



Kinetic study of a commercial lipase for hydrolysis of semi-refined oil of anchovy (*Engraulis ringens*)

[Estudio cinético de una lipasa comercial para la hidrólisis de aceite semirrefinado de anchoa (*Engraulis ringens*)]

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Resumen

Las lipasas por su naturaleza ecológica y versatilidad catalítica, son ideales para su aplicación en la industria de hidrólisis del aceite de pescado debido a su propiedad selectiva, que permite conservar los ácidos grasos poliinsaturados (AGPI's) en la estructura lipídica. El objetivo de esta investigación fue determinar la actividad y parámetros cinéticos de una lipasa comercial AY AMANO "30SD" además, los valores de temperatura y tiempo para lograr un grado óptimo de hidrólisis en aceite semi-refinado de anchoveta. Los experimentos se llevaron a cabo en un minireactor encamisado con un volumen de trabajo de 400 mL (aceite- agua-enzima) con control de temperatura y pH 7.00, concentración de enzima 350 U/mL y agitación 160 rpm. Se utilizó un diseño factorial 3x3 y la metodología de superficie de respuesta. Los resultados obtenidos del estudio de la enzima fueron: actividad = $37\ 384,55 \pm 395,07$ U/g y parámetros cinéticos: $K_m = 7,98$ g/L y $V_{m\acute{a}x.} = 0,038887$ g/Lxmin. Correspondientemente se obtuvieron los siguientes parámetros óptimos: Grado de hidrólisis 4.01%, temperatura 46.86 °C y tiempo de hidrólisis 90 minutos, con un nivel de confianza 95% ($p < 0.05$). Conclusiones: El estudio permitió caracterizar cinéticamente la lipasa comercial y determinar el grado óptimo de hidrólisis del aceite semi-refinado de anchoveta.

Palabras clave: Aceite de anchoveta; lipasa; hidrólisis.

Abstract

Lipases due to their ecological nature and catalytic versatility, are ideal for their application in the fish oil hydrolysis industry due to their selective property, which allows the preservation of polyunsaturated fatty acids (PUFAs) in the lipid structure. The objective of this research was to determine the activity and kinetic parameters of a commercial AY AMANO "30SD" lipase, as well as the temperature and time values to achieve an optimal degree of hydrolysis in semi-refined anchovy oil. The experiments were carried out in a jacketed minireactor with a working volume of 400 mL (oil-water-enzyme) with temperature control and pH 7.00, enzyme concentration 350 U/mL and stirring 160 rpm. A 3x3 factorial design and the response surface methodology were used. The results obtained from the study of the enzyme were: activity = $37\ 384.55 \pm 395.07$ U/g and kinetic parameters: $K_m = 7.98$ g/L and $V_{max.} = 0.038887$ g/Lxmin. Correspondingly, the following optimal parameters were obtained: Degree of hydrolysis 4.01%, temperature 46.86 °C and hydrolysis time 90 minutes, with a confidence level of 95% ($p < 0.05$). Conclusions: The study allowed us to kinetically characterize the commercial lipase and determine the optimum degree of hydrolysis of the semi-refined anchovy oil.

Keywords: Anchovy oil; lipase; hydrolysis.

1. Introduction

The fish oil industry in Peru has gained relevance since the discovery of its beneficial properties that its consumption provides due to its high content of polyunsaturated fatty acids (PUFAs) of the $\omega - 3$ chain. (Valenzuela B. et al., 2012),

In recent years the consumption of PUFA's in the human diet has increased but in higher concentrations, for which the way of consuming these essential fatty acids has been innovated in the form of concentrated supplements for people lacking in these components (Lopez H., 2016), this has developed over time techniques that allow obtaining high concentrations of polyunsaturated fatty acids (PUFAs) of the ω -3 chain, which are predominant eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) (Quian, et al., 2020).

