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USING OF FILAMENTOUS FUNGI IN BIODEGRADATION OF OIL WASTE CONTAMINATED SOIL

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ABSTRACT

This study investigated the ability of four fungi isolated from oil polluted soil to utilize Petroleum hydrocarbon. The fungal isolates obtained in this study *Aspergillus niger*, *Aspergillus fumigatus*, *Fusarium avanecium* and *Penicllium funiculosum* were found to be more predominant in the polluted soil. In the present study, significant differences in the percent of oil degrading fungi were evident among the time of biodegradation. Dry weights of utilizing Petroleum hydrocarbon as a carbon and energy source was determined. There were no significant increased in dry weights of fungi as the 7 days of incubation. The highest percentage loss of Petroleum hydrocarbon concentration by the axenic cultures of fungi was 95% with *A. niger* after 28 days of treatment. The highest percentage loss of Petroleum hydrocarbon concentration by the mixed cultures of fungi were 90% with A. *niger and A. fumigatus*, but the lowest loss of Petroleum hydrocarbon calculated in mixed four fungal strains (*A. niger, A. fumigatus, P. funiculosum* and *F. avaneceium*) to 70%.

KEYWORDS: pollution, petroleum hydrocarbon, biodegradation, fungi.

1. INTRODUCTION

The term Petroleum has been used as asynoym to crude oil, Crude oil, a dark sticky liquid, is a complex mixture of varing molecular weight which is used for the preparation of Petroleum products.[1] Crude oil contains more than 30 parent polyaromatic hydrocarbons (PAHs), and it contains hundreds of different hydrocarbon compounds such as Paraffins, naphthenes, aromatics as well as organic sulfur compounds, organic nitrogen compounds and oxygen containing hydrocarbons (Phenols). Some fractions of crude oil are toxic for living organisms. Microbial breakdown of hydrocarbon pollutants is generally a very slow proceed,

but it could be optimum biodegradation can only occur if the right environmental conditions such as PH, temperature, nutrients and relevant microbial consortia are present.[2], [3] Showed that the petroleum did not persist for long periods in the most soils even when relatively large quantities of petroleum have spilled. This is probably due to large part to the initial degradation by the action of sunlight followed by microbial attack when the oil sinks. *Fusarium sp*. F092 was isolated based on the ability to degrade Chrysene under saline conditions.[4] This ability of fungi due to their ability to synthesize relatively unspecific enzymes involved in cellulose and lignin degradation, which are capable of degrading high molecular weight, complex or more recalcitrant compounds, including aromatic structures.[5]

The aim of this work is to determine the ability of indigenous fungi to utilize crude oil as carbon source and for growth thus degrading both petroleum fractions in axenic and mixed cultures.

2. MATERIAL AND METHODS

2.1. Samples of Crude Oil

Petroleum hydrocarbon was supplied by AI-Dora refinary in Iraq. It was transferred to laboratory in dark glass bottle closed tightly and kept in a cold and dark place until to use.

2.2. Collection of Soil Samples

Non- contaminated soil samples were obtained from a cultivation area near AI-Dora refinary pipes during 2017. All samples of soils were taken randomly from upper surface of soil at depth 5-10 cm. The samples were then transferred to laboratory in sterile nylon sacs. The soil was air dried for a week and then sieved through 2mm mesh.

2.3. Selection of Fungal Strains

Soil fungi were estimated by soil dilution plate count method[6] every week and compared with zero time. From each sample, 1 g of soil was dissolved in 9 ml of distilled water and serial dilutions were prepared for each sample. Diluted samples were transferred to petri dishes and then mixed with potato dextrose agar (PDA) media containing chloaramphenicol (250 mg/L). The Petri dishes were incubated in incubator with 28°C for seven days. Then, different fungal colony was isolated and cultured separately in PDA.[7] Fungal specimens were examined under light microscope after preparations and identified using morphological characters and taxonomical keys provided in the mycological keys.[8]

