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Enzymatic esterification of ethanol and oleic acid — a kinetic study

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Abstract

The formation of ethyl oleate from ethanol and oleic acid using a free or immobilised *Rhizomucor miehei* lipase as catalyst was evaluated in a biphasic system. Based on a $2^{(3)}$ factorial design previously developed [1], it was possible to study the reaction kinetics. The data obtained indicated that the reaction follows a Michaelis–Menten kinetics and it is described by the ternary complex mechanism. Based on the proposed model, the kinetic constants to the esterification reaction, without considering substrate inhibition, were determined. The kinetic results showed that the free lipase had the same affinity for both substrates ($K_{m(Et)} = 1.79$ M, $K_{m(Ol)} = 1.80$ M) while the lipase in its immobilised form had higher affinity for oleic acid ($K_{m,app(Et)} = 1.20$ M, $K_{m,app(Ol)} = 1.16 \times 10^{-8}$ M). It was also verified that the specificity was higher in the immobilised lipase system ($K_{s,app(Et)} = 2.90$ mmol h^{-1} mg^{-1} M^{-1}) than in the free one ($K_{s(Et)} = 0.637$ mmol $h^{-1}mg^{-1}$ M^{-1}). Diffusional effects were detected for low ethanol and oleic acid concentrations, when using the enzyme in its immobilised form, and related to the effectiveness factor. The integrated Michaelis–Menten equation coupled to the kinetic constants obtained, accord well with experimental results.

Keywords: Esterification; *Rhizomucor miehei* lipase; Immobilisation; Kinetic constants; Ternary complex mechanism

1. Introduction

Lipases (triacylglycerol ester hydrolase, EC 3.1.1.3) are enzymes that in nature catalyse hydrolysis of triacylglycerols. However, it is now well established that those enzymes may also catalyse esterification and interesterification reactions, if the aqueous medium is replaced by an organic or an aqueous/organic medium [2–4]. Compared to aqueous media, biphasic systems present some advantages such as the reduction of substrate and/or product inhibition, the solubilisation of hydrophobic compounds, and the possibility of shifting thermodynamic equilibrium towards the reaction synthesis.

Several lipases have been studied with industrial purposes being the *Rhizomucor miehei* used in different areas such as cosmetic [5,6], food [6,7] and pharmaceutical [8,9] industry. This enzyme is a single polypeptide chain protein made up of 269 residues (molecular weight of an unmodified chain is 29,472) and is an α/β type protein. Its active site is composed by the catalytic triad Ser144, His257, and Asp203 [10]; the catalytic serine is protected by a lid

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and the enzyme activity is greatly increased in the presence of a lipid–water interface, a phenomenon known as interfacial activation [11].

The lipase can be used in its free or immobilised form having the latter some advantages such as ease recovery and re-use, greater stability of the enzyme, and possibility of continuous operation.

In order to identify the optimal conditions to perform the ester synthesis, it is useful to know the reaction kinetics and the constants that describe the kinetic behaviour.

In this paper, the kinetic of the ethanol–oleic acid esterification reaction, catalysed by a free or immobilised *R. miehei* lipase, was studied. The estimated kinetic constants were used to develop a model that describes the experimental behaviour.

2. Materials and methods

2.1. Enzyme and chemicals

A commercial lipase from *R. miehei*, Palatase M 1000 L, a kind gift of Novo-Nordisk (Baegsvaerd, Denmark), was used in the ethanol–oleic acid esterification reactions.

The immobilisation support, Accurel EP700, from Akzo (Obernburg, Germany) was a kind gift of S.C.I.E. Irmãos Planas Almasque Lda (Lisbon, Portugal).

All the chemicals were of analytical reagent grade, from Merck (Damstadt, Germany).

2.2. Enzyme immobilisation

The lipase was immobilised by adsorption on Accurel EP700, a polyamide support with a particle size of 350–1000 μm, a void volume of 75%, and a pore size of 50–300 nm. For the esterification reactions, 1 ml of the lipase solution (10.2 mg of protein) was added to 300 mg of the immobilisation support. After being vortex-mixed for 1 min, the enzyme-support contact time was 1 h [12]. The preparation was then washed with potassium phosphate solution (100 mM) and vacuum-filtered.

