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Kinetic study on the enzymatic esterification of octanoic acid and hexanol by immobilized *Candida antarctica* lipase B

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ABSTRACT

This study investigates reaction kinetics of the esterification of octanoic acid and hexanol into hexyl octanoate, catalyzed by an immobilized *Candida antarctica* lipase (Novozym® 435). The product is considered natural and used as a fresh vegetable and fruity flavour additive in food, cosmetic and pharmaceutical products. The reaction is performed in *n*-decane as the solvent, to improve enzyme stability and to increase the reaction yield. The influence of substrate concentration on hexyl octanoate synthesis is investigated over a wide range up to 2 M. The observed bi-substrate inhibition pattern follows a Ping-Pong bi-bi mechanism with dead-end inhibition by both substrates and, based on the proposed model, the kinetic constants of the esterification reaction are estimated. These parameters are verified to be intrinsic – neither external nor internal mass transfer resistances are significant for the examined reaction system – and are essential to extend analysis to a large-scale process and for a wide range of operating conditions. The progress of the reaction is also observed and the kinetic model is validated by fitting experimental progress curves with two different concentrations of biocatalyst. Effects of biphasicity of the reaction system, inhibition by the ester produced and the influence of the reverse reaction have been also evaluated.

1. Introduction

Esters of carboxylic acids and alcohols have a great commercial importance because of their applications in several industrial fields. In particular, short-chain aliphatic esters are mainly used as flavours and fragrance compounds in the food, cosmetic and pharmaceutical industries. Their fruity notes make them suitable as additives in fruit juices, cheeses, baked goods, candies, beverages and ice creams [1]. Likewise, esters produced from long-chain fatty acids and short-chain alcohols are used in the food, cosmetic and pharmaceutical industries; in addition, they have applications in detergent industries [2], as plasticizers for PVC, solvents, diesel additives, water-resisting agents, lubricants and in hydraulic fluids [3,4].

Traditionally, esters are prepared by chemical synthesis with inorganic catalysts – alkali metals, mineral acids, ion-exchange resins, Lewis acids, metals and their oxides – requiring post-treatments because of the poor selectivity and undesirable side reactions [3]. It has been shown that biocatalysis can offer significant environmental savings due to a decreased use of hazardous chemicals and more efficient use of resources [5,6]. Consequently, owing to the need to develop methods for the production of such esters from cheaper and more available raw materials and to the growing demand for "green" and natural products, biocatalysis is an attractive alternative for the synthesis of esters from natural precursors.

Biocatalysis has achieved important improvements in the application of lipases – E.C.3.1.1.3 – as catalysts to hydrolyse or synthesize esters [7–10]. Lipases are conventionally defined as enzymes which catalyze the triacylglycerol hydrolysis to glycerol and free fatty acids [11], but at low water content, the reverse reaction of esterification is thermodynamically favoured [12]. This reversal of their original hydrolytic activity is usually obtained by using them in organic solvents at low water activity or in solvent-free media composed of the mixture of substrates while removing the water formed. Synthesis of esters in the presence of various organic solvents is well documented and many studies have investigated the influence of different solvents [11,13–20]. It should be noted that water must be present at sufficient concentration to activate the biocatalyst, but not too much in order to avoid hydrolysis

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[21,22]. Much research has been conducted in this field and has it been extensively reviewed by for example Gandhi [23], Yahya et al. [22], Paiva et al. [12] and Hou and Shimada [24].

The main obstacle to the wider application of lipase is the biocatalyst cost. As a means of reducing it, lipase immobilization on an inert support can be advantageous to allow the easy recycling of the biocatalyst. However, the cost of the biocatalyst depends on a number of factors, for instance scale of production [25]. As of now, lipases are finding greater use in industry such as for biodiesel production which will certainly help to bring the costs down and open the door for other applications using the same biocatalyst.

In order to make the most efficient use of the catalyst, identifying the optimal conditions to perform the ester synthesis is important. To do so it is helpful to evaluate the reaction kinetics and the constants describing the kinetic behaviour. Several theoretical models have been proposed to explain lipase-catalyzed esterification in organic solvents. Kinetic studies on processes using immobilized enzymes as catalysts were relatively scarce before 2000 and have become important only in the last decade. Paiva et al. [12] reported an overview of the possible kinetics and mechanisms of reactions catalyzed by immobilized lipase. Enzymatic models that are based on the application of simple Michaelis–Menten kinetics are valid for most simple enzymatic reactions. Nevertheless, when a two-substrate two-product reaction occurs, the general scheme "bi-bi" has to be taken in account. This scheme includes two possible mechanisms: Ping-Pong (non-sequential) and ternary complex (sequential). The latter type can be ordered or random [11,26]. The Ping-Pong bi-bi mechanism for lipase-catalyzed esterification and transesterification reactions in organic media is the generally accepted model [2,13,14,17,27–31], where the reaction is thought to occur via an acyl-enzyme. When enzymatic systems are used, it is often necessary to consider inhibition effects of one or both the substrates. These phenomena are the result of inactive binary or ternary complexes between the enzyme and other substrates in the medium, competing with the main substrate. With regard to esterification, some reports have not found any inhibition phenomena [1,28,29,32]. Other studies have identified an inhibition by only alcohol [13,14,31,33–35] or only acid [11,16,19]. A few publications have dealt with the inhibition by both substrates; this effect has been found in reactions between short-chain acid and short-chain alcohol such as isovaleric acid and ethanol [17], butyric acid and isoamyl alcohol [30]; long-chain acid and short-chain alcohol such as oleic acid and *n*-butanol [2] and oleic acid and ethanol [32]; short-chain acid and long-chain alcohol such as butanoic acid and oleyl alcohol [2]; long-chain acid and long-chain alcohol such as oleic acid and cetyl alcohol [36] and oleic acid and oleyl alcohol [2].

