Synthesis of Ethyl Propionate Catalyzed by Poly(N-AEAAm-co-AAc)-cl-MBAm Hydrogel-Immobilized Lipase of Bacillus coagulans MTCC-6375

Shamsher Singh Kanwar,[1] Rajeev Kumar Kaushal,[1] Amit Aggarwal,[2] Sandeep Chauhan,[3] Swapandeep Singh Chimni,[4] Ghansham Singh Chauhan[3]

[1]Department of Biotechnology, Himachal Pradesh University, Summer Hill, Shimla-171 005, India
[2]Department of Biotechnology, University of Rajasthan, Jaipur, India
[3]Department of Chemistry, Himachal Pradesh University, Summer Hill, Shimla-171 005, India
[4]Department of Chemistry, Guru Nanak Dev University, Amritsar-143 005, India

ABSTRACT: A purified alkaline thermotolerant bacterial lipase of *Bacillus coagulans* MTCC-6375 was efficiently immobilized onto poly(N-AEAAm-co-AAc-cl-MBAm)-hydrogel at pH 8.5 and at temperature 55°C in 16 h. The hydrogel-bound matrix possessed 1.04 U/g (matrix) lipase activity with a specific activity of 1.8 U/mg of protein. The immobilized lipase resulted in formation of 52.5 mM of ethyl propionate (52% conversion) at 55°C in 9 h in n-nonane. Ethanol and propionic acid when used in a ratio of 300 : 100 mM, respectively, in n-nonane along with 10 mg of hydrogel-bound lipase resulted in optimal synthesis of ethyl propionate (82.5 mM). Addition of molecular sieves (3 Å, 0.7 g/reaction volume) further enhanced the conversion rate to 82.4% resulting in 83.5 mM of ethyl propionate. Incubation temperature below or above 55°C had a marked effect on the synthesis of ethyl propionate. However, esterification performed in n-heptane at 65°C resulted in 87.5 mM of ethyl propionate with a conversation rate of 89.3%. © 2007 Wiley Periodicals, Inc. J Appl Polym Sci 105: 1437–1443, 2007

Key words: hydrogels; lipase; esterification

INTRODUCTION

Enzyme immobilization has emerged as a promising tool to enhance the stability and efficiency of enzymes. In nonaqueous solvents, enzymes can catalyze reactions that are difficult or impossible to carry out in water, become more stable and can exhibit altered selectivity.[1] Lipases (E.C. 3.1.1.3) have been successfully immobilized on a variety of matrices for performing esterification and trans-esterification reactions in organic solvents. For the last few years, there has been an increasing interest in the use of enzymes for the biosynthesis of molecules in organic media.[2–4] The lipases tend to retain their catalytic potential as hydrolases or esterases in aqueous or organic media. However, the behavior of enzymes in organic phase is different from that in the aqueous phase. Most proteins are poorly soluble in organic solvents, and it is often necessary to immobilize enzyme onto a suitable porous matrix that provides an increased interfacial surface area, easy separation of catalyst, and reuse of immobilized enzyme. From an industrial point of view, immobilized lipases offer economic incentives of enhanced thermal and chemical stability, ease of handling, easy recovery, and reuse relative to nonimmobilized forms.[5–7] There is paucity of literature on use of lipase of *B. coagulans* in organic synthesis. The newer enzymes including lipase(s) sourced from thermotolerant or thermophilic microorganisms have inherent advantage to work at enhanced temperatures; which also enable greater solubility of the reactants or products.

An appropriate solvent is always necessary to solublize the substrates, and to partition the substrates and products in different phases.[8] Immobilization also increases enzyme's thermal stability.[9] Moreover, the solvent might be necessary in case of cosoluble substrates (liquid or solids and liquids), for ester synthesis or trans-esterification.[6,7,10–15] Polarity of the solvent phase exerts a great influence on water solubilization and distortion of H-bonds.[16] Water activity associated with dehydrated/immobilized enzyme plays several roles in enzyme structure and functions.[17] The enzyme sometimes may be inactive in dehydrated systems.[18] Moreover, production of acidic or basic species, or direct addition of exchangers, such as zeolites[19,20] or salt hydrates[21] can affect the performance of biocatalyst in organic solvents. The impact of such species may be particularly important

when using forms of enzymes where buffer salts are present in relatively low concentrations, as is the case of immobilized preparations.[22] Thus, it is essential to study the effect of various physical factors as well as solvents to perform the desired esterification.

