Kinetic study of the enzymatic synthesis of 2-Phenyl-ethyl acetate in discontinuous tank reactor

Asunción M. Hidalgo, Arturo Sánchez, José Luis Gómez, Elisa Gómez, María Gómez, María Dolores Murcia*

Department of Chemical Engineering, University of Murcia, Campus de Espinardo, 30100, Spain

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2-phenyl-ethyl acetate, 2-phenyl alcohol, vinyl acetate, Novozym® 435, transesterification, kinetics

ABSTRACT

There is increasing interest in new processes to produce aromatics labeled as "natural" and, as a consequence, enzymatic processes, in some organic solvent or in solvent-free medium, have

been developed. This paper offers a kinetic study of lipase-catalyzed transesterification for synthesizing 2-phenyl-ethyl acetate in a batch reactor, using Novozym® 435 and hexane as solvent. Influence of five variables on the conversion is studied. A pseudo-first order kinetic model, derived as a simplification of a Bisubstrate Ping-Pong kinetics, is presented. The amount of catalyst was the variable most affecting kinetic and yield, while initial substrate concentration had little effect on the reaction kinetic. Yield increased gradually as the molar ratio increased, and was more pronounced with vinyl acetate than 2-phenethyl alcohol. Michaelis constants of both substrates and maximum reaction rate, for the range of assayed experimental conditions of temperature, stirring speed and enzyme concentration, were determined and compared with others in the literature. Finally, the proposed kinetic model was validated with high correlation coefficients.

1. Introduction

Flavoring agents and fragrances are among the prime additives used by the food, pharmaceutical, personal care, beverages and drug industries worldwide. According to an inform of marketsandmarkets.com, published on march 2018, the flavors and fragrances market was valued at USD 20.55 Billion in 2016 and is projected to reach USD 24.13 Billion by 2022, at a compound annual growth rate (**CAGR**) of 2.67% during the forecast period from 2017 to 2022.[1] Generally, these chemical products are obtained by esterification/transesterification of phenyl alcohol at high temperatures in the presence of mineral acids, using a Lewis acid ($ZnCl_2$, $FeCl_3$) as catalyst. However, acid catalysts are difficult to separate and the cost of waste management may be excessive.[2-5]

One alternative is to use an enzymatic reaction since such reactions are mild and only small amounts of by-products are produced due to the substrate specificity of the enzyme. The most common enzymes used are lipases, because they work in organic solvents and are capable of carrying out several reactions (alcoholysis, esterification, transesterification and inter-esterification).[6,7] Furthermore, immobilized lipase processes enhance stability, the ease of separation from the reaction mixture and reusability.[8]

Kinetic studies of lipase-catalyzed esterification have been carried out with enzymes of different origins and substrates (acids and alcohols) of various chain lengths and structures, either in solvent-free systems or in the presence of an organic solvent.[9-11] Most kinetic studies developed for the lipase-catalyzed synthesis of esters have considered the direct esterification of a variety of alcohols using an acid and have described a Ping-Pong Bi-Bi kinetic mechanism with inhibition by both substrates.[12-14]

The presence of 2-phenyl-ethyl acetate is related to the rose note of many aromas.[2] Transesterification of 2-phenyl-ethyl alcohol with vinyl acetate as an acyl group donor has been carried out using the immobilized lipase Novozym® 435 of *Candida antarctica*. The product obtained together with the fragrant ester is vinyl alcohol, which immediately tautomerizes to acetaldehyde, so that the balance moves towards the formation of the ester.[15-17] Kuo et al.[3], using Novozym® 435 at enzyme concentration of 50 mg mL^{-1} and substrate concentrations of 50 mM for both substrates, studied the enzymatic synthesis of natural rose aroma-active 2-phenyl-ethyl acetate (*P*) by means of the transesterification reaction between 2-phenyl ethanol (*A*) and vinyl acetate (*B*) in hexane, obtaining the following kinetic parameters: V_{max} = 399 mM min^{-1}, K_M^A =2220 mM and K_M^B = 1720 mM. Based on the calculated kinetic parameters, the affinity of the

enzyme towards vinyl acetate seems to be greater than that towards 2-phenyl ethanol, since K_M^B is lower than K_M^A. Also, the sum of both Michaelis constants, $K_M^A + K_M^B = 3940$ mM, is much higher than the 50 mM substrates concentration, which can be used to simplify the Ping-Pong equation, as it is proposed later in the present work.

These results agree with the Ping-Pong mechanism, which assumes that the acyl donor is the first substrate that binds to the lipase.[18] The experimental equipment used consists of an orbital shaking bath and a sealed glass tube of 3 mL (diameter 1.5 cm) as reactor.

In a recent publication, Bansodeet al.[14] studied the synthesis and kinetic of isoamyl butyrate using Novozym® 435 as biocatalyst in a conventional stirred batch reactor of 50 mL capacity. The best fitting model using non-linear regression was obtained for Ping-Pong Bi-Bi with alcohol inhibition. The following values were obtained: specific activity of enzyme K_E = 28.027 mmol min^{-1} g^{-1}, Michaelis constants K_M^A = 636 mM and K_M^B = 657 mM and inhibition constant K_{ib} = 22 mM. The Michaelis constants (K_M^A and K_M^B) indicate that the enzyme has nearly equal specificity for both alcohol and acid.

In view of the different results previously obtained, which depend not only on the medium, but on the experimental equipment used, in this study, the production of 2-phenyl-ethyl acetate was carried out in a discontinuous tank reactor to obtain the intrinsic values of the kinetic constants. The influence of different variables (enzyme concentration, substrate ratio, stoichiometric substrate quantities, temperature and stirring speed) on the conversion was also studied. Finally, the fitting of the experimental data suggested an irreversible first order kinetic model.

2. Materials and Methods

2.1. Chemicals

Vinyl acetate ($C_4H_6O_2$), molecular weight 86.09 g/mol (≥99.0%), CAS number (108-05-4), 2-phenyl-ethanol ($C_8H_{10}O$), molecular weight 122.16 g/mol (≥99.0%), CAS number (60-12-8) and 2-phenylethyl acetate ($C_{10}H_{12}O_2$), molecular weight 164.20 g/mol (≥99.0%), CAS number (103-45-7) were obtained from Sigma-Aldrich. Hexane (C_6H_{14}) for gas chromatography, molecular weight 88.18 g/mol (≥98.0%), CAS number (110-54-3), used as solvent, was purchased from Panreac.

