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Effect of Chain Length of Alcohol on the Lipase-Catalyzed Esterification of Propionic Acid in Supercritical Carbon Dioxide

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Abstract The esterification of propionic acid was investigated using three different alcohols, namely, isopropyl alcohol, isobutyl alcohol, and isoamyl alcohol. The variation of conversion with time for the synthesis of isoamyl propionate was investigated in the presence of five enzymes. Novozym 435 showed the highest activity, and this was used as the enzyme for investigating the various parameters that influence the esterification reaction. The Ping-Pong Bi-Bi model with inhibition by both acid and alcohol was used to model the experimental data and determine the kinetics of the esterification reaction.

Keywords Supercritical carbon dioxide · Novozym 435 · Esterification · Primary alcohol · Secondary alcohol · Ping-Pong Bi-Bi mechanism

Introduction

Esters are primarily used as flavors in food products and are obtained as extracts from naturally occurring materials, but they are not sufficient to fulfill the growing demands. Therefore, chemical synthesis of esters is used. But this is not considered as food grade, and chemical synthesis can also lead to formation of side products. Thus, enzyme-catalyzed synthesis of esters appears to be a viable alternative to chemical synthesis.

Lipases (*triacylglycerol ester hydrolases*, EC 3.1.1.3) are enzymes that can catalyze esterification [1–25], transesterification [24–30], and hydrolysis [25, 31] reactions. Catalysis without solvent (solvent-free) has been investigated [14–18], but the direct contact of enzyme with substrate at high concentration will generally deactivate the enzyme, and therefore, a solvent is used for enzyme-catalyzed reactions. Because water is a product of esterification, it is not used as a solvent for lipase-catalyzed esterification. Enzymes show better stability in nonaqueous organic solvents with log *P* (logarithm of partial coefficient in an octanol–water two-phase system) value greater than two [13], as these solvents will not strip off the essential water that is needed for enzyme to be active.

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Thus, organic solvents [1–13, 27–29] and supercritical carbon dioxide ($ScCO_2$) [10–19, 21–27] have been used as the reaction medium.

Supercritical fluids (ScF) are fluids above their critical temperature and critical pressure and possess properties between that of gases and liquids. The densities of ScF are comparable with liquids, and the diffusivities are comparable with gases resulting in lower mass transfer resistance. An additional benefit of using supercritical fluids along with enzymatic catalysis is that it provides a medium for the recovery of products or reactants. Thus, the downstream processing can be in a single stage, and the supercritical solvent can be removed by depressurizing the system. Among many supercritical fluids that can be used for the enzymatic reaction, $ScCO_2$ is often used because of its moderate supercritical properties (T_c=31.1 °C and P_c=7.38 MPa). The temperature range used for employing supercritical carbon dioxide in processing is compatible with the use of enzymes as catalysts. It is also nontoxic, nonflammable, and easily available. The log *P* value of $ScCO_2$ is 2.0 at 11.8 MPa and 50 °C [13] and, therefore, can be used for lipase-catalyzed reactions. The initial rates for esterification of oleic acid and ethanol [10], stearic acid and ethanol [13], oleic acid and *n*-octyl alcohol [16], oleic acid and oleyl alcohol [18], and acetic anhydride and isoamyl alcohol [24] are all higher in $ScCO_2$ than in organic solvents.

The Ping-Pong Bi-Bi kinetic model with inhibition is frequently used to model the experimental data in lipase-catalyzed esterification [2–4, 7, 11, 17, 20, 23, 25] and transesterification [25–30]. Many researchers have successfully used the Ping-Pong Bi-Bi model to predict the lipase-catalyzed reaction in $ScCO_2$ [11, 17, 23, 25–27], in reverse micelles [20], and in a mixture of $ScCO_2$ with ionic liquids [30].

The objective of this investigation is to study the kinetics of the synthesis of isopropyl propionate (IPP), isobutyl propionate (IBP), and isoamyl propionate (IAP) in $ScCO_2$ and the effect of chain length of alcohols on the synthesis of these esters. Thus, the esters synthesized in this study are IPP, IBP, and IAP, which are used as banana and pineapple flavors in food industries.

Materials and Methods

Materials

Isopropyl alcohol, isobutyl alcohol, acetone, and hexane were purchased form Merck Mumbai India, and isoamyl alcohol, propionic acid, and chlorobenzene were purchased form S.D. Fine Chem., India. All solvents used were of high-performance liquid chromatography grade, and the chemicals used were at least 99% pure. Novozym 435 and Lipolase 100T were generously donated by Novo Nordisk, Denmark; lipase from *Candida rugosa* and lipase from *porcine pancreas* were purchased from Sigma Aldrich, and *hog pancreas* lipase was bought from Fluka Chemie AG, Switzerland. All enzymes were preserved at 4 °C, but they are kept in a desiccator with silica gel at 20°°C for 12 h to remove excess water from the enzyme. Carbon dioxide (99%) from Vinayaka Gases (India) was used after passing the gas through a bed of silica gel. All chemicals were filtered through a microporous membrane before use.

