Lipase catalyzed transesterification of ethyl butyrate synthesis in *n*-hexane— a kinetic study

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Abstract Kinetics of lipase catalyzed transesterification of ethyl caprate and butyric acid was investigated. The objective of this work was to propose a reaction mechanism and develop a rate equation for the synthesis of ethyl butyrate by transesterification using surfactant coated lipase from *Candida rugosa*. The reaction rate could be described in terms of Michaelis–Menten equation with a Ping-Pong Bi–Bi mechanism and competitive inhibition by both the substrates. The values of kinetic parameters computed were $V_{\max} = 2.861 \mu\text{mol}/\text{min}/\text{mg}$; $K_{m(\text{acid})} = 0.0746 \text{ M}$; $K_{m(\text{ester})} = 0.125 \text{ M}$; $K_i \text{ acid} = 0.450 \text{ M}$. This study indicated a competitive enzyme inhibition by butyric acid during lipase catalyzed transesterification reaction. Experimental observations had clearly indicated that the substrates as well as product act as dead-end inhibitors.

Keywords *Candida rugosa* · Transesterification · Lipase · Enzyme concentrations · Flavor esters

Introduction

Short chain esters and alcohols were widely used as flavor and fragrance compounds in food industry (Schwab et al. 2008; Rajendran et al. 2009). Esters were responsible for the aroma of many fruits, e.g. banana, apple and pineapple and had many potential applications in the food industry (Mahapatra et al. 2009; Shu et al. 2011; Pires-Cabral et al. 2010). Consequently, these esters had commercial significance in fragrance, cosmetics, food and pharmaceutical industries. Since, the common sources of natural flavor compounds were not sufficient enough to meet these market demands and often their direct extraction from plants involves expensive and low-yielding processes (Berger 2009), new enzymatic synthesis and their optimization to obtain these flavor molecules, provide an important alternative for the food industry (Schrader et al. 2004, Serra et al. 2005). Enzymatic synthesis provides an attractive and environmentally more benign alternative to the conventional chemical approaches used for the production of high quality alkanolamides. Current commercial use of enzymes, together with new applications, will continue to play a vital role in maintaining and enhancing the quality of life while protecting the environment for future generations.

Lipase catalyzed esterification and transesterification reactions for flavor esters have numerous food applications. Lipases, which are considered to be natural by the food legislation agencies, had been widely investigated for ester synthesis, mainly in organic solvents, due to their enhanced solubility in hydrophobic substrates and eliminating side reactions caused by water (Langrand et al. 1990). The nature of organic solvent affects the enzymatic synthesis by causing inactivation/inhibition by directly interacting with the enzyme or with the diffusible substrates/product, or the

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water layer at the vicinity of the enzyme (Vijay Kumar and Narsimha Rao 2003). Lipase mediated synthesis of flavor esters under solvent-free conditions had significant importance in both food and pharmaceutical industries due to the avoidance of toxic solvent and elimination of its recovery during the operation (Guvenc et al. 2002). Lipase catalyzed synthesis of flavor esters by transesterification reactions is influenced by a number of parameters such as molar ratio, reaction time, temperature, substrate concentration, and amount of immobilized enzyme.

Short-chain flavor esters had been generally produced by free and immobilized lipases from various sources in organic solvents (Pires-Cabral et al. 2007; Dave and Madamwar 2006). Lipases had been successfully used to catalyze esterification (Thakar and Madamwar 2005; Rodriguez-Nogales et al. 2005; Santos and Castro 2006). Further, interesterification reactions were aimed at the production of flavoring esters for food, pharmaceutical and cosmetic applications. Among a wide variety of immobilization supports, surfactant coated method was tested for the synthesis of ethyl butyrate.

Lipase immobilization was known to allow better operation control, easier product recovery, flexible reactor design, and, in some cases, enhanced operational stability (Santos and Castro 2006).

Although higher conversion yields were achieved using organic solvent, their toxicity is generally a problem for many industrial applications. Moreover, some commonly employed organic solvents were too expensive to allow an industrial scale-up. Literature had suggested few studies concerning to the production of flavor and fragrance short-chain esters in solvent-free systems by enzymatic synthesis (Chowdary et al. 2000). In the present study, efforts were made to synthesize ethyl butyrate by transesterification using surfactant coated lipase from *Candida rugosa* in *n*-hexane. The Ping-Pong Bi–Bi model had been proposed to explain several experimental lipase-catalyzed reaction kinetics (Pires-Cabral et al. 2009; Bezbradica et al. 2006; Yadav and Lathi 2004; Romero et al. 2007; Yadav and Devi 2004; Yadav and Triverdi 2003). In the Ping-Pong Bi–Bi mechanism, the first step in the lipase-catalyzed reaction consists of the binding of the fatty acid to the enzyme. An acyl-enzyme intermediate was then formed and a water molecule was released. Subsequently, the alcohol binds to the acyl-enzyme complex and the ester was formed. After the release of the ester, another fatty acid could bind to the enzyme.