2.4. Determination of the Fungal Growth Ability under Petroleum Hydrocarbon Pollution

The growth assay was used to find the resistant fungal species to crude oil contamination of the soil. The assay was conducted by comparing the growth rates of fungal strains, as colony diameter, on the crude oil contaminated and controls petri dishes. Test dishes were prepared by adding crude oil to warm PDA solution. In order to have 2% concentration of Petroleum hydrocarbon in all plates, the solution was thoroughly mixed with a magnetic stirrer, right before it was added to the plates. Pure PDA was used in control plates. All dishes were incubated with 7 mm plugs of fungal mycelia taken from agar inoculums plate. The dishes were incubated at 28°C in an incubator. Fungal mycelia extension on the plates (colony diameter) was measured using with measuring tape after 7 days and compared with control plates. Determination of dry weight of mycelia of fungal strains by harvested after 7 days incubation in flasks containing liquid mineral salts media amended with petroleum hydrocarbon and compared with other flasks without containing petroleum hydrocarbon (control) on filter paper by filtration and dried in the oven Memert-854 (Schwabach- Holand) with 85°C.

2.5. Extraction of Crude Oil from Soil

The extraction of crude oil from soil was conducted according to the methods used by[9] with slight modified. Two grams of soil was mixed with 10 ml of CH_2Cl_2 and shaken firmly. The sample was centrifuged 5000 g for 10 min to precipitate the soil, and the solvent phase was removed. This solvent extraction was repeated twice. The solvent was vaporized during 24 h and the amount of oil was measured using the gravimetric method and its reduction was compared with zero time samples. Two samples from each replicate were taken for crude oil extraction and further preparations. After the oil was extracted using the mentioned method, the extract residue was dissolved in 5 ml n-hexane and filtered. The sample was loaded to 1x 25 cm column filled with 20 cm Florsil and 5 cm anhydrous Na_2SO_4. The column was pre-washed with n-hexane. 30 ml of n-hexane was used as mobile phase to release aliphatic fractions and then 30 ml of n-hexane/ dichloromethane (1:1, v/v) was used to release aromatic fractions. The aromatic fractions were collected and the solvent was evaporated. The residue was weighed to determine the amount of total aromatic fractions in each sample. The residue was dissolved in 5 ml acetonitrile and analyze by gas chromatography.

2.6. Biodegradation Studies and TPH (Total Petroleum Hydrocarbons) Extraction

Growth and degradation studies over a time course were carried out using[10] method, 2 ml of AI-Dora crude oil (as a sole source of carbon and energy)/ 98 ml mineral salts medium in 250 ml flasks. The liquid mineral salts media then inoculated with 7mm disk from the mycelia of the old 7 days fungi colony isolated. The control flasks were not inoculated with mycelia of fungi isolated.

All flasks were covered with non- absorbent cotton wool and incubated at 28°C an incubator. The flasks were shaken manually at regular intervals to allow adequate mixing and homogeneity of the contents. The experimental setup was monitored for a period of 28 days. After 7 days of time interval, the flask was taken out and microbial activities were stopped by adding 1% 1N HCL for extraction of crude oil 50 ml culture broth was mixed with 50ml petroleum ether: acetone (1:1) in to a separating funnel and was shaken vigorously to get a single emulsified layer. Acetone was then added and shaken gently break the emulsification, which resulted in three layers. Top layer was a mixture of petroleum ether, crude oil and acetone; clumping cells make the middle layer and the bottom aqueous layer contains acetone, water in soluble form. The lower two layers were separated out while top layer containing petroleum ether and acetone was taken out in a clean beaker. The extracted oil was passed through anhydrous sodium sulphate to remove moisture.

