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Determination the Optimum Conditions of the Activity and Stability of Lipase Extracted from Sunflower Germinated Seeds

Alaa M. Dh. Al-Haidari^{*1}, Ibrahim S. Alsaadawi¹ and Saad Hussein Khudhair²

¹Department of Biology, College of Science, Baghdad University, Bagdad, Iraq

²Al Karkh University of Science, Baghdad, Iraq

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Abstract

The present study was conducted to determine the optimum conditions required for lipase enzyme activity extracted from germinated sunflower seeds, including temperature, pH, agitation, time of incubation, enzyme concentration, substrate type, and concentrations of mineral salts and EDTA. Optimum pH, temperature and time of incubation required for lipase stability were also determined. The results showed that the optimum lipase activity (3.251U/ml) was found at 30 °C and pH 7 after 20 minutes of incubation when using 1 ml lipase enzyme with 0.02 ml of CaCl₂ (10 mM) at 100 rpm of agitation and in the presence of olive oil as the substrate for enzyme reaction. EDTA appeared to have inhibitory effects, while Ca⁺² and Mg⁺² have stimulatory effects on lipase activity. The values of lipase activity, total activity, and specific activity measured under optimum conditions were increased by 36.99%, 36.95%, and 38.21% over control, respectively. The enzyme showed stability at a temperature ranged between 30 to 50 °C, pH between 7 to 8, and time of incubation between 10 to 40 minutes. These results suggest that lipase enzyme extracted from germinated sunflower seeds have stability that depends on pH, temperature, and incubation period, which enables it to be used in different industries.

Keywords: Lipase, sunflower, Germinated seeds, Enzyme activity and stability.

تحديد الظروف المثلى لفعالية واستقرارية انزيم اللايباز المستخلص من بذور زهرة الشمس النابتة

الاء محمد ظافر الحيدري^{*1}, ابراهيم شعبان السعداوي¹, سعد حسين خضير²

¹ قسم علوم الحياة، كلية العلوم، جامعة بغداد، بغداد، العراق.

² جامعة الكرخ للعلوم، بغداد، العراق.

الخلاصة

تم في هذه الدراسة تحديد الظروف المثلى لفعالية انزيم اللايباز المستخلص من بادرات بذور نبات زهرة الشمس والتي تتضمن درجة الحرارة والاس الهيدروجيني والمدة الزمنية للحضن وتركيز الانزيم الامثل للتفاعل ونوع المادة الاساس الاكثر تأثيرا وكذلك تراكيز الاملاح المعدنية وال EDTA التي تؤثر في فعاليته، فضلا عن العوامل المؤثرة في استقرارية الانزيم كالأس الهيدروجيني ودرجة الحرارة ومدة التحضين. اوضحت النتائج ان الفعالية المثلى للانزيم كانت عند 30 °م وأس الهيدروجيني 7 بعد 20 دقيقة من الحضن عند استخدام واحد مل من الانزيم مع 0.02 مل لكلوريد الكالسيوم بتركيز 10 ملي مولر عند 100 دورة بالدقيقة ومع وجود زيت الزيتون كمادة اساس للتفاعل الانزيمي، وكان لمركب EDTA تأثيرا مثبطا بينما كان لأيونات الكالسيوم

*Email: alaadhafar2015@gmail.com

والمغنيسيوم تأثيراً محفزاً لفعالية الانزيم. ان قيم كل من فعالية الانزيم والفعالية الكلية والفعالية النوعية قد ازدادت عند استخدام الظروف المثلى بنسبة 27%، 27.99% و 27.65% عن معاملة المقارنة (الظروف المستخدمة من قبل Rahman et al., 2005) على التوالي عند استخدام نفس القيم لانزيم اللابيز، زيت الزيتون، كلوريد الكالسيوم والاس الهيدروجيني. اوضح الانزيم استقرارية عند درجات حرارة تراوحت بين 30 الى 50 °م واس هيدروجيني تراوح بين 7 و 8 ومدة حضن بين 10 الى 40 دقيقة، اوضحت تلك النتائج ان انزيم اللابيز المستخلص من البذور النابتة لزهرة الشمس يمتلك استقرارية للاس الهيدروجيني والحرارة وفترة الحضن، الامر الذي يشير الى امكانية استخدامه في الصناعات المختلفة.

Introduction

Lipases (triacylglycerol ester hydrolase, EC3.1.1.3) are enzymes that catalyze the total or partial hydrolysis of fats and oils, releasing glycerol and free fatty acids [1]. The purified enzyme is used in a wide range of industries, such as food industries [2], detergent industries [3], and biodiesel production from wastes and non-edible vegetables oil, with the aims of reducing the high cost of biodiesel production and other uses [4-7].

