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BIODEGRADATION OF PAHS BY CONSORTIUM MICROORGANISMS ISOLATED FROM OIL CONTAMINATED SOIL

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ABSTRACT

Different two isolates of microorganisms were isolated from oil contaminated soil (Baghdad/Al-Dora refinery site). Then these isolates were identified as *Bacillus* sp. and *Aspergillus parasiticus* according to their morphological characteristics. These isolates were tested for their ability to degrade naphthalene (500ppm) by methylene blue and naphthalene residue by HPLC, results revealed that naphthalene was degraded with the rate of degradation (70, 45 and 61)% and (75, 50 and 72)% when treated with (*Bacillus* sp., *Aspergillus parasiticus* and co-cultures of *Bacillus* sp.-*Aspergillus parasiticus*) respectively, after incubating in a shaker incubator 120rpm at 30C^{o} for 8days.

KEYWORDS: Naphthalene biodegradation, *Bacillus*, *Aspergillus parasiticus.*

INTRODUCTION

Naphthalene is an organic compound with chemical formula $C_{10}H_8$. It is the simplest polycyclic aromatic hydrocarbon, consists of a fused pair of benzene rings (Kuppusamy *et al.*, 2016; Li *et al.*, 2016).

The microorganisms used for degradation should be indigenous to the contaminated area or site. Five isolates belong to *Staphylococcus* sp. *Corynebacterium* sp. *Pseudomonas* sp. *Bacillus* sp. and *Micrococcus* sp. were significantly able to degrade naphthalene (Messiah and Lal, 2014; Sharma, 2014).

Recently, soil fungi have been studied regarding their ability to degrade (PAHs) and produce ligninolytic enzymes under micro aerobic and very low-oxygen conditions (Varjani *et al.*,

2015). Species belong to *Aspergillus* spp., *Trichocladium canadense*, and *Fusarium oxysporum* able to degrade PAHs (Varjani and Upasani, 2016).

Co-cultures of bacteria and fungi have been found to be effective in both the mineralization and degradation of PAHs and the bacterial consortium *Bacillus* sp. and *Penicillium janthinellum* were able to grow on naphthalene as a sole source of carbon and energy (Sajna *et al.*, 2015).

MATERIALS AND METHODS

Sampling for bacteria isolation

Suspension of soil contaminated with crude oil (Baghdad/Al-Dora refinery site) was heat-treated (80ºC for 10min) and individually placed on nutrient agar plates. After 24hr of incubation at 30ºC, colonies were recovered and purified by streaking on fresh nutrient agar (Alnasser *et al.*, 2007).

Sampling for fungal isolation

Suspension of soil contaminated with crude oil was placed on plates containing PDA medium. After 5days of incubation at 25ºC, isolate was recovered and purified till ensuring purity of fungal isolate. Then isolate 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 isolate was diagnosed due to their morphological characteristics by utilizing the lactophenol solution, and then slides were recognized under the microscope.

Methylene blue (Redox indicator)

A suitable amount of mineral salt medium preparing with some modification (Ameen *et al.*, 2014) was poured in Erlenmeyer flasks (100ml) containing 2ml of Tween 80 then volume flasks were completed to 25ml with the same media, (pH 7.0), 2drops of methylene blue as prepared by Wilson and Jones (1993) and 500ppm naphthalene were added after autoclaved media at 121^0C for 15min. Three types of the microbial consortia (bacteria, fungi and co-cultures of bacteria-fungi) were inoculated. Flasks for fungi were inoculated with diameter 9mm of selected fungal isolates, while bacterial flasks were inoculated with 5% of bacterial suspension and flasks with co-cultures of bacteria-fungi were inoculated with the same

amounts of bacteria and fungi. All samples were repeated with the negative control, then flasks were incubated in a shaker incubator 120rpm at 30C^{o} for 8days.

After the incubation period, growth culture was filtrated and centrifuging at 10000rpm for 15min. Supernatant was analyzed by spectrophotometer at 609nm and the percentage of degradation was calculated by a formula as below (Hari, 2003).

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

Estimation of naphthalene 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 279nm.

$$\text{Degradation} = \frac{\text{Initial Conc. of naphthalene - naphthalene Conc. after incubation}}{\text{Initial Conc. of naphthalene}} \times 100$$

RESULTS AND DISCUSSION

Identification of microorganisms

Morphological diagnoses of pure isolates were characteristics and determined as a genus *Bacillus* by microscopy (Gram and spore staining) (Figure 1).