These techniques that allow obtaining high concentrations of PUFA's have been investigated by various authors, for example molecular distillation (Cerón, Cardona, & Toro, 2012), formation of urea complexes (Gamez-Meza et al., 2003; Nagachinta, and Akoh, 2012), separation by chromatography (Dillon, Aponte, Tarozo, & Huang, 2013), extraction by supercritical fluids (Sahena et al., 2009), among others. The results of the applications of these techniques lead to use of chemical solvents, high costs in equipment and complex stages, on the other hand, the concentrates obtained from these techniques are mostly of synthetic structure not assimilable in the human body and with formation of undesirable components in the process, while the enzymatic applications to obtain concentrated PUFA's in the form of triacylglycerols (TAG) with greater digestibility (Solaesa, Sanz, Falkeborg, Beltran and Guo, 2016) have displaced these conventional and chemical methods, giving rise to an eco-friendly process with mild conditions of pH, temperature, low energy cost and low consumption of enzymes, resulting in purer products.

The enzymatic application using lipases of fungal origin to achieve these concentrates is based on their selective properties used for hydrolysis and alcoholysis reactions (Correa et. Al., 2017) where the preference of lipase for fatty acid components is of order decreasing: palmitoleic acid> oleic acid> = palmitic acid> stearic acid> EPA> DHA, allowing the PUFA's to be preserved in the lipid structure (Hamed, H. et al., 2018), this hydrolysis process is complemented with other methods to achieve high concentrations of PUFA (Bonilla-Méndez & Hoyos-Concha, 2018), these have been raised in various investigations. For example, Kahveci and Xu (2011) report in their research the use of salmon oil substrate, achieving the concentration of EPA that went from 5.46% to 10.00% and DHA increasing 2.8 times using lipase *Candida rugosa*, Miranda K. et al. (2013) reported the use of a *Candida antarctica* Lipase for the production of structured diacylglycerols with 89.37% PUFA of the omega-3 chain (EPA, DHA and DPA) using fish oil as substrate, Valverde L. et al. (2014) reported an increase in EPA that went from 19% to 61% and a DHA that went from 22% to 69% using Lipozyme TL-IM and QLG lipase with sardine and tuna oil substrate, Aarthy M. et. al. (2016) achieved an increase in EPA from 16.9% to 30.4% and DHA from 6.5% to 9.6% using cod, sardine, salmon and shark oil as substrate catalyzing the reaction with a *Cryptococcus sp.* lipase.

Therefore, the objective of this research is to study a commercial lipase of fungal origin *Candida cylindracea* for its application in a process of hydrolysis of semi-refined Peruvian anchovy oil, in order to verify the effect that commercial lipase has in the process of hydrolysis evaluating the response variable in% Degree of hydrolysis.

2. Materials and Methods

2.1 Substratum

20 L batch of semi-refined anchovy oil (*Engraulis ringens*) provided by the COLPEX INTERNATIONAL SAC refinery, located in the port of Supe, Peru.

2.2 Enzyme

For the enzymatic hydrolysis process, the commercial enzyme AY "AMANO" 30SD was used, acquired from the company AMANO ENZYME, it is a triacylglycerol hydrolase lipase manufactured by a unique fermentation process with a selected strain of *Candida cylindracea*. This enzyme hydrolyzes short, medium and long fatty acids of 1, 2 and 3 positions of triacylglycerol and is applicable to processing of fats and oils. This lipase has a declared activity of 38,600 U / g, the optimal operating conditions reported by the company AMANO Enzyme are: pH 7.00, temperature 50 °C and inactivation temperature 80 °C (AMANO ENZYME, 2019).

2.3 Determination of lipid activity

The lipid activity of the enzyme AY "AMANO" 30SD, was determined following the test method for lipases at pH 7.0, used by the supplier AMANO ENZYME (2019), using olive oil emulsion with a 2% solution of a synthetic polymer soluble in water and 40 mL of phosphate citrate buffer at pH 7.0 controlled with a Thermo scientific pH meter brand ORION model A211. The mixing of the emulsion was carried out in a IKA Brand homogenizer - model RW20.

