Deakin Research Online

This is the published version:


Available from Deakin Research Online:

http://hdl.handle.net/10536/DRO/DU:30047716

Reproduced with the kind permission of the copyright owner.

Copyright : 2008, Akadémiai Kiadó
ENZYMATIC SYNTHESIS OF ISOPROPYL MYRISTATE USING IMMOBILIZED LIPASE FROM BACILLUS CEREUS MTCC 8372

M. L. VERMA¹, G. S. CHAUHAN² and S. S. KANWAR¹*

¹Departments of Biotechnology, Himachal Pradesh University, Summer-Hill, Shimla 171 005, India
²Departments of Chemistry, Himachal Pradesh University, Summer-Hill, Shimla 171 005, India

(Received: 10 January 2008; revised: 29 February 2008; accepted: 2 May 2008)

A purified alkaline thermo-tolerant bacterial lipase from Bacillus cereus MTCC 8372 was immobilized on a Poly (MAc-co-DMA-cl-MBAm) hydrogel. The hydrogel showed approximately 94% binding capacity for lipase. The immobilized lipase (2.36 IU) was used to achieve esterification of myristic acid and isopropanol in n-heptane at 65 ºC under continuous shaking. The myristic acid and isopropanol when used at a concentration of 100 mM each in n-heptane resulted in formation of isopropyl myristate (66.0 ± 0.3 mM) in 15 h. The reaction temperature below or higher than 65 ºC markedly reduced the formation of isopropyl myristate. Addition of a molecular sieve (3 Å X 1.5 mm) to the reaction mixture drastically reduced the ester formation. The hydrogel bound lipase when repetitively used to perform esterification under optimized conditions resulted in 38.0 ± 0.2 mM isopropyl myristate after the 3rd cycle of esterification.

Keywords: Bacillus cereus MTCC 8372, lipase, Poly (MAc-co-DMA-cl-MBAm) hydrogel, immobilization, isopropyl myristate synthesis, organic solvent and gas liquid chromatography analysis

Introduction

Fatty acid esters were used in many chemical reagents, medicines, cosmetics or foods by taking advantage of their particular properties [1–4]. These esters can be synthesized by condensation reaction (reverse hydrolysis) of a fatty acid

* Corresponding author; Telefax: 91-177-2831948; E-mail: kanwarss2000@yahoo.com
and an alcohol. Many esters are industrially manufactured by chemical methods because chemical methods involve high temperature or high pressure; it is difficult to esterify unstable substances, such as polyunsaturated fatty acids, ascorbic acid, and polyols. Regio-specific acylation of alcohols requires protection and deprotection steps that increase the cost of the product synthesized \[5\]. Moreover, the esters intended for use in food, fragrances, flavors etc require restricted use of certain chemicals.

Over the last couple of years lipases (EC 3.1.1.3), one of the most important group of hydrolytic enzymes are in the lime-light because of their ability to achieve reverse esterification in water free/ water restricted media or organic solvents. In water limited media or organic solvents enzymes can catalyze reactions that are difficult or impossible to carry out in water \[6\]. Lipase catalyzed esterification has the advantage of mild reaction conditions, one step synthesis without protection and deprotection steps and reusability of the immobilized biocatalyst. Lipase catalyzed synthesis of ester also fulfill the requirement of consumers for natural products. Short chain fatty acid esters synthesized using lipases give fruity flavors \[2, 7–9\].

The immobilization of lipase on a suitable matrix confers stability to the enzyme against temperature, pH and organic solvents \[6\]. The use of tailor-made synthetic hydrogel/matrices are gaining importance as they can be made to possess hydrophilic or hydrophobic character \[10–15\]. Generally, lipase catalyzed reactions are carried out in hydrophobic solvents like \(n\)-alkanes. The enzymatic synthesis of esters essentially requires appropriate knowledge of effects of various physical factors and solvent engineering to obtain efficient conversion of reactants into the desired ester product.