In spite of several kinetic studies, the information to perform an appropriate analysis for a later industrial scale up continues to be quite limited. Also, most of the studies deal with ester synthesis from very short- or long-chain substrates, or to investigate different immobilization techniques, or to obtain qualitative information on effects of different solvents, lengths of alcohol and acid chains, additions of water and lipase origins.

The topic of the current paper, the esterification of octanoic acid and hexanol has not received much attention. The product of the reaction between octanoic acid and hexanol is hexyl octanoate, which has a special relevance in food processing as a natural flavour enhancer incorporated in a wide range of aromas such as apple, banana, cider, grape and melon [37]. This reaction has been studied using cutinase [37] or free lipases in free-solvent systems [38] and aqueous miniemulsion systems [39], but there are no reports on immobilized lipase-catalyzed kinetics in organic solvents. For this reason, the kinetic study of the enzymatic synthesis of a long-chain ester has been rigorously carried out in the present work. The reaction has been catalyzed by the commercial immobilized lipase Novozym® 435, mainly used in production of aromatic esters [40–44], in transesterification of oils to fatty acid methyl or ethyl esters (FAME/FAEE) [45,46] and in esterification of free fatty acids in pretreatments of waste cooking oils [47,48]. Recently, Novozym® 435 was also compared to other commercial lipase preparations in the synthesis of flavour esters and it showed to be more suitable than other biocatalysts in most cases [49]. The product of the reaction between octanoic acid and hexanol is hexyl octanoate, which has a special relevance in food processing as a natural flavour enhancer.

The aim of this study is an experimental investigation of the lipase-catalyzed esterification kinetics and the suggestion of a mechanism in order to determine the kinetic constants. The study is organized as follows: firstly, the effect of substrate concentrations on the initial reaction rate has been assessed by initial-rate measurements, pointing out the possible inhibitory effects of both substrates; then, the esterification kinetics has been modelled by the Ping-Pong bi-bi mechanism and the kinetic parameters have been evaluated; finally, the kinetic model was validated by reaction tests carried out for long times, by taking in account also the influence of the biphasicity, the inhibitory effect of the ester produced and the reverse reaction.

2. Material and methods

2.1. Enzyme and chemicals

The commercial immobilized lipase Novozym® 435 from *Candida antarctica* lipase B on a macroporous acrylic resin (Lewatit OC 1600), a kind gift from Novozymes A/S (Denmark), was used for the hexanol–octanoic acid esterification. The specific surface area is 95.50 m^2/g, the average pore diametre is 179.2 Å and it has a nominal activity of 7000 PLU/g. One propyl laurate unit (PLU) is defined as the number of μmol of *n*-propyl laurate obtained in the standard test corresponding to the esterification of lauric acid with *n*-propyl alcohol, after 15 min at atmospheric pressure [50]. All chemicals were of analytical reagent grade purchased from Merck Schuchardt OHG (Germany). Substrates were used without any pre-treatment.

2.2. Esterification reaction

Esterification is a bi-substrate bi-product reaction $A + B \leftrightarrow P + Q$ (A: octanoic acid, B: hexanol, P: hexyl octanoate, Q: water). This reaction was carried out in *n*-decane as solvent, because it has the logarithm of the octanol/water partition coefficient (log *P*) of 5.6, which gives the suitable conditions for a high reaction yield [51].

Batch reactions were performed in glass vials of 4 ml with a working volume of 2 ml containing different substrate–concentration mixtures in *n*-decane. The vials were placed in a thermo-shaker (HLC Bovenden, Germany) and substrates and solvent were loaded. The reaction mixture was incubated at 35 °C with shaking (600 rpm). The biocatalyst was added to initiate the reaction.

The effect of substrate concentration on the reaction rate was investigated by esterifying varying-initial concentrations of octanoic acid (from 0.1 M to 2 M) and varying-initial concentrations of hexanol (from 0.1 M to 2 M), with 2.5 g/l of biocatalyst. Samples of 100 μl volume were taken at different times to study the change in composition. The progress of esterification was monitored by determining the ester concentration by HPLC. Low conversions (<10%) were used to minimize the possible inhibition by products (hexyl octanoate and water) and to consider the linear region of the reaction rate. Initial reaction rates were determined from the slope of the initial linear portions of the ester concentration versus time plots.

Other tests were performed with ester addition together with substrates at the start of the reaction to verify if ester (product) is or not an inhibitor for the esterification.

Since esterification rate depends on amount of biocatalyst [4,16,17,19,30,36,52], tests with equimolar substrates (0.2, 0.5, 0.75, 1, 2 M) were performed at 35 °C with two different amounts of Novozym 435 (2.5 g/l and 5 g/l). In order to evaluate the relevance of the reverse reaction (hydrolysis), tests with different concentrations (from 0.1 M to 2 M) of water and ester as substrates were carried out in decane at 35 °C with 2.5 g/l of biocatalyst under stirring (600 rpm). These experiments were carried out for longer times in order to obtain the progress curves.

All experiments (reactions) were done in duplicates.

2.3. Analysis of samples

Aliquots of the reaction mixture were withdrawn periodically and centrifuged by an Eppendorf MiniSpinPlus centrifuge at 14,500 rpm for 30 s to remove catalyst residues. The ester concentration was essayed by HPLC using the UltiMate 3000 Dionex (Luna C18 column from Phenomenex, ID 4.6 mm, L 250 mm, particle size 5 μm) and the software Chromeleon®. The method of detection was UV at 210 nm. The compounds were eluted by gradient elution with an eluant system of methanol and water, both containing 0.05% of acetic acid, starting at 85% methanol for 7 min, followed by 90% methanol for 3 min and ending with 100% methanol for 10 min [53]. The flow rate of mobile phase was 1 ml/min.

