ISSN: 0067-2904

Biodegradation of Naphthalene by *Aspergillus flavus* Before and After Exposing to UV Light

Duha Bahaa Mohammed Al Fayaad[1], Mohammed A.Al-A'adhmi[1], Yaaroub Faleh Al Fatlawy[2*]

[1]Ministry of Science and Technology, Baghdad, Iraq
[2]College of Science, University of Baghdad, Baghdad, Iraq

Abstract

Aspergillus flavus was tested for its ability to degrade naphthalene by using solid mineral salts medium (SMS) with different concentrations 100, 300, 500 ppm of naphthalene. Results showed that 100ppm was the best concentration consumed by the fungal test then 300ppm and 500ppm the results for secondary test by using Liquid Mineral Salts Medium (LMSM) 95% of degradation for 100ppm then75% for 300ppm and 30% of degradation for 500ppm then the fungal test was tested for its ability to produce lignolytic enzymes results revealed that lignin peroxidase enzyme was only produced .then fungal test exposed to U.V light and the result showed after 10 minutes of U.V light exposure the degradation ratio were 91% for 100ppm then 79% for 300ppm and 73% for 500ppm.

After 20 minutes of U.V light exposure the results showed 93% for 100ppm, 84% for 300ppm and 80% for 500ppm.

Keywords: Biodegradation, Naphthalene, *Aspergillus flavus* , U.V. Light

التفكك الحيوي للنفثالين بواسطة فطر *Aspergillus flavus* قبل وبعد التعرض للاشعة فوق البنفسجية

ضحى بهاء محمد الفياض[1]، محمد عبدالوهاب الاعظمي[1]، يعرب فالح الفتلاوي[2*]

[1]وزارة العلوم والتكنلوجيا، بغداد، العراق
[2] كلية العلوم، جامعة بغداد، بغداد، العراق

الخلاصة

تم اختبار الفطر *Aspergillus flavus* في قدرته على تحلل النفثالين باستخدام وسط الأملاح المعدنية الصلبة (SMS) بتراكيز مختلفة 100، 300 ، 500 جزءبالمليون من النفتالين .وأظهرت النتائج أن تركيز 100 جزء بالمليون كان أفضل تركيز يستهلكها الفطر المختبري من 300 جزء بالمليون و 500 جزء بالمليون بينما اظهرت نتائج الاختبار الثانوي باستخدام وسط الاملاح المعدنية السائل (LMSM) 95٪ من التحلل لل 100 جزء بالمليون ثم75 ٪ لل 300 جزء بالمليون و30 ٪ من التحلل 500 للجزء بالمليون بعدها تم اختبار الفطر لقدرته على انتاج الانزيمات المفككة للكنين.

أظهرت النتائج أن إنزيم lip هو الانزيم الوحيد الذي انتج واستخلص في هذه التجربة .ثمتم تعريض الفطر لضوء الأشعة فوق البنفسجية وأظهرت النتيجة بعد 10 دقيقة من التعرض لضوء الأشعة فوق البنفسجية 91٪ لل 100 جزء بالمليون ثم 79 ٪ لـ 300 جزء بالمليون و73 ٪ لل .500 جزء بالمليون وبعد 20 دقيقة من التعرض للضوء U.V أظهرت نتائج التحلل 93 ٪ لل100 جزء بالمليون ،84 ٪ لل 300 جزء بالمليون و 80٪ لل 500 جزء بالمليون.

*Email:Dr.yaaroub@Yahoo.com

Introduction

Filamentous fungi play an important role in degradation and detoxification of polycyclic hydrocarbons, including condensed aromatic ring systems as well as other xenobiotic compounds, present in polluted environments. Some of these compounds are very harmful and carcinogenic [1].

Filamentous fungi have been reported to colonize the solid substrate and tolerate high concentration of toxic compounds [2]. Species belonging to the genera *Trichoderma harizianum* , *Fusarium avaneceum ,Penicillium chrysogenum, , Aspergillus niger* and *Aspergillus flavus* are some examples of this kind of fungi that have been recently described as tolerant to pollutants such as B(a)Pyrene, anthracene, naphthalene [3].

