# Extraction, purification and characterization of lipoxygenase from *Pleurotus ostreatus.*

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**Abstract:**
Lipoxygenase was extracted from the cup of *Pleurotus ostreatus* ( Jaq : Fr ) oyster mushroom for the first time in Iraq, and purified homogeneously through precipitation with 40% saturation of $(NH_4)_2SO_4$ as a partial purification then loaded on DEAE-Cellulose (Diethyl amino ethyl Cellulose) ion-exchange chromatography column and then the highly active elution parts have been passed through gel filtration column with Sephacryl S-300 as a final purification with 804 (U/mg protein) specific activity, 11.32 fold of purification and 36.54% yield . The molecular weight of the enzyme was estimated to 74 KDa by gel filtration Sephacryl S-300 column and the isoelectric point for enzyme was 5.3. The optimal pH for lipoxygenase activity and stability were 8 and 6-8.5 respectively, and the optimal temperature for the activity and stability of the enzyme were 30 and 10-45 respectively. Also the activation energy necessary for Lipoxygenase to convert lionleic acid to product and for enzyme denaturalization were calculated to 9.674 and 28.087 Kilo calorie/mole respectively.

**Keyword:** *Pleurotus ostreatus*, Oyster mushroom, Purification, Lipoxygenase.

# استخلاص، تنقية وتوصيف انزيم الليبوكسيجينيز من الفطر *Pleurotus ostreatus*

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**الخلاصة:**
تم استخلاص إنزيم الليبوكيسجينيز من الجسم الثمري للفطر المحاري *Pleurotus ostreatus* ( Jaq : Fr ) لأول مرة في العراق، وتمت تنقية الأنزيم جزئيا بواسطة الترسيب بكبريتات الامونيوم بنسبة اشباع 40% ثم التنقية النهائية بتحميله في عمود التبادل الايوني DEAE-Cellulose (Diethyl amino ethyl Cellulose بعدها مررت الأجزاء المستردة ذات الفعالية العالية للانزيم في عمود الترشيح الهلامي بالسيفاكريل S-300 وكانت الفعالية النوعية للإنزيم 804 (وحدة/ملغم بروتين) منقى 11.32 مرة بحصيلة انزيمية 36.54%. كان الوزن الجزيئي للإنزيم 74 كيلودالتن بطريقة الترشيح الهلامي بعمود السيفاكريل S-300 ، وعينت نقطة التعادل الكهربائي 5.3. قدرت الأس الهيدروجيني الأمثل لفعالية الانزيم وثباته 8 و 6-8.5 على التوالي ، بينما كانت درجة الحرارة المثلى لفعالية الانزيم وثباته 30 و 10-45 درجة مئوية على التوالي. وتم أيضا حساب طاقه التنشيط للإنزيم اللازمة لتحويل حامض اللينوليك إلى ناتج و طاقه التنشيط اللازمة لمسخ الإنزيم 9.674 و 28.087 كيلو سعرة/ مول على التوالي.

