Properties of Poly(AAc-*co*-HPMA-*cl*-EGDMA) Hydrogel-Bound Lipase of *Pseudomonas aeruginosa* MTCC-4713 and Its Use in Synthesis of Methyl Acrylate

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ABSTRACT: Microbial lipases (E.C. 3.1.1.3) are preferred biocatalysts for the synthesis of esters in organic solvents. Various extracellular thermoalkaliphilic lipases have been reported from *Pseudomonas* sp. In the present study, a purified alkaline thermoalkalophilic extracellular lipase of *Pseudomonas aeruginosa* MTCC-4713 was efficiently immobilized onto a synthetic poly(AAc-*co*-HPMA-*cl*-EGDMA) hydrogel by adsorption and the bound lipase was evaluated for its hydrolytic potential towards various *p*-nitrophenyl acyl esters varying in their C-chain lengths. The bound lipase showed optimal hydrolytic activity towards *p*-nitrophenyl palmitate (*p*-NPP) at pH 8.5 and temperature 45°C. The hydrolytic activity of the hydrogel-bound lipase was markedly enhanced by the presence of Hg^{2+}, Fe^{3+}, and NH_4^+ salt ions in that order. The hydrogel-immobilized lipase (25 mg) was used to perform esterification in various *n*-alkane(s) that resulted in ~ 84.9 m*M* of methyl acrylate at 45°C in *n*-heptane under shaking (120 rpm) after 6 h, when methanol and acrylic acid were used in a ratio of 100 m*M*:100 m*M*, respectively. Addition of a molecular sieve (3Å × 1.5 mm) to the reaction system at a concentration of 100 mg/reaction vol (1 mL) resulted in a moderate enhancement in conversion of reactants into methyl acrylate (85.6 m*M*). During the repetitive esterification under optimum conditions, the hydrogel-bound lipase produced 71.3 m*M* of ester after 10th cycle of reuse. © 2007 Wiley Periodicals, Inc. J Appl Polym Sci 104: 183–191, 2007

Key words: *Pseudomonas aeruginosa* MTCC-4713 lipase; hydrogel; immobilization; esterification

INTRODUCTION

Enzymes are versatile biocatalysts, capable of catalyzing diverse and unique reactions that are highly specific. In today's world, enzymes find great use in a large number of fields such as food, dairy, pharmaceutical, detergent, textile, and cosmetics industries. Lipases (EC 3.1.1.3) have gained importance over proteases and amylases, especially in the field of organic synthesis. In the last few years, there has been an increasing interest in the use of enzymes for the biosynthesis of molecules in organic media.[1–3] Many lipases when used in organic solvents catalyzed a number of useful reactions including esterification,[4–9] transesterification, regioselectivity, acylation of glycols and menthols as well as synthesis of peptides,[10,11] and other chemicals.[12–17] Enzymes are being examined intensively as catalysts for the preparation of new classes of reagents, especially sugars, chiral synthons, metabolites, food components, flavor/fragrance compounds, and biomedically important esters.

Among bacteria, the lipases from *Pseudomonas* species exhibit the highest versatility, reactivity, and stability in catalyzing the reactions in organic phase.[18–20] Many esters are industrially manufactured by chemical methods. However, chemical methods involve high temperature and high pressure, and often it is difficult to esterify unstable substances,[21] which enhances the manufacturing cost. In contrast, lipase-catalyzed condensation requires mild condition without protection and deprotection steps. Lipases have been successfully immobilized on a variety of matrices/supports for performing esterification and transesterification reactions in organic solvents.[22] The use of immobilized biocatalysts offers many processing advantages over free enzyme. The synthetic hydrogels, which possess good efficiency for binding of lipases could be a tailor, made with hydrophobic or hydrophilic characteristics.[23–26] The porous nature of the hydrogel allows the solvent, reactants as well as the product to diffuse freely that enables the substrate to interact with the enzyme easily.[27] The enzyme sometimes may be inactive in a completely dehy-

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drated system.[28] Thus, it is essential to study the effect of various physical parameters to perform the desired hydrolysis or esterification reactions. In the present study, a weakly hydrophilic hydrogel was used for immobilization of a lipase of *P. aeruginosa* MTCC-4713 and the hydrogel-bound lipase was further evaluated for performing synthesis of methyl acrylate in an organic medium. Methyl acrylate is used chiefly as a comonomer (with acrylonitrile) in making acrylic and nonacrylic fibers, cleaning agents, antioxidants, amphoteric surfactants, dispersion for paints, inks, adhesions, and Vitamin B1. It is also used in making aqueous resins and dispersions for textiles and papers.

