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Kinetics of Enzymatic Synthesis of Cinnamyl Butyrate by Immobilized Lipase

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Abstract This work illustrates the enzymatic synthesis of cinnamyl butyrate by esterification of butyric acid and cinnamyl alcohol. Experiments were performed to study the various operating parameters such as molar ratio, enzyme concentration, temperature, and speed of agitation. Also, the suitable kinetic model for esterification reaction was predicted and the various kinetic parameters were determined. It has been observed that the experimental results agree well with the simulated results obtained by following the ping-pong bi-bi mechanism with dead-end inhibition by both the substrate acid and alcohol. The highest 90% conversion of butyric acid was observed after 12 h at the following reaction conditions: substrate molar ratio 1:2 (butyric acid/cinnamyl alcohol), temperature 50 °C, enzyme loading 2% (with respect to the weight of the substrates), and agitation speed 250 rpm. Diffusional mass transfer limitations between substrate and enzyme surface do not show significant effect on reaction kinetics. Enzyme reusability study reveals that it retains 85% of its catalytic activity after five consecutive cycles.

Keywords Esterification · Flavor synthesis · Cinnamyl butyrate · Enzymatic catalysis · Immobilized lipase · Kinetic study · Ping-pong bi-bi mechanism

Introduction

The short-chain esters having sweet-smelling aroma are commonly used as a flavoring agent in most of the food products like beverages, candies, jellies, jams, confectionery, dairy products, and chocolates and ice creams [1]. The long-chain esters derived from fatty acids are mostly used in cosmetics, pharmaceuticals, and surfactants [2], and also as plasticizers, lubricants, diesel additives, and water-resistant agents, and in hydraulic fluids and as reaction media [3, 4].

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Many short-chain fatty acid esters exhibit characteristic fruity smell such as ethyl butyrate [5] (pineapple or strawberry flavor), methyl butyrate [6] (pineapple or apple flavor), butyl butyrate [7] and butyl isobutyrate [8] (pineapple flavor), isoamyl isovalerate [9] (apple flavor), and isoamyl acetate [10] and isoamyl butyrate [11, 12] (banana flavor). Also, some cinnamyl esters possess characteristic aroma and are used in various cosmetic products. One of the cinnamyl esters, i.e., cinnamyl acetate, is synthesized by transesterification of cinnamyl alcohol and vinyl acetate using chemical and enzyme as a catalyst [13–15]. Cinnamyl butyrate has also wide applications in pharmaceutical, flavor, and cosmetic industry. Belsito et al. presented the detailed toxicological and dermatological studies of cinnamyl butyrate along with other cinnamyl esters [16], which show that cinnamyl butyrate possesses the nearly similar test results as compared to cinnamyl acetate and could be considered as a potential candidate instead of cinnamyl acetate. However, the synthesis part of the cinnamyl butyrate is not well explored in the literature. As per authors' knowledge, there are no articles reported on the detailed synthesis of cinnamyl butyrate. Hence, this work reports an insight to the synthesis of cinnamyl butyrate.

Ester synthesis using enzymes as a catalyst proved to be much better than traditional methods due to their mild reaction conditions, high efficiency and high regio- and stereoselectivity, minimizes the use of hazardous chemicals [17]. Various groups of lipase enzyme have been identified and used to catalyze esterification and transesterification reactions due their robust and high catalytic activity. It can be used in both free and immobilized forms [18–23], but the latter is mostly utilized due to its high operational stability, easy recovery, and high reusability [24, 25]. Thus, biocatalysis is a promising alternative for the production of esters from considerably cheaper and abundant raw materials. Esterification reaction in the presence of organic solvents is widely reported in the literature along with its influence on the course of the reaction [26–34]. The reaction temperature along with the other parameters has a significant effect on the reaction rate due to their thermal sensitivity [35]. In the production of esters using biocatalyst, it is important to determine the optimal conditions of reactions for the maximum efficiency of the process along with its reaction kinetics. Many hypothetical models have been proposed to elucidate the kinetics of the esterification reaction catalyzed by an immobilized enzyme in the organic media [36]. The enzyme-catalyzed reactions containing a single substrate generally follow the simple Michaelis–Menten mechanism, and the bisubstrate reaction mainly follows two types of mechanisms: the ping-pong mechanism (non-sequential) and the ternary complex mechanism (sequential) [26, 37]. It is necessary to study the inhibition effect by substrates in the enzyme-catalyzed reaction. The inhibition caused by one or both the substrates was due to the formation of inactive ternary complex with the enzyme. Considering enzyme-catalyzed esterification, some literature reports the inhibition by only one substrate, by acid [26, 29, 32, 38], or by alcohol [14, 27, 31, 34, 39–41] and others do not find any inhibitory effect by both the substrates [1, 42–44]. Some literature also reports inhibition by both the substrates [2, 11, 30, 44, 45].

