Methacrylic Acid and Dodecyl Methacrylate (MAc-DMA) Hydrogel for Enhanced Catalytic Activity of Lipase of *Bacillus coagulans* MTCC-6375

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ABSTRACT: An alkaline thermotolerant bacterial lipase of *Bacillus coagulans* MTCC-6375 was purified and immobilized on a methacrylic acid and dodecyl methacrylate (MAc-DMA) hydrogel. The lipase was optimally bound to the matrix after 20 min of incubation at 55°C and pH 9 under shaking conditions. The matrix-bound lipase retained approximately 50% of its initial activity at 70–80°C after 3 h of incubation. The immobilized lipase was highly active on medium chain length *p*-nitrophenyl acyl ester (C: 8, *p*-nitrophenyl caprylate) than other *p*-nitrophenyl acyl esters. The presence of Fe^{3+}, NH_4^+, K^+, and Zn^{2+} ions at 1 m*M* concentration in the reaction mixture resulted in a profound increase in the activity of immobilized lipase. Most of the detergents partially reduced the activity of the immobilized lipase. The immobilized lipase performed ~62% conversion in 12 h at temperature 55°C. © 2006 Wiley Periodicals, Inc. J Appl Polym Sci 100: 1420–1426, 2006

Key words: *Bacillus coagulans* MTCC-6375; hydrogel; esterification and recycling

INTRODUCTION

Lipases (triacylglycerol hydrolase EC 3.1.1.3) are enzymes that catalyze the hydrolysis of fats and ester bonds in triacylglycerol to give fatty acids, diacyl glycerols, monoacyl glycerols, and glycerol. Over the last couple of years, there has been an increasing interest in the use of enzymes for the biosynthesis of molecules in organic media.[1–8] The application of lipases as catalysts in organic synthesis has been paid much more attention because of its several advantages to chemists. The lipases obtained from *Pseudomonas*, *Candida*, and *Rhizomucor* spp have been extensively studied, but there is limited information on lipase(s) obtained from *Bacillus* spp. The behavior of enzymes in organic phase is different from that in the aqueous phase. Most proteins are poorly soluble in organic solvents, and it is often necessary to immobilize enzyme on to a suitable porous matrix that provides an increased interfacial surface area, increased enzyme stability, easy separation of catalyst, and reuse of immobilized enzyme. From an industrial point of view, immobilized lipases generally offer the economic incentives of enhanced thermal and chemical stability, ease of handling, recovery, and reuse relative to nonimmobilized forms.[9,10] Lipases are versatile biocatalysts for a variety of reactions such as hydrolysis of fats, fat modification, and syntheses of glycerides and esters.[1,9] The hydrogel of alginate, carrageenan, acrylamide etc also retains some water essential for catalytic activity of lipase. The immobilization of lipase has been extensively studied and many matrices have been evaluated,[9,11–14] but activities of immobilized lipases were generally lower than those for other immobilized enzymes.[10,15]

Because of their good mechanical strength and chemical functionality, new synthetic polymers have great potential as support materials for enzyme immobilization.[12] Different sol–gel materials have been used to entrap lipases, yielding immobilized lipases with 80-fold esterification activity compared with free enzyme.[11] Phyllosilicate sol–gel matrix-immobilized lipase has also been used as biocatalyst for esterification of lauric acid with octanol for five cycles of esterification without significant loss of activity of biocatalyst.[13] We have shown that immobilization of lipase of *Bacillus coagulans* MTCC-6375 on silica increased the stability of bound lipase at enhanced temperature (50°C) than the free lipase at 45°C and also enhanced

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enzyme activity at an acidic pH (pH 5.5) than at pH 8.5 for the free lipase.[14] In the present study, we report a novel polymeric network as support for lipase immobilization. A moderately hydrophilic and weakly ionizable monomeric acid, i.e., methacrylic acid (MAc) has been hydrophobically modified by copolymerization with dodecyl methacrylate (DMA) in the presence of *N*, *N*-methylene-bisacrylamide (MBA) to obtain tailor-made polymeric support for lipase immobilization.

