Developing the rate equations for two enzymatic Ping-Pong reactions in series: Application to the bio-synthesis of Bis(2-ethylhexyl) azelate

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ABSTRACT

In this work, the rate equations of two simultaneous bisubstrate Ping-Pong in series reactions have been developed for the first time. To obtain these equations, the approximation to the stationary state has been applied and, for the total balance of enzyme, all the intermediate complexes of the two reactions, which are present simultaneously in the reaction medium, have been taken into account.

To check the kinetic equations obtained, the synthesis of bis(2-ethylhexyl) azelate by transesterification from diethyl azelate and 2-ethylhexanol, in the presence of the immobilized lipase Novozym$^{@}$ 435, has been used as reaction model. The reaction has been carried out in solvent-free conditions in a batch reactor. A design model of the reactor has also been developed and solved by applying a numerical method. The model equations have been implemented in user's software in Visual Basic for Applications. In that software a routine, based on a very well-known procedure to minimize the sum of the square errors, has also been implemented. By using this software, the model parameters have been determined by fitting the experimental data to the model. A high determination coefficient was obtained, which validated both the kinetic equations and the reactor's design model.

Keywords: Kinetic equations; bisubstrate; Ping-Pong in series; dicarboxylic esters; lipase; bis(2-ethylhexyl) azelate.

1. INTRODUCTION

Along the last century, the field of enzymatic reactions has experimented an important increase and, as a consequence, most of the mechanisms and kinetic equations involved in the enzymatic processes have been well established. Currently, not only a high number of publications in the high impact journals, but also excellent books, on the more common mechanisms and kinetic equations related with mono-substrate or bi-substrate enzymatic reactions, with or without inhibition, can be consulted [1, 2]. But new reactions of interest continuously appear and new mechanisms and kinetic equations must be developed to better understand their behaviour.

In this sense, it is observed that, in the last years, important efforts have been made to find alternatives to traditional lubricants. In this way, esters of branched alcohols and dicarboxylic acids have shown good properties to be used as an alternative friendly with the environment, because they are biodegradable and can be obtained through enzymatic processes, which are very selective and don't need high level of energy consumption.

Between these esters, adipates and sebacates have been some of the more commonly used [3-12], being the direct esterification between the dicarboxylic acid and the alcohol, or the transesterification from other ester, the more common ways to be obtained. The reaction is carried out, generally, in the presence of a bio-catalyst, in solvent free systems or in not aqueous solvents. As the main characteristic, it must be pointed out that the overall reaction takes place by way of an intermediate semi-ester, which is synthesized in a first reaction and after, in a second reaction, act as an in situ new substrate to give, finally, the di-ester, which is the product of interest. As a result, the global process can be considered as the coupling of two consecutive reactions, which is a new field to be studied, as it has been made in this work.

Some examples of these reactions are, for the adipates, the bio-synthesis of bis(2-ethylhexyl) adipate, [3], dilauryl adipate, [5], dimethyl adipate, [6] and dioctyl adipate, [7], and for sebacates, bis(2-ethylhexyl) sebacate and bis(3,5,5-trimethylhexyl) sebacate, [4]. Despite the adipic and sebacic acids have been the first dicarboxylic acids used to obtain esters with lubricant properties, recently the azelaic acid has been also tested, because most of its esters can be used in the plasticizer industries, [13, 14, 15].

As bio-catalysts, the most commonly used were Novozym$^{@}$ 435 and Lipozyme$^{@}$ IM, [3, 4]. Also, lipase from *Candida rugosa* immobilised on Mg, Zn and Ni of layered double hydroxides, [6], and an immobilized lipase from *Thermomyces lanuginosus*,

[12], were used. And related to the reaction conditions, most of these studies were carried out in "solvent-free" medium, [8, 12], which makes it possible to obtain the pure final product without the solvent removal, reducing the cost of the process.

Regarding the kinetics of these processes practically no works can be found that provide detailed reaction mechanisms and kinetic equations. Most of the studies, based on the application of the Surface Response Methodology, [5, 9, 10, 15], have been developed to optimize the experimental conditions of the bio-synthesis of these compounds. Also, some other optimization studies, based on the neural network theory, have been applied [7, 14]. Until now, only one work has been found where, from data of initial reaction rates, a bisubstrate ping-pong mechanism with inhibition by methanol was proposed [9]. Although this is, probably, the first study about the esters of dicarboxylic acids that proposes a classic kinetic equation, the equation derived from the mechanism only takes into account the final product, and the intermediate semi-ester was not considered.

Some other reactions catalysed by lipases to obtain esters from monocarboxylic acids and monohydroxylic alcohols have been studied, [16-19], and, in most of them, the bisubstrate ping-pong equation has been used.

Other aspect that must be taken into account is the determination of the kinetic parameters of the kinetic model for these reaction types. In this sense, some works [20, 21], where problems that include the bisubstrate ping-pong kinetics are solved, are of interest, mainly the second one [21], where the group of non-linear differential equations of the model are solved by means of numerical calculation.

In this work, taken into account that the first substrate to be attached to the enzyme must be an acyl donor, the kinetic equations of two simultaneous bisubstrate Ping-Pong in series reactions have been developed by the first time. To obtain these equations, the approximation to the stationary state has been applied and, for the total balance of enzyme, all intermediate complexes of the two reactions, which are present simultaneously in the reaction medium, have been taken into account. Additionally, to check the kinetic equations obtained, the synthesis of bis(2-ethylhexyl) azelate by transesterification from diethyl azelate and 2-ethylhexanol, in the presence of the immobilized lipase Novozym$^{@}$ 435, has been used as reaction model, and the experimental results obtained in a batch reactor have been fitted to the model, using user's software in Visual Basic for Applications, with good degree of agreement.

2. THEORY: PING-PONG IN SERIES REACTIONS. MECHANISM AND KINETIC EQUATIONS

2.1. Reactions

According to the nomenclature adopted in this work, and taken into account that the reaction chosen to check the kinetic model has been the enzymatic synthesis of bis(2-ethylhexyl) azelate, by transesterification from diethyl azelate and 2-ethylhexanol, the reactions that take place can be represented as follows:

Reaction 1; $A + B \xrightarrow{Lipase} R\uparrow + P$ Eq. (1)

Reaction 2; $A + P \xrightarrow{Lipase} R\uparrow + Q$ Eq. (2)

In this reaction scheme the product *R*, ethanol in the reaction type selected, disappears from the reaction medium by vaporization due to the operational temperature chosen, 70 ºC, which makes the reactions irreversible. A more detailed scheme of the reaction where the chemical structures are shown can be seen in Figure 1.