In this work, the aim was to synthesize cinnamyl butyrate by an immobilized enzyme as a catalyst in the presence of hexane as organic media. The detailed experimental investigation was performed to study the effect of various parameters such as a substrate molar ratio, enzyme loading, temperature, and speed of agitation. Also, the kinetic model was fitted and kinetic constants are calculated by solving the ping-pong bi-bi rate equation.

Material and Methods

Enzyme and Chemicals

Immobilized enzyme *Candida antarctica* lipase B (CAL-B) was provided by Fermenta Biotech, Ltd., Thane, Mumbai, as *Fermase CALB 10000*, having the particle size 300–500 μm, and immobilized on polyacrylate resin beads using covalent bonding. All other chemicals such as cinnamyl alcohol, butyric acid, ethanol, hexane, *p*-nitrophenyl acetate, chloroform, isoamyl alcohol, sodium phosphate, acetone, gum arabic, 2-propanol, and sodium chloride were procured from reputed firms and used directly without further purifications.

Experimental

All reactions were carried out in a 50-cm^3 baffled glass reactor equipped with an overhead stirrer having the provision to control speed. The desired quantity of the reactants was measured and transferred to the reactor. The rector was further sealed using vacuum grease. A known amount of solvent (hexane) is also added to the reaction to improve the enzyme activity as well as to act as the reaction medium. The whole reactor assembly was immersed in a water bath equipped with a temperature controller to maintain the desired temperature in the order of ±2 °C. The reaction is initiated by the addition of a measured amount of enzyme as a catalyst, and the addition of enzyme is considered to be the start of the reaction. As water is the byproduct of esterification reactions, molecular sieves were added to act as a scavenger for water. Samples were withdrawn periodically from reaction mass, centrifuged using a centrifuge machine at the speed of 8000 rpm for 5 min. The supernatant liquid was then transferred to the fresh vial and analyzed using gas chromatography and standard titrimetric analysis method.

Statistical Analysis

Statistical analysis is a very useful technique to analyze, interpret, and summarize the experimental data. Data obtained from the results were analyzed using single-factor ANOVA with the help of Microsoft Office Excel. All the experiments were carried out in triplicate, and the data is reported as mean ± SD. p values less than 0.05 are considered to be significant.

Gas Chromatography Analysis

Gas chromatography (Chemito GC 8610) equipped with a flame ionization detector (FID), packed column 5% OV 101 (1/8 in. diameter, 3 m length). Nitrogen was used as a carrier gas at the flow rate of 30 cm^3 min^{-1}, and air and hydrogen are used at the flow rate of 300 and 20 mL min^{-1}, respectively. Both the injector and detector were held at a constant temperature of 280 °C. Initially, 1 μL of the sample was injected at 50 °C oven temperature and raised up to 250 °C with the rate of 17 °C min^{-1} and held for 1 min. An internal standard method was used to determine the percent conversion of acid using *n*-dodecane as an internal standard to calculate the standard relative response of the detector.

Titrimetric Analysis

The change in concentration of acid during the reaction course was monitored by determining the acid value of the samples using a standard titrimetric method reported in our previous work [46]. The analysis was performed by transferring 0.10 to 0.20 g of the sample in a 50-cm^3 conical flask. Ten milliliters of neutralized ethanol was added to the flask, gently shaken to mix the contents in ethanol, and titrated against the potassium hydroxide solution using phenolphthalein as an acid-base indicator. The end point was determined by a change in color from colorless to slightly pink.

The progress of the reaction was measured on the basis of the determination of percent conversion of the acid. The acid value was determined using the following formula:

$$\text{Acid value} = \frac{56.1 \times N \times V}{\text{W}}$$

where N is the normality of KOH required to neutralize the acid, V is the volume of KOH in milliliters required to neutralize the acid, and W is the weight of the sample in grams.

Determination of Enzymatic Activity

Enzyme assay was performed to determine the activity of the enzyme using the standard protocol. The activity of free and immobilized CALB was assayed spectrophotometrically with *p*-nitrophenyl acetate as a substrate [47]. The reaction mixture consisted of 2.0 cm^3 of buffer (1 g dm^{-3} gum arabic, 0.15 M NaCl, and 0.1 M sodium phosphate, pH 7.0) and 0.1 cm^3 of 15 mmol dm^{-3} *p*-nitrophenyl acetate dissolved in 2-propanol. The mixture was prewarmed at 40 °C, and then 100 mg of the enzyme was added. After 5 min of incubation, the reaction was stopped by adding 2.0 cm^3 of Marmur solution (chloroform/isoamyl alcohol, 24:1) followed by centrifugation at 10,000 rpm for 5 min at 4 °C. The clear supernatant was taken off and measured at 405 nm in a UV/Vis spectrophotometer. One enzyme unit is defined as the amount of the enzyme that liberates 1 μmol of *p*-nitrophenol per min at pH 7.0 and 40 °C. The activity of the enzyme used was found to be 10.653 U g^{-1}.