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**Introduction:**

*Pleurotus ostreatus*, commonly known as oyster mushroom is a group of higher fleshy fungi belonging to the Basiodiomycetes. It is regarded as one of the major edible mushrooms cultivated in different countries [1]. *Pleurotus* is considered a rich source of proteins that make up 5% of the weight of material fresh fungus, which is equivalent to 20_40% by weight of dry matter and has high content of vitamin C and B complex [2], also is rich in elements Ca, P, Fe, K and being a poor for Na, therefore is recommended for balanced diet and people with high blood pressure or heart patients [3]. Oyster mushroom can be cultivated in any type of lignocellulose material like straw, sawdust, rice hull, etc. for human consumption as food due to its good flavor and highly nutrient content or to extract compound such as carbohydrate, proteins and enzymes in several applications [4,5,6]. The aroma contributes significantly to the characteristic flavor of mushroom, the aroma compounds has been investigation and identified as $C_8$ compound 3-octanol, 2-octen1-ol, 3-octanone, 1-octen-3-one and 1-octen-3-ol. Among these aroma compound 1-octen-3-ol, known as raw mushroom or mushroom alcohol, is contained in al type of mushroom and is considered to be the most important aroma compound for the flavor of edible mushroom [7]. It has been observed that the biogeneration of 1-octen-3-ol was due to oxygenation by a lipoxygenase of linoleic acid [8]. Lipoxygenase (LOX; linoleate:oxygen oxidoreductase, EC 1.13.11.12) comprised a class of non-heme iron-containing dioxygenases, which consider as a key enzyme in the oxidative degradation of lipid, discovered in 1928 by Haas and Bohn. LOX is widely distributed in plant, mammals, algae, bacteria and fungus [9]. This enzyme acts on polyunsaturated fatty acid with *cis,cis*-1,4-pentadiene system, but geometrical configuration with *cis-trans* and *trans-trans* renders this fatty acid inactive or inhibitors to lipoxygenase catalysis [10,11]. Tressl, et al. [12] reported that the 13-hydroperoxy-9Z,11E-ctadecadienoic acid [13-Z,E-HPOD) first results from the hydroperoxidation of linoleic acid by LOX then is converted subsequently into 1-octen-3-one and 10-carbon compounds, and the former is finally reduced by alcohol oxidoreductase to 1-octen-3-ol. It is clear that the initial products of fatty acid oxygenation by lipoxygenase are hydroperoxides, these hydroperoxides themselves are odorless and tasteless and are not the direct cause of off-odors and flavors produced in food due to storage or processing. However, but they readily decompose to produce a variety of compounds include: aldehydes, ketones and alcohols, acids, epoxides and polymeric materials which contribute to the off-odors especially the carbonyl compounds, for instance, they are responsible for the undesirable 'beany', 'green' and 'grassy' flavours [13]. Other investigations propose that a alcohols and aldehydes, compounds are used for flavouring foods [14].

**Aims of study:**

**1**- Extraction and purification of lipoxygenase from *P. ostreatus*.
**2**- Studying the characteristics of enzyme

**Materials and Methods:**

**Collection of sample:** The sample used in this study was from Dr. Abdullah and cultured with assist him in Plant Protection Department – Agriculture College / Tikrit University.

**Buffer of enzyme extraction:** It prepared by using (0.02M, pH 7) sodium phosphate buffer solution as assessed in [15]

**Assay of enzyme activity:** It is measured as method described by [9]. **One unit of LOX activity is defined as an increase in absorbance of 0.001 at 480 nm/min/mg of protein under assay conditions.**

**Estimation of protein concentration:** Protein concentration was estimated by a dye binding method that used from Bradford [16].

**Purification of lipoxygenase:** Enzyme purified firstly by precipitation with 40% saturation of $(NH_4)_2SO_4$ as partial purification, according to [17], then used ion exchange DEAE–Cellulose column with dimension (40×2)cm as described by [18], and used gel filtration Sephacryl S-300 column with dimension (50×2)cm as mention in [19], as a final purification steps.

**Studying the characteristics of purified enzyme:**

1- **Determination of molecular weight:** Molecular weight of the purified enzyme was appointed in a way of gel filtration as mention by [20].

2- **Determination of the Isoelectric point (pI):** Used the method that described by [21] in preparation of solutions and determination of Isoelectric (pI).
3- **Determination of optimal pH for enzyme activity and stability:** It is estimated according to [22].
4- **Determination of the optimal temperature for the activity and stability of the enzyme:** It is determined as described by [23].

**Estimated of The Activation Energy:** the activation energy was estimated according to Arrhenius equation of [23].

**Result and Discussion:**

**Enzyme Extract:**

Extract was obtained from the homogenizing of fruiting body of *Pleurotus ostreatus* due to lipoxygenase enzyme is intracellular, and regarded as a crud ezyme. The specific activity was 70.97U/mg, fold was 1 and yield was 100% as shown in table 1. This result was agree with [9] while was different with [19] and [20]. The difference in specific activity of enzyme extracted with same method due to difference in the ionic power according to enzyme's sources with same the extraction solution [24].

