Synthesis of Geranyl Butyrate with the Poly(acrylic acid-*co*-hydroxy propyl methacrylate-*cl*-ethylene glycol dimethacrylate) Hydrogel Immobilized Lipase of *Pseudomonas aeruginosa* MTCC-4713

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ABSTRACT: Microbial lipases (E.C. 3.1.1.3) are the preferred biocatalysts for the synthesis of various fragrance compounds, such as linalool acetate, citronellal acetate, and geranyl acetate, in organic solvents over chemical synthesis. In this study, a purified alkaline extracellular lipase of *Pseudomonas aeruginosa* MTCC-4713 was efficiently immobilized onto a synthetic poly(AAc-*co*-HPMA-*cl*-EGDMA) hydrogel by surface adsorption, and the bound lipase was evaluated for its hydrolytic potential toward various *p*-nitrophenyl acyl esters, which differed in their C-chain length. Among four series of hydrogels prepared by the variation of the concentrations of monomer and crosslinker, two hydrogels, namely, I_{5d} and I_{20d}, that exhibited relatively higher protein (lipase activity) bindings were selected to perform hydrolytic and synthetic (geranyl butyrate) reactions in aqueous and organic solvents. The hydrogel-bound lipase was highly hydrolytic toward *p*-nitrophenyl ester (C: 16; *p*-nitrophenyl palmitate). The hydrogel-immobilized lipase was quite stable and retained approximately 57.6% of its original hydrolytic activity after the fifth cycle of reuse under optimized conditions (pH 8.5, 65°C). The hydrogel-immobilized lipase when used to perform the esterification of geraniol/butyric acid (400 : 100 m*M*) in *n*-heptane resulted in 98.8 m*M* geranyl butyrate at 65°C under shaking (120 rpm) after 15 h of reaction time. The addition of a molecular sieve (3 Å × 1.5 mm) to the reaction system at a concentration of 100 mg per reaction volume (1 mL) resulted in the complete conversion of the reactants into geranyl butyrate. © 2008 Wiley Periodicals, Inc. J Appl Polym Sci 110: 2681–2692, 2008

Key words: enzymes; esterification; hydrogels

INTRODUCTION

Lipases (E.C. 3.1.1.3) are versatile biocatalysts that have been used to catalyze a range of reactions, such as the esterification, amidation, and transesterification of esters and organic carbonates.[1] They are highly stable under adverse conditions, such as organic solvents and high temperatures. The applications of lipase include the production of food additives, chiral intermediates, and pharmaceutical products.[2] Lipase-catalyzed condensation reactions in organic solvents are useful for the synthesis of esters.[3–8] In the last few years, there has been increasing interest in the use of enzymes for the biosynthesis of molecules in organic media.[9–11] At present, many esters are industrially manufactured by chemical methods. However, chemical methods involve high temperatures or high pressures; it is difficult in many cases to esterify unstable substances, such as polyunsaturated fatty acid, ascorbic acid, and polyols.[12] Furthermore, the regiospecific acylation of alcohol requires protection and deprotection steps.[13] Such steps are likely to cause a rise in the manufacturing costs of the esters. In case of esters derived for use in food as additives, chemical impurities are not desired.

Many lipase-catalyzed esterification or condensation reactions have been developed by the use of a variety of lipases of microbial origin.[14–19] Such reactions are possible in organic solvents. However, in a lipase-catalyzed reversible reaction (esterification), the direction and equilibrium of the reaction are determined by the concentration or activities of the substrates and products, the temperature, the pressure, and so on.[13] The conversion in the transesterification reaction with another ester as a substrate is quite high compared to that achievable in a condensation reaction.[20,21] However, acetaldehyde forms a Schiff base with the lipase.[22] In contrast, a condensa-

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tion/esterification reaction has the advantages that the free carboxylic acid can be directly esterified to reduce the cost and that the reaction affords only a harmless byproduct, that is, water, which greatly simplifies the purification steps.

Furthermore, immobilized lipases offer the economic incentives of enhanced thermal and chemical stability, ease of handling, and easy recovery and reuse relative to nonimmobilized forms.[23,24] Immobilization also facilitates the dispersal of enzymes on a solid surface to provide a greater interfacial area and accessibility relative to the use of enzyme powder in low-water reaction media. Lipase-catalyzed reactions, however, have a major inconvenience: the conversions are relatively low compared with traditional chemical processes when crude commercial enzymes are used. It would be an advantage to use a purified immobilized lipase to perform esterification reactions in organic media. Geranyl butyrate (3,7-dimethyl 2,6-octadien-1-yl butyrate) is one of the most frequently used terpenoid fragrance materials, with a flowery roselike composition that does not discolor soaps. This ester is frequently used in perfumes and cosmetics such as lipsticks with or without the conventional ones. In flavor (for rose, orange oil, and lemon type) compositions, geranyl butyrate is used in small quantities to accentuate citrus notes. The geraniol molecule possesses a double bond, and its esterification with a fatty acid in the presence of a strong catalyst such as a mineral acid requires protection and deprotection steps. Alternatively, the synthesis of geranyl butyrate from geraniol and butyric acid can be achieved by an immobilized biocatalyst (lipase), which could be easily separated from the reaction mixture. In this study, an extracellular thermoalkaliphilic purified lipase of *Pseudomonas aeruginosa* MTCC-4713 immobilized on synthetic hydrogel(s) was used for the optimal synthesis of geranyl butyrate.

EXPERIMENTAL

Chemicals and reagents

The chemicals, which included $NaNO_3$, K_2HPO_4, KCl, $MgSO_4{\cdot}7H_2O$, $FeSO_4{\cdot}7H_2O$, $(NH_4)_2SO_4$, *n*-nonane, and methanol and butyric acid (S. D. Fine Chemicals, Ltd., Hyderabad, India); *p*-nitrophenyl palmitate (*p*-NPP), *p*-nitrophenyl laurate (*p*-NPL), *p*-nitrophenyl acetate (*p*-NPA), *p*-nitrophenyl caprylate (*p*-NPC), *p*-nitrophenyl formate (*p*-NPF), and *n*-pentadecane (Lancaster Synthesis, White Lund Morecambe, UK); 2-propanol and acetone (Qualigens Fine Chemicals, Mumbai, India); acrylic acid (AAc), 2-hydroxy propyl methacrylate (HPMA), ammonium persulfate (APS), *n*-pentane, *n*-hexane, and *n*-heptane (Merck, Ltd. Mumbai, India); geraniol (National Chemicals, Nutan Gujarat Industrial Estate, India); Triton X-100 (Sigma, St. Louis, MO); and Tris-HCl (Ranbaxy, New Delhi, India) were analytic grade and were used as received.