Reusability Study of Enzyme

The reusability study was carried out to test the catalytic efficiency of the biocatalyst at the optimum reaction conditions. After every cycle, the catalyst was recovered by simple filtration, washed with acetone (5 mL × 2 mL), dried in an oven for 30 min at 40 °C, kept overnight in a desiccator, and used for the next reaction cycle. The catalytic activity was determined after every cycle by using the above mentioned procedure.

Determination of Kinetic Constant

The kinetics of esterification reaction of cinnamyl alcohol and butyric acid were done by analyzing initial rates (determined by an initial slope method) at various acid and alcohol concentrations. The data obtained was fitted with Michaelis–Menten kinetics with a ping-pong bi-bi mechanism by a nonlinear regression (NLR) method using Microsoft Excel 2013. The values of kinetic constants were calculated by using minimization of $\sum_i \sqrt{1 + \text{MSR}_i{}^2}$, where MSR_i is the mean square residual between theoretical and experimental data.

Results and Discussion

Effect of Enzyme Loading

Effect of enzyme loading on conversion of butyric acid was studied at different enzyme concentrations ranging from 1 to 3% by keeping all other parameters constant. The results obtained are represented in Fig. 1.

A significant change in the conversion was observed when enzyme concentration was varied from 0.84 to 2% while with a further increase in enzyme concentration, the initial rate as well as a total conversion of acid showed a marginal change. The increase in conversion can be explained by an increase in acyl–enzyme complex formation due to increased catalytic sites at higher enzyme loading. The saturation effect at elevated enzyme concentration was observed due to a reduction in the formation of enzyme–substrate complexes due to the external mass transfer limitations that arise from an excess of enzyme concentration. Also, due to the reversible nature of the esterification reaction, the forward reaction becomes less dominant after attaining the equilibrium conditions. The effect of enzyme concentration on conversion in the case of enzymatic reactions was well reported and found to be in agreement with the reported literature [22, 48]. As the conversion obtained at an enzyme concentration of 2 and 3% does not have any significant difference, 2% enzyme loading is considered to be optimum and further experiments were carried out using the same enzyme loading.

Effect of Temperature Variation

Enzymes are very sensitive to temperature, and little fluctuation in reaction temperature above optimum range may lead to enzyme deactivation resulting in lower enzyme activity [35]. The

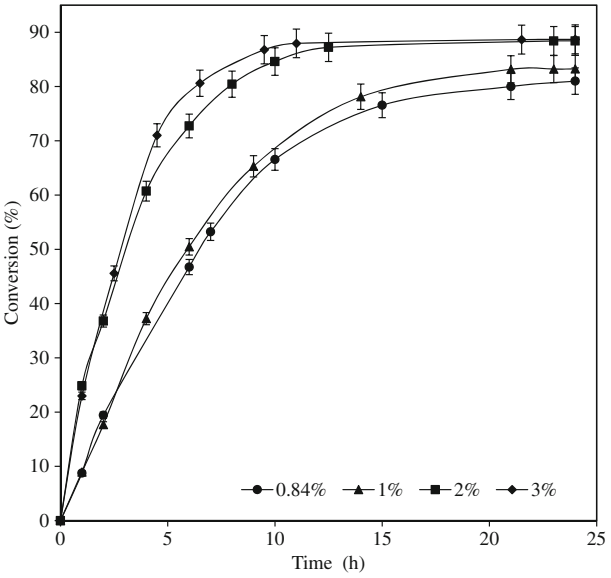

Fig. 1 Effect of enzyme loading on percent conversion of butyric acid at a molar ratio of 1:2, reaction temperature of 50 °C, and speed of agitation of 250 rpm

effect of temperature variation on percentage conversion was studied in the temperature range of 40–60 °C, and the results obtained are represented in Fig. 2. An increase in percent conversion was observed with an increase in temperature from 40 to 50 °C, and a further increase gives a slight increase in the conversion of the reaction. At higher reaction temperature, the solubility of the substrates increases in the solvent favoring more diffusion of a substrate molecule to the enzyme surface due to reducd mass transfer limitations of the system. The increased diffusion of substrates favors the formation of more acyl–enzyme complexes which directly influences the reaction rate as well as conversion profile of the reaction. Although the rate of diffusion is proportional to the temperature, a marginal difference was observed in the conversion obtained at 50 and 60 °C. Hence, 50 °C is considered as the optimum reaction temperature. All the further reactions were carried out at the same temperature.