MATERIALS AND METHODS

Chemicals and reagents

$NaNO_3$, K_2HPO_4, KCl, $MgSO_4 \cdot 7H_2O$, $FeSO_4 \cdot 7H_2O$, and $(NH_4)_2SO_4$ (S.D. Fine-Chem, Hyderabad, India), yeast-extract, gum acacia, and Tris buffer (HIMEDIA Laboratory, Mumbai, India); sucrose (MERCK, Mumbai, India); *p*-nitrophenyl formate (*p*-NPF), *p*-nitrophenyl acetate (*p*-NPA), *p*-nitrophenyl caprylate (*p*-NPC), *p*-nitrophenyl laurate (*p*-NPL), and *p*-nitrophenyl palmitate (*p*-NPP, Lancaster Synthesis, England); 2-propanol and Triton X-100 (Qualigens Fine Chemicals, India); $MgCl_2$; acrylic acid (AAc), 2-hydroxy propyl methacrylate (HPMA), ammonium persulphate (APS) and ethylene glycol dimethacrylate (EGDMA; MERCK, Mumbai, India); methanol and acetone (Qualigens Fine Chemicals, India); *n*-nonane, *n*-pentane, *n*-hexane, *n*-heptane, and *n*-hexadecane (Lancaster Synthesis, England) were used as received.

Microorganism

The *Pseudomonas aeruginosa* isolate designated MTCC-4713 was obtained from the Department of Biotechnology, Himachal Pradesh University, Shimla (India).

Production of lipase by *Pseudomonas aeruginosa* MTCC-4713

Seed culture of *Pseudomonas aeruginosa* MTCC-4713 was prepared by inoculating 50 mL of broth with a loop-full of culture. The culture was allowed to grow for 48 h at 55°C under shaking at 165 rpm. Thereafter, 10% (v/v), 48-h-old seed culture was used to inoculate 1000 mL of the production medium (50 mL each in 250-mL capacity Erlenmeyer flasks). The seeded production medium was incubated at 55°C and 165 rpm for 48 h (Orbitek shaking incubator, AID Electronics, Chennai, India).

Purification of lipase

The culture broth was centrifuged after 48-h postinoculation at 10,000 × *g* for 10 min at 4°C (SIGMA 3K30,

Germany). The cell pellet was discarded and the supernatant was filtered through Whattman paper No. 1. The protein content was measured by a standard method.[29] This filtrate/broth was henceforth referred as crude lipase. The required amount of ammonium sulfate was added to the crude lipase to achieve 80% (w/v) saturation. The contents were mixed thoroughly and kept overnight at 4°C. The precipitates pelleted by centrifugation at 12,000 × *g* at 4°C for 30 min were reconstituted in 10 mL of Tris buffer (pH 7.5) and were extensively dialyzed against the same buffer. Finally, the lipase activity was assayed and the concentrated lipase preparation was stored at −20°C till further use. The purification of the dialyzed lipase enzyme was performed on a DEAE-cellulose column (Amersham Pharmacia, Sweden) as described previously.[30] The fractions were analyzed for lipase activity and protein. The fractions showing lipase activity were pooled and stored at −20°C. The specific activity of the purified enzyme was compared with the crude enzyme and fold purification was calculated.

Assay of lipase activity

Lipase assay was performed by a colorimetric method.[31] The stock solution (20 m*M*) of *p*-NPP was prepared in 2-propanol. The reaction mixture comprised 75 μL of *p*-NPP stock solution and 5 μL of crude/purified enzyme or 10 mg of immobilized matrix. The final volume of this reaction mixture was made to 3 mL with 0.05*M* Tris buffer, pH 7.5 for free, and 8.5 for bound lipase. The test tubes were incubated for 10 min at 55°C under continuous shaking in water-bath incubator. Appropriate control with a heat-inactivated enzyme (5 min in boiling water bath) was included with each assay. The absorbance of *p*-nitrophenol released was measured at A_{410}. The unknown concentration of *p*-nitrophenol released was determined from a reference curve of *p*-nitrophenol (2–50 μg/mL final concentrations in 0.05*M* Tris buffer, pH 7.5 for free enzyme, and pH 8.5 for immobilized enzyme assay). Each of the assays was performed in duplicate, and mean values were presented. The protein was assayed by a standard method.[29] One unit (U) of lipase activity was defined as micromole(s) of *p*-nitrophenol released per minute by one milliliter of free enzyme or per gram of immobilized enzyme under standard assay conditions. Specific activity was expressed as micromole(s) of the *p*-nitrophenol released per minute by one milligram of protein.

Immobilization of lipase on hydrogels

Synthesis of hydrogels network

The hydrogel employed for the immobilization of lipase was based on AAc. The hydrogel was obtained

when AAc was copolymerized with HPMA in water : acetone (1 : 1, by volume) using APS as an initiator. AAc (2 mL) was taken in 4.0 mL of a solvent system comprising acetone/water (1 : 1, v/v) along with a fixed concentration of initiator APS (1 mM) and known concentration of a crosslinker EGDMA (1%, w/v). The reaction was designed to have AAc : HPMA in a ratio of 5 : 5 (v/v) and EGDMA (15%, w/w) with respect to total weight of the monomer. The reaction mixture was heated in water bath at 50°C when APS was used as initiator for 30 min. Insoluble products were separated from the reaction mixture by filtration. Sol fraction, if any, trapped in the body of the network was separated from the synthesized network by polarity gradient method by treating the network with water, methanol, and acetone, separately, in Soxhlet apparatus from solvent of higher to lower polarity. The polymer was dried in an air oven at 40°C for 24 h to obtain a constant weight (xerogel). The matrix-bound lipase was evaluated to study the effect of pH, temperature, thermal stability, and specificity towards hydrolysis of *p*-nitrophenyl esters of varying C-chain lengths besides synthesis of methyl acrylate in organic medium.

