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Baghdad Science Journal **Vol.15(1)2018**

DOI: http://dx.doi.org/10.21123/bsj.2018.15.1.0043

Biodegradation of Anthracene Compound by Two Species of Filamentous Fungi

***Duha Bahaa Mohammed*[1]** ***Adnan H. Abbas*[2*]** ***Eman H. Abed*[3]**
***Ahmed M. Abed Ali*[4]**

Received 26/9/2017
Accepted 7/1/2018

Abstract:

Isolation and identification fungi of *Emericella nidulans* and *Aspergillus flavus* from a pinkish and yellowish artificial clay, by using potato dextrose agar (PDA)**.** Results revealed that *E. nidulans* was the best for degrading anthracene (92.3%) with maximum biomass production (3.7gm/l), compared to *A. flavus* with the rate of degradation (89%) and biomass production of (1.2gm/l), when methylene blue was used as redox indicator after incubating in a shaker incubator 120rpm at 30C^{o} for 8days. Results indicated that *E. nidulans* has a high ability of anthracene degradation with the rate of (84%), while *A. flavus* showed the lower level with (77%) by using HPLC.

Keywords: Filamentous fungi, Anthracene biodegradation, *Emericella nidulans, Aspergillus flavus.*

Introduction:

Polycyclic aromatic hydrocarbons (PAHs) are prevalent environmental pollutants. Uptake of anthracene via numerous routes in the body imposes toxicity to the skin, blood, intestine and the lymphatic system, thus leading to tumors, edema, itching and the upsurge of fluids in tissues (1, 2).

Numerous procedures were utilized to expel PAHs, as physical treatment, chemicals and microbial degradation using bacteria, fungi, algae and yeasts (3, 4).

Anthracene is a solidify PAH ($C_{14}H_{10}$), comprising about three combined Benzene rings. It will be utilized to prepare the Red Dye Alizarin and other dyes. Anthracene may be colorless, but display a blue (400-500)nm peak fluorescence under ultraviolet light (5).

Many literatures show that filamentous fungi are playing an important role in removing of PAHs and even more effective than some bacteria. Increasing non-ligninolytic fungi, *Penicillium simplicissmum* and species belonged to the genera *Trichoderma, Fusarium, Penicillium, Stachybotrys, Aspergillus, Cladosporium, Mortierella, Beauveria* and *Engyodontium*, have been recently described as tolerant to pollutants such as PCBs and chlorobenzoic acids (6).

[1, 2, 3, 4] Ministry of Science and Technology, Environment and Water Directorate, Baghdad, Iraq.
[*]Corresponding author: adn927an@gmail.com

Materials and Methods:

The source of fungal isolates

Fungal isolates were isolated from a yellowish and pinkish artificial clay.

Purification of isolated fungi

Fungus isolates were purified by repeat culturing on plates containing PDA medium, till ensuring purity of fungal isolates. Then isolates subsequently cultured on PDA slants and allowed to grow for 5-7days stored at 4°C as stock cultures.

Identification of fungal isolates

A colony of fungal isolates were diagnosed due to their morphological characteristics by utilizing the lactophenol solution, then slides were recognized under the microscope.

Methylene blue (Redox indicator)

Erlenmeyer flasks (Volume 100ml) were used in this experiment containing a suitable amount of mineral salt medium preparing by Ameen *et al*., (2014) with some modification (7), containing 25µl of Tween 80, then volume flasks were completed to 25ml with the same media, (pH 7.0), 15µl of methylene blue as prepared by Wilson and Jones (8) and 100ppm anthracene were added after autoclaved media at 121^{0}C for 15min. Flasks were inoculated with diameter 7mm of selected fungal isolates (One isolate for each flask). Flasks were incubated in a shaker incubator 120rpm at 30C^{o} for

8days. All experiments were done in duplicate and control for each isolate. After the incubation period, growth culture was filtrated to separate biomass, followed by centrifugation at 10000rpm for 15min. Supernatant was analyzed by spectrophotometer at 609nm and the percentage of degradation was calculated by a formula as below (9).

$$\% \text{ of degradation} = 1 - \frac{\text{Absorbance of treated sample}}{\text{Absorbance of control}} \times 100$$

Estimation of anthracene concentration by HPLC

After a period of incubation, the growth was harvested at 10000rpm for 20min and filtered through Whatman No. 1. Add 10ml of hexane to 25ml of supernatant, then mixing for 30min by using separate funnel, then 1ml from the upper phase (Hexane) was transferred to a sterile tube for HPLC analysis, which achieved with reverse-phase column C18 (Syknm Chromatography Products, Germany). Separation was accomplished by isocratic elution in (Acetonitrile: Water) (70:30), with a flow rate 1.0ml/min and UV absorbance detector set at 254nm.

$$\text{Degradation} = \frac{\text{Initial conc. of anthracene - Anthracene conc. after incubation}}{\text{Initial conc. of anthracene}} \times 100$$

Estimation of biomass

Twenty-five ml of growth media was centrifuged with speed 10000rpm at 4^0C for 20min, then supernatant was discarded and biomass washed 2-3 times with distilled water, re-centrifuge under the same conditions. Dry weight was estimated after drying at 80^0C for 24h (10)**.**

Results and Discussion:

Identification of fungal isolates

Morphological characteristics of fungal colonies by using a dissecting microscope, were the initial identification according to their macroscopic and microscopic features. Results showed that most active isolates were identified as *E. nidulans* and *A. flavus* (11) [Fig. 1, 2 (A&B)].

