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Synthesis of Pine-Apple Flavor by Celite-Immobilized Lipase

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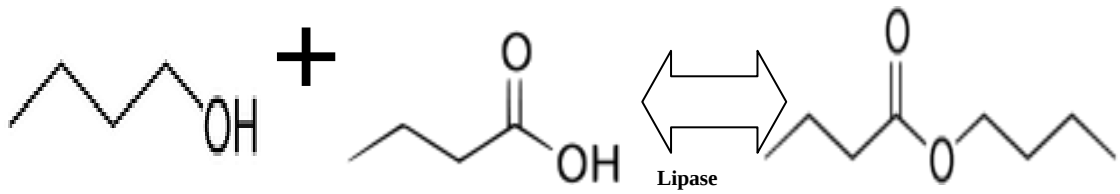
ABSTRACT

Alkaline-thermotolerant lipase from *Bacillus licheniformis* MTCC-10498 was purified and immobilized on Celite-545 matrix. The matrix shows a binding efficiency of 80% for lipase of *B. licheniformis* with a specific activity 1.02 U/mg of protein. The immobilized lipase shows 50% activity up to 70°C. This immobilized lipase was checked for its efficacy in the synthesis of butyl butyrate, a flavor ester. The formation of ester was highest when butyric acid and butyl alcohol were as in an equimolar ratio (100 mM each) in n-hexane under continuous shaking (120 rpm) along with 20 mg celite-bound lipase at 45°C in 5 h. The addition of molecular sieves (50 mg) to the reaction cocktail promoted the ester yield. The immobilized lipase was reused up to 8th cycle. The immobilized lipase when used for the esterification of butyl alcohol and butyric acid under above-optimized conditions resulted in butyl butyrate (91.6%) conversion in n-hexane.

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

1. INTRODUCTION

Lipases (triacylglycerol acyl-hydrolases EC 3.1.1.3) are an important group of enzymes that catalyze the breakdown of oils and fats, with the subsequent release of free fatty acids, acylglycerols, and glycerol [1, 2]. In addition, lipases can catalyze the hydrolysis and synthesis of a broad range of natural and unnatural 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]. Lipase-catalyzed reactions, generally have low conversions than chemical processes, if crude commercial enzyme preparations are employed which results in low volumetric productivities less pure product. Such a drawback can be coupled with biocatalyst inhibition by-products and/or substrates and biocatalyst inactivation by heat (thermal inactivation) or by chemical inactivation [6, 7]. To overcome such limitations, the immobilization of lipases is often recommended [5, 8, 9]. *Bacillus licheniformis* MTCC-10498 used in the present study was isolated from hot-water spring of Tattapani, Mandi, Himachal Pradesh (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]. Butyl butyrate is in high demand as a component of pineapple flavour in food, beverage and pharmaceutical industries [13]. Considering the high demand of butyl 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 butyl butyrate yield [14].



Butyl alcohol Butyric acid Butyl butyrate

Fig. 1: Synthesis of butyl butyrate using lipase.

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2. Material and Methods

2.1 Chemicals

Butyl alcohol (98% pure) and butyric acid (98% pure) were obtained from Merck Chemical 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 Chemical 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 (bacterial) supernatant obtained after 36 h of lipase production was used for purification of lipase activity. Ammonium sulphate in appropriate amount was added to the supernatant to achieve 80% saturation [15]. Thoroughly mixed content kept at 4 ºC overnight to maximize the precipitation. There after the precipitate (protein-ammonium sulphate conjugate) was sedimented by centrifugation at 12,000 X g for 30 min at 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 of 2 h so as to completely remove ammonium sulphate. Finally the lipase was assayed in the dialyzate and was further concentrated using freeze-drying technique. The concentrated lipase was stored at -20ºC until further used. The dialyzate was assayed for protein content and lipase activity.

2.2.3 Hydrophobic interaction (Octyl-Sepharose) chromatography

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 fractions showing lipase activities were pooled (14 ml) and quantified. At each stage of the purification procedure, the yield of lipase and fold purification was determined. Purified lipase was stored at -20 ºC until subsequent use.