2.4. Kinetic constant determination

The initial reaction rates obtained at various acid and alcohol concentrations were fitted to Michaelis–Menten kinetics with Ping-Pong bi-bi mechanism by a nonlinear regression method (NLR) with the aid of the software Microsoft Excel 2010. The values of kinetic constants were computed by the minimization of the $\sum_i \ln\sqrt{1+\mathrm{MSR}_i{}^2}$, where MSR is the mean square residual between theoretical and experimental data.

3. Results and discussion

3.1. Effect of substrates

Substrate effects on reaction rate were investigated, maintaining one substrate at constant concentration while varying the concentration of the other reactant.

The concentration of ester versus time was plotted for each experimental condition. A linear trend was observed for the first 6–10 min and its slope – divided by the concentration of biocatalyst loaded – gave the specific initial rate for each test. Then, initial reaction rates were plotted versus the concentration of the variable substrate for each group. The inhibition by both alcohol and acid can be seen clearly in Figs. 6 and 7 (Section 4). Here, the decrease of initial reaction rate at increasing alcohol and acid concentration was evident for every acid concentration and for low alcohol concentrations, respectively.

The maximum initial rate was reached when the A/B ratio was between 2:0.75 and 2:1.

Therefore, a bi-substrate inhibition has been observed in this work. In both cases, inhibition was due to the formation of dead-end or non-productive complexes between substrates and enzyme. This statement is supported by the Hanes plots (see Figs. 1 and 2). When acid concentration was constant (Fig. 6), its increase led to increasing maximum reaction rates and to inhibitory effects occurring at higher alcohol concentrations. Instead, when alcohol concentration was constant (Fig. 7), the inhibitory effect of the acid was more evident at low alcohol concentrations, because when A/B ratios were high it needs to consider not only the competitive inhibition by A but also the effect of the lower pH of the reaction mixture.

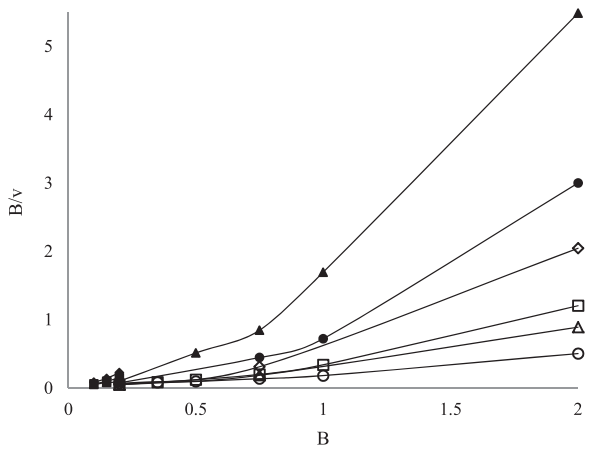

Fig. 1. Hanes plot with constant acid concentration. ♦A = 0.1 M; ■ A = 0.15 M; -▲- A = 0.2 M; ● A = 0.35 M; ◊ A = 0.5 M; □ A = 0.75 M; △ A = 1 M; ○ A = 2 M.

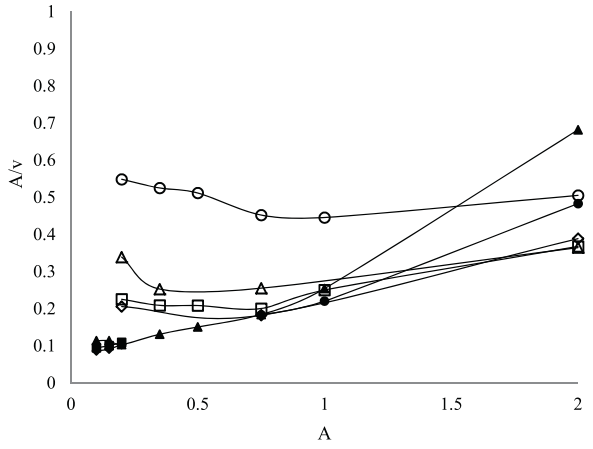

Fig. 2. Hanes plot with constant alcohol concentration. ♦B = 0.1 M; ■ B = 0.15 M; -▲- B = 0.2 M; ● B = 0.35 M; ◊ B = 0.5 M; □ B = 0.75 M; △ B = 1 M; ○ B = 2 M.

3.2. Effect of ester addition on esterification reaction

In order to investigate the presence of inhibition by the ester produced, hexyl octanoate at different concentrations was added to the substrates (octanoic acid + hexanol) at the start of the reaction. The initial rate was evaluated and compared with data obtained under the same operating conditions, but without initial addition of ester. Results showed that ester did not have any significant inhibitory effect on the esterification. For example, comparison between initial rates obtained with initial addition of ester (0.2 M) and without addition of ester is evident in Fig. 3 for different substrate concentrations.

Also Martinelle and Hult [14] detected no inhibition caused by the ester (ethyl octanoate) on *Candida antarctica* lipase B immobilized on polypropylene.

3.3. External and internal mass-transfer limitations

Since the reaction has been catalyzed by an immobilized lipase on a porous support, it needs to be checked if kinetic constants were intrinsic or affected by diffusional internal and external mass-transfer phenomena.

