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Biotechnological Utilisation of Fusel Oil, A Food Industry By-Product

A Kinetic Model on Enzymatic Esterification of *i*-Amyl Alcohol and Oleic Acid by *Candida antarctica* Lipase B

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Summary

Fusel oil is a by-product of distilleries, its main component is *i*-amyl alcohol, which can form ester compounds. Esterification of oleic acid and *i*-amyl alcohol by *Candida antarctica* lipase B (Novozym 435 preparation) in *n*-heptane solvent was studied in this work. Ping--pong bi-bi mechanism (inhibition phenomena taken into account) was applied as a complex kinetic model. The parameters of the model were determined by numerical methods. It was found that four-parameter model fitted well with the experimental results and described properly the enzymatic reaction.

Key words: fusel oil, esterification, lipase, ping-pong bi-bi mechanism

Introduction

The environmental impact of biotechnological utilisation of food industry and agricultural wastes and by--products is twofold: the utilisation for manufacture of value-added products on the one hand, and the environmentally safe, waste-free biotechnological process using mild conditions on the other. Fusel oil is a by-product of distilleries, its average composition is 10 % ethanol, 13 % *n*-propanol, 15 % *i*-butanol, 51 % *i*-amyl alcohol, 11 % miscellaneous alcohols and water. Nowadays fusel oil is usually burned to complete the energy demand of distilleries (*1*). However, its utilisation in a form of *e.g.* ester compound with long-chain acids results in a biolubricant. These fatty acids can be obtained from fats and oils by hydrolysis, and oleic acid is found in almost every type of triglyceride source.

Esterification of fusel oil with oleic acid using sulphuric acid as a catalyst was studied earlier (*2*) and the product was considered as 'biolubricant' since the raw material was of biological origin and the product is biologically degradable. However, it may contain acid in traces (from the catalyst), which is not advantageous in certain lubricating applications. Therefore, it would be more favourable to use enzyme as a catalyst instead of acid. Among enzymes, lipases are found suitable biocatalysts for esterification reactions. Before developing a complete method for enzymatic manufacture of this biolubricant, a detailed kinetic analysis of the reaction mechanism should be carried out.

Esterification reactions by lipase in non-conventional media have been studied in our laboratory for long (*3–6*). Enzymatic esterification of fatty acids and ingredients of fusel oil was studied by Gulati *et al*. (*7*) using lipase from *Aspergillus tereus*. They found that in *n*-hexane solvent the alcohols were able to react with the fatty acids (miristic acid, palmitic acid, stearic acid), except with oleic acid. Using other lipase preparations (*Candida antarctica, Candida rugosa, Rhizomucor miehei*, porcine pancreas), however, made the oleic acid esterification with

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similar low molecular mass alcohols possible. In Table 1 the results published on the kinetics of enzymatic esterification of oleic acid with short-chain alcohols in organic solvents are summarized.

Garcia *et al*. (*8*) studied the kinetics of *i*-propyl oleate formation by *Candida antarctica* lipase. The model used was an ordered bi-bi type containing 13 kinetic parameters, so it seemed too complicated, because it had high uncertainty. Esterification of butyl alcohol by *Candida rugosa* lipase was studied by Zaidi *et al*. (*9*), where ping-pong bi-bi mechanism was assumed in the kinetic model with 5 parameters. However, the range of substrate concentration measured was quite narrow (0.1–1.0 mol/L), and the error of the modelling was found very high (28 %).

Immobilized *Rhizomucor miehei* lipase was applied for ethyl oleate synthesis by Oliveira *et al*. (*10*). In order to describe the kinetics, random bi-bi model was used which contained 4 parameters. However, the parameter values differed 15 orders of magnitude, implying that the effect of a certain parameter is nearly negligible compared to the others.

Esterification of ethyl alcohol and oleic acid by immobilized *Rhizomucor miehei* lipase was also studied by Goddard *et al.* (*11*). Michaelis-Menten model was used for the description of the reaction; however, different kinetics was used in each alcohol concentration, which is considered as a pseudo-one-substrate model.

