Short-Chain Ester Synthesis by Transesterification Employing Poly (MAc-*co*-DMA-*cl*-MBAm) Hydrogel-Bound Lipase of *Bacillus Coagulans* MTCC-6375

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ABSTRACT: Lipases (E.C. 3.1.1.3) have been extensively used to achieve various esterification reactions in water-restricted or water-free media. In the present study a purified thermotolerant alkalophilic extracellular lipase of *Bacillus coagulans* MTCC-6375 has been efficiently immobilized onto a synthetic *poly* (MAc-*co*-DMA-*cl*-MBAm) hydrogel by surface absorption, and the bound lipase was used to perform short-chain fatty acid ester synthesis in *n*-alkane(s). The hydrogel bound lipase resulted in approximately 67 m*M* of isoamyl acetate at 55°C in *n*-heptane under shaking in 15 h when vinyl acetate:isoamyl alcohol was used in a ratio of 100 m*M*:100 m*M*. Addition of a molecular sieve (3 Å × 1.5 mm) to the reaction system at a concentration of 25–500 mg per reaction volume had deleterious effect on the conversion of reactants to isoamyl acetate (64 m*M*). During the repetitive esterification under optimal conditions, the hydrogel bound lipase produced 31.3 m*M* of ester after fourth cycle of reuse. Use of methanol and 2-propanol (instead of isoamyl alcohol) resulted in 78.2 and 64.9 m*M* of methyl acetate and 2-propyl acetate, respectively, under the optimized conditions in *n*-heptane © 2008 Wiley Periodicals, Inc. J Appl Polym Sci 109: 1063–1071, 2008

Key words: *Bacillus coagulans* MTCC-6375; lipase; hydrogel; immobilization

INTRODUCTION

Immobilized enzymes are used in many commercialized products for higher yields. The lipases (E.C. 3.1.1.3) that constitute a most versatile group of enzymes have been used for performing esterification and trans-esterification reactions in organic solvents. In the last few years, there has been an increasing interest in the use of enzymes for the biosynthesis of molecules in organic media.[1–5] A few extracellular thermoalkaliphilic lipases have been reported from *Bacillus* sp. Lipase catalyzed reactions, however, have a major inconvenience that the conversions are relatively low when compared with traditional chemical processes if crude commercial enzymes are employed. Thus purification of the enzymatic activity is an important aspect to study various properties of the pure enzyme.[6] Chelating agents like EDTA inhibits the metallolipases while others do not.[7–8] Structural elucidation of lipases has shown that catalytic site of most lipases resembles that of serine proteases.[9] Immobilized lipases offer economic incentives of enhanced thermal and chemical stability, ease of handling, easy recovery, and reuse relative to nonimmobilized forms.[5,10–12] Immobilization also increases enzyme's thermal stability.[13] Besides the nature/characteristics of the immobilization matrices/supports, the effect of solvents on enzyme activity, specificity, and stability have been extensively studied and many solvent-parameters have been compared to develop a simple strategy for choosing the appropriate solvent for each enzymatic reaction.[14–16] A solvent is necessary for solublizing the substrates, for partitioning the substrates and products in different phases[17] as well as for ester synthesis or trans-esterification.[13,18,19]

Esters of short chain fatty acids and alcohols are known as flavor and fragrance compounds that are used in food, beverage, cosmetic, and pharmaceutical industries. Currently, most of the flavor compounds are provided by traditional methods such as chemical synthesis or extraction from natural sources. Isoamyl acetate occurs naturally in the volatile portion of banana fruit and cocoa bean, and is used in perfumes/fragrances, as a flavoring agent in nonalcoholic beverages, ice cream, candy, baked goods, and chewing gum; a solvent for old oil colors, tannins, nitrocellulose, lacquers, paints, formaldehyde, synthetic resins and waxes, celluloid and camphor; for masking unpleasant odors and to perfume shoe polish. Butyl

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acetate is often employed in combination with *n*-butanol in paints, because *n*-butanol enhances the resistance to blushing and increases the solvency in many cases. It is also used as a solvent for various nitrocellulose lacquer systems, in perfume manufacture and in the production of pharmaceuticals and flavoring agents. Many acetate and butyrate esters are components of natural flavors. The reports on the production of acetate esters are scarce in various organic solvents. Because of the toxicity of acetate on lipase activity in enzymic acetylation, the use of acetic acids as acyl donor in transesterification and direct esterification reactions was previously attempted with little or no success.[20] In the present study, a synthetic *poly* (MAc-*co*-DMA-*cl*-DMAm) hydrogel bound lipase of *Bacillus coagulans* MTCC-6375 has been employed in organic media to synthesize isoamyl acetate and a few other short-chain esters by transesterification.

EXPERIMENTAL

Chemicals and reagents

N,*N*-Methylene bisacrylamide (MBAm), methacrylic acid (MAc), dodecyl methacrylate (DMA), benzoyl peroxide, methanol, isopropanol, and acetone were procurede from Qualigens Fine Chemicals, Mumbai, India; $NaNO_3$, K_2HPO_4, KCl, $MgSO_4.7H_2O$, $FeSO_4.7H_2O$, $(NH_4)_2SO_4$ (S.d. Fine-Chem, Mumbai); yeast-extract, gum acacia (HIMEDIA Laboratory, Mumbai); molecular sieve (3 Å × 1.5 mm), sucrose (MERCK, Mumbai, India); *p*-nitrophenyl palmitate (*p*-NPP), vinyl acetate, isoamyl acetate, *n*-pentane, *n*-hexane, *n*-heptane, *n*-nonane, *n*-hexadecane (Lancaster Synthesis, England); 2-propanol, Triton X-100 (Qualigens Fine Chemicals, India); and isoamyl alcohol (Sarabhai Chemicals, India). All these chemicals were of analytical grade and were used as received.

