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The *Candida rugosa* lipase catalyzed synthesis of amyl isobutyrate in organic solvent and solvent-free system: A kinetic study

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Abstract

The *Candida rugosa* lipase catalyzed esterification of butyric acid with amyl alcohol in isooctane and in solvent-free system was studied. Nearly complete conversion (>95%) of substrates was achieved using low enzyme amount of 0.5% (w/v) at 45 °C. The initial rates of esterification were attempted to correlate with concentrations of substrates by various bisubstrate kinetic models. The reaction rate of esterification in isooctane could be described with a ping–pong bi–bi mechanism and inhibition by amyl alcohol. Obtained specificity constants indicate that lipase from *C. rugosa* has higher affinity towards acid substrate. The rate of esterification in solvent-free system could not be described with applied bisubstrate models probably due to denaturation of lipase in absence of solvent at high concentrations of both substrates. Nevertheless, the maximum initial rate in solvent-free system was higher than corresponding values in isooctane which indicates that solvent-free system has good perspectives for industrial utilization at lower *S*/*E* ratios.

Keywords: *Candida rugosa* lipase; Ping–pong bi–bi kinetics; Alcohol inhibition; Amyl isobutyrate; Esterification

1. Introduction

Esters of carboxylic acids and alcohols have notable commercial significance because they have wide application in cosmetics, food and pharmaceutical industry. Although these important products are manufactured mostly by the method that includes use of aggressive chemical catalysts, development of the research area of enzymatic esterification is very propulsive during previous two decades [1,2]. Numerous reports of achieving high yields of esters with various lipases (triacylglycerol hydrolases) of microbial origin have been published recently [3–5]. The enzymatic ester synthesis was prevalently performed in various organic media, but synthesis in solvent-free systems is also investigated because of considerable simplification of downstream processing and reduced environmental hazard [6–8]. Contributions in development of enzyme immobilization methods, which ensure multiple application of enzyme with retained activity, are well documented [1,2,9–11]. Another important direction in optimization of enzymatic ester synthesis is monitoring and controlling the water activity in reaction mixture [6,12,13].

The thorough knowledge of kinetics is of great importance, not only in order to elucidate mechanism of this reaction, but because reliable information about the rate of product formation and changes in experimental systems are necessity for the design of suitable reactors and later industrial scale-up. Several studies focused on kinetics of enzymatic esterification have been performed and different models were proposed. Chulalaksananukul et al. carried out ester synthesis using lipase from *Mucor miehei* and proposed ping–pong bi–bi model with dead-end inhibition by alcohol [14]. The same model was proposed by Hazarika et al. for synthesis of ethyl oleate with porcine pancreatic lipase [15] and by Yadav and Lathi for synthesis of butyl isobutyrate with the commercial immobilized lipase from *Candida antarctica* [16]. On the other hand, in the case of the synthesis of citronellol laurate the random order bi–bi model with inhibition by lauric acid was reported [17]. Arcos et al. proposed the model that does not include any kind of inhibition [18]. Ping–pong bi–bi kinetics with inhibition by both substrates was observed in studies with lipase from *Burkholderia cepacia* [11] and with the lipase from *Rhizomucor miehei* [19]. Garcia et al. developed kinetic model for entire course of the reaction based on ordered

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bi–bi mechanism of esterification with the inhibition by both substrates and both products [20].

As it can be seen from this brief overview, investigations of kinetics of lipase-catalyzed synthesis of esters were carried out with lipases of different origin and with alcohols and acids of various chain-lengths and structures. Additionally, there are differences in type of agitation of reaction mixture, and type of reaction system (solvent-free or reaction in organic solvent). Besides, majority of studies were performed with immobilized lipases, so it is necessary to consider the influence of additional physical phenomena, such as mass transfer effect and changes of lipase conformation during immobilization, on reaction rate. Due to effect of all these factors, various types of bisubstrate enzyme kinetic models have been proposed and great discrepancies between observations on substrate inhibition exist.

