

**Poly aromatic hydrocarbons degradation by lignin peroxidase produced from local isolate *Aspergillus terreus* SG777**

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**Abstract**

Lignin peroxidase produced from *A. terreus* SG777 precipitated with 80% ammonium sulfate saturation then desalting by dialysis against Tris buffer solution and partially purified by ion exchange chromatography using DEAE- cellulose. The specific activity reached 56.67U/mg with purification fold was 5.4 times and 13.7% recovery. The optimum pH for activity and stability was (3). The maximum activity was observed at 40°C and the enzyme maintained the activity when it was incubated at (25-35)°C.

Degradation of poly aromatic hydrocarbons when incubated with 65.51 U/ml lignin peroxidase at (40°C) for 4 hr compared with the Refinery treatment. Results showed (97.54%) removal efficiency of Di benzo (a, h) anthracene by lignin peroxidase, while only (54.37%) was treated by Refinery treatment. Fluorene and anthracene degradation (95.98%) and (83.51%) respectively, while the fluorene and anthracene degradation treated by Refinery treatment only 84.51% and (46.83%) respectively.

The aim of this study was degradation of poly aromatic hydrocarbons by using partial purification lignin peroxidase.

**Keyword** Environment and Safety in Oil Industries, *Aspergillus*, lignin peroxidase, poly aromatic hydrocarbons

**Introduction**

A chief hazard for the environment and in many cases its poly cyclic aromatic hydrocarbons; they comprise risk to human and animal health. Poly cyclic aromatic hydrocarbons have caused a significant concern due to their toxicity and carcinogenicity. Therefore the increased consumption of fossil fuels, their incidence in the environment has gradually increased since last (10 –150) years [1].

Poly cyclic aromatic hydrocarbons anthropogenic source are becoming more significant with increasing industrial processes like petroleum and carbon manufactures, wood treatment plants, and many others activities in which burning of fuels or organic materials is included. The maximum PAHs levels have been so far detected in petroleum gasification sites [2].

The oxidation of PAHs is catalyzed by lignin peroxidase which have an important role in the first attack on high molecular weight PAHs, contains at least four benzene rings in soil [3].

One of the most important ligninolytic enzymes is lignin peroxidase glycosylated, heme containing enzymes which functionally need  $H_2O_2$  for the oxidation of lignin associated aromatic structures [4]. Numerous reports have exposed that LiP from *Phanerochaete chrysosporium* is directly implicated in the degradation of different xenobiotic compounds and dyes [5].

The aim of this study to degradation poly aromatic hydrocarbons by lignin peroxidase which produce from locale isolate *Aspergillus terreus* SG777 with high efficient and short time compared when we use the microorganism itself.

## **Methods**

### **1. Lignocellulosic Substrate**

Lignocellulosic agro-industrial wastes such as Banana stalks and Corn stover were obtained from some farms in Baghdad. Another substrate such as Wheat and Rice were obtained from market respectively and Bush horse which contains (Soybean, Corn, kernels and Barley) obtained from Equestrian Club in Jadriya – Baghdad. The substrates were crushed into small pieces, oven dried ( $50^\circ C$ ), and ground to 40 mm mesh particle size and stored in air-tight plastic jars.

### **2. Inoculum preparation**

A pure culture of the indigenous strain *A.terreus* SG-777 which produces all extracellular ligninolytic enzymes, isolated from horse manure was used for the present study [6]. Inoculums were prepared by growing the fungus in Kirk's basal salt medium [7]. Hundred ml of media it was added in Erlenmeyer flasks (500ml) .The medium was supplemented with Millipore filtered ( $0.22\mu m$ ) glucose (0.5%). Spores of *A.terreus* S.G-777 added to the media and incubation at ( $30^\circ C$ ) for (5 to 10) days to obtain homogenous spore suspensions of fungi ( $1 \times 10^6$  spores / ml) to use as inoculum [8].