2.2.4 Assay of lipase activity and Unit of lipase activity

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

2.3 Esterification reactions

2.3.1 Preparation of standard profile of butyl butyrate

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

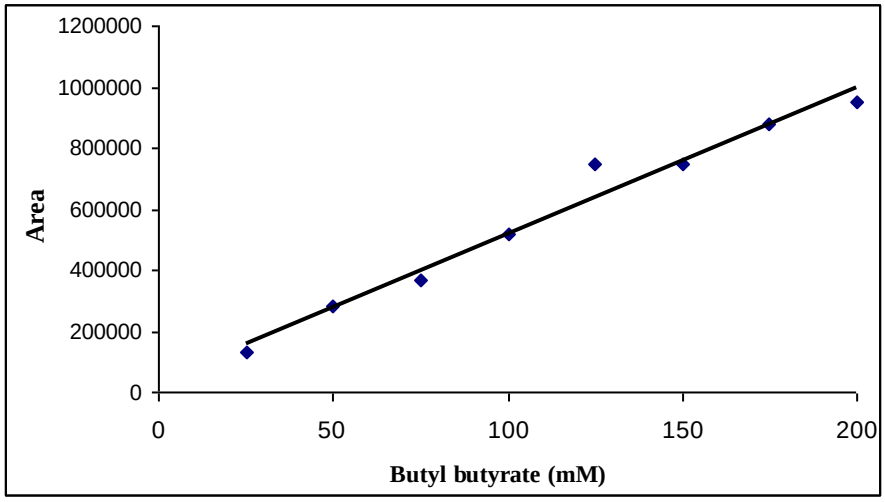

Fig. 2: Standard profile of butyl butyrate in n-hexane.

2.3.2 Synthesis of butyl 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 butyl butyrate formed was determined by GLC analysis.

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

The effect of the relative molar ratio of butyl alcohol and butyric acid on the synthesis of butyl butyrate was determined by keeping the concentration of one of the reactants (butyl 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 butyl 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 butyl 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 reaction mixture comprising 100: 100 (mM) butyl alcohol and butyric acid at 50 ºC in 6 h reaction time.

2.3.5 Effect of reaction temperature for synthesis of butyl butyrate

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

2.3.6 Effect of reaction time for synthesis of butyl butyrate

The reaction mixture was 1ml contained bound lipase 20 mg and 100 mM concentration of ethyl alcohol and butyric acid in n-hexane in 10 ml plastic capped glass vial. The reaction mixture was incubated at 45 ºC in an incubator under shaking conditions (120 rpm) 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 butyl butyrate.

2.3.7 Effect of addition of molecular sieves on a synthesis of butyl 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. Butyl butyrate synthesized in each case was determined by GLC.

2.3.8 Effect of salt ions on ester synthesis

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 butyl butyrate

The formation of butyl 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 cycle of ester synthesis under similar conditions.

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

Under optimized conditions, the 2 ml reaction volume was scaled up to a 50 ml reaction volume. The esterification was perfomed 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 and immobilization

The production broth when inoculated with 10% (v/v) of 36 h old seed culture produced 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 was subjected to extensive dialysis against Tris buffer (0.05 M, pH 8.0). This dialyzate was loaded on an 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 butyl butyrate by manipulating various reaction parameters.

3.2 Esterification reactions

3.2.1 Effect of relative proportions of reactant on synthesis of butyl butyrate

The formation of ester was highest when butyric acid and alcohol was used as 100: 100 mM in n-hexane under continuous shaking after 6 h at 50 ºC (Fig. 3). In the subsequent reactions, same concentration of reactants was employed. Amount of butyl butyrate was estimated from a standard profile of pure butyl 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 of 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 that optimal synthesis of ethyl laurate and ethyl propionate at an equimolar proportion of reactants (100 mM each) in n-nonane [19]. In another study, effect of acetic acid concentration on esterification reaction using lipase SP435 was studied [20].

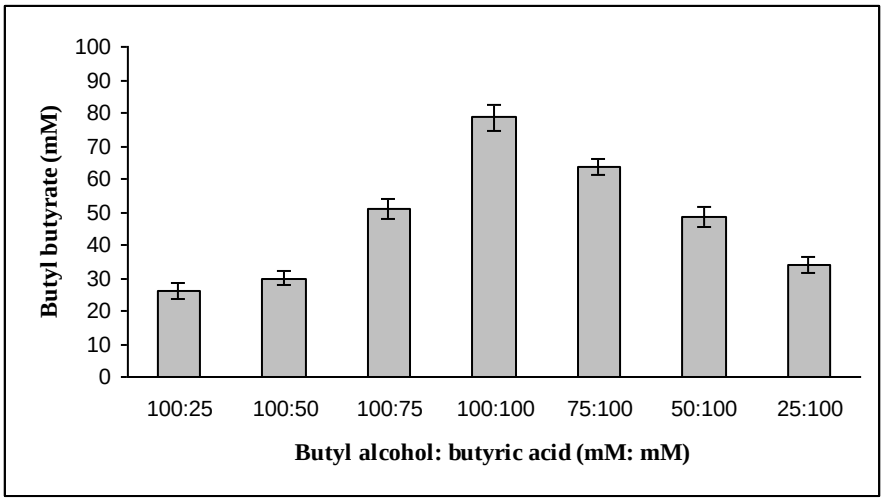

Fig. 3: Effect of relative proportion of reactants butyl butyrate.

