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EFFECT OF TEMPERATURE ON THE KINETICS OF ENZYMATIC HYDROLYSIS OF SARDINE OIL BY DIFFERENT LIPASES

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Abstract: In the present work, the effect of temperature on the partial enzyme-catalysed hydrolysis of sardine oil by three different lipases was studied: *Candida rugosa* AY-30, *Rhizopus oryzae* FAP-15 and *Burkoldheria cepacian* PS-CI. The reaction was carried out in glass flasks containing an emulsion of sardine oil:phosphate buffer solution of pH 7.0 (40:60 v/v) and 0.05 g of the different enzymes. The resulting mixtures were placed on magnetic stirring plates inside of an incubator at 20, 30, 40, 50 and 60°C. Samples were withdrawn at different reaction times. To describe the global hydrolysis, a reversible Michaelis-Menten model was considered, which includes an "α-factor" that considers the loss of enzymatic activity due to thermal denaturing. PS-CI lipase exhibited both the greatest enzymatic activity and thermal stability, reaching a hydrolysis degree of 0.468 at 24 h. The kinetic parameters of the models were estimated by non-linear regression with restrictions in a MATLAB® platform (R2018b), with a coefficient of determination (R^2_{prom}>0.93).

Keywords: hydrolysis, kinetic parameters, Michaelis-Menten reversible model, enzymatic activity

INTRODUCTION

Sardine oil has been of great interest due to its high content of n-3 polyunsaturated fatty acids (n-3 PUFA) (Sidhu, 2003), which are considered essential because they are only obtained through the diet (Hernández *et al.,* 2016; Baeza *et al.,* 2016). The consumption of n-3 PUFA are vital, since they are involved in the process of retina and brain formation in foetuses, as well as the production of eicosanoids. In addition, its consumption has been related to the prevention of coronary, neuromuscular, immunological, diabetes, allergenic, depression and cancer diseases (Makrides *et al.,* 1996; Mathew, 2002, Piñeiro

et al., 2013; Castellanos and Rodríguez, 2015; Hallaban *et al.,* 2016; Shahidi and Ambigaipalan, 2018, Rimm *et al.,* 2018; Damascus *et al.,* 2017; Wang and Huang, 2015). Due to the multiple benefits of n-3 PUFA, research has been conducted towards the production of acylglycerols (AG) containing these fatty acids for pharmaceutical and nutraceutical applications (Gunstone, 2003). Most edible oils are constituted of triacylglycerols (TAG), but different investigations have focused on the production of oils containing mainly of diacylglycerols, since it has been shown that their consumption contributes to weight loss, lower liver fat content, to the reduction of body fat, to the improvement of lipid metabolism favouring oxidation and to the reduction of TAG storage in adipose tissue (Maki KC *et al.,* 2002; Yuan *et al.,* 2010; Kamphuis *et al.,* 2003). On the other hand, monoacylglycerols are recognized for being excellent emulsifiers and they have food, cosmetic and pharmaceutical applications (Hernández and Otero, 2008; Fregolente *et al.,* 2007). Enzymatic processes for lipid modification have multiple advantages compared to conventional chemical processes, such as mild reaction conditions of pH, temperature and reduced energy cost, low enzyme consumption, high catalytic efficiency and a wide range of fatty acid selectivity of lipases leading to specific and pure products (Antczak *et al.,* 2009; Shimada *et al.,* 2001). There are different enzymatic methods to produce FA rich in n-3 PUFA, such as lipase-catalyzed esterification, glycerolysis and interestification reactions (Noriega *et al.,* 2013; Miranda *et al.,* 2012; Chen *et al.,* 2017; Correa *et al.,* 2017), but one of the most promising methods is the partial enzymatic hydrolysis of fish oil (Cheong LZ *et al.,* 2007; Bhandari *et al.,* 2015; Morales *et al.,* 2018; Mohammadi *et al.,* 2015). Lipases are commonly used to catalyze enzymatic reactions in AG production, and are recognized as the most important group of biocatalysts in biotechnology due to their versatility (Hasan *et al.,* 2006). Lipase-catalyzed enzymatic hydrolysis has shown to be a direct and selective method for the production of n-3 PUFA concentrates, however, the presence of double bonds in *cis* carbon-carbon conformation in n-3 PUFA, particularly in the acids eicosapentanoic (EPA) and docosahexanoic (DHA) cause folds in the hydrocarbon chains, causing stearic obstacles. Due to this stearic hindrance effect, the enzymatic active sites cannot reach the ester bonds of these fatty acids attached to glycerol, which normally hinders the hydrolysis of EPA and DHA (Kahveci and Xu, 2011; Okada and Morrisey, 2007). Different researchers have reported in their studies that lipases have different modes of action for the hydrolysis of specific fatty acids depending on their source of origin (Salihu *et al.,* 2011; Wanasundara and Shahidi, 1998; Yamane, 1987). According to Paiva *et al.* (2000) the composition of the lid that covers the active site, the geometry of the catalytic amino acids, the structure and the dynamics of the opening of the lid of the active site are the characteristics that confer functional characteristics to lipases. Enzymatic hydrolysis is usually carried out in the presence of buffer solutions because the decrease in pH caused by the free fatty acids produced can decrease the catalytic activity of lipases (Fu *et al.,* 1995; Avelar *et al.,* 2013).