Microorganism and purification of the lipase

P. aeruginosa MTCC-4713 was obtained from the Department of Biotechnology, Himachal Pradesh University (Shimla, India). The culture was maintained by repeated subculturing at 55°C in a mineral-based broth. The mineral-based broth contained, 3.0 g/L $NaNO_3$, 0.1 g/L K_2HPO_4, 0.5 g/L KCl, 0.5 g/L $MgSO_4{\cdot}7H_2O$, 0.01 g/L $FeSO_4{\cdot}7H_2O$, 5.0 g/L sucrose, and 1.0% (v/v) cottonseed oil (pH 8.5). The seed culture was prepared by the inoculation of 50 mL of broth with a loopfull of culture that was incubated for 48 h at 55°C under shaking (250 rpm). Thereafter, 7.5% (v/v) 48-h-old seed culture was used to inoculate 500 mL of the production broth (50 mL each in 250-mL Erlenmeyer flask). This production broth was incubated at 55°C and 250 rpm for 48 h. The culture broth was centrifuged at 10,000 g for 20 min at 4°C (Sigma 3K30, Harz, Germany). The cell pellet was discarded, and the supernatant was retained. The supernatant was filtered through Whattman paper no. 1 filter (Whatman International Ltd., Maidstone, England), and its protein content was measured.[25] The filtrate, henceforth referred to as crude lipase, was used to obtain purified lipase by successive dialysis and Diethyl amino ethane–cellulose anion-exchange chromatography.[9]

Assay of lipase activity

The lipase assays were performed by a colorimetric method[26] with *p*-NPP, as described previously.[9] Each of the assays was performed in triplicate, and mean values are presented. One unit of lipase activity was defined as the amount of free enzyme (in milliliters) or immobilized enzyme (in grams, including the weight of matrix) that released 1 μmol of *p*-nitrophenol from *p*-NPP at 55°C for free enzyme or at 65°C for immobilized enzyme in 1 min. Specific activity was expressed as micromoles of the *p*-nitrophenol released per minute by 1 mg of protein.

Synthesis of the hydrogel networks

The hydrogels used for the immobilization of lipase were based on AAc. The hydrogels were obtained when AAc was copolymerized separately with HPMA with APS as an initiator. Ethylene glycol dimethacrylate (EGDMA) was used as crosslinker to produce a different series of hydrogels. In brief, AAc (2 mL) was added to 4.0 mL of a solvent system composed of acetone and water (1 : 1 v/v) along with fixed concentration of initiator APS (1 m*M*) and a known concentration of crosslinker EGDMA (1%,

TABLE I
Networks (Xerogels) with Various Concentrations of AAc/HPMA with EGDMA as a Crosslinker

Matrix (hydrogel)	AAc/HPMA (volume ratio)	EGDMA (% w/w)
I_{5a}	5 : 1	5
I_{10a}	5 : 1	10
I_{15a}	5 : 1	15
I_{20a}	5 : 1	20
I_{5b}	5 : 2	5
I_{10b}	5 : 2	10
I_{15b}	5 : 2	15
I_{20b}	5 : 2	20
I_{5d}	5 : 4	5
I_{10d}	5 : 4	10
I_{15d}	5 : 4	15
I_{20d}	5 : 4	20
I_{5e}	5 : 5	5
I_{10e}	5 : 5	10
I_{15e}	5 : 5	15
I_{20e}	5 : 5	20

w/w). The reaction was designed to have AAc/HPMA in ratios of 5 : 1, 5 : 2, 5 : 4, and 5 : 5 (w/w). In the other four reaction mixtures, only the concentration of crosslinker was varied (5, 10, 15, and 20%, w/w) with respect to the total weight of the monomers (Table I). The reaction mixture was heated in a water bath at 50°C for 30 min with APS as the initiator. Thus, four different series of hydrogels were synthesized. Each of the dried networks was synthesized as a single piece. The sol fraction, if any, trapped in the body of the network was separated from the synthesized networks by a polarity gradient method by the treatment of the networks with water, methanol, and acetone separately in a Soxhlet apparatus from a solvent of higher polarity to one of lower polarity. The polymers were dried in an air oven at 40°C for 24 h to obtain a constant weight.

Immobilization of the purified lipase on different matrices (hydrogels)

The dried networks were broken into smaller pieces and grinded manually in a pestle and mortar to obtain a fine powder composed of particles 0.2–0.3 mm in diameter. Different completely dried matrices (10 mg of each) were incubated with purified lipase (200 μL; 0.05*M* phosphate buffer, pH 7.5) at 8°C for 20 h in separate glass vials. The supernatant was decanted, and its volume, hydrolytic activity, and protein (unbound) were recorded for each of the matrices. The immobilized protein in different matrices was determined by the subtraction of unbound protein in the supernatant from the total protein used for immobilization in comparison to the total enzyme units in 200 μL of purified lipase incubated with each of the matrices. The matrices that showed efficient binding/adsorption of the protein (lipase) were prepared in bulk (6 g each) for further study.

Properties of the hydrogel-immobilized lipase

Among various hydrogel matrices, the matrices possessing relatively higher bound protein (i.e., lipase) were selected (I_{5d} and I_{20d}). These two matrices were further studied to evaluate the effects of pH, temperature, thermostability, and specificity of the bound lipase toward the hydrolysis of *p*-nitrophenyl esters with various C-chain lengths.

Effect of the preincubation temperature on the immobilized lipase

The activity of the hydrogel (I_{5d} and I_{20d})-immobilized lipase (20 mg) was assayed separately by the incubation of the reaction mixture up to 3 h in glass tubes at temperatures ranging from 35 to 75°C in a water-bath incubator under shaking conditions. The lipase activity was assayed at 55°C after 10 min of incubation.

Thermostability of the I_{5d}- and I_{20d}-immobilized lipases

The thermostability of the hydrogel (I_{5d} and I_{20d})-immobilized lipase (20 mg) was examined at an optimized temperature of 65°C. The immobilized lipase (1 : 1, diluted in Tris buffer) was preincubated separately in an aqueous environment (0.05*M* Tris buffer, pH 8.5) at 35, 45, 55, 65, and 75°C up to 3 h with continuous shaking in a water-bath shaker. Subsequently, the lipase activities of the immobilized matrices were determined at 65°C after 10 min of incubation.

