J. Agric. Sci. Mansoura Univ., 27 (9): 6381 - 6391, 2002

PURIFICATION AND IMMOBILIZATION OF LIPASE PRODUCED FROM *Pseudomonas aeruginosa*

El-Hawary, Fatma I.; Aida H. Afify; I. I. Ismail and Amal Y.M.I. El-Shafey
Dept. of Microbiology, Fac. of Agric., Mansoura Univ., Mansoura, Egypt

ABSTRACT

The lipases of *P. aeruginosa* were isolated, immobilized and characterized. They were applied to hydrolysis of crude oils i.e. cotton oil, maize oil, palm oil, sunflower oil and olive oil. The lipase produced by *P. aeruginosa* was precipitated from the growth media using by cold acetone and was immobilized on celite. The characteristics of free and immobilized lipases were studied as follows:

The optimum temperature for lipases activity was 37°C for free lipase, whereas it was 44°C for immobilized one. The optimum pH value for free and immobilized lipases was 7.4. The stability to pH was higher in the immobilized enzyme than the free one. Immobilized lipase was stable between pH 6.8-7.8 and free lipase between 7.2-7.6. Both free and immobilized lipases were stable at 40°C but immobilized one was more stable at (40-50°C). The optimum substrate concentrations for free and immobilized lipases were 0.25% and 0.30%, respectively. Free and immobilized lipases were able to hydrolyse all natural oils tested. Olive oil was the most hydrolyzed one than other oils.

Keywords: Immobilized lipase, oils, *Pseudomonas aeruginosa*

INTRODUCTION

Lipases of microorganisms are now extensively studied primarily because of their possible biotechnological applications. *P. cepacia* lipase was purified by precipitating with Tris-HCl buffer at pH 6.5, centrifuged, dialyzed against the same buffer. The final solution was loaded at 4°C on DEAE-Sepharose column, active fractions were pooled and dialyzed against ammonium acetate buffer at pH 7.0 (Gaelle *et al.*, 1997). At the same line Omar *et al.* (1987) used acetone to precipitate lipase. While Sugiura *et al.* (1977) *purified P. fluorescens* lipase by ethanol precipitation (65-85%) from the culture broth. Also, Nadkarni, (1971) performed the purification of *P. aeruginosa* lipase by means of ammonium sulfate and gel filtration techniques. Mencher and Alford (1967) used solid ammonium sulfate to 35% saturation for precipitation, the precipitate was collected by centrifugation and dissolved in phosphate buffer. Sephadex-G100 column was used for fractionation.

The immobilization of enzymes is a technique makes their use on an industrial scale more applicable. This is because immobilized enzymes offer certain processing advantages over free enzymes such as ease of separation from the product, localization within a reactor, improved stability, activity retention, continuous operation over extended time periods, and the possibility of obtaining superactivity. There are different materials can used to immobilize enzymes such as Sol-Gel (Manfred *et al.*, 1996) immobilized lipase from *P. cepacia*, Yoshitsugu and Hideo (1992) immobilized lipase from *P. fluorescens* on macroporous anion exchange resin using glutaraldhyde to

enhance the adsorption. Jei-Fu *et al.* (1990) studied six different types of materials including polyvinyl choloride (PVC), chitosan, chitin, agarose, sepharose and trisacyl for their lipase coupling efficiencies. Lipase could be immobilized efficiently on chitin to yield a high specific lipolytic activity.

Furthermore, in this respect the characteristics of free and immobilized lipases were studied. The characteristics were factors affect reaction rates i.e temperature, pH. Many workers supported that, Nielson (1985) replaced the pancreatin by microbial enzymes in partial hydrolysis of food fats and monoglycerides production. Langrand *et al.* (1988) reported that thirteen commercial lipase preparations were checked for their ability to catalyse the formation of flavour esters (isoamyl or geranyl acetate, propionate and butyrate) by either direct esterification or ester soluolysis in n-heptane. The formation of isoamyl or geranyl butyrates and propionates by direct esterification was catalyzed by the majority of the tested lipases. Acetic acid esters were more difficult to obtain. Transesterification reactions were found to be a good alternative way for ester synthesis.

Accordingly, the aim of work: immobilization of lipase, characterization of free and immobilized enzymes, study of thermal stability and hydrolysis of natural oils.

