Properties and Application of Poly(methacrylic acid-*co*-dodecyl methacrylate-*cl*-*N*,*N*-methylene bisacrylamide) Hydrogel Immobilized *Bacillus cereus* MTCC 8372 Lipase for the Synthesis of Geranyl Acetate

Madan Lal Verma, Shamsher Singh Kanwar

Department of Biotechnology, Himachal Pradesh University, Shimla 171005, India

Received 2 August 2007; accepted 10 April 2008
DOI 10.1002/app.28539
Published online 10 July 2008 in Wiley InterScience (www.interscience.wiley.com).

ABSTRACT: A range of fatty acid esters is now being produced commercially with immobilized microbial lipases (glycerol ester hydrolases; EC) in nonaqueous solvents. In this study, a synthetic hydrogel was prepared by the copolymerization of methacrylic acid and dodecyl methacrylate in the presence of a crosslinker, *N*,*N*-methylene bisacrylamide. A purified alkaline thermotolerant bacterial lipase from *Bacillus cereus* MTCC 8372 was immobilized on a poly(methacrylic acid-*co*-dodecyl methacrylate-*cl*-*N*,*N*-methylene bisacrylamide) hydrogel by an adsorption method. The hydrogel showed a 95% binding efficiency for the lipase. The bound lipase was evaluated for its hydrolytic potential toward various *p*-nitrophenyl acyl esters with various C chain lengths. The bound lipase showed optimal hydrolytic activity toward *p*-nitrophenyl palmitate at a pH of 8.5 and a temperature of 55°C. The hydrolytic activity of the hydrogel-bound lipase was enhanced by Hg^{2+}, Fe^{3+}, and NH_4^+ ions at a concentration of 1 m*M*. The hydrogel-bound lipase was used to synthesize geranyl acetate from geraniol and acetic acid in *n*-heptane. The optimization of the reaction conditions, such as catalyst loading, effect of substrate concentration, solvent (*n*-pentane, *n*-hexane, *n*-heptane, *n*-octane, and *n*-nonane), reaction time, temperature, molecular sieve (3 Å × 1.5 mm) and scale up (at 50-mL level), was studied. The immobilized lipase (25 mg/mL) was used to perform an esterification in *n*-alkane(s) that resulted in the synthesis of approximately 82.8 m*M* geranyl acetate at 55°C in *n*-heptane under continuous shaking (160 rpm) after 15 h when geraniol and acetic acid were used in a ratio of 100 : 100 m*M*. The addition of a molecular sieve (3 Å × 1.5 mm) to the reaction system at a concentration of 40 mg/mL in reaction volume (2 mL) resulted in an increase in the conversion of reactants into geranyl acetate (90.0 m*M*). During the repetitive esterification under optimum conditions, the hydrogel-bound lipase produced ester (37.0 m*M*) after the eighth cycle of reuse. When the reaction volume was scaled up to 50 mL, the ester synthesized was 58.7 m*M* under optimized conditions. © 2008 Wiley Periodicals, Inc. J Appl Polym Sci 110: 837–846, 2008

Key words: esterification; hydrogels; proteins

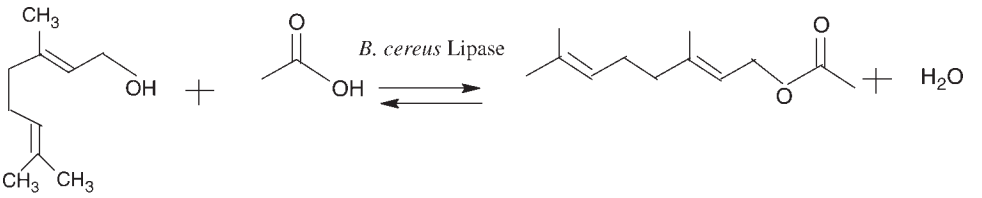
INTRODUCTION

Many esters are used as natural fragrances. Traditional extractions from plant materials and direct biosynthesis by fermentation are the two generally used methods for flavor and fragrance production.[1] Natural flavor esters extracted from plant materials are often either too scarce or too expensive for commercial use. For industrial use, flavors are usually produced by chemical synthesis and are not considered natural products. Thus, their market value is lower than that of esters from natural sources. Such esters, however, may be considered natural when produced by lipase-mediated synthesis.[2] Nowadays, the synthesis of flavor compounds by biotechnological processes plays an increasing role in the food industry. This is the result, among other things, of scientific advances in biological processes, which make use of microorganisms or enzymes as an alternative to chemical synthesis, combined with recent developments in analytical techniques.[3,4]

Lipases of microbial origin are the most versatile enzymes and are known to bring about a range of bioconversion reactions,[5] which include hydrolysis, interesterification, esterification, alcoholysis, acidolysis, and aminolysis.[6–10] Their unique characteristics include substrate specificity, stereospecificity, regioselectivity, and the ability to catalyze a heterogeneous reaction at the interface of water-soluble and water-insoluble systems.[11,12] Microbial lipases have an enormous biotechnological potential because they provide alternative methods to chemical asymmetric

Correspondence to: S. S. Kanwar (kanwarss2000@yahoo.com).

Contract grant sponsor: Department of Biotechnology, Ministry of Science and Technology, Government of India [through a research fellowship (Department of Biotechnology-Senior Research Fellowship) to M.L.V.].



Geraniol Acetic acid Geranyl acetate

Scheme 1

synthesis. A range of fatty acid esters is now being produced commercially with immobilized lipases in nonaqueous solvents.[13–15] The interest in industrial processes that use lipase biocatalysts to synthesize more such esters is still growing because of their important and multiple applications.

