Application of Lipase Purified from *Aspergillus fumigatus* in the Syntheses of Ethyl Acetate and Ethyl Lactate

Akshita Mehta, Chetna Grover, Kamal Kumar Bhardwaj, and Reena Gupta*

Department of Biotechnology, Himachal Pradesh University, Summer Hill Shimla (H.P.) 171 005, INDIA

Abstract: Microbial lipases are used for the synthesis of various short chain esters such as octyl acetate, methyl salicylate, ethyl acetate and ethyl lactate. In this study, a purified lipase of *Aspergillus fumigatus* was utilized for the synthesis of two esters i.e. ethyl acetate and ethyl lactate. The purified lipase from *Aspergillus fumigatus* performed esterification of ethanol and acetic acid (at a molar ratio of 1:1) when incubated at 40°C under shaking (130 min−1) for 12 h resulting in the formation of ethyl acetate (89%). In case of ethyl lactate maximum esterification (87.32%) was achieved when ethanol and lactic acid (500:100 mM) was used in heptane resulting in the synthesis of ethyl lactate at 40°C under shaking (120 rpm) after 12 h of reaction time. These esters of short chain carboxylic acid and alcohols belong to the highly important natural aroma compounds and are used as green solvents in food and pharmaceutical industry.

Key words: ethyl acetate, ethyl lactate, *Aspergillus fumigatus*, esterification, lipase

1 Introduction

Lipases (triacylglycerol ester hydrolases E.C. 3.1.1.3) are ubiquitous enzymes that catalyse the breakdown of fats and oils with subsequent release of free fatty acid, diacylglycerol, monoacylglycerol and glycerol. They are found in animals, plants, fungi and bacteria and are of considerable physiological significance and industrial potential. However, the most suitable sources for lipase production are the microorganisms which can produce high quality lipases in lower cost and shorter time. Fungi are more preferred sources of lipases because of their stability at different temperature and pH. Purification of enzymes allows successful determination of their primary amino acid sequence and three-dimensional structure. Purified lipase preparations are needed in industries employing the enzymes for the biocatalytic production of food additives (flavour modifying enzymes), industrial reagents (glyceride hydrolyzing enzyme), stain removing agents (detergent additives), digestive drugs, diagnostic enzymes in medical applications, nutraceuticals, surfactants and additives in cosmetics.

Esters of short chain carboxylic acid and alcohols belong to the highly important natural aroma compounds. The production of natural aroma compounds by fermentation and enzymatic reactions in aqueous, organic solvent, or novel environmentally friendly solvent media has become a widely-studied field of research in the last few years. Ethyl acetate is used in the nail polish remover and in decaffeinating tea, coffee and cigarettes. It is also used in the perfumes because it evaporates quickly leaving the scent on the skin. It is found as a solvent in the cosmetic products. It is widely used as an extraction agent, intermediate in the pharmaceuticals. Ethyl acetate is an effective poison for use in entomology as its vapours are a respiratory tract irritant which can kill the insects quickly. It is also used as a carrier solvent for herbicides.

Ethyl lactate finds industrial applications in specialty coatings, inks, cleaners because of its high performance and versatility. Due to its low toxicity, ethyl lactate is a popular choice across many different scenarios. Ethyl lactate is a flavouring agent present in cabbage, peas, vinegar, bread, roasted chicken, butter, blackberry, pineapple, raspberry and various wines and spirits. Ethyl lactate also known as lactic acid ethyl ester, is a monobasic ester formed from lactic acid and ethanol, commonly used as a solvent. As a “green” solubilizing agent, ethyl lactate has many advantages compared with other organic-based solubilizing agents.

With the great recent interest for natural products, the flavour industry is more and more interested in the use of...
biotechnology to produce natural flavours. This study deals with the syntheses of ethyl acetate and ethyl lactate from purified lipase from Aspergillus fumigatus.

