Kinetic Study for the Ethanolysis of Fish Oil Catalyzed by Lipozyme[®] 435 in Different Reaction Media

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Abstract: The ethanolysis of fish oil in various reaction medium (tert-pentanol, n-hexane and solvent free system) catalyzed by the immobilized commercial lipase Lipozyme[®] 435 (*Candida Antarctica*) at atmospheric pressure has been studied in this work. The effect of some kinetic parameters, such as the amount of lipase, temperature and the initial reactant molar ratio ethanol:oil on monoacylglyceride and ethyl ester yield has been analyzed. Experimental data were successfully correlated by a simple kinetic model based on the elementary reactions proposed in this work. At high initial reactant molar ratio the three elementary steps can be considered as irreversible. However the reaction rate constants ratio for the deacylation of monoglyceride to glycerol decreased by decreasing the molar ratio ethanol:oil. The reaction rates are slower in n-hexane as reaction medium compared to tert-pentanol and a solvent-free system, at the experimental conditions essayed in this work. In this last case, ethanol acts as solvent for reaction and as reactant.

Key words: fish oil, lipase, ethanolysis, kinetic parameters

1 Introduction

Fish oil is one of the main sources of polyunsaturated omega-3 fatty acids (n-3 PUFA) such as eicosapentaenoic acid (20:5 n-3, EPA) and docosahexaenoic acid (22:6 n-3, DHA). An adequate intake of omega-3 fatty acids helps to prevent cardiovascular diseases, cancer, and brain disorders¹. The use of lipases to transform fish oil into derivatives rich in omega-3 fatty acids is an attractive alternative to the conventional chemical methods since lipases can operate under mild conditions, which is preferable for polyunsaturated fatty acids that can easily undergo oxidation. Enzymatic alcoholysis of fish oil by using 1,3-specific lipases is a simple method to obtain 2-MAG significantly enriched in EPA and DHA². Furthermore the ethyl esters formed in the ethanolysis reaction are currently used as dietary supplement³. The use of organic solvents for transesterification reactions has several objectives such as ensuring a homogeneous reaction mixture for reactants and products and increase of diffusion rate by reducing the viscosity of the reaction medium, minimizing thus mass transfer problems⁴.

The aim of this work is the study of the ethanolysis reaction of fish oil at atmospheric pressure and in different reaction media, catalyzed by the commercial immobilized lipase *Candida antarctica* (Lipozyme[®] 435). The most common solvents used in transesterification are hydrophobic organic solvents: hexane, n-heptane, petroleum ether, cyclohexane, 2-butanol and tert-butanol⁵. One of the most useful solvents studied in the literature is tert-butanol since it is only moderately polar (log P = 0.35)⁴. However, tert-butanol has a quite high melting point (25°C) increasing the risk of solvent crystallization⁶.

In this work, the alcoholysis has been performed in the absence of any organic solvent and in two different organic solvents as reaction medium, tert-pentanol and n-hexane. Tert-pentanol is also a tertiary alcohol and it is more hydrophobic than tert-butanol, log P = 0.96, being suitable as solvent for this kind of reactions⁵. It has already been successfully used in enzymatic glycerolysis and it has been recently used with good results in the ethanolysis of fish oil catalyzed by different immobilized lipases including *Candida antarctica* B (CALB) immobilized by anion ex-

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change and hydrophobic adsorption³⁾. The main drawback of pure tert-pentanol is the high cost compared to tert-butanol⁶⁾. In most of the literature, no explanation is provided about the amount of solvent necessary to create a homogeneous system in the ethanolysis process. In this work, the amount of solvent was based on previous work on liquid-liquid equilibria for the ethanolysis systems of fish oil in both organic solvents⁷⁾. This way, the amount of solvent added to create a homogeneous phase could be optimized. Solvents may also influence the residual water of the enzyme varying its catalytic activity. However, the *Candida antarctica* lipase B used in this work has been described to be still active at very low water content in the system⁸⁾. In addition, it has also a high resistance to alcohol deactivation⁹⁾.

In this work, the effect of some kinetic parameters such as the ethanol:oil initial molar ratio (4:1 – 76:1), reaction temperature (293.2 – 323.2 K) and the amount of lipase (2.5 – 10 wt% based on total weight of substrates) has been investigated. Experimental data were correlated satisfactorily by a simple kinetic model based on the elementary reaction that may occur in this system.

2 Materials and methods

2.1 Materials

The refined fish oil, a mixture of tuna and sardine oil, was kindly provided by AFAMSA. Ethanol was purchased from Merck KGaA with a purity of 99.9% and a water content of 0.05%. Tert-pentanol was obtained from Merck with a purity of $\geq 99\%$ and a water content of 0.065%. *n*-Hexane was purchased from Lab-Scan Analytical Sciences with a purity of $\geq 99\%$ and a water content of 0.01%. Products were stored over activated 3 Å molecular sieves to keep them dried. The lipase used in this work was Lipzyme[®] 435 from *Candida antarctica* immobilized on a macroporous hydrophobic acrylic resin (food grade). It was kindly donated by Novozymes (Bagsvaerd, Denmark).

