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Modeling of enzymatic esterification kinetics with respect to the substrates ratio

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Enzymatic esterification in reversed micelle system is presented. The initial reaction rate has its local maximum at equimolar initial ratio of alcohol to acid for each one of the studied acid concentrations. Modelling of this phenomenon is made based on Michaelis-Menten equation for Ping-Pong Bi-Bi mechanism. One variable in this equation changes with the initial acid concentration while the other is set to alter with the ratio of alcohol to acid and its deviation from the determined optimal value of 1. The observed inhibition by the acid is considered. The effect of acid dilution when the initial water concentration in the reversed micelle system is increased is also taken into account. The kinetic parameters are determined graphically. The modelled rate dependences on the substrates ratio are compared to the measured data. Suggestions for further model development are made.

Key words: enzymatic esterification, Ping-Pong Bi-Bi kinetic model, substrate inhibition, *Candida rugosa* lipase, reversed micelles.

INTRODUCTION

The kinetics of many fatty ester syntheses catalysed by fungal lipases (free or immobilised) has been shown to follow Ping-Pong Bi-Bi mechanism [1]. This mechanism was postulated for reactions in biphasic organic-aqueous systems with solvent [2, 3], in solvent-free systems [4] or in reversed micelle solvents [5−7]. Regarding microemulsion reaction network, some elaborated theoretical models were proposed which took into account the partitioning of the substrates between the phases [5, 8]. For biphasic systems, the effect of the organic solvents polarity was mathematically described through dissociation constants for the substrates [2] or by their thermodynamic activities [4]. However, substrate inhibition was included only in the latter case taking into account a competitive inhibition by the alcohol solely.

The enzymatic esterification that proceeds in the fastest way at one and the same ratio (despite the change in both substrates concentrations) was previously studied by us [9]. Although such phenomenon has not been directly stated anywhere in literature, similar relationship can be revealed if some data are carefully examined. Thus in *n*-heptane Novozyme 435 catalyses best the ethyl acetate synthesis at an ethanol molar excess of *ca.* 4.5 [10], and *i*-amyl oleate at about an equimolar ratio of the substrates [3].

In the present article, we propose an approach to modelling the enzymatic synthesis, which proceeds with optimal rates at a constant initial ratio of alcohol to acid in spite of the change in their concentrations.

EXPERIMENTAL

Materials and methods

The studied reversed micelle system (RMS) consisted of substrates, oleic acid and *i*-amyl alcohol dissolved in *i*-octane, all p.a. (Merck or Sigma-Aldrich). The enzyme, CRL (*Candida rugosa* lipase, TypeVII, Sigma), was incorporated inside the reversed micelles formed by the quaternary ammonium salt, cetyl pyridinium chloride, CPC (Sigma) under injection of a known amount of an aqueous buffer solution.

The effects of substrates and water concentrations on the initial esterification rate were examined in kinetic series following titrimetrically (alcoholic 0.1 mol·dm^{-3} KOH/*phenolphthalein*) the consumption of the free oleic acid [11]. Some esterifications were performed in duplicate, the rates determined deviated from each other by a relative error of 1%. CRL was used as received and the initial rates were referred to g {solid}. The protein content in the solid CRL preparation was assayed according to Sigma Diagnostics, Procedure No. 690 and it was found to be ≈ 14%. In the same origin CRL preparation, ≈8% protein was measured by Zaidi *et al*. [12].

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In the studies (if otherwise stated) the following RMS parameters were held constant: CRL − 3 g {solid}·dm^{-3}; CPC − 0.115 mol·dm^{-3}; aqueous buffer type - K_2H/KH_2 phosphate − 0.05 mol·dm^{-3}; pH 6.88−7.09; temperature − 35°C; stirring rate − ~ 70 rpm.