The enzyme-mediated production of the esters was found to be more cost effective than the chemical synthesis. Although lipase catalyzed transesterification process has commercial significance, the utilization of the enzyme is not extensive because of its high cost. Future

developments in low cost production and purification technologies would lower the cost of these enzymes for the increased commercial applications. Moreover, lipase catalysis in organic media confers other advantages: easy enzyme and product recovery, the occurrence of new reactions were not possible in water because of kinetic or thermodynamic restrictions (i.e., the water effect in transesterification reactions), and increased enzyme stability (Sharma and Kanwar 2014).

The focus of the present work was the kinetic study of ethyl butyrate (a banana/pineapple flavor ester) production by esterification, using *n*-hexane, catalyzed by *Candida rugosa* lipase immobilized as surfactant coated lipase. The inhibitory effect of both substrates (ethanol and butyric acid) on lipase activity was also investigated. The kinetic data was fitted to substrate-inhibition and Ping-Pong Bi–Bi inhibition kinetic models.

Materials and methods

Enzymes

The lipase from *Candida rugosa* (CRL) Type-VII and porcine pancreatic lipase (PPL) Type-II were procured from Aldrich Chemicals (Milwaukee, WI, USA) and Sigma (St. Louis, MO, USA) respectively. Lipase *Mucor javanicus* was procured from Amano pharmaceuticals. Co. Ltd (Nagoya, Japan).

Chemicals

Butyric, valeric, hexanoic, octanoic, octanol, ethyl butyrate, ethyl caprate (ethyl decanoate) were procured from Aldrich chemicals (Milwaukee, WI, USA). Ethyl stearate was procured from Sigma Chemicals Co. (St. Louis, MO, USA). Tributyrin (Glycerol tributyrinate) was from Merck (Darmstadt, Germany). Methanol, ethanol, butanol, *n*-hexane, *n*-heptane, *n*-octane, isooctane, petroleum ether (Boiling point 40–60 and 60–80 °C) and molecular sieves (3 Å) were procured from SD Fine-Chem Ltd. (Mumbai, India). Phenolphthalein and sodium hydroxide, chloroform, dichloromethane, tetrahydrofuran were procured from SISCO Research Laboratories Pvt Ltd. (Mumbai, India). Ethylmethyl ketone was procured from Loba Chemie Pvt Ltd. (Mumbai, India). All chemicals used were of AR grade.

Lipase assay and protein determination

Lipase assay was determined spectrophotometrically using tributyrin emulsion as the substrate (Basri et al. 1995a).

Further, total protein was estimated using modified Lowry method and tri nitrobenzene sulfonate (TNBS) as a standard (Basri et al. 1995b). One unit of hydrolytic activity has been defined as 1 μmol of butyric acid released per minute per mg enzyme under the standard assay conditions. The hydrolytic activity of *Candida rugosa* was 32,000 U/g; *Porcine Pancreas* was 40,000 U/g of enzyme and the hydrolytic activities of *Mucor javanicus* was 11,000 U/g of enzyme, respectively. All the experiments were performed in duplicate and the average of the results were reported.

Preparation of surfactant-coated lipase

The surfactant-coated lipase was prepared according to the methods given elsewhere (Kamiya et al. 1995). Five hundred milligram of lipase and 500 mg of surfactant were mixed with 500 ml of 0.1 M phosphate buffer solution (pH 7.0) and sonicated for 20 min. The preparation was stored at 4 °C for 24 h. Subsequently, the solution was centrifuged and the translucent solution was lyophilized (−40 °C) for 2 h. A white powder was obtained.

Transesterification reaction

Transesterification reactions were carried out in 100 ml conical flasks equipped with stoppers. Ethyl caprate and butyric acid (acidolysis), as the substrates (equimolar concentration) in 10 ml *n*-hexane was added to the flasks. The surface coated lipase from *Candida rugosa* enzyme was also added to initiate the reaction. Flasks were incubated at 37 °C for 96 h of incubation time at 150 rpm on a rotary shaker (Remi Instruments, Model No CIS −24, Mumbai, India).

