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Kinetic cutinase-catalyzed esterification of caproic acid in organic solvent system

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ABSTRACT

Practical application of any chemical reaction requires the knowledge of its kinetics; in particular if one wishes to be able to describe a chemical reactor over an extended range of reaction conditions or if one intends to optimize the reaction conditions, a suitable kinetic model must be obtained. In order to ensure that the model is applicable over a wide range of experimental conditions it should be based on a mechanistic scheme describing the fundamental steps involved in the reaction; the development of these kind of models can also be used to provide insight into the processes that are taking place.

A kinetic study, using experiments carried out in a batch stirred reactor, has been made for the enzymatic esterification of caproic acid with ethyl alcohol catalyzed by *Fusarium solani pisi* cutinase. Different acid and alcohol concentrations (whilst also varying the acid/alcohol molar ratio) were tested and the results were used to identify the best reaction scheme to describe the results obtained over an extended range of conditions. Several different approaches were used to identify the most adequate mechanistic model, namely by resorting to the quasi stationary state and the rate-limiting hypothesis. The main kinetic characteristics observed in esterification reaction were found to follow an ordered Ping-Pong Bi–Bi mechanism but different modifications were used o ensure that the kinetic model was applicable over the entire range of experimental conditions that were covered.

1. Introduction

Studies of various enzymes (mostly lipases and esterases) in enzymatic synthesis of short chain acid esters, compounds, which have very significant application in cosmetic, pharmaceutical and food industries, increased last few decades [1–4]. Enzyme catalyzed esterification is an interesting option when compared to chemical synthesis as it has the advantages of being able to be carried-out under mild reaction conditions, and to ensure the high quality and purity of the products; also enzymes have been considered as natural components by food regulatory agencies [5–7].

Fusarium solani pisi cutinase activity in hydrolysis, esterification and transesterification has been extensively exploited in recent years and several applications in different industrial fields have been proposed [8]. Numerous report of using cutinases in different reaction media, often dissolved in aqueous solution but also suspended as a powder or immobilized, have been reported [8,9]. Immobilization has been commonly obtained by adsorption onto solid supports [10–19] or encapsulation in reverse micelles of sodium bis(2-ethylhexyl)sulfosuccinate (AOT) [20–23], phosphatidylcholine [24], or cetyltrimethylammonium bromide (CTAB) [25]. The cutinase was used in fundamental studies on the hydrolysis of triglycerides [26], transesterification [15–17], in studies of esterification reactions [27–31] and in order to clarify cutinase mechanism of reaction regarding stereo-selectivity and specificity [32,33]. The understanding of the reaction kinetics is of importance not only to explain in the analysis of the mechanism of the reaction, but also because the information about the rate of product formation and changes in experimental system are essential for the design of appropriate reactor and later industrial scale up.

Modeling of cutinase-catalyzed reaction for transesterification [18,21,22] and esterificaton reaction [24] has been reported. A Ping-Pong Bi–Bi mechanism with competitive inhibition by the alcohol was proposed by Serralha et al. [18] for the alcoholysis of butyl acetate with hexanol in isooctane catalyzed by cutinase immobilized on NaY zeolite. Ping-Pong Bi–Bi mechanism was also proposed for the same reaction in AOT/isooctane reverse micellar system by Carvalho et al. [22] and by Sebastião et al. [21] and Pinto-Sousa et al. [24] for the esterification reaction in phosphatidylcholine reversed miccelar system. Although these representative examples of research work focused on the kinetic analysis of cutinase-catalyzed reaction, mostly performed on transesterification and esterification reactions in reversed micellar systems, no detailed kinetic model for esterification reaction in organic solvent, namely in *iso*-octane, by lyophilized cutinase, was presented, despite the catalytic potential of cutinase in organic solvent system. However,

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Nomenclature

Ac	acid
Al	alcohol
Es	ester
E	free enzyme
$[E]_t$	total enzyme concentration (mM)
EAc	enzyme–acyl complex
EAc2	enzyme–acyl complex with two bound acid molecules
EAc3	enzyme–acyl complex with three bound acid molecules
EAl	enzyme–alcohol complex
K_{a1}, K_{a2}, K_{a3}, K_{b1}, K_{b2}, K_{b3}	equilibrium constants
K_i	inhibition coordination constant
k_{a1}, k_{a2}, k_{a3}, k_{b1}, k_{b2}, k_{b3}	rate constants ($mM^{-1}\ min^{-1}$)

the development of a detailed kinetic model that is capable of describing the reaction over an extended range of reactions conditions is of paramount importance for practical applications, namely for the optimisation of reaction systems.

Cutinases are a group of enzymes that can be considered as a link between esterases and lipases. As small carboxylic ester hydrolases, the sub-family of cutinases consists of 20 members based on amino-acid sequence similarity, which display hydrolytic activity on cutin polymers and efficiently hydrolyze soluble esters and emulsified triacylglycerols. Cutinase belongs to the family of serine hydrolases containing its catalytic serine centre at the middle of a sharp turn between a β-strand and a α-helix [14,34]. The *F. s. pisi* catalytic triad, *Ser-120*, *Asp-175* and *His-188*, is accessible to the solvent and it can possibility to accommodate different substrates. The *Asp-175* hydrogen binds to *His-188*, thus promoting the interaction of the imidazole ring with *Ser-120*. The histidine acts as a base, deprotonating the serine to generate a very nucleophilic alkoxide (–O–) group (Fig. 1). The serine in the active centre of the enzyme is a very strong nucleophile, which attacks the carbonyl group of the acid, forming a stable tetrahedral intermediate acyl–enzyme complex. The acyl–enzyme complex is stabilized by the oxyanion hole [12]. Water is then released and the structure reverts to the planar carbonyl flat plane acyl–enzyme intermediate. The alcohol acts afterwards as a new nucleophile and links to the tetrahedral intermediate. Subsequently, as the final step, the resolution of tetrahedral complex yields the ester and the free enzyme. This reversible reaction was shown to follow a Ping-Pong Bi–Bi mechanism [8,35].

