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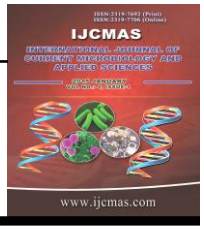

Original Research Article

Ability of some fungi isolated from a sediment of Suq-Al Shuyukh marshes on biodegradation of crude oil

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A B S T R A C T

Nineteen filamentous fungi isolated from the upper surface of sediments in four stations in Suq-Al Shuyukh marshes in Thi- Qar governorate, Iraq, three stations M_1, M_2, M_3 in AI-Sanaf marsh and M_4 in AI-Hammar marsh by using dilution method. The results showed that Deuteromycota were more dominant genera with 94.5% and the second dominant genera were Ascomycota with 2.5% and the third dominant genera were Zygomycota with 2.5%. *Aspergillus niger* and *Fusarium solani* were more frequancy with 100% in all samples, but *Aspergillus fumigates, Penicillium funiculosum* were 83% and the percent of *A. flavus* reached to 33%. The remaining 3 fungal species isolated with a very low frequency with 16%. The results showed that the different sites in present study were no high effect on fungi diversity in sediments of marshes because the similarity of environmental conditions in study sites. In the present study, significant differences were recorded in the ability of fungi, *A. niger*, *F. solani*, *A. fumigatus*, *P. funiculosum* to biodegradation of crude oil. The highest percentage loss of concentration of crude oil by the axenic cultures of *A .niger* reached to 95% after 28 days of treatment. But The highest percentage loss of concentration of crude oil calculated with mixed cultures of *A. niger* and *A. fumigatus* with 90%, but the lowest loss of concentration of crude oil calculated in mixed four fungal strains (*A. niger*, *A. fumigatus*, *P. funiculosum* and *F. solani*) with 70%.

Keywords

Biodegradation; crude oil; fungi; pollution ; sediments

Introduction

Southern marshes in Iraq were one of the big moister land locations in the world, distributed with *Phragmites* sp. and *Typha* sp. plants. Abdullah (1983) referred that these marshes were rich reservoir to many different organisms. Partow (2009) isolated three species of Ascomycetes from Phragmites in southern marshes' water in Iraq were *Zopfiella leucotricha*, *Z. karachiensis*, *Strattonia mesopotamica* (Abdullah *et al.*, 2010) isolated 67 species of fungi from the sediments of southern marshes in Iraq. In the same time Abdel-Hafez *et al.* (1977) isolated 92 species from salt marshes in Egypt. Muhsin and Booth (1987) were studied of associated fungi of six species in salt marshes plants in

Canada and isolated 26 species of fungi from Deuteromycota and three species from Ascomycota during this study, but Kohlmeyer and Volkmann-Kohlmeyer (1995) were isolated new species from Deuteromycota was *Trichoderma emedullar.*

Fungi play an important role in aquatic ecosystem beside other decomposers in the degradation of dead plant materials as well as materials from animal and transferred to basic elements in food chain Heald and Odum (1970). Moreover fungi and other microorganisms have the ability to degrade several pollutants including crude oil in the aquatic ecosystem and utilize them as a nutrient sources Davis and Westlake (1979). They may also metabolize such as pollutants to substrates with low harmful effect on the environment (Cerniglia *et al.,* 1991; Sutherlands, 1999; Boonchan *et al.,* 2000).

Crude oil consists of four main groups of hydrocarbons including aliphatics, aromatic, resins and asphaltines (Colwell and Walker, 1997). Some fractions of crude oil are toxic for living organisms. However various microorganisms are able to use some crude oil fractions are a sole carbon source and change these component to non- toxic materials such as CO2 and H2O (Ewis *et al.,* 1998), 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 (Chaineau *et al.,* 2000). Atlas (1981) 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. The individual aliphatic, olefinic and naphthenic compounds are mostly susceptible to attack by microorganisms Atlas and Bartha (1992), such as 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 (Potin *et al.,* 2004).

AL-Dossari *et al.* (2001) reported the capability of *Aspergillus terreus* and *Acremonium killense* strains isolated from the surface sediments of Shatt Al-Arab River in degradation of a mixture of five polycyclic aromatic hydrocarbons in laboratory. More recently, Al-Dossari (2008) reported high degradative ability displayed by isolates of *Aspergillus niger*, *A. terreus*, *Paecilomyces* sp. and *Acremonium* sp. isolated from sediments of southern marshes of Iraq against crude oil *in vitro*.

Materials and Methods

Six sediments samples were collected from three stations (M1, M2, M3) in Al-Sanaf and one station (M4) in Al-Hammar marshes during April 2012 (Fig. 1). The method used for collection of the sediment samples was the same as described previously by Hohnk (1972).