**Purification of lipoxygenase:**

The results of *pleurotus ostreatus* lipoxygenase purification are mentioned in table 1.

**1- Partial purification by concentrated with ammonium sulfate:**

The crud enzyme was concentrated by 40% saturation of $(NH_4)_2SO_4$, the enzyme specific activity in this step was 91.91U/mg, and the fold of purified enzyme was (1.34) with 76% yield. The findings of this step agree with [9] and [25]. The salt balances the charges existing on protein surface, and withdraws the layer of water surrounding it, thus decreasing its dissolvable nature and increasing the protein concentration [19].

**2- Final purifications:**

**A. Ion exchange chromatography.**

Enzyme solution that concentration by $(NH_4)_2SO_4$ was loaded on DEAE–Cellulose column and eluted with linear salt gradient (0.1-0.6 M) NaCl solutions, six peaks of protein noted in the eluted fractions, one of these peak belong to activity of lipoxygenase which is the peak at 55-67 fractions that eluted with 0.2M NaCl as observed in figure 1. Specific activity increased in this step to 160.96U/mg, and the fold of enzyme was 2.27 with 60% yield. This result was accord with [9] and [19] where the researcher achieved 64.3% and 57.58% yield respectively.

**Table 1**- purification steps of lipoxygenase extracted from *pleurotus ostreatus*

| No | Steps of purification | Volume (ml) | Activity (U/ml) | Protein Conc. (mg/ml) | Specific activity (U/mg) | Total activity | Purific-ation fold | Yield (%) |
|---|---|---|---|---|---|---|---|---|
| **1** | Crude enzyme | 75 | 440 | 6.2 | 70.97 | 33000 | 1 | 100 |
| **2** | 40% saturation $(NH_4)_2So_4$ | 20 | 1250 | 13.6 | 91.91 | 25000 | 1.34 | 76 |
| **3** | Ion exchange chromato-graphy by DEAE–Cellulose | 30 | 660 | 4.1 | 160.96 | 19800 | 2.27 | 60 |
| **4** | Gel filtration chromatog-raphy by Sephacryl S-300 | 30 | 402 | 0.5 | 804 | 12060 | 11.32 | 36.54 |

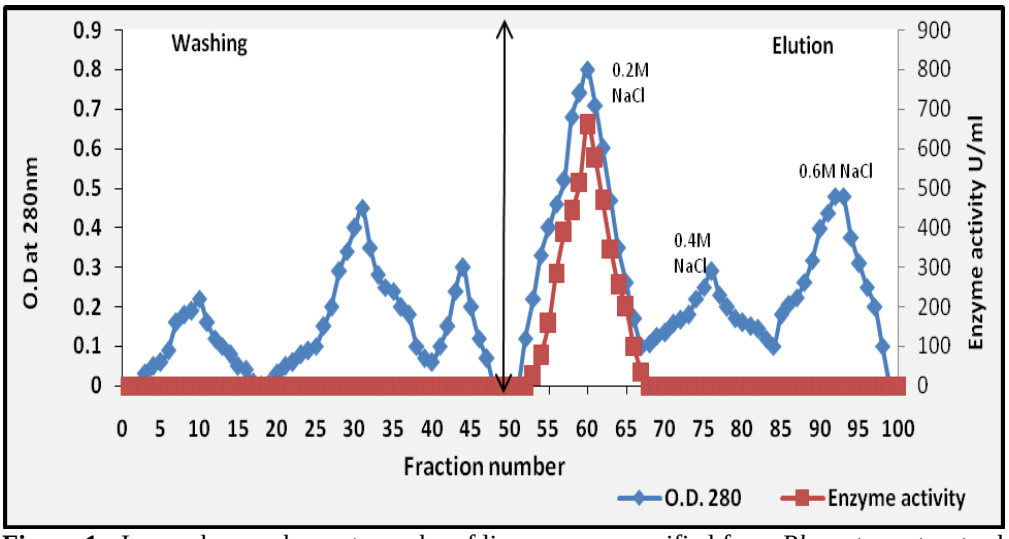


**Figure 1-** Ion exchange chromatography of lipoxygenase purified from *Pleurotus ostreatus* by DEAE-Cellulose column (40×2) equilibrated with (0.02M,pH7) sodium phosphate buffer.