Immobilized lipase from Candida antarctica lipase B (CALB) supported on a DVB/methacrylate co-polymer resin Lewatit® VP OC 1600 and marketed under the name Novozym® 435 was donated by Novozymes Spain S.A and used as biocatalyst. Its declared activity is 10000 PLU/g, where one unit corresponds to the synthesis of 1 μmol per minute propyl laureate from lauric acid and 1-propanol. Other chemicals were of analytical grade and were used without further purification.

2.2. Experimental system and analytical equipment.

Two reactors were simultaneously used. The experiments were carried out in a glass reactor with a capacity of 50 mL. The reactor has three upper openings, of which the central one was used to introduce the solution and the catalyst, and the other two lateral upper openings were used for taking samples. The catalyst is introduced into the reaction medium inside a nylon mesh porous bag, which prevents the catalyst from being released and lost.

Stirring was by means of a magnetic bar, which allows the speed to be regulated, while eliminating diffusion limitations. The reactor is equipped with a water coil, connected to a thermostatic bath which allows a constant reaction temperature.

The samples analysis was carried out in a high-quality gas chromatograph model "Agilent 7820-A GC System" (Agilent Technologies).

2.3. Experimental method

Solutions of 50 mL of the desired concentration of both reagents were prepared using hexane as solvent. Once the thermostatic bath reaches the chosen temperature, the solution is introduced into the reactor and the magnetic stirring is started. The catalyst, previously weighed, is placed into the nylon mesh and introduced into the reactor through its upper opening. This represents the beginning of the reaction time, and samples were taken at 0, 25, 50, 85, 120 and 160 minutes. Samples were analyzed in the gas chromatograph. The following operational variables were studied in different series:

- Temperature: 30, 35, 40 °C. Catalyst concentration: 0.35 mg mL^{-1}; initial concentration: 50 mM; molar ratio: 1:1 and stirring speed 300 rpm.
- Stirring speed: 100, 200, 300, 500 rpm. Catalyst concentration: 0.35 mg mL^{-1}; initial concentration: 50 mM; molar ratio: 1:1 and temperature: 40 °C.
- Catalyst concentration: 0.1, 0.2, 0.35, 0.5, 0.6 mg mL^{-1}. Molar ratio: 1:1; initial concentration: 50 mM; temperature: 40 °C; and stirring speed 300 rpm.
- Initial concentration: 25 mM, 50 mM, 100 mM. Catalyst concentration: 0.35 mg mL^{-1}; molar ratio: 1:1; temperature: 40 °C; and stirring speed 300 rpm.

- Molar ratio: 1:1, 1:2, 1:4, 2:1, 4:1. Catalyst concentration: 0.35 mg mL^{-1}; initial concentration: 25 mM; temperature: 40 °C; and stirring speed 300 rpm.

Duplicate experiments were carried out and the average values were obtained. The maximum calculated standard deviation was 2.16% for all the data.

2.4. Analytical method

The reaction was followed by gas chromatography using a capillary column (dimensions: 30m x 0.320mm x 0.25μm) and a flame ionization detector (250 °C). The flow rate of N_2 (gas) was 1.10 mL min^{-1}. A 1 μL aliquot of the reaction sample was introduced by a split injector at 250 °C. Following the indications of other authors[2], the oven temperature ramp was set at 2 °C min^{-1} from 164 °C to 180 °C. The product was identified by comparing the retention times of the reaction samples with known standards. The corresponding calibration curve gave the following equations:

[2-phenethyl alcohol] (mM) = area/0.0232 (r = 0.9991)

[2-phenylethyl acetate] (mM) = area/0.0207 (r = 0.9989)

3. Kinetic model

2-Phenylethyl acetate (*P*) was obtained by a transesterification process between 2-phenylethanol (*A*) and vinyl acetate (*B*) in the presence of the catalyst Novozym® 435 lipase. The results were analyzed based on the conversion of the 2-phenylethanol with time which, according to the used nomenclature, is defined as follows:

$$X_A = \frac{C_{A0} - C_A}{C_{A0}} \qquad (1)$$

3.1. Model equations

3.1.1. Model hypothesis and reaction rate

Previous studies[3,16] described the reaction synthesis of 2-phenylethyl acetate and geranyl acetate as a bisubstrate Ping-Pong mechanism.

If this mechanism is accepted as hypothesis, the reaction rate can be written as follows[19,20]:

$$v = \frac{V_{\max} C_A C_B}{K_M^A C_B + K_M^B C_A + C_A C_B} \qquad (2)$$

Taking into account that the molar stoichiometry of the reaction is 1:1, if it is started from stoichiometric amounts of both substrates and if, in addition, we consider that the sum of both substrate constants is much higher that the concentration of substrate (*A*) [3], equation (2) for the reaction rate equation can be simplified as follows:

$$v = kC_A \qquad (3)$$

Where k is the pseudo-first order apparent constant (min^{-1}), that can be written as:

$$k = \frac{V_{\max}}{K_M^A + K_M^B} \qquad (4)$$

Otherwise, the maximum reaction rate of the enzymatic reaction, V_{max}, is a function of the enzyme concentration according to the following equation:

$$V_{\max} = K_E C_E \qquad (5)$$

By combining equations 4 and 5, the pseudo-first order kinetic constant must vary linearly with the enzyme concentration as follows:

$$k = KC_E \tag{6}$$

where K ($L \cdot g^{-1} \cdot min^{-1}$), can be written as:

$$K = \frac{K_E}{K_M^A + K_M^B} \tag{7}$$

Besides, since the transesterification reaction was carried out in a stirred thermally insulated batch reactor, it is assumed that:

- The reactor behaves as a complete mixing reactor.

- The reaction takes place under isothermal conditions, so that the kinetic parameters are kept constant in each of the different experiments.