3.2.2 Effect of biocatalyst load on the synthesis of butyl 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 butyl alcohol: butyric acid (100 mM: 100 mM) in n-hexane was performed (Fig. 4). The formation of ester remained more or less the same with an increase in concentration of matrix bound lipase under continuous shaking condition (120 rpm) after 6 h at 50 ˚C. In the subsequent esterification reactions, 20 mg of matrix-bound lipase was used for bio-catalysis.

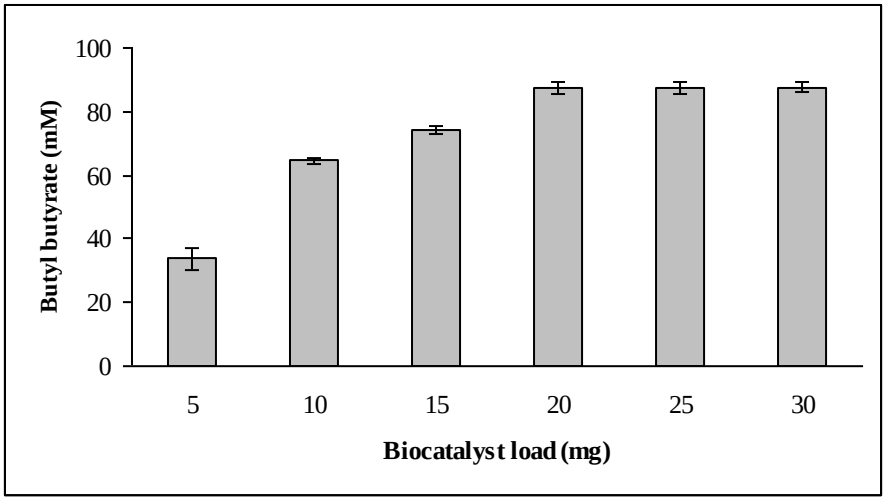

Fig. 4: Effect of biocatalyst load on the synthesis of butyl butyrate.

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

The effect of change in the reaction temperature on the synthesis of butyl butyrate by immobilized lipase was studied up to 35-75 ˚C. Maximum synthesis (87.8 mM) of butyl butyrate was obtained at 45˚C after 6 h (Fig. 5).

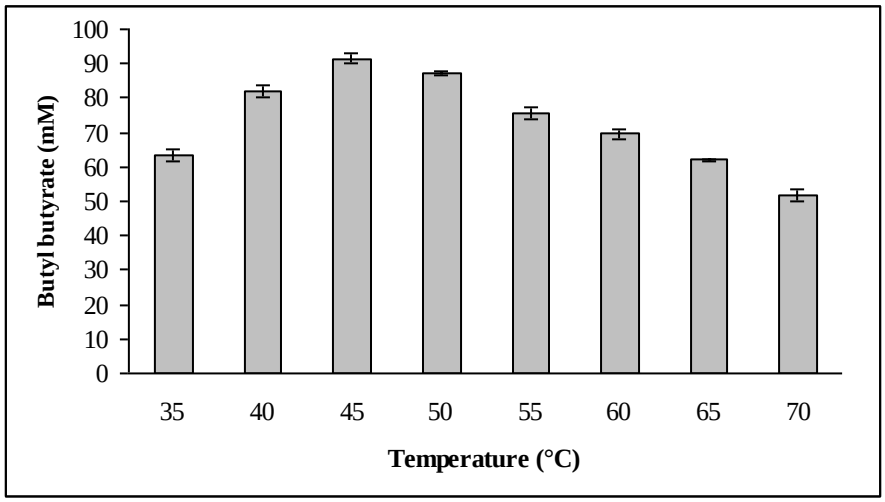

Fig. 5: Effect of reaction temperature on the synthesis of butyl butyrate.