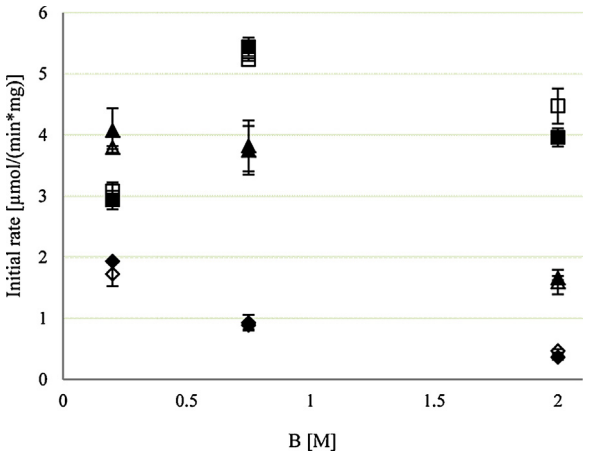

Fig. 3. Initial rate with and without initial addition of ester. ♦ $A = 0.2$ M, $E = 0$ M; ▲ $A = 0.75$ M, $E = 0$ M; ■ $A = 2$ M, $E = 0$ M; ◊ $A = 0.2$ M, $E = 0.2$ M; Δ $A = 0.75$ M, $E = 0.2$ M; □ $A = 2$ M, $E = 0.2$ M.

With regards to external mass transfer, diffusivity coefficients of octanoic acid and hexanol in *n*-decane ($D^0_{1,2}$) were estimated to be 1.89×10^{-6} cm^2/s and 2.08×10^{-6} cm^2/s, respectively, using the Wilke and Chang [54] correlation (Eq. (1)):

$$D^0_{1,2} = 7.4 \times 10^{-8} \frac{T \cdot M_2^{0.5}}{\eta_2 V_1^{0.6}} [=] \quad (\text{cm}^2/\text{s}) \tag{1}$$

where subscripts 1 and 2 are referred to diffusing species and solvent, respectively; T (K) is the operating temperature; η_2 (cP) is the viscosity of solvent; V_1 (cm^3/mol) is the molar volume of diffusing species at boiling point under normal conditions. With the conservative hypothesis of Sherwood's number ($k_L \cdot d_p / D^0_{1,2}$) equal to 2 – valid for non-agitated systems – the mass transfer coefficient k_L was calculated as 9.43×10^{-5} cm^2/s for octanoid acid and 1.04×10^{-4} cm^2/s for hexanol. In addition, diffusion time t_D and characteristic reaction time t_r were evaluated by Eqs. (2) and (3) [16,30].

$$t_D = \frac{D^0_{1,2}}{{k_L}^2} \tag{2}$$

$$\frac{1}{t_r} = \frac{v(S_0)}{S_0} \tag{3}$$

In Eq. (3), the highest observed initial reaction rate was considered as $v(S_0)$, according to a conservative criterion. The characteristic reaction time (73 min) was much greater than the diffusion times (3.5 min for octanoic acid and 3.2 min for hexanol). Consequently, the reaction was the rate determining step and this assessment is supported by a safe approach in computation of reaction time and diffusion time.

Thiele's observable modulus was evaluated by Eq. (4) [55] to assess the magnitude of possible diffusional limitations inside biocatalyst pores:

$$\Phi = \frac{v_0}{D_{\text{eff}} S_0} \left(\frac{V_p}{A_p} \right)^2 \tag{4}$$

where v_0 is the maximum initial reaction rate observed in our tests; S_0 is the limiting-substrate concentration corresponding to v_0; D_{eff} is the effective diffusion coefficient for substrate; V_p and A_p denote the particle volume and external surface area, respectively.

Thiele's modulus was equal to 0.0323 and 0.0293 when effective diffusion coefficient of acid and alcohol were considered, respectively. Since in both cases Thiele's modulus was lower than 0.3, internal mass-transfer limitation was negligible [55].

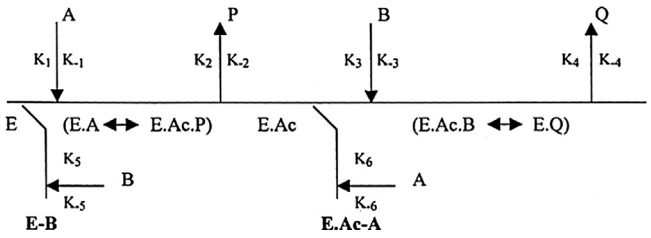

Fig. 4. Reaction mechanism of enzymatic esterification with inhibition by substrates (A = Acid, B = Alcohol, P = Ester, Q = Water, E = Enzyme, Ac = Acyl group).

4. Kinetic modeling – determination of kinetic parameters

Experimental data allow qualitative aspects of the reaction to be highlighted, however in order to be used for design, prediction and to find the reaction mechanism, it must be expressed in mathematical terms by the kinetic equation. In this work, the most accepted Ping-Pong bi-bi model [12,56] with inhibition by both the substrates was used and fitted to the experimental data. The reaction mechanism is illustrated in Fig. 4, where both substrates form dead-end complexes (E-B; E.Ac-A) with enzyme forms.

Eq. (5) describing this model is non-linear with five parameters: maximum reaction rate $V_{\max}$, Michaelis constant of the acid K_{mA} and of the alcohol K_{mB}, inhibition constant of the acid K_{iA} and of the alcohol K_{iB} [57,58].

$$v = \frac{V_{\max}[A][B]}{[A][B] + K_{mA}[B]\left\{1 + ([B]/K_{iB})\right\} + K_{mB}[A]\left\{1 + ([A]/K_{iA})\right\}} \tag{5}$$

The five parameters that minimize $\sum_i \ln \sqrt{1 + \text{MSR}_i^2}$ are reported in Table 1, with a mean standard deviation less than 8%.

