Synthesis of Methyl Butyrate Catalyzed by Lipase from Aspergillus fumigatus

Manpreet Kaur, Akshita Mehta, and Reena Gupta*

Department of Biotechnology, Himachal Pradesh University, Summerhill, Shimla, 171 005, INDIA

Abstract: Lipase is a potential biocatalyst and can be exploited for various applications such as food, pharmaceutical, oleochemistry, organic chemistry, biofuels and in detergent industries. In the present study, lipase from Aspergillus fumigatus was purified to homogeneity by SDS and Native PAGE and evaluated as biocatalyst for the synthesis of methyl butyrate which is a flavor ester. A purification fold of 6.96 was achieved by using Octyl Sepharose column chromatography. Methyl butyrate was synthesized by transesterification of vinyl butyrate with methanol, in a medium containing n-hexane as a solvent. The molar ratio of 2:2 (vinyl butyrate:methanol) was found to be optimum for the synthesis of methyl butyrate. The yield of methyl butyrate was maximum when reactants were incubated for 16 h at an incubation temperature of 40°C. The maximum yield (86%) of ester was obtained with 30 µg/ml of purified lipase.

Key words: Aspergillus fumigatus, lipase, flavor, ester, methyl butyrate

1 Introduction

Lipases (triacylglycerol acyl hydrolases, E.C. 3.1.1.3) are a class of enzymes, which catalyze the hydrolysis of long chain triglycerides to glycerol and fatty acids. In addition, lipases catalyze the hydrolysis and trans-esterification as well as the synthesis of esters and exhibit enantio-selective properties. Microbial lipases have immense potential in industrial applications. Biocatalysis shows a distinct advantage over the chemical route in terms of process simplification, quality of product and reduction in waste formation.

Purified lipases are indispensable catalysts for valuable transformations in the field of oleochemistry, organic chemistry, biofuels, and pharmaceutical sectors. Lipases have been purified from animal, plant, fungal and bacterial sources using variety of methods involving ammonium sulphate precipitation, ion exchange chromatography followed by gel filtration and hydrophobic interaction chromatography. Short-chain fatty acid esters are commonly used in the food, beverage, cosmetic and pharmaceutical industries as flavorings or fragrances due to their typical fruity smells and high volatilities. Natural flavor esters extracted from plant materials are often too scarce or expensive for industrial use and chemical synthesis often involves environmentally harmful production processes and lacks substrate selectivity, which may produce racemic mixtures with undesired side products that reduce synthesis efficiency and increase downstream costs.

Recently, Candida antarctica lipase B (CALB) has been used for the synthesis of fatty acid ethyl esters. Lipase has been found to be efficient for the esterification of β-sitostanol. Ethyl oleate has been synthesized by using a lipase from Rhizopus microspores. Lipase from Bacillus has been employed for the synthesis of methyl oleate and methyl butyrate. Methyl butyrate (MB) is the methyl ester of butyric acid having a characteristic sweet and fruity odor like that of apples and pineapples. It occurs in many plant products in minute quantities and in pineapple oil. Methyl butyrate is of immense importance which is widely used in manufacturing of flavors, pesticides, adhesives, pharmaceuticals, plasticizers, solvents of paints, polymerization monomers. It can also be used as emulsifier in the food and cosmetic industries. Methyl butyrate has numerous food applications such as in the synthesis of modified triacylglycerols, emulsifiers, peptides and oligosaccharides. Methyl butyrate is produced by the distillation of essential oils extracted from various plants, but is also manufactured on an industrial scale for use in perfumes and flavoring industries. The synthesis of methyl butyrate through Rhizopus oryzae NRRL 3562 lipase has been studied.

*Correspondence to: Reena Gupta, Department of Biotechnology, Himachal Pradesh University, Summerhill, Shimla- 171 005, INDIA
E-mail: reenagupta_2001@yahoo.com
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M. Kaur, A. Mehta, and R. Gupta

2 Experimental

2.1 Materials

The materials used were potato dextrose agar (PDA), p-nitrophenyl benzoate (p-NPB), p-nitrophenol (p-NP), isopropanol, Tris-HCl buffer, vinyl butyrate, methanol, n-hexane, etc. All the chemicals used in present investigation were of analytical grade and of high purity either procured from Sigma–Aldrich (USA) or HIMEDIA (Mumbai, India).

2.1.1 Fungal isolate

Lipase producing Aspergillus fumigatus was procured from the Department of Biotechnology, Himachal Pradesh University, Shimla. It was isolated from oil-contaminated soil samples from HRTC workshop, Taradevi, Shimla, H.P. The fungal strain was maintained on potato dextrose agar and incubated at 37°C for 3 days and stored at 4°C in refrigerator.