### **3. Enzyme production**

kirk's basal medium modified by using (5g) from Bush horses consist of (Soybean , Corn, kernels and Barley) as a substrate instead of glucose , the substrate moistened by Kirk's basal salt medium . All the flasks were autoclaved and inoculated with (4ml) ( $1 \times 10^5$  spore/ml) homogenous suspension of strain *A.terreus* SG-777. The inoculated flasks were incubated at ( $30^\circ C$ ) for 10 days. Culture flasks were harvested by (100ml) of (100mM) sodium succinate buffer, the flasks were shaken (120 rpm) for 30min. The mixture was filtered through a Watman No.1 filter paper and then centrifuged at ( $3000 \times g$ ) for (10min). The clear supernatants were keep at ( $4^\circ C$ ) to determine the activities of LiP, MnP and lacc [9].

#### 4. Lignin peroxidase activity

Lip assay was performed by using (2.6ml) reaction mixture containing 1ml buffer of pH 3, (1ml) of (4mM) veratryl alcohol (3,4 -dimethoxybenzylalcohol), (500 $\mu$ L) of the (1mM) H<sub>2</sub>O<sub>2</sub> , and (100 $\mu$ L) of the crude enzyme .A blank contained 100  $\mu$ L of distilled water instead of crude enzyme . The absorbance was read after 10 min. reaction interval at (310nm) ( $E_{310} = 9300M^{-1}cm^{-1}$ ). Enzyme activity unit was defined as  $\mu$ M of veratraldehyde formed veratryl alcohol per min. [10].

Lignin peroxidase enzyme activity was expressed as U/ml .An international unit IU (or U) is defined as the amount of enzyme, which catalyzed the transformation of 1 micromole of substrate per minute under standard conditions. This was calculated using the formula [11]:

$$\text{Enzyme Activity (U/ml)} = (A \times V) / (t \times \epsilon \times v)$$

Where :

A= Absorbance at corresponding wavelength

V= Total volume of reaction mixture (ml)

v= enzyme volume (ml)

t= Incubation time (min.)

$\epsilon$ = Corresponding Extinction coefficient ( $M^{-1} cm^{-1}$ ).

#### Protein concentration

The protein concentration was estimated according to Lowry method [12].

#### 5. Optimization of lignin peroxidase enzyme production

To further enhance the lignin peroxidase enzyme production capability of fungi, different nutritional substrate and different physical parameters (incubation time, moisture level, inoculum size, pH and temperature) were optimized according method [13].

#### 6. Purification of lignin peroxidase

Under optimum condition the Lip which produce from selected isolate *A. terreus* SG-777 was purification by following steps were carried out.

- 1- Lignin peroxidase produced from *A. terreus* SG777 precipitated with (80%) ammonium sulfate saturation.
- 2- Desalting by dialysis against Tris buffer solution.
- 3- Partially purified by ion exchange chromatography using DEAE- cellulose.

#### 7. Degradation poly aromatic hydrocarbons (PAHs)

**Sample collected:** Two samples (10 l) as polluted water with PAHs were collected from remnants of AL- Dora Refinery /Baghdad. The samples types included (first sample, before entering the

processing unit and the second sample after mechanical, physical chemical and biological treatment).

**Extraction of PAHs from water:**The extraction of PAHs was done according to [14] as following: Five hindered milliliters from the first sample (before entering the processing unit ) was taken in separator funnel and extracted by equal volume from n- hexane by vigorous shaking for 3 min. The organic phase was approved through anhydrous sodium sulphate to eliminate traces of water contents.

The extract volume were concentrated by evaporated at room temperature overnight to near dryness , then added 1 ml acetonitrile as another solvent . Sample was analytic by using High performance liquid chromatography (HPLC) technique under condition [Column Reprisil 100, C18 dimension (25 x0.46) cm , mobile face (Acetonitrile – water) flow rate (1.2 ml/min.), column temperature (30 C°) and wave length (245nm)] [15].

The latest sample was after biological treatment extract and analysis under same condition to compare with the result of treatment by enzyme using in this study.