Naphthalene is a volatile organic compound (VOC) with $C_{10}H_8$ that is a widespread atmospheric contaminant .It is the simplest polycyclic aromatic hydrocarbon.Ultra violet light
has been shown to be lethal and mutagenic in a variety of organisms, including fungi. The correlation between the quantity of energy absorbed by DNA and the observed biological effects (survival and mutation frequency) are illustrated in the wavelength region between 254 and 320 nm. U.V. irradiation was found to be best for the improvement of strains like *Aspergillus niger* for maximum production of various enzymes [4]. In recent years, attempts have been made for the overproduction of microbial enzymes by induced mutagenesis. Suntornsuk and Hang [5] have reported that the strain improvement in *Rhizopus oryzae* by UV resulted in the production of more glucoamylase by a mutant than the parent strain. Mutational experiments were performed to produce morphological mutants from *Aspergillus wentii* by UV and X ray irradiation. The purpose of the present investigation is to know whether an selected fungal strain was still active in degradation between wild and UV mutants after exposing it to UV light.

Materials and methods

Testing of the isolate for naphthalene biodegradation

Primary Test

Fungal isolate was tested for its ability to degrade naphthalene using solid mineral salts medium and 0.1 ml from naphthalene solution containing (100,300,500ppm) was spreader on solid medium agar plates then methanol evaporated by left plates for 1 hr inside sterile hood, white thin layer formed, then the plates were inoculated with fungal disk 7mm from7 days old culture of fungal isolate. The plates were incubated at 28C^o for 7 days to allow growth of test fungi. They were then examined for their growth formation around the fungal test. The growth diameter was measured in each case. [6].

Good: Radial growth rate (50-80mm)
Moderat: Radial growth rate (20-49mm)
Weak: Radial growth rate (1-19mm)

Secondary test

The fungal isolate was grown in 100 ml Erlenmeyer flasks containing 25 ml liquid mineral salts medium, pH was adjusted to 7, then autoclaved at 121^{0}C for 15 min, the sterilized media were inoculated with fungal disk (7mm)of *A. flavus* from7 days old culture of fungal isolate with 100,300,500ppm naphthalene separately. Duplicate for each concentrate of isolate and control, then flasks were incubated in shaker incubator 130rpm for 7days at 30^{0}C [7] and naphthalene residue was determined.

Testing of the isolate for lignolytic enzymes production

Czapek-Dox agar medium was employed for the screening of lignolytic enzymes activities. 0.0025% azure B (w/v) and 0.0025% phenol red (w/v) were used to detect lignin peroxidase [8] and manganese peroxidase [9], respectively. Laccase activity was tested by adding 0.05% (w/v) guaiacol to Czapek's Medium [10]. Fungal discs (7 mm) were taken from the periphery of 7- day old cultures grown on Czapek-Dox agar plates. Discs were inoculated onto triplicate plates containing the screening media. Plates were incubated at 30°C for 7 days in a static incubator. The colored zone produced from chromogenic substrate metabolism was noticed.

- Oxidation of phenol red forms green zones.
- Oxidation of azure B forms yellow zones.
- Oxidative polymerization of guaiacol forms reddish brown zones.

Determination of naphthalene concentration by HPLC

After incubation, the flasks were removed and the cultures centrifuged at 10000 rpm for 20 min, then filtered through whatman no.1, 10ml of hexane was added to 25 ml of mineral medium and was shaken for 30 min in separate funnel then 1ml of upper phase (hexane) was shifted to the sterile tubes and was used for HPLC analysis [11]. HPLC analyses were performed with reverse-phase column C18 (Syknm Chromatography Products, Germany). Separation was achieved by isocratic elution in acetonitrile: water (70:30) respectively, with a flow rate of 1.0 ml/min and UV absorbance detector set at 279 nm.

Biodegradation% = initial concentration of naphthalene **-** naphthalene concentration after incubation periods **/** initial concentration of naphthalene *100 [9].

Fungal inoculums preparation

Inoculums suspension of selected fungal isolate was prepared from periphery 7 days old culture grown on PDA slants, the colonies was covered with 5 ml of Tween 20 (5%). The inoculum were achieved by carefully rubbing the colonies with a sterile loop, the slants were then shaken vigorously for 15 min with a vortex and then transferred to a new sterile tube then the inoculum was transferred to a sterile syringe attached to a sterile filter Millipore 0.22µm .The suspension was filtered and collected in a sterile tube.

This procedure removed the majority of the hyphae producing inoculums mainly composed of spores [12].

UV Mutagenesis

The best natural selectant *Aspergillus flavus* was grown on potato dextrose agar (PDA) medium for 7 days at 30°C. Fungal spore suspensions were distributed into sterilized petri plates (2 ml in each plate).

