Biocatalytic Hydrolysis of Triglycerides Using a Plant Lipase Extracted from Oil Seeds

Sefha Farida [1*] And **Abouseoud Mahmoud**

[1] Materials and Environment Laboratory, Department of Methods and Environment Engineering, Faculty of Technology, University Yahia Fares, Medea 26000, Algeria, sefha.farida@univ-medea.dz

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Abstract:
The objective of this study was to evaluate the hydrolytic activity of a lipase extracted from a vegetable source and more precisely, that found in oilseeds. The first step was chosen the vegetable source which gave a maximum lipase activity. After screening, the crude enzyme extract underwent partial purification. In the second step, the effects of certain physico-chemicals parameters on the lipase activity have been studied. The analysis of the influence of the operating conditions (pH, temperature, the concentration of the substrate "triglycerides" and the concentration of enzyme) on the initial rate of the lipase enzymatic reaction is an essential step in any enzymatic study. Among four vegetable sources analyzed quantitatively by an enzymatic assay, castor seeds "*Ricinus communis* L", have a significant catalytic power compared to other sources and a stability of activity. These seeds are well adapted to our study. The effects of physico-chemical agents on the hydrolysis of olive oil using partially purified enzyme fractions of ricin lipase "*Ricinus communis* L" show that the neutral pH 7.0±0.055 and the temperature 40 ±0.056 °C were the most favorable to catalyze 3.8 10^{-2} M of the substrate using 0.6 g of enzyme. Enzymatic hydrolysis using *Ricinus communis* L lipase gave a hydrolysis yield of 58% for a period of 30 min. The K_M and V_M values of the partially purified lipase for triglycerides hydrolysis were found to be 0.016 M and 90.9 µmol/min.

Keywords: Triglycerides; Hydrolysis; Lipase; *Ricinus communis* L; Yield.

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I. Introduction

Fats or lipids are very energetic compounds, consisting mainly of triglycerides (TG). They are not only a source of energy but an important raw material for the industry in the preparation of fatty acids [1]. There are many types of methods to release free fatty acids out of triglyceride structure such as acidic hydrolysis, alkaline hydrolysis, or enzymatic hydrolysis [2,3].

* Corresponding authors: SEFHA Farida, E-mail: sefha.farida@univ-medea.dz

[4,5]

The application of enzymatic catalysis to chemical processes decreases the use of harmful products in the environment and thus reduces the cost of treatment. Enzymes offer a distinct advantage due to their high catalytic turn-over power, specificity, biodegradability and limited by-product formation

The transformation of triglycerides to fatty acids and glycerol is done by enzymes called hydrolases. Among the hydrolases that can catalyze the transformation reaction of triglycerides, lipases [6].

Lipases or triacylglycerol ester hydrolase (EC 3.1.1.3) are widely distributed in nature [7], are generally found in animals, plants and microorganisms. They have an important physiological role in fat metabolism in the presence of water. The hydrolysis reactions of the ester bonds of hydrophobic substrates take place at the lipid / water interface [8,9]. Depending on the reaction conditions in which they are found, in particular the water content of the medium, lipases can also carry out esterification (reaction between an acid and an alcohol), transesterification (between an ester and an alcohol) or interesterification reactions (reaction between two esters) [10,11,12]. According to the works of some authors, plants or plant biomass represent a very abundant source for extracting lipases [13,14]. Which are extracted from oilseeds are simple to perform at low cost, easily exploitable and has high specificity [15]. Fernanda et *al* found that the high hydrolysis conversions can be achieved under mild conditions and low times using vegetable lipases [16].

Our study aims to explore the catalytic potential of a vegetable lipase extracted from plant seeds. For this purpose, the hydrolysis reaction of lipids has been studied. Focus will be given to the determination and implementation of the best conditions to enhance the implementation of lipase in order to conduct the reactions in the most favorable conditions to achieve optimal speed and yield.

II. Materials and Methods

II.1. Chemicals and materials

Acetone (essay ≥ 99.9%) was purchased from Sigma-Aldrich. Buffer solutions were from Fluka. Ethanol, NaOH and calcium chloride ($CaCl_2$) were from Riedel-de Haën. Castor seeds, Lin seeds, Ngella Sativa seeds and Back Nigella seeds were purchased from a local market.