2 Materials and Methods

2.1 Materials

2.1.1 Chemicals

Potato dextrose agar (PDA), tributyrin, para-nitrophenyl palmitate (p-NPP), para-nitrophenyl acetate (p-NPA), para-nitrophenyl benzoate (p-NPBenz), para-nitrophenyl butyrate (p-NPB), para-nitrophenol (p-NP), Tween 80, Tween 60, Tris buffer, peptone, galactose, NaCl, iso-propanol, CaCl₂, Bradford reagent and all other chemicals used in the present study were either procured from Himedia Laboratory Ltd., Mumbai, India or Sigma-Aldrich, Steinheim, Germany; Sigma Chemicals Co., USA. All the chemicals used were of analytical grade.

2.1.2 Biological Material

Lipase producing fungal isolate Aspergillus fumigatus was procured from the Department of Biotechnology, Himachal Pradesh University, Shimla. It was isolated from oil-contaminated soil samples from HRTC workshop, Tara Devi, Shimla, Himachal Pradesh.

2.2 Methods

2.2.1 Maintenance of strain and inoculum preparation

The fungal strain was maintained on potato dextrose agar plate and incubated at 37°C for 3 days in incubator and stored at 4°C in refrigerator. 2.4 × 10⁵ spores were collected from three to four days old culture grown on PDA plates using sterilized inoculation loop in 2 mL of sterile buffer saline and added to the 50 mL production medium.

2.2.2 Production of lipase

The lipase producing fungal strain was grown in the medium containing peptone (18.0 g/L), galactose (15.0 g/L), sodium chloride (5.0 g/L), calcium chloride (1 g/L) and Tween 80 (10 mL/L). The inoculum was added to 50 mL sterile production medium and incubated for 3 days under shaking condition at 150 rpm at 45°C. The culture broth was analyzed for lipase activity.

2.2.3 Assay of lipase enzyme

Lipase activity was assayed by the method given by Winkler and Stuckmann (1979) by measuring the micromoles of para-nitrophenol (p-NP) released from para-nitrophenyl benzoate (p-NP-Benz).

2.2.4 Lipase activity

One unit (U) of lipase activity was defined as the amount of enzyme required to release one micromole of p-NP from the substrate (p-NP-Benz) per minute under standard assay conditions.

2.2.5 Protein estimation

The concentration of protein was estimated by dye binding method using standard bovine serum albumin (BSA).

2.2.6 Specific activity of lipase

One unit of specific activity was defined as the activity of the enzyme in units per mg of protein content.

2.3 Purification of lipase from fungal isolate Aspergillus fumigatus

The enzyme was purified by hydrophobic interaction chromatography. The crude enzyme (3 mL) after dialysis was loaded onto the column of octyl sepharose column equilibrated with 0.1M Tris-HCl buffer (pH 9.0). The column was eluted with Tris-HCl buffer. The adsorbed protein was eluted with the gradients of ammonium sulfate. All eluted fractions were assayed both for lipase activity as well as for their protein content.

2.4 Esterification process for the synthesis of ethyl acetate by purified lipase

2.4.1 Synthesis and analysis of ethyl acetate by gas chromatography/mass spectrometry (GC/MS)

The esterification study was performed with purified lipase in DMSO (5 mL reaction volume). The biocatalyst (20 µg/mL) was added to the reaction mixture containing an appropriate concentration of reactants (100 mM of acetic acid and 100 mM of ethanol) incubated at 35°C under shaking (130 min⁻¹) for 9 h. The reaction mixture was assayed for the presence of ethyl acetate by GC/MS (Central Instrumentation Lab, Panjab University, Chandigarh) using a sample of 1 µL.

2.5 Optimization of reaction conditions for the synthesis of ethyl acetate

2.5.1 Effect of molarity of ethanol on the synthesis of ethyl acetate

The esterification studies were performed with lipase in water-saturated DMSO (5 mL reaction volume). The biocatalyst (20 µg/mL) was added to the reaction mixture containing 100 mM acetic acid and different concentrations of ethanol (50, 75, 100 and 120 mM). The esterification was carried out for 9 h at 35°C under continuous shaking and the amount of ester formed was determined by GLC.

