and stable at pH >7.0 [17].

The efficiency of immobilized lipase prepared by the three binary immobilization protocols is shown in Table 1. The binary immobilized lipase prepared by the method III yielded a higher total coupled protein (287.2 μg/g-chitosan) than that prepared by the method I (123.2 μg/g-chitosan) and by the method II (281.6 μg/g-chitosan). The reason for the low protein loading yield obtained for the binary immobilized lipase prepared by the method I might be due to the conjugation of EDC with glutaraldehyde since EDC possessed primary amino groups. The activity of the binary immobilized lipase prepared by the method III (13.8 U/g-chitosan) was higher than that prepared by the method I (2.7 U/g-chitosan) and by the method II (10.1 U/g-chitosan). Eventhough, the total protein loading was nearly equal for the immobilized lipase prepared by methods II and III, a higher lipase activity was observed in the case of method III. It might be due to the deactivation of some of the protein already cross-linked to the support during the activation process in method II. The activity yield for the binary immobilized lipase prepared by the method III (91.5%) was also higher than that prepared by the methods I (43.4%) and II (71.0%). Therefore, it was decided to follow this protocol (method III) of binary immobilization of lipase to chitosan and study the performance of the immobilized lipase in the hydrolysis of *p*-NPP.

The efficiency of the binary immobilized lipase is compared with that of the immobilized lipase prepared by activation with EDC and cross-linking with glutaraldehyde in Table 2. The binary immobilized lipase showed a higher protein loading of 287.2 μg/g-chitosan and activity of 13.8 U/g-chitosan. The activity yield (91.5%) showed a slight decrease as compared to the lipase immobilized by activation (98.5%). It could be presumed that the lipase bound to chitosan through EDC activation was slightly denatured and some of the active sites were not available for the second stage lipase binding by cross-linking

Table 2
Yields of protein loading and activity of the immobilized enzyme prepared by the binary method, by activation with EDC and by cross-linking with glutaraldehyde

Method of immobilization	Protein loading (μg/g-chitosan)	Protein loading yield (%)	Lipase activity (U/g-chitosan)	Specific activity (U/mg-protein)	Activity yield (%)
Activation with EDC	86.4	15.2	4.3	49.8	98.5
Cross-linking with glutaraldehyde	162.1	28.4	6.98	42.6	84.2
Binary	287.2	25.2	13.8	46.2	91.5
Free lipase	–[a]	0	4.8[b]	50.5	100

[a] Protein content of free lipase solution was 95 μg/ml.
[b] Activity of the free lipase is expressed for 1 ml (0.5%).

with glutaraldehyde. A similar decrease of activity yield was also evident for the lipase immobilized to chitosan by cross-linking with glutaraldehyde follow by activation with EDC (method II) as shown in Table 1.

3.2. Effect of pH and temperature on the activity of immobilized enzyme

Effect of pH on the residual activity of lipase immobilized by the binary method was studied by varying the pH of the reaction medium from 5 to 13 and the pH profile is shown in Fig. 3. Following the binary immobilization, the optimum pH shifted from 8 to 9 when compared to the free enzyme. Generally, binding of enzymes to polycationic supports would result in an acidic shift in the pH optimum [18]. Although chitosan belonged to the polycationic support, the pH optimum of the immobilized enzyme exhibited a basic shift. The EDC reagent used to activate the OH groups made the chitosan support anionic and the pH optimum shifted towards basic. A similar shift in pH optimum of *C. rugosa* lipase immobilized on PVC, sepharose, chitin and agarose [3] and celite [19] has also been reported in the literature. In spite of the shift in the pH, the immobilized enzyme retained a residual activity similar to that of the free lipase (Fig. 3).

The temperature dependence of the hydrolytic activity of free and immobilized lipase is shown in Fig. 4. The optimum reaction temperature (30 °C) of the lipase was not altered by immobilization. The binary immobilized lipase demonstrated higher residual activities above 30 °C as compared to the free lipase. However, complete denaturation of the enzyme occurred at 60 °C. The temperature profile for the binary immobilized lipase was broader indicating that the enzyme activity became less dependent on the temperature after immobilization.

3.3. Stability of the immobilized enzyme

The variation of the residual activity of the free and the binary immobilized lipase with pH is shown in Fig. 5. The binary immobilized lipase was stable in the pH range from 5 to 8. Free lipase was stable in the pH range from 6 to 7. This indicated that immobilization appreciably improved the stability of lipase in the alkaline region.

