Kinetic modelling and kinetic parameters calculation in the lipase-catalysed synthesis of geranyl acetate.

M.D. Murcia, M. Gómez, E. Gómez, J.L*. Gómez, A.M. Hidalgo, A. Sánchez, P. Vergara

Chemical Engineering Department. University of Murcia. Spain.

*Corresponding author. Email: carrasco@um.es. Phone: +34 868887351. Fax: +34 868884148.

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Abstract

The interest in new natural products used in cosmetic industry has increased the research in order to synthesize those compounds. The aim of this work is to assess the reaction of the transesterification between geraniol and vinyl acetate for the enzymatic synthesis of geranyl acetate, using Novozym® 435 as catalyst, as well as to obtain a kinetic model for the bioprocess and the values of the kinetic parameters. Five experimental series have been performed with variation of: enzyme amount, concentrations of geraniol and vinyl acetate in molar ratio 1:1, stirring rate, temperature and, finally, substrates molar ratio. A conversion of geranyl acetate of 98.4% has been obtained in the best experimental conditions and, also, high conversions were obtained for most of the other conditions assayed. It has been concluded that the catalyst amount is the most significant variable on the reaction kinetics and the reaction yield and that both the stirring rate and the initial substrates concentrations have no significant

influence. Finally, a Ping Pong Bisubstrate kinetic model has been applied and its kinetic parameters have been determined by using an improved version of a procedure developed by authors in a previous work. The values obtained for the Michaelis constants shown that, for the equal substrates concentrations series, the Ping-Pong model can be simplified to a pseudo-first order kinetic model, which has been confirmed, with high correlation coefficients, by fitting the experimental results of this series to the simplified model using the "Curve Expert" software.

1. INTRODUCTION

Geraniol is a monoterpene alcohol linked to a hydroxyl group. It is commercially very important because it is present in the vast majority of essential oils extracted from aromatic plants. Is, therefore, one of the most important alcohols in the aromas industry and the fragrances, and it has anti-infective, immunostimulant properties, bactericides, and acts as a pesticide agent with low toxicity (Marquez, 2004; Rojas et al., 2009). Against the benefits of this alcohol, geraniol has a certain degree of toxicity, which limits its use in the direct application in cosmetic products. However if this alcohol is transformed into its ester, geranyl acetate could be used in the cosmetics industry presenting the benefits of geraniol and without contributing with any toxicity to the resulting product (Tang et al., 2012; Xiong et al., 2014).

Geranyl acetate is widely used in industry mainly due to its organoleptic properties, being one of the most valuable geranyl esters. It presents a sweet fruity flavour and rose and lavender aroma, being classified as an edible scent so that its use is also allowed in the food industry (Xiong et al., 2014). It can be found directly also in the essential oils of some food such as lemon, almond or ginger, and among its main uses is the food

industry, manufacture of detergents, cosmetics and perfumes, and pharmaceutical industry (Chen et al., 2002).

Geranyl acetate can be isolated by vacuum fractionation from essential oils. The limited availability of these natural raw materials makes this method not suitable for large-scale industrial production. As an alternative, these esters may be produced by chemical synthesis and enzymatic extraction or catalysis (biocatalysis). Enzymatic synthesis presents numerous advantages for the production of different types of substances due to: greater specificity in the formation of products, better recovery and purification after the synthesis, lower energy consumption and lower by-products formation. Several enzymes can be used in biocatalysis, such as lipases, which are especially prominent due to their great versatility. Lipases can catalyze reactions of triacylglycerol hydrolysis, inter and transesterification, ester synthesis, alcoholysis, aminolysis, peroxidation, epoxidation, etc. (García et al., 1996; García et al., 2000; Sovová and Zarevúcka, 2003; Yadav and Devi, 2004; Camacho et al., 2007; Gupta et al., 2013)

Lipases are often used in the chemical industry to obtain products with high added value, as in the case of cosmetics. Furthermore, in an organic medium they provide a high yield in relation to the synthesis of an organic compound (Badgujar and Bhanage, 2014) and, also, they can work in solvent-free systems (Bódalo et al., 2008; Bódalo et al., 2009; Gómez et al., 2011a). Lipases from different sources have been used to study the synthesis of geranyl acetate: lipase from *Pseudomonas sp.* (Yee and Akoh, 1996), from *Mucor miehei* (Chulalaksananukul et al., 1992; Chulalaksananukul et al., 1993), from *Pseudomonas fluorescens* (Xiong et al., 2014), or lipase from *Candida rugosa* (Rosa et al., 2017).

Among all lipases, and despite it has not been used until now in the synthesis of geranyl acetate, the most commonly known and used currently is 1,3-lipase from *Candida antartica B*, commercially known as Novozym® 435, due to the company that commercializes it, Novozymes. This type of enzyme is considered according to the regulations of the FDA (Food and Drugs Administration) as a GRAS (Generally Recognized as Safe) enzyme (Rivera-Pérez and García-Carreño, 2007).

Although many papers have studied the transesterification of geraniol to obtain geranyl acetate with different enzymes (free or immobilized) and in different medium (ionic liquid, supercritical CO_2) only a few studies have been found about the kinetic of the reaction. The transesterification reaction has been described by a pseudo first-order reaction, by the Michaelis-Menten kinetics of single substrate (Mahapatra et al., 2009). Other authors have proposed a mechanism for the transesterification synthesis of geranyl acetate based on the Ping-Pong Bi-Bi model without substrate and product inhibition. A kinetic model was established and the corresponding optimal kinetic constants were calculated (Xiong et al., 2014).

The purpose of this research is to study the reaction of the transesterification between geraniol and vinyl acetate for the enzymatic synthesis of geranyl acetate in a hexane medium using Novozym® 435 as catalyst. Besides, a Ping Pong Bisubstrate mechanism has been applied and its kinetic parameters have been calculated by using an improved version of a procedure developed by authors in a previous work. From the values of the kinetic parameters, and for the series with substrates molar ratio 1:1, a pseudo-first

order kinetic model, derived as a simplification of the Ping Pong mechanism, has been used and validated with high correlation coefficients.