The emulsion and buffer solution were placed in a minireactor coupled to a LAUDA model A 12 heating thermostat and with a Thermolyne Nuova II stirrer model S18520-26 magnetic stirrer, the equipment was programmed at temperature of 37 °C, it was kept for 15 minutes, then the 10 mL of enzyme were added in the following ratio: 1: 6000, 1: 7000 and 1: 8000.

Finally, the reaction time was finished, 5 ml of acetone and ethanol solution were used in a 1: 1 ratio. The samples were drawn at the following times 0, 5, 10, 15, 20, 25, 30, 35, 40 and 45 minutes. The samples were titrated with 0.05N NaOH to find the amount of carboxylic fatty acids formed. The enzymatic activity was determined by measuring the free fatty acids formed during hydrolysis at various times, following equation:

$$\frac{\text{umol free fatty acids}}{\text{ml sample}} = \frac{[\text{NaOH } 0.05 \text{ N ml (sample-blank)}]}{\text{Total sample}} \times N \times 1000 \quad (1)$$

This Eq (1) was then used to determine the linearity range graphically by the formation of micromoles of fatty acids per minute over a period of time.

2.4 Determination of kinetics parameters

Olive oil emulsion was used with a 2% PVA solution (polyvinyl alcohol) at a fat concentration of 137.40 g/L - 251.90 g/L shown in table 1. The determination of the kinetics parameters of the enzyme was carried out at a temperature of 37 °C, a pH of 7.0 and a 1: 7000 solution of AY "AMANO" 30 SD enzyme.

The selection of the model to find the enzyme kinetics in oil hydrolysis was based on the Michaelis-Menten model catalyzed by a single substrate enzyme. According to model given by Michaelis-Menten, where the reaction rate could be determined for reactions catalyzed by enzymes using the equation:

$$V = \frac{V_{\text{máx}} \times [S]}{K_m + [S]} \quad (2)$$

Where:

$V_{m\acute{a}x}$ = reaction speed (grams of free fatty acids formed / grams sample * minute)

K_m = Michaelis-Menten constant (grams of free fatty acids formed / minute)

[S] = Substrate concentration (grams of oil).

The kinetic parameters (K_m and $V_{m\acute{a}x}$) Could be determined according to linear regression curve presented by Lineweaver and Burk, (1934):

$$\frac{1}{V} = \frac{K_m}{V_{m\acute{a}x}} \times \frac{1}{[S]} + \frac{1}{V_{m\acute{a}x}} \quad (3)$$

Where graphing the inverses of the Lineweaver and Burk equation $1/V$ vs $1/[S]$, we will obtain the slope of this curve that gives us the values of $K_m/V_{m\acute{a}x}$ and the intercept gives us $1/V_{m\acute{a}x}$.

Table 1. Preparation of substrate concentration to determine the kinetic parameters 'Km' and 'Vmax'

N° Experience	Concentration [So] (g/L)	Emulsion (mL)	Buffer (mL)
1	137.40	15.00	30.00
2	160.30	17.50	27.50
3	183.20	20.00	25.00
4	206.10	22.50	22.50
5	229.00	25.00	20.00
6	251.90	27.50	17.50

2.5 Effect of Temperature

The determination of the effect of temperature on lipid activity was carried out following the methodology described by Gentili et al. (1996) using the lipase isolated from *Candida Cylindracea*, working in the temperature range (35 °C – 80°C) at pH 7.00 for 30 minutes, analyzing the lipid activity by the titrimetric method of free carboxylic acids released in the hydrolysis described by Camacho (2014).

2.6 Thermal stability

The thermal stability of the enzyme AY "AMANO" 30 SD influenced by temperature was determined, incubating the enzyme at pH 7.00 in a range of temperatures and measuring the activity after 1 hour of incubation with the methodology described by Mohd Hussin, Attan, & Wahab (2020). The incubation temperatures were as follows: 35, 40, 45, 50, 55, 60, 70 and 80 °C.