Experimental base of the model

In Fig. 1, the effects of the initial concentration of the acid and of the initial ratio R = mol [alcohol]$_0$/mol [acid]$_0$ (Fig. 1a) and of the water, *i.e.* the aqueous buffer (Fig. 1b) on the initial reaction rate (V_0) are shown. For [oleic acid]$_0$ = 0.1 mol·dm^{-3} at R = 0.5 and R = 0.75 (Fig. 1a), the initial CPC concentration was reduced 3-fold (to 0.0383 mol·dm^{-3}) in order to establish a stable RMS of W_0 = 30 (W_0 = mol H_2O/mol CPC). In Fig. 1a it is well seen that for each acid concentration the rate has its local maximum at R = 1. The aim of this work is to consider this phenomenon 'optimal rates at a constant substrates ratio' into well known kinetic model for Ping-Pong Bi-Bi mechanism (Eqn. (1)):

$$\frac{1}{V_0} = \frac{1}{V_{\max}} + \frac{K_{mB}}{V_{\max}} \times \frac{1}{B} + \frac{K_{mA}}{V_{\max}} \times \frac{1}{A} \qquad (1).$$

For the purpose, we propose a modification of the variable for the alcohol concentration *A*, in the model equation (1).

Moreover, from the data represented in Fig. 1b, it is seen that the initial water concentration in the RMS-volume, [H_2O]$_0$, affects significantly the reaction rate. For highest [oleic acid]$_0$ = 0.4 mol·dm^{-3} and W_0 = 30 ([H_2O]$_0$ = 3.45 mol·dm^{-3}) the rates decreased (Fig. 1a), obviously due to the inhibition caused by the acid. However, increasing the water concentration the rates increased substantially (Fig. 1b). At [H_2O]$_0$ = 4.60 mol·dm^{-3} (W_0 = 40) the rates approached the highest values gained at [oleic acid]$_0$ = 0.3 mol·dm^{-3}. The effect of substrates dilution by water is evident and it diminishes the inhibition effect. It can be concluded that both substrates influence the reaction rate through their concentrations with respect to the dispersed aqueous phase of the reversed micelles where the enzyme molecule is incorporated and where the reaction proceeds. In eq. 1, the variables for the substrates concentrations *A* and *B* should be transformed taking into account the dilution effect of the water initially present in the RMS.

Model development

Transformation of the variables A and B in order to consider the effect of the initial water. The first transformation consists in normalisation of both initial substrates concentrations, *A* and *B*, to the initial water concentration in the RMS-volume.