2.2 Ethanolysis reaction

The mixture of fish oil and ethanol with and without the presence of an organic solvent (tert-pentanol and *n*-hexane) was incubated at different temperatures in an orbital stirrer water bath. The temperature was controlled with a precision of ± 0.5 K. Based on previous studies on liquid-liquid equilibrium for the ethanolysis system, the reaction mixture contains 30 wt% of the organic solvent to ensure a homogeneous reaction medium from the beginning of the reaction. The reaction was started by the addition of the lipase. At selected time intervals, the reaction mixture was withdrawn and filtered through a microfilter (0.45 μ m, Sartorius RC) to stop the reaction by removing the lipase. All samples were stored at -18°C prior to analysis and ana-

lyzed at least by duplicate.

2.3 Analysis of reaction products

The lipid profile (MAG, DAG, TAG and EE) was analyzed by a normal-phase high-performance liquid chromatography (NP-HPLC). Separations were carried out at room temperature in a Lichrospher Diol column (5 mm, 4 mm $\times$ 250 mm) and detection was performed by an ELSD (Agilent Technologies 1200 Series Model, Santa Clara CA, United States) at 35°C . The method and calibration procedure have been previously described in detail¹⁰⁾.

The glycerol formed in the enzymatic alcoholysis has been determined by High-Temperature Gas Chromatography (HT-GC). A Hewlett Packard gas chromatograph (HP 6890 Series GC System) equipped with a flame ionization detector (FID), a fused silica capillary column of 30m $\times$ 0.25 mm i.d. coated with a 0.25 mm film thickness of 65% Phenyl Methylpolysiloxane (65HT) as a stationary phase and an Agilent Technologies 7683B Series automatic injector has been used for this analysis. The GC method has been previously described in detail⁷⁾.

The free fatty acid (FFA) content of the fish oil has been determined according to the AOCS Official Method Ca 5a-40 using an automatic titrator Methrom (Titrand 605)¹¹⁾. The FFA content was found to be $0.23 \pm 0.015\%$ expressed as percentage of oleic acid. Due to the low free fatty acid content, FFA neutralization was not significant.

The fatty acid profile of the fish oil used in this work and different MAG fractions has been determined by gas chromatography by following the AOAC method¹²⁾. Details of the gas chromatograph method can be found elsewhere¹³⁾.

3 Results and discussion

3.1 Ethanolysis reaction of fish oil

Figure 1 shows a typical time course of the product composition in the ethanolysis reaction of fish oil with Lipzyme[®] 435 in the presence of tert-pentanol as reaction medium at 303.2 K, ethanol:oil molar ratio 76:1 with a 10 wt% lipase content based on the weight of reactants.

The production of fatty acid ethyl esters is very rapid at the beginning of the reaction and then it becomes slower. The intermediate DAG is accumulated for a little while only in small amounts (less than 5 mole%) and then it is rapidly deacylated to MAG. The production of MAG reaches a maximum in 2–3 h reaction time. According to Esteban *et al.*¹⁴⁾, technical grade Novozym 435 prefers TAG as substrate, since the deacylation of MAG to glycerol becomes important only when the TAG content in the reaction medium is very low (after 2–3 h reaction time). This can be also observed in Fig. 1 on the lipid profile of TAG, MAG and glycerol. At the reaction conditions studied in this work, no free fatty acids were observed in the reaction

Fig. 1 Components profile in the ethanolysis of fish oil in tert-pentanol with lipase Lipozyme® 435: Δ triglyceride, + diglyceride, ○ monoglyceride, * ethyl esters, □ glycerol (ethanol:oil = 76:1, 10 wt% lipase based on weight of reactants, 303.2 K). Continuous lines represent the kinetic model.

medium (<0.1%). Muñoz *et al.*¹⁵⁾, also detected no FFA (<0.5%) when absolute ethanol (0.5% water) and absolute dry ethanol (0.02% water) were used in the alcoholysis of cod liver oil with ethanol by the lipase Novozyme 435 at 72:1 molar ratio ethanol:oil.

3.2 Kinetic model

The enzyme kinetics and reaction mechanism for transesterification systems have been recently review by Gog *et al.*¹⁶⁾. The most commonly used mechanism to describe the alcoholysis of triglycerides is the Ping-Pong Bi-Bi mechanism¹⁶⁾. Alcohol and substrate inhibition of enzyme are often considered⁹⁾. In many of these studies only initial changes of substrates were considered to estimate the kinetic parameters of the model and formation of di- and monoglycerides are not considered¹⁷⁾. Pilarek and Szewczyk proposed a kinetic model for 1,3-specific enzymatic alcoholysis of triacylglycerols based on mass balances for each individual component assuming that the enzymatic alcoholysis of acyl bounds is an irreversible reaction following the Ping-Pong Bi-Bi mechanism with competitive inhibition by alcohol¹⁷⁾. Other authors analyze different lipase-catalyzed reactions by response surface methodology^{18–20)}. Camacho *et al.*²¹⁾ proposed a mechanistic model based on the elementary reactions that may occur in the process. In this work, a simple model similar to the one proposed by Oliveira *et al.*²²⁾, has been considered. The main difference is that mono- and diglycerides have been taken into account since they were detected under the reaction con-

ditions studied (Fig. 1).