Microorganism

The *Bacillus coagulans* MTCC-6375 isolate was obtained from Department of Biotechnology, Himachal Pradesh University, Summer Hill, Shimla (India).

Maintenance and subculturing of the *Bacillus coagulans*

The microbial culture was maintained by repeated subculturing at 35°C on a Mineral Based (MB) broth supplemented with 0.5% (w/v) sucrose and 1.0% (v/v) of cottonseed oil as a sole carbon source (pH 7.0).

Production of lipase by *Bacillus coagulans*

Seed culture was prepared by inoculating 50 mL of broth with a loop-full of culture. The culture was allowed to grow for 36 h at 45°C under shaking condition at 160 rpm (Orbitek shaking incubator, AID Electronics, Madras, India). Thereafter, 5% (v/v), 36-h-old seed culture was used to inoculate 500 mL of the production medium (50 mL each in 250 mL capacity Erlenmeyer flasks). The seeded production medium was incubated at 45°C and 250 rpm for 60 h (Orbitek shaking incubator, AID Electronics, Madras, India). Three batches of 500 mL each were used to obtain crude lipase.

Preparation of crude lipase

The culture broth was centrifuged at 10,000 × *g* for 10 min at 4°C (SIGMA 3K30, Germany). The cell-pellet was discarded and the supernatant was retained. The supernatant was filtered through Whattman paper No. 1. The protein was assayed by a standard method.[21] This filtrate/broth preparation was henceforth referred as crude lipase. Lipase assay was performed by a colorimetric method.[22] The stock solution (20 m*M*) of *p*-nitrophenyl palmitate (*p*-NPP) was prepared in HPLC grade 2-propanol. The reaction mixture comprised of 75 μL of *p*-NPP stock solution and 20 μL of crude/purified enzyme or 20 mg of immobilized matrix (weight of matrix included). The final volume of this reaction mixture was made to 3 mL, with 0.05*M* Tris buffer (pH 8.5). The test tubes were incubated for 10 min at 45°C under continuous shaking in water-bath-incubator. The residual lipase activity was blocked with addition of chilled acetone: ethanol (1:1) mixture. 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 410 nm (Shimadzu UV/Visible spectrophotometer, Japan). 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 8.5). Each of the assays was performed in duplicate and mean values were presented. One unit (U) of lipase activity was defined as μmole(s) of *p*-nitrophenol released per min by one mL of free enzyme or one gram of immobilized enzyme (weight of matrix included) under standard assay conditions. Specific activity was expressed as μmole(s) of the *p*-nitrophenol released per min per mg of protein.

Precipitation of lipase

The cell-free production broth obtained after 60 h postinoculation was used for purification of lipase. The required amount of ammonium sulfate was added to the cell-free broth to achieve 60% (w/v) saturation. The contents were mixed thoroughly and kept over night at 4°C. Thereafter, broth was centrifuged at 10,000 × *g* at 4°C for 10 min. The supernatant

was discarded, and precipitates reconstituted in Tris buffer (pH 8.5) were extensively dialyzed against the same buffer to remove ammonium sulfate. 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 an anion-exchange (DEAE-cellulose) column (Amersham Pharmacia, Sweden) as described below.

Anion exchange column chromatography

A column (12 × 2 cm, Vt = 50.2 cm^3) was packed with preswollen DEAE-cellulose (Sigma Chem., USA). The matrix was activated sequentially with 0.1*N* HCl and 0.1*M* NaOH. Subsequently, the column was further washed with 0.05*M* phosphate buffer (pH 8.5) containing 1.0*M* KCl. The column was equilibrated with 0.05*M* phosphate buffer (pH 8.5) and the dialyzed enzyme was loaded on the column. Eluted buffer (with proteins) was recycled three times. Twelve fractions were eluted using 0.05*M* phosphate buffer (pH 8.5) followed by another 13 fractions that were eluted using 0.05*M* phosphate buffer (pH 8.5) containing 1.0*M* KCl (fraction volume 3 mL). The eluted fractions were analyzed both for the lipase activity as well as for the total protein content. The fractions showing lipase activity were pooled and stored at −20°C. The specific activity of the purified enzyme was compared with that of the crude enzyme and fold purification was calculated.

Synthesis of hydrogel

The hydrogel *poly*(MAc-*co*-DMA-*cl*-MBAm) 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 benzyol peroxide (30 mg) as an initiator.[5] 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, isoamyl alcohol, 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.

Immobilization

Purified lipase (8 mL; 35.6 U; 0.52 mg protein/mL) was incubated with 2 g of preswollen *poly*(MAc-*co*-DMA-*cl*-MBAm) hydrogel for 18 h at 4°C. The matrix was preswollen in Tris buffer (0.05*M*, pH 8.5) for 16 h at 8°C. The lipase-bound matrix was filtered through Whattman No.1 filter paper. The bound lipase activity as well as percent protein binding was determined (by estimating unbound protein in the filtrate). Total unbound protein in filtrate was deducted from total protein incubated with the matrix.