Effect of the pH of the reaction buffer on the hydrogel (I_{5d} and I_{20d})-immobilized enzyme

The effect of pH of the reaction buffer on the catalytic potential of the hydrogel (I_{5d} and I_{20d})-bound lipase was assayed by the incubation of 20 mg of immobilized lipase in Tris buffer (0.05*M*) adjusted at pH values of 5.5, 6.5, 7.5, 8.5, and 9.5. The lipase activity was assayed at 65°C after 10 min of incubation.

Stability of the I_{5d}- and I_{20d}-hydrogel immobilized lipases at various pH's at 65°C

The stability of immobilized lipase in buffer at various pH's was examined at an optimized temperature of 65°C. The immobilized lipase preparations were preincubated at room temperature (ca. 25°C) separately in aqueous environment (0.05*M* Tris buffer, pH's = 5.5, 6.5, 7.5, 8.5, and 9.5) up to 1 h with continuous shaking. Thereafter, the lipase activity was assayed at 65°C after 10 min of incubation.

Effect of the C-chain length of acyl ester (substrate) on the I_{5d}- and I_{20d}-hydrogel-immobilized lipase activity

The hydrogel (I_{5d} and I_{20d})-immobilized lipase (20 mg) was reacted with each of the five *p*-nitrophenyl esters (20 m*M* stock prepared in 2-propanol). The *p*-nitrophenyl esters were *p*-NPF, *p*-NPA, *p*-NPC, *p*-NPL, and *p*-NPP. The hydrolytic activity of bound lipase was determined under standard assay conditions at an optimized temperature and pH of 65°C and 8.5, respectively.

Reusability of the immobilized lipase for hydrolytic activity toward *p*-NPP

The reusability of the I_{5d} and I_{20d} hydrogels to perform repetitive hydrolysis of *p*-NPP in an aqueous system was evaluated. The hydrogel-bound lipase (20 mg) was added to 2.9 mL of reaction buffer (0.05*M* Tris, pH 8.5, and 0.1%, w/v gum acacia) and kept for 10 min at 65°C. Thereafter, 80 μL of *p*-NPP stock (20 m*M* prepared in 2-propanol) was added, and again incubation was further performed at 65°C for 10 min followed by the lipase assay. The matrix was recovered after decantation of the reaction buffer, subjected to two washings with 0.05*M* Tris buffer (3 mL for each wash) at room temperature, and reused for another cycle of hydrolysis in fresh reaction buffer. The reusability of the immobilized lipase was determined for up to eight cycles of hydrolysis of *p*-NPP. The residual activity after each cycle of hydrolysis was determined in comparison to the activity recorded in the first cycle of each matrix. Out of the two matrices (I_{5d} and I_{20d}), the hydrogel-bound biocatalyst that retained a relatively higher hydrolytic activity was used to perform ester synthesis (geranyl butyrate) in *n*-alkanes.

Geranyl butyrate synthesis with the I_{20d}-hydrogel-immobilized lipase

Esterification studies were performed with the I_{20d}-hydrogel-bound lipase (50 mg) in a reaction volume of 1 mL. Only the I_{20d}-hydrogel-bound biocatalyst, which showed better hydrolytic activity toward *p*-NPP than the other (I_{5d} hydrogel) biocatalyst, was selected for its ability to synthesize geranyl butyrate in an organic medium. The reaction mixture contained appropriate an amount of reactants in *n*-heptane unless specified otherwise. All reactions were performed in triplicate in Teflon-stoppered glass vials (5-mL capacity) under shaking (120 rpm), and mean values are presented.

Assay of geranyl butyrate by gas–liquid chromatography (GLC)

We prepared a reference profile of geranyl butyrate (25–200 m*M*) by making the final volume 1 mL in *n*-heptane. The reference curve was plotted between the molar concentration (millimolar) of geranyl butyrate and the corresponding area under the peak (retention time = 1.0 min). The presence of geranyl butyrate was detected by analysis of the sample (2 μL) by GLC. The GLC (Michro-9100, Netel Chromatographs, Thane, India) was programmed for an oven temperature of 250°C, an flame ionization detector temperature of 280°C, and an injector temperature of 280°C (holding time = 2 min). The GLC was equipped with a 10% SE Chrom WHP column (2 m, diameter = 1/8 in., and mesh size = 80–100). N_2 was used as a carrier gas (flow rate = 30 mL/min).

Effect of the relative molar ratio of the reactants on the geranyl butyrate synthesis

We determined the effect of relative molar ratio of geraniol and butyric acid on the synthesis of geranyl butyrate by keeping the concentration of one of the reactants (butyric acid) at 100 m*M* and varying the concentration of the second reactant, geraniol (50–600 m*M*), in a reaction volume of 1 mL in *n*-heptane. The esterification was carried out with the I_{20d}-matrix-bound lipase (50 mg) at 65°C for 15 h. It was determined that geranyl butyrate was formed.

Optimization of the reaction time for the synthesis of geranyl butyrate by the immobilized lipase

The reaction mixture (1 mL) contained 50 mg of I_{20d}-bound lipase, 400 m*M* (final concentration) geraniol, and 100 m*M* butyric acid in *n*-heptane. The reaction mixture was incubated at 65°C in a water-bath incubator for up to 21 h. The reaction mixture was sampled (50 μL) at intervals of 3 h and subjected to the detection of geranyl butyrate.

Temperature optimization for the esterification reaction

The reaction mixture (1 mL) contained 50 mg of hydrogel-bound lipase, 400 m*M* geraniol, and 100 m*M* butyric acid in *n*-heptane. The reaction mixture was incubated at 45, 55, 65, and 75°C in a water-bath incubator for up to 15 h. That geranyl butyrate formed in each case was determined. The temperature for the optimal synthesis of ester was determined for subsequent study.

TABLE II
Purification Profile of Lipase

Type of preparation	Volume (mL)	Activity (U/mL)	Total activity (U)	Protein (mg/mL)	Total protein (mg)	Specific activity (U/mg)	Fold purification
Crude extract	600	0.80	479.4	12.4	7440.0	0.06	1.0
Dialyzed lipase	10	4.48	44.8	4.2	42.0	1.06	16.6
DEAE-purified lipase	15	2.69	40.4	1.0	15.3	2.64	41.3

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

In the reaction mixture, *n*-heptane, which was initially used as a solvent phase, was replaced with *n*-alkanes with various C-chain lengths, that is, *n*-pentane, *n*-hexane, *n*-nonane, and *n*-pentadecane. The immobilized lipase was added to the aforementioned reaction mixture to perform the esterification. The reaction was carried out for 15 h at 65°C. Geranyl butyrate so formed was assayed by GLC. The *n*-alkane that gave best amount of geranyl butyrate was selected for further study.