In previous work made by de Barros et al. [30] *F. s. pisi* cutinase expressed in *Saccharomyces cerevisiae* showed a high potential for the synthesis of short chain ethyl esters in *iso*-octane, an organic solvent recognized as a safe ingredient for use in the food and beverage industrial processes by FDA. Among the advantages of using the organic solvent media for enzymatic ester synthesis are the increased solubility of non-polar substrates and products and the shifting the thermodynamic equilibrium of the reaction to favour ester synthesis over hydrolysis. Log *P* (the partition coefficient between water and 1-octanol) is generally used to describe the solvent hydrophobicity. It has been reported that solvent with intermediate log *P* (around 4) are suitable for esters synthesis [36]. Log *P* of *iso*-octane is 4.5 and by previous work it was shown that it favours the esterification reaction, reason for which it was chosen for this work.

Since mechanism based kinetic models are usually better for interpolating and extrapolating purposes, the goal of this study is to estimate the kinetic parameters of this system in terms of a Ping-Pong Bi–Bi model and to analyse possible refinements that can be introduced in this model to improve the description of the reaction system with the aim of obtaining a model that can be used over a sufficiently wide range of operating conditions so as to allow the optimization of a bioreactor for this reaction.

As a model system the production of ethyl caproate from caproic acid and ethanol has been used. Ethyl caproate is a flavour compound that is incorporated in a wide range of aromas such as: apple, apple green apple, banana, beer, butter, cognac, herbal, pineapple and wine [37].

2. Materials and methods

2.1. Enzyme and chemicals

F. s. pisi cutinase wild-type was biosynthesized by recombinant *S. cerevisiae* SU50 strain as described by Calado et al. [38].

Caproic acid (C_6) (99.0%, Fluka, Germany) and ethanol abs. (VWR, Germany) were used for ester synthesis, while *iso*-octane (99.5%, Fluka, Germany) was used as organic solvent and n-decane (VWR, Germany) was used as an internal standard for gas chromatography (GC). Sodium sulfate anhydride (Acros, Geel, Belgium) was used to dry *iso*-octane as organic media before and after esterification reactions. Saturated salt solution of sodium chloride (Panreac, Spain) was used for equilibration of enzyme and substrates. All other chemicals used were of analytical grade.

2.2. Purification and lyophilization of cutinase

The isolation and purification of cutinase excreted by recombinant *S. cerevisiae* SU50 strain were carried out by expanded bed adsorption (EBA) [39].

The pool of elution fractions exhibiting the highest cutinase activity was firstly dialyzed against distilled water and then frozen at −80 °C and lyophilized (B.BRAUN Biotech. International CHRIST Alpha 2-4) overnight. Lyophilized cutinase preparations were characterized by measuring the cutinase esterolytic activity and protein content [40–42] and stored at −20 °C before used in esterification reactions.

2.3. Characterization of the cutinase preparations

The cutinase estereolytic activity was assayed using a spectrophotometric method based on monitoring the hydrolysis of p-nitrophenylbutyrate (p-NPB) to nitrophenol (p-NP), a yellow compound easily identify and quantify by the absorbance at 400 nm [40]. One unit of cutinase estereolytic activity was defined as the amount of enzyme required to convert 1 μmol of p-NPB to p-NP per 1 min, at 30 °C and pH 8. The extinction coefficient of p-NP was considered to be 1.84×10^4 ($M^{-1}\ cm^{-1}$), as indicated by the supplier (Sigma).

The protein concentration was determined by the method of PEARCE (BCA assay) with reference to a standard, the Bovine Serum Albumin (BSA) (Merck) [42].

Specific activities of lyophilized cutinase preparations were of 170 U mg^{-1}. These enzyme samples when loaded in SDS electrophoresis gel showed a single band of 22 kDa [41].

2.4. Methods for monitoring substrate and ester concentrations

The concentrations of ethanol, caproic acid and ethyl caproate were determined using a Hewlett-Packard model 5890 gas chromatograph, equipped with a flame ionization detector (FID). A WCOT Fused Silica coating CP Chirasil-Dex CB column, 25 m × 0.25 mm, DF = 0.25 (Varian Inc.) was used to separate the components in the reaction mixture. n-Decane was used as an internal standard in the computation of ethyl caproate and respective

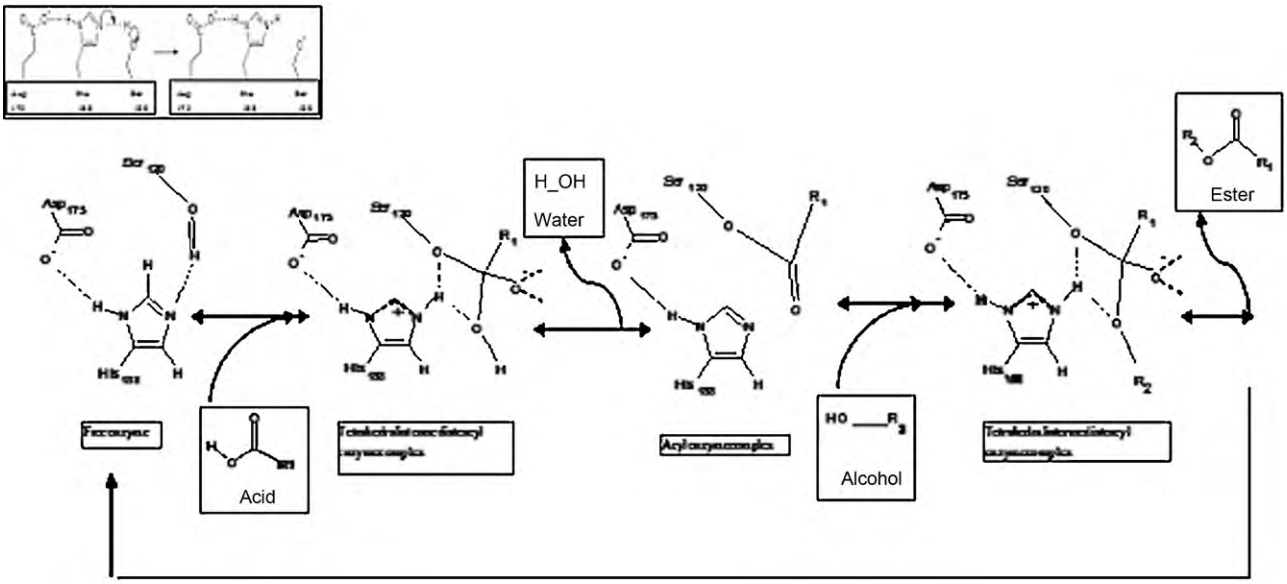

Fig. 1. Schematized mechanism for the esterification catalyzed by cutinase.

substrates concentrations in the reaction media. Nitrogen was used as carrier gas. The oven temperature was held at 50 °C for 4 min before being raised to 160 °C for 1.67 min at 15 °C min^{-1}; the injector temperature was set at 200 °C and the detector temperature was set at 250 °C.

