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# Effect of temperature on the kinetics of the enzymatic esterification of n-3 polyunsaturated fatty acids and glycerol

# Efecto de la temperatura en la cinética de esterificación enzimática de ácidos grasos poliinsaturados n-3 y glicerol

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## Abstract
In this work, the effect of temperature on the kinetics of esterification of polyunsaturated fatty acids (PUFA) and glycerol catalyzed by *Candida antarctica* lipase was studied for the production of structured acylglycerols in a batch reactor. A concentrate of n-3 PUFA was prepared by chemical hydrolysis of Menhaden oil followed by urea precipitation. Experiments were conducted at 40, 50, and 60°C using the stoichiometric ratio of 3 mol of PUFA per mol of glycerol. At 60°C the overall esterification yield was 51.8%. The activation energy was estimated in 28.611 kJ mol$^{-1}$. To describe the overall esterification kinetics, a Michaelis-Menten model with reversible effects was used. Esterification kinetics of the structured acylglycerols obeyed a kinetic model based on the ordered-sequential multi-product multi-substrate mechanism. The kinetic parameters of both models were obtained by a non-linear regression algorithm using the MATLAB® software (R2018b). The average coefficient of determination of the models was $R^2_{avg}$=0.93.
*Keywords*: acylglycerols, enzymatic esterification, kinetic parameters, n-3 PUFA.

## Resumen
En el presente trabajo se estudió el efecto de la temperatura sobre la cinética de esterificación de ácidos grasos poliinsaturados (AGPI) y el glicerol catalizada por la lipasa de *Candida antarctica* para la producción de acilgliceroles estructurados en un reactor en lotes. Se preparó un concentrado de AGPI n-3 por medio de hidrólisis química del aceite de arenque seguido de una precipitación con urea. Los experimentos se realizaron a 40, 50 y 60°C, usando una relación estequiométrica de 3 moles de AGPI por mol de glicerol. A 60°C el rendimiento en la esterificación global fue de 51.8%. La energía de activación estimada fue de 28.611 kJ mol$^{-1}$. Para describir la cinética de esterificación global se utilizó un modelo de Michaelis-Menten con efectos reversibles. La cinética de esterificación de los acilgliceroles estructurados obedeció a un modelo cinético basado en un mecanismo secuencial ordenado multi-sustrato multi-producto. Los parámetros cinéticos de ambos modelos fueron obtenidos por un algoritmo de regresión no-lineal por medio del programa de MATLAB® (R2018b). El coeficiente de determinación promedio de los modelos fue de $R^2_{prom}$=0.93.
*Palabras clave*: acilgliceroles, esterificación enzimática, parámetros cinéticos, AGPI n-3.

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# 1 Introduction

The n-3 polyunsaturated fatty acids (PUFA) are considered essential for human life. This is because the human body cannot synthesize them, and thus they must be provided through the diet (Baeza *et al*., 2014; Hernández *et al*., 2016; Khan *et al*., 2023). The n-3 PUFA are important components that provide flexibility, fluidity, and permeability to cell membranes, which in turn promotes cardiovascular health. Essential fatty acids are also important because they are involved in the process of brain and retina formation in fetuses. They also participate in the production of eicosanoid acids, which are regulators of inflammation, fever, thrombosis and blood vessel dilatation (Calder, 2013). The consumption of n-3 PUFA has been related to the prevention of coronary heart, neuromuscular, and immune diseases, diabetes, allergenic reactions, depression and cancer (Piñeiro *et al*., 2013; Castellanos & Rodriguez, 2015; Hallaban *et al*., 2016; Shahidi & Ambigaipalan, 2018; Kołodziej *et al*., 2023). The multiple benefits of the consumption of n-3 PUFAs have encouraged research focused on the synthesis of acylglycerols (AG) containing these FFA for pharmaceutical and nutraceutical applications (Xie *et al*., 2023). A way to produce AG containing n-3 PUFA is by direct esterification of the PUFA and glycerol catalyzed by lipases (Zhao *et al*., 2011; Rivero *et al*., 2020; Mbatia *et al*., 2011; Von der Haar *et al*., 2014; Castejón & Señoráns, 2019; Zhu *et al*., 2024). Lipases can hydrolyze lipids in aqueous medium and catalyze the binding of FFA to glycerol in a virtually anhydrous medium. Therefore, it is important to determine the effect of water on enzyme reactions, since water is a by-product of the esterification reaction. On the other hand, temperature affects enzymatic reactions by increasing the reaction rate up to an optimal point, beyond which enzymatic activity may decline due to enzyme denaturation. (González *et al*., 2010).