MATERIALS AND METHODS

The micro-organisms:

Seven bacterial isolates of lipolytic enzyme ones; previously isolated by El-Shafey (1999) from the soil of Agric. Res. Sta. , Fac. of Agric. Mansoura Univ., Mansoura. They were identified as: strain 1 belonging to *Pseudomonas aeruginosa* strain 2 belonging to *Pseudomonas* sp., strain 3 belonging to *Pseudomonas fluorescens*, while strain 4, 5 & 6 belonging to *Micrococcus* sp. (I, II & III), respectively and strain 7 belonging to *Bacillus cereus*. The most active strains , namely, *P. aeruginosa, P. fluorescens* and *B. cereus* were chosen during this work .

Crude enzyme:

Crude enzyme was prepared according to the method of Omar *et al.* (1987); under the optimal growth conditions (Afify, *et al.*, 2002); after the fermentation period the culture broth was centrifuged at 1500 xg for 20 min. Cold acetone was added to the supernatant to a final concentration of 80% (v/v) and left over night. The precipitate formed was collected by centrifugation at 6600 xg for 30 min. and finally washed twice with cold acetone. The resultant precipitate was either dissolved in 100 ml of Tris-HCl buffer (pH 7.4) and kept in refrigerator until using (stock solution) or dried under vacuum over night. The precipitate obtained was used as crude enzyme.

Estimation of lipase activity:

Lipase activity was estimated as described by Oi *et al.* (1969) with some modifications as follows: The reaction mixture contained 5.0 ml of 5.0% olive oil emulsion in 7% gum acacia, 5.0 ml of 0.2 M Tris-HCl buffer (pH 7 4). 2.0 ml 0.2 M $CaCl_2$, 1.0 ml enzyme solution and 2.0 ml glass distilled water.

After the incubation period under the assay conditions, the total amount of liberated fatty acids was titrated against 0.01 mol/L NaOH. The blank was an assay mixture containing boiled enzyme lipase calculated according to Xia *et al.* (1996). Enzyme unit is defined as the amount of enzyme that liberates 1 µmol free fatty acids (FFA) from olive oil in 1 min under the analytical conditions.

Enzyme immobilization:

Lipase was immobilized according to the method described by Goderis *et al.* (1987) as follows:

One gram celite (BDH Ltd., England) was added to 10 ml stock solution (100 U enzyme /ml of buffer), mixed thoroughly. Water phase of the buffer was subsequently removed by evaporating at under cooling for 15 hours. The resultant was used as immobilized enzyme.

Thermal stability:

One milliliter of enzyme solution or equivalent amount of immobilized enzyme were added to Tris-HCl buffer (pH 7.4) for free and immobilized enzyme and heated in test tubes to various temperatures (40, 50, 60 and 70°C) for 30 min. After incubation the solutions were cooled immediately in ice bath for 5 min. The residual activity was determined as described by (Iwai and Tsujisaka, 1974).

Effect of substrate concentration:

The effect of substrate concentration on free and immobilized enzymes activity was studied using 1 ml of enzyme solution and the same quantity of immobilized enzyme. The concentrations of olive oil used were 0.05, 0.10, 0.15, 0.20, 0.25, 0.30 and 0.35% (w/v). The reaction was carried out at the optimum pH and the optimum temperature of each enzyme used.

Hydrolysis of natural oils:

According to the method described by Omar *et al.* (1987) with modification; one milliliter of enzyme solution or equivalent amount of immobilized enzyme was added to reaction mixture containing (5 ml of different oils) i.e. sunflower; palm; cotton seed; maize and olive oils and 5 ml of Tris-HCl with optimum pH of each enzyme. The mixture was incubated at optimum temperature of each enzyme for 10 min. with shacking. The reaction was terminated by adding 20 ml of acetone/ethanol mixture (1: 1 v/v) and then the free fatty acids liberated were titrtated with 0.01 N NaOH solution.

Determination of protein:

The protein content was determined by the method of Lowry *et al.* (1951).

RESULTS AND DISCUSSION

Partial purification of enzyme:

The crude lipase from *P. aeruginosa* was precipitated by cold acetone, dialysed and lyophilized. The recovery of enzymatic activity was high (81.23%) in acetone precipitate (Table 1) and the specific activity increased by 4.8 fold with 81.23% remained activity and 16.8% yield of protein.