Effect of pH of reaction buffer on hydrogel-immobilized enzyme

Effect of pH of the reaction buffer on catalytic potential of hydrogel-bound lipase was assayed by incubating immobilized lipase (50 mg) under shaking (100 rpm) in Tris buffer (0.05M) adjusted at pH 6.5, 7.0, 7.5, 8.0, 8.5, and 9.0. The lipase activity was assayed at 55°C after 10 min of incubation.

Effect of incubation temperature on hydrogel-immobilized lipase

The activity of hydrogel-bound lipase (50 mg) was assayed separately by incubating the reaction mixture taken in glass tubes at temperature ranging from 35 to 75°C in water-bath incubator under shaking condition (120 rpm). The lipase activity was assayed at 55°C after 10 min of incubation.

Stability of immobilized lipase at varying pH

Stability of the immobilized lipase (50 mg) in buffers (0.05M Tris buffer, pH 6.5, 7.0, 7.5, 8.0, 8.5, and 9.0) of varying pH was examined at optimized temperature of 45°C. Immobilized lipase was preincubated separately at each of the selected pH for 1 h under continuous shaking. Thereafter, the activity of immobilized enzyme was determined at 55°C under standard assay conditions.

Thermostability of hydrogel-immobilized lipase

Thermostability of the immobilized lipase (50 mg) was examined at optimized pH of 8.5. Immobilized-lipase was preincubated at 35, 45, 55, and 65°C for 3 h. Subsequently, lipase activity of immobilized-biocatalyst was determined under standard assay conditions.

Effect of C-chain length of acyl ester (substrate) on immobilized lipase

The hydrogel-immobilized lipase (50 mg) was separatly reacted with each of the five *p*-nitrophenyl esters (20 mM stock prepared in 2-propanol). The nitrophenyl esters were *p*-NPF (1 : 0), *p*-NPA (2 : 0), *p*-NPC (8 : 0), *p*-NPL (12 : 0), and *p*-NPP (16 : 0). The hydrolytic activity of bound lipase was determined under standard assay conditions at optimized temperature and pH of 45°C and pH 8.5.

Effect of salt-ions on hydrolytic activity of immobilized lipase

The effect of various salt ions (FeCl$_3$, AlCl$_3$, NH$_4$Cl, CoCl$_2$, MgCl$_2$, and HgCl$_2$) on the hydrolytic activity of immobilized lipase towards *p*-NPP was evaluated by preincubating the hydrogel-bound biocatalyst (50 mg) with each of the selected salt ions at 1 mM final concentration in the reaction buffer (pH 8.5; 0.05M Tris buffer). The residual lipase activity in each case was determined and expressed as relative activity with respect to the control (buffer without salt ion). The lipase activity was assayed after 30 min incubation at 45°C.

Methyl acrylate synthesis employing hydrogel-immobilized lipase

The esterification studies were performed by using hydrogel-bound lipase in a reaction volume of 1.5 mL in *n*-heptane. The synthesis of methyl acrylate was studied by separately taking different amounts of immobilized lipase (25, 50, 75, 100, and 125 mg) in the reaction mixture (100 mM methanol : 100 mM acrylic acid) to perform the esterification studies in *n*-heptane at 45°C for 15 h under continuous shaking. Methyl acrylate synthesized in each case was determined by gas chromatography (GC).

A reference profile was prepared using varying concentrations of methyl acrylate by making final volume to 1.5 mL in *n*-heptane. The reference curve was plotted between the molar concentration of methyl acrylate (20–120 mM in *n*-heptane) and the corresponding area under the peak. The reaction mixture was assayed for the presence of methyl acrylate using a sample size of 2 μL. The GC (Michro-9100, Netel Chromatographs, India) equipped with a steel column (Chrom WHP column; length: 2 m; diameter: 1/8 in.;

10% SE-30; solid support Chrom WHP; mesh size: 80–100) was programmed at oven temperature 100°C, FID temperature 120°C, and injector temperature 120°C. N_2 was used as a carrier gas (flow rate 30 mL/min).

Optimization of amount of biocatalyst for esterification studies

The synthesis of methyl acrylate was studied by taking different amount of immobilized lipase (25–125 mg/reaction vol in duplicates) in 1.5 mL of reaction mixture containing 100 mM each of methanol and acrylic acid in n-heptane at 65°C for 15 h under shaking (120 rpm).

Effect of relative proportion of reactants on methyl acrylate synthesis

The effect of relative proportion of methanol and acrylic acid on synthesis of methyl acrylate was determined by keeping the concentration of one of the reactants, i.e., methanol at 100 mM and varying the concentration of second reactant acrylic acid (25–100 mM) in a reaction volume of 1.5 mL in n-heptane. The esterification was carried out in the presence of 25 mg of matrix-bound lipase at 45°C in Teflon stoppered-glass vials for 15 h under continuous shaking (120 rpm). The methyl acrylate formed in each of the combinations of the reactants was determined by GC analysis.