The study the enzymatic esterification reaction may follow two alternative approaches. The first approach consists of an overall analysis in terms of a overall esterification (GE) value. In the second approach, a stepwise reaction mechanism is proposed and the resulting kinetic models are solved numerically. This is done for each type of AG present in the medium; namely, mono-, di- and triacylglycerols. Noriega *et al*. (2013) studied the effect of temperature, molar ratio and reaction time in the esterification of PUFA to glycerol by using a statistical experimental design with a rotatable central composition. During the experiments, water was eliminated from the medium by means of molecular sieves. The authors determined the optimal conditions for the formation of each acyl glycerol of interest. Mathematical modeling is a useful tool that helps elucidate the relevant phenomena occurring in a process (Thuy *et al*., 2023). Upon validation, the models may be employed to analyze, scale up, and optimize the process in terms of a specific response variable, such as productivity or efficiency (Valle *et al*., 2024; Albalate *et al*., 2023). The models must be capable of describing quantitatively the relevant phenomena occurring in the system and thus predict the experimental data with reasonably accuracy (Du *et al*., 2016). The goal of this work was two-fold. First, to test the effect of temperature on the GE value under controlled laboratory conditions; and second, to determine a kinetic model for the formation of monoacylglycerols (MAG), diacylglycerols (DAG) and triacylglycerols (TAG) by direct esterification of n-3 PUFA into glycerol catalyzed by a *Candida antarctica* lipase in a batch reactor.

# 2 Methods

## 2.1 Reagents

For the reaction medium, Menhaden oil (Sigma-Aldrich), food-grade glycerol (J.T. Baker) and tert-butylhydroquinone (TBHQ, Dresen Quimica, Mexico), as an antioxidant were used. The catalyst used was *Candida antarctica* NV-435 (Novo Nordisk Denmark) immobilized on a macroporous acrylic resin. For high-performance thin-layer chromatography (HPTLC) analysis, HPTLC-HLF plates (Uniplate) of 10x10 cm with silica gel, analytical-grade hexane and ethyl ether (Sigma-Aldrich), glacial acetic acid (Fermont), and reactive-grade iodine (Fermont) were used. To quantify MAG, DAG, and TAG, a standard curve was generated using lipid standards (Sigma-Aldrich) containing a mixture of cis-9-monoolein, cis-9-1,2-diolein, cis-9-1,3-diolein, and cis-9-triolein.

## 2.2 Isolation and concentration of PUFA

The procedure described by Correa *et al*. (2017) was followed to obtain a PUFA concentrate from Menhaden fish oil (Sigma-Aldrich): 100 g of oil were hydrolyzed with 200 mL of KOH 7 M in 70% ethanol, heating to reflux for one hour. The aqueous phase was acidified down to pH 1.0 with concentrated HCl, then the free fatty acids (FFA) were extracted twice with 200 mL of hexane. After solvent evaporation, FFA (15 g) were mixed with urea (25 g) in an Erlenmeyer flask with 100 mL ethanol (95%) and heated until the mixture turned to a light yellow clear homogeneous solution. The mixture was transferred to centrifuge tubes and rapidly cooled by immersion in chilled water for crystal formation. The crystals were removed

by centrifugation at 6000x*g* for 15 min at 0°C. The supernatant was transferred into a separator funnel and acidified to pH 4.0, then the PUFA were extracted with 1:1 hexane:water for 30 min using a glass stirrer. Finally, the organic phase (hexane) containing the PUFA was separated and rotary evaporated at 40 rpm, 40°C, using a vacuum of 25 mmHg.

### *2.3 Enzymatic esterification*

Enzymatic esterification was carried out by duplicate in 25 mL Celstir jacketed flask reactors (Corning) connected to a water-heated circulator (Polyscience) for temperature control and placed over magnetic plates for continuous stirring at 200 rpm. Inside each jar, 3000 mg of glycerol and PUFA were placed in a stoichiometry ratio of 3 mol PUFA/mol glycerol. The reactant mixture was stirred for 5 min until the medium reached a constant temperature, then 150 mg of enzyme (5% w/w) were added to initiate the esterification reaction. The experimental runs were performed at 40, 50, and 60°C. Samples of 15 $\mu$L were withdrawn from the reactor at pre-specified reaction time values (0, 0.33, 0.66, 1.0, 1.33, 1.66, 2.0, 2.5, 4.0, 6.0 and 8.0 h). Samples were analyzed to determine the content of FFA by titration with NaOH. Samples of 5 $\mu$L were collected simultaneously from the reactor to determine AG by HPTLC. To prevent oxidation of PUFAs, 0.6 mg of tert-butylhydroxyquinone (TBHQ) was added to the samples and flushed with UHP nitrogen. Whenever the reaction flasks were opened, UHP nitrogen gas was flushed into the reaction flasks to evacuate the oxygen present in the system.