2.2 Methods

2.2.1 Production of lipase

The lipase producing fungal strain was grown in the medium containing peptone (18.0 g), galactose (15.0 g), sodium chloride (5.0 g), calcium chloride (1.0 g), tween 80 (10 ml/l) pH 10.0, in 1 L distilled water. The inoculum was added to 50 ml sterile production medium and incubated for 3 days under shaking condition at 150 rpm at 45°C.

2.2.1.1 Enzyme assay

Lipase activity was assayed by the method of Winkler and Stuckmann (1979).2, 2.2.1.2 Unit of lipase

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

2.2.2 Purification of enzyme

The enzyme was purified by using hydrophobic interaction chromatography by following the methodology of Mehta et al., (2018).2

2.2.2.1 Protein estimation

The concentration of protein was estimated by dye binding method (Bradford, 1976) using standard bovine serum albumin (BSA).

2.2.3 Esterification process for the synthesis of methyl butyrate by purified lipase

Methyl butyrate synthesis was carried out in screw-capped vials by using purified lipase in 3 ml reaction volume. The reaction mixture consisted of different molar concentrations of methanol (1-5 M) and vinyl butyrate (2 M) in n-hexane. Reaction was initiated by addition of lipase (40 µL or 20 µg/mL) purified from Aspergillus fumigatus. Thereafter, the reaction mixtures were incubated at 40°C under shaking (120 min⁻¹) for 12 h.

2.2.4 Analysis of methyl butyrate by gas liquid chromatography (GLC)

The reaction mixture was assayed for the presence of methyl butyrate by gas liquid chromatography (GLC) 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 130°C, injection temperature of 140°C and FID temperature of 150°C.

2.2.5 Optimization of reaction conditions for the synthesis of methyl butyrate

2.2.5.1 Effect of alcohol molarity on the synthesis of methyl butyrate

The effect of methanol molarity on methyl butyrate synthesis was studied by maintaining the concentration of vinyl butyrate constant i.e. 2 M and varying the concentration of methanol (1-5 M) in n-hexane to complete the reaction volume 3 mL. The reaction was initiated by adding 20 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.

2.2.5.2 Effect of reaction time on synthesis of methyl butyrate

The reaction mixture comprised purified lipase, vinyl butyrate and methanol in optimized molar ratio in solvent (n-hexane) to complete the reaction mixture volume 3 mL. The glass vials were incubated in a shaker for 4, 8, 12, 16, 20 and 24 h at 40°C.

2.2.5.3 Effect of amount of biocatalyst on synthesis of methyl butyrate

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

2.2.5.4 Effect of amount of biocatalyst on synthesis of methyl butyrate

The synthesis of methyl butyrate was studied by taking different amounts of purified lipase (10-60 µg/mL) in reaction mixture (3 mL) using optimized molar concentration of reactants, time and temperature.
3 Results and discussion

Lipase from A. fumigatus was purified by ammonium sulphate precipitation and Octyl Sepharose column chromatography and resulted in 6.96-fold purification with specific activity of 14.34 Umg⁻¹.

3.1 Optimization of reaction conditions for synthesis of methyl butyrate

3.1.1 Effect of methanol molarity on the synthesis of methyl butyrate

In present study, maximum conversion of 81.4% was obtained with 2M methanol in 2M vinyl butyrate (Fig. 1). However, an increase in methanol concentration had an inhibiting effect on the synthesis of methyl butyrate, only 65.7% conversion was achieved by using 5M methanol. The difference in methanol molarity towards the yield of methyl butyrate may be attributed to either steric hindrance or electronic effect of substrate on the purified lipase or specificity of purified lipase toward the substrate.

The optimum molar ratio of 2:4 (salicylic acid:methanol) for the synthesis of methyl salicylate (70.25%) in DMSO by using silica bound lipase from Geobacillus sp. has been reported⁹. 90% conversion of ethyl oleate with 1:2 molar ratio (oleic acid:ethanol) of substrates by using lipase from Rhizopus microsporus has been reported recently⁹.