Method

Seven-milliliter 316 stainless steel reactors were used for the lipase-catalyzed enzymatic reaction in $ScCO_2$. Each reactor, loaded with alcohol, propionic acid, and enzyme, was

pressurized to an initial pressure of 100 bar at 25 °C, using chilled CO_2 from the cylinder with a high pressure pump. The pressurized reactors were then immersed in a water bath maintained at a desired temperature with fluctuations less than 0.5 °C. All the reactions were conducted at a constant density of CO_2 (0.82 $g cm^{-3}$) at various temperatures. Even though the pressure was higher at every temperature increment, the density of the system remained constant.

The concentration range considered for the study was high enough for experimental detection with minimal error and low enough for the substrate to be completely soluble in $ScCO_2$. While the solubility and phase equilibrium data [32] of hexanoic acid and butyl acetate in supercritical carbon dioxide are available, to the best of our knowledge, no literature is available on the solubilities of propionate studied here. Therefore, a two-reactor system was designed to investigate the effect of solubility/mixing in the reaction [14, 19]. The enzyme was added into one reactor, and acid and alcohol were added to the other reactor. Thus, there was no physical contact between the reactants and the enzyme. The concentration of the reactants and the enzyme in the two-reactor setup was taken to be identical to the concentrations in the single reactor. The reactors were then individually pressurized. After pressurization, they were connected through a small steel tube, and the connecting valves were opened and incubated at the required temperatures. It should be pointed out that the substrates and the enzyme are not in physical contact with each other in the two-reactor setup. Therefore, the substrate and enzyme can come in contact with each other only through $ScCO_2$. The conversion obtained in the two-reactor system was identical to the conversion obtained in the single reactor system indicating the system is completely soluble in $ScCO_2$.

After the desired reaction time, the reactor was depressurized and the contents were eluted in 2 cm^3 of eluent. Hexane was used as eluent for isopropyl propionate and isoamyl propionate, while acetone was used as an eluent for isobutyl propionate. The enzyme was removed by centrifugation at 4,000 rpm for 2 min, and 1 cm^3 of the supernatant reaction mixture was analyzed by gas chromatography. Many reactions were repeated in triplicate, and the reproducibility of results for conversion was within 2%.

Experiments were also conducted to determine the stability and re-usability of the enzyme. After each cycle, the reaction mixture was removed, and the enzyme was rinsed with hexane and filtered and the solvent evaporated before being used with fresh substrates. The retention of the activity of the enzyme after repeated use was assessed in terms of conversion at the end of each cycle. The enzyme retained almost complete activity for 3 cycles, and this stability may be due to immobilization and low water content.

Analysis

A gas chromatograph (Varian CP-3800) with a flame ionization detector was used for the detection and quantification of alcohol, acid, and esters. The compounds were separated in a VF-5 ms (5% phenyl, 95% dimethyl polysiloxane, 30 m×0.25 mm×1 μm) capillary column with helium (UHP grade) as the carrier gas at 0.4 mL min^{-1}. A constant split ratio of 50 was maintained for better resolution of the analytes. The column oven temperature was initially maintained at 40 °C for 6 min, then ramped at a rate of 5 °C min^{-1} to 150 °C and held for 2 min. The injector and detector temperature was kept at 200 and 220 °C, respectively. The esters were synthesized chemically and purified and used for calibration. Twenty-five microliters of chlorobenzene was added as the internal standard to the supernatant obtained before injection. The retention times of isopropyl alcohol, acetone, hexane, isobutyl alcohol, propionic acid, isoamyl alcohol, isopropyl propionate, chlorobenzene, isobutyl propionate, and isoamyl propionate were 6.4, 6.5, 8.1, 8.9, 12.1, 12.5, 13,

16.8, 17.3, and 20.9 min, respectively. The amount of reactants and products was quantified by calculating the area under the peaks of standard samples.

