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BIODEGRADATION OF SOME HYDROCARBONS IN SUEZ GULF PETROLEUM CRUDE OIL BY *FUSARIUM OXYSPORUM*

AFIFI, M. M[a] and BAYOUMI, R. A[b]

[a] *Botany and Microbiology Dept., Faculty of Science, Al-Azhar University, Assiut 71524, Egypt.*

[b] *Botany and Microbiology Dept., Faculty of Science (Boys), Al-Azhar University, Cairo, Egypt.*

Abstract

Among fifty fungal isolates were isolated from crude oil polluted soil samples collected from different localities in Upper Egypt (Assuit), *Fusarium oxysporum* C61 was selected for crude oil biodegradation due to of its high metabolic activity. The identification process of fungal genera and species resulted in fifty fungal isolates belonging to twenty six genera presented as follows: ten strains of *Aspergillus*, nine strains of *Penicillium* and one strain of *Absidia, Cunninghamella, Eupenicillium, Fusarium, Nectria, Phoma* and *Syncephallastrum*. The influence of various, pH values, nitrogen sources, phosphrous sources, amino acids and vitamins were investigated to obtain optimal crude oil biodegradation. The results were found to be, pH 8, sodium nitrate, ammonium phosphate, phenylalanine, and vitamin B1, respectively. The chemical compositions of the residual crude oil were analyzed by gas chromatography mass spectrometer (GC-MS). The ability of fungal isolates to degrade crude oil as sole carbon and energy source under all optimal conditions reveled changes in some components of crude oil (not shown). Analysis of hydrocarbon components of the crude oil substrate left after the growth of *F. oxysporium* (referred to as residual crude oil) resulted in the presence of tridecanes, pentadecanes, hexadecanes, and dodecanese in reaction products, which brought about greater reduction in peak of crude oil components, relative to those of the control (undegraded).

Keywords: Petroleum crude oil; Biodegradation; Hydrocarbons; *Fusarium oxysporium*.

Introduction

The study of hydrocarbon degradation by microorganisms has received emphasis because of the increased incidence of petroleum based- pollution (Atlas, 1991; Bartha, 1986; Margesin and Schinner, 1997). Information by hydrocarbon degradation is needed to determine how microorganisms might be feasibly utilized in the removal of these pollutants from the environments. The microbiological decontamination of the oil- polluted soil is claimed to be an efficient economic and versatile alternative to physicochemical treatment (Bartha, 1986; Atlas, 1991). Also, Polychlorinated dibenzo-p-dioxins and dibenzofurans (PCDDs/DFs), including 2,3,7,8-substituted isomers were present in samples of shellfish and fish, and ambient air collected from Masan Bay, and Masan City, South Korea (Im *et al.*,

2004). A petroleum hydrocarbon (PHCs) continues to be used as the principle source of energy and hence they are stilling global environmental pollutants of interest. The world crude oil production is approximately 3 billion tons/year, with roughly 70 % of this production transported by sea. A part from accidental contamination of ecosystem, the vast a mounts of oil sludge generated in refineries from water oil separation systems and accumulation of waste oily materials in crude oil storage tank bottoms pose great problems because of the extensive disposal methods (Ferrari *et al.,* 1996; Vasudevan and Rajaram, 2001).Despite decades of research, successful bioremediation of petroleum hydrocarbon contaminated soil remains a challenge. Petroleum hydrocarbons enter surface and subsurface soils from accidental spills of crude oil and fuels from the large pipeline networks buried below the surface. These subsoil conduits carry large volumes of crude petroleum offshore and on-shore, from oil exploration and production field operations to refineries to bulk storage terminals. The amount of crude oil spilled on land due to pipeline failures is estimated to be 40.000 barrels per year (1.680.000 gallons) or 70 % of all oil discharged to soil and water bodies (Salanitro, 2000). The technology has been usefully employed in the removal of a class of organic pollutants known as hydrocarbons; primarily being used to promote the removal of these compounds from soil, industrial wastewater, surface water reservoirs, and ground water aquifers (Lehmann, 1998). As it is well known, the Red Sea suffers from oil pollution especially around the oil fields in the Suez Gulf. The exposure of water in this area to many types of petroleum crude oil contaminations has drawn our thinking to study the possibility of the crude oil biodegradation by fungal strains isolated from pollutant source.

The purposes of this work were to (i) collect a number of aerobic culturable hydrocarbon degrading fungal isolates from different soil samples collected from upper Egypt (Assuit), (ii) identify of the hydrocarbon utilizing fungal isolates by morphological characteristics according to standard keys, (iii) investigate the biodegradation activity of the selected fungal strains by mean dry weight and selection of the most potent one and (iv) investigate some parameters controlling the growth and biodegradation of some hydrocarbons in crude oil by best one fungal isolate.