Isolation of fungi

One technique are applied to isolated

fungi from the surface (15–30 cm) depth of sediments samples by using dilution plate method Al-Nasrawi (2012). Four types of media were used for isolation of fungi, Potato dextrose agar (PDA), Malt extract agar (MEM), Yeast extract agar (YEA) and Mineral salts medium (MSM). Each medium were supplemented with 250 mg I-1 chloramphenicol to suppress bacterial growth. Plates and media were incubated at 25 ℃ in the dark. Single colonies were picked from the plates under a dissecting microscope and transferred to appropriate media to allow fungal development. Stock cultures were maintained on the potato dextrose agar slant, subcultured periodically and stored at 4 ℃. Mineral salts medium containing (gI-1): K2HPO4, 1.71; KH2PO4, 1.32; NaNO3, 0.42; MgSO4.7H2O, 0.42; CaCl2, 0.02 was used for the induction experiments. All media were autoclaved at 120 ℃ min.

General and specific taxonomic references were used for the identification of fungal species (Barnett, 1962; Ellis, 1971; Ellis, 1976; Raper and Fennel, 1973; Moustafa, 1982; Moubasher and Al-Subai, 1987; Pitt, 1991; Klich and Pitt, 1992. Crude oil at 2% was used as a carbon source for the biodegradation.

Biodegradation studies and TPH (Total petroleum Hydrocarbons) Extraction

Growth and degradation studies over a time course were carried out using method (Mittal and Singh, 2009). 2 ml of crude oil from Al- Nasiriya (Al-Katea field) in Iraq as a sole source of carbon and energy / 98 ml mineral salts media in 250 ml flasks. The liquid mineral salts medium 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 colony isolated. All flasks were covered with non- absorbent cotton wool and incubated at 25°C an incubator MIR 153 (Sanyo-Japan). 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 of culture broth was mixed with 50ml petroleum ether: acetone (1:1) in a separating funnel and was shaken vigorously to get a single emulsified layer. Acetone was then added and shaken gently to 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 mixed with crude oil 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 70co water bath Bs11 (Jeo Tech-Korea) to approximately 1 ml. The gravimetric estimation of 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 John and Okpokwasili (2012).

% degradation = a-b / a x100

Where, 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 : 30m, id: 0.24mm). 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, where 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.

Determination ability of mixed fungal isolate to degradation crude oil

Growth and degradation studies over a time course were carried out using method of Mittal and Singh (2009) too, but inoculated the liquid mineral salts media with mixed the old 7 day fungi isolated. The control flasks were not inoculated with mycelia of fungi isolated.

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.

Result and Discussion

Nineteen filamentous fungi isolated from the upper surface of sediments in four stations in Suq-Alshuyukh marshes in Thi-Qar governorate, Iraq (Table 1).

The results showed that Deuteromycota were more dominant genera with 95.5% included seventeen species belong to 4 genus, and the second genera were Ascomycota with 2.5%, included one species belong one genus and the third genera were Zycomycota with 25%, included one species belong one genus (Table 2). *Aspergillus niger*, *Fusarium solani* were more frequency with 100%, while *A. fumigatus* and *Penicillium funiculosum* were moderate frequency with 83%, but *A. flavus* and *Alternaria alternata* were low frequency with 33%.

The remaining 3 fungal species were isolated with a very low frequency with 16 % (Table 3). The differences in numbers of species isolated from sediments on media due to the faster growth of Deuteromycota when compare with Ascomycota and Zycomycota.

The results showed that the axenic cultures of fungi degraded the crude oil in mineral salts medium. The highest percentage loss of concentration of crude oil by the axenic cultures of *A. niger* and *A. fumigatus* with 95% and 75%, respectively after 28 days of treatment (Table 4, Fig. 2, 3) and these figures 2 and 3 showed disappearance of large number of bands when compared with control (untreated crude oil) (Fig. 4).

These results was similar to the findings of George-Okafor *et al.* (2009) which showed that *Aspergillus versicolor* and *Aspergillus niger* exhibited

biodegradation of hydrocarbons higher than 98 %. And the results in the present study were similar to the finding of Al-Dossari (2008) which showed that *A. niger* exhibited highly degradation of crude oil with 85.4% than other studied fungi.