Effect of Substrate Concentration on Reaction Kinetics

Reactions are carried out by keeping the concentration of one reactant constant while varying the concentration of the other reactant. It was found that there is a significant influence of substrate concentration on the conversion of butyric acid and also on the rate of reaction. Initially, the butyric acid concentration was kept constant and the concentration of cinnamyl alcohol was varied in the range of 1.84 to 7.36 mol dm^{-3} which was always in excess compared to the concentration of acid. It was observed that with an increase in the concentration of alcohol (Fig. 3), the initial reaction rate increases rapidly but, at higher reaction time, the reaction rate gets reduced. It clearly shows the inhibition of enzyme with respect to alcohol at higher concentrations. The increase in conversion with an increase in substrate concentration was due to the increase in the formation of acyl–enzyme complex resulting in more product formation. The enzyme inhibition at higher substrate concentrations leads to a decrease in the

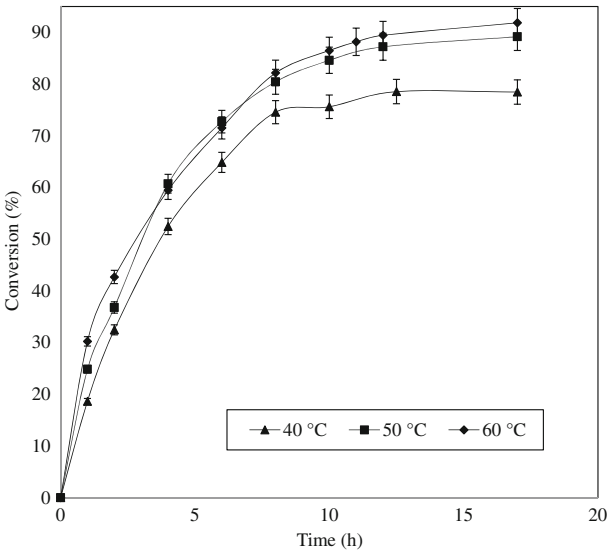

Fig. 2 Effect of reaction temperature on percent conversion of butyric acid at a molar ratio of 1:2, enzyme loading of 2%, and speed of agitation of 250 rpm

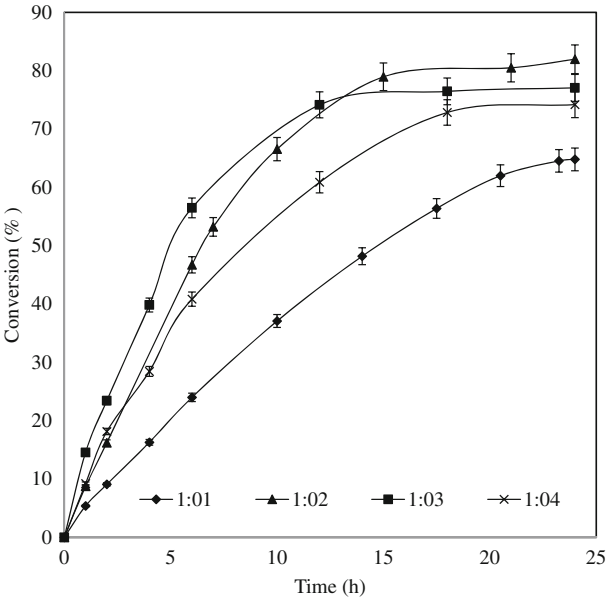

Fig. 3 Effect of substrate concentration on percent conversion of butyric acid at a reaction temperature of 50 °C, enzyme loading of 0.84%, and speed of agitation of 250 rpm

conversion. The reaction data shows that either single substrate or both the substrates can be responsible for the inhibitory action of enzyme, reducing the progress of the reaction.

To investigate the inhibitory effect of both the substrates, experiments were carried out individually by keeping one substrate constant and varying the concentration of the other. The Lineweaver–Burk double reciprocal plot was studied with varying concentrations of alcohol at constant acid concentration and by varying acid concentrations at constant alcohol concentration to determine the inhibitory effect of cinnamyl alcohol and butyric acid, respectively. The results obtained are represented in Fig. 4a, b. From the Lineweaver–Burk plot, it is clear that the reaction rate is substrate inhibited by both the substrates at higher substrate concentrations. The inhibition effect of acid, as well as alcohol with the formation of the dead-end complex, is well reported in the literature [2, 11].

The explanation of the mechanism using the random bi-bi mechanism is ruled out, as enzymatic esterification reactions follow the formation of intermediate active acyl–enzyme complex. In this study, the experimental findings were correlated and explained using a ping-pong bi-bi model, considering the inhibitory effect by both the substrates.

