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Synthesis of 2-ethylhexyl-2-ethylhexanoate catalyzed by immobilized lipase in *n*-hexane: A kinetic study

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Abstract

A direct esterification reaction for the synthesis of 2-ethylhexyl-2-ethylhexanoate from 2-ethylhexanoic acid and 2-ethyl-1-hexanol in *n*-hexane as solvent, and Novozym 435, as catalyst, has been carried out. The effect of concentrations of 2-ethylhexyl-2-ethylhexanoate, water, 2-ethylhexanoic acid and 2-ethyl-1-hexanol on ester yield, the initial forward esterification reaction and the reverse reaction rates have been investigated. The experimental results show that the esterification reaction proceeds with a Ping-Pong Bi-Bi mechanism and that 2-ethylhexanoic acid inhibits the reaction.

Kinetic parameters were calculated based on this model as follows: $R_{max} = 37.59\ mmol\ h^{-1}\ g^{-1}$, $K_{mAl} = 1.51\ M$, $K_{mAc} = 0.78\ M$, $K_{iAc} = 1.55\ M$. The calculated parameters were used to estimate the theoretical initial reaction rate. The calculated initial reaction rates from kinetic parameters for concentrations of less than 0.1 M 2-ethylhexanoic acid show good agreements with the experimental data.

Keywords: Esterification; Novozym 435; Ping-Pong mechanism; Bi-Bi mechanism; Kinetic; 2-Ethylhexanoic acid

1. Introduction

The ability of enzymes to catalyze chemical reactions in organic solvents is well established [1–3]. Many esterification reactions, carried out in the presence of enzymes, have been reported [4–6]. Esters are compounds with many applications mainly in food, detergents, pharmaceuticals and cosmetic industries, e.g., as flavoring and fragrance agents. The main advantages of using lipase as catalysts for these kinds of reactions are high yields, mild working conditions (in comparison with inorganic acid catalysts), easy recovery, and reusability of the catalyst (i.e. an immobilized enzyme) [7–9]. 2-Ethylhexyl-2-ethylhexanoate has been used in cosmetic or pharmaceutical preparations for improving the spreading behavior of the oil, particularly on the human skin [10].

The catalytic esterification of 2-ethylhexanoic acid with 2-ethyl-1-hexanol in supercritical CO_2 has been reported elsewhere [11]. The effect of pressure (150–250 bar), temperature (75–140 °C), flow rate of CO_2 (0.36–0.72 g min^{-1}), mole ratio of the alcohol to the acid (0.5–2), and the type of catalyst (Amberlyst® 15 as a strong solid acid-catalyst, zirconium oxide as a Lewis acid-catalyst, and Novozym 435 as an enzymatic catalyst) has been evaluated. The ester, 2-ethylhexyl-2-ethylhexanoate, was continuously synthesized with 100% selectivity and 40% conversion using zirconium oxide, while the enzymatic catalysis gave no significant conversion (3%) due to acid inactivation of the enzyme. Amberlyst® 15 preferentially catalyzed the dehydration of 2-ethyl-1-hexanol to produce 2-ethyl-1-hexene. High temperatures favored this reaction, so at 140 °C and 150 bar, the conversion to alkene was 99%. This method lacks enough selectivity and has a low conversion for esterification reaction when Amberlyst® 15 is used.

In order to identify optimum conditions for performing the ester synthesis, it is useful to know the reaction kinetics and rate constants [12]. There are two prevalent models to describe the enzymatic mechanism and to get kinetic information. Some reactions have been explained with Michaelis–Menten model [12,13]. The other common model is Ping-Pong mechanism, which has been used by many researchers to calculate kinetic

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parameters and describe the mechanism of different reactions [2,4,9,14,15].

Despite the fact that several kinetic studies are reported, the information needed for process design [16], and optimization of the reaction under investigation are limited [9]. Therefore, in this work, the lipase-catalyzed synthesis of 2-ethylhexyl-2-ethylhexanoate from 2-ethylhexanoic acid and 2-ethyl-1-hexanol was investigated and a kinetic model is presented.

