Biodegradation of Naphthalene by Local Fungal Isolates

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Abstract

Three hundred and twelve (312) local fungal isolates were isolated from sixty four (64) different contaminated soil samples with oil wastes at different periods, using potato dextrose agar (PDA).the fungal isolates were tested for its ability to degrade naphthalene .Primary and secondary screening were done using solid (MSM) and liquid (MSM) with 100ppm naphthalene and pH 7 respectively. Results from Primary screening showed that 25 isolates gave good growth, 47 gave moderate growth, 66 gave weak growth and 174 were never growing. According to above results 25 fungal isolates were tested for its ability to degredade naphthalene using liquid mineral media (MSM) pH7,100ppm naphthalene and incubated at 30 ^{0}C 120rpm for 7 days. Reduction of naphthalene concentration was estimated by using HPLC and Horiba analysis. Results showed that Ti, Mi, Ru isolates appeared highest ability to consume naphthalene (87.7), (88.1), (87.6) % respectively. According to Macro and Microscopic featured the isolates Ti, Mi, Ru were subsequently identified as *Aspergillus niger, Trichoderma viridi* and *Fusarium verticelloides* respectively.

Keywords: *Aspergillus niger; Trichoderma viridi; Fusarium verticelloides*; Biodegradation; Naphthalene.

التفكك الحيوي للنفثالين بواسطة عزلات فطرية محلية

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الخلاصة

عزلت 312 عزلة فطرية من من 64 نموذج تربة ملوثة بالمخلفات النفطية وبفترات مختلفة باستخدم وسط PDA. اختبرت قابلية العزلات الفطرية على تفكيك النفثالين .حيث اجريت غربلة اولية وثانوية باستخدام وسط الاملاح المعدنية الصلب والسائل ذو تركيز ppm100 نفثالين ورقم هيدروجيني 7 .اظهرت نتائج الغربلة الاولية ان 25 عزلة اعطت نمو جيد ,47 كان نموها متوسط ,66 كان نموها ضعيف في حين 174 عزلة لم تنمو على الوسط .وفقا للنتائج اعلاه اختبرت قابلية 25 عزلة في تفكيك النفثالين باستخدام وسط الاملاح المعدنية السائل ذو تركيز ppm100 نفثالين ورقم هيدروجيني 7 وحضن بالحاضنة الهزارة بسرعة rpm120 وبدرجة 30^{0}C لمدة 7 ايام .قدر اختزال تركيز النفثالين باستخدام جهازي Horiba and HPLC analysis .اظهرت النتائج ان العزلات T1,Mi2,Ru هي افضل العزلات باستهلاك النفثالين حيث كانت نسب التفكيك على التوالي % (87.6) , (88.1), (87.7) بعدها شخصت مظهريا ومجهريا باستخدام المجهر

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المركب وكانت على التوالي *Aspergillus niger*, *Trichoderma viridi*, *Fusarium verticelloides*,

Introduction

Microorganisms including species of bacteria, fungi and algae had the capability of degrading polycyclic aromatic hydrocarbons (PAHs) of up to 5 aromatic rings have been isolated from various oil contaminated soil .While there is a diverse group of microorganisms with the capability of degrading LMW PAHs such as Naphthalene phenanthrene and anthracene, only a limited number have been identified as able to degrade HMW PAHs such as benzo(a)pyrene [1] In soils, PAH degrading microorganisms are common members of the indigenous microbial population One of PAHs was Naphthalene, can be used as a sole carbon and energy source [2]. Bacteria and fungi are the predominant microorganisms able to degrade PAHs through one of two mechanisms. The first is when a PAH contaminant is used as a source of carbon and energy, and the second is by co-metabolism. Co-metabolism is an important process for the degradation of HMW PAHs, as well as for some PAH mixtures [3]. Microbial PAH degradation pathways can be aerobic or anaerobic, and the enzymes can have broad substrate specificity [4].A wide variety of fungi, both ligninolytic and non-ligninolytic, have the capability of degrading PAHs, but in contrast to bacteria, fungi typically do not use PAHs as sole carbon and energy sources, but co-metabolically transform them to less toxic products [5]. Fungal PAH degradation relies upon two enzyme systems including cytochrome P-450 monooxygenases and lignin peroxidises [6]. Cytochrome P-450 monooxygenases are complex, membrane bound, multicomponent systems which can have broad substrate specificities [5].

