Application of Lipase Immobilized on Nylon-6 for the Synthesis of Butyl Acetate by Transesterification Reaction in *n*-Heptane

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ABSTRACT: A purified alkaline thermotolerant bacterial lipase from *Bacillus coagulans* BTS-3 was immobilized on nylon-6 matrix activated by glutaraldehyde. The matrix showed ~ 70% binding efficiency for lipase. The bound lipase was used to perform transesterification in *n*-heptane. The reaction studied was conversion of vinyl acetate and butanol to butyl acetate and vinyl alcohol. Synthesis of butyl acetate was used as a parameter to study the transesterification reaction. The immobilized enzyme achieved ~ 75% conversion of vinyl acetate and butanol (100 mmol/L each) into butyl acetate in *n*-heptane at 55°C in 12 h. When alkane of C-chain lower or higher than *n*-heptane was used as an organic solvent, the conversion of vinyl acetate and butanol to butyl acetate decreased. During the repetitive transesterification under optimal conditions, the nylon bound lipase produced 77.6 mmol/L of butyl acetate after third cycle of reuse.
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Key words: *Bacillus coagulans* BTS-3; lipase; nylon-6; immobilization; transesterification; butyl acetate

INTRODUCTION

Lipases (E.C. 3.1.1.3) are versatile catalysts, which have been employed to catalyze a range of reactions such as esterification, amidation, and transesterification of esters as well as organic carbonates. They are highly stable under adverse conditions such as organic solvents, high temperature, etc. Applications of lipase include production of food additives, chiral intermediates, and pharmaceutical products.[1] Lipases-catalyzed condensation in an organic solvent is useful for the synthesis of esters.[2–6] In the last few years, there has been an increasing interest in the use of enzymes for the biosynthesis of molecules in organic media.[7–9] At present, many esters are industrially manufactured by chemical methods. However, chemical methods involve high temperature or high pressure; it is difficult in many cases to esterify unstable substances such as polyunsaturated fatty acids, ascorbic acid, and polyols. Furthermore, regiospecific acylation of alcohol requires the protection and deprotection steps.[10] Such steps are likely to cause a rise in manufacturing costs of the esters. In case of esters derived for use in food as additives, chemical impurities are not desired.

Many esterification or condensation reactions have been developed by employing a variety of lipases of microbial origin.[11–14] In a lipase-catalyzed reversible reaction (esterification), direction and equilibrium of the reaction are determined by the concentration or activities of substrates and products, temperature, pressure, etc. In the lipase-catalyzed alcoholysis, methyl, ethyl, and vinyl esters have been widely used as substrates. Among the esters, vinyl ester has been extensively employed as a substrate because the by-product vinyl alcohol is almost irreversibly converted to acetaldehyde to increase the conversion.[15–18] However, acetaldehyde forms a Schiff base with the lipase.[19] (Scheme 1).

n-Butyl acetate is an important solvent in the paint industry. It is often employed in combination with *n*-butanol in paints, as *n*-butanol enhances the resistance to blushing and also reduces the viscosity of the solution. Because of its low water absorption, its high resistance to hydrolysis, and its high solvency, *n*-butyl acetate can also be employed as an extractant in the manufacture of pharmaceutical preparations, and as an ingredient of cleaners, essences, and fragrances.

The microbial lipases that constitute a most versatile group of enzymes have been successfully immobilized on a variety of matrices/supports for performing esterification and transesterification reactions in

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Scheme 1

organic solvents. Immobilized lipases offer economic incentives of enhanced thermal and chemical stability, ease of handling, easy recovery, and reuse relative to nonimmobilized forms.[6,20] Immobilization also facilitates dispersal of enzyme on a solid surface to provide a greater interfacial area and accessibility relative to the use of enzyme powder in low water reaction media. In terms of enzyme activity, the primary effect of the water activity include modulating the balance (equilibrium) between hydrolysis and synthetic processes[21–23] as well as hydration state of an enzyme, the latter regulates plasticity and catalytic activity.[24]

The present study is focused on the effect of various parameters on nylon immobilized lipase-catalyzed synthesis of butyl acetate in *n*-heptane as a model system for the study of transesterification.