Use of hydrogels as supports for enzyme immobilization has attracted the attention of scientists and technologists worldwide.[23,24] Acrylic acid (AAc) and N-aminoethyl acrylamide (N-AEAAm) based polymer absorbs water and are strongly hydrophilic. The balance of polymer–polymer and polymer–solvent interaction determines the solubility of a polymer in a solvent. In the present study, authors have evaluated the effect of various parameters on the formation of ethyl propionate using a strongly hydrophilic poly(N-AEAAm-co-AAc-cl-MBAm)-hydrogel-immobilized purified lipase of a moderately thermotolerant Bacillus coagulans MTCC-6375. Ethyl propionate is used to manufacture various propionates, which are used in the reduction of pharmaceuticals, antifungal agents, agrochemicals, plasticizers, rubber chemicals, dyes, etc. This ester possesses fruity rum odor and is used in perfumery and fragrances.

EXPERIMENTAL

Chemicals

N,N-methylene bisacrylamide (MBAm), N-amino ethyl acrylamide (N-AEAAm), ammonium per sulfate (APS), acrylic acid (AAc), 2-propanol, various n-alkanes (Merck, Germany), and p-nitrophenyl palmitate (p-NPP, Lancaster Synthesis, England) were purchased and used as received.

Microorganism and lipase

The Bacillus coagulans MTCC-6375 was obtained from Department of Biotechnology, Himachal Pradesh University, Summer Hill, Shimla (India). The purified lipase of Bacillus coagulans MTCC-6375 was used for immobilization on poly(N-AEAAm-co-AAc-cl-MBAm)-hydrogel. One unit of lipase was the amount of enzyme that produced one micromole of p-nitrophenol per minute by hydrolysis of p-NPP at 45°C (55°C in case of immobilized enzyme) under assay conditions. The lipase sourced from B. coagulans MTCC-6375 was purified by ammonium sulfate precipitation/dialysis, DEAE-cellulose anion exchange and hydrophobic interaction (Octyl-Sepharose) chromatography to obtain a highly homogeneous protein.

Hydrophobic column chromatography

The prepacked Octyl-Sepharose column ($V_t = 5$ cm^3) was equilibrated with 5 mL of elution-buffer (50 mM sodium phosphate, pH 7.2) at a flow rate of 1.0 mL/min followed by washing with 10 mL of start-buffer

(50 mM sodium phosphate, 1.0M ammonium sulfate, pH 7.2). Crude lipase (2.4 mg protein) was loaded on the column. The column was developed with elution-buffer (50 mM sodium phosphate, pH 7.2). The eluted fractions (2 mL) were assayed both for lipase activity as well as their protein content.[25] Fractions showing lipase activity under a peak were pooled (6 mL). The column was regenerated with 5 mL of distilled water followed by 5 mL of start-buffer.

Synthesis of hydrogel network

Poly(N-AEAAm-co-AAc-cl-MBAm) hydrogel was prepared by copolymerizing AAc (0.138M) and N-AEAAm (0.138M) in distilled water (20 mL) and APS (200 mg) was used as an initiator. The reactants were vigorously stirred and allowed to stand for 30 min at 25°C. The polymerization was achieved by warming the mixture at 60°C for 2 h in water bath. The polymer was washed thrice with excess of warm distilled water (60°C) to remove unreacted compounds. The polymer was dried in a vacuum oven at 30°C to get a constant weight. Ten grams of dried polymer was suspended in 20 mL of water along with AAc (0.138M), a crosslinker MBAm (400 mg), and APS (200 mg). The suspension was mixed and warmed at 60°C for 1 h to obtain a crosslinked network. The unreacted components were removed by extensive washing with distilled water and methanol. Thereafter, the network was completely dried in vacuum oven to obtain a xerogel. The xerogel was used subsequently for immobilization of the lipase of B. coagulans MTCC-6375. The xerogel had an S_w (swelling capacity in water) value of 250 in distilled water.

Assay of hydrolytic activity

The hydrolytic activity of free or immobilized lipase was assayed by a standard colorimetric method using p-NPP as a substrate.[26] Stock solution (20 mM) of p-NPP was prepared in 2-propanol. The reaction mixture comprised of 75 μL of p-NPP stock solution and 5 μL of free or 10 mg of immobilized matrix. The final volume of this reaction mixture was made to 3 mL with 0.05M Tris buffer, pH 7.5 for free and 8.5 for bound lipase. The test tubes were incubated for 10 min at 55°C under continuous shaking in water-bath-incubator. Appropriate control with a heat-inactivated enzyme (5 min in boiling water bath) was included with each assay. The absorbance of p-nitrophenol released was measured at A_{410}. The unknown concentration of p-nitrophenol released was determined from a reference curve of p-nitrophenol (2–50 μg/mL final concentrations in 0.05M Tris buffer, pH 7.5 for free enzyme and pH 8.5 for immobilized enzyme assay). Each of the assays was performed in duplicate and mean values were presented. Specific

activity was expressed as μmole(s) of the *p*-nitrophenol released per min by 1 mg of protein.