In the present work, the effect of temperature on the partial enzymatic hydrolysis of sardine oil catalyzed by three different lipases was studied: *Candida rugosa* AY-30, *Rhizopus oryzae* FAP-15 and *Burkoldheria cepacia* PS-CI.

MATERIALS AND METHODS

REACTANTS

Fresh sardine oil was used, which was subjected to alkaline refining, bleached and deodorized in order to meet the

recommendations of fish oil refining (Noriega *et al.,* 2009). Triton X-100 was used as emulsifier (Electrophoresis reagent, from Sigma Aldrich), tert-butylhydroquinone (TBHQ, purchased from Dresen Chemical) as antioxidant and a phosphate buffer solution (0.1 M, pH 7). Free form of *Candida rugosa* AY-30, *Rhizopus oryzae* FAP-15 and immobilized *Burkoldheria cepacia* PS-CI lipases were employed as biocatalysts and they were provided by Amano Pharmaceutic Co. Total hydrolysis was determined by titrations, using sodium hydroxide as titrating solution (ACS grade; Fermont PA Cert) and phenolphthalein as indicator.

ENZYMATIC HYDROLYSIS

Enzymatic hydrolysis reactions were carried out in glass flasks (4 cm in diameter and 7 cm in height), containing a mixture of 12 mL of sardine oil with TBHQ (0.5% w/w) and 18 mL of phosphate buffer solution (0.1 M, pH 7). The resulting mixture was homogenized by the addition of triton as an emulsifier (0.7% w/w). A solution of the soluble lipases was prepared in 3 mL of acetate buffer at 5% w/w oil, this was added to the reaction flask to start the enzymatic reaction. Reaction flasks were set on magnetic stirring plates within an incubator. Experimental runs were performed at different operating temperatures (20, 30, 40, 50 and 60°C) and samples were withdrawn at different times during 24 h. Each experimental run was conducted in duplicate.

Free fatty acids (FFA) produced were analyzed by titrating the samples with a 0.01N NaOH solution in ethanol using phenolphthalein as indicator. Total hydrolysis (TH) was calculated with the following equation (Rooney and Weatherley, 2001):

$$TH = \frac{V_{NaOH} * 10^{-3} * M_{NaOH} * MM}{W_t * f} \quad (1)$$

Where V_{NaOH} is the volume of NaOH solution required in the titration; M is the concentration of NaOH; MM is the average molecular weight of the fatty acids present in the oil; W_t is the sample weight and f is the mass fraction of oil at the start of the reaction.

ENZYMATIC MODEL

There are diverse researches on the kinetic study of enzyme-catalysed hydrolysis of oils. Some of them are based on mechanisms such as Michaelis-Menten (Redondo *et al.,* 1995; Sharma *et al.,* 2014), inhibition (Plazeres *et al.,* 1993; Quian *et al.,* 2011; Bhandary *et al.,* 2013), reaction at the water-oil interface (Al-Zuhair *et al.,* 2003; Brijwani and Vadlani, 2010; Jaime *et al.,* 2017), chemical kinetics (Knezevic *et al.,* 1998; Morales *et al.,* 2018) and even more complex enzymatic mechanisms such as multi-substrate multi-product ping-pong (Hermansyah *et al.,* 2006; Phuah *et al.,* 2012).