2.5. Enzymatic esterification

The esterification of acid and alcohol by cutinase was carried out in *iso*-octane as organic solvent. Unless otherwise stated a typical esterification reaction for ethyl esters synthesis was carried out in 7 ml of working volume inside a 10 ml flasks capped with rubber (EPDM stoppers, black, Sigma-Aldrich, Germany) so as to minimize the evaporation or loss of volatile compounds. Reaction medium and enzyme were pre-equilibrated separately at 30 °C, for 72 h, inside closed vessels containing saturated NaCl aqueous solution to control water activities (a_w = 0.75). Alcohol, acid and n-decane were mixed thoroughly in *iso*-octane before the addition of enzyme (lyophilized form). The first sample (zero point of the reaction) was collected before enzyme addition. The enzymatic ester synthesis was performed in an incubator (AGITORB 160E, Aralab, Portugal) at 30 °C and cutinase activity of 240 ± 10 U ml^{-1} reaction mixture (r.m.). The experimental setup enabled us to run multiple parallel experiments using a multiple magnetic stirrer (S.B.S. Instruments A-23) and the simultaneous operation of 6–12 reactors. A control run, for each set of experimental conditions, was performed using at least one blank experiment (without the enzyme) running in parallel for each enzymatic ester synthesis. Experiments were performed at least in duplicate and the experimental error was estimated as less than 8%. The calibration curves that were obtained allowed us to estimate accurately the concentration of caproic acid above 30 mM; bellow this value there is a significant error in the determination. However, the experimental caproic acid concentration were usually above 30 mM. The fact that the calibration curve is not accurate below 30 mM may explain why the model has more difficult in fitting experiments with low caproic acid concentrations. During the reaction, a magnetic stirring set at 300 rpm was used. Samples were withdrawn periodically using a needle, without destroying the rubber cap. The reaction yield was calculated according to the molar ratio between the ethyl ester and respective limiting substrate, alcohol or acid.

2.6. Kinetic model

The base Ping-Pong Bi–Bi mechanism was used as a starting point and modifications have been introduced so as to improve the descriptive ability of the model. For each kinetic model the material balances for ethanol, caproic acid, ethyl caproate, water and, in the case of the base model for the enzyme containing species, were derived and the corresponding set of differential equations was numerically solved using the Euler's method. The model was fitted to the experimental data (all the available experiments were used simultaneously) by a least squares procedure where the objective function consisted of the sum of the square of relative errors of the concentrations of ethanol, caproic acid, ethyl caproate measured in all experiments for all times; this corresponded to a total of 807 data points distributed over 18 different experiments with different initial concentrations. The experimental conditions, for the set of experiments used in the fitting procedure are given in Table 1. This procedure was carried-out using a Microsoft Excel 2003 spreadsheet and the estimation of the parameters was carried-out using the Solver tool.

3. Results and discussion

3.1. Base kinetic model

The esterification of caproic acid by ethanol catalyzed by cutinase is an enzymatic reaction system which involves two reac-

Table 1
Experimental conditions for the set of experiments used in the fitting of the different models. All concentrations are given in mM. Enzyme concentration is equal to 0.1 mM in all runs.

Run	Ethanol conc.	Carpoic acid conc.	Run	Ethanol conc.	Carpoic acid conc.
1	99	87	10	104	99
2	188	196	11	165	197
3	204	175	12	160	178
4	201	140	13	167	164
5	214	127	14	122	172
6	202	100	15	110	181
7	232	74	16	92	183
8	216	45	17	229	106
9	105	196	18	121	95

Table 2
Kinetic constant obtained for the synthesis of ethyl caproate catalyzed by cutinase.

	Complete model	Quasi stationary state model	Rate limiting step model
k_{a1} ($mM^{-1}\ min^{-1}$)	1.67	0.876	
k_{-a1} ($mM^{-1}\ min^{-1}$)	0.022	2.42	
K_{a1}	76[a]	0.36[a]	0.39
k_{b1} ($mM^{-1}\ min^{-1}$)	0.274	0.026	0.024
k_{-b1} ($mM^{-1}\ min^{-1}$)	7.12	0.0020	0.073
Objective function	302,053	226,802	207,729

[a]Computed as $K_{a1} = k_{a1}/k_{-a1}$.

tants and two products. As most of the kinetic studies on the cutinase-catalyzed synthesis of esters have described the reaction as following a Ping-Pong Bi–Bi kinetic mechanism with inhibition by the substrates, this reaction mechanism was taken as the basis for this work. Taking into account that for this reaction it is necessary that an acyl–enzyme bond is formed, the first step will be the formation of the active enzyme–substrate complex between cutinase and the caproic acid. The sequence of steps involved in the reaction can be schematized as in Fig. 1.

According to this scheme, and assuming that the first two steps occur rapidly, we can write the general mechanism by following reaction steps.

$$E + Ac \underset{k_{-a1}}{\overset{k_{a1}}{\rightleftarrows}} EAc + H_2O \quad (1)$$

$$EAc + Al \underset{k_{-b1}}{\overset{k_{b1}}{\rightleftarrows}} Es + E \quad (2)$$

where k_{a1}, k_{-a1}, k_{b1}, k_{-b1} are the rate constants for the different steps.

Assuming that these reactions have simple order kinetics and since all reactions were carried-out in closed vessels (thus allowing us to neglect evaporation of any of the components in the mixture), the material balances for each of the species involved were written, leading to the following set of differential equations:

$$\frac{d[E]}{dt} = -k_{a1}[E][Ac] + k_{-a1}[EAc][H_2O] + k_{b1}[EAc][Al] - k_{-b1}[E][Es] \quad (3)$$

$$\frac{d[Ac]}{dt} = -k_{a1}[E][Ac] + k_{-a1}[EAc][H_2O] \quad (4)$$

$$\frac{d[H_2O]}{dt} = k_{a1}[E][Ac] - k_{-a1}[EAc][H_2O] \quad (5)$$

$$\frac{d[Al]}{dt} = -k_{b1}[EAc][Al] + k_{-b1}[E][Es] \quad (6)$$

$$\frac{d[Es]}{dt} = k_{b1}[EAc][Al] - k_{-b1}[E][Es] \quad (7)$$

$$\frac{d[EAc]}{dt} = k_{a1}[E][Ac] - k_{-a1}[EAc][H_2O] - k_{b1}[Ac][Al] + k_{-b1}[E][Es] \quad (8)$$

As a first step the complete model described by these equations, and the relevant initial conditions for each of the runs that were performed, was fitted, using the four kinetic rate constants as the fitting parameters, to the experimental data available. The parameters obtained by the fitting are presented in Table 2.