Swelling capacity

Swelling capacity of the dry hydrogel (xerogel) was determined by incubating the preweighed (50 mg) dry matrix (xerogel) in distilled water for 1 h at 55°C. The hydrogel was sedimented by centrifugation (10,000 × *g* for 5 min), aqueous phase was decanted completely, and the swollen matrix (hydrogel) was reweighed. The swollen matrix possessed 5.5 times of its original weight.

Ester synthesis employing immobilized lipase

A reference curve was calibrated between molar concentration (m*M*) of vinyl acetate and the corresponding area under the peak (retention time 0.48 min). A sample size of 2 μL was used for GLC analysis. The GLC (Michro-9100, Netel Chromatographs, Thane, India) was programmed for oven temperature 100°C, FID temperature 160°C, and injector temperature 160°C. The assay of vinyl acetate was performed on 15% SE30 Chromo WHP column (2 m × 1.8 in.) using N_2 as a carrier gas (flow rate 30 mL/min).

Determination of amount of isoamyl acetate synthesized

After the completion of esterification at specified time interval, the reaction mixture was withdrawn (2–5 μL) and subjected to analysis of residual vinyl acetate. The amount of residual vinyl acetate was subtracted from the original amount of vinyl acetate to determine the amount of vinyl acetate consumed. The amount of ester produced in the transesterification reaction was considered to be equivalent to the amount of vinyl acetate consumed in the transesterification reaction.

Effect of relative proportion of reactants on isoamyl acetate synthesis

The effect of concentration and relative molar ratio of isoamyl alcohol and vinyl acetate on synthesis of isoamyl acetate was determined by keeping the concentration of one of the reactants (isoamyl alcohol or vinyl acetate) at 100 m*M* and varying the concentration of second reactant (25–100 m*M*) in a reaction volume of 1 mL in *n*-heptane. The esterification was carried out using matrix-bound lipase (20 mg) at 55°C in Teflon stoppered-glass vials for 21 h under continuous shaking (160 rpm). The isoamyl acetate formed in

TABLE I
Production of Crude Lipase by *B. coagulans*

Batch	Volume (mL)	Lipase activity (U/mL)	Specific activity (U/mg)
1	500	1.62	1.16
2	500	1.84	1.26
3	500	1.72	1.14

each of the combinations of the reactants was determined by GC analysis.

Effect of increasing concentration of biocatalyst on trans-esterification

The effect of varying concentrations of hydrogel-bound lipase on ester formation was evaluated by increasing the concentration of immobilized-biocatalyst (i.e., 20, 40, 60, and 80 mg) in the reaction mixture comprising 100 m*M* each of isoamyl alcohol and vinyl acetate in *n*-heptane incubated at 55°C. The trans-esterification reaction was performed for 21 h under continuous shaking.

Optimization of incubation time on synthesis of isoamyl acetate

The hydrogel-immobilized lipase (20 mg) was washed twice, in 1 mL of *n*-heptane (solvent) at room temperature. Thereafter, the matrix was recovered by decantation of *n*-heptane, and used to catalyze the esterification of isoamyl acetate. The reaction mixture (1 mL) contained 20 mg of bound lipase, 100 m*M* (final concentration) of isoamyl alcohol, and 100 m*M* vinyl acetate in *n*-heptane in a Teflon-stoppered glass vial (5 mL capacity). The reaction mixture was incubated at 55°C in a water-bath-incubator under shaking conditions (160 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 analysis for the formation of isoamyl acetate.

Effect of reaction temperature

Temperature for the esterification reaction was optimized by carrying out the reaction at room temperature, 25, 35, 45, 55, 65, and 75°C for 15 h in *n*-heptane using 20 mg immobilized enzyme. The isoamyl acetate formed in each case was determined by GC analysis.

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*-octane, *n*-nonane, *n*-hexadecane, and *n*-heptadecane. The immobilized lipase (20 mg) was added to the above-mentioned reaction mixture to perform the esterification. The reaction was carried out for 15 h at 55°C, with continuous shaking. The isoamyl acetate formed during the reaction was analyzed by GC analysis.

Effect of addition of the molecular sieve on synthesis of isoamyl acetate

A molecular sieve was selected to study its effect on the synthesis of isoamyl acetate by immobilized lipase. To the above reaction mixture prepared in *n*-heptane, varying amount (25–500 mg) of molecular sieve was added. The esterification was carried out in duplicate by adding 20 mg of immobilized lipase at 55°C, with continuous shaking for 15 h. Isoamyl acetate synthesized in each case was determined by GC.

Reusability of immobilized lipase in continuous cycles of esterification for synthesis of isoamyl acetate

The formation of isoamyl acetate from isoamyl alcohol and vinyl acetate (100 m*M* of isoamyl alcohol and 100 m*M* of vinyl acetate) catalyzed by immobilized lipase in *n*-heptane was use to check the retention of catalytic (esterase) activity of hydrogel-immobilized enzyme. The immobilized lipase was assayed for 4 cycles of 15 h each, for isoamyl acetate formation. After each cycle of esterification, the immobilized enzyme was washed twice for 5 min each in 1 mL *n*-heptane at room temperature. Thereafter, *n*-heptane was decanted and matrix was reused for fresh cycle of ester synthesis under similar conditions.