2. MATERIALS AND METHODS

2.1. Chemicals

Geraniol ($C_{10}H_{18}O$) (98%), Vinyl acetate ($C_4H_6O_2$) that contains 3-20 ppm hydroquinone as inhibitor (≥ 99%), Geranyl acetate ($C_{12}H_{20}O_2$) (≥ 97%) were obtained from Sigma-Aldrich. n-Hexane (95%) was purchased from Panreac Aplichem. Novozym® 435 (immobilized *Candida antarctica* lipase B), was kindly provided by Novo Nordisk AS (Copenhagen, Denmark). Other chemicals were of analytical grade and were used without further purification.

2.2. Experimental procedure

Experiments were conducted in a jacketed batch reactor of 50 ml total volume. The experimental procedure was always the same, the amount of vinyl acetate and geraniol needed for each experiment together with n-hexane until complete the final volume of 50 mL were placed in the reactor. It is important to take into account that, since very volatile compounds were used, once the solution was prepared the reagents were quickly returned to the fridge to avoid losses by compounds volatilization.

At the same time, the reactor was connected to the thermostatic bath adjusting the temperature at the desired value and waiting for the system to stabilize. The enzyme

amount needed in each test was weighed and introduced into a porous nylon mesh carefully closed to ensure there was no enzyme loss, which is essential in order to prevent the introduction of enzyme particles into the column of the chromatograph, once analysis of the sample takes place. Besides, it is important to adjust the height of the mesh inside the reactor, avoiding placing it at the bottom and achieving a good contact of the enzyme with the solution.

When the selected temperature for each test was reached, the solution was introduced into the reactor and a sample of 800 microliters was taken at time 0 for the corresponding analysis and determination of the initial assay concentration. The required stirring rate is adjusted for each experiment, and then the mesh was introduced with the enzyme. Samples were taken at 20, 40, 80, 120 and 160 minutes. It is important to note that, once the samples were taken, the vial and the tank reactor are quickly closed to avoid losses by vaporization. Samples were stored in the fridge before their analysis by gas chromatography.

All the experiments were done in duplicates and average values are presented in this work. The maximum calculated standard deviation was 1.92 and the medium standard deviation for all the data was 0.24.

2.3. Samples analysis

Geraniol and geranyl acetate concentrations were monitored by capillary column GC, using a 7820A Agilent chromatograph equipped with a flame ionization detector (FID). The injection system was split-splitless. A silica capillary column (30 m x 0.32 mm x

0.25 µm) was used. Injector and detector temperatures were set at 250 ºC. Oven temperature was programmed 164 to 180 ºC at a rate of 2 ºC/min. The carrier gas was nitrogen at a flow rate of 1.1 mL/min.

The quantification was based on external calibration using standard solutions of geraniol and geranyl acetate, over the range of 10-50 mM. Retention times for geraniol and geranyl acetate were 3.27 and 3.98 min respectively. The calibration curves for both compounds were the following ones:

$$A_{Gol} = 66.667\ C_{Gol}\ (mM),\ r = 0.9989$$

$$A_{GAc} = 74.074\ C_{GAc}\ (mM),\ r = 0.9986$$

2.4. Experimental series

Five experimental series were done, varying in each one a different operational variable: enzyme amount, substrate concentration in the molar ratio 1:1, stirring, temperature and molar ratio with fixed geraniol concentration at 50 mM. All the conditions for the different experiments are summarized in Table 1. The enzyme amount in series 3 and 4 was lower than the regular one fixed in 50 mg in order to clearly observe the influence of the selected operational variable.

3. KINETIC MODEL

3.1. Reaction

The reaction that takes place consists in the synthesis of geranil acetate by the transesterification reaction between vinyl acetate and geraniol in the presence of the Novozym® 435 lipase catalyst and in a medium of hexane. The reaction stoichiometry is 1:1. One mol of geraniol reacts with one mol of vinyl acetate to give one mol of geranyl acetate and one mol of vinyl alcohol, which tautomerizes very fast to vinyl aldehyde and displaces the reaction to the formation of geranyl acetate.

3.2. Proposed mechanism and reaction rate

From the study of the reaction and the fitting of the experimental data using the "Curve Expert" program, it has been found that the kinetics of the reaction fit well to a pseudo-first order kinetics in all the series where the substrates molar ratio was keep constant and equal to 1:1. This is not a typical kinetics of enzymatic reactions, so it should be a simplification of a more general and true kinetics. According to some references in the literature where other lipases where used (Xiong et al., 2014; Badgujar and Bhanage, 2014), the reaction follows a Ping-Pong Bisustrate mechanism. If this mechanism is assumed the reaction rate can be written as follows:

$$r = \frac{V_m C_{Gol} C_{VAc}}{K_{MVAc} C_{Gol} + K_{MGol} C_{VAc} + C_{Gol} C_{VAc}} \quad (1)$$

The maximum reaction rate, V_m , can be expressed as a function of the amount of enzyme:

$$V_m = k_E m_E \quad (2)$$

Being:

k_E = specific activity of enzyme (mM min^{-1} mg^{-1})

m_E = enzyme amount (mg)

3.3. Reaction rate: particular situations

Taking into account that the stoichiometry of the reaction is 1:1, in the experimental series with stoichiometric amounts of both substrates it is verified that:

$$C_{Gol} = C_{VAc} \tag{3}$$

Under these conditions, equation (1) can be simplified as follows:

$$r = \frac{V_m C_{Gol}}{K_{MVAc} + K_{MGol} + C_{Gol}} \tag{4}$$

In addition, if the following condition is verified:

$$K_{MVAc} + K_{MGol} \geq C_{Gol} \tag{5}$$

Then, equation (4) is reduced to a first order apparent kinetics:

$$r = \frac{V_m}{K_{MVAc} + K_{MGol}} C_{Gol} \tag{6}$$

Equation (6) can be written in abbreviated form as:

$$r = k_0 C_{Gol} \tag{7}$$

Where the apparent constant, k_0 (min^{-1}), of pseudo-first order is:

$$k_0 = \frac{V_m}{K_{MVAc} + K_{MGol}} \tag{8}$$

By substituting the value of V_m given by equation (2) in equation (8):

$$k_0 = \frac{k_E m_E}{K_{MVAc} + K_{MGol}} = k\, m_E \tag{9}$$

From all the exposed before and if the particular conditions previously established are given, the pseudo-first order apparent constant (k_0) must vary linearly with the enzyme amount (m_E) according to equation (9).