Determination of kinetic constants

Reactions were carried up to 3 h with periodic sampling (1–3 h). The initial rates were calculated from the linear portions of the plots of product concentration *versus* reaction time by fitting the time course of the reaction to a linear function by regression, and determining the slope of the tangent to the curve. The effects of alcohol and acid or ester concentration on the reaction rate were investigated by esterification or transesterification of various concentrations of acid (ranging from 0.02 to 0.8 M) with various initial concentrations of alcohol or ester (0.05–0.8 M) and vice versa. The initial reaction rates obtained were fitted to Michaelis–Menten kinetics with Ping-Pong Bi–Bi mechanism by nonlinear regression (Chulallaksnanukul et al. 1992) using Microsoft Excel software 5.0.

$$\frac{V}{V_{\max}} = \frac{(EC)(HA)}{K_m(EC) * (HA) * \left\{1 + \frac{HA}{K_i}\right\} + K_m(EC) * (HA) + (EC) * (HA)} \quad (1)$$

where, $V_{\max}$: maximum rate of a reaction; (EC): initial ethyl caprate concentration; K_i : inhibition constant of butyric acid; K_m (EC) and (BA): Michaelis constants of ethyl caprate and butyric acid (M).

Determination of equilibrium constant (K_o)

The equilibrium constants in transesterification reaction were determined using Eq. (2).

$$K_o = \frac{(PES) * (a_w)}{(SES) * (SAC)} \quad (2)$$

where, (PES) = the product ester (M); (a_w) = Thermodynamic water activity; (SES) = Substrate ester (M); (SAC) = Acid substrate (M).

Gas chromatography (GC)

Esters of ethyl butyrate were analyzed using GC (Shimadzu GC 14 B), equipped with FID and Carbowax 20-M column (3 m length, 3.175 i.d). The column temperature was programmed at 45 °C for 1 min and then increased to 175 °C at the rate of 10 °C per minute. Finally, the column was maintained for 10 min at that temperature. Further, injection port and detector temperatures were maintained at 200 and 250 °C respectively. Nitrogen was the carrier gas (30 ml/min). Ethyl stearate was used as the internal standard for the quantification of transesterification reactions.

Gas chromatography–mass spectrometry (GC–MS)

The samples were analyzed using a Shimadzu 17 A-GC chromatograph equipped with a QP-5000 (Quadrupole) mass spectrometer. A sample volume of 0.1 ml (ethyl butyrate) was mixed with 2 ml hexane, and 0.5 μl sample was injected to Capillary column (DBwax, 30 m $\times$ 0.2 mm ID, film thickness 0.25 μm). Helium was the carrier gas at a flow rate of 2 ml per minute. Injection port and detector temperatures were maintained at 200 and 250 °C respectively. For ethyl butyrate, the column temperature was programmed at 45 °C for 1 min and then increased to 175 °C at the rate of 10 °C per minute, and at that temperature the column was maintained for 10 min. Splitting ratio was 1:50; and the ionization voltage was 70 eV. The compounds were identified by matching their

fragmentation pattern in mass spectra with those of NIST library and standard.

Results and discussion

Choice of solvent

The nature of organic solvent affects the enzymatic synthesis by causing inactivation/inhibition by directly interacting with the enzyme or with the diffusible substrates/product, or the water layer at the vicinity of the enzyme (Kumar and Rao 2003). The results of transesterification of ethyl caprate and butyric acid using the lipase from *Candida rugosa* in different solvents with and without buffer saturation at 37 °C for 96 h of incubation time was studied. A progressive increase in the percent esterification with increase in log *P* value has been observed. Although, there was an increasing trend in the percentage transesterification with an increase in log *P* value, there was no marked correlation between these two parameters especially at higher log *P* values. It was believed that more water soluble (hydrophilic) solvents such as Dioxane (log *P* 1.1), Tetrahydrofuran (0.48), Chloroform (2), strip off the essential hydration layer present on the enzyme surface leading to enzyme denaturation. On the other hand, water immiscible solvents such as cyclohexane (3.2), *n*-hexane (3.5), *n*-heptane (4.0), isooctane (4.5) do not affect the micro aqueous layer around the enzyme, thereby retaining the activity. Earlier reports indicated that an optimum amount of water greater than that found in a dry solvent may be required to increase the lipase activity (Chulalaksnanukul et al. 1992). Thus, buffer saturated solvents (0.01 M phosphate buffer, pH 7) were used for an esterification reaction. However, the percent esterification did not increase with buffering of the reaction media. Maximum percent esterification of 90 was obtained in *n*-hexane after 96 h of reaction time at 37 °C and 10 g/l of enzyme concentration, and hence, is chosen for further study. It was also well known that *n*-hexane could be used for food applications.