Soluble porcine pancreatic lipase was applied for the ethyl oleate synthesis by Hazarika *et al*. (*12*). They assumed ping-pong mechanism as well, containing 4 parameters. However, the range of substrate concentration studied was even narrower (0.3–0.8 mol/L) than in the case of Zaidi *et al*. (*9*).

Description of the correct kinetics on the particular esterification reaction is even more difficult due to the various possible inhibition effects. As it is shown in Table 1, ethyl alcohol as a substrate was in all cases considered as inhibitor, while in the esterifications with other alcohols, both substrates were regarded as inhibitors.

To summarize, it seems from Table 1 that the kinetic models/parameters published so far cannot be considered as a proper, detailed kinetic description of the enzymatic process for oleic acid esterification with short chain alcohols. Moreover, no data have been found on esterification with *i*-amyl alcohol. Therefore, our aim was to elaborate a proper, sophisticated model for this particular reaction.

Theory

Kinetics of enzymatic reactions can be described by the well-known Michaelis-Menten model. For reactions having 2 substrates and 2 products (bi-bi reactions), its application is quite complicated since various mechanisms can be considered according to the order and rate of binding both substrates to the enzyme active sites and releasing the products from them (random, ordered, ping-pong, *etc.*).

Since majority of the kinetic studies suggest ping-pong bi-bi mechanism for the enzymatic esterification of oleic acid and short-chain alcohols, we have also considered it as an initial point for the description. According to Cleland (*13*) the ping-pong bi-bi mechanism can be outlined as follows:

$$A + E \underset{k_2}{\overset{k_1}{\rightleftarrows}} \left(\frac{EA}{FP}\right) \underset{k_4}{\overset{k_3}{\rightleftarrows}} P + F + B \underset{k_6}{\overset{k_5}{\rightleftarrows}} \left(\frac{FB}{EQ}\right) \underset{k_8}{\overset{k_7}{\rightleftarrows}} Q + E \qquad /1/$$

In the first step the enzyme *E* reacts with substrate *A* (oleic acid) forming an *AE* enzyme-substrate complex, which is transformed into *FP* modified complex by an internal rearrangement. Product *P* (water) comes off the complex, then the modified enzyme molecule *F* is able to react with substrate *B* (*i*-amyl alcohol), forming a new enzyme-substrate complex *FB*. It is transformed into an enzyme-product complex *EQ*, and then *Q* (*i*-amyl oleate) moves to the bulk solution. Finally, enzyme *E* becomes free and can react with another substrate *A* molecule. In Eq. 1 the reaction rate constants are shown (k_1-k_8), among them k_1, k_3, k_5 and k_7 belong to the forward direction of the reaction, while the others to the backward direction

Table 1. Kinetic studies on esterification of oleic acid

Alcohol	Enzyme	Model	Inhibition	Reference	Note
i-propyl alcohol	Novozym 435 *Candida antarctica*	ordered bi-bi 13 parameters	both S and P competitive	(*8*)	too many parameters fitted one measurement; high error of fitting
butyl alcohol	immobilised *Candida rugosa*	ping-pong bi-bi 5 parameters	both acid and alcohol	(*9*)	error is 28 %; narrow range of substrate concentration (0.1–1.0 mol/L)
ethyl alcohol	immobilised *Rhizomucor miehei*	random bi-bi 4 parameters	alcohol	(*10*)	Difference between the parameters is 15 orders of magnitude; quasi-one-substrate kinetics
ethyl alcohol	immobilised *Rhizomucor miehei*	Michaelis-Menten 2+1 parameters	alcohol	(*11*)	Different kinetics for each alcohol concentration; pseudo-one-substrate kinetics
ethyl alcohol	soluble, from porcine pancrease	ping-pong bi-bi 4 parameters	alcohol	(*12*)	Narrow range of substrate concentration (0.3–0.8 mol/L)

(with negative sign). Summarising the steps, the formation rate of the product can be written as follows:

$$v = \frac{(k_1k_3k_5k_7AB - k_2k_4k_6k_8PQ)E_0}{E + (EA + FP) + F + (FB + EQ)} \qquad /2/$$

where E_0 is the initial enzyme concentration and E is the actual enzyme concentration (the other capital letters mean the concentration of the particular compound). This model, however, is too complicated to be applied in practice. To simplify the situation, the main parameters influencing the rate of product formation are selected, as follows (*14*): the concentration of substrate *A* (first)/*i*-amyl alcohol; the concentration of substrate *B* (second); enzyme concentration; and the amount of products formed altogether.