II.2. Preparation of the crude lipase extract

In the present work, the lipase extracted from vegetable sources and in particular those contained in the seeds of oil sources (castor seeds, Lin seeds, nigella sativa seeds and black nigella seeds).

The seeds previously peeled were crushed in a mortar. After the addition distilled water, the samples were vigorously vortexed until the ground material dissolved. The crude lipase extract (CLE) thus obtained was filtered on a gauze. For each plant material, a crude lipase extract was obtained.

II.3. Preparation of olive oil emulsion

With stirring, 20 g of gum arabic were poured in rain in 1L of distilled water previously brought to the boil. When the gum arabic was dispersing, the solution allowed stirring in an ice-filled crystallizer until the solution clarified. Using a homogenizer, the olive oil emulsion was prepared by adding olive oil (it all depends on the desired substrate concentration) in the gum arabic solution, the mixture was stirred 4 times for 1 minute, stopping for 30 seconds between two successive agitations to prevent the emulsion from heating up.

II.4. Enzymatic activity essay

For each seed source, lipase assay was carried out under standard conditions (ambient temperature, pH 7.0), the reaction medium used was prepared according to the following procedure [17]: in two Erlenmeyer flasks: 5ml of olive oil emulsion, 3ml of buffer solution pH 7.0 and 0.5ml of $CaCl_2$ were introduced. The first Erlenmeyer flask was used as a control, and 3ml of the crude lipase extract (standard test) was added to the second Erlenmeyer flask. After 30 min, the reaction was stopped by adding 5 ml of the acetone-ethanol solution (1: 1, v / v). Both media was titrated with 0.05M NaOH solution. The hydrolytic activity of lipases defined as the amount of enzyme required to produce 1mol of free fatty acid (n_{ffa}). Thus, the hydrolysis yield is estimated by the number of moles of acid released

II.5. Partial purification of the crude lipase extract

The partial purification of the crude lipase extract was carried out according to this procedure described in several works [18,19]. All the operations of the isolation of the enzyme were carried out at 4 °C [20]. Three volumes of cold acetone solvent at 4°C were poured drop wise into one volume of crude lipase extract placed under stirring in the crushed ice. Once the acetone was poured completely, the mixture (crude extract-acetone) is left in the fridge for 2 hours and then a white precipitate form. Its recovery was done by centrifugation at 3000 rpm for 5 minutes. The pellet recovered was dried at room temperature; the powder obtained is the partially purified lipase (PPL). Isolation was at 4 ° C, so this temperature is sufficient for its conservation.

II.7. Effect of the influence of the reaction conditions on the kinetics of triglyceride hydrolysis

II.7.1. Effect of pH

The reaction medium contains 5 ml of the prepared emulsion (contains 0.5 ml of olive oil), 0.5 ml of the $CaCl_2$ solution and 3 ml of the buffer solution varying from pH 4.0 to pH 9.0. At

room temperature and with continuous stirring (1000 rpm), the reaction was started after the addition of 0.1 g of the purified lipase. After 30 min, the reaction was stopped by the addition of 5 ml of the acetone-ethanol solution (1: 1, v / v). The media are titrated with 0.05M NaOH solution.

II.7.2. Effect of temperature

The operating procedure will be repeated, but this time the temperature of the reaction medium was varied between 20-70 °C using a thermostatic bath.

II.7.3. Effect of substrate concentration

One of the first measurements taken in any kinetic study was the determination of the reaction rate as a function of the reactant concentration. Thus, to determine the rate of hydrolysis of olive oil by lipase, it is first necessary to carry out a kinetic study by varying the concentration of substrate in the medium.

Then, the operating protocol to be followed was the same as above, except that the concentration of the substrate (olive oil) was varied between 0.02M and 0.23M.

II.7.4. Effect of the enzyme concentration

The last parameter that remains to be studied was the enzyme concentration. The evolution of the enzymatic reaction when the mass of the enzyme was reduced from 0.1 to 0.8 g was examined under the optimal conditions (pH, T and [Substrate]). The same experimental protocols are maintained.

II.8. Hydrolysis yield

In order to evaluate the effect of the enzyme on triglycerides for the different reaction media, the same reaction medium without enzyme extract, called: controls, was carried out in parallel. The hydrolysis yield (Hy %) of triglycerides was determined by the semi-purified lipase according to the following rule:

$$Hy\ (\%) = \frac{(n_{ffa} - n_{ffa0}) * M}{m_{TG_0}} * 100 \qquad (1)$$

Hy: Hydrolysis yield.
N_{ffa0}: number of moles of initial free fatty acid (µmole).
N_{ffa}: number of moles of free fatty acid after the reaction (µmole).
m_{TG0}: mass of initial triglycerides (g).
M: average molar mass of triglycerides (g / mole).