2.2.- Mechanism

For each one of the two reactions given by Eqs. (1) and (2), and taking into account that the first substrate that is attached to the enzyme must be an acyl donor (the azelate), a bisubstrate Ping-Pong mechanism is proposed, As a consequence, the overall process is formed by two bisubstrate Ping-Pong reactions in series according to the following scheme:

Reaction 1:

Step 1: $E + B \underset{k_{-1}}{\overset{k_1}{\rightleftarrows}} EB$ Eq. (3)

Step 2: $EB \xrightarrow{k_2} E^* + R$ Eq. (4)

Step 3: $E^* + A \underset{k_{-3}}{\overset{k_3}{\rightleftarrows}} E^*A$ Eq. (5)

Step 4: $E^*A \xrightarrow{k_4} E + P$ Eq. (6)

Reaction 2:

Step 5: $E + P \underset{k_{-5}}{\overset{k_5}{\rightleftarrows}} EP$ Eq. (7)

Step 6: $EP \xrightarrow{k_6} E^{**} + R$ Eq. (8)

Step 7: $E^{**} + A \underset{k_{-7}}{\overset{k_7}{\rightleftharpoons}} E^{**}A$ Eq. (9)

Step 8: $E^{**}A \xrightarrow{k_8} E + Q$ Eq.(10)

2.3.- Enzyme: Intermediate complexes and total mass balance

The total mass balance of the enzyme is given by the sum of the free enzyme and all the enzyme complexes. This sum must be expressed as a function of the free enzyme, *E*.

To obtain the relationships between the different complexes of the enzyme and the free enzyme, *E*, the approximation to the stationary state has been applied as follows:

2.3.1.- Complexes involved in reaction 1

From steps 1 and 2:

$$\frac{d[EB]}{dt} = k_1[E][B] - k_{-1}[EB] - k_2[EB] = 0 \qquad \text{Eq. (11)}$$

From Eq. (11):

$$[EB] = \frac{k_1[E][B]}{k_{-1} + k_2} \qquad \text{Eq. (12)}$$

From steps 2 and 3:

$$\frac{d[E^*]}{dt} = k_2[EB] - k_3[E^*][A] + k_{-3}[E^*A] = 0 \qquad \text{Eq. (13)}$$

From steps 3 and 4:

$$\frac{d[E^*A]}{dt} = k_3[E^*][A] - k_{-3}[E^*A] - k_4[E^*A] = 0 \qquad \text{Eq. (14)}$$

By summing Eq. (13) and (14):

$$k_2[EB] - k_4[E^*A] = 0 \qquad \text{Eq. (15)}$$

From Eq. (15) and (12):

$$[E^*A] = \frac{k_1 k_2[E][B]}{k_4\left(k_{-1} + k_2\right)} \qquad \text{Eq. (16)}$$

By substituting Eq. (12) and (16) in Eq. (13), for $[E^*]$ it is obtained:

$$[E^*] = \frac{(k_1 k_2 k_4 + k_1 k_2 k_{-3})}{k_3 k_4 (k_{-1} + k_2)} \frac{[E][B]}{[A]} \qquad \text{Eq. (17)}$$

From Eq. (12), (16) and (17), the following auxiliary parameters are defined:

$$a_1 = \frac{k_1}{k_{-1} + k_2} \qquad \text{Eq. (18)}$$

$$a_2 = \frac{k_1 k_2}{k_4 (k_{-1} + k_2)} \qquad \text{Eq. (19)}$$

$$a_3 = \frac{k_1 k_2 k_4 + k_1 k_2 k_{-3}}{k_3 k_4 (k_{-1} + k_2)} \qquad \text{Eq. (20)}$$

And, from Eq. (12), (16) and (17) and these new parameters, the sum of the complexes involved in reaction 1 is obtained:

$$\sum_{\text{Reaction}1} Complexes = [E]\left((a_1 + a_2)[B] + a_3 \frac{[B]}{[A]} \right) \qquad \text{Eq. (21)}$$

2.3.2.- Complexes involved in reaction 2

From steps 5 and 6:

$$\frac{d[EP]}{dt} = k_5[E][P] - k_{-5}[EP] - k_6[EP] = 0 \qquad \text{Eq. (22)}$$

From Eq. (22):

$$[EP] = \frac{k_5[E][P]}{k_{-5} + k_6} \qquad \text{Eq. (23)}$$

From steps 6 and 7:

$$\frac{d[E^{**}]}{dt} = k_6[EP] - k_7[E^{**}][A] + k_{-7}[E^{**}A] = 0 \qquad \text{Eq. (24)}$$

From steps 7 and 8:

$$\frac{d[E^{**}A]}{dt} = k_7[E^{**}][A] - k_{-7}[E^{**}A] - k_8[E^{**}A] = 0 \qquad \text{Eq. (25)}$$

By summing Eq. (24) and (25):

$$k_6[EP] - k_8[E^{**}A] = 0 \qquad \text{Eq. (26)}$$

From Eq. (26) and (23):

$$[E^{**}A] = \frac{k_5 k_6[E][P]}{k_8 (k_{-5} + k_6)} \qquad \text{Eq. (27)}$$

By substituting Eq. (23) and (27) in Eq. (24), for $[E^{**}]$ it is obtained:

$$[E^{**}] = \frac{(k_5 k_6 k_8 + k_5 k_6 k_{-7})}{k_7 k_8 (k_{-5} + k_6)} \frac{[E][P]}{[A]} \qquad \text{Eq. (28)}$$

From Eq. (23), (27) and (28), the following auxiliary parameters are defined:

$$b_1 = \frac{k_5}{k_{-5} + k_6} \qquad \text{Eq. (29)}$$

$$b_2 = \frac{k_5 k_6}{k_8 (k_{-5} + k_6)} \qquad \text{Eq. (30)}$$

$$b_3 = \frac{k_5 k_6 k_8 + k_5 k_6 k_{-7}}{k_7 k_8 (k_{-5} + k_6)} \qquad \text{Eq. (31)}$$

And, from Eq. (23), (27) and (28) and these new parameters, the sum of the complexes involved in reaction 2 is obtained:

$$\sum_{\text{Re}action\,2} Complexes = [E]\left((b_1 + b_2)[P] + b_3 \frac{[P]}{[A]} \right) \qquad \text{Eq. (32)}$$

2.3.3.-Total mass balance of enzyme

If $[E_0]$ is the total concentration of enzyme, its total mass balance is given by:

$$[E]_0 = [E] + \sum_{\text{Re}action\,1} Complexes + \sum_{\text{Re}action\,2} Complexes \qquad \text{Eq. (33)}$$