Optimization of reaction time for synthesis of methyl acrylate

The reaction mixture (1.5 mL) contained 25 mg of hydrogel-bound lipase, 100 mM (final concentration) each of methanol, and 100 mM (final concentration) of acrylic acid in n-heptane in a Teflo$n$-stoppered glass vial (5-mL capacity). The reaction mixture was incubated at 45°C in a water-bath incubator under shaking (120 rpm) up to 21 h. The reaction mixture was sampled (2 μL) in duplicate at an interval of 3 h and subjected to analysis by GC for the formation of methyl acrylate. The reaction time that gave best result was selected for further studies.

Optimization of temperature for esterification reaction

The reaction mixture (1.5 mL) contained 25 mg of hydrogel-bound lipase, 100 mM of methanol, and 100 mM of acrylic acid in n-heptane in a Teflon-stoppered glass vial (5-mL capacity). The reaction mixture was incubated at 45, 55, and 65°C in a water-bath incubator under shaking (120 rpm) up to 6 h. The methyl acrylate formed in each case was determined. The temperature, at which maximum synthesis of ester was recorded, was selected for subsequent studies.

Effect of C-chain length of solvent (alkane)

In the reaction mixture, n-heptane that was initially employed as a solvent phase was replaced with n-alkanes of varying C-chain length, i.e., n-pentane, n-hexane, n-nonane, and n-hexadecane. The immobilized lipase (25 mg) was added to the above-mentioned reaction mixture prepared in any of the chosen n-alkanes and the reaction was carried out for 6 h at 45°C under continuous shaking (120 rpm). Methyl acrylate so formed was assayed by GC. The n-alkane that gave best amount of methyl acrylate was selected for further studies.

Effect of addition of the molecular sieve on synthesis of methyl acrylate

A molecular sieve (3Å × 1.5 mm) was selected to study its effect on the synthesis of methyl acrylate by immobilized lipase. To the above-mentioned reaction mixture prepared in n-heptane, varying amount (25–150 mg/reaction vol of 1.5 mL) of molecular sieve was added. The esterification was carried out in duplicate by adding 25 mg of immobilized lipase at 45°C under shaking (120 rpm) for 6 h. Methyl acrylate synthesized in each case was determined.

Reusability of immobilized lipase in continuous cycles of esterification for synthesis of methyl acrylate

The formation of methyl acrylate from methanol and acrylic acid (100 mM : 100 mM) catalyzed by immobilized lipase in n-heptane was used to check the retention of catalytic (esterase) activity of hydrogel-immobilized enzyme. The repetitive esterification was performed up to 10 cycles for 6 h each to synthesized methyl acrylate at 45°C in n-heptane. After each cycle of esterification, the immobilized enzyme was washed twice for 5 min each in 2 mL of n-heptane at room temperature. Thereafter, n-heptane was decanted and matrix was reused for another cycle of esterification.

RESULTS

Purification of lipase

The harvested cell-free broth had a lipase activity of 1.56 U with protein concentration of 16.5 mg/mL (specific activity 0.1 U/mg). The dialyzate showed lipase activity of 2.25 U/mL (specific activity 0.24 U/mg). The anion-exchange chromatography of the dialyzed lipase on DEAE-cellulose column resulted in a single peak. The fractions showing lipase activity were pooled (21 mL) and assayed for lipase and protein content (2.04 U/mL; protein 0.53 mg/mL; specific activity 3.9 U/mg). The purified lipase was used for immobilization by adsorption on hydrogels (Table I).

TABLE I
Purification Profile of Lipase

Preparation	Volume (mL)	Activity (U)	Total activity (U)	Protein (mg/mL)	Total protein (mg)	Specific activity (U/mg)	Fold purification
Crude extract	300.0	1.56	469.2	16.5	4950.0	0.10	1.0
Dialyzed lipase	11.0	2.25	24.8	9.5	104.0	0.24	2.5
DEAE-purified lipase	21.0	2.04	42.9	0.53	11.0	3.90	40.9

Immobilization of purified lipase

The hydrogel showed 78.6% binding of purified lipase of *P. aeruginosa* MTCC-4713. The enzyme (200-μL lipase $\approx$ 2.04 U) incubated with the hydrogel contained 0.52 mg/mL of protein.

Effect of pH of the reaction buffer on hydrolytic activity of hydrogel-immobilized lipase

When immobilized lipase was subjected to 1-h preexposure at any of the selected pH (pH 7.0–9.0) at 45°C, the immobilized biocatalyst showed maximum residual hydrolytic activity after preexposure at pH 8.0 (Fig. 1).

Effect of preincubation temperature on hydrogel-immobilized lipase

The effect of preincubation temperature on the hydrolytic reaction was studied separately at temperatures ranging 35–75°C, by maintaining the pH of the Tris buffer (0.05M) at 8.5 for the immobilized lipase (Fig. 2). A temperature of 45°C for immobilized enzyme was found to give maximum hydrolytic activity for the *p*-NPP.

