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Binary immobilization: a newer approach for immobilizing lipase from a thermophilic sp. of *Thermomyces lanuginosus*

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We report binary immobilization of *Thermomyces lanuginosus* lipase enzyme using chitosan as the support. This method of enzyme immobilization is better than cross-linked enzyme aggregate (CLEA) in terms of better enzyme recovery and separation. This method of immobilization resulted in an increase in the thermostability of the binary immobilized lipase as against the crude free enzyme. This preparation could be used for nearly 15 consecutive cycles with 80-100% efficiency. Reusability of the immobilized enzyme makes it an economical alternative to the traditional way. Immobilized lipases in particular are a modern catalytic tool for various industrially significant reactions and applications.

Keywords: Binary; Chitosan; Immobilization; Reusability; *Thermomyces lanuginosus* lipase; Thermostability

In binary immobilization, the enzyme is cross-linked to a polymeric material like chitosan *via* both the hydroxyl and amino groups in presence of a cross linking agent[1]. Chitosan is an amine polysaccharide obtained from alkaline deacetylation of chitin, a non elastic and nitrogenated polysaccharide, which is found on the walls of fungi and outer skeleton of arthropods such as insects, crustaceans and beetles[2]. Chitosan has been reused as immobilization support either for physical adsorption or crosslinking of enzymes[1,3,4]. Currently, chemically aminated lipase from *Candida antartica*, *Thermomyces lanuginosus* and *Rhizomucor meihei* were used to improve their immobilization on octyl-glyoxyl agarose beads[5]. The immobilized lipase possessed higher activity than free lipase at higher temperatures[16,17]. Another strategy for immobilization of a versatile enzyme lipase B from *Candida antarctica* (CaLB) was reported in which optimized entrapment of CaLB in sol-gel matrices by the response-surface methodology that enable competent process development[18]. Further studies were proceeded in which immobilized lipase were prepared with magnetic core shell structure for application of heterogeneous interesterification of palm stearin[19]. As standard immobilization of the enzyme lipase from *Thermomyces lanuginosus* have the potential applications in transesterification reactions in which chitosan matrix were evaluated by different strategies[20,21]. Here, in the present study chitosan is used as the support for binary immobilization using lipase enzyme. The objective of the present study was to evaluate other better possibilities of *Thermomyces lanuginosus* lipase immobilization. Using this method there is a better enzyme recovery and separation. This method of enzyme immobilization is better than CLEA as enzyme recovery and separation is more efficient. This method of immobilization resulted in an increase in the thermostability of the binary immobilized lipase as against the crude free enzyme.

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Materials and Methods

Microorganism and culture condition

Lipase production from *Thermomyces lanuginosus* was carried out in medium containing (% *w/v*) sunflower oil (emulsified with 2% gum acacia), 8.0 mL; sorbitol, 2.0; CSL, 1.0; Tween-80, 0.4; $CaCl_2.2H_2O$, 0.10; $MgSO_4.7H_2O$, 0.50; KCl, 0.50, pH 9.0, inoculated with 5×10^6 spores/50 mL and incubated at 45°C , 200 rpm for 96 h. The fermentation broth was filtered and centrifuged to remove cell debris and biomass before concentration *via* 10 kDa cellulose acetate membrane (Millipore). The five fold concentrated retentate was used for immobilization.

Enzyme immobilization

Physical adsorption

Chitosan flakes (2.0 g) were mixed with 5.0 mL of 50 mM Tris HCl buffer (pH 9.0) and kept at room temperature for 2 h. Concentrated lipase (5.0 mL) was

added to it and the suspension thoroughly mixed at 100 rpm for 6 h at 45°C. The support was filtered off and washed twice with 50 mM Tris HCl buffer (pH 9.0) to remove unbound enzyme.

Binary immobilization

The procedure of (Hung *et al.*[1] 2003) was used. Immobilization followed by cross linking using EDC was attempted both sequentially and simultaneously. The effect of detergents (SDS, Tween -80 and Triton X-100) was evaluated at a concentration of 100 mg on the cross linking and immobilization. The reusability of the immobilized preparation was evaluated by triolein hydrolysis.

Analytical techniques

Lipase assay

The procedure of Winkler and Stuckman using p-nitro phenyl palmitate was used[6]. One unit of lipase is the amount of enzyme required to release one μmole of free phenol from the substrate per mL per min under the standard assay conditions.