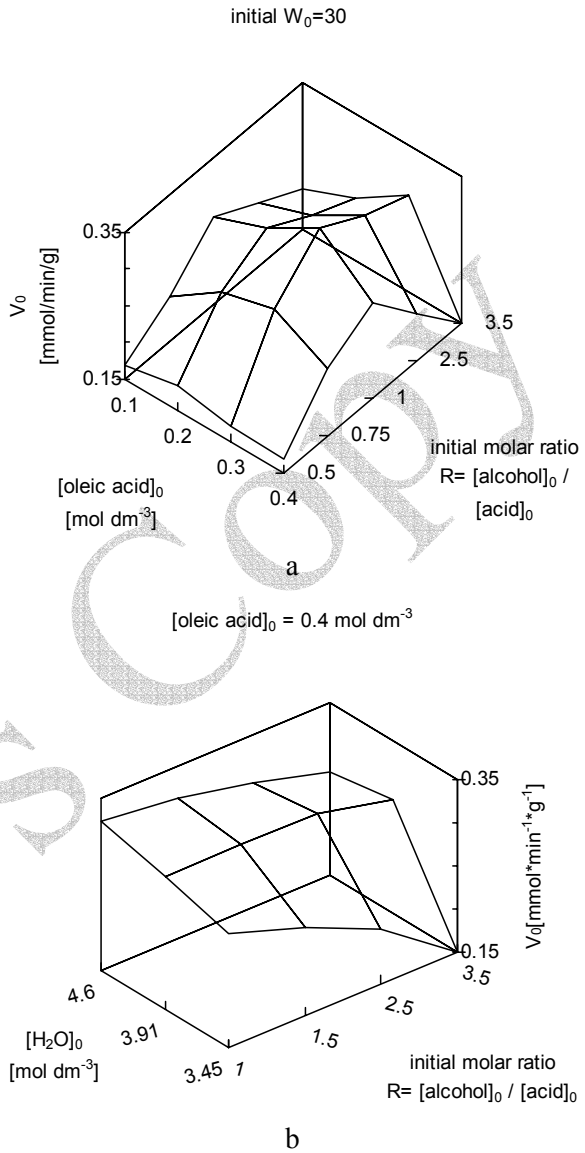

Fig. 1. Effect of the initial concentrations of the substrates (a) and of the water (b) on the initial reaction rate, V_0. CPC and CRL concentrations were as mentioned in Experimental, except for [oleic acid]$_0$ = 0.1 mol·dm^{-3} at R = 0.5 and R = 0.75 where CPC-concentrations were 0.0383 mol·dm^{-3} and CRL, 1 g·dm^{-3}. [Oleic acid]$_0$ = 0.4 mol·dm^{-3}, R = 3.5 and [H_2O]$_0$ = 3.45 mol·dm^{-3} was not measured.

The reason is, that the substrates concentrations with respect to the dispersed aqueous phase of the RMS can not be initially settled and known as they are consequent upon the spontaneous processes of micelle formation and mass exchange between the reversed micelles and the continuous hydrocarbon phase where the substrates are dissolved. The variables *B* and *A* in Eqn. (1) are transformed into B^* and A^* and the following expressions (2) and (3) are assigned:

$$B^* = \frac{[oleic\ acid]_0}{[H_2O]_0} \qquad (2),$$

i.e. B^* is the initial molar ratio of the oleic acid to the water in the RMS-volume. B^* is dimensionless variable. As it can be calculated from the data in Fig. 1, the effect of B^* is studied for five B^*_i-values: 0.0299, 0.0614, 0.0899, 0.1053, and 0.1189;

$$A^* = \frac{[i-amyl\ alcohol]_0}{[H_2O]_0} \qquad (3),$$

i.e. A^* is the respective dimensionless variable for the initial molar ratio of the *i*-amyl alcohol to the water in the RMS-volume.

Further transformation of the variable A^* in order to consider the effect of the initial substrates ratio. As already discussed, for each one experimental B^*_i, the highest initial rate, $V_{0,ij}$, was measured when $A^*_j = B^*_i$, *i.e.* always at R = 1. In Fig. 1 two areas can be distinguished. In the series $B^*_1 = A^*_j > A^*_{j-1} > A^*_{j-2} > \ldots$ (R decreases from 1 to 0.5), $V_{0,ij} > V_{0,ij-1} > V_{0,ij-2} >$ as a result of the reduction in the initial alcohol concentration. In the series $B^*_1 = A^*_j < A^*_{j+1} < A^*_{j+2} < \ldots$ (R increases from 1 to 3.5), $V_{0,ij} > V_{0,ij+1} > V_{0,ij+2} > \ldots$ due to the inhibition by the alcohol.

On this experimental base, further transformation of the variable A^* is made in order to consider the described effect of the initial substrates ratio. Instead of A^* in the model Eqn. (1) we propose a new variable A_R which represents a relationship between the two substrates according to the following expression:

$$A_R = e^{-\frac{|A^* - B^*|}{A^*}} \qquad (4).$$

Each one value $A_{R,ij}$ can be directly calculated from the known initial concentrations in the RMS, [oleic acid]$_0$, [*i*-amyl alcohol]$_0$, and [H_2O]$_0$ using expressions (2) and (3). The power on the right side of Eqn. (4), $\frac{|A^* - B^*|}{A^*}$, is involved in the following two equalities:

- for each $A^* < B^*$, $\frac{|A^* - B^*|}{A^*} = \frac{B^*}{A^*} - 1$ (4');

- for each $A^* > B^*$, $\frac{|A^* - B^*|}{A^*} = 1 - \frac{B^*}{A^*}$ (4'').