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

A molecular sieve (3 Å × 1.5 mm, Merck, Darmstadt, Germany) was selected to study its effect on the synthesis of geranyl butyrate by immobilized lipase. To the previous reaction mixture prepared in *n*-heptane, various amounts (25–500 mg/mL) of the molecular sieve were added. The esterification was carried out with the immobilized lipase at 65°C for 15 h. The geranyl butyrate synthesized in each case was determined.

RESULTS

The harvested cell-free broth was subjected to successive fractionation by salting out, extensive dialysis, and anion-exchange chromatography on a DEAE–cellulose column. The protein concentration and the lipase (hydrolytic) activity at every step were determined to find out the specific activity and fold purification of the lipase of *P. aeruginosa* MTCC-4713 (Table II). The cell-free broth (600 mL) had a lipase activity of 0.8 U with a protein concentration of 12.4 mg/mL (specific activity = 0.064 U/mg at 48 h post inoculation). The protein (lipase) was optimally precipitated at 80% (w/v) ammonium sulfate saturation. The precipitate reconstituted in 10 mL of Tris buffer (0.05*M*, pH 8.5) was extensively dialyzed against the same buffer. The dialyzate showed a lipase activity of 4.48 U (specific activity = 1.06 U/mg). The anion-exchange chromatography of the dialyzed lipase on the DEAE–cellulose column resulted in a single peak. The fractions possessing lipase activity were pooled (15 mL, 2.69 U, 1.02 mg of protein/mL, specific activity = 2.64 U/mg).

The fractionation and anion-exchange chromatography of the crude lipase resulted in 16.6 and 41.3-fold purification of the bacterial lipase. The purified lipase was used in subsequent studies.

Protein-binding efficiency of the hydrogels and their characteristics

Four series of hydrogels prepared by the variation of the concentration of the monomer AAc and the copolymer HPMA (AAc/HPMA = 5 : 1–5 : 5 w/w) and the crosslinker (EGDMA = 5–20% w/w) showed an increase in protein binding with a corresponding increase in the concentration of EGDMA at a fixed ratio of AAc to HPMA (Table III). Thus, it was a decrease in the porosity of the polymer (hydrogel) that increased the efficiency of the hydrogel to bind the protein (lipase) of *P. aeruginosa* MTCC-4713. A similar trend was observed in the series of four hydrogels prepared by increases in the concentration of the crosslinker (EGDMA) from 5 to 20% at 10% concentration of crosslinker (EGDMA). A series of four hydrogels (I_{5d}, I_{10d}, I_{15d}, and I_{20d}) synthesized by the use of a 15% w/w concentration of EGDMA produced hydrogels (Table III) with more or less similar porosity and protein-binding efficiency (95.2–96.7%). A further increase in the concentration of crosslinker to 20% (w/w) resulted in a hydrogel with a low protein-binding efficiency when

TABLE III
Protein-Binding Efficiency and Hydrolytic Activity of Various Hydrogels

Hydrogel series	Protein binding efficiency (%)	Activity (U/g)
I_{5a}	59.0	0.345
I_{10a}	89.0	0.403
I_{15a}	91.8	0.432
I_{20a}	96.6	0.533
I_{5b}	52.3	0.475
I_{10b}	72.5	0.547
I_{15b}	85.8	0.576
I_{20b}	94.5	0.734
I_{5d}	96.2	1.137
I_{10d}	95.8	0.950
I_{15d}	95.2	1.130
I_{20d}	96.7	1.490
I_{5e}	43.7	0.734
I_{10e}	71.1	0.720
I_{15e}	90.3	0.576
I_{20e}	92.6	0.446

TABLE IV
Optimization of Protein Concentration for Immobilization onto the Hydrogel Support

Protein (mg/mL)	Hydrogel	Bound protein (mg/g)	Binding efficiency (%)
0.2	I_{5d}	2.2	11.2
	I_{20d}	4.6	23.4
0.4	I_{5d}	14.0	36.7
	I_{20d}	15.1	39.0
0.6	I_{5d}	21.1	35.6
	I_{20d}	26.3	43.8
0.8	I_{5d}	41.0	51.6
	I_{20d}	39.4	49.8
1.0	I_{5d}	67.0	67.0
	I_{20d}	71.6	71.6
1.2	I_{5d}	107.0	89.3
	I_{20d}	110.0	92.5
1.4	I_{5d}	115.0	82.4
	I_{20d}	124.0	88.7
1.6	I_{5d}	105.0	65.7
	I_{20d}	128.0	80.5
1.8	I_{5d}	96.1	53.5
	I_{20d}	123.0	68.4
2.0	I_{5d}	57.3	28.9
	I_{20d}	84.4	44.4

the copolymer was used at a concentration of 5–10% (w/w). Among all of the hydrogel polymers, the hydrogel (I_{20d}) prepared with a 20% concentration of HPMA and a 15% (w/w) concentration of crosslinker (EGDMA) possessed the highest protein-binding efficiency (96.7%) and bound lipase activity (1.49 U/g). Another hydrogel (I_{5d}) prepared with a 5% concentration of HPMA and a 15% (w/w) concentration of crosslinker (EGDMA) exhibited a slightly lower protein-binding efficiency (96.2%) and bound lipase activity (0.95 U/g) than the hydrogel I_{20d}. Thus, hydrogels I_{5d} and I_{20d} were selected for further study to evaluate the hydrolytic and esterification abilities of these hydrogel-bound lipases/biocatalysts in aqueous and organic media.

Optimization of the protein (lipase) concentration for immobilization onto the I_{5d} and I_{20d} hydrogels

The effect of various concentrations of protein (0.2–2.0 mg/mL) available for binding onto hydrogels I_{5d} and I_{20d} was determined, and the binding efficiency of each of the hydrogels for protein was calculated (Table IV). An increase in the concentration of protein from 0.2 to 1.2 mg/mL with respect to a fixed amount of hydrogel resulted in a corresponding increase in bound protein dissolved with hydrogels I_{5d} and I_{20d}. Binding efficiencies of 89.3 and 92.5% for protein were recorded for hydrogels I_{5d} and I_{20d}, respectively. Any further increase in protein concentration in the corresponding buffer prompted declines in the amount of bound protein and binding efficiency, which were quite gradual in the case of the I_{20d} hydrogel but very drastic in the case of the I_{5d} matrix.