In the present study, to describe the total enzymatic hydrolysis of sardine oil, a reversible Michaelis-Menten model was considered that includes an activity term "α" that considers the loss of enzymatic activity due to the effect of temperature. For data analysis and modelling, the following considerations were taken into account: (a) Water is not considered within the reaction mechanism or in the kinetic model because it is a substrate that is found in excess in the reaction medium, (b) in the reaction there are reversible effects caused by the enzymatic esterification due to the FFA produced by the hydrolysis of the oil, (c) to reduce the number of variables in the reaction, FFA were considered as a single product whose molecular weight is the average of the fatty acids present in the sardine oil (MM = 286.66 g mol^{-1}), (d) the non-enzymatic effects of the reaction are not considered in the model, because these are negligible with respect to the enzymatic effect , (e) each molecule of hydrolysed sardine oil can lead to three

molecules of FFA, thus, the model considers a cubic term in the product concentration, and, (f) the enzyme concentration is much lower than that of the substrates, and then, the variation with respect to time of the enzyme-substrate complex tends to zero.

According to the above stated, the reaction mechanism for the total enzymatic hydrolysis is described as follows:

$$E + AG \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} E * AG \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} E + 3AGL \quad (2)$$

After establishing the kinetic equations for each compound, and solve the corresponding algebraic calculations, the following enzymatic model was obtained:

$$v = \frac{dP}{dt} = \frac{\alpha(K_{mP}V_1[S] - K_{mS}V_2[P]^3)}{K_{mS}K_{mP} + K_{mP}[S] + K_{mS}[P]^3} \quad (3)$$

Equation 2 is an adjusted model published by Bisswander (2002), where α is the activity of the enzyme, K_{mP} is the Michaelis constant for the product (FFA), K_{mS} is the Michaelis constant for the substrate (FA), V_1 is the maximum rate of formation and V_2 is the maximum rate of the reverse reaction.

EFFECT OF TEMPERATURE

In an enzyme-catalysed reaction, the reaction rate increases as a function of temperature according to the Arrhenius equation; however, since they are proteins, after a certain temperature the enzymes can present deformations in their structure, which causes losses in enzymatic activity. The temperature at which the catalytic activity is maximum is called the optimum temperature. In different studies, models have been established to describe the loss of activity caused by temperature considering a denaturation equation that is analogous to the Arrhenius equation (Ferreiro *et al.,* 2019). Kinetics principles indicate that the initial reaction rate increases, however, in the case of enzymes, losses of catalytic activity can occur as a consequence of thermal denaturation.

The proposed kinetic model includes the term "α", which considers the loss of activity caused by thermal denaturation. The model used that describes the loss of activity with respect to time as a function of temperature was based on the equation reported by Voll *et al.* (2011) to describe the loss of activity due to clogging caused by an immobilized lipase. The model considers two conditions to determine the change in activity as a function of the operating temperature (T) in the enzymatic hydrolysis reaction:

$$\text{If } T \leq T_{opt} \quad \therefore \quad \frac{d\alpha}{dt} = 0; \quad (4)$$

$$\text{If } T > T_{opt} \quad \therefore \quad \frac{d\alpha}{dt} = k_d \alpha^n (T - T_{opt})^q;$$

$$n = 1 \text{ or } 2 \quad q = 1 \text{ or } 2 \quad \text{where} \quad \alpha_0 = 1 \quad (5)$$

where T_{opt} is the optimum temperature of the enzyme and k_d is a deactivation constant.

To calculate the activation energy, an equation analogous to that of Arrhenius was considered:

$$V_1 = Ae^{-\frac{E_a}{RT}} \quad (6)$$

Where V_1 is the maximum rate of product formation, A is the pre-exponential factor, E_a is the activation energy and R is the universal gas constant.