3.1.2 Effect of incubation time on synthesis of methyl butyrate

In present study, maximum yield of methyl butyrate i.e. 84.2% was recorded at incubation time of 16 h (Table 1). With further increase in incubation time, no increase in conversion was observed. Reaction time gives an insight into the performance of an enzyme as the reaction progresses, which will be helpful to determine the shortest time necessary for obtaining good yield and so enhancing cost-effectiveness of the process and will vary with the reaction conditions. Synthesis of methyl butyrate using immobilized lipase from Rhizopus oryzae NRRL 3562 showed maximum molar conversion with reaction time of 14 h¹⁰. However, another study showed maximum conversion for methyl butyrate (93.9%) catalyzed by lipase B immobilized on magnetic nanoparticles from Candida antarctica under an incubation time of 8 h¹¹. In another study, chloramphenicol was converted to chloramphenicol propionate (98%) by using lipase from Bacillus amylobiqaufaciens at incubation time of 8 h¹². A recent study on esterification of potato starch with oleic acid showed the maximum synthesis of starch oleate at 24 h of incubation¹³. In present study, maximum molar conversion i.e. 84.5% was observed at 40°C after which there was a decline in the conversion rate with only 51.1% conversion at 55°C (Table 2). This suggested that at higher temperature, the conversion rate is controlled by reaction temperature. In lipase catalyzed reactions, temperature significantly influences both the initial rate of the reaction and stability of the enzyme. Enzyme stability decreases with the increase in temperature above a certain range. In contrast, at lower temperature the reaction rate is limited by mass transport phenomenon. In a previous study, the maximum yield of methyl salicylate i.e. 73.64% by using silica immobilized lipase from Geobacillus sp. was recorded at 55°C, after which there was a decline in conversion rate with only 51.98% at 60°C.⁵ While another study done on production of flavor esters by lipase B from Candida antarctica im-

![Fig. 1 Effect of molarity of methanol on the synthesis of methyl butyrate.](image)

Table 1 Effect of incubation time on the synthesis of methyl butyrate.

| Incubation time (h) | Ester yield (%) |
|---------------------|-----------------|
| 4                   | 58.6            |
| 8                   | 72.9            |
| 12                  | 81.9            |
| 16                  | 84.2            |
| 20                  | 74.6            |
| 24                  | 68.9            |

Table 2 Effect of incubation temperature on the synthesis of methyl butyrate.

| Incubation temperature (°C) | Ester yield (%) |
|----------------------------|-----------------|
| 35                         | 78.8            |
| 40                         | 84.5            |
| 45                         | 75.3            |
| 50                         | 67.6            |
| 55                         | 50.2            |
mobilized on magnetic nanoparticles showed that for methyl butyrate, the conversion remained constant from 15-40°C and decreased at 45°C. A recent study showed that the synthesis of red pitaya seed oil esters catalyzed by lipozyme was achieved at an optimum temperature of 50.5°C.

### 3.1.4 Effect of amount of protein on synthesis of methyl butyrate

In present study, maximum synthesis of methyl butyrate i.e. 86% was obtained with 30 μg/mL of purified lipase (Fig. 2). Upon increasing the enzyme amount further, the molar conversion was decreased which might be due to difficulty in maintaining uniform suspension of the biocatalyst at higher enzyme concentration. The excess enzyme did not contribute to the increase in the percentage conversion. Among all variables, effect of amount of enzyme contributes to attain higher molar conversions. In a previous study, a molar conversion of 70.42% was reported by using 80 U of silica immobilized lipase from *Rhizopus oryzae* NRRL 3562. Another study showed that methyl salicylate was synthesized by using 20 mg/mL silica bound lipase from *Geobacillus* sp.

### 3.2 Percent yield of methyl butyrate formed

The formation of ester methyl butyrate was analyzed through GLC by comparing the retention time (Rt 2.50 min) of the test sample (Fig. 3) to the retention time of the standard run for methyl butyrate (Rt 2.43) as shown in Fig. 4. On optimizing different parameters such as molar ratio, incubation time, temperature and amount of lipase, the yield of methyl butyrate was found to be 86% which was calculated from the area covered under the peak. In a previous study, approximately 90% conversion of methyl butyrate and ethyl butyrate has been achieved by lipase B from *Candida antarctica* immobilized on magnetic nanoparticles. In another study, 70.42% synthesis of methyl butyrate has been reported by immobilized *Rhizopus oryzae* NRRL 3562 lipase.

### Conclusion

In present study, a 6.68 fold purified lipase was used for the synthesis of methyl butyrate. The biocatalyst studied in this work, promoted efficient biosynthesis of methyl butyrate. The optimal conditions for synthesizing methyl butyrate by *A. fumigatus* lipase resulted in a substrate conversion of 86%. Further studies will be conducted for the synthesis of other flavor esters and their reaction conditions like optimal temperature, biocatalyst amount and others parameters that affect the esterification reactions as well as to evaluate the utilization of this biocatalyst in a solvent-free system.