The lipase from *Candida rugosa* has wide application in oil hydrolysis, transesterification, esterification and enantioselective biotransformation due to its low price and number of immobilization procedures developed with this enzyme [21–25]. Nevertheless, kinetics of oil hydrolysis catalyzed with this enzyme is well documented [26], but data about kinetics of the esterification are scarce.

The aim of this investigation was selection of bisubstrate enzyme kinetics that describes the dependence between initial velocity and concentrations of substrates. The comparison of kinetics in two different reaction media (solvent-free system and reaction performed in isooctane) was made in order to investigate the influence of the solvent on the mechanism of reaction.

2. Experimental

2.1. Materials

C. rugosa lipase (triacylglycerol hydrolase, EC 3.1.1.3) was provided by Sigma (St. Louis, USA). The lipase was a crude preparation with activity of 860 U g^{-1} solid based on olive oil emulsion method [26] and 10% protein based on Lowry method for protein assay [27]. Isooctane was provided by Fluka (Buchs, Switzerland). Amyl alcohol and isobutyric acid were provided by Farmitaliana Carlo Erba (Milano, Italy). All chemicals were of 99% or higher purity.

2.2. Methods

Ester synthesis was carried out in stoppered flasks (100 ml) in isooctane. The reaction mixture containing enzyme and 0.25 M of both substrates was diluted up to the volume of 10 ml with isooctane and was incubated on a shaker at 150 rpm and at 45 °C unless otherwise specified. Each experimental point was obtained by performing reaction in separate flasks. All of the experiments were carried out in duplicate.

The progress of esterification was monitored by determination of the residual acid content by titration against sodium hydroxide using phenolphthalein as an indicator and mixture of ethanol and diethyl ether (1:1) as a quenching agent. The ester formed was calculated as being equivalent to acid consumed. This was tested by determination of ester concentration on Varian 3400 gas chromatograph equipped with a DB-1 capillary column and a flame ionization detector. Nitrogen was used as a carrier gas. The initial reaction rates were determined from the slope of the initial linear portions of the plots of ester concentration versus time. Initial rates and yields determined by GC analysis and titrimetry were in good agreement.

3. Results and discussions

3.1. The effect of reaction conditions

The temperature of the ester synthesis was varied in the range of 35–55 °C. The experiment was carried out with 0.5% of lipase and 10 μl of added water. The reaction curves are presented in Fig. 1. The highest initial rate (30.7 μmol min^{-1} g^{-1}) and yield of esters after 48 h (87.4%) were observed in experiment carried out at 45 °C. Both, decrease and increase of temperature of 10 °C led to decrease of initial rate of ester synthesis to approximately one third of the highest value. Obtained temperature optimum is considerably lower than temperature optimums for commercial immobilized lipase from *C. antarctica* or *R. miehei* [3,17,20], but it is higher than optimum for lipases of animal origin [4,28]. All further experiments were carried out at 45 °C.

The effect of the water concentration on the lipase activity was studied in the range of 0–0.5% (v/v). The reaction temperature was 45 °C and the enzyme concentration was 0.5% (w/v). The reaction curves are presented in Fig. 2. The highest initial rate of synthesis and the highest yield of esters were determined at a water concentration of 0.1% (v/v). The initial rates of ester synthesis at other water concentrations were significantly lower. Therefore, all further experiments were performed at a water concentration of 0.1% (v/v).

The effect of enzyme concentration was studied in the range of 0.1–0.7% (w/v), at 45 °C and 0.1% of water. The results are illustrated in Fig. 3. It can be seen that there is a strong correlation between the concentration of the biocatalyst and both the initial rate of ester synthesis and the yield of ester after 48 h. At the concentration of enzyme of 0.5% the highest initial rate was determined (30.7 μmol min^{-1} g^{-1}). Decreasing the enzyme

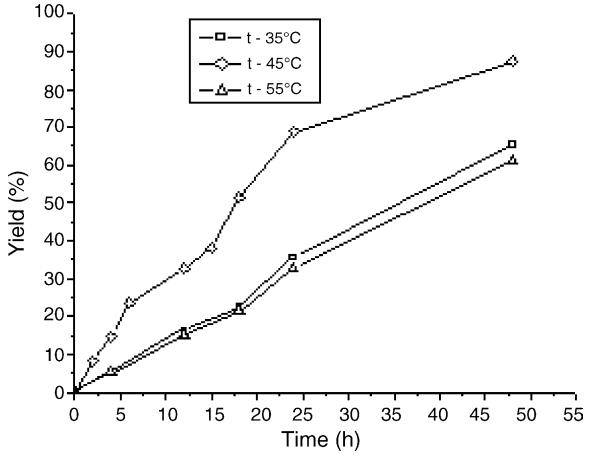

Fig. 1. The effect of reaction temperature on the synthesis of amyl isobutyrate.