Isopropyl myristate is used as one of the important additives in high grade cosmetics and topical medicinal preparations. In this study, we are reporting synthesis of isopropyl myristate by using a hydrogel immobilized alkalophilic lipase of \(B.\ cereus\) MTCC 8372 (Scheme 1).

\[
\text{Isopropyl alcohol} + \text{Myristic acid} \xrightarrow{\text{lipase}} \text{Isopropyl myristate}
\]

Scheme 1
Materials and Methods

Chemicals

* N, N-methylene bisacrylamide (MBAm), methacrylic acid (MAc), dodecyl methacrylate (DMA), methanol and acetone were procured from Qualigens Fine Chemicals, Mumbai, India; p-nitrophenyl palmitate (p-NPP) and various n-alkanes (n-pentane, n-hexane, n-heptane, n-octane and n-nonane) were from Lancaster Synthesis, England; isopropyl myristate was obtained from Acros Organics (New Jersey, USA); myristic acid, isopropanol and molecular sieve (3 Å X 1.5 mm) were procured from E. Merck Pvt. Ltd., Mumbai, India. All chemicals were of analytical grade and were used as received.

Production of lipase by B. cereus MTCC 8372

The *Bacillus cereus* isolate designated MTCC 8372 was obtained from Department of Biotechnology, Himachal Pradesh University, Shimla (India). Seed culture of *B. cereus* MTCC-8372 was prepared by inoculating 50 ml of broth with a loop-full of culture. The culture was allowed to grow for 36 h at 55 ºC under shaking at 160 rpm. Thereafter, 10% (v/v), 36 hour old seed culture was used to inoculate 1000 ml of the production medium (50 ml each in 250 ml capacity Erlenmeyer flasks). The seeded production medium was incubated at 55 ºC under shaking (160 rpm) for 48 h (Orbitek shaking incubator, AID Electronics, Chennai, India).

Purification of lipase

The culture broth was centrifuged after 48 h post inoculation at 10,000 × g for 10 min at 4 ºC (SIGMA 3K30, Germany). The cell-pellet was discarded and the supernatant was filtered through Whatman paper No. 1. The protein content was measured by a standard method [16]. This filtrate broth was henceforth referred as crude lipase. The required amount of ammonium sulfate was added to the crude lipase to achieve 80% (w/v) saturation. The contents were mixed thoroughly and kept over night at 4 ºC. The precipitates sedimented by centrifugation at 12,000 × g at 4 ºC for 30 min were reconstituted in minimum amount of Tris buffer (0.05 M, pH 8.5) and were extensively dialyzed against the same buffer. The puri-
fication of the dialyzed lipase enzyme was performed on an Octyl-Sepharose column (Amersham Pharmacia, Sweden) as described previously [17]. The fractions were analyzed for lipase activity and protein. The fractions showing lipase activity were pooled and stored at –20°C. The specific activity of the purified enzyme was compared with the crude enzyme and the rate purification was calculated.

**Enzyme Assay**

The lipase was assayed by a colorimetric method using p-NPP [18]. The reaction mixture contained 80 µl of p-NPP stock-solution (20 mM p-NPP prepared in isopropanol), 20 µl of test sample (lipase) and Tris buffer (0.05 M, pH 8.5) to make a final volume of 3 ml. The reaction mixture was incubated at 55 ºC for 10 min in a water-bath and lipase activity was assayed at 410 nm. The reaction was stopped by keeping the reaction mixture in –20 ºC for 7 min. Each of the assays was performed in triplicate, unless otherwise stated and mean values were recorded. One unit (IU) of lipase activity was defined as micromole(s) of p-nitrophenol released per minute by hydrolysis of p-NPP by one ml of soluble enzyme at 55 ºC or 1 g of hydrogel-bound enzyme (weight of matrix included) at 65 ºC under assay conditions.