Recently, the development of lipase-based technologies for the synthesis of novel compounds has rapidly expanded the uses of these enzymes.[16] An increasing number of lipases with suitable properties are becoming available, and efforts are underway to commercialize biotransformation and syntheses based on lipases.[16] The advantages of an enzymatic route over chemical methods and efficient lipase-catalyzed condensation in nonaqueous media, that is, organic solvents, has attracted much attention.[17] Most lipases can act in a wide range of pH's and temperatures, although alkaline bacterial lipases are more common.[18–20] Various features of the reaction selectivity of lipases are modulated by exogenous factors, such as choice of cosubstrates/reactants, water activity, pH, metal ions, temperature, and immobilization.[21–23]

In this study, a synthetic hydrogel was used for the immobilization of a *Bacillus cereus* MTCC 8372 lipase, and the bound lipase was further evaluated for the synthesis of geranyl acetate in organic media (Scheme 1). Geranyl acetate is a natural organic compound that is classified as a monoterpene. It is a colorless liquid with a pleasant floral or fruity aroma. Geranyl acetate is a natural constituent of more than 60 essential oils, including ceylon citronella, palmarosa, lemon grass, petit grain, neroli, geranium, coriander, carrot, and sassafras. It can be obtained by the fractional distillation of essential oils. Geranyl acetate is used primarily as a component of perfumes for creams and soaps and as a flavoring ingredient. It is used particularly in rose formulations where a sweet fruity or citrus aroma is desired.

EXPERIMENTAL

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, Ltd., Hyderabad, India); yeast extract, gum acacia, and Tris buffer (Himedia Laboratory, Ltd., Mumbai, India); sucrose and the molecular sieves (3 Å × 1.5 mm; Merck, Ltd., Mumbai, India); *p*-nitrophenyl formate (*p*-NPF), *p*-nitrophenyl acetate (*p*-NPA), *p*-nitrophenyl caprylate (*p*-NPC), *p*-nitrophenyl laurate (*p*-NPL), *p*-nitrophenyl palmitate (*p*-NPP), *n*-pentane, *n*-hexane, *n*-heptane, *n*-octane, and *n*-nonane (Lancaster Synthesis, White Lund, Morecambe, England); 2-propanol, acetic acid, and Triton X-100 (Qualigens Fine Chemicals, Mumbai, India); $MgCl_2$, *N,N*-methylene bisacrylamide (MBAm), methacrylic acid (MAc), dodecyl methacrylate (DMA), benzyol peroxide, geraniol, acetone, and isopropyl alcohol (Qualigens Fine Chemicals, Mumbai, India); and geranyl acetate and geraniol (Sigma-Aldrich, Steinheim, Germany) were used as received.

Microorganism

B. cereus isolate, designated MTCC 8372, was obtained from the Department of Biotechnology, Himachal Pradesh University (Shimla, India).

Production of lipase by *B. cereus* MTCC 8372

We prepared a seed culture of *B. cereus* MTCC 8372 by inoculating 50 mL of broth with a loopfull of culture. The culture grew for 36 h at 55°C under continuous shaking (160 rpm). Thereafter, a 10% (v/v), 36-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 for 48 h at 55°C and 160 rpm (Orbitek shaking incubator, AID Electronics, Chennai, India).

Purification of the lipase

The culture broth was centrifuged after 48 h after inoculation at 10,000 × g for 10 min at 4°C (Sigma 3K30, Harz, Germany). The cell pellet was discarded, and the supernatant was filtered through a Whatman no. 1 filter (Whatman International Ltd., Maidstone, England). We henceforth refer to this filtrate broth as the 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 sedimented by centrifugation at 12,000 × g at 4°C for 30 min were reconstituted in a minimum amount of Tris buffer (0.05*M*, pH 8.5) and were extensively dialyzed against the same buffer. Finally, the lipase activity was assayed,[24] and the concentrated lipase preparation was stored at −20°C until further use. The purification of the dialyzed lipase enzyme was performed on an octyl sepharose column (Amersham Pharmacia, Uppsala, Sweden) as described previously.[25] The fractions were analyzed for lipase activity and protein content by standard methods.[26] 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 the fold purification was calculated.

Assay of lipase activity

The lipase was assayed by a colorimetric method with *p*-NPP.[24] The reaction mixture contained 80 μL of *p*-NPP stock solution (20 m*M* *p*-NPP prepared in isopropyl alcohol), 20 μL of the test sample (lipase), and Tris buffer (0.05*M*, pH 8.5) to make final volume of 3 mL. The reaction mixture was incubated at 55°C for 10 min in a water bath. We stopped the reaction by keeping the reaction mixture at −20°C for 7 min. Appropriate control with a heat-inactivated enzyme (5 min in a boiling-water bath) was included with each assay. The absorbance of *p*-nitrophenol released was measured at A_{410} (absorbance read at 410 nm). 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 triplicate, unless otherwise stated, and the mean values were recorded. One unit (1 IU) of lipase activity was defined as the micromoles of *p*-nitrophenol released per minute by the hydrolysis of *p*-NPP by 1 mL of soluble enzyme or 1 g of hydrogel-bound enzyme (weight of matrix included) at 55°C under assay conditions. The specific activity was expressed as micromoles of the *p*-nitrophenol released per minute by 1 mg of protein.

Immobilization of the lipase on the hydrogel

Synthesis of the hydrogel

The synthetic hydrogel was obtained with MAc–DMA–MBAm in a ratio of 1 : 3 : 5, as reported recently.[27] The synthetic polymeric matrix was washed consecutively with different solvents (water, methanol, and acetone) to remove unreacted compounds. The matrix was finally dehydrated in an oven at 50°C to achieve a constant weight (xerogel). The swelling capacity (S_w) of the xerogel in distilled water was found as follows:

$$S_w = \frac{W_2 - W_1}{W_1}$$

where W_1 is the weight of the xerogel (mg) and W_2 is the weight of the hydrogel (mg), that is, the net weight of the polymeric matrix (xerogel) after it was suspended in an excess volume of water for 1 h at 55°C.

Immobilization procedure

The xerogel (3.5 g), pre-equilibrated in an excess volume of Tris buffer (0.05*M*, pH 8.5), was incubated with purified lipase (6.0 mL ≈ 0.59 IU/mL; 4.2 mg/mL protein) at 8°C for 20 h in a glass vial. The volumes of the supernatant and unbound protein[18] and the lipase activity[16] were estimated. The weight of the enzyme-incubated matrix was estimated, and the bound lipase activity was assayed with 20 mg of matrix. The bound protein in the hydrogel was determined by subtraction of the unbound protein in the supernatant from the total protein used for immobilization.