They were exposure to UV light in a "Dispensing – Cabinet" fitted with TUP 40w Germicidal lamp which has about 90% of its radiation at 2540-2550 .Optimum dose required to get maximum mutants was arrived by exposing the organism for different periods of time (10 and 20min) at the distance of 20 cm from the UV source. Each UV exposed spore suspension was stored in dark overnight to avoid photo reactivation.

UV treated fungal spore suspensions of 0.1 ml was inoculated into petri plate containing potato dextrose agar medium. The plates were incubated for 7 days at 30°C. Then developed fungal strains whose survival rate was less than 1%.and tested for degradable enzymes production [13].

Results and Discussion

Testing of the isolate for naphthalene biodegradation befor and after exposing to U.V.light

Primary Test

The disappearance of Naphthalene from the solid MSM, suggested being consumed by the fungal isolate, but no growth on this medium was observed by survival of a white thin layer of naphthalene on the surfaces of the dishes indicating the inability of the isolate to consume this compound. This may be attributed to the reason that fungal colony are not growing on this medium because they do not possess the ability to degrade this compound as a result of lack specialist enzymatic system, or due to the absence of metabolic capacity .Other reason could be the low solubility of this compound which reduce the availability to microorganism and then cannot be attacked. [14].

Identical study done by [15] found failure of fungal isolates to grow on solid MSM with anthracene which attributed to the degradation of compound containing more than one cycle and more resistant to oxidative enzymes Figure-1.