Results and Discussion

Screening of Enzyme with Isoamyl Propionate

Lipases like Novozym 435 from *Candida antarctica* [3, 15, 24–26, 28–30], *C. rugosa* lipase [3, 4, 7, 8], Lipozyme RM IM [13, 16, 17, 24, 28, 29], *Mucor miehei* [10–13, 21, 23, 27], crude *hog pancreas* lipase [14, 15, 19], Lipolase 100T, [9, 15], Lipozyme TL IM [28, 29], Lipozyme IM 20 [3, 6], *Mucor* sp.[1], *Burkholderia cepacia* [2], crude *porcine pancreas* [3], and Randozyme SP-435 [5] have been effectively used for esterification and transesterification. In this study, we considered five different enzymes, namely, Novozym 435, Lipolase 100T, and lipases from *C. rugosa*, *porcine pancreas*, and *hog pancreas* for the synthesis of isoamyl propionate. The effect of enzyme loading and change in conversion with time was determined. The results obtained for the variation in conversion with time are plotted in Fig. 1. Reaction was performed for 24 h with 459 mmol L^{-1} of propionic acid and 314 mmol L^{-1} of isoamyl alcohol with 10 mg of enzyme loading at 50 °C. The conversion was nearly 40% in the presence of Novozym 435, while conversions were less than 3% in the presence of other enzymes. To explore whether higher concentrations of substrate are inhibiting the enzyme activity, the effect of enzyme loading was studied for the above system. The enzyme loading was increased up to 50 mg (which is equivalent to 21% (*w*/*w*) considering only the weight of the substrate, propionic acid). The results obtained are shown in Fig. 2 and the trends obtained are similar. The initial reaction rate is nearly seven times in the presence of Novozym 435 compared to other enzymes. Thus, based on the effect of enzyme loading and conversion with time, Novozym 435 is the best enzyme (among the enzymes studied) for isoamyl propionate synthesis. Therefore, Novozym 435 was used for studying the effect of various other parameters on esterification.

Reaction with Different Chain Lengths of Alcohol

The synthesis of esters with different chain lengths of acids and alcohols is important. Therefore, the effect of chain length of alcohol for enzymatic esterification has been investigated in this study. Three different branched alcohols, namely, isopropyl alcohol, isobutyl alcohol, and isoamyl alcohol were investigated. Apart from chain length, these alcohols can be categorized into two categories, namely, primary alcohol and secondary alcohol. Isopropyl alcohol is a secondary alcohol, while isobutyl and isoamyl are primary alcohols. The typical kinetic expressions of these reactions are:

$$\underset{\text{Propionic acid(R1)}}{CH_3CH_2COOH} + \underset{\text{Isopropyl alcohol(R2)}}{(CH_3)_2CHOH} \rightleftarrows \underset{\text{water (P1)}}{H_2O} + \underset{\text{Isoprophyl propionate (P2)}}{CH_3CH_2COOCH(CH_3)_2}$$

$$\underset{\text{Propionic acid(R1)}}{CH_3CH_2COOH} + \underset{\text{Isobutyl alcohol (R2)}}{(CH_3)_2CHCH_2OH} \rightleftarrows \underset{\text{water (P1)}}{H_2O} + \underset{\text{Isobutyl propionate(P2)}}{CH_3CH_2COOCH_2CH(CH_3)_2}$$

$$\underset{\text{Propionic acid(R1)}}{CH_3CH_2COOH} + \underset{\text{Isoamyl alcohol (R2)}}{(CH_3)_2CH(CH_2)_2OH} \rightleftarrows \underset{\text{water (P1)}}{H_2O} + \underset{\text{Isoamyl propionate (P2)}}{CH_3CH_2COO(CH_2)_2CH(CH_3)_2}$$

To study the effect of chain length, the concentration of 287 mmol L^{-1} for each alcohol was taken with equimolar propionic acid. The conversion of propionic acid was the highest

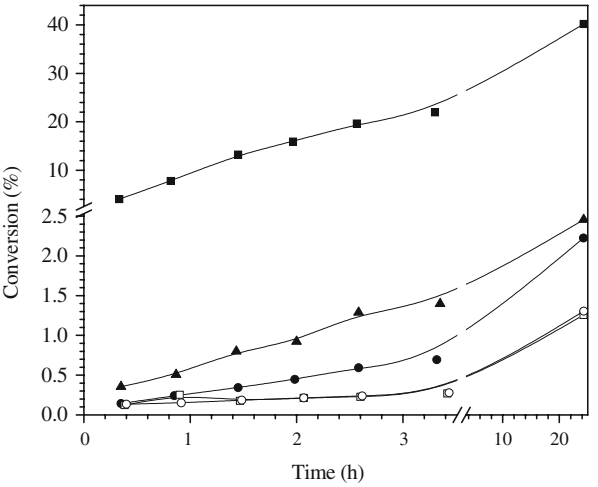

Fig. 1 Effect of different enzymes on the conversion of propionic acid in isoamyl propionate, with 459 mmol L^{-1} of propionic acid and 314 mmol L^{-1} of isoamyl alcohol with 10-mg enzyme loading at 50 °C. *Filled square*, Novozym 435; *filled circle*, Lipolase 100T; *filled triangle*, lipase from *Candida rugosa*; *empty square*, lipase from *porcine pancreas*; *empty circle*, *hog pancreas* lipase

for isobutyl alcohol and the lowest for isopropyl alcohol. These results show that the reaction rate is higher for the reaction with primary alcohol than that with secondary alcohol. The reaction rate decreases with an increase in chain length of primary alcohols. The kinetics of this reaction will be discussed later.