Triolein hydrolysis

Reaction mixture was prepared using triolein (1.0 mL), Tris HCl buffer (10.0 mL, 50 mM, pH 9.0) and immobilized lipase (500 mg) and incubated for 12 h at 45°C, 100 rpm in a shaking water bath. After the reaction, immobilized lipase was removed washed with Tris HCl buffer (50 mM, pH 9.0) and reused. For analysis of the reaction products, 0.1 mL HCl (6.0 mM) and 0.5 mL hexane were added to 0.5 mL of the reaction mixture and centrifuged. To 0.5 mL of the supernatant, 0.1 mL of copper pyridine reagent was added. This mixture was homogenized and absorbance read at 715 nm using UV/VIS spectrophotometer to determine % conversion of fatty acid (X).

$$X\,(\%) = \frac{\text{Initial concentration of fatty acid} - \text{concentration of fatty acid (t)}}{\text{Initial concentration of fatty acid}} \times 100$$

Morphology studies

Scanning Electron Microscopy was carried out to evaluate the morphology of the binary immobilized lipase on chitosan.

Results and Discussion

Immobilization

Chitosan has been reported by several researchers as a good support for immobilizing lipases by physical adsorption, crosslinking and binary immobilization[3,4]. In the present investigation, by physical adsorption 73.1% of lipase could be immobilized on chitosan (Table 1). Though, the entrapment level of lipase on chitosan is reported to vary between 43-50% depending upon the level of chitosan[7], however, a higher immobilization was recorded in the present case. When binary immobilization was carried out initially using chitosan as the support, by simultaneous immobilization, only 34.93% immobilization was recorded (Table 1), however, activation by EDC followed by crosslinking with chitosan proved to be a better method with 59.6% immobilization efficiency.

Hung *et al.*[1] (2003) also reported that sequential activation followed by crosslinking resulted in higher enzyme immobilization. This is due to the binding of lipase on both hydroxyl and amino groups of activated chitosan. Very high efficiency of lipase immobilization efficiency was recorded when SDS and Tween 80 were added during immobilization procedure. In this case, with SDS there was an increase of nearly 40% in immobilization efficiency with 97.86% binary immobilization observed (Table 2). This increase in immobilization efficiency is most probably due to the increase in enzyme activity by fixing the opening form of lipase by surfactants[8].

The surface morphology of the unactivated chitosan as observed as SEM is presented in (Fig. 1A). The immobilized chitosan (Fig. 1B) on the other hand shows visible protuberances indicating the crosslinking of the lipase with the hydroxylated

Table 1 — Binary immobilization by simultaneous and sequential crosslinking

Immobilization support (2.0 g)	Total lipase units (IU)	Lipase units adsorbed (IU)	Percent Immobilization (%)
Simultaneous cross linking and immobilization	1654.50	577.9	34.93
Sequential cross linking and immobilization	1654.50	986.0	59.60

Table 2 — Effect of surfactants on binary immobilization

Additives	Total lipase units (IU)	Lipase units adsorbed (IU)	Percentage immobilization (%)
None	1654.50	986.00	59.60
SDS	1654.50	1619.10	97.86
Triton X-100	1654.50	511.20	30.90
Tween-80	1654.50	1138.30	68.80

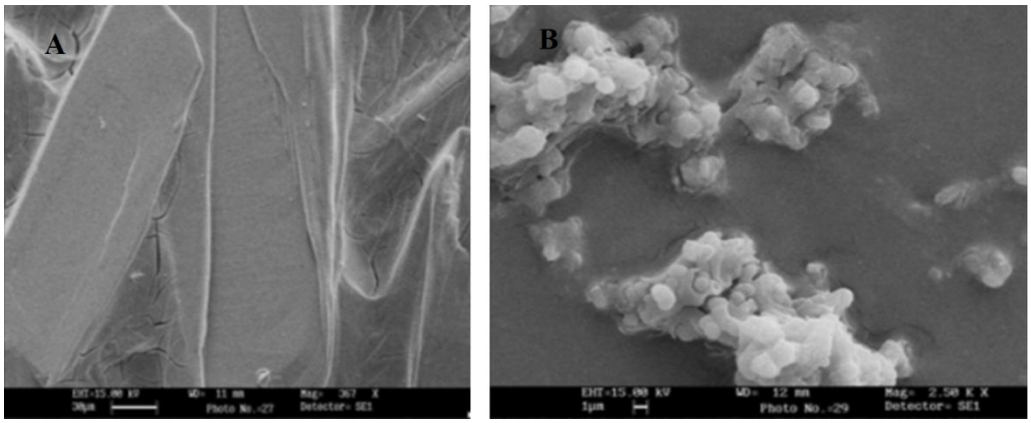

Fig. 1 — Scanning electron micrograph (SEM) of the (A) Untreated chitosan (control); and (B) Binary immobilized lipase on chitosan

and aminated surface in presence of EDC and glutaraldehyde.

Reusability of immobilized lipase

The binary immobilized form of *T. lanuginosus* lipase could carry out 15 consecutive triolein hydrolysis cycles with upto 80-100% efficiency. There was very negligible loss in activity in the repeated cycles with no significant variation noted for the enzyme activity between the first 8 cycles. On the other hand, the physically adsorbed form was reusable for only 3 cycles. Hung *et al*.[1] (2003) and Chiou *et al.*[3] (2004) reported nearly similar results with cross linked immobilized *Candida rugosa* lipase for 10 cycles.