The mechanism assumed to consist the following steps: the substrate butyric acid [A] binds first to the enzyme [E] surface to form the acid–lipase complex [EA] which then, on isomerization, forms the acyl–enzyme intermediate [$E^{\#}$] with the release of water [P] molecule. In the second step, the cinnamyl alcohol [B] reacts to the acyl–enzyme binary complex to form another complex [$E^{\#}B$], which, on isomerization, gives the enzyme–ester complex [EQ], which finally gives ester [Q] and enzyme [E]. The dead-end inhibition complexes [$E^{\#}A$] and [EB] are formed when the enzyme reacts with acid and alcohol, respectively. The general schematic representation is illustrated in Fig. 5 [49].

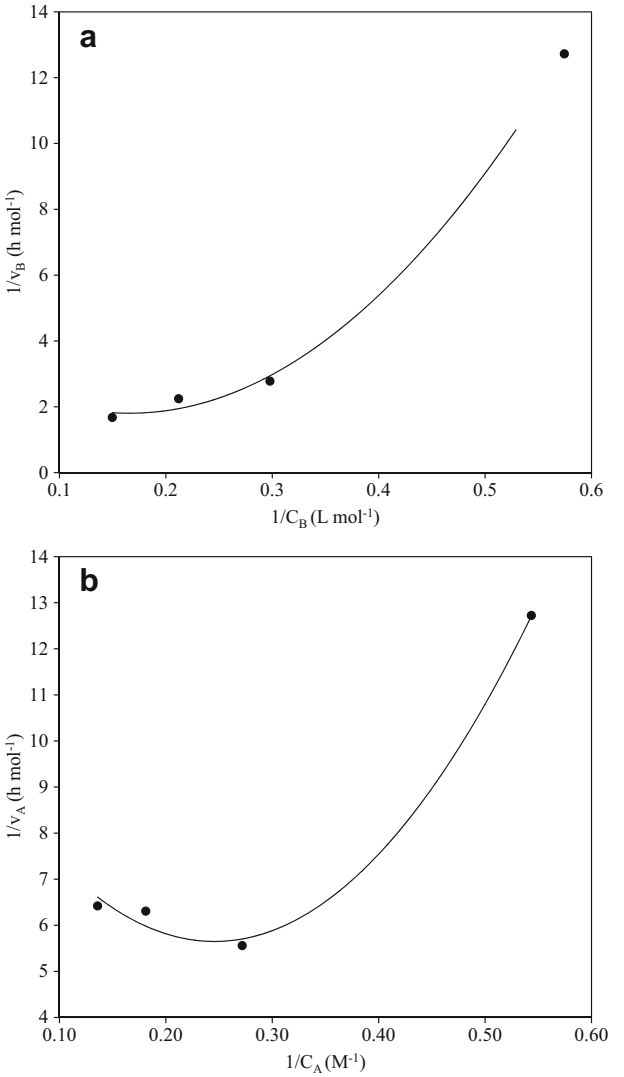

Fig. 4 **a** The Lineweaver–Burk double reciprocal plot for different concentrations of cinnamyl alcohol (1.84–7.36 M) at constant butyric acid concentration (1.84 M). **b** The Lineweaver–Burk double reciprocal plot for different concentrations of butyric acid (1.84–7.36 M) at constant cinnamyl alcohol concentration (1.8 M)

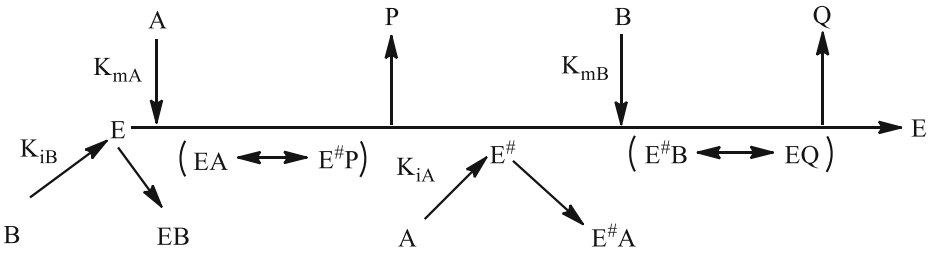

Fig. 5 The general schematic representation of the ping-pong bi-bi mechanism with inhibition by both substrates

The results obtained in this study were validated with the kinetic expression for this mechanism. The rate equation for the bisubstrate ping-pong bi-bi mechanism is represented by Eq. 1 [49]

$$v = \frac{V_{\max}[\mathrm{A}][\mathrm{B}]}{[\mathrm{A}][\mathrm{B}] + K_{\mathrm{MA}}[\mathrm{B}]\left(1 + \frac{[\mathrm{B}]}{K_{\mathrm{iB}}}\right) + K_{\mathrm{MB}}[\mathrm{A}]\left(1 + \frac{[\mathrm{A}]}{K_{\mathrm{iA}}}\right)} \quad (1)$$