Lipases activity is the total ability of lipases to hydrolyze lipids and fats and produce fatty acids and glycerol. The activity was found to be affected by various factors, such as temperature, pH, agitation, time of incubation, enzyme concentration, type of substrate, minerals and EDTA. Lipase stability is also affected by time of incubation, pH and temperature. The optimum of these factors was found to be varied among the enzymes and their varieties in the same plant species [8, 9]. Therefore, it has become necessary to determine the optimum aforementioned factors in order to obtain the higher specific activity and stability of the tested enzyme.

In an earlier work [10], lipase was extracted from sunflower seeds by Tris-HCl buffer at 72 h after germination. Purification of the enzyme was also made; however the characteristics of the enzyme were not investigated. Therefore, the present study was conducted to determine the optimum temperature, pH, agitation, time of incubation, enzyme concentration, type of substrate, minerals and EDTA which are required for maximum lipase activity. Incubation time, pH and temperature for maximum lipase stability were also determined.

Materials and Methods

Lipase extraction and purification

Lipase was extracted from sunflower germinated seeds after 72 h of seeds germination and purified by procedure outlined by earlier work [10]. The enzyme was used to determine the optimum value of the following factors.

1. Effect of incubation time on lipase activity

The reaction mixture consisting of enzyme, oil emulsion substrate, and CaCl_2 was incubated at 5, 10, 20, 30, 40, 50, 60, 70, and 80 minutes in a shaker water bath, then the enzyme activity was measured to determine the optimal time for incubation [11].

2. Effect of pH on lipase activity

pH of the reaction mixture that could support maximal lipase activity was determined by adjusting the pH of reaction mixture to 4, 5, 6, 7, 8, and 9 using different types of buffers, including Tris-HCl buffer (pH 8, 9), phosphate buffer (pH 6, 7), and acetate buffer (pH 4, 5). Then, the lipase activity was measured.

3. Effect of incubation temperature on lipase activity

Optimal incubation temperature for maximal lipase activity was evaluated by incubating the reaction mixture at different temperatures of 25, 30, 35, 40, 45, and 50 °C, then lipase activity was measured [11].

4. Effect of enzyme concentration on lipase activity

Optimal enzyme concentration which supports maximal lipase activity was evaluated using different concentrations of lipase (0.25, 0.5, 0.75, 1, 1.25, 1.5, 1.75, and 2 ml), then lipase activity was measured [11].

5. Effect of agitation on lipase activity

The effect of agitation on lipase activity was studied by the incubation of the mixture of reaction at 0, 25, 50, 75, 100, 125, and 150 rpm in shaker water bath. Lipase activity was determined at the end of the incubation period [11].

6. Effect the type of substrate (oils) on lipase activity

The effects of various types of substrates on lipase activity were tested. Sunflower oil, soy beans oil, coconut oil, and walnut oil were added to the mixture of reaction instead of olive oil and incubated in shaker water bath, then the activity of lipase was measured. The lipase activity from olive oil was used as a standard value for the tested oils [11].

7. Effects of mineral salts and EDTA on lipase activity

Salts of CaCl_2 , MgCl_2 and KCl as well as EDTA, at concentrations of 0, 5, 10, 15 and 20 mM, were added to the reaction mixture to test their effects on lipase activity. The activity was measured by spectrophotometer at 715 nm at the end of the incubation period [11].

Lipase activity with and without using optimal conditions

Lipase activity was measured using the optimal conditions obtained from the previous experiments (1 ml enzyme concentration, 2.5 ml olive oil emulsion, 0.02 ml CaCl_2 , 20 minutes incubation time, 30°C, pH at 7, and 100 rpm of agitation). Conditions utilized by Rahman *et al.*[11] were used for comparison.

Factors affecting lipase stability

1. Effects of temperature on lipase stability

The enzyme solution was incubated in water bath at temperatures between 30 to 90°C for 30 minutes. At the end of each incubation period, the enzyme was rapidly cooled at 0°C for 10 min and tested by adding it to the reaction mixture (olive oil emulsion, buffer, and others). After the end of incubation period, the absorbance was read at 715 nm to determine the enzyme activity.

2. Effects of pH on lipase stability

The effects of pH on lipase stability was determined by incubating 1 ml of enzyme in various buffer solutions ranged from 3 to 9, including acetate buffer with pH 4 and 5, phosphate buffer with pH 6 and 7, and Tris- HCl buffer with pH 8 and 9, in shaker water bath. Lipase activity was then measured [11]

3. Effects of time on lipase stability

Time is a very important factor that affects lipase stability. One ml of the enzyme was incubated in water bath for different times (0, 10, 20, 30, 40, 50, 60, 70, and 80 minutes). Then, the enzyme was tested by adding it to the reaction mixture (olive oil emulsion, buffer, and others). After the end of incubation period, the absorbance was read at 715 nm to determine the enzyme activity.