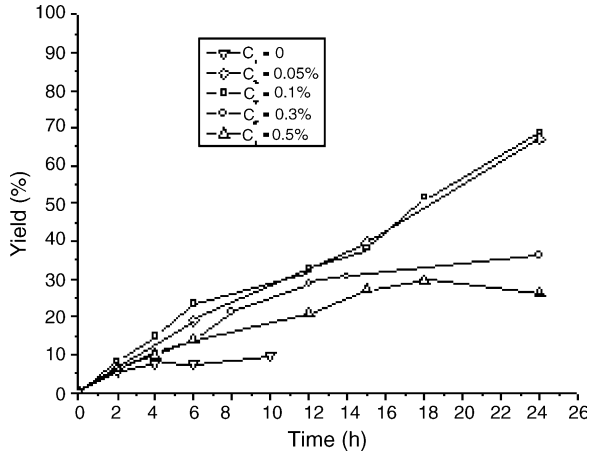

Fig. 2. The effect of quantity of added water on the synthesis of amyl isobutyrate.

concentration to 0.3% and 0.1% led to a substantial decrease of the initial rates, while the increase of concentration led to slight decrease. All further experiments were carried out with 0.5% (w/v) of lipase from *C. rugosa* in order to reach maximum initial rate and utilization of enzyme activity.

3.2. The effect of substrate molar ratio

One of the most frequently utilized ways of increasing the yield of esterification is performing the reaction with the excess of nucleophil. A drawback of this strategy is that it is often followed with a decrease of the initial rate. In order to investigate the influence of the excess of one substrate on the initial rate of amyl isobutyrate synthesis, reaction was performed at various substrate molar ratios.

As it can be seen from Fig. 4, the highest initial rates were achieved at acid:alcohol molar ratio 4:1. In the experiment with a slightly lower excess of acid (molar ratio 2:1), initial rates were higher than in the experiment with equimolar conditions, but rapid decrease of rate at high concentrations of substrates indicates that certain inhibition occurs. Since the initial rates are considerably lower at excess of alcohol, it is plausible inhibition was caused by amyl alcohol.

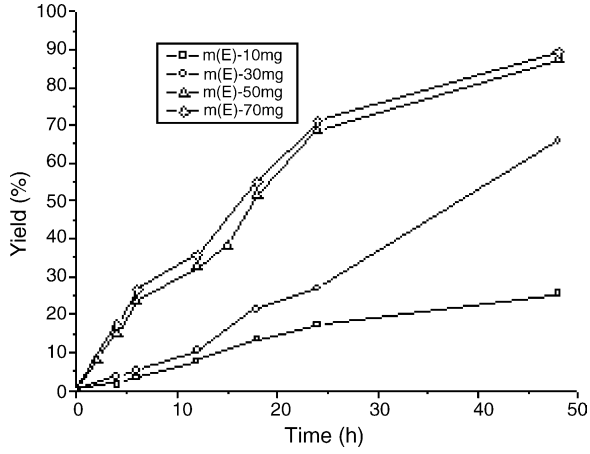

Fig. 3. The effect of lipase concentration on the synthesis of amyl isobutyrate.