2.7 Hydrolysis reaction and experimental design

The experimental tests were carried out according to a 3x3 factorial design. The effects of the independent variables (temperature and time) in the liberation of μ moles of fatty acids/ml of sample x min were evaluated. Statistical analysis was obtained in the STATISTICA software.

The enzymatic hydrolysis reaction was carried out in a 500 mL mini-reactor with a working volume of 400 mL dilution (1:1) semi-refined oil-water, where the operating conditions were constant magnetic stirring of 160 rpm. and an enzyme concentration of 350 U/g.

Table 2. Experimental Design - varying the relationship of Temperature (°C) and Reaction Time (minutes)

Assay	Independent Variables		Encoded	
	Temperature (°C)	Time (minutes)	T	t
1	50	60	0	0
2	40	60	-1	0
3	50	90	0	1
4	50	60	0	0
5	60	90	1	1
6	60	30	1	-1
7	40	90	-1	1
8	40	30	-1	-1
9	50	30	0	-1
10	60	60	1	0
11	50	60	0	0
12	40	60	-1	0
13	50	90	0	1
14	50	60	0	0
15	60	90	1	1
16	60	30	1	-1
17	40	90	-1	1
18	40	30	-1	-1
19	50	30	0	-1
20	60	60	1	0

2.8 Degree Hydrolysis (DH)

The degree of hydrolysis (%) was determined by measuring the acidity of the hydrolyzed oil and the oil before hydrolyzing at different times as shown in table 2, using a 0.025N NaOH base according to NTP 209.005: 1968 method. (revised 2016). The saponification index was evaluated for the non-hydrolyzed oil according to NTP 209.058: 1980 method (revised 2016). The blank was determined for each treatment, the degree of hydrolysis (%DH) was calculated according to following equation:

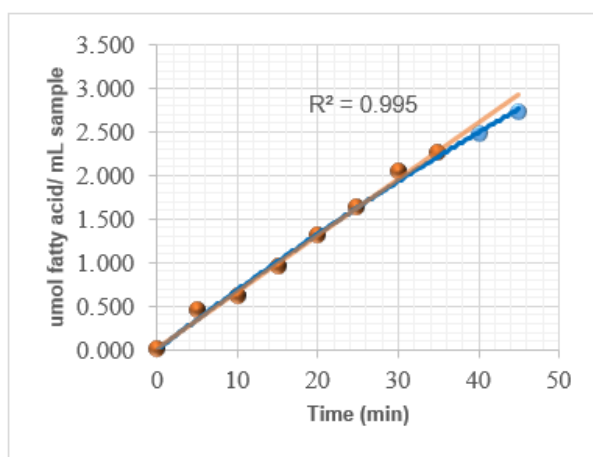
$$\% \text{Degree Hydrolysis} = \frac{\text{Acid Value (hydrolysed oil-Blank)}}{\text{Saponification Value (original oil)-Acid value(original oil)}} \times 100 \quad (4)$$

From this Eq (4) we can obtain the hydrolyzed fatty acids on the Ester index (saponification value - acid value), which allows a better approximation of the average molecular weight of the triglycerides present in the oil (Bhandari, Chaurasia, & Singh, 2017).