In both cases the power represents a measure of how much A^* deviates from B^*, *i.e.* how much the substrates molar ratio deviates from its optimum value of 1. Otherwise, the new variable can be represented as follows:

$$A_R = e^{-\frac{|A^* - B^*|}{A^*}} = e^{-\left|1 - \frac{1}{R}\right|} = e^{-\frac{|R-1|}{R}} \qquad (4''').$$

The present exponential form is chosen to limit up the A_R-value when $A^* = B^*$ (R = 1). A_R can be maximally equal to 1 only if $A^* = B^*$ (R = 1).

Using the new variable A_R, Eqn. (1) is transformed into Eqn. (5):

$$\frac{1}{V_0} = \frac{1}{V_{max}} + \frac{K_{mB^*}}{V_{max}} \times \frac{1}{B^*} + \frac{K_{mA_R}}{V_{max}} \times \frac{1}{A_R} \qquad (5),$$

where the variable B^* is defined according to Eqn. (2), and the variable A_R, – according to Eqn. (4).

Our arguments that the variable A_R can introduce correctly the observed effect of the initial substrates ratio on V_0 (Fig. 1a) are pointed as follows:

- A_R is maximal (= 1) for each pair $A^*_j = B^*_i$ (each R = 1) and according to Eqn. (5) the initial rate, V_0, will have its local maximum depending only on B^*_i-value.

- In the series $B^*_1 = A^*_j > A^*_{j-1} > A^*_{j-2} >$ (R decreases from 1 to 0), A_R diminishes and tends to 0 at $A^*_j << B^*_i$. In this way the decrease in V_0 with the alcohol reduction is described.

- In the series $B^*_1 = A^*_j < A^*_{j+1} < A^*_{j+2} \ldots$ (R increases above 1), A_R diminishes. Thus the decrease in V_0 caused by inhibition by the alcohol can be described. It has to be mentioned that the values $A^*_j >> B^*_i$ are not allowed due to system restrictions. It is known that large amounts of the alcohol (co-surfactant) cause an increase in the interface curvature and reversed micelles too small in size do not suit the enzyme [13, 14].

The dependence of the measured V_0 on the new variable A_R is illustrated in Fig. 2 for two of the studied B^*_i-values: 0.0614 and 0.1189. The values $V_{0,i}$ raise from 0 to $V_{max,i}$ with A_R altered from 0 to 1. A_R tends to 0 when R tends to 0 (4'''), which means no alcohol in the system, so it is logical to have no reaction rate. On the other hand, due to the discussed system restrictions, it is not advisable to raise the alcohol concentration very much; R is recommended to be below 9 [15]. It can be calculated that for R up to 9 A_R has great sensibility to the change in R. Thus, by means of the variable A_R the effect of the alcohol concentration can be considered, including its inhibition effect.

The dependences like those shown in Fig. 2 can be further transformed into the known linear forms of the Michaelis-Menten equation, and the kinetic parameters in Eqn. (5), Michaelis-Menten constants

K_{mB^*} and K_{mA_R}, and V_{max} can be graphically determined.

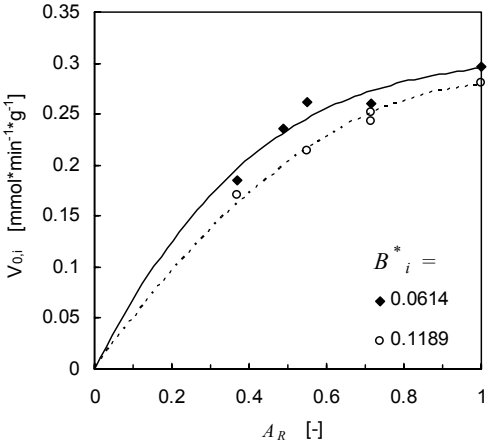

Fig. 2. Dependence of the measured rates on the new variable A_R (Eqn. (4)).