The variation of the conversion with the chain length of the alcohol can be attributed to the influence of various factors such as the molecular size of the alcohol, solubility of the alcohol in the supercritical fluid, and affinity of the lipase for the particular alcohol. This phenomenon could also be explained in terms of the binding energy that is released when a substrate binds at the active site [9]. Only a few of the substrates that bind at the active site can release a sufficient amount of binding energy required for effecting a change in conformation of lipase to a form that is a more efficient catalyst (known as the induced fit model for enzyme action) [1, 33, 34]. More than one active site in the overall lipase

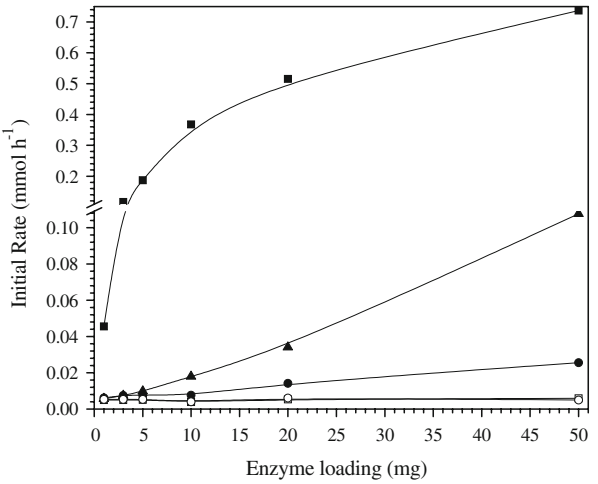

Fig. 2 Effect of enzyme loading with different enzymes on the initial rate during the synthesis of isoamyl propionate with 459 mmol L^{-1} of propionic acid and 314 mmol L^{-1} of isoamyl alcohol at 50 °C. The reaction time was 30 min for Novozym 435 and 90 min for other enzymes. See Fig. 1 for significance of symbols

molecule is possible, as observed in the literature [9, 35], and two acyl-binding pockets in the active site of the lipase [36] have also been postulated. There is an existence of more than one type of active site in the overall lipase molecule, one for smaller substrates and the other that can accommodate the larger substrates [9].

The variation of conversion with the chain length of alcohol has been investigated earlier in organic solvents. For example, various esters have been synthesized in cyclohexane using propionic, butyric, and caproic acids, as well as methanol, ethanol, allyl, butanol, isoamyl, geraniol, citronellol, and farnesol alcohols. The conversions were dependent on the affinity of the lipase toward the acids (short or long) or the alcohols (linear or branched and short or long) [1]. The presence of side chain and unsaturation in alcohol reduce the conversion and the affinity of the *Mucor* sp. lipase with increasing chain length of alcohol [1]. Similarly, the esterification of lauric acid with various C1–C18 aliphatic alcohols by Lipolase 100L in isooctane and the highest reaction rate were observed with *n*-butyl alcohol [9].

The effect of chain length of acetate for the transesterification to geranyl acetate with immobilized lipase from *M. miehei* shows an increase in the rate of lipase activity from methyl acetate (C3) to propyl acetate (C5), then it decreases continuously to octyl acetate (C10) [27]. Blattner et al. [23] studied the effect of chain length of alcohol for enzymatic esterification of lauric acid with encapsulated *C. antarctica* in $ScCO_2$. The study showed that, in $ScCO_2$, there is an increase of the esterification reaction rate when the chain length of the alcohol increases from ethanol to 1-butanol. This maximum is followed by a sharp decline and a slow increase towards the long chain alcohols. For transesterification of methyl acetoacetate with different alcohols with Novozym 435 in toluene, the conversions were higher for primary alcohol than with secondary alcohol. The conversions decreased with increase in the chain length for both primary alcohol and secondary alcohol [29]. For the enzymatic esterification of butyric acid with different primary and secondary alcohols, conversions with secondary alcohols were lower than that obtained with primary alcohol. The conversion reduced with the chain length of alcohol for both primary and secondary alcohols [37]. The conversion and reaction rate of *p*-nitrophenyl esters decreases with increasing chain length for *p*-nitrophenyl butyrate to *p*-nitrophenyl palmitate for hydrolysis reaction in $ScCO_2$ [31].

Effect of Enzyme Loading

The effect of enzyme loading was investigated with 287 mmol L^{-1} of both acid and alcohol at 50 °C. Figure 3 shows the results obtained for the variation in initial rate with enzyme loading. There is a sharp increase in the initial rate with an increase in enzyme loading up 10–15 mg (4–6% (*w*/*w*), 5-7.5 gmol^{-1} of alcohol). The initial rate followed the order: isobutyl propionate > isoamyl propionate > isopropyl propionate.