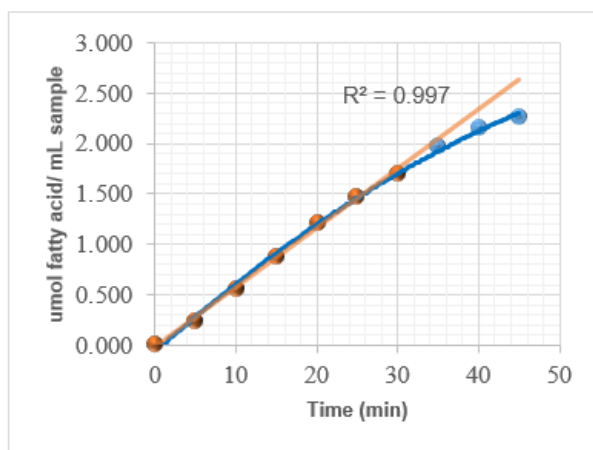
3. Results and discussions

3.1 Determination of enzyme activity

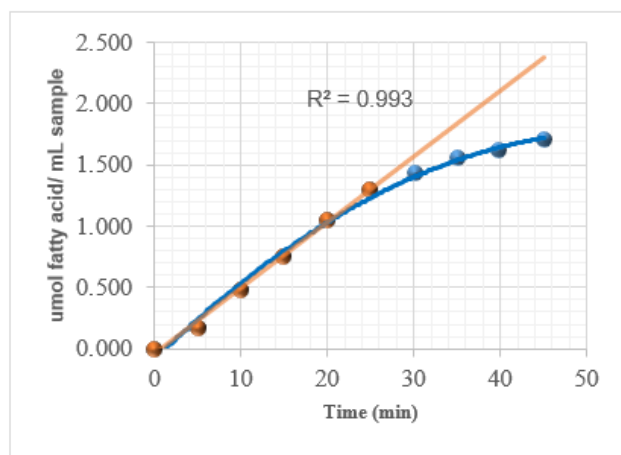
The activities of the enzyme AY "AMANO" 30SD at dilutions 1: 6000, 1: 7000 and 1: 8000, the linearity range was found graphically at the times of 35, 30 and 25 minutes respectively (Figure 1), which resulted with activities of 36,972.79 U/g, 37,420.38 U/g and 37,760.48 U/g, U being the amount of micromole equivalents of fatty acid released per minute, working with a 12.5% olive oil emulsion substrate, the three experiences were found at the same reaction conditions with pH 7.00 and constant stirring of 160 RPM.



(a) 1:6000



(b) 1:7000



(c) 1:8000

Figure 1. Determination of the linearity range of the enzyme AY "AMANO" 30SD

It is necessary to indicate that in the literature different activities will be found for the lipase isolated from *Candida Cylindracea*, as in the case of Wanasundara and Shahidi (1998) where they obtain an activity of 22,500 U / g using tributyrin substrate at pH 8.0, which It is confirmed that the conditions used for the enzymatic activity determination tests influence the results (Fernández-Jerí, Yadira, et al., 2013).

Therefore, it is important to corroborate the result of enzymatic activities with the analysis sheets sent by the supplier and to compare the methods used. In this investigation, an average activity of $37,384.55 \pm 395.07$ U/g was obtained, being within the range indicated by the analysis card of this commercial enzyme, which indicates that the enzyme should not have an activity less than 30,000 U/g.

3.2 Determination of kinetic parameters

The kinetic parameters of the enzyme AY "AMANO" 30SD (K_m and V_{max} .) Were determined using the linear regression curve of Lineweaver and Burk, (1934) as a graphic tool. Figure 2 shows the equation obtained from the linear regression of the experiences in table 1.

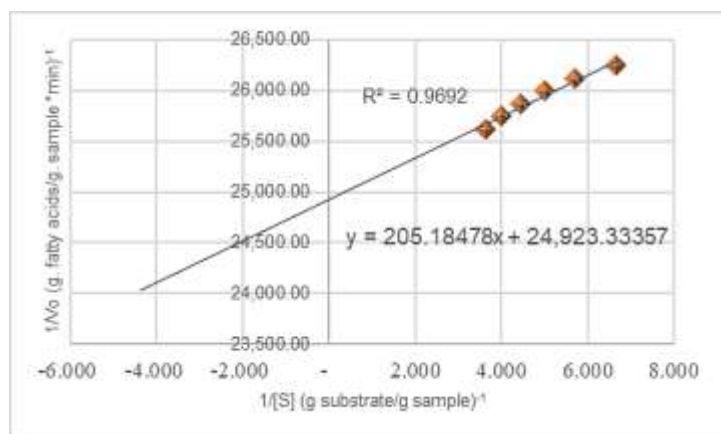


Figure 2. Lineweaver-Burk graph for the determination of the kinetic parameters of the enzyme AY "AMANO" 30 SD.