Kinetic parameters determination. Lineweaver-Burk transformations $\frac{1}{V_{0,i}} \propto \frac{1}{A_R}$ (B^*_I = const) based on experimental data (Fig. 1a and 1b) are represented in Fig. 3.

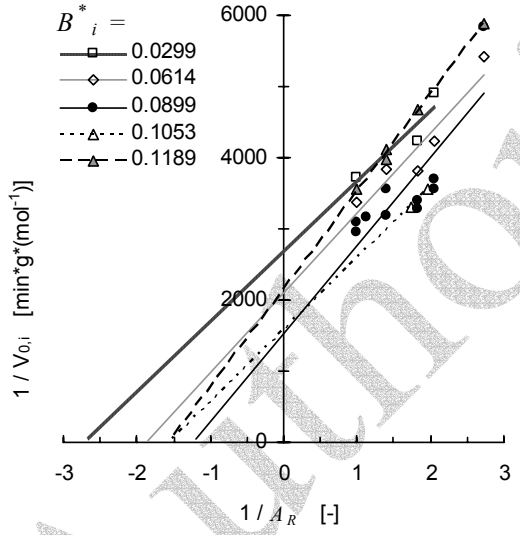

Fig. 3. Lineweaver-Burk linearisation $\frac{1}{V_{0,i}} \propto \frac{1}{A_R}$ for different fixed B^*_i.

The slopes, intercepts and correlation coefficients of the lines obtained are listed in Table 1.

Table 1. Characterisation of the lines obtained from Lineweaver-Burk transformations $\frac{1}{V_{0,i}} \propto \frac{1}{A_R}$ in Fig. 3.

B^*_i	Slope, min·g·mol^{-1}	Intercept, min·g·mol^{-1}	Correlation coefficient, R^2
0.0299	996.16	2676.6	0.8511
0.0614	1128.6	2105.9	0.8974
0.0899	1238.4	1544.0	0.6833
0.1053	1003.2	1576.7	1
0.1189	1372.2	2153.9	0.9789

It is seen that the slope is not constant but it rises with B^*. This is due to the competitive inhibition effect caused by the acid. The linear dependence of slopes on B^* is shown in Fig. 4a and the following equation 6 is drawn:

$$\frac{K_{mA_R}}{V_{max}} \times \left(1 + \frac{B^*}{K_{iB^*}}\right) = \frac{K_{mA_R}}{V_{max}} + \frac{K_{mA_R}}{V_{max}} \times \frac{B^*}{K_{iB^*}} = \\ = 926.2 + 2732 \times B^* \quad (6),$$

where K_{iB^*} is inhibition constant of the acid under the form of the variable B^*.

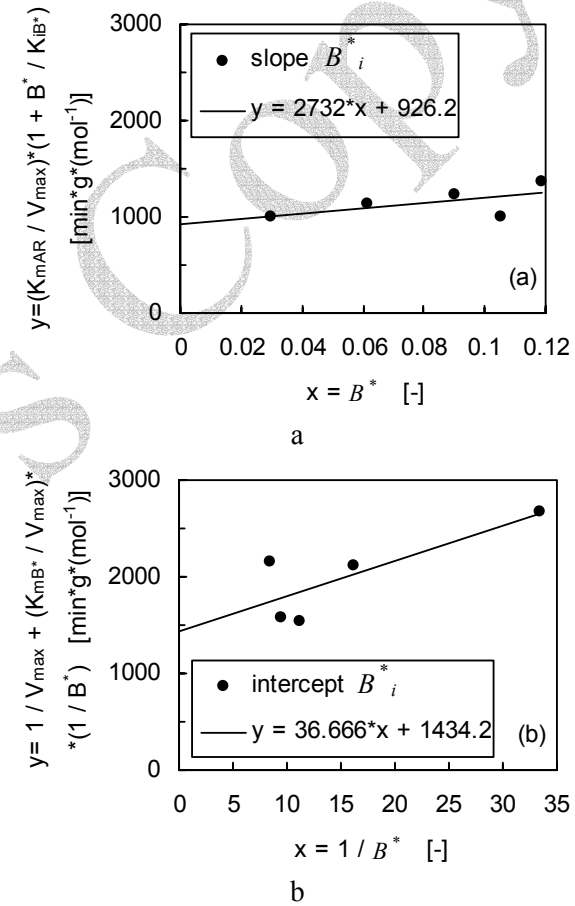

Fig. 4. Replot of slopes (a) and intercepts (b) of the lines in Fig. 3.