2. Materials and methods

2.1. Materials

The immobilized lipase, Novozym 435 from *Candida antarctica*, supported on a macroporous acrylic resin with a water content of 1–2 w/w% was kindly provided by Novo Nordisk, Denmark. 2-Ethyl-1-hexanol, *n*-hexane, *n*-decane, *p*-toluenesulfonic acid (PTSA), and toluene supplied by Merck. 2-Ethylhexanoic acid was purchased from Tat Chemical Co. (Isfahan, Iran) with purity of >99% verified by capillary GC-FID. Due to difficulties with finding a supplier of pure 2-ethylhexyl-2-ethylhexanoate, the compound was synthesized from 2-ethyl-1-hexanol and 2-ethylhexanoic acid, in toluene as solvent and PTSA as catalyst, the purity of the ester was confirmed by capillary GC-FID (i.e. > 99%).

2.2. Synthesis procedure

Synthesis of 2-ethylhexyl-2-ethylhexanoate was carried out in a batch stirred tank reactor made of Pyrex glass with a spherical geometry and 250 mL capacity. The reactor equipped with a magnetic stirrer, containing 2-ethyl-1-hexanol, *n*-decane (as an internal standard), and *n*-hexane was placed in a thermostatic water bath providing a constant temperature to within ±0.1 °C. A reflux condenser was used to prevent loss of the reacting compounds. When the reaction temperature reached the set value, the acid and the lipase as a catalyst were added. Aliquot samples of 200 μL of the reaction mixture were withdrawn at various time intervals and the concentrations of product and reactants were determined using GC-FID to calculate the amount of conversion. In order to stop the reaction, after withdrawal from the reactor, the samples were cooled immediately in refrigerator to avoid any further reaction. Average uncertainty of the reported data was found to be less than 8%.

The Novozym 435 concentration was kept constant at 2 g of enzyme/L of *n*-hexane volume in all experiments throughout the present reaction series. In a previous work [17], no improvement in esterification conversion was observed with increasing the stirring rate. Therefore, all experimental runs were performed with a stirrer speed of 200 rpm.

2.3. Determination of kinetic parameters

The time course of 2-ethylhexyl-2-ethylhexanoate synthesis was plotted and the slope of the tangent to the curve at the start of the reaction was taken as the initial reaction rate. It was expressed as mmol of the product (i.e. 2-ethylhexyl-2-ethylhexanoate) per hour per gram of immobilized enzyme (mmol h^{-1} g^{-1}). In order to calculate the kinetic parameters, the initial reaction rate data were fitted with the proposed rate equation by non-linear regression analysis using the Matlab computer program.

2.4. Analytical methods

The reaction mixtures were analyzed with a gas chromatograph (GC-17 Shimadzu) equipped with a flame ionization detector (GC-FID) and a BP21-FFAP capillary column with 25-m length and 0.52-mm i.d. Nitrogen was used as the carrier gas at a total flow rate of 29 mL/min. The initial temperature and time of 50 °C and 0.0 min were set, respectively. After injection of the sample, the temperature of the column oven was linearly raised to 110 °C with the rate of 10 °C min^{-1} and then with the rate of 15 °C min^{-1} to the final temperature of 190 °C. Finally, the oven temperature was kept constant at 190 °C for 2 min. The GC injection port and detector temperatures were set at 230 and 260 °C, respectively. Retention times of the peaks were as follows: *n*-decane, 1.72 min; 2-ethyl-1-hexanol, 6.35 min; 2-ethylhexyl-2-ethylhexanoate, 8.99 min; 2-ethylhexanoic acid, 10.54 min. Quantitative data obtained using the internal standard method based on the peak area of the standard solutions and *n*-decane as an internal standard.

3. Results and discussion

The effect of temperature, concentration of 2-ethyl-1-hexanol, 2-ethylhexanoic acid, 2-ethylhexyl-2-ethylhexanoate, and water on forward reaction and the effect of concentrations of 2-ethylhexyl-2-ethylhexanoate and water on reverse reaction were investigated.