MATERIALS AND METHODS

Chemicals and reagents

Vinyl acetate, butanol, and butyl acetate (MERK, Mumbai, India), glutaraldehyde, *n*-hexane, *n*-heptane, *n*-octane, *n*-pentadecane, and *p*-nitrophenyl palmitate (Lancaster Synthesis, England) have been used as received.

Preparation of lipase from *Bacillus coagulans* BTS-3

The thermophilic and alkaliphilic lipase producing bacterium, *B. coagulans* BTS-3, was originally isolated from kitchen waste of a sweet shop.[25] It was grown in a series of 250 mL Erlenmeyer flasks containing 50 mL production medium [yeast extract {0.5% (w/v)}, peptone {0.5% (w/v)}, NaCl {0.05% (w/v)}, $CaCl_2$ {0.005% (w/v)}, refined mustard oil {0.1% (v/v) with 0.5% (w/v) gum arabica}] at a temperature of 55°C, when pH was adjusted to 8.5, for 48 h. The cells were removed from culture broth by centrifugation at 10,000 g for 20 min at 4°C. To 500 mL of cold supernatant, ammonium sulfate was added with constant stirring to achieve 70% saturation. It was subsequently centrifuged at 10,000 g for 40 min and the precipitate was reconstituted in 3 mL of 0.1 mol/L Tris HCl buffer (pH 8.5), the sample was dialyzed against same buffer overnight to remove the ammonium sulfate. The dialysate was loaded on the DEAE-Sepharose column that resulted in a single peak whose lipase activity[26] and protein concentration[27] was determined by standard methods. The DEAE-Sepharose column purified fraction was referred to as purified lipase (PL).[25]

Immobilization of lipase on nylon-6

The PL from *B. coagulans* BTS-3 was immobilized on nylon-6. Increasing amount of protein (2.28–22.8 μg) was added to a fixed amount of matrix (50 mg). Nylon-6 particles were partially hydrolyzed with 6*N* HCl for 30 min, rinsed with distilled water, and contacted with buffered 2.5% glutaraldehyde solution at pH 7.0 for 1 h. Finally, the glutaraldehyde-treated nylon particles were rinsed with buffer and contacted with a purified enzyme at 4°C for overnight. The supernatant was decanted, its volume and protein was estimated. The immobilized protein was determined by subtracting unbound protein obtained in the supernatant from the total protein used for immobilization.

Enzyme assay

The activity of free lipase was assayed by a colorimetric method[26] and immobilized lipase was assayed as previously described.[25] One unit (U) or IU of lipase activity was defined as micromole(s) of 4-nitrophenol released by 1 mL of free enzyme or 1 g of immobilized matrix at 55°C under assay conditions.

Transesterification process for the synthesis of butyl acetate by nylon-6 immobilized lipase

Preparation of standard profile of butyl acetate

A reference profile was prepared using varying concentrations of butyl acetate (25–150 mmol/L) in *n*-heptane. The reference curve was plotted between the molar concentration (mmol/L) of butyl acetate and the corresponding area under the peak (retention time 0.64 min).

Analysis of butyl acetate synthesis by GC

The sample size of 2 μL was used for GLC analysis. The sample was analyzed with GC, using a packed

column (10% SE-30 chrom WHP, 2 m, ''1 × 8'' India, 80–100 mesh size) and flame ionization detector with nitrogen as carrier gas at a flow rate of 30 mL/min. Temperatures used were 150°C for the oven and 160°C for the detector and injector.

Effect of molar concentration and ratio of reactants on transesterification

The effect of molar concentration and molar ratio of butanol and vinyl acetate on synthesis of butyl acetate was determined by keeping the concentration of one of the reactants (butanol/vinyl acetate) at 100 mmol/L and varying the concentration of second reactant (25–100 mmol/L) in a reaction volume of 1 mL in *n*-heptane. The transesterification was carried out with 50 mg of matrix-bound lipase at 55°C in Teflon stoppered-glass vials for 15 h under continuous shaking. The butyl acetate formed in each of the combinations of the reactants was determined by GLC analysis with a sample size of 2 μL.