Effect of substrate concentration

The effect of substrate concentration on the synthesis of ethyl butyrate using lipase from *Candida rugosa* was depicted in Fig. 1. It could be observed from the figure that the maximum transesterification (%) was achieved at 0.05 M substrate concentration. With an increase in substrate concentration, the esterification yields decreased from 90% at 0.05 M to 12% at 1.0 M. The increase in ester concentration increased the %transesterification. This decreasing trend could be due to enzyme inactivation

Fig. 1 Effect of substrate and ester concentrations using 10 g/l enzyme concentration at 37 °C for 96 h of incubation time in *n*-hexane

(Chulalaksnanukul et al. 1990) or inhibitory action of the substrate. Unlike ethanol, ethyl caprate would not accumulate at the vicinity of the enzyme due to its lesser polarity and thus would not disturb the essential water layer around the enzyme surface. These results had indicated that inhibition of butyric acid is the prime reason for the low conversions rather than the enzyme inactivation by ethyl caprate. The highest percent esterification was obtained at lower substrate concentrations. However, the maximum ester concentration (0.25 M) was obtained at 0.5 M substrate concentration and thus the influence of other parameters was studied at a substrate concentration of 0.5 M.

Effect of immobilized enzyme concentration

The effect of enzyme concentration on percent transesterification was investigated. The percent esterification increased linearly with an increase in enzyme concentration and a maximum of 83% was achieved at 15 g/l. The increase in enzyme concentration (up to 20 g/l) resulted in either a marginal increase or no change in percent esterification (84%) or it remained constant. Therefore, the effect of other parameters on percent esterification was studied at this enzyme concentration.

Effect of temperature

The effect of temperature on the synthesis of ethyl butyrate was depicted in Fig. 2. The percent esterification increased linearly with an increase in the reaction temperature up to 50 °C, which gave 92.6% Esterification at 96 h of Incubation time. However, by increasing the temperature beyond 50 °C, the percent esterification reduction to 58%

Fig. 2 Influence of reaction temperature at 0.5 M substrate concentration, 10 g/l enzyme concentration at 96 h of incubation time in *n*-hexane

at 70 °C was noticed. The reaction rate was found to increase up to a temperature of 50 °C (0.54 $\mu\text{mol/min/mg}$ enzyme) and remained constant up to 65 °C but at 70 °C a decrease in the reaction rate (0.473 $\mu\text{mol/min/mg}$ enzyme) was observed. The percent esterification decreased to 80% and 58% at 65 °C and 70 °C respectively from 92.6% at 50 °C at 96 h of incubation time. Therefore the temperature selected was 50 °C.

Effect of molar ratio

The influence of excess substrate concentrations on the reaction rate was studied. In this study, ethyl caprate concentration was kept constant at 0.05, 0.1, 0.2, 0.3, 0.4 and 0.5 M, while butyric acid concentrations varied from 0.02

Fig. 3 Dependence of reaction rate as a function of butyric acid concentration at each fixed ethyl caprate concentration using 10 g/l enzyme at 50 °C in *n*-hexane

to 0.8 M and vice versa. The results depicted in Fig. 3 showed that the increase in butyric acid concentration led to increased reaction rate and reached a maximum at 0.2 M, and a further increase in acid concentration (up to 0.8 M) resulted in the decreased rate in all the ethyl caprate concentrations tested. It may be due to the excess butyric acid concentration inhibiting the bi-substrate reaction. However, the maximum percent esterification (95%) was obtained at equimolar ratio of acid and ester at 96 h of incubation time. On the other hand, the proportional increase in percent transesterification in ethyl caprate concentration at any given butyric acid concentration indicated no inhibitory effect of ethyl caprate, Fig. 4.