Results and Discussion

1- Effects of incubation periods (time) on lipase activity

The results showed that 20 minutes of incubation for the mixture of the enzyme and substrate in shaker water bath exerted the maximum activity (1.87 U/ml). The lipase enzyme remained active until 30 minutes then the activity declined sharply (Figure-1). The decline in enzyme activity after 30 minutes of incubation could be either due to the decrease in the substrate availability or the catabolizing repression of the enzyme.

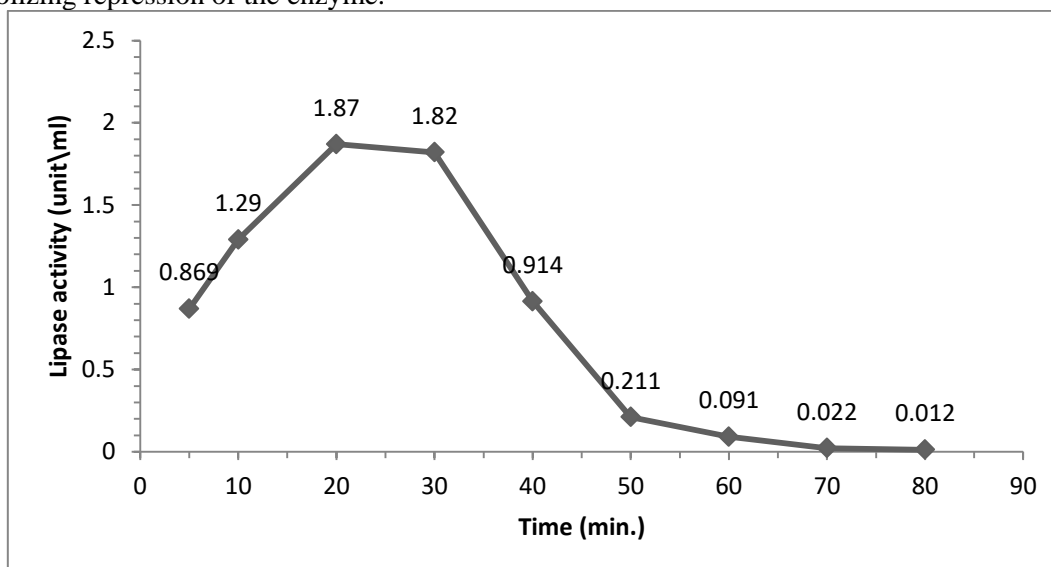


Figure 1- Effects of incubation time on the activity of lipase extracted from germinating sunflower seeds.

2. Effects of pH on lipase activity

Lipase showed the highest activity (2.3 U/ml) at pH 7. This suggests that the lipases extracted from sunflower seeds prefer neutral pH conditions, giving the maximum activity when Tris-HCl buffer at pH 7 was used in the experiments (Figure-2). Several investigators reported that oil seeds of different plant species contain acid, alkaline, or even neutral lipases. Madhikar *et al.* [8] demonstrated that the enzyme activity in sunflower germinated seeds was increased with increasing pH, with maximum activity observed at pH 7. Sagioglu and Arabaci [12] showed that the maximum activity of sunflower lipase was achieved at pH 7.5. Muto and Beevers [13] found that castor bean seeds have two types of lipases, acid lipase and alkaline lipase, with maximum activity at pH 5 and 9, respectively. Eze and Chilaka [14] suggested that white melon seeds contain acid lipase and alkaline lipase with optimum activity at pH 4.5 and 7.5, respectively. The optimum pH for lipase activity depends on the source of enzyme, the substrate used, and the components of the assay reaction [14].

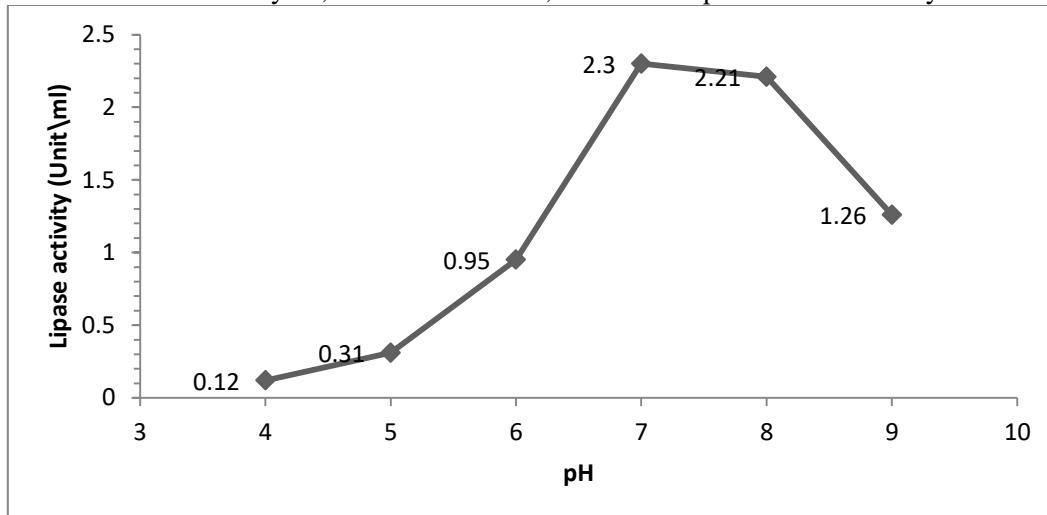


Figure 2-Activity of lipase extracted from germinating sunflower seeds under the effects of different pH ranges using different buffers.