Lipase-catalyzed ethanolysis of triglycerides takes places yielding one molecule of fatty acid ethyl ester and a glyceride containing one fewer ester bond at each step, that is DAG, MAG and glycerol as last product⁹⁾:

Hydrolysis reaction has not been taken into account since no FFA were detected (<0.1%). All the kinetic reactions can be considered as pseudo homogeneous catalyzed reactions. The three reversible reactions have been considered as elementary reactions; therefore forward and reverse reactions are expected to follow a second order kinetic, being k_1 , k_2 and k_3 the forward rate constant and k_{-1} , k_{-2} and k_{-3} the reverse rate constants for the lipase catalyzed reaction. In this kinetic study the ethanol:oil molar ratio exceeded to a large extent the stoichiometric ratio; therefore the consumption of ethanol during the ethanolysis reaction can be considered as negligible and the molar concentration of ethanol along the reaction was considered constant and it has been included in the forward rate constant. Concentrations of reaction products are expressed in ethanol and solvent-free basis. The reactions involved in the ethanolysis system are the following:

$$\begin{aligned} \frac{d n_{\text{TAG}}/n_{\text{total}}}{dt} &= -k_1 x_{\text{TAG}} + k'_{-1} x_{\text{DAG}} x_{\text{EE}} \\ \frac{d n_{\text{DAG}}/n_{\text{total}}}{dt} &= k_1 x_{\text{TAG}} - k'_{-1} x_{\text{DAG}} x_{\text{EE}} - k_2 x_{\text{DAG}} + k'_{-2} x_{\text{MAG}} x_{\text{EE}} \\ \frac{d n_{\text{MAG}}/n_{\text{total}}}{dt} &= k_2 x_{\text{DAG}} - k'_{-2} x_{\text{MAG}} x_{\text{EE}} - k_3 x_{\text{MAG}} + k'_{-3} x_{\text{GLY}} x_{\text{EE}} \\ \frac{d n_{\text{EE}}/n_{\text{total}}}{dt} &= k_1 x_{\text{TAG}} - k'_{-1} x_{\text{DAG}} x_{\text{EE}} + k_2 x_{\text{DAG}} - k'_{-2} x_{\text{MAG}} x_{\text{EE}} \\ &\quad + k_3 x_{\text{MAG}} - k'_{-3} x_{\text{GLY}} x_{\text{EE}} \\ \frac{d n_{\text{GLY}}/n_{\text{total}}}{dt} &= k_3 x_{\text{MAG}} - k'_{-3} x_{\text{GLY}} x_{\text{EE}} \end{aligned} \quad [1]$$

where n_{TAG} , n_{DAG} , n_{MAG} , n_{EE} and n_{GLY} are the moles of triacylglycerols, diacylglycerols, monoacylglycerols, ethyl esters and glycerol respectively and n_{total} is the total number of moles excluding the solvent and the ethanol to express concentrations in a solvent-ethanol-free basis. The effective forward and reverse rate constant are obtained by solving the set of differential equations simultaneously. In this work, the differential equations were solved numerically with a fourth-order Runge-Kutta method and by reducing the experimental kinetic data minimizing the following objective function (O.F.):

$$\text{O.F.} = \frac{\sum_{\text{all samples}} \sum_{i=1}^n \text{abs}(x_{i,\text{exp}} - x_{i,\text{calc}})}{n_{\text{samples}}} \cdot 100 \quad [2]$$

by using the Simplex-Nelder-Mead method. The subscripts

i refers to the different components in the ethanolysis system: TAG, DAG, MAG, EE and glycerol. The subscripts “exp” and “calc” refer to the experimental and calculated mole fraction of the different components for each experimental kinetic data (n_{samples}). In this work, the effect of the organic solvent used as reaction medium, amount of lipase, reaction temperature and initial molar reactant ratio on the kinetic parameters was studied. As the regioisomers of DAG and MAG could not be distinguished with the applied analytical procedure, no difference was made between them in the model. The effective rate constants include the catalyst concentration and the rate constant for the catalyzed reaction can be expressed as a function of the lipase concentration, C :

$$k' = k \cdot C \quad [3]$$

The effect of temperature on the reaction rate has been described by the Arrhenius type equation:

$$k' = k_0 \exp\left(-\frac{E_a}{RT}\right) \quad [4]$$

where k_0 is the preexponential factor, E_a is the activation energy of the reaction and R is the gas constant. The continuous lines in Fig. 1 represent the results obtained with the kinetic model proposed in this work. It can be observed that the experimental data were correlated satisfactorily.

3.3 Ethanolysis reaction in tert-pentanol as reaction medium

A total of 9 ethanolysis kinetic experiments were performed in tert-pentanol as organic solvent ensuring a homogeneous medium from the beginning of the reaction. The amount of tert-pentanol in the liquid phase was 30 wt% in all experiments. The effect of lipase amount, reaction temperature and initial reactant molar ratio was analyzed and kinetic parameters were obtained for each kinetic experiment.

3.3.1 Effect of the amount of catalyst

The effect of the lipase loading was studied by varying the lipase concentration from 2.5 to 10 wt% based on total weight of reactants (oil + ethanol). The reaction temperature was fixed at 293.2 K, and the initial reactant molar ratio (ethanol:oil) was 76:1. An increase in the lipase loading leads to an increase in the reaction rate due to an increase in the total number of active sites of the lipase. This can be observed in Fig. 2 where the MAG profile at different lipase

Fig. 2 MAG profile in ethanolysis reaction in tert-pentanol at different lipase loadings (○ 10 wt%; □ 5 wt%; △ 2.5 wt%); initial molar ratio 76:1, $T = 293.2$ K. Continuous lines represent the kinetic model.

concentrations has been represented. By increasing the amount of lipase the maximum in the MAG concentrations is reached sooner and MAG degrades faster to glycerol. At 10 wt% catalyst loading the maximum in the MAG content in the reaction medium is reached at 120 minutes, whereas this value is delayed until 360 min and 480 min at 5 wt% and 2.5 wt%, respectively. Table 1 presents the effective forward and reverse reaction rates for the three consecutive reactions described in Equations 1 and the corresponding objective function (Ec. 2).