The amount of immobilised protein was determined by a modified Folin assay [13].

2.3. Lipase activity assays

Lipase-catalysed esterifications in the biphasic system were performed in 100 ml Erlenmeyer flasks containing a total volume of 20 ml at 30°C and a shaking rate of 150 rpm. Different buffer solutions (depending on the pH of each assay) (100 mM), ethanol and free or immobilised lipase composed the aqueous phase, while oleic acid was used as both the acid substrate and the extractant solvent. Samples of the lower phase were taken and the ethanol concentration, in this phase, was analysed by GC using a Philips chromatograph with flame ionisation detector ($T_{col} = 165$°C, $T_{inj} = 170$°C, and $T_{det} = 230$°C), being the samples injected in a Porapack Q column.

The initial reaction rates were estimated from the slope of plots of ethanol consumed (taking into account that the ethanol was also physically extracted) against time and reported as mole per liter of alcohol consumed per hour.

2.4. Factorial design methodology

The effect of ethanol and oleic acid concentration, as well as pH, on the lipase specific activity was studied in a previous work [1] using a $2^{(3)}$ fractional factorial design [14] expanded further to a central composite design (CCD) [15] (Table 1). The initial rates obtained were used to calculate the coefficients of a second order polynomial equation, which allowed to obtain the response surfaces shown in Oliveira et al. [1].

The correlation coefficient between the experimental results and the ones given by the model were 0.966 and 0.967 for the free and the immobilised lipase system, respectively. Since the calculated val-

Table 1
Parameters studied on the factorial design [1]

Factor	Level				
	−2	−1	0	+1	+2
(1) pH	4.0	4.75	5.5	6.25	7.0
(2) Oleic acid (M)	0.2	0.65	1.1	1.55	2.0
(3) Ethanol (M)	0.2	0.65	1.1	1.55	2.0

Constant conditions: $T = 30$°C; $[\text{Enzyme}]_{free} = 10.2$ mg of protein ml^{-1}; $[\text{Enzyme}]_{immob.} = 3.09$ mg g^{-1} of support.

ues of F ($F_{\text{free enzyme}} = 1.835$ and $F_{\text{immobilised enzyme}} = 1.371$) were smaller than the F critical tabulated values, the results are statistically significant.

2.5. Kinetic constants determination

The values of kinetic constants were computed by nonlinear regression by means of the MsDEV/RNLIN routine based on MINPACK routines developed by More et al. [16], on 82 (for free enzyme system) and 217 (for immobilised enzyme system) data points estimated from the response surfaces obtained in the previous work [1].

3. Results and discussion

The effect of ethanol and oleic acid concentrations on the esterification reaction, catalysed by a free or immobilised *R. miehei* lipase, was optimised in a previous work [1] by using the factorial design methodology. Based on the response surfaces obtained, multiple combinations of ethanol–oleic acid concentrations can be achieved. These results were used in this paper to study the kinetic of the enzymatic esterification reaction.

3.1. Initial esterification rate studies for the free and the immobilised lipase system

3.1.1. Free enzyme

The reaction kinetics using the free lipase (10.2 mg of protein/ml of lipase solution) was investigated by studying the effect of both substrates (ethanol and oleic acid) concentrations on the initial esterification rate.

The initial rate increased with the increase in the ethanol concentration until 1.25 M. Above this value, a drop in the initial rate was observed indicating an ethanol inhibitory effect. This fact could be explained by the high ethanol solubility in the aqueous solution, which can cause enzyme inhibition or inactivation, probably due to lipase tridimensional structure modification [17].

The effect of oleic acid on the initial esterification rate, at fixed ethanol concentrations, showed a similar behaviour. At high oleic acid concentrations (> 1.25 M), an inhibitory effect was also observed, which could be due to lipase inhibition originated by the acid substrate and/or to mass transfer diffusional limitations. It should also be taken into consideration that the increase of oleic acid concentration lead to an organic–aqueous phases ratio increase and consequently, the interfacial area will be not the same in all cases.

The results described above showed that the reaction follows a Michaelis–Menten kinetic with substrate inhibition, which was confirmed by the Lineweaver–Burk plot (1/initial rate vs. 1/[substrate]) where for high substrate concentrations the curve went sharply upwards indicating substrate inhibition.