Optimization of incubation time for transesterification

At first, the nylon-6 immobilized lipase (50 mg) was washed twice in 1 mL of *n*-heptane (solvent) at room temperature. Thereafter, the matrix was recovered by decantation of *n*-heptane and used to catalyze the transesterification of butyl acetate. The reaction mixture (1 mL) contained 50 mg of bound lipase, 100 mmol/L concentration of butanol, and 100 mmol/L concentration of vinyl acetate in *n*-heptane in a Teflon-stoppered glass vial (5 mL capacity). The reaction mixture was incubated at 55°C in a water-bath-incubator under shaking conditions up to 18 h. The reaction mixture was sampled (2 μL) in duplicate at an interval of 3 h, and subjected to analysis by GLC for the formation of butyl acetate.

Effect of C-chain length of solvent (alkane) on transesterification

In the reaction mixture, *n*-heptane, initially employed as a solvent phase, was replaced with *n*-alkanes of varying C-chain length i.e., *n*-pentane, *n*-hexane, *n*-heptane, and *n*-pentadecane. The immobilized lipase (50 mg) was added to the 100 mmol/L of both reactants with each of the solvent mentioned earlier to perform the transesterification. The reaction was carried out for 12 h at 55°C with continuous shaking and the sample size loaded was 2 μL for GLC analysis of butyl acetate formed.

Effect of temperature on transesterification

Temperature for the reaction was optimized by carrying the reaction at 45, 50, 55, 60, and 65°C for 12 h in *n*-heptane using 50 mg immobilized enzyme with 100 m*M* each of the reactants. The butyl acetate formed in each of the combinations of the temperature was determined by GLC analysis with a sample size of 2 μL.

Reusability of immobilized lipase in continuous cycles of transesterification for synthesis of butyl acetate

The formation of butyl acetate from butanol and vinyl acetate (100 mmol/L concentration of each) catalyzed by immobilized lipase in *n*-heptane was used to check the retention of catalytic (esterase) activity of nylon-immobilized enzyme. The immobilized lipase was assayed for three cycles of 12 h each, for butyl acetate formation. After each cycle of transesterification, the immobilized enzyme was washed twice for 5 min each in 1 mL *n*-heptane at room temperature. Thereafter *n*-heptane was decanted and matrix was reused for fresh cycle of ester synthesis under similar conditions.

RESULTS AND DISCUSSION

Purification of bacterial lipase

The cell free broth (500 mL) had a lipase activity of 0.95 U/mL (protein 1.1 mg/mL). The protein was optimally precipitated at 70% (w/v) ammonium sulfate saturation. The precipitate reconstituted in Tris buffer, pH 8.5 was extensively dialyzed against the same buffer. The dialyzed fraction showed lipase activity of (4.2 U/mL, 0.7 mg protein/mL, and specific activity 6.0 U/mg). The chromatography of the dialyzed lipase on DEAE-Sepharose column resulted in a single peak. The fractions showing lipase activities were pooled (1.2 U/mL, 0.02 mg of protein/mL, and specific activity 55 U/mg). The DEAE-column PL showed 61-fold purification.

Protein binding efficiency of the immobilized lipase

The PL of *B. coagulans* BTS-3 was optimally immobilized/bound to glutaraldehyde activated nylon-6. Enzyme [total protein 11.4 μg] when incubated with support (50 mg) gave maximum binding efficiency showing 70% of protein binding and 1.2 U/g of enzyme binding (Fig. 1). Previously, a protein loading of ~15 mg/g was reported for lipase from *Candida rugosa* immobilized on nylon-6.[28] Recently, it was found that 77% of protein in supernatant from *C. rugosa* was immobilized onto the kaolin.[29]