3.4. Discontinuous reactor: mass balance

The transesterification reaction is carried out in a thermostatic discontinuous stirred tank reactor, where the following hypotheses can be assumed:

- The reactor behaves like 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.

According to these hypotheses, if r is the geraniol disappearance rate, taking into account that the terms of inlet and outlet are 0 due to the discontinuous system, the mass balance can be expressed as:

$$Accumulation = -Disappearance \tag{10}$$

The mass balance equation (10) can be formulated as:

$$\frac{dC_{Gol}}{dt} = -r \tag{11}$$

For equation (11) the value of the reaction rate is given by Eq. (1) and the initial conditions are:

$$t = 0; \qquad C_{Gol} = C_{Gol0}; \qquad C_{VAc} = C_{VAc0} \tag{12}$$

In particular, if condition (5) is verified, the mass balance differential equation is reduced to:

$$\frac{dC_{Gol}}{dt} = -k_0 C_{Gol} \tag{13}$$

Being the initial condition:

$$t = 0; \qquad C_{Gol} = C_{Gol0} \tag{14}$$

By integrating equation (13) with this initial condition, it is obtained:

$$C_{Gol} = C_{Gol0} e^{-k_0 t} \quad (15)$$

By defining geraniol conversion as:

$$X = \frac{C_{Gol0} - C_{Gol}}{C_{Gol0}} \quad (16)$$

It is obtained:

$$C_{Gol} = C_{Gol0}(1 - X) \quad (17)$$

From equations (15) and (17), the conversion of geraniol is given by:

$$X = 1 - e^{-k_0 t} \quad (18)$$

Equation (18) provides the dependence of the conversion over time for the series with substrates molar ratio 1:1 when the condition expressed in Eq. (5) is verified, and allows analyzing the reaction progress for geraniol. If the above simplifications and assumptions are satisfied for the reaction under study, the conversion data must fit well to equation (18) and additionally, the pseudo-first order apparent constant, k_0, must linearly vary with enzyme concentration, as predicted by equation (9).

4. RESULTS AND DISCUSSION

In this section, the influence of the different operational variables on the reaction progress and the fitting of the experimental data to the kinetic model are analyzed in order to verify its validity. For this purpose, the Curve Expert V1.3 software was used and the kinetic parameters were obtained.

4.1. Influence of enzyme amount

Firstly, enzyme amount has been varied between 10 and 50 mg as it can be observed in Table 1. All the experiments have been done under the same conditions of temperature, stirring and substrates concentration. The reaction volume was kept constant at 50 mL.

Table 1. Experimental conditions for the different series.

Enzyme amount (mg)	Temperature (ºC)	$[Geraniol]_0$ (mM)	$[Vinyl\ acetate]_0$ (mM)	Molar ratio	Stirring (rpm)
Series 1: variation of enzyme amount					
10 20 30 40 50	30	50	50	1:1	300
Series 2: variation of initial substrates concentrations with molar ratio 1:1					
50	30	12.5 25 50 75	12.5 25 50 75	1:1	300
Series 3: variation of stirring					
30	30	50	50	1:1	100 200 400
Series 4: variation of temperature					
20	25 30 35	50	50	1:1	300
Series 5: variation of substrates molar ratio					
50	30	50 50 50	25 50 75	1:0.5 1:1.0 1:1.5	300

Figure 1 shows the experimental conversion and the calculated ones using equation (18) of the proposed kinetic model.

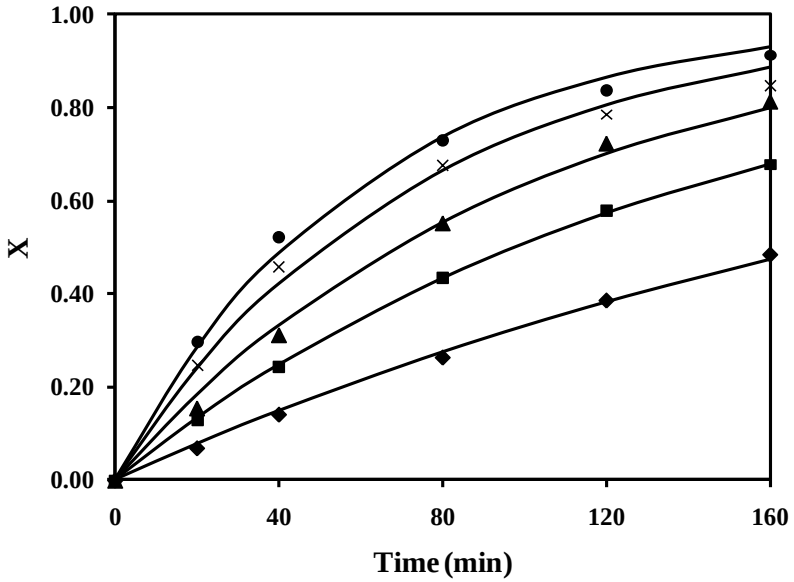

Figure 1. *Experimental and theoretical conversions of geraniol versus time. Experimental conditions: [Geraniol]$_0$ = 50 mM, molar ratio [Geraniol]$_0$:[Vinyl acetate]$_0$ = 1:1, T = 30 ºC, stirring = 300 rpm, V = 50 mL. Enzyme amount = (♦) 10, (■) 20, (▲) 30, (x) 40, (●) 50 mg and (−) model.*

As it can be expected, when enzyme amount is increased the geraniol conversion is also increased. The highest values of geraniol conversion were reached using also the highest enzyme amount. By comparing experimental and calculated conversion shown in Figure 1, it can be concluded that the fitting of experimental data to equation (18) is really good, as confirmed by the correlation coefficients obtained, higher than 0.99 for all experiments of this series.