Hydrolytic properties of the hydrogel-immobilized lipase

The matrix-bound lipase was evaluated to study the effect of pH, temperature, thermal stability, salt ions, and specificity toward the hydrolysis of *p*-nitrophenyl esters with various C chain lengths and for the synthesis of geranyl acetate in organic media.

Effect of the pH reaction buffer

The effect of the pH of the reaction buffer on the catalytic potential of the hydrogel-bound lipase was assayed by incubation of the immobilized lipase in Tris buffer (0.05*M*, with 0.4%, v/v Triton X-100 and 0.1% w/v gum acacia) adjusted to pH values of 7.0, 7.5, 8.0, 8.5, 9.0, and 9.5. The lipase activity was assayed at 55°C after 10 min of incubation.

pH stability studies

The stability of the immobilized lipase in buffers with various pH values was examined at an optimized temperature (55°C). The immobilized lipase was preincubated separately in an aqueous environment (0.05*M* Tris buffer, pH's = 7.0, 7.5, 8.0, 8.5, 9.0, and 9.5) with up to 1 h of continuous shaking in a water-bath shaker incubator. Thereafter, the activity of the immobilized enzyme was determined at 55°C under standard assay conditions.

Effect of the incubation temperature on the lipase

We assayed the activity of the bound lipase separately by incubating the reaction mixture taken in glass tubes at temperatures ranging from 35 to 75°C in a water-bath shaker incubator under continuous shaking (160 rpm). The lipase activity was assayed at 55°C after 10 min of incubation.

Thermostability studies

The thermostability of the immobilized lipase (50 mg) was examined at an optimized pH of 8.5. The immobilized lipase was preincubated at 35, 45, 55, 65, and 75°C for 3 h. Subsequently, the lipase activity of the immobilized biocatalyst was determined under standard assay conditions.

Effect of the incubation time on the hydrolytic activity of the hydrogel-immobilized lipase

The hydrolytic reaction of the immobilized enzyme was carried out up to 10 h at 55°C, and lipase activity was recorded after every 2 h.

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

The hydrogel-immobilized lipase was reacted separately with each of the five *p*-nitrophenyl esters (20 m*M* stock prepared in 2-propanol). The nitrophenyl esters were *p*-NPF, *p*-NPA, *p*-NPC, *p*-NPL, and *p*-NPP. The hydrolytic activity of the bound lipase was determined under standard assay conditions at an optimized temperature (55°C) and pH (8.5).

Effect of salt ions on the hydrolytic activity of the hydrogel-immobilized lipase

The effect of various salt ions ($FeCl_3$, $AlCl_3$, NH_4Cl, $CoCl_2$, $MgCl_2$, and $HgCl_2$) on the hydrolytic activity of the immobilized lipase was evaluated by the preincubation of each of the selected salt ions at 1 m*M* final concentration in the reaction buffer (pH 8.5, 0.05*M* Tris). The residual lipase activity in each case was determined and expressed as relative activity with respect to the control (without salt ion). The lipase activity was assayed after 10 min of incubation at 55°C.

Geranyl acetate synthesis with the hydrogel-bound lipase

The esterification studies were performed with the hydrogel-bound lipase in a reaction volume of 2 mL in *n*-heptane. The geranyl acetate synthesized in each case was determined by gas–liquid chromatography (GLC).

A reference profile was prepared with various concentrations of geranyl acetate in *n*-heptane with a final volume of 2 mL. The reference curve was plotted between the molar concentration of geranyl acetate (20–100 m*M* in *n*-heptane) and the corresponding area under the peak. The reaction mixture was assayed for the presence of geranyl acetate with a sample size of 2 μL. The GLC instrument (Michro-9100, Netel Chromatographs, Thane, India) equipped with a steel column (Chrom WHP column, length = 2 m, diameter = $\frac{1}{8}$ in., 10% SE-30, solid support Chrom WHP, mesh size = 80–100) was programmed for a first oven temperature of 90°C, a ramp rate of 30°C/min, a second oven temperature of 250°C, ISO2 of 2 min, an injector temperature of 260°C, and a flame ionization detector (FID) temperature of 270°C. N_2 was used as a carrier gas (flow rate = 30 mL/min).

Effect of the biocatalyst load for the esterification studies

We studied the synthesis of geranyl acetate by placing different amounts of the immobilized lipase (12.5–62.5 mg/mL in duplicates) in 2 mL of a reaction mixture containing 100 m*M* each of geraniol and acetic acid in *n*-heptane at 55°C for 15 h under shaking (160 rpm). GLC analysis was done for the quantification of the geranyl acetate synthesis.

Effect of the relative proportion of the reactants on the geranyl acetate synthesis

We determined the effect of the relative proportion of geraniol and acetic acid on the geranyl acetate synthesis by keeping the concentration of one of the reactants, that is, geraniol, at 100 m*M* and varying the concentration of the second reactant, that is, acetic acid (25–100 m*M*), in a 2-mL reaction volume in *n*-heptane. The esterification was carried out with 25 mg/mL of the matrix-bound lipase at 55°C in Teflon-stoppered glass vials for 15 h under continuous shaking (160 rpm). The geranyl acetate formed in each of the combinations of the reactants was determined by GLC analysis.

Reaction time studies for the synthesis of geranyl acetate

The reaction mixture (2 mL) contained 25 mg/mL hydrogel-bound lipase and 100 m*M* (final concentration) each geraniol and acetic acid in *n*-heptane in a Teflon-stoppered glass vial (5-mL capacity). The reaction mixture was incubated at 55°C under shaking (160 rpm) for up to 21 h. The reaction mixture was sampled (2 μL) in duplicate at 3 h intervals and subjected to analysis by GLC for geranyl acetate formation. The reaction time that gave the best result was selected for further studies.