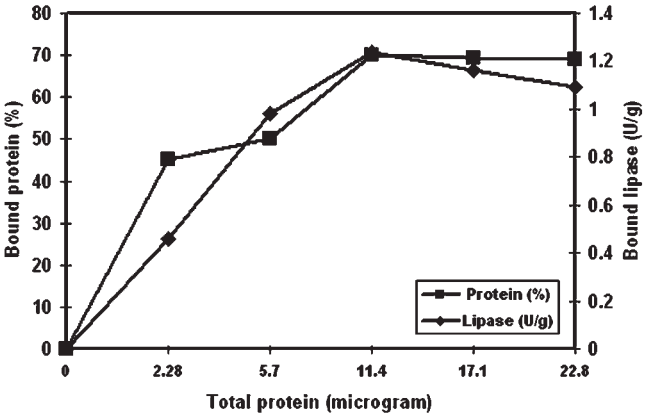

Figure 1 Effect of protein concentration on immobilization of lipase from *Bacillus coagulans* BTS-3 on nylon-6.

Effect of kinetic parameters on transesterification reaction catalyzed by nylon-6 immobilized lipase

Effect of relative concentration of reactants on transesterification

The effect of varying concentrations of butanol and vinyl acetate on synthesis of butyl acetate was evaluated by keeping the concentration of one of the reactants at 100 m*M* in *n*-heptane. A fixed concentration of vinyl acetate (100 mmol/L) and an increased concentration of butanol (25–100 mmol/L) at 55°C under continuous shaking for 15 h increased the synthesis of butyl acetate gradually (10–95 mmol/L). However, at equimolar concentration of both the reactants (100 mmol/L), the formation of butyl acetate increased markedly (Fig. 2). But when the concentration of butanol was fixed (100 mmol/L) and the concentration of vinyl acetate was changed from 100 to 25 mmol/L in *n*-heptane, a sharp decrease in the synthesis of butyl acetate was noticed. Thus, formation of butyl acetate was maximum when 100 mmol/L vinyl alcohol and butanol were used each in *n*-heptane under continuous shaking after 12 h at 55°C. In the subsequent reactions, 100 mmol/L of each of the reactants was employed to achieve transesterification. Recently, it was reported that optimal synthesis of ethyl propionate by a synthetic hydrogel bound-lipase of *Pseudomonas aeruginosa* BTS-2 was achieved when acid and ethanol were used in an equimolar ratio (100 mmol/L) in the reaction mixture.[14] In our recent study, when lipase from *B. coagulans* BTS-3 was immobilized on polyethylene and employed for the synthesis of ethyl propionate, the esterification efficiency increased from 11.8% to 98% on increasing the concentration of the reactants, i.e., ethanol and propionic acid from 100 to 300 mmol/L.[30] Earlier, lipase immobilized on silica aerogels was used in catalytic transesterification of 1-octanol with vinyl laurate. The reactants used in a molar ratio 3 : 5 produced 2.4 mmol/L of octyl laurate.[31]

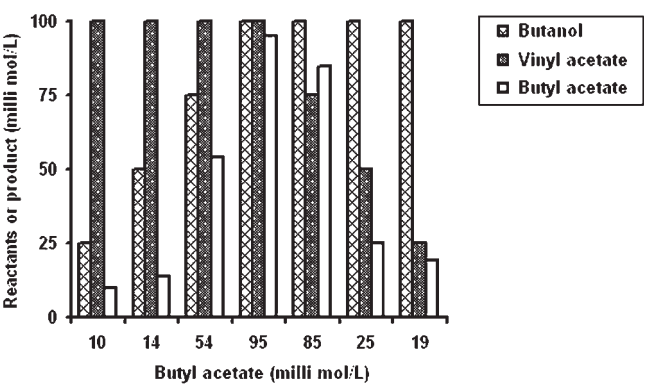

Figure 2 Effect of molar concentration of reactants on synthesis of butyl acetate in *n*-heptane.