Effect of the incubation temperature on the stability of the hydrogel-bound lipase

The effect of heat on the hydrolytic activity of the I_{5d}- and I_{20d}-hydrogel-bound enzymes was studied by the variation of the incubation temperature from 35 to 75°C (Fig. 1) in a water-bath shaker. Both hydrogel-bound biocatalysts showed an increase in their hydrolytic activities when the temperature was increased from 35 to 75°C (Table V). However, the I_{20d}-hydrogel-bound lipase possessed the maximum hydrolytic activity toward *p*-NPP at 65°C, which was approximately 37% more than that of the I_{5d}-matrix-bound lipase. Any further increase in the temperature up to 75°C resulted in a drastic decrease in the activities of the bound lipase supported on the I_{5d} or I_{20d} hydrogel.

After the prolonged (3 h) incubation of hydrogel-bound lipase at each of the previous temperatures, both biocatalysts showed increased hydrolytic activity toward *p*-NPP at 45 and 55°C, and the enzymatic activity was highest at 55°C (than at 45 or 65°C, with activities of 79.4 and 73.0%, respectively) for the I_{5d}- and I_{20d}-hydrogel-bound lipases, respectively.

Effect of the pH of the reaction buffer on the I_{5d}- and I_{20d}-immobilized lipases

The effect of pH of the reaction buffer on the hydrolytic activity of the bound lipase (20 mg) was evaluated by the adjustment of the pH in the range 5.5–9.5 (Table VI). A pH of 8.5 for the I_{5d}- and I_{20d}-immobilized lipases was found to be optimum for the hydrolysis of *p*-NPP (Fig. 2). The immobilized matrix

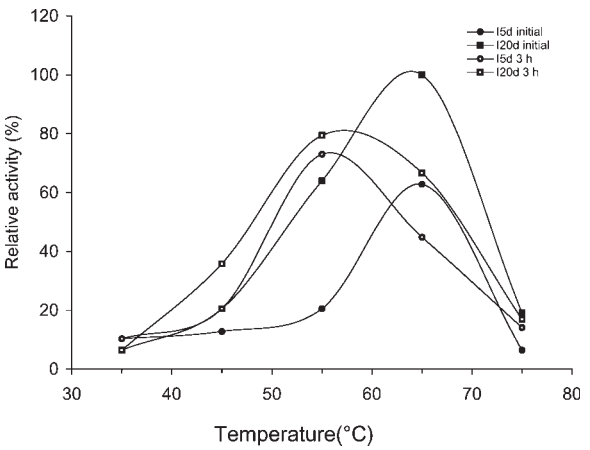

Figure 1 Effect of the incubation temperature on the hydrolytic activity/stability of the hydrogel-bound lipase. The relative activities were calculated with respect to the maximum lipase activity recorded for the I_{20d}-hydrogel-bound lipase at 65°C.

TABLE V
Effect of the Incubation Temperature on the Hydrolytic Activity/Stability of the Hydrogel-Bound Lipases

Temperature (°C)	Hydrogel	Initial activity (U/g)	Initial relative activity (%)[a]	Activity at 3 h (U/g)	Relative activity at 3 h (%)[a]
35	I_{5d}	0.058	10.3	0.058	10.3
	I_{20d}	0.036	6.4	0.036	6.4
45	I_{5d}	0.072	12.8	0.115	20.5
	I_{20d}	0.115	20.5	0.201	35.8
55	I_{5d}	0.115	20.5	0.410	73.0
	I_{20d}	0.360	64.0	0.446	79.4
65	I_{5d}	0.353	62.8	0.252	44.8
	I_{20d}	0.562	100.0	0.374	66.6
75	I_{5d}	0.036	6.4	0.079	14.1
	I_{20d}	0.108	19.2	0.095	16.4

[a] Relative activity was calculated with respect to the maximum lipase activity recorded for hydrogel I_{20d} at 65°C.

showed a 60% increase in hydrolytic activity in comparison to that at pH 7.5. A prolong pre-exposure (3 h) of the both of the hydrogel-bound biocatalysts at a pH of 7.5 resulted in maximal retention of residual lipase activity for the hydrolysis of *p*-NPP.

Effect of the C-chain length of acyl ester on the I_{5d}- and I_{20d}-hydrogel-immobilized lipases

Each of the hydrogel-immobilized lipases (20 mg) was used to catalyze the hydrolysis of various *p*-nitrophenyl esters differing in their C-chain lengths (Table VII). The I_{5d}- and I_{20d}-immobilized lipase was highly hydrolytic toward a relatively longer *p*-NPP ester (Fig. 3).

Reusability of the immobilized lipase for hydrolytic activity toward *p*-NPP

The use of both hydrogel-bound lipase preparations (20 mg) for the hydrolysis of *p*-NPP in repetitive cycles showed a marked retention in the catalytic activity of each of the biocatalysts during eight cycles of hydrolysis of *p*-NPP (Table VIII). The I_{5d}- and I_{20d}-hydrogel-bound biocatalysts retained more than half of its original activity after the fourth (54.1%) and seventh cycles (50.4%) of reuse (Fig. 4). Thus, the I_{20d}-hydrogel-bound lipase retained a greater biocatalytic activity than the other lipase. This biocatalyst was thus chosen for esterification in organic media.

Effect of the relative molar ratio of the reactants on the synthesis of geranyl butyrate by the I_{20d}-immobilized lipase

When the I_{20d}-bound lipase was used to perform esterification at various molar concentrations (ratios) of the reactants, an increase in the molar ratio of geraniol to butyric acid from 100 : 100 to 600 : 100 m*M* resulted in a maximum conversion (98.7 m*M*) of

TABLE VI
Effect of the pH of the Reaction Buffer on the Hydrolytic Activity of the Hydrogel-Bound Lipases

pH	Hydrogel	Initial activity (U/g)	Initial relative activity (%)[a]	Activity at 3 h (U/g)	Relative activity at 3 h (%)[a]
5.5	I_{5d}	0.094	27.2	0.112	32.4
	I_{20d}	0.093	26.8	0.043	12.4
6.5	I_{5d}	0.108	31.2	0.144	41.6
	I_{20d}	0.115	33.2	0.151	43.6
7.5	I_{5d}	0.216	62.4	0.389	112.4
	I_{20d}	0.244	70.5	0.482	139.3
8.5	I_{5d}	0.346	100.0	0.230	66.5
	I_{20d}	0.345	99.7	0.308	89.0
9.5	I_{5d}	0.151	43.6	0.173	50.0
	I_{20d}	0.151	43.6	0.094	27.2

[a] The 100% activity was equivalent to 0.346 U/g obtained at a pH of 8.5 for the reaction buffer (0.05*M* Tris buffer).