Michaelis constants combine the kinetic constants reported in Fig. 4, according to the Eqs. (6) and (7):

$$K_{mA} = \frac{(k_{-1} + k_2)k_4}{k_1(k_2 + k_4)} \tag{6}$$

$$K_{mB} = \frac{(k_{-3} + k_4)k_2}{k_3(k_2 + k_4)} \tag{7}$$

Since K_{mA} is higher than K_{mB}, biocatalyst has a greater affinity for the alcohol than for the acid. Moreover, the K_{iB} is greater than K_{iA}, meaning that octanoic acid has a greater inhibitory effect than hexanol.

The graphic illustration of obtained Ping-Pong bi-bi model is depicted in Fig. 5.

Comparisons of the experimental and calculated – through the model – initial rates using parameters from Table 1 are shown in Figs. 6 and 7. The model fits the data very well.

5. Validation of the kinetic model at long times

5.1. Conversion profiles at long times and effect of biocatalyst amount

A set of tests were carried out for long times (up to 6 h) with a molar *A*:*B* ratio of 1:1 and two different amounts of biocatalyst loaded before starting the reaction. As the substrate concentrations increased, reaction was slower because of inhibitory effects and the possible reversibility of the reaction. This is evident in plots of conversion versus time in Figs. 8 and 9.

Reaction was very fast and when acid and alcohol concentrations were low (0.2 M), a conversion of 90% was reached within 1 h and 2 h with 5 g/l and 2.5 g/l of biocatalyst, respectively.

Comparing initial data for the same substrate concentrations, it can be observed that there was a linear relationship, in line with what other authors have found. As expected, conversion rate with

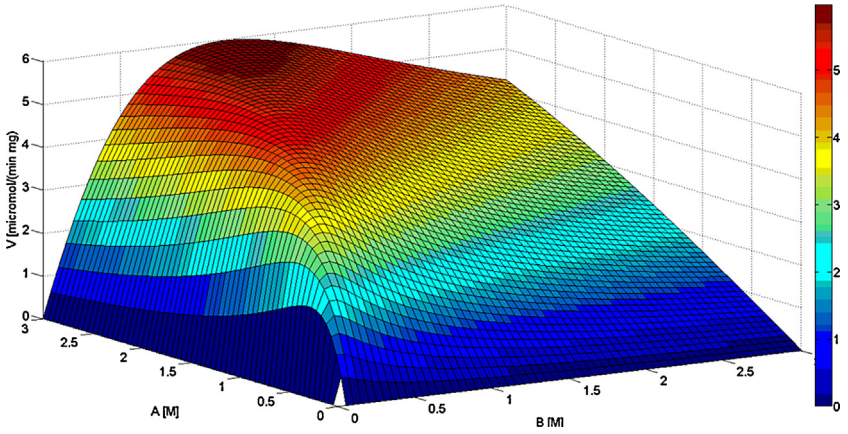

Fig. 5. Illustration of obtained Ping-Pong bi-bi model.

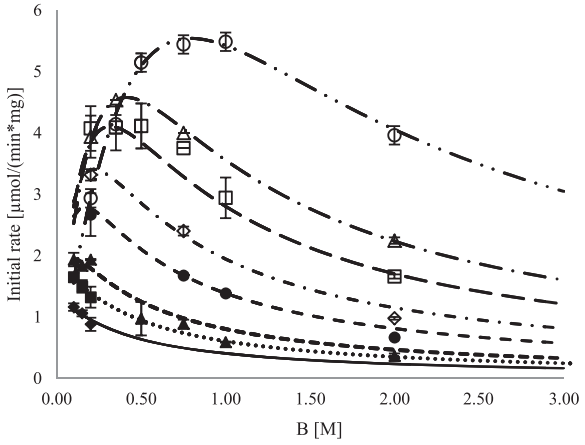

Fig. 6. Experimental and calculated initial rates with constant octanoic acid concentration. Experimental data: ♦ A = 0.1 M; ■ A = 0.15 M; ▲ A = 0.2 M; ● A = 0.35 M; ◊ A = 0.5 M; □ A = 0.75 M; Δ A = 1 M; ○ A = 2 M. Model simulation: – A = 0.1 M; * * * A = 0.15 M; – – – A = 0.2 M; — A = 0.35 M; – · – · A = 0.5 M; – – – A = 0.75 M; – · – · A = 1 M; – ·· – ·· A = 2 M.

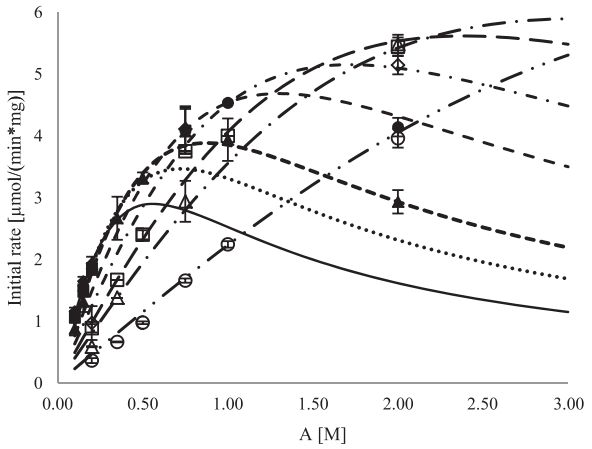

Fig. 7. Experimental and calculated initial rates with constant hexanol concentration. Experimental data: ♦ B = 0.1 M; ■ B = 0.15 M; ▲ B = 0.2 M; ● B = 0.35 M; ◊ B = 0.5 M; □ B = 0.75 M; Δ B = 1 M; ○ B = 2 M. Model simulation: – B = 0.1 M; * * * B = 0.15 M; – – – B = 0.2 M; — B = 0.35 M;– · – · B = 0.5 M; – – – B = 0.75 M; – · – · B = 1 M; – ·· – ·· B = 2 M.