EXPERIMENTAL

Chemicals

N, *N*-methylene bisacrylamide, dodecyl methacrylate, isopropanol (Qualigens Fine Chemicals, India), methacrylic acid, (S. D. Fine Chemicals, India), benzoyl peroxide (Sarabhai Chemicals, India), acetone (Qualigens Fine Chemicals, India), *p*-nitrophenol, *p*-nitrophenyl palmitate, *p*-nitrophenyl acetate, *p*-nitrophenyl caprylate, *p*-nitrophenyl laurate, *p*-nitrophenyl formate, *n*-nonane (Lancaster Synthesis, UK), Tween 20, Tween 80, Triton X-100 (Sigma Chemical Co, St. Louis, MO), *N*-cetyl-*N*, *N*, *N*-trimethylammonium bromide (CTAB, Merck, Germany), and sodium dodecyl sulfate (SDS, SRL Mumbai, India) have been used as received. All these chemicals were of analytical grade.

Microorganism and lipase

The *B. coagulans* MTCC-6375 was obtained from Department of Biotechnology, Himachal Pradesh University, Summer Hill, Shimla (India). The purified lipase of *B. coagulans* MTCC-6375 was used for immobilization on methacrylic acid and dodecyl methacrylate (MAc-DMA) hydrogel. Hydrolytic activity of lipase was expressed as micromole of *p*-nitrophenol produced per minute by hydrolysis of *p*-nitrophenyl palmitate at 45°C by 1 mL of free (soluble) enzyme or at 55°C by 1 g of MAc-DMA-immobilized enzyme (including weight of matrix) under assay conditions. The lipase sourced from *B. coagulans* MTCC-6375 was purified by successive salting out and DEAE cellulose anion-exchange chromatography as previously described.[14,15] The protein was estimated by a standard method.[16]

Synthesis of hydrogel network

MAc-DMA polymeric network was prepared by copolymerizing methacrylic acid (23.6 m*M*) and dodecyl methacrylate (6.85 m*M*) in acetone (4 mL), in the presence of a crosslinker *N*, *N*-methylene bisacrylamide (376 mg) and benzoyl peroxide (30 mg) as an initiator. The reactants were vigorously stirred and transferred inside an airtight glass vial under vacuum. The vial was sealed and heated at 80°C for 30 min in a water bath. Insoluble product (polymer) was separated from the reaction mixture by filtration to remove unreacted soluble constituents. Successive washings with water, methanol, and acetone in that order separated the unreacted compounds trapped in the body of the polymer. The polymer was dried completely in an air-oven at 40°C for 24 h to obtain a xerogel.

Assay of hydrolase activity

The hydrolytic activity of free or immobilized lipase was assayed by a standard colorimetric method, using *p*-nitrophenyl palmitate (*p*NPP) as a substrate.[17] A 20 m*M* stock solution *p*NPP was prepared in HPLC grade isopropanol. The reaction mixture containing 75 μL of *p*NPP stock-solution and 2.20 mL of Tris buffer (0.05*M*, pH 8.5) was preincubated at 45°C (soluble enzyme) or 55°C (hydrogel bound enzyme) for 10 min. Thereafter, reaction was started by adding either 5 μL of soluble or 50 mg of hydrogel-bound lipase. The reaction mixture was incubated at 45°C (soluble enzyme) or 55°C (hydrogel bound enzyme) for 10 min in a water-bath. Adding 1 mL of chilled acetone : ethanol mixture (1 : 1, kept at −20°C over night) stopped further lipase-catalyzed reaction. Control containing heat-inactivated (5 min in boiling water bath) enzyme (in duplicate) was also incubated with each assay. The A_{410} (Shimadzu UV/Visible Spectrophotometer, Japan) of heat-inactivated lipase was subtracted from the absorbance of the corresponding test sample. The unknown concentration of *p*-nitrophenol released was determined from a reference curve of *p*-nitrophenol (2–20 μg/mL in 0.05*M* Tris buffer, pH 8.5). Each of the assays was performed in triplicate unless otherwise stated and mean values were presented. The released *p*-nitrophenol was recorded at A_{410}.

Effect of coupling pH on immobilization of lipase

The effect of buffer pH on immobilization of lipase on hydrogel was studied by incubating the matrix (50 mg) with lipase (dissolved in Tris buffer of different pH 5.7–10) at 55°C for 20 min under continuous shaking (160 rpm). The buffer was removed by decantation and the matrix was subjected to three washings of 5 min each with excess of 0.05*M* Tris, pH 8.5. The lipase activity was assayed in the matrix thereafter at 55°C.