The thermal stabilities of the free and the binary immobilized lipase in terms of the residual activities are compared in Fig. 6. Lipase immobilized by the binary

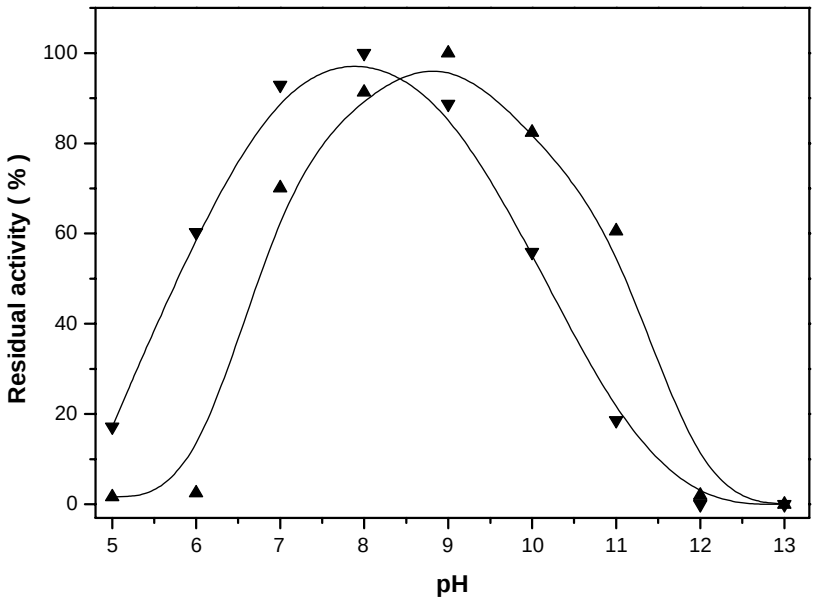

Fig. 3. Effect of substrate pH on residual activity of free (▼) and binary immobilized (▲) lipase.

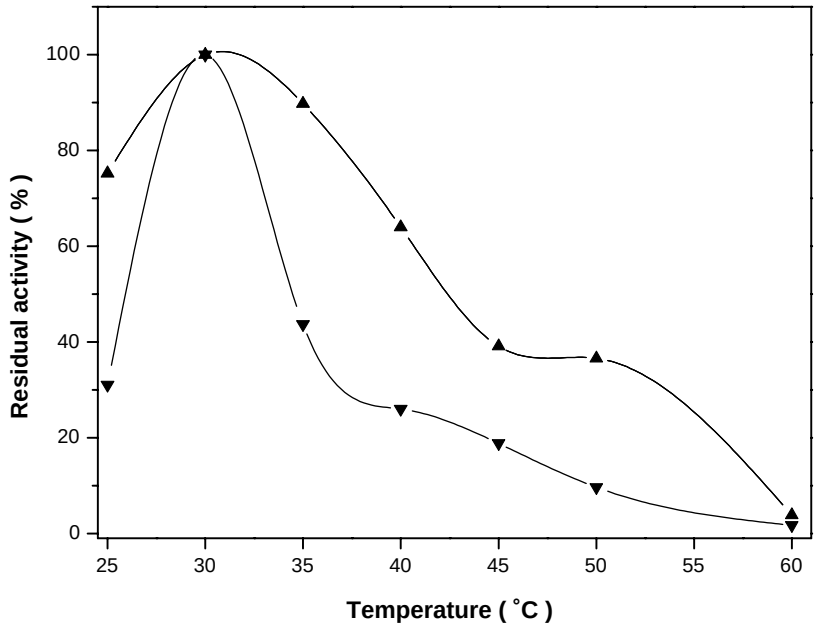

Fig. 4. Effect of reaction temperature on the residual activity of free (▼) and binary immobilized (▲) lipase.

method remained fully active up to 40 °C. However, inactivation of the enzyme occurred on treatment at higher temperatures. Free lipase remained stable only up to 30 °C. At 60 °C, the residual activity of the binary immobilized lipase was 23% as against 12% for free lipase. The binary method of immobilization has considerably increased the thermal stability of lipase.

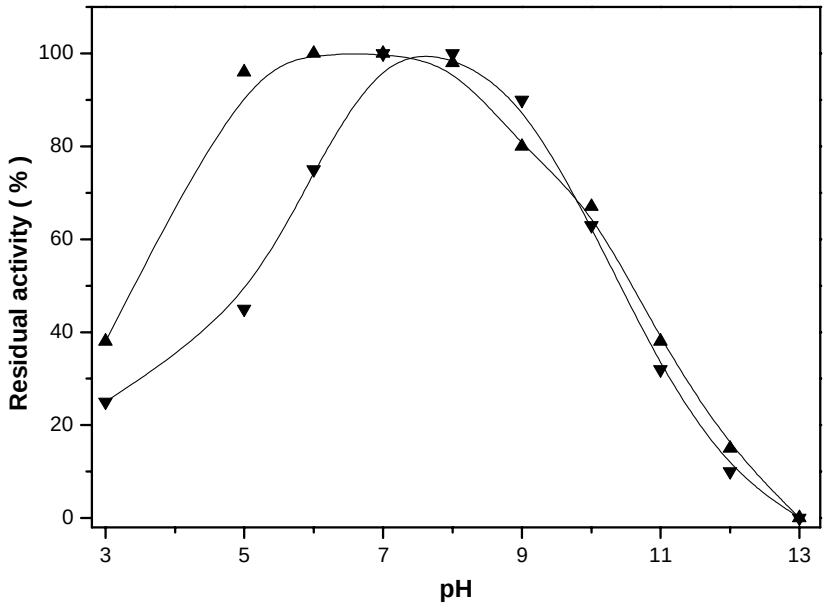

Fig. 5. pH stability of free (▼) and binary immobilized (▲) lipase.