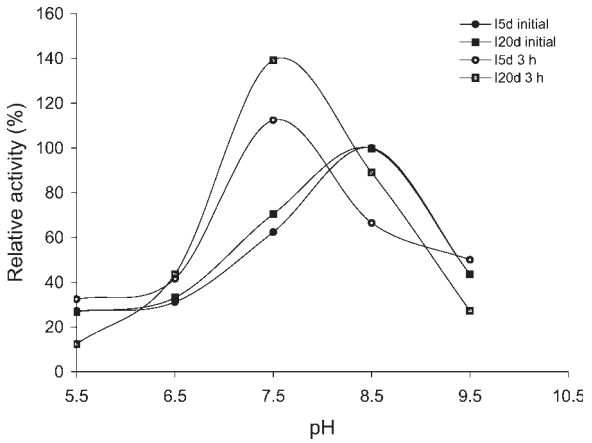

Figure 2 Effect of the pH of the reaction buffer on the hydrolytic activity of the hydrogel-bound lipase. The 100% activity was equivalent to 0.346 U/g of polymer support obtained at a pH of 8.5 for the reaction buffer (0.05*M* Tris).

reactants into geranyl butyrate at 400 : 100 m*M* geraniol/butyric acid over 15 h at 65°C (Fig. 5).

Effect of the reaction time on the synthesis of geranyl butyrate by the I_{20d}-hydrogel immobilized lipase

The kinetics of the immobilized-lipase-catalyzed synthesis of geranyl butyrate was studied up to 21 h at 65°C in *n*-heptane under continuous shaking (Fig. 6). The synthesis of geranyl butyrate increased increasing reaction time up to 15 h (99.1 m*M*) and gradually decreased thereafter. Thus, in the subsequent esterification reactions, a reaction time of 15 h at 65°C for the matrix I_{20d}-immobilized lipase was considered optimum for the synthesis of geranyl butyrate.

Effect of the temperature on the synthesis of geranyl butyrate

When esterification was performed at temperatures ranging from 45 to 75°C at a molar concentration of 400 : 100 m*M* for geraniol/butyric acid, 98.2 m*M* geranyl butyrate was synthesized over 15 h at 65°C in *n*-heptane (Fig. 7). Reaction temperatures above or below 65°C prompted a gradual decline in the amount of ester synthesized.

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

The amount of geranyl butyrate synthesized by the use of immobilized lipase in the presence of *n*-alkanes of relatively shorter C-chain length than *n*-heptane resulted in a marked decrease in ester (Fig. 8). The alkanes with C-chain lengths greater than that of *n*-heptane resulted in a drastic decline in the amount of geranyl butyrate. Thus, geranyl butyrate was optimally produced in *n*-heptane (98.8 m*M*) over 15 h at 65°C.

Effect of the molecular sieve on the synthesis of geranyl butyrate

The addition of a molecular sieve to the reaction mixture promoted the synthesis of geranyl butyrate by the I_{20d}-immobilized lipase in *n*-heptane to 100% (Fig. 9). The maximum amount of ester (100 m*M*) was produced over 15 h at 65°C when 100 mg of molecular sieve was added to the reaction mixture. The molecular sieve, when included at a concentration below or higher than 100 mg per reaction volume, had a declining effect on the ester synthesis.

DISCUSSION

Over the last decade, interest in industrial processes using lipases as biocatalysts has been growing because of their important and multiple applications.[2,27,28] In this study, a series of hydrophobic matrices (hydrogels), differing in the concentration of a copolymer (HPMA), were used for the immobilization of an alkaline lipase from thermophilic *P. aeruginosa* MTCC-4713. The experimental data established that *P. aeruginosa* MTCC-4713 lipase, when immobilized on an I_{5d} or I_{20d} hydrogel, exhibited a relatively higher hydrolytic activity at an alkaline

TABLE VII
Effect of the C-Chain Length of Acyl Ester (Substrate) on the Hydrolytic Activity of the I_{5d}- and I_{20d}-Immobilized Lipases

	I_{5d}-hydrogel-bound lipase		I_{20d}-hydrogel-bound lipase	
p-Nitrophenyl ester	Activity (U/g)	Relative activity (%)[a]	Activity (U/g)	Relative activity (%)[a]
p-NPP	0.531	98.9	0.537	100.0
p-NPL	0.322	60.0	0.215	40.0
p-NPC	0.212	39.5	0.232	43.2
p-NPA	0.102	19.0	0.129	24.0
p-NPF	0.088	16.4	0.098	18.2

[a] The 100% activity of hydrogel-bound lipase was equivalent to 0.54 U/g at 65°C.

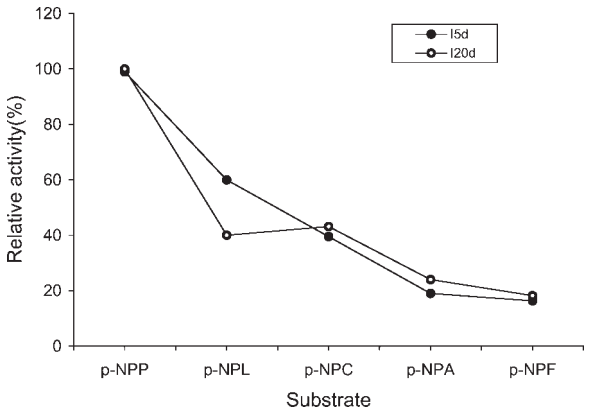

Figure 3 Effect of the C-chain length of acyl ester (substrate) on the hydrolytic activity of the I_{5d}- and I_{20d}-hydrogel-immobilized lipases. The 100% activity of the hydrogel-bound lipase was equivalent to 0.54 U/g at 65°C.