Immobilization kinetics of the lipase

The matrix (50 mg) taken in a glass vial was suspended in 0.05*M* Tris buffer pH 8.5 containing lipase with continuous shaking (160 rpm) at 55°C. At intervals, the supernatant was sampled (5 μL) and assayed for residual (unbound) lipase activity. The bound fraction was determined by subtracting residual activity

from the original lipase activity used during the process of immobilization.

Effect of protein concentration on immobilization of lipase

Concentration of total protein (as lipase) in the coupling buffer (0.05*M* Tris buffer, pH 9.0) was varied between 0.25 and 2.0 mg/mL so as to determine the optimal concentration of protein with respect to fixed amount of matrix (100 mg) to achieve efficient binding of the protein.

Thermostability of free and immobilized lipase

Thermostability of the free lipase was evaluated at 70°C and that of hydrogel-immobilized (50 mg) lipase was examined at 70 and 80°C. Immobilized and free lipases were incubated separately in 0.05*M* Tris buffer of pH 8.5 up to 5 h with continuous shaking (160 rpm). At intervals, the residual lipase activities of the free and immobilized enzymes were determined under standard assay conditions. Residual activity in the nonincubated sample was taken as 100% (2.46 U/g for immobilized enzyme).

Effect of acyl chain length on immobilized lipase activity

The MAc-DMA-immobilized lipase (50 mg) was reacted with any of the five *p*-nitrophenyl esters (substrates) dissolved in isopropanol. The *p*-nitrophenyl esters were *p*-nitrophenyl formate (1:0), *p*-nitrophenyl acetate (2 : 0), *p*-nitrophenyl caprylate (8 : 0), *p*-nitrophenyl laurate (12 : 0), and *p*-nitrophenyl palmitate (16 : 0). The released *p*-nitrophenol was measured at A_{410}.

Effect of detergents on activity of immobilized lipase

The effect of a few common detergents on the activity of immobilized lipase was studied by incubating 50 mg of hydrogel-immobilized lipase separately in 1% (w/v) solution of the selected detergents for 20 min at 55°C. Thereafter, the solution containing detergent was removed by decantation. The matrix was washed thrice with excess of 0.05*M* Tris buffer, pH 8.5, and this matrix was assayed at 55°C for residual lipase activity. The detergents used in this study included Tween 20, Tween 80, CTAB, SDS, and Triton X-100.

Effect of metal ions on immobilized lipase activity

To evaluate the effect of various metal ions on lipase activity, chloride salts of various elements (Al^{3+}, K^+, Na^+, Zn^{2+}, NH_4^+, Hg^{2+}, Mg^{2+}, Co^{2+}, Mn^{2+}, Fe^{3+} and Cu^{2+}) and sodium salt of Mo^{2-} ions were used to study their effect on the activity of hydrogel-bound lipase. The selected salt-ions were separately included in the reaction mixture at a final concentration of 1 m*M*. The lipase activity was assayed after 10 min incubation at 55°C and A_{410} values were recorded.

Hydrolytic activity of immobilized lipase during recycling

The hydrolysis of *p*-nitrophenyl palmitate was performed in 3 mL of reaction buffer at 55°C by employing 50 mg of recycled immobilized lipase up to 10 cycles. After every cycle of hydrolysis of *p*-nitrophenyl palmitate, the reaction mixture was discarded by decantation and the matrix was washed thrice in 0.05*M* Tris buffer, pH 8.5. This matrix was reused for next cycle of hydrolysis of *p*-nitrophenyl palmitate under similar conditions.

Repetitive synthesis of ethyl acetate using immobilized lipase in continuous cycles of esterification

The formation of ethyl acetate from acetic acid and ethanol (1:3, by vol) catalyzed by immobilized lipase in *n*-nonane was used as a model system to check the efficiency/ability of the bound lipase to perform repetitive esterification under optimized conditions. The immobilized biocatalyst was repetitively used for 5 cycles of esterification. Reaction mixture (2 mL) contained 150 m*M* of ethanol, 50 m*M* of acetic acid, and 50 mg of MAc-DMA-immobilized enzyme. All esterification reactions were performed for 12 h at 55°C in a water bath under continuous shaking (120 rpm) in Teflon-lined closed glass vials (5 mL capacity). After completion of an esterification reaction, the immobilized biocatalyst was washed thrice for 5 min each in *n*-nonane (2 mL) and the wash was removed by decantation. The hydrogel-bound enzyme was reused for fresh cycle of esterification under similar conditions.