Determination of kinetic constants

It has been shown that lipases from *Rhizomucor miehei* and Porcine pancreas follow a Ping-Pong Bi-Bi mechanism with competitive inhibition by the acyl acceptor in organic solvent system (Chulallaksanakul et al. 1992). The study was focused on the determination of kinetic constants for the transesterification reaction between ethyl caprate and butyric acid. From the plots of double reciprocal initial rates of transesterification *versus* reciprocal butyric acid concentration, Fig. 5, a set of parallel lines were seen at lower butyric acid concentration in the range of 0.05 and 0.1 M. As the acid concentration increased, the slope increased and the value of intercept ($1/V_{\text{max}}$) decreased. These results suggested an assumed Ping-Pong Bi-Bi mechanism with typical competitive inhibition by one of the substrates (in this case butyric acid) (Chulallaksanakul et al. 1992; Vijay Kumar and Narsimha Rao 2003; Chulallaksanakul et al. 1990). The shapes of the

Fig. 4 Dependence of reaction rate as a function of ethyl caprate concentration at each fixed butyric acid concentration using 10 g/l enzyme concentration at 50 °C in *n*-hexane

Fig. 5 Double reciprocal plot of initial rate versus butyric acid concentration at each fixed concentration of ethyl caprate

curves indicate the inhibition at higher concentrations of hexanoic acid, and also, the inhibition was much higher at lower ethyl caprate concentrations. These results suggested that the binding of butyric acid ($K_{m(\text{acid})}$: 0.0766 M) could be much stronger than the binding of ethyl caprate to the lipase ($K_{m(\text{ester})}$: 0.210 M). This kind of inhibition has been reported earlier in the acyl transfer reaction catalyzed by lipase B from *Candida antarctica* in organic solvent system with competitive inhibition by alcohol. During transesterification reaction, the lipase may react with acid to yield dead-end lipase-acid complex or an ester to form another lipase-ester complex. Lipase-ester complex is further converted to an acyl-enzyme intermediate. The interaction of acyl-enzyme with acid led to the release of ester (product) and free lipase. Chulallaksnanukul et al. (1992) have reported the reaction mechanism for the synthesis of geranyl acetate with competitive inhibition by geraniol. The results obtained suggested that a similar scheme of mechanism might be operative for the synthesis of ethyl caprate by transesterification reaction.

To determine kinetic parameters, the experimental data (85 data points) were fitted into non-linear regression using Eq. (1), to evaluate Michaelis–Menten kinetic constants as follows: $K_{m(\text{ester})}$: 0.125 M; $K_{m(\text{acid})}$: 0.0746 M; $K_i(\text{ester})$: 2.34 M; $K_i(\text{acid})$: 0.450 M; $V_{\max}$: 2.861 $\mu\text{mol/min/mg}$ of enzyme. The activation energy (E_a) of system was 12 kJ/mol, enthalpy of a reaction (ΔH) was 2.987 KCal/mol, free energy of activation (ΔG) was -4.89 KCal/mol and the equilibrium constant (K_o), which is calculated from Eq. (2), was 1213.345/mol at 50 °C. The K_i value of ester (2.34 M) was much higher than the K_i value of acid (0.450 M), suggesting that the acid inhibition would be more pronounced than that of the ester.

Fig. 6 Influence of incubation time on synthesis of ethylbutyrate in solvent-free system using 0.05 mol substrate and different enzyme concentrations at 50 °C

Where, (PES) = the product ester(M); (a_w) = Thermodynamic water activity; (SES) = Substrate ester(M); (SAC) = Acid substrate(M).

Transesterification in solvent-free system

It was desirable to carry out the reactions in solvent-free systems. The elimination of organic solvent simplifies the downstream processing and led to the development of ‘natural’ flavor product (Trani et al. 1991). Such esters found extensive food applications and hence, it was idealistic to carry the reactions composed solely of substrates. Therefore, efforts were made to synthesize ethyl butyrate in solvent-free medium. The effect of incubation time and enzyme concentration on the synthesis of ethyl butyrate was depicted in Fig. 6, indicated that there was an increase in percent esterification with increase in enzyme concentration and incubation time. The maximum percent esterification of 76.56 was obtained at 10% (w/w) of enzyme concentration for 120 h of incubation period. There was no significant increase in percent esterification with further increase in enzyme concentration beyond 15% (w/w).

Conclusion

The surfactant coated lipase from *Candida rugosa* was suitable for the synthesis of ethyl butyrate by transesterification. Esterification reaction was affected by higher substrate concentrations. Esterification increased with the increased enzyme concentration. However, the esterification yields were affected by higher butyric acid concentrations but not by ethyl caprate. The esterification yields decreased with increasing reaction temperature, indicating that lower temperatures are favorable for esterification. The

calculated Michaelis–Menten parameters indicated that the acid inhibition (K_i acid: 0.450 M) was more pronounced than that of the ester (ethyl caprate) (K_i ester: 2.347 M). The maximum percent esterification of 96 was obtained at 0.5 M substrate concentration (1:1), 10 g/l enzyme concentration at 50 °C and 96 h of incubation time.

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