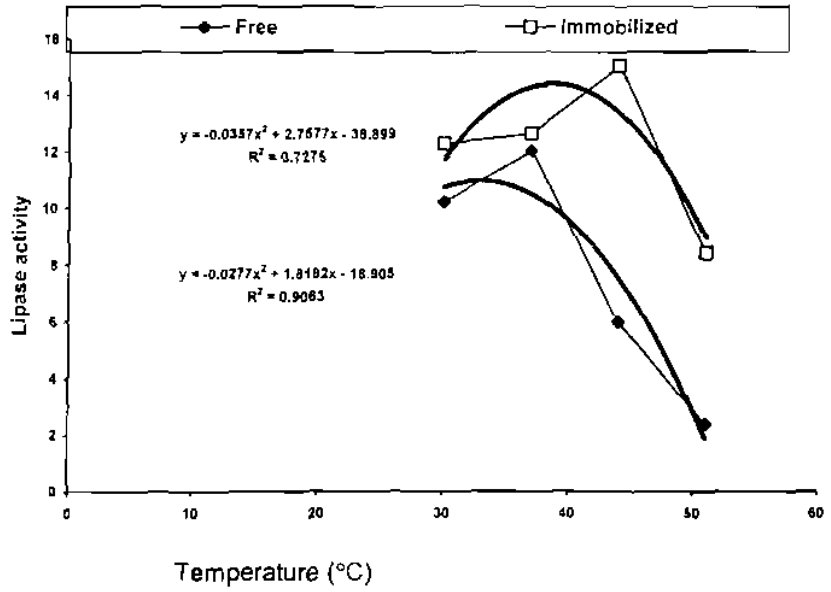
Table 1. Scheme for the partial purification of lipase from culture supernatant of *Pseudomonas aeruginosa*.

Fractions	Total protein (mg)	Total activity (u)	Specific activity u/mg	Recovery (%)	Purification (fold)
Culture supernatant	160.55	4164.72	25.94	100.00	1.0
Acetone precipitate	26.98	3383.00	125.35	81.23	4.8

Characteristics of free and immobilized lipases:

1.Temperature reaction:

The variations of the initial reaction rates with temperature is shown in Fig. (1) for the free and immobilized lipases preparations derived from *P. aeruginosa*. For the free enzymes, the reaction rate increased from 30 to 37°C.



Lipase activity = µmol FFA / ml / min

Fig. 1. Effect of temperature on free and immobilized lipase activity

Further increase of temperature than 44°C decreased the enzyme activity. On the other hand, the maximal reaction rate for immobilized enzyme was observed at 44°C. The decrease of reaction rate was noticed above this temperature and at 51°C it was much slower but still higher than for the free lipase. The fitting of temperature curves opayed the statistical formula:

$Y = - 0.01770 + 0.48434 x + 0.00286 x^2 - 0.00023 x^3$

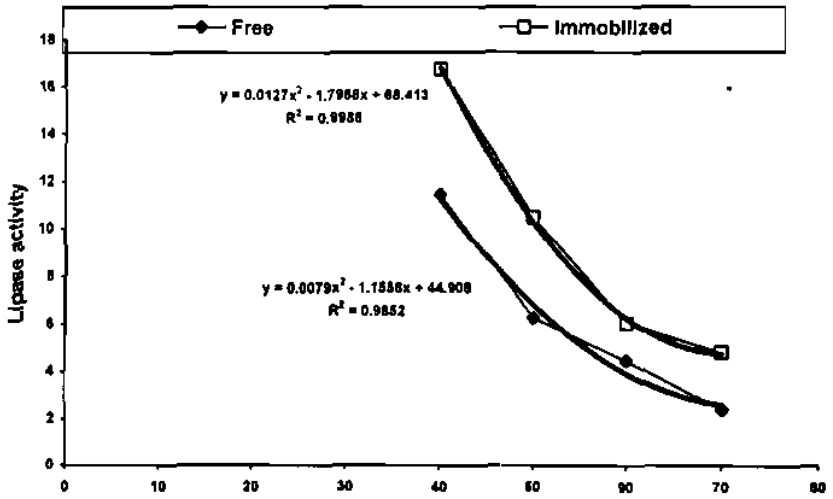
with correlation coefficient 0.094352 for free lipase Fig. (1).