Effect of protein concentration on immobilization of lipase

Concentration of total protein (as lipase) in the coupling buffer (0.05*M* Tris, pH 8.5) was varied between 0.25 and 2.0 mg/mL so as to determine the optimal concentration of protein for a fixed amount of matrix (10 mg).

Optimization of parameters for synthesis of ethyl propionate

The effect of various factors such as reaction time, relative molar concentration of reactants, addition of molecular sieve, reaction temperature and C-chain length of the solvent (alkanes) on the rate of synthesis of ethyl propionate was consecutively evaluated.

Analysis of ethyl propionate by GLC

The GLC (Michro-9100, Netel Chromatographs, India) was programmed for oven temperature 75–200°C, ramp rate 20°C/min, FID temperature 220°C, injector temperature 210°C and holding time of 2 min at 200°C. The assay of ethyl propionate using 2 μL sample was performed on a 300 cm × 0.32 mm ID, bonded phase-fused silica BP624 moderately polar SGE capillary column using N_2 as a carrier gas (flow rate 30 mL/min). A reference profile was prepared by using 2 μL of varying concentrations of ethyl propionate (50–500 m*M*) prepared in *n*-nonane or *n*-heptane. The curve was plotted between the molar concentration (in m*M*) of ethyl propionate and the corresponding area under the peak (retention time 0.77 min). The percent conversion achieved during the esterification reaction was determined as given below.

$$\text{Conversion} = \frac{\text{Area under the peak of ester formed at limiting concentration of reactant(s)}}{\text{Reference area under the peak of same ester at similar concentration}} \times 100$$

Effect of incubation time on synthesis of ethyl propionate by free and immobilized lipase

The reaction mixture (1.5 mL) contained 10 mg of hydrogel-bound lipase, 100 m*M* each of ethanol and propionic acid in *n*-nonane in a Teflon-stoppered glass vial (5 mL capacity). The reaction mixture was incubated at 65°C in a water-bath-incubator shaker (160 rpm) for 15 h. The solvent phase (2 μL) was sampled in duplicate at an interval of 3 h and subjected to analysis by GLC for the formation of ethyl propionate.

Effect of molar concentration of reactants on ethyl propionate synthesis

The effect of molar ratio of ethanol and propionic acid on synthesis of ethyl propionate was determined by keeping the concentration of one of the reactants (ethanol or propionic acid) at 100 m*M* and varying the concentration of other reactant (100–400 m*M*) in a reaction volume of 1.5 mL in *n*-nonane. The esterification was carried out by using 10 mg of matrix-bound lipase at 55°C in Teflon stoppered-glass vials for 9 h under continuous shaking. The ethyl propionate formed in each of the combinations of the reactants was determined by GLC.

Effect of addition of molecular sieves on synthesis of ethyl propionate

Molecular sieves of 3 Å were used to study their effect on the synthesis of ethyl propionate by immobilized lipase. To the above reaction mixture prepared in *n*-nonane, varying amount (100–900 mg) of molecular sieves was added. The esterification was carried out in duplicate by adding 10 mg of immobilized lipase at 55°C for 9 h. Ethyl propionate synthesized in each case was determined.

Effect of solvents varying in C-chain length on esterification

In the reaction mixture (1.5 mL) *n*-nonane that was initially employed as a solvent phase was replaced with *n*-alkane of varying C-chain length, i.e., *n*-pentane, *n*-hexane, *n*-heptane, and *n*-octane. The immobilized lipase (10 mg) was added to the above-mentioned reaction mixture to perform the esterification. The reaction was carried out for 9 h at 55°C.

Effect of reaction temperature on esterification

The reaction mixture (1.5 mL) containing ethanol and propionic acid (300 : 100 m*M*) in *n*-heptane and 10 mg of bound lipase was incubated at each of the selected temperature (35–75°C) in a water bath incubator-shaker (160 rpm) for 9 h in Teflon-lined stoppered glass-vials. The amount of ester produced was determined by GLC.