The enzymatic hydrolysis reaction of sardine oil was carried out at different temperatures (20, 30, 40, 50 and 60°C). Theoretically, the reaction rate increases as a function of temperature, but this is not appreciated at high temperatures due to enzymatic denaturation, for this reason it was considered a determining factor when determining the maximum rates (V) where:

$$V_{1(20°C)} < V_{1(30°C)} < V_{1(40°C)} < V_{1(50°C)} < V_{1(60°C)} \quad (7)$$

$$V_{2(20°C)} < V_{2(30°C)} < V_{2(40°C)} < V_{2(50°C)} < V_{2(60°C)} \quad (8)$$

For practical purposes, maximum rates will be considered to be the only kinetic parameters that change as a function of temperature.

GIBBS ENERGY

The formula for calculating the Gibbs free energy change in a reaction is given by

$$\Delta_r G' = \Delta_r G'^{\circ} + RT\ln(Q) \quad (9)$$

Where $\Delta_r G'^{o}$ is the change in standard Gibbs free energy, R is the universal gas constant, and Q is the ratio between the products and substrates of the reaction. The change in standard Gibbs energy is calculated from the equilibrium constant:

$$\Delta_r G'^{\circ} = RT\ln\left(K'_{eq}\right) \quad (10)$$

When the reaction reaches steady state, the catalytic efficiencies in both directions of the reaction are equal, K'_{eq} is a constant represented by the ratio between the concentrations of the products and the substrates at equilibrium (Alberty *et al.,* 2011, Noor *et al.,* 2013). This can be expressed according to the Haldane relation:

$$K'_{eq} = \frac{V_1 K_{mP}}{V_2 K_{mS}} \quad (11)$$

The equilibrium constant was calculated at the different operating temperatures, as was the change in the standard Gibbs energy.

ESTIMATION OF KINETIC PARAMETERS

The experimental data were analysed by means of the adjustment of the reversible Michaelis-Menten model (Eq. 3), including an activity term "α" that considers the loss of activity due to temperature (Eq. 5), using the software MATLAB® (R2018b), for which the corresponding programming codes were developed. The parameters of the kinetic model were estimated simultaneously by means of a non-linear regression method with restrictions (eqs. 4, 5, 7 and 8) considering all the experimental data at the different temperatures. The activation energy was determined by adjusting the Arrhenius equation (Eq. 6) considering the values obtained from the maximum rate of formation (V_1) and the standard Gibbs free energy (Eq. 10) was calculated from the Haldane's relation (Eq. 11). Iterations of the parameters were carried out continuously until the sum of the squared errors between the models and the experimental data was the minimum. The regression analysis to the reaction data under the established conditions was validated by the coefficient of determination (R squared).

RESULTS AND DISCUSSION

Figure 1 describes the total hydrolysis kinetics of sardine oil with lipase *Candida rugosa* AY-30, which reached a degree of hydrolysis of 0.2238 at a temperature of 40°C after 24h of reaction. However, at a temperature of 50°C or higher, a lower degree of hydrolysis was obtained due to larger loss of activity due to thermal denaturation. Different reports about hydrolysis of various oils where *Candida rugosa* lipases were used, have concluded optimum operating temperatures are around 35 and 37°C (Bhandari *et al.,* 2013; Sharma *et al.,* 2013; Bhandari *et al.,* 2017). Those results ranged the optimum temperature of 36.14°C calculated by fitting the kinetic parameters for AY-30 lipase. Despite a loss of activity occurred at 40°C for AY-30 lipase, a higher conversion was achieved due to the residual enzymatic activity and a higher reaction rate with respect to the observed at 30°C. The deactivation model (Eq. 5) exhibited a better

adjustment considering quadratic effects in the activity and in ΔT (n=2 and q=2). The values of the calculated kinetic parameters are presented in Table 1. The rate of loss in AY-30 lipase activity is lower due to the low value of the deactivation constant (k_d) of 1.14 X 10^{-2} K^{-1}, compared to that calculated for FAP-15 lipase of 0.123 K^{-1}. The calculated values for the activation energy and the pre-exponential factor are 57.12 kJ mol^{-1} and 3.296 X 10^{11} mM h^{-1}, respectively (Fig. 4a). In comparison to other works, Sharma *et al.* (2014) studied the hydrolysis of tuna oil catalysed by *Candida antarctica* lipase B, attaining an E_a of 26.1 kJ mol^{-1}; from their findings, Jaime *et al.* (2017) obtained an E_a of 18.07 kJ mol^{-1} for the hydrolysis of olive oil catalysed by a *Candida rugosa* lipase, whereas, Pronk *et al.* (1992) calculated the activation energy for a *Candida rugosa* lipase in a membrane reactor of 21.3 kJ mol^{-1}.