From Table 1 it can be observed that the reaction rate constants also increase by increasing the amount of lipase. Reaction constants corresponding to the reverse reaction can be considered negligible. Therefore, at high initial reactant molar ratio the three consecutive steps can be considered as irreversible. The regression of the kinetic data has been also performed considering the three steps as irreversible. Similar values of the forward reaction rates were obtained (data not presented) with similar objective function values (7.31, 8.76 and 8.27 for 2.5, 5 and 10 wt%, respectively). From the values shown in Table 1 it can be

Table 1 Effective reaction rate constants in the ethanolysis system in tert-pentanol at different lipase loading (MR = 76:1; $T = 293.2$ K).

Lipase, % wt	k_1'	k_{-1}'	k_2'	k_{-2}'	k_3'	k_{-3}'	O.F.
2.5	$4.48 \cdot 10^{-3}$	$2.06 \cdot 10^{-10}$	$6.24 \cdot 10^{-2}$	$1.98 \cdot 10^{-9}$	$4.00 \cdot 10^{-4}$	$2.07 \cdot 10^{-8}$	8.27
5	$9.61 \cdot 10^{-3}$	$1.92 \cdot 10^{-8}$	$9.98 \cdot 10^{-2}$	$2.00 \cdot 10^{-7}$	$8.76 \cdot 10^{-4}$	$2.14 \cdot 10^{-6}$	8.73
10	$1.62 \cdot 10^{-2}$	$2.07 \cdot 10^{-7}$	$1.57 \cdot 10^{-1}$	$1.96 \cdot 10^{-6}$	$1.40 \cdot 10^{-3}$	$2.09 \cdot 10^{-5}$	6.41

Fig. 3 Forward effective reaction rate constants as a function of lipase loading ($\circ$ k'_1 ; $\square$ k'_2 ; $\triangle$ k'_3). Reaction temperature 293.2 K, initial molar ratio 76:1, tert-pentanol as reaction medium.

observed that the effective forward reaction rate k'_2 is the highest of all the forward reaction rates. Therefore as soon as DAG are formed in the first step they are consumed to produce MAG and the corresponding ethyl esters. This can also be observed in Fig. 1 where DAG were only present in the reaction medium at the very beginning of the process. MAG content increases until a maximum is reached since k_1 is higher than k_3 . When all the TAG are consumed the decrease in the MAG content starts since the value of k_3 is the lowest of all the forward reaction rates.

Based on equation 3, the reaction rate constant can be obtained by plotting the effective rate constant versus the catalyst concentration. Figure 3 shows the effective forward reaction rates (k'_1 , k'_2 and k'_3) as a function of the catalyst concentration. A linear dependence on lipase concentration can be observed where the slope is the rate constant at 293.2 K and ethanol:oil molar ratio of 76:1. Similar behavior has been observed in the methanolysis of *Brassica carinata* oil catalyzed by potassium hydroxide²³. The values of the reaction rate constant are shown in Fig. 3 with the corresponding regression coefficient. From the values of the reaction rate constant can be concluded that the effect of enzyme concentration is more pronounced in the DAG consumption to form MAG and the corresponding ethyl ester than in the other two reactions.

3.3.2 Effect of reaction temperature

A series of ethanolysis reactions has been performed varying the temperature in the range of 293.2 to 323.2 K. The initial reactant molar ratio was fixed at 76:1 with a lipase loading of 10 wt%. All the reaction rates increased with increasing temperature. As an example, Figure 4

Fig. 4 Effect of temperature on MAG (open symbols) and ethyl esters (filled symbols) content in the reaction medium: $\circ$, $\bullet$ 293.2 K; $\square$, $\blacksquare$ 303.2 K; $\triangle$, $\blacktriangle$ 313.2 K; $\diamond$, $\blacklozenge$ 323.2 K. Initial reactant molar ratio = 76:1, catalyst loading 10 wt%. Continuous lines represent the kinetic model.

shows the MAG profile and the ethyl ester profile at the four temperatures studied in this work. The maximum of MAG content is reached sooner with the reaction temperature. The heat of reaction is for many transesterification systems generally small; therefore the equilibrium conversion observed for the ethyl esters is essentially temperature independent (Fig. 4). For comparison, Fjerbaeck *et al.*⁴, reported an estimated heat of the reaction of -18.5 kJ/mol fatty acid methyl esters (FAME) at 25°C.

The effective reaction rate constants are listed in Table 2 with the corresponding objective function. At each reaction temperature, the slowest reaction rate of the forward steps was the reaction from monoglyceride to glycerol, in contrast to the reaction from diglyceride to monoglyceride. Due to large excess of ethanol, the three consecutive steps can be considered irreversible, specially the two first steps. The effective reverse reaction rate k'_{-3} (from glycerol to monoglyceride) seems to be the most sensitive to the reaction temperature (see Table 2). The increase of k'_{-3} from 293.2 to 323.2 K is more than tenfold, whereas the increase in the effective forward reaction rates (k'_1 , k'_2 and k'_3) is around threefold.