3.1.2. Mass balance and model integration

Since it is a closed system, the mass balance for 2-phenylethanol is reduced by equaling the terms of accumulation and disappearance, according to the following equation:

$$\frac{dC_A}{dt} = -v \tag{8}$$

If $v$is the 2-phenyl ethanol disappearance rate defined in equation (3), equation (8) can be written as:

$$\frac{dC_A}{dt} = -kC_A \tag{9}$$

Integrating equation (9) with the following initial conditions:

$$t = 0; \qquad C_A = C_{A0} \tag{10}$$

gives:

$$\ln \frac{C_A}{C_{A0}} = -kt \tag{11}$$

and taking into account the conversion definition given in equation (1), the conversion of *A* is given by:

$$X_A = 1 - e^{-kt} \tag{12}$$

Finally, by combining equations (6) and (12), the conversion of *A* can be written as:

$$X_A = 1 - e^{-KC_E t} \tag{13}$$

Both equations (12) and (13) provide the progress of the reaction with time using different enzyme concentrations when the two reagents are in a 1:1 molar ratio.

3.2. Intrinsic kinetic parameters

3.2.1. Obtaining V_{max} and checking the model

From equation (2) the reaction rate, for the series where the reagents are in stoichiometric relation and for the initial values, can be rewritten as follows:

$$v_0 = \frac{V_{\max} C_{A0}}{K_M^A + K_M^B + C_{A0}} \tag{14}$$

By linearizing equation (14) we obtain:

$$\frac{1}{v_0} = \frac{K_M^A + K_M^B}{V_{\max}} \frac{1}{C_{A0}} + \frac{1}{V_{\max}} \tag{15}$$

According to equation (15), from the linear fitting of $1/v_0$ *versus* $1/C_{A0}$, for different initial concentrations of both substrates at constant molar ratio of 1:1, it is possible to obtain the values of the sum of the Michaelis constants, $K_M^A + K_M^B$, and the maximum reaction rate, $V_{\max}$. This allows us to accept or reject the hypothesis that the sum of both substrate constants is much higher than the concentration of substrate *A*.

But, for the application of equation (15), values of initial reaction rate for the different values of initial substrate concentration are necessary. Theoretically, these values could be obtained by applying the pseudo-first order kinetic equation (3) to the initial concentration of substrate, *A*, using the value of the kinetic constant, *k*, obtained in the fitting of experimental conversions of this series to equation (12), as follows:

$$v_0 = kC_{A0} \tag{16}$$

Also other procedures, including graphical methods, can be used to obtain the initial reaction rate, as it is shown later in section 4.4.

3.2.2. Michaelis constants

For the series with different substrate concentrations, the following relation between the initial concentrations can be established:

$$n = \frac{C_{A0}}{C_{B0}} \tag{17}$$

From equation (2), the initial reaction rate is given by:

$$v_0 = \frac{V_{\max} C_{A0} C_{B0}}{K_M^A C_{B0} + K_M^B C_{A0} + C_{A0} C_{B0}} \tag{18}$$

From equation (18) the inverse of the initial reaction rate can be obtained as follows:

$$\frac{1}{v_0} = \frac{K_M^A C_{B0} + K_M^B C_{A0}}{V_{\max} C_{A0} C_{B0}} + \frac{1}{V_{\max}} \qquad (19)$$

Taking into account the relation established in equation (17), equation (19) can be rewritten as:

$$\frac{1}{v_0} = \frac{K_M^A + K_M^B n}{V_{\max}} \frac{1}{C_{A0}} + \frac{1}{V_{\max}} \qquad (20)$$

As explained above, the values of the initial reaction rate can be obtained by using equation (16), and the values of the parameter k are obtained in the fitting of the conversions of this experimental series to equation (12). And, by substituting equation (16) in equation (20):

$$\left(\frac{V_{\max}}{k} - C_{A0} \right) = K_M^A + K_M^B n \qquad (21)$$

And finally, by using the value of $V_{\max}$ obtained as indicated in section 3.2.1, and by representing ($V_{\max}/k - C_{A0}$) *versus* n, the values of K_M^A and K_M^B can be obtained.

3.2.3. Activation Energy

In chemical kinetics, the effect of temperature on the rate constant is described by the Arrhenius equation:

$$k = k_0 e^{-E_a/RT} \qquad (22)$$

By linearizing this equation, the values of activation energy, E_a, and frequency factor, k_0, can be obtained.

4. Results and Discussion

To study the reaction kinetics, different experimental series were carried out in which temperature, stirring speed, enzyme concentration, initial substrate concentrations with molar ratio 1:1 and initial substrate concentrations with different molar ratios were varied. The experimental results are presented below, together with the theoretical results obtained by fitting to the proposed kinetic model.

4.1. Temperature variation

The results of temperature variation assays are depicted in Figure 1 where dots are the experimental data and continuous lines are the values calculated with the model. Table S1 of supporting information represents the values of the kinetic constant, k, and correlation coefficient, r, for the different experiments.

Figure 1 shows that the conversion increases when the temperature increases, according to equation (22). Enzymes usually present an optimal temperature range above which they are denatured, which negatively affects the reaction rate and hence conversion. Figure 1 shows that this optimum value was not reached because, for the three assayed temperatures, the conversion increased. The literature describes that Novozym®435 is able to work at high temperatures[21], presenting optimal values up to 90 ºC. In the present study, it was not possible to work at high temperatures due to the high volatility of both substrate and product. For this reason, a temperature of 40 ºC was chosen as the optimal for the following experimental series.

Through the linear representation of equation (22) a value of 27.3 $kJmol^{-1}$ was obtained for the activation energy, E_a, with a correlation coefficient of 0.9654. The obtained activation energy is

of the same order as that obtained by Uppenberg et al.[22] for this kind of reaction (between 3.762 and 36.72 kJmol^{-1}).

4.2. Stirring speed variation

Experimental and fitted data for different stirring speeds are shown in Figure 2.The experimental conversion values were fitted to equation (12); dots are the experimental data and continuous lines the values calculated with the model. The values of the kinetic constant, k, and the correlation coefficients are shown in Table S2 of supporting information.

It is well known than external diffusional limitations can be reduced when stirring increases.[16]As it can be seen in Figure 2, there is a big conversion difference as stirring speed increases from 0 rpm to 100 rpm, while the differences between 100 and 500 rpm were not so important. Therefore, 300 rpm has been chosen as the optimal stirring speed of the reaction because values below 300 rpm provided lower conversion values and stirring above 500 rpm led to an excess of turbulence that can affect enzyme activity.

4.3. Enzyme concentration variation

Once temperature and stirring had been fixed at 40 ºC and 300 rpm, respectively, variations in enzyme concentration were studied. Figure 3 presents the experimental data and those fitted to equation (12), while Table S3 in supporting informationshows the values of the kinetic constants and their correlation coefficients for the different enzyme concentrations.