**B. Gel filtration chromatography:**

It is the second step in purification, the enzyme solution passed through Sephacryl S-300 column, and eluted by (0.02M, pH 7) sodium phosphate buffer solution. The eluted fraction contained one peak of protein at (16-32) fractions that containing activity of lipoxygenase as shown in figure 2. The specific activity was 804U/mg), and the fold of purification was 11.32 with 36.54% yield. Our result was fit in with [26] where obtain 36% yield when used Sephadex G-200, while [9] achieved 57.03 yield when used Sephadex G-100.

Purification by ion exchange chromatography and gel filtration chromatography are two of the classical methods of purifying used by many researcher as a final step of purification.

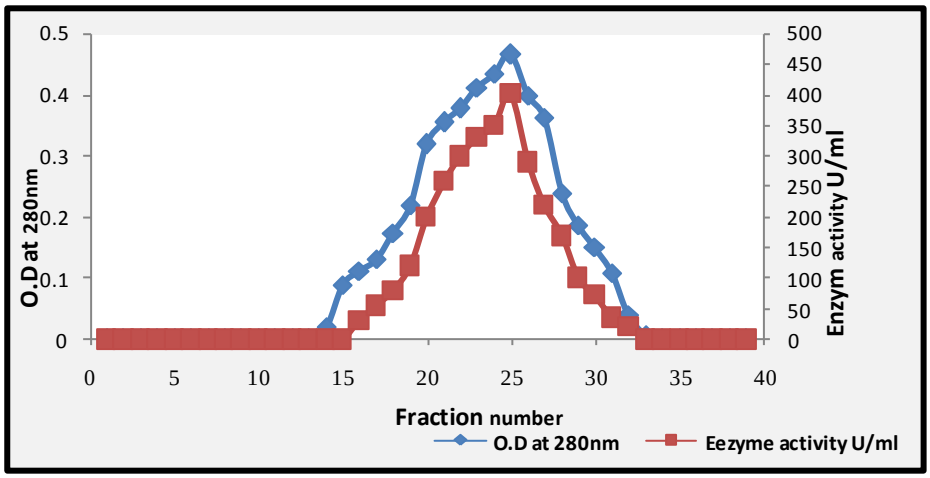


**Figure 2-** Gel filtration chromatography of lipoxygenase purified from *Pleurotus ostreatus* by Sephacryl S-300 column (50×2) equilibrated with (0.02M,pH7) sodium phosphate buffer.

**Characterization of purified enzyme:**

**1- Enzyme molecular weight:**

The enzyme molecular has been estimated by Gel Filtration with Sephacryl S-300 column of (50×2 cm) with (0.02M, pH 7) Sodium phosphate buffer under the same condition of enzyme separation. The ratio of elution volume (Ve) of every standard protein was divided on the elution volume of blue dextran. When Lipoxygenase enzyme has been passed through the column under the same condition, the ratio of elution volume (Ve) to the void volume (Vo) was 1.57 as displayed in Figure 3. When this value has been projected on the linear relation between the ratio of elution volume to the void volume of the standard proteins of known molecular weight, it comes out that the molecular weight of

Lipoxygenase was 74000 Daltons. This result was accord with [15] and dis agree with [9] who, extract lipoxygenase from fungi and [19] who extract the same enzyme from plant.
Perez-Gilabert *et al.* [27], report that most of lipoxygenase extract from fungus have a molecular weight range of (60000-120000) Daltons. The Lipoxygenase enzyme molecular weight varies according to the method used to estimated it, enzyme source such as the molecular weight of lipoxygenase from plant differs from animal, bacteria and fungus, this difference resulting from heredity and ecology factor.