Initial reaction rate can be described by including only the first three factors, taken into account the fact that product concentration is 0 at the beginning of the reaction:

$$v = \frac{V_m}{1 + \frac{K_A}{[A]} + \frac{K_B}{[B]}} \qquad /3/$$

If substrate inhibition is considered as well, the three-parameter equation should be completed with another constant (*15*) and the following equation can be used:

$$v = \frac{V_m}{1 + \frac{K_A}{[A]}\left(1 + \frac{[B]}{K_{iB}}\right) + \frac{K_B}{[B]}} \qquad /4/$$

This model contains four parameters which can be further supplemented by the product inhibition factors. In this way two more parameters are added into the equation (Eq. 5). In this equation K'_{AB} parameter is the rate of the apparent product formation, the ping-pong parameter of the reaction:

$$v = \frac{V_m}{1 + \left(\frac{K_A}{[A]} + \frac{K'_{AB}\cdot[P]}{[A]\cdot[B]}\right)\left(1 + \frac{[B]}{K_{iB}}\right) + \frac{K_B}{[B]}\cdot\left(1 + \frac{[P]}{K_{iP}}\right)} \qquad /5/$$

where

$$V_m = \frac{k_3k_7}{k_3 + k_7}, \quad K_A = \frac{k_7(k_2 + k_3)}{k_1(k_3 + k_7)}, \quad K_B = \frac{k_3(k_6 + k_7)}{k_5(k_3 + k_7)},$$

$$K_{iP} = \frac{k_3}{k_4}, \quad K_{iB} = \frac{k_5}{k_6} \quad \text{and} \quad K'_{AB} = \frac{k_2k_4(k_6 + k_7)}{k_1k_5(k_3 + k_7)}$$

In our case, water is one of the products in esterification. But it is not only a product, a small amount of water should be present initially in the reaction mixture to keep in an active formation of the enzyme structure. At the beginning of the reaction, however, the initial concentration of water does not change significantly, therefore water content (*P*) can be considered as constant (its effect is negligible). Thus the equation can be simplified and a five-parameter equation can be obtained:

$$v = \frac{V_m}{1 + \left(\frac{K_A}{[A]} + \frac{K_{AB}}{[A]\cdot[B]}\right)\left(1 + \frac{[B]}{K_{iB}}\right) + \frac{K_B}{[B]}} \qquad /6/$$

It can be seen that the difference between the four- and five-parameter equations (Eqs. 4 and 6) is the K_{AB} factor. Its influence is significant only in the case when concentrations of both substrates are very low. However, we do not plan to carry out measurements under these conditions, thus it is assumed that no significant difference will be observed in the modelling results obtained by using the two systems.

Materials and Methods

Novozym 435 preparation was used in the esterification reaction. It is a commercial *Candida antarctica* lipase B (EC 3.1.1.3. triacylglycerol acylhydrolase) immobilized on a macroporous acrylic resin with a water content of 1–2 % by mass. The enzyme was provided as a gift by Novo Nordisk A/S (Denmark). The nominal activity of the catalytic preparation was approximately 7000 propyl laurate units (PLU) per gram. One PLU is defined as the number of μmol of *n*-propyl laurate obtained in the standard test corresponding to the esterification of lauric acid with *n*-propyl alcohol, after 15 min at atmospheric pressure.

The chemicals ethanol, *n*-heptane and oleic acid were purchased from Reanal (Hungary), diethyl ether from Spectrum 3D (Hungary), *i*-amyl oleate from Fluka (USA) and *i*-amyl alcohol from Sigma (USA). All the chemicals used were of analytical grade.

Experiments were carried out in a New Brunswick G24 shaking incubator in 100-cm^3 flasks using *n*-heptane solvent containing the substrates (oleic acid and *i*-amyl alcohol) in various concentrations. The water level was adjusted to 0.1 % by mass (optimal concentration) before the measurements, taking into account the water content of the chemicals used.