These enzymes catalyze the formation of arene oxides by incorporating one atom of the oxygen molecule into the PAH, while the second atom is reduced to water [6].

White rot fungi such as *Phanerochaete chrysosporium* produce extracellular lignin peroxidises as well as manganese peroxidises and laccases which play a role in PAH degradation [7]. Lignin peroxidises in particular have been shown to initiate free radical attack upon PAHs by transferring one electron to form an aryl cation radical which can be oxidized to form a quinone and subsequently undergo ring fission [8]. PAHs which can be oxidized by lignin peroxidises include anthracene, pyrene, perylene, benzo(a)pyrene and benz(a)anthracene [9]the aim of this study was designed as a detective about the active isolates in consumption of naphthalene**.**

Materials and methods:

Samples collection:

The soil samples used for the isolation of fungi were collected from 18 different regions of contaminant sites with oil waste at different periods. The samples were collected randomly from the superficial layer of soil (5-10cm) in depth, using
pre-sterilized spatula and were transferred into sterilized glass bottle .the samples were then air dried, sieved (2.0mm), and stored at 4^0C until use.

Isolation of fungi:

The fungal isolates from the soil samples were isolated by serial dilution of PDA (potato dextrose agar) medium. At pH7 Suspension of 1 g (dry weight) of soil sample in 10 ml of sterile distilled water was prepared. 1 ml from each soil suspension was then diluted serially (10^{-2}–10^{-6}) .dilutions used were from (10^{-2}– 10^{-5}) then 0.1 ml from each dilution was spread on PDA plates for the estimation of fungal population. The plates were incubated at 28°C for 4-7 days. Each colony grown was selected and maintained on PDA medium and stored at 4^oC for further work [10].

Purification of isolated fungi:

The isolated fungi were purified by point inoculating them on plates containing PDA medium. The fungi were purified by repeated point inoculation. After ensuring purity, the cultures were sub-cultured on PDA slants, allowed to grow for 5-7 days, and subsequently stored at 4°C

Screening of the isolates for Naphthalene biodegradation

Primary screening

Sixty-four fungal isolates were tested for their ability to degrade naphthalene using 0.1 ml (100 ppm) naphthalene stock solution mix with solid mineral salts medium then the plates were inoculated with fungal disk 7mm for 7 days old culture of fungal isolates. The plates were incubated at $28C^0$ 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. [11]

Secondary screening:

The fungal isolates that gave best growth formation in primary screening were selected and were grown in 100 ml Erlenmeyer flasks containing 25 ml liquid mineral salts media at pH 7, then 100ppm naphthalene was added per flask and autoclaved at $121C^0$ for 15 min, the sterilized media were inoculated with one fungal disk (7mm) from 7 days old culture of fungal isolates. Duplicate for each isolate and control, then flasks were incubated in shaker incubator 120rpm for 7days at $30C^0$ [12].

Degradation of naphthalene

Fungal growth means that the isolates consume naphthalene which mean degradation of this compound to more polar derivatives and that was clearly by primary screening (on sold media) and secondary screening (in liquid media)

Results and discussion

A total of 312 isolates were found, different species belongs to Ascomycetes and Deuteromycetes were isolated from oil contaminated soil [13] this concluded Positive for the occurrence of fungi in oil contaminated soil

Screening of the isolates for naphthalene biodegradation

Primary screening

The percentage of positive growth isolates was found to be 44.23% in samples collected from oil contaminated soil, while 55.77% of isolates show negative growth.