**Degradation PAHs by lignin peroxidase:** Degradation of PAHs which founded in water sample bringing from remnants AL- Dora Refinery /Baghdad conducted by modified the method of [16] were the reaction mixture for sample consist of : 10 ml sodium tartarate buffer (100 mM) , 50 ml water sample, (1ml contain 65.51 U/m) of purified Lip enzyme and 5ml H<sub>2</sub>O<sub>2</sub> (1mM) . A blank contained 1ml of distilled water instead of purified Lip enzyme. Both the sample and blank incubated at (40°C) with shaken at (80rpm) for 4 hr. The extraction and analytic for tow mixtures conducted for results determined.

## **Results**

### **1. Optimization of lignin peroxidase enzyme production**

Lignin peroxidase enzyme production from *A. terreus* SG 777 under optimum conditions (banana stalks ,1:5 w/v moisture level,192 hrs incubation time , inoculums size 1x10<sup>5</sup>, pH 4.5 and 35C°incubation temperature).

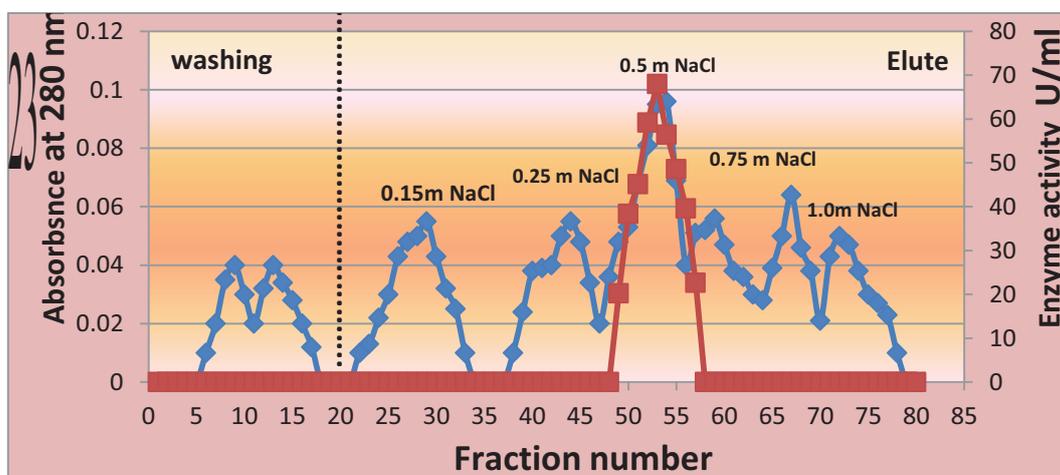
### **2. Purification of lignin peroxidase**

Lignin peroxidase from *A. terreus* SG 777 produced at optimum conditions, supernatant was collected from the medium, which gave specific activity 10.45 U/mg and concentrated by salt using 80%ammonium sulfate saturation. And then enzyme solution was desalting by using the dialysis tube against Tris buffer .The results showed the increases of the specific activity of enzyme reached to 23.88 U/mg and purification fold was 2.29 with 43.4 % yield (table 1).

The enzyme solution after concentration step was passed through the DEAE-Cellulose ion exchange column that already equilibrated with the Tris-HCl buffer (10 mM, pH=7.5). Absorbance is

measured for washing parts (positively charged proteins) at the wave length of 280 nm. When the arrival of the absorbance of the line of zero, binding protein (negative proteins) were eluted with (Tris – HCl buffer (10 mM), pH=7.5 supplement with Nacl (0.15-1) M).

The results in figure (1) showed two peaks of protein appeared in the wash step and no one of them showed enzyme activity. Six protein peaks appeared in the elution step and only one peak of them showed lignin peroxidase activity concentrated in fraction parts (50-58) eluted by 0.5M sodium chloride. Fractions which have enzyme activity collected and concentrated. The specific activity of enzyme reached (56.67U/mg) with (5.4) purification fold and enzyme yield (13.7%) (table 1).