The operational parameters during the kinetic experiments were 200 rpm and 30 °C. A mass of 0.01 g of immobilized lipase preparation was added to the incubated homogeneous reaction mixture to initiate the enzymatic reaction. Samples in duplicates were taken from the reaction mixture regularly.

Analysis

Water content of the chemicals and reaction mixtures was measured by Mettler DL35 type automatic Karl-Fisher titrator.

The concentrations of ester (formed) and oleic acid (consumed) were determined by an HP 5890 type GC. The parameters used during the determination are listed as follows: FFAP capillary column (Macherey-Nagel, Germany), 25 m×0.32 mm i.d., with 0.50 μm film thickness; FID detector; N_2 carrier gas 4.2 cm^3/min; N_2 auxiliary gas 45.8 cm^3/min; H_2 gas 35 cm^3/min; air 120 cm^3/min; injector temperature 250 °C; detector temperature 250 °C; temperature of the column 150 °C (isothermal). The total determination time necessary was 30 min.

Results and Discussion

Reaction rate determination

Experiments using substrate concentrations in the range of 0.4–6.0 and 0.2–2.0 mol/L of *i*-amyl alcohol and

oleic acid, respectively, were carried out at 30 °C temperature, with an optimal initial water content of 0.1 % by mass (determined earlier) (*16*) in *n*-heptane solvent. Progress curves of oleic acid consumption as a function of time were measured.

Gregory-Newton method was used to calculate the initial reaction rates from the data of progress curves (*17*). The initial reaction rate data were modified taking into account the amount of enzyme used. Thus the reaction rate values were obtained as $\mu mol/(s \cdot g_{enzyme})$ and summarised in Table 2.

Table 2. Initial reaction rate data [$\mu mol/(s \cdot g_{enzyme})$] using various oleic acid (c_{OA}) and *i*-amyl alcohol concentrations (c_{iAA}) (all the concentrations in mol/L)

c_{OA} \ c_{iAA}	0.4	1.0	2.0	4.0	6.0
0.2	4.90	5.13	5.45	5.57	3.50
0.5	7.91	7.91	8.26	7.13	7.31
1.0	9.68	12.56	12.80	9.69	10.80
1.5	11.25	15.15	18.45	15.30	13.72
2.0	9.78	14.46	17.65	14.50	14.31

Effect of immobilization on the mass transfer

Since immobilised enzyme preparation was used in the experiments, it was important to decide, before the detailed kinetical analysis, whether the reaction rates measured were the real values of enzymatic reaction or influenced significantly by the diffusion rates of the compounds (from the bulk phase to the solid particle and *vice versa*).

The reaction and the diffusion take place simultaneously and the rate-limiting step is always the one which is slower. Using immobilised lipase preparations, rate of diffusion is usually not the limiting step (*18*).

In our measurements, a method described by Yadav and Lathi (*19*) was applied to determine the rate-limiting step, using the Weisz-Prater criteria. This method is based on the calculation and comparison of the two relevant relaxation times. The ratio of the relaxation time of biocatalysis rate, t_r and that of the diffusion rate, t_d shows which process should be considered as the limiting step.

The relaxation times can be defined as follows:

$$t_r = \frac{C_0}{r(C_0)} \quad \text{and} \quad t_d = \frac{D}{(k_{SL})^2} \qquad /7/$$

Oleic acid, having slower diffusivity, was chosen for the calculations, and the highest reaction rate-substrate concentration value-pair was taken from Table 2. Thus t_r was calculated as:

$$t_r = \frac{C_0}{r(C_0)} 2^5 \text{ s} \qquad /8/$$

Diffusion constant (D) of oleic acid in *n*-heptane was determined according to Shibel method (*20*):

$$D = k \frac{T}{\eta_B V_S^{1/3}} \qquad /9/$$

V_S molar volume density was estimated from its critical volume (V_C):

$$V_S = 0.285 V_C^{1.048} \qquad /10/$$

V_C of oleic acid is 1152 cm^3/mol, thus V_S is obtained as 460 cm^3/g. In this way D diffusion coefficient is calculated as $1.61 \cdot 10^{-5}$ cm^2/s.