The petroleum ether and acetone was evaporated on a 70°C water bath to approximately 1 ml. The gravimetric estimation of residual oil left after biodegradation was made by weightening the quantity of oil in a tared beaker. The percentage degradation of the crude oil was determined as described by[11]

% degradation= a-b / a x100

Were a: is the weight of crude oil control, b: is the weight of crude oil remaining in the each case. After weighing the quantity of oil in a beaker, the beaker rinsed twice with 2 ml methylene chloride. The rinses were added to vial and the total n- alkanes and aromatic concentrations were determined by Gas chromatography (GC-FID Shimadzu 2014) with a Tc-5 capillary column (length: 30 m, id: 0.24 mm). The carrier gas was helium delivered at a constant rate of 1.5 ml min- 1 with a column pressure of 100 KPa and interface temperature of 280°C The temperature program was started at 60°C and increased at 10°C min-1 to 280°C were it was maintained for 10-20 min to allow lake eluting compounds to exit the column. The injection volume was 2 µL and the injector temperature was maintained at 280°C.

2.7. Statistical Analysis

The present study conducted an ANOVA (analysis of variance) which was performed on all the treatments and done using the SPSS (version 10.0) package to determine whether or not, a significance difference.

3. RESULTS AND DISCUSSION

3.1. Isolated Fungi

Study on the fungal species showed that *A. niger, A. fumigatus, F. avaneceium and P. funiculosum* were the common fungi, with high frequency in the petroleum polluted soil (Table 1), Table 1 explains that the frequency of *A. niger, F. avaneceium* reached to 100% and the frequency of *A. fumigates, P. funiculosum* reached to 83%. But in the same time the other fungi frequency reached to 16-33%, these results refer the adaption of all fungal strains above to petroleum compounds and degradation a wide range to these compounds.[5,12] It seems that petroleum pollution could not inhibit the growth and variation of fungal strains in petroleum polluted soil. It seems that the fungal species used oil compounds as nutrients and crude oil pollution cause to increase fungal growth.[13]

In the same time the organic compounds in soil were activated increase the growth of fungi and increases excreted extracellular enzymes and decrease of soil pH more than in liquid media and finally increases in biodegradation of crude oil.[12], [13] Reported that *A. flavus* and *P. notatum* are capable of growth and utilize the crude oil more than the other tested fungi.

Table (1): Fungal strains isolated from soil contaminated with 2% crude oil.

Numbers of fungal species appear	Fungal species	Frequency %
6	*A spergillus niger*	100
5	*A.fumigatus*	83
2	*A. flavus*	33
1	*A. versicolor*	16
2	*alternaria alternate*	33
6	*Fusarium avaneceium*	100
5	*Penicillum funiculosum*	83
1	*Rhizopus stolinifer*	16

3.2. Fungal Growth Ability under Crude Oil Pollution

The growth ability of the isolated fungal strains was carried out under 2% concentration of crude oil and was expressed as diameter of the colony (Table 2). This table shows that the all above-mentioned fungi were resistant to crude oil pollution. Among the studied fungi, *A.*

niger showed the highest resistance to 2% crude oil pollution (with 8.5 cm diameter of colony after 7 days growth), and three fungal strains including *F. avaneceium* (5.9 cm), *A. fumigatus* (4.5 cm) and *P. funiculosum* (3.6 cm) were also relatively resistant ones. The colony diameters were determined after 4 and 7 days in the 2% concentration of crude oil polluted PDA media.

Table (2): Effect of crude oil on colony diameter to fungal strains.

Fungi	concentration %	4th day / c	7th day /cm
A. niger	**2**	**8.5**	**8.5**
	Control	**7.6**	**8.5**
A. fumigatus	**2**	**0.8**	**4.5**
	Control	**4.3**	**6.7**
F. avaneceium	**2**	**5.1**	**5.9**
	Control	**6.7**	**7.3**
P. funiculosum	**2**	**0.9**	**3.6**
	Control	**0.9**	**2.3**

In the same time the results showed that all above mentioned fungi were resistant to crude oil pollution when the determined dry weight of these fungal strains. Among the studied fungi, *A. niger* showed the highest resistance to 2% crude oil pollution (with 1.20 gm dry weight of mycelia after 7 days growth) and three fungal strains including *F. avaneceium* (0.81 gm). *A. fumigatus* (0.61 gm) and *P. funiculosum* (0.56 gm) were also resistance when compare with control (Table 3). No significance differences were observed in dry weight with crude oil during utilization by all mycelial fungal after 7 days of incubation.