Kinetics of transesterification by nylon-6 immobilized lipase

The kinetics of transesterification reaction for synthesis of butyl acetate catalyzed by immobilized lipase was studied up to 18 h at 55°C in *n*-heptane under continuous shaking. The synthesis of butyl acetate increased with an increase in the reaction time till 12 h and started decreasing thereafter (Fig. 3). The concentration of butyl acetate formed was estimated by using reference curve of butyl acetate prepared in *n*-heptane. At 12 h, ~93 mmol/L of butyl acetate was produced. Thus, in the subsequent transesterification reactions, a reaction time of 12 h at 55°C for nylon immobilized-lipase was considered optimum. Earlier, in our recent study, the hydrogel-immobilized lipase of *P. aeroginosa* catalyzed the esteri-

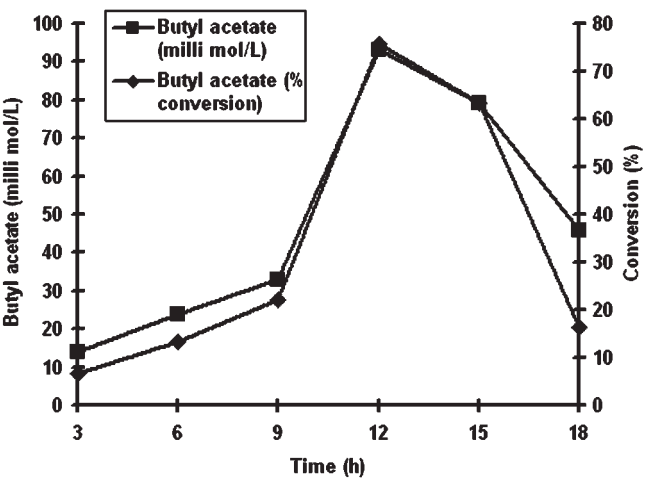

Figure 3 Effect of incubation time on transesterification using nylon-bound lipase.

fication of ethanol and propionic acid into ethyl propionate in a short period of 3 h at 65°C.[14]

Effect of C-chain length of solvent (*n*-alkane) on transesterification

The transesterification reaction was catalyzed by using immobilized lipase in the presence of a series of *n*-alkanes of relatively shorter C-chain length than *n*-heptane. The synthesis of butyl acetate decreased with a decrease in the C-chain length of the alkanes. The synthesis of butyl acetate was maximum (93 mmol/L) at 55°C under continuous shaking, when transesterification was performed in the presence of *n*-heptane as a solvent (Fig. 4). In the presence of a higher C-chain length alkane, *n*-pentadecane also, synthesis of butyl acetate was very less. As an *n*-alkane with a lower or higher C-chain length was used as a solvent, a gradual decrease in the rate of butyl acetate synthesis was noticed. In general, as the log *P* value of an *n*-alkane decreases corresponding to decrease in the C-chain length of the alkanes, the hydrophobicity of the alkanes also decreases in that order. Earlier, butyl acetate synthesis by lipase-catalyzed transesterification has been reported in hexane.[32] In a recent study, using hydrogel-immobilized lipase of *P. aeruginosa* BTS-2 enhanced synthesis of ethyl propionate was reported in *n*-nonane[14] while use of silica-immobilized lipase of *B. coagulans* BTS-3 for synthesis of ethyl propionate gave maximum conversion in *n*-hexane.[33] In our another recent study, use of alkanes of 7–9 carbon chain lengths as a solvent system resulted in 54–61% conversion of reactants into ethyl laurate by immobilized lipase of *B. coagulans* MTCC-6375.[34]

Effect of temperature on transesterification reaction by nylon-6 immobilized enzyme

The effect of change in temperature on transesterification reaction by immobilized lipase was also studied. A maximum conversion of butanol and vinyl acetate to butyl acetate (75.69%) was achieved at 55°C in 12 h (Fig. 5). A further increase in the reaction temperature led to a decreased conversion rate and the amount of butyl acetate produced. The alteration in temperature of reaction mixture might interfere with the porosity, hydrophobic character, and diffusion of the reactants and/or products at the catalytic site of enzyme. It appeared that temperature has an important effect on the physical state of substrate dispersion in an organic solvent. Higher temperature and liquefaction could make the substrate more acceptable to the enzyme.[35] In a recent study, maximum conversion of reactants into ethyl laurate by hydrogel-immobilized lipase of *B. coagulans* was achieved at a temperature of 65°C.[34]