**Synthesis of hydrogels network**

The hydrogel was obtained by using MAc:DMA:MBAm in the ratio of 1:3:5 as reported recently [13]. 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 ºC to achieve a constant weight (xerogel). The swelling capacity ($S_w$) of xerogel in distilled water was defined as follows:

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

$W_1$: Weight of xerogel in mg  
$W_2$: Weight of hydrogel in mg i.e. net weight of polymeric matrix (xerogel) after suspending it in excess volume of water for 1 h at 55 ºC.
Immobilization procedure

The xerogel (1.0 g) pre-equilibrated in excess volume of Tris-buffer (0.05 M, pH 8.5) was incubated with purified lipase (4.0 ml) at 8 ºC for 20 h in a glass vial. The volume of the supernatant, unbound protein [16] and lipase activity [18] were estimated. The weight of enzyme-incubated matrix was estimated and bound lipase activity was assayed using 20 mg of matrix. The bound protein in matrix was determined by subtracting unbound protein in the supernatant from the total protein used for immobilization.

Esterification process for the synthesis of isopropyl myristate by hydrogel immobilized lipase

Preparation of standard profile of isopropyl myristate

A reference profile was prepared using varying concentrations of isopropyl myristate (25–100 mmol/L) in n-heptane. The reference curve was plotted between the molar concentration (mmol/L) of isopropyl myristate and the corresponding area under the peak (retention time 1.54 min).

Analysis of isopropyl myristate synthesis by Gas Liquid Chromatography (GLC)

The sample size for the GLC analysis was 2 µl. The sample was analyzed with GLC, using a packed column (10% SE-30 Chrom WHP, 2 meter length, mesh size 80–100, internal diameter 1/8 inches, Netel Chromatograph, Thane, India). Nitrogen was used as the carrier gas (30 cm³/min). Temperature set for column/oven, injector and flame ionization detector was 250 ºC, 260 ºC and 270 ºC respectively.

Esterification of isopropanol and myristic acid

Isopropyl myristate synthesis was performed by using 100 mM isopropanol, 100 mM myristic acid and hydrogel bound lipase (50 mg) taken in 1.0 ml of n-heptane. The reaction was performed at 65 ºC for 18 h along with heat inacti-
vated hydrogel bound lipase as a control. All esterification reactions were performed in teflon-lined glass vials (5 ml capacity) using 50 mg (= 2.36 IU) of matrix bound lipase under shaking (160 rpm). Each of the reactions was performed in triplicate and average values were calculated. Samples (10 µl) were withdrawn at intervals and analysed by GLC for presence of isopropyl myristate.

Optimization of parameters for synthesis of isopropyl myristate

The effects of various factors such as reaction time, relative molar concentration of reactants, C-chain length of the solvent (alkane), amount of molecular sieve and reaction temperature on the rate of synthesis of isopropyl myristate were separately evaluated. All esterification reactions were performed in triplicate using 50 mg of hydrogel bound lipase per reaction volume (1 ml) under shaking conditions (160 rpm).

Effect of relative molar concentrations of reactants on ester synthesis

The effect of relative molar ratio of isopropanol and myristic acid on synthesis of isopropyl myristate by immobilized lipase was studied by keeping the concentration of one of the reactants (isopropanol or myristic acid) at 100 mM and varying the concentration of other reactant (25–100 mM) in the reaction mixture in n-heptane. The esterification was carried out for 15 h under shaking conditions using hydrogel bound lipase and the amount of ester formed was determined by GLC.

Effect of reaction time on synthesis of isopropyl myristate

The reaction mixture contained hydrogel immobilized lipase and 100 mM each of isopropanol and myristic acid in n-heptane. The glass vials were incubated at 65 °C in a water bath incubator shaker for 18 h. At 3 h intervals, the solvent phase (2 l) was sampled and analyzed by GLC for the presence of isopropyl myristate.
Effect of temperature on ester synthesis

The effect of temperature (55, 65 and 75 °C) on the synthesis of isopropyl myristate was studied. The reaction mixture containing isopropanol and myristic acid (100 mM: 100 mM) in \( n \)-heptane and hydrogel immobilized lipase was incubated at each of the selected temperatures for 15 h. The amount of ester synthesized was determined.