As it can be observed the higher the enzyme concentration is, the greater the conversion. When the pseudo-first order kinetic constant was plotted against enzyme concentration, C_E, the linearity of the obtained fitting confirmed that the proposed kinetic model is valid. A value of K

= 0.0494 ($L \cdot g^{-1} \cdot min^{-1}$) was obtained by fitting to equation (6) with a correlation coefficient of 0.9982. Finally, conversion values for all the experimental points of this series were fitted to equation (13). The results depicted in Figure 4 comply with the expected behaviour. A new value of K = 0.047 ($L \cdot g^{-1} \cdot min^{-1}$) was obtained with a correlation coefficient of 0.9982, representing a difference of 4.8% with respect to the one obtained by linearization to equation (6) of the pseudo-first order kinetic constant.

4.4. Initial substrate concentration 1:1 variation

Figure 5 shows the result of the experimental fitting of the experimental conversion data to Eq. (12) for the series of equal initial substrate concentrations. The points correspond to the experimental data and the solid lines to the values calculated by the model.

As expected for a pseudo-first order kinetics, the initial concentration of the substrates had not significant influence on the conversion. The values of k and the corresponding values for the correlation coefficients are shown in Table S4 of supporting information. The average value of k was 0.0165min^{-1}.

Although the value of k decreased slightly with increasing concentrations, the variation was not significant, and so all the conversion data can be fitted to equation (12), with an unique value of k = 0.0164 min^{-1}, very close to the average value, and a correlation coefficient of 0.9975.

To obtain the values of the initial reaction rate for this series, a graphical method shown in Figure 6 has been used. In Figure 6, the concentration values for the product P, for the different initial concentrations of A and times of less than 10 minutes, were plotted, and a family of

straight lines was obtained. From the slope of the line for each initial concentration the initial reaction rate, v_0, was calculated. The values are included in Table S5 of supporting information.

According to equation (15), in Figure 7 the inverse of the initial reaction rate,$1/v_0$, was plotted *versus* the inverse of the initial concentration, $1/C_{A0}$, and from the slope and the intercept the following values were obtained with a correlation coefficient of 0.9999:

$$\frac{K_M^A + K_M^B}{V_{\max}} = 59.02 \text{ min}; \qquad \frac{1}{V_{\max}} = 0.0609 \text{ min mM}^{-1}$$

And, from these values, the following maximum reaction rate and sum of Michaelis constants were obtained:

$$V_{\max} = 16.42 \text{ mM min}^{-1}; \qquad K_M^A + K_M^B = 969.1 \text{ mM}$$

This result agrees with the hypothesis that the sum of the Michaelis constants is much greater than the concentration of substrate *A*, but it should not be used for initial concentrations higher than 100 mM because this would produce an error higher than 10%. This may explain the slight decrease in the value of the constant *k* when there was an increase in the initial concentration. However, the error would hardly reach 2.5% with an initial concentration of 25 mM.

4.5. Molar ratio variation

4.5.1. Vinyl acetate concentration variation

Figure 8 shows the fitting of the experimental conversion data to equation (12) for the series of variation of initial vinyl acetate concentrations, while maintaining the initial 2-phenylethanol concentration constant. As it can be seen in the figure, although the substrates are not in molar

ratio 1:1 in this series, the pseudo-first order rate equation has proven to be also valid and an excellent fitting to equation (12) was obtained. The points correspond to the experimental data and the continuous lines to the values calculated by the model, as in the previous series. The values of the constant *k* and the values of the correlation coefficient are shown in Table S6 of supporting information. In addition, an average *k* was calculated.

The values of *k* for n < 1 do not change much when the initial concentration of vinyl acetate increases with respect to the initial 2-phenyl ethanol concentration. It is possible to make a single fitting of these data to equation (12), with an average value of $k = 0.0182$ $min^{-1}$and a correlation coefficient of 0.9981.

Otherwise, as Figure 9 shows, from the slope and intercept obtained in the fitting of experimental data to equation (21) and the value of V_{max} calculated in section 4.4, the following values of both Michaelis constants were obtained:

$$K_M^B = 96.757 \text{ mM}$$

$$K_M^A = 836.01 \text{ mM}$$

The sum of both constants is 932.8 mM, which implies a difference of 3.75% with respect to the sum obtained in section 4.4 using equation (15). Bansode et al.[14], in the synthesis of isoamyl butyrate, using immobilized lipase and applying a Ping Pong Bi Bi model, obtained a value of 636 mM for the Michaelis constant of isoamyl alcohol, close to the one obtained for the 2-phenylethanol in this work.

4.5.2. 2-Phenylethanol concentration variation

Figure 10 shows the results of fitting the experimental data conversion to equation (12) for the series of variation of initial 2-phenylethanol concentration keeping constant the initial concentration of vinyl acetate. Again, the points correspond to the experimental data and the solid lines to the values calculated by the model. Values of the constant *k* and the correlation coefficient are shown in Table S7 of supporting information.

As it can be seen in Table S7, in this series there are significant differences in the values of the parameter *k*, and a single fitting cannot be applied. In a similar way, in a study of 2-phenylethyl acetate synthesis Kuo et al.[15] showed that increasing the concentration of 2-phenylethanol, while keeping the vinyl acetate concentration constant, increased the conversion, as shown in this work. This can be a consequence of the Le Chatelier principle, because the excess of 2-phenylethanol can displace the equilibrium to the formation of 2-phenylethyl acetate.

5. Conclusions

The transesterification reaction between 2-phenylethanol and vinyl acetate for the enzymatic synthesis of 2-phenylethyl acetate (rose aroma), using Novozym® 435 as catalyst, was carried out in hexane medium. Five experimental series were performed and the influences of the different operational variables were analyzed. A pseudo-first order kinetic model, obtained as a simplification of a Ping-Pong Bisubstrate kinetics, was proposed and validated with high correlation coefficients.

A maximum conversion of 2-phenylethyl acetate of 95.42% was obtained at 120 min in the optimal experimental conditions: 0.6 mg mL^{-1} of enzyme, 40ºC, 300 rpm and substrate concentrations of 100 mM and 25 mM of 2-phenylethanol and vinyl acetate, respectively, the last one being the limiting reagent.