III. Results and Discussions

III.1. Choice the source of lipase

Table 1 summarizes the results of lipase activities for different sources of plant seeds. The difference in the number of moles between the two media (with lipase and without lipase) determines the enzymatic activity for each source:

Table 1: Enzymatic activity for different plant sources of lipase

Sources of seeds	n_{ffa} (µmol)	Enzymatic activity (U)
Castor	240	8
Nigella (black seeds arabia)	220	7.33
Lin	100	3.33
Black Nigella (Habba Sawda)	85	2.83

From this table, it appears that the castor seed lipase (*Ricinus communis* L) has the highest hydrolytic activity compared to that obtained by nigella seeds (*Nigella sativa*) and lin seeds (*Linum usitatissimum* L). Castor seeds were easily peeled and ground. On the other hand, nigella and flax seeds were hard, which maked it difficult to isolate the enzyme. This evaluation made it possible to assess the effectiveness of these lipases before the implementation of the enzymatic reactions.

Much work has been carried out using plant lipases for lipid hydrolysis (triglycerides) and for other applications [14,16,21,22].

Based on these results, castor seeds were chosen for further study.

III.3. Effect of pH

The activity profile shown in figure 2, suggests that the performance of the hydrolysis of olive oil by lipase was influenced by the nature of the environment (acid, neutral or alkaline), where it was observed that the pH increase causes an increase in hydrolysis efficiency to some extent and then decreases. In the neutral medium (pH = 7±0.055), the hydrolysis of olive oil yield is higher than that recorded by the other pH values. The similar result was found by commercial lipase CRL type VII from Candida rugosa (activity ≥ 700 units/mg solid) [23]. Serri et *al* showed that the optimum pH for the hydrolysis of palm oil by CRL was 7.5 [24].

Based on these results, we can say that the amino acid residues of the active site of the enzyme molecule which have ionizable groups (some will bind and positioning the substrate, the other part in the reaction and used in most maintaining the conformation of the enzyme), are pH sensitive (the same substrate) and have different ionization states depending on the pH value.

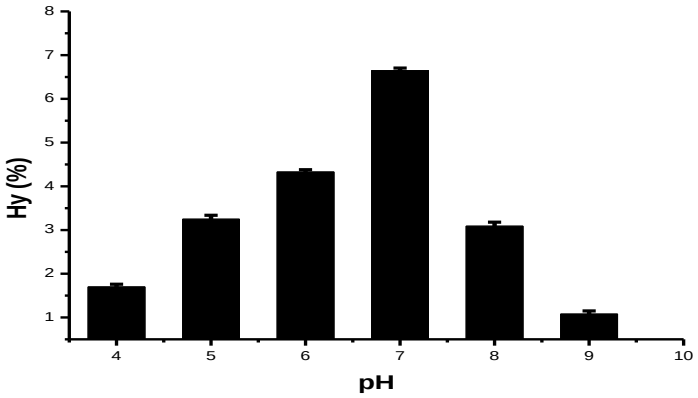

Figure 1: Effect of pH on the hydrolysis yield of triglycerides by lipase ([TG] = 0.2M; T_{amb}; m_E = 0.1g; t = 30min).

III.4. Effect of temperature

Figure 3 shows the hydrolysis yields calculated for each temperature. Effect of reaction temperature on the hydrolysis yield of triglycerides by castor lipase (*Ricinus communis* L) has two opposing effects: On the one hand, the increase in temperature causes the increase in the molecules agitation which will then increase the frequency of collision between the triglyceride and the lipase and consequently increase the hydrolysis yield, this stage is between 20 ° C and 40 ° C. But on the other hand, above 40°C, the hydrolysis yield decreased with the increase in temperature, this phenomenon was due to the denaturation of the lipase which alters the protein structure and thus changes the active lipase conformation to the inactive lipase conformation.