In the same time the similar results were record by Farid (2012) in their study obtained that the fungus *Penicillium chrysogenum* loss the percentage of concentration of crude oil in axenic culture to 76% after a month period. The highest percentage loss of crude oil concentration by the mixed cultures of *A. niger* and *A. fumigatus* with 90% after 28 days of treatment (Table 5, Fig. 5), 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 Mancera-Lopez *et al.* (2007). The in vitro growth test of the isolated fungi showed aspecies-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. Crude oil consists of saturated and aromatic hydrocarbons and asphaltic compounds of varying molecular weight, complexity, and degree of susceptibility to microbial oxidation Raymond and Davis (1960). Mycelial organisms can penetrate insoluble substances such as crude oil and this increase the surface are available for microbial attack Davis and Westlake (1979).

The results showed that 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 treatment (Table 5, Fig. 6, 7). These results due to the reduction of fugal growth because many factors such as the competition and antagonisms (Mancera-Lopez *et al.*, 2007). And in the same time the statistical methods obtained significantly in biodegradation of crude oil by these fungi.

This result was similar to findings of Al-Dossari (2008) which show that mixed four fungi isolated exhibited decreases in biodegradation of crude oil. However, Mancera-Lopez *et al.* (2007) found that fungi *Penicillium funiculosum* and *Aspergillus sydowii* were loss TPHs concentration to 86, 81% respectively, and study of Okoro and Amund (2010) on the biodegradation potential of hydrocarbon, they had been shown that the fungus *Aspergillus fumigatus* can removed of PAHs with 80% after 120 days of exposure, and the same result was obtained by Colombo *et al.* (1996) in their study reported that *A. terres* and *Fusarium* sp. were the percent degradation to aliphatic compounds reached to 100 %. No significant difference was observed in the changes in pH values obtained on crude oil during utilization by all fungal isolates from 0h 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 after 28 days of incubation (Table 6).

The reduction in pH of the cultures fluid in flasks within 28 day incubation period confirmed chemical changes of the hydrocarbon substrates which must have been precipitated by microbial enzymes

Atlas and Bartha (1972). Hydrogen ion concentration is a major variable governing the activity and composition of fungi. Many species can metabolise 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 hydrocarbon often leads to production of organic acids and other metabolic products Nwachukwu and Ugoji (1995). Thus organic acids probably produced account for the reduction in pH levels Oboh *et al.* (2006).

Table.1 List of fungi isolated from the upper surface of sediments in different stations in Al-Sanaf and Al-Hammar marshes

Fungi species	Stations			
	M_1 *	M_2	M_3	M_4
A.niger Tighem	+	+	+	+
A.fumigatus Fresenius	+	+	+	+
A.flavus Link		+		+
A.versicolor (Vuill)Triqboschi		+		
A.candidus Link		+		
A.nidulans (Eidam) Vuill		+		
A.ustus Thom		+		+
A.wentii Wehmer		+		+
A.restrictus G.Smith		+		
A.kanagawaensis Nehira		+		
Alternaria alternate Keissler	+		+	
Fusarium solani Link	+	+	+	
Pencillium funiculosum Thom	+	+	+	+
P.brevicompactum Dierckx				+
P.raistrickii G.Smith				+
P.bilaii Chalabuda		+		+
Pencillium sp.				+
Phoma sp.				+
Rhizopus stolinifer (Ehrenb:Fr.)Vuill	+			

*M_1,M_2,M_3 :Al-Sanaf marsh,M_4 :Al-Hammar marsh.

Table.2 Frequancy of fungi genera by using pour plate

Fungi species	Genus number	Frequency %
Deuteromycota	4	94.5*
Ascomycota	1	2.5
Zycomycota	1	2.5

* Frequancy of fungi genera = Total number of fungi genera / Total number to all fungi genera x100

Table.3 Frequency of strains isolated from the upper surface of sediments

Fungal species	Numbers of fungal species appear	Frequency %
Aspergillus niger Tighem	6	100
A.fumigatus Fresenius	5	83
A. flavus Link	2	33
A. versicolor (Vuill)Triqboschi	1	16
Alternaria alternate Keissler	2	33
Fusarium solani Link	6	100
Penicillum funiculosum Thom.	5	83
Rhizopus stolinifer (Ehrenb:Fr.)Vuill	1	16

Table.4 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

Table.5 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+	7	60
Pf+Fs	14	65
	21	70
	28	70

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

Table.6 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

Fig.1 Location map of Al-Sanaf and Al-Hammar marshes showing the sampling stations