TABLE I
Summary of the Purification of the Lipase of *B. cereus* MTCC 8372

Purification step	Volume (mL)	Lipase activity (IU/mL)	Protein (mg/mL)	Specific activity (IU/mg)	Fold purification
Crude lipase	90.0	0.12	0.90	0.13	1.0
Ammonium sulfate precipitation	10.0	0.19	0.60	0.32	2.5
Dialyzate	20.0	0.60	0.15	4.0	30.77
Octyl sepharose chromatography	6.0	0.59	0.14	4.2	32.30

Effect of the temperature on the esterification reaction

The reaction mixture (2 mL) contained 25 mg/mL hydrogel-bound lipase and 100 m*M* each geraniol and acetic acid in *n*-heptane in a Teflon-stoppered glass vial (5-mL capacity). The reaction mixture was incubated at 45, 55, 65, and 75°C under shaking (160 rpm) for up to 15 h. The geranyl acetate formed in each case was determined. The temperature at which the maximum synthesis of ester was recorded was selected for subsequent studies.

Effect of the C chain length of the solvent (alkane)

In the reaction mixture, *n*-heptane, which was initially used as the solvent phase, was replaced with *n*-alkanes with various C chain length, that is, *n*-pentane, *n*-hexane, *n*-heptane, *n*-octane, and *n*-nonane. The immobilized lipase (25 mg/mL) was added to the previous reaction mixture prepared in each of the chosen *n*-alkanes, and the reaction was carried out for 15 h at 55°C under continuous shaking (160 rpm). The geranyl acetate formed was assayed by GLC. The *n*-alkane that gave best amount of geranyl acetate was selected for further studies.

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

A molecular sieve (3 Å × 1.5 mm) was used to study its effect on the synthesis of geranyl acetate by the immobilized lipase. To the previous reaction mixture prepared in *n*-heptane, various amounts (12.5–62.5 mg/mL) of molecular sieve were added. The esterification was carried out in duplicate by the addition of 25 mg/mL immobilized lipase at 55°C under shaking (160 rpm) for 15 h. The geranyl acetate synthesized in each case was determined.

Reusability of the immobilized lipase in batch cycles of esterification for the synthesis of geranyl acetate

The formation of geranyl acetate from geraniol and acetic acid (100 : 100 m*M*) catalyzed by the immobilized lipase in *n*-heptane was used to check the retention of the catalytic activity of the hydrogel-immobilized enzyme. The repetitive esterification was performed for up to eight cycles of 15 h each to synthesize the geranyl acetate at 55°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 the matrix was reused for another cycle of esterification.

Bioprocess development at the 50-mL level for the geranyl acetate synthesis

Under optimized conditions, the 2-mL reaction volume was scaled up to a 50-mL reaction volume. The esterification was performed in a 250-mL capped flask at 55°C for 30 h under shaking, and the geranyl acetate synthesized was assayed by GLC.

RESULTS

Purification of lipase

The harvested cell-free broth had a lipase activity of 0.12 IU with a protein concentration of 0.90 mg/mL (specific activity = 0.13 IU/mg). The dialyzate showed a lipase activity of 0.60 IU/mL (specific activity = 4.0 IU/mg). Hydrophobic interaction chromatography of the dialyzed lipase on the octyl sepharose column resulted in a single peak. The fractions showing lipase activity were pooled (Table I) and assayed for lipase and protein content (6.0 mL, 0.59 IU/mL, protein = 0.14 mg/mL, specific activity = 4.2 IU/mg). The purified lipase was used for immobilization by adsorption on the hydrogel.

Immobilization of the purified lipase

The hydrogel showed a 95% binding/retention of purified lipase of *B. cereus* MTCC 8372. The poly (methacrylic acid-*co*-dodecyl methacrylate-*cl*-*N*,*N*-methylene bisacrylamide) [poly(MAc-*co*-DMA-*cl*-MBAm)] matrix possessed an S_w value of 3.46.

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

When the immobilized lipase was subjected to 1 h of pre-exposure at each of the selected pH values (pH = 7.0= 9.5) at 55°C, the immobilized biocatalyst

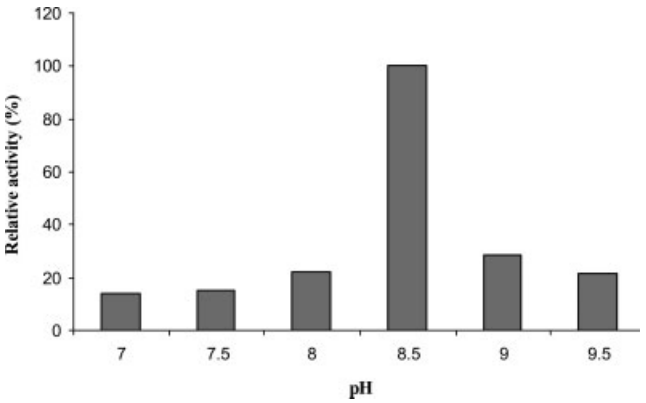


Figure 1 Effect of the pH on the hydrolytic activity of the immobilized lipase.

showed a maximum residual hydrolytic activity after pre-exposure at pH 8.5 (Fig. 1).

Stability of the immobilized lipase at various pH's

The immobilized lipase was found to be most stable at pH 8.5 (Fig. 2).

Effect of the preincubation temperature on the hydrogel-immobilized lipase

We studied the effect of the preincubation temperature for the hydrolytic reaction separately at temperatures ranging from 35 to 75°C by maintaining the pH of the Tris buffer (0.05*M*) at 8.5 for the immobilized lipase. A temperature of 55°C for the immobilized enzyme was found to give maximum hydrolytic activity for the *p*-NPP (Fig. 3).

Thermostability of the hydrogel-immobilized lipase

The immobilized lipase was separately incubated at each of the preselected temperatures (35, 45, 55, 65, and 75°C) for 3 h in 3 mL of Tris buffer (pH = 8.5, 0.05*M*). The immobilized lipase was found to be most stable at 55°C (Fig. 4).