Table (3): Effect of crude oil on mycellial dry weight to fungal strain.

Fungi	Control/gm	dry weight/gm
A. niger	**0.64**	**1.20**
A. fumigatus	**0.51**	**0.61**
F. avaneceium	**0.39**	**0.81**
P. funiculosum	**0.02**	**0.56**

The same result was obtained by[14], in their study on the changes of mycelium dry weight of *A. niger, A. flavus, Curvularia lunata, Rhizopus sp*. and *Trichoderma sp*. on media containing different concentrations of crude oil (0.5, 1.0, 2.0 ml), the results showed in this study that *Trichoderma sp*. exhibited an increasing mycelium dry weight with increase in crude oil concentrations while *A. niger* dry weight reached to 2.68 mg in 2ml concentration, but the lowest dry weight was calculated with *Rhizopus sp*. (with 2.11 mg).

In the present study, a significance differences in he percent of oil degrading fungi were evident among the time of biodegradation were. biodegradation (Figure 1). This Figure explain that the highest percent of biodegradation in soil was reached to 95% after 21 days, but the lowest percent of biodegradation was recorded in zero time.

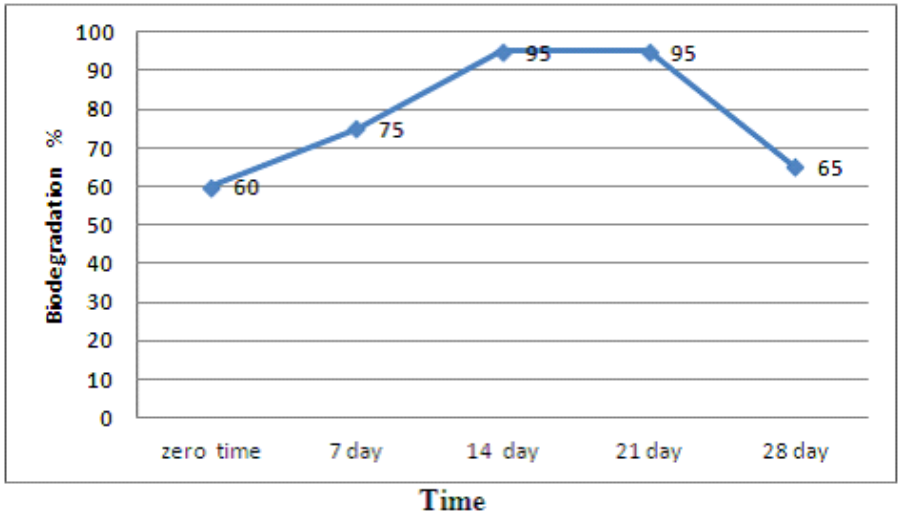

Figure (1): Average of biodegradation in soil after contaming with crude oil.

On the other hand, there will also be increasing in numbers of certain microorganisms especially those capable of degrading the hydrocarbons.[15] A higher numbers of fungi reached to 14.3 x10 5 in soils after 28 days incubation, but the lowest numbers of fungi reached to 1.6 x105 in zero time (Figure 2).[16] Had shown that a change in the number of microbes during biodegradation was the simplest way to measure their activity. The same result was obtained by[17] in this study shown that the numbers of oil degrading fungi were more than the numbers of oil degrading bacteria in the soil and changes in the flora of soil fungi following oil waste application. In spite the growth conditions available in the present study were different from those present in the natural environment therefore, it was difficult to interpret the counts in the terms of natural situation. However, the microbial counts were a direct indicator of petroleum biodegradation activity.[18]