According to equation (9), the pseudo-first order apparent constant (k_0) must linearly vary with the enzyme amount. Figure 2 shows that the obtained correlation is very high, which leads us to affirm that the proposed model is adequate.

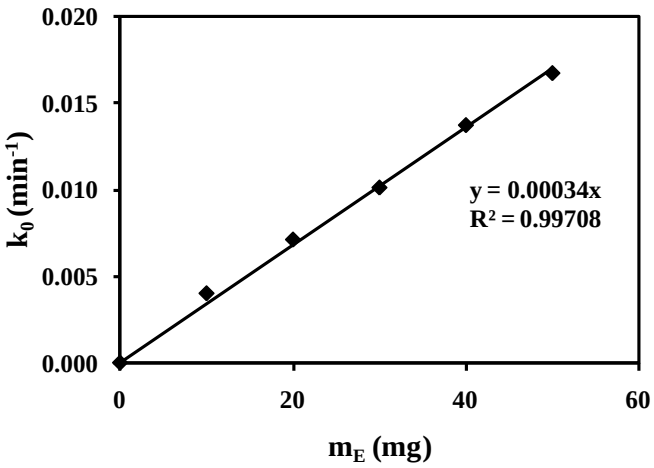

Figure 2. *Fitting of k_0 with m_E, following equation (9).*

The obtained correlation is:

$$k_0 = 0.00034 m_E (min^{-1}) \quad R^2 = 0.9971 \tag{19}$$

Additionally, and by combining equation (9) and (18) the following equation is obtained:

$$X = 1 - e^{-k m_E t} \tag{20}$$

Regarding equation (20), the product of time and enzyme amount ($m_E\ t$) can be used as a new independent variable, whereby said equation predicts, for this series of enzyme variation, a exponential dependence of the conversion versus this new combined variable. The corresponding fitting has been made and the result is shown in Figure 3 and, as it can be seen, the expected behavior is fulfilled and all the conversions corresponding to the five enzyme values tested are grouped in a single curve.

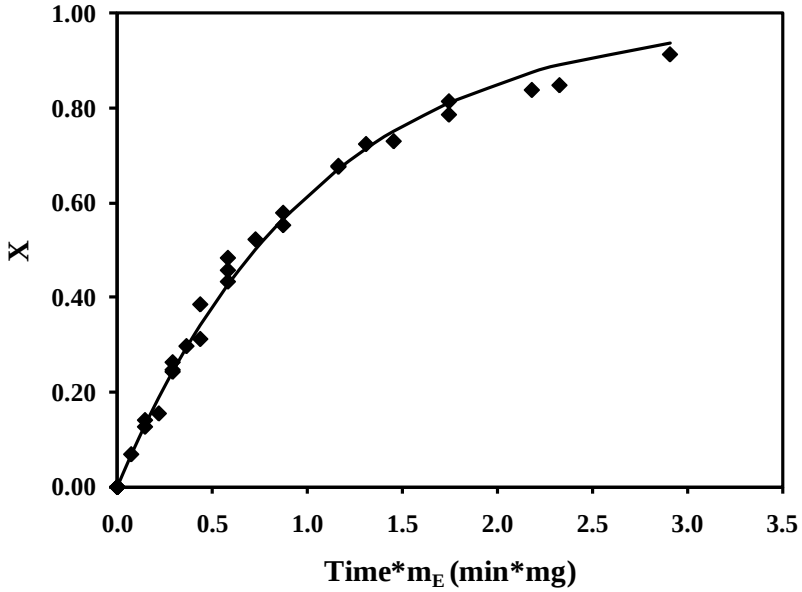

Figure 3. *Fitting of x with m_E t, following equation (20).*

The result of the fitting performed with the Curve-Expert software is shown in equation (21).

$$X = 1 - e^{-0.00035 m_E t} \qquad (21)$$

The correlation coefficient of the fitting was 0.9973, with a standard deviation of 0.022. It is important to highlight that the values for k_0 obtained in equations (19) and (21) are practically equals, which proves again that the proposed kinetic model is accurate.

4.2. Influence of molar substrate concentrations with molar ratio 1:1

In this series, assays have been done for four different substrates concentrations (12.5, 25, 50 and 75 mM) with a molar ratio of 1:1. As in the previous series, experimental data and the calculated from the fitting to a pseudo-first order kinetic are shown in Figure 4. This figure shows that no significant variation in the geraniol conversion is

obtaining when substrate concentration is modified and, by this, for the fitting to the kinetic model, average conversion values of the four experiments were used.

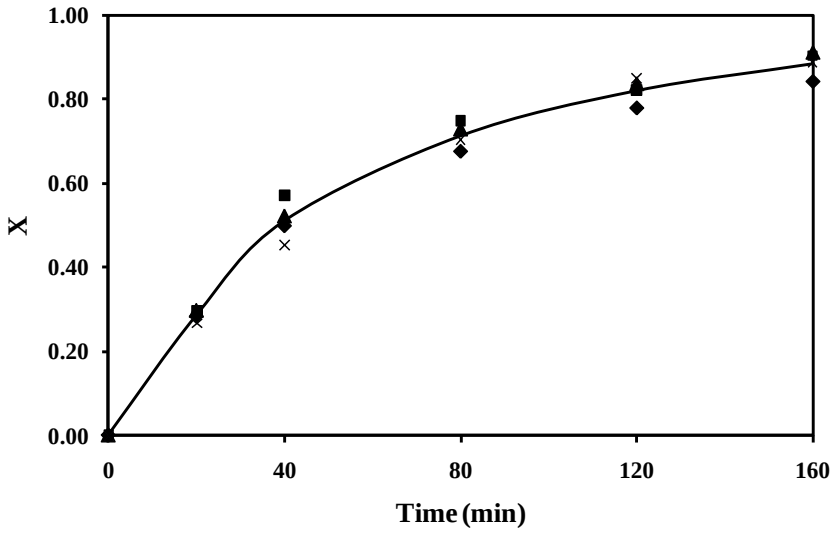

Figure 4. *Experimental and theoretical conversions of geraniol versus time. Experimental conditions: enzyme amount = 50 mg, molar ratio [Geraniol]$_0$:[Vinyl acetate]$_0$ = 1:1, T = 30 ºC, stirring = 300 rpm, V = 50 mL. Initial substrate concentrations = (♦) 12.5, (■) 25, (▲) 50, (x) 75 mM and (−) model.*

