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Ability of Some Soil Fungi in Biodegradation of Petroleum Hydrocarbon

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Abstract This study investigated the abilities of four fungi isolated indigenously polluted soil to utilize Petroleum hydrocarbon. Of all the fungal isolates obtained in this study Aspergillus niger, Aspergillus fumigatus, Fusarium solani 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 and pH of utilizing Petroleum hydrocarbon as a carbon and energy source. There was no significant increased in dry weights of fungi as the 7 days of incubation. The pH values decreased in fungal cells metabolized after 28 days of incubation. The PH value in F. solani culture was above when compare with another fungi and these PH reached to 5.8 after 28 days 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. solani) to 70%.

Keywords: *pollution, petroleum hydrocarbon, biodegradation, fungi*

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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). The leakage of crude oil into soil damages the biological systems residing in the soil, including microorganisms and plants [2]. 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 [3], as well as the pollution of petroleum hydrocarbons caused a major changes in the physical and chemical properties of the soil. It is an environmental concern, because contaminated soils may be unsuitable for agricultural, industrial, or recreational use and also potential sources for surface and ground water contamination [4]. [5] 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 [6], and can degrade the aliphatic fraction in crude oil [7]. However whether it degrades all fractions and aliphatic degradative pathway in crude oil are not yet clearly understood. These 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 [8]. In Iraq, oil fields which have a wide distributions in many sites linked together by a wide net of transfer pipes to carry the oils to all refiners and exporting ports in north and south of Iraq but the accidents destroy these pipes so, a large quantity of oils run over the soil and cause soil pollution.

The crude oil molecules have a limited move and attach strongly at the soils surface therefore we must use the biological treatment to remove these pollutants because the crude oil hydrocarbons is staying at the upper six foots of the soil surface near the plants roots [9].

There are other factors which play important role in increasing the activation of the microorganisms and degradation of crude oil such as the mixing of contaminated and uncontaminated soil.

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-Nasiriya (AI-Katea field) 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 the AI-Nasiriya city refinery pipes during 2013. The refinery may encounter risk of contamination by the products of refinery during oil transportation or pipe line leakage.

In the north zone of this area, there is a wide salty desert and a big salt marshes in AL-Nasirya city. On windy days, the salty soil from the desert moves to the cultivation area by wind. As the result the salty soil covers the surface of the cultivation area and mixes with cultivation soil. The salty area may be widening in the next decades. 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. The soil texture was determined by hydrometer methods and the pH (Hydrogen ion concentration) was determined by dissolving 1gm of soil in 5 ml of distilled water. The mixture was stirred well and allowed to stand for 30 minutes.

Petroleum hydrocarbon was added to the soil in the final concentration of 2% (w/w) and mixed to make uniform contaminated soil and incubated for 28 days under laboratory conditions.

2.3. Selection of Fungal Strains

Soil fungi were estimated by soil dilution plate count method [10] 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 MIR 153 (Sanyo-Japan) with 25°C for seven days. Then, different fungal colony were isolated and cultured separately in PDA [11]. Fungal specimens were examined under light microscope after preparations and identified using morphological characters and taxonomical keys provided in the mycological keys [12].

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 were conducted by comparing the growth rates of fungal strains, as colony diameter, on the crude oil contaminated and control 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 5 mm plugs of fungal mycelia taken from agar inoculums plate. The dishes were incubated at 25°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. PH was determined with pH meter HI 98103 (Hanna- Germany).

2.5. Extraction of Crude Oil from Soil

The extraction of crude oil from soil was conducted according to the methods used by [13] with slight modified. Two grams of soil was mixed with 10 ml of CH_2Cl_2 and shaken firmly. The sample was centrifuged ICE Centra-8R (USA) 3000 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 sample.

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 relase 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 [14] method, 2 ml of AI-Nasiriya 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 5mm 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 25°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 wile 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 BS11 (Jeo Tech- Korea) to approximately 1 ml. The gravimetric estimation of residual oil left after biodegradation was made by weighing the quantity of oil in a tared beaker. The percentage degradation of the crude oil was determined as described by [15] % 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 tared 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. solani 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. solani 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 result refer the adaption of all fungal strains above to petroleum compounds and degradation a wide range to these compounds [8,16]. It seem 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 [17]. And 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 [16]. [18] 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 strain isolated from soil contaminated with 2% crude oil

Numbers of fungal species appear	Fungal species	Frequency %
6	A spergillus niger Tighem	100
5	A.fumigatus Fresenius	83
2	A. flavus Link	33
1	A. versicolor (Vuill) Triqboschi	16
2	Alternaria alternate Keissler	33
6	Fusarium solani Link	100
5	Penicillum funiculosum Thom	83
1	Rhizopus stolinifer (Ehrenb: Fr.) Vuill	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 show that the all above-mentioned fungi are 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. solani (5.9 cm) , A. fumigatus (4.5 cm), P. funiculosum (3.6 cm) and 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. And in the same time the results showed that all above mentioned fungi are 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. solani (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 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. solani	2	5.1	5.9
	Control	6.7	7.3
P. funiculosum	2	0.9	3.6
	Control	0.9	2.3

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

Fungi	Control/cm	Treatment/cm
A. niger	0.64	1.20
A. fumigatus	0.51	0.61
F. solani	0.39	0.81
P. funiculosum	0.02	0.56

On the other hand, there will also be increasing in numbers of certain microorganisms especially those capable of degrading the hydrocarbons [19]. 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. A higher numbers of fungi reached to 14.3 x10 5 in soil after 28 days incubation, but the lowest numbers of fungi reached to 1.6 x105 in zero time (Figure 2). [20] 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 [21] 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 [22].