Effect of addition of molecular sieves on synthesis of isopropyl myristate

The molecular sieve (3Å × 1.5 mm) was added to the reaction mixture containing 100 mM each of isopropanol and myristic acid in \( n \)-heptane. The esterification was carried out by using immobilized lipase at 65 °C for 15 h. Isopropyl myristate synthesized in each case was determined by GLC.

Effect of various solvents (alkanes) on synthesis of isopropyl myristate

In the reaction mixture (1.0 ml), the \( n \)-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, \( n \)-heptane, \( n \)-octane and \( n \)-nonane. The hydrogel bound lipase was added to the reaction mixture and reaction was run for 15 h at 65 °C.

Repetitive use of hydrogel immobilized lipase for ester synthesis

The immobilized lipase was used for the synthesis of isopropyl myristate in \( n \)-heptane repetitively upto 6 cycles of 15 h each at 65 °C. After first use of immobilized lipase, the biocatalyst was recovered by decanting the reaction mixture, washed thrice in excess of \( n \)-heptane and this biocatalyst was used to catalyze fresh esterification reaction under optimized conditions. The amount of ester synthesized was monitored after each esterification reaction by GLC.
Results

Purification of bacterial lipase

The cell free broth (360 ml) had a lipase activity of 0.73 IU/ml (protein 16.4 mg/ml). The protein was optimally precipitated at 80% (w/v) ammonium sulfate saturation. The precipitate reconstituted in Tris buffer (0.05 M, pH 8.5) was extensively dialyzed against the same buffer. The dialyzed fraction showed lipase activity of (1.19 IU/ml, 8.0 mg protein/ml, and specific activity 0.15 IU/mg). The chromatography of the dialyzed lipase on Octyl Sepharose column resulted in a single peak. The fractions showing lipase activities were pooled (1.24 IU/ml, 0.8 mg of protein/ml and specific activity 1.55 IU/mg). The Octyl Sepharose chromatography resulted in 35.2 fold purified lipase.

Protein binding efficiency of the synthetic hydrogel

This matrix efficiently bound to the enzyme (94% protein binding). The hydrogel bound lipase exhibited an activity 4.72 IU.g⁻¹ matrix (weight of hydrogel included). The hydrogel bound lipase was washed thrice in n-heptane and dehydrated at room temperature to obtain a constant weight. The poly (MAc-co-DMA-c1-MBAm) matrix possessed $S_w$ value of 3.68.

Effect of relative concentration of reactants on esterification

The effect of varying concentrations of each of the reactants on ester formation was evaluated by keeping the concentration of one of the reactants at 100 mM in n-heptane (Figure 1). At a fixed concentration of myristic acid (100 mM), an increase in the concentration of isopropanol (25–100 mM) at 65 °C under continuous shaking, the synthesis of ester remained more or less the same (32.0 ± 0.2–35.0 ± 0.4 mM). However, at equimolar concentration of both reactants (100 mM), the formation of isopropyl myristate increased markedly. But when the concentration of isopropanol was fixed (100 mM) and the concentration of myristic acid was elevated from 25.0 ± 0.2 to 75.0 ± 0.4 mM in n-heptane, a sharp increase in the synthesis of ester was noticed. In the subsequent reactions, 100 mM of each of the reactants were employed to achieve esterification.
Effect of time course on the ester synthesis

The effect of reaction time on synthesis of isopropyl myristate using hydrogel immobilized lipase was studied at 65 ºC in n-heptane under continuous shaking (Figure 2) up to 18 h. The synthesis of ester was time dependent and a maximum amount of isopropyl myristate (66.0 ± 0.3 mM) was produced after 15 h of reaction when isopropanol and myristic acid were used at 100 mM concentration each in n-heptane.