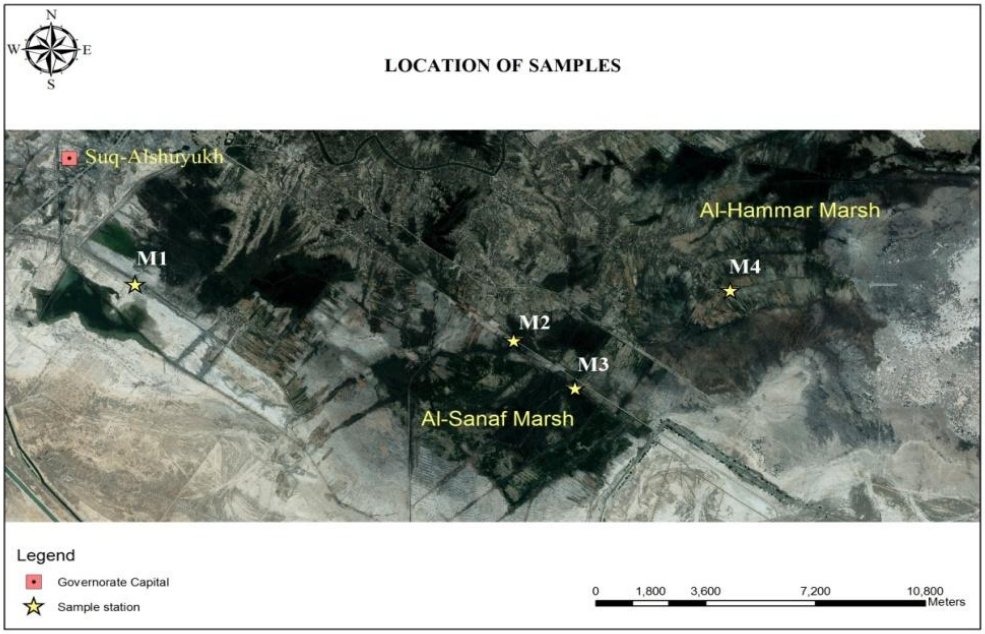


Fig.2 GC chromatogram of crude oil after a 28day exposure to a pure culture of *A. niger*

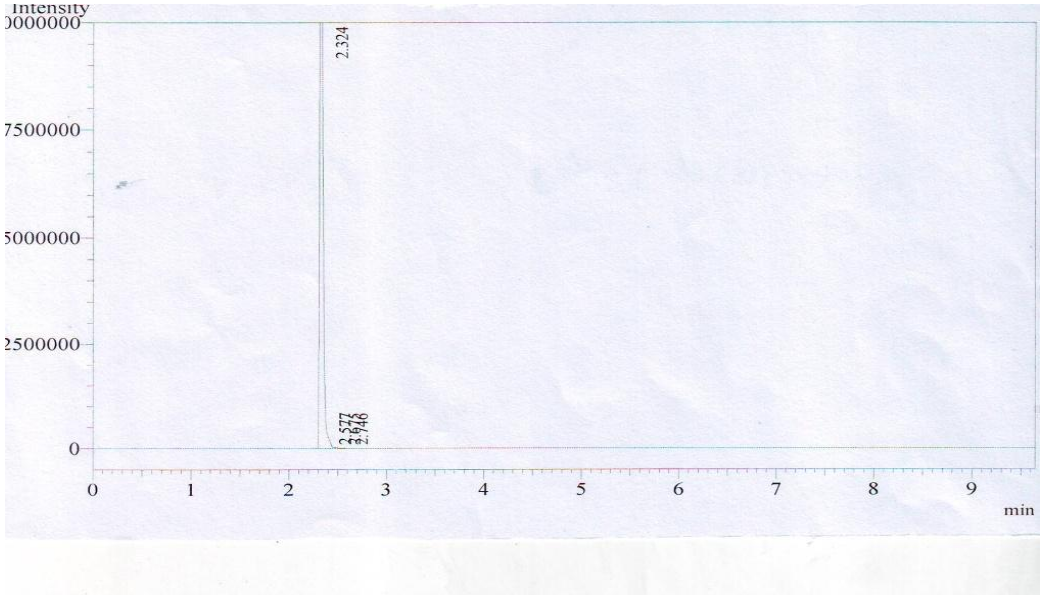


Fig.3 GC chromatogram of crude oil after a 28 day exposure to a pure culture of *A. fumigatus*