3. Effects of incubation temperature on lipase activity

Lipases extracted from sunflower germinated seeds showed maximum activity (2.29 U/ml) at a temperature of 30 °C (Figure-3). This result agrees with the study of Ezema [15] who found that the two types of lipase extracted from white melon had optimum activity at 30 °C. Other studies reported that maximum activity of lipases extracted from white melon and soya bean germinated seeds was achieved at temperatures of 40 and 24 °C, respectively [14, 16]. Different earlier studies showed that the optimum temperature for maximum enzyme activity was 30 °C, as in cellulose extracted from a local isolate of *Pantoea* spp. [17] and peroxidase extracted from *Brassica oleracea* Var. [18].

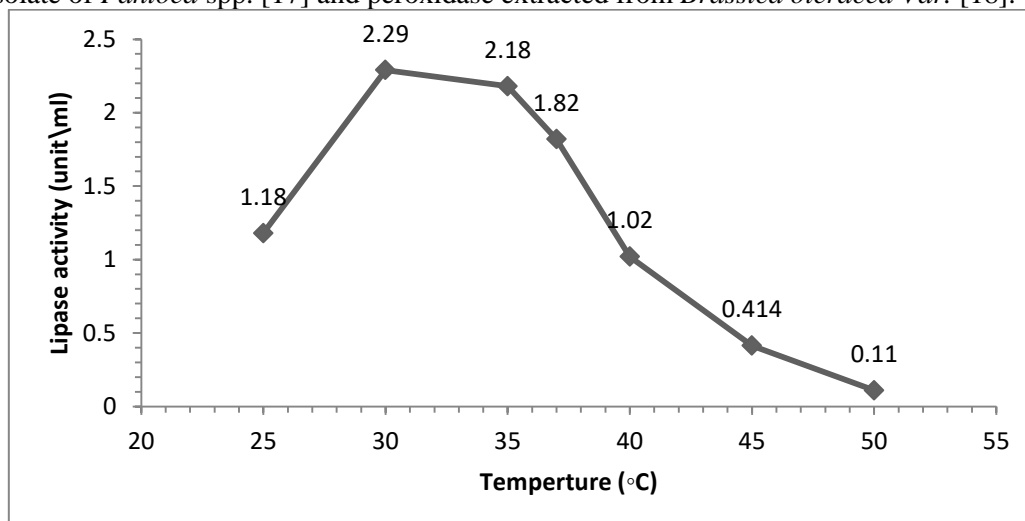


Figure 3-Activity of lipase extracted from germinating sunflower seeds under different incubation temperatures.

4. Effects of enzyme concentration on lipase activity

The results presented in Figure-4 shows that lipase activity was increased gradually with increasing the concentration of the enzyme, until it reached 2.36 U/ml at a concentration of 1.75 ml, then remained constant even when lipase concentration was increased to 2 ml. Also, the results showed that the activity was 2.31 U/ml at 1 ml, while it was 2.36 U/ml at 1.75 ml of enzyme. This implies that there is a little (0.05 U/ml) increase in lipase activity when 1.75 ml of enzyme is used. However, this increase is not considered as economically valuable. Therefore, 1 ml of lipase concentration was the optimum concentration of enzyme that exerted the highest activity (2.31 U/ml). The increase of enzyme concentrations will increase the rate of reaction as more enzymes causes more colloid with substrate molecules [19], but further increasing lipase concentration has no further effect on the rate of hydrolysis as all enzyme molecules are saturated with substrate molecules [20].