The temperature influence on the reaction rate was found to follow an Arrhenius type dependence (Eq 4). The activation energy and the preexponential factor for each consecutive reaction can be calculated by plotting the logarithm of the effective calculated reaction rate constant versus the reciprocal of absolute temperature (Fig. 5). These values are presented in Table 3. According to the tendency in the effective reaction rates, the highest value

Table 2 Effective reaction rate constants in the ethanolysis system in tert-pentanol at different reaction temperatures (MR = 76:1; 10 wt% catalyst loading).

T, K	k_1'	k_{-1}'	k_2'	k_{-2}'	k_3'	k_{-3}'	O.F.
293.2	$1.62 \cdot 10^{-2}$	$2.07 \cdot 10^{-7}$	$1.57 \cdot 10^{-1}$	$1.96 \cdot 10^{-6}$	$1.40 \cdot 10^{-3}$	$2.09 \cdot 10^{-5}$	6.41
303.2	$2.26 \cdot 10^{-2}$	$4.52 \cdot 10^{-7}$	$2.08 \cdot 10^{-1}$	$1.92 \cdot 10^{-6}$	$2.41 \cdot 10^{-3}$	$9.05 \cdot 10^{-5}$	8.76
313.2	$3.38 \cdot 10^{-2}$	$5.50 \cdot 10^{-7}$	$4.31 \cdot 10^{-1}$	$1.98 \cdot 10^{-6}$	$2.78 \cdot 10^{-3}$	$3.27 \cdot 10^{-4}$	9.36
323.2	$4.26 \cdot 10^{-2}$	$4.23 \cdot 10^{-7}$	$5.67 \cdot 10^{-1}$	$2.05 \cdot 10^{-6}$	$4.54 \cdot 10^{-3}$	$2.83 \cdot 10^{-4}$	10.10

Fig. 5 Arrhenius plot of effective reaction rates: $\circ$ k_1' ; $\square$ k_2' ; $\triangle$ k_3' ; $\diamond$ k_{-3}' . Lipase loading 10 wt%, initial molar ratio 76:1, tert-pentanol as reaction medium.

found for the activation energy corresponds to the reaction from glycerol to monoglyceride, being the most sensitive step to the reaction temperature. This behavior has been also described by Nouredini and Zhu²⁴⁾, in the study of methanolysis of soybean oil with sodium hydroxide. These authors also found for the third reaction step that the forward reaction has lower activation energy than the reverse reaction (21.68 kJ/mol versus 41.31 kJ/mol)²⁴⁾. Not much data of activation energy for ethanolysis reaction of oil by lipases has been reported in the literature. Chester-

field *et al.*⁹⁾ reported a value for the activation energy of 17.3 kJ/mol for the global reaction rate of ethanolysis of waste cooking oil to fatty acid ethyl esters in the temperature range between 297 to 320 K, catalyzed by Novozym 435.

3.3.3 Effect of the initial reactant molar ratio.

To determine the effect of ethanol:oil initial molar ratio, ethanolysis reactions were carried out at different reactant molar ratios at 10 wt% lipase loading and 303.2 K. The ethanol:oil molar ratio was varied in the range 4:1-76:1. **Figure 6** shows the MAG and ethyl ester profiles at the different initial molar ratios. The ethanolysis reaction rate increased by increasing the excess of ethanol in the reaction medium. Regarding the MAG content, it can be observed that when the molar ratio ethanol:oil decreased, the maximum reached in the MAG content in the reaction mixture also decreased. When the ethanol:oil ratio decreased from 76:1 to 4:1, the maximum MAG content decreased from 24% to 16%. The same behavior in the production of MAG when varying the molar reactant ratio in the ethanolysis of different type of oils catalyzed by lipases has been found in the literature^{15, 25, 26)}.

The experimental kinetic data at different reactant molar ratio were correlated with the model proposed in this work. The forward and reverse reaction rates are presented in **Table 4** with the corresponding objective function. At each molar ratio, the reaction from diglyceride to monoglyceride is the fastest of all the steps. In general, the forward reaction rate constants decreased by decreasing the initial reactant molar ratio, while the opposite is observed for the reverse reaction rate constants. Although, the conversion of TAG to DAG and of DAG to MAG can be considered as irreversible, due to the low value of the effective reverse

Table 3 Activation energies and preexponential factors for the ethanolysis reaction in tert-pentanol at initial molar ratio 76:1 with a lipase loading of 10 wt%.

Step	1	2	3	-3
E_a (kJ/mol)	26.10	35.98	28.91	72.32
k_o	$7.33 \cdot 10^2$	$3.88 \cdot 10^5$	$2.08 \cdot 10^2$	$2.23 \cdot 10^8$
r^2	0.9932	0.9632	0.9603	0.8699

Step: 1: triglyceride to diglyceride; 2: diglyceride to monoglyceride; 3: monoglyceride to glycerol; -3 glycerol to monoglyceride.

Fig. 6 Effect of initial molar ratio ethanol:oil on MAG (open symbols) and ethyl esters (filled symbols) content in the reaction medium: ○, ● 76:1; □, ■ 38:1; △, ▲ 10:1; ◇, ◆ 4:1. Reaction temperature = 303.2 K, catalyst loading 10 wt%, tert-pentanol as reaction medium. Continuous lines represent the kinetic model.

reaction rates for these two first steps compared with the corresponding effective forward reaction rates. However, the ratio k_3/k_3' (i.e. the equilibrium constant), corresponding to the deacylation of monoglyceride to glycerol, monotonously decreased with decreasing the ethanol:oil molar ratio. Its values were 26.7, 8.8, 5.4 and 1.6 for the initial ethanol:oil molar ratios of 76:1, 38:1, 10:1 and 4:1, respectively. This behavior can be clearly observed in Fig. 6 where the MAG profiles are flatter at molar ratio of 4:1 than 76:1 because the monoglyceride consumption decreases with decreasing initial molar ratio.