RESULTS

The lipase protein in the harvested broth (0.27 U, 8.2 mg of protein/mL) was optimally precipitated at
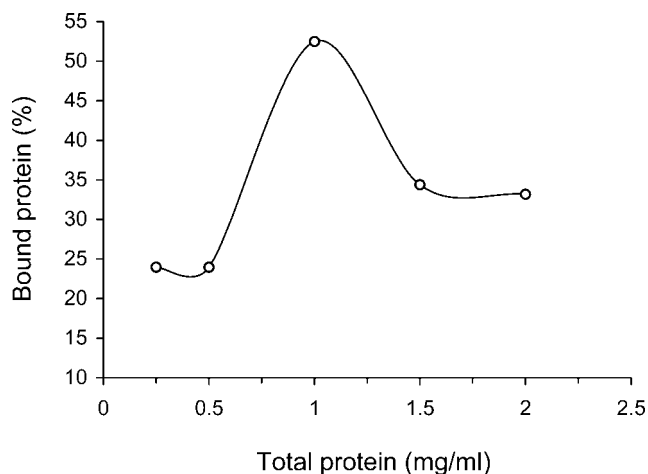
Figure 1 Effect of protein concentration on immobilization.

60% (w/v) ammonium sulfate saturation. The precipitates were reconstituted in a minimum volume of $0.05M$ Tris buffer, pH 8.5 and were extensively dialyzed against the same buffer. The dialyzed enzyme showed an activity of 3.39 U/mL, specific activity of 0.67 U/mg. The anion exchange chromatography of lipase on DEAE-cellulose column resulted in two peaks (results not shown). The fractions showing lipase activity were pooled (5.52 U/mL, protein 3.2 mg/mL, specific activity 1.73 U/mg) and subjected to freeze drying by lyophilization. The DEAE-anion exchange chromatography resulted in ~ 75.3-fold concentration of lipase. Hydrophobic interaction (Octyl-Sepharose) chromatography was performed to obtain a highly homogeneous protein possessing specific activity of 1.98 U/mg and 76.4-fold purification. The purified lipase showed the presence of a distinct band of ~ 103 kDa on 10% SDS-PAGE (result not shown). The efficient binding of the protein onto the hydrogel was obtained at 1.0 mg/mL concentration with respect to 10 mg of hydrogel matrix (Fig. 1).

Protein binding efficiency and hydrolytic activity of the hydrogel

The purified lipase of *B. coagulans* MTCC-6375 was optimally immobilized/bound on to a strongly hydrophilic poly(*N*-AEAAm-*co*-AAc-*cl*-MBAm)-hydrogel that retained 52% (~1.01 U/g matrix) of the total protein used for immobilization (Fig. 1). The hydrogel-bound matrix possessed 145 U/g (of matrix) lipase activity with a specific activity of 18 U/mg protein.

Effect of incubation time on synthesis of ethyl propionate

The kinetics of immobilized-lipase catalyzed the synthesis of ethyl propionate that was studied for 15 h

at 55°C in *n*-nonane under continuous shaking (Fig. 2). The synthesis of ethyl propionate increased with increase in the reaction time till 9 h and remained more or less static thereafter. At 9 h, 52.5 m*M* of ethyl propionate was produced with a conversion rate of 52%. Thus in the subsequent esterification reactions, a reaction time of 9 h at 55°C for poly (*N*-AEAAm-*co*-AAc-*cl*-MBAm)-hydrogel immobilized-lipase was considered optimum for the synthesis of ethyl propionate.

Effect of molar ratio of reactants on synthesis of ethyl propionate

When esterification was performed in by varying the molar ratio of the reactants, an increase in molar ratio of ethanol to propionic acid from 1 : 1 to 3 : 1 resulted in 80% conversion of reactants into ethyl propionate (82.5 m*M*) in 9 h at 55°C. However, when concentration of ethanol was fixed at 100 m*M*, an increase in the molar ratio of propionic acid from 100 to 400 m*M* drastically decreased the formation of ethyl propionate (Fig. 3).