### *2.4 Analysis of produced acylglicerols*

For the analysis of products of the reaction, aliquots of 5 $\mu$L were withdrawn from the reaction flask and diluted in 1 mL of a chloroform-methanol (2:1 v/v) mixture. This procedure guaranteed a concentration of 10 $\mu$g sample/$\mu$L solution, as was previously standardized. The analysis of 1 $\mu$L of the diluted samples was performed on a 10x10 cm HPTLC silica gel plates (Si 60 Merck). The samples were placed 1 cm from the lower edge of the plate, and separated 1 cm from each other. The HPTLC analysis was carried out into a vertical glass chamber with double channel (CAMAG) in the presence of the mobile phase of hexane:ethyl ether (38:62 v/v, with 1% glacial acetic acid) (Pino & Noriega, 2011). The plate was immersed into the channel with the mobile phase for the chromatographic elution, until the mobile phase front displaced about 8.5 cm, or 1.0 cm before reaching the upper edge. The plate was then removed from the chamber and allowed to evaporate the solvents inside a laboratory fume hood. The plates were developed by spraying a iodine solution (0.5% w/v in ethanol), until a uniform light-yellow color was observed. The solvents were evaporated at 4°C by 45 minutes, and the developed plates were scanned with 300 dpi resolution. The images were analyzed by means of the ImageJ 1.45 software to obtain the corresponding chromatogram (Schneider *et al*., 2012). For quantification of FFA, mono-, di- and triacylglycerols, a standard curve was previously obtained by using a standard lipid mixture (Sigma-Aldrich) containing cis-9-monoolein, cis-9-1.2-diolein, cis-9-1.3-diolein and cis-9-triolein. An additional standard curve for free linoleic acid was also prepared. To determine the GE, 15 $\mu$L samples were collected during the enzymatic reaction and diluted in 2-propanol. The FFA in the samples were titrated with 0.05 M NaOH using phenolphthalein as indicator. The GE value was estimated from the following equation:

$$GE = \left(1 - \frac{C_{FFA}}{C_{0FFA}}\right) \times 100 \tag{1}$$

where $C_{0FFA}$ and $C_{FFA}$ are the initial and actual concentration of FFA in the sample, respectively.

## 3 Results and discussion

### *3.1 Proposed enzymatic kinetic models and estimation of kinetic parameters*

#### *3.1.1 Overall esterification (GE)*

To describe the GE, a Michaelis-Menten model with reversible effects was proposed. The following assumptions were made: (a) PUFA is considered as the only substrate in the reaction since it is the major FFA present, (b) the water produced by the chemical reaction remains in the solution and thus makes the enzymatic esterification reversible, (c) the enzyme concentration is much smaller than that of the substrates; furthermore, the concentration of the enzyme-substrate complex is constant with time, and d) the reactor is perfectly mixed.

The rate of consumption of PUFA is given by (Bisswanger, 2017):

$$\frac{dC_{PUFA}}{dt} = v_{PUFA} = \frac{K_{mW}V_1C_{PUFA} - K_{mPUFA}V_2C_W}{K_{mPUFA}K_{mW} + K_{mW}C_{PUFA} + K_{mPUFA}C_W} \tag{2}$$

where $K_m$ is the Michaelis-Menten constant, $V$ is the maximum reaction rate, and $V_1$ is the maximum rate of product formation. This parameter is a function of temperature and can be described by the Arrhenius equation:

$$V_1 = A\exp\left(\frac{-E_a}{RT}\right) \tag{3}$$

Table 1. Definition of kinetic parameters and simplified rate equations from kinetic model for reactions between PUFA and glycerol to produce mono-, di- and triacylglycerols in the presence of water.