By substituting Eq. (21) and (32) in Eq. (33), the following expression for the mass balance of enzyme is obtained:

$$[E]_0 = [E]\left(1 + (a_1 + a_2)[B] + a_3 \frac{[B]}{[A]} + (b_1 + b_2)[P] + b_3 \frac{[P]}{[A]} \right) \qquad \text{Eq. (34)}$$

Eq. (34) can be reordered as follows:

$$[E]_0 = [E] \frac{(a_1 + a_2)}{[A]} \left(\frac{1}{(a_1 + a_2)}[A] + [A][B] + \frac{a_3}{(a_1 + a_2)}[B] + \frac{(b_1 + b_2)}{(a_1 + a_2)}[A][P] + \frac{b_3}{(a_1 + a_2)}[P] \right)$$

Eq. (35)

From Eq. (35), four new parameters, three of them with the same units that a Michaelis constant and another one dimensionless, can be defined as follows:

$$K_{MA} = \frac{1}{(a_1 + a_2)} \qquad \text{Eq. (36)}$$

$$K_{MB} = \frac{a_3}{(a_1 + a_2)} \qquad \text{Eq. (37)}$$

$$K_{AP} = \frac{(b_1 + b_2)}{(a_1 + a_2)} \qquad \text{Eq. (38)}$$

$$K_{MP} = \frac{b_3}{(a_1 + a_2)} \qquad \text{Eq. (39)}$$

And, by substituting Eq. (36), (37), (38) and (39) in Eq. (35), the total balance of enzyme can be written as:

$$[E]_0 = [E]\frac{(a_1 + a_2)}{[A]}\left(K_{MA}[A] + K_{MB}[B] + K_{MP}[P] + [A][B] + K_{AP}[A][P]\right) \qquad \text{Eq. (40)}$$

2.4.- Reaction rate of substrates disappearance and products formation

2.4.1.- *Disappearance rate of substrate B*

According to the step 1, the disappearance rate of *B* is given by:

$$r_B = k_1[E][B] - k_{-1}[EB] \qquad \text{Eq. (41)}$$

By substituting Eq. (12) in Eq. (41) it is obtained:

$$r_B = \frac{k_1 k_2}{k_{-1} + k_2}[E][B] \qquad \text{Eq. (42)}$$

By dividing Eq. (42) by $[E]_0$, and taken into account Eq. (40):

$$\frac{r_B}{[E]_0} = \frac{\frac{k_1 k_2}{k_{-1} + k_2}[E][B]}{[E]\frac{(a_1 + a_2)}{[A]}\left(K_{MA}[A] + K_{MB}[B] + K_{MP}[P] + [A][B] + K_{AP}[A][P]\right)} \qquad \text{Eq. (43)}$$

By multiplying the left and right members of Eq. (43) by $[E]_0$, removing the concentration of free enzyme, $[E]$, in the right member, and by multiplying numerator and denominator by $[A]$ and dividing by $(a_1 + a_2)$, it is obtained:

$$r_B = \frac{\frac{k_1 k_2}{(k_{-1} + k_2)(a_1 + a_2)}[E]_0[A][B]}{K_{MA}[A] + K_{MB}[B] + K_{MP}[P] + [A][B] + K_{AP}[A][P]} \qquad \text{Eq. (44)}$$

Usually, the specific activity of enzyme is given related to its mass unit and not to its concentration. By this, if E_0 is the total amount of enzyme in the system, in mass units, M_E its molecular weight and V_R the reactor volume, the following relationship is verified:

$$[E]_0 = \frac{E_0}{M_E V_R} \qquad \text{Eq. (45)}$$

By substituting Eq. (45) in Eq. (44) it is obtained:

$$r_B = \frac{\frac{k_1 k_2}{(k_{-1}+k_2)(a_1+a_2)M_E V_R} E_0[A][B]}{K_{MA}[A]+K_{MB}[B]+K_{MP}[P]+[A][B]+K_{AP}[A][P]} \qquad \text{Eq. (46)}$$

The specific activity of enzyme in reaction 1 is defined as:

$$k_{cat1} = \frac{k_1 k_2}{(k_{-1}+k_2)(a_1+a_2)M_E V_R} \qquad \text{Eq. (47)}$$

And, by substituting Eq. (47) in Eq. (46), the following final expression for the disappearance rate of the substrate *B* is obtained:

$$r_B = \frac{k_{cat1} E_0[A][B]}{K_{MA}[A]+K_{MB}[B]+K_{MP}[P]+[A][B]+K_{AP}[A][P]} \qquad \text{Eq. (48)}$$

2.4.2.- *Formation rate of product Q*

The final product *Q* is formed in step 8 and its generation rate is given by:

$$r_Q = k_8[E^{**}A] \qquad \text{Eq. (49)}$$

Taken into account the value given in Eq. (27) for complex $E^{**}A$, it is obtained:

$$r_Q = \frac{k_5 k_6}{k_{-5}+k_6}[E][P] \qquad \text{Eq. (50)}$$

By dividing the left and right terms of Eq. (50) by $[E]_0$, and according to Eq. (40), it is follows:

$$\frac{r_Q}{[E]_0} = \frac{\frac{k_5 k_6}{k_{-5}+k_6}[E][P]}{[E]\frac{(a_1+a_2)}{[A]}\left(K_{MA}[A]+K_{MB}[B]+K_{MP}[P]+[A][B]+K_{AP}[A][P]\right)} \qquad \text{Eq. (51)}$$

Now, by multiplying the left and right members of Eq. (51) by $[E]_0$, removing the concentration of free enzyme, [*E*], in the right member, and by multiplying numerator and denominator by [*A*] and dividing by (a_1+ a_2), it is obtained:

$$r_Q = \frac{\frac{k_5 k_6}{(k_{-5}+k_6)(a_1+a_2)}[E]_0[A][P]}{K_{MA}[A]+K_{MB}[B]+K_{MP}[P]+[A][B]+K_{AP}[A][P]} \qquad \text{Eq. (52)}$$

Taken into account the value of $[E]_0$ given in Eq. (45):

$$r_Q = \frac{\dfrac{k_5 k_6}{(k_{-5}+k_6)(a_1+a_2)M_E V_R} E_0[A][P]}{K_{MA}[A]+K_{MB}[B]+K_{MP}[P]+[A][B]+K_{AP}[A][P]} \qquad \text{Eq. (53)}$$

Now, by defining the specific catalytic activity of enzime reaction 2 as:

$$k_{cat2} = \frac{k_5 k_6}{(k_{-5}+k_6)(a_1+a_2)M_E V_R} \qquad \text{Eq. (54)}$$