5 g/l of biocatalyst (Fig. 9) was twice of the conversion with 2.5 g/l (Fig. 8), and also the final conversion was the same, i.e. this does not depend on initial enzyme amount. This was further confirmed in Fig. 10, where initial rate was plotted versus catalyst concentration at different substrate concentrations.

5.2. Fitting progress curves

The proposed Eq. (5) with kinetic parameters in Table 1 was integrated and used to fit experimental data in the progress curves.

Even if the mean standard deviation over all experiments was low (5.1% and 5.5% when 2.5 g/l and 5 g/l of biocatalyst were used respectively), the model fit the experimental data for the first 45 min better (mean standard deviation of 2.64% and 4.10%), whereas the experimental product concentration after 45 min is slower than that expected (mean standard deviation of 7.77% and 6.48%). This may be due to the observed agglomeration of biocatalyst particles, that reduces the active sites exposed to the substrates. Agglomeration has been reported by Foresti and Ferreira [59] for

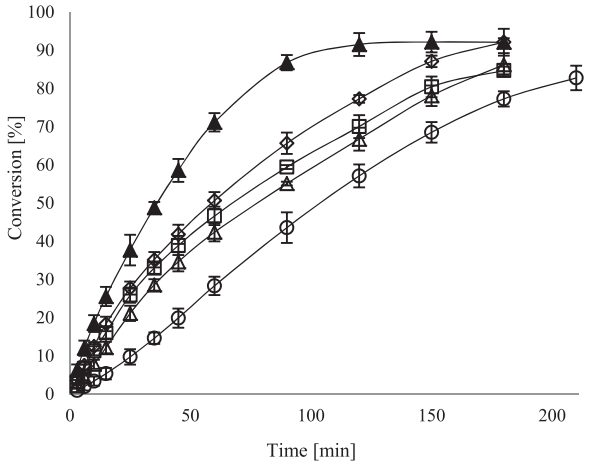

Fig. 8. Plot of conversion versus time with different substrate concentrations and 2.5 g/l of biocatalyst. -▲- A = B = 0.2 M; ◊ A = B = 0.5 M; □ A = B = 0.75 M; Δ A = B = 1 M; ○ A = B = 2 M.

Table 1
Kinetic constants of Ping-Pong bi-bi model.

Parameter	V_{max} [μmol/ min·mg$_{cat}$]	K_{mA} [mol/l]	K_{mB} [mol/l]	K_{iA} [mol/l]	K_{iB} [mol/l]
Value	264.4	17.09	1.205	0.173	0.355

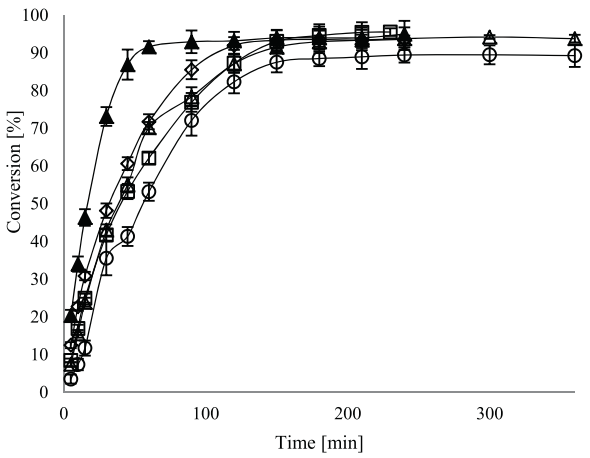

Fig. 9. Plot of conversion versus time with different substrate concentrations and 5 g/l of biocatalyst. -▲- $A = B = 0.2$ M; ◊ $A = B = 0.5$ M; □ $A = B = 0.75$ M; △ $A = B = 1$ M; ○ $A = B = 2$ M.

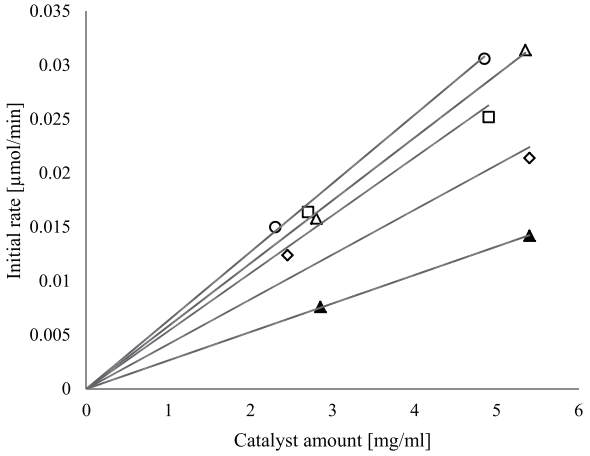

Fig. 10. Plot of initial rate versus catalyst concentration at different substrate concentrations. ▲ $A = B = 0.2$ M; ◊ $A = B = 0.5$ M; □ $A = B = 0.75$ M; △ $A = B = 1$ M; ○ $A = B = 2$ M.

an immobilized *Candida antarctica* lipase B. Moreover, equilibrium concentrations of ester was lower than the model which leads to 100% conversions.

It can also be observed that the model fitting was very good when substrate concentrations were low, but the fit decreased with increasing substrate concentrations (except with substrate concentration of 2 M and 2.5 g/l of biocatalyst), as shown in Table 2:

Table 2
Mean standard deviation percentage for tests at long times.

$A = B$ [M]	Biocatalyst concentration [g/l]	Mean stand. dev. [%]
0.2	2.5	1.31
	5	0.99
0.5	2.5	2.95
	5	4.26
0.75	2.5	4.83
	5	5.86
1	2.5	6.16
	5	6.43
2	2.5	2.63
	5	9.68

Different hypothesis were considered and verified to better understand the behaviour of the reaction system at long times:

- as water was produced, its increased concentration led to a second phase where a certain amount of the substrate hexanol was solubilized;
- the enzyme was inactivate by the acid;
- the reverse reaction has to be taken in account.