| **Variable** | $\mathbf{V_{eG}}$ | $\mathbf{V_{hMAG}}$ | $\mathbf{V_{eMAG}}$ | $\mathbf{V_{hDAG}}$ | $\mathbf{V_{eDAG}}$ | $\mathbf{V_{hTAG}}$ | $\mathbf{K_{mG}}$ | $\mathbf{K_{mMAG}}$ | $\mathbf{K_{mDAG}}$ |
|---|---|---|---|---|---|---|---|---|---|
| **Definition** | $\frac{k_1k_2}{k_{-1}+k_2}$ | $\frac{k_{-2}k_{-3}}{k_{-2}+k_3}$ | $\frac{k_4k_5}{k_{-4}+k_5}$ | $\frac{k_{-5}k_{-6}}{k_{-5}+k_6}$ | $\frac{k_7k_8}{k_{-7}+k_8}$ | $\frac{k_{-8}k_{-9}}{k_{-8}+k_9}$ | $\frac{k_{-1}}{k_1}$ | $\frac{k_{-4}}{k_4}$ | $\frac{k_{-7}}{k_7}$ |
| **Variable** | **A** | **B** | **C** | **D** | **E** | | | | |
| **Definition** | $V_{hMAG}C_{MAG}C_W$ | $V_{eG}C_GC_{PUFA}$ | $V_{hDAG}C_{DAG}C_w$ | $V_{eMAG}C_{MAG}C_{PUFA}$ | $V_{hTAG}C_{TAG}C_W$ | | | | |
| **Variable** | **F** | **X** | | | | | | | |
| **Definition** | $V_{eDAG}C_{DAG}C_{PUFA}$ | $(C_W + K_{mDAG}C_{DAG} + K_{mMAG}C_{MAG} + K_{mG}C_G)C_{PUFA}$ | | | | | | | |
| **Variable** | $\mathbf{V_{PUFA}}$**, -** $\mathbf{V_W}$ | | $\mathbf{V_G}$ | $\mathbf{V_{MAG}}$ | $\mathbf{V_{DAG}}$ | | $\mathbf{V_{TAG}}$ | | |
| **Definition** | $\frac{(A-B+C-D+E-F)}{X}$ | | $\frac{(A-B)}{X}$ | $\frac{(C+B-A-D)}{X}$ | $\frac{(E+D-C-F)}{X}$ | | $\frac{(F-E)}{X}$ | | |

where $A$ is the pre-exponential factor (mM $h^{-1}$), $E_a$ is the activation energy (kJ $mol^{-1}$), $R$ it is the ideal gas constant (kJ $K^{-1}$ $mol^{-1}$) and T is the temperature (K). To analyze the effect of the temperature on GE, the $E_a$ value was determined. The numerical solution of Equation (2) provided the numerical values of $C_{PUFA}$ as a function of time, which were further used in Equation (1) to determine GE.

### *3.1.2 Kinetics of acylglycerol production*

To describe the formation of acylglycerols, an enzymatic kinetic model based on an ordered-sequential multi-product multi-substrate mechanism was considered. The following assumptions were made: (a) the water produced during esterification causes hydrolysis effects, (b) two enzyme complex structures ($E^*$) are formed between each reaction step, before the respective sub-product is obtained, (c) the initial water concentration in the system is negligible ($C_{W0}$= 0), (d) a single substrate (PUFA) was considered with a molecular weight of 286.86 g $mol^{-1}$ based on the composition of FFA analyzed by GC according to the work reported by Noriega *et al*. (2013), (e) the decrease in enzyme activity was considered negligible attributed to the high stability and prolonged activity at elevated temperatures demonstrated by the lipase from *Candida antarctica*, and (f) the enzyme concentration is much lower than the substrate concentration, and thus the concentrations of all species in which the enzyme is present do not vary with time. The reaction mechanism is as follows:

$$G + PUFA + E \underset{k_{-1}}{\overset{k_1}{\rightleftarrows}} E_1^* \underset{k_{-2}}{\overset{k_2}{\rightleftarrows}} E_2^* \underset{k_{-3}}{\overset{k_3}{\rightleftarrows}} MAG + W + E \tag{4}$$

$$MAG + PUFA + E \underset{k_{-4}}{\overset{k_4}{\rightleftarrows}} E_3^* \underset{k_{-5}}{\overset{k_5}{\rightleftarrows}} E_4^* \underset{k_{-6}}{\overset{k_6}{\rightleftarrows}} DAG + W + E \tag{5}$$

$$DAG + PUFA + E \underset{k_{-7}}{\overset{k_7}{\rightleftarrows}} E_5^* \underset{k_{-8}}{\overset{k_8}{\rightleftarrows}} E_6^* \underset{k_{-9}}{\overset{k_9}{\rightleftarrows}} TAG + W + E \tag{6}$$

Where PUFA, G, MAG, DAG and TAG are the substrates and products in the reaction, $E$ is the free enzyme, $E_i^*(i = 1,\ldots,6)$ are the enzyme complexes and $k_i$ $(i = -9,\ldots,9)$ are kinetic constants.

