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Synthesis of Banana Flavor by Celite Immobilized Bacterial Lipase

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ABSTRACT

A purified alkaline thermotolerant lipase was immobilized on the Celite-545 matrix. The matrix shows a binding efficiency of 80 % for lipase of *B. licheniformis* with a specific activity of 1.02 U/mg of protein. The immobilized lipase shows 50 % activity up to 70 °C. Isoamyl butyrate (banana flavor) was synthesized by the esterification reaction of isoamyl alcohol and butyric acid in an n-hexane medium. Under optimal experimental conditions, a maximum ester conversion percentage of 91.1 % was reached after 50 min, for the esterification reaction performed at equimolar ratio alcohol: acid at 100 and 100 mM of each substrate. The product (isoamyl butyrate) was confirmed by Gas chromatography (GC). The application of the biocatalyst prepared showed to be a fruitful strategy for flavor ester synthesis in a non-aqueous medium. The addition of molecular sieves (50 mg) to the reaction cocktail promoted the ester yield. The immobilized lipase was reused the up to 8thcycle.

Keywords: *Bacillus licheniformis* MTCC-10498, Celite-545, immobilized lipase, esterification.

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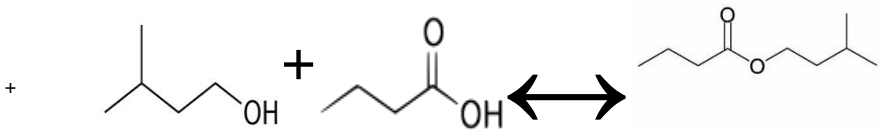
1.Introduction

Lipases are a group of biocatalysts that catalyze the breakdown of oils and fats, with subsequent release of free fatty acids, acylglycerol, and glycerol [1, 2]. In addition, lipases can catalyze the hydrolysis and synthesis of a broad range of esters, while retaining high enantio or regioselectivity [3]. This combination of broad substrate range and high selectivity makes lipases ideal catalysts for organic synthesis [4, 5]. The use of lipases to carry out esterification alleviates the necessity of a wide variety of complex post-reaction separation processes and thus leads to lower overall operation costs [6]. However, lipase- catalyzed reactions, have, herewith a major inconvenience mainly associated with low conversions when compared to traditional chemical processes, if crude commercial enzyme preparations are employed. These intrinsically low volumetric productivities may lead to products quantitatively less pure than those obtained via chemical synthesis. Such a drawback can be coupled with biocatalyst inhibition byproducts and/or substrates and biocatalyst inactivation by heat (thermal inactivation) or by chemical inactivation [6, 7]. To overcome such limits the immobilization of lipases is often recommended [5, 8, 9]. *Bacillus licheniformis* MTCC-10498 used in the present study was isolated from the hot- water spring of Tattapani, Mandi (India). The microbial lipase from *Bacillus licheniformis* MTCC-10498 was purified and successfully immobilized on Celite-545 [10, 11] by physical adsorption rendering active and stable immobilized preparation [12]. Isoamyl butyrate is in high demand as a component of pineapple flavor in the food, beverage, and pharmaceutical industries [13]. Considering the high demand for isoamyl butyrate as a pineapple-flavoring agent, an attempt has been made to synthesize (Fig. 1) this ester by optimizing various reaction parameters for maximization of isoamyl butyrate yield [14].



Isoamyl alcohol Butyric acid Lipase Isobutyl butyrate

Fig. 1 Lipase catalyzed synthesis of isoamyl butyrate.

2.Material and Methods

2.1 Chemicals

Isoamyl alcohol (98% pure) and butyric acid (98% pure) were obtained from MerckChemical Co. (Darmstadt, Germany). Molecular sieves (3A˚ x 1.5mm) were purchased from E. Merck (India) Limited, Worli, Mumbai, India. Celite-545 (matrix) was purchased from SD Fine limited, Mumbai, India. All other chemicals were of analytical grade and obtained from Hi-Media (Mumbai, India).

2.2 Enzyme preparation

2.2.1 Enzyme precipitation

The cell-free supernatant obtained after 36 h of lipase production was used for the purification of bacterial lipase. A required amount of ammonium sulphate was added to the supernatant to achieve 80% saturation [15]. The contents were thoroughly mixed and kept at 4 ºC overnight to achieve maximum precipitation after the precipitate (protein- ammonium sulphate conjugate) was sedimented by centrifugation at 12,000 X g for 30 minat 4 ºC. The precipitates were reconstituted in a minimal volume of buffer (Tris 0.05 M; pH 8.0).