3.1. The effect of temperature

The effect of temperature on the initial esterification reaction rate is shown in Fig. 1. It can be seen that the initial reaction rate increases with temperature from 30 to 45 °C. However, a further increase in temperature from 50 to 70 °C reduced the ini-

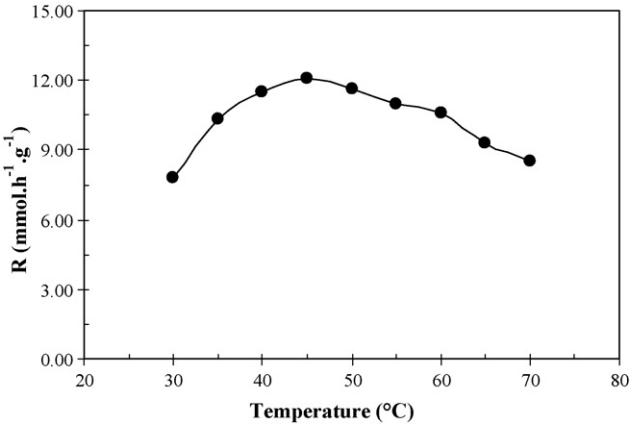

Fig. 1. The effect of temperature on the initial reaction rate of 2-ethylhexanoic acid and 2-ethyl-1-hexanol at the equal concentration of 0.5 M.

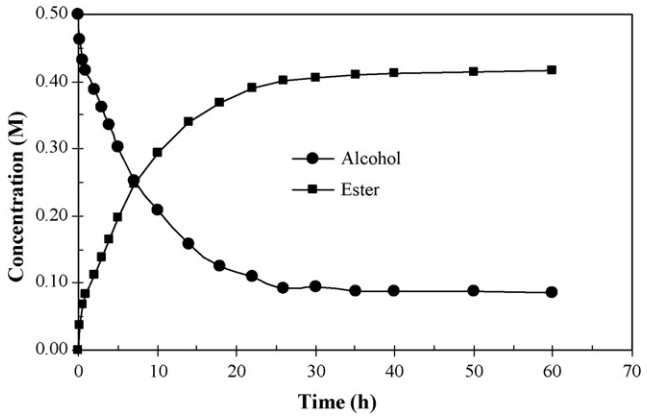

Fig. 2. Time course of 2-ethylhexyl-2-ethylhexanoate synthesis. Reactants concentrations = 0.5 M; immobilized Novozym 435 concentration = 2 g/L.

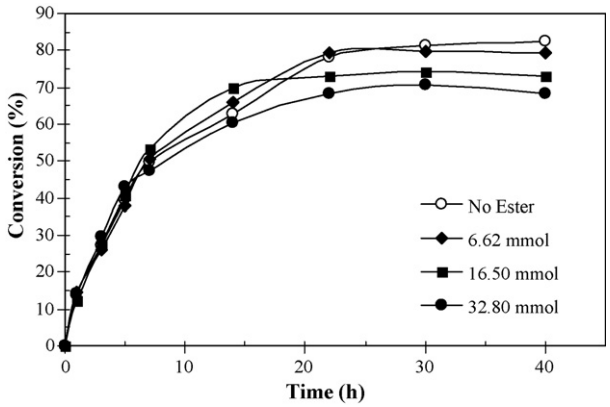

Fig. 3. The effect of 2-ethylhexyl-2-ethylhexanoate content on the synthesis of 2-ethylhexyl-2-ethylhexanoate. Reactants concentrations = 0.4 M; immobilized Novozym 435 concentration = 2 g/L.

tial reaction rate. Temperature affects the enzyme stability [18], and thus the reaction thermodynamic equilibrium and kinetics. These factors may interact leading to the observed trend. The enzyme thermal denaturation also decreases the initial reaction rate at high temperatures [18]. Therefore, all further experiments were carried out at the optimum temperature of 45 °C.

3.2. Synthesis of 2-ethylhexyl-2-ethylhexanoate

The time course of reactant consumption and product formation at initial equimolar concentrations (i.e. 0.5 M) is shown in Fig. 2. Reaction rates are high within the short time of 5 min from the start, slowing down as the reactants are consumed. A maximum esterification percentage of 83% is obtained at a reaction time of 40 h. At the beginning, 2-ethylhexanoic acid promptly reacts with 2-ethyl-1-hexanol, producing 2-ethylhexyl-2-ethylhexanoate and water. This reaction reaches equilibrium within 40 h.