Effect of molecular sieves on synthesis of ethyl propionate

Addition of molecular sieves to the reaction mixture markedly enhanced the amount of ethyl propionate synthesized by poly(*N*-AEAAm-*co*-AAc-*cl*-MBAm)-hydrogel bound biocatalyst (Fig. 4). Approximately, 83.5 m*M* of ethyl propionate was synthesized (conversion rate of 82.4%) in 9 h in *n*-nonane at 55°C when 0.7 g of molecular sieves were added into the reaction mixture (1.5 mL). Any further increase in
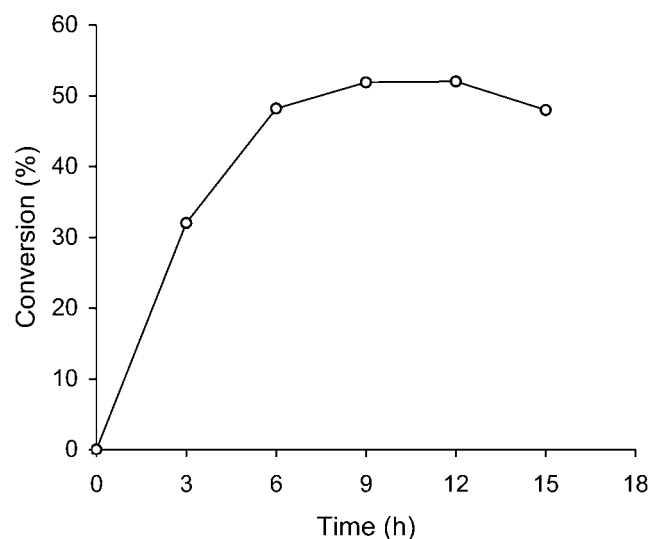

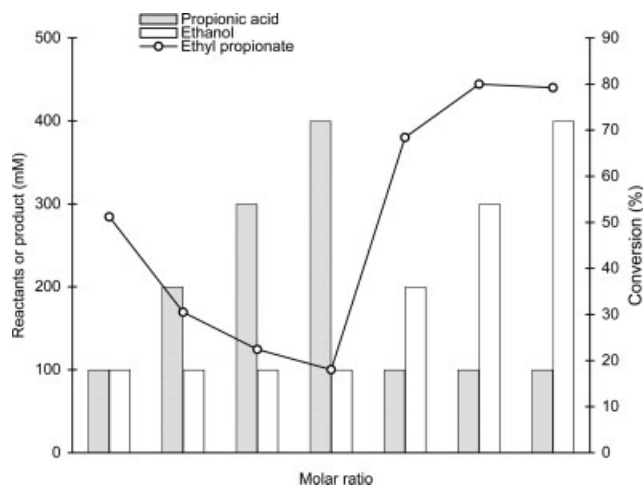**Figure 2** Kinetics of formation of ethyl propionate by immobilized lipase.

Figure 3 Effect of molar ratio of reactants on synthesis of ethyl propionate.

the concentration of molecular sieves had a declining effect on the ester formation.

Effect of C-chain length of solvent (alkane) on synthesis of ethyl propionate

The concentration of ethyl propionate synthesized by employing immobilized lipase in the presence of alkanes of relatively shorter or longer C-chain length than *n*-heptane tend to decrease the amount of ethyl propionate produced (Fig. 5). Thus use of *n*-heptane was considered in the subsequent reaction.

Effect of reaction temperature on esterification

The esterification was optimally (87.3 m*M* ethyl propionate) achieved representing a 89.3% conversion at
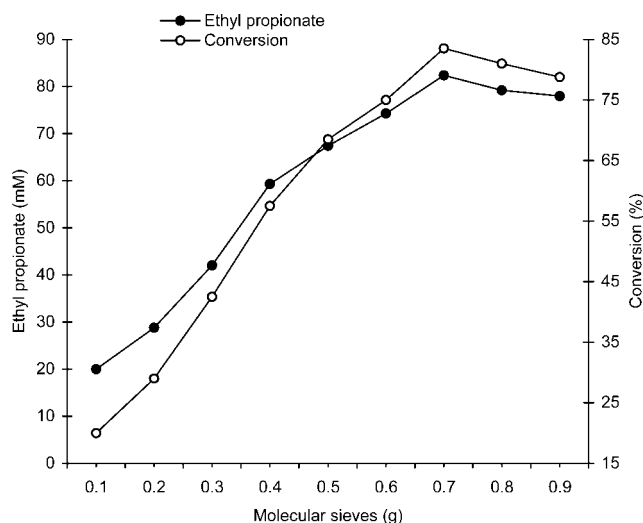

Figure 4 Effect of concentration of molecular sieves on esterification.
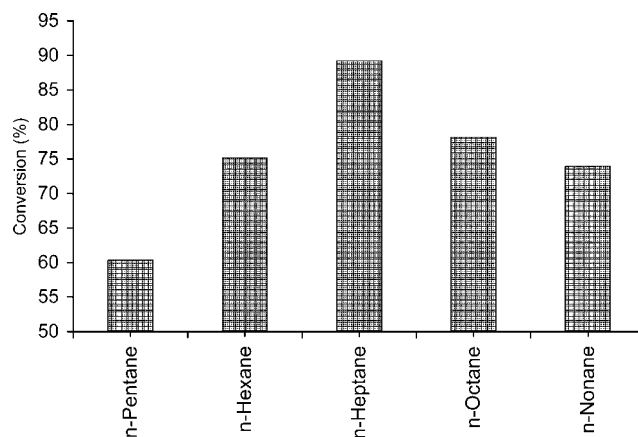

Figure 5 Effect of organic solvent on esterification.