Effect of various alcohols on synthesis of respective acetate ester by transesterification

Various alcohols that included methanol, ethanol, 1-propanol, 2-propanol, 1-butanol, 2-butanol, and 1-hexanol were used instead of isoamyl alcohol to perform transesterification using vinyl acetate (as an acetate donor) for synthesis of respective alcohol-ace-

TABLE II
Summary of Purification of Lipase

Fraction	Volume (mL)	Lipase activity (IU)	Total lipase activity	Protein content (mg/mL)	Total protein	Specific activity (IU/mg)	Fold purification	Yield %
Crude	500.0	1.7	850.0	1.5	750.0	1.1	1.0	100.0
$(NH_4)_2SO_4$	12.0	22.5	270.0	0.6	7.2	37.5	34.1	31.8
Dialyzed	12.0	28.5	342.0	0.5	6.0	57.0	51.8	40.2
DEAE-purified	10.0	35.6	356.0	0.5	5.0	71.2	64.7	41.9

tate ester in *n*-heptane at 55°C for 21 h, under shaking conditions using hydrogel-bound biocatalyst. The amount of each of the alcohol-acetate ester was indirectly determining the amount of vinyl acetate used during the transesterification reaction.

RESULTS

Purification of *B. coagulans* lipase

The production broth seeded with *B. coagulans* produced 1.62 to 1.84 U/mL of lipase (Table I) at 60 h postinoculation. The protein was optimally precipitated at 60% (w/v) of ammonium sulfate saturation. The precipitates reconstituted in 12 mL of Tris buffer (0.05*M*, pH 8.5) were extensively dialyzed against the same buffer. The dialyzate (12 mL) showed lipase activity of 28.5 U (specific activity 57.0 U/mg; Table II). The anion-exchange chromatography of the dialyzed lipase on DEAE-Cellulose column resulted in a single peak. The fractions showing lipase activity were pooled and assayed for lipase and protein (35.6 U, 0.5 mg of protein/mL, specific activity 71.2 U/mg). The anion exchange chromatography resulted in approximately 42-fold purified lipase.

Effect of relative proportions of reactants on esterification

The effect of varying molar concentration of each of the reactants on ester formation was evaluated by keeping the concentration of one of the reactants at 100 m*M* in *n*-heptane. The formation of ester was highest when vinyl acetate and isoamyl alcohol were used at 100 m*M*:100 m*M* in *n*-heptane under continuous shaking condition after 21 h at 55°C (Fig. 1). In the subsequent reactions, same concentration of the reactants was employed to achieve transesterification. Amount of isoamyl acetate synthesized was estimated from a standard profile of vinyl acetate prepared in *n*-heptane.

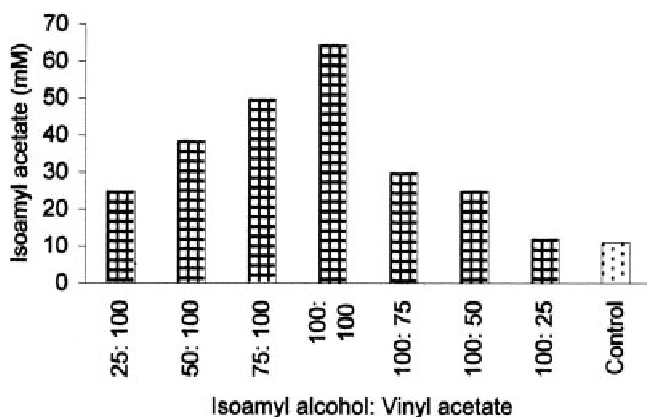

Figure 1 Effect of relative proportion of reactants on synthesis of isoamyl acetate.

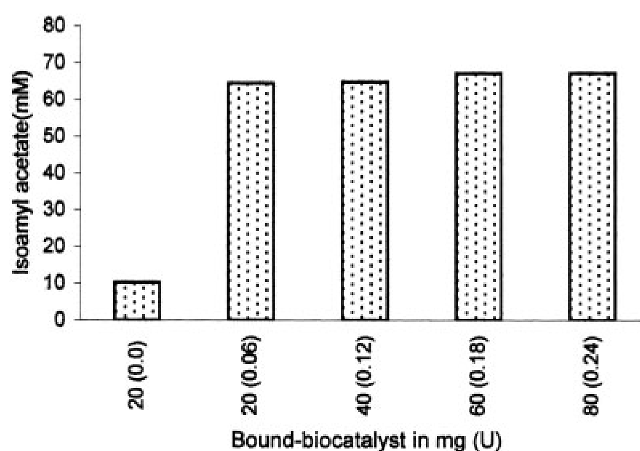

Figure 2 Effect of amount of biocatalyst on isoamyl acetate synthesis.

Effect of amount of biocatalyst on isoamyl acetate synthesis

The trans-esterification reaction with isoamyl alcohol: vinyl acetate (100 m*M*:100 m*M*) in *n*-heptane was performed at 55°C (Fig. 2). The formation of ester remained more or less the same with an increase in concentration of hydrogel-bound lipase under continuous shaking condition after 21 h at 55°C. In the subsequent transesterification reactions, 20 mg of hydrogel bound lipase was used for biocatalysis.

Kinetics of synthesis of isoamyl acetate

The effect of reaction time on synthesis of isoamyl acetate using immobilized lipase was studied at a temperature of 55°C in *n*-heptane under shaking condition up to 21 h. The synthesis of the ester was time dependent and a maximum amount of isoamyl acetate (64.5 m*M*) was produced after 15 h of reaction when isoamyl alcohol and vinyl acetate were used at 100 m*M* each in *n*-heptane (Fig. 3). Thus, in subsequent reaction a reaction time of 15 h at 55°C was considered

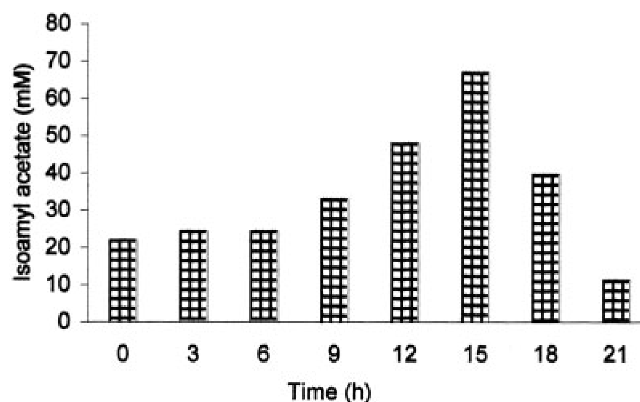

Figure 3 Effect of reaction time on synthesis of isoamyl acetate.