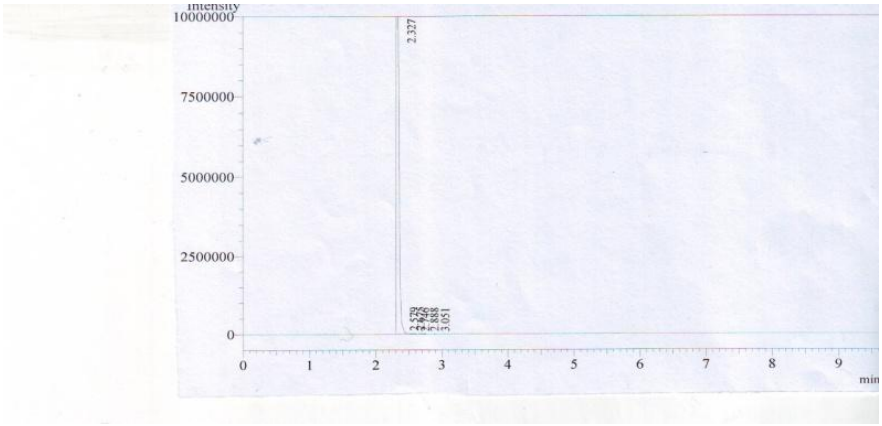
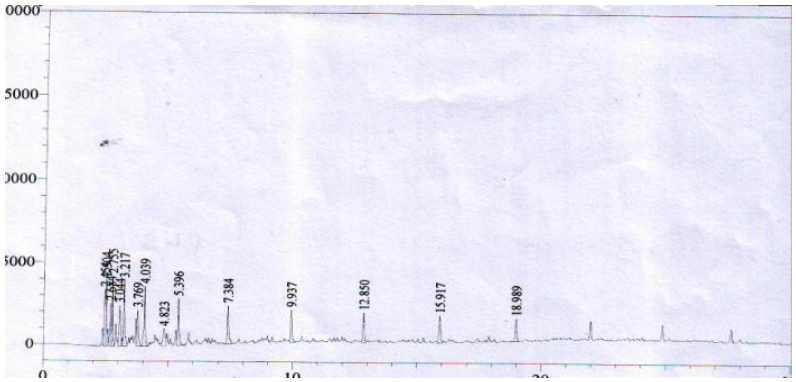


Fig.4 GC chromatogram of untreated crude oil (control)



Time(min)

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

Fig.5 Biodegradation of crude oil by mixed culture of *A. niger* and *A. fumigates* after 28 day incubation

Fig.6 GC chromatogram of crude oil after a 28 day exposure to a pure culture of *A.niger*, *A. fumigatus*. *F. solani* and *P. funiculosum*

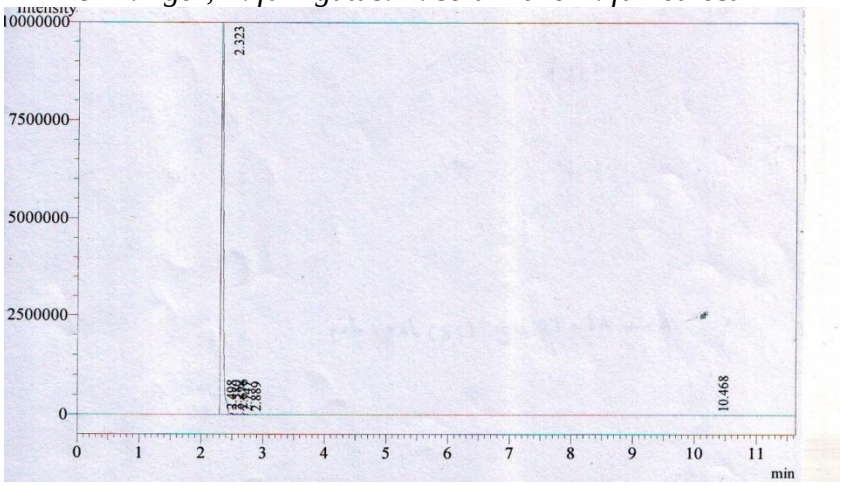


Fig.7 Biodegradation of crude oil by *A. niger*, *A. fumigatus*, *F. solani* and *P. funiculosum* after 28 day incubation

The differences of stations in present study are no high affected in fungi diversity in sediments because the similarty of environmental conditions such as temperature, salinity and hydrogen ion concentration and found of Phragmites sp. and Typha sp. plants. However after the war 2003 in Iraq, the marshes were restoration of waters and human activities were increases with specific used of catch boats then increases pollutants in these sites with hydrocarbons pollutants and probability to reach these compounds to waters from plants after dead and degradation. And the same time the southern marshes of Iraq were anatural filter of agriculture, industrial pollutants residue whene reached these pollutants with Tigris and Euphraties waters.

However fungi play an important role to degradation during by excretion extracellular enzymes such as cellulose, Laccase and other compounds. However crude oil, insecticides and herbicidies were degradation by A.niger, F.solani, Penicillium sp. and Trichoderma lignorum Al-Jawhari (1998), Ravelet *et al.* (2000), Mohamed *et al.* (2012), Mtui (2012).

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