The reaction mixture (2 μL) was sampled and assayed for ethyl acetate by GLC programmed at 40–200°C with a ramp rate of 20°C/min (10% SE-30 Chromosorb WHP column, Michro-9100 GLC, Netel Chromatographs, India). Nitrogen was used as a carrier gas at a flow rate of 30 cm^3/min.

Time-dependant formation of ethyl acetate by hydrogel-bound lipase

The reaction mixture (3.0 mL) containing 150 m*M* of ethanol and 50 m*M* of acetic acid in *n*-nonane was catalyzed by the addition of 50 mg of hydrogel-bound lipase at 55°C under continuous shaking (120 rpm) for 21 h. At periodic intervals, 2 μL of solvent system was

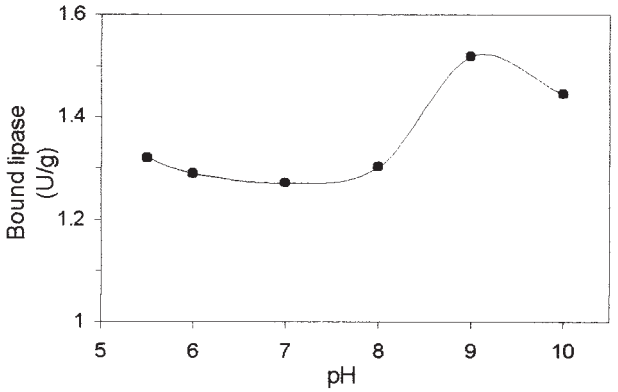

Figure 1 Effect of coupling pH on immobilization of lipase onto MAc-DMA matrix. The effect of pH of coupling buffer on binding of protein (lipase) onto hydrogel was studied by incubating the matrix (50 mg) with lipase (dissolved in Tris buffer of different pH 5.7–10) at 55°C for 20 min under continuous shaking (160 rpm).

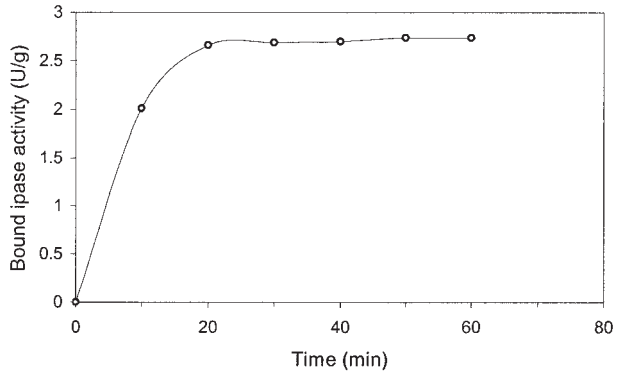

Figure 2 Effect of incubation time on immobilization of lipase. The matrix (50 mg) taken in a glass vial was suspended in 0.05*M* Tris buffer, pH 8.5, containing lipase under shaking (160 rpm) at 55°C. At intervals, the supernatant was sampled (5 μL) and assayed for residual (unbound) as well as bound-lipase activity.

withdrawn and analyzed for the formation of ethyl acetate by GLC.

RESULTS

The protein (with high lipase activity) in the harvested broth (0.27 U/mL of lipase, 8.2 mg of protein/mL) was optimally precipitated at 60% (w/v) ammonium sulfate saturation. The precipitates reconstituted in a minimum volume of 0.05*M* Tris buffer, pH 8.5, and were extensively dialyzed against the same buffer. The dialyzed enzyme showed an activity of 3.4 U/mL, specific activity of 0.67 U/mg. The anion-exchange chromatography of lipase on DEAE-cellulose column resulted in two peaks (results not shown). The fractions showing lipase activity were pooled (5.5 U/mL, protein 3.2 mg/mL, specific activity 1.7 U/mg) and subjected to freeze drying by lyophilization. The DEAE-anion exchange chromatography resulted in approximately 75-fold concentration of lipase.