Effect of reaction time on hydrolytic activity of immobilized lipase

The hydrolytic reaction of immobilized enzyme was carried out at 55°C and activity was taken after every 3 h up to 21 h. Maximum activity was obtained at 3 h and it reduced up to 50% at 7 h (Fig. 3).
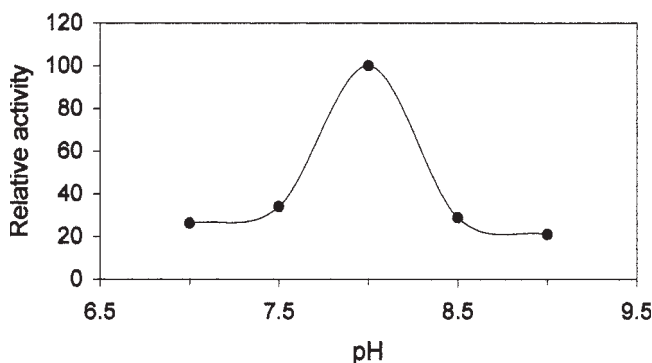
Effect of C-chain length of acyl ester (substrate) on immobilized lipase

The immobilized lipase was used to catalyze the hydrolysis of various *p*-nitrophenyl esters differing in their C-chain lengths. The bound-lipase was highly hydrolytic towards a relatively longer C-chain length ester (*p*-NPP) than the other ones (Table II).

Effect of salt ion on immobilized lipase

The effect of various salt ions (Fe^{+3}, Al^{+3}, NH_4^+, Co^{+2}, Mg^{+2}, and Hg^{+2}) on the hydrolytic activity of immobilized lipase was evaluated by including each of the selected salt ions at 1 mM final concentration in the reaction buffer (pH 8.5) for immobilized enzyme (Table III). The lipase activity of the bound lipase was enhanced in the presence of NH_4^+ (132.5%), Fe^{+3} (163.5%), and Hg^+ (226.9%). In contrast, Al^{+3}, Co^{+2}, and Mg^{+2} antagonized the hydrolytic activity of the bound lipase.

Optimization of esterification conditions using immobilized lipase

The synthesis of methyl acrylate was studied by taking different amount of immobilized lipase (25–125 mg/reaction vol in duplicates) in 100 mM methanol : 100 mM acrylic acid at 45°C for 15 h in *n*-heptane under shaking (120 rpm; Table IV). The maximum synthesis of methyl acrylate (80 mM) was obtained with 25 mg of immobilized lipase. All subsequent
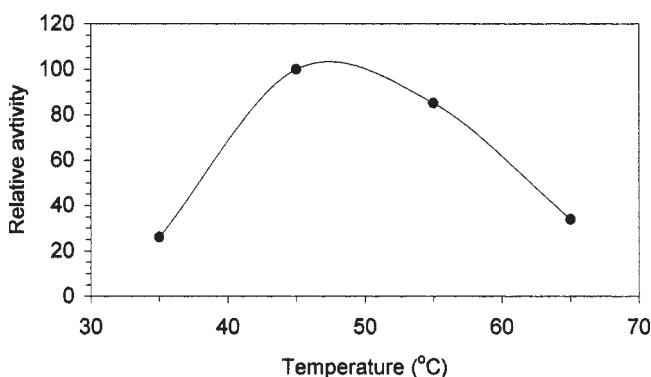

Figure 1 Effect of pH on activity of hydrogel-bound lipase.

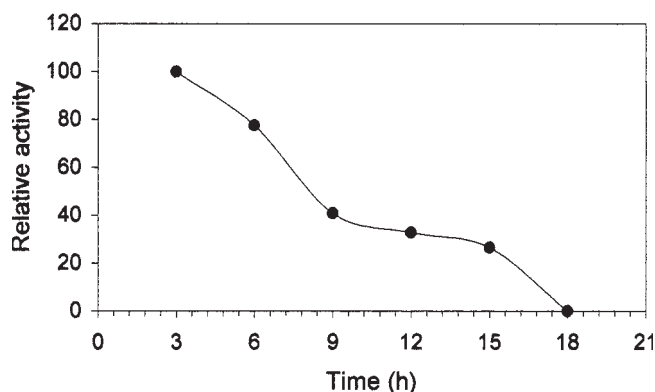**Figure 2** Effect of preincubation temperature on immobilized lipase.

Figure 3 Effect of incubation time on immobilized lipase at 55°C.

esterification studies were performed in triplicate by employing 25 mg of hydrogel-bound lipase in Teflon-gasketed glass-vials (5-mL capacity) in a water-bath incubator shaker (120 rpm). The amount of ester synthesized was presented as mean values.

Effect of temperature on esterification reaction

The esterification was performed using 100 mM methanol : 100 mM acrylic acid at selected temperatures (45–65°C) for 15 h in n-heptane. The maximum ester synthesis (80.6 mM) was recorded at 45°C. At 55 and 65°C, there was a decrease in the yield of ester produced (Fig. 4).