The intercepts are proportional to $1/B^*$ as shown in Fig. 4b and the following linear equation is drawn:

$$\frac{1}{V_{\max}} + \frac{K_{mB^*}}{V_{\max}} \times \frac{1}{B^*} = 1434.2 + 36.666 \times \frac{1}{B^*} \quad (7).$$

Including the inhibition observed, Ping-Pong Bi-Bi model for the studied reaction (Eqn. (5)) is transformed in the final form:

$$\frac{1}{V_0} = \frac{1}{V_{\max}} + \frac{K_{mB^*}}{V_{\max}} \times \frac{1}{B^*} + \frac{K_{mA_R}}{V_{\max}} \times \left(1 + \frac{B^*}{K_{iB^*}}\right) \times \frac{1}{A_R} \quad (8).$$

From equations (6) and (7) the kinetic parameters for the studied reaction are determined and listed in Table 2. As in parameters determination are involved experiments of $[H_2O]_0$ = 3.45−4.6 mol·dm^{-3}, according to Eqn. (2) K_{mB}* corresponds to [oleic acid]$_0$ = 0.0882−0.1176 mol·dm^{-3}. According to Eqn. (4'''), K_{mA_R} corresponds to R = 0.696. Taking into account the protein content of the lipase preparation used, ≈14%, the determined value of V_{max} can be recalculated to be 0.30 mol·h^{-1}·g^{-1} {protein}, which is comparable to published data for oleate esters produced by nylon-immobilised *Candida rugosa* lipase [12]. Thus, for butyl oleate enzymatic syntesis, V_{max} was determined to be 0.19 mol·h^{-1}·g^{-1} {protein}, and the ratio $K_{m(alcohol)}/K_{m(acid)}$ corresponded to R = 0.5.

Table 2. Kinetic parameters in Ping-Pong Bi-Bi model with inhibition (Eqn. (8)) for the studied esterification reaction in RMS [a].

V_{max}, mmol·min^{-1}·g^{-1}	K_{mB}*	K_{iB}*	K_{mA_R}	R corresponding to K_{mA_R} (Eqn. (4'''))
0.6973	0.0256	0.3390	0.6458	0.696

a - RMS consisted of CPC − 0.115 mol·dm^{-3}; CRL − 3 g·dm^{-3}; $[H_2O]_0$ = 3.45−4.6 mol·dm^{-3}.

Comparison between experimental and model data. Suggestions for further model development. Comparison between experimental and model values of the initial reaction rate is shown in Fig. 5a–d. The model data describe well the trend of rate dependence on R, which is the goal of the proposed modelling approach. However, the model Eqn. (8) describes poorly the rate decrease at [oleic acid]$_0$ = 0.4 mol·dm^{-3} (Fig. 5c). It is due to the fact that inhibition effect on $1/V_{max}$ has not been introduced. As seen in Fig. 3 at B* = 0.1189 the rate decreases (intercept in Fig. 4b is increased). Dependence on B*, $\frac{1}{V_{max}} \times \left[1 + f\left(B^*\right)\right]$, has to be involved when experiments at B* > 0.1189 are carried out.