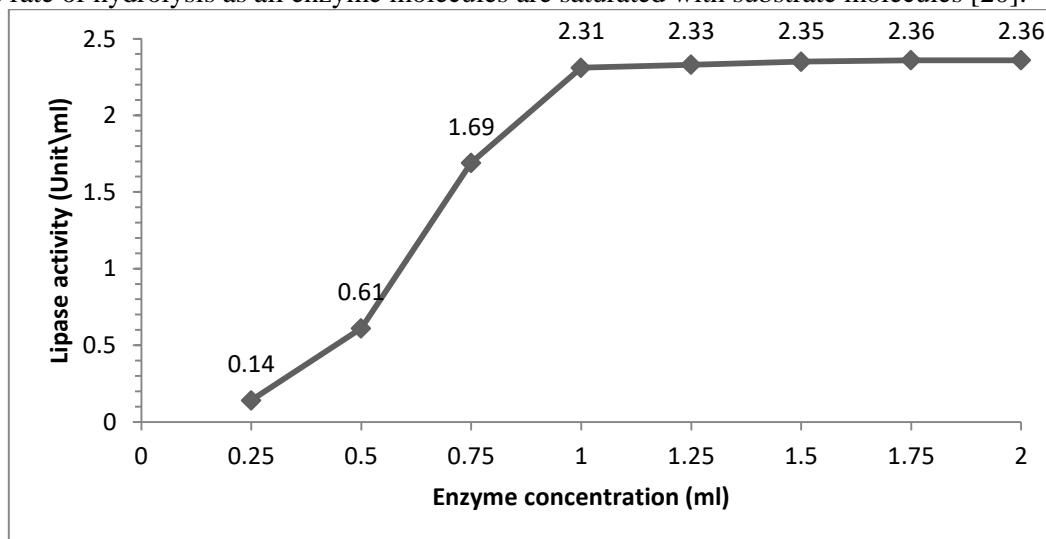


Figure 4-Effects of different enzyme concentrations on the activity of lipase extracted from germinating sunflower seeds.

5. Effects of agitation on lipase activity

The results presented in Figure-5 show that agitation at 100 rpm was quite sufficient to yield optimum lipases activity (2.31 U/ml), since the activity remained almost the same at 125 and 150 rpm. Agitation rate may affect the viability of the enzyme to the substrate during the reaction of lipase activity, while higher agitation rate may affect the structure of the enzyme [21].

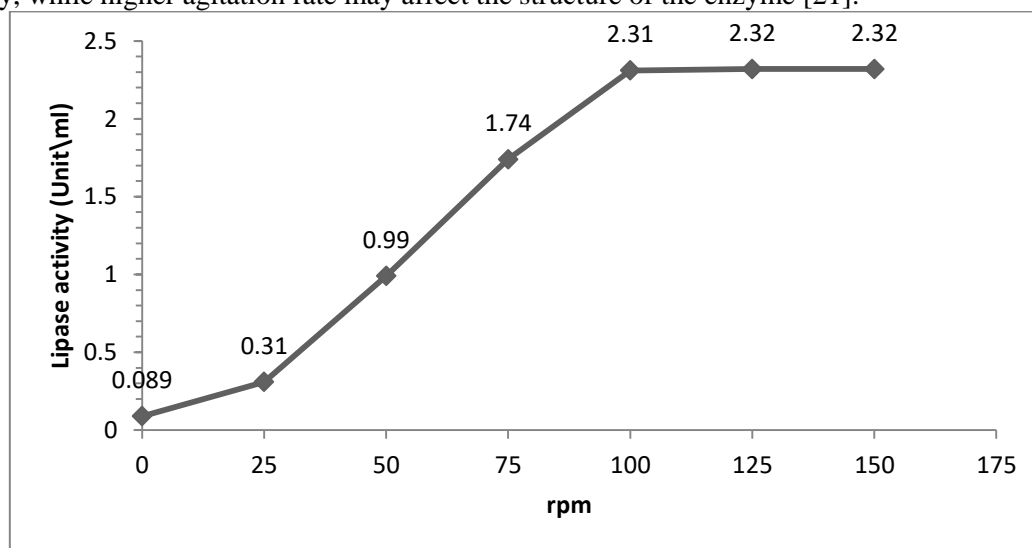


Figure 5-Activity of lipase extracted from germinating sunflower seeds under the effects of different Agitation rate (rpm).

6. Effects of types of substrate on lipase activity

Figure-6 shows that different types of oils used in the mixture of reaction caused different values of lipase activity. Olive oil exerted the highest value of activity (2.31 U/ml), followed by walnut oil (1.98 U/ml), while coconut oil had the lowest value (0.28 U/ml).

It was reported that the activity of lipase increases or decreases according to the type and concentration of oil used [22]. The oil substrate may change the physical and chemical characters of lipase enzyme and higher oil concentration may not be suitable for lipase activity or even becomes inhibitory to lipase enzyme.