The *Rhizopus oryzae* lipase FAP-15 reached a higher degree of hydrolysis of 0.2448 at a temperature of 30°C after 24 h of reaction. According to the fitting model, it is estimated that the optimal operating temperature of the FAP-15 lipase is 38.26°C. Khaskheli *et al.* (2015) studied the hydrolysis of castor oil catalysed by a *Rhizopus oryzae* lipase and indicated an optimum temperature of 37°C, however, other investigations have used lipases of the same origin and have mentioned higher conversions at higher temperatures (around 40°C) (Mendez *et al.,* 2009; Li *et al.,* 2015). From our results, the activation energy and pre-exponential factor values for FAP-15 lipase were 47.17 kJ mol^{-1} and 5.377 X 10^{19} mM h^{-1}, respectively (Fig. 4b). Different investigations have reported E_a values similar to those obtained in the present work (Kim and Chung, 1989; Méndez *et al.,* 2009).

Figure 3 shows that the lipase PS-CI from *Burkoldheria cepacia* was the one that exerted the highest activity, reaching a total hydrolysis of 0.468 at a temperature of 60°C after 24h of reaction. In comparison to AY-30 and FAP-15 lipases, PS-CI was the only immobilised enzyme used in this study, and that fact can explain its greater thermal stability and not an apparent loss of activity caused by thermal denaturation. Different studies have claimed that the immobilization process confers benefits to the enzyme, namely, greater thermal stability and longer catalytic useful life (Bousque *et al.,* 2000; Fishman *et al.,* 2001; Murty and Muniswaran, 2002). PS-CI lipase exhibited activation energy and pre-exponential factor values of 30.82 kJ mol^{-1} and 3.623 X 10^7 mM h^{-1} (Fig. 4c). Sharma *et al.* (2015) calculated the activation energy of the hydrolysis of tuna, salmon and herring oils catalysed by the immobilized *Candida antarctica* lipase B and obtained values of 26.1, 16.6 and 32.1, respectively. Pogaku *et al.* (2011), obtained E_a values between 17.78 and 23.01 kJ mol^{-1} in the study of the different steps of a transesterification catalysed by an immobilized enzyme.

The standard Gibbs free energy change at the different operating temperatures was calculated for the enzymes FAP-15, AY-30 and PS-CI, obtaining average values of -28.93, -26.03 and 38.45 kJ mol^{-1}, respectively. Negative values indicates that reaction is going towards hydrolysis, and that is also explained by the values of the Michaelis constants, there is more affinity of the different lipases to the substrates ($K_{mS}<K_{mP}$). Ferreiro *et al.* (2019), evaluated the hydrolysis reaction of cotton, olive and palm kernel oil, catalysed by a *Geotrichum candidum* lipase, and obtained $\Delta_r G'°$ of -7.48, -7.47 and -6.32 kJ mol^{-1}, respectively. The difference of the results obtained in both investigations are attributed to the different models used, which affect the degree of reaction kinetics and, as well as the dimensions of the kinetic parameters used in the Haldane relation (Eq. 11).

The simultaneous adjustment of the total hydrolysis models for the lipases at the different temperatures exerted good agreement with the experimental data, this is validated by the coefficient of determination (R^2_{avg}>0.93). The determination of the activation energies by means of the direct adjustment of the Arrhenius equation at the maximum rates of formation obtained from the lipases, AY-30, FAP-15 and PS-CI, was validated by the determination coefficients of 0.96, 0.90 and 0.97, respectively.