And, by substituting Eq. (54) in Eq. (53), the following final expression for the formation tate of the product *Q* is obtained:

$$r_Q = \frac{k_{cat2} E_0[A][P]}{K_{MA}[A]+K_{MB}[B]+K_{MP}[P]+[A][B]+K_{AP}[A][P]} \qquad \text{Eq. (55)}$$

2.4.3.- *Reaction rate of intermediate product P*

The reaction used to check the model has been carried out in a discontinuous tank reactor. By this, the reaction rate of the intermediate product *P* can be obtained from a total mass balance of azelate, as follows. If $[B]_0$ is the initial concentration of diethyl azelate, its total mass balance at time t is given by:

$$[B]+[P]+[Q]=[B]_0 \qquad \text{Eq. (56)}$$

From Eq. (56) it is obtained:

$$\frac{d[B]}{dt}+\frac{d[P]}{dt}+\frac{d[Q]}{dt}=0 \qquad \text{Eq. (57)}$$

The three derivatives that appear in Eq. (57) are the accumulation of *B*, *P*, and *Q*, respectively, in the reactor along time. And it is known that in a tank reactor the accumulation of a product is equal to its generation rate, but the accumulation of a substrate is equal to its negative disappearance rate. So, Eq. (57) can be rewritten as:

$$-r_B + r_P + r_Q = 0 \qquad \text{Eq. (58)}$$

From Eq. (58), the reaction rate of the intermediate product *P* is obtained:

$$r_P = r_B - r_Q \qquad \text{Eq. (59)}$$

2.4.4.- *Disappearance rate of substrate A*

In the same way that for the product *P*, and taking into account that only one molecule of 2-ethylhexanol is necessary to give a molecule of the intermediate product,

but two molecules of 2-ethylhexanol are necessary to give a molecule of the final product *Q*, the following mass balance for *A* is obtained:

$$[A]+[P]+2[Q]=[A]_0 \qquad \text{Eq. (60)}$$

From Eq. (60):

$$\frac{d[A]}{dt}+\frac{d[P]}{dt}+2\frac{d[Q]}{dt}=0 \qquad \text{Eq. (61)}$$

Taking into account that the substrate *A* disappears, and from the relationships between the accumulations of *A*, *P* and *Q* and their reaction rates:

$$-r_A+r_P+2r_Q=0 \qquad \text{Eq. (62)}$$

Finally, by combining Eq. (62) and (59) the reaction rate of *A* is obtained:

$$r_A=r_B+r_Q \qquad \text{Eq. (63)}$$

2.5.- Discontinuous tank reactor: design equations

2.5.1.- *Diffusional limitations: effectiveness factors*

The reaction chosen to check the model has been carried out in a discontinuous stirred tank reactor and, by this, the external diffusional limitations have been considered negligible. But, regarding the internal limitations, as an immobilized derivative of enzyme has been used they must be taken into account. With this purpose, an estimate, based on the theoretical solution of the effectiveness factor for the first order kinetics, [22], is proposed for each one of the two reactions. With these hypotheses, both effectiveness factors are given, respectively, by the following equations:

$$\eta_1=3\left(\frac{1}{\Phi_1 th(\Phi_1)}-\frac{1}{\Phi_1^2}\right) \qquad \text{Eq. (64)}$$

$$\eta_2=3\left(\frac{1}{\Phi_2 th(\Phi_2)}-\frac{1}{\Phi_2^2}\right) \qquad \text{Eq. (65)}$$

In equations (64) and (65), Φ_1 y Φ_2 are, respectively, the values of the Thièle modulus, given by the following expressions:

$$\Phi_1 = \sqrt{\frac{R_C^2 V_{m1}}{K_{M1} D_{S1}}} \qquad \text{Eq. (66)}$$

$$\Phi_2 = \sqrt{\frac{R_C^2 V_{m2}}{K_{M2} D_{S2}}} \qquad \text{Eq. (67)}$$

In equations (66) and (67) R_C is the radius of the bio-catalytic particle, and K_{M1}, K_{M2}, D_{S1} *and* D_{S2} are, respectively, the equivalent average Michaelis constants and average diffusivities for each one of the two reactions. In addition, V_{m1} and V_{m2} are the maximum reaction rates of reactions 1 and 2, which depend on the specific activity of the enzyme, k_{cat1} and k_{cat2}, for each one of the two reactions, and the total enzyme amount, E_0, which is the same for both reactions. So, equations (66) and (67) can be rewritten as:

$$\Phi_1 = \sqrt{\frac{R_C^2 k_{cat1} E_0}{K_{M1} D_{S1}}} = \sqrt{\frac{R_C^2 k_{cat1}}{K_{M1} D_{S1}}} \sqrt{E_0} \qquad \text{Eq. (68)}$$

$$\Phi_2 = \sqrt{\frac{R_C^2 k_{cat2} E_0}{K_{M2} D_{S2}}} = \sqrt{\frac{R_C^2 k_{cat2}}{K_{M2} D_{S2}}} \sqrt{E_0} \qquad \text{Eq. (69)}$$

2.5.2.- *Discontinuous tank reactor: mass balances*

Taken into account all the above paragraphs, the mass balances in the reactor, for substrates and products, are given by the following equations:

$$\frac{d[A]}{dt} = -\left(\eta_1 r_B + \eta_2 r_Q\right) \qquad \text{Eq. (70)}$$

$$\frac{d[B]}{dt} = -\eta_1 r_B \qquad \text{Eq. (71)}$$

$$\frac{d[P]}{dt} = \eta_1 r_B - \eta_2 r_Q \qquad \text{Eq. (72)}$$

$$\frac{d[Q]}{dt} = \eta_1 r_Q \qquad \text{Eq. (73)}$$

With the initial conditions:

$$t = 0; [A] = [A]_0; [B] = [B]_0; [P] = 0; [Q] = 0 \qquad \text{Eq. (74)}$$

3. MATERIALS AND METHODS

3.1. Chemicals

Diethyl azelate (90%), 2-ethylhexyl alcohol (99.6%), methyl myristate (99%) and n-heptane (>96%), were purchased from Sigma Aldrich. Bis(2-ethylhexyl) azelate (75%) was purchased from TCI Europe NV.

The lipase-based bio-catalyst, Novozym@ 435 was kindly provided by Novozymes Spain S.A.

Other reagents and products were of analytical grade.