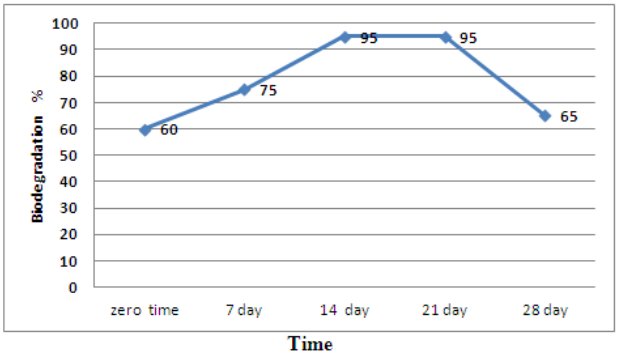

Figure 1. Average of biodegradation in soil after treatment with crude oil

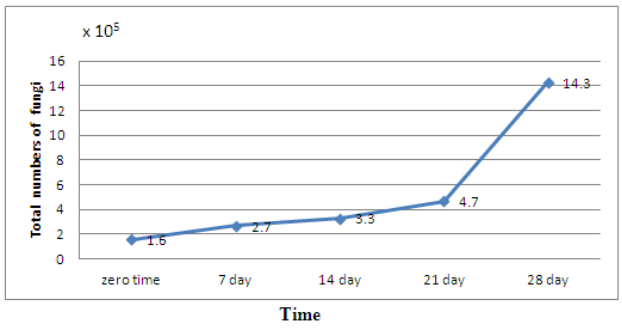

Figure 2. Total numbers of fungi isolated from soil after treatment with crude oil

The same result was obtained by [18], 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) . No significant differences were observed in the changes in pH values obtained with crude oil during utilization by all fungal isolates from 0hour to the 28th days of incubation. P. funiculosum had the lowest pH of 5.1 after 28 days of incubation, but the F. solani had the highest pH value of 5.8 (Table 4) . The reduction in pH in cultures fluid within 28 days incubation period confirmed chemical changes of the hydrocarbon substrates which must have been precipitated by microbial enzymes [23]. Hydrogen ion concentration is amajor variable govering the activity and composition of fungi. Many species can metabolize over a wide pH range from the highly acidic to alkaline extremes. Thus the insensitivity of the fungi to high hydrogen ion concentration and narrow pH range of most bacteria account for the sharp drop in pH. Microbial degradation of hydrocarbons often leads to production of organic acids and other metabolic products [24]. Thus organic acids probably produced account for the reduction in pH levels [25].

Table 4. Changes in pH produced by fungal strains during utilization of crude oil

Time (days)	Fungi			
	A. niger	A. fumigatus	F. solani	P. funicuolosum
0	7.0	7.0	7.0	7.0
7	6.2	6.3	6.8	6.4
14	6.1	6.1	6.4	6.2
21	5.6	5.8	6.0	5.2
28	5.6	5.7	5.8	5.1

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 fungus 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 [26] which showed that Aspergillus versicolor and Aspergillus niger exhibited biodegradation of hydrocarbons higher than 98%. The result was obtained by [21] 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 [27] found that fungi Penicillium funiculosum and Aspergillus sydowii were loss TPHs concentration to 86, 81% respectively, and the same result was obtained by [28] in their study reported that A. fumigatus cultures were removed over 80% of PAHs after 120 days of exposure.

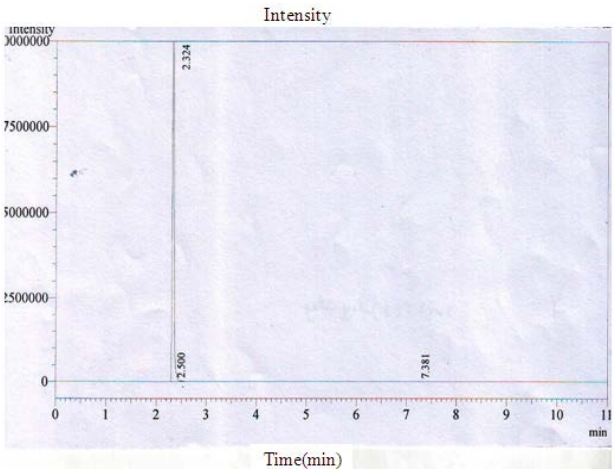

Figure 3. GC chromatogram of crude oil in a soil after 28 day incubation

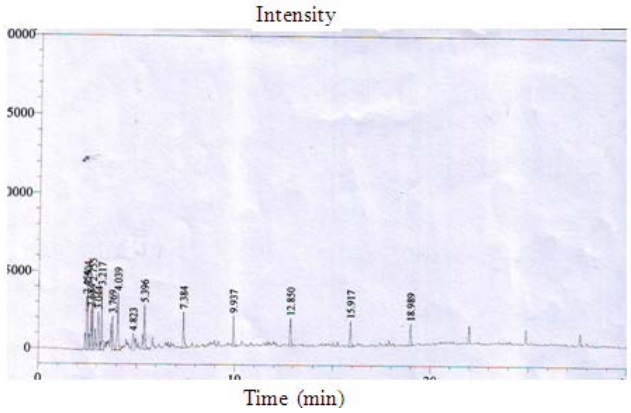

Figure 4. GC chromatogram of untreated crude oil (control)