CONCLUSIONS

The effect of temperature on the partial enzymatic hydrolysis of sardine oil catalysed by three different lipases was studied. To describe the reaction, a reversible Michaelis-Menten model was considered with an "α" term to consider activity losses as a function of temperature. After 24 h, lipases AY-30, FAP-15 and PS-CI reached degrees of hydrolysis of 0.2238, 0.2448 and 0.468, at temperatures of 30, 40 and 60°C, respectively. The free lipases, AY-30 and FAP-15, exhibited activity losses at temperatures of 40°C or higher due to denaturation of the enzyme, whereas the immobilised lipase PS-CI showed greater thermal stability. Despite the complex composition of n-3 PUFA present in sardine oil, a good degree of hydrolysis was obtained in comparison to other studies. The partial hydrolysis of sardine oil with the different lipases is desirable for the purposes of this investigation, which is to produce oils containing mono- and diacylglycerols rich in n-3 PUFA.

The proposed models presented a good adjustment to the experimental data, this was validated by the determination coefficients (R^2). Interesting kinetic parameters were determined, such as the standard Gibbs free energy, the activation energies, and optimal operating temperatures for the different lipases were proposed. The kinetic parameters obtained are a good reference for the design and scaling of reactors for this type of reaction.

NOMENCLATURE

α	Enzymatic activity, [-]
A	Pre-exponential factor, [mM h^{-1}]
$\Delta_r G'$	Gibbs free energy change, [kJ mol^{-1}]
$\Delta_r G'^{o}$	Gibbs standard free energy change, [kJ mol^{-1}]
E_a	Activation energy, [kJ mol^{-1}]
f	Mass fraction, [-]
TG	Total hydrolysis, [-]
k_d	Deactivation constant, [K^{-1}]
K_{eq}	Equilibrium constant, [-]
K_{mP}	Michaelis constant for product, [mM^3]
K_{mS}	Michaelis constant for substrate, [mM]
M	Molar concentration, [mol L^{-1}]
Mw	Molecular weight, [mol g^{-1}]
n y q	Exponential constants, [-]
R	Ideal gas constant, 8.3144 [J K^{-1} mol^{-1}]
T_{opt}	Optimum temperature, [°C]
v	Reaction rate, [mM h^{-1}]
V	Maximum rate, [mM h^{-1}]
V_{NaOH}	NaOH volume, [mL]
W_t	Sample weight, [g]

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HIGHLIGHTS

- Experimental runs were carried out at different temperatures (20, 30, 40, 50 and 60°C) to study the effect of temperature on the partial enzymatic hydrolysis of sardine oil catalysed by three different lipases: *Candida rugosa* AY-30, *Rhizopus oryzae* FAP-15 and *Burkoldheria cepacian* PS-CI.
- A Michaelis-Menten model with reversible effects was proposed to describe the total hydrolysis of sardine oil and a model was established to consider the loss of enzymatic activity caused by thermal denaturation.
- Important kinetic parameters such as the change in standard Gibbs free energy, activation energy (E_a), pre-exponential factor (A), Michaelis constants (K_m) and maximum rates (V) were calculated.
- The models propose optimum operating temperatures of 36.14°C for lipase AY-30 and 38.26°C for lipase FAP-15.
- The parameters of the models were estimated by simultaneously fitting the models to the experimental data by means of a non-linear regression with restrictions in a MATLAB® platform (R2018b). The fit of the models was validated by the coefficients of determination (R^2)