At 70˚C, there was a marked decrease (52 mM) in the ester synthesis, which might be on account of denaturation of the lipase. At 75 ˚C there was no ester synthesis. The effect of reaction time on the synthesis of butyl 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 butyl butyrate (91.6 mM) was produced after 5 h of reaction when butyl alcohol and butyric acid were used at 100 mM each in n-hexane (Fig. 6). Thus in subsequent reaction a reaction time of 5 h was considered optimum to perform synthesis of butyl butyrate using bound lipase. In a similar study butyl acetate was synthesized by celite immobilized recombinant CS-2 lipase in 10 h at 55˚C [19].

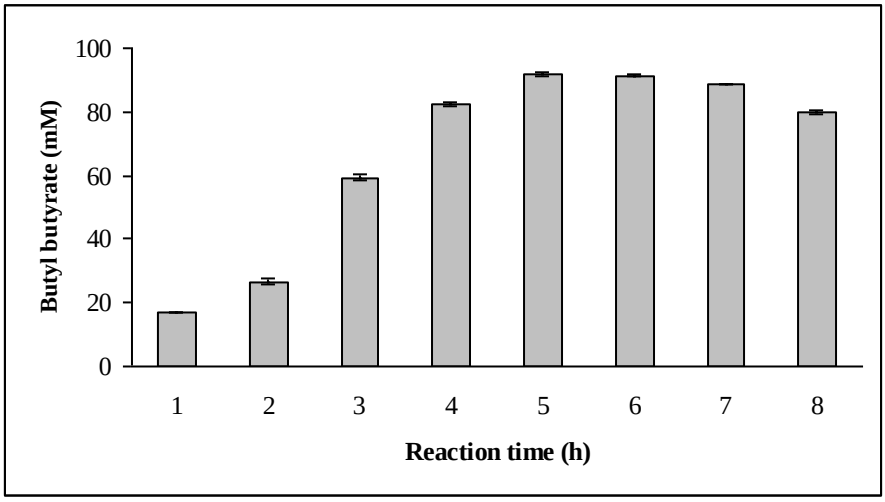

Fig. 6: Effect of reaction time on the synthesis of butyl butyrate.

3.4 Effect of addition of molecular sieves on esterification

The esterification reaction resulted in formation of water as a by-product of the reaction, and its removal using a molecular sieves (3Å) might enhance the synthesis of ester by pushing the reaction equilibrium in the forward direction. It appeared that an increase in the concentration of molecular sieve (25-50 mg) provided a corresponding increase in physically active surface area of the molecular sieve, which readily absorbed water and thus promoted forward reaction kinetics [20]. However, when the effect of molecular sieve was studied by adding a molecular sieves (<50 mg per reaction volume), a gradual decline (91.6 to 90.3mM) in the amount of ester formed was noticed (Fig. 7). Thus addition of molecular sieves at 50 mg was found to be optimum for the synthesis of butyl butyrate.

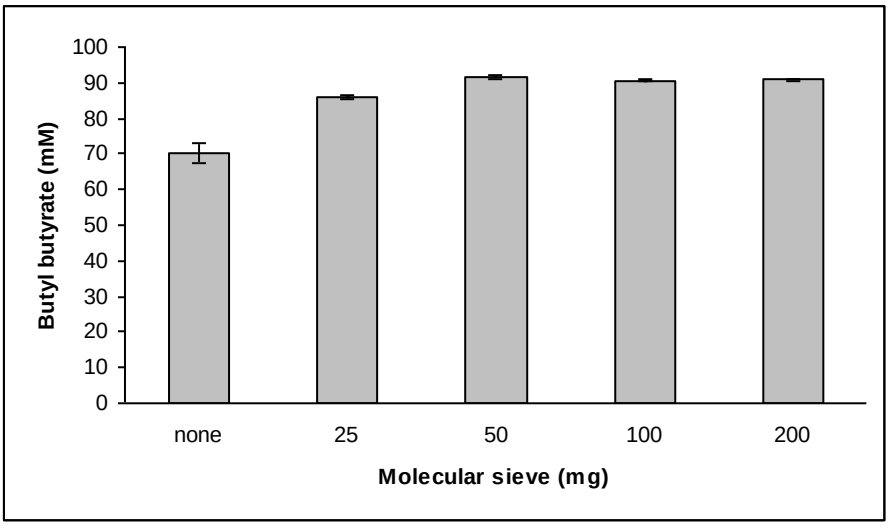

Fig. 7: Effect of molecular sieves on the synthesis of butyl butyrate.