Effect of reaction time for synthesis of methyl acrylate

The kinetics of immobilized-lipase catalyzed synthesis of methyl acrylate was studied up to 21 h at 45°C in n-heptane. The synthesis of methyl acrylate increased with time till 6 h and gradually declined thereafter. At 6 h, $\sim$ 78.3 mM of methyl acrylate was produced. Thus, in the subsequent esterification reactions, a reaction time of 6 h at 45°C for immobilized lipase was considered optimum for the synthesis of methyl acrylate (Fig. 5).

Effect of C-chain length of solvent (alkane) on synthesis of methyl acrylate

A maximum amount of (84.9 mM) methyl acrylate was synthesized when n-heptane was employed as a solvent system (Table V). With increasing C-chain

TABLE III
Effect of Salt Ions on Immobilized Lipase on Immobilized Lipase

Salt ion	Relative activity (%)
Fe^{3+}	163.6
Co^{2+}	38.2
Mg^{2+}	97.6
Al^{3+}	34.9
NH_4^+	132.5
Hg^{2+}	226.9
No ions	100.0

length of the solvent system, the yield of ester decreased drastically. Esterification performed in n-hexane (16 mM) or n-hexadecane (16.4 mM) gave a very low yield of methyl acrylate.

Effect of proportional concentration of reactants on synthesis of methyl acrylate

When esterification was performed by varying the molar concentration (ratio) of the reactants, an increase in molar ratio of methanol to acrylic acid from 25 mM : 100 mM to 100 mM : 100 mM resulted in synthesis of 83.5 mM of methyl acrylate in 6 h at 45°C (Table VI). However, when concentration of methanol was fixed at 100 mM, a relative increase in the concentration of acrylic acid from 25 to 100 mM resulted in a drastic decrease in the amount of methyl acrylate formed.

Effect of molecular sieve on synthesis of methyl acrylate

Addition of molecular sieve to the reaction mixture gradually enhanced the amount of methyl acrylate synthesized by immobilized-lipase (Table VII). An improved ester synthesis (85.5 to 85.6 mM) was recorded at 75–100 mg of molecular sieve. Further increase into concentration of molecular sieve resulted in a gradual decline of methyl acrylate synthesis.

Reusability of immobilized lipase in continuous cycles of esterification

Immobilized matrix (25 mg) was repetitively used up to 10 cycles of esterification using both the reactants in n-heptane at a final concentration of 100 mM each

TABLE II
Effect of C-Chain Length of the Substrate on Hydrolytic Activity of Immobilized Lipase

Effect of substrate	Relative activity (%)
p-NPP	100.00
p-NPL	80.72
p-NPC	25.00
p-NPA	9.67
p-NPF	4.20

TABLE IV
Optimization of Amount of Biocatalyst for Ester Synthesized

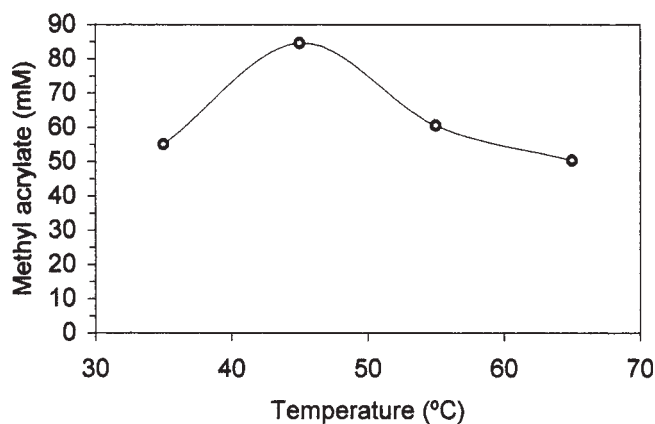| Immobilized biocatalyst (mg)	Methyl acrylate formed (mM)
25	80.0
50	79.8
75	77.7
100	76.9
125	72.3

Figure 4 Effect of reaction temperature on the synthesis of methyl acrylate.

(methanol : acrylic acid). The esterification was performed at 45°C for 6 h for each cycle. After 10th cycle, the hydrogel-bound lipase retained 71.4% of its initial esterification activity (Fig. 6).

DISCUSSION

In recent time, lipases have emerged as key enzymes in swiftly growing biotechnology, owing to their multi-faceted properties, which find usage in a wide array of industrial applications, such as food technology, detergent, chemical industry, and biomedical science.[32–35] In the present study, a weakly hydrophilic synthetic matrix (hydrogel) was used for immobilization of an alkaline lipase of a thermophilic *P. aeruginosa* MTCC-4713. The experimental data established that lipase of *P. aeruginosa* MTCC-4713 was efficiently immobilized on the hydrogel used in the present study. Previously, a protein loading of ∼ 15 mg/g was reported for lipase immobilized on Nylon-6.[32] *Bacillus* GK8 was immobilized on silica, and it took only 30 min to bind maximally.[33] Lipases that are hydrolytic at alkaline pH are conventionally used in detergents and laundry. Most lipases
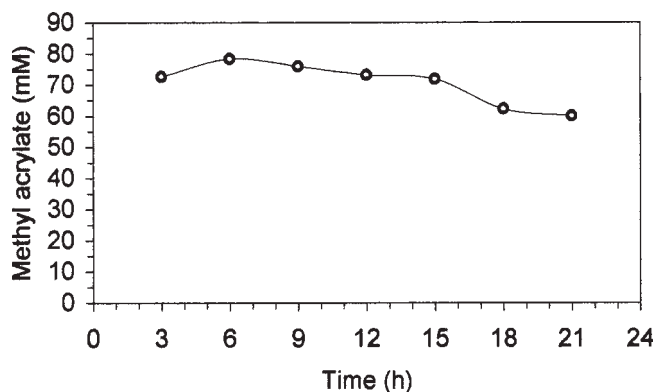