2.2.2 Salt removal by dialysis

The precipitates transferred into a dialysis apparatus were dialyzed against Tris buffer (0.05M, pH 8.5) at a regular interval toto to completely remove ammonium sulphate. Finally, the lipase was assayed in the dialysate and was further concentrated using the freeze-drying technique. The concentrated lipase was stored at -20ºC until further use. Thedialysate was assayed for protein content and lipase activity.

2.2.3 Hydrophobic interaction (Octyl-Sepharose) chromatography

A column of pre-swollen Octyl Sepharose (Sigma Chemical Co., MO, Saint Louis, USA) was packed (V_t = 25 $cm^{3)}$ in a sintered glass column. Equilibration was done using 20 ml of start buffer (mixture of 50 mM sodium phosphate and 1.0 M ammonium-sulphate,pH 8.0) at a flow rate of 1.0 ml/ min. The dialyzed lipase was loaded on the column. The elution was performed with 20 ml of elution buffer (50 mM Sodium phosphate, pH 7.2). All fractions (3 ml) were assayed for both lipase activity and protein content. The fraction showing lipase activities was pooled (14 ml) and quantified. Fold purification, as well as the yield of lipase, was determined at each stage of the purification procedure. Purified lipase was stored at -20 ºC until subsequent use.

2.2.4 Assay of lipase activity and Unit of lipase activity

The lipase activity was assayed using p-nitrophenyl palmitate (p-NPP), a chromomeric substrate. Lipase activity of crude lipase, purified or matrix-bound lipase wasassayed employing a modified colorimetric method [16]. A standard quantitative assay fordetermining the protein content in a solution was used [17]. The enzyme activity was defined as a □mole (s) of *p*-nitrophenol released per min by one ml of free enzyme or per mg of the immobilized enzyme (weight of matrix included) under standard assay conditions. Specific activity was expressed as □a mole (s) of the *p*-nitrophenol released permin per mg of protein.

2.3 Esterification reactions

2.3.1 Preparation of standard profile of isoamyl butyrate

A reference curve was plotted between the molar concentration of isoamyl butyrate and the area under the peak (retention time 1.0-1.3 min). A sample size of 2 µl was used for GLC analysis. The GLC (Micro-9100, Netel chromatographs, was programmed for oven temperature 150 ºC, injector 160 ºC, and FID temperature 170 ºC. The assay of isoamyl butyrate was performed on a 10% SE Chromo WHP packed column (2 meters X
1.8 inches) using N_2 as a carrier gas (flow rate 30 ml/min). After the completion of the esterification reaction at specified time intervals, the reaction mixture was withdrawn (2 µl) and subjected to analysis of isoamyl butyrate by GLC.

2.3.2 Synthesis of isoamyl butyrate

The celite-bound lipase was washed in 1 ml of n-hexane (solvent) at room temperature. Thereafter the matrix was recovered by decantation of n-hexane and used to immobilize lipase onto it. The esterification was carried out using Celite bound lipase by manipulating various

physical and kinetic parameters (relative proportions of reactants, temperature, time, molecular sieve, salt ions, and reusability). The isoamyl butyrate formedwas determined by GLC analysis.

2.3.3 Effect of the relative proportion of reactant on isoamyl butyrate synthesis

The effect of the relative molar ratio of isoamyl alcohol and butyric acid on the synthesis of isoamyl butyrate was determined by keeping the concentration of one of the reactants (isoamyl alcohol or butyric acid) at 100 mm and varying the concentration of the second reactant (25-100mm) in a reaction volume of 2 ml in n-hexane. The esterification was carried out using matrix-bound lipase (20 mg) at 50°C in a 10 ml plastic-capped glass vial for 6 h under continuous shaking. The isoamyl butyrate formed in each of the combinations of the reactants was determined by GLC analysis.

2.3.4 Effect of bound lipase concentration for the synthesis of isoamyl butyrate

The effect of bound lipase concentration on ester formation was evaluated by enhancing the concentration of bound lipase (5, 10, 15, 20, 25, and 30 mg) in the reactionmixture comprising 100: 100 (mM) isoamyl alcohol and butyric acid at 50 ºC in 6 h reactiontime.

2.3.5 Effect of reaction temperature for the synthesis of isoamyl butyrate

Temperature for the esterification reaction was studied at 35, 40, 45, 50, 55, 60, 65, 70, and 75 ºC for 6 h in n-hexane using 20 mg bound lipase. The isoamyl butyrate formedin each case was determined by GLC analysis.