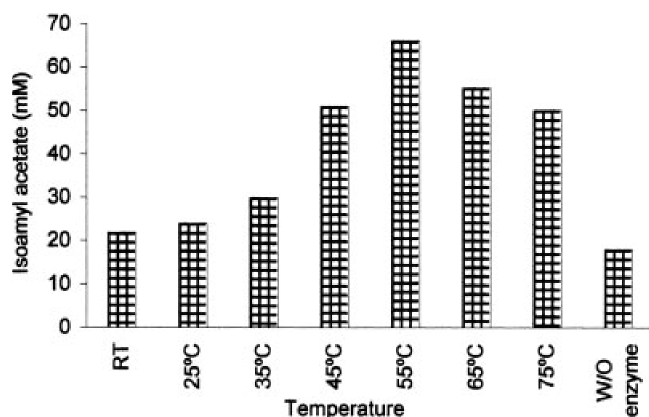

Figure 4 Effect of reaction temperature on synthesis of isoamyl acetate.

optimum to perform synthesis of isoamyl acetate using hydrogel-bound lipase.

Effect of temperature on synthesis of isoamyl acetate

The effect of change in the reaction temperature on the synthesis of isoamyl acetate by immobilized lipase was also studied. Maximum synthesis (64.6 m*M*) of isoamyl acetate was obtained at 55°C after 15 h (Fig. 4). At 65°C, there was a marked decrease (55.2 m*M*) in the ester synthesis, which might be on account of denaturation of the lipase. At 75°C there was a further decline (50.1 m*M*) in the amount of ester produced.

Effect of solvent (*n*-alkane) on the ester synthesis

Use of *n*-pentane, *n*-hexane, *n*-hexadecane, or *n*-heptadecane reduced the amount of ester formed under similar conditions (Fig. 5). The use of *n*-heptane, *n*-octane, and *n*-nonane resulted in 63.7, 63.2, and 66.2 m*M* of isoamyl acetate. Thus, the maximum conversion of reactants into ester was recorded in *n*-nonane (66.2 m*M*) at 55°C under shaking after 21 h.

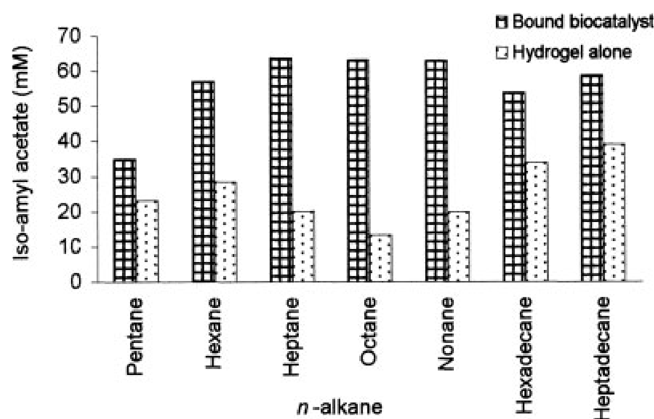

Figure 5 Effect of solvent (*n*-alkane) on synthesis of isoamyl acetate.

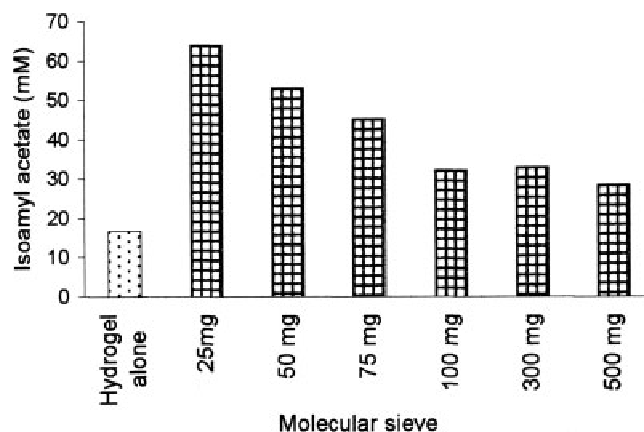

Figure 6 Effect of molecular sieve on synthesis of isoamyl acetate.

Effect of addition of molecular sieve on transesterification

The transesterification reaction resulted in formation of water as a by-product of the reaction, and its removal using a molecular sieve might enhance the synthesis of ester by pushing the reaction equilibrium in the forward direction. However, when the effect of molecular sieve was studied by adding a molecular sieve (25–500 mg per reaction volume), a gradual decline (64.0–28.4 m*M* isoamyl acetate) in the amount of ester formed was noticed (Fig. 6). Thus, addition of a molecular sieve had a deleterious effect on the transesterification reaction.