3.2. Experimental procedure

The reaction was carried out under solvent free conditions. Experiments were conducted in a jacketed stirred batch reactor of 50 cm^3 total volume. The stirring speed was fixed at 350 rpm and 70 ºC was the operating temperature chosen. A total amount of 20 g of the two substrates, 2-ethylhexanol and diethyl azelate, was added to the reactor in 2:1 molar ratio, due to the stoichiometry of the overall reaction. After, when the operating temperature was reached, the required enzyme amount was added. The reaction progress was followed by taking samples of 50 µl which were diluted in heptane and analyzing the concentrations of residual substrates and products until a reaction time of 210 minutes.

3.3. Analytical method

The analytical method used is an adaptation of a previous one from our research group [23]. So, the analysis of substrates and products has been carried out in an Agilent 7820A GC, with detector of flame ionization (FID) and a silica capillary column (30m ×0.32mm×0.25 µm). The analysis conditions were: Injector temperature 250 °C, detector temperature 300 °C and split ratio 2:1. Nitrogen was used as carrier gas, at a flow rate of 1 mL min^{-1}. Oven temperature was kept constant at 80 °C for 1 min, increased to 120 °C at a rate of 75 °C min^{-1} and, after 1 min, increased again to 290 °C,

at 20 °C min^{-1}, temperature which was maintained for 3.5 min. Diluted samples of 1 μL were injected, being 14 minutes the total time of the analysis. Methyl myristate was used as internal standard in the determination of both substrates and the final product, being the intermediate product determined by difference from the total mass balance.

3.4. Experimental planning

As the enzymatic reaction was carried out in a solvent free medium and the substrates must be in 2:1 molar ratio, due to the stoichiometry of the reaction, the only parameters to be varied are the enzyme amount, the temperature and the stirring speed. The enzyme amount is the most important of the above three variables, because is the bio-catalyst and its variation has significant influence on the reaction rate. By this, and because the objective of this experimental work is not the optimization of the enzymatic reaction but to be able to check the kinetic model, only an experimental series for three enzyme amounts has been carried out. In this series, the total enzyme amount in the reactor has been 0.25, 0.50 and 1.00 g., respectively. Duplicate runs were made in all assays and the temperature was kept constant at 70ºC and the stirring speed in 350 rpm.

3.5.-Solving the model.

The differential equations (70) to (73) are not linear, and their solution can only be obtained by using numerical methods. In this work, the finite differences method has been applied and the following discrete equations have been obtained:

$$[A]_{t+\Delta t} = [A]_t - \left(\eta_1 r_B + \eta_2 r_Q\right)_t \Delta t \qquad \text{Eq. (75)}$$

$$[B]_{t+\Delta t} = [B]_t - \left(\eta_1 r_B\right)_t \Delta t \qquad \text{Eq. (76)}$$

$$[P]_{t+\Delta t} = [P]_t + \left(\eta_1 r_B - \eta_2 r_Q\right)_t \Delta t \qquad \text{Eq. (77)}$$

$$[Q]_{t+\Delta t} = [Q]_t + \left(\eta_2 r_Q\right)_t \Delta t \qquad \text{Eq. (78)}$$

These equations have been solved as indicated in section 4.2.

4.- RESULTS AND DISCUSSION

4.1.- Progress curves

The experimental results obtained are depicted in Figures 2A to 2C, for 0.25, 0.50 and 1.00 g of enzyme, respectively. In these Figures, the progress curves of the concentrations of both substrates, as well as the intermediate product, the final product and the sum of the different compounds of azelate, are shown. The dots are the experimental values and the solid lines are the ones calculated with the model.

Regarding Fig. 2A, it can be seen that, with 0.25 g of enzyme, after a total reaction time of 210 minutes, significant amounts of 2-ethylhexanol and the intermediate product remains in the reaction medium, and total conversion to the final product is not reached. On Fig. 2B it can be observed that an increasing of the enzyme amount until 0.50 g improves the reaction's yield, but still no total conversion to the final product is obtained. Finally, by using 1.00 g of enzyme, total conversion to the final product is practically reached, as it is shown on Fig. 2C.

Additionally, the conversions of substrates and products can be defined as follows:

$$Conversion\ of\ substrate\ A = X_A = \frac{[A]_0 - [A]}{[A]_0} \qquad \text{Eq. (79)}$$

$$Conversion\ of\ substrate\ B = X_B = \frac{[B]_0 - [B]}{[B]_0} \qquad \text{Eq. (80)}$$

$$Conversion\ of\ P\ from\ substrate\ B = X_P = \frac{[P]}{[B]_0} \qquad \text{Eq. (81)}$$

$$Conversion\ of\ Q\ from\ substrate\ B = X_Q = \frac{[Q]}{[B]_0} \qquad \text{Eq. (82)}$$

The progress curves with time of these different conversions are shown in Figs. 3 and 4. In Fig. 3A and 3B, the conversion of 2-ethylhexanol and diethyl azelate, respectively, have been plotted against time for the three amounts of enzyme assayed. As it can be seen, almost total conversion of diethyl azelate is reached with the three amounts of enzyme, but for the 2-ethylhexanol only total conversion is reached when 1 g of enzyme is used. This is because the diethyl azelate, which initial concentration must be half of the one of 2-ethylhexanol, is almost totally consumed in the reaction 1. But when practically no residual amounts of diethyl azelate are present in the reaction medium, significant amounts of 2-ethylhexanol must remain, to react with the intermediate *P* to obtain the final product *Q*.

In Fig. 4A and 4B, the conversions of the intermediate product, *P*, and the final product, *Q*, are shown. As it is shown in Fig. 4A, there is an increasing in the conversion of P during the first minutes, reaching a maximum after and, finally, decreasing. For a total reaction time of 210 minutes, a value of zero is only reached when 1 g of enzyme is used, which indicates that practically no amount of the intermediate product remains. In the same way, in Fig. 4B it can be observed that the conversion of *Q* increases as the enzyme amount increases, and the highest value is reached when 1 g of enzyme is used.

4.2.- Fitting the model: parameters determination

To check the model, the experimental data must be fitted to their equations, and the kinetic parameters must be calculated. But the differential equations of the model are strongly non-linear and they cannot be integrated analytically, which complicates the determination of the parameters of the model by direct methods of minimization of the error. In this sense, as indicated in the Introduction section, reference [21] can help us, because in that work a similar problem, that also includes the bisubstrate ping-pong kinetics, was solved.

As in the above work, user's software in Visual Basic for Applications has been developed. The software fits the experimental data to the model and calculates the model parameters by minimizing the sum of square error by using the optimization routine developed by Nelder and Mead [24]. Eight has been the total number of parameters to calculate: The two values of the specific activity of the enzyme, each one for each reaction, the four constants in the denominator of the rate equations, and two additional parameters to estimate the effectiveness factor. Table 1 summarizes the values obtained for these parameters in the fitting of the experimental data to the model.