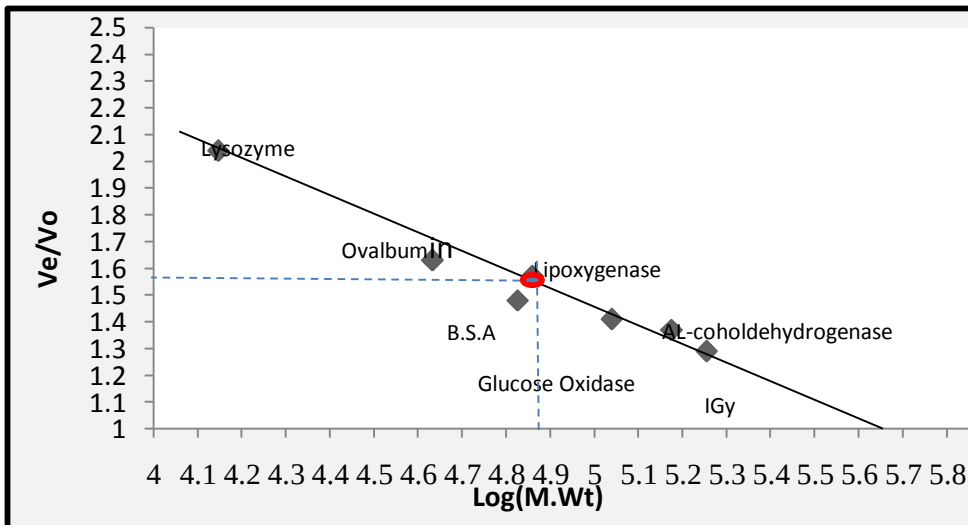


**Figure 3-** Selectivity curve for lipoxygenase Molecular Weight Determination by Gel Filteration Using Sephacryl S-300 Column Purified from *Pleurotus ostreatus.*

**2- Isoelectric point for The Enzyme (pI):**

The separation and specifying of (pI) for *P. ostreatus* lipoxygenase enzyme have appeared as one protein band after coloring the gel with Commassie Brilliant Blue R-250 as show in figure 4. The Iso electric point for the Lipoxygenase was 5.3 when it has been estimated by Iso electric focusing which depends on progressive hydrogen number (pH) of the gel that is stable and sustainable due to the charged small particles (Ampholytes). Our result was agree with [17] found it 5.8, while it different with [9], [19] and [20] found it equal to 6.1. It is noteworthy that the Iso electric point (pI) varies according to the enzyme's source. If the sources are vegetarian, animal or microbiological, yeast, mold and bacteria [28].

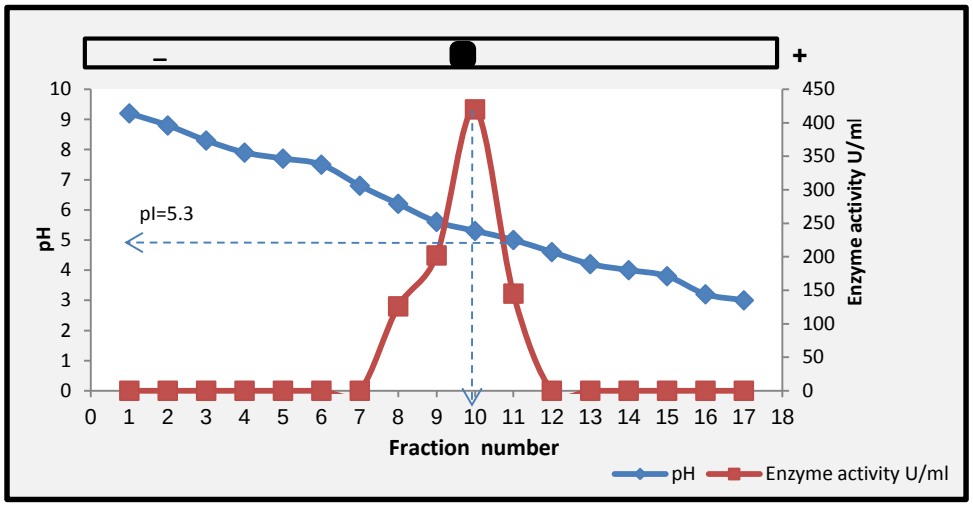


**Figure 4-** The iso electric point of purified lipoxygenase from *Pleurotus ostreatus* by PAGE (lipoxygenase Iso electric point = 5.3).

