



## USE OF FUNGI IN BIOREMEDIATION OF CONTAMINATED SITES WITH HYDROCARBONS

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### Abstract

Contamination of petroleum hydrocarbons is considered as one of the major environmental concerns facing the world nowadays. Bioremediation is a successful method to deal with such type of contamination. This study includes two steps of treatment the first is consisted of isolation and diagnosis of the most growing and widespread fungi in soil samples contaminated with crude oil taken from the Al-kask and Al-Qayara oil refineries, (28) fungal isolates belonging to the genus of *Penicillium sp.* and (23) fungal isolates of the *Aspergillus sp.*, the genera were isolated from a total of (64) isolates that are isolated from soils contaminated with crude oil. While the second step is a study of the ability of isolated fungal genera to break down Poly Aromatic Hydrocarbons (PAHs) which are found in crude oil concentrations (5%, 10%) that pollute soil samples as a source of carbon and energy, and to enhance processes by adding some auxiliary materials to fungus metabolism, such as humic acid. The analysis of samples by the GC device (Gas Chromatography) showed a clear decrease in concentrations of PAHs compared to non-injected soil samples with fungi genera (*Penicillium sp.*, *Aspergillus sp.*). This is considered as an evidence of the fungal ability to break down the (PAHs). *Penicillium sp.* is more efficient in breaking down both polluted concentrations of crude oil (5%, 10%) and with removal rates reached to 32.10% and 25.50% for both concentrations respectively, whereas removal rates by *Aspergillus sp.* isolates of the same polluted concentrations were 19.28% and 13.72%, respectively. The addition of the humic acid with fungus gives huge effectiveness for fungal genera (*Penicillium sp.*, *Aspergillus sp.*) to break down (PAHs) where the removal percentage increased to 56.70% at concentration (5%) and 44.50% for concentration (10%) by *Penicillium sp.* In contrast, the removal percentage by genus *Aspergillus sp.* enhanced with humic acid was 38.42% and 32.91% at the two concentrations (5%) and (10%), respectively.

**Keyword:** Fungi, Poly Aromatic Hydrocarbons (PAHs), Humic acid.

### Introduction

The technological development that occurred during the past few years led to an increase in the demand for oil derivatives and consequently to a significant increase in the consumption of those derivatives in the world (Spellman, 2015). Oil is extracted from the ground in huge quantities and then transport through transport pipelines especially extending over long distances by land and sea transportation to different regions where it is consumed as a source of energy or a basic raw material enters in many different industries, during these different stages in dealing with oil, starting from the extraction and transportation operations reaching to production processes cause accidents and errors resulting in environmental pollution (Ullah *et al.*, 2015; Gall *et al.*, 2015).

Crude oil is a complex mixture of a large number of hydrocarbon compounds and other non-hydrocarbon consist of different chemical elements, the chemical composition of the oil varies between the types. However, the common feature that binds most of the hydrocarbon compounds the presence of carbon and hydrogen atoms in it. The difference in the properties of oil result of the distinct ability of the carbon element to bond with other elements to form simple or complex molecular formations (Speight, 2017).

The hydrocarbon composition of the crude oil is the basis in chemical classification into aliphatic and aromatic parts. Crude oil shows a great difference in appearance, viscosity and color from one oil field to another (Sari *et al.*, 2018). Knowing the composition, concentration, and characteristics of crude oil helps to determine appropriate methods for eliminating from pollution that cause (Wang *et al.*, 2017).

Contamination of the soil with crude oil and its derivatives causes physical, chemical and biological damage to the soil because it contains many toxic compounds with relatively high concentrations such as hydrocarbons, benzene gasoline, cyclones, cycloalkane rings and toluene, etc., which can remain for a long time in the soil (Dindar *et al.*, 2015; Di martino *et al.*, 2012).

Soil contaminated with hydrocarbons is considered a serious environmental problem, which has received great attention from the world over the past decades because it leads to accumulation of hydrocarbon pollutants in the tissues of animals and plants then transmission to humans through the food chain, which may cause death or hereditary mutations (USEPA, 2011).

Bioremediation, it is one of the successful and far-reaching means for treating oil-contaminated soils and its derivatives, relatively low cost and their final products are not harmful to the environment consisting of water vapor and carbon dioxide (Kumar *et al.*, 2011; Mansur *et al.*, 2015). Bioremediation is method friendly of the environment and use it in treatment will avoid harmful side effects that potential result from physical and chemical methods (Ibrahim *et al.*, 2016; Abioye, 2011).