Experimental and predicted values for some esterifications, which have not been used in the parameters determination procedure, are com-pared in Table 3. The model proposed is sensitive to the increase in $[H_2O]_0$ through the variable B^* (Eqn. (2)). This means that the model is sensitive to the acid dilution by the dispersed aqueous phase. However, the second variable, A_R (Eqn. (4''')), does not depend on water. The model is not sensitive to the dilution of the alcohol-substrate, which needs further resolution. As it has been discussed, some esterifications in Fig. 1a required a special condition, lower CPC-concentration, 0.0383 mol·dm^{-3} and subsequently lower water concentration, $[H_2O]_0$ = 1.15 mol·dm^{-3}, in order to keep W_0 = 30. These data can be also modeled using the kinetic parameters in Table 2 if the variables are recalculated for $[H_2O]_0$ = 3.45 mol·dm^{-3} where the parameters are valid. The recalculated variables $(B^*)'$ and $(A^*)'$ should keep the following ratio constant:

$$\frac{\left(B^*\right)'}{B^*} = \frac{\left(A^*\right)'}{A^*} = \frac{\left([H_2O]_0\right)' = 3.45\ mol \cdot dm^{-3}}{[H_2O]_0 \quad = 1.15\ mol \cdot dm^{-3}} \quad (9).$$

The recalculated variables, experimental and predicted (in Eqn. (8)) rates are shown in Table 4. The comparison shows good approximation of modeled to measured rates.

Table 3. Experimental and predicted values of the initial reaction rate[a].

Initial concentrations in RMS, mol·dm^{-3}			Variables in Eqn. (8)		V_0, mmol·min^{-1}·g^{-1}	
[oleic acid]$_0$	[alcohol]$_0$	$[H_2O]_0$	B^*	A_R	experimental	predicted
0.3083	0.3	3.45	0.0894	0.9727	0.3391	0.3281
0.3015	0.3	4.60	0.0655	0.9951	0.3088	0.3221
0.4126	0.4	4.60	0.0897	0.9689	0.3232	0.3277
0.4115	0.4	5.175	0.0795	0.9717	0.3162	0.3255

a - Predicted values are calculated upon model Eqn. (8) and parameters in Table 2.

Table 4. Experimental and predicted values of the initial reaction rate[a].

initial concentrations in RMS, mol·dm^{-3}			variables in Eqn. (8)		V_0, mmol·min^{-1}·g^{-1}	
[oleic acid]$_0$	[alcohol]$_0$	$[H_2O]_0$	$(B^*)'$	$(A_R)' = A_R$	experimental	predicted
0.1113	0.050	1.15	0.2904	0.2932	0.1682	0.1347
0.1043	0.075	1.15	0.2721	0.6764	0.2115	0.2477
0.1043	0.150	1.15	0.2721	0.7375	0.2659	0.2609

a - Predicted values are calculated upon model Eqn. (8) and parameters in Table 2.