The model was found to provide a reasonable description of the data but presented significant deviations in the runs where either the acid or the alcohol concentrations were near 200 mM.

In particular, the reaction rate seemed to be underestimated when the acid concentration was in the range from 160 to 200 mM and somewhat overestimated when the concentration of alcohol was in the range from 175 to 200 mM. This implied that, in order to have a better description of the reaction under all conditions covered by the experiments some modifications should be introduced in the overall scheme.

However, working with this set of equations posed a series of issues that had to be dealt with. Since the concentrations of the various species have different orders of magnitude, in particular since the enzyme concentration is much smaller than that of the other species present, and since the reactions proceed at different speed, the numerical integration of Eqs. (3)–(8) requires a relatively small integration step and, consequently, very lengthy integrations. In order to circumvent this difficulty we have tried to apply a set of approximating assumptions that would allow us to obtain a global reaction rate law. Although using a global reaction law has the disadvantage of not allowing us to compute the individual steps separately, it has the distinct advantage of giving a more concise description of the reactor and being easier to adapt for the final goal of optimising reaction conditions in a bioreactor.

To obtain a global reaction rate some additional assumptions have to be made. On one hand we tried the quasi stationary state assumption and on the other hand we used the rate-limiting step hypothesis.

We will start by considering that a quasi stationary state approach can be used and, thus the derivatives of the species involving the intermediates (Eqs. (3) and (8)) are zero. This assumption is usually adequate when the intermediate species are very reactive and, looking at the reaction rate constants that were estimated using the complete model, this might not be the case since the reaction rate constant for the second reaction is actually smaller than for the first one. It should be noted that the relative rate of the two reactions cannot be ascertained just by looking at the values of the kinetic rate constants, since all of these reactions are the second order and the concentrations of the various species are quite different. In fact for this hypothesis to be applicable it is sufficient that the concentration of the intermediate is kept sufficiently low during the course of the reaction, so that its derivative can be taken as zero. Thus, albeit bearing in mind the inherent and possible limitations of this approach, the advantages of having a global kinetic rate law are significant and we will see what the application of this assumption provides in terms of the overall fitting to the experimental data.

From the quasi stationary state assumption the relation between the enzyme–acyl concentration and the concentration of the free enzyme can be computed.

$$[EAc] = \frac{k_{a1}[E][Ac] + k_{-b1}[E][Es]}{k_{b1}[Al] + k_{-a1}[H_2O]} \quad (9)$$

Taking into account that the total amount of enzyme that was added to the reaction mixture is fixed we can obtain a relation between the concentrations of the two enzyme species and the total enzyme concentration

$$E_t = \left(1 + \frac{k_{a1}[Ac] + k_{-b1}[Es]}{k_{b1}[Al] + k_{-a1}[H_2O]}\right)[E] \quad (10)$$

where E_t is the concentration of enzyme that was added. From Eqs. (9) and (10) we can obtain

$$[E] = \frac{k_{b1}[Al] + k_{-a1}[H_2O]}{k_{a1}[Ac] + k_{-b1}[Es] + k_{b1}[Al] + k_{-a1}[H_2O]} E_t \quad (11)$$

and

$$[EAc] = \frac{k_{a1}[Ac] + k_{-b1}[Es]}{k_{a1}[Ac] + k_{-b1}[Es] + k_{b1}[Al] + k_{-a1}[H_2O]} E_t \quad (12)$$

A global rate equation can be obtained.

$$r = \frac{k_{a1}k_{b1}[\mathrm{Ac}][\mathrm{Al}] - k_{-a1}k_{-b1}[\mathrm{Es}][\mathrm{H_2O}]}{k_{a1}[\mathrm{Ac}] + k_{-b1}[\mathrm{Es}] + k_{b1}[\mathrm{Al}] + k_{-a1}[\mathrm{H_2O}]}\mathrm{E}_t \quad (13)$$

According to this model a global reaction stoichiometry was used and the concentrations of the various species were computed according to the rate equation given in Eq. (10).

$$\mathrm{Ac} + \mathrm{Al} \rightarrow \mathrm{Es} + \mathrm{H_2O} \quad (14)$$

The fitting of this model to all experimental data, as it was done for the complete model, estimates a set of parameters that are given in Table 2.

Looking at the parameters in Table 2 we can see that the approximate model actually provides a better description of the experimental data, as seen by the fact that the objective function is around 2/3 of the objective function for the complete model. This fact may be explained by at least to reasons: on one hand the complete model is probably more prone to errors in the numerical integration procedure, since it generates to a rather stiff set of differential equations and, on the other hand, the approximation involved in the quasi steady state approximation may actually compensate for some of the shortcomings of the base model.

Another possible approach to develop a global reaction rate equation is to use the rate-limiting step assumption. This alternative approach has the advantage of supplying equations that are usually much simpler than the ones that are obtained by the quasi stationary state assumption and is easier to apply to more elaborate reaction schemes.

Under this approach we will consider that the first step of the overall reaction (described by Eq. (1)) is very fast when compared to the second step and can always be assumed to be in equilibrium. Looking at the kinetic rate constants estimated by the complete model we can see that, although it seems likely that the second step is the rate limiting one (depending, of course, on the actual concentrations of the various species), since the reaction rate constant for the first step is larger than for the second one, the reverse reaction rate constant for the first step is not particularly high, which might limit the applicability of this assumption, although it would seem better that quasi stationary state.