65°C in the presence of 0.7 g of molecular sieve in *n*-heptane (Fig. 6).

DISCUSSION

A variety of fatty acid esters are now being produced commercially using immobilized lipase in nonaqueous solvents.[3,6,7,10,27–30] Compared to conventional chemical synthesis from alcohols and carboxylic acids using mineral acids as a catalyst, the use of lipases as biocatalysts to produce these high value-added fatty acid esters in organic media offers significant advantages.[31–34] These include use of any hydrophobic substrate, higher selectivity, milder processing conditions, and ease of product isolation and enzyme reuse.[5,14,15,35] In the present study, a hydrophilic poly(*N*-AEAAm-*co*-AAc-*cl*-MBAm)-hydrogel efficiently immobilized onto a purified alkaline lipase form a thermotolerant *B. coagulans* MTCC-6375. The hydrogel exhibited a very high swelling in aqueous phase. The immobilized lipase was subsequently used to study the effect of various physical
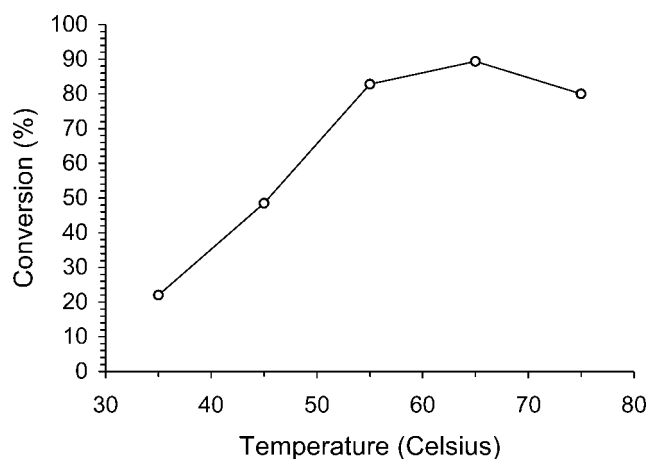

Figure 6 Effect of reaction temperature on formation of ethyl propionate.

parameters on the synthesis of ethyl propionate in a relatively inert solvent system (n-alkane). Various features of reaction selectivity of lipases are modulated by exogenous factors, such as type of organic solvent, choice of cosubstrates/reactants, water activity, pH, temperature, and immobilization.[33]

There is paucity of literature on properties of lipase of *B. coagulans* and its potential application(s) in organic solvents for performing esterification reactions. The lipase sourced from *B. coagulans* has been found to be stable in many organic solvents, including various alcohols, alkanes and common solvents.[6,7] The immobilized lipase efficiently catalyzed the esterification of ethanol and propionic acid into ethyl propionate in 9 h at 65°C under optimized conditions. Temperature has an important effect on the physical state of substrate dispersion also. Higher temperature leads to liquefaction that makes substrate more diffusible and easily acceptable to enzyme.[36] Moreover, in the present study, *B. coagulans* MTCC-6375 lipase bound to a hydrophilic hydrogel exhibited a good thermotolerance at 65°C than the free lipase that possessed an optimum temperature of 45°C[6] for hydrolysis of *p*-NPP. It appeared that chemical characteristics of poly(N-AEAAm-*co*-AAc-*cl*-MBAm) network exerted greater influence on immobilized-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 substrate or products.[37,38] In a previous study, lipase immobilized onto poly(2-hydroxyethyl methacrylate-*co*-methyl methacrylate)-matrix gave a higher yield for both hydrolysis and esterification activity as compared to other polymers.[23] Novozyme-435 immobilized on an acrylic resin when used for synthesis of isopropyl laurate exhibited its maximum activity at 60°C.[30] At higher temperature (>65°C), thermal deactivation of the enzyme became more pronounced thus affecting the conversion of reactants into ester. Lipozyme-IM that is another important enzyme for esterification showed optimal synthesis of oleic acid esters at 50°C.[27]

The esterification of ethanol and propionic acid by immobilized lipase from *B. coagulans* MTCC-6375 was enhanced when molar concentration of the hydrophobic reactant, i.e., ethanol was increased from 1 : 1 to 3 : 1 (ethanol : propionic acid) in the reaction mixture. On other hand, an increase in the molar concentration in favor of propionic acid brought about a decrease in the amount of ethyl propionate synthesized. The copolymer moiety N-AEAAm, a constituent of the porous hydrogel-network bears one highly basic amino group, which might have interacted with the amino acid residues at the active site of the enzyme.