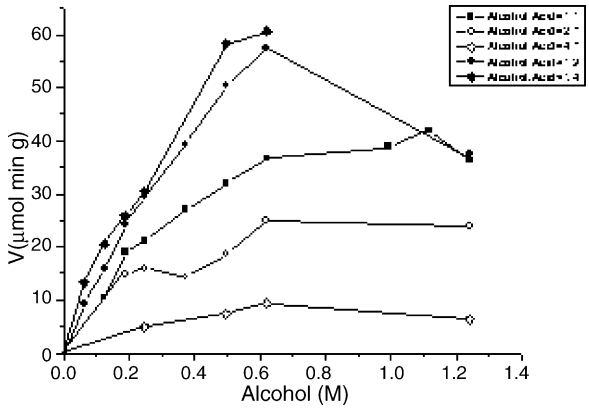

Fig. 4. The effect of substrate molar ratio on the initial rate of the esterification.

In previous study by Garcia et al. [20], focused on investigating effect of substrate molar ratio on initial rate of cetyl oleate synthesis, an excess of alcohol accelerated ester synthesis. The highest rates were observed at acid/alcohol ratio 1:10. A majority of studies on molar substrate ratio was concentrated on its effect on yield of esterification. In a study of synthesis of amyl acetate, excess of acid drastically decreased yield of esters, while excess of alcohol was beneficial up to acid/alcohol ratio 1:2 [29]. Similar results were obtained in a study of synthesis of isoamyl isovalerate, in which optimum acid/alcohol ratio was 1:1.5 [4].

In order to make proper comparison with related studies (which were prevalently focused on the yield of product), additional experimental series was performed in which the effect of substrate molar ratio on final yield of esterification was investigated. The highest final yield of product (nearly 100%) was obtained at acid:alcohol molar ratio 2.5. The results imply that in a synthesis of amyl isobutyrate by the lipase from *C. rugosa* nucleophilic substrate (amyl alcohol) acted as inhibitor. Inhibitory effect of alcohol has been previously reported in several studies, and proposed mechanism of inhibition includes formation of non-reactive dead-end complex between enzyme and alcohol [16].

3.3. Kinetic studies

Initial rates of ester synthesis were determined for various concentrations of acid and alcohol. Alcohol concentration was varied in range 0.06–1.25 M, while acid concentration was between 0.12 and 2.5 M. Higher acid concentrations were applied because excess of acid exhibited favorable effect on reaction rate in a previous part of a study.

After analysis of related literature reports and results of previous part of the study, two kinetic models were tested: ping–pong bi–bi mechanism with alcohol inhibition and ordered bi–bi mechanism with alcohol inhibition [30]. Equations that describe these models are:

$$v = \frac{V_{\max}[\mathrm{Al}][\mathrm{Ac}]}{[\mathrm{Al}][\mathrm{Ac}] + K_{\mathrm{Al}}[\mathrm{Ac}] + K_{\mathrm{Ac}}[\mathrm{Al}] + \frac{K_{\mathrm{Ac}}}{K_{\mathrm{i,Al}}}[\mathrm{Al}]^2} \quad (1)$$

$$v = \frac{V_{\max}[\mathrm{Al}][\mathrm{Ac}]}{K_{\mathrm{d,Ac}}K_{\mathrm{Al}} + [\mathrm{Al}][\mathrm{Ac}] + K_{\mathrm{Al}}[\mathrm{Ac}] + K_{\mathrm{Ac}}[\mathrm{Al}] + \frac{K_{\mathrm{Ac}}}{K_{\mathrm{i,Al}}}[\mathrm{Al}]^2} \quad (2)$$

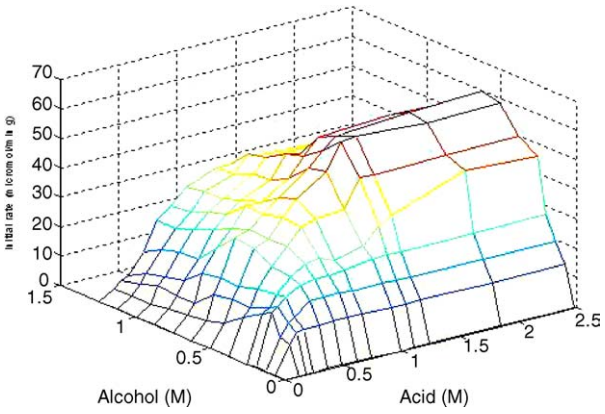

Fig. 5. The dependence of initial rate on concentrations of both substrates.