Under these assumption the reaction rate will be limited by the rate of the second reaction

$$r = k_{b1}(([\mathrm{EAc}][\mathrm{Al}]) - ([\mathrm{E}][\mathrm{Es}]/K_{b1})); \quad K_{b1} = \frac{k_{b1}}{k_{-b1}} \quad (15)$$

And the concentrations of the species involving the enzyme can be computed using the equilibrium condition for the first reaction step

$$[\mathrm{EAc}] = K_{a1}\frac{[\mathrm{E}][\mathrm{Ac}]}{[\mathrm{H_2O}]}; \quad K_{a1} = \frac{k_{a1}}{k_{-a1}} \quad (16)$$

As above, the overall balance to the enzyme that was added to the reactor

$$[\mathrm{E}]_t = [\mathrm{E}] + [\mathrm{EAc}] = [\mathrm{E}]\left(1 + K_{a1}\frac{[\mathrm{Ac}]}{[\mathrm{H_2O}]}\right) \quad (17)$$

$$[\mathrm{E}] = \frac{[\mathrm{E}]_t}{1 + K_{a1}([\mathrm{Ac}]/[\mathrm{H_2O}])} \quad (18)$$

Combining all these equations leads to the following global reaction rate equation

$$r = \frac{k_{b1}[\mathrm{E}]_t(K_{a1}([\mathrm{Ac}][\mathrm{Al}]/[\mathrm{H_2O}]) - ([\mathrm{Es}]/K_{b1}))}{1 + K_{a1}([\mathrm{Ac}]/[\mathrm{H_2O}])}$$

$$= \frac{k_{b1}[\mathrm{E}]_t(K_{a1}[\mathrm{Ac}][\mathrm{Al}] - [\mathrm{Es}][\mathrm{H_2O}]/K_{b1})}{[\mathrm{H_2O}] + K_{a1}[\mathrm{Ac}]} \quad (19)$$

Again the complete model will involved computing the transient material balances for the relevant species using this rate equation.

The model was solved for all experimental conditions that were used, as before, and the estimated kinetic parameters are, again, shown in Table 2.

The data on Table 2 shows that the rate limiting step approximation provides a better description of the reaction, as shown by the fact that its objective function is around 10% lower that for the quasi-steady state one. The rate limiting approximation, however, does not allow the simultaneous estimation of k_{a1} and k_{-a1}, since the first step is considered to be in equilibrium. However, comparing the values of K_{a1} for quasi stationary state model ($K_{a1} = 0.36$) and rate limiting step model assumption $K_{a1} = 0.39$ we can see that the constants that were estimated are quite similar (Table 2).

Also, from the fittings of the model to the experimental data, we can see that both models are able to describe in a reasonable way the experimental data although some significant discrepancies are observed, again, as for the complete model, namely for the cases where either the acid or the alcohol concentrations are higher. As explained above we will introduce some refinements in the model may be required to describe the experimental data over the entire range of experimental conditions that were used. We will now discuss these refinements. Since the quasi stationary state assumption and the rate limiting step approximation give comparable results, and since deriving global rate equations for more complex models is significantly easier within the rate limiting step approximation and this has also provided a better fitting to the experimental data, we will use the latter hereafter.

3.2. Refinements of the model

We will address the discrepancies observed for higher alcohol and acid concentrations in two different ways.

On one hand, according to previous studies for the cutinase-catalyzed esterification reaction [18] the alcohol can react with enzyme and form dead-end a complex which is unable to participate in the reaction. Thus, the rate-limiting global reaction rate was modified to take into account the reversible formation of an enzyme–alcohol complex, which was taken as being totally inactive for the reaction at hand.

On the other hand, it has been observed, for the hydrolysis of triglycerides, that the enzyme was able to bind up to 3 of product molecules (which can be assumed to be the free acid molecules) [43]. In the case of the hydrolysis, the binding of product molecules implied a certain degree of product inhibition; however, in this work, if the enzyme is, in fact, able to bond additional reactant molecules this could imply an increase in the apparent order of the reaction in relation to the reactant in question. Again the rate limiting step approximation was used to obtain global reaction rate equations.

3.2.1. Second order approximation

Using the assumption that two molecules of acid could be bound to the active site of the enzyme we can write the following equations:

$$\mathrm{E} + \mathrm{Ac} \rightarrow \mathrm{EAc} + \mathrm{H_2O}; \quad K_{a1} = \frac{k_{a1}}{k_{-a1}} \quad (20)$$

$$\mathrm{EAc} + \mathrm{Ac} \rightarrow \mathrm{EAc2} + \mathrm{H_2O}; \quad K_{a2} = \frac{k_{a2}}{k_{-a2}} \quad (21)$$

$$\mathrm{EAc} + \mathrm{Al} \rightarrow \mathrm{E} + \mathrm{Es}; \quad K_{b1} = \frac{k_{b1}}{k_{-b1}} \quad (22)$$

$$\mathrm{EAc2} + \mathrm{Al} \rightarrow \mathrm{EAc} + \mathrm{Es}; \quad K_{b2} = \frac{k_{b2}}{k_{-b2}} \quad (23)$$

With this reaction scheme the overall reaction rate will be given by the summation of the contributions of the two different reactions

$$r = k_{b1}(([EAc][Al]) - ([E][Es]/K_{b1})) + k_{b2}(([EAc2][Al]) - ([EAc][Es]/K_{b2})) \quad (24)$$

Assuming again, that both the reactions involving the formation of enzyme–acyl complexes, Eqs. (22) and (23) can be assumed to be near equilibrium we can write

$$[EAc] = K_{a1}\frac{[E][Ac]}{[H_2O]} \quad (25)$$

$$[EAc2] = K_{a2}\frac{[EAc][Ac]}{[H_2O]} = K_{a1}K_{a2}\frac{[E][Ac]^2}{[H_2O]^2} \quad (26)$$

$$[E]_t = [E] + [EAc] + [EAc2] = [E]\left(1 + K_{a1}\frac{[Ac]}{[H_2O]} + K_{a1}K_{a2}\frac{[Ac]^2}{[H_2O]^2}\right) \quad (27)$$

$$[E] = \frac{[E]_t}{(1 + K_{a1}([Ac]/[H_2O]) + K_{a1}K_{a2}([Ac]^2/[H_2O]^2))} \quad (28)$$

Substituting Eqs. (25)–(28) into Eq. (24) the following expression for the global rate law is obtained:

$$r = \frac{k_{b1}[E]_t(K_{a1}([Ac][Al]/[H_2O]) - [Es]/K_{b1}) + k_{b2}[E]_t(K_{a1}K_{a2}([Ac]^2[Al]/[H_2O]^2) - K_{a1}([Ac][Es]/[H_2O])/K_{b2})}{1 + K_{a1}([Ac]/[H_2O]) + K_{a1}K_{a2}([Ac]^2/[H_2O]^2)} \quad (29)$$

where $K_{a1} = k_{a1}/k_{-a1}$, $K_{a2} = k_{a2}/k_{-a2}$, $K_{b1} = k_{b1}/k_{-b1}$, and $K_{b2} = k_{b2}/k_{-b2}$ are equilibrium constants for Eqs (20)–(23).