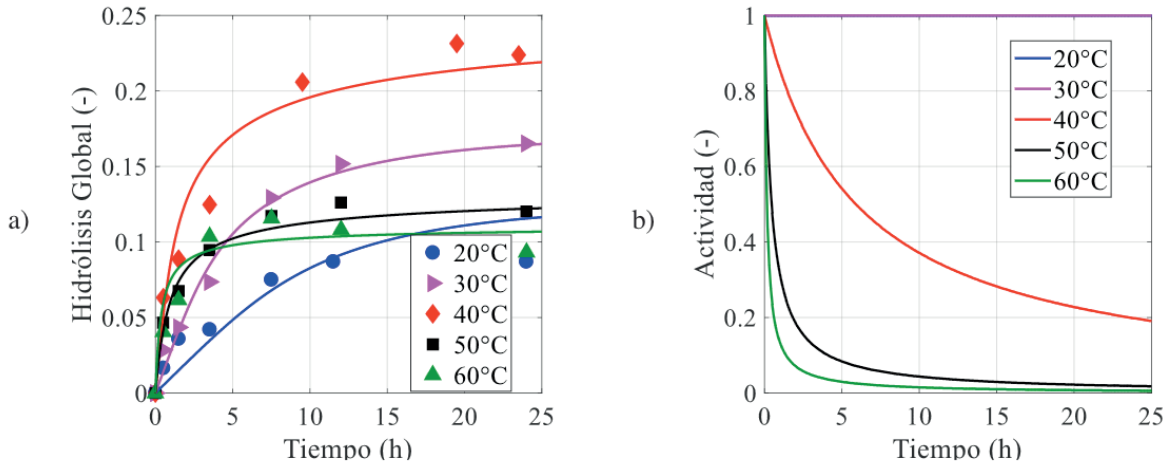

Figure 1. a) Kinetics of total enzymatic hydrolysis of sardine oil catalysed by lipase AY-30 at different temperatures and b) enzymatic activity. Experimental data (symbols) and proposed models (solid lines).

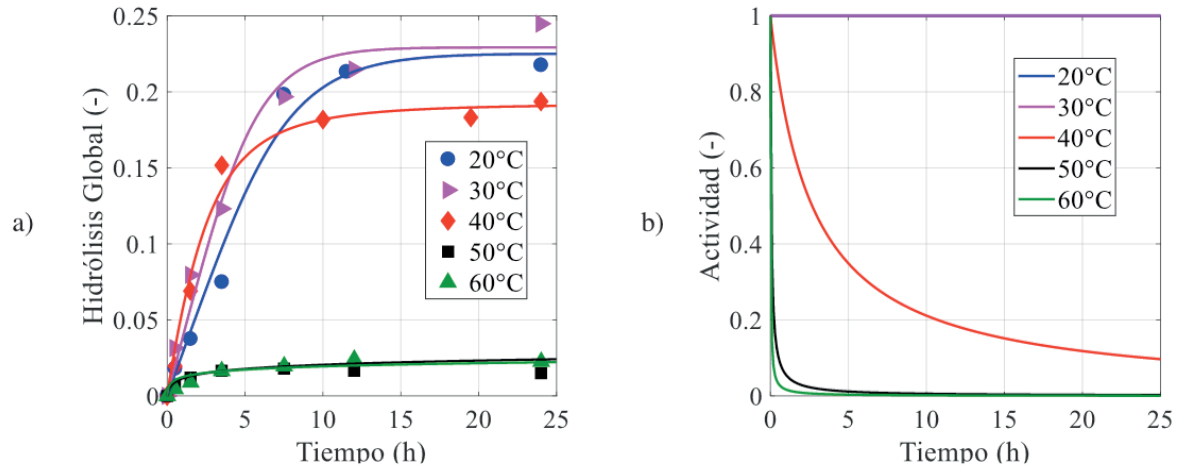

Figure 2. a) Total enzymatic hydrolysis kinetics of sardine oil catalysed by FAP-15 lipase at different temperatures and b) enzymatic activity. Experimental data (symbols) and proposed models (solid lines).