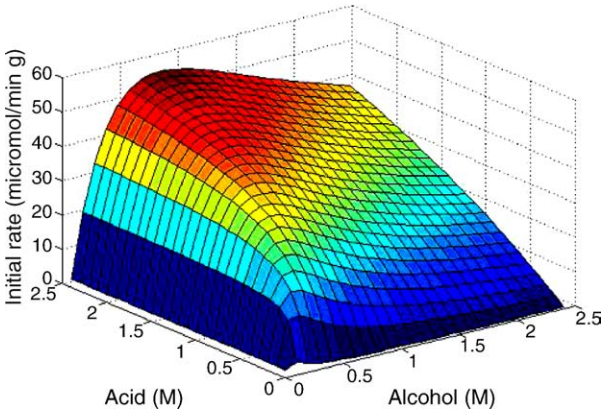

Fig. 7. The illustration of obtained ping–pong bi–bi model.

where v is the initial reaction rate, V_{max} the maximum reaction rate, K_{Al} and K_{Ac} Michaelis constants of amyl alcohol and isobutyric acid, and $K_{i,Al}$ is the amyl alcohol inhibition constant.

Fig. 5 represents dependence of initial rate of amyl isobutyrate synthesis on concentrations of both substrates. In Fig. 6A and B, effects of concentrations of each substrate on initial rate are depicted by representative curves at different fixed concentrations of other substrate. The effect of the increase of alcohol concentration on initial rate in Figs. 5 and 6A confirmed hypothesis of inhibitory effect of amyl alcohol on lipase from *C. rugosa* since the decrease of initial rate with increase of alcohol concentration above 0.6 M can be observed. On the other hand, the increase of concentration of isobutyric acid until 2.5 M did not cause any decrease of initial rates, and curves in Fig. 6B represent typical Michaelis–Menten kinetics. Therefore, the maximum initial rate of 60 μmol min^{-1} g^{-1} was achieved at 2.5 M of acid and 0.625 M of alcohol.

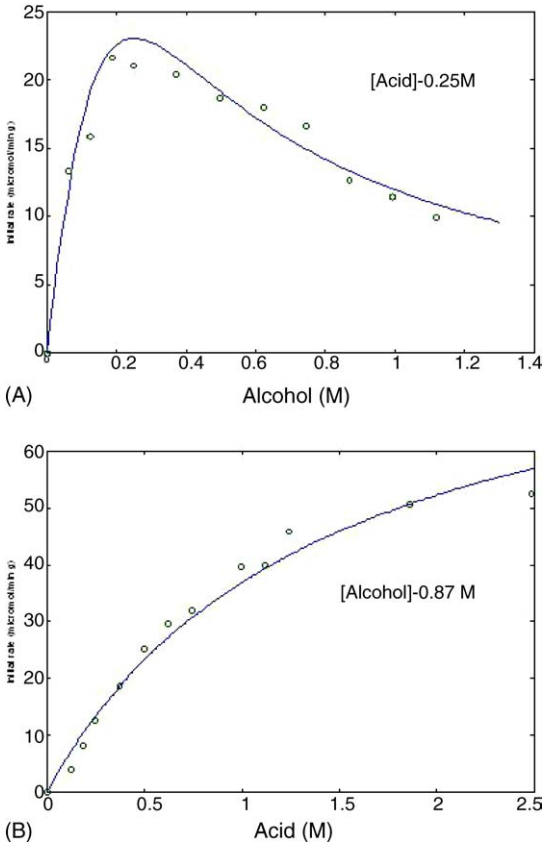

Fig. 6. The ping–pong bi–bi model curves at representative fixed concentrations of acid (A) and alcohol (B).