3.2.2. Third order approximation

This model was further extended to consider that up to three acid molecules could bind to the enzyme, as described by the equations below

$$E + Ac \rightarrow EAc + H_2O; \quad K_{a1} = \frac{k_{a1}}{k_{-a1}} \quad (30)$$

$$EAc + Ac \rightarrow EAc2 + H_2O; \quad K_{a2} = \frac{k_{a2}}{k_{-a2}} \quad (31)$$

$$EAc2 + Ac \rightarrow EAc3 + H_2O; \quad K_{a3} = \frac{k_{a3}}{k_{-a3}} \quad (32)$$

$$EAc + Al \rightarrow E + Es; \quad K_{b3} = \frac{k_{b1}}{k_{-b1}} \quad (33)$$

$$EAc2 + Al \rightarrow EAc + Es; \quad K_{b1} = \frac{k_{b2}}{k_{-b2}} \quad (34)$$

$$EAc3 + Al \rightarrow EAc2 + Es; \quad K_{b3} = \frac{k_{b3}}{k_{-b3}} \quad (35)$$

Following the same procedure as above the final rate equation is obtained as

$$r = \frac{k_{b1}[E]_t(K_{a1}([Ac][Al]/[H_2O]) - [Es]/K_{b1}) + k_{b2}[E]_t(K_{a1}K_{a2}([Ac]^2[Al]/[H_2O]^2) - K_{a1}([Ac][Es]/[HlO])/K_{b2}) + k_{b3}[E]_t(K_{a1}K_{a2}K_{a3}([Ac]^3[Al]/[H_2O]^3) - K_{a1}K_{a2}([Ac]^2[Es]/[H_2O]^2)/K_{b3})}{1 + K_{a1}([Ac]/[H_2O]) + K_{a1}K_{a2}([Ac]^2/[H_2O]^2) + K_{a1}K_{a2}K_{a3}([Ac]^3/[H_2O]^3)} \quad (36)$$

where $K_{a1} = k_{a1}/k_{-a1}$, $K_{a2} = k_{a2}/k_{-a2}$, $K_{a3} = k_{a3}/k_{-a3}$, $K_{b1} = k_{b1}/k_{-b1}$, $K_{b2} = k_{b2}/k_{-b2}$, and $K_{b3} = k_{b3}/k_{-b3}$, are equilibrium constants for Eqs. (30)–(35).

Table 3
Values of kinetic and equilibrium constants for the six models.

	K_{a1} (mM^{-1})	k_{b1} ($mM^{-1}\,min^{-1}$)	$1/K_{b1}$ (mM)	$K_{a1}K_{a2}$ (mM^{-2})	k_{b2} ($mM^{-1}\,min^{-1}$)	$1/K_{b2}$ (mM)	$K_{a1}K_{a2}K_{a3}$ (mM^{-3})	k_{b3} ($mM^{-1}\,min^{-1}$)	$1/K_{b3}$ (mM)	K_i (mM^{-1})	F_{obl}
First order rate limiting step	0.368	0.252	0.067								206,370
First order rate limiting step inhib. by alcohol	0.368	0.252	0.067							1×10^{-10}	206,370
Second order rate limiting step	0.225	0.404	0	0.003	0.003	46.8					197,849
Second order rate limiting step inhib. by alcohol	0.369	0.376	0	0.004	0.002	83.8				3.39×10^{-3}	197,451
Third order rate limiting step	0.128	0.640	0.006	1.15×10^{-4}	2.79×10^{-4}	234	2.06×10^{-5}	0.025	4954		195,349
Third order rate limiting step inhib. by alcohol	0.229	0.651	0.013	2.01×10^{-4}	2.77×10^{-4}	280	3.45×10^{-5}	0.025	5961	5.83×10^{-3}	194,226

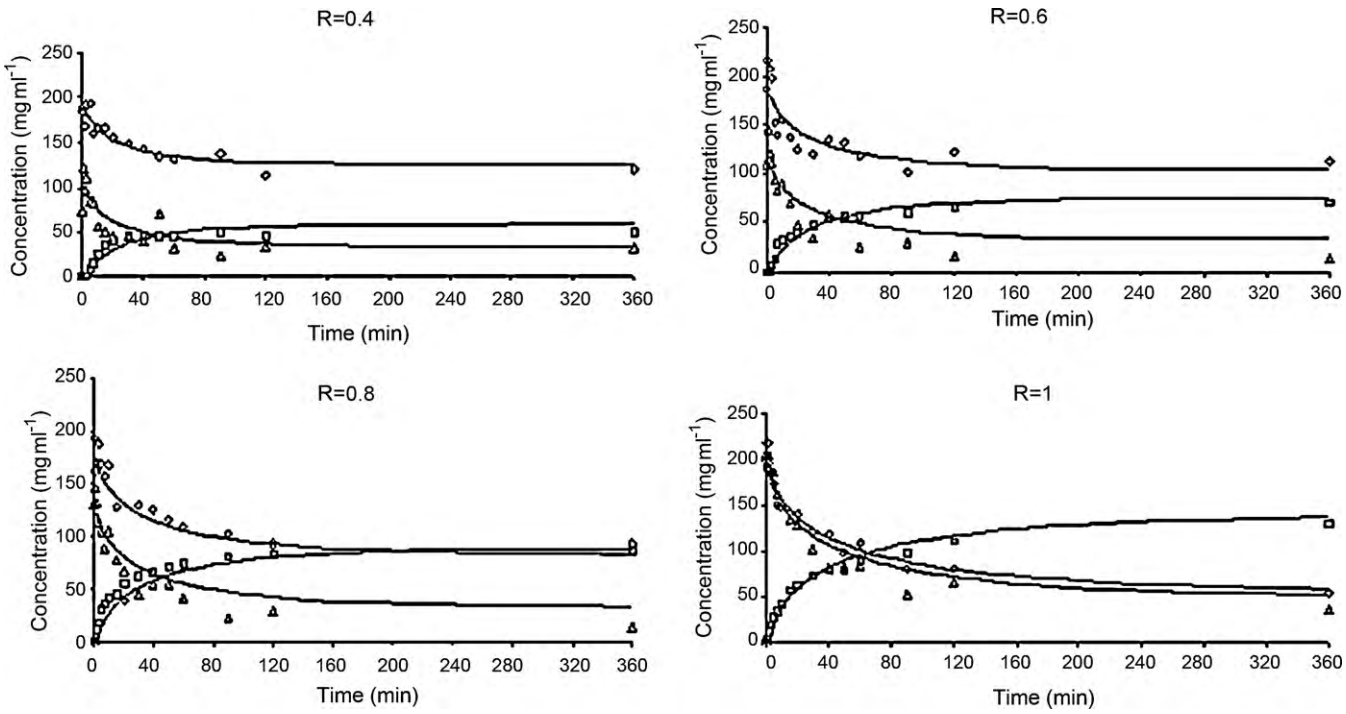

Fig. 2. Experimental results for different alcohol/acid (R) molar ratio, obtained using a fixed concentration of caproic acid (200 mM) at a different concentration of alcohol. △, ethanol; □, ethyl caproate;◇, caproic acid. Lines correspond to the kinetic model.