The mass transfer coefficient can be calculated (based on the Sherwood number) from the diffusion coefficient and the particle size:

$$k_{SL} = 2D/d \qquad /11/$$

The average diameter of Novozym 435 immobilised lipase preparation is 0.06 cm, thus the value of the mass transfer coefficient is $5.3 \cdot 10^{-4}$ cm/s.

The relaxation time for the diffusion is calculated as:

$$t_d = \frac{D}{(k_{SL})^2} = \frac{1.61 \cdot 10^{-5} / (cm^2/s)}{(5.3 \cdot 10^{-4} / (cm/s))^2} = 55.9 \text{ s} \qquad /12/$$

Comparing the values of t_r and t_d it can be concluded that diffusion rate is three orders of magnitude higher than the reaction rate, thus the rates measured in the enzymatic process can be considered as the real reaction rates.

Kinetic analysis

In Fig. 1 the initial reaction rates are presented as a function of oleic acid (substrate 1) concentration, while in Fig. 2 the same data are shown as a function of *i*-amyl alcohol (substrate 2) concentration. It can be clearly seen that while oleic acid has a slight inhibition effect, *i*-amyl alcohol considerably inhibits the enzymatic reaction.

In the first step of the kinetic analysis the traditional linearization (graphic) methods were applied. Reciprocal data of the initial rates were plotted against the reciprocal data of the initial substrate concentrations (both) (Fig. 3). It can be seen in Fig. 3b that in lower substrate concen-

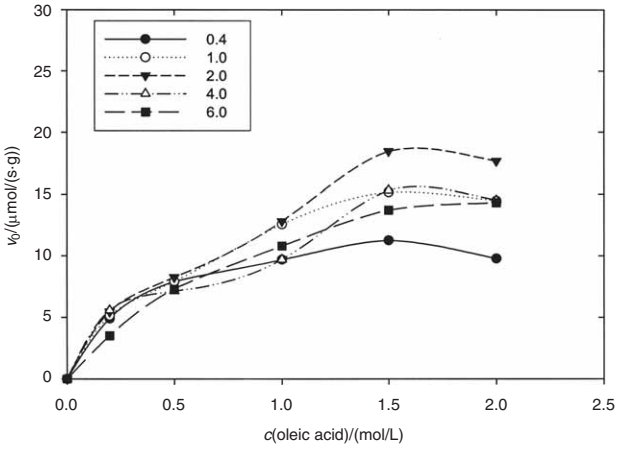

Fig. 1. Initial reaction rates as a function of oleic acid concentration at different *i*-amyl alcohol concentrations in mol/L

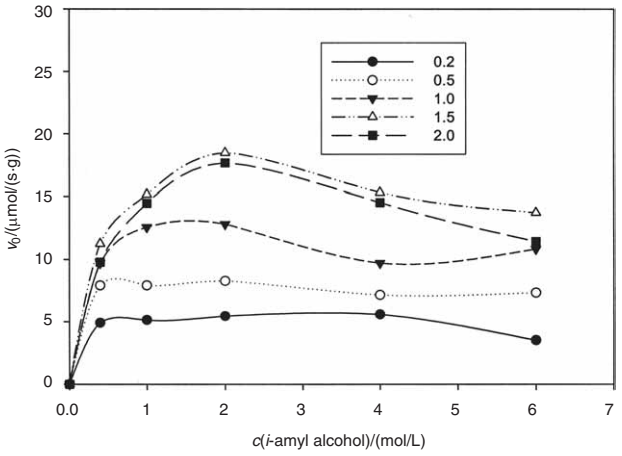

Fig. 2. Initial reaction rates as a function of *i*-amyl alcohol concentration at different oleic acid concentrations in mol/L

trations the lines are parallel, implying ping-pong bi-bi mechanism. However, in higher substrate concentrations the lines keep steeply upwards towards the ordinate, which means that inhibition (by the alcohol compound) occurred. Thus the kinetic parameters cannot be determined graphically and the mechanism of inhibitions cannot be doubtlessly decided.