A similar kind of optimal enzyme loading has been reported in the literature [19, 24–26]. The initial rate and conversion increases with increasing enzyme loading below the optimal enzyme loading but has a very marginal increase in the reaction rate with increasing enzyme loading above this optimal value. The conversion of isoamyl butyrate with crude *hog pancreas* lipase varies up to an enzyme loading of 6% (*w*/*w*) in $ScCO_2$ [19]. The optimal enzyme loading of 3% (*w*/*w*) and 7% (*w*/*w*) was observed for esterification and transesterification synthesis of butyl butyrate in $ScCO_2$ with Novozym 435 [25]. The synthesis of isoamyl acetate from isoamyl alcohol and acetic anhydride using Novozym 435 in $ScCO_2$ shows that there is an appreciable increase in the initial rate and conversion up to an enzyme loading of 6.25 gmol^{-1} of alcohol [24].

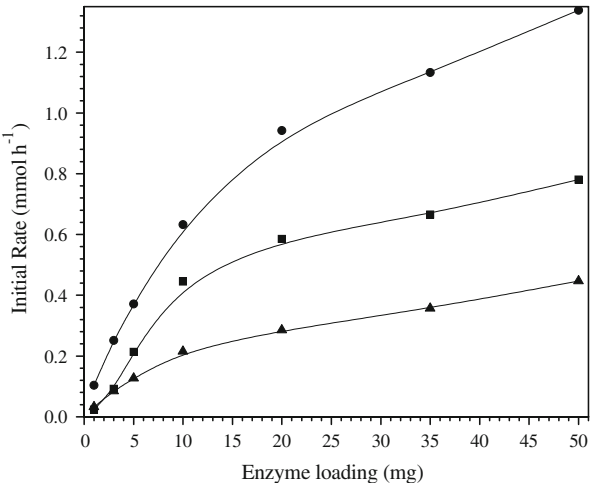

Fig. 3 Effect of Novozym 435 loading on the initial rate at 50 °C. The concentrations of propionic acid and alcohol were 287 mmol L^{-1}. *Filled triangle*, isopropyl propionate; *filled circle*, isobutyl propionate; *filled square*, isoamyl propionate

Effect of Temperature

The effect of temperature was investigated with Novozym 435 with 10 mg of enzyme loading and with the concentration of 287 mmol L^{-1} for both acid and alcohol. Results obtained are plotted in Fig. 4. At lower temperatures, there is an increase in the initial rate with temperature, and the conversion reaches a maximum value around 50–55 °C. A further increase in temperature decreases the reaction rate. Chulalaksananuku et al. showed that the enzyme stability decreased with temperature due to thermal denaturation above 40 °C for the immobilized lipase from *M. miehei* in $ScCO_2$ [27]. As temperature increases above 40 °C, the extent of esterification and reaction rate decreases for the synthesis of isoamyl acetate from isoamyl alcohol and acetic anhydride in the presence of Novozym 435 in $ScCO_2$ [24]. An optimum temperature of 40 °C at 20 MPa and 50 °C for 10 MPa was observed for the esterification of oleic acid and 1-octanol with Lipozyme RM IM in $ScCO_2$

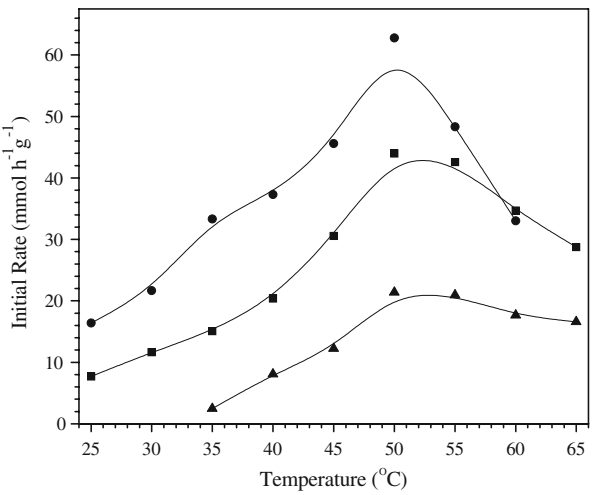

Fig. 4 Effect of temperature on the initial rate with 10 mg of Novozym 435. The concentrations of propionic acid and alcohol were 287 mmol L^{-1}. See Fig. 3 for significance of symbols