Effect of physical parameters on immobilization of lipase on MAc-DMA matrix

The effect of pH of the coupling buffer on binding of the protein (lipase) onto the hydrogel support was studied by using coupling buffers (0.05*M* Tris) with pH in the range of 5.5–10.0. The protein (lipase) prepared in coupling buffer of pH 9.0 showed maximum binding to the MAc-DMA hydrogel (Fig. 1).

Moreover, the binding of the lipase onto the MAc-DMA hydrogel was very rapid as maximum immobilization of protein on to the MAc-DMA hydrogel was achieved after 20 min incubation at 55°C (Fig. 2). Thereafter, the amount of bound lipase remained more or less the same. Thus, an incubation time of 20 min was considered optimum for immobilization of soluble lipase (taken in a 0.05*M* coupling buffer, pH 9.0) on to MAc-DMA hydrogel. Moreover, the optimal binding of the available protein (lipase) was achieved at 55°C when the total protein concentration in the coupling buffer was kept at 1 mg/mL (Fig. 3). At 45°C, although maximum binding of the protein on to the MAc-DMA hydrogel occurred at 1 mg/mL concentration, yet the bound-protein was less than that recorded at 55°C.

To determine the thermal sensitivity of the bound lipase, the hydrogel-immobilized lipase was incubated at 70 and 80°C separately in a water bath shaker. The bound lipase appeared to be quite stable at both the tested temperatures as it retained approximately half of its initial activity after 3 h incubation at 70–80°C (Fig. 4). In contrast, the free lipase lost ~50% of its total activity after 20 min incubation at 70°C. Thus,

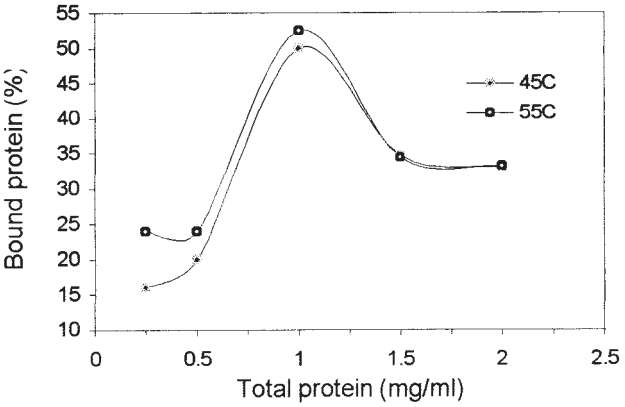

Figure 3 Effect of protein concentration on immobilization of lipase. Concentration of total protein (as lipase) in the coupling buffer (0.05*M* Tris buffer, pH 9.0) was adjusted at 0.25–2.0 mg/mL with respect to a fixed amount of xerogel (100 mg dry weight).

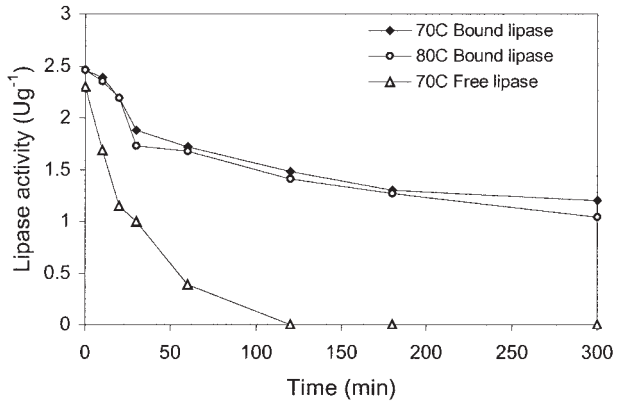

Figure 4 Thermostability of immobilized lipase. Immobilized and free lipases were incubated separately in 0.05*M* Tris buffer, pH 8.5 up to 5 h under shaking (160 rpm). At intervals, the residual lipase activities of the free (70°C) and immobilized biocatalysts (70 and 80°C) were determined.