2.3.6 Effect of reaction time for synthesis of isoamyl butyrate

The reaction mixture 1ml contained 20 mg of bound lipase and 100 mM final concentration of isoamyl alcohol and butyric acid in n-hexane in a 10 ml plastic capped glass vial. The reaction mixture was incubated at 45 ºC in an incubator under shaking conditions (120 rpm) for up to 8 h. The reaction mixture was sampled (2 µl) in duplicate at an interval of 1 h and subjected to analysis by GLC for the formation of isoamyl butyrate.

2.3.7 Effect of addition of molecular sieves on the synthesis of isoamyl butyrate

To the above reaction mixture prepared in n-hexane, varying amounts (25-300 mg) of molecular sieves were added. The esterification was carried out in duplicate by adding 20 mg of bound lipase at 45 ºC with continuous shaking for 5 h. Isoamyl butyrate synthesized in each case was determined by GLC.

2.3.8 Effect of salt ions on ester synthesis

The effects of salt ions were studied by pre-incubating salt ions at a concentration of 1 mM in 1 ml n-hexane. Each salt ion (50 µl) was mixed with the reaction mixture.

2.3.9 Reusability of bound lipase on the synthesis of isoamyl butyrate

The formation of isoamyl butyrate from butyric acid and ethyl alcohol (100: 100mM) with bound lipase was assayed for 8 cycles of 5 h each. After each cycle of esterification, the bound lipase was washed twice for 5 min each in 1ml n-hexane at room temperature. Thereafter, n-hexane was decanted and the matrix was reused for a fresh cycleof ester synthesis under similar conditions.

2.3.10 Bioprocess development at the 50 ml level for the isoamyl butyrate

Under optimized conditions, the 2 ml reaction volume was scaled up to a 50 ml reaction volume. The esterification was performed in a 250 ml capped flask at 45°C for 6 h under shaking and GLC assayed the ester synthesized.

3. Results and discussion

3.1 Enzyme purification andimmobilization

The production broth when inoculated with 10% (v/v) of 36 h old seed culture produced an optimal amount of lipase activity (2.0 U/ml), specific activity (1.01 U/mg), and protein (1.88 mg/ml) at 72 h post-inoculation under optimized conditions. The culture broth rendered cell-free by centrifugation at 10,000 x g at 4°C for 10 min was filtered through Whatman filter paper no.1 and assayed for lipase activity using p-NPP. The cell-free broth when salted out resulted in maximal lipase activity in the pellet obtained using 80% (w/v) of ammonium sulphates. The sedimented precipitates were reconstituted in 10 ml of Tris buffer (0.05 M, pH 8.0). This suspension was transferred into a dialysis bag wassubjected to extensive dialysis against Tris buffer (0.05 M, pH 8.0). This dialyzate was loaded on the octyl-sepharose column and used for immobilization. The swelling capacity of the Celite matrix was 1.6 fold. The purified lipase (2.0 U/ml; protein content 0.0612 mg and specific activity 32.66 U/mg; was used for immobilization by adsorption on Celite-
545. The Celite showed 80% binding of purified lipase. The immobilized lipase of *B. licheniformis* MTCC-10498 possessed a specific activity of 26.12 U/mg. The above lipase was then subjected to check its potential in the synthesis of isoamyl butyrate by manipulating various reaction parameters.

3.2 Esterification reactions

3.2.1 Effect of relative proportions of the reactant on the synthesis of isoamyl butyrate

The formation of ester was highest when butyric acid and alcohol were used as 100:100 mM in n-hexane under continuous shaking after 6 h at 50 ºC. In the subsequent reactions, the same concentration of reactants was employed. The amount of isoamyl butyrate was estimated from a standard profile of pure isoamyl butyrate (Merck, Germany).In past, we have reported that optimal synthesis of ethyl cinnamate occurred at an equimolar proportion of reactants (100 mM each) in DMSO [18]. A relative excess molar concentration of either acid or alcohol would denature or precipitate the protein, and such an effect would inactivate the biocatalyst and thus would decrease the ester synthesis. Also, it was likely that excess acid might have partially inactivated the bound lipase because of charge alteration/charge rearrangement at the catalytic site of the Celite-bound biocatalyst. In past, we have reported the optimal synthesis of ethyl laurate and ethyl propionate at an equimolar proportion of reactants (100 mM each) in n-nonane [19]. In another study, the effect of acetic acid concentration on esterification reaction using lipase SP435 was studied[20].