According to equation (18) geraniol conversion is not dependent on substrates concentrations and only depends on time and the pseudo-first order kinetic constant, k_0. This result agrees with the one expected for a first order kinetic under isothermal conditions, where the conversion is not a function of the substrate concentrations and therefore, after the obtained results, we can affirm again that the proposed simplified kinetic model is adequate.

In addition, as a consequence of the molar ratio 1.1, the exact reaction rate can be simplified and expressed for this series by equation (4) and, from it, the initial reaction rate is given as follows:

$$r_0 = \frac{V_m C_{Gol0}}{K_{MVAc} + K_{MGol} + C_{Gol0}} \quad (22)$$

From equation (22), the inverse of the initial reaction rate can be obtained:

$$\frac{1}{r_0} = \frac{K_{MVAc} + K_{MGol}}{V_m} \frac{1}{C_{Gol0}} + \frac{1}{V_m} \quad (23)$$

Equation (23) predicts a linear variation between the inverse of the initial reaction rate and the inverse of the initial concentration of geraniol. Also, from the slope and intercept of the above linear relationship, the values of V_m and the sum $K_{MVAc} + K_{MGol}$ can be obtained. To be able to apply equation (23), data of initial reaction rate are necessary, which is not easy due that the progress curve is not a linear function of time. By this, an improved version of a procedure developed in a previous paper by authors (Gomez et al., 2011b) was used. It is based, firstly, on the use, in the linearized form of the Ping-Pong kinetic equation, of several average reaction rates for different initial substrate concentrations, obtained at different reaction times, to obtain a collection of apparent kinetic parameters. After that, it has been observed a linear dependence with time of the collection of apparent kinetic parameters obtained at different time intervals, so their extrapolation to time zero to obtain the true kinetic parameters is possible. With this purpose, and taken into account equation (11) between the accumulation and the reaction rate in a discontinuous reactor, average values of reaction rate, *r(t)*, at time 20, 40, 80, 120 and 160 minutes, have been obtained as:

$$r(t) = \frac{C_{Gol0} - C_{Gol}}{t} \quad (24)$$

The inverse of these values has been plotted versus the inverse of initial concentrations of geraniol, as shown in Figure 5 (A), and a family of straight lines have been obtained, each one corresponding to each value of the reaction time, with high correlation coefficients.

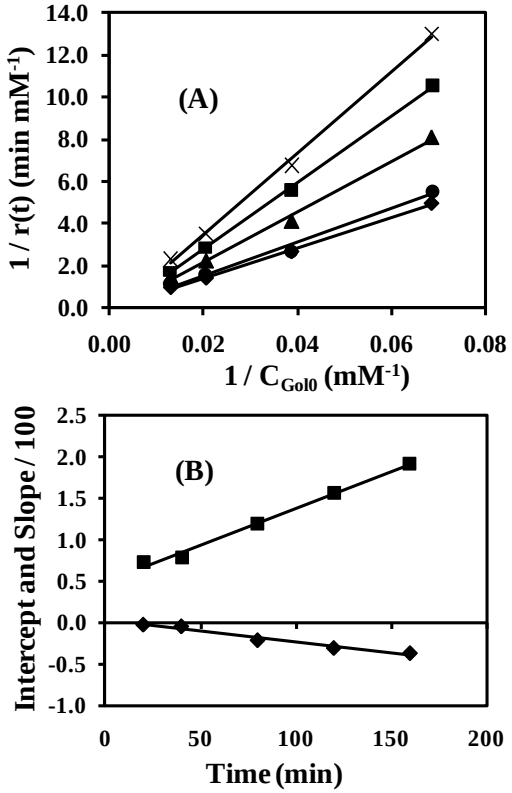

Figure 5. (A): *Inverse of average reaction rate at time t, r(t), versus 1 / C_{Gol0}. Experimental conditions: enzyme amount = 50 mg, molar ratio [Geraniol]$_0$:[Vinyl acetate]$_0$ = 1:1, T = 30 ºC, stirring = 300 rpm, V = 50 mL. Reaction time (minutes): (♦) 20, (●) 40, (▲) 80, (■) 120, (x) 160.* ***(B):*** *Intercept (♦) and Slope / 100 (■) versus time.*

The intercept and slope obtained for each fitting are the apparent values, at time t, of the inverse of V_m and the ratio between the sum of K_{MVAc} + K_{MGol} and V_m , respectively.

After that, these apparent values have been plotted versus time, as it can be seen in Figure 5 (B). From these new fittings, two relationships between the apparent parameters and the reaction time are obtained, which for time zero gives the true values of these parameters as follows:

$$Intercept = \frac{1}{V_m} = 0.023\ mM^{-1}\ min \tag{25}$$

$$Slope = \frac{K_{MVAc} + K_{MGol}}{V_m} = 49.090\ min \tag{26}$$

And, from the equations above, the following values are obtained:

$$V_m = 43.48\ mM\ min^{-1} \tag{27}$$

$$K_{MVAc} + K_{MGol} = 2134.35\ mM \tag{28}$$

Others authors, (Kuo et al., 2014), in the synthesis of 2-phenilethyl acetate by transesterification using lipase of *Candida rugosa*, obtained a value of 2420 mM for the sum of Michaelis constant, very close with to one obtained in this work. Taken into account that the value of C_{Gol} varies from 25 to 75 mM, the value obtained for Michaelis constants sum is 28 times higher than the highest value of C_{Gol}, which demonstrates that the condition expressed in equation (5) is verified and, as a consequence, the approximation to a pseudo-first order reaction for the series with molar ratio 1:1 is valid.