Peak labeled	Compounds
2,7	Naphthaline and PAH_s.
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

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

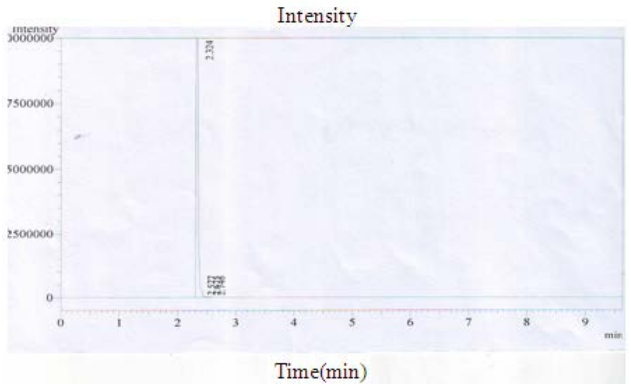

Figure 5. GC chromatogram of crude oil after a 28 day exposure to a pure culture of *A. niger*

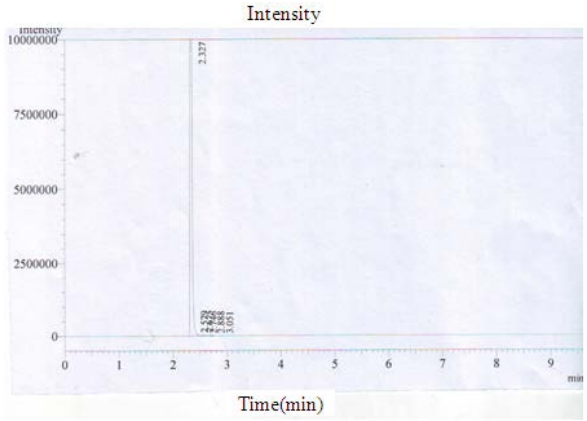

Figure 6. GC chromatogram of crude oil after a28 day exposure to a pure culture of *A. fumigates*

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 [27]. The in 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 [29]. but the lowest loss of crude oil calculated in mixed four fungal strains (A. niger + A. fumigatus + P. funiculosum + F. solani) 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 refer low fragmentation of crude oil. These result due to the reduce of fungal growth because many facters such as the competition and antagonasms [27]. The results were obtained by [30] In their study reported that mixed four fungi isolated exhibited decreases in biodegradation of crude oil.

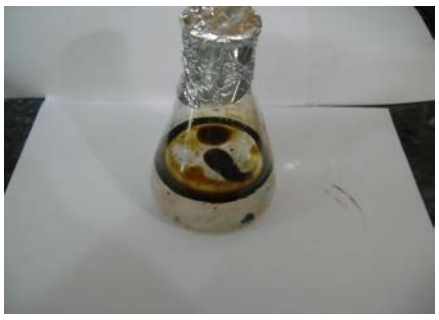

Figure 7. Biodegradation of crude oil by mixed culture of *A. niger* and *A. fumigates* after 28 day incubation

Table 6. Biodegradation of crude oil by using gravimetric method

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

An: Aspergillus niger
Af: Aspergillus fumigates
Fs: Fusarium solani
Pf: Penicillium funiculosum

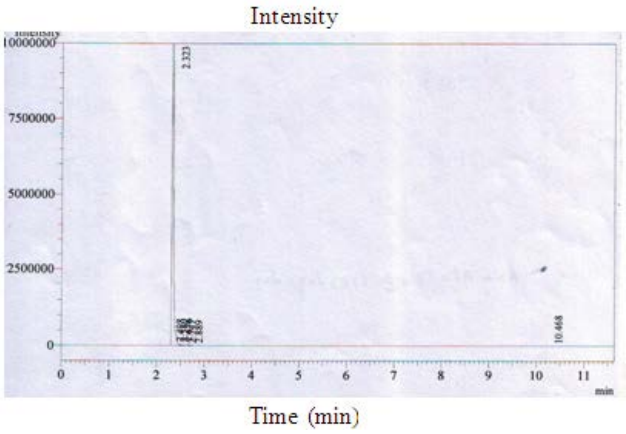

Figure 8. GC chromatogram of crude oil after a 28 day ecposure to a pure culture of A. niger, A. fumigates, F. solani and P. funivulosum

Figure 9. Biodegradation of crude oil by A. niger, A. fumigates, F. solani and P. funivulosum 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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