3.5 Effect of salt ions on ester synthesis

Most of the salt ions had inhibitory effect on ester synthesis. The maximum inhibition was done by mercuric ions (Hg^{2+}) as evident from Fig. 8. The salts of the heavy metals such as Fe^{2+}, Zn^{2+}, Hg^{2+}, Fe^{3+} strongly inhibited the lipase of Pseudomonas aeruginosa KKA-5, suggesting that they were able to alter the enzyme conformation [22]. Divalent cations such as Co2+, Ni2+, Hg2+ and Sn2+ drastically inhibited the lipase activity [23].

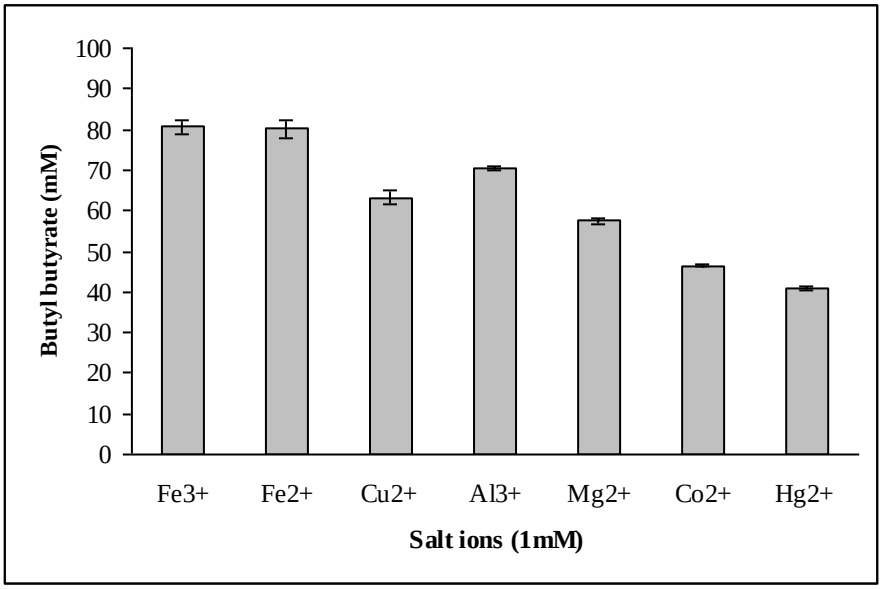

Fig. 8: Effect of salt ions on the synthesis of butyl butyrate.

3.2.7 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 butyl butyrate after 8th cycle of esterification (Fig. 9). In each cycle esterification was performed for 5 h. In a previous study on silica immobilized lipase was used up to 13 cycles in the synthesis of ethyl propionate [23]. In another study using hydrogel bound lipase was used for 4 cycles [18].

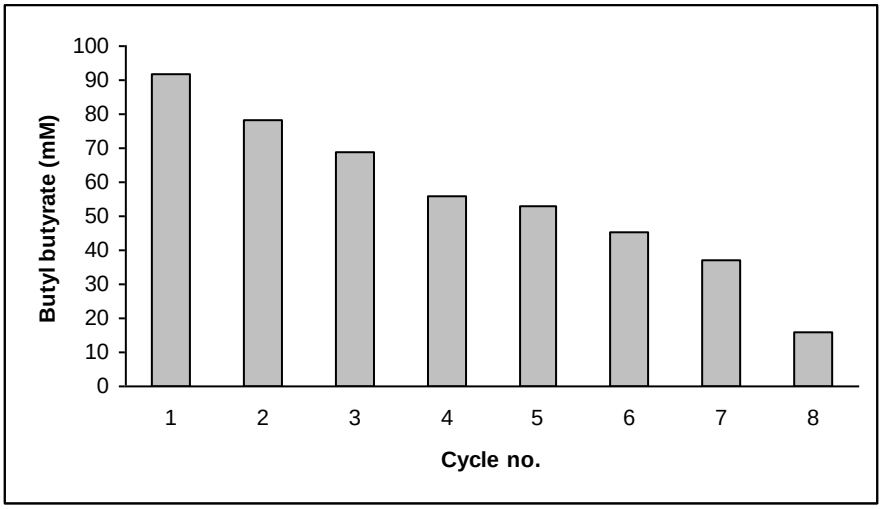

Fig. 9: Reusability of bound lipase on the synthesis of butyl butyrate.

3.2.8 Bioprocess development at the 50 ml level for butyl 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 91.6 mM (Table 1). In an earlier study, in the synthesis of geranyl acetate [24] the conversion was noticed as 30.16 mM.

Table 1: Bioprocess development for the synthesis of butyl butyrate.

Volume (ml)	Butyl butyrate synthesized (mM)
5	91.6± 0.6
10	90.8± 0.1
50	90.7± 0.2

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