The data from the initial rate measurements (Fig. 6) were used for the optimization of parameters in Eqs. (1) and (2). Parameters were optimized by the least square error estimation using MatLab 5.2 software and models were tested by exact probability test (Fisher test). The F-values were 1.4236 and 1.7856 for ping–pong model and ordered bi–bi model, respectively. Since theoretical F-value for applied experimental design and significance value of 0.05 is 1.4585, ping–pong bi–bi model was selected for describing kinetics of amyl isobutyrate synthesis. Graphic illustration of obtained ping–pong bi–bi model is depicted in Fig. 7. The ping–pong bi–bi model with alcohol inhibition was previously reported in several studies concerning enzymatic esterification with substrates of various chain lengths and lipases of different origin [14–16]. Values of equation parameters in Eq. (1) and the specificity constants ($K_s = k_{cat}/K_M$) obtained after processing of experimental data are given in Table 1.

High value of $K_{s,Ac}/K_{s,Al}$ (12.5) indicate that lipase from *C. rugosa* has higher affinity towards acid substrate. The higher affinity towards acid substrate was observed previously in synthesis of butyl isobutyrate [16], and in a several studies focused on a synthesis of ethyl oleate [15,31–33]. It must be emphasized that in each of mentioned studies ratio $K_{s,Ac}/K_{s,Al}$ was lower than 12.5 which indicates that lipase from *C. rugosa* has more

Table 1
The kinetic constants from ping–pong bi–bi model with alcohol inhibition

Parameter	Value
V_{max} (μmol min^{-1} g^{-1})	167
K_{Al} (mol dm^{-3})	0.75
K_{Ac} (mol dm^{-3})	0.06
$K_{i,Al}$ (mol dm^{-3})	0.02
$K_{s,Ac}$ (dm^3 min^{-1} g^{-2})	55.7×10^{-3}
$K_{s,Al}$ (dm^3 min^{-1} g^{-2})	4.45×10^{-3}

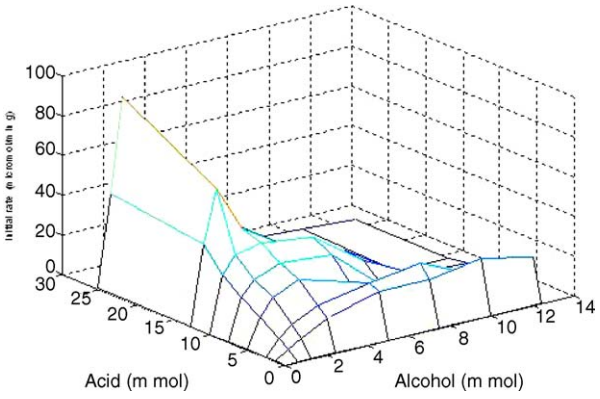

Fig. 8. The dependence of initial rate of solvent-free esterification on concentrations of both substrates.

pronounced affinity towards acid substrate than enzymes applied in cited articles.

Prior to kinetic studies of amyl isobutyrate synthesis in solvent-free system, the optimum temperature and amount of added water was determined in same way as for reaction in isooctane (results not shown). The optimum temperature was 45 °C and the optimum amount of added water was 50 μl. Higher amount of added water is necessary probably due to the fact that destruction of water layer around enzyme by both substrates is more intensive because they have direct access to the enzyme in absence of solvent.

In a kinetic study, molar quantity of isobutyric acid was varied between 1.25 and 25 mmol, while molar quantity of amyl alcohol was varied between 0.625 and 12.5 mmol. The same amount of enzyme as in experiment with isooctane was applied. The obtained results are illustrated in Fig. 8.

It can be seen from surface plot in Fig. 8 that the increase of the quantity of alcohol has similar effect on the rate of the ester synthesis as in the system with organic solvent (Fig. 5), i.e. inhibition of lipase by amyl alcohol is evident. On the other hand, isobutyric acid exhibited the same influence on kinetics of ester synthesis only in area of small molar quantities of alcohol (less than 5 mmol). At molar quantities of alcohol higher than 5 mmol, a steep decrease of initial rates occurs with increase of acid molar quantity above 2.5 mmol. Due to this fact, besides ping–pong bi–bi model with alcohol inhibition, additional model was tested in this experimental series—ping–pong bi–bi model with inhibition by both substrates [19]. However, after subjecting models to exact probability test both models had to be rejected. Such a poor agreement of experimental results with kinetic models, which proved to be adequate in numerous studies (including the first experimental series of this study) indicates that some other phenomena occurs in solvent-free system without affecting the mechanism of enzymatic reaction. Possible explanation could be based on the fact that both substrates have certain polarity, and therefore the ability of binding water molecules and damaging monomolecular water layer around lipase molecule. This binding is probably severe in system without organic solvent because concentrations of both substrates in microenvironment of enzyme molecule are higher. The five-times higher optimum amount of added water in solvent-free system compared with the system with isooctane corroborates this assumption. Therefore, the steep decrease of initial rates of ester synthesis in solvent-free system at high concentrations of both substrates is most likely a consequence of rapid denaturation of enzyme due to destruction of monomolecular water layer. Consequently, ping–pong bi–bi model could not describe experimental results adequately because it does not include enzyme denaturation.