In order to identify the reaction mechanism of the formation of ethyl oleate in the biphasic system, a graphical representation of Michaelis–Menten equation of ethanol concentration/initial rate (or oleic acid/initial rate) against ethanol (or oleic acid) concentration (Hanes plot) [18] at several fixed values of oleic acid (or ethanol) concentration, was used (in this case, as in the subsequent ones, the range of substrate concentrations that led to inhibition was not considered).

For both substrates, straight lines with an interception point at the second quadrant were observed allowing to identify the reaction mechanism as being of the BiBi type, involving formation of a ternary complex [18], as shown in Fig. 1. In this model, the two substrates (Ol and Et) are bound to the lipase, in either a specific or a random order, to form an L–Et–Ol complex, which then reacts to give rise to the products.

Previously, it has been suggested that the action of this enzyme follows a two-step reaction mecha-

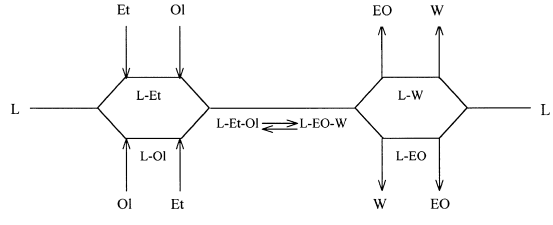

Fig. 1. Ternary complex mechanism (L — lipase; Et — ethanol; Ol — oleic acid; EO — ethyl oleate; W — water).

nism, usually named to as Ping-Pong. For example, this mechanism was described for enzymatic esterification reactions similar to the one studied in this work, as is the case of the ethanol–oleic acid reaction in n-hexane [19], the oleic acid and methanol [20] or oleic acid and octanol [21] reactions in organic medium. The ternary complex mechanism was referred for the resolution of racemic glycidol through esterification with butanoic acid in organic media [22].

3.1.2. Immobilised enzyme

Esterification reaction using the lipase in its immobilised form (3.09 mg of immobilised protein/g of support) was also evaluated.

When fixed oleic acid concentrations were used, the increase of the ethanol concentration proportionally enhanced the initial rate and consequently, no evidence of inhibition by ethanol was found in the range values studied. This behaviour could be related to the fact that in the immobilised system, the enzyme was not in direct contact with the alcohol. Also, the immobilised enzyme probably allows a more effective ethanol extraction with concomitant reduction in inhibitory effects.

When fixed ethanol concentration were used, the increase of oleic acid concentration did not lead to a significant decrease on the initial rate, as in the free lipase system, probably due to the microenvironment associated to the location of the hydrophobic support at the aqueous–organic interface.

However, the use of immobilised enzymes also led to diffusional effects because of the presence of an additional solid phase. This created problems particularly for low ethanol and oleic acid concentrations.

3.2. Kinetic constants

The rate equation for the ternary complex mechanism, considering that the experiments were performed under conditions in which the influence of the products can be neglected, is given by [18]

$$v = \frac{V_{max}[Et][Ol]}{K_{(Et)}K_{m(Ol)} + K_{m(Ol)}[Et] + K_{m(Et)}[Ol] + [Et][Ol]} \quad (1)$$

where v is the initial reaction rate; [Ol] and [Et] are the concentrations of oleic acid and ethanol, respectively; V_{max} $(= k_{cat}E)$ is the maximum velocity or limiting rate; $K_{m(Ol)}$ and $K_{m(Et)}$ are the Michaelis constants for oleic acid and ethanol, respectively; and $K_{(Et)}$ is the dissociation constant of the ethanol–lipase complex. In immobilised enzyme system, the kinetic constants will be noted as "apparent" (app) since the interaction between the support and the two substrates, which can result in different substrate concentrations near the enzyme, was not accounted.

Janssen et al. [23] suggested that this equation (Eq. 1) could not be a correct model to describe the initial rates since it does not consider the presence of water, which is one of the products of esterification reaction and is present in the reaction medium since the beginning. However, their studies showed no statistical justification to include an extra parameter, corresponding to the referred product, in the kinetic equation.