The lipase from *Candida antarctica* is a non regiospecific lipase, however it behaves as 1,3 specific at a great excess of ethanol²⁷. According to this, at the highest molar ratio studied in this work (76:1), the maximum observed in the MAG production should correspond to 2-MAG accumulation. However, after reaching this maximum, the 2-MAG content was decreasing. This behavior was also observed in the literature considering two different approaches (i) acyl-

migration can happen converting the 2-MAG into 1(3)-MAG and the 1,3 specific *Candida antarctica* could act to form glycerol as the final degradation product and ethyl esters or (ii) *Candida antarctica* does not act as specific since reaction medium conditions have been changed, being the 2-MAG the only substrate for the lipase^{14, 15}.

According to Irimescu *et al.*²⁸, the regiospecificity of the lipase depends on the type of reaction and the initial composition of the reaction medium. Table 5 shows the fatty acid profile of the MAG fraction at the maximum reached in the MAG profile during the reaction course at the different molar ratios. Since the lipase behaves as 1,3 specific at a great excess of ethanol, the fatty acid profile reported at the highest molar ratio studied in this work would correspond to the fatty acid profile at the *sn*-2 position. The main difference in the fatty acid profile at the maximum MAG content was found in the content of DHA, probably because it is preferable bound at the *sn*-2 position in the triacylglyceride¹⁰. At molar ratios higher than 8:1 the mole percentage of DHA in the MAG fraction is around 45%, however at molar ratio of 4:1 this percentage decreases down to 29%. This support the loss of regiospecificity of *Candida antarctica* at low ethanol:oil molar ratio.

3.4 Ethanolysis reaction in other reaction media

Ethanolysis reaction was also conducted in hexane and in a solvent-free system to compare the behavior of Lipzyme® 435 in other reaction media. Ethanolysis was performed at a molar ratio of 76:1 with a lipase loading of 10 wt% at three different temperatures in *n*-hexane and in a solvent-free reaction medium. Figure 7 shows the component profile for the reaction products in *n*-hexane at 303.2 K. The behavior is qualitatively similar to the one described in Fig. 1 for tert-pentanol as reaction medium, although the kinetics in *n*-hexane are slower. For a better comparison, Fig. 8 shows the ethyl ester and the MAG profiles in the three reaction media. The reaction products profile in the solvent-free system is similar to the profile obtained during the reaction in tert-pentanol. Due to the high excess of ethanol used in these kinetic experiments, there are no mass transfer limitations by poor immiscibility of fish oil and ethanol. Ethanol acts as both solvent reaction medium and reactant. Although, two phases exist at beginning of the ethanolysis⁷, the change in the polarity of the reaction

Table 4 Effective reaction rate constants in the ethanolysis system in tert-pentanol at different initial molar ratio ethanol:oil with a lipase loading of 10 wt% and T = 303.2 K.

MR	k_1'	k_{-1}'	k_2'	k_{-2}'	k_3'	k_{-3}'	O.F.
76:1	$2.26 \cdot 10^{-2}$	$4.52 \cdot 10^{-7}$	$2.08 \cdot 10^{-1}$	$1.92 \cdot 10^{-6}$	$2.41 \cdot 10^{-3}$	$9.05 \cdot 10^{-5}$	8.76
38:1	$1.99 \cdot 10^{-2}$	$2.00 \cdot 10^{-5}$	$1.60 \cdot 10^{-1}$	$3.29 \cdot 10^{-6}$	$4.62 \cdot 10^{-3}$	$5.24 \cdot 10^{-4}$	11.74
8:1	$8.24 \cdot 10^{-3}$	$1.62 \cdot 10^{-5}$	$1.06 \cdot 10^{-1}$	$8.77 \cdot 10^{-6}$	$3.57 \cdot 10^{-3}$	$6.62 \cdot 10^{-4}$	7.65
4:1	$3.84 \cdot 10^{-3}$	$1.69 \cdot 10^{-4}$	$7.88 \cdot 10^{-2}$	$7.10 \cdot 10^{-5}$	$6.20 \cdot 10^{-3}$	$3.83 \cdot 10^{-3}$	13.86

Table 5 Fatty acid (FA) distribution of the fish oil and at the maximum of MAG at different initial molar ratio ethanol:oil (MR) in tert-pentanol and in different reaction media (*n*-hexane, solvent-free system).