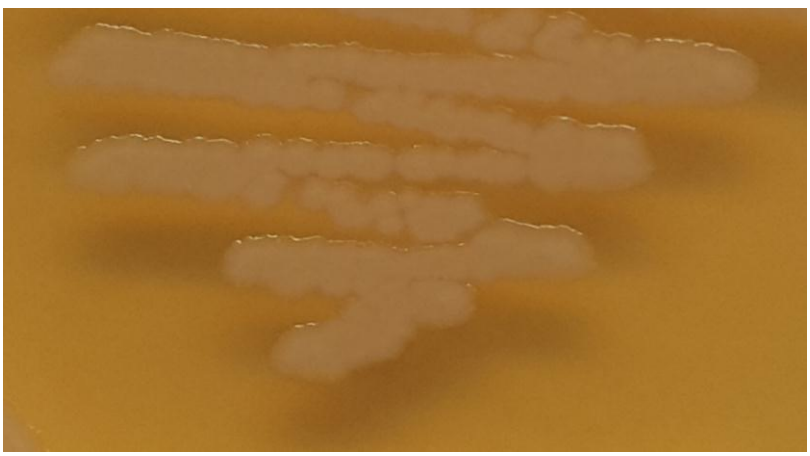

Fig. 1: Morphological feature of *Bacillus* genus.

While morphological characteristics of fungal isolate were done by using a dissecting and initial identification according to their macroscopic and microscopic features. Results showed that active isolate were identified as *A. parasiticus* (Samson *et al*., 2000) (Figure 2).

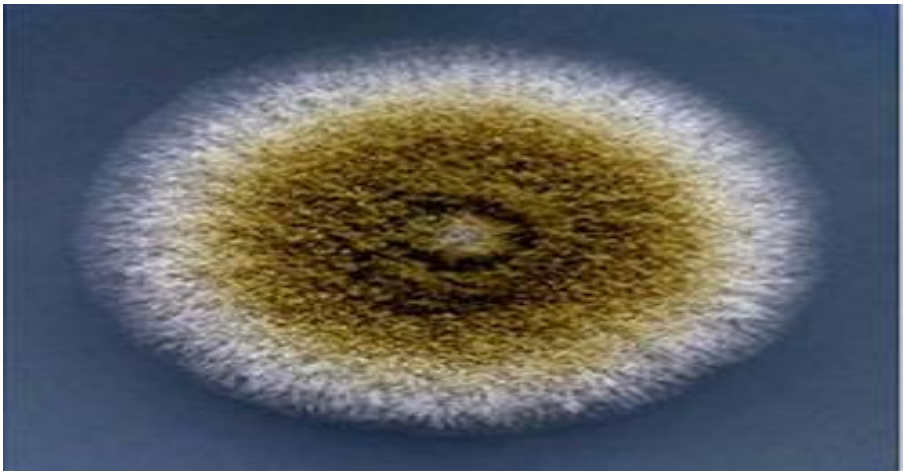

Fig. 2: Morphological feature of *Aspergillus parasiticus.*

Biodegradation of naphthalene

The disappearance of naphthalene under different conditions of treatment (inoculated with *Bacillus* sp., *Aspergillus parasiticus* and co-cultures of *Bacillus* sp.-*Aspergillus parasiticus*) with OD at 609nm (0.289, 0.529 and 0.375) were (70, 45 and 61)%, respectively (Table 1) in a shaker incubator 120rpm at 30C^{o} for 8days, by using methylene blue as a reduction agent to the residue of naphthalene (Figure 3).

Table 1: Reduction of methylene blue and percentage of naphthalene degradation by *Bacillus* sp. *and A. parasiticus* after 8days in shaker incubator 120rpm at 30^{0}C.

No.	Isolates	Reduction of methylene blue (OD)	Degradation %
1	Control negative	0.961	
2	*Bacillus* sp.	0.289	70
3	*A. parasiticus*	0.529	45
4	Mixed	0.375	61

Control negative: (media, methylene blue and naphthalene)