As expected, the concentration of catalyst was proven to be the most significant variable, with the first order apparent kinetic constant varying linearly with the enzyme concentration. On the other hand, it was observed that the initial substrate concentration has no significant influence. However, variations in the substrates molar ratio had an effect, being more pronounced as the 2-phenyethanol increased with respect to vinyl acetate. Temperature affected the enzymatic process, and the maximum value tolerated by the catalyst was not reached due to the high volatility of the products, because the use of pressurized reactors is recommended in future application of the process.

A methodology to calculate the kinetic parameters, able to be applied in other similar processes, has been established, and the Michaelis constants of both substrates and the maximum reaction rate, for the range of assayed experimental conditions of temperature (40 C), stirring speed (300 rpm) and enzyme concentration (0.35 mg mL^{-1}), were determined (K_M^A = 836.01 mM , K_M^B = 96.757 mM and V_{max}= 16.42 mM min^{-1}). The proposed kinetic model was validated with correlation coefficients higher than 0.99 for all experimental series.

The enzymatic synthesis of rose aroma could be developed on a larger scale and the knowledge gained herein of the kinetics of the reaction would help in the scaling up and design of the reactor to obtain aromas by biocatalytic synthesis.

FIGURES

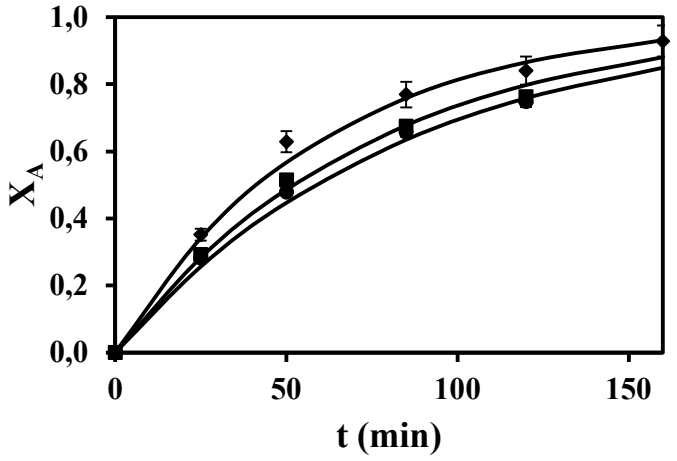

Figure 1. Experimental (points) and theoretical conversions (lines)*versus* time, according to equation (12).Temperature (°C): (♦)40, (■)35, (●)30; catalyst concentration: 0.35 mg mL^{-1}; initial concentration: 25 mM; molar ratio: 1:1; and stirring speed: 300 rpm.

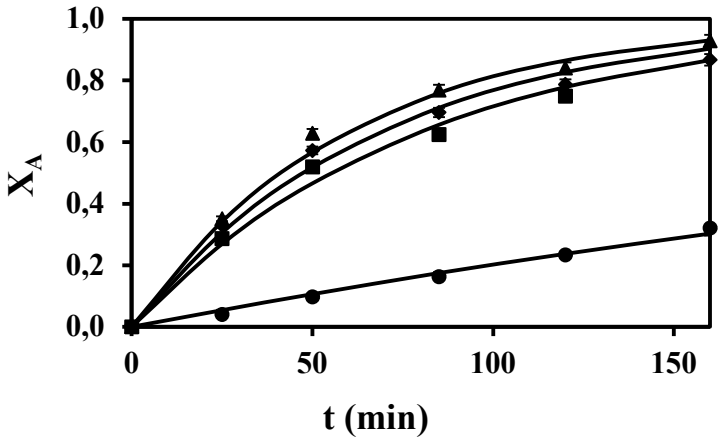

Figure 2. Experimental (points) and theoretical conversions (lines) *versus* time, according to equation (12).Stirring speed (rpm): (▲)300,(♦)500, (■)100, (●)0; catalyst concentration: 0.35 mg mL^{-1}; initial concentration: 25 mM; molar ratio: 1:1; and temperature 40 °C.

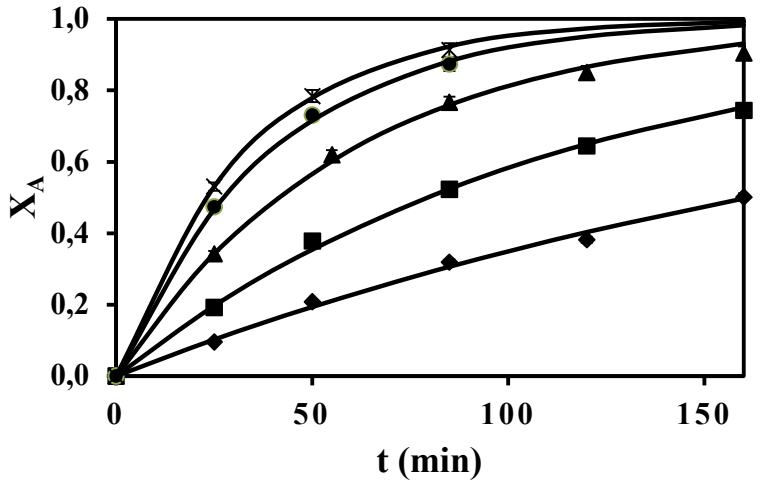

Figure 3. Experimental (points) and theoretical conversions (lines) *versus* time, according to equation (12).Catalyst concentration (mg mL^{-1}): (♦) 0.1, (■)0.2,(▲)0.35,(●)0.5,(x)0.6; molarratio: 1:1; initial concentration: 50 mM; temperature: 40 °C; and stirring speed: 300 rpm.