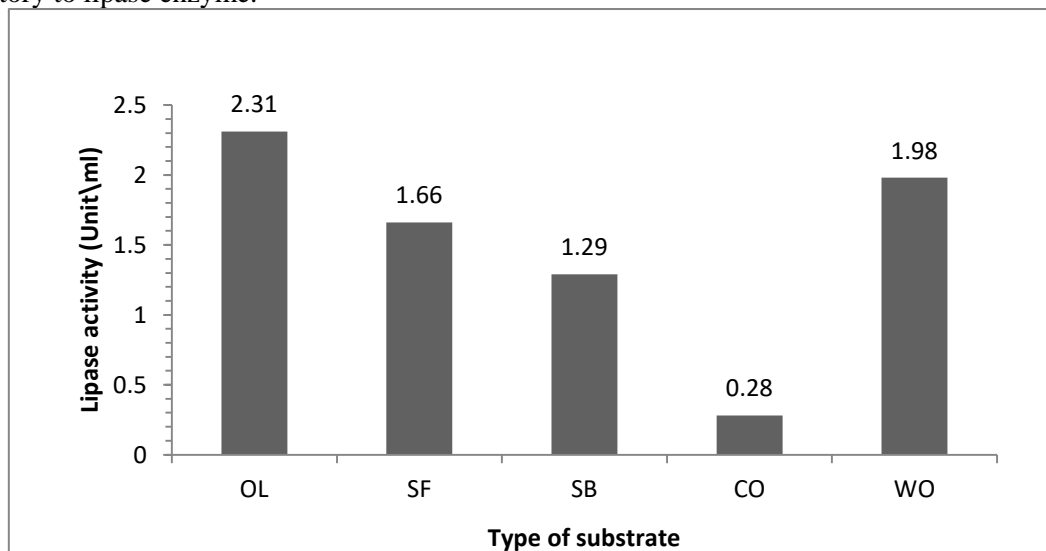


Figure 6-Effects of different types of oils (substrate) on the activity of lipase extracted from germinating Sunflower seeds. OL: Olive oil, SF: Sunflower oil, SB: Soya beans oil, CO: Coconut oil and WO: Walnut oil.

7. Effects of mineral salts and EDTA on lipase activity

The effects of mineral salt ions and EDTA on lipase activity were different according to the type and concentration of mineral salts and EDTA used (Figure-7). The results showed that CaCl_2 , MgCl_2 and KCl at 10 mM caused an increase in residual lipase activity, which reached 146.5%, 119.5%, and 109.8%, respectively, while EDTA at 10 mM caused a decrease in residual lipase activity, reaching 86.2%. Therefore, EDTA manifested significant inhibiting effects on lipase activity at a concentration of 10 mM, as well as at other concentrations. This inhibitory effect may be due to the effect of EDTA on the interaction between the enzyme and substrate (oil). The inhibitory effects of EDTA, which acts as chelating agent, on lipase are also reported in seeds of different plant species, such as African bean [23], castor bean [24], white melon [14], and mustard and rape [25].

The higher activity of lipases caused by Ca^{+2} is also reported by Madhikar *et al.* [8] who studied the lipases of sunflower germinated seeds, while Kermasha and Van de Voort [26] reported that Ca^{+2} has an inhibitory effect on lipases extracted from French bean. The presence of Ca^{+2} and Mg^{+2} increases the activity at low concentrations, while Hg^{+2} and EDTA decrease the activity of lipase extracted from Soybean seeds [16].

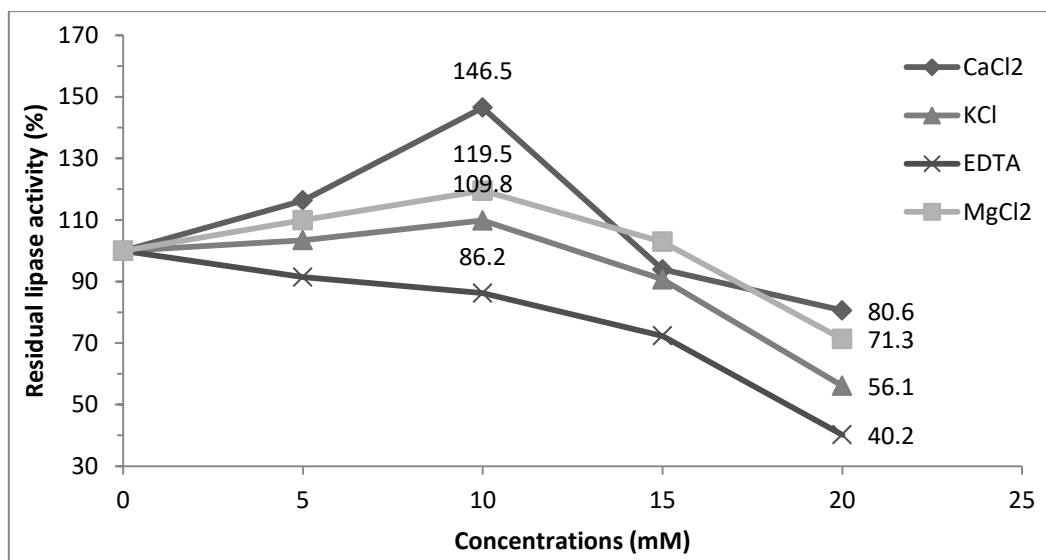


Figure 7-Effects of different concentrations of mineral salts and EDTA on the activity of lipase extracted from germinated sunflower seeds.