5.3. Effect of the biphasic reaction medium

Thermodynamic calculations revealed that only when substrate concentration was 0.75 M, 1 M and 2 M, the concentration of the water produced during the esterification led to a biphasic reaction medium after more than 1 h. Instead, when substrate concentration was 0.2 M and 0.5 M, the highest concentration of water – at the end of the reaction – was less than its solubility limit in the reaction mixture. Foresti et al. [60] presented a biphasic model, which explicitly considered the presence of a second phase in ethyl oleate synthesis by an immobilized *Candida antarctica B*. This approach was considered also in esterification of octanoic acid and hexanol in this work. Chemical reaction and mass transfer between liquid phases (organic phase and aqueous phase) were considered, but the biphasic approach was not found to give a better fitting at long times. The traditional monophasic approach and the new biphasic approach represented experimental data with the same fitting. Therefore, the presence of water as second liquid phase did not influence the behaviour at long times in our operating conditions, probably because the volume of water produced was very small in relation to the organic phase volume. In fact, the highest concentration of water was 2 M – if reaction with $A = B = 2$ M would be complete – which corresponds to 36 μl of water in 2 ml of reaction volume (0.018, v/v). The amount of hexanol solubilized in this low volume of water was negligible and the aqueous phase was not enough to influence the progress curves.

5.4. Enzyme denaturation by substrates

Enzyme denaturation by substrates has been included in the kinetic model by Bezbradica et al. [18,31] in free-solvent systems. Nevertheless, in our operating conditions the presence of the solvent provided a sheltering effect and no enzyme denaturation took place, insomuch as the model expressed in Eq. (5) with enzyme inactivation by acid expressed in Eq. (8) had the best fitting with experimental data when the constant of enzyme deactivation k_D was zero-value.

$$-\frac{d[E]}{dt} = k_D[E][A] \tag{8}$$

5.5. Reverse reaction

The reverse reaction of esterification (hydrolysis) was not experimentally relevant in our operating conditions. Since the esterification reaction reached a final conversion of more than 90%, an analysis of hydrolysis at initial rate would be inaccurate. Ester concentration was monitored for long times and its variation had standard deviations less than 3% depending probably on experimental errors when samples are prepared to be analyzed. Consequently, any change in ester concentration is comparable with the experimental error and it is not possible to evaluate any reverse reaction constant accurately.

Nevertheless, the Ping-Pong bi-bi model expressed in Eq. (5) can be modified in to Eq. (9) [57] to take into account the contribution

Table 3
Initial octanoic acid, alcohol and decane concentrations.

Octanoic acid [M]	Hexanol [M]	Decane [M]
0.2	0.2	4.8
0.5	0.5	4.4
0.75	0.75	4.0
1	1	3.7
2	2	2.2

of the reverse reaction.

$$v = \frac{V_f V_r([A][b] - ([P][Q]/K_{eq}))}{V_r[A][B] + V_r K_{mA}[B]\left\{1 + ([B]/K_{iB})\right\} + V_r K_{mB}[A]\left\{1 + ([A]/K_{iA})\right\}} + \frac{V_f K_{mQ}[P]}{K_{eq}} + \frac{V_f K_{mP}[Q]}{K_{eq}} + \frac{V_f K_{mQ}[A][P]}{K_{eq}K_a} + \frac{V_f[P][Q]}{K_{eq}} + \frac{V_r K_{mA}[B][Q]}{K_q} \quad (9)$$

The parameters introduced with this reversible model are the reverse maximal velocity V_r, the equilibrium constant K_{eq}, the Michaelis constants for the products K_{mP} and K_{mQ}, the dissociation constant for acid substrate $K_A(=(k_{-1}/k_1))$ and the dissociation constant for water $K_Q(=(k_4/k_{-4}))$. V_f is the forward maximal reaction and it is the same of V_{max} in Eq. (5).

Since it was not possible to accurately make hydrolysis analysis at initial rates, the equilibrium constant and the kinetic constants of esterification have been estimated by fitting Eq. (9) with progress curves of esterification (whose equivalent conversion profiles are showed in Figs. 8 and 9) by a non-linear regression method. The best fitted kinetic parameters led to a very good fitting when initial substrate concentrations were 0.2 M, 0.5 M, 0.75 M and 1 M, whereas a different behaviour was observed when substrate concentration was increased from 1 M to 2 M (data not shown). This can be due to the different polarity of the reaction medium. In fact, increasing substrate concentrations correspond to decreasing solvent (decane) concentration, as shown in Table 3. When both substrate concentrations were 0.2–1 M, solvent concentration was higher than substrate concentration. Instead, when both substrate concentration was 2 M, decane concentration was comparable to substrate concentration. This led to a change in polarity of the reaction system which made the proposed kinetic model inaccurate in all range 0.2–2 M.

Therefore, the non-linear regression method was applied without taking into account data at 2 M substrate concentration. The reversible model – with equilibrium and kinetic constants reported in Table 4 – fit very well the experimental data (see Figs. 11 and 12). Mean standard deviation was 0.76% and 1.05% with 2.5 g/l and 5 g/l of biocatalyst, respectively, whereas maximum standard deviation was 4.45% and 4.20% with 2.5 g/l and 5 g/l of biocatalyst, respectively.