where v is the initial rate of the reaction, $V_{\max}$ is the maximum velocity, [A] and [B] are the initial concentrations of the two substrates, K_{MA} is the Michaelis–Menten constant for substrate [A], K_{MB} is the Michaelis–Menten constant for substrate [B], K_{iA} is the inhibition constant for [A], and K_{iB} is the inhibition constant for [B]. The values of the kinetic constants were estimated by the nonlinear regression method using Microsoft Excel 2013 using minimization of the sum of squares. The reaction rate data for esterification reaction was fitted with the predicted data obtained by the ping-pong bi-bi mechanism. The experimental data was further used to validate the kinetic model, and it is observed that the reaction follows a ping-pong bi-bi mechanism with dead-end inhibition by butyric acid as well as cinnamyl alcohol. Figure 6 represents the variation in initial rates plotted against the concentration of alcohol and compared with the simulated rates predicted by the ping-pong bi-bi mechanism. The kinetic parameters obtained using the initial reaction rate are summarized in Table 1. The values of inhibition constants reveal that the inhibitory effect of cinnamyl alcohol is greater than the inhibition effect observed by butyric acid. And, the greater value of the Michaelis constant suggests that enzyme has the greater affinity towards the butyric acid over cinnamyl alcohol.

Effect of Mass Transfer

In a heterogeneous system containing immobilized enzyme as a catalyst, the reaction rate might be affected by external or internal mass transfer limitations. The external mass transfer limitations can be overcome by increasing the speed of agitation. Experiments were carried out at the different speeds of agitation in the range of 150–450 rpm by keeping all the other parameters constant, and the results obtained are depicted in Fig. 7.

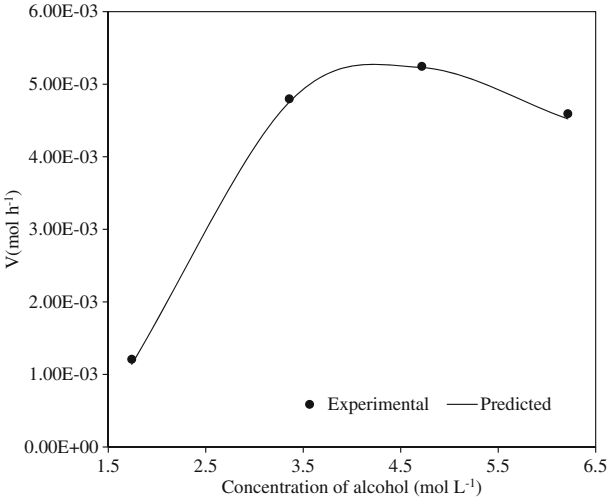

Fig. 6 Comparison of experimental initial rates with simulated rates

Table 1 Kinetic parameters for the esterification reactions of butyric acid with cinnamyl alcohol

Parameter	V_{max} (mol h^{-1})	K_{mA} (mol dm^{-3})	K_{mB} (mol dm^{-3})	K_{iA} (mol dm^{-3})	K_{iB} (mol dm^{-3})	SSE
Value	0.0072	0.0004	0.1549	0.1263	0.0001	1.2*E*−08

It can be seen that there is external mass transfer limitations in a heterogeneous system. As the speed of agitation increases the overall reaction conversion increases up to certain level and a further increase leads to a decrease in percent conversion. Here, a difference in the rate of reaction and conversion obtained at 250 and 350 rpm was very low which indicates that the external mass transfer resistance is negligible after 250 rpm. The decrease is possibly due to breakage of enzyme bonding with the immobilized surface resulting in the loss of enzymatic activity. The evaluation of diffusional mass transfer and internal diffusivity coefficient was done by using theoretical calculations to determine the controlling mechanism.

In the case of enzyme immobilized on the spherical porous beads, it is necessary to determine the effect of external diffusional mass transfer at the surface of the catalyst and the internal diffusional mass transfer through the pores of the catalyst. The effect of external mass transfer on the rate of the reaction was determined at 250 rpm with the help of the dimensionless Damköhler number (D_a) and the external effectiveness number (η) represented by Eqs. 2 and 3 respectively [50].

$$D_a = \frac{V_{max}''}{K_{sl} \cdot [A]_b} \tag{2}$$

$$\eta = \frac{\text{observed reaction rate}}{\text{reaction rate obtained without external mass transfer limitations}} \tag{3}$$

where V_{max}'' is the maximum reaction rate per unit surface area of biocatalyst (mol dm^{-2} s^{-1}), K_{sl} is the solid–liquid mass transfer coefficient, and $[A]_b$ is the concentration of butyric acid in bulk.