The disappearance of Naphthalene from solid MSM, suggesting consumed by the fungal isolates, but no growth on this medium was observed by survival of a white thin layer of naphthalene on the surfaces of the dishes indication of the inability of the isolates from the consumption of this compound.

The results show 25 fungal isolates gave good growth on solid MSM by used naphthalene as a sole source of carbon and energy (50-80mm diameter of fungal growth), 47 fungal isolates was Moderate growth (20-49mm diameter of fungal growth), 66 weak growth (10-19mm diameter of fungal growth) and 174 fungal isolates were never growing on Naphthalene solid media figure 1 A, B, C, and D, table 1.

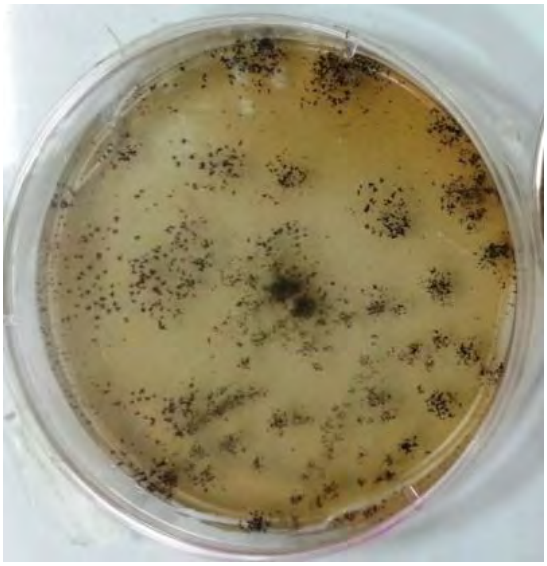
identical study done by [14] found fail of fungal isolates to grow on solid MSM with anthracene which attribute the degradation of compound containing more than one cycle is more resistant to oxidative enzymes. Based on the previously, (25) fungal isolates that showed "good" growth on solid MSM containing naphthalene were selected for a subsequent study.

The disappearance of Naphthalene from solid MSM, suggesting consumed by the fungal isolates, but no growth on this medium was observed by survival of a white thin layer of naphthalene on the surfaces of the dishes indication of the inability of the isolates of consumption of this compound. This may be attributed to the reason that fungal colonies are not growing on this medium because they do not possess the ability to degrade this compound as a result of the lack of enzymatic system specialist, or due to the absence of metabolic capacity other reason low solubility of this compound which reduce availability to microorganisms and finally may be consume the simple oxidative products that have the ability to antioxidant of this compound [15].

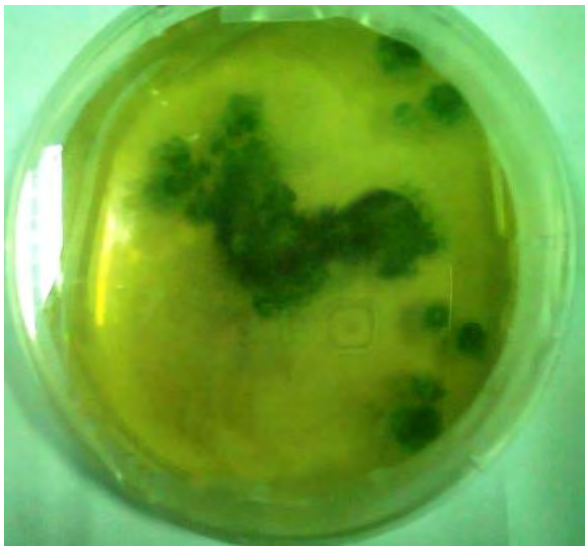
Table 1- The ability of fungal isolates on the growth on solid MSM containing pure naphthalene 100ppm after incubation period 7 days at 280C.