[16]. Optimum temperature for the synthesis of isoamyl butyrate is 55 °C for Novozym 435 and 50 °C for *hog pancreas* lipase and Lipolase 100T in $ScCO_2$ [15]. The optimum temperature for enzymatic transesterification of castor oil with ethanol and methanol is 50 and 45 °C, respectively, with Novozym 435 in $ScCO_2$ [26]. The optimum temperature is in the range of 45–50 °C for the esterification of butanol with butyric acid and transesterification of butanol with ethyl butyrate using Novozym 435 in $ScCO_2$ [25]. The optimum temperature was reported as 45 °C for esterification of myristic acid with ethanol using crude *hog pancreas* lipase in $ScCO_2$ [14]. An optimum temperature of 40–45 °C was reported for the synthesis of isoamyl laurate and isoamyl stearate by esterification in $ScCO_2$ [22]. An optimum temperature of around 50 °C was also reported for the hydrolysis of *p*-nitrophenyl esters in acetonitrile and $ScCO_2$ [31]. The optimum temperature obtained in this study is similar to the optimum temperature of 40–60 °C for the synthesis of isoamyl acetate [24], isoamyl butyrate [19], ethyl palmitate [38], lavandulyl acetate [39], ethyl myristate [14], octyl palmitate [40], and geranyl acetate [27] in $ScCO_2$.

The activation energy can be calculated for the range below the optimum temperature and was found to be 118, 43, and 56 kJ mol^{-1} for the synthesis of isopropyl propionate, isobutyl propionate, and isoamyl propionate, respectively. The activation energy was 45 and 70 kJ mol^{-1} at 10 and 20 MPa, respectively, for the esterification of oleic acid and 1-octanol with Lipozyme RM IM in $ScCO_2$ [16]. Similarly, the activation energies for esterification of butyric acid and transesterification of ethyl butyrate to butyrate in $ScCO_2$ were 32 and 25 kJ mol^{-1}, respectively [25]. The activation energies observed in this study are comparable to those obtained for biocatalytic reactions as reported for the esterification in the synthesis of tetrahydrofurfuryl butyrate (47 kJ mol^{-1}) [41] and butyl laurate (45 kJ mol^{-1}) [7].

Kinetics of Reaction

The kinetics of reaction was studied at 50 °C with 10 mg enzyme loading for all three alcohols (isopropyl alcohol, isobutyl alcohol, and isoamyl alcohol) with propionic acid at various concentrations. All results are plotted in Fig. 5. To study the effect of chain length, the concentration of 287 mmol L^{-1} alcohol was taken with equimolar propionic acid. The

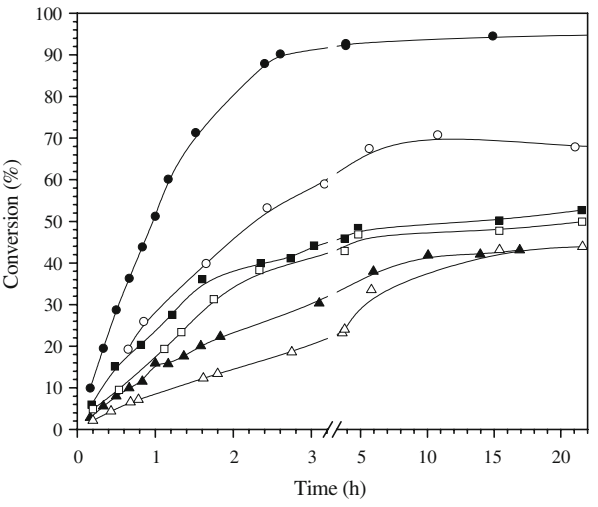

Fig. 5 Effect of initial substrate concentration on the conversion of propionic acid with 10 mg of Novozym 435 at 50 °C. *Filled circle*: propionic acid 143 mmol L^{-1}, isobutyl alcohol 143 mmol L^{-1}; *filled square*: propionic acid 287 mmol L^{-1}, isobutyl alcohol 287 mmol L^{-1}; *filled triangle*: propionic acid 547 mmol L^{-1}, isobutyl alcohol 442 mmol L^{-1}; *empty circle*: propionic acid 174 mmol L^{-1}, isoamyl alcohol 119 mmol L^{-1}; *empty square*: propionic acid 287 mmol L^{-1}, isoamyl alcohol 287 mmol L^{-1}; *empty triangle*: propionic acid 287 mmol L^{-1}, isopropyl alcohol 287 mmol L^{-1}

effect of chain length was not altered and was the same as that observed earlier. The conversion of propionic acid was highest for isobutyl alcohol and lowest for isopropyl alcohol. The effect of concentration of the substrate on the conversion of propionic acid was also investigated with three different concentrations for isobutyl propionate and two different concentrations for isoamyl propionate. In the case of both propionates, it was observed that the conversion of propionic acid was higher for lower substrate concentration. More than 95% conversion of propionic acid was observed at lower equimolar concentration of 143 mmol L^{-1} for isobutyl propionate synthesis. Similarly, 65% conversion of propionic acid and nearly 100% conversion of isoamyl alcohol were observed when the initial concentrations were 174 and 119 mmol L^{-1} for propionic acid and isoamyl alcohol, respectively. The results indicate that there is deactivation of enzyme at higher concentrations of substrates due to substrate inhibition.