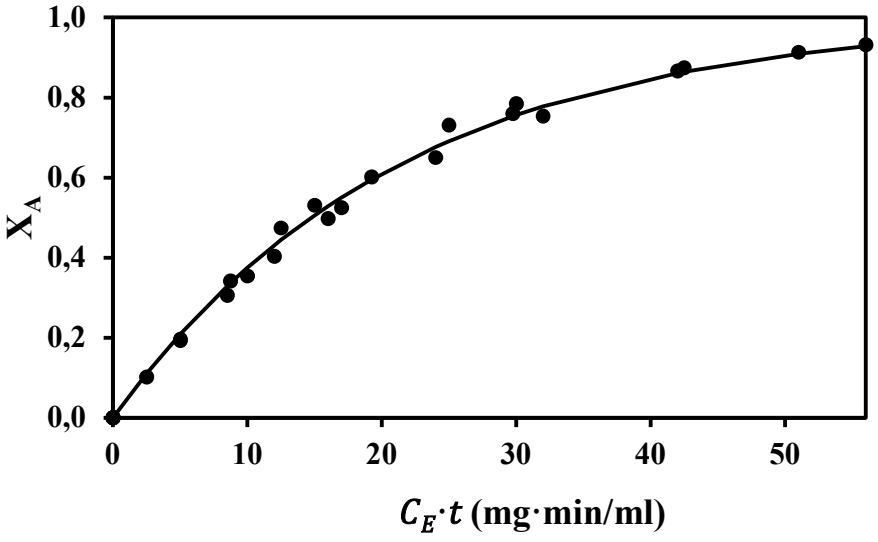

Figure 4. Fitting of X_A with $C_E \cdot t$, according to equation (13), for the data of Figure 3.

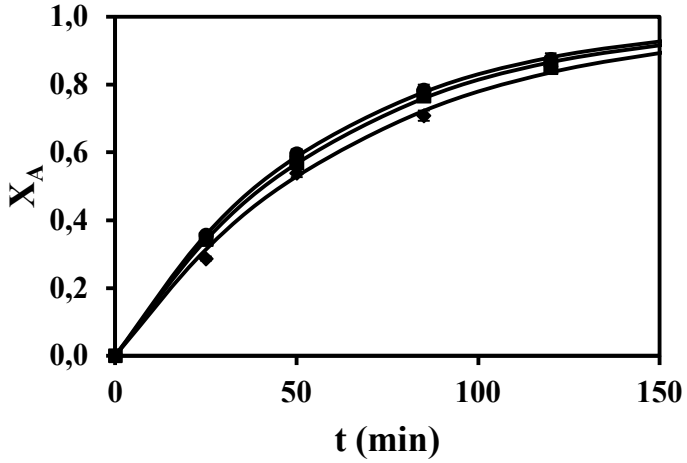

Figure 5. Experimental (points) and theoretical conversions (lines)*versus* time, according to equation (12).Initial concentration (mM): (♦)25, (■)50, (●)100.Catalyst concentration: 0.35 mg mL^{-1}; molar ratio: 1:1; temperature: 40 °C; and stirring speed: 300 rpm.

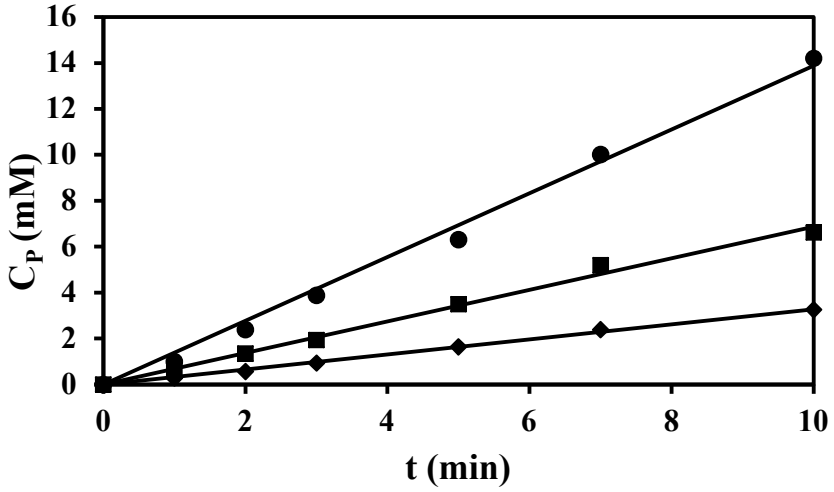

Figure 6. Concentration of2-phenylethylacetate (points)*versus* time. The slope of the line represents the initial reaction rate. Initial concentration (mM): (♦)25, (■)50, (●)100.Catalyst concentration: 0.35 mg mL^{-1}; molar ratio: 1:1; temperature: 40 °C; and stirring speed: 300 rpm.

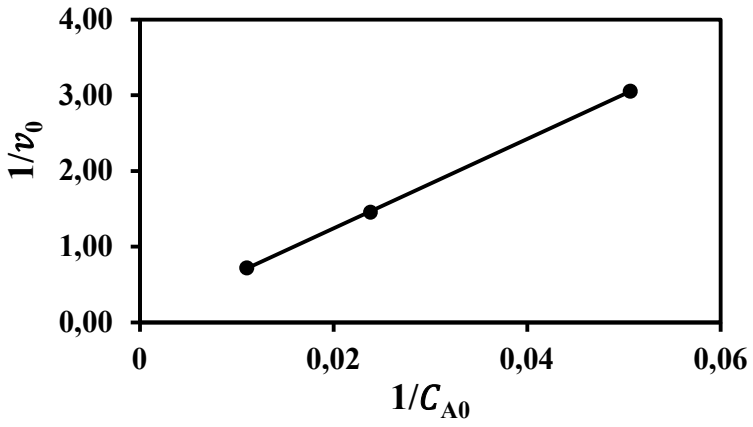

Figure 7. Representation of $1/v_0$ *versus* $1/C_{A0}$, according to equation (15).

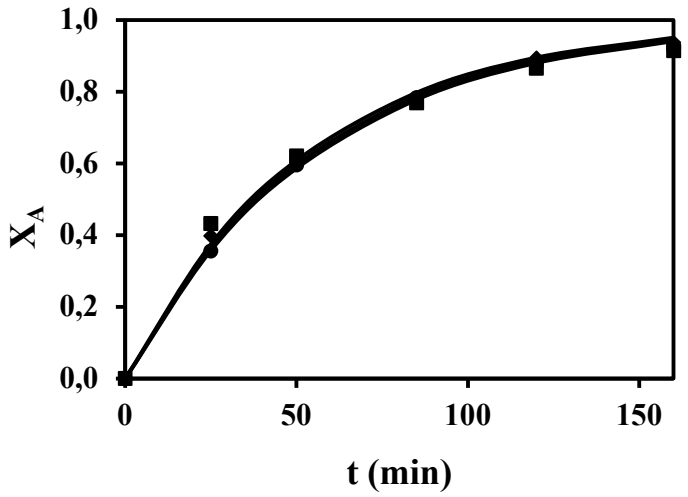

Figure 8. Experimental (points) and theoretical conversions (lines) *versus* time, following equation (12).Parameter$n = (C_{A0}/C_{B0})$: (♦)1/4, (■)1/2, (●)1; catalyst concentration: 0.35 mg mL^{-1}; initial concentration of 2-phenethyl alcohol: 25 mM temperature: 40 °C; and stirring speed: 300 rpm.