While, $Y = 0.01595 - 0.36381\ x + 0.04582\ x^2 - 0.00069\ x^3$ with correlation coefficient 0.96611 for immobilized lipase Fig. (1).

These results are in agreement with those obtained by Pencreac'h *et al.* (1997) who reported that free enzyme reaction rate increased from 25 to 45°C while from 45 to 50°C for the immobilized lipase purified from *P. cepacia*. Also, Lavayre and Baratti (1982) found that the apparent optimum temperature of free and immobilized pancreatic lipase were 32°C and 40°C, respectively.

2.Thermal stability:

The results in Fig. (2) showed that at 40°C, both free and immobilized lipases were stable and total initial activity was recorded after 30 min. In contrast, after 30 min. of incubation at 70°C the remaining activity of the free and immobilized lipase represented 21% and 25% from initial activity, respectively. These results indicated that immobilized lipase produced by *P. aeruginosa* was more stable than free lipase at 70°C. The free enzyme was completely stable at 40°C for 30 min., while 60%, 40% of the activity was retained at 50°C and 60°C for 30 min., respectively. On the other hand, immobilized enzyme was completely active at 40°C while 95%, 60% of the activity retained at 50°C and 60°C for 30 min., respectively. The results also show that both lipases lost their activities rapidly at temperature above 60°C.,



Temperature (°C)

Lipase activity = µmol FFA / ml / min

Fig. 2 . Thermal stability of free and immobilized lipase.

The fitting of thermal stability curves opayed the statistical formula:

$Y = 0.01025 + 1.36779\ x - 0.038270\ x^2 + 0.00028\ x^3$ with correlation coefficient 0.98103 for free lipase Fig. (2).

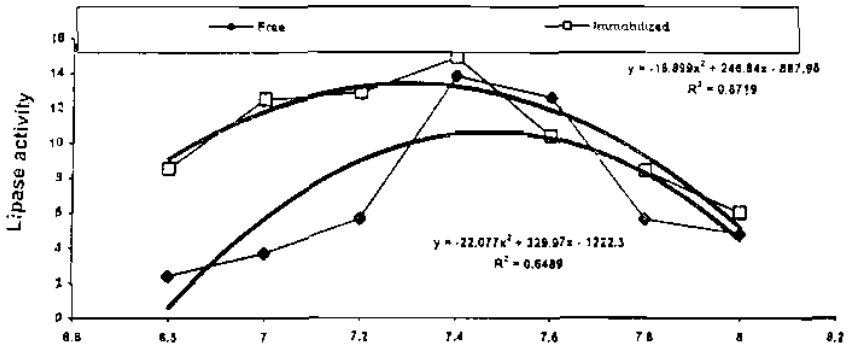
While, Y = 0.00228 + 2.10577 x -0.05974 x^2 + 0.00044 x^3 with correlation coefficient 0.99956 for immobilized lipase Fig. (2).

These results are similar to those obtained by El-Shafei and Rezkallah (1997) who reported that the activity is lost completely at 80°C for lipase produced from *Bacillus cereus*, while Songgang *et al.* (1997) results with *Pseudomonas pseudoalcaligenes* indicated that lipase was stable below 60°C, lost 40% of its activity at 70°C. 10% of its activity remained at 80°C. Aiso, Kim *et al.* (1994) found that the lipase from a thermophilic *Bacillus* species purified to homogenity from the culture supernatant had high thermostability and no loss of activity upon incubation at 60°C for 30 min. Also, Kurashige *et al.* (1989) found that. the thermal stability of *Pseudomonas fluorescens* lipase was remarkably improved by immobilization on celite.

These results contrast those indicated by Pencreac'h *et al.* (1997) who found that at 50°C both free and immobilized lipases from *Pseudomonas cepacia* were stable and more than 95% of the initial activity was recovered after one hour of incubation.

3.pH reaction:

The results in Fig. (3) show that pH control from 6 8 to 8.00 slightly enhanced the lipase activity The lipase activity reacned its maximum value at the pH range from 7.4-7 6 for free lipase but at pH 7 0-7.6 for immobilized lipase. The optimum lipase activity for both two enzymes was at pH 7.4. The reaction was carried out at 37°C for free lipase and 44°C for immobilized lipase prepared from *P. aeruginosa*



Lipase activity = µmol FFA / ml / min

Fig. 3 . Effect of pH on free and immobilized lipase activity.