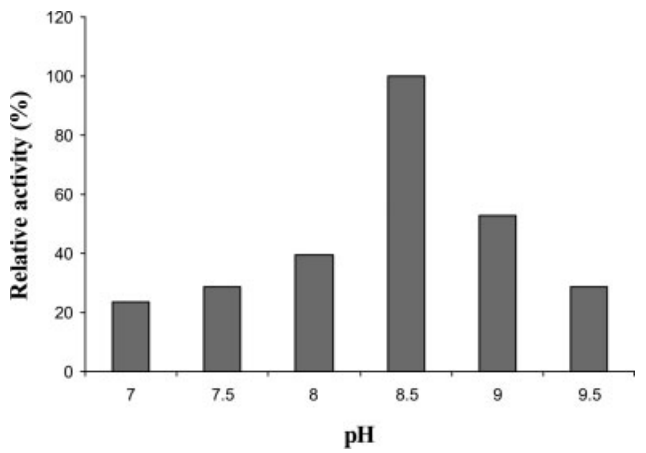


Figure 2 Stability of the immobilized lipase at various pH's.

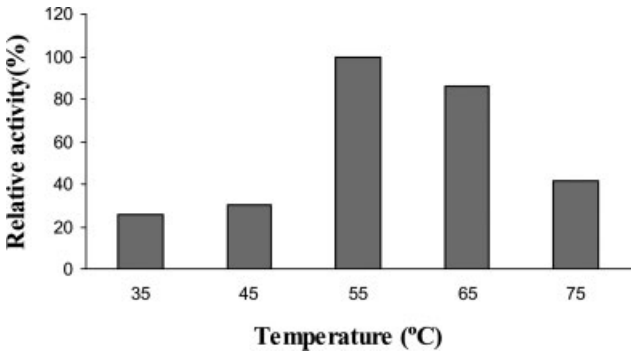


Figure 3 Effect of the preincubation temperature on the hydrolytic activity of the immobilized lipase.

Effect of the incubation time on the hydrolytic activity of the hydrogel-immobilized lipase

The hydrolytic reaction of the immobilized enzyme was carried out for up to 10 h at 55°C, and the activity was recorded every 2 h. Maximum activity was obtained at 2 h (Fig. 5), and it decreased up to 50% at 6 h.

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

The immobilized lipase was used to catalyze the hydrolysis of various *p*-nitrophenyl esters with different C chain lengths. The immobilized lipase was highly hydrolytic toward a relatively longer C-chain-length ester (*p*-NPP) than toward the other ones (Fig. 6). This indicated a preferential specificity of the *B. cereus* lipase toward longer carbon-chain-length substrates.

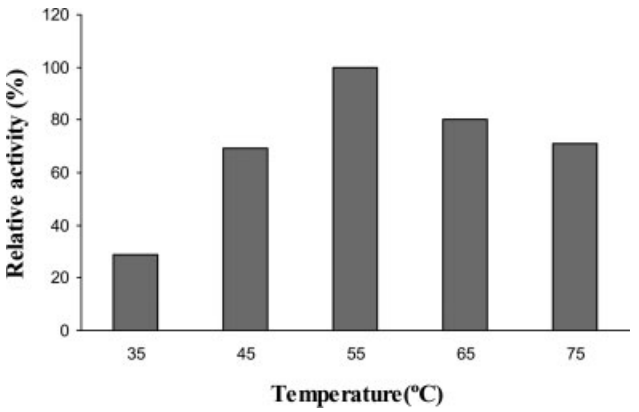


Figure 4 Thermostability of the immobilized lipase.

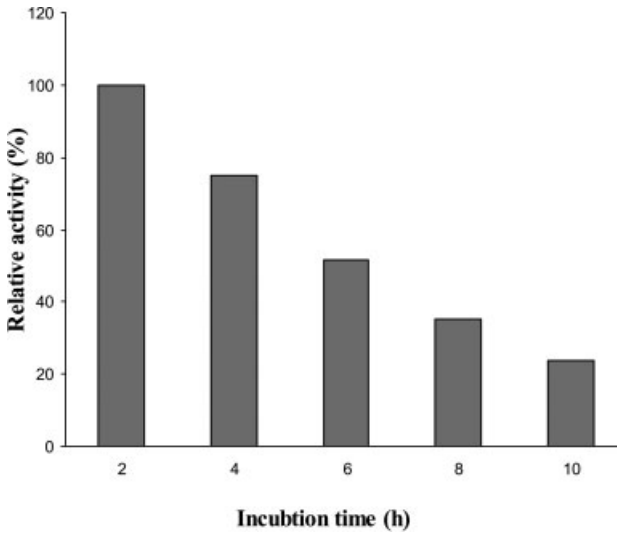


Figure 5 Effect of the incubation time on the immobilized lipase at 55°C.

Effect of salt ions on the hydrogel-immobilized lipase

The lipase activity of the bound lipase was enhanced (Table II) in the presence of NH_4^+ (121.61%), Fe^{+3} (130%), and Hg^{+2} (188.71%). In contrast, the presence of Al^{+3}, Co^{+2}, and Mg^{+2} antagonized the hydrolytic activity of the bound lipase.

Optimization of the esterification conditions with the immobilized lipase

We studied the synthesis of geranyl acetate by placing different amounts of immobilized lipase (12.5–62.5 mg/mL in duplicates) in 100 m*M* each of geraniol and acetic acid at 55°C for 15 h in *n*-heptane under shaking (160 rpm). The maximum synthesis of geranyl acetate (82.74 m*M*) was obtained with 25 mg/mL immobilized lipase (Table III). All subsequent esterification studies were performed in triplicate by the use of the hydrogel-bound lipase (25 mg/mL) in Teflon-gasketed glass vials (5-mL capacity) in a incubator shaker (160 rpm). The amount of ester synthesized is presented as a mean value.