3.3. The effect of products concentration on the esterification reaction rate

In order to study the inhibition effect of the products on the esterification reaction rate; several experiments were carried out under conditions shown in Table 1. The inhibition was investigated by adding small amounts of 2-ethylhexyl-2-ethylhexanoate or water at the beginning of the reaction.

Table 1
Different concentrations of the products chosen for the study of the product inhibition in synthesis of 2-ethylhexyl-2-ethylhexanoate from 2-ethylhexanoic acid and 2-ethyl-1-hexanol (substrates were in equimolar concentration of 0.4 M)

C_{ester} (M)	C_{water} (M)	Conversion (%)
0	0	82.6
0.13	0	79.5
0.33	0	72.9
0.66	0	68.4
0	0.33	63.6
0	0.66	47.8
0	0.98	36.3

Figs. 3 and 4 show the effect of ester and water concentrations on the synthesis of 2-ethylhexyl-2-ethylhexanoate catalyzed by the Novozym 435, respectively. When the ester concentration is increased (Fig. 3), conversion of the reactants to products is decreased. Most of the experimental works reported on the esterification reaction have shown that esters have not any kind of inhibition on lipases and it is not considered as an enzyme inhibitor. Therefore, this decrease in ester conversion can be explained by the equilibrium displacement toward the reactants [7].

When the concentration of water is increased (Fig. 4), a significant reduction in conversion and the initial reaction rate is observed. This can be attributed to the shifting of the equilibrium towards the reactants and also on the inhibition effect of water and/or due to enzyme inactivation [19]. The addition of water to the system probably increases the thickness of water layer around the enzyme and causes the diffusion problems [15]. Organic reactants and products, which have poor solubility in aqueous media, have difficulties diffusing through the water layer to and from the active site of the enzyme. Therefore, the decreasing trend in the initial reaction rate and the conversion reveals the inhibition due to water inactivation of the enzyme and displacement of the equilibrium towards the

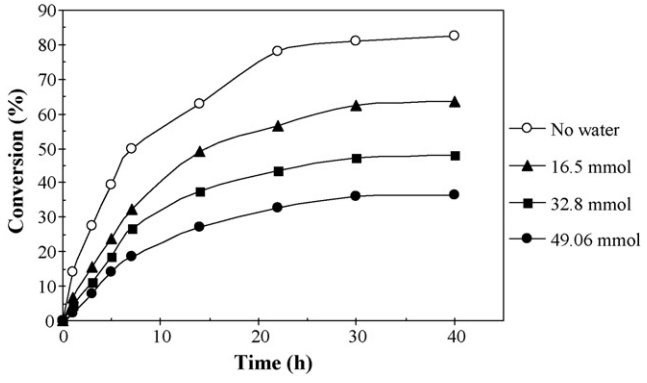

Fig. 4. The effect of water on the synthesis of 2-ethylhexyl-2-ethylhexanoate. Reactants concentrations = 0.4 M; immobilized Novozym 435 concentration = 2 g/L.

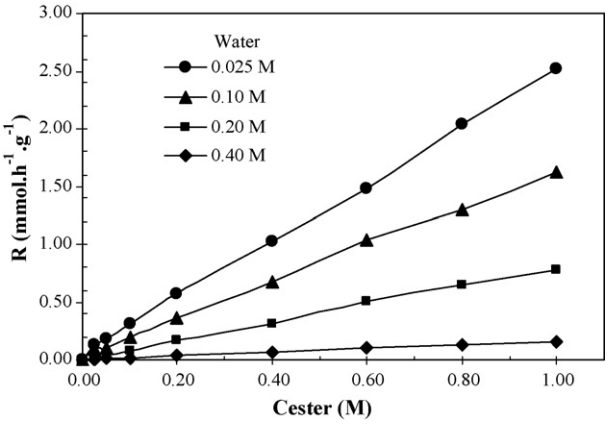

Fig. 5. The initial hydrolysis reaction rate of 2-ethylhexyl-2-ethylhexanoate at various concentrations of ester and constant concentration of water.