Based on the reaction mechanism represented by equations (4) through (6), mass balance equations were prepared for each species present in the system (Cheirsilp *et al*., 2007), where $V_e$ and $V_h$ are the maximum rates of esterification and hydrolysis, respectively, and $K_G$, $K_{MAG}$ and $K_{DAG}$ are the equilibrium constants of each reaction step. The definition of the kinetic parameters and the reaction rate equations obtained are disclosed in Table 1.

Glycerol concentration ($C_G$) was calculated using a mass balance based on the stoichiometry of the reaction (Eq. 4, 5 and 6) considering the concentrations of mono-, di- and triacylglycerols obtained in HPTLC analysis by the following equation:

$$C_G = C_{0G} - (C_{MAG} + C_{DAG} + C_{TAG}) \tag{7}$$

where $C_{0G}$ is the initial molar concentration (M) of G and $C_G$, $C_{MAG}$, $C_{DAG}$ and $C_{TAG}$ are the molar concentration of G, MAG, DAG and TAG, respectively.

### *3.1.3 Estimation of kinetic parameters*

The experimental data were used to fit the kinetic model (Table 1, Eq. 2) by means of a computer program written within the MATLAB (R2018b) software. The model parameters were simultaneously estimated for all temperatures tested in the experiments using a non-linear regression method with constrains and the assumption that the maximum rates ($V_{eG}$, $V_{hMAG}$, $V_{eMAG}$, $V_{hDAG}$, $V_{eDAG}$ and $V_{hTAG}$) and kinetic constants ($K_{mG}$, $K_{mMAG}$ and $K_{mDAG}$) obey the Arrhenius equation (Eq. 3). The numerical values

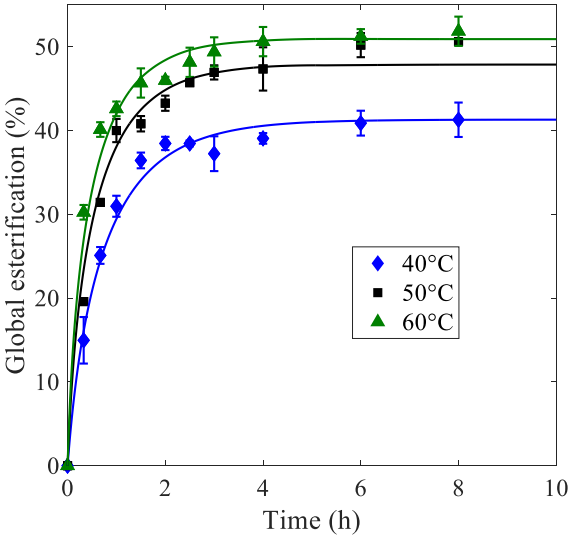


Figure 1. Kinetic of global esterification. Experimental data (symbols), reversible Michaelis-Menten model (solid lines).

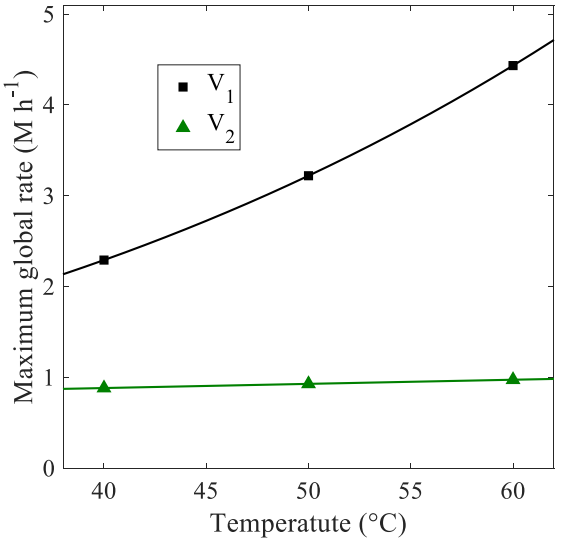


Figure 2. Effect of temperature on maximum global rates of reaction. Subscript 1, esterification; subscript 2, hydrolysis.

of the model parameters were computed iteratively as the ordinary differential equations describing the mass balance of each species in the mixture were solved as a function of time. The iterations were conducted until the sum of the squared errors between the model predictions and the experimental data in terms of the species concentrations reached a minimum value, and the parameter values did not vary significantly from one iteration to the next. The coefficient of determination (R-squared) was used as the criterion for convergence.