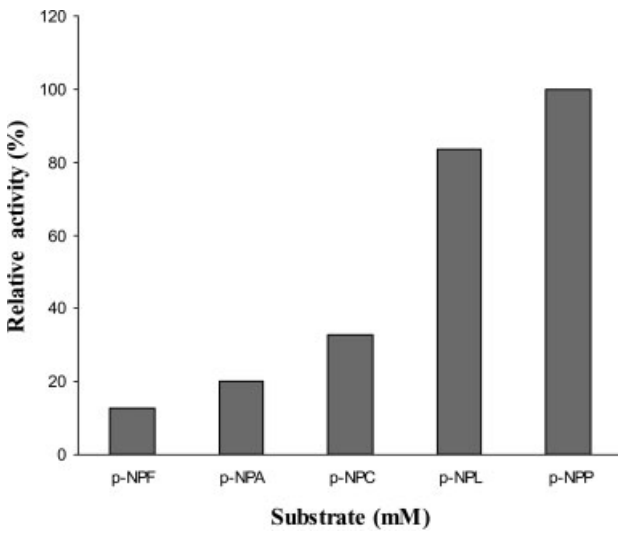


Figure 6 Effect of the C chain length of the acyl esters on the immobilized lipase.

TABLE II
Effect of the Salt Ions on the Immobilized Lipase

Salt ion	Relative activity (%)
No ions	100
Hg^{2+}	188.71
Fe^{2+}	130
NH_4^+	121.61
Al^{3+}	52.26
Mg^{2+}	95.81
Co^{2+}	69.03

Effect of the proportional concentration of the reactants on the synthesis of geranyl acetate

We evaluated the effect of various concentrations of each of the reactants on ester formation by keeping the concentration of one of the reactants at 100 m*M* in *n*-heptane. At a fixed concentration of acetic acid (100 m*M*), with an increase in the concentration of geraniol (25–100 m*M*) at 55°C under continuous shaking, the synthesis of ester increased markedly from 26.79 to 70.11 m*M*. However, when the concentration of geraniol was fixed (100 m*M*), and the concentration of acetic acid was enhanced from 25 to 100 m*M* in *n*-heptane, a sharp increase in the synthesis of ester was noticed from 19.53 to 74.19 m*M*. However, at equimolar concentrations of both reactants (100 m*M*), the formation of geranyl acetate (82.76 m*M*) was observed to be at a maximum (Table IV). In subsequent reactions, 100 m*M* of each of the reactants was used to achieve esterification.

Effect of the temperature on the esterification reaction

The esterification was performed with 100 m*M* each geraniol and acetic acid at selected temperatures

TABLE III
Effect of the Biocatalyst Concentration on the Synthesis of Geranyl Acetate

Biocatalyst concentration (mg/mL)	Geranyl acetate (m*M*)
12.5	75.89
25.0	83.74
37.5	74.85
50.0	74.45
62.5	72.98

TABLE IV
Effect of the Molar Concentrations of the Reactants on the Synthesis of Geranyl Acetate

Geraniol : acetic acid (m*M*)	Geranyl acetate (m*M*)
Matrix control	0.00
25 : 100	26.79
50 : 100	52.09
75 : 100	70.11
100 : 100	82.76
100 : 75	74.19
100 : 50	48.91
100 : 25	19.53

(45–75°C) for 15 h in *n*-heptane. Maximum ester synthesis (83 m*M*) was recorded at 55°C (Table V). At 45 and 75°C, there was a decrease in the yield of ester produced.

Effect of the reaction time on the synthesis of geranyl acetate

The kinetics of the immobilized-lipase-catalyzed synthesis of geranyl acetate was studied for up to 21 h at 55°C in *n*-heptane. The geranyl acetate synthesis increased with time up to 15 h and gradually declined thereafter. At 15 h, approximately 82.7 m*M* geranyl acetate was produced (Table VI). Thus, in subsequent esterification reactions, a reaction time of 15 h at 55°C for immobilized lipase was considered optimum for the synthesis of geranyl acetate.

Effect of the C chain length of the solvent (alkane) on the synthesis of geranyl acetate

A maximum amount of geranyl acetate (83.60 m*M*) was synthesized when *n*-nonane was used as a solvent system (Table VII). With increasing C chain length of the solvent system, ester synthesis gradually increased. The esterification performed in *n*-heptane (82.24 m*M*) or *n*-octane (82.53 m*M*) gave almost the same concentration of geranyl acetate.

Effect of the molecular sieve (3 Å × 1.5 mm) on the synthesis of geranyl acetate

The addition of the molecular sieve to the reaction mixture gradually increased the amount of geranyl acetate synthesized by the immobilized lipase (Table VIII). Improved ester synthesis (81.0–90.0 m*M*) was recorded at 10–40 mg/mL molecular sieve. A further increase of the concentration of the molecular sieve to 50 mg/mL or above resulted in a gradual decline of geranyl acetate synthesized.

TABLE V
Effect of the Reaction Temperature on the Synthesis of Geranyl Acetate

Temperature (°C)	Geranyl acetate (m*M*)
45	43
55	83
65	80
75	63

TABLE VI
Effect of the Reaction Time on the Synthesis of Geranyl Acetate

Reaction time (h)	Geranyl acetate (m*M*)
0	0.00
3	29.0
6	48.0
9	62.0
12	78.0
15	82.7
18	80.6
21	79.0

Reusability of the immobilized lipase in continuous cycles of esterification

The immobilized matrix (25 mg/mL) was repetitively used in batch cycles for up to eight cycles of esterification with both of the reactants in *n*-heptane at a final concentration of 100 m*M* each geraniol and acetic acid. The esterification was performed at 55°C for 15 h for each cycle. The hydrogel-bound lipase retained 37.0 m*M* (Table IX) of its initial esterification activity after the eighth cycle.

Bioprocess development at the 50-mL level for the geranyl acetate synthesis

Under optimized conditions, a 2-mL reaction volume was scaled up to the 50-mL level in a 250-mL Teflon-gasket-capped flask. When the reaction volume was increased from 2 to 50 mL, the time required to achieve a certain conversion (58.7m*M*) also increased from 15 to 30 h (Table X). The conversion was low (30.16 m*M*) even after 45 h.