The maximum yield is therefore recorded for an optimal temperature of 40 ±0.056 °C. Ejedegba et *al* found the maximum activity of lipase isolated from coconut (*Cocos nucifera linn*) seed was obtained at 35 °C [25]. Nguyen et al. hydrolyzed Virgin Coconut Oil using *Candida rugosa* lipase at 40 °C [26].

Maximum enzyme activity of lipase purified from a psychrotrophic bacterium was observed at 37◦C using rice bran oil as substrate [27].

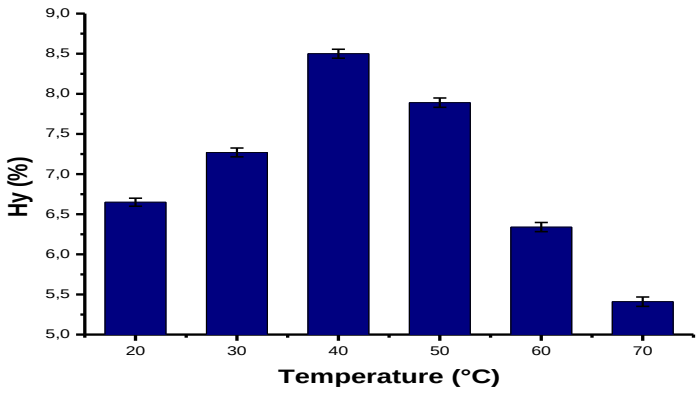

Figure 2: Effect of temperature on the hydrolysis yield of triglycerides by lipase

([TG] = 0.2M; pH=7.0; m_E = 0.1g; t = 30min).

III.5. Effect of substrate concentration

Figure 4 shows the product development kinetics.

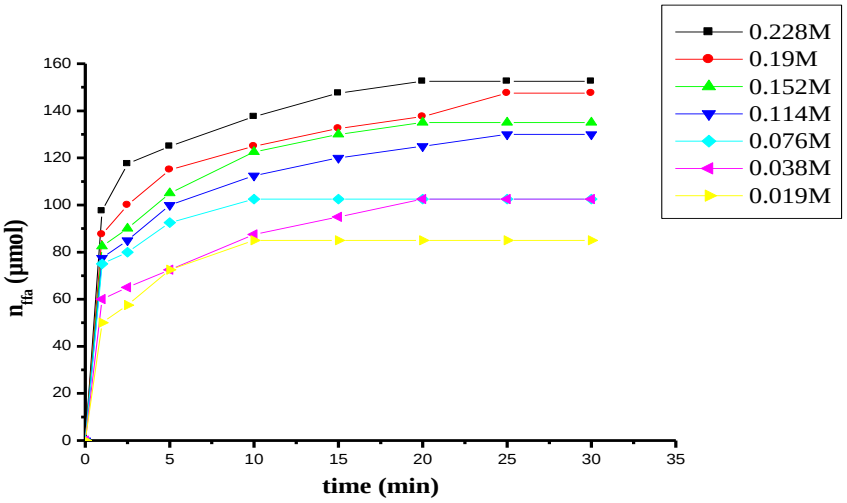

Figure 3: Kinetics of formation of free fatty acid for each substrate concentration (pH = 7.0, T = 40 ° C; m_E = 0.1g, t = 30min).

The optimization criteria are:

- Lipase activity (number of moles of acid released / min).
- Hydrolysis yield ([acid] / [substrate] * 100).

So, to determine the optimal substrate concentration; the initial velocity and the hydrolysis yield are plotted as a function of the substrate concentration

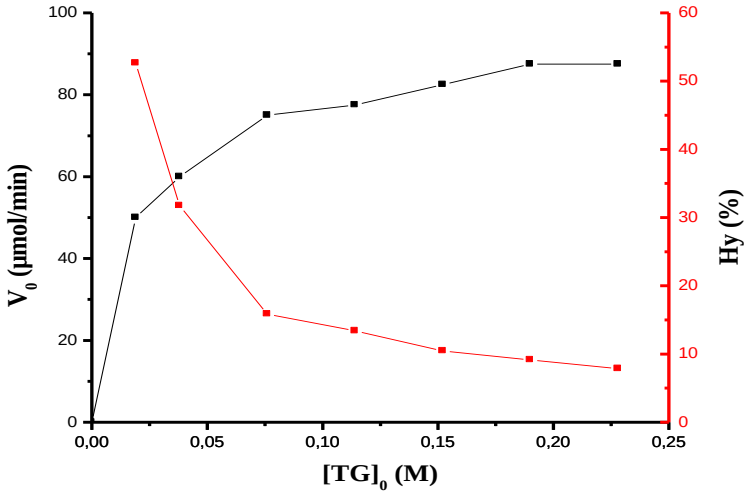

Figure 4: Variation of the initial reaction velocity and the hydrolysis yield as a function of substrate concentration (pH=7.0; T=40°C; m_E=0.1g; t=30min).