The fitting of pH curves opayed the statistical formula:

Y = 0.00067 - 169.29649 x + 45.78043 x^2 - 3.06892 x^3 with correlation coefficient 0.75233 for free lipase Fig. (3).

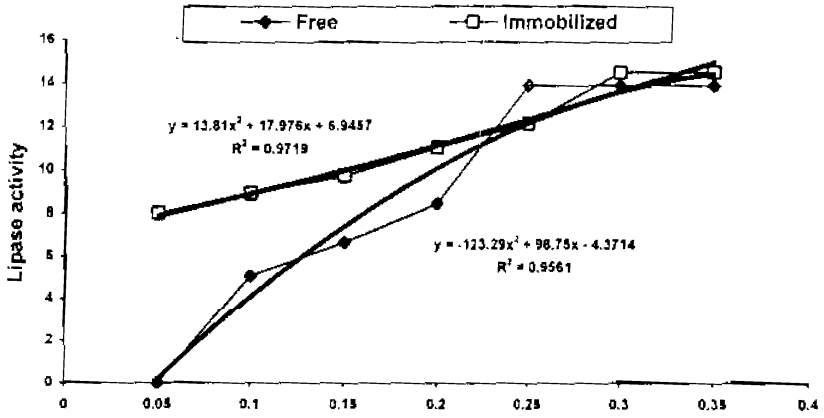
While, Y = -0.00056-112.70089 x + 31.54259 x^2-2.17184 x^3 with correlation coefficient 0 94635 for immobilized lipase Fig. (3).

It is clear that the immobilized enzyme has the higher correlation than free enzyme in optimum temperature, thermal stability and pH

With regard to the results obtained by Kosugi and Suzuki (1992) the optimum pH of *P. fluorescens* lipase was pH 4.0 and 6.0 for the immobilized and soluble lipases, respectively Qiaoqin *et al.* (1992) reported that pH 9.2 was the optimal pH for the free lipase activity from bacteria strain F-1903 isolated from Fujian soil.On the other hand. Yoshitsugu and Hideo (1992) found that, the optimum pH of lipase was 4.0 for the immobilized lipase and pH 6.0 for the soluble free one, and free lipase can be promoted at strake alkaline reaction. Also, in the same line Pabai *et al* (1995) suggested that the free lipase from *P fragi* CRDA 323 activity reached its optimum at the pH range 8.5-9.0. Castellar *et al* (1996) reported that very alkaline pH 9 0 was the best pH for free lipase activity from *Chromobacterium viscosum* and *P glumae*.

4.Substrate concentration:

The effect of different concentrations of olive oil on lipase activity was studied. The rate of olive oil hydrolysis was measured at substrate concentrations from 0.05% to 0.35% The reaction for free and immobilized lipases from *P. aeruginosa* was carried out at the optimum pH and temperature of each enzyme. Fig. (4) show that the maximum activity was obtained at 0.25% and 0.30% olive oil, for free and immobilized lipase, respectively. Then, the reaction reached its steady state. Data show also that immobilized enzyme was more active than free enzyme at higher substrate concentration. This may be due to that, soluble lipase might be inhibited severely by the substrate at higher concentrations, whereas immobilization of lipase reduced substrate inhibition (Kwon and Rhee, 1984).



Lipase activity = µmol FFA / ml / min

Fig. 4 .Effect of substrate concentration on activity of free and immobilized lipase.

The fitting of substrate concentration curves opayed the statistical formula:

$Y = -0.23348 + 9.40873\ x + 366.17749\ x^2 - 793.53535\ x^3$ with correlation coefficient 0.96963 for free lipase Fig. (4).

While, $Y = 0.89576 + 129.26349\ x - 553.98266\ x^2 + 861.01010\ x^3$ with correlation coefficient 0.94469 for immobilized lipase Fig. (4).

These equations showed that the immobilized enzyme show lower correlation coefficient than free enzyme.