Reusability of immobilized enzyme for ester synthesis

The bound lipase when repetitively used to perform esterification at 55°C under optimized conditions in *n*-nonane resulted in 67.2, 50.6, 39.6, and 31.3 m*M* isoamyl acetate after first, second, third, and fourth cycle of transesterification (Fig. 7). In each cycle transesterification was performed for 21 h.

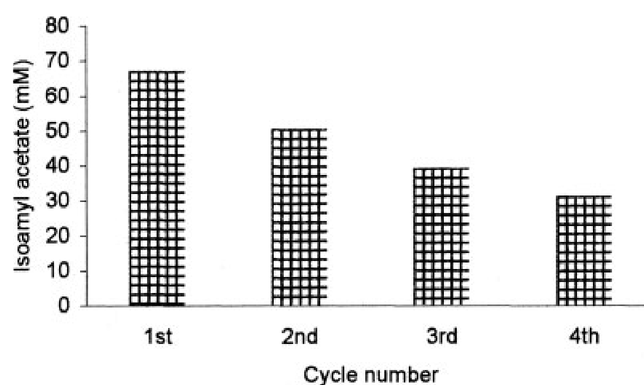

Figure 7 Reusability of immobilized lipase for isoamyl acetate synthesis.

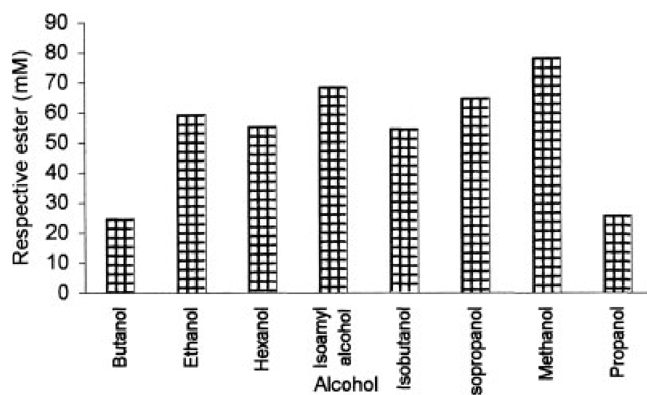

Figure 8 Effect of various alcohols on synthesis of respective ester.

Effect of various alcohols on synthesis of respective acetate esters by transesterification

Various alcohols that included methanol, ethanol, 1-propanol, 2-propanol, 1-butanol, 2-butanol, and 1-hexanol were used instead of isoamyl alcohol to perform transesterification using vinyl acetate (as an acetate donor) for synthesis of respective alcohol-acetate esters in *n*-heptane at 55°C for 21 h under shaking (Fig. 8). Use of methanol and 2-propanol resulted in 78.2 and 64.9 m*M* of methyl acetate and 2-propyl acetate, respectively. Thus, it appeared that use of methanol resulted in a relatively higher transesterification. The recorded results indicated that there was no relationship between the C-chain lengths of the alcohol used in transesterification reaction using *B. coagulans* bound-lipase.

DISCUSSION

Lipase is a ubiquitous activity possessed by most of the organisms. Many bacteria, fungi, and yeasts produce extracellular lipases. Newer diverse microbial sources of lipases have been reported in the recent years.[5,23–25] In the present study an extracellular alkaline lipase of *Bacillus coagulans* MTCC-6375 was purified and immobilized on to a *poly*(MAc-*co*-DMA-*cl*-MBAm) hydrogel.

The purified lipase was efficiently immobilized on hydrogel. The hydrogel-bound lipase was subsequently employed to synthesize isoamyl acetate and similar esters under optimized conditions in a water free organic solvent system. Ester synthesis in the water-free media/organic solvents could be achieved very easily.[25–27] Thermostable lipases can overcome the problem of degradation of lipase at high temperature. Immobilized lipases generally offer economic incentives of enhanced thermal and chemical stability, ease of handling, recovery, and reuse relative to nonimmobilized forms.[10,28] Various features of reaction selectivity of lipases are modulated by exogenous factors such as choice of cosubstrates/reactants, water activity, pH, temperature, and immobilization.[29–32] A variety of fatty acid esters are now being produced commercially using immobilized lipase in nonaqueous solvents.[33–35]

The synthesis of isoamyl acetate in the presence of bound lipase of *B. coagulans* appeared to be optimum (61.2%) when isoamyl alcohol and vinyl acetate were used at a concentration 100 m*M*:100 m*M* each in *n*-heptane at 55°C in 21 h. A relative excess molar concentration of either acid or alcohol would denature or precipitate protein, and such effect would inactivate the biocatalyst and thus would decrease the ester synthesis. Also, it was likely that excess of acid might have partially inactivated the bound lipase because of charge alteration/charge rearrangement at the catalytic site of the hydrogel-bound biocatalyst. Recently, we have reported optimal synthesis of ethyl laurate and ethyl propionate at an equimolar proportion of reactants (100 m*M* each) in *n*-nonane.[36,37] In another study, effect of acetic acid concentration on esterification reaction using lipase SP435 was studied.[38] High concentration of acetic acid (0.4 to 0.7*M*) inhibited SP435 lipase activity, resulting in low conversion yields for acetate esters. The presence of fatty acid (acetic) can damage the hydrolytic layer-protein interaction of the enzyme structure causing lipase deactivation during esterification process. Thus, *B. coagulans* lipase is also vulnerable to high concentration of lauric acid in reaction medium just like other lipases. Because of toxicity of (acetic) acid on lipase activity in enzymatic acetylation, the use of acids as an acyl donor in transesterification and direct esterification reactions was previously attempted with little or no success.[20]