Studies have increased in recent years about the use of fungi in the bioremediation of soils contaminated with crude oil because these organisms have extracellular enzymes capable of degrade hydrocarbons as a sole source of carbon. Fungi have the ability to live and grow rapidly under harsh environmental conditions (George-Okafor *et al.*, 2009; Olukunle *et al.*, 2012). (Kota *et al.*, 2014) Studied the possibility of fungi in treating soil contaminated with hydrocarbons and chose fungal species for his study

*Aspergillus versicolor*, *Bionectria ochroleuca*, *Trichoderma virens*, *Aspergillus flavus* and *Penicillium chermisinum* his results showed a high ability of fungi to degrade hydrocarbons.

The main objective of this research was treat soil contaminated with crude oil, and to emphasize the use of bioremediation as a new method of treatment through:

- Use appropriate fungi species for the decomposition process.
- Using the most appropriate environmental conditions in the treatment process.
- Using some stimulants for the bioremediation process such as humic acid.

### Materials and Methods

#### Design experience

100g of contaminated soil samples with different concentrations (5%, 10%) of crude oil are added in a sterile beaker 250 ml, then adding a spore suspension from fungal genera selected for treatment. The same coefficients were carried out for the soil contaminated concentrations, with three replications for each contaminated soil sample. Sterile distilled water was added to each sample to achieve 60% of the field capacity of the original soil sample. Humic acid was added to the selected beakers. The samples were incubated at a temperature of 28 °C for 40 days and then measured the concentration of (PAH<sub>s</sub>) using Gas chromatography device.

#### Sample collection

##### Soil samples contaminated with crude oil

Soil contaminated with crude oil samples were collected from the Al-Kask refinery, and the Al-Qayara refinery located in the city of Mosul–Iraq. Collected (5) samples from each site polluted with crude oil. The samples were taken at a depth of (0-15 cm) and mixed so that it represented the study sites.

##### Soil samples not contaminated with crude oil

Soil samples not contaminated with crude oil were collected from the Al-Qayara refinery. The samples are taken randomly from (3) sites and at a depth (0-15 cm). All samples are mixed and represented soil characteristics at the study site.

#### Isolation and diagnosis of fungi from soil contaminated with crude oil

The collected soil samples were mixed homogeneously and passed through a sieve (2 mm) to remove gravel and other unwanted ingredients. The dilution method and plates used to isolate the fungi by taking (1) g of contaminated soil and adding it to the test tube contain (9) ml of sterile distilled water to obtain dilution ( $10^{-1}$ ), then (1) ml of dilution ( $10^{-1}$ ) was transferred to a second test tube containing (9) ml of sterile distilled water to obtain the dilution ( $10^{-2}$ ). Add (1) ml from dilution ( $10^{-2}$ ) to a third test tube contain (9) ml of sterile distilled water to obtain dilution ( $10^{-3}$ ), after obtaining the three dilutions ( $10^{-1}$ ,  $10^{-2}$ ,  $10^{-3}$ ) transferred (1) ml of both dilutions ( $10^{-1}$ ) and ( $10^{-3}$ ) to sterile Petri dishes that contain

(10) ml from sterile PDA medium. This process was performed with three replications per concentration. The dishes were incubated at 28 °C for 7 days (Meysami and Baheri, 2003). The most common and widespread fungal genera in Petri dishes were isolated and then diagnosed by microscope in order to use in bioremediation.

#### Physical and chemical tests of soil sample not contaminated with crude oil

Soil sample not contaminated with crude oil subject to routine tests such as pH, electrical conductivity, bicarbonate, calcium, magnesium, chlorine, and field capacity using the methods described by (Richards, 1954), Estimation of the organic matter according to the method described by (Walkley-Black, 1934), and soil texture according to Method (Gupta, 2000).

#### Prepare the spore suspension of fungi

Spore suspension of fungi is prepared from the incubated Petri dishes, which includes both fungal genera specified for bioremediation (*Penicillium sp.*, *Aspergillus sp.*). (10) ml of sterile distilled water is added to each Petri dish contain colonies for only one fungal genera. The spores were scraped from the surface of fungal colonies by (Loop) and then transported to a sterile conical flask containing a sterile gauze cap to prevent the fungus hyphae and the PDA medium residues from passing to the flask. Then, drops from sterile (Tween 80) are added to prevent spores clump and distribution it uniformly in the conical flask (Ho and Ko, 1997; Balakrishnan *et al.*, 2013). The number of fungal spores was calculated by haemocytometer and it was  $2.1 \times 10^6$  spore/ml for *Penicillium sp.* and  $4.4 \times 10^6$  spore/ml for *Aspergillus sp.*