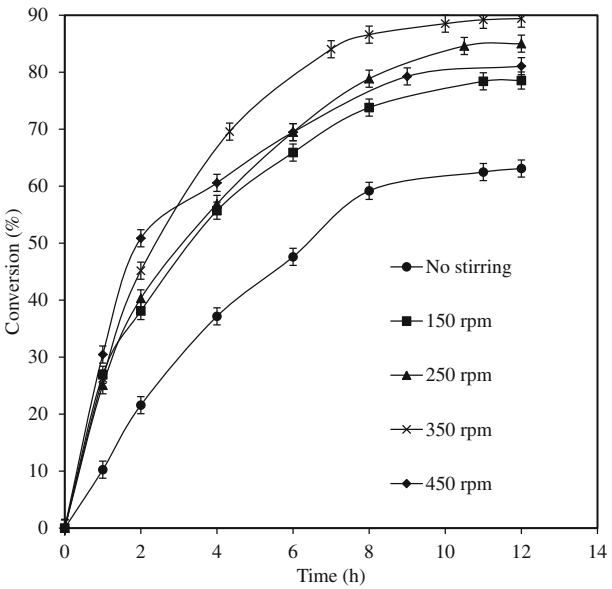

Fig. 7 Effect of speed of agitation on percent conversion of butyric acid at a molar ratio of 1:2, reaction temperature of 50 °C, and enzyme loading of 2%

Many authors calculated the solid–liquid mass transfer coefficient by considering the limiting value of the Sherwood number, Sh = 2, which is generally used for the non-agitated system (natural convection), but in the given agitated system, a more accurate solid–liquid mass transfer coefficient was determined according to Eq. 4 [51].

$$K_{\mathrm{sl}} \cdot \mathrm{Sc}^{2/3} = 0.13 \left[\frac{\left(\frac{P}{V_{\mathrm{L}}}\right) \cdot \mu}{\rho^2} \right]^{1/4} \tag{4}$$

where Sc is the Schmidt number, P is the power intake (W), ρ is the density of the continuous phase (kg m^{-3}), V_{L} is the total volume of the solution (cm^3), and μ is the dynamic fluid viscosity (mPa s^{-1}).

The terms involved in the above equation was calculated with the help of some reported equations (Eqs. 5–9) for shake flask with some modifications in average energy dissipation and modified power number correlations [52, 53].

$$\frac{P}{V_{\mathrm{L}}} = \varepsilon \cdot \rho \tag{5}$$

$$\varepsilon = \mathrm{Ne}' \cdot n^3 \cdot d^5 \cdot {V_{\mathrm{L}}}^{-2/3} \tag{6}$$

$$\mathrm{Ne}' = 70 \cdot Re^{-1} + 25 \cdot Re^{-0.6} + 1.5 \cdot Re^{-0.2} \tag{7}$$

$$Re = \frac{n \cdot d^2 \cdot \rho}{\mu} \tag{8}$$

$$\mathrm{Sc} = \frac{\mu}{\rho \cdot D} \tag{9}$$

where ε is the average energy dissipation rate (W kg^{-1}), Ne′ is the modified power number, n is the speed of agitation (rps), d is the diameter of the impeller (m), R_e is the Reynolds number, and D is the diffusivity of cinnamic acid (cm^2 s^{-1}).

The diffusion coefficient of solute in *n*-hexane was calculated with the help of Wilke–Chang equation modified and generalized in terms of latent heat of vaporization by Sitaraman et al. [54]. The generalized form of Wilke–Chang equation is represented in Eq. 1.

$$D = 5.4 \times 10^{-8} \left(\frac{{M_{\mathrm{s}}}^{1/2} \cdot {L_{\mathrm{s}}}^{1/3} \cdot T}{\eta \cdot {V_{\mathrm{m}}}^{0.5} \cdot L^{0.3}} \right)^{0.93} \tag{10}$$

where M_{s} is the molar mass of the solvent, L_{s} is the latent heat of vaporization of solvent at normal boiling point (cal g^{-1}), V_{m} is the molecular volume (m^3 mol^{-1}), L is the latent heat of vaporization of solute at normal boiling point (cal g^{-1}), and T is the temperature (K).

The surface area of the biocatalyst needed for the calculation of the maximum rate of reaction per unit surface area of the catalyst was 116.72 m^2 g^{-1} (provided by Fermenta Biotech, Ltd., Mumbai). By calculating all the factors associated in Eqs. 4–10, the value of Damköhler number and external effectiveness factor can be calculated from Eqs. 2 and 3. The value of Damköhler number is found to be 5.49×10^{-11}. If the value D_{a} 1, the external diffusional mass transfer has a negligible effect on the given system [50]. Hence, it was confirmed that the rate of the reported system is not influenced by any external diffusive mass

transfer limitations. Also, as the concentration of acid in the reaction mass is nearly equal to the concentration at the surface of the catalyst, the external effectiveness factor tended to 1. It also implies that the rate is not limited by external mass transfer [50]. Hydrodynamic parameters of the batch reactor for esterification reaction are represented in Table 2.