The thermodynamic constant K_{eq} should depend only on reaction and operating temperature and it can be calculated from thermodynamic information on standard Gibbs free energy and enthalpy of formation of octanoic acid, hexanol, hexyl octanoate and water [61], according to Eqs. (10) and (11):

$$K_{eq}(298.15\,\mathrm{K}) = e^{-(\Delta G_f^0/R\cdot 298.15)} \quad (10)$$

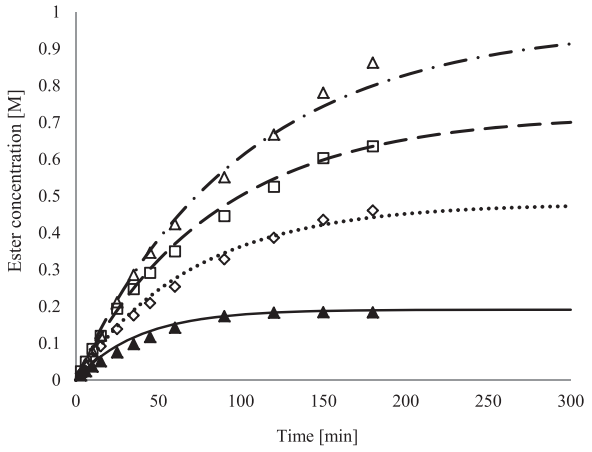

Fig. 11. Comparison of experimental and reversible model prediction in progress curves with 2.5 g/l of biocatalyst and initial substrate concentrations of 0.2 M, 0.5 M, 0.75 M and 1 M. Experimental data: ▲ $A=B=0.2$ M; ◊ $A=B=0.5$ M; □ $A=B=0.75$ M; Δ $A=B=1$ M. Model simulation: – $A=B=0.2$ M; * * * $A=B=0.5$ M; – – – $A=B=0.75$ M; – · – · $A=B=1$ M.

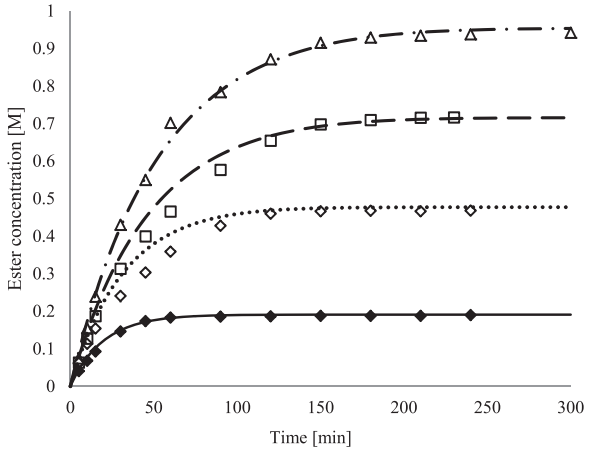

Fig. 12. Comparison of experimental and reversible model prediction in progress curves with 5 g/l of biocatalyst and initial substrate concentrations of 0.2 M, 0.5 M, 0.75 M and 1 M. Experimental data: ▲ $A=B=0.2$ M; ◊ $A=B=0.5$ M; □ $A=B=0.75$ M; Δ $A=B=1$ M. Model simulation: – $A=B=0.2$ M; * * * $A=B=0.5$ M; - - - $A=B=0.75$ M; – · – · $A=B=1$ M.

$$K_{eq}(T) = K_{eq}(298.15\,\mathrm{K}) \cdot e^{(\Delta H_f^0/R)((1/298.15)-(1/T))} \quad (11)$$

The equilibrium constant at 35 °C (operating temperature) calculated by Eqs. (10) and (11) is low and equal to 2.1×10^{-3}, so hydrolysis is thermodynamically favoured. Nevertheless, the esterification in organic solvents can be shifted towards the product side by decreasing the concentration of water, one of the products, in the reaction mixture [62]. As a consequence, the equilibrium constant of esterification carried out in decane was higher (equal to 438.6, Table 4) and esterification was favoured.

Table 4
Kinetic parameters of reversible Ping-Pong bi-bi model (substrate concentration 0.2–1 M).

Parameter	V_f [μmol/min·mg$_{cat}$]	V_r [μmol/min·mg$_{cat}$]	K_{eq} [/]	K_{mA} [mol/l]	K_{mB} [mol/l]	K_{mP} [mol/l]	K_{mQ} [mol/l]	K_A [mol/l]	K_Q [mol/l]	K_{iA} [mol/l]	K_{iB} [mol/l]
Value	264.4	3.509	438.6	17.09	1.205	0.187	5.856	1.094	3.068	0.173	0.355

6. Conclusion

The enzymatic hexanol–octanoic acid esterification was carried out by immobilized lipase to produce a natural flavour compound. The aim of the investigation was to better understand the behaviour of the reactive system through an analysis of the reaction kinetics. In particular, effects of substrate concentration and biocatalyst amount have been investigated. In addition, experimental data were used to evaluate kinetic constants with a view to a scaled-up process. The reaction was found to be inhibited by both the substrates and the proposed mathematical model – Ping-Pong bi-bi – showed good agreement with the experimental data. In addition, a linear reaction rate increase with biocatalyst amount was observed. The progress of the reaction was investigated towards equilibrium and the contribution of the reverse reaction was considered. Conversion of 90% was reached within only one hour when both substrate concentrations were 0.2 M and biocatalyst amount was 5 g/l. In all other experimental conditions, almost complete conversion of substrates was obtained within four hours. The results indicate that the model developed is adequate to describe the kinetics of the lipase-catalyzed esterification of octanoic acid and hexanol up to both substrate concentration 1 M. Extrapolations over this concentration would be inaccurate because of changes in polarity and a different model or different kinetic parameters have to be taken into account.

Finally, it has been confirmed that reaction was carried out in conditions of kinetic-limited regime and neither external nor internal diffusional limitations took place.

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