Figure 1. Effect of relative proportion of reactants on synthesis of isopropyl myristate. Values are mean ± S.D. of 3 replicates and test values are significant at the level of p > 0.05; Student’s t-test as comparable with respect to each other

Effect of reaction temperature on the isopropyl myristate synthesis

The effect of reaction time on synthesis of isopropyl myristate using hydrogel immobilized lipase was studied at 65 ºC in n-heptane under continuous shaking (Figure 2) up to 18 h. The synthesis of ester was time dependent and a maximum amount of isopropyl myristate (66.0 ± 0.3 mM) was produced after 15 h of reaction when isopropanol and myristic acid were used at 100 mM concentration each in n-heptane.

Effect of reaction temperature on the isopropyl myristate synthesis

The effect of change in the reaction temperature on the synthesis of isopropyl myristate by immobilized biocatalyst was studied (Figure 3). An increase in the reaction temperature to 75 ºC decreased the conversion rate but the amount of ester produced was still higher than the amount of ester produced at 55 ºC. 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 75 ºC, there was no further increase in the ester synthesis, which might be on account of denaturation of the lipase as well as alteration in the 3-D structure of lipase. Thus isopropyl myristate synthesis was 66.0 ± 0.4 mM achieved at 65 ºC in 15 h in a batch reaction.

Acta Microbiologica et Immunologica Hungarica 55, 2008
Effect of addition of molecular sieve

The esterification reaction results in formation of water as a byproduct 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 presence of a molecular sieve was studied by adding molecular sieve from 25 to 100 mg per reaction volume (1 ml), a drastic decrease in the synthesis of isopropyl myristate was noticed (Figure 4).

Figure 2. Effect of reaction time on synthesis of isopropyl myristate. Values are mean ± S.D. of 3 replicates and test values are significant at the level of p > 0.05; Students t-test as comparable with respect to each other.

Figure 3. Effect of reaction temperature on synthesis of isopropyl myristate. Values are mean ± S.D. of 3 replicates and test values are significant at the level of p > 0.05; Students t-test as comparable with respect to each other.
Effect of solvent (n-alkane) on the ester synthesis and repetitive esterification by bound lipase

The n-pentane was highly volatile and its complete evaporation at the selected temperature (65 °C) was noticed (Figure 5). Use of n-octane or n-nonane reduced the amount of ester formed under similar conditions. The maximum conver-
sion of reactants into ester (66.0 ± 0.4 mM) was recorded in n-heptane at 65 °C under shaking. The hydrogel bound lipase when repetitively used to perform esterification (38.0 ± 0.2 mM) under optimized conditions after 3rd cycle of esterification (Figure 6).

**Discussion**

Lipase is a versatile enzyme with many potential industrial applications; it has been used for the modification of fats and oils and the synthesis of flavor esters and food additives. A variety of fatty acid esters are now being produced commercially using immobilized lipase in nonaqueous solvents. In the present study, an extracellular alkaline lipase of *Bacillus cereus* MTCC 8372 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 isopropyl myristate under optimized conditions in a water free organic solvent system. The hydrogel, besides providing water needed for enzyme activity can also absorb water produced during esterification reaction, thus enhancing the conversion to product(s).

Recently, we have reported that optimal synthesis of ethyl propionate by a synthetic hydrogel bound lipase of *P. aeruginosa* BTS-2 was achieved when fatty acid and alcohol were used in an equimolar ratio (100 mM each) in the reaction mixture [9]. In another study, effect of acetic acid concentration on esterification...
reaction using lipase SP435 was studied [19]. 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. cereus MTCC 8372 lipase is also vulnerable to high concentration of myristic acid in reaction medium just like other lipases. Due to 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]. It appeared that such a decrease in the ester formation might be because of change brought about by excess concentration of polar propionic acid in the charge distribution at the catalytic site of lipase comprised a triad of serine, aspartic (or glutamic) acid and histidine; serine being a highly conserved residue in various lipases including lipase obtained from P. aeruginosa because its catalytic activity is strongly inhibited by PMSF [21].