pH, as was evident by its repetitive use for hydrolysis of *p*-NPP. It appeared that a decrease in porosity of the synthetic hydrogels, which resulted from crosslinking with EGDMA, accounted for an increase in the hydrolytic activity toward *p*-NPP. This could be explained on the basis of better retention of the *P. aeruginosa* (30 kDa) lipase, which was much smaller than the lipase obtained from *Bacillus coagulans* MTCC 6375 (103 kDa) or *B. coagulans* BTS-2. Lipases that are hydrolytic at alkaline pH are conventionally used in detergents and laundry. However, if a lipase retains it activity in acidic and alkaline environments, its usefulness can be extended to achieve catalysis under mild acidic conditions also. Most lipases can act in a wide range of pH and temperature values, although alkaline bacterial lipases are more common.[8,9,28] Lipase immobilized onto poly(hydroxy ethyl methacrylate methyl methacrylate) gave a higher yield for both hydrolysis and esterification activity compared to other polymers.[29]

Various features of the reaction selectivity of lipases are generally influenced by various exogenous factors, such as choice of cosubstrates/reactants, water activity, pH, temperature, and immobilization.[30–33] The lipase of *P. aeruginosa* after immobilization on hydrogel I_{20d} was relatively more thermotolerant (65°C) than the free lipase (55°C). The temperature optimum for the immobilized form was 10°C higher than the free form. It appeared that temperature had an important effect on the physical state of substrate dispersion, too. A higher temperature and liquefaction could be assumed to make the substrate more acceptable to the enzyme.[34] It was likely that the structure of lipase immobilized onto the hydrogel became more fluid and open at elevated temperatures. However, prolonged exposure of bound biocatalyst at selected temperatures revealed higher hydrolytic activities of the I_{5d}- and I_{20d}-hydrogel-bound lipases at 55°C than at 65°C, which might have been on account of some leaching of enzyme out of the hydrogel support. The leaching of the enzyme/lipase appeared to occur as the optimum pH of the hydrogel-immobilized lipases at pH 8.5 moved after 3 h to pH 7.5, which appeared to be same as that of the free *P. aeruginosa* lipase. This observation of partial leaching of hydrogel-bound lipase was further supported by the data obtained during the repetitive hydrolysis of *p*-NPP over seven repeated cycles of hydrolysis, when the hydrolytic activity of bound lipase was reduced to about 50% of the original activity.

Specialized biocatalysts with distinguishing chemical characteristics, such as the I_{5d} the I_{20d} hydrogels used in this study, might have greater influence on the lipase activity and selectivity by virtue of support matrix effects directly on enzyme conformation or microenvironment, differences in substrate diffusion rates, or physiochemical interaction directly with the substrate and/or products as previously postulated.[35,36] In a previous study, lipase immobilized onto a poly(2-hydroxyethyl methacrylate-*co*-methyl methacrylate) matrix gave a higher yield for

TABLE VIII
Reusability of the I_{5d}- and I_{20d}-Hydrogel-Immobilized Lipases for the Hydrolysis of *p*-NPP

	I_{5d}-hydrogel-bound lipase		I_{20d}-hydrogel-bound lipase	
Cycle number	Activity (U/g)	Relative activity (%)[a]	Activity (U/g)	Relative activity (%)[a]
1	0.396	99.9	0.399	100.0
2	0.324	81.2	0.331	83.0
3	0.288	72.2	0.288	72.0
4	0.216	54.1	0.252	63.0
5	0.175	43.9	0.230	57.6
6	0.112	28.1	0.216	54.0
7	0.108	27.1	0.201	50.4
8	0.072	18.1	0.108	27.0

[a] The 100% activity of hydrogel-bound lipase was equivalent to 0.4 U/g of matrix at 65°C with *p*-NPP.

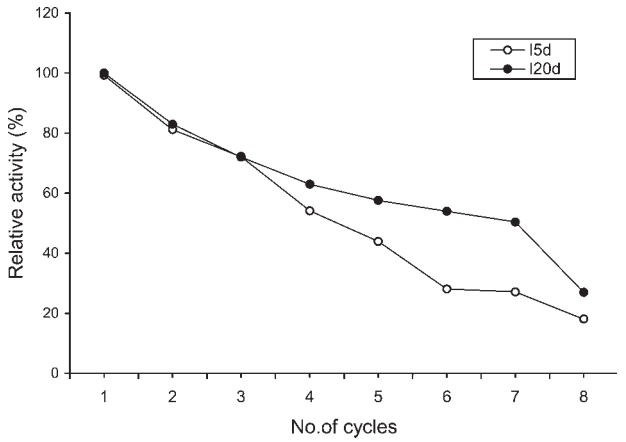

Figure 4 Reusability of the I_{5d}- and I_{20d}-hydrogel-immobilized lipases for the hydrolysis of *p*-NPP. The 100% activity of the hydrogel-bound lipase was equivalent to 0.4 U/g of matrix at 65°C with *p*-NPP.

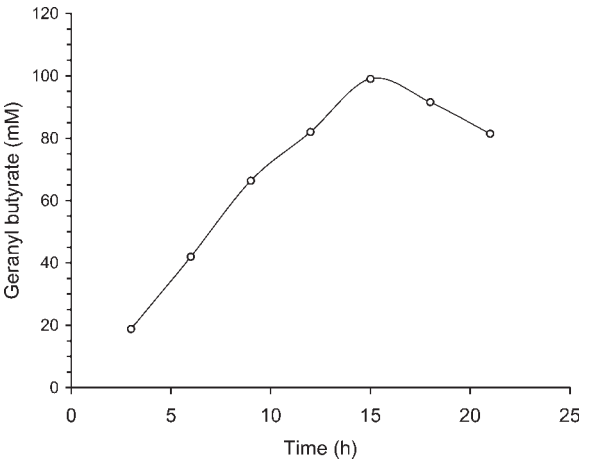

Figure 6 Effect of the reaction time on the synthesis of geranyl butyrate.

both hydrolysis and esterification activity compared to other polymers.[29] Moreover, immobilization also facilitated the dispersal of enzyme on a solid surface to provide a far greater interfacial area and accessibility of substrate relative to the use of enzyme powders in low-water reaction media.[37] The emergence of commercial applications of lipases for the preparation of value-added specialty products from lipids or by esterification/transesterification would be dependent on the understanding and controlling of reaction selectivity, which is defined as comparative differences in rate of reaction toward different substrates.[38]

A variety of fatty acid esters are now produced commercially with immobilized lipases in nonaqueous solvents.[2,39,40] In this study, a poly(acrylic acid-copolymer-hydroxy propyl methacrylate-cross-linked-ethylene glycol dimethacrylate) hydrogel immobilized alkaline lipase of thermophilic *P. aeruginosa* MTCC-4713 was used to catalyze the esterification of geraniol and butyric acid into geranyl butyrate over a period of 15 h at 65°C. The esterification of geraniol and butyric acid by immobilized lipase from *P. aeruginosa* was further enhanced when the molar concentration of the hydrophobic reactant, that is, geraniol, was increased in the reaction mixture. On other hand, an increase in the molar concentration in favor of butyric acid brought about a decrease in the amount of geranyl butyrate synthesized.