#### Preparing soil samples contaminated with crude oil

The non-polluted soil samples taken from Al-Qayara refinery, are mixed and used to make soil samples contaminated with different concentrations from crude oil. Quantities from crude oil are added to the dry soil sample after sterilization by autoclave at a temperature of 121 degrees Celsius and pressure 1 atmosphere for 15 minutes then dry in oven at a temperature of 70 degrees Celsius. Two types from soils contaminated with crude oil are made artificially with concentrations (5%, 10%) in order to conduct a bioremediation on these samples from soils contaminated with crude oil.

#### Bioremediation of soils contaminated with crude oil

Samples from soils contaminated with different concentrations of crude oil taken in sterile beakers (250 ml). Then, these samples were injected with spore suspension of fungi genera used in the treatment. The additives were added according to schedule (1), The beakers were covered with sterile cotton and placed in the incubator at a temperature of 28 °C for 40 days and the contents of the beakers were mixed under sterile conditions every three days in order to ventilate the mixture.

#### Sample analysis

The Samples are analyzed by Gas Chromatography device model Shimadzu (2010, Japan).

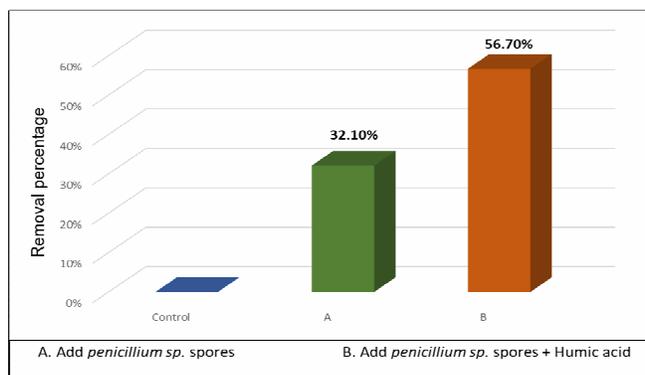
**Table 1 :** shows the addition of fungal species (*Penicillium sp.*, *Aspergillus sp.*) and humic acid to soils samples contaminated with different concentrations of crude oil.

<i>Penicillium sp.</i>							
Concentration of crude oil in the soil (%)	Soil amount (gm)	Replications	Sterile distilled water (Acts 60% of soil field capacity) (ml)	Type of addition	Quantity (ml)	Spore suspension (ml)	Notes
5	100	3	21.9	-----	-----	2.1	The amount of sterilized distilled water according to method (Ghanem <i>et al.</i> , 2016) and (Milton and Rachakonda, 2005)
5	100	3	18.5	Humic acid	3.4	2.1	
5	100	3	24	-----	-----	-----	Control
10	100	3	21.9	-----	-----	2.1	The amount of sterilized distilled water according to method (Ghanem <i>et al.</i> , 2016) and (Milton and Rachakonda, 2005)
10	100	3	18.5	Humic acid	3.4	2.1	
10	100	3	24	-----	-----	-----	Control
<i>Aspergillus sp.</i>							
5	100	3	23	-----	-----	1	The amount of sterilized distilled water according to method (Ghanem <i>et al.</i> , 2016) and (Milton and Rachakonda, 2005)
5	100	3	19.6	Humic acid	3.4	1	
5	100	3	24	-----	-----	-----	Control
10	100	3	23	-----	-----	1	The amount of sterilized distilled water according to method (Ghanem <i>et al.</i> , 2016) and (Milton and Rachakonda, 2005)
10	100	3	19.6	Humic acid	3.4	1	
10	100	3	24	-----	-----	-----	Control