The kinetic parameters were obtained graphically with the equation of the curve, which are: the Michaelis-Menten constant (K_m) of 7.98 g/L and the maximum speed (V_{max}) of 0.038887 g/L \times min with an $R^2 = 0.9692$, varying the concentrations of the oil emulsion in the reaction mixture between 137.40 g/L - 251.9 g/L, working at a temperature of 37 °C and pH 7.00.

The constants of the kinetic parameters of the *Candida cylindracea* lipase from the company SIGMA ALDRICH were reported by Sharma, A. et. al. (2013) resulting in a $K_m = 2.57$ g/L and a $V_{max} = 9.82$ g/Lxmin, where there is notably a significant difference in the Michaelis-Menten constant, this is due to fact that there is a greater affinity of the active sites of the enzyme with the substrate used and the maximum speed it reaches is totally higher, so it is inferred that a higher degree of hydrolysis will be obtained in less working time.

3.3 Effect of temperature

As shown in figure 3, the activity of the enzyme AY "AMANO" 30 SD was highly sensitive after 60 °C, decreasing its activity at higher temperatures. The 100% activity was observed at the optimum temperature of 50 °C, which confirms with reference to technical data sheet sent by the supplier of the enzyme. On the other hand, authors such as Gentili et al. (1997), report in their research that the lipase isolated from *Candida cylindracea* presents 100% of its maximum activity at 45 °C, which differs 5 °C from the results obtained in this research, this is due to method used and the conditions (substrate: tributyrin, pH 7.4) to determine lipid activity.

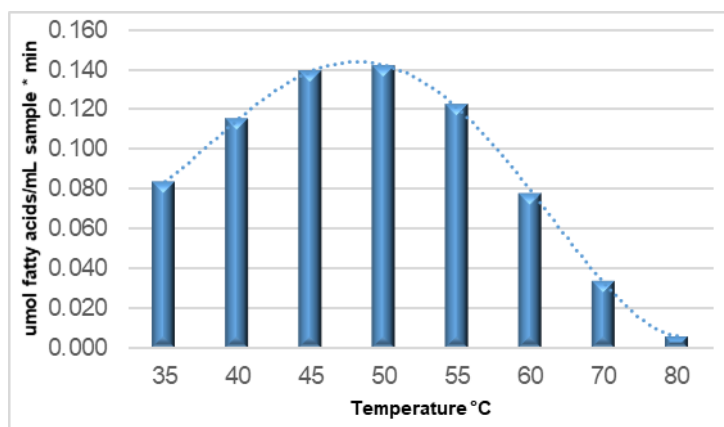


Figure 3. Effect of temperature using enzyme AY "AMANO" 30SD

The effect of temperature on the enzyme according to figure 3, showed a sharp decrease in activity from a temperature of 55 °C, preserving and showing significant activity only in the range of 35 - 50 °C, while at temperatures of 60-80 °C the enzyme maintains its activity only up to 17.60%. The low working temperatures that this effect demonstrates give us favorable advantages for greater energy savings and mild conditions during the biocatalysis process where they do not harm the quality of the oil, as mentioned by Dobrev et al. (2014) and Martínez-Corona et al., (2019).

3.4 Thermal Stability

Figure 4 shows at a temperature of 50 °C the enzyme maintains the residual activity up to 55.40% and gradually loses it until reaching 80 °C, leaving only 2.5% of its residual activity. Therefore, the results show that the enzyme was very stable in the range 35 - 45 °C where it maintains a residual activity of 97.5% to 89.5% respectively.