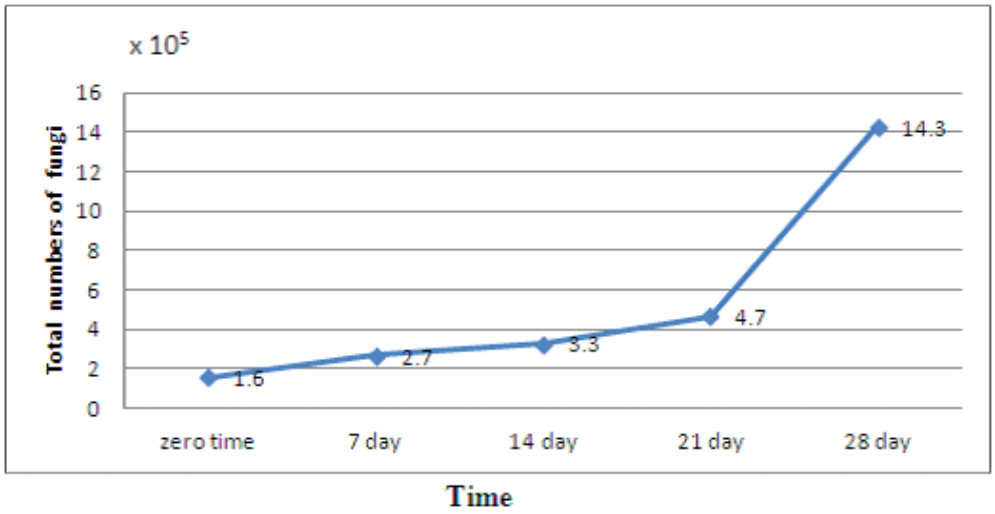

Figure (2): Total numbers of fungi isolated from soil after contaming with crude oil.

3.3. Biodegradation

The residual crude oil in soil resulting from biodegradation was measured by gas chromatography technique (GC). After 28 days growing of fungal strains in crude oil contained soil, biodegradation of crude oil was determined (Figure 3). This Figure revealed disapper many peaks when compared with untreated crude oil (Figure 4). The results showed that the axenic culture of fungi degraded the crude oil in mineral salts media. The highest percentage loss of crude oil concentration by the axenic cultures of fungi was 95, 75% by the fungi *A. niger, A. fumigatus*) after 28 days of biodegradation (Table 5, Figure 5, Figure 6) and these Figures showed disapper large number of bands when compared with untreated crude oil (control) (Figure 4). This result was similar to the findings of[19] which showed that *Aspergillus versicolor* and *Aspergillus niger* exhibited biodegradation of hydrocarbons higher than 98%. The result was obtained by[20] in their study obtained that the fungus *Penicillium chrysogenum* loss of crude oil concentration percentage in axenic culture to 76% after a month period. However[21] found that fungi *Penicillium funiculosum* and *Aspergillus sydowii* were loss TPHs concentration to 86, 81% respectively, and the same result was obtained by[22] in their study reported that *A. fumigatus* cultures were removed over 80% of PAHs after 120 days of exposure.