Figure 5 Effect of reaction time on the synthesis of methyl acrylate by hydrogel-immobilized lipase.

TABLE V
Effect of C-Chain Length of Solvents (Alkanes)
on the Synthesis of Methyl Acrylate

Organic solvent	Methyl acrylate (m*M*)
n-Pentane	0.0
n-Hexane	16.0
n-Heptane	84.9
n-Nonane	46.7
n-Hexadecane	16.4

can act in a wide range of pH and temperature, although alkaline bacterial lipases are more common.[24–26,34] Lipase immobilized onto poly(HEMAc-*co*-MMAm) gave a higher yield for both hydrolysis and esterification activity compared to the use of other polymer-immobilized biocatalysts.[23]

Various features of reaction selectivity of lipases are modulated by exogenous factors such as choice of cosubstrates/reactants, water activity, pH, salt ions, temperature, and immobilization.[34–38] The hydrogel-bound lipase of *P. aeruginosa* MTCC-4713 was optimally active at pH 8.0 and temperature 45°C. Recently, lipase from a mutant strain of *Corynebacterium* sp. was immobilized and pH for the assay of immobilized lipase was found to be the same (8.0) as that of a soluble enzyme. However, lipase from *Bacillus coagulans* MTCC-6375 immobilized on silica showed a relatively higher activity at pH 5.5.[25] The free enzyme of *B. coagulans* BTS-3 was stable within a pH range of 8.0–10.5.[39] Earlier, in a study, *Bacillus* sp. lipase was stable in the pH range of 7.0–10.0 with optimum pH 8.0.[40] Lipase of *P. aeruginosa* after immobilization on hydrogel retained good thermotolerance at 55°C. In a recent study, the optimum temperature for immobilized lipase from the *B. coagulans* MTCC-6375 was found to be 50°C.[30] Immobilization of lipase from *Candida rugosa* on chitosan showed optimum reaction temperature of 30°C while immobilization of lipase from same organism on kaolin showed highest activity at 40°C. Generally, the temperature above ambient promotes liquefaction of reactants and also tend to make the substrate more diffusible and hence easily acceptable to the enzyme.[41] At 65°C, there was a decrease in the activity of *P. aeruginosa* MTCC-4713 lipase, which might be on account of denaturation

TABLE VI
Effect of Proportional Concentration
of Reactants on Esterification

Methanol (m*M*)	Acrylic acid (m*M*)	Methyl acrylate (m*M*)
25	100	76.6
50	100	77.4
75	100	80.8
100	100	83.5
100	75	59.9
100	50	12.6
100	25	0.0

TABLE VII
Effect of Molecular Sieve on Methyl Acrylate Synthesis

Molecular sieve (mg)	Methyl acrylate (mM)
25	72.5
50	83.7
75	85.5
100	85.6
300	83.4
500	76.4

of the lipase. Heat-promoted protein unfolding also led to the loss of enzymatic activity. It was shown that the immobilized preparations were much more stable than the soluble enzyme when immobilization of lipase from *Bacillus thermocatenulatus* was done on hydrophobic supports, maintaining 100% of the activity at 65°C *Bacillus* GK8 lipase when immobilized on HP-20 beads retained complete activity at 60°C.[40] On the other hand, a lipase from *C. rugosa* when immobilized on chitosan showed 23% residual activity at 60°C.

The hydrogel-bound lipase of *P. aeruginosa* MTCC-4713 was highly hydrolytic towards longer C-chain length ester (*p*-NPP) than the shorter ones. The *p*-NPP was also more efficiently hydrolyzed by free *B. coagulans* enzyme.[39] This indicated a preferential specificity of *B. coagulans* lipase towards longer carbon chain length substrates, as reported previously for a thermoalkaliphilic lipase from *Bacillus* sp.[42] In another study, a lipase from psychrotrophic *Pseudomonas* sp. displayed highest activity towards C: 10 acyl groups of *p*-nitrophenyl esters.[43] Recently, immobilized lipase from *B. coagulans* BTS-1 was found to be more hydrolytic to a medium C-length ester than shorter or longer C-length esters.[25] Previously, a purified lipase from *Pseudomonas cepacia* immobilized on a commercially available microporous polypropylene support showed a higher activity with *p*-NPA and very low with *p*NPP.[44] The hydrolytic activity of the hydrogel-bound lipase of *P. aeruginosa* MTCC-4713 was promoted/enhanced in the presence of a few salt ions that included Hg^{2+}, Fe^{3+}, and NH_4^+ ions in that order. In contrast, Al^{+3}, Co^{+2}, and Mg^{+2} antagonized the hydrolytic activity of this hydrogel-bound lipase. Exposure of Ca^{2+} ions to an extracellular lipase of *P. aeruginosa* 2D was reported to cause 360% increase in the lipase activity but the presence of Hg^{2+} and Co^{2+} strongly inhibit the activity.[45] Hg^{2+}, Al^{3+}, Mn^{2+}, and Co^{2+} ions exerted a drastic decline in lipase activity of *Rhizopus oryzae*.[21]