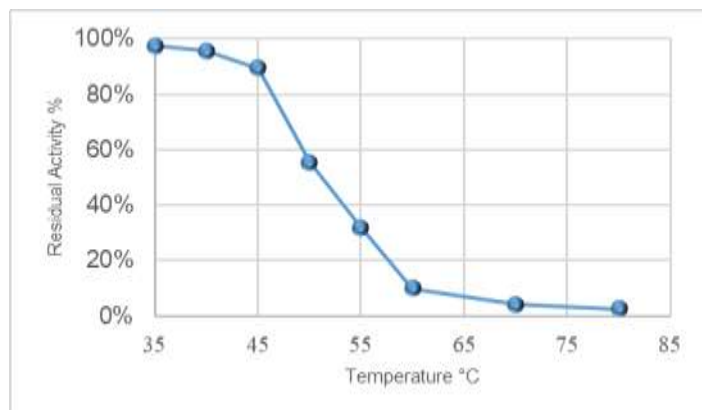


Figure 4. Thermal stability of enzyme AY "AMANO" 30SD

This profile of residual activity Vs Temperature is similar to those presented by other authors with respect to fungal lipases as in the case of Coca, Janny, et. al., (2001) with the lipase extracted from *Aspergillus niger* showing a thermal stability in the range of 30-40 °C, maintaining a residual activity of 98-90% respectively. Another lipase of fungal origin is the extracellular lipase from *Marinobacter sp.* studied by Fernández-Jerí, et.al (2013) who report its thermal stability of 37-45 °C, in the same way Benjamin & Pandey (1998), report the thermal stability of lipases isolated from *Candida rugosa* DMS 203 in a range of 35-40 °C. The thermal stability of the enzyme is affected at temperatures higher than 50 °C as mentioned by Diestra Balta, Margarito Aguilar, Vega Paulino, & Castillo Calderón (2015) in their research, due to weakening of intermolecular forces that help to preserve the structure three-dimensional of the enzymes, which a sudden change in temperature translates into the loss of the catalytic capacity.

It should be noted that it has been shown that the *Candida cylindracea* enzyme studied in this research has the same thermal behavior as other lipases of fungal origin.

3.5 Enzymatic hydrolysis

The enzymatic hydrolysis of the semi-refined anchovy oil was started at pH 7.00 conditions and constant stirring. The first hour of the reaction there was a color change in the oil, becoming darker and cloudy due to hydrolysis and autoxidation of the oil in a high concentration of water, thus influencing the organoleptic modifications (Tena et al., 2018).



Figure 5. Appearance of semi-refined anchovy oil in the hydrolysis process

The response surface methodology was used to study the degree of hydrolysis (% DH) catalyzed by a commercial lipase in semi-refined anchovy oil. The results are shown in Table 3.

Table 3. Result of %DH for the enzyme AY "AMANO" 30SD

Assay	Independent variables		Dependent Variables
	Temperature (°C)	Time (minutes)	%DH
1	50	60	3.870
2	40	30	3.271
3	50	30	3.404
4	60	30	2.406
5	40	60	3.404
6	50	60	3.870
7	60	60	2.539
8	40	90	3.737
9	50	90	3.803
10	60	90	2.738
11	50	60	3.936
12	40	30	3.337
13	50	30	3.404
14	60	30	2.472
15	40	60	3.470
16	50	60	3.936
17	60	60	2.539
18	40	90	3.670
19	50	90	3.936
20	60	90	2.871

The results obtained in the experimental design were used to obtain the quadratic models that express the DH(%) as a function of the dependent variables. The equation Eq (5) obtained has $R^2 = 0.9726$, indicating that model explains 97.2569% of the variability in the% of Degree of Hydrolysis, according to Missau et al. (2014) mentions for biotechnological processes, this R^2 is acceptable due to great variability in bioprocesses.

$$DH (\%) = -12.7604 + 0.677476 \cdot T + 0.0177718 \cdot t - 0.00720143 \cdot T^2 - 0.0000283333 \cdot Tt - 0.0000793254 \cdot t^2 \quad (5)$$

Where:

- DH (%) = is percentage of degree of hydrolysis
- T = temperature values
- t = time respectively.