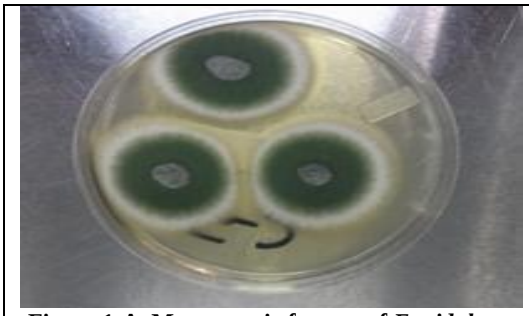

Figure 1-A. Macroscopic feature of *E. nidulans*, after growing on the PDA medium at 28°C for 7days.

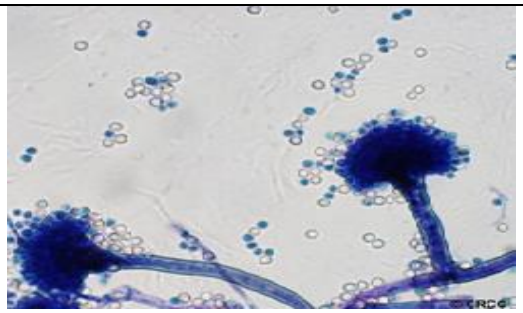

Figure 1-B. Microscopic feature of *E. nidulans*, (Staining with lactophenol solution) showing conidial head (40x).

Figure 2-A. Macroscopic feature of *A. flavus* after growing on the PDA medium at 28°C for 7 days.

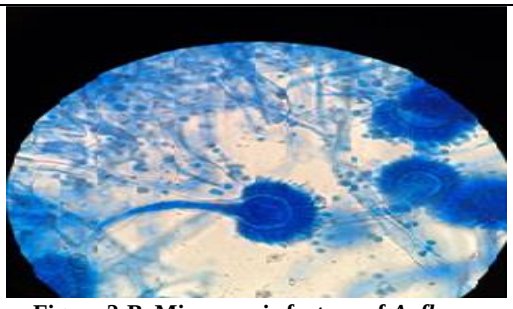

Figure 2-B. Microscopic feature of *A. flavus* (Staining with lactophenol solution) showing conidial head (40x).

Biodegradation of anthracene

The period of incubation was the most important factor that affects degradation processes (12) and the optimum condition for anthracene degradation (100ppm), by *E. nidulans* and *A. flavus* isolated

from artificial clay, were incubated in a shaker incubator 120rpm at 30C^{o} for 8days.

The result showed that increasing concentration of a water soluble synthetic compound (Tween 80) improved the biodegradation and bioremediation of toxic pollutants. Muthusamy *et al.*, (13), just as (1, 2.5 and 5) gm/l in the media create gradual increasing in decane biodegradation (55.0, 74.5 and 79.3)%, respectively, at 20°C. Whereas 10gm/l of tween 80 caused decreases in biodegradation (57.2)% compared to 5gm/l (79.3)% at the same degree.

We can be inferred that low nutrients, low pH, accumulation of metabolites resulting from oxidation processes, lack oxygen and ventilation, were the reasons of decreasing anthracene degradation after 8days (14).

Ravelet *et al.*, (15) recorded that Zygomycota *Mucor racemosus* was able to degrade more than 40% of anthracene after 8days of incubation. This result elucidates that biodegradation happened due to the higher surface area (Fungal mycelium), which increased both mechanical and enzymatic contacts with an insoluble substrate such as anthracene and invade a larger volume of soil (16). At the same time, Mohammed *et al.*, (17) verified that degradation of anthracene by fungi are more practical than bacterial degradation.

A- Methylene blue method

The statistical results showed that *E. nidulans* was significantly the better isolate in degradation of anthracene compound by using methylene blue as a reduction agent to the residue of anthracene, with an OD 0.2 and rate 92.3%, compared to an OD of *A. flavus* 0.3 and rate of degradation was 89% (Fig. 3) (Table 1).

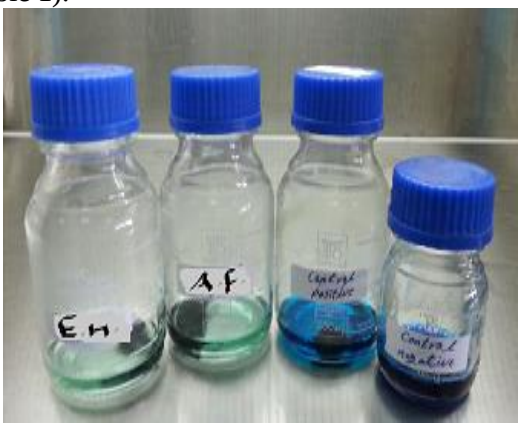

Figure 3. Reduction of methylene blue (Redox indicator) by *E. nidulans* and *A. flavus* after 8days of incubation in shaker incubator 120rpm at 30^{0}C.