3.2.3. Alcohol inhibition

Each of these schemes was changed so as to incorporate the possibility of reversible inhibition by the alcohol, according to

$$(\mathrm{i})\ \mathrm{E} + \mathrm{Al} \rightarrow \mathrm{EAl}; \tag{37}$$

$$[\mathrm{EAl}] = K_i[\mathrm{E}][\mathrm{Al}] \tag{38}$$

$$[\mathrm{E}]_t = [\mathrm{E}] + [\mathrm{EAl}] + [\mathrm{EAc}] + \cdots = [\mathrm{E}]\left(1 + K_i[\mathrm{Al}] + K_{a1}\frac{[\mathrm{Ac}]}{[\mathrm{H_2O}]} + \cdots\right) \tag{39}$$

$$[\mathrm{E}] = \frac{[\mathrm{E}]_t}{1 + k_I[\mathrm{Al}] + K_{a1}([\mathrm{Ac}]/[\mathrm{H_2O}]) + \cdots} \tag{40}$$

$$r = \frac{k_{b1}[\mathrm{E}]_t(K_1([\mathrm{Ac}][\mathrm{Al}]/[\mathrm{H_2O}]) - [\mathrm{Es}]/K_{b1}) + \cdots}{1 + K_i[\mathrm{Al}] + K_{a1}([\mathrm{Ac}]/[\mathrm{H_2O}]) + \cdots} \tag{41}$$

where K_t is inhibition coordination constant.

As it can be seen, this kind of inhibition leads only to the inclusion, in the denominator or the reaction rate law of the corresponding term.

Table 3 summarizes the values of kinetic and equilibrium constants for the six models that were tested.

The first observation that can be made is that the inclusion of the alcohol inhibition in the first order model does not produce

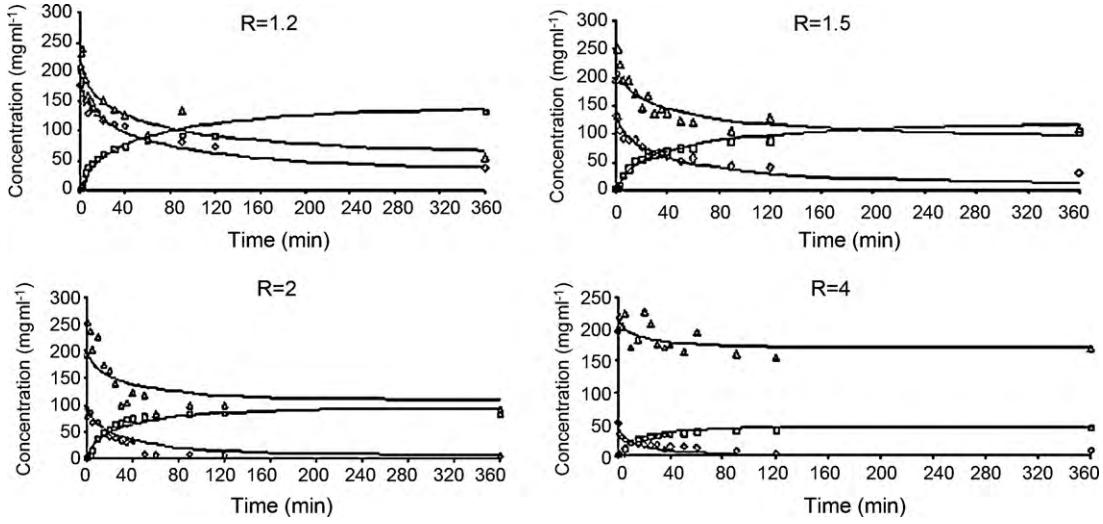

Fig. 3. Experimental results for different alcohol/acid (R) molar ratio, obtained using a fixed concentration of ethanol (200 mM) at a different concentration of caproic acid. △, ethanol; □, ethyl caproate; ◇, caproic acid. Lines correspond to the kinetic model.

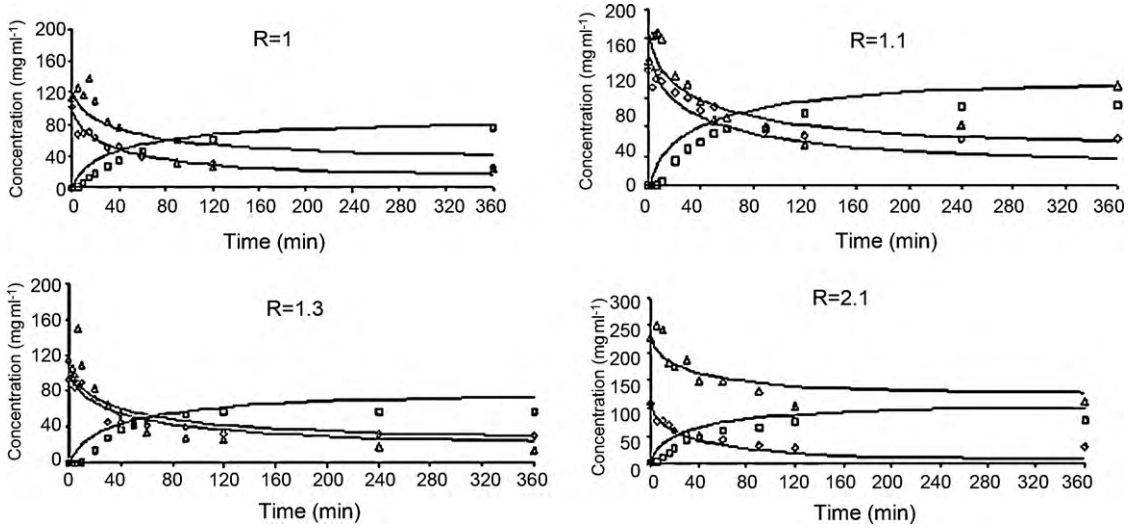

Fig. 4. Experimental results for different alcohol/acid (R) molar ratio, obtained using a concentration lower than 120 mM of alcohol and acid. △, ethanol; □, ethyl caproate; ◊, caproic acid. Lines correspond to the kinetic model.

any significant improvement in the description of the experimental data.