Internal diffusional mass transfer was theoretically evaluated through the observable Thiele modulus (ϕ) represented in Eq. 11 [50].

$$\phi = \frac{\nu_{obs}}{D_{eff} \cdot [A]_0} \cdot \left(\frac{R}{3}\right)^2 \tag{11}$$

$$D_{eff} = D \cdot \frac{\varepsilon_p}{\tau} \tag{12}$$

where V_{obs} is the observable reaction rate per unit mass of biocatalyst (mol mg^{-1} s^{-1}), R is the particle radius (m), D_{eff} is the effective diffusivity (cm^2 s^{-1}), $[A]_0$ is the initial concentration of butyric acid, τ is the tortuosity factor of enzyme, and ε_p is the particle porosity.

Values for the ratio of particle porosity versus tortuosity factor, and bulk density of Fermase CALB 10000 were 0.25 and 0.453 g cm^{-3}. The value of observable Thiele modulus was found to be 1.01×10^{-9}. For the values of $\phi \leq 0.3$, the internal effectiveness factor approaches 1 [50] and there is a negligible effect of internal diffusional mass transfer on reaction rate. The obtained values are in correlation with the internal effectiveness factor which indicates that internal diffusional mass transfer resistance had no significant effect on the reaction rate of the system. Considering overall diffusion limited mass transfer, it can be concluded that the rate of reaction is only governed by enzyme kinetics, via ping-pong bi-bi mechanism.

Enzyme Reusability

Enzyme reusability was studied by performing th esterification reaction at optimum reaction conditions, which includes enzyme loading 2%, temperature 50°C, molar ratio 1:2, and speed of agitation 250 rpm. The percent conversion was calculated by considering the conversion of the first cycle as 100%. The results obtained are represented in Fig. 8. The marginal change in percent conversion was observed after four consecutive cycles. The conversion was decreased from 88.4 to 75.84% after the 4th cycle. The decrease in conversion might be due to the loss of enzymatic activity during the reaction course.

Table 2 Hydrodynamic parameters for the esterification reaction of butyric acid with cinnamyl alcohol

Hydrodynamic parameter	Value
ρ, kg m^{-3}	655
d, m	0.02
n, rps	5.83
V_L, m^{-3}	17.13×10^{-6}
μ, Pa s^{-1}	3.15×10^{-3}
D_a, m^2 s^{-1}	5.49×10^{-11}
ε, W kg^{-1}	1.539
R_e	48,518
N_e'	0.2133
Sc	6.881×10^{-3}
P/V_L, W m^{-3}	1008.4147
K_{sl}, m s^{-1}	1.6938×10^{-5}

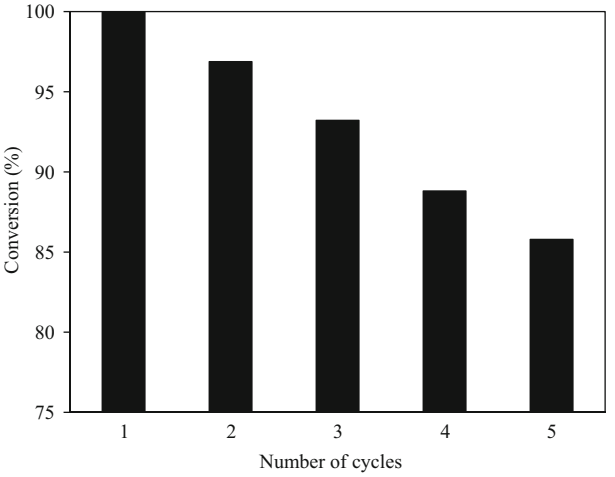

Fig. 8 Effect of reusability of catalyst on percent conversion of butyric acid at a molar ratio of 1:2, reaction temperature of 50 °C, enzyme loading of 2%, and speed of agitation of 250 rpm. The conversion for the first cycle (88.4%) is considered as a 100% to determine the relative conversion for subsequent cycles after 10 h

Conclusion

The current study investigated the immobilized enzyme (Fermase CALB 10000) catalyzed synthesis of cinnamyl butyrate by esterification of butyric acid and cinnamyl alcohol in hexane. This study also illustrated the effects of various reaction parameters on the reaction progress, and the parameters are optimized using a basic single factor at a time method. It was observed that the molar ratio of acid to alcohol of 1:2, enzyme loading of 2%, reaction temperature of 50 °C, and speed of agitation of 250 rpm are found to be the optimum for the maximum conversion. The kinetic model for esterification reaction was developed and fitted based on the experimental initial rate data, and the kinetic parameters were determined. Based on the findings in this study, it was proved that the esterification reaction follows the ping-pong bi-bi mechanism with dead-end inhibition by both cinnamyl alcohol and butyric acid at higher concentrations. The diffusional mass transfer limitations were studied, and it was found that internal as well as external mass transfer limitations do not play any significant role in the given esterification reaction and the reaction is mainly governed by enzyme kinetics.

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