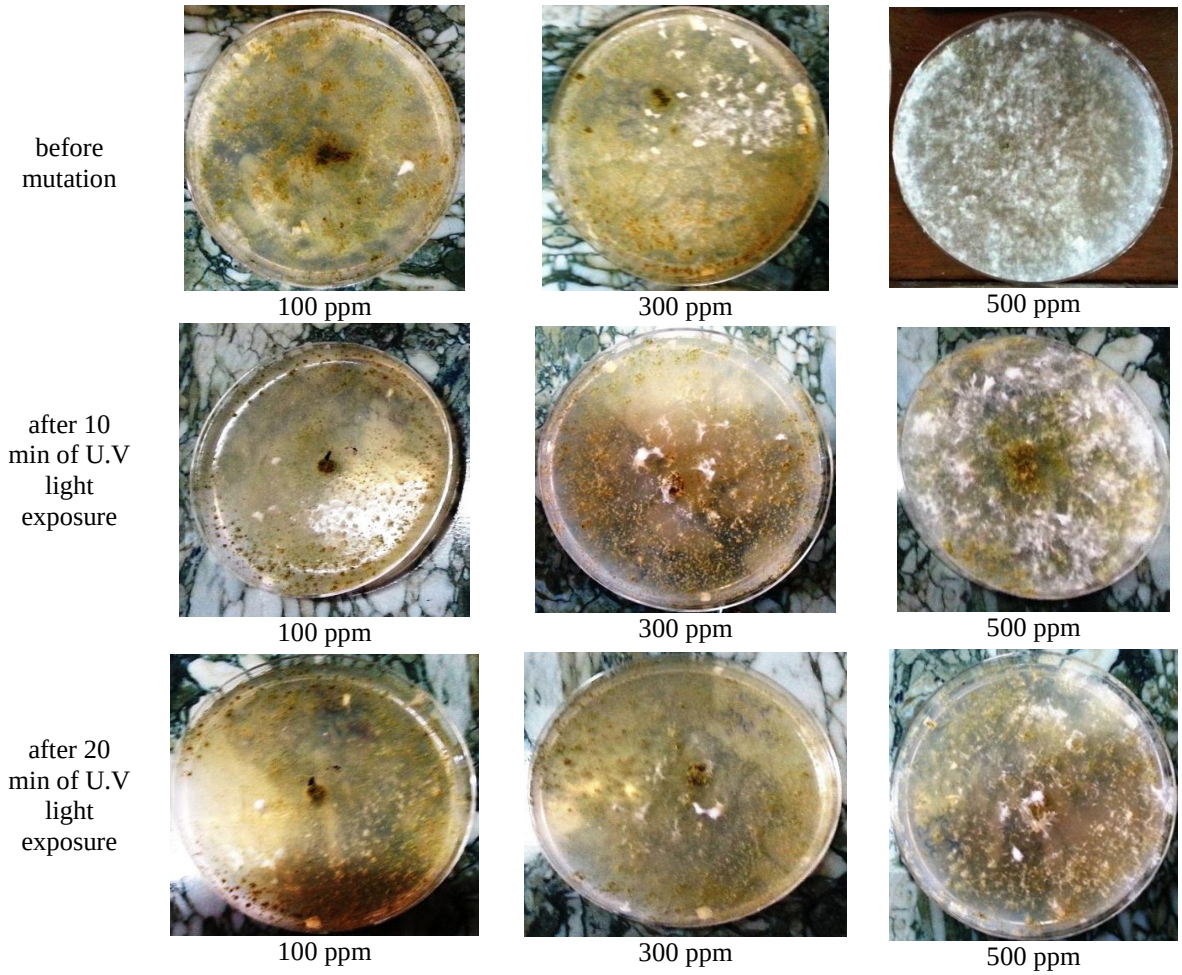


Figure 1-The ability of fungal isolate growth on solid MSM ,pH 7 containing pure naphthalene 100,300,500ppm after incubation period of 7 days at 30^0C.

Secondary Test

The results showed variation in the capacity of the fungal isolate *Aspergillus flavus* in consumption of naphthalene in the liquid mineral salts media**.** In normal state before mutation the degradation occur in 100 and 300 ppm but in 500ppm results not well. After exposing to U.V. light the degradation increase in all concentrations especially after 20 min.

Earlier study with *A. niger* in which increased time of UV exposure (>20 minutes) resulted in decreased alpha amylase production [16, 17].

Other studied showed that *A. fumigatus* loose its ability to lip enzyme production after 30 min of U.V light exposure [18] Table-1.

Table 1- Naphthalene biodegradation by *Aspergillus flavus* in liquid MSM with 100,300,500ppm naphthalene, pH 7 and incubated for 7 days, 120 rpm at 30^0C

S.no.	percentage of biodegradation %			
	concentration(ppm)	100	300	500
1	control	6.1	5.4	6.4
2	*Aspergillus flavus* before mutation	95	75	30
3				
4	after 10 min of U.V light exposure	91	79	73
5	after 20 min of U.V light exposure	93	84	80

Testing of the isolate for lignolytic enzymes production

In the current study, it was found that fungal isolate *Aspergillus flavus* befor and after exposing to U.V.light could produce degradable enzyme for naphthalene on solid media**.**Oxidation of azure B to form yellow zones indicating Lip enzyme production**,** Oxidation of phenol red to form green zones

indicating Mnp enzyme production and oxidative polymerization of guaiacol to form reddish brown zones indicating Lac enzyme production, these last two enzymes never showed in tested fungal isolate Figure-2. In this figure also showed that after exposed to U.V.light in 20 min the diameter of yellow zone increase which mean increase secretion of degradable enzyme Lip.

These results agreed with the study of [19] that used *Aspergillus terreus* and other fungal isolates to biodegradate of some polycyclic aromatic hydrocarbons when fungal isolates grew on solid media containing phenol red, azure B and guaiacol separately. Color removal by filamentous fungi has been attributed to be mainly due to biosorption to the mycelium [20].