A higher temperature and liquefaction tended to make the substrate more diffusible and, hence, easily acceptable to the enzyme.[34] Also, such an effect might have increased the diffusion of butyric acid and geraniol onto the catalytic site and the diffusion of geranyl butyrate synthesized away from the catalytic site of lipase. Moreover, the use of alkanes of C-chain length shorter and greater than *n*-heptane

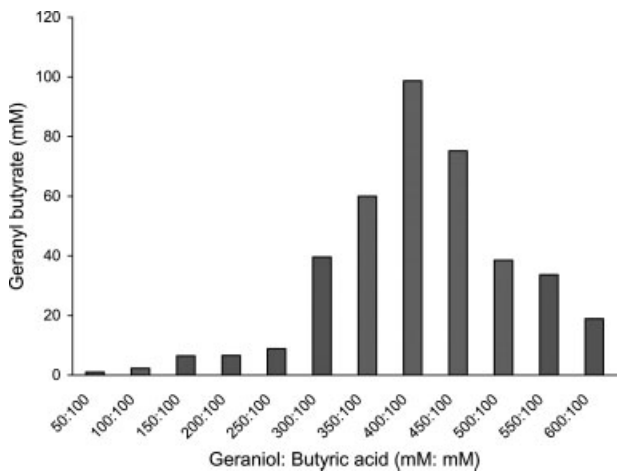

Figure 5 Effect of the proportion of the reactants on the synthesis of geranyl butyrate by the hydrogel-bound lipase.

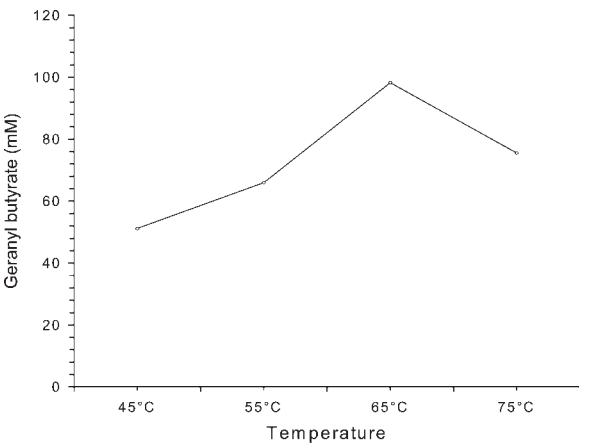

Figure 7 Effect of the reaction temperature on the synthesis of geranyl butyrate.

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 geranyl butyrate synthesis was noticed. As the log *P* value of an *n*-alkane increased corresponding to 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 that appeared to be very important for the modulation of the catalytic activity of the *P. aeruginosa* MTCC-4713 lipase.

Water that is produced as a byproduct of the esterification reactions performed by biocatalysts in organic media has several adverse effects on the reaction and enzyme activity/performance,[41] which result in a decreases in the rate of enzyme activity, as seen in this study. Molecular sieves, which are an important class of synthetic adsorbents, possess high porosity with pores of uniform size and molecular dimensions.[42] 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 achieved maximal synthesis of ester (geranyl butyrate). It appeared that an increase in the concentration of the molecular sieve provided a corresponding increase in the physically active surface area that readily absorbed water and thereby prevented the inhibition of forward reaction kinetics. Such an improvement in the rate of esterification was previously reported for the esterification of lauric acid and geraniol in the presence of a molecular sieve.[43] Thus, this study showed that the immobilization of the lipase sourced from *P. aeruginosa* MTCC-4713 on a synthetic poly(AAc-*co*-HPMA-*cl*-EGDMA) hydrogel was quite stable, as this hydrogel (I_{20d}) retained hydrolytic activity toward the *p*-NPP during repetitive hydrolysis. Also, this hydrogel-bound lipase efficiently achieved esterification of geraniol and butyric acid at 65°C in *n*-heptane. The ability of the hydrogel-bound lipase of *P. aeruginosa* MTCC-4713 to perform esterification of other fragrance esters shall be evaluated in subsequent studies.

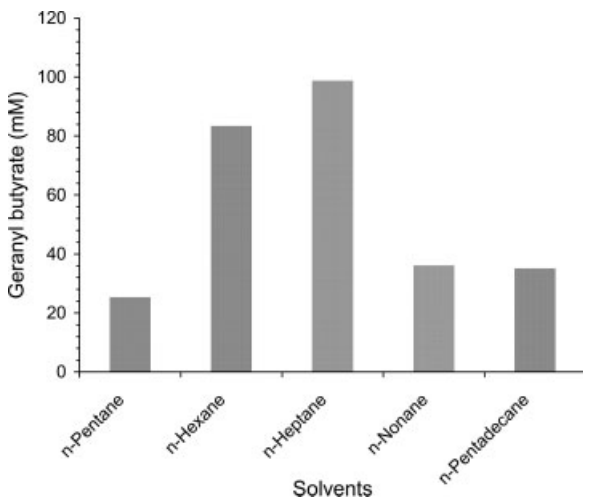

Figure 8 Effect of the C-chain length of *n*-alkane on the synthesis of geranyl butyrate.

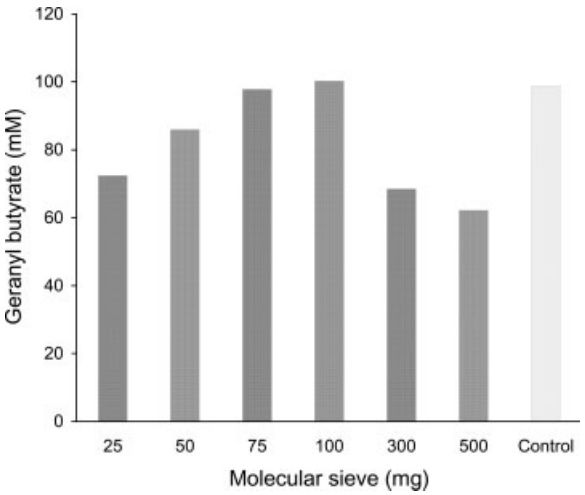

Figure 9 Effect of the addition of a molecular sieve on the synthesis of geranyl butyrate. The control refers to the reaction performed in the absence of molecular sieves.

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