**3- The optimal pH for enzyme activity and stability:**

**A. Optimum pH for lipoxygenase activity:**

Figure 5- shows the activity of purified lipoxygenase enzyme at different pH values ranging from 3.0 to 10.5, it is found the optimum pH for the activity of enzyme under study was 8.0, it has been noticed that the enzyme activity decreases at less than pH 7 and more than pH 9, also the enzyme activity disappears completely at pH values ranging from 5 downwards and 10.5 upwards. The findings achieved for this study was agree with [15] and [20] found it 8 and 7.5 respectively, and disagree with [9] found it equal to 6. The optimum pH varies according to the animal, vegetarian or microorganism source from which enzyme is purified [30].

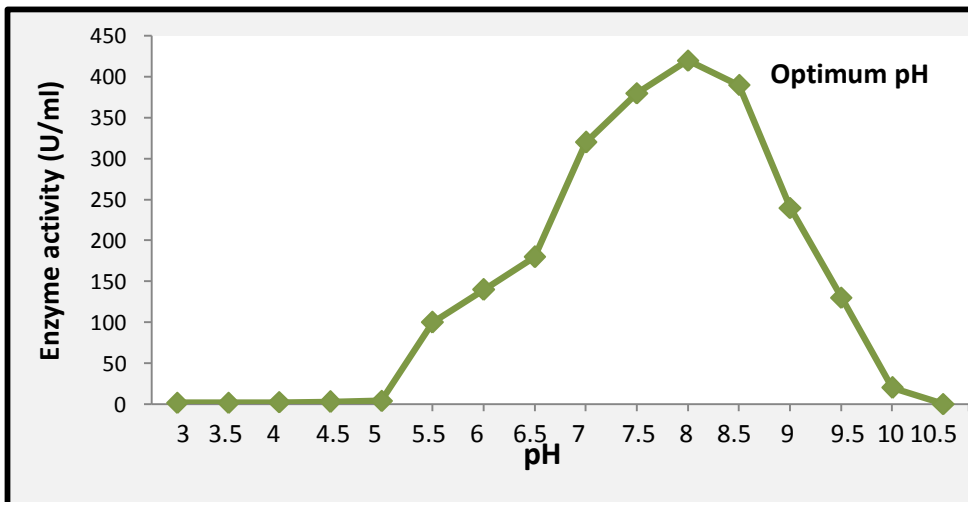


**Figure 5-** Optimum pH for Lipoxygenase enzyme activity purified from *Pleurotus ostreatus* at pH range (3-10.5) by using linoleic acid as a substrate.

**B. Optimum pH for lipoxygenase stability:**

To study the pH effect on purified Lipoxygenase stability, the enzyme has been incubated for 20 minute with buffer solutions whose pH range 3-10.5, then estimated the remaining activity as mentioned in figure 6. It has come out that optimum pH of the enzyme stability was 6-8.5, and has been noticed that the enzyme stability decreased at pHs out of this range. The results may give a conclusion that lipoxygenase of *P. ostreatus* was more stable in pH close to neutral than in alkaline or acidic, where the activity of *P. ostreatus* lipoxygenase reduced at pHs 5.5 downwards and 9 upwards. This result was concord with [19] and [26] found it ranged 6-8.5 and 6-9 respectively.

This decrease in enzyme activity is attributed to the effect of pH on the enzyme molecular structure to the extent that it changes the enzyme second and third structure as well as form of the active site [31].