Also, on Fig.5, the effectiveness factors estimated for the two reactions and the three enzyme concentrations are shown and, from their values, it seems that significant internal limitations exist in both reactions. In this figure it can be seen also, that the effectiveness factor for the first reaction is higher than that for the second one, which can be explained because for the second reaction the diffusion of substrates into the catalytic particles is more difficult, due to the previous presence of both substrates and products of the first reaction.

Additionally, in Figures 2 to 4, previously commented, the solid lines are the values calculated by the software for the different concentrations and conversion above

mentioned. Finally, on Fig. 6, all concentrations calculated with the model have been plotted against the experimental ones and, as it can be seen, a good linear correlation has been obtained, with a determination coefficient of 0.9925, which validates the model.

5. CONCLUSIONS

In this work, the kinetic equations of two simultaneous bisubstrate Ping-Pong in series reactions have been developed by the first time. To obtain these equations, the approximation to stationary state has been applied and, for the total balance of enzyme, all intermediate complexes of the two reactions, which are present simultaneously in the reaction medium, have been taken into account.

To check the kinetic equations obtained the synthesis of bis(2-ethylhexyl) azelate by transesterification from diethyl azelate and 2-ethylhexanol, in the presence of the immobilized lipase Novozym$^{@}$ 435, has been used as reaction model, and the reaction has been carried out in solvent free conditions in a batch reactor.

A design model of the reactor has been also developed and solved by applying a numerical method. The model equations have been implemented in user's software in Visual Basic for Applications and a routine, based on a very well-known procedure to minimize the sum of the square errors, has been also implemented.

By using this software, the model parameters have been determined by fitting the experimental data to the model. A high determination coefficient was obtained, which validated both the kinetic equations and the reactor's design model.

ACKNOWLEDGEMENT

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NOMENCLATURE

a_1	Auxiliary parameter defined in equation 18
a_2	Auxiliary parameter defined in equation 19

a_3	Auxiliary parameter defined in equation 20
A	Alcohol (2-Ethylhexanol)
b_1	Auxiliary parameter defined in equation 29
b_2	Auxiliary parameter defined in equation 30
b_3	Auxiliary parameter defined in equation 31
B	Acyl donor (Diethyl azelate)
D_{S1}	Average diffusivity in equation 66
D_{S2}	Average diffusivity in equation 67
E	Enzyme (Lipase)
E_0	Total enzyme amount in the reactor
E^*	Modified form of enzyme in reaction 1
E^{**}	Modified form of enzyme in reaction 2
EB	Intermediate complex of enzyme and B in reaction 1
EP	Intermediate complex of enzyme and P in reaction 2
E^*A	Intermediate complex of modified enzyme and A in reaction 1
$E^{**}A$	Intermediate complex of modified enzyme and A in reaction 2
k_1	Direct kinetic constant of the reversible step 1
k_{-1}	Reverse kinetic constant of the reversible step 1
k_2	Kinetic constant of the irreversible step 2
k_3	Direct kinetic constant of the reversible step 3
k_{-3}	Reverse kinetic constant of the reversible step 3
k_4	Kinetic constant of the irreversible step 4
k_5	Direct kinetic constant of the reversible step 5
k_{-5}	Reverse kinetic constant of the reversible step 5
k_6	Kinetic constant of the irreversible step 6
k_7	Direct kinetic constant of the reversible step 7
k_{-7}	Reverse kinetic constant of the reversible step 7
k_8	Kinetic constant of the irreversible step 8
k_{cat1}	Specific activity of enzyme in reaction 1
k_{cat2}	Specific activity of enzyme in reaction 2
K_{AP}	Constant multiplying [A][P] in the denominator of rate equation
K_{MA}	Michaelis constant before [A] in the denominator of rate equation
K_{MB}	Michaelis constant before [B] in the denominator of rate equation
K_{MP}	Michaelis constant before [P] in the denominator of rate equation

K_{M1}	Average Michaelis constant in equation 66
K_{M2}	Average Michaelis constant in equation 67
M_E	Molecular weight of enzyme
P	Intermediate product (Ethyl, 2-ethylhexyl azelate)
Q	Final product (Bis(2-ethylhexyl) azelate)
r_A	Disappearance rate of A
r_B	Disappearance rate of B
r_P	Formation rate of P
r_Q	Formation rate of Q
R	Subproduct released in reaction 1 and 2 (Ethyl alcohol)
R_C	Radius of the catalytic particle
t	Time
Δt	Time increment
V_{max}	Maximum reaction rate
V_R	Reactor volume
X_A	Conversion of substrate A
X_B	Conversion of substrate B
X_P	Conversion to P from substrate B
X_Q	Conversion to Q from substrate B
$[A]$	Concentration of A
$[A]_0$	Initial concentration of A
$[B]$	Concentration of B
$[B]_0$	Initial concentration of B
[E]	Concentration of enzyme
$[E]_0$	Total concentration of enzyme
$[P]$	Concentration of P
$[P]_0$	Initial concentration of P
$[Q]$	Concentration of Q
$[Q]_0$	Initial concentration of Q
Φ_1	Thièle modulus for reaction 1
Φ_2	Thièle modulus for reaction 2
η_1	Effectiveness factor for reaction 1
η_2	Effectiveness factor for reaction 2

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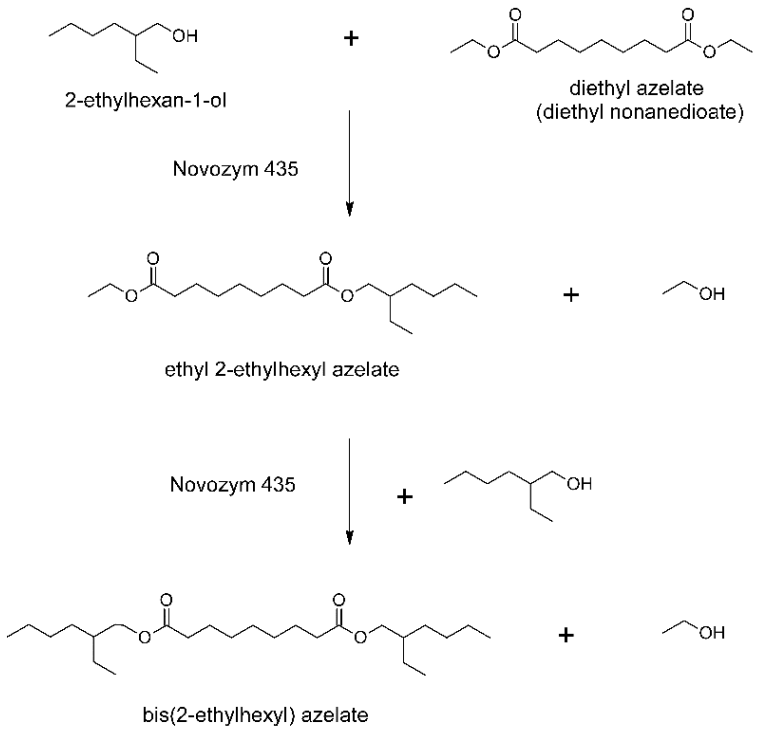
***Declaration of Interest Statement**