2.5.2 Effect of temperature on the synthesis of ethyl acetate

The effect of temperature (35, 40, 45 and 50°C) on the synthesis of ethyl acetate was studied. The amount of ester formed was analyzed by GLC.

2.5.3 Effect of incubation time on the esterification reaction

The reaction mixture comprised purified lipase, acetic acid and ethanol in optimized molar ratio in solvent (heptane) to complete the reaction mixture volume 5 mL. The glass vials were incubated in a shaker for 6, 9, 12, 15 and 18 h at 40°C.
2.6 Synthesis and analysis of ethyl lactate by GC

The esterification studies were performed with lipase in water-saturated heptane (5 mL reaction volume). The biocatalyst (60 μL) was added to the reaction mixture containing an appropriate concentration of reactants ethanol (500 mM) and lactic acid (100 mM) incubated at 40°C under shaking for 12 h. The reaction mixture was assayed for the presence of ethyl lactate by GC using a sample of 2 μL. The GC was equipped with a packed-column (12% SE-30 Chrom WHP, 2 m length, mesh size 80-100, internal diameter 0.32 cm, Netel chromatographs, Thane, India). Nitrogen was used as a carrier gas (20 mL min⁻¹). GC was programmed for oven temperature of 170°C, injection temperature of 200°C and FID temperature of 200°C.

2.7 Optimization of reaction conditions for the synthesis of ethyl lactate ester

2.7.1 Effect of molarity of ethanol on synthesis of ethyl lactate

The effect of ethanol molarity on ethyl lactate synthesis was studied by maintaining the concentration of acid (lactic acid) constant i.e. 100 M and varying the concentration of ethanol (100-500 mM) in heptane to complete the reaction volume 5 mL. The reaction was initiated by adding 30 μg/mL of protein and incubating the reaction mixture at 40°C for 12 h. The esterification was carried out using purified lipase under standard conditions.

2.7.2 Effect of incubation time on synthesis of ethyl lactate

The reaction mixture comprised purified lipase, lactic acid and ethanol in optimized molar ratio in solvent (heptane) to complete the reaction mixture volume 5 mL. The glass vials were incubated in a shaker for 4, 8, 12, 16, 20 and 24 h at 40°C.

2.7.3 Effect of incubation temperature on the synthesis of ethyl lactate

The effect of optimum incubation temperature (35, 40, 45, 50 and 55°C) on the synthesis of ethyl lactate was studied by using optimized molar concentration of reactants and incubation time. The amount of ester synthesized was determined from the standard profile of ethyl lactate.

2.7.4 Effect of amount of protein on synthesis of ethyl lactate

The effect of different amounts of purified enzyme (10-60 μg/mL) on the synthesis of ethyl lactate using optimized molar concentration of reactants, time and temperature was studied.

3 Results and Discussion

3.1 Purification of lipase from fungal isolate Aspergillus fumigatus

The enzyme was purified using octyl sepharose column. The specific activity of pooled enzyme fractions was observed to be 14.34 U mg⁻¹ with a fold purification of 6.96²².

3.2 Synthesis of ethyl acetate

In sample, fragment ion at 41 and 63 m/z as shown in Fig. 1 was compared with GC/MS analysis of standard ethyl acetate whose fragment ion was at 43, 61 and 88 m/z (Fig. 2). The esterification performance was dependent on the alcohol structure with maximum activity occurring with the primary alcohols²³. In an earlier study, the ethyl acetate was formed from ethanol and acetic acid applying immobilized Candida antarctica lipase B enzyme at 50°C²⁴. Similarly, esterification studies were carried out using ethanol and acetic acid using immobilized lipase of Staphylococcus pasteurii. GC analysis showed 98% conversion of the reactants to ethyl acetate within 24 hours of incubation²⁵. Lipase from Candida antarctica lipase B showed 92.08% conversion or synthesis of ethyl lactate in the presence of reactants²⁶.