		Fatty acid composition at the maximum in the MAG fraction					
FA MR	Fish oil	Tert-pentanol ^a				<i>n</i> -Hexane ^b	Solvent free ^b
		4:01	8:01	38:01	76:01	76:01	76:01
C14:0	4.7 ± 0.1	4.0 ± 0.2	3.4 ± 0.2	4.5 ± 0.3	4.8 ± 0.5	6.0 ± 0.7	5.0 ± 0.5
C16:0	23.3 ± 0.2	20.7 ± 0.3	19 ± 1	18.2 ± 0.7	17.6 ± 0.8	18.5 ± 0.9	17.5 ± 0.9
C16:1	6.9 ± 0.1	6.5 ± 0.3	5.6 ± 0.3	6.2 ± 0.3	6.8 ± 0.5	6.5 ± 0.2	6.6 ± 0.4
C18:0	6.1 ± 0.1	6.0 ± 0.2	2.1 ± 0.2	1.5 ± 0.6	1.3 ± 0.3	1.6 ± 0.7	0.6 ± 0.2
C18:1n-9	18.4 ± 0.1	18.2 ± 0.4	12.4 ± 0.7	10.9 ± 0.7	11.2 ± 0.5	11.2 ± 0.5	12 ± 2
C18:1n-7	3.0 ± 0.1	1.4 ± 0.5	1.1 ± 0.2	1.3 ± 0.3	0.9 ± 0.3	0.7 ± 0.1	0.6 ± 0.2
C18:2n-6	2.4 ± 0.1	2.5 ± 0.2	2.3 ± 0.1	2.7 ± 0.6	2.1 ± 0.3	2.5 ± 0.3	3.1 ± 0.2
C18:3n-3	0.7 ± 0.1	n.d.	n.d.	n.d.	n.d.	0.4 ± 0.2	n.d.
C18:4n-3	1.1 ± 0.1	1.0 ± 0.2	1.4 ± 0.2	1.9 ± 0.6	1.0 ± 0.3	0.5 ± 0.1	0.7 ± 0.1
C20:1n-9	1.9 ± 0.1	1.5 ± 0.3	1.2 ± 0.3	n.d.	n.d.	n.d.	n.d.
C20:3n-3	2.0 ± 0.1	1.9 ± 0.1	1.2 ± 0.2	1.7 ± 0.4	1.4 ± 0.3	1.6 ± 0.3	1.2 ± 0.2
C20:5n-3	6.5 ± 0.1	6.1 ± 0.2	3.4 ± 0.2	5.1 ± 0.4	6.1 ± 0.3	5.4 ± 0.4	5.6 ± 0.2
C22:5n-3	1.6 ± 0.1	1.5 ± 0.1	1.9 ± 0.3	2.0 ± 0.2	2.2 ± 0.3	2.1 ± 0.2	1.1 ± 0.5
C22:6n-3	21.4 ± 0.2	28.7 ± 0.8	45 ± 1	44 ± 2	45 ± 2	43 ± 2	46 ± 1
SFA	34.1 ± 0.4	30.7 ± 0.7	25 ± 1	24 ± 2	24 ± 2	26 ± 2	23 ± 2
MUFA	30.2 ± 0.4	27 ± 2	20 ± 2	18 ± 1	19 ± 1	18.4 ± 0.8	19 ± 3
PUFA	35.7 ± 0.8	42 ± 2	55 ± 2	57 ± 4	57 ± 4	56 ± 4	58 ± 2

SFA: total saturated fatty acids; MUFA: total monounsaturated fatty acids; PUFA: total polyunsaturated fatty acids; n.d no detected; a = 303.2 K; b = 293.2K.

mixture because of the production of ethyl esters and monoglycerides favors a kinetic control of the ethanolysis process. In any case, due to the initial immiscibility of both reactants, the alcoholysis in the presence of a solvent would be recommended when the alcoholysis reaction is carried out at industrial scale, where the mass transfer problems could become more important. The maximum in the MAG content is reached at 120 min, approximately, in solvent-free system and tert-pentanol reaction medium, whereas this maximum was reached at 360 min in *n*-hexane. This can be related to the hydrophobicity of the support of Lipozyme[®] 435 and the different solvent polarity. Tert-pentanol is more polar than *n*-hexane ($\log P_{\text{hexane}} = 3.5$ and $\log P_{\text{tert-pentanol}} = 0.96$). Piyattheerawong *et al.*⁽⁸⁾, observed that CALB was able to maintain its active conformation after being in contact with dry ethanol. Therefore tert-pentanol and solvent-free medium at high molar ratio (76:1) do not cause any activity loss despite their polarity. However, Lipozyme[®] 435 is immobilized in a hydrophobic support and *n*-hexane could be preferably adsorbed in the support blocking an easy access of the reactants, mainly

the polar ethanol, to the active site of the lipase. **Table 5** shows similar fatty acid profiles with a high DHA content obtained for different reaction media in the MAG fraction at the maximum reached.

Ethanolysis kinetic data in a solvent-free system and in *n*-hexane have been correlated to the model proposed in this work (**Table 6**). The two first steps can be considered as irreversible since the effective reverse reaction rates are very small compared to the forward reactions. However, the effective forward and reverse reaction rates of the third step were of the same order (see the MAG profiles **Fig. 8**). **Figures 9** and **10** show the Arrhenius plots for *n*-hexane and solvent-free systems, and the corresponding activation energies are tabulated in **Table 7**.

4 Conclusions

The ethanolysis reaction catalyzed by the commercial enzyme Lipozyme[®] 435 was carried out at different conditions to determine the effect of some kinetic variables on

Fig. 7 Components profile in the ethanolysis of fish oil in *n*-hexane with lipase Novozym 435: Δ triglyceride, + diglyceride, $\circ$ monoglyceride, * ethyl esters, $\square$ glycerol (ethanol:oil = 76:1, 10 wt% lipase based on weight of reactants, 303.2 K). Continuous lines represent the kinetic model.