## 3.2 *Effect of temperature*

Table 2. Kinetic parameters determined for the global esterification reaction of PUFA to glycerol at different temperatures.

| Parameter | Temperature (° C) | | | Units |
|---|---|---|---|---|
| | 40 | 50 | 60 | |
| $K_{mPUFA}$ | 1.554 x$10^{-2}$ | 1.076 x$10^{-2}$ | 7.713 x$10^{-3}$ | M |
| $K_{mW}$ | 4.215 x$10^{-3}$ | 2.509 x$10^{-3}$ | 1.557 x$10^{-3}$ | M |
| $V_1$ | 2.292 | 3.220 | 4.434 | M $h^{-1}$ |
| $V_2$ | 0.883 | 0.929 | 0.975 | M $h^{-1}$ |

### 3.2.1 *Overall esterification*

Figure 1 shows the evolution of GE with time when the temperature was set to 40, 50 and 60 °C. It is noted that the Michaelis-Menten model showed good agreement with the experimental data, with an average value of the determination coefficient: $R^2_{avg}$=0.98. The value of GE increased with temperature, achieving 41.2, 50.6, and 51.8% at 40, 50, and 60°C, respectively, after 8 h of reaction. These results were similar to those observed with other lipases. Figure 2 shows that the maximum overall esterification rates $V_1$ and $V_2$ increased with temperature, but the value of $V_2$ was less sensitive to temperature than the value of $V_1$. The values of $V_1$ were plotted according to the linearized form of the Arrhenius equation and the values of $E_a$ and the pre-exponential term A were obtained. This resulted in the values of 28.61 kJ $mol^{-1}$ and 1.35x$10^5$ M $h^{-1}$, respectively. The calculated $E_a$ falls within the values obtained in other studies of esterification, hydrolysis, and transesterification reactions (Jamie *et al*., 2017). The decrease of the Michaelis constants ($K_m$) with temperature (Table 2), suggests that the enzyme had a greater affinity for the substrate at higher operating temperatures.

The presence of water in the medium causes reversible effects on the reaction because lipases can hydrolyze lipids in an aqueous medium. In a previous study, enzymatic esterification under similar conditions was investigated, but molecular sieves were added to the medium to isolate the water produced during the reaction, achieving an GE close to 70% after 10 h of reaction (Correa *et al*., 2017). On the other hand, some works implemented different methods to remove or isolate the produced water during esterification reaction, resulting GE higher than 90% (Noriega *et al*., 2013).

### 3.2.2 *Acylglycerols production*

Figure 3 shows the concentrations of the acylglycerols produced during the reaction. It is noted that MAG were the main product obtained during enzymatic esterification at all temperatures tested in the experiments. The proposed enzymatic model showed good agreement with the experimental data for all