The results shown in figure 5 prove that for the more reagents, more the reaction velocity is high (V). But V no longer increases proportionally when the substrate concentration increases, then ceases to increase beyond 0.15M. This behavior results from a saturation effect: when V no longer varies even if the mass of the substrate increases, the system is saturated by the substrate. The physical interpretation is that each enzyme molecule in the reaction mixture has its substrate binding site occupied by the substrate.

The intersection of the two curves gives the optimum of the substrate ($3.8\ 10^{-2}$M), because it gives better activity and a good hydrolysis yield (35%).

Sibel F. et *al* found the plotting of initial velocity versus substrate concentration indicated that the lipase isolated from *Candida rugosa* was not inhibited up to the substrate concentration of 50% (v/v) [28].

The behavior of "$V_0 = f([TG]_0)$" is similar to that of a classic Michaelis-Menten kinetics. The initial reaction velocity is therefore given by the following relationship:

$$V_0 = V_M \frac{[TG]_0}{K_M + [TG]_0} \quad (2)$$

V_M = Maximum velocity.

V_0 = Initial velocity.

K_M = Michaelis-Menten constant.

$[TG]_0$ = initial concentration of triglycerides.

Then from this hyperbolic representation (figure 5), we can determine the kinetic parameters V_M and K_M such that: V_M is the maximum reaction velocity and K_M is the Michaelis-Menten constant which is the value of $[TG]_0$ for $V = V_M/2$.

The graphical determination of V_M and K_M from Figure 5 is not always accurate, and other methods have been proposed, they are based on a linearization of the Michaelis-Menten equation (2), the best known is the double inverse representation according to Lineweaver-Burck [29].

The Lineweaver-Burk linearization allows us to confirm whether the kinetics are Michaelian or not and to access the V_M and K_M parameters. So, we can determine the V_M and K_M parameters using the double inverse graphic representation $1/V_0 = f(1/[TG]_0)$ from Lineweaver-Burk.

So equation (2) becomes:

$$\frac{1}{V_0} = \frac{1}{V_M} + \frac{K_M}{V_M} * \frac{1}{[TG]_0} \quad (3)$$

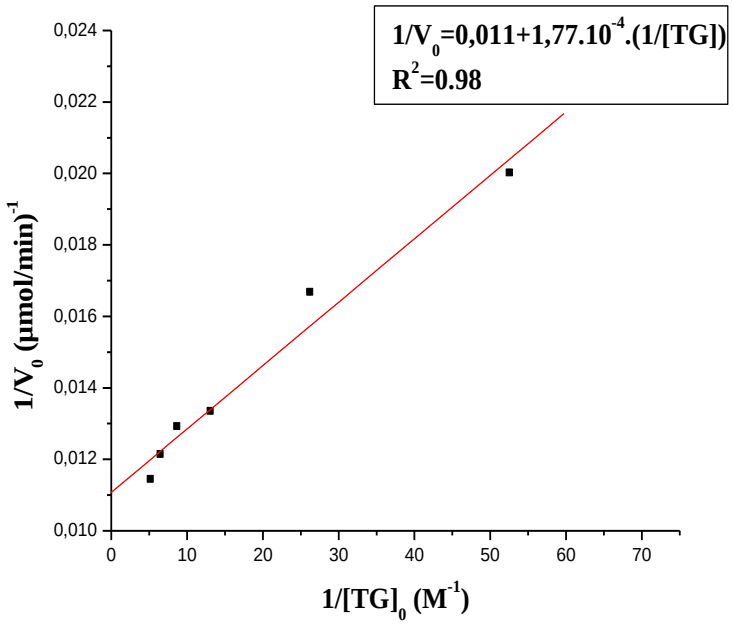

Figure 5: Lineweaver-Burck double inverse representation

The values of kinetic constants obtained from the Lineweaver-Burck linearization illustrated in Figure 5 are shown in the table 2

Table 2: kinetics constants determined by Lineweaver-Burck linearization.