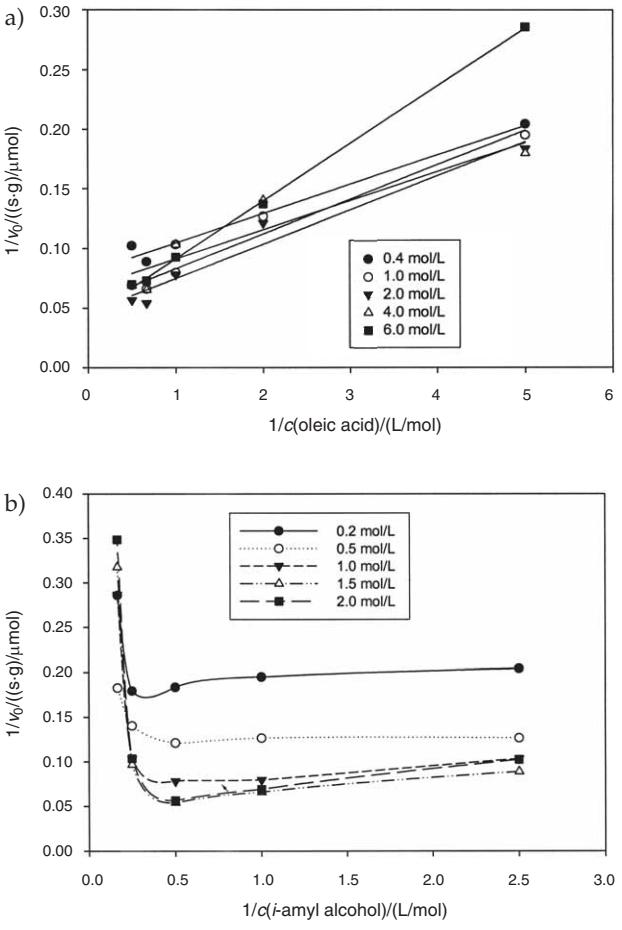

Fig. 3. Lineweaver-Burk linearization of the reaction rate. Substrate concentration data for a) oleic acid and b) *i*-amyl alcohol

Ping-pong bi-bi kinetic models having 3 (Eq. 3), 4 (Eq. 4) and 5 (Eq. 6) parameters were used for the description of the enzymatic esterification and the parameters were calculated based on the experimental data. In the calculation, a variation of simplex method was applied, namely the Nelder-Mead method, which is more sensitive for the initial values of the parameters and slower than the original simplex method, but it provides more accurate final results (*21*).

Since the method is sensitive for the initial values of the parameters, a two-step method was elaborated. The principle of the complete modelling system is outlined in Fig. 4. In the first step, the values of kinetic parameters were estimated by a special programme, applying a simplified model (Lucenz 4 programme by A.G. Clark, New Zealand) with no inhibition, using the experimental data. Thus the instability of the method was eliminated. The estimated kinetic parameters obtained were then used as initial parameters for the extended kinetic model. Having checked the kinetic results by comparing the experimental data, the program either refused the results and started another circle, modifying one or more parameters according to the Nelder-Mead method, or accepted the results.

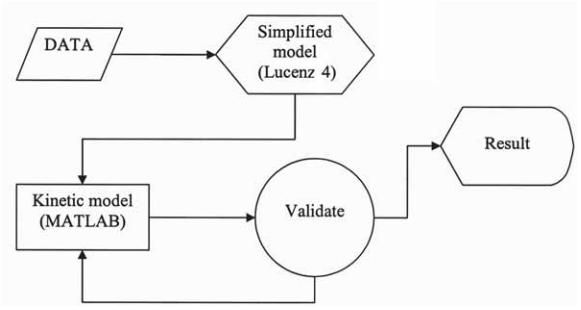

Fig. 4. Scheme of the two-stage parameter determination

During the procedure, the programme was run on data belonging to only one acid concentration, otherwise divergence occurred. In this way the parameters were obtained for every acid concentration, which were used in a final run of the programme, where errors were minimised.

In Table 3 values of the determined parameters are summarized, and in Fig. 5 the experimental and calculated initial reaction rates are shown. It can be seen from the value of R^2, that the 4-parameter model (taking into account the inhibitions, as well) described better the kinetics of enzymatic *i*-amyl oleate synthesis than the 3--parameter model.