Compression of lipase activity and specific activity measured under optimum and non- optimum conditions

The results indicated that the optimum conditions for the specific lipase activity are quite different from conditions adopted by Rahman *et al.*, [11]. Temperature, time of incubation, and agitation rate were found to be obviously decreased in the present optimum conditions, compared with in the conditions utilized by Rahman (Table-1). The other factors remained the same.

The results also showed that lipase activity, total activity, and specific activity values measured by optimum conditions were increased by 36.99%, 36.95% and 38.21%, respectively, over values achieved by Rahman (Table-2). The enzyme concentration, the amount of olive oil, and the amount of CaCl₂ in both conditions were almost the same.

Table 1-Reaction of lipase activity according to Rahman *et al.* [11] as compared to that achieved via the present optimum conditions.

Factors	Rahman <i>et al.</i> condition	Optimum conditions
Enzyme concentration	1 ml	1 ml
Olive oil emulsion	2.5 ml	2.5 ml
CaCl ₂	0.02 ml	0.02 ml
Incubation time	30 min.	20 min.
Temperature	37°C	30°C
pH	7	7
Agitation	200 rpm	100 rpm

Table 2-Lipase activity in sunflower germinated seeds measured by Rahman *et al.*[11] as compared to that achieved via the present optimum conditions.

Parameters	Studied values	
	Rahman <i>et al.</i> conditions	Optimum conditions
Protein concentration (mg/ml)	2.754	2.730
Total proteins(mg)	22.032	21.840
Activity (U/ml)	2.373	3.251
Total activity(U)	18.990	26.008
Specific activity (U/mg)	0.861	1.190

Factors effecting lipase stability

1. Effects of temperature on lipase stability

The results indicated that lipase extracted from sunflower germinated seeds was stable at a temperature between 30 to 50°C, then the activity started to decline sharply, but still detectable below 50 °C, indicating that the enzyme is thermo-stable (Figure-8).

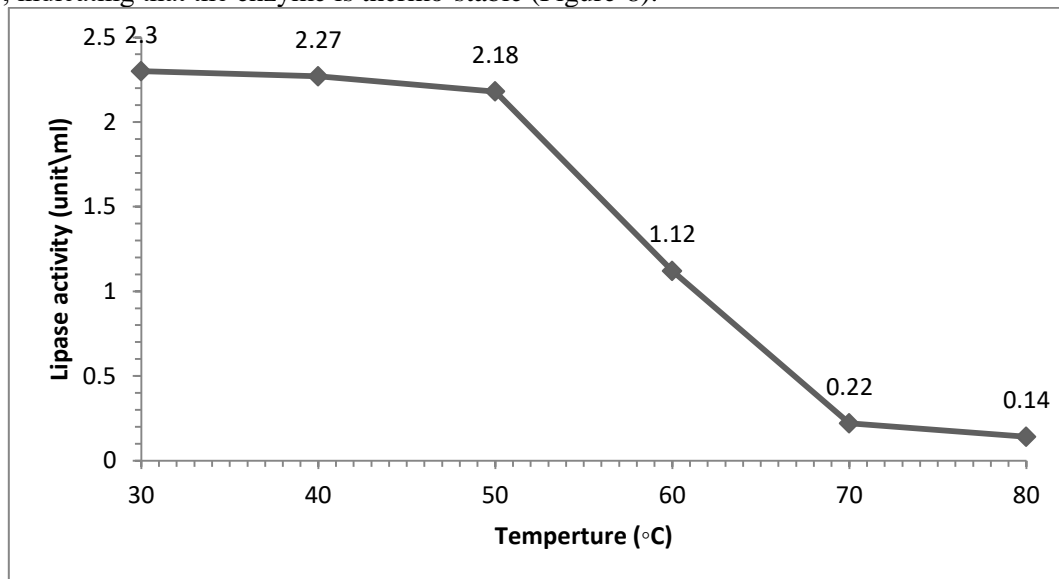


Figure 8-Effects of temperature on stability of lipase extracted from sunflower germinated seeds.

2. Effects of pH on lipase stability

Figure-9 indicates that lipase was stable at pH 7 - 8 with activity values of 2.29 U/ml and 2.3 U/ml, respectively. This suggests that the enzyme was stable in neutral and weak alkaline solutions.