DISCUSSION

In this study, a synthetic matrix (hydrogel) was used for the immobilization of an alkaline lipase of thermophilic *B. cereus*. The experimental data established

TABLE VII
Effect of the C Chain Length of the Solvents (Alkanes) on the Synthesis of Geranyl Acetate

Organic solvent (*n*-alkane)	Geranyl acetate (m*M*)
n-Pentane	42.07
n-Hexane	48.17
n-Heptane	82.24
n-Octane	82.53
n-Nonane	83.60

TABLE VIII
Effect of the Molecular Sieve on the Synthesis of Geranyl Acetate

Molecular sieve (mg/mL)	Geranyl acetate (m*M*)
Control	81.0
10	83.0
20	84.0
30	87.0
40	90.0
50	81.5
60	79.5

that the lipase of *B. cereus* was efficiently immobilized on the hydrogel used in this study.

Exogenous factors, such as pH and temperature, modulate the reaction selectivity of lipases.[28] The hydrogel-bound lipase of *B. cereus* was optimally active at a pH of 8.5 and a temperature of 55°C. The lipase of *B. cereus* after immobilization on the hydrogel retained good thermotolerance at 55°C. The immobilization of lipase from *Candida rugosa* on chitosan showed an optimum reaction temperature of 30°C, whereas the immobilization of lipase from the same organism on kaolin showed the highest activity at 40°C. Generally, temperatures above ambient promote the liquefaction of the reactants and tend to make the substrate more diffusible and, hence, easily acceptable to the enzyme.[29] At 75°C, there was decrease in the activity of the *B. cereus* lipase, which might have been due to the denaturation of the lipase. Heat promoted protein unfolding and, thus, led to a loss of enzymatic activity. It was shown that immobilized preparations were much more stable than the soluble enzyme when the immobilization of lipase from *Bacillus thermocatenulatus* was done on hydrophobic supports, with 100% of the activity maintained at 65°C. *Bacillus* GK8 lipase, when immobilized on HP-20 beads, retained complete activity at 60°C.[30] 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 *B. cereus* MTCC-8372 was highly hydrolytic toward a longer C-chain-length ester (*p*-NPP) than toward the shorter ones. In another study, a lipase from a psychrotrophic *Pseudomonas* species displayed the highest activity toward C : 10 acyl groups of *p*-nitrophenyl esters.[31] Recently, immobilized lipase from *Bacillus coagulans* BTS-1 was found to be more hydrolytic toward a medium-C-length ester than toward shorter or longer C length esters.[26] Previously, a purified lipase from *Pseudomonas cepacia* immobilized on a commercially available microporous polypropylene support showed a higher activity with *p*-NPA and a very low activity with *p*-NPP.[32] The hydrolytic activity of the hydrogel-bound lipase of *B. cereus* was promoted/enhanced in the presence of a few salt ions, which 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. The exposure of Ca^{2+} ions to an extracellular lipase of *Pseudomonas aeruginosa* 2D was reported to cause a 360% increase in the lipase activity, but the presence of Hg^{2+} and Co^{2+} strongly inhibited the activity.[33] Hg^{2+}, Al^{3+}, Mn^{2+}, and Co^{2+} ions exerted a drastic decrease in the lipase activity of *Rhizopus oryzae*.[34]

TABLE IX
Repetitive Use of the Hydrogel-Bound Enzyme for the Synthesis of Geranyl Acetate

Cycle number	Geranyl acetate (m*M*)
Control	82.0
1	80.0
2	77.0
3	73.0
4	66.0
5	57.0
6	51.0
7	43.0
8	37.0

TABLE X
Bioprocess Development for the Synthesis of Geranyl Acetate at the 50-mL Level

Time (h)	Geranyl acetate (m*M*)
15	51
30	58.7
45	30.16

The application of lipases as biocatalysts to produce these high-value-added fatty acid esters in organic media have offered significant advantages,[34–38] which include the use of any hydrophobic substrate, higher selectivity, milder processing conditions, and ease of product isolation and enzyme reuse.[39,40] In this study, the poly(MAc-*co*-DMA-*cl*-MBAm)-hydrogel-immobilized alkaline lipase of a thermophilic *B. cereus* MTCC 8372 was used to catalyze the esterification of geraniol and acetic acid into geraniol acetate over a period of 15 h at 55°C. When *n*-alkane, with a shorter C chain length than *n*-nonane, was used as a solvent, a gradual decrease in the rate of geraniol acetate synthesis was noticed. As the log *P* (partition coefficient between *n*-octanol and water) value of an *n*-alkane increased with an increase in the C chain length of the alkanes, the hydrophobicity of the alkane, that is, the solvent, also increased in that order, and this appeared to be very important for the modulation of the catalytic activity of the *B. cereus* lipase.

Esterification is generally a water-limited reaction because the equilibrium catalyzed by hydrolytic enzymes is in favor of hydrolysis.[41] Water inhibits

the reaction also when an immobilized enzyme with a hydrophilic nature of support is used,[42] which results in a decrease in the rate of enzyme activity, as seen in this study. When the amount of the desiccant/molecular sieve in the reaction mixture was increased to 40 mg/mL in the reaction mixture, the *B. cereus* hydrogel-immobilized lipase showed an increase in synthesis of geranyl acetate. Thus, presence of the molecular sieve in the reaction mixture invariably prevented the inhibitory effects of the accumulation of water on the esterification reaction between acetic acid and geraniol. It appeared that an increase in the concentration of the molecular sieve provided a corresponding increase in the physically active surface area of the molecular sieve, which readily absorbed water and, thus, promoted forward reaction kinetics. The esterification of geraniol and acetic acid by the immobilized lipase from *B. cereus* was further enhanced when the molar concentration of the hydrophobic reactant, that is, geraniol, was increased from 25 : 100 to 100 : 100 (geraniol/acetic acid) in the reaction mixture.

Thus, this study established that the immobilization of the lipase obtained from *B. cereus* MTCC 8372 on the poly(MAc-*co*-DMA-*cl*-MBAm) hydrogel was quite stable, as the hydrogel retained hydrolytic activity toward the *p*-NPP and achieved repetitive esterification in *n*-heptane for a number of cycles.

The authors acknowledge the Bioinformatics Centre at Himachal Pradesh University (Shimla, India) for making available its Internet facility.