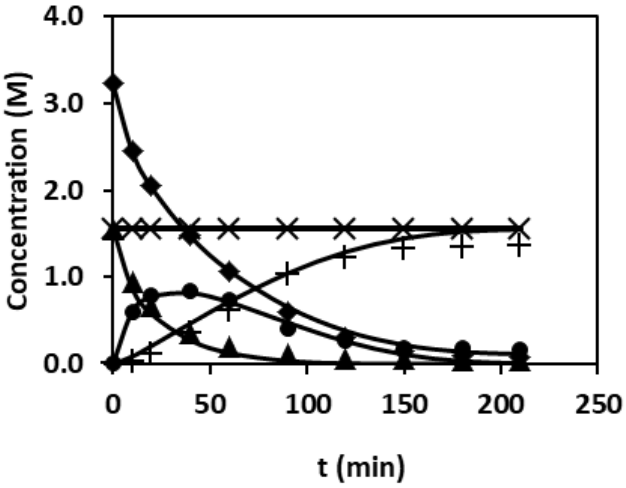
Declaration of interests

☒ The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

☐The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:

Graphical Abstract





Variation of substrates and products concentrations along the reaction time:

♦ [2-Etilhexanol] (Mol L^{-1}); ▲ [Diethyl azelate] (Mol L^{-1}); ● [Ethyl 2-ethylhexyl azelate] (Mol L^{-1}); **+** [Bis(2-ethylhexyl) azelate] (Mol L^{-1}); **X** [Sum of azelates]; (**−**) kinetic model.