Stability of the binary immobilized lipase

The immobilized preparation is often more thermostable as compared to the crude form[9,10]. In the present case, the binary immobilized lipase of *T. lanuginosus* retained 88.0% residual activity (RA) as against 43.1% RA for crude lipase even after 24 h of incubation at 60°C. The binary immobilized lipase exhibits nearly 2.04 fold higher stability at this temperature. At even higher temperature of 70°C, this pattern was repeated where the crude lipase becomes inactive after 24 h. However, the binary immobilized lipase exhibited 52% RA, thereby exhibiting significant thermostability (Fig. 2A). Alloue *et al.*[11] (2008) and Yi *et al*.[15] (2009) also reported increased thermal stability for immobilized *Yarrowia lipolytica* and *Candida rugosa* lipases. It is most likely that like immobilization by adsorption[11,12], binary immobilization also increases the shielding of the enzymatic protein conformation and its resistance to thermal denaturation.

There are several reports stating that, depending on the support used, the pH optimum may also shift of the immobilized support[13,14]. Yi *et al.*[15] (2009)

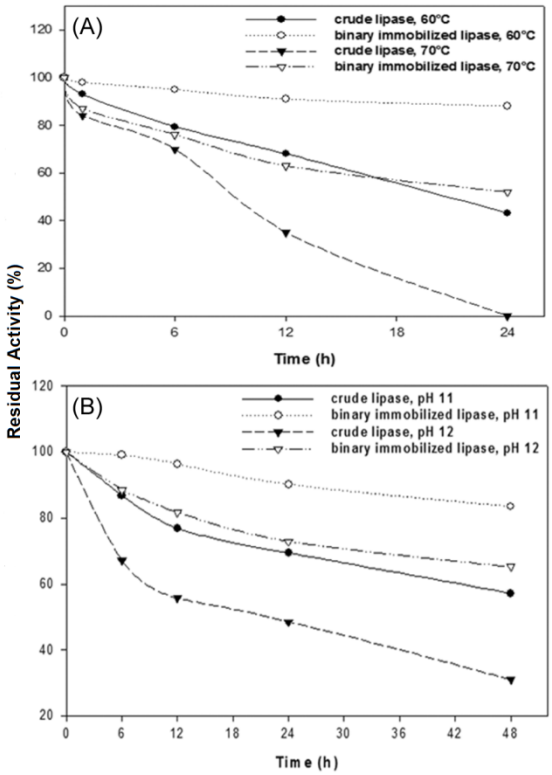

Fig. 2 — Comparison of (A) Temperature stability for 24 h at 60 and 70°C for crude and binary immobilized lipase; and (B) pH stability for 48 h at pH 11.0 and 12.0 for crude and binary immobilized lipase

reported that a shift towards the alkaline side may be noticed when chitosan is used along with glutaraldehyde for crosslinking and immobilization of the enzyme but in this case there was no such shift and the optimum remained at 9.0. In terms of pH stability, at highly alkaline pH of 11, more than 26.3% increase in residual activity was noted for immobilized lipase as against crude lipase after 48 h incubation (Fig. 2B). At even higher alkaline pH of

12.0, the binary immobilized lipase exhibited 65.2% RA as against only 31.0% by crude native lipase after 48 h. Even at acidic pH of 4.0, there was 24.5% increase in relative activity of the binary immobilized form as compared to the crude lipase form. Similarly, Yi *et al.*[15] (2009) reported increased pH stability for immobilized *Candida rugosa* lipase. The improved thermo and pH stability helps in increasing the variety of industrial catalysis where this lipase may be used.

Kinetic studies

Double reciprocal graphs when plotted showed the effect of substrate concentration on lipase activity for both free and binary immobilized lipase forms using p-NPP as substrate. Michaelis-Menten kinetics were calculated using increasing concentration of para nitro phenyl palmitate where K_m is the Michaelis constant and V_{max} is the maximum rate. Higher K_m (0.71 µM) was observed for binary immobilized lipase as against only 0.4 µM for free crude lipase. Also the rate of reaction for immobilized lipase (2380.9 µM gm^{-1} min^{-1}) was nearly 17 times higher as against free crude lipase (142.8 µM mL^{-1} min^{-1}). K_{cat} of 5.95 sec^{-1} for crude free lipase was much lower than K_{cat} of 55.89 sec^{-1} for immobilized lipase.

Conclusion

Thus it is concluded that the binary immobilized *Thermomyces lanuginosus* lipase is very stable form with significant reusability. Sequential activation followed by crosslinking in presence of surfactants increases the immobilization efficiency. In terms of reusability, this preparation could be used for nearly fifteen consecutive cycles with 100- 80% efficiency. Besides this, binary immobilization results in an increase in thermal stability and pH stability due to higher enzyme stabilization and also a higher rate of reaction.

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