Immobilization on a poly (AAc-co-HPMA-co-EGDMA) hydrogel improved the thermal stability of the P. aeruginosa lipase [15]. It appeared that temperature had an important effect on the physical state of substrate dispersion in an organic solvent. Higher temperature and liquefaction can be assumed to make the substrate more acceptable for the enzyme [22]. It was likely that the structure of lipase immobilized on hydrogel became more fluid at an elevated temperature. Such an effect might have increased the diffusion of myristic acid and isopropanol on to the catalytic site and diffusion of isopropyl myristate synthesized away from the catalytic site of lipase. It appeared that specialized support matrices with distinguishing chemical character such as the one used in the present study might have greater influence on lipase activity and selectivity by virtue of support matrix effects directly on enzyme conformation or micro-environment, differences in substrate diffusion rates or physiochemical interactions directly with substrate and/or products [23, 24]. Moreover, immobilization techniques facilitated the dispersal of enzyme on a solid surface to provide far greater interfacial area and accessibility of substrate relative to the use of enzyme powders in low water reaction media [25]. The emergence of commercial use of lipases for preparing increased value specialty products from lipids or by esterification/ transesterification will be dependent on better understanding and controlling of reaction selectivity [26].

The alteration in temperature of reaction mixture might interfere with the porosity, hydrophobicity character and diffusion of the reactants and/or products at the catalytic site of enzyme or hydrogel. At 75 °C, there was no further increase in the ester synthesis, which might be caused by denaturation of the lipase as well as by alteration in its 3-D structure. Esterification is generally a water-limited re-
action because the equilibrium catalyzed by hydrolytic enzymes is in favor of hydrolysis [27]. Esterification of isopropanol and myristic acid by hydrogel immobilized lipase of B. cereus MTCC 8372 in the presence of a molecular sieve, however, bought improvement in the present study. The addition of a molecular sieve or silica gel usually improved the equilibrium conversion [13–15, 28], yet in many cases negative effects such as the formation of di-ester and degradation of unstable substrates have also been reported [29–31]. Therefore, an appropriate combination of solvent and the amount of desiccant/molecular sieve existed for improvement of the equilibrium conversion in most of lipase-catalyzed condensation reactions [3]. Previously, for many esterification reactions between fatty acids and alcohols in organic media, appreciable improvements were reported in the rate of esterification in the presence of molecular sieves [13–15, 28, 32, 33].

The choice of an appropriate solvent system keeping the reactants dissolved while not interacting with the enzyme, matrix/support or with any of the reactants, and not readily evaporating at the temperature of catalysis is very important in achieving efficient esterification. In the present study, the use of n-heptane (log P value = 4) appeared to be most suitable for ester synthesis in a water free system. As an n-alkane with a lower or a higher C-chain length than n-heptane was used as solvent, a gradual decrease in the rate of isopropyl myristate synthesis was noticed. In general, as the log P value of an n-alkane decreased corresponding to decrease in the C-chain length the hydrophobicity of the alkanes also decreased in that order. The presence of a highly hydrophobic environment keeping the reactants dissolved appeared to be more suitable for performing no synthesis of isopropyl myristate by the hydrophobic-hydrogel bound lipase of B. cereus MTCC 8372.

In the present study we have shown that a systematic approach could be devised by the optimization of physical parameters and by the selection of solvent engineering to obtain 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, industrially useful enzymes must be very rugged and shall retain their catalytic activity in the presence of organic solvents, temperatures above ambient and shall also maintain high conversion rate. This is amongst very few studies of use of a synthetic hydrogel bound extra-cellular alkaline lipase of a thermoalkaliphilic B. cereus MTCC 8372 for the synthesis of a medium chain length ester in an organic solvent. The efficacy of this lipase to catalyze direct esterification and transesterification reactions in organic solvents require to be explored in our future studies.
Acknowledgements

The financial grant in the form of Senior Research Fellowship given by Department of Biotechnology, Ministry of Science and Technology, Govt. of India, New Delhi to one of the authors (Mr. Madan Lal Verma) is thankfully acknowledged. The authors also thankfully acknowledge the Bioinformatics centre, H. P. University, Shimla for providing the web resource facility.

References