Figure1
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Figure 1

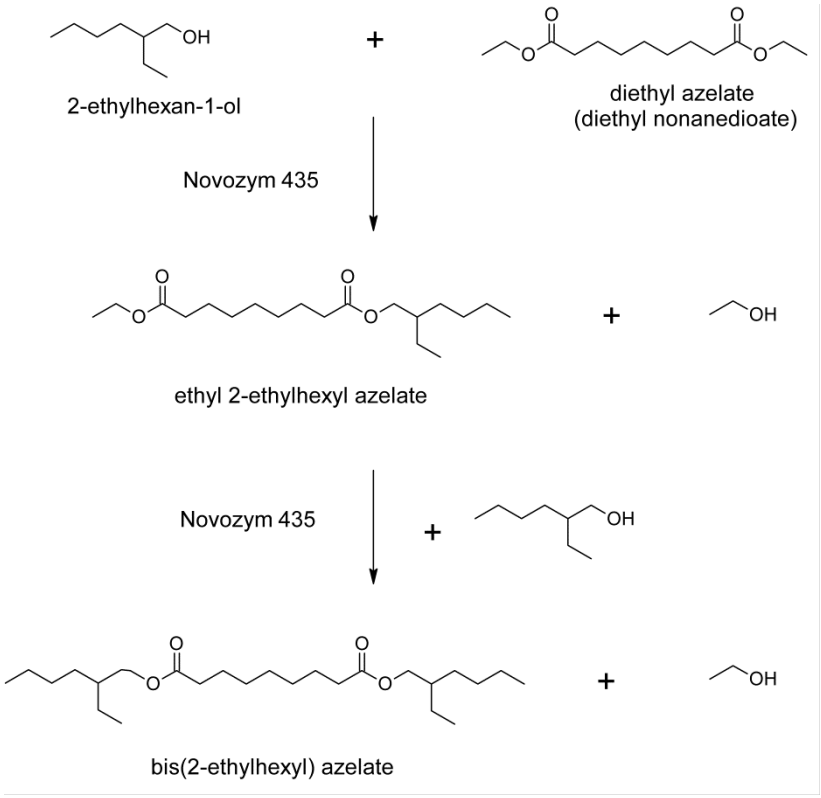

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Figure 2

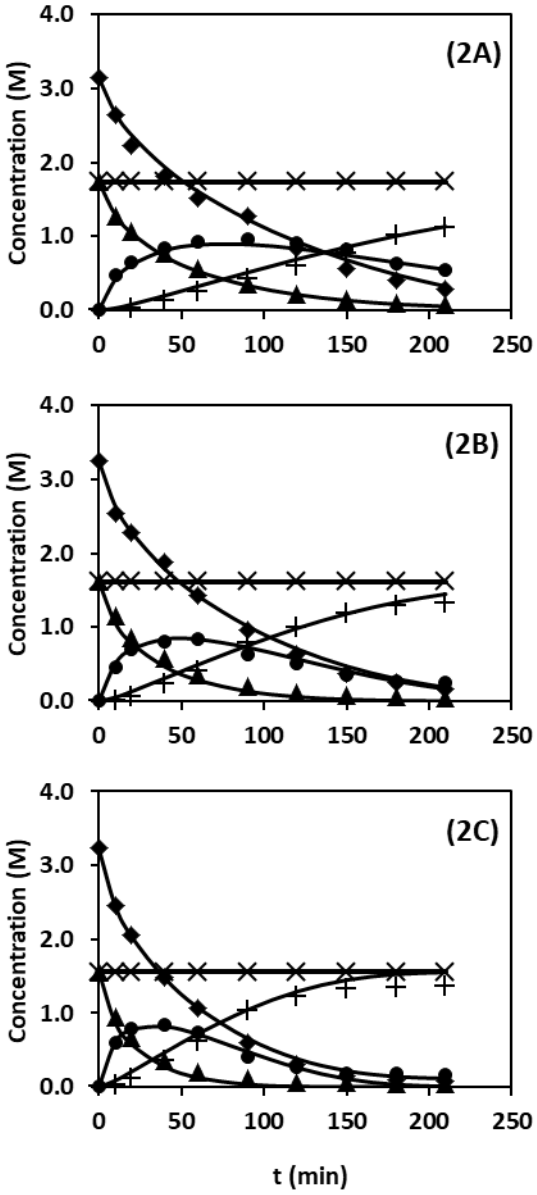

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Figure 3

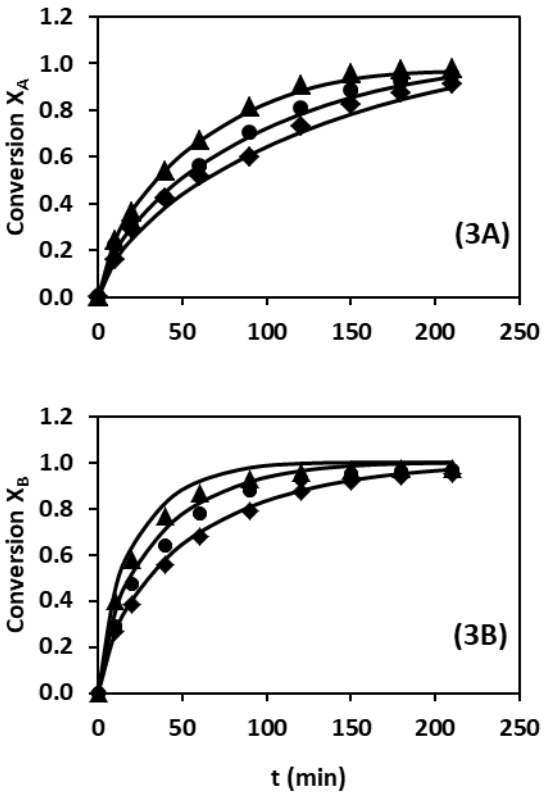

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Figure 4

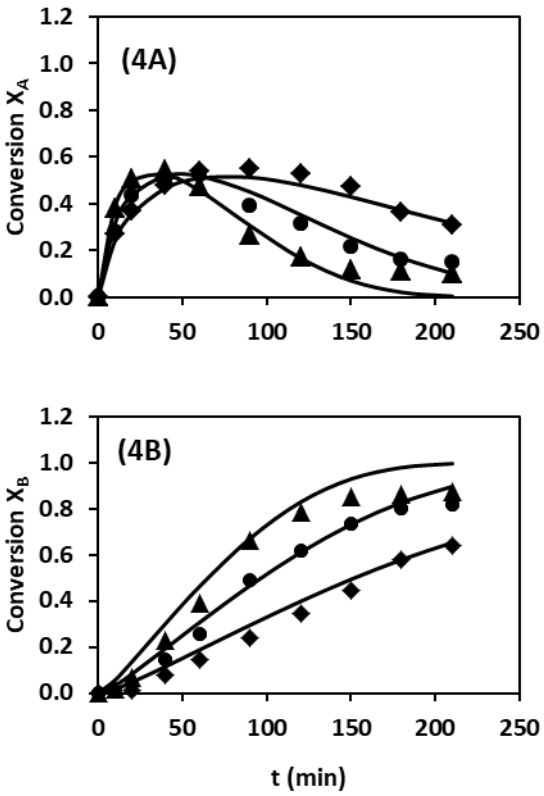

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Figure 5

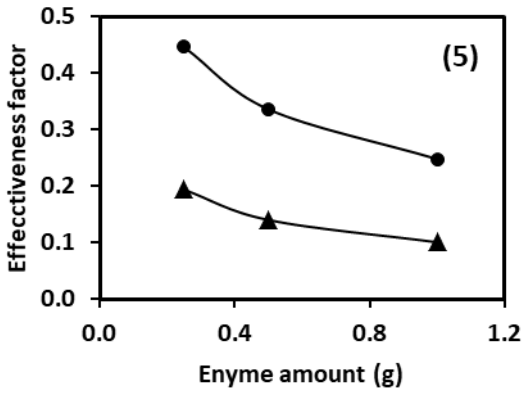

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Figure 6

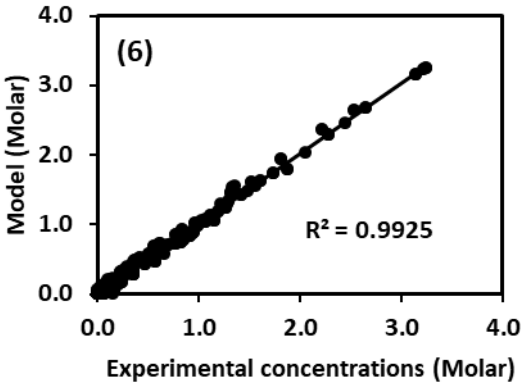

Figure captions

Figure 1: Reaction scheme of the biocatalytic synthesis of Bis(2-ethylhexyl) azelate

Figure 2: Influence of enzyme amount on the variation of substrates and products concentrations along the reaction time. Fig. 1A : 0.25 g enzyme; Fig. 1B : 0.50 g enzyme; Fig. 1C: 1.00 g enzyme. ♦ [*A*] (Mol L^{-1}); ▲ [*B*] (Mol L^{-1}); ● [P] (Mol L^{-1}); + [*Q*] (Mol L^{-1}); **X** Σ([*B*]+[*P*]+[*Q*]); (−) model.

Figure 3: Influence of enzyme amount on the variation of conversions of substrates *A* and *B* along the reaction time. Fig. 2A : substrate *A*; Fig. 2B :substrate *B*. ♦ 0.25 g enzyme; ● 0.50 g enzyme; ▲ 1.00 g enzyme: (−) model

Figure 4: Influence of enzyme amount on the variation of conversions of products *P* and *Q* along the reaction time. Fig. 3A : intermediate product *P*; Fig. 3B : final product *Q*. ♦ 0.25 g enzyme; ● 0.50 g enzyme; ▲ 1.00 g enzyme; (−) model

Figure 5: Effectiveness factors for the two reactions and the different amounts of enzyme: ● Reaction 1; ▲Reaction 2.

Figure 6: Molar concentrations calculated by the model against the experimental ones.

Table1

Table 1: Parameters of the model and their units

Parameters of model	Values	Units
k_{cat1}	2.039	*Mol* L^{-1} *min*$^{-1}$ g_{enzyme}^{-1}
k_{cat2}	2.172	*Mol* L^{-1} *min*$^{-1}$ g_{enzyme}^{-1}
K_{MA}	3.385	*Mol* L^{-1}
K_{MB}	0.378	*Mol* L^{-1}
K_{MP}	3.067	*Mol* L^{-1}
K_{AP}	9.217	*Dimensionless*
$\sqrt{\frac{R^2 k_{cat1}}{K_{M1} D_{S1}}}$	11.367	$g_{enzyme}^{-1/2}$
$\sqrt{\frac{R^2 k_{cat2}}{K_{M2} D_{S2}}}$	31.123	$g_{enzyme}^{-1/2}$