3.3 Optimization of reaction conditions of esterification process of ethyl acetate

3.3.1 Effect of molarity of ethanol on the synthesis of ethyl acetate

It was observed that the maximum yield of ethyl acetate was observed when ethanol was used at a concentration of
100 mM in n-heptane under continuous shaking for 9 h at 35°C (Fig. 3). In a previous study, at a fixed concentration of acetic acid, an increase in the concentration of alcohol promoted the synthesis of ester.

3.3.2 Effect of temperature on the esterification reaction

Maximum yield (86.2%) of ethyl acetate was obtained at 40°C after that there was decrease in the ester yield (Fig. 4). Higher temperature also affects the three-dimensional structure of the enzyme, which could lead to thermal deactivation.

3.3.3 Effect of incubation time on the synthesis of ethyl acetate

The overall conversion increased to 89% after 12 h of incubation (Fig. 5). The synthesis of ester is time dependent, 98% Conversion of the reactants to ethyl acetate was achieved after 24 h of reaction using *Staphylococcus pasteuri* lipase immobilized on calcium alginate beads.

3.4 Esterification process for the synthesis of ethyl lactate by purified lipase

In sample, retention time of ethyl lactate was found to be similar with standard ethyl lactate (retention time 0.50) (Fig. 6). Ethyl lactate synthesis was maximum (87%) within 12 h. As reported earlier an increment in ethyl lactate yield was observed with increasing molar ratio of ethanol to acid, but the trend was reversed when the ratio reached a certain value this might due to the fact that increasing ethanol shifted the reaction equilibrium towards product formation. In a previous study, the ethyl lactate synthesis efficiency increased depending on temperature and reached approximately 74% at 50°C.

3.5 Optimization of reaction conditions for the synthesis of ethyl lactate

3.5.1 Effect of molarity of ethanol on synthesis of ethyl lactate

The 300 mM ethanol showed the maximum yield (87.2%) of ethyl lactate as shown in Table 1. The molar ratio of the substrates strongly affected the rate of this reversible reaction. The molar ratio 1:3 was found to be optimum for the synthesis of ethyl ferulate which gave 2.36 moles/L of ethyl ferulate. The hydrogel-immobilized lipase from *Pseudomonas aeruginosa* in the ratio of 400:100 mM in heptane resulted in 98.8 mM geranyl butyrate. When the concentrations of ethanol and propionic acid were increased in equal proportions from 100 to 300 mM for esterification, the esterification efficiency increased from 11.8% to 98% for immobilized lipase from *Bacillus coagulans* BTS-1.

3.5.2 Effect of incubation time on synthesis of ethyl lactate

It was observed that maximum conversion of 87.3% was obtained after 12 h of incubation (Table 2). A reaction time of 26.87 h gave maximum yield (24.32%) of ethyl lactate synthesized from commercial lipase (Novozyme 435) under optimal conditions. Conversion of 92% was achieved in the synthesis of pentyl 2-methylpropanoate using *Candida rugosa* lipase in a relatively short time of 26 h at 35°C. The synthesis of geranyl butyrate using *Pseudomonas aeruginosa* MTCC-4713 immobilized lipase was increased by increasing reaction time to 15 h (99.1 mM).

3.5.3 Effect of incubation temperature on the synthesis of ethyl lactate

The effect of temperature on the enzymatic esterification is shown in Fig. 7. 87.3% yield of ethyl lactate was achieved at 40°C in 12 h in a reaction. This might be on
Syntheses of Ethyl Acetate and Ethyl Lactate by Purified Lipase

J. Oleo Sci. 69, (1) 23-29 (2020)

account of denaturation of the lipase as well as alteration in the 3-D structure of lipase\[^{20}\]. In an earlier study, the synthesis efficiency of ethyl lactate using lipase from Candida antartica increased and reached approximately 74% at 50°C\[^{10}\]. The commercial lipase, Novozym 435 showed yield of ethyl lactate up to 25.13% at temperature of 55°C and rotation speed of 150 rpm\[^{20}\]. The highest lactic acid conversion achieved was 0.63 in the membrane reactor at 50°C, while the lactic acid conversion was 0.37 in the batch reactor using Rhizomucor miehei lipase under the same operating conditions\[^{35}\].