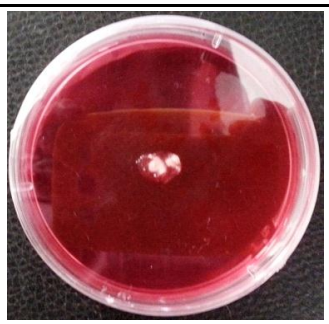
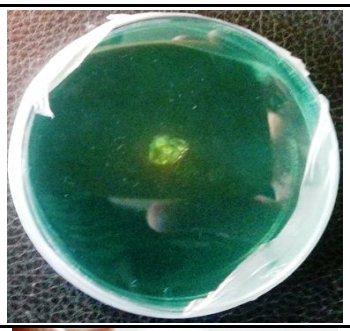
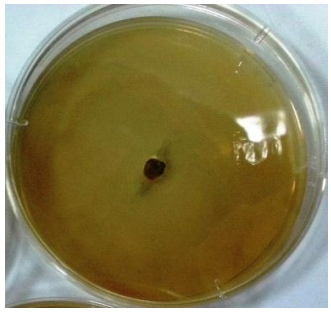
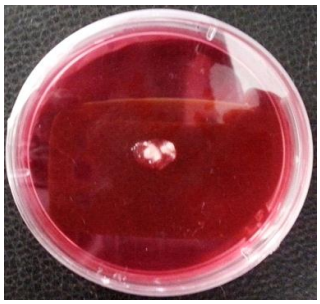
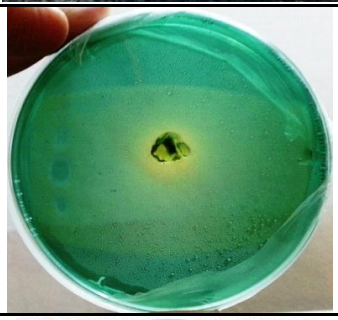
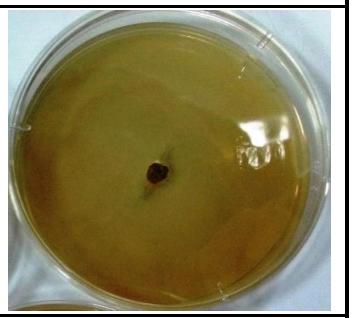
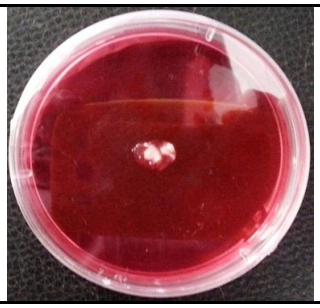
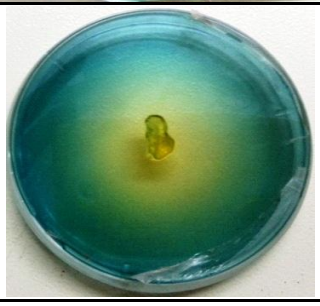
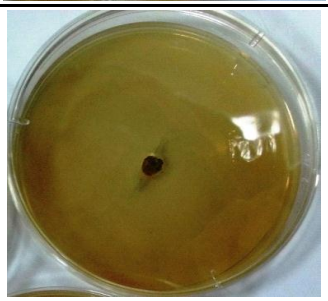
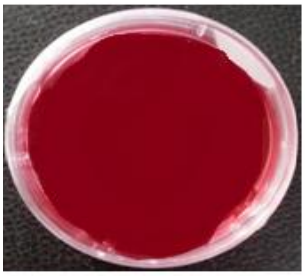
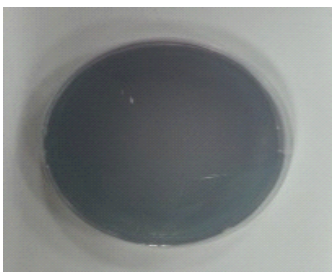
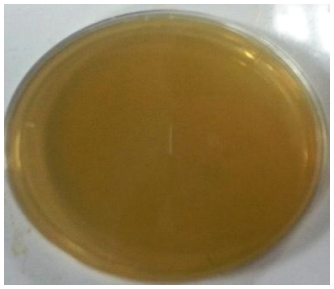
Fungal isolate	**Lignolytic enzymes production**		
	A	**B**	**C**
Lignolytic enzymes productions ***Aspergillus flavus*** **before mutation**			
after 10 min of U.V light exposure			
after 20 min of U.V light exposure			
Control			

Figure 2-Ligninolytic enzymes production on solid media by *Aspergillus flavus* before and after mutation when incubation at 28^0C for 7 days. A: no Mnp production. B: Lip production by oxidation of Azure B to form yellow zone. C: no Lac production.

References

1. D'Annibale A, Rosetto F, Leonardi V, Federici F, Petruccioli M **2006**.Role of autochthonous filamentous fungi in bioremediation of a soil historically contaminated with aromatic hydrocarbons. *Appl. Environ. Microbiol.*, 72(1), pp: 28-36.

2. Li X, Lin X, Yin R, Wu Y, Chu H, Zeng J, and Yang T. **2010**. Optimization of laccase-mediated benzo[a]pyrene oxidation and the bioremedial application in aged polycyclic aromatic hydrocarbons-contaminated soil. *J Health Sci*., 56, pp:534–540.

3. Tigini, V., Prigione V., Di, Toro S., Fava, F., and Varese, G.C. **2009**. Isolation and characterization of polychlorinated biphenyl (PCB) degrading fungi from a historically contaminated soil. *Mycology Journal*, 8, p:5.