**Fig. (1) Ion exchange chromatography for lignin peroxidase purification from *A.terreus* SG-777 isolate by using DEAE-cellulose column (2.5×16cm) equilibrated with Tris-HCl buffer (10 mM ,pH 7.5 ), eluted with Tris –HCl buffer with NaCl (0.15 -1 M) in flow rate 0.8 ml /min., 4ml for each fraction.**

**Table (1): The purification steps of lignin peroxidase from *A. terreus* SG777 isolate.**

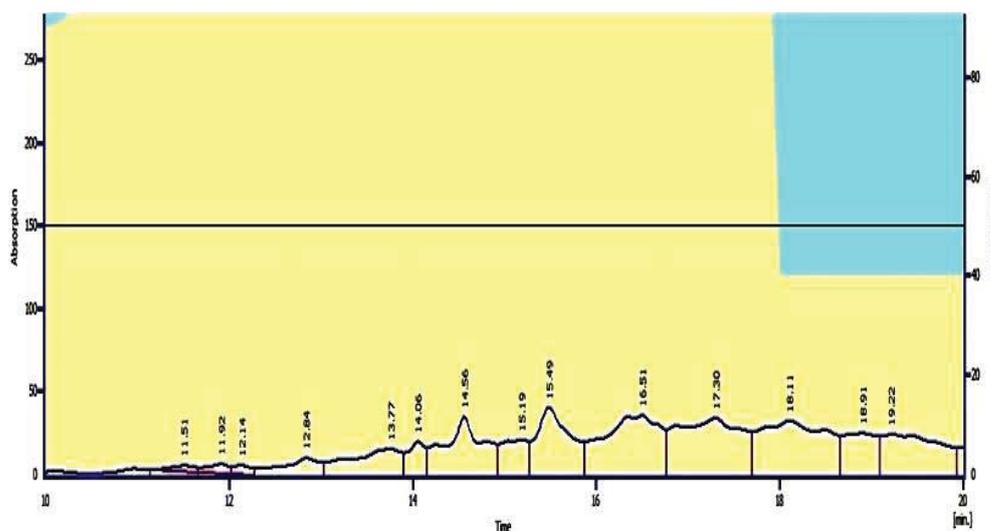
Purification step	Volume (ml)	Enzyme activity (U/ml)	Protein concentration (mg/ml)	Specific activity (U/mg)	Total activity (U)	Purification fold	Yield %
Crude enzyme	50	99.35	9.5	10.45	4967.5	1	100
Precipitation with 80% saturation of (NH <sub>4</sub> )SO <sub>4</sub> And desalting by dialysis	12	179.8	7.53	23.88	2157.6	2.29	43.4
Ion exchange chromatography (DEAE-Cellulose) and enzyme concentration by sucrose.	10	68	1.2	56.67	680	5.4	13.7

### 3 .Poly aromatic hydrocarbons degradation

**Detection of PAHs in water:** The results in the table (2) and figure (2) showed six types of poly aromatic hydrocarbons compound include (Naphthalene, Fluorene, Anthracene, Pyrene, Chrysene and Dibenzo (a, h) anthracene) depending on the retention time for each one on the water sample from remnants of AL- Dora Refinery.

**Table (2) Poly aromatic hydrocarbons detection in water sample by RP-HPLC.**

No.	PAH	R <sub>t</sub> value (min)
1	Naphthalene	12.16
2	Fluorene	13.33
3	Anthracene	13.81
4	Pyrene	14.49
5	Chrysene	14.97
6	Dibenzo(a,h)anthracene	16.24