S.no	Growth Development of Isolates	Isolates Number
1-	Good	25
2-	Moderate	47
3-	Weak	66
4-	No growth	174

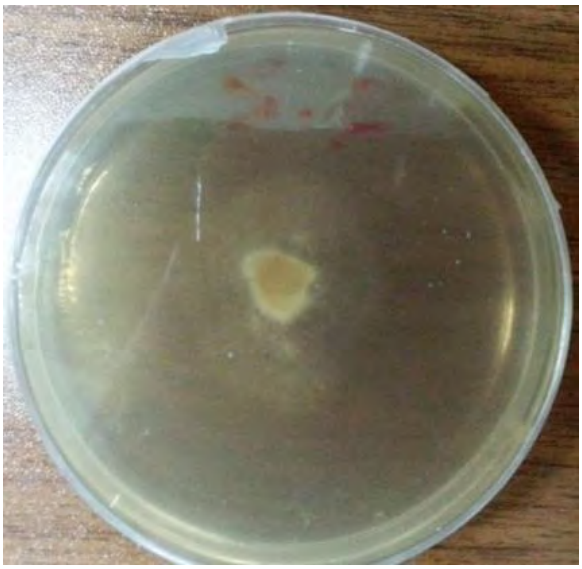
Good: - Diameter of Fungal Growth (50-80mm)
Moderate: - Diameter of Fungal Growth (20-49mm)
Weak: - Diameter of Fungal Growth (10-19mm)



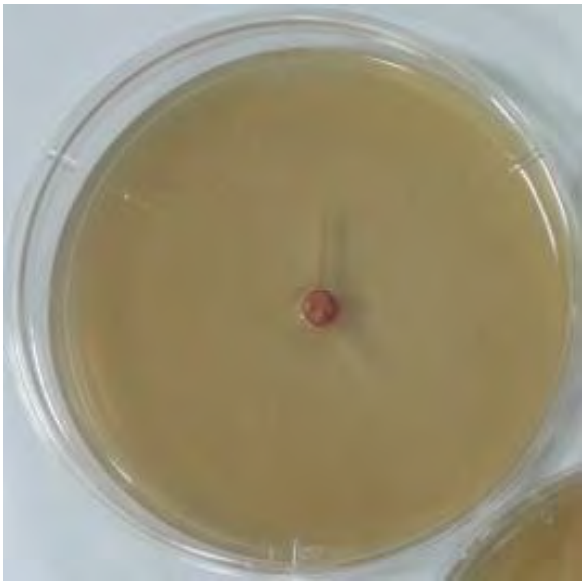
A-Good Fungal Growth



B-Moderate Fungal Growth



C-Weak Fungal Growth



D- No Growth

Figure 1-A, B, C and D- Fungal growth on solid media containing 100ppm Naphthalene after 7 days at 28^{o}C

Secondary screening

(25) Selected fungal isolates that displayed "good" growth in solid media containing Naphthalene test its ability to degrade naphthalene after a period of incubation for 7 days in shaker incubator 120 rpm at 30^{o}C, the results showed the ability of all fungal isolates to degrade naphthalene table 2.

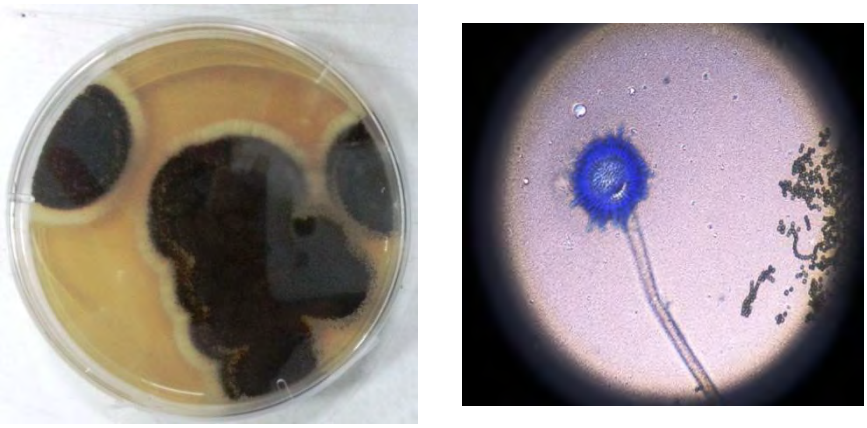
Table 2- Growth of fungal isolates resulted from primary screening in liquid mineral salt medium with 100ppm naphthalene, pH 7, 7 days, 120 rpm.