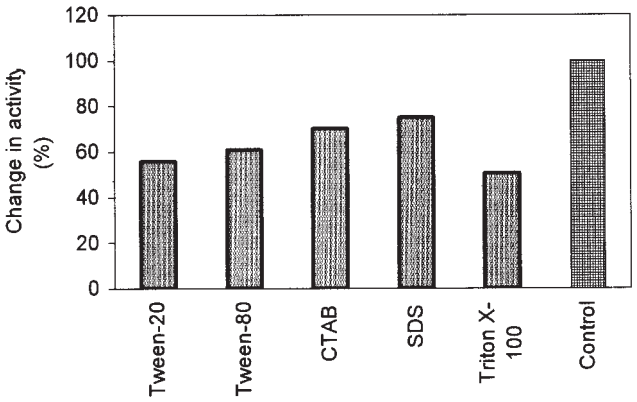

Figure 5 Effect of detergents on activity of immobilized lipase. Hydrogel-bound lipase was incubated separately in 1% (w/v) solution of the selected detergent for 20 min at 55°C. Thereafter, the detergent was removed by decantation and matrix was washed thrice with excess of 0.05*M* Tris buffer, pH 8.5. This matrix was assayed at 55°C for residual lipase activity.

immobilization of the lipase onto the hydrogel improved its stability at enhanced temperature.

Effect of acyl chain length on activity of immobilized lipase

The MAc-DMA-immobilized lipase was specifically highly hydrolytic towards medium C-chain length acyl ester (C: 8, *p*-nitrophenyl caprylate) instead of relatively shorter or longer C-chain length esters (Table I).

Effect of detergents on immobilized lipase activity

The effect of each of the selected detergents on the activity of immobilized lipase was studied. Although most of the detergents tested in the present study markedly decreased the activity of the immobilized lipase (Fig. 5), yet Triton X-100 had the maximum denaturing effect on the MAc-DMA hydrogel-immobilized lipase. Thus, the catalytic activity of the bound lipase was highly sensitive to the denaturing effect of most of the detergents used in this study.

TABLE I
Effect of Acyl Chain Length on Hydrolytic Activity of Immobilized Lipase of *B. coagulans* MTCC-6375

p-Nitrophenyl acyl substrate	Lipase activity (U/g)
p-Nitrophenyl formate	1.66
p-Nitrophenyl acetate	1.71
p-Nitrophenyl caprylate	2.50
p-Nitrophenyl laurate	1.70
p-Nitrophenyl palmitate	1.70

A 20 m*M* stock solution *p*-nitrophenyl acyl ester (substrate) was prepared in *iso*-propanol. The reaction mixture containing 75 μL of *p*NPP stock-solution, and 2.20 mL of Tris buffer (0.05*M*, pH 8.5) was preincubated at 55°C for 10 min. The hydrolytic reaction was performed by addition of 50 mg of hydrogel-bound lipase at 55°C for 10 min and the lipase activity was calculated thereafter.

Effect of salt-ions on immobilized lipase activity

The presence of some of the salt ions (Mo^{2-}, Fe^{3+}, NH_4^+, K^+ and Zn^{2+}) markedly increased the lipase activity. The presence of Fe^{3+} ions resulted in a maximum lipase activity (2.46 U/g) that was ~285% more than the control value. However, presence of Al^{3+}, Hg^{2+}, Mg^{2+}, Co^{2+}, and Mn^{2+} decreased the hydrolytic activity of immobilized lipase (Fig. 6).

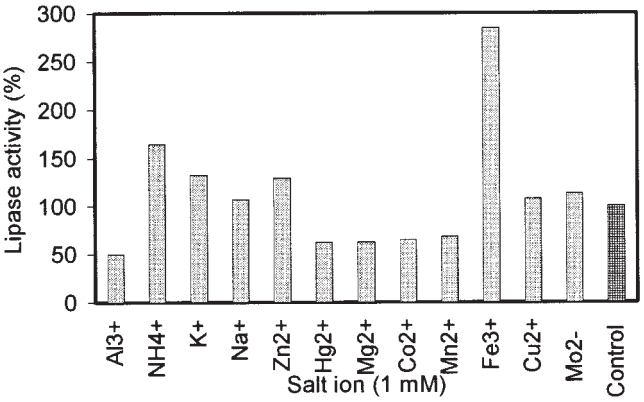

Figure 6 Effect of various metal-ions on activity of immobilized lipase. The selected salt-ions (1 m*M*) were separately incubated with 50 mg of bound-lipase in 0.05*M* Tris buffer, pH 8.5 at 55°C for 10 min under shaking (120 rpm). The buffer was decanted, and matrix was washed thrice in excess of same buffer and used for hydrolysis of *p*NPP at 55°C for 10 min. The residual lipase activity was assayed thereafter.