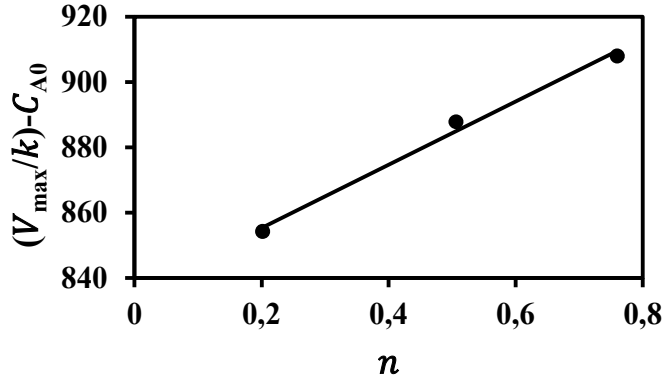

Figure 9. Representation of ($V_{max}/k - C_{A0}$) *versus* n, according to equation (21).

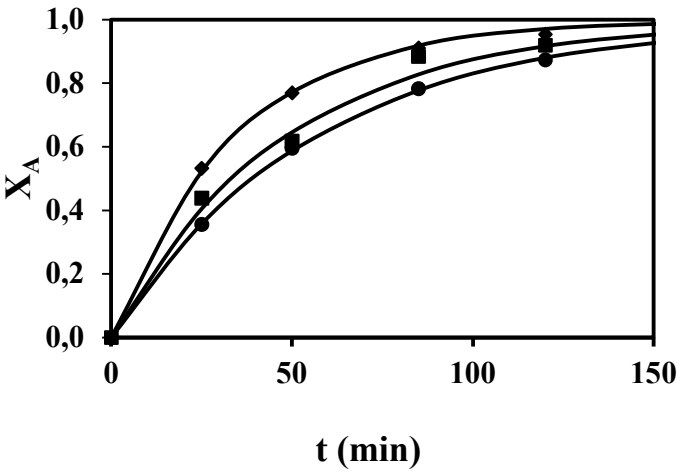

Figure 10. Experimental (points) and theoretical conversions (lines) *versus* time, following equation (12).Parameter $n = (C_{A0}/C_{B0})$: (♦)4, (■)2, (●)1; catalyst concentration: 0.35 mg mL^{-1}; initial concentration of vinyl acetate: 25 mM; temperature: 40 °C; and stirring speed: 300 rpm.

ASSOCIATED CONTENT

Supporting Information.

Table S1. Kinetic constants and correlation coefficients for temperature variation series.

Table S2. Kinetic constants and correlation coefficients for stirring speed variation series.

Table S3. Kinetic constants and correlation coefficients for enzyme concentration variation series.

Table S4. Kinetic constants and correlation coefficients for initial substrate concentration variation in molar ratio 1:1.

Table S5. Initial rates and correlation coefficients for initial substrate concentration variation in molar ratio 1.1.

Table S6. Kinetic constants and correlation coefficients for initial vinyl acetate concentration variation.

Table S7. Kinetic constants and correlation coefficients for initial 2-phenylethanol variation.

AUTHOR INFORMATION

Corresponding Author

*Asunción M. Hidalgo, Email: ahidalgo@um.es. Tel.: +34868887355; fax: +34868884148

Author Contributions

The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript.

Notes

The authors declare no competing financial interest.

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ABBREVIATIONS

A: 2-phenylethanol

B: vinyl acetate

C_A: 2-phenylethanol concentration, (mM)

C_{A0}: initial 2-phenylethanol concentration, (mM)

C_B: vinyl acetate concentration, (mM)

C_{B0}: initial vinyl acetate concentration, (mM)

C_E: enzyme concentration, (g L^{-1})

C_P: 2-phenylethyl acetate concentration, (mM)

E_a: activation energy, (J mol^{-1})

k: pseudo-first order kinetic constant, (min^{-1})

k_0: frequency factor, (min^{-1})

K: constant defined in equation (7), ($L \cdot g^{-1} \cdot min^{-1}$)

K_E: specific activity of enzyme, (mmoles min^{-1} g^{-1})

K_M^A :Michaelis constant of 2-phenylethanol, (mM)

K_M^B :Michaelis constant of vinyl acetate, (mM)

R: gas universal constant, (8.3143 J $^oK^{-1}$ mol^{-1})

P: 2-phenylethyl acetate

T: temperature, (^oK)

v: reaction rate, (mM·min^{-1})

v_0: initial reaction rate, (mM·min^{-1})

V_{max}: maximum reaction rate, (mM·min^{-1})

X_A: conversion,

REFERENCES

[1] https://www.marketsandmarkets.com/Market-Reports/flavors-fragrance-market

[2] Yadav G and Mehta P, Heterogeneous catalysis in esterification reactions: preparation of phenethyl acetate and cyclohexyl acetate by using a variety of solid acidic catalysts. *Ind. Eng. Chem. Res.* **1994**, 33, 2198–2208.

[3] Kuo, C. H.; Chiang, S. H.; Ju, H. Y.;Chem, Y. M.; Liao,M. Y.; Liu, Y. C.; Shieh, C. J.Enzymatic synthesis of rose aromatic ester(2-phenylethyl acetate) by lipase. *J. Sci. Food Agric.***2012**, *92*, 2141-2147.

[4] Wang, Y.; Zhang, D. H.; Zhang, J. Y.; Chen, N.;Zhi, G. Y. High-yield synthesis of bioactive ethyl cinnamate by enzymatic esterificationof cinnamic acid. *Food Chem*. **2016**, *190*, 629-633.

[5] Kim, H.; Park, C. Enzymatic synthesis of phenethyl ester from phenethylacohol with acyl donor. *Enzyme Microb. Tech.* **2017**, *100,* 37-44.

[6] Sharma, S.;Kanwar, S. S. Organic solvent tolerant lipases and applications. *Sci. World J.*2014 (Article ID 625258).