In order to develop equation which include denaturation effects, kinetic model which includes first-order denaturation by both substrates has been tested (Eq. (3)).

$$v = \frac{V_{\max}(1 - k_{\mathrm{d}}[\mathrm{Al}][\mathrm{Ac}])[\mathrm{Al}][\mathrm{Ac}]}{[\mathrm{Al}][\mathrm{Ac}] + K_{\mathrm{Al}}[\mathrm{Ac}] + K_{\mathrm{Ac}}[\mathrm{Al}] + \frac{K_{\mathrm{Ac}}}{K_{i,\mathrm{Al}}}[\mathrm{Al}]^2} \quad (3)$$

where k_{d} is the rate constant of enzyme denaturation.

Although this model was in somewhat better agreement with experimental results then both previously tested models, it had to be rejected after performing statistical analysis.

The highest initial rate in solvent-free system (93 μmol $\mathrm{min}^{-1}\,\mathrm{g}^{-1}$) was reached at 25 mmol of acid and at 1.25 mmol of alcohol. This value is significantly higher than corresponding value in system with isooctane (60 μmol $\mathrm{min}^{-1}\,\mathrm{g}^{-1}$) which indicates that solvent-free system has good prospects for ester synthesis, but at slightly lower amounts of substrates in order to avoid denaturation.

4. Conclusions

In this work, the feasibility of using lipase from *C. rugosa* as a catalyst for amyl isobutyrate synthesis in isooctane and solvent-free system has been demonstrated. Nearly complete conversion (>95%) of the amyl alcohol to ester could be achieved using low enzyme amount of 0.5% (w/v) at 45 °C, when the acid:alcohol ratio was 2.5.

The reaction in organic solvent was well approximated by ping–pong bi–bi kinetic model with inhibition by amyl alcohol. No evidence of enzyme inhibition by isobutyric acid was present up to 2.5 M.

On the other hand, the bisubstrate kinetic models with substrate inhibition did not predict well the reaction performed in solvent-free system, implying either the different reaction mechanisms, or significant enzyme denaturation. Nevertheless, the highest obtained initial rate of amyl isobutyrate synthesis in solvent-free system were higher than corresponding value in the synthesis performed in isooctane, implying that the ester synthesis in solvent-free system can be very fruitful at low *S*/*E* ratios. Considering the industrial importance of the esters, we think that the obtained results could lead to better process designs for production of the flavor compounds in the future.

References

[1] R.M. Yahia, W.A. Anderson, M. Moo-Young, Enzyme Microb. Technol. 23 (1998) 438.
[2] X. Malcata, H. Reyes, H. Garcia, C. Hill, C. Amundson, Enzyme Microb. Technol. 14 (1992) 426.
[3] S. Hari Krishna, B. Manohar, S. Divakar, S.G. Prapulla, N.G. Karanth, Enzyme Microb. Technol. 26 (2000) 131.