Esterification is generally a water-limited reaction because the equilibrium catalyzed by hydrolytic enzymes is in favor of hydrolysis.[39,40] The esterification of ethanol and propionic acid by hydrogel-immobilized lipase of *B. coagulans* MTCC-6375 in the absence of a water scavenger/molecular sieve exhibited ~ 80% esterification. However, addition of molecular sieves further enhanced the rate of esterification to 83.5%. Activated molecular sieves or salt hydrates can be added to the system to remove the water produced by the reaction.[29] Thus, presence of molecular sieves in the reaction mixture invariably prevented the inhibitory effects of accumulation of water (as an end-product) on the esterification reaction between propionic acid and ethanol. 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. Such an improvement in the rate of esterification has been previously reported for the esterification of lauric acid and geraniol,[41] synthesis of isopropyl laurate,[30] formation of ethyl acetate,[14] and ethyl laurate[15] in the presence of molecular sieves.

The choice of an appropriate solvent system that keeps the reactants dissolved, did not react with the enzyme, matrix/support or any of the reactants, and also did not readily evaporate at the temperature of catalysis is very important in achieving efficient esterification. In the present study, the use of *n*-heptane (log *P* value = 3.5) with a C-chain length shorter than *n*-nonane further enhanced the rate of esterification. In general, as the log *P* value of an *n*-alkane increased corresponding to an increase in the C-chain length of the alkanes (log *P* value for *n*-pentane, *n*-hexane, *n*-heptane, *n*-octane, and *n*-nonane is 3, 3.5, 4, 4.5, and 5, respectively), the hydrophobicity of the alkanes also increased in that order. Thus, *n*-heptane that was less hydrophobic than *n*-nonane appeared to be more suitable to synthesize ethyl propionate employing hydrophilic poly(N-AEAAm-*co*-AAc-*cl*-MBAm)-hydrogel bound lipase of *B. coagulans* MTCC-6375.

The present study concluded that poly(N-AEAAm-*co*-AAc-*cl*-MBAm)-hydrogel-immobilized-lipase of *B. coagulans* MTCC-6375 efficiently esterified ethanol and propionic acid into ethyl propionate at a temperature of 65°C in a short period of 9 h in *n*-heptane. Moreover, addition of moleculars sieve while employing a highly hydrophilic poly(N-AEAAm-*co*-AAc-*cl*-MBAm)-matrix was essential to achieve maximum/efficient rate of esterification.

References

1. Klibanov, A. M. Nature 2001, 409, 241.
2. Gargouri, M.; Drouet, P.; Legoy, M. D. J Biotechnol 2002, 92, 259.
3. Castillo, E.; Pezzotti, F.; Navarro, A.; Lopez-Munguia, A. J Biotechnol 2003, 102, 251.
4. Noel, M.; Combes, D. J Biotechnol 2003, 102, 23.

5. Malcata, F. X.; Reyes, H. R.; Garcia, H. S.; Hill, C. G.; Admunson, C. H. J Am Oil Chem Soc 1990, 67, 890.

6. Kanwar, S. S.; Srivastva, M.; Ghazi, I. A.; Chimni, S. S.; Kaushal, R. K.; Joshi, G. K. Acta Microbiol Immunol Hugarica 2004, 51, 57.

7. Kanwar, S. S.; Verma, H. K.; Pathak, S.; Kumar, Y.; Verma, M. L.; Chauhan, G. S. Acta Microbiol Immunol Hungarica 2006, 53, 195.

8. Reslow, M.; Adelkreutz, P.; Mattiasson, B. Eur J Biochem 1988, 177, 313.

9. Zaks, A.; Klibanov, A. M. Science 1984, 224, 1249.

10. Cambou, B.; Klibanov, A. M. J Am Chem Soc 1984, 106, 2687.

11. Zaks, A.; Klibanov, A. M. J Biol Chem 1988, 263, 3194.

12. Boyer, J. L.; Gilot, B.; Guirand, R. In Recent Progress en Genie des Procedes, Nouvelles Applications de La methodologie de Genie des Procedes; Storck, A.; Grevillot, G, Eds.; Lavoisier Technique et Documentation: Paris, France, 1987; p 7.