3.5.4 Effect of amount of protein on synthesis of ethyl lactate

The overall conversion increased to 87.32% with an increase in enzyme loading up to 30 µg/mL (Fig. 8). But further increase in amount of enzyme has declined the conversion to 71%. This might be due to the fact that excess amount of enzyme may increase the viscosity of solution and reduce the mass transfer\[^{30}\].

4 Conclusion

The present study used Aspergillus fumigatus lipase to carry out the synthesis of two esters (ethyl acetate and ethyl lactate) using carboxylic acid and alcohols. The 87.32% yield of ethyl lactate was achieved with ethanol and lactic acid and 89% yield of ethyl acetate was observed with ethanol and acetic acid. The synthesized esters (ethyl lactate and ethyl acetate) can be used in high added-value products due to their low toxicity. They can also be used in artificial fruit essence and give artificial flavours such as pineapple, bananas and strawberry in confectionary, ice-creams, cakes etc.

Acknowledgements

The financial support from Department of Biotechnology, Ministry of Science and Technology, Govt. of India, to Department of Biotechnology, Himachal Pradesh University, Shimla (India), is thankfully acknowledged. Fellowship granted to Ms. Akshita Mehta from DEST (Department of Environment, Science and Technology) Himachal Pradesh in the form of Project Fellow is also thankfully acknowledged.

Conflicts of Interests

The author(s) declare(s) that there is no conflict of in-

Table 1  Effect of molarity of ethanol on the synthesis of ethyl lactate.

| Ethanol molarity (mM) | Ester yield (%) |
|----------------------|----------------|
| 100                  | 67.5 ± 0.8     |
| 200                  | 78.1 ± 1.0     |
| 300                  | 87.2 ± 0.3     |
| 400                  | 63.8 ± 0.3     |
| 500                  | 55.2 ± 0.9     |

Values are mean ± SD of three observations.

Table 2  Effect of incubation time on the synthesis of ethyl lactate.

| Incubation time (h) | Ester yield (%) |
|---------------------|----------------|
| 4                   | 67.2 ± 0.6     |
| 8                   | 79.8 ± 0.9     |
| 12                  | 87.3 ± 1.0     |
| 16                  | 74.1 ± 0.8     |
| 20                  | 62.5 ± 0.6     |
| 24                  | 55.1 ± 0.9     |

Values are mean ± SD of three observations.

Table 3

| GLC report showing peak of a)ethyl lactate(b)ethyl lactate(standard) synthesized from ethanol and lactic acid using lipase from Aspergillus fumigatus. |
|-------------------------------------------------------------------------------------------------|
| ![Graph](image.png)                                                                                |

![Graph](image.png)
Fig. 7 Effect of incubation temperature on the synthesis of ethyl lactate.

Fig. 8 Effect of amount of enzyme on synthesis of ethyl lactate.

interests regarding the publication of this article.

References

1) Pandey, A. Biodiesel. in Handbook of Plant Based Biofuels. Pandey, A. ed. CRC press, Boca Raton, USA. pp. 29-44 (2009).

2) Jaeger, K.E.; Eggert, T. Lipases for biotechnology. Curr. Opin. Biotechnol. 13, 390-397 (2002).

3) Gopinath, S.C., Anbu, P.; Lakshmipriya, T.; Hilda, A. Strategies to characterize fungal lipases for applications in medicine and dairy industry. BioMed Res. Int. 3, 11-15 (2013).