The kinetic constants in the Eq. (1) were estimated by the Hanes plot [18] and by the nonlinear regression method [16]. Similar kinetic constant values were obtained by the two methods (Table 2). The Michaelis constant values showed that the free enzyme has the same affinity with both substrates, which is also similar to the one observed in the immobilised system with respect to ethanol. However, the apparent Michaelis constant for oleic acid ($K_{m,app(Ol)}$) was lower than the $K_{m(Ol)}$ obtained in the other system, showing that the lipase in its immobilised form has a higher affinity for the acid substrate. The enzyme specificity was higher in the immobilised lipase system, especially with respect to oleic acid, as shown by the values of the specificity constant ($K_s = k_{cat}/K_m$).

A different affinity behaviour for the same esterification reaction, was observed with the lipase microencapsulated in phosphatidylcholine microemulsions [24]. In this case, the $K_{m,app}$ values were higher (lower affinity) than the ones observed for the free lipase system, which was explained by steric hindrances caused by surfactant membrane and/or conformational changes in the enzyme protein.

The proposed model (Eq. 1) and the kinetic constants obtained (Table 2) were applied to the data points estimated from the response surfaces for the

Table 2
Kinetic constants obtained for the esterification reaction catalysed by the *R. miehei* lipase

	Free lipase		Immobilised lipase
	Hanes plot	Nonlinear regression	Nonlinear regression
V_{max} (M h^{-1} mg^{-1})[a,b]	0.049	0.057	80.174
$K_{m(Et)}$ (M)[a]	1.49	1.79	1.20
$K_{m(Ol)}$ (M)[a]	1.48	1.80	1.16×10^{-8}
$K_{(Et)}$ (M)[a]	0.409	0.396	9.46×10^{7}
$K_{s(Et)}$ (mmol h^{-1} mg^{-1} M^{-1})[a]	0.662	0.637	2.90
$K_{s(Ol)}$ (mmol h^{-1} mg^{-1} M^{-1})[a]	0.666	0.633	3.00×10^{8}

[a] The kinetic constants are apparent for the immobilised lipase.
[b] V_{max} is expressed per milligram of free or immobilised protein, respectively, for the free or the immobilised enzyme system.

free and the immobilised lipase. From Fig. 2, it can be observed that, in the range without inhibition, the model predicts satisfactorily the estimated data. However, this behaviour was not found for the immobilised enzyme if using, for example, an oleic acid concentration of 0.5 M. In this case, the results obtained by Michaelis–Menten model diverge to the ones estimated from the response surface (Fig. 3) showing that, as expected, significant diffusional resistances are present when using low substrate concentrations. The calculated effectiveness factor values, defined as the ratio of the initial rates estimated from the response surface and those expected by Michaelis–Menten kinetics, were lower than 1 (not shown here), until an ethanol concentration around 1.2 M, which is in agreement with the differences observed.

3.3. Modelling of enzymatic esterification reaction

The kinetic results were also applied to model the performance of the batch reactor used in this work. With this purpose, the Michaelis–Menten equation described above (Eq. 1) was integrated giving rise to the following equation

$$k_{cat}\frac{E_T t}{V} = [Et]_0 X - K_{m(Et)}\ln(1-X) - K_{m(Ol)}\frac{[Et]_0}{[Ol]_0}\ln(1-X) + \frac{K_{(Et)}K_{m(Ol)}}{[Ol]_0}\left(\frac{1}{1-X}-1\right) \quad (2)$$

where X is the conversion degree, E_T is the total amount of enzyme, t is the time, and V is the liquid volume. The experimental results, obtained for initial

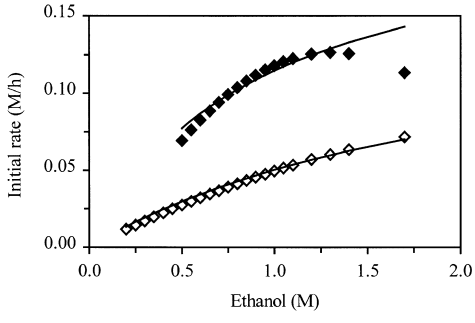

Fig. 2. Comparison of model prediction (—) and data points obtained from the free (♦) and the immobilised (◇) enzyme response surface, at 1.1 M of oleic acid concentration. $[Enzyme]_{free} = 10.2$ mg/ml; $[Enzyme]_{immob.} = 3.09$ mg/g support.