13. Nishio, T.; Chicano, T.; Kamimura, M. Agric Biol Chem 1988, 52, 1203.

14. Kanwar, S. S.; Chauhan, G. S.; Chimni, S. S.; Kumar, Y.; Rawat, G. S.; Kaushal, R. K. J Appl Polym Sci 2006, 100, 1420.

15. Kanwar, S. S.; Kaushal, R. K.; Verma, M. L.; Kumar, Y.; Chauhan, G. S.; Gupta, R.; Chimni, S. S. Indian J Microbiol 2005, 45, 187.

16. Lanne, C.; Boeren, S.; Vos, K.; Veeger, C. Biotechnol Bioeng 1987, 30, 81.

17. Drapron, R. In Properties of Water in Foods; Simates, D.; Multon, J. L., Eds.; Martinus Nijhoff: Dodrecht, 1985; p 171.

18. Goldberg, M.; Thomas, D.; Legoy, M. D. Eur J Biochem 1990, 190, 603.

19. Fontes, N.; Patridge, J.; Halling, P. J.; Barreiros, S. Biotechnol Bioeng 2002, 77, 296.

20. Harper, N.; Barreiros, S. Biotechnol Prog 2002, 18, 1451.

21. Fontes, N.; Harper, N.; Halling, P. J.; Barreiros, S. Biotechnol Bioeng 2003, 82, 802.

22. Vidinha, P.; Harper, N.; Micaeto, N. M.; Lourenco, N. M. T.; Gomes de Silva, M. D. R.; Cabral, J. M. S.; Afonso, C. A. M.; Soares, C. M.; Berreiros, S. Biotechnol Bioeng 203, 85, 442.

23. Esa, N. B. M.Studies on the Suitability of Poly (2-Hydroxy Methacrylate-*co*-methyl methacrylate) as a Matrix for the Immobilization of Lipase, Ph.D. Thesis; 1996; Universiti Putra: Malaysia.

24. Kaushal, R. K.; Sen, U.; Kanwar, S. S.; Chauhan, G. S.In Proceedings of the IXth Int Workshop Bioencapsulation, Illkirch, Strasbourg, France, May 25–27, 2003.

25. Lowry, O. H.; Rosenbrough, N. J.; Farr, A. L.; Randall, R. J. J Biol Chem 1951, 193, 265.

26. Winkler, U. K.; Stuckmann, M. J Bacteriol 1979, 138, 663.

27. Habulin, M.; Krmelj, V.; Knez, Z. J Agri Food Chem 1996, 44, 338.

28. Pandey, A.; Benjamin, S.; Soccol, C. R.; Nigam, P.; Krieger, N.; Soccol, V. T. Biotechnol Appl Biochem 1999, 29, 199.

29. Sekeroglu, G.; Fadiloglu, S.; Ibanoglu, E. J Sci Food Agri 2002, 82, 1516.

30. Sekeroglu, G.; Fadiloglu, S.; Ibanoglu, E. Turkish J Eng Env Sci 2004, 28, 241.

31. Klibanov, A. M. Chem Tech 1986, 16, 354.

32. Dordick, J. S. Enzyme Microbiol Technol 1989, 11, 194.

33. Bornscheuer, U. T. Enzyme Microbiol Technol 1995, 17, 578.

34. Sheldon, R. A.In: Enzymatic Reactions in Organic Media; Koskinen, A. M. P.; Klibanov, A. M., Eds.; Blackie Academic: Glasgow, 1996; p 267.

35. Yahya, A. R. M.; Anderson, W. A.; Moo-Young, M. Enzyme Microb Technol 1998, 23, 438.

36. Kontkanen, H.; Tenkanen, M.; Fagerstrom, R.; Reinikainen, T. J Biotechnol 2004, 108, 51.

37. Goncalves, A. P. V.; Lopes, J. M.; Lemos, F.; Ribeiro, F. R.; Prazeres, D. M. F.; Cabral, J. M. S.; Aires-Barrow, M. R. Enzyme Microb Technol 1997, 20, 93.

38. Pouilloux, Y.; Abro, S.; Vanhove, C.; Barrault, J. J Mol Catal A: Chem 1999, 149, 243.

39. Halling, P. J Curr Opin Chem Biol 2002, 4, 74.

40. Jack, K. Esterification in Organic Solvent: Effect of Water on Immobilized Enzyme Activity and Performance 1991, Final year project report, Reading University: UK.

41. Mustafa, U. U. Trends J Agri Forestry 1998, 22, 573.