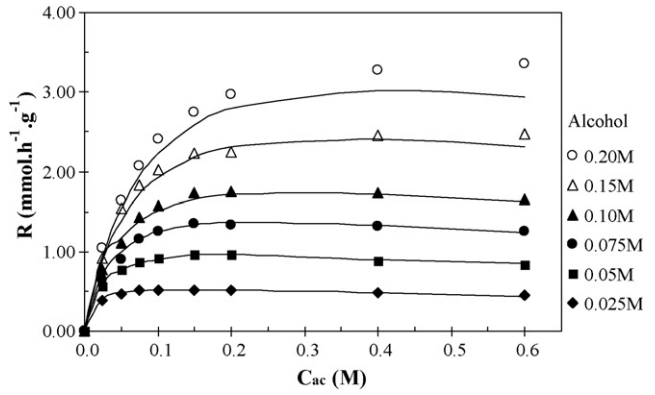

Fig. 7. The initial esterification reaction rate data. Solid lines are calculated from Eq. (2) using the kinetic parameters given in Table 1. Symbols represent the experimental data.

reactants. Enzyme activity depends on the microaqueous layer around the enzyme, and it can be modified by the dissolution of reactants or products in this layer. In fact, the extent of esterification reaction decreases with an increase in the water content at the thermodynamic equilibrium state [3,5,20].

The hydrolysis of esters was also studied. Figs. 5 and 6 show the effect of 2-ethylhexyl-2-ethylhexanoate and water concentrations on acid and alcohol formation, respectively. Based on these data, water concentration significantly decreases the initial reaction rate and has a considerable inhibition and/or inactivation effect on the enzyme activity [19], but ester concentration has no inhibition effect on the initial reaction rate of the ester hydrolysis.

3.4. The effect of the reactants concentration on the esterification initial reaction rate

The effect of 2-ethyl-1-hexanol and 2-ethylhexanoic acid concentrations on the initial reaction rates were investigated by esterifying various fixed initial quantities of 2-ethyl-1-hexanol with different concentrations of 2-ethylhexanoic acid and vice versa. Similar to the procedure reported in the literature [9,14]. Fig. 7 shows that when the concentration of 2-ethylhexanoic acid was increased, the initial reaction rate also increased, reaching a maximum at the acid concentration of 0.2 M. Concentrations above 0.2 M did not increase the initial reaction rate. This behavior can be an indication of inhibition effect of 2-ethylhexanoic acid on the enzyme activity. In agreement with the latter, subsequent increase in 2-ethyl-1-hexanol concentration led to an increase in the initial reaction rate in the concentration range of alcohol studied. This means that 2-ethyl-1-hexanol concentration has no inhibition effects on the enzyme activity.

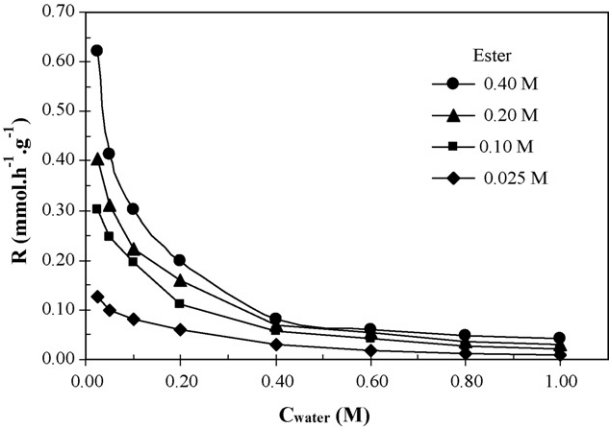

Fig. 6. The initial hydrolysis reaction rate of 2-ethylhexyl-2-ethylhexanoate at various concentrations of water and constant concentration of ester.

3.5. Kinetics and mechanism of the reaction

Most of the kinetic studies on the lipase-catalyzed synthesis of esters have considered the direct esterification of alcohols with acids, and have described a Ping-Pong Bi-Bi kinetic mechanism with inhibition by both or one of the reactants [4,15].