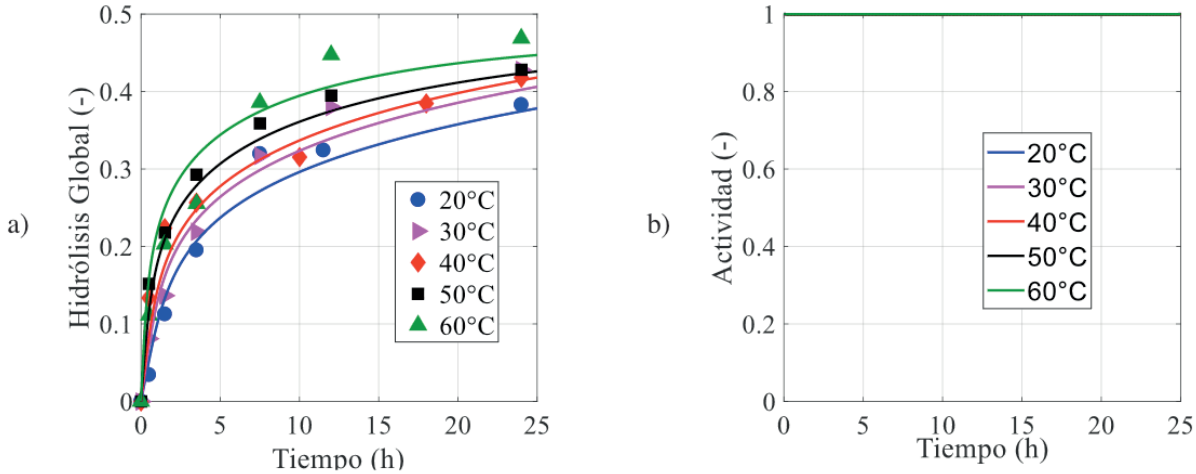

Figure 3. a) Kinetics of total enzymatic hydrolysis of sardine oil catalszed by lipase PS-CI at different temperatures and b) enzymatic activity. Experimental data (symbols) and proposed models (solid lines).

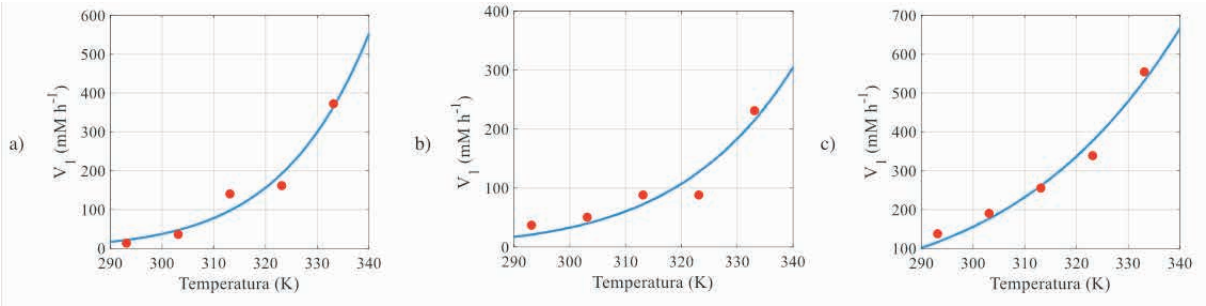

Figure 4. Effect of temperature on the maximum production rate constant in global hydrolysis (V_1). a) AY-30, b) FAP-15 and c) PS-CI. Values determined from the determined parameters (symbols) and model of the Arrhenius equation (solid line).

Parámetro	AY-30	FAP-15	PS-CI	Unidades
K_{mS}	$5.92x10^{-2}$	$3.27x10^{-4}$	$5.8x10^{-3}$	mM
K_{mP}	423.10	95.82	98.86	mM^3
$V_{1(20°C)}$	13.02	36.84	137.64	mM h^{-1}
$V_{1(30°C)}$	36.15	50.25	190.11	mM h^{-1}
$V_{1(40°C)}$	140.35	87.91	255.07	mM h^{-1}
$V_{1(50°C)}$	161.25	87.92	338.17	mM h^{-1}
$V_{1(60°C)}$	371.80	230.78	554.27	mM h^{-1}
$V_{2(20°C)}$	8.38	144.37	0.28	mM h^{-1}
$V_{2(30°C)}$	8.51	185.47	0.68	mM h^{-1}
$V_{2(40°C)}$	8.64	577.73	1.22	mM h^{-1}
$V_{2(50°C)}$	68.74	578.70	5.07	mM h^{-1}
$V_{2(60°C)}$	326.75	589.99	9.23	mM h^{-1}
E_a	57.12	47.17	30.82	kJ mol^{-1}
k_d	$1.14x10^{-2}$	0.123	-	K^{-1}
T_{opt}	36.14	38.26	-	°C
A	$3.296x10^{11}$	$5.344x10^{19}$	$3.623x10^{7}$	mM h^{-1}
$\Delta_r G'^{o}_{avg}$	-26.03	-28.93	-38.45	kJ mol^{-1}

Table 1. Kinetic parameters determined for the total enzymatic hydrolysis reaction of sardine oil.