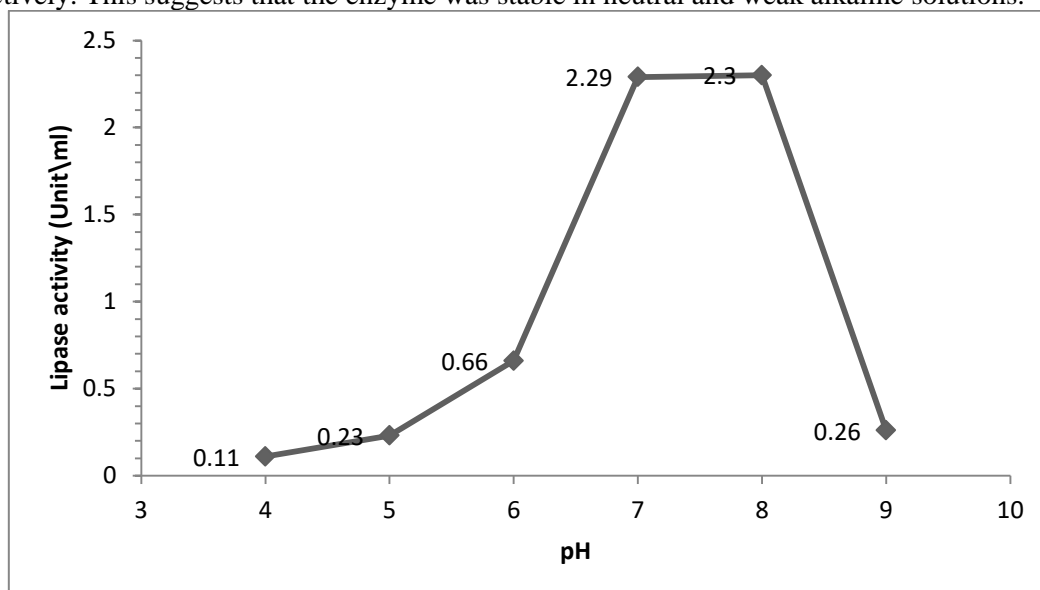


Figure 9-Effects of pH value on stability of lipase extracted from germinating sunflower seeds.

3. Effects of time on lipase stability

The residual activity of the tested lipase was found to be stable at time ranged 10 - 40 minutes, then decreased gradually (Figure-10). This result suggests that lipase enzyme is less stable for a long period of time.

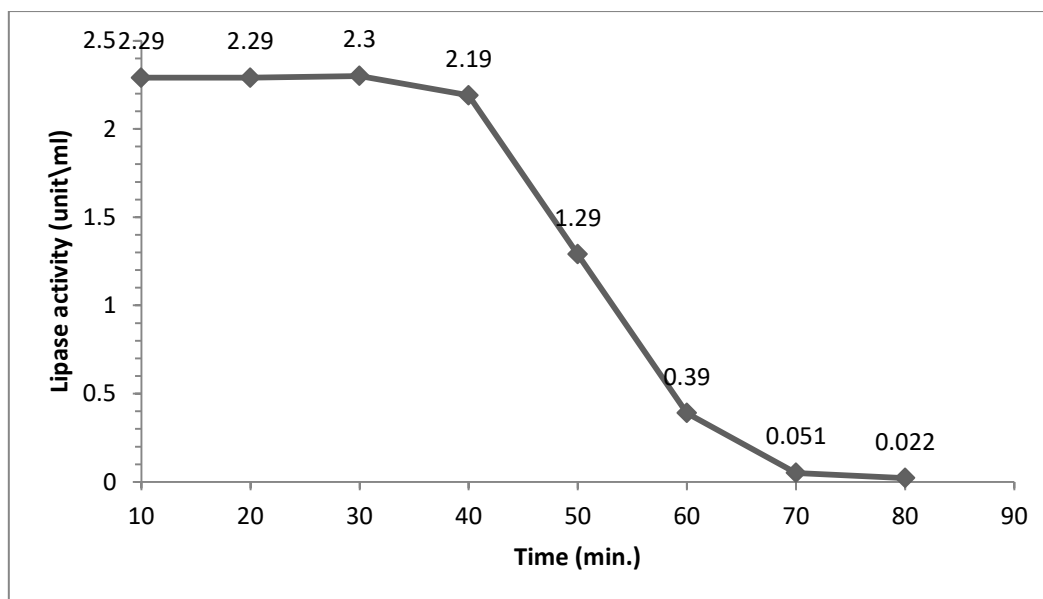


Figure 10-Effects of time on the stability of lipase extracted from germinated sunflower seeds.

Conclusions

Based on the results, the optimum conditions for lipase extracted from sunflower seeds were determined and found to cause increased lipase activity, total activity, and specific activity by 36.99%, 36.95% and 38.21%, respectively, over the values obtained via the conditions used by Rahman *et al.* [11]. The enzyme showed stability at a temperature ranged between 30 and 50 °C, pH between 7 and 8, and time of incubation between 10 and 40 minutes. These results suggest that lipase enzyme extracted from germinated sunflower seeds has stability to pH, temperature, and incubation period, which enables its utilization in different industries.

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