References

1. Yadav, G. D.; Trivedi, A. Enzyme Microb Technol 2003, 32, 783.
2. Abbas, H.; Comeau, L. Enzyme Microb Technol 2003, 6270, 1.
3. Knorr, D. Food Biotechnol 1987, l, 225.
4. Christen, P.; López-Munguía, A. Food Biotechnol 1994, 8, 167.
5. Vulfson, E. In Lipases: Their Structure, Biochemistry, and Application; Wooley, P.; Peterson, S. B., Eds.; Cambridge University Press: Cambridge, England, 1994; p 271.
6. Jaeger, K.; Ransac, S.; Dijktra, C.; Colson, C.; Heuvel, M.; Misset, O. FEMS Microbiol Rev 1994, 15, 29.
7. Pandey, A.; Benjamin, S.; Soccol, C.; Nigam, P.; Krieger, N.; Soccol, V. Biotechnol Appl Biochem 1999, 29, 119.
8. Nagao, T.; Shimada, Y.; Sugihara, A.; Murata, A.; Komemushi, S.; Tominaga, Y. J Am Oil Chem Soc 2001, 78, 167.
9. Kim, I.; Kim, H.; Lee, K.; Chung, S.; Ko, S. J Am Oil Chem Soc 2002, 79, 363.
10. Kim, Y.; Bae, J.; Oh, B.; Lee, W.; Choi, J. Biores Technol 2002, 82, 57.
11. Brockman, H.; Borgstorm, B. Lipases; Elsevier: Amsterdam, 1984.
12. Jaeger, K.; Reetz, T. TIBTECH 1998, 16, 396.
13. Macrae, A. R.; Roel, E. L.; Brand, H. M. Seifen Oele Fette Wachse 1984, 116, 201.
14. Shimada, Y.; Hirota, Y.; Baba, T.; Sugihara, A.; Moriyama, S.; Tominaga, Y.; Terai, T. J Am Oil Chem Soc 1999, 76, 713.
15. Zaidi, A.; Gainer, J. L.; Carta, G.; Mrani, A.; Kadiri, T.; Belarbi, Y.; Mir, A. J Biotechnol 2002, 93, 209.
16. Liese, A.; Seelbach, K.; Wandrey, C Industrial Biotransformations; Wiley-VCH, Weinheim, 2000.
17. Sheldon, R. Chem Commun 2001, 2399.
18. Kanwar, S. S.; Verma, H. K.; Pathak, S.; Kaushal, R. K.; Kumar, Y.; Verma, M. L.; Chimni, S. S.; Chauhan, G. S. Acta Microbiol Immunol Hung 2006, 53, 195.
19. Kanwar, S. S.; Verma, M. L.; Maheshwari, C.; Chauhan, S.; Chimni, S. S.; Chauhan, G. S. J Appl Polym Sci 2007, 104, 183.
20. Harun, A.; Basri, M.; Ahmad, M. B.; Salleh, A. B. J Appl Polym Sci 2004, 92, 3381.
21. Bornscheuer, U. T.; Yamane, T. Enzyme Microb Technol 1994, 16, 864.
22. Klien, R. R.; King, G.; Moreau, R. A.; Mcneill, G. P.; Villeneuve, P.; Hass, M. J. J Am Oil Chem Soc 1997, 74, 1401.
23. Rhee, J. S.; Kwon, S. J. J Microbiol Biotechnol 1998, 8, 191.
24. Winkler, U. K.; Stuckmann, M. J Bacteriol 1979, 138, 663.
25. Kanwar, S. S.; Ghazi, I. A.; Chimni, S. S.; Joshi, G. K.; Rao, G. V.; Kaushal, R. K.; Gupta, R.; Punj, V. Protein Exp Purif 2006, 46, 421.
26. Bradford, M. M. Anal Biochem 1976, 72, 248.
27. Kanwar, S. S.; Chauhan, G. S.; Chimni, S. S.; Kumar, Y.; Rawat, G. S.; Kaushal, R. K. J Appl Polym Sci 2006, 100, 1420.
28. Lee, C. H.; Parkin, K. L. Biotechnol Bioeng 2001, 75, 219.
29. Sunna, A.; Hunter, L.; Hutton, C. A.; Bergquist, P. L. Enzyme Microb Technol 2000, 31, 472.
30. Rashid, N.; Shimada, Y.; Ezaki, S.; Atomi, H.; Imanaka, T. Appl Environ Microbiol 2001, 67, 4064.
31. Pancreac'h, G.; Leullier, M.; Baratti, J. C. Biotechnol Bioeng 1997, 56, 181.
32. Goncalves, A. P. V.; Lopes, J. M.; Lemos, F.; Ribeiro, F. R.; Prazeres, D. M. F.; Cabial, J. M. S.; Aires-Barrow, M. R. Enzyme Microb Technol 1997, 20, 93.
33. Kaushal, R. K.; Sen, U.; Kanwar, S. S.; Chauhan, G. S. Proceedings of the IXth International Workshop on Bioencapsulation, Illkirch, Strasbourg, France, 2003.
34. Arcos, J. A.; Bernabe, M.; Otero, C. Biotechnol Bioeng 1998, 57, 505.
35. Dordick, J. S. Enzyme Microb Technol 1989, 11, 194.
36. Bornscheuer, U. T. Enzyme Microbiol Technol 1995, 17, 578.
37. Sheldon, R. A. In Enzymatic Reactions in Organic Media; Koskinen, A. M. P.; Klibanov, A. M., Eds.; Blackie: Glasgow, 1996; p 267.
38. Malcata, F. X.; Reyes, H. R.; Garcia, H. S.; Hill, C. G.; Admunson, C. H. J Am Oil Chem Soc 1990, 67, 890.
39. Yahya, A. R. M.; Anderson, W. A.; Moo-Young, M. Enzyme Microb Technol 1998, 23, 438.
40. Halling P. J. Enzyme Microb Technol 1984, 16, 513.
41. Jack, K. Appl Microb Biotechnol 1991, 64, 763.
42. Mustafa, U. U. Trends J Agric Forestry 1998, 22, 573.