**Results and Discussion**

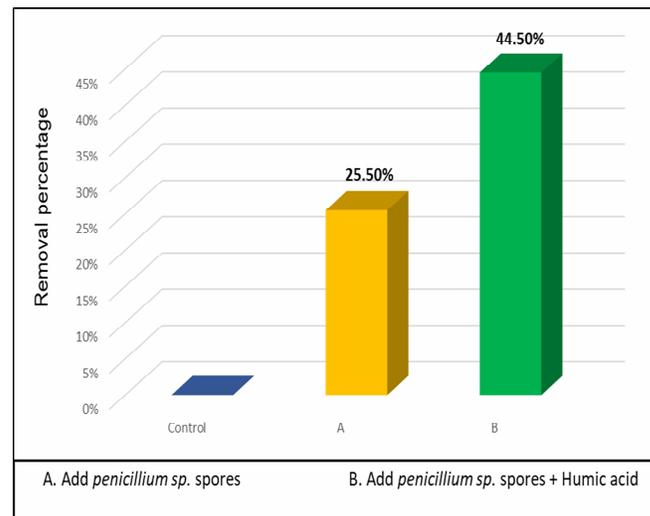
The results showed that the most dominant fungal genera in polluted soil samples was (*Penicillium sp.*, *Aspergillus sp.*). Many studies indicated that the fungal genera (*Penicillium sp.*, *Aspergillus sp.*) have the ability to degrade hydrocarbon compounds (Hussaini *et al.*, 2008), fungal genera (*Penicillium sp.*, *Aspergillus sp.*) were chosen to be used in the bioremediation of soils contaminated with crude oil and enhanced their ability to degrade hydrocarbon compounds by adding some stimulation to its metabolic activity. The results obtained showed ability of fungal genera selected to grow and spread in contaminated soil samples with different concentrations of crude oil (5%, 10%), but to varying degrees depending on the concentration of contamination.

The results showed that *Penicillium sp.* added to the soil contaminated with crude oil at a concentration of (5%) caused removal of (PAHs) 32.10%, while the removal percentage increased to 56.70% when added humic acid and fungus together to contaminated soil as shown in Figure (1)



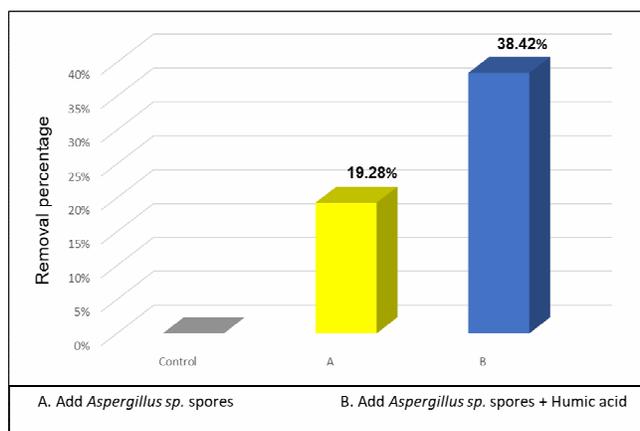
**Fig. 1:** The removal percentage of (PAHs) from contaminated soil with crude oil of concentration (5%).

Figure (2) showed the removal percentage of (PAHs) from the contaminated soil samples with a concentration (10%) of crude oil, the removal percentage was 25.50% when adding *Penicillium sp.* alone, while this percentage increased to 44.50 % When added humic acid with *Penicillium sp.*



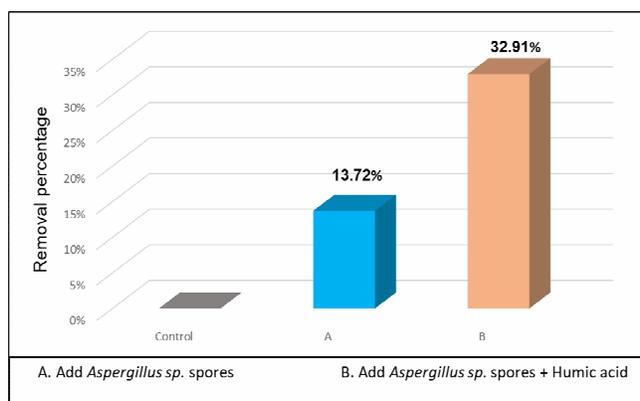
**Fig. 2:** The removal percentage of (PAHs) from contaminated soil with crude oil of concentration (10%).

The Figure (3) showed the achieved removal percentage from adding *Aspergillus sp.* alone to the contaminated soil sample with a concentration (5%) of crude oil, the percentage was 19.28%, while the percentage increased to 38.42% when adding humic acid with *Aspergillus sp.*



**Fig. 3:** The removal percentage of (PAHs) from contaminated soil with crude oil of concentration (5%).

On the other hand, the Figure (4) showed the removal ratios of (PAHs) from the contaminated soil sample with a concentration (10%) of crude oil after adding *Aspergillus sp.* to it, the ratio was 13.72% when adding *Aspergillus sp.* alone while increasing to 32.91% when added humic acid with *Aspergillus sp.*



**Fig. 4:** The removal percentage of (PAHs) from contaminated soil with crude oil of concentration (10%).

The results during all phases showed clear decrease in the concentration of (PAHs) compounds from soils samples contaminated with different concentrations of crude oil when added the fungal genera (*Penicillium sp.*, *Aspergillus sp.*) alone, also when added humic acid with fungal genera, but percentage of removal (PAHs) different depending on the type of treatment conducted on contaminated soil samples and concentration of crude oil in the soil samples.