On the contrary, inclusion of higher order coordination of the acid molecules always improved the description of the experimental data, as it can be seen by the improvements in the objective function which measures the deviation between the experimental and the computed data points. In fact, the inclusion of the second order term produced a decrease of the objective function of around 5% and a further inclusion of a third order term reduced the objective function around 1%.

The apparent equilibrium constants for the various reaction steps were fitted as the inverse of the equilibrium constant for reasons related to the minimization process. It can be seen that the esterification involving the enzyme with only a single acid molecule bound is very much displaced towards the products, since $1/K_{b1}$ is always very small, regardless of the model being used. However, as the number of acid molecules bound to the enzyme increase the reaction seems to be increasingly displaced towards the reactants.

The fittings also show that $K_{a2} < K_{a1}$, which indicates that enzyme is much less receptive for the binding of a second acid molecule and that the binding of a third molecule is even less favourable.

Inclusion of the inhibition by alcohol shows that only a marginal improvement on the quality of the fitting is obtained, although this improvement is close to 0.5% in the case when the starting model is the one with the third order kinetic term. We can then conclude that, for the more detailed mechanism some alcohol inhibition does, in fact, occur. This inhibition is described as a competitive binding of alcohol to enzyme decrease the number of position where acid could react and form enzyme–acyl complex and in these way less for product formation.

In view of these results the third order model with alcohol inhibition was accepted as the better model and it was applied to studies of the alcohol/acid molar ratio.

It should be noted that all the modelling was performed assuming a pseudo-homogeneous system. Although the reaction proceeds with the formation of water, which is not mixable with the organic solvent and may induce the appearance of an aqueous phase, the amount of water produced is relatively small and, although it may segregate in the isooctane, the enzyme is still fully dispersed in the organic medium and no complications due to the formation of an aqueous phase were expected nor were observed.

The importance of role of water from a kinetic point of view in organic microaqueous media was reported by Monot et al. [44]. It has been found that distribution of water was in organic solvent very small, then the other part of water was adsorbed by the enzyme and for the greatest part it contributed to the formation of a discrete water phase. However, water removal had a little effect on the final yield of esterification. In fact by the study of Monet et al. showed the basically similar mechanism for the reaction carried out with solid hydrated enzyme in organic solvent system with no discrete aqueous phase (system described in this work) and for biphasic aqueous enzyme solution/organic solvent system.

3.3. *Effect of alcohol/acid molar ratio*

The quality of the fittings was checked by looking at two of the series of experiments that were carried out at different alcohol/acid molar ratio. Fig. 2 shows the results of the fitting of the third order model with alcohol inhibition. A good fitting was achieved despite some deviations that are observed in relation to the alcohol concentration and which can be attributed to some evaporation of the alcohol during the experiment, a fact that was not taken in account by the model.

A set of experimental data for fixed alcohol concentration is depicted in Fig. 3.

In some cases it was noticed that if the concentration of alcohol was lower than 100 mM there is deviation in model predicting and experimental data (Fig. 4). The similar behavior was explained by Carvalho et al. [8] in cutinase-catalyzed transesterification with small concentration of hexanol due to the cutinase deactivation and denaturation.

The most significant deviations between the model computations and the experimental data were observed for higher concentration of the substrates.

As it can be seen in Fig. 5, after 15 min a sharp decrease in the reaction rate is observed. This may be due to the high concentration of acid, active site of enzyme could be saturated with acid molecules, that could change microenvironment of the enzyme and lead to inhibition effect [21,24,25] and it was not completely accounted for by the kinetic model that was used.

The inhibition effect of hexanoic (caproic) acid above 150 mM was referred for reverse micellar system [25] in reaction esterification.

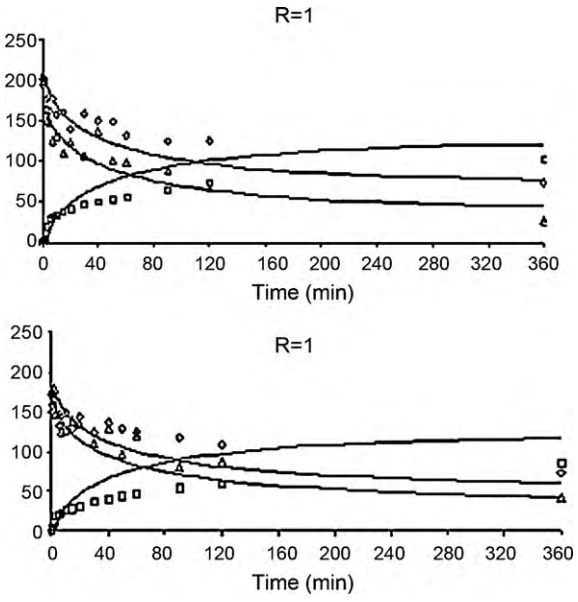

Fig. 5. Experimental results for alcohol/acid molar ratio $R = 1$, [alcohol] = 200 mM. △, ethanol; □, ethyl caproate; ◊, caproic acid. Lines correspond to the kinetic model.

4. Conclusions

A third order a Ping-Pong Bi–Bi mechanism with inhibition by alcohol was proposed to describe the esterification reaction of ethanol with caproic acid in organic medium and it was shown to be able to adequately describe the results over the entire range of conditions that were used in this study. Although the improvements made to the base model by the introduction of the second and third order bonding of acid molecules to the enzyme, as well as the inclusion of the inhibition by alcohol, improved the overall ability of the kinetic model to describe the experimental results, minor issues are still not completely resolved in the cases where the substrate concentration is high. Eventually the inclusion of terms corresponding to further inhibitions by products may have to be included in order to compensate for these differences.

Nevertheless the model was shown to be able to describe with reasonable accuracy the experimental results over the entire range of conditions and may be useful for reactor design and optimization, namely if the reactor is to be operated using the multiple additions of substrates.

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