Fig. 8 Effect of reaction medium on MAG (open symbols) and ethyl esters (filled symbols) content in the reaction medium: $\circ$, $\bullet$ tert-pentanol; $\square$, $\blacksquare$ *n*-hexane; $\triangle$, $\blacktriangle$ solvent-free system. Initial reactant molar ratio = 76:1, reaction temperature = 293.2 K, catalyst loading 10 wt%. Continuous lines represent the kinetic model.

Table 6 Effective reaction rate constants in the ethanolysis system in *n*-hexane and in solvent-free system at different reaction temperatures (ethanol:oil = 76:1, lipase loading = 10 wt%).

T, K	k_1'	k_{-1}'	k_2'	k_{-2}'	k_3'	k_{-3}'	O.F.
<i>n</i> -Hexane							
293.2	$5.09 \cdot 10^{-3}$	$2.06 \cdot 10^{-11}$	$5.46 \cdot 10^{-2}$	$1.80 \cdot 10^{-12}$	$9.52 \cdot 10^{-4}$	$1.62 \cdot 10^{-4}$	10.44
303.2	$7.03 \cdot 10^{-3}$	$1.78 \cdot 10^{-11}$	$7.28 \cdot 10^{-2}$	$2.13 \cdot 10^{-11}$	$1.48 \cdot 10^{-3}$	$1.92 \cdot 10^{-4}$	8.21
323.2	$1.55 \cdot 10^{-2}$	$3.33 \cdot 10^{-11}$	$1.69 \cdot 10^{-1}$	$3.07 \cdot 10^{-11}$	$3.55 \cdot 10^{-3}$	$8.60 \cdot 10^{-4}$	5.93
Solvent-free system							
293.2	$2.26 \cdot 10^{-2}$	$2.37 \cdot 10^{-11}$	$1.44 \cdot 10^{-1}$	$1.94 \cdot 10^{-10}$	$1.47 \cdot 10^{-3}$	$8.14 \cdot 10^{-4}$	9.15
303.2	$2.64 \cdot 10^{-2}$	$2.39 \cdot 10^{-11}$	$1.77 \cdot 10^{-1}$	$1.85 \cdot 10^{-10}$	$1.78 \cdot 10^{-3}$	$9.77 \cdot 10^{-4}$	8.89
323.2	$4.07 \cdot 10^{-2}$	$2.73 \cdot 10^{-11}$	$1.96 \cdot 10^{-1}$	$2.07 \cdot 10^{-10}$	$2.20 \cdot 10^{-3}$	$1.48 \cdot 10^{-3}$	8.59

the MAG and EE production. In a typical reaction course the initial production of EE is very rapid, the DAG is accumulated for a little while and then it is rapidly deacylated to MAG. The MAG production reaches a maximum and it is deacylated to glycerol. The experimental kinetic data were satisfactorily correlated by a simple kinetic model based on the elementary reactions proposed in this work. From this model, kinetic parameters, such as the forward and reverse reaction rate constants and activation energies were evaluated. At high ethanol:oil molar ratio values, the three reaction steps can be considered as irreversible due to the high values of the effective forward reaction rate constants compared to the values of the reverse reaction rate constants.

The reaction rate was found to increase with increasing amount of lipase. The MAG maximum is reached faster but MAG also degrades faster to glycerol. The higher the reaction temperature, the sooner the maximum MAG content is reached. The maximum MAG content was decreased by decreasing the initial ethanol:oil molar ratio. Additionally, the ratio of the forward and reversed reaction rate constants for the third step (deacylation of monoglyceride to glycerol) decreased with decreasing the molar ratio ethanol:oil.

At high initial ethanol:oil molar ratio (76:1) the reaction rate is similar in a tert-pentanol as reaction medium and in a solvent-free system, where ethanol acts as both solvent reaction medium and as reactant; the reaction rate in *n*-

Fig. 9 Arrhenius plot of effective reaction rate: ○ k'_1 ; □ k'_2 ; △ k'_3 ; ◇ k'_{-3} . Lipase loading 10 wt%, initial molar ratio 76:1, *n*-hexane as reaction medium.

Fig. 10 Arrhenius plot of effective reaction rate: ○ k'_1 ; □ k'_2 ; △ k'_3 ; ◇ k'_{-3} . Lipase loading 10 wt%, initial molar ratio 76:1, solvent-free system.

Table 7 Activation energies and preexponential factors for the ethanolysis reaction in *n*-hexane and in a solvent free system at initial molar ratio 76:1 with a lipase loading of 10 wt%.

Step	1	2	3	-3
<i>n</i> -Hexane				
E_a (kJ/mol)	29.65	30.16	34.62	45.83
k_o	$9.92 \cdot 10^2$	$1.25 \cdot 10^4$	$1.39 \cdot 10^3$	$2.01 \cdot 10^4$
r^2	0.9947	0.9878	0.9995	0.9309
Solvent-free				
E_a (kJ/mol)	15.65	7.53	10.42	15.89
k_o	$1.36 \cdot 10^1$	$0.33 \cdot 10^1$	$1.08 \cdot 10^{-1}$	$5.46 \cdot 10^{-1}$
r^2	0.9893	0.8792	0.9813	0.9969

Step:1: triglyceride to diglyceride; 2: diglyceride to monoglyceride; 3: monoglyceride to glycerol; -3 glycerol to monoglyceride.

hexane is slower. This can be related with the hydrophobicity of the support of Lipozyme® 435 and the different solvent polarity, *n*-hexane being non-polar.

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