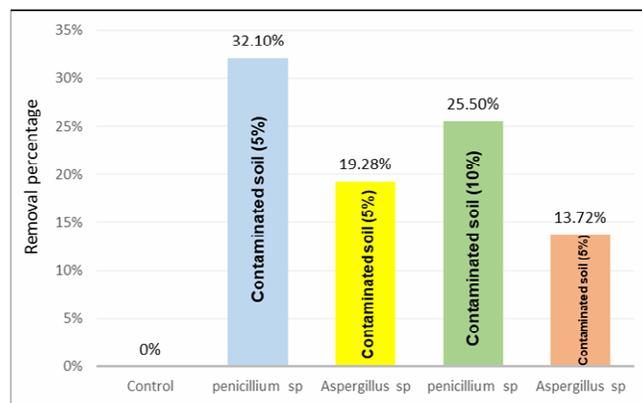
Also the all results showed that whenever an increase of crude oil concentrations in soil samples is accompanied with a decrease of removal percentage of (PAHs) by *Penicillium sp.* and *Aspergillus sp.* because hydrocarbon pollutants act to inhibit the enzymatic activity of microorganisms as the level of pollution increase (Alrumman *et al.*, 2015). These results were agreed with (Hadibarata and Tachibana, 2009) who mentioned that an increase of crude oil concentrations effects on decomposed quantities from it because the ability of fungi to break down hydrocarbons is inversely affected with the concentrations of crude oil.

The reason for (PAHs) concentrations decline from soils samples contaminated with crude oil after injecting fungal genera (*Penicillium sp.*, *Aspergillus sp.*) to the soil samples because these fungi use hydrocarbons as the sole source of carbon and energy to build their cell wall and

increase their biomass and subtract carbon dioxide and water as a final product.

Fungi genera have an effective ability to break down hydrocarbon compounds into small parts by their extracellular enzymatic systems that secrete special enzymes to break down hydrocarbon compounds (Mohsenzadeh *et al.*, 2012; Vanishree *et al.*, 2014). Humic acid added with fungal genera (*Penicillium sp.*, *Aspergillus sp.*) to soils samples had a great effect in reducing the concentration of (PAHs) compounds because it is considered as an effective adsorption factor for organic pollutants, including hydrocarbons. In addition, it increases the ionic exchange capacity of the added medium and provides the necessary nutrients to the growth and spread of microorganisms in the soil (Pukalchik *et al.*, 2019).

Figure (5) showed the ability of the fungal genera (*Penicillium sp.*, *Aspergillus sp.*) to degrade the (PAHs), the results showed a clear difference between two fungal genera in breaking down efficiency of (PAHs), therefore the removed percentage of (PAHs) is different too. *Penicillium sp.* showed higher efficiency in degrade (PAHs) compounds of crude oil for both polluted concentrations (5%, 10%) and removal rate was 32.10% and 25.50%, respectively, while *Aspergillus sp.*, was lower efficiency to degrade (PAHs) compounds at a rate of 19.28 % For concentration (5%) and 13.72% for concentration (10%). These results are different from observation of (Sari *et al.*, 2019) during his study on the efficacy of fungal genera (*Penicillium sp.*, *Aspergillus sp.*) in degrade (PAHs) compounds for crude oil samples taken from Siak Petroleum Company in Riau/Indonesia. His results showed that *Aspergillus sp.* have higher efficiency in the degrade (PAHs) than *Penicillium sp.*. All these results indicate that the biodegradation of crude oil depends on the type of fungus genera and concentration of crude oil (Ghanem *et al.*, 2016).



**Fig. 5:** Comparing the removal percentage of (PAHs) by the fungal genera from soil polluted with crude oil.

## Conclusions

Fungi organisms have the ability to survive in soils contaminated with crude oil especially fungal genera (*Penicillium sp.*, *Aspergillus sp.*) because they have extracellular enzyme systems that degrade hydrocarbon compounds as a sole source of carbon and energy to build their cell wall. Fungi Can be used in bioremediation of soils contaminated with crude oil and enhancing its ability to degrade hydrocarbons by adding some stimulation to its metabolic activity such as humic acid.

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