4) Patel, N.; Rai, D.; Shivam, Shahane, S.; Mishra, U. Lipases: Sources, production, purification, and applications. Recent Pat. Biotechnol. 13, 45-56 (2019).

5) Thakur, S. Lipases, its sources, properties and applications: a review. Int. J. Sci. Eng. Res. 3, 1-29 (2012).

6) Trichel, H.; Oliveira, D.; Mazutti, M.; Luccio, M.D.; Oliveira, J.V. A review on microbial lipases production. Food Bioproc. Tech. 3, 182-196 (2010).

7) Ko, W.H.; Wang, I.T.; Ann, P.J. A simple method for the detection of liolytic microorganism in soil. Soil Bio. Biochem. 37, 597-599 (2005).

8) Verma, M.L.; Kanwar, S.S. Lipases. in Encyclopedia of Industrial Biotechnology. Wiley online library. pp. 50-65 (2010).

9) Kumar, A.; Kanwar, S.S. Catalytic potential of a nitrocellulose membrane immobilized lipase in aqueous and organic media. J. Appl. Polym. Sci. 124, 37-44 (2012).

10) Melani, N.B.; Tambourgi, E.B.; Silveira, E. Lipases: From production to applications. Sep. Purif. Rev. Doi. 10.1080/15422119.2018.1564328 (2019).

11) Alvaraez-Macarie, E.; Baratti, J. Short chain flavor ester synthesis by a new esterase from Bacillus licheniformis. J. Mol. Catal. A 10, 377-383 (2000).

12) Dordick, J.S. Enzymatic catalysis in monophasic organic-solvents. Enzyme Microb. Technol. 1, 194-211 (1989).

13) de Souza, M.C.M.; dos Santos, K.P.; Freire, R.; Barreto, A.C.H.; Fechine, P.B.A.; Goncalves, L.R.B. Production of flavor esters catalyzed by Lipase B from Candida antarctica immobilized on magnetic nanoparticles. Braz. J. Chem. Eng. 34, 681-690 (2017).

14) Cantone, S.; Hanefeld, U.; Basso, A. Biocatalysis in non-conventional media-ionic liquids, supercritical fluids and the gas phase. Green Chem. 9, 954-971 (2007).

15) Pereira, C.S.M.; Silva, V.M.T.M.; Rodrigues, A.E. Ethyl lactate as a solvent: Properties, applications and production processes – a review. Green Chem. 13, 2658-2671 (2011).

16) Inaba, C.; Maekawa, K.; Morisaka, H.; Kuroda, K.; Ueda, M. Efficient synthesis of enantiomeric ethyl lactate by Candida antarctica lipase B(CALB)- displaying yeasts. Appl. Microbiol. Biotechnol. 83, 859-864 (2009).

17) Guo, J.; Jia, S. Effects of enzymes on ester production during the course of Chinese liquor fermentation as discussed by correlation analysis and path analysis. J. Inst. Brew. 120, 565-570 (2014).

18) Koutinas, M.; Yangou, C.; Osório, N.M.; Ioannou, K.; Canet, A.; Valero, F.; Ferreira-Dias, S. Application of commercial and non-commercial immobilized lipases for biocatalytic production of ethyl lactate in organic solvents. Bioresour. Technol. 247, 496-503 (2017).

19) Sneath, P.H.A. Gram negative rod and cocci. in Bergey’s Manual of Determinative Bacteriology (Sneath, H.A.; Mair, N.S.; Sharpe, M.E.; Holt, J.G. eds.), Lippincott Williams & Wilkins, Philadelphia. pp. 189-190 (1986).

20) Winkler, U.K.; Stickmann, M. Glycogen, hyaluronate, and some other polysaccharides greatly enhance the formation of exolipase by Serratia marcescens. J. Bacteriol. 138, 663-670 (1979).

21) Bradford, M.M. A rapid and sensitive method for the quantitation of microgram quantities of protein utiliz-
ing the principle of protein-dye binding. *Anal. Biochem.* 72, 248-254 (1976).