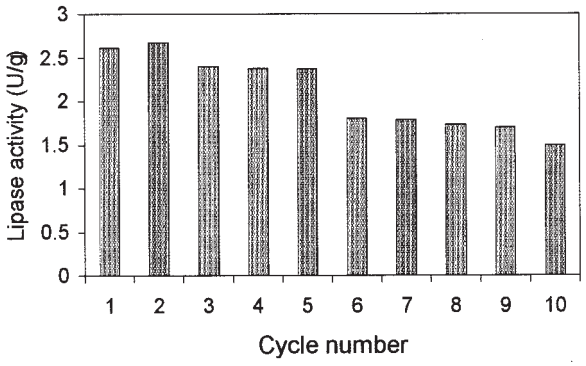

Figure 7 Reusability profile of immobilized lipase for hydrolysis of *p*NPP. The hydrolysis was performed at 55°C by employing 50 mg of immobilized lipase. After hydrolysis of *p*NPP, the reaction mixture was discarded by decantation and the matrix was washed thrice in 0.05*M* Tris buffer, pH 8.5. This matrix was reused for next cycle of hydrolysis under similar conditions.

Hydrolytic activity of immobilized lipase during recycling

The hydrolysis of *p*-nitrophenyl palmitate was performed by employing recycled immobilized lipase. After 10th cycle of reuse, the MAc-DMA-immobilized lipase retained approximately 56% of its initial hydrolytic activity (Fig. 7).

Effect of reaction time on rate of esterification

The formation of ethyl acetate from ethanol and acetic acid was maximally observed at 12 h at 55°C under continuous shaking in *n*-nonane (Fig. 8).

Reusability of immobilized lipase for synthesis of ethyl acetate

The reuse of MAc-DMA-immobilized biocatalyst showed a gradual decline in the catalytic activity for the synthesis of ethyl acetate in *n*-nonane (Table II). The rate of conversion of ethanol and acetic acid to ethyl acetate ranged between 61.6 and 44.3% during 5 cycles of reuse of the immobilized lipase.

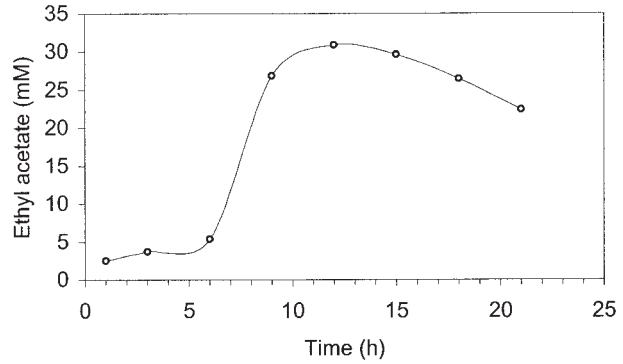

Figure 8 Time-dependent formation of ethyl acetate in *n*-nonane. 3.0 mL of reaction mixture containing 150 m*M* of ethanol and 50 m*M* of acetic acid in *n*-nonane was catalyzed by 50 mg of hydrogel-bound lipase at 55°C under shaking (120 rpm).

TABLE II
Reusability of MAc-DMA-Immobilized Lipase for Synthesis of Ethyl Acetate

Cycle number	Ethyl acetate formed (m*M*)	Conversion (%)
1	30.8	61.6
2	28.8	57.6
3	24.7	49.3
4	24.1	48.3
5	22.2	44.3

The reaction mixture contained 150 m*M* of ethanol, 50 m*M* of acetic acid, 50 mg of MAc-DMA-immobilized enzyme and *n*-nonane to make a final volume of 3.0 mL. Esterification was performed for 12 h at 55°C under continuous shaking (120 rpm) in Teflon-lined closed glass vials. After completion of reaction, the immobilized biocatalyst was retained and washed thrice for 5 min each in *n*-nonane (2 mL). This hydrogel-bound lipase was reused for fresh cycle of esterification under similar conditions.