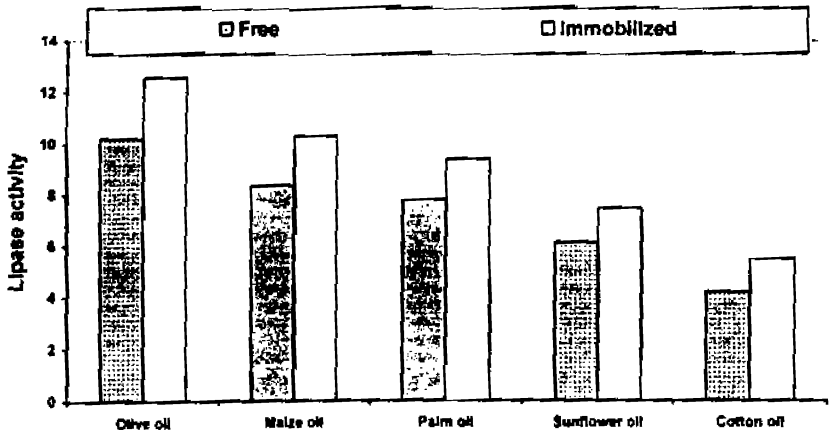
Elwan *et al.* (1977 and 1987) found that the optimum substrate concentration for free lipase from *B. stearothermophilus* and *Penicillium chrysogenum* was 0.2%.

Also, Kosugi and Suzuki (1992) reported that the extent of final hydrolysis by the *P. fluorescens* soluble lipase notably decreased at higher substrate concentrations, whereas, the extent of final hydrolysis by the immobilized lipase was high even at higher substrate concentrations. Data show also that immobilized enzyme was more active than free enzyme at higher substrate concentration.

5.Hydrolysis of natural oils:

The ability of *P. aeruginosa* lipase on hydrolyzing different oils was tested by incubating each oil with the free and immobilized enzyme. Under the optimal conditions then, the free fatty acids liberated were determined.

Fig. (5) shows that free and immobilized lipase of *P. aeruginosa* hydrolyzed various natural oils with different extents depending on the variety of oil. They can be arranged as follows with free and immobilized enzyme: olive oil, maize oil, palm oil, sunflower oil and cotton oil, respectively. It can be deduced that free and immobilized lipase hydrolyzed olive oil more than all natural oils, but free lipase hydrolyzed these oils slightly less than the immobilized lipase.



Lipase activity = µmol FFA / ml / min

Fig. 5 . Hydrolysis of natural oils by lipases.

These results are in agreement with those obtained by Kokusho *et al.* (1982) found that *Alcaligenes* sp. No. 679 lipase could hydrolyse the tested natural fats and oils to the same degree as olive oil. Also, Omar *et al.* (1987) who stated that *Humicola lanuginosa* No. 3 lipase was capable of hydrolyzing broad range of fats and oils. Plant oil was found to be most rapidly hydrolyzed oil, while castor oil was poorly hydrolyzed.

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تنقية وتحميل إنزيم الليبيز المنتج بواسطة ميكروب *Pseudomonas aeruginosa*

فاطمة إبراهيم الهوارى، عايدة حافظ عفيفي ، إسماعيل إبراهيم إسماعيل، أمل يحيى الشافعي
قسم الميكروبيولوجى – كلية الزراعة – جامعة المنصورة – المنصورة – مصر

في هذا البحث تمت دراسة خصائص إنزيم الليبيز لميكروب *Pseudomonas aeruginosa* المنقى جزئيا بواسطة الأسيتون البارد والمثبت على السليت *celite* وقد أظهرت النتائج زيادة درجة الحرارة المثلى للإنزيم المثبت (٤٤°م) عن مثيلة الحر (٣٧° م)، كذلك لوحظ زيادة ثبات الإنزيم المثبت للـ *pH* (٦ر٨-٨ر٧) عن الإنزيم الحر (٢ر٧-٦ر٧)، كما لوحظ ارتفاع درجة الثبات الحراري للإنزيم المثبت (٤٠-٥٠° م) عن مثيلة الحر (٤٠° م) التركيز الأمثل من مادة التفاعل هو ٢٥ر%، ٣٠ر% لكلا الإنزيمين على الترتيب وبدراسة قدرة الإنزيمين على تحليل بعض أنواع الزيوت فكان لهما القدرة على تحليل بعض الزيوت (بذرة القطن – الذرة – النخيل – عباد الشمس – الزيتون) ولكن وجد أن زيت الزيتون أكثر تحللا عن باقي الزيوت الأخرى سواء كان الإنزيم في الصورة الحرة أو في الصورة المثبتة ٠