However, the 5-parameter model is not more accurate than the 4-parameter one (value of R^2 is the same), therefore it is not reasonable to use the more complicated model. This theory was checked by using *F*-statistics (*22*), where the significance level used was p=0.05. As the result shows the 3-parameter model was significantly different compared to the 4- and 5-parameter models, while the difference between the 4- and 5-parameter models was found not significant.

Table 3. Parameters of the kinetic models

Parameters	Three--parameter model	Four--parameter model	Five--parameter model
V_m/(μmol/(s·g))	23.5	30.8	29.9
K_A/(mol/L)	0.86	0.65	0.55
K_B/(mol/L)	0.19	0.58	0.53
K_{iB}/(mol/L)	–	3.2	2.7
K_{AB}/(mol/L)	–	–	0.055
R^2 (–)	0.952	0.975	0.975
ARE*/%	12.2	3.1	2.8

*average relative error

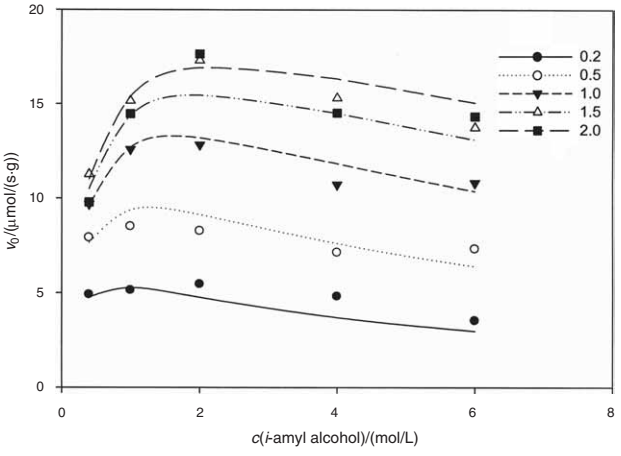

Fig. 5. Initial reaction rates as a function of *i*-amyl alcohol concentration at different oleic acid concentrations in mol/L; dots are the measured values, lines are calculated from the 4-parameter model

The results of the modelling were intended to be compared with other literature data, however no similar results were found in the published material. Either the substrates of the esterification, or the enzyme applied were different, thus the parameters were not possible to compare.

Conclusions

Kinetic model for description of the enzymatic esterification of oleic acid and *i*-amyl alcohol has been, according to our best knowledge, elaborated and presented for the first time in literature. The model is based on the ping-pong bi-bi mechanism and not only the product, but substrate inhibition is taken into account, as well. The model fitted well to the experimental data (checked by R^2 values), though the measurements covered an extremely wide concentration range.

The kinetic model containing the determined parameters can be used in a particular enzymatic esterification reaction for calculations of the optimal conditions of various aspects, like highest yield, lowest acid residue, lowest amount of enzyme, shortest reaction time, *etc.* Moreover, we are planning to apply the model for bioreactor design to realise continuous enzymatic *i*-amyl oleate synthesis by lipase.

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List of symbols

A, B	substrate concentrations (mol/L)
C_0	substrate concentration in the liquid phase (mol/L)
D	diffusion coefficient of the substrate in the liquid bulk phase (cm^2/s)
d	particle size (cm)
E	enzyme concentration (g/L)
F	concentration of modified enzyme (g/L)
K_A, K_B, K_P, K_Q	Michaelis-Menten constants (mol/L)
K'_{AB}, K_{AB}	ping-pong parameters (mol/L)
K_{eq}	equilibrium constant (–)
K_{iB}, K_{iA}, K_{iQ}, K_{iP}	inhibition constants (mol/L)
k_{SL}	mass transfer coefficient of solid-liquid interface (cm/s)
k_x	reaction rate constants
P, Q	product concentrations (mol/L)
$r(C_0)$	reaction rate (mol/(L·s))
T	temperature (K)
t_d	relaxation time of diffusion rate (s)
t_r	relaxation time of biocatalysis rate (s)
v	initial reaction rate (μmol/(s·g))
V_c	critical volume (cm^3/mol)
V_m	maximum reaction rate (μmol/(s·g))
V_S	molar volume density (cm^3/g)
η_B	viscosity (mPa (cP))

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