According to the Ping-Pong Bi-Bi kinetic mechanism the acyl donor (i.e. the acid) binds first to the free enzyme forming a noncovalent enzyme–acid complex which is transformed by a unimolecular isomerization reaction to an enzyme–acyl intermediate with the concomitant release of the first product (i.e. water). In a second step, the second reactant, in this case, 2-ethyl-1-hexanol, binds to the binary enzyme–acyl complex and forms a tertiary complex enzyme–acyl–alcohol. This complex is also isomerized by a unimolecular reaction to an enzyme–ester complex, followed by the release of the product, 2-ethylhexyl-2-ethylhexanoate. The enzyme changes to its initial conformation. The rate equation for this kind of mechanism with inhibition effect of one of the reactants is given by Segel [20] as:

$$R = \frac{R_{\max}[\mathrm{Al}][\mathrm{Ac}]}{K_{\mathrm{mAl}}[\mathrm{Ac}](1 + [\mathrm{Ac}]/K_{\mathrm{iAc}}) + K_{\mathrm{mAc}}[\mathrm{Al}] + [\mathrm{Al}][\mathrm{Ac}]} \quad (1)$$

where R is the initial reaction rate, $R_{\max}$ is the maximum initial reaction rate and K_{mAc} and K_{mAl} are the binding constants (Michaelis constants) for both reactants, 2-ethyl hexanoic acid (Ac) and 2-ethyl-1-hexanol (Al), respectively. K_{iAc} is the 2-ethylhexanoic acid inhibition constant. Eq. (1) describes the initial reaction rate without considering any effect of products.

In order to determine the kinetic parameters of this reaction, several experiments were done at the constant 2-ethyl-1-hexanol

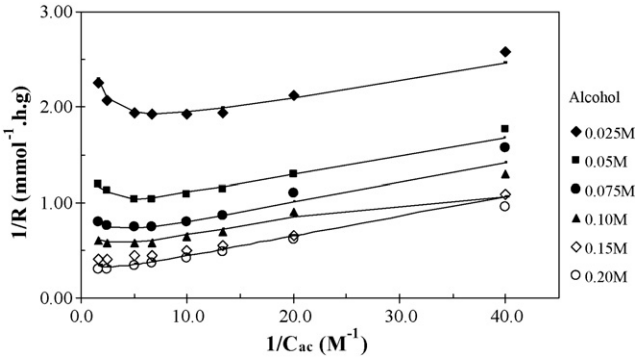

Fig. 8. Reciprocal of the initial reaction rate vs. reciprocal of 2-ethylhexanoic acid concentration (C_{ac}). Solid lines are calculated using Eq. (2).

concentration and different 2-ethylhexanoic acid concentrations. Reciprocal initial reaction rates (R^{-1}) were plotted versus the inverse acid concentration (C_{ac}^{-1}) (Lineweaver–Burk plot) for several initial 2-ethyl-1-hexanol concentrations. Results are shown in Fig. 8. 2-Ethyl-hexanoic acid inhibition was confirmed since at low values of the inverse of its concentration the lines are curved up as shown in Fig. 8. It was also established that 2-ethyl-1-hexanol was not a Novozym 435 inhibitor; since its initial reaction rate increased as 2-ethyl-1-hexanol concentration increased as shown in Fig. 9. This mechanism is characterized by parallel lines in the Lineweaver–Burk (double reciprocal) representation at concentrations in which there is no inhibition, as it happens in the present system. The results shown in Figs. 8 and 9 support a Ping-Pong Bi-Bi mechanism with 2-ethylhexanoic acid inhibition. The acid inhibition effect can be explained by the hypothesis that acid may react with acyl–enzyme complex to yield a dead end complex that cannot transfer acyl moiety to the alcohol [14]. In other words, there are two possible explanations for the observed decrease in the bioactivity with varying acid concentration. One possible explanation is the dramatic decrease of the pH of the mixture. The other explanation for observed decrease in initial reaction rate is competitive inhibition by the reactants [4].