3.2.2 Effect of biocatalyst loadon the synthesis of isoamyl butyrate

The effect of varying concentrations of biocatalyst on ester formation was evaluated by increasing the concentration of the Celite-bound lipase (*i.e.*, 5, 10, 15, 20, 25, and 30 mg) in the reaction system. The esterification reaction with isoamyl alcohol: butyric acid (100 mM: 100 mM) in n-hexane was performed. The formation of ester remained more or less the same with an increase in the concentration of matrix-bound lipase under continuous shaking conditions (120 rpm) after 6 h at 50 ˚C. In the subsequent esterification reactions, 20 mg of matrix-bound lipase was used for bio-catalysis.

3.2.3 Effect of reaction temperature and reaction time for isoamyl butyrate synthesis

The effect of change in the reaction temperature on the synthesis of isoamyl butyrate by immobilized lipase was studied up to 35-75 ˚C. Maximum synthesis (91.1 mM) of isoamyl butyrate was obtained at 45 ˚C after 6 h. At 70 ˚C, there was a decrease (52 mM) in the ester synthesis, which might be on account of the denaturation of the lipase. At 75 ˚C there was a marked decrease in ester synthesis. The effect of reaction time on thesynthesis of isoamyl butyrate using immobilized lipase was studied at a temperature of 45
˚C in n-hexane under shaking conditions up to 8 h. The synthesis of the ester was time- dependent and a maximum amount of isoamyl butyrate (91.6 mM) was produced after 5 h of reaction when isoamyl alcohol and butyric acid were used at 100 mM each in n-hexane. Thus in subsequent reaction, a reaction time of 5 h was considered optimum to perform synthesis of isoamyl butyrate using bound lipase. In a similar, study isoamyl acetate was synthesized by celite-immobilized recombinant CS-2 lipase for 10 h at 55˚C [19].

3.2.4 Effect of addition of molecular sieves on esterification

The esterification reaction resulted in the formation of water as a by-product of the reaction, and its removal using molecular sieves (3Å) might enhance the synthesis of the ester by pushing the reaction equilibrium in the forward direction. It appeared that an increase in the concentration of the molecular sieve (25-50 mg) provided a corresponding increase in the physically active surface area of the molecular sieve, which readily absorbed water and thus promoted forward reaction kinetics [20]. However, when the effect of the molecular sieve was studied by adding a

molecular sieve (<50 mg per reaction volume), a gradual decline (91.6 to 90.3mM) in the amount of ester formed was noticed. Thus the addition of molecular sieves at 50 mg was found to be optimum for the synthesis of isoamylbutyrate.

3.2.5 Effect of salt ions on ester synthesis

Most of the salt ions had an inhibitory effect on ester synthesis. The maximum inhibition was done by mercuric ions (Hg^{2+}). The salts of the heavy metals such as Fe^{2+}, Zn^{2+}, Hg^{2+}, and Fe^{3+} strongly inhibited the lipase of Pseudomonas aeruginosa KKA-5, suggesting that they were able to alter the enzyme conformation [22]. Divalent cations suchas Co2+, Ni2+, Hg2+, and Sn2+ drastically inhibit lipase activity [23].

3.2.6 Reusability of immobilized enzyme for ester synthesis

The bound lipase when repetitively used to perform esterification at 45 ^{o}C under optimized conditions resulted in 15.7 mM isoamyl butyrate after the 8th cycle of esterification. In each cycle esterification was performed for 5 h. In a previous study, silicaimmobilized lipase was used for up to 13 cycles in the synthesis of ethyl propionate [23]. In another study using hydrogel bound lipase was used for 4 cycles [18].

3.2.7 Bioprocess development at the 50 ml level for isoamyl butyrate synthesis

Under optimized conditions, a 2 ml reaction volume was scaled up to the 50 ml Teflon capped flask. When the reaction volume was increased from 2 to 50 ml under optimized conditions the conversion was 86.7 mM. In an earlier study, in the synthesis of geranyl acetate [24], the conversion was noticed as 30.16 mM.