[4] G.V. Chowdary, M.N. Ramesh, S.G. Prapulla, Process Biochem. 36 (2000) 331.
[5] X.L. He, B.-Q. Chen, T.-W. Tan, J. Mol. Catal. B: Enzym. 18 (2002) 333.
[6] M. Goldberg, F. Parvaresh, D. Thomas, M.-D. Legoy, Enzyme Microb. Technol. 12 (1990) 976.
[7] T. Garcia, N. Sanchez, M. Martinez, J. Aracil, Enzyme Microb. Technol. 25 (1999) 584.
[8] H. Ghamgui, M. Karra-Chaabouni, Y. Gargouri, Enzyme Microb. Technol. 35 (2004) 355.
[9] E. Katchalski-Katzir, D.M. Kraemer, J. Mol. Catal. B: Enzym. 10 (2000) 157.
[10] M. Szczesna-Antczak, T. Antczak, M. Rzyska, S. Bielecki, J. Mol. Catal. B: Enzym. 19–20 (2002) 261.
[11] S. Maury, P. Buisson, A. Perrard, A.C. Pierre, J. Mol. Catal. B: Enzym. 32 (2005) 193.
[12] S. Bloomer, P. Aldercreutz, B. Mattiasson, Enzyme Microb. Technol. 14 (1992) 546.
[13] P.J. Halling, Enzyme Microb. Technol. 16 (1994) 178.
[14] W. Chulalaksananukul, J.-S. Condoret, D. Combes, Enzyme Microb. Technol. 14 (1992) 293.
[15] S. Hazarika, P. Goswami, N.N. Dutta, A.K. Hazarika, Chem. Eng. J. 85 (2002) 61.
[16] G.D. Yadav, P.S. Lathi, Biochem. Eng. J. 16 (2003) 245.
[17] G.D. Yadav, P.S. Lathi, J. Mol. Catal. B: Enzym. 27 (2004) 109.
[18] J.A. Arcos, G.A. Hill Jr., C. Otero, Biotechnol. Bioeng. 73 (2001) 104.
[19] S. Hari Krishna, N.G. Karanth, Biochim. Biophys. Acta 1547 (2001) 262.
[20] T. Garcia, A. Coteron, M. Martinez, J. Aracil, Chem. Eng. Sci. 55 (2000) 1411.
[21] J.M. Guisan, P. Sabuquillo, R. Fernandez-Lafuente, G. Fernandez-Lorente, C. Mateo, P.J. Halling, D. Kennedy, E. Miyata, D. Re, J. Mol. Catal. B: Enzym. 11 (2001) 817.
[22] T.-C. Hung, R. Giridhar, S.-H. Chiou, W.-T. Wu, J. Mol. Catal. B: Enzym. 26 (2003) 69.
[23] Z. Knezevic, G. Kukic, M. Vukovic, B. Bugarski, B. Obradovic, Process Biochem. 39 (2004) 1377.
[24] H.W. Yu, J.C. Wu, C.B. Ching, Chirality 17 (2005) 16.
[25] H.W. Yu, J.C. Wu, C.B. Ching, Biotechnol. Lett. 26 (2004) 629.
[26] Z. Knezevic, S. Siler-Marinkovic, L. Mojovic, Appl. Microbiol. Biotechnol. 49 (1998) 267.
[27] O.H. Lowry, N.J. Resebrough, A.L. Farr, R.J. Randall, J. Biol. Chem. 193 (1951) 265.
[28] D.T. Lai, C.J. O'Connor, J. Mol. Catal. B: Enzym. 6 (1999) 411.
[29] S. Hari Krishna, S. Divakar, S.G. Prapulla, N.G. Karanth, J. Biotechnol. 87 (2001) 193.
[30] H. Segel, Enzyme Kinetics: Behavior and Analysis of Rapid Equilibrium and Steady-State Enzyme Systems, John Wiley & Sons, New York, 1975.
[31] A.M. Brzozowsky, U. Derevenda, Z.S. Derevenda, G.G. Godson, D.M. Lawson, J.P. Turkenberg, F. Bjorkling, B. Huge-Jensen, S.A. Patkar, L. Thim, Nature 351 (1991) 491.
[32] A.C. Oliveira, M.F. Rosa, M.R. Aires-Barros, J.M.S. Cabral, J. Mol. Catal. B: Enzym. 11 (2001) 999.
[33] A. Marty, W. Chulalaksananukul, R.M. Willemot, J.S. Condoret, Biotechnol. Bioeng. 39 (1992) 273.