Once the Ping-Pong mechanism was confirmed, the kinetic parameters of the Eq. (1) were calculated by a multiple regression fitting of the experimental data. The results are shown in

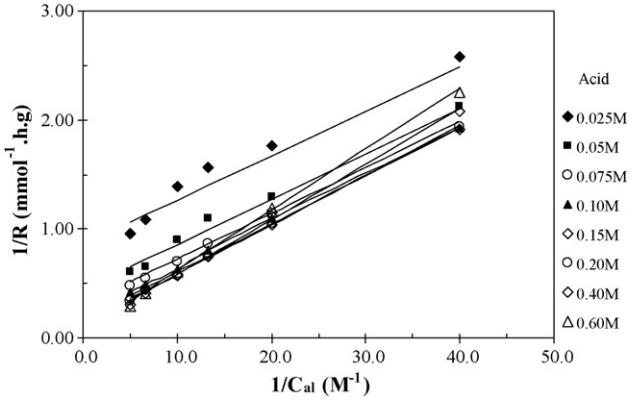

Fig. 9. Reciprocal of the initial reaction rate vs. reciprocal of 2-ethyl-1-hexanol concentration (C_{al}). Solid lines are calculated using Eq. (2).

Table 2
Kinetic parameters for the synthesis of 2-ethylhexyl-2-ethylhexanoate from 2-ethylhexanoic acid and 2-ethyl-1-hexanol

Parameter	Value	Units
R_{max}	37.59	mmol h^{-1} g^{-1}
K_{mAl}	1.51	M
K_{mAc}	0.78	M
K_{iAc}	1.55	M

Table 2. Based on the calculated kinetic parameters, affinity of the enzyme towards 2-ethylhexanoic acid seems to be greater than of 2-ethyl-1-hexanol, since K_{mAc} was lower than K_{mAl}. In other words, the relatively large value of K_{mAl} shows the alcohol inhibition is much less than the acid inhibition. Therefore, the effect of the alcohol inhibition is not shown in Eq. (1). These results agree to a Ping-Pong Bi-Bi mechanism, which assumes that the acyl donor is the first reactant that binds to the lipase. Zaidi et al. [4] studied the effect of alcohol chain-length and found that the Michaelis constant for the long-chain alcohols were higher than the short-chain alcohols.

Thus, the final kinetic equation for the enzymatic synthesis of 2-ethylhexyl-2-ethylhexanoate from 2-ethylhexanoic acid is as follows:

$$R = \frac{37.59[\mathrm{Al}][\mathrm{Ac}]}{1.51[\mathrm{Ac}](1 + [\mathrm{Ac}]/1.55) + 0.78[\mathrm{Al}] + [\mathrm{Al}][\mathrm{Ac}]} \quad (2)$$

This kinetic equation was used to estimate the initial reaction rates for the investigated intervals of the reactant concentrations. Symbols in Fig. 7 show the experimental data obtained from the synthesis of 2-ethylhexyl-2-ethylhexanoate and the estimated trend (i.e. solid curves) calculated with the Eq. (2). At lower concentration of reactants, predictions were quite close to the experimental values. Only when the concentration of both reactants was 0.2 M, the calculated initial reaction rates were lower than the experimental data. This may be related to the inhibition of the produced water, which causes a severe inactivation of the Novozym 435 or the acid inhibition of the reaction.

4. Conclusion

Enzymatic esterification reaction of 2-ethylhexyl-2-ethylhexanoate can be described by Ping-Pong Bi-Bi mechanism with 2-ethylhexanoic acid inhibition for concentrations over 0.1 M. This can be observed from estimated parameters as follows: $R_{max} = 37.59$ mmol h^{-1} g^{-1}, $K_{mAl} = 1.51$ M, $K_mAc = 0.78$ M, $K_{iAc} = 1.55$ M. These values demonstrate a higher affinity of Novozym 435 for the acid rather than the alcohol ($K_{mAc} < K_{mAl}$). The calculated parameters were used to estimate theoretical initial reaction rates. The initial reaction rates, calculated from the kinetic parameters, show a good agreement with the experimental data at the acid concentrations of less than 0.1 M. At the acid concentrations of 0.2 M or higher a difference between calculated and the experimental data is observed which might be due to the acid inhibition of the reaction and/or produced water.

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