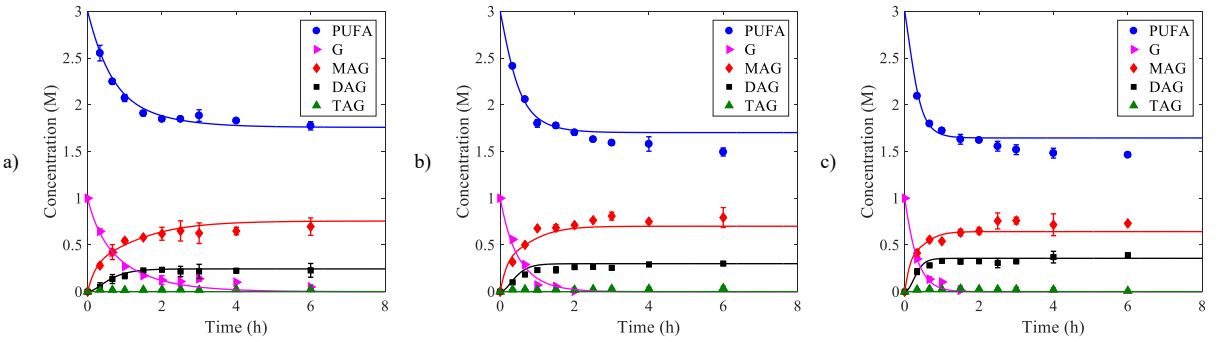


Figure 3. Kinetics of the production of acylglycerols during the enzymatic esterification of PUFA and glycerol at different temperatures: a) 40°C, b) 50°C and c) 60°C. Experimental data (symbols) and ordered sequential multi-product and multi-substrate model (solid lines).

Table 3. Kinetic parameters calculated for the esterification reaction of PUFA to glycerol for the formation of acylglycerols at different temperatures.

| **Parameter** | **Temperature (° C)** | | | **Units** |
|---|---|---|---|---|
| | 40 | 50 | 60 | |
| $K_G$ | 0.689 | 1.138 | 1.832 | $M^{-2}$ |
| $K_{MAG}$ | $3.269x10^{-4}$ | $5.913x10^{-4}$ | $1.032x10^{-3}$ | $M^{-2}$ |
| $K_{DAG}$ | $3.269x10^{-4}$ | $5.913x10^{-4}$ | $1.032x10^{-3}$ | $M^{-2}$ |
| $V_{hMAG}$ | $1.460x10^{-5}$ | $2.671x10^{-5}$ | $4.711x10^{-5}$ | $M^{-1}\ h^{-1}$ |
| $V_{eG}$ | 1.083 | 2.379 | 4.983 | $M^{-1}\ h^{-1}$ |
| $V_{hDAG}$ | 3.907 | 6.008 | 9.0038 | $M^{-1}\ h^{-1}$ |
| $V_{eMAG}$ | 0.890 | 1.963 | 4.128 | $M^{-1}\ h^{-1}$ |
| $V_{hTAG}$ | 2.128 | 4.093 | 7.756 | $M^{-1}\ h^{-1}$ |
| $V_{eDAG}$ | $1.251x10^{-9}$ | $4.643x10^{-9}$ | $1.592x10^{-8}$ | $M^{-1}\ h^{-1}$ |

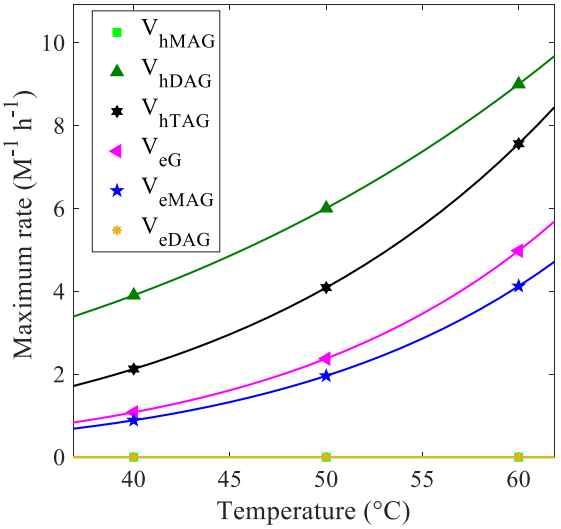


Figure 4. Effect of temperature on maximum rate parameters of reaction stages. Subscript e, esterification; subscript h, hydrolysis.

components of the enzymatic reaction during time ($R^2_{avg}$=0.93). The estimated kinetic parameters are reported in Table 3, where it can be observed that the maximum rate ($V_{eG}$, $V_{hMAG}$, $V_{eMAG}$, $V_{hDAG}$, $V_{eDAG}$ and $V_{hTAG}$) increases with temperature for each stage. These parameters were analyzed considering the different stages of the enzymatic reaction (Eq 4, 5, and 6). In the first reaction stage for the production of MAG (Eq 4), the hydrolysis values ($V_{hMAG}$) are negligible compared to the esterification parameter ($V_{eG}$) which is reflected in the elevated production of MAG in the medium. Cheirsilp *et al.* (2007) utilized a similar model to the one established in this study to describe an enzymatic glycerolysis reaction catalyzed by an immobilized lipase. The kinetic parameters they calculated also indicated a higher tendency towards G esterification rather than MAG hydrolysis. In the second stage of reaction for the production of DAG (Eq 5), the hydrolysis parameter ($V_{hDAG}$) was the highest value of the whole reaction (Fig. 4), indicating the difficulties for the production of DAG caused by the tendency to hydrolyze to form MAG. Water molecules produced during the reaction can reach concentrations close to 2.5% w/w, considering a GE of 50%, which may favor the formation of a specific acylglycerol. Watanabe *et al.* (2005) investigated the esterification of conjugated linoleic acid and found that a water concentration of 1% w/w favored the formation of MAG compared to DAG. Finally, in the last reaction stage for the production of TAG (Eq 6), the highest rate of DAG esterification was insignificant compared to TAG hydrolysis ($V_{eDAG} \ll V_{hTAG}$). This is supported by the small production of TAG during the reaction. Previous studies used molecular sieves