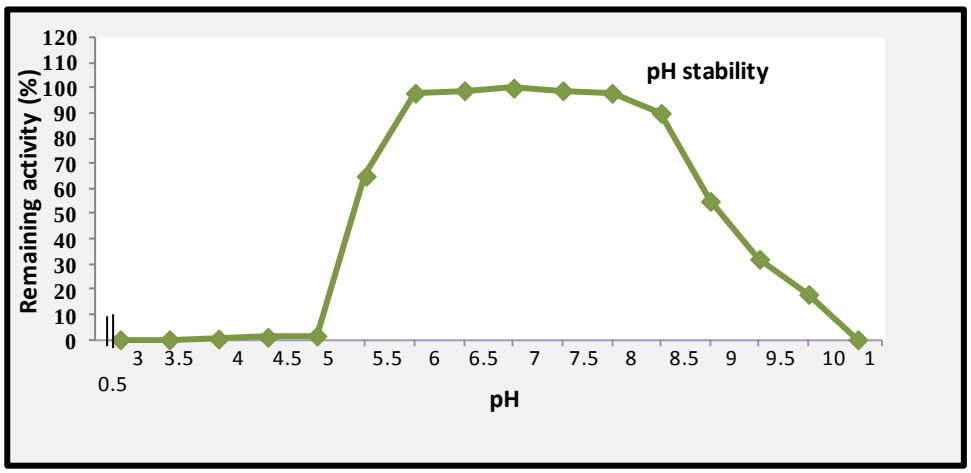


**Figure 6-** Optimum pH for Lipoxygenase enzyme activity purified from *Pleurotus ostreatus* at pH range (3-10.5) by using linoleic acid as a substrate.

**4- The optimal temperature for enzyme activity and stability:**

**A. Optimum Temperature for Lipoxygenase Enzyme:**

Lipoxygenase enzyme reactions have been conducted under (5-90) $^0$C. Figure 7 presented an increase in enzyme activity at high temperatures, this activity has reached its utmost 440U/ml at 30 $^0$C, then it has progressively decrease by the increase in temperature. The activity has totally disappeared at 75 $^0$C. The result achieved from this study was concord with [20], and disagree with [9], [19] and [26]. Indeed, the optimum temperature for the enzyme activity is not a stable and distinguishing characteristic of the enzyme because it depends on experimental conditions and on the enzyme low stability therein. If the enzyme reaction is long, the enzyme activity will decrease as a result of the temperature effect on the enzyme structure. The more time the reaction takes, more effect of temperature will happen [23].

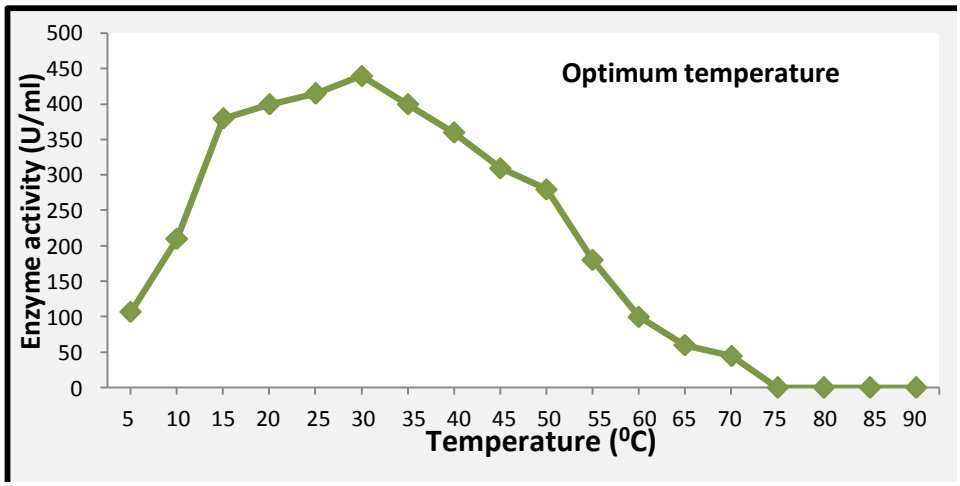


**Figure 7-** Optimum Temperature for Lipoxygenase enzyme activity purified from *Pleurotus ostreatus* at temperature range (5 – 90) by using linoleic acid as a substrate.