4.3. Influence of stirring

In this series, the effect of the stirring variation on the conversion of the enzymatic reaction for three specific cases: 100 rpm, 200 rpm and 400 rpm, has been studied. The experimental and theoretical results are shown in Figure 6.

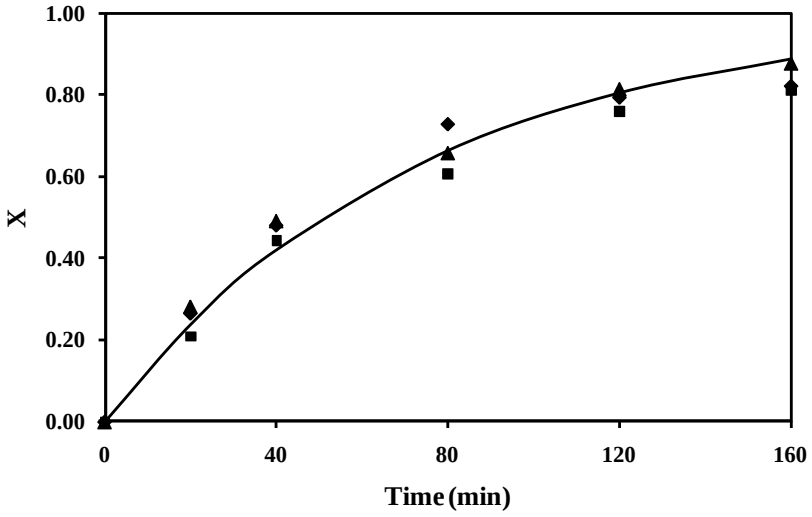

Figure 6. *Experimental and theoretical conversions of geraniol versus time. Experimental conditions: enzyme amount = 30 mg, [Geraniol]$_0$ = 50 mM, molar ratio [Geraniol]$_0$:[Vinyl acetate]$_0$ = 1:1, T = 30 ºC, V = 50 mL. Stirring = (♦) 100, (■) 200, (▲) 400 rpm and (−) model.*

No apparent change appears as a consequence of varying the stirring rate, which can be interpreted as the absence of external diffusional limitations in the studied interval.

4.4. Influence of temperature

Temperature influence was studied from the values of 25 to 35 ºC. Results are presented in Figure 7.

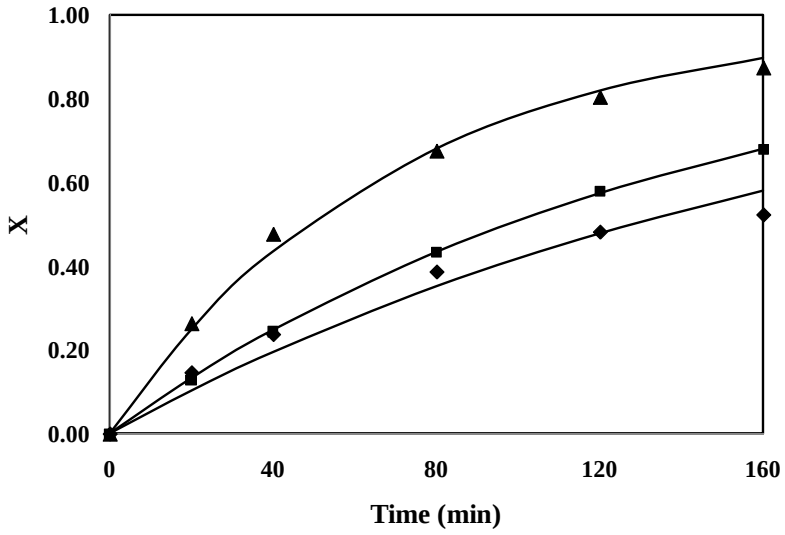

Figure 7. *Experimental and theoretical conversions of geraniol versus time. Experimental conditions: enzyme amount = 20 mg, [Geraniol]$_0$ = 50 mM, molar ratio [Geraniol]$_0$:[Vinyl acetate]$_0$ = 1:1, stirring = 300 rpm, V = 50 mL. Temperature = (♦) 25, (■) 30, (▲) 35 ºC and (−) model.*

In this experimental series several problems can take place, because when working with volatile compounds the higher the temperature at which they are exposed, the greater the probability of compounds losses by evaporation. For this reason, it was decided not to exceed the temperature of 35 ºC and, additionally, to work with the lowest amount of enzyme, 20 mg, so that the observed differences could be attributed mainly to the influence of temperature. Also, it must be taken into account that enzymes are active in a limited range of temperatures, so that the enzymatic activity presents an optimal temperature (Peña et al., 2004) and, if possible, high temperatures must be avoided.

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

$$k_0 = A \cdot e^{-E_a / RT} \qquad (29)$$

From linearization of equation (29) the following equation is obtained:

$$ln\,k_0 = ln\,A - \frac{E_a}{R}\left(\frac{1}{T}\right) \tag{30}$$

The representation of equation (30) is depicted in Figure 8. From the fitting of equation (30) the values of activation energy, E_a, and frequency factor, A, are obtained.