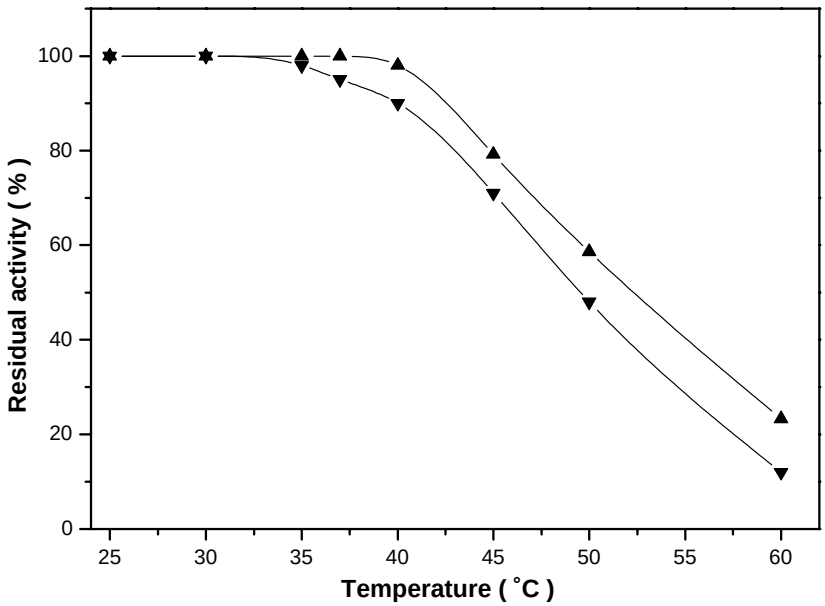

Fig. 6. Thermal stability of free (▼) and binary immobilized (▲) lipase.

3.4. Reuse and storage stability of the immobilized lipase

Lipase immobilized by the binary method was used repeatedly (10 times) to hydrolyze *p*-NPP, and the reusability was examined because of its importance for repeated applications in a batch or a continuous reactor. Residual activity of the free and the immobilized lipase at repeated use is shown in Fig. 7. After 10 reuses, lipase immobilized by the binary method

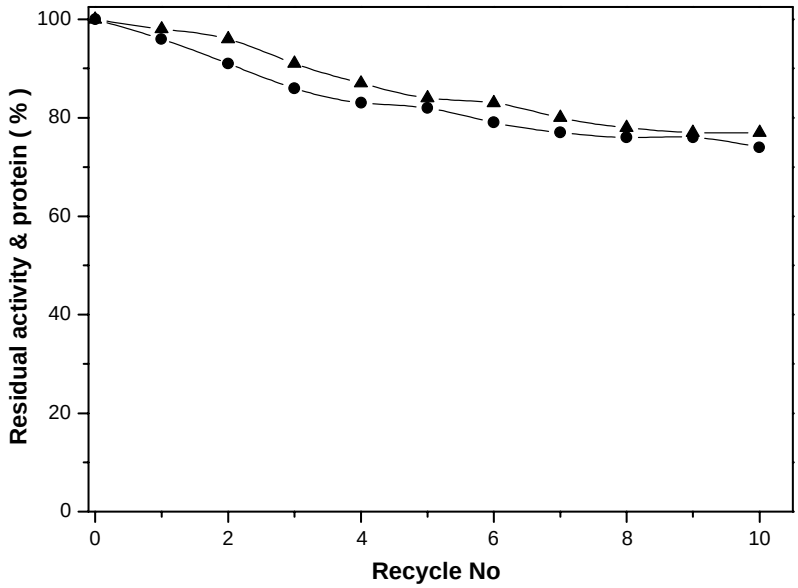

Fig. 7. Effect of repeated use on residual protein (▲) and activity (●) at 30 °C and pH 9.