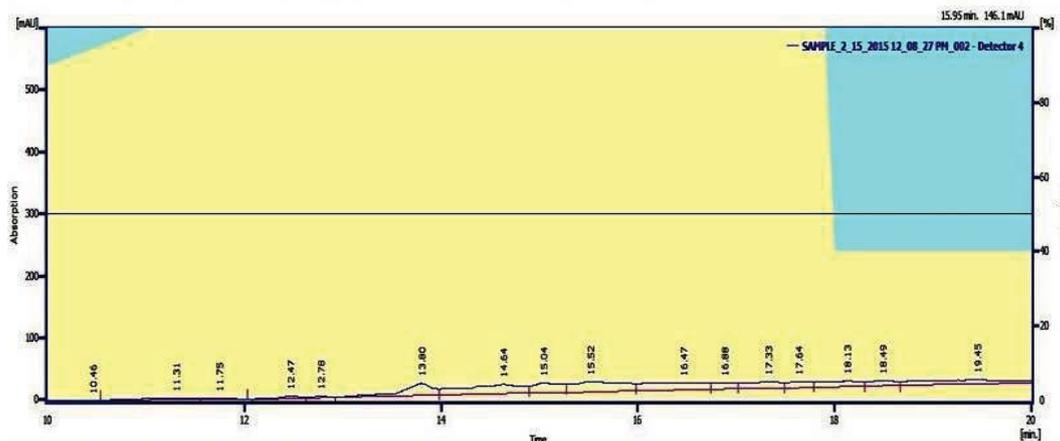


**Fig. (2) Chromatogram for mix PAHs detection on the water sample from remnants of AL- Dora Refinery.**

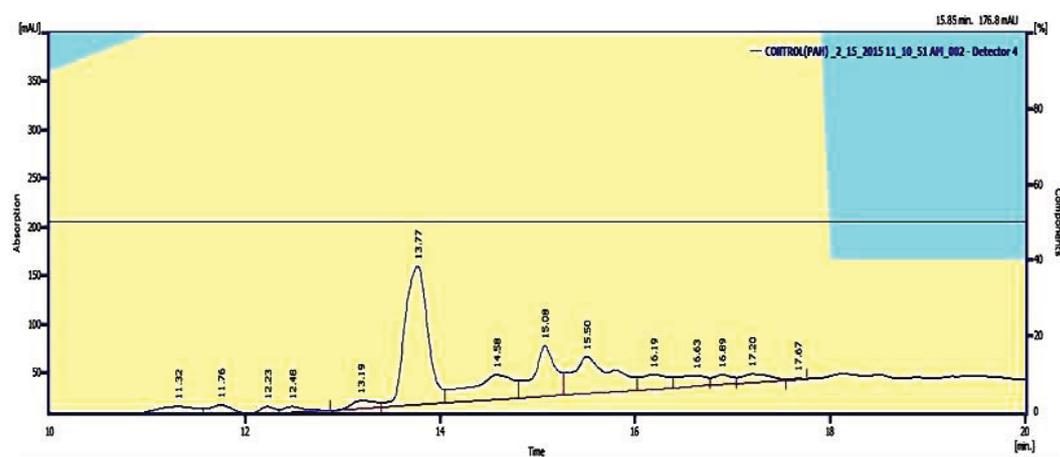
**Degradation PAHs by lignin peroxidase:** After confirming the existence of six aromatic compounds in water samples tested, they treated by adding lignin peroxidase. According to the decrease in peak area for each PAHs, results in the table (3) and figure (3) showed the degradation PAHs when incubated with (65.51U/m) lignin peroxidase at (40°C) for 4 hrs compared with control figure (4) and the Refinery treatment figure (5), which showed low decrease in peak area for PAHs presence.