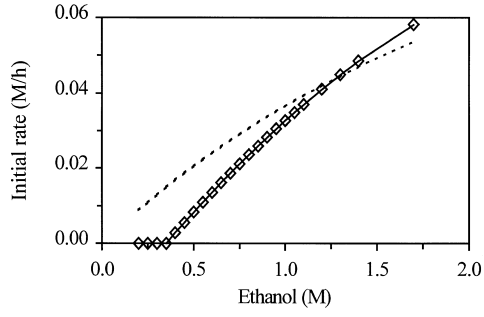

Fig. 3. Comparison of kinetic model prediction, considering (—) or not (- - - -) the effectiveness factor, and data points (◇) obtained from the immobilised lipase response surface at 0.5 M of oleic acid concentration. $[Enzyme]_{immob.} = 3.09$ mg/g support.

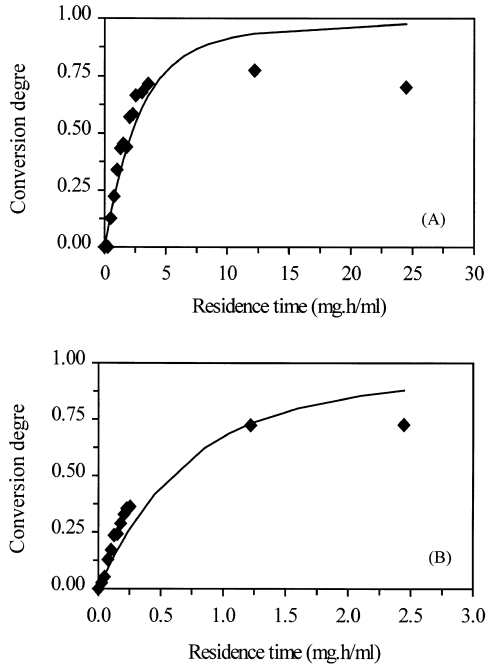

Fig. 4. Modelling of the experimental results obtained using 0.65 M of ethanol and 1.55 M of oleic acid concentration in the presence of the free (A) and the immobilised (B) lipase. The symbols represent the experimental data points and the line is the model prediction. $[\text{Enzyme}]_{\text{free}} = 10.2$ mg/ml; $[\text{Enzyme}]_{\text{immob.}} =$ 3.09 mg/g support.

concentrations of ethanol ($[\text{Et}]_0$) and oleic acid ($[\text{Ol}]_0$) of 0.65 and 1.55 M, respectively, were then adjusted by applying the integrated rate equation (Eq. 2). In the free lipase system (Fig. 4A), a good correlation between the model and the experimental data was observed for conversion degrees below the equilibrium. When this point was reached, the curve did not describe the experimental data since the inverse reaction was not considered in the present study.

For the immobilised lipase system (Fig. 4B), the model did not describe the experimental data as well as in the previous case probably due to the effects that arise from the immobilisation.

4. Conclusions

A free or immobilised *R. miehei* lipase was used as catalyst in the ethanol–oleic acid esterification reaction, which was carried out in an aqueous–organic biphasic system. It was proposed that the reaction follows a Michaelis–Menten kinetics and it is described by the ternary complex mechanism. The kinetic constants were estimated by using a non-linear regression method (without considering substrate inhibition). For the free enzyme, the following values for $V_{\max}$, $K_{\text{m(Et)}}$, $K_{\text{m(Ol)}}$, and $K_{\text{(Et)}}$ were obtained: 0.057 M h^{-1} mg^{-1} of protein, 1.79, 1.80 and 0.396 M, respectively. When the immobilised enzyme was used, the apparent kinetic constants were also estimated. The ethanol apparent Michaelis constant ($K_{\text{m,app(Et)}}$) was similar to the $K_{\text{m(Et)}}$ obtained in the system with the free enzyme, while the immobilised enzyme shows higher affinity for the oleic acid. The estimated kinetic constants described successfully the initial rate vs. substrate concentration performances. However, in the immobilised lipase system, the existence of diffusional resistances especially at low ethanol and oleic acid concentrations was detected.

The kinetic constants obtained were applied in the integrated Michaelis–Menten equation in order to predict the experimental observations. For both enzyme systems, a good correlation between the experimental data and the model was obtained before achieving the equilibrium of the esterification reaction.

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