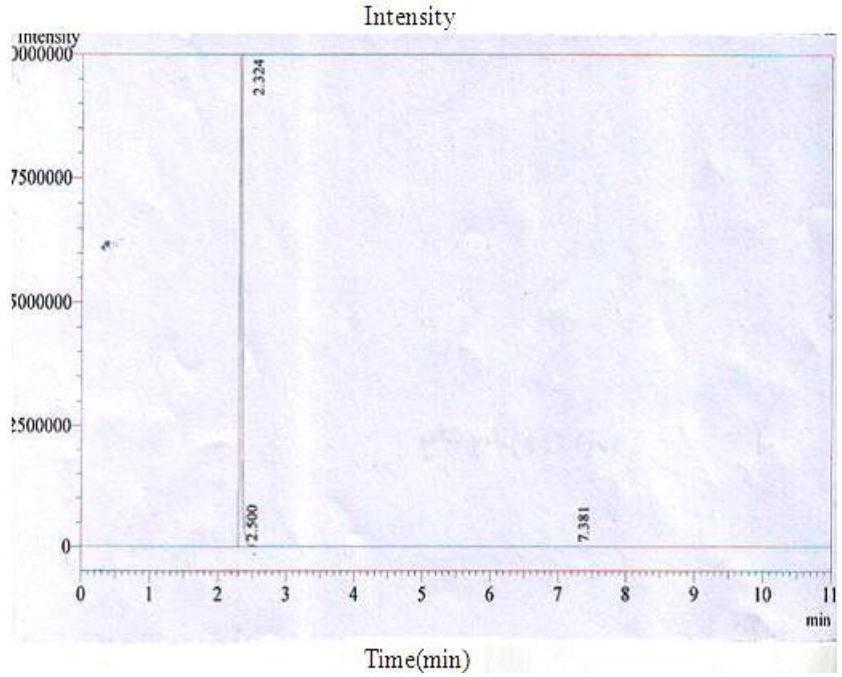

Figure (3): GC chromatogram of crude oil in a soil after 28 day incubation.

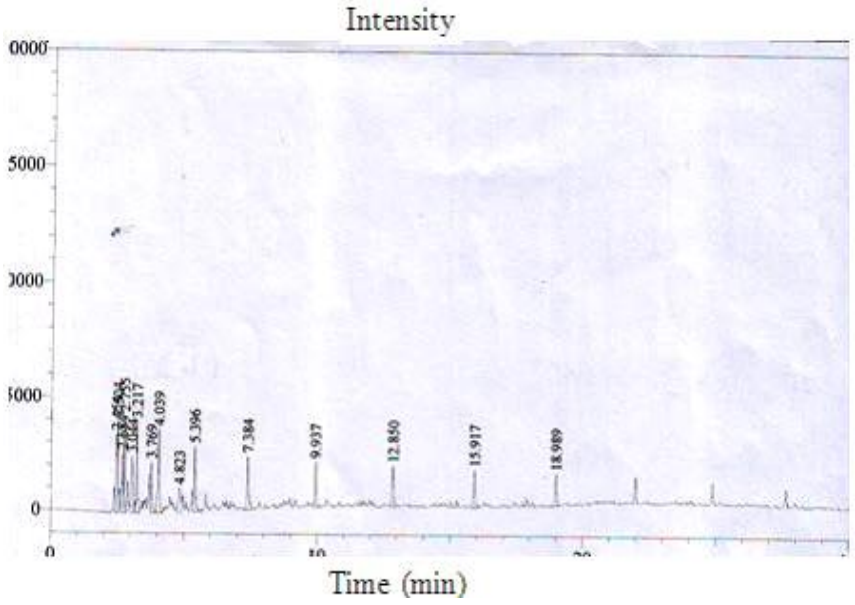

Figure (4): GC chromatogram of untreated crude oil (control).

Peak labeled	Compounds
2,7	Naphthaline and PAHs.
3	Nitrogen, sulfur and oxygen containing Hydrocarbon.
4,5,9,12,15,18	Paraffin and cyclane.

Table (5): Biodegradation of crude oil by using gravimetric method.