[7] Bialecka-Florjanczyk, E.;Krzyczkowska, J.;Stolarzewicz, I.;Kapturowska, A. Synthesis of 2-phenylethyl acetate in the presence of *Yarrowialipolytica* KKP 379 biomass. *J. Mol. Cat. B-Enzym.***2012**, *74*, 241-245.

[8] Cao, L.;Langen, L.; Sheldon, R. A.Immobilised enzymes: carrier-bound or carrier-free?*Curr.Opin. Biotech.***2013**,*14(4)*, 387-394.

[9] Liu, K. J.; Huang, Y.R. Lipase-catalyzed production of a bioactive terpene ester in supercritical carbon dioxide. *J.Biotechnol.***2010**,*146*, 215-220.

[10] Yadav, G.; Dhoot, S. Immobilized lipase-catalysed synthesis of cinnamyl laureate in non-aqueous media. *J. Mol. Cat. B-Enzym.***2009**, *57*, 34-39.

[11] Sun, S.; Shan, L.; Liu, Y.;Jin, Q.; Song, Y.; Wang, X. Solvent-free enzymatic synthesis of feruloylated diacylglycerols and kinetic study. *J. Mol. Cat. B-Enzym.* **2009**, *57,* 104-108.

[12] Badgujar, K. C.;Bhanage, B. M. Application of lipase immobilized on the biocompatibleternary blend polymer matrix for synthesis of citronellyl acetate in non-aqueous media: Kinetic modeling study. *Enzyme Microb. Tech.* **2014**, *57*, 16-25.

[13] Gawas, S. D.; Jadhav, S. V.; Rathod, V. K. Solvent free lipase catalysed synthesis of ethyl laureate: optimization and kinetic studies. *Appl.Biochem.Biotechnol.* **2016**, *180*, 1428-1445.

[14] Bansode, S. R.;Hardikar, M. A.; Rathod, V. K. Evaluation of reaction parameters and kinetic modeling for Novozym® 435 catalyzed synthesis of isoamyl butyrate. *J. Chem.Technol.Biotechnol.* **2017**,*92,* 1306-1314.

[15] Kuo, C. H.; Chen G. J.;Chem, C. I.; Liu, Y. C.; Shieh, C. J. Kinetics and optimization of lipase-catalyzed synthesis of rose fragrance 2-phenylethyl acetate through transesterification.*Process Biochem.* **2014**,*49*, 437-444.

[16] Xiong, J.; Huang, Y.; Zhang, H.; Hou, L.;Lipase-catalyzed transesterification synthesis of geranyl acetate in organic solvents and its kinetics. *Food Sci.Technol. Res.***2014**,*20(2)*,207-216.

[17] Xiong, J.; Huang, Y. J.; Zhang, H. Lipase-catalyzed transesterification synthesis of citronellyl acetate in a solvent-free system and its reaction kinetics. *Eur. Food Res. Technol.* **2012**,*235,* 1-5.

[18] Yadav, G. D.; Lathi, P. S. Lipase catalyzed transesterification of methyl acetoacetate with n-butanol. *J. Mol.Catal. B.***2005**, *32,* 107-113.

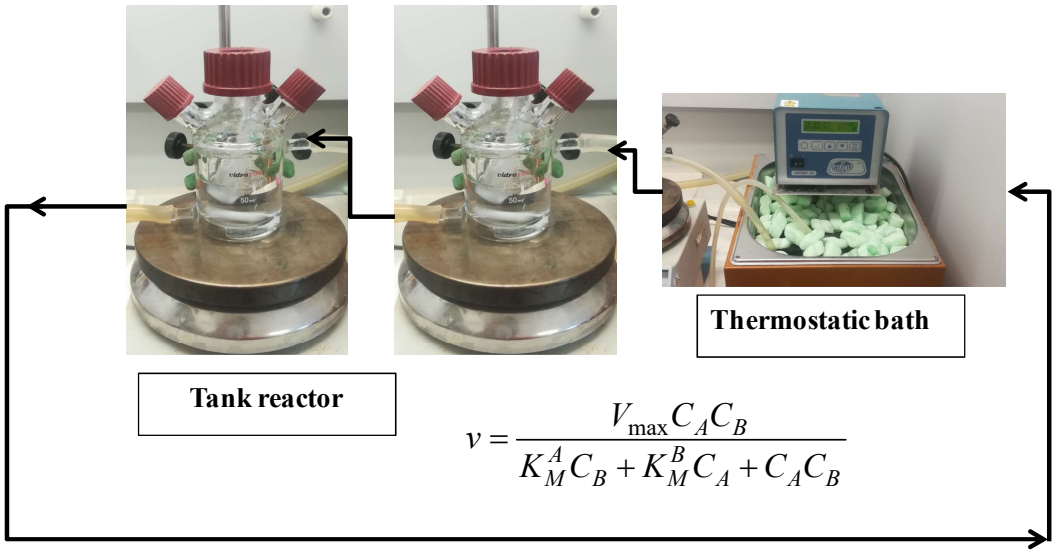
[19] Gómez, J. L.;Bódalo, A.; Gómez, E.; Hidalgo, A. M.; Gómez, M.A new method to estimate intrinsic parameters in the Ping-pong bisustrate kinetic: Application to the oxipolymerization of phenol. *Am. J.Biochem.Biotechnol.* **2005**,*2,* 115-120.

[20] Gómez, J. L.;Bódalo, A.; Gómez, E.;Bastida, J.; Hidalgo, A. M.; Gómez, M.A covered particle deactivation model and an expanded Dunford mechanism for the kinetic analysis of the immobilized SBP/phenol/hydrogen peroxide system. *Chem.Eng. J.* **2008**, *138*, 460-473.

[21] Kirk, O.; Christensen, M. W. Lipases from *Candida Antarctica*: Unique Biocatalysts from Unique Origin. *Organic Process Research & Development.* **2002**,*6*, 446-451.

[22] Uppenberg, J.;Patkar, S.;Bergfors, T.; Jones, T. A.; Crystallization and preliminary X-ray studies of lipase B from *Candida antarctica*. *J. Mol. Biol.***1994**,*235,* 790-792.

TOC


Thermostatic bath
Tank reactor
$v = \frac{V_{\max} C_A C_B}{K_M^A C_B + K_M^B C_A + C_A C_B}$