to sequester the water produced by the reaction which resulted in a considerable amount of TAG produced during enzymatic esterification. This indicates that the presence of water significantly reduced TAG formation (Correa *et al*., 2017). Bornadel *et al*. (2013) investigated the enzymatic esterification of oleic oil catalyzed by lipase NV-435. Despite the authors removed water from the medium by using vacuum, they obtained a scarce production of TAG during the first 15 hours of reaction.

Table 4. Activation energies and pre-exponential factors obtained at maximum velocities of the model based on the ordered-sequential mechanism.

| **Parameter** | **A** ($M^{-1}$ $h^{-1}$) | $\mathbf{E_a}$ (kJ $mol^{-1}$) |
|---|---|---|
| $V_{hMAG}$ | $4.354x10^3$ | 50.806 |
| $V_{eG}$ | $1.188x10^{11}$ | 66.188 |
| $V_{hDAG}$ | $4.267x10^6$ | 36.200 |
| $V_{eMAG}$ | $1.116x10^{11}$ | 66.534 |
| $V_{hTAG}$ | $3.178\ x10^9$ | 55.001 |
| $V_{eDAG}$ | $3.159\ x10^9$ | 110.320 |

The $E_a$ and pre-exponential factor of the Arrhenius equation were obtained for each step of the reaction. The results are depicted in Table 4. The highest $E_a$ value obtained (110.32 kJ $mol^{-1}$) was for $V_{eDAG}$, which also reflect the difficulty for the production of TAG. The other values of $E_a$ are very similar and compared to those reported in other enzymatic studies (Zhang *et al*., 2020).

## Conclusion

*Candida antarctica* (NV-435) lipase was able to produce acylglycerols with n-3 PUFA by enzymatic esterification at different temperatures. The GE increased with temperature, reaching 51.8% at 60°C and 8 h of reaction. A reversible Michaelis-Menten model described with good accuracy the kinetics of the GE at all temperatures tested in the experiments. An enzymatic kinetic model based on an ordered-sequential mechanism satisfactorily described the simultaneous formation of each acylglycerol ($R^2_{avg}$=0.93). Water produced during the reaction caused significant reversible effects, reduced considerably the formation of TAG, and favored the production of MAG; in addition to the highest value $E_a$ for esterification of DAG. Based on the kinetic parameters calculated in the models, the substrate ratio, reaction time, and temperature can be varied to favor the formation of a specific structured acylglycerol in a batch reactor. This study can be considered as a reference for the design and scale-up of similar bioprocesses.

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### *Nomenclature*

| | |
|---|---|
| $C$ | Molar concentration, M |
| $E_a$ | Activation energy, kJ $mol^{-1}$ |
| $k$ | Rate constant, $M^{-1}$ $h^{-1}$ |
| $K$ | Equilibrium constants, $M^{-2}$ |
| $K_m$ | Michaelis constant, M |
| $R$ | Gas constant, 8.3144 J $K^{-1}$ $mol^{-1}$ |
| $T$ | Temperature, K |
| $v$ | Reaction rate, M $h^{-1}$ |
| $V$ | Maximum overall rates, M $h^{-1}$ |
| $V_e$, $V_h$ | Maximum rates of esterification and hydrolysis stages, respectively ($M^{-1}$ $h^{-1}$) |

Subscripts

| | |
|---|---|
| AG | Acylglycerols |
| DAG | Diacylglycerols |
| FFA | Free Fatty Acids |
| G | Glycerol |
| MAG | Monoacylglycerols |
| PUFA | Polyunsaturated fatty acids |
| TAG | Triacylglycerols |
| W | Water |
| 1,2,3, etc. | Consecutive reaction |

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