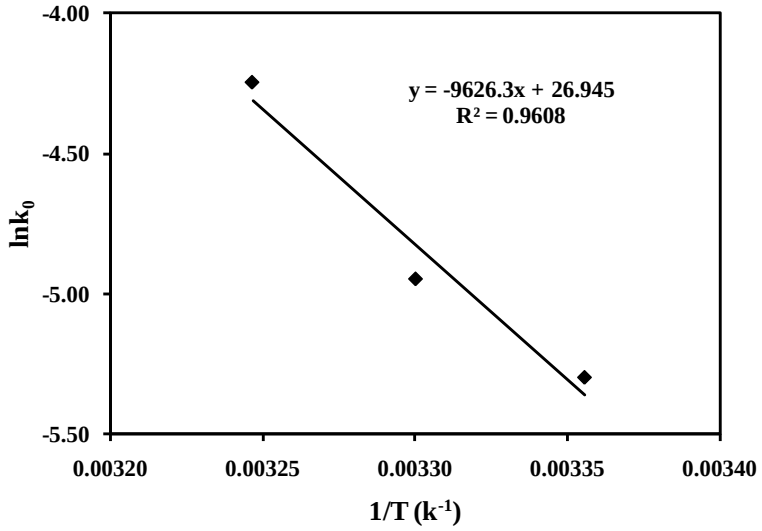

Figure 8. *Fitting of first order kinetic constant logarithm versus temperature inverse according to Arrhenius equation*

$$ln\,k_0 = 26.945 - 9626.3\left(\frac{1}{T}\right) \tag{31}$$

$$E_a = 19.060\ kcal\ mol^{-1},\ A = 5.04\ e^{11}\ min^{-1} \tag{32}$$

This value of activation energy is 10 times higher than the one obtained by Xiong, (Xiong et al., 2014), which is 1.845 kcal mol^{-1} working with a *Pseudomonas fluorescens*

lipase. Badgujar, (Badgujar and Bhanage, 2014), working with *Pseudomonas cepacia* lipase, obtained that the activation energy, for crude and immobilized enzyme, was 19.9 and 13.76 kcal mol^{-1} respectively, very close to the one obtained in this work. Besides, others authors, (Uppengerg et al., 1994), obtained a value between 3.762 and 36.72 kJ mol^{-1} for these reaction types.

4.5. Influence of molar ratio

Finally, in the last experimental series the relation of substrate concentration has been varied. In this study, geraniol concentration was kept constant at the value of 50 mM, and vinyl acetate concentration was varied between 25 and 75 mM. Results are depicted in Figure 9 and, as it can be seen, the maximum conversion value (98.4 %) is attained with the molar ratio 1:1.5, so a slight excess of vinyl acetate, which acts as limiting reagent, is recommended to achieve higher conversion values.

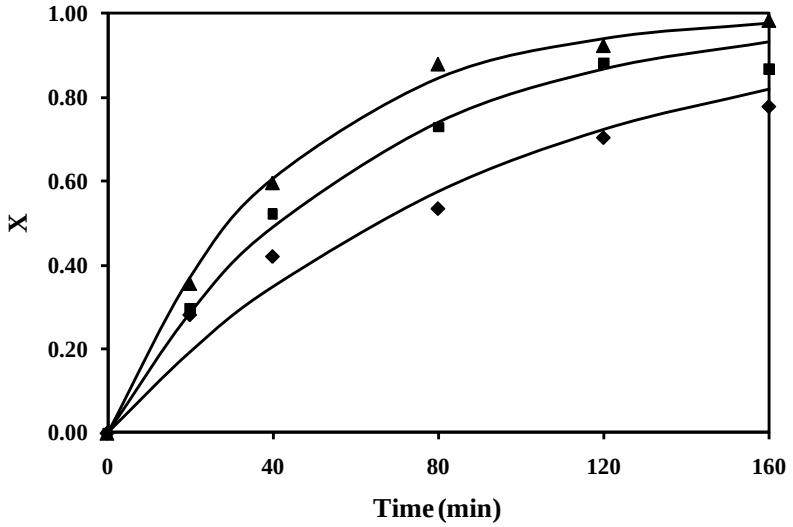

Figure 9. *Experimental and theoretical conversions of geraniol versus time. Experimental conditions: enzyme amount = 50 mg, $[Geraniol]_0$ = 50 mM, T = 30 ºC, stirring = 300 rpm, V = 50 mL. Molar ratio $[Geraniol]_0$:$[Vinyl\ acetate]_0$ = (♦) 1:0.5, (■) 1:1, (▲) 1:1.5 and (−) model.*

The fitting of the experimental data to the pseudo-first kinetic model is not as good as in the previous series. The reason is that, being not equals the initial concentrations of both substrates; it is not possible to simplify the Ping Pong kinetic equation to a first order one. The worst fitting is obtaining for 25 mM vinyl acetate and 50 mM geraniol because the obtained conversion is smaller and in this case vinyl acetate is the limiting reagent. This result confirms that the pseudo-first order model is a good approximation only under equal substrates concentrations.

In a similar way to the second series, the data of this series can be used, combined with the results obtained for V_m in section 4.2, to obtain the individual values of both Michaelis constant, K_{MVAc} and K_{MGol}, as described by Gomez (Gomez et al., 2011b). Now, since the molar ratio is not 1:1, the complete Ping-Pong kinetic equation must be used, which applied to the initial reaction rate results as follows:

$$r_0 = \frac{V_m C_{Gol0} C_{VAc0}}{K_{MVAc} C_{Gol0} + K_{MGol} C_{VAc0} + C_{Gol0} C_{VAc0}} \qquad (33)$$

Taken into account that in this series C_{Gol0} was kept constant, the inverse of the initial reaction rate is a linear function of the inverse of C_{VAc0} , as follows:

$$\frac{1}{r_0} = \frac{K_{MVAc}}{V_m}\frac{1}{C_{VAc0}} + \left(\frac{K_{MGol}}{V_m C_{Gol0}} + \frac{1}{V_m}\right) \qquad (34)$$

From the slope and intercept of equation (34), and the value of V_m previously determined in section 4.2, the individual values of the Michaelis constant can be obtained. As in 4.2, average values of reaction rate at different times, *r(t)*, were

calculated and their inverse was plotted versus the inverse of C_{VAc0} and a new family of straight lines was obtained, as it can be seen in Figure 10. After that, the different values of the slope and intercept obtained in the fitting were plotted versus time and fitted to linear functions of time.