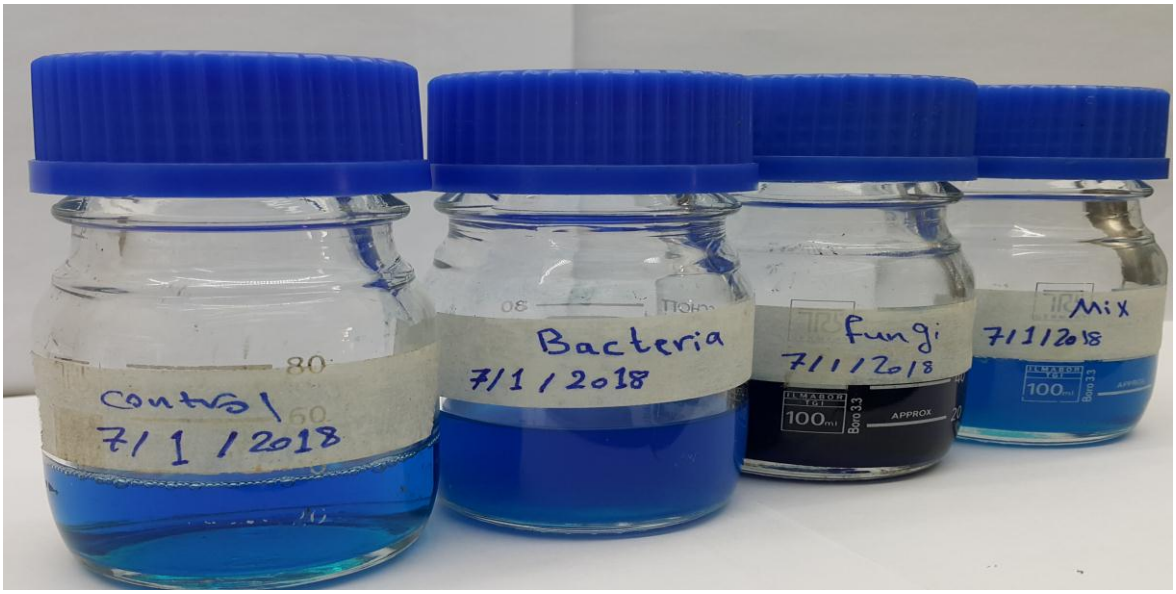

Fig. 3: Reduction of methylene blue (Redox indicator) by *Bacillus* sp. and *A. parasiticus* after 8days of incubation in shaker incubator 120rpm at 30^0C.

The ability of these isolates induce a color change in the medium is presumably due to the reduction of the indicator by the oxidized products of hydrocarbon degradation or due to the microorganism growth, which utilizes oxygen for their metabolism (Hari, 2003), the results agreed with Svobodova *et al*., (2006) who reported in experiment that *Phanerochaete chrysosporiumn* and *Irpex lacteus* could degrade (81.32 and 83.45)% of naphthalene, respectively after 8days of incubation in the presence of methylene blue. In this experiment, it should be noted that after 10days of incubation, the medium containing methylene blue gradually become darker and greener. Genus *Bacillus* was reported to be involved in the degradation of aliphatic (Cybulski *et al.*, 2003) and polycyclic aromatic (Kazunga and Aitken, 2000) hydrocarbons. The role of *Bacillus* spp. in the degradation of complex hydrocarbons has been characterized as that of secondary degraders, using metabolites produced by the primary hydrocarbon degraders (Chaillan *et al.*, 2004).

Microorganisms have been found to degrade PAHs as carbon and energy sources to carbon dioxide and water, as a means of reducing PAH toxicity and as co-metabolic substrates (Perelo, 2010). *Bacillus* sp. strain DHT, isolated from oil contaminated soil, grew and produced biosurfactant when cultured in the presence of hydrocarbons, including crude oil, diesel oil, hexadecane, naphthalene, pyrene, dibenzothiophene, salicylate, catechol and phenanthrene as the sole carbon sources at (30-45)°C. However, no growth occurred in toluene, phenol, 2-hydroxyquinoline and carbazole (Kumar *et al.*, 2007).

Determination of naphthalene degradability by HPLC

The results of naphthalene degradation (500ppm) by using HPLC showed the high ability of *Bacillus* sp. in naphthalene degradation with 75%, while *A. parasiticus* was capable to be degraded with 50%, whereas the mixture of *Bacillus* sp. and *A. parasiticus* revealed high ability of degradation of naphthalene with ratio 72% (Table 2).

Table 2: Degradation of naphthalene (500ppm) by *Bacillus* sp. and *A. parasiticus* in liquid mineral salts medium after 8days in shaker incubator 120rpm at 30^0C.

No.	Microorganisms	Anthracene degradation %
1	Control negative	1
2	*Bacillus* sp.	75
3	*A. parasiticus*	50
4	Mixed	72

Control negative: (media without inoculum)

Many researchers used different bacterial isolates for naphthalene biodegradation. The efficiency of naphthalene degradation was evaluated by High Performance Liquid Chromatography (HPLC) analysis. Kafilzadeh *et al*., (2011) isolated *Bacillus* sp., *Pseudomonas* sp., *Corynebacterium* sp., *Staphylococcus* sp. and *Micrococcus* sp. from waste oil samples. These isolates were able to degrade naphthalene after one week of incubation with the rate of (86, 80, 77, 69 and 58)%, respectively.

The fungal species *Phanerochate chrysosporium*, *Trametes versicolor*, *Pleurotus ostreatus* have the ability to degrade anthracene with the rate of degradation (77.5, 69.6 and 72.3)%, respectively, when fungal species incubated in 130rpm at 30^0C (Atagana, 2009).

The concentration of a PAH also has a crucial impact on the potential success of microbial degradation. If PAH concentrations were too low, the genes necessary for enzyme production may not be induced and the enzymes necessary for degradation may not be produced (Van Hamme, 2004). However, if PAH concentrations are too high, toxic effects can be exerted upon the cellular and toxic metabolites may accumulate in the growth medium (Morasch *et al.*, 2001). While the fungus *Trametes versicolor* degraded 62% of 300ppm phenanethrene when the fungal isolate incubated for 10 days (Levin and Forchiassin, 2003).

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