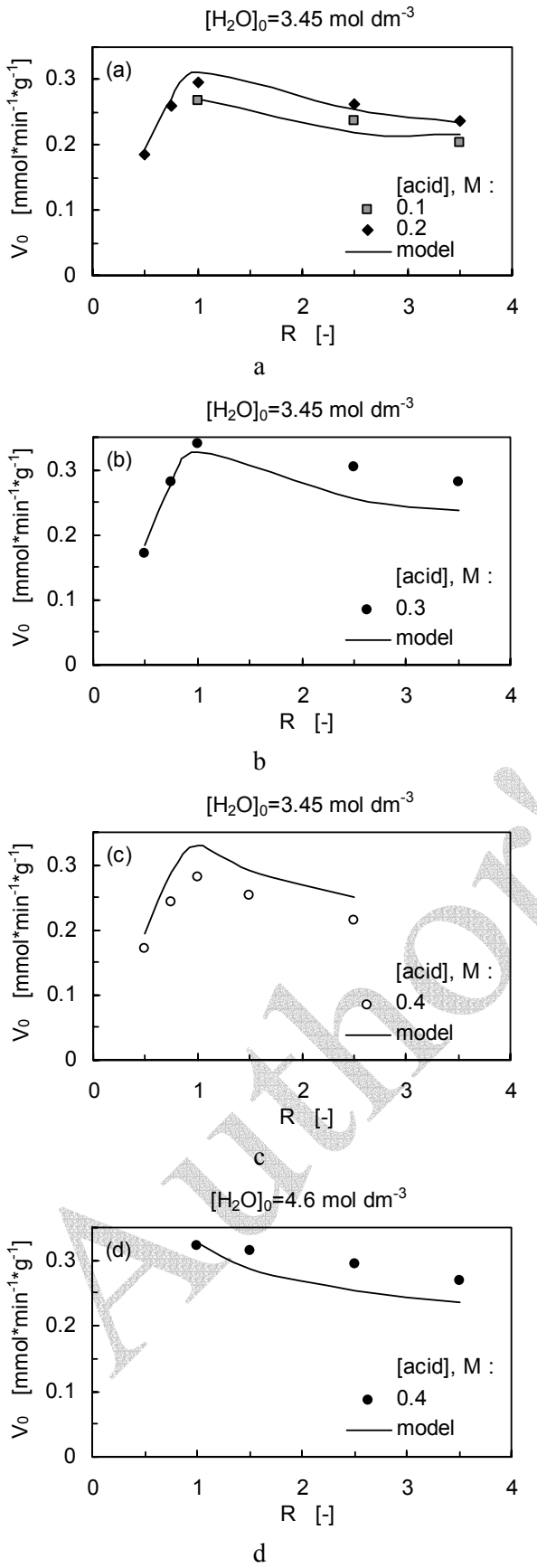

Fig. 5. Comparison between experimental and model values of reaction rate for different initial concentrations of acid, alcohol, and water in RMS (a)–(d).

CONCLUSIONS

Esterification reaction in RMS, characterised by optimal rates achieved when keeping constant the initial molar ratio of alcohol to acid, is modeled. The modeling is based on the Michaelis-Menten equation for Ping-Pong Bi-Bi mechanism. One variable in this equation is conventional and changes with the concentration of the acid while the other alters with the ratio of alcohol to acid and its deviation from the optimal value is experimentally determined. By this transformation, the effect of the alcohol concentration including its inhibition effect, which causes the rate decrease above the optimal ratio, is considered together in one variable. This simplifies the model equation. The approach could be applied to other reaction systems of similar catalytic behavior, i.e. the highest rates at constant substrates ratio.

In the studied reaction inhibition by the acid is observed and considered in the model equation. The effect of acid dilution when the initial water concentration in RMS is increased is also taken into account. The modeled rate dependences on the substrates ratio correspond well to the measured data. The model needs further evolution with respect to dilution effect on the alcohol-substrate. The inhibition effect of the acid-substrate also needs future experimental research and model refinement.

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МОДЕЛИРАНЕ НА КИНЕТИКАТА НА ЕНЗИМНА ЕСТЕРИФИКАЦИЯ ПО ОТНОШЕНИЕ НА СЪОТНОШЕНИЕТО НА СУБСТРАТИТЕ

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(Резюме)

Изследвана е реакция на ензимна естерификация в система с обърнати мицели. За всяка една от изследваните концентрации на киселината е установен локален максимум в профила на началната скорост на реакцията, съответстващ на еквимоларното начално съотношение на алкохола към киселината. Предложено е моделиране на това явление. Моделното описание се основава на трансформиране на уравнението на Михаелис-Ментен за реакции, протичащи по „Пинг-понг“ механизъм. Едната от променливите в модифицираното уравнение е свързана с началната концентрация на киселината, докато другата променлива отразява съотношението на алкохола към киселината и се явява количествена мярка за това, с колко то се различава от експерименталната оптимална стойност 1. Отчетен е наблюдаваният ефект на субстратно инхибиране от киселината. Под внимание е взет ефектът на разреждане на киселината при повишаване на концентрацията на водата в системата с обърнати мицели. Кинетичните параметри в моделното описание на изследваната ензимна естерификация са определени графично. Експерименталните и моделните зависимости на началната скорост на реакцията от началното молно съотношение на субстратите са сравнени. Направени са предложения за допълнително подобряване на моделното описание.