For DH (%) with the enzyme AY "AMANO" 30SD it is influenced by temperature and time as shown by the Pareto diagram represented in figure 6, which is a response from the analysis of variance, where the effects AA, A, BB and AB have a negative effect, but not significant, but the effect B: time has a positive value and meaning, which means that an increase in time increases the degree of hydrolysis in a positive way.

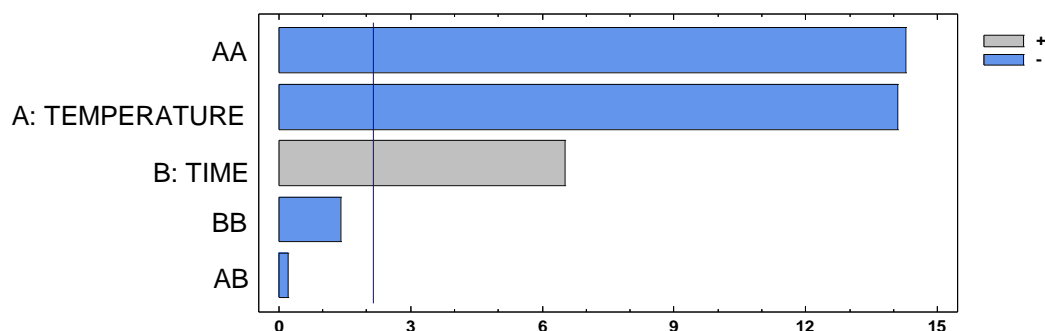


Figure 6. Standardized Pareto Diagram for Degree of Hydrolysis

Equation influenced only by significant factors ($p < 0.05$):

$$DH (\%) = -12.7604 + 0.677476 \cdot T + 0.0177718 \cdot t - 0.00720143 \cdot t^2 \quad (6)$$

From the analysis of variance, it is determined that the variable temperature factor (T) in the degree of hydrolysis (%DH) is highly significant ($p < 0.05$), reaching the highest percentage of degree of hydrolysis. According to table 3, for the conditions set out in the experiments, the values range from 2,406% to 3,936% obtained after hydrolysis.

3.6 DH% optimization

DH optimization (%) was achieved by applying the STATISTICA software where the optimal factors for degree of hydrolysis (%) were graphically obtained. Temperature: 46.86 °C and Time: 90 minutes, reaching an optimal DH value (%) of 4.01.

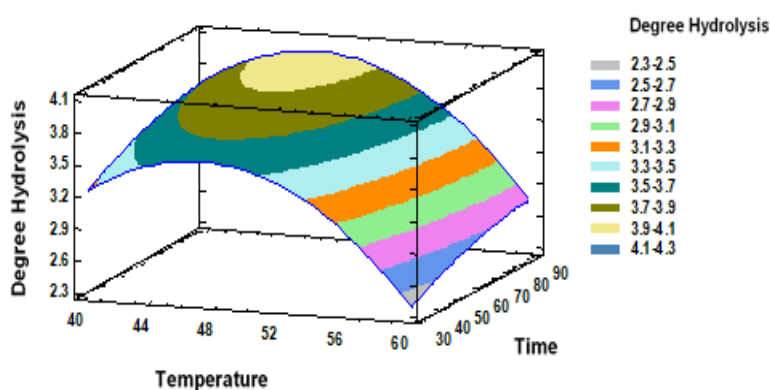


Figure 7. Optimization of the Hydrolysis Degree of semi-refined anchovy oil with a commercial lipase AY AMANO "30SD"

4. Conclusions

The study of the commercial lipase AY "AMANO" 30SD, allowed to obtain relevant information on its characteristics for its application and to achieve a process of hydrolysis of semi-refined anchovy oil. Using the response surface methodology, the optimal degree of hydrolysis was determined is 4.01%, and the factors of temperature at 46.86 °

C and reaction time at 90 minutes with an enzyme concentration of 350 U/g significantly influence, modelling an equation significant at a 95% confidence level ($p < 0.05$).

5. Acknowledgments

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