S.no	Isolates Names	Naphthalene remaining (ppm) by HPLC	TPH remaining (ppm) by Horiba
1.	DB1	24.5	25
2.	DB2	41.6	42.3
3.	T1	12.4	12.9
4.	T2	22.3	22.9
5.	DS1	31	31.9
6.	DS2	56.85	57.1
7.	DS3	23.1	24,61
8.	DS4	34.54	35.4
9.	DR1	51.68	52
10.	DR2	36.0	37.9
11.	SC	22.9	23.2
12.	MI1	19.9	20.1
13.	MI2	11.9	12.2
14.	MI3	25.9	26.2
15.	JG	18.4	18.9
16.	Y1	22.1	22.8
17.	Y2	27.8	29.4
18.	MA	31.5	35.9
19.	DM	19.5	20.1
20.	D601	22.4	23.7
21.	D602	34.22	37.2
22.	DA1	32.6	33.7
23.	DA2	52.68	53.3
24.	RU	12.3	12.8
25.	SH	30.05	31.5

According to secondary screening results, we select the most active sites isolates which were (T1, MI2 and RU).

Identification of Fungal Isolates

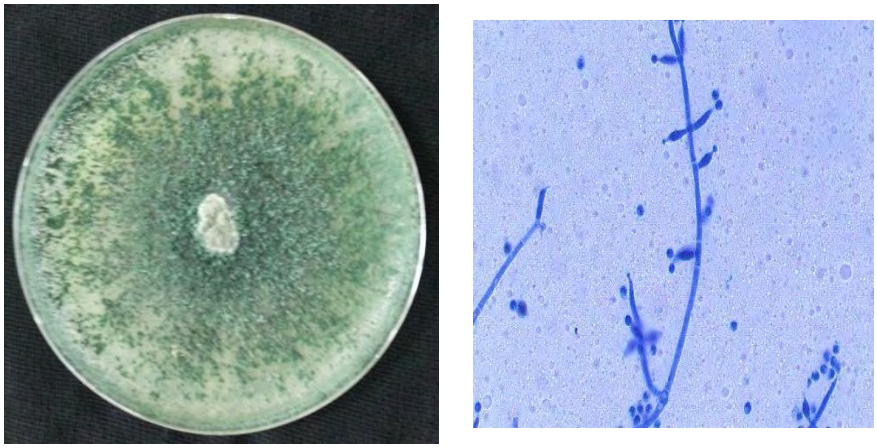
The initial examination of the fungal colonies developing were done by using a dissecting microscope, and attended the slides for these colonies for the purpose of study their characteristics under an optical microscope compound. The results show that most active isolates were *Aspergillus niger*, *Trichoderma viridi, Fusarium verticelloides*, respectively [16] figure 2a and b, 3a and b and 4a and b.



a. Macroscopic feature of *Aspergillus niger*

b. Microscopic feature of *Aspergillus niger*

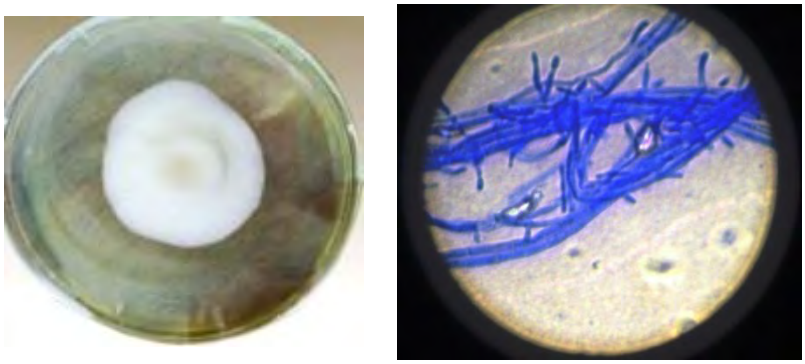
Figure 2a and b- Macroscopic and microscopic features of *Aspergillus niger*