Fungi	Time (days)	Percent of biodegradation %
	7	55
A. niger	14	60
	21	60
	28	95
	7	60
A. fumigatus	14	65
	21	75
	28	75
	7	35
F. avaneceium	14	45
	21	55
	28	55
	7	25
P. funiculosum	14	35
	21	60
	28	65

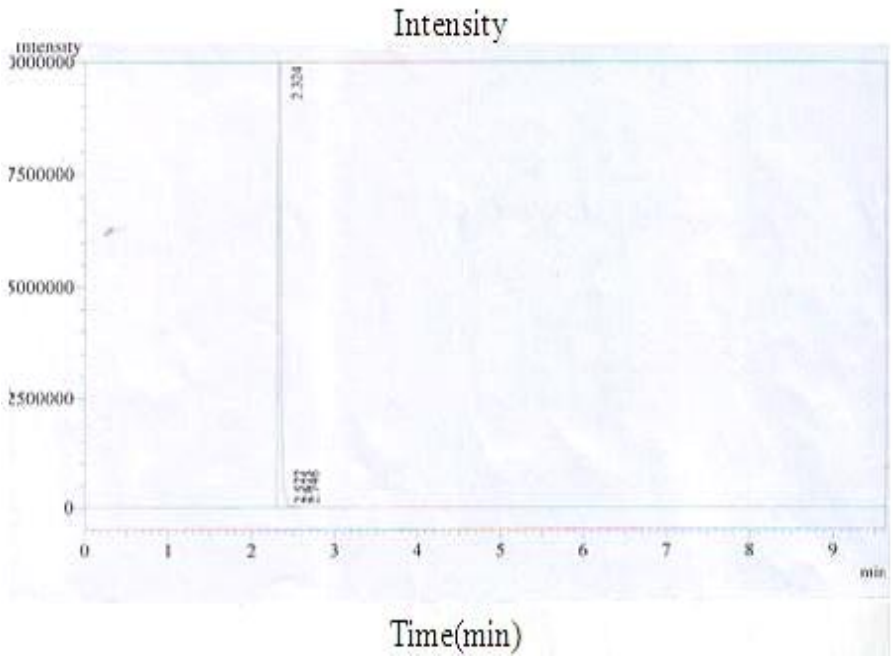

Figure (5): GC chromatogram of crude oil after a 28 day exposure to a pure culture of *A. niger*.

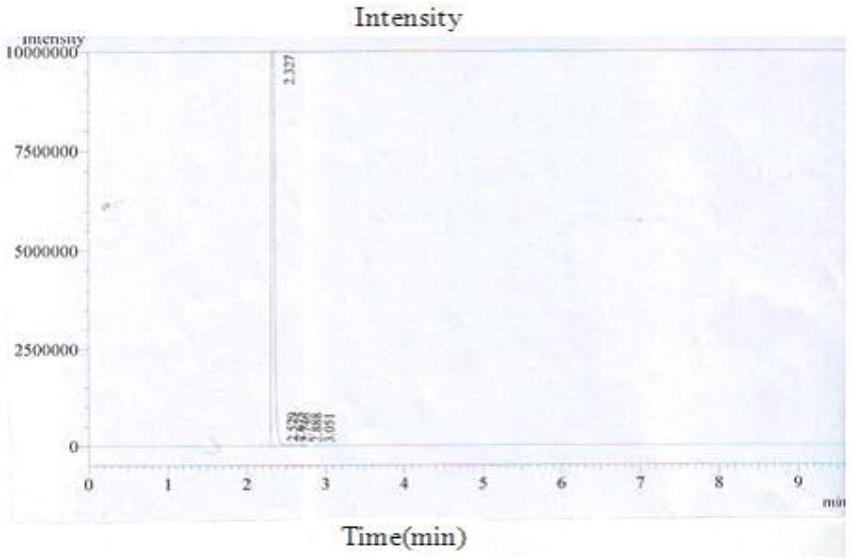

Figure (6): GC chromatogram of crude oil after a28 day exposure to a pure culture of *A. fumigates.*