A variety of fatty acid esters are now produced commercially using immobilized lipases in nonaqueous solvents.[33,46] When compared with conventional chemical synthesis from alcohols and carboxylic acids using mineral acids as a catalyst, the use of lipases as biocatalysts to produce these high value-added fatty acid esters in organic media offered significant advantages[47–50] that included the use of any hydrophobic substrate, higher selectivity, milder processing conditions, and the ease of product isolation and enzyme reuse.[51,52] In the present study, the poly(AAc-*co*-HPMA-*cl*-EGDMA)-hydrogel-immobilized alkaline lipase of a thermophilic *P. aeruginosa* MTCC-4713 was used to catalyze esterification of methanol and acrylic acid into methyl acrylate in a period of 6 h at 45°C. Moreover, the use of alkanes of C-chain length shorter and greater than *n*-heptane decreased the rate of esterification. As *n*-alkane with a shorter C-chain length was used as a solvent, a gradual decrease in rate of methyl acrylate synthesis was noticed. It was observed that as the log P value of an *n*-alkane increased corresponding to the increase in the C-chain length of the alkanes, the hydrophobicity of the alkane, i.e., solvent also increased in that order, and that appeared to be very important for modulation of catalytic activity of the *P. aeruginosa* MTCC-4713 lipase.

Esterification is generally a water-limited reaction because the equilibrium catalyzed by hydrolytic enzymes is in favor of hydrolysis.[53] Water also inhibits the reaction besides when an immobilized enzyme with a support, which has hydrophilic nature is used[54] resulting in a decrease in the rate of enzyme activity, as seen in the present study. When the amount of the molecular sieve in the reaction mixture was increased to 100 mg in the reaction mixture, the *P. aeruginosa* MTCC-4713 hydrogel-immobilized-shows increase in synthesis of ester (methyl acrylate) $\sim$ 85.6 mM. Thus, presence of molecular sieve in the reaction mixture invariably prevented the inhibitory effects of accumulation of water on the esterification reaction between acrylic acid and methanol. It appeared that an increase in the concentration of the molecular sieve provided a corresponding increase in the immobilized biocatalyst.
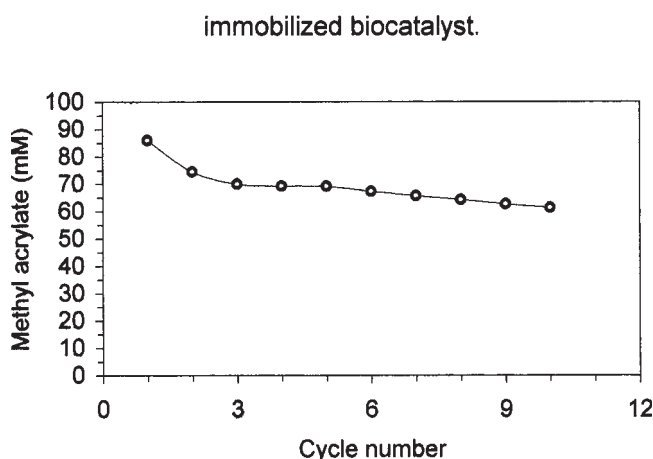

Figure 6 Repetitive synthesis of ester by immobilized biocatalyst.

physically active-surface area of molecular sieve that readily absorbed water, that otherwise inhibited the forward reaction kinetics. An improvement in the rate of esterification has been previously reported for the esterification of lauric acid and methanol in the presence of a molecular sieve.[55] The esterification of methanol and acrylic acid by immobilized lipase from *P. aeruginosa* was further enhanced when molar concentration of the hydrophobic reactant, i.e., methanol was increased from 25 : 100 to 100 : 100 (methanol:acrylic acid) in the reaction mixture. Thus, the present study established that the immobilization of the lipase sourced from *P. aeruginosa* MTCC-4713 on poly(AAc-*co*-HPMA-*cl*-EGDMA)-hydrogel was quite stable as the hydrogel retained hydrolytic activity towards the *p*-NPP as well as achieved repetitive esterification in *n*-heptane for a number of cycles. The use of hydrogel-immobilized lipase of *P. aeruginosa* MTCC-4713 may be further explored in preparing medically important esters in organic media besides industrially important short- or long-chain esters by condensation or transesterification.

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