a. Macroscopic feature of *Trichoderma viridi*

b. Microscopic feature of *Trichoderma viridi*

Figure 3a and b -Macroscopic and microscopic features of *Trichoderma viridi*



a. Macroscopic feature of *Fusarium verticelloides*

b. Microscopic feature of *Fusarium verticelloides*

Figure 4a and b- Macroscopic and microscopic features of *Fusarium verticelloides*

References:

1. Juhasz, A.L and Naidu R, 2000. Bioremediation of high molecular weight polycyclic aromatic hydrocarbons: a review of the microbial degradation of benzo[a]pyrene.Int Biodeterior Biodegrad 45:57–88 .

2. Uz, I., Y.P. Duan, and A. Ogram. **2000**. Characterization of the naphthalene-degrading bacterium, *Rhodococcus opacus* M213. *FEMS Microbiol. Lett.* 185: 231-238

3. Chauhan, A., Fazlurrahman, J. Oakeshott, and R. Jain. **2008**. Bacterial metabolism of polycyclic aromatic hydrocarbons: strategies for bioremediation. *Indian J. Microbiol.*48: 95-113.

4. Habe, H., and T. Omori. **2003**. Genetics of polycyclic aromatic hydrocarbon metabolism in diverse aerobic bacteria. *Biosci. Biotechnol., Biochem.* 67: 225-243.

5. Labana, S., M. Kapur, D. Malik, D. and Jain R.K.**2007**. Diversity, biodegradation and bioremediation of polycyclic aromatic hydrocarbons. In *Environmental Bioremediation Technologies*, ed. S.N. Singh and R.D. Tripathi. Berlin Heidelberg: Springer-Verlag.

6. Cerniglia, C.E.**1997**. Fungal metabolism of polycyclic aromatic hydrocarbons: past, present and future applications in bioremediation. *J. Industr. Microbiol. Biotechnol.*19: 324-333.

7. Sutherland, J.B. **1992**. Detoxification of polycyclic aromatic hydrocarbons by fungi. *J. Ind.Microbiol. Biotechnol.* 9: 53-61.

8. Cerniglia, C.E., J.R. Althaus, F.E. Evans, J.P. Freeman, R.K. Mitchum, and S.K. Yang. **1983**. Stereochemistry and evidence for an arene oxide-NIH shift pathway in the fungal metabolism of naphthalene. *Chem. Biol. Interact*: 119-132.

9. Haemmerli, S.D., M.S. Leisola, D. Sanglard, and A. Fiechter. **1986**. Oxidation of benzo(a)pyrene by extracellular ligninases of *Phanerochaete chrysosporium*. Veratryl alcohol and stability of ligninase. *J. Biol. Chem.* 261: 6900-6903.

10. Persson, Y., Frankki, S., Haglund, P. and Tysklind, M. **2000**. Degradation of polycyclic aromatic hydrocarbons (PAHs) in contaminated soils *Journal of Hazardous Materials.*166(2): 80-93

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

12. Bartha, R. **1986**. Biotechnology of petroleum pollutant biodegradation. *Microb. Ecol.* 12:155-172.

13. Maheshwari ,R. ,Bharadwaj , G. and Bhat ,M.**2000** .Thermophilic fungi , their physiology and enzymes .*Microbiology and Molecular Biology Review* 63: 461-488 .

14. 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: 244-252.

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

16. Samson, R.A., Hoekstra, E.S., Frisvad, J.C. and Filtenborg, O. **2000**. *Introduction to food-borne fungi*. 6th ed. Centraalbureau voor Schimmelcultures, Netherlands 389 pp.