**B. Thermal Stability of Lipoxygenase:**

The result of incubation Lipoxygenase enzyme at (5-90) $^0$C for 20 minute was shown in figure 8, where the enzyme nearly retains its full activity at (10-45) $^0$C. Then the enzyme activity decreases progressively when the temperature increases to more than 45 $^0$C. At 75 $^0$C lipoxygenase lost its full activity. This indicated that *Pleurotus ostreatus* lipoxygenase is moderately sensitive enzyme to thermal coefficients. The result achieved from our study was identical to the finding of [20] and [27]. All Research refer that lipoxygenases from different sources are stable at temperature range 10-60 $^0$C [9], [25] and [32].

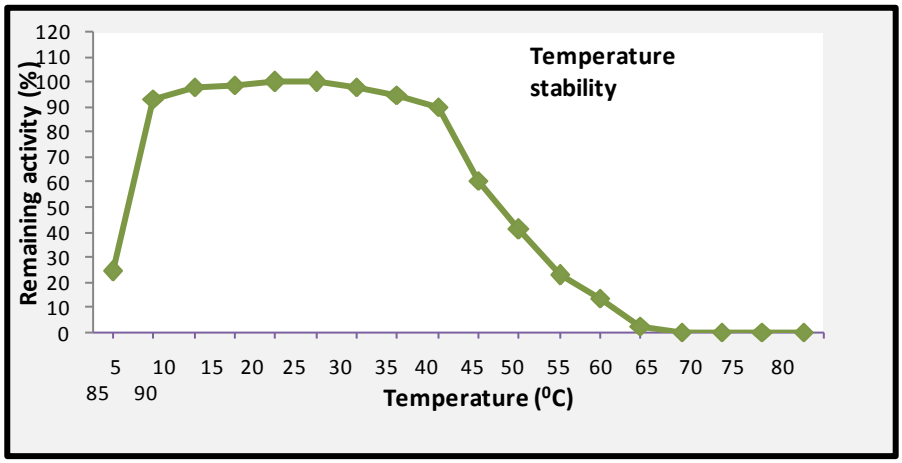


**Figure 8-** Optimum Temperature for Lipoxygenase enzyme stability purified from *Pleurotus ostreatus* at temperature range (15 – 90) by using linoleic acid as a substrate.

**The Activation Energy of Lipoxygenase:**

The Ea necessary for the enzyme to convert the substrate to product has been calculated according to Arrhenius equation as expressed in figure 9, the Ea necessary for lipoxygenase to convert lionleic acid to product was 9.674 Kilo calorie/mole, this value within the range mentioned by [29], which is 6-18 Kilo calorie/mole for most enzyme reaction, we could say that the lipoxygenase enzyme has high catalyzing efficiency to convert linoleic acid to an output. while the Ea for enzyme denaturalization has been calculated as 28.087 Kilo calorie/mole, this value gives an idea about the enzyme non stability at high temperatures. The Ea necessary for enzyme denaturalization ranged 40-175 Kilo calorie [29]. This findings was agreed with [19] and [20].

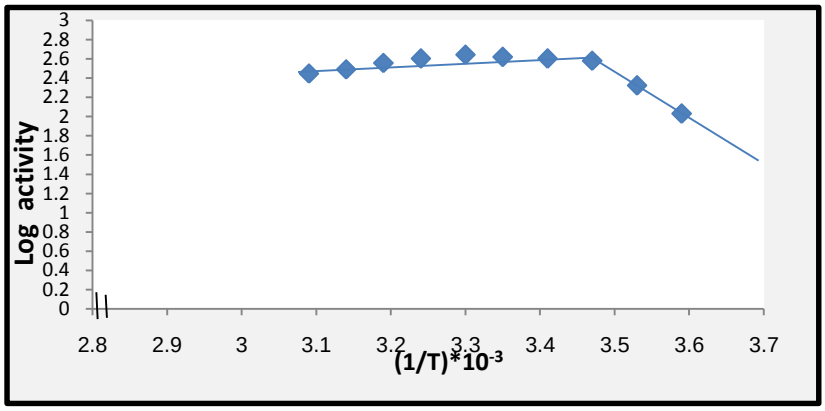


**Figure 9-** Arrhenius curve to determination the activation energy for Lipoxygenase activity of *Pleurotus ostreatus* when use linoleic acid as a substrate.

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