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References

1. Vulfson EN (1994). Industrial application of lipases: lipases-there structure, biochemistry application. Woolley P, and Peterson SB (Eds.). Cambridge University Press, Cambridge, pp 271-288
2. Castro HF, Mendes AA, Santos JC and Aguiar CL (2004). Modificação de óleose gorduras por biotransformação. Quim Nova 27:146-156
3. Pandey A, Benjamin S, Soccol CR, Nigam PN, Krieger, Soccol VT (1999). The realm of microbial lipases in biotechnology. Biotechnol Appl Biochem 29:119–131
4. Lai DT and O'Connor JO (1999). Studies of short chain alkyl esters catalyzed by goat pregastric lipases. J Mol catalysis B Enzymatic 6:411-420
5. Villeneuve P, Muderhwa JM, Graille JM, and Haas MJ, (2000). Customizing lipases for biocatalysis: A survey of chemical, physical and molecular biological approaches. J Mol catalysis B Enzymatic 9:113-148
6. Yahya ARM, Anderson WA and Moo-Young M (1998). Ester synthesis in lipase-catalyzed reactions. Enzyme Microb Technol 23:438-450
7. Halling PJ (1984). Effect of water on equilibria catalyzed by hydrolytic enzymes in biphasic reaction systems. Enzyme Microb Technol 6:513-516
8. Balca˜o VM, Paiva AL, Malcata FX. (1996). Enzyme Microb Technol 18:392-416
9. Tischer W, Kasche V (1999) Immobilized enzymes: crystals or carriers? TrendsBiotechnol 17: 326-335
10. Kumar A, Kanwar SS (2011). Synthesis of ethyl ferulate in the organic mediumusing Celite-immobilized lipase. Biores Technol 102: 2162-2167
11. Peng R, Lin J, Wei D (2011). Synthesis of isoamyl acetate in n-Heptane by the recombinant CS-2 lipase immobilized on kieselguhr. Afr J Food Sci Technol 2: 59-66
12. Oliveira PC, Alves GM, Castro HF (2000). Immobilization studies and catalytic properties of microbial lipases onto styrene-divinylbenzene copolymer. Biochem Eng J 5:63-71
13. Varma MN and Madras G (2008). Kinetics of synthesis of isoamyl butyrate by esterification and transesterification in supercritical

carbon dioxide. J Chem Technol Biotechnol 83:1135-1144

14. Anschau, Andréia & Aragão, Vitor & Porciuncula, Barbara & Kalil, Susana & Burkert, Carlos & Burkert, Janaína. (2011). Enzymatic Synthesis Optimization of Isoamyl Butyrate. Journal of the Brazilian Chemical Society. 22. 2148-2156. 10.1590/S0103-50532011001100018.
15. Scopes RK (1987). Ammonium sulfate precipitation table. In: Protein purification:principle and practice. Springer-Verlag, New York, pp. 45-54
16. Winkler UK, Stuckmann M (1979). Glucogen, hyaluronate, and some other polysaccharides greatly enhance the formation of exolipase by *Serratia marcescens.* J Bacteriol 138:663-670
17. Lowry OH, Rosebrough NJ, Farr AL, Randall RJ (1951). Protein measurement withthe Folin-Phenol reagents. J Biol Chem 193:265-275
18. Sharma CK, Chauhan GS, Kanwar SS (2011). Synthesis of medically important ethyl cinnamate ester by porcine pancreatic lipase immobilized on poly (AAc-*co*- HPMA-*cl*-EGDMA) hydrogel. J Appl Polym Sci 121: 2674–2679
19. Peng R, Lin J and Wei D (2011). Co-expression of an organic solvent tolerant lipaseand its cognate foldase of Pseudomonas aeruginosa CS-2 and the application of theimmobilized recombinant lipase. Applied Microbiol Biotechnol, 165(3-4): 926-937
20. Izumi T, and Eda Y (1995). Enzymatic synthesis of (*R*)- and (*S*)-2-cyclohexane-1-ol. J Chem Technol Biotechnol. 62: 25–29
21. Wehtje E, Kaur J, Adlercreutz P, Chand S & Mattiasson B (1997). Water-activity controlling enzymatic esterification processes. Enzyme and Microbial Technol 21: 502-510
22. Peng R, Lin J and Wei D (2011). Synthesis of isoamyl acetate in *n*-heptane by the recombinant CS-2 lipase immobilized on kieselguhr. African J of Food Sc and Technology 2: 59-66
23. Kuwabara K, Watanabe Y, Adachi S, Nakanishi K and Mutsuno R (2003). Synthesis of 6-O-unsaturated acyl L-ascorbates by immobilized lipase in acetone in the presence of molecular sieve. Biochem Eng J 16: 17-23
24. Sharon C, Furugoh S, Yamakido T, Ogawa H, Kato, Y (1998). Purification and characterization of a Pseudomonas aeruginosa KKA-5 lipase from and its role in castor oil hydrolysis. J Ind Microb Biotech 20, 304-307