DISCUSSION

Lipases are currently receiving increased attention because of their potential applications in biotechnology-based industries like medicine, dairy, beverage, food, artificial flavors/fragrances, and detergents.[18,19] During the last decade, screening of large number of microorganisms has resulted in obtaining many microorganism(s) possessing a variety of enzymes catalyzing syntheses of fine chemicals in organic/water-restricted solvents.[20] Among bacteria, *Pseudomonas*, *Rhizomucor*, and *Bacillus* spp are the most important lipase-producing genera. In last few years, emphasis has been laid on purification and characterization of various microbial lipases. *Bacillus coagulans* MTCC-6375 lipase was purified to homogeneity by employing successive techniques of ammonium sulfate precipitation, DEAE-cellulose anion-exchange chromatography, and gel-permeation, yielding 75-fold of purification. Purified native lipase displayed optimum activity at pH 8.5 and temperature 45°C.[14] The pH, metal ions, and detergents have been important environmental factors that tend to modulate activity of most free and immobilized enzyme preparations.[11,14,21,22]

In the present study, *B. coagulans* MTCC-6375 lipase was found to efficiently bind onto poly (MeAcAc-*co*-DMA)-*cl*-*N*,*N*-MBA i.e., MAc-DMA support under standardized conditions. The matrix displayed optimum lipase activity at pH 8.5 and temperature 55°C. The reusability of this matrix was significantly boosted following 2nd cycle of *p*-NPP hydrolysis. This increase in lipase activity might be attributed to prolonged interfacial activation of immobilized lipase. The MAc-DMA-

immobilized lipase retained more than 50% of its original esterase activity after 10th cycle of reuse. In our present study, the thermostability of *B. coagulans* MTCC-6375 lipase was also enhanced upon immobilization on the present matrix, which showed 48.0% retention in original lipase activity after 5 h of continuous incubation at 80°C. Some thermostable lipases have been previously reported from many bacteria, including *Bacillus* spp.,[23,24] *B. coagulans*,[25] *B. cereus*,[26] and *B. stearothermophilus*.[21] One of the most notable thermostable enzymes from a *Bacillus* strain showed a maximum activity at 60°C and retained 100% of the original activity after being held at 75°C for 30 min.[27] The half-life of this enzyme was 8 h at 75°C, and the enzyme retained at least 90% of the original activity after being incubated at 60°C for 15 h. Lipase from *B. coagulans* BTS-3 was found to retain 75% of its activity at 70°C at 20 min of incubation time.[25] A few other highly thermostable lipases have also been reported in literature.[23,24,28–30] An extra cellular lipase isolated from a *Bacillus* spp.[23,24] possessed an optimum activity at 50°C with a half-life of 15 min at 75°C. This enzyme was stable in the presence of various oxidizing or reducing agents, surfactants, chelating agents, and also in certain organic solvents.

Thermal and operational stability of many lipases can be significantly enhanced by immobilization.[11,31,32] In the present study, immobilization of lipase onto the hydrogel markedly enhanced its stability at 70–80°C, whereas the free enzyme lost approximately half of its activity at 70°C. In a previous report, *C. antarctica* lipase B could be thermally stabilized by immobilization; however, the native enzyme and the covalently immobilized preparation appeared to follow different modes of thermal deactivation.[31]

Compared with conventional chemical synthesis from alcohols and carboxylic acids using mineral acids as a catalyst, the use of lipases as biocatalysts to produce high value-added fatty acid esters in organic media may offer many significant advantages.[33] These include the use of hydrophobic substrates, higher selectivity, milder processing conditions, and the ease of product isolation and enzyme reuse. The real esterification potential of the *B. coagulans* lipase in organic media might be explored by synthesizing a series of short and long chain fatty acid esters. The short chain esters of methyl ketones and fatty acids constitute aroma and flavor of natural.[34] In our present study, the MAc-DMA-immobilized lipase-catalyzed esterification of acetate and ethanol under optimized conditions resulted in a maximum yield of 31 m*M* (61.6%) in a model system using *n*-nonane as a solvent. The potential applications of lipase of *B. coagulans* MTCC-6375 to synthesize various esters by esterification and *trans*-esterification are currently being explored. A range of fatty acid esters is now being produced commercially using immobilized lipases in nonaqueous solvents.[35–37] The interest in industrial processes employing lipase as a biocatalyst to synthesize more such esters is still growing because of its important and multiple applications.

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