Kinetics constants / Model	V_M (µmol/min)	K_M (M)
Linear representation (Lineweaver-Burck)	90,9 ± 3,71.10^{-4}	0,0160 ± 1,47.10^{-5}

The V_M value obtained from the inverse of the intercept. The Michaelis -Menten constant (K_M) reflects the affinity of the enzyme for its substrate, the lower value represents the high affinity of the enzyme for the substrate enzyme. It's also specific to each enzyme source and substrate, such as the work of Lydia Toscano et *al* who reported the Michaelis-Menten parameters K_M and V_M of extracellular lipase produced from Trichoderma harzianum by solid state to be 6,6 mmol/L and 7,5 U/mL [30]. However, Sherly Rusli et *al* also reported that the Michaelis -Menten constant of Commercial enzyme Lipomod 034P (L34P) from *Candida rugosa* using semi-refined sunflower as source of triglycerides was 121,26 mol/L [31]. The R-squared (0,98) is a measure of how well

a linear regression model fits a dataset, this value indicates that Lineweaver-Burke plot it almost perfectly linear. Consequently, we can conclude that hydrolysis of the triglyceride from olive oil source by the lipase extracted from castor beans followed Michaelis-Menten kinetics.

III.6. Effect of enzyme concentration

The numbers of moles of the product are noted in function of enzyme mass. The results are illustrated in figure 6 suggest that:

For a given substrate mass, the study of variations of product moles number as a function of enzyme mass shows that this variation is not linear. The curve has a hyperbolic shape corresponding to the fact that beyond a certain enzyme mass, the entire substrate is in the form of an enzyme-substrate complex; it follows that the product moles number in function of this complex concentration remains constant.

Then the hydrolysis yield for pH = 7.0±0.055, T = 40±0.056 °C, [TG] = 3.8 10^{-2}M and m_E = 0.6±0.049g is 58%. The study of Florencia et *al* using 0.05 g lipase powder (LP) extracted from castor bean seed and 0.20 sunflower oil:aqueous phase (w/w) without gum arabic gave 20.47 ± 7.19 mmolFFA/goil·gLP·h [32].

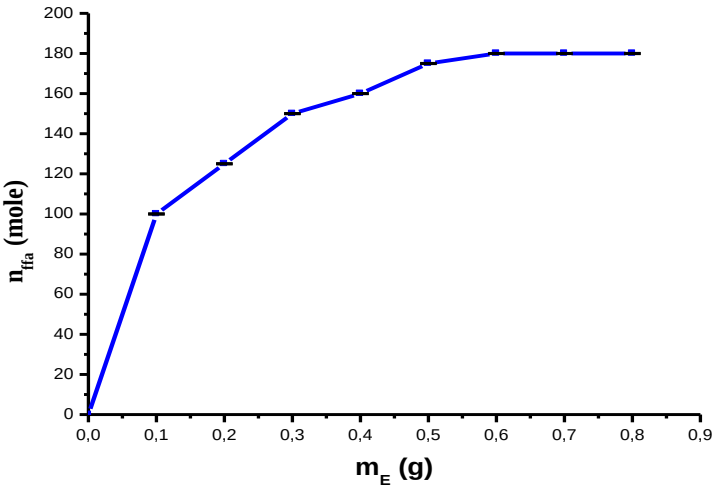

Figure 6: Variations of product moles number as a function of enzyme mass (pH = 7.0, T = 40 ° C, [TG] = 3.8 10^{-2}M, t = 30min).

IV. Conclusion

The main objective of our work was originally to assess the hydrolysis yield of lipids by lipase extracted from a plant source. Subsequently, the experimental strategy made it possible to optimize the conditions under which one can have a maximum hydrolysis yield at an acceptable speed.

Hydrolysis was carried out with castor lipase "*Ricinus communis* L". Tests show that this plant source has maximum lipase activity compared to other sources. It was shown during this study that the physicochemical factors (pH, temperature, the substrate concentration "*olive oil*" and the

enzyme concentration) had a direct influence on the speed of the process and on the hydrolysis yield.

The results showed that the lipase extracted from the castor seeds gives its maximum activity point in a neutral medium (pH 7.0) and at a temperature of 40 ° C. One enzymatic unit of lipase can transform up to 3.8 micromoles of substrate into product per minute.

V. References

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