The highest percentage loss of crude oil concentration by the mixed cultures of fungi was 90% with *A. niger and A. fumigatus* after 28 days of biodegradation (Table 6, Figure 7), This Figure refer disapper large quantity of crude oil after incubation and in the same time this Figure refer large fragmentation of crude oil. these greater capacity to remove crude oil due to the adaptation of these fungi to the pollutant composition, as well as to the enzymatic systems of the fungi[23] in the vitro growth test of the isolated fungi showed a species-specific response. All of the studied fungal strains were able to growth in 2% v/v oil pollution and therefore could be useful for the remediation of light soil pollution. Results of the research showed that the amounts of crude oil were decreased in the presence of the studied fungal strains considerably. It means that the fungal strains were able to degrade crude oil and consumption of its components. Mycelial organisms can penetrate insoluble substances such as crude oil and this increase the surface are available for microbial attack[24] but the lowest loss of crude oil calculated in mixed four fungal strains (A. *niger* + *A. fumigatus* + *P. funiculosum* + *F. avaneceium*) to 70% after 28 days of biodegradation (Table 6). (Figure 8) observed disappeared large number of bands when compared with untreated crude oil (control) (Figure 4). (Figure 9) observed disapper large quantity of crude oil after incubation and in the same time this Figure refers low fragmentation of crude oil. These result due to the reduce of fungal growth because many facters such as the competition and antagonasms.[24] The results were obtained by[25] In their study reported that mixed four fungi isolated exhibited decreases in biodegradation of crude oil.

Figure (7): Biodegradation of crude oil by mixed culture of *A. niger* and *A. fumigates* after 28 day incubation.

Table (6): Biodegradation of crude oil by using gravimetric method.

Fungi	Time (days)	Percent of biodegradation %
	7	5.0
An +Af	14	55
	21	60
	28	90
	7	45
An + Pf	14	60
	21	60
	28	75
	7	55
An + Fa	14	60
	21	75
	28	75
	7	60
Af+Fa	14	60
	21	60
	28	75
	7	30
Af+Pf	14	65
	21	75
	28	85
	7	50
Pf+Fa	14	60
	21	65
	28	80
	7	60
An + Af+ Pf+ Fa	14	65
	21	70
	28	70

An: *Aspergillus niger* Af: *Aspergillus fumigates* Fs: *Fusarium avaneceium* Pf: *Penicillium funiculosum.*

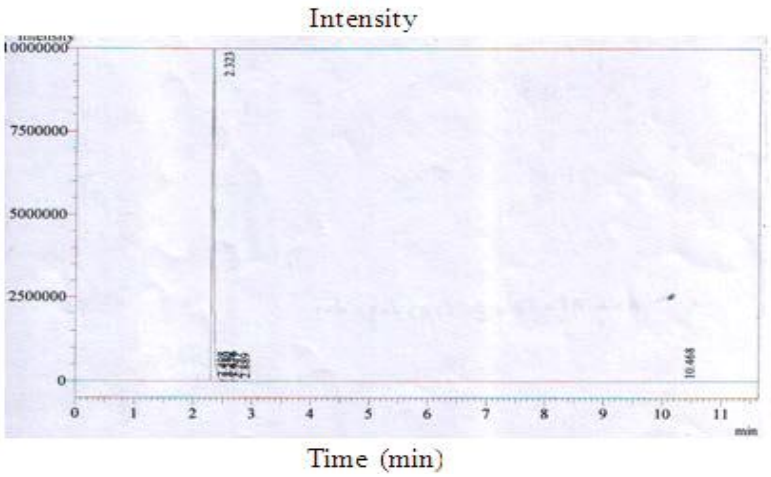

Figure (8): GC chromatogram of crude oil after a 28 day ecposure to a pure culture of *A. niger, A. fumigates, F. avaneceium* and *P. funiculosum.*

Figure (9): Biodegradation of crude oil by *A. niger, A. fumigates, F. avaneceium* and *P. funiculosum* after 28 day incubation.

4. CONCLUSION

The data obtained in the present study investigation advanced our knowledge of petroleum hydrocarbon resistance in mixed culture of *A. niger and A. fumigates* isolated from soil and may make promising candidates for further investigations regarding their ability to remove petroleum hydrocarbon from contaminated environments.

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