**Table (3) :Comparison between enzyme treatment and refinery treatment of PAHs component in water samples.**

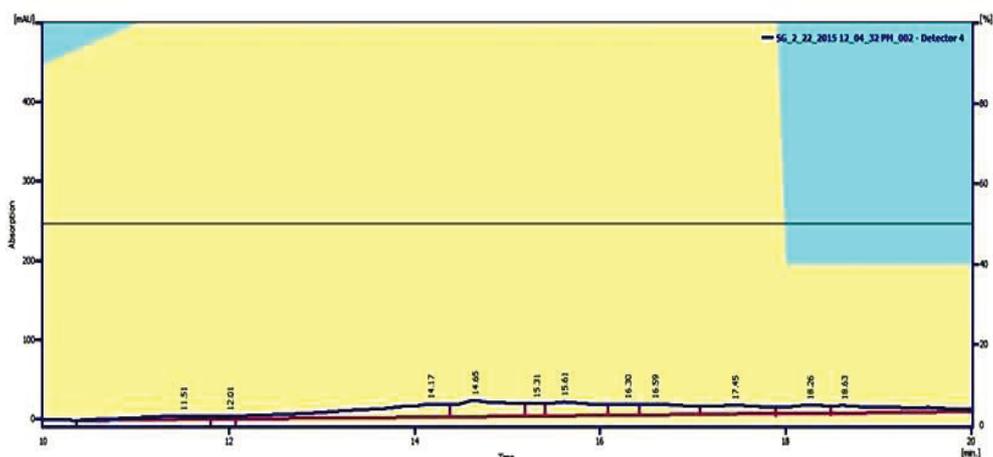
PAHs	Rt (min)	Peak area		
		Control	Enzyme treatment	Refinery treatment
Naphthalene	12.16	66.355	42.582	53.380
Fluorene	13.33	138.764	23.136	35.122
Anthracene	13.81	2332.396	416.178	1258.333
Pyrene	14.49	817.534	638.840	804.893
Chrysene	14.97	814.362	326.436	200.467
Dibenzo(a,h) anthracene	16.24	292.839	0.000	275.998



**Fig. (3) Chromatogram for poly aromatic hydrocarbons degradation by lignin peroxidase for 4hrs.**



**Fig. (4) Chromatogram for control sample contains the PAHs without lignin peroxidase.**



**Fig. (5) Chromatogram for PAHs after Refinery treatment of water sample.**

Di benzo (a, h) anthracene were degraded completely (100%) by lignin peroxidase, while only (5.75%) was reduced by Refinery treatment. Fluorene and anthracene were degraded about (83.33%) and (82.16%) respectively, while the fluorene and anthracene degradation when treated by Refinery treatment were (74.7%) and (46%) respectively.

Naphthalene and pyrene degradation were (35.83%) and (21,86%) respectively and there are also highest degradation compared with Refinery treatment were (19.54%) and (1.55%) respectively .Only chrysene was lower degradation by lignin peroxidase (59.92%) than the degradation by Refinery treatment (75.38%) as seen in the table (4).

From these results observed the Lip enzyme for PAHs degradation more efficiencies than Refinery treatment which use the microorganism in biological treatment.

**Table (4) Poly aromatic hydrocarbons degradation by lignin peroxidase and Refinery treatment after four hour.**

PAHs	Poly aromatic degradation % after 4 hours by	
	Lignin peroxidase	Refinery treatment
Naphthalene	35.83	19.55
Fluorene	83.33	74.7
Anthracene	82.16	46
Pyrene	21.86	1.55
Chrysene	59.92	75.38
Dibenzo(a,h) Anthracene	100	5.7

### Feasibility

- 1-The cost treatment by investigated enzyme is less than current treatment used in the refinery .
- 2-Decrease the time of degradation.
- 3-Reduce the methods of treatment.
- 4-Decrease of energy consumption.
- 5-Prevent the contamination with microorganisms.

### Conclusion

- Lignin peroxidase was extracted and concentrated by ammonium sulfite, then purified by ion exchange using DEAE - cellulose (Column chromatography).
- Optimum temperature for LiP activity was at 40°C, and the enzyme was stable between 25 - 35°C after 30 min . Whereas the optimum pH for LiP activity and stability was 3 at 40°C.
- Purified LiP was more efficient for dyes removal than crude LiP or H<sub>2</sub>O<sub>2</sub>.
- Purified LiP exhibited higher removal efficiency for poly aromatic hydrocarbons in comparison with to treatment in AL-Dorah Refinery.

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