4. Kang,. S.W, Ko E.H, Lee J.S and Kim S.W. **1999**.Overproduction of beta-glucosidase by *Aspergillus nigher* mutant from lignoocellluosic biomass. *Biotechnol. Lett*., 21, pp:647-650.

5. Suntornsuk W and Hang YD **2008** Strain improvement of *Rhizopus oryzae* for production of l (+)-lactic acid and glucoamylase. *Lett. Appl.Microbiol*., 19, pp:249–252.

6. Anupama M. and Padma S. **2009** Studies on biodegradaction of crude oil by Aspergillus niger, S. *The South Pacific Journal of Natural Science*, 27, pp:57-60.

7. Bartha, R. **1986**. Biotechnology of petroleum pollutant biodegradation. *Microbial Ecology*. 12, pp: 155-172.

8. Archibald F. and Roy B. **1992**. Production of manganic chelates by laccase from the lignin-degrading fungus Trametes (Coriolus) versicolor. *Appl. Environ. Microbiol*., 58, pp: 1496–1499.

9. Kuwahara, M., Glenn, J., Morgan, M. and Gold, M**. 1984**.Separation and characterization of two extracellular H2O-dependent oxidases from lignolytic cultures of *Phanerochaete chrysosporium*. *FEBS Lett*., 169, pp: 247–250.

10. Kiiskinen L. and Saloheimo M. **2004**. Molecular cloning and expression in Saccharomyces cerevisiae of a laccase gene from the Ascomycete Melanocarpus albomyces. *Appl. Environ. Microbiol*., 70, pp: 137-144.

11. Loser, C., Seidel, H., Zehnsdarf, A. and Stoltmeister, U. **1998**. Microbial Degradation of Hydrocarbons in Soil During Aerobic/anaerobic Changes and Under Purely Aerobic Conditions. *Appl. Microbiol. Biotechnol*., 49, p:631.

12. Eva.P, and Emillia.M. **2001**. Inoculum Standardization for Antifungal Susceptibility Testing of Filamentous Fungi Pathogenic for Humans. *Clin Microbiol*. 39(4), pp: 1345–1347**.**

13. Amany Z. and Abd El-Aziz B. **2013**. Improvement of Kojic Acid Production by a Mutant strain of *Aspergillus flavus* Microbiology Department, National Center for Radiation Research and Technology, Atomic Energy Authority, Nasr City, Cairo, Egypt. *Journal of Natural Sciences Research*., 3(4), pp:31-41

14. Mollea, C., Bosco, F. and Ruggeri, B. **2005**. Fungal biodegradation of naphthalene: *Microcosms studies, Chemosphere*, 60, pp: 636–643.

15. Toledo, F. L., Calvo, C., Rodelas, B. and Lopez, L.G. **2006**. Selection and identification of bacteria-fungi isolated from waste crude oil with polycyclic aromatic hydrocarbons removal capacities. *Systematic and Appli. Microbial*., 29, pp: 244-252.

16. Varalakshmi KN, Kumudini BS, Nandhini BN,Solomon J, Suhas R,Mahesh B and Kavitha AP **2009** Production and characterization of alpha amylase from *Aspergillus niger* JGI 24 isolated in Bangalore. *Polish J. Microbiol*. 58, pp:29-36.

17. Prabakaran M., Thennarasu V. and Ayeswariya Mangala R. **2009**. Comparative studies on the enzyme activities of wild and mutant fungal strains isolated from sugarcane field Department of Zoology, K.N.G. Arts College for Women (Autonomous), Centre for Advanced Studies in Botany, University of Madras, Guindy Campus,*Indian Journal of Science and Technology*, 2(11).

18. Viniegra G., Favela T., Aguilar N. and Romerofoonez J. **2003**. Advantages of fungal enzyme production in solid state over liquid fermentation systems. *Biochem. Eng. J*., 13, pp:157-167.

19. Ali I., Neveen M. K and Mohamed N. A., **2012**. Biodegradation of some polycyclic aromatic hydrocarbons by *Aspergillus terreus*. Department of Botany, Faculty of Science, *Journal of Cairo University, Egypt*., 6(16), pp: 3783-3790.

20. Yang Q.Y, Yang M., Pritsch K, Yediler A.,Hagn A., Scloter M and Kettrup A **2003**.Decolorization of synthetic dyes and production of manganese-dependent peroxidases by new fungal isolates. *Biotechnol. Lett*., 25, pp:701-713.