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### Conflicts of Interests

The author(s) declare(s) that there is no conflict of interests regarding the publication of this article.
References

1) 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).

2) Homaei, A. Enzyme immobilization and its application in the food industry. in Advances in Food Biotechnology (Ravishankar, R. ed.) First ed. John Wiley & Sons, Ltd., pp. 145-164 (2016).

3) Rihani, A.; Tchati, L.; Soumati, B. Isolation and identification of lipase producing fungi from local olive oil manufacture in East of Algeria. Sci. Study Res: Chem Eng. Biotechnol. Food Ind. 19, 013-022 (2018).

4) Sahay, S.; Chouhan, D. Study on the potential of cold-active lipases from psychrotrophic fungi for detergent formulation. J. Genet. Eng. Biotechnol. 16, 319-325 (2018).

5) Brault, G.; Shareck, F.; Hurtubise, Y.; Lépine, F.; Doucet, N. Short-chain flavor ester synthesis in organic media by an E. coli whole-cell biocatalyst expressing a newly characterized heterologous lipase. PLoS One 9, e91872 (2014).

6) de Oliveira, U.M.F.; de Matos, L.J.B.L.; de Souza, M.C.M.; Pinheiro, B.B.; dos Santos, J.C.S.; Gonçalves, L.R.B. Effect of the presence of surfactants and immobilization conditions on catalysts’ properties of Rhizomucor miehei lipase onto chitosan. Appl. Biochem. Biotechnol. 184, 1263-1285 (2018).

7) Dill, L.P.; Kochepka, D.M.; Krieger, N.; Ramos, L.P. Synthesis of fatty acid ethyl esters with conventional and microwave heating systems using the free lipase B from Candida antarctica. Biocat. Biotransform. 37, 25-34 (2018).

8) Hakalin, N.L.S.; Molina-Gutiérrez, M.; Prieto, A.; Jesús Martínez, M. Optimization of lipase-catalyzed synthesis of β-sitostanol esters by response surface methodology. Food Chem. 261, 139-148 (2018).

9) Martínez-Ruiz, A.; Tovar-Castro, L.; García, H.S.; Saucedo-Castañeda, G.; Favela-Torre, E. Continuous ethyl oleate synthesis by lipases produced by solid-state fermentation by Rhizopus microsporus. Biore sour. Technol. 265, 52-58 (2018).

10) Chopra, N.; Kaur, J. Point mutation Arg153-His at surface of Bacillus lipase contributing towards increased thermostability and ester synthesis: insight into molecular network. Mol. Cell Biochem. 443, 159-168 (2018).

11) Garlapati, V.K.; Banerjee, R. Solvent-free synthesis of flavor esters through immobilized lipase mediated trans-esterification. Enzyme Res. 2013, 1-6 (2013).

12) de Souza, M.C.M.; dos Santos, K.P.; Freire, R.M.; Barreto, A.C.H.; Fechine, P.B.A.; Gonçalves, L.R.B. Production of flavor esters catalyzed by lipase b from Candida antarctica immobilized on magnetic nanoparticles. Brazilian J. Chem. Eng. 34, 681-690 (2018).

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

14) Bradford, M.M. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Chem. 72, 248-254 (1976).

15) Bhardwaj, K.K.; Saun, N.K.; Gupta, R. Immobilization of lipase from Geobacillus sp. and its application in synthesis of methyl salicylate. J. Oleo Sci. 66, 391-398 (2017).

16) Dong, F.; Li, L.; Lin, L.; He, D.; Chen, J.; Wei, W.; Wei, D. Trans-esterification synthesis of chloramphenicol esters with the lipase from Bacillus amyloliquifaciens. Molecules 22, 1523-1534 (2017).

17) Mayilvahanan, A.; Ramchary, A.; Niraikulam, A.; Kuppuswami, G.M.; Ramudu, K.N. A green process for starch oleate synthesis by Cryptococcus sp. MTCC 5455 lipase and its potential as an emulsifying agent. Starch 71, 1700325 (2019).

18) Abdullah, A.; Gani, S.S.S.; Hin, T.Y.Y.; Haiyee, Z.A.; Zaidan, U.H.; Kassim, M.A.; Halmi, M.I.E. Lipase-catalyzed synthesis of red pitaya (Hylocereus polyrhizus) seed oil esters for cosmeceutical applications; process optimization using response surface methodology. RSC Adv. 9, 5599-5609 (2019).