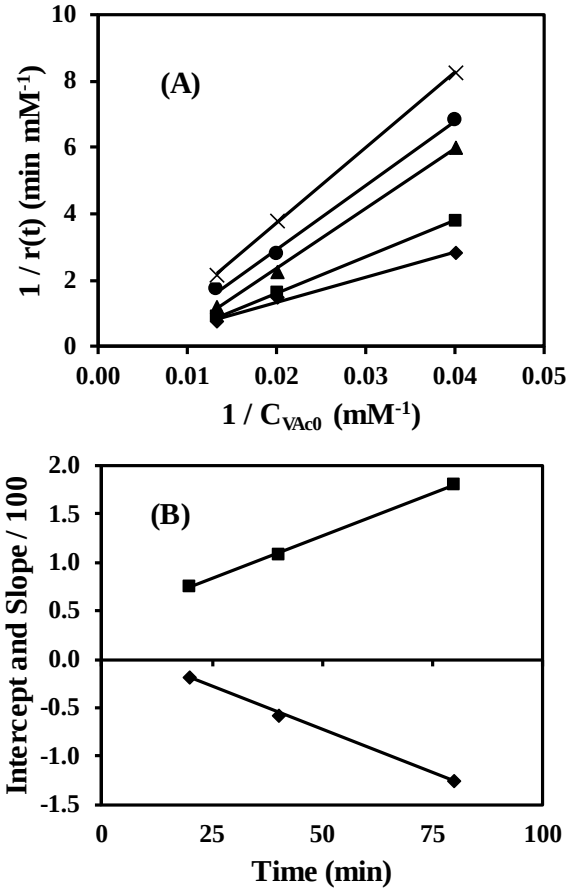

Figure 10. (A)*: Inverse of average reaction rate at time t, r(t), versus 1 / C_{VAc0}. Experimental conditions: enzyme amount = 50 mg, [Geraniol]$_0$ = 50 mM, Molar ratio variable, T = 30 ºC, stirring = 300 rpm, V = 50 mL. Reaction time (minutes): (♦) 20, (■) 40, (▲) 80, (●) 120, (x) 160.* ***(B):*** *Intercept (♦) and Slope / 100 (■) versus time.*

Finally, from the extrapolation to time zero of these linear relationships, the following true values of the slope and intercept were obtained:

$$Slope = \frac{K_{MVAc}}{V_m} = 40.490\ min \tag{35}$$

$$Intercept = \frac{K_{MGol}}{V_m C_{Gol0}} + \frac{1}{V_m} = 0.166\ mM^{-1}\ min \tag{36}$$

And, from the above relationships and the value of V_m, the following individual values of the Michaelis constants are obtained:

$$K_{MVAc} = 1760.42\ mM \tag{37}$$

$$K_{MGol} = 311.74\ mM \tag{38}$$

From these values, a new sum of the Michaelis constants can be obtained:

$$K_{MVAc} + K_{MGol} = 2072.16\ mM \tag{39}$$

The difference between the sum given by equation (28) and the one given by equation (39) is only of 3 %, which confirms that both, the individual constant and the sum, are reliable. Moreover, other author (Kuo et al., 2014) obtained for the K_{MVAc} constant a value of 2310 mM which, despite not being obtained for the same reaction and with the same lipase, is of the same order of magnitude than the one calculated in this work.

5. CONCLUSIONS

The transesterification reaction between geraniol and vinyl acetate for the enzymatic synthesis of geranyl acetate, using Novozym® 435 as catalyst, has been carried out in hexane medium. Five experimental series have been performed and the influence of the different operational variables has been analyzed. A kinetic model, based in a Ping Pong Bisubstrate mechanism, has been proposed which, for the different series with equal substrate concentrations, can be simplified to a pseudo-first order kinetic model.

A maximum conversion of geranyl acetate of 98.4% has been obtained in the best experimental conditions: 50 mg of enzyme amount, 30ºC, 300 rpm and substrate concentrations of 50 mM and 75 mM of geraniol and vinyl acetate, respectively, being the last one the limiting reagent.

It has been proven, as expected, that the amount of catalyst is the most significant variable, varying linearly the first order apparent kinetic constant with the enzyme amount. On the other hand, it has been observed that both the stirring rate and the initial substrate concentration have no significant influence. As for temperature, Arrhenius parameters have been obtained and it was observed that compounds loss could occur for temperature values higher than 35 ºC due to their high volatility

Finally, the proposed kinetic model has been validated with high correlation coefficients and the kinetic parameters of the Ping-Pong equation, Vm, K_{MVAc} and K_{MGol}, were calculated. Also, it has been proven that the simplification to a pseudo-first order kinetic is a good approximation under equal substrate concentrations, but the complete Ping-Pong equation must be used when the concentrations are different.

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Nomenclature

A:	frecuency factor of Arrhenius law, (min^{-1})
A_{GAc}	geranil acetate area in the chromatogram
A_{Gol}	geraniol area in the chromatogram
C_{Gol}:	geraniol concentration, (mM)
C_{Gol0}:	initial geraniol concentration, (mM)
C_{VAc}:	vinyl acetate concentration, (mM)
C_{VAc0}:	initial vinyl acetate concentration, (mM)
E_a:	activation energy, (kcal mol^{-1})
GAc	geranil acetate
Gol:	geraniol
k_E:	specific constant of enzyme, mM min^{-1} mg^{-1}
k_0:	first order apparent kinetic constant, (min^{-1})
K_{MVAc}:	vinyl acetate Michaelis constant, (mM)
K_{MGol}:	geraniol Michaelis constant, (mM)
m_E:	enzyme amount, (mg)
r:	geraniol disappearance rate, (mM min^{-1})
$r(t)$:	average geraniol disappearance rate after a time t, (mM min^{-1})
R:	ideal gas universal constant, (kcal K^{-1} mol^{-1}))
T:	temperature (K)
VAc:	vinyl acetate
V_m:	maximum reaction rate, (mM min^{-1})
X:	conversion of geraniol