22) Mehta, A.; Grover, C.; Gupta, R. Purification of lipase from *Aspergillus fumigatus* using Octyl Sepharose column chromatography and its characterization. *J. Basic Microbiol.* 58, 857-866 (2018).

23) Rajendran, A.; Palanisamy, A.; Thangavelu, V. Lipase catalyzed ester synthesis for food processing industry. *Braz. Arch. Biol. Technol.* 52, 207-219 (2009).

24) Csanadi, Z.; Kurdi, R.; Belafi-Bako, K. Ethyl acetate synthesis in gas phase by immobilized lipase. *Hung. J. Ind. Chem.* 40, 39-44 (2012).

25) Dukhande, M.; Pawar, A. Biosynthesis of flavoring compound- ethyl acetate using immobilized lipase produced by *Staphylococcus pasteuri*. *Int. J. Res. Eng. Technol.* 2, 59-63 (2015).

26) Verma, M.L.; Azmi, W.; Kanwar, S.S. Enzymatic synthesis of isopropyl acetate by immobilized *Bacillus cereus* lipase in organic medium. *Enzyme Res.* doi.org/10.4061/2011/919386 (2011).

27) Yong, Y.P.; Al-Duri, B. Kinetic studies on immobilized lipase esterification of oleic acid and octanol. *J. Chem. Technol. Biotechnol.* 65, 239-248 (1996).

28) Paiva, A.L.; Balcao, V.M.; Malcata, F.X. Kinetics and mechanisms of reactions catalyzed by immobilized lipases. *Enzyme Microb. Technol.* 27, 187-204 (2000).

29) Sun, J.; Jiang, Y.; Zhou, L.; Gao, J. Optimization and kinetic study of immobilized lipase-catalyzed synthesis of ethyl lactate. *Biocatal. Biotransfor.* 28, 279-287 (2010).

30) Saun, N.K.; Narwal, S.K.; Dogra, P.; Chauhan, G.S.; Gupta, R. Comparative study of free and immobilized lipase from *Bacillus aerius* and its application in synthesis of ethyl ferulate. *J. Oleo Sci.* 63, 911-919 (2014).

31) Kanwar, S.S.; Gehlot, S.; Verma, M.L.; Gupta, R.; Kumar, Y.; Chauhan, G.S. 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. *J. Appl. Polym. Sci.* 110, 2681-2692 (2008).

32) Kumar, S.; Ola, R.P.; Pahuji, S.; Kaushal, R.; Kanwar, S.S.; Gupta, R. Thermostability and esterification of a polyethylene- immobilized lipase from *Bacillus coagulans* BTS-3. *J. Appl. Polym. Sci.* 102, 3986-3993 (2005).

33) Knezevic-Jugovic, Z.; Bezbradica, D.; Jakov, Z. Lipase catalyzed synthesis of flavor esters in non-aqueous media: Optimization of the yield of pentyl 2-methylpropanoate by statistical analysis. *J. Serb. Chem. Soc.* 73, 1139-1151 (2008).

34) Yadav, G.D.; Lathi, P.S. Intensification of enzymatic synthesis of propylene glycol monolaurate from 1, 2-propanediol and lauric acid under microwave irradiation : Kinetics of forward and reverse reactions. *Enzyme Microb. Technol.* 38, 814-820 (2006).

35) Nigiz, F.U.; Hilmioglu, N.D. *Rhizomucor miehei* lipase-immobilized sodium alginate membrane preparation and usage in a pervaporation biocatalytic membrane reactor. *Chem. Biochem. Eng. Q.* 30, 381-391 (2016).

36) Gawas, S.D.; Jadhav, S.V.; Rathod, V.K. Solvent free lipase catalysed synthesis of ethyl laurate: Optimization and kinetic studies. *Appl. Biochem. Biotechnol.* 180, 1428-1445 (2016).