The effect of concentration of the substrate on inhibition was investigated by a kinetic study. The initial rate of reaction for the synthesis of propionate was studied for all three alcohols. The results are plotted in Fig. 6a–c for the syntheses of isopropyl propionate, isobutyl propionate, and isoamyl propionate, respectively. The variation in the initial rate was investigated at 50 °C with 10 mg of enzyme loading in the concentration ranges of 32–1,493, 26–1,238, and 22–1,049 mmol L^{-1} of isopropyl alcohol, isobutyl alcohol, and isoamyl alcohol, respectively, with six different propionic acid concentrations, i.e., 38, 77, 128, 257, 382, and 511 mmol L^{-1}. Figures 6a–c shows that the qualitative trends in initial rate with the range of concentrations are similar for the synthesis of all three propionates.

With an increase in alcohol concentration, at lower acid concentration, the initial rate increases and then decreases. The optimum alcohol concentration at which maximum initial rate can be obtained increases with an increase in acid concentration. Due to this, the initial rate does not decrease at higher acid concentrations. There is a continuous decrease in initial rate with propionic acid concentration from the starting range of concentration studied with lower alcohol concentration for all three alcohols. At higher alcohol concentration, the initial rates show an optimum value and similar trends are obtained for all the alcohols. The results obtained show that both the substrate acid and alcohol cause inhibition for these esterification reactions. Most of the lipase-catalyzed reactions can be explained by the Ping Pong Bi-Bi mechanism. This mechanism has been reported in the literature in organic solvents [2–7, 11, 12, 27, 29] as well as in $ScCO_2$ [11, 23, 25–27]. It has been successfully used for modeling esterification [2–7, 11, 12, 23, 25], trans-esterification [25–27, 29, 30], and hydrolysis [25, 31] reactions.

Most of the studies have reported only the inhibition by alcohol [4, 11, 23], but this assumption is applicable only for reactions conducted with short chain alcohols and long chain acids. Short chain acids like butyric acid [6] and propionic acid (as observed in this study) inhibit the enzyme, and thus, this inhibition should also be considered.

According to the Ping-Pong Bi-Bi mechanism, in the esterification reaction, propionic acid (R1) reacts with enzyme to form an enzyme–propionic acid complex, which then isomerizes to acyl–enzyme intermediate and releases water as the first product (P1). Acyl–enzyme binds with alcohol (R2) to form another binary complex that isomerizes to a corresponding alcohol propionate–enzyme complex, which finally releases the ester propionate (P2) and enzyme. The alcohol is represented by a reactant (R2) and the product is represented by a product (P2). The model equation for the initial rate of esterification reaction, V, is [25, 26]:

$$V = \frac{V_{\max}[C_{R1}][C_{R2}]}{[C_{R1}][C_{R2}] + K_{R2}[C_{R1}]\left(1 + \frac{[C_{R1}]}{K_{IR1}}\right) + K_{R1}[C_{R2}]\left(1 + \frac{[C_{R2}]}{K_{IR2}}\right)}$$