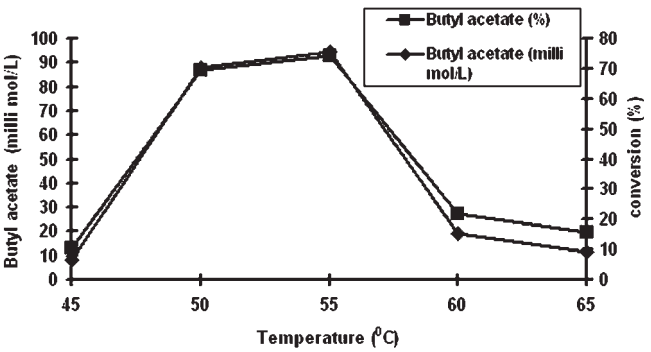

Figure 5 Effect of reaction temperature on transesterification.

Reusability of immobilized enzyme for transesterification

The bound lipase was repetitively used to perform transesterification under optimized conditions in *n*-heptane. It resulted in synthesis of 77.67 mmol/L butyl acetate after third cycle of transesterification (Fig. 6) and in each cycle, transesterification was

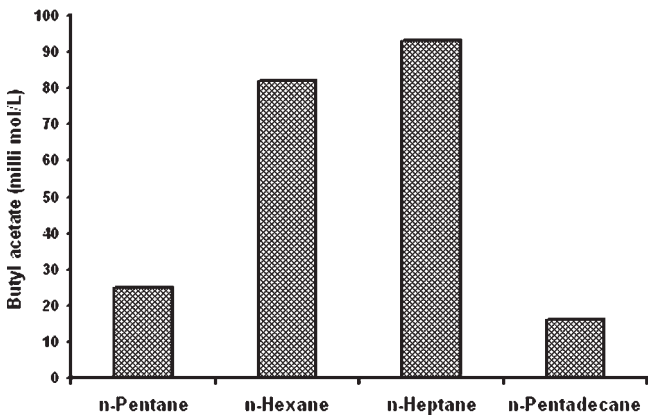

Figure 4 Effect of various *n*-alkanes on transesterification using nylon-immobilized lipase.

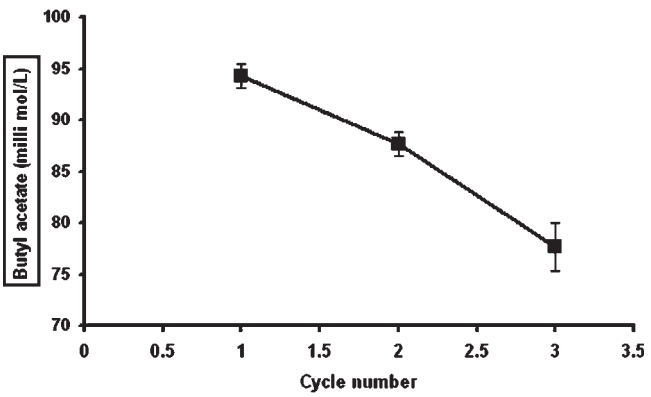

Figure 6 Reusability of immobilized lipase for transesterification.

performed for 12 h at 55°C. In our recent study, immobilized lipase from *P. aeroginosa* retained 30% of its original catalytic activity after five cycles of reuse for esterification of ethanol and propionic acid into ethyl propionate.[14]

CONCLUSIONS

The present study concluded that nylon-6 immobilized lipase of *B. coagulans* BTS-3 efficiently performed transesterification reaction for synthesis of butyl acetate from vinyl acetate in *n*-heptane at 55°C in 12 h, molar ratio of reactants being 100 mmol/L each. Moreover, the immobilized enzyme had good reusability in performing continuous transesterification.

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