In the esterification reaction, the hydrogel bound lipase of *B. coagulans* optimally produced isoamyl acetate at 55°C in 15 h. However, further increase in the reaction temperature to 65 or 75°C decreased the esterification rate. The alteration in temperature of reaction mixture might interfere with the porosity, hydrophobic character, and diffusion of the reactants and/or products at the catalytic site of enzyme or hydrogel. At temperature more than 55°C, there was no further increase in the amount of ester synthesized, which might be on account of denaturation of the lipase as well as alteration in the native structure of lipase. Recently, the hydrogel-bound lipase of *Bacillus coagulans* MTCC-6375 has been used for optimal synthesis of ethyl laurate at 65°C in 21 h.[36] Also, a synthetic hydrogel immobilized lipase of *P. aeruginosa* BTS-2 was optimally produced ethyl propionate when acid and alcohol was used in an equimolar (100 m*M*) ratio in the reaction system.[37]

The use of alkanes of C-chain length shorter and longer than *n*-heptane decreased the rate of esterification. When *n*-alkane with a shorter C-chain length was used as a solvent, a gradual decrease in rate of

isoamyl acetate synthesis was noticed. It was observed that as the log *P* value of an *n*-alkane increased corresponding to increase in the C-chain length of the alkanes (log *P* value for *n*-pentane, *n*-hexane, *n*-heptane, and *n*-nonane is 3, 3.5, 4, and 5, respectively), 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 hydrogel-bound *B. coagulans* lipase. Thus, it appeared that choice of an appropriate solvent system was critical for the synthesis of ester using hydrogel-immobilized lipase of *Bacillus coagulans*. A relatively higher hydrophobicity appeared to be an important property to be built into a synthetic hydrogel for increasing the catalytic activity of bound lipase of *B. coagulans*. Higher the log *P*, the more hydrophobic is the solvent. In a recent study using hydrogel-immobilized lipase of *P. aeruginosa* BTS-2, we have shown a similar effect that resulted in an enhanced synthesis of ethyl propionate in *n*-nonane.[39] However, previously reported lipase of *P. aeruginosa* was more efficient in synthesis of ester as indicated by approximately 94% conversion of reactants into ester. In another study, hydrogel-bound *B. coagulans* MTCC-6375 lipase could achieve 61% conversion of reactants into ethyl laurate.[36] This lower conversion into ester might be due to use of a higher carbon chain length fatty acid (lauric acid, C 12) than the one used previously (propionic acid, C 3). It appeared that *B. coagulans* MTCC-6375 lipase is inherently less efficient in performing esterification than the *Pseudomonas* lipase as in the present study approximately 64–67 m*M* of isoamyl acetate could be produced by manipulating various physical and kinetic parameters.

Water and alcohol that are produced as by-product(s) of the esterification or trans-esterification reaction, respectively, performed by biocatalysts in organic media have several adverse effects on the reaction and enzyme activity/performance. Thus, accumulation of (vinyl) alcohol as a by-product of the trans-esterification reaction would inhibit the forward reaction unless is simultaneously removed out off the reaction system. Esterification of isoamyl alcohol and vinyl acetate by hydrogel-immobilized lipase of *B. coagulans* in the absence of a molecular sieve exhibited an increase in amount of ester synthesized. When a molecular sieve was added in the reaction mixture, and the effect of its concentration in the reaction system on rate of trans-esterification by hydrogel-bound lipase was studied, little change in the conversion of vinyl acetate and isoamyl alcohol into isoamyl acetate was recorded. Thus, presence of molecular sieve in the reaction mixture was redundant to improve the trans-esterification between isoamyl alcohol and vinyl acetate. An improvement in the rate of esterification after addition of a molecular sieve have been previously reported for the esterification of lauric acid and geraniol,[16] isoamyl alcohol, and propionic acid by hydrogel-bound lipase of *P. aeruginosa* BTS-2[36] and ethyl laurate by hydrogel-bound lipase of *Bacillus coagulans* MTCC-6375.[37]

The present study showed that *poly*(MAc-*co*-DMA-*cl*-MBAm) hydrogel-immobilized-lipase of *Bacillus coagulans* efficiently performed esterification of isoamyl alcohol and vinyl acetate into isoamyl acetate at a temperature of 55°C, addition of molecular sieve in conjunction with a hydrophobic matrix caused a decline in the rate of esterification, and an equi-molar ratio of isoamyl alcohol as well as vinyl acetate was necessary for achieving an optimal rate of trans-esterification. Foregoing discussion indicated that a systematic approach could be devised on the basis of solvent engineering to favor optimal synthesis of an ester of interest. Many studies have shown that smaller fatty acids are involved in inhibition of enzyme at higher concentration. Generally, commercial enzymes must be very rugged to remain active for prolonged duration when exposed to an adverse environment. This study is among very few reports on the use of a synthetic hydrogel bound extracellular alkaline lipase of a moderately thermotolerant *B. coagulans* for the synthesis of isoamyl acetate and a few other alcohol-acetate esters, of short chain lengths in a water free organic solvent system. The efficacy of this lipase to catalyze interesterification and transesterification reactions in organic solvents/water restricted/controlled media are still required to be explored in future studies.