The ability of these isolates to induce the color change in the media is presumably due to a reduction of the indicator by oxidized products of hydrocarbon degradation or due to the fungal growth, which utilizes oxygen for their metabolism (9). Results showed that *Penicillium simplicissimum*, *Phanerochaete chrysosporium* and *Irpex lacteus*, were degraded anthracene (100µM) after 61days of incubation, with ratio approximately (86, 40 and 38)%, respectively (6).

Table 1. Reduction of methylene blue and percentage of anthracene degradation by *E. nidulans and A. flavus* after 8days in shaker incubator 120rpm at 30^{0}C.

No.	Fungal isolates	Reduction of methylene blue (OD)	Degradation %
1	Control negative	2.396	
2	Control positive	2.354	1.75
3	*Emericella nidulans*	0.2	92.3
4	*Aspergillus flavus*	0.3	89

Control negative: (media, methylene blue and anthracene)
Control positive: (media, methylene blue and inoculum)

B- Estimation of biomass method

The results showed variation in the capacity of the fungal isolates in the consumption of anthracene in the liquid mineral salts media. It was found that the pink colony (*E. nidulans*) was the best isolate for anthracene degradation with the rate of 84% and yield maximum biomass 3.7gm/l, while the yield maximum biomass of yellow colony (*A. flavus*) was 1.2gm/l, with the rate of anthracene degradation reaching to 77% (Table 2).

Table 2. Biomass yield and anthracene degradation by *A. flavus* and *E. nidulans* isolates in liquid mineral salts medium with 100ppm anthracene after 8days in shaker incubator 120rpm at 30^{0}C

No.	Fungal isolates	Biomass yield gm\l	Anthracene degradation %
1	Control	3.80	1.752
2	*Emericella nidulans*	3.7	84
3	*Aspergillus flavus*	1.2	77

Control: (media without inoculum)

Results showed that there is a relationship between rates of anthracene consumption concentrations and biomass production. *E. nidulans* was the best isolate that decreases anthracene concentration and produces maximum biomass. Garapati and Mishra (18) were used *Penicillium* sp. and *Aspergillus versicolor* upon growth in liquid medium containing 500ppm of crude oil, they recorded that these isolates increase in biomass (2.3 and 2.56)gm/l, respectively, according to decrease in crude oil concentration with a rate of degradation (76.5 and 78.4)%, respectively.

Biomass of *Stereum ostrea* and *Phanerochaete chrysosporium* growing in liquid medium contains 1% anthracene under shaking conditions, were initially slow for 4days, then picked up and remained steady at 8days of incubation. *Stereum ostrea* produced maximum biomass of 1.89gm/flask (25ml) after 8days of incubation with anthracene degradation 62.3%, while the fungus *Phanerochaete chrysosporium* gave 1.78gm/flask (25ml) with anthracene degradation 56.6% over the same period of incubation (19).

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التفكك الحيوي لمركب الانثراسين باستخدام نوعين من الفطريات الخيطية

ضحى بهاء محمد[1] عدنان حنون عباس[2] ايمان حسين عبد[3] أحمد محسن عبدعلي[4]

[1، 2، 3، 4] وزارة العلوم والتكنولوجيا، دائرة البيئة والمياه، بغداد، العراق.

الخلاصة:

عزلت وشخصت عزلتين من الفطريات *Emericella nidulans* و *Aspergillus flavus* من الطين الاصطناعي الوردي والاصفر باستخدام وسط اكار الديكستروز والبطاطا. توصلت النتائج الى ان عزلة *E. nidulans* هي الافضل في تفكيك الانثراسين بنسبة 93% وانتاج كتلة حيوية بنسبة 3.7 غم/لتر مقارنة بعزلة *A. flavus* المفككة للانثراسين بنسبة 89% وانتاج 1.2 غم/لتر من الكتلة الحيوية عند استخدام صبغة المثيل الازرق كعامل مختزل، بعد حضنها في حاضنة هزازة بسرعة 120 دورة/دقيقة في درجة حرارة 30 درجة مئوية لمدة 8 أيام. كذلك اشارت النتائج الى ظهور قابلية عالية في تفكيك الانثراسين بنسبة 84% في حال استخدام العزلة *E. nidulans* بينما اظهرت العزلة *A. flavus* مستويات واطئة من تفكك الانثراسين بنسبة 77% باستخدام HPLC.

الكلمات المفتاحية: الفطريات الخيطية، التفكك الحيوي للانثراسين، الفطر *Emericella nidulans*، *Aspergillus flavus*.