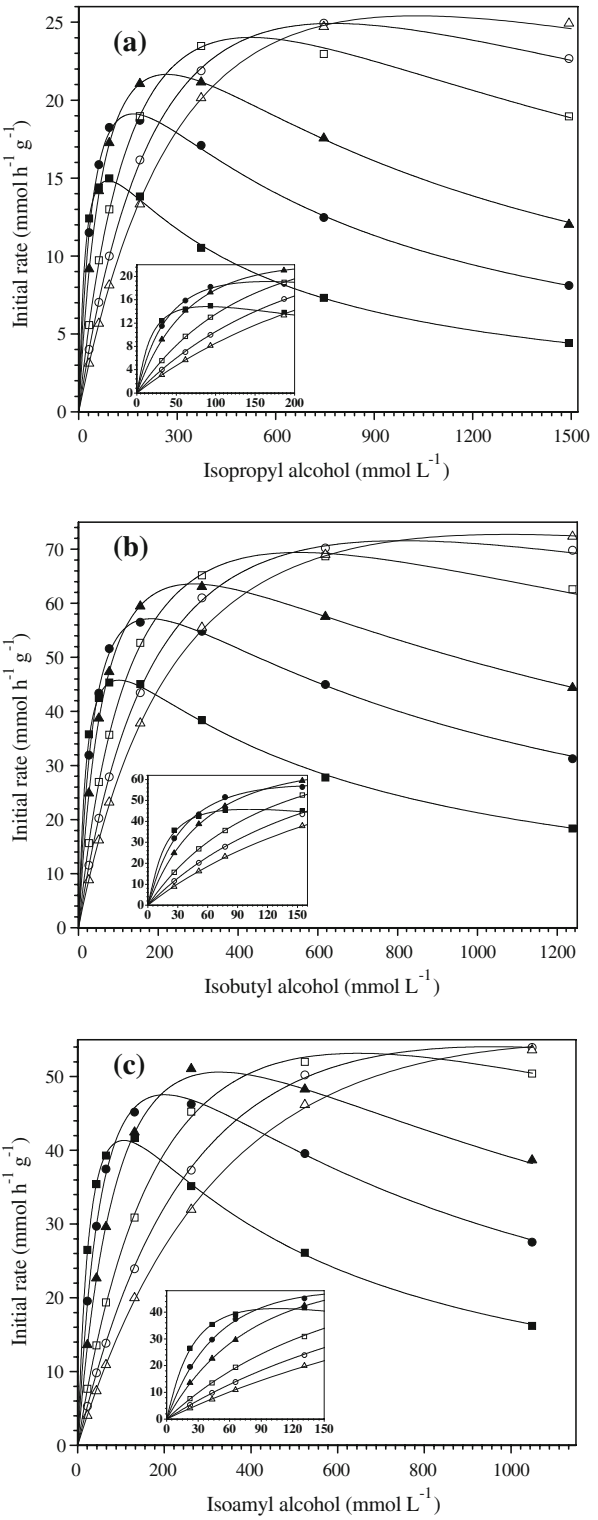

Fig. 6 Effect of concentration of acid and alcohol in **a** isopropyl propionate synthesis, **b** isobutyl propionate synthesis, and **c** isoamyl propionate synthesis at 50 °C with 10 mg of Novozym 435 loading. The values predicted by the model are represented by *solid lines*. Legends represent concentration of propionic acid: *filled square*, 38 mmol L^{-1}; *filled circle*, 77 mmol L^{-1}; *filled triangle*, 128 mmol L^{-1}; *empty square*, 257 mmol L^{-1}; *empty circle*, 382 mmol L^{-1}; *empty triangle*, 511 mmol L^{-1}

where V_{max} is the maximum initial velocity of esterification reaction; K_{R1} and K_{R2} are Michaelis constants; and K_{IR1} and K_{IR2} are dissociation constants for the corresponding reactant and product, respectively. All the parameters in the above equations are estimated using an optimization program in Matlab® 6.5. The parameters obtained for all the three alcohols are tabulated in Table 1. There is a good agreement between the experimental data and model prediction for all three alcohols.

The synthesis of isopropyl propionate by the secondary alcohol has the lowest V_{max} compared to that obtained for the synthesis of isobutyl propionate and isoamyl propionate using the primary alcohols. Among isobutyl propionate and isoamyl propionate, the synthesis of the former has a higher V_{max} compared to the synthesis of the latter. Thus, the synthesis of the propionate with an alcohol of smaller chain length is higher than that obtained with an alcohol of longer chain length. The ratio, K_{R1}/K_{R2}, defines the acid affinity for enzyme and a higher value shows higher affinity [4]. The calculated values for IPP, IPB, and IAP are 4, 2.3, and 2.1, respectively, which show that Novozym 435 affinity for propionic acid decreases in the presence of alcohol of longer chain length. The inhibition constant for alcohol is higher than the inhibition constant of the acid by an order of magnitude, which shows that propionic acid strongly inhibits the enzyme for all three alcohols.

Conclusions

Isopropyl propionate, isobutyl propionate, and isoamyl propionate were synthesized successfully using five different lipases in supercritical carbon dioxide. Novozym 435 was found to be most effective among the five enzymes studied. The optimum enzyme loading of 4–6% (*w*/*w*) and the optimum temperature of 50–50 °C were observed for the synthesis of isopropyl propionate, isobutyl propionate, and isoamyl propionate in the presence of Novozym 435 in supercritical carbon dioxide. The enzymatic esterification of propionic acid was faster with primary alcohols (isobutyl alcohol, isoamyl alcohol) than that observed with the secondary alcohol (isopropyl alcohol). The initial rate of reaction decreases with an increase in chain length of the primary alcohol, and thus, the conversion obtained for the synthesis by isobutyl alcohol was higher than that obtained for the synthesis by isoamyl alcohol. The Ping-Pong Bi-Bi model with inhibition by both acid and alcohol was able to predict the initial rate data for the synthesis of all three propionates.

Table 1 Kinetic parameters for the esterification of propionic acid with different alcohols.

	Isopropyl propionate	Isobutyl propionate	Isoamyl propionate
V_{max} (mmol $g^{-1}h^{-1}$)	53	122	104
K_{R1} (mmol L^{-1})	56	35	21
K_{R2} (mmol L^{-1})	14	15	10
K_{IR1} (mmol L^{-1})	14	24	9
K_{IR2} (mmol L^{-1})	226	238	123

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