References

1. Gargouri, M.; Drouet, P.; Legoy, M. D. J Biotechnol 2002, 92, 259.
2. Castillo, E.; Pezzotti, F.; Navarro, A.; Lopez-Munguia, A. J Biotechnol 2003, 102, 251.
3. Noel, M.; Combes, D. J Biotechnol 2003, 102, 23.
4. Tejo Sekeroglu, G.; Fadiloglu, S.; Ibanoglu, E. Turk J Eng Environ Sci 2004, 28, 241.
5. Kanwar, S. S.; Verma, H. K.; Kumar, Y.; Chimni, S. S.; Chauhan, G. S. ACTA Microbiol et Immunol Hungarica 2006, 53, 195.
6. Kumar, S.; Kikon, K.; Upadhyay, A.; Kanwar, S. S.; Gupta, R. Protein Expr Purif 2005, 41, 38.
7. Hiol, A.; Donzo, M. D.; Druet, D.; Comeau, L. C. Enzyme Microb Technol 1999, 25, 80.
8. Arreguin-Espinosa, R.; Arreguin, B.; Gonzalez, C. Biotechnol Appl Biochem 2000, 31, 239.
9. Winkler, F.K.; D'Arcy, A.; Hunziker, W. Nature 1990, 343, 771.
10. Malcata, F. X.; Reyes, H. R.; Garcia, H. S.; Hill, C. G.; Admunson, C. H. J Am Oil Chem Soc 1990, 67, 890.
11. Kanwar, S. S.; Kaushal, R. K.; Jawed, A.; Chimni, S. S.; Punj, V.; Asian J Microbiol Biotechnol Environ Sci 2004, 6, 53.
12. Kanwar, S. S.; Srivastva, M.; Ghazi, I. A.; Chimni, S. S.; Kaushal, R. K.; Joshi, G. K. Acta Microbiol et Immunol Hugarica 2004, 51, 57.
13. Zaks, A.; Klibanov, A. M. Science 1984, 224, 1249.
14. Lanne, C.; Boeren, S.; Vos, K.; Veeger, C. Biotechnol Bioeng 1987, 30, 81.

15. Mustafa, U. U. Trends J Agric Forestry 1998, 22, 573.
16. Basri, A. R.; Harun, A.; Ahmad, M. B.; Razak, C. N. A.; Salleh, A. B. J Appl Polym Sci 2001, 82, 1404.
17. Reslow, M.; Adelkreutz, P.; Mattiasson, B. Eur J Biochem 1988, 177, 313.
18. Boyer, J. L.; Gilot, B.; Guirand, R.In Recent progress en genie des procedes, Nouvelles applications de La methodologie de genie des procedes ed. Storck A & Grevillot G. 1987, 7.
19. Nishio, T.; Chicano, T.; Kamimura, M. Agric Biol Chem 1988, 52, 1203.
20. Guvenc, A.; Kapucu, N.; Mehmetoglu, U. Proc Biochem 2002, 38, 379.
21. Lowry, O. H.; Rosenbrough, N. J.; Farr, A. L.; Randall, R. J. J Biol Chem 1951, 193, 265.
22. Winkler, U. K.; Stuckmann, M. J Bacteriol 1979, 138, 663.
23. Tan, T.; Zhang, M.; Wang, B.; Ying, C.; Deng, L. Proc Biochem 2003, 39, 459.
24. Gupta, R.; Gupta, N.; Rathi, P. Appl Microbiol Biotechnol 2004, 64, 763.
25. Celia, E.; Cernia, E.; Palocci, C.; Soro, S.; Turchet, T. J. Supercrit Fluids 2005, 33, 193.
26. Srivastva, S.; Modak, J; Madras, G. Ind Eng Chem Res 2002, 41, 1940.
27. Zaidi, A.; Gainer, J. L.; Carta, G.; Mrani, A.; Kadiri, T.; Belarbi, Y.; Mir, A. J Biotechnol 2002, 93, 209.
28. Yahya, A. R. M.; Anderson, W. A.; Moo-Young, M. Enzyme Microbiol Technol 1998, 23, 438.
29. Bornscheuer, U. T.; Yamane, T. Enzyme Microbiol Technol 1994, 16, 864.
30. Klien, R. R.; King, G.; Moreau, R. A.; Mcneill, G. P.; Villeneuve, P.; Hass, M. J. J Am Oil Chem Soc 1997, 74, 1401.
31. Rhee, J. S.; Kwon, S. J. J Microbiol Biotechnol 1998, 8, 191.
32. Lee, C. H.; Parkin, K. L. Biotechnol Bioeng 2001, 75, 219.
33. Macrae, A. R.; Roel, E. L.; Brand, H. M. Seifen Oele Fette Wachse 1984, 116, 201.
34. Pandey, A.; Benjamin, S.; Soccol, C. R.; Nigam, P.; Krieger, N.; Soccol, V. T. Biotechnol Appl Biochem 1999, 29, 199.
35. Kaushal, R. K.; Sen, U.; Kanwar, S. S.; Chauhan, G. S. Proceedings of the IXth International Workshop on Bioencapsulation, Strasbourg, France, 2003.
36. Kanwar, S. S.; Verma, H. K.; Kaushal, R. K.; Verma, H. K.; Kumar, Y., Chimni, S. S.; Chauhan, G. S. World J Microbiol Biotechnol 2005, 21, 1037.
37. Kanwar, S. S.; Kaushal, R. K.; Jawed, A.; Chimni S. S. Indian J Biochem Biophys 2005, 42, 233.
38. Claon, P.; Akoh, C. Enzyme Microbiol Technol 1994, 16, 835.
39. Kanwar, S. S.; Kaushal, R. K.; Verma, M. L., Kumar, Y.; Chauhan, G. S.; Gupta, R.; Chimni, S. S. Indian J Microbiol 2005, 45, 187.