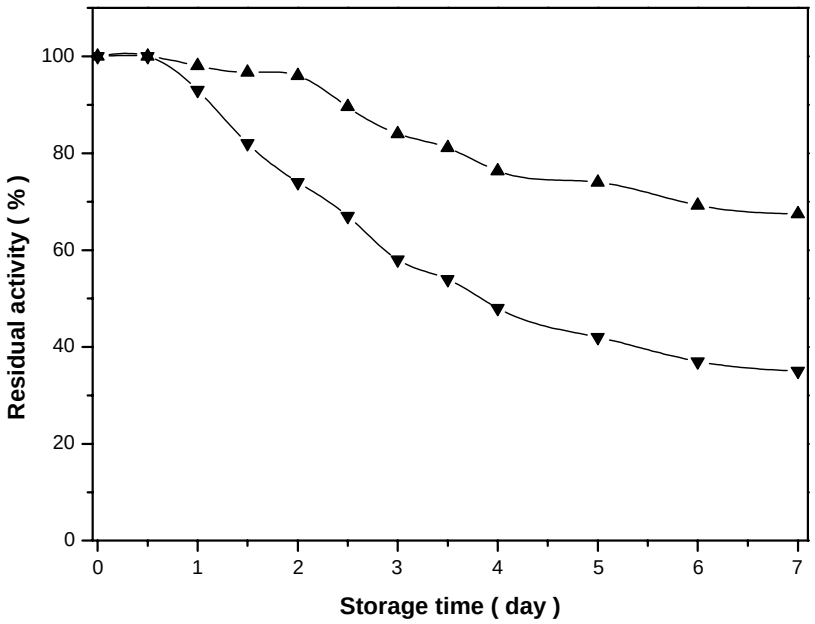

Fig. 8. Storage stability of free (▼) and binary immobilized (▲) lipase at 25 °C.

retained 74% residual activity. Activity retention of 11% after three reuses [19], 27% after seven reuses [20] and 10.5% after seven reuses [21] was reported in the literature. In comparison, the activity retained by the binary immobilized lipase, in the present study, was higher.

The storage stability of the binary immobilized lipase compared with that of the free lipase is shown in Fig. 8. After 7 days of storage at 25 °C, the residual activity of the binary immobilized lipase was 67%, while that of the free lipase was 35% indicating that the immobilization procedure has considerably increased the storage stability.

3.5. Kinetics of hydrolysis

The Michaelis–Menten kinetics of the hydrolytic activity of the free and the binary immobilized lipase was investigated using varying initial concentrations of *p*-NPP as the substrate. Michaelis constant, K_m and maximum reaction velocity, V_{max} as evaluated from the double reciprocal plot are shown in Table 3. The V_{max} value of 96.1 U/mg-protein exhibited by lipase immobilized to chitosan beads by the binary method was found to be higher than that of free lipase (92.5 U/mg-protein). If the V_{max} values could be regarded as the actual hydrolytic activity of free or immobilized lipase under substrate non-limited conditions, the maximum activity yield of the immobilized lipase could be calculated to be 104%. The K_m value determined for the immobilized lipase (18.01 mM) was about 11-fold higher than that of the free lipase (1.67 mM), which indicated a lower affinity towards the substrate. This increase in K_m might be either due to the structural changes in the enzyme induced by the binary immobilization procedure or due to the lower accessibility of the substrate to the active sites [22].

The energy of activation (E_a) of lipase binding to chitosan was also determined by the Arrhenius equa-

Table 3
Activation energy (E_a) and kinetic parameters (K_m and V_{max}) for free and binary immobilized lipase

Types	E_a (kcal/gmol)	V_{max} (U/mg-protein)	K_m (mM)
Binary immobilized lipase	15.3	96.1	18.01
Free lipase	38.6	92.5	1.67

tion and is given in Table 3. The E_a of the immobilized enzyme (15.3 kcal/gmol) was much lower than that of the free enzyme (38.6 kcal/gmol), suggesting that the enzyme has significantly higher affinity for the chitosan's active sites and lower sensitivity to temperature [21].

4. Conclusions

Lipase was immobilized to chitosan beads by a binary method in which lipase was first linked to the hydroxyl groups of chitosan activated with EDC followed by cross-linking more lipase to the amino group of chitosan using glutaraldehyde. Optimum pH of the immobilized lipase was shifted to a higher value, while the optimum temperature of free and the immobilized enzyme remained unchanged. Binary immobilization method yielded the highest protein loading and activity of 287.2 μg/g-chitosan and 13.8 U/g-chitosan, respectively. The activity is highest in comparison with the immobilized lipase prepared by activation with EDC (4.3 U/g-chitosan) and by cross-linking with glutaraldehyde (6.98 U/g-chitosan). The protein coupling and activity yields were 25.2 and 91.5%, respectively. Thermal and pH stabilities and reusability of the lipase increased considerably by the binary method of immobilization. The kinetic constants V_{max} and K_m and the energy of activation (E_a) were determined to be 96.1 U/mg-protein, 18.01 mM and 15.3 kcal/gmol, respectively. The energy activation is lower than that of free lipase. The results indicated that this method is a promising technique for large-scale preparation of immobilized lipases for industrial applications.

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