Materials and methods

Fungal strains:

Different fungal colonies isolated from petroleum crude oil polluted soil samples were collected from different localities in upper Egypt (Assuit). The fungal isolates

were identified on the basis of morphological characteristics and microscopic examination with the help of keys of (Raper and Fennell, 1965; Booth, 1971; Ellis, 1971; 1976; Pitt, 1979; Domsch *et al.*, 1980; Moubasher, 1993; Pitt and Hocking, 1997; Barnett and Hunter, 1999). The identified fungal strains were maintained routinely in the laboratory on potato dextrose agar (PDA) slants at 4°C.

Characterization of Crude oil:

The physico-chemical properties of Suez gulf petroleum co. (GUPCo) was determined according to standard methods by Bayoumi and Abdullh, (2004). The GUPco was characterized by the flowing: Density, 0.9456; specific gravity, 0.9465, API, 18; Kinematic viscosity (cSt), 16.73; Pour point, 18 (C); sulfur content, 1.77 (wt%); asphaltene content, 3.89 (wt%); resin content, 18.15 (wt%), oil content 77.95 (wt%).

Culture conditions:

The various fungal strains were inoculated by 7-days old cultures on PDA. For oil degradation studies, 0.2 (v/v), crude oil was added to 100 ml culture medium in a series of 250 ml flasks, with the petroleum crude oil serving as supplemented carbon and energy sources. The media used were mineral salts medium (MSM), which contained (g/l): KH_2PO_4, 1; $MgSO_4.7H_2O$, 0.5; KCl, 0.05. Two ml of crude oil sample were emulsified gently with 2 gm of Arabic gum in a clean porcelain mortar. The previously heated media was added gently until a homogenous suspension was obtained then inoculated and incubated at 28°C for 5 days. Control was flasks containing uninoculated medium. The biomass produced was recovered by filtration through pre-extracted, dried, and weighed by Whitman filter paper (No.113). The mycelium residues were washed with 50 ml diethyl ether to remove oil residues. The mycelium was dried at 60°C to a constant weight, being cooled in desiccators before dry weight determination. Mean ± SD of dry weight has been carried out for three replicates.

To optimize crude oil biodegradation mainly, various; pH values (2, 4, 6, 8, and 10); nitrogen sources (calcium nitrate, ammonium chloride, ammonium. nitrate, potassium nitrate, and silver nitrate); phosphorous sources (sodium monohydrogen phosphate, sodium dihydrogen phosphate, potassium monohydrogen phosphate, ammonium. phosphate and potassium dihydrogen phosphate); amino acids (phenylalanine, asparagine, methionine, aspartic acid, lysine and isoleucine); and vitamins (vitamin B6, Vitamin B1, folic acid, and riboflavin), were investigated.

Crude oil basal salts broth (COBSB) medium was adjusted at various pH values in the flasks (250 ml) only 100 ml for each one by using HCl-NaOH buffer. Each treatment of sodium, phosphorous, amino acid, and vitamin was added to COBSB medium in concentration 0.1% (w/v) in triplicate flasks.

Hydrocarbon recovery and analysis:

Residual crude oil in each flask were extracted with chloroform (33 ml), and concentrated by evaporation under a nitrogen flow (Bayoumi and Abdallah, 2004). After solvent evaporation, the dried extract was re-dissolved in 0.5 ml chloroform, 0.1 aliquots was analyzed by SHIMADZU-QP 5050A GC-MS.

The injection temperature was 300ºC and the hydrocarbon fractions were fractionated on a Perkin Elmer Sigma capillary column (50 cm x 0.5 mm). The oven temperature was programmed from 75 to 300ºC, increasing of 5ºC min^{-1}. The carrier gas was N_2 at a flow rate 10 ml min^{-1}.Identification of hydrocarbon components of the crude oil was by comparison with retention times of hydrocarbon standards, while the extent of degradation (%) was calculated by comparing the decrease in the peak areas relative to those of the control (undegraded).

Data analysis:

The data obtained for each parameter were evaluated and submitted for analysis of variance by F. test. The mean value was compared at 5% level of significance using the last significance differences (LSD) test (Gomez and Gomez, 1984).

Results and discussion

Preliminary experiments showed that the fungus *Fusarium oxysporum* C61 could metabolize crude oil among fifty tested fungal strains.

Table (1) depicts a summary of identification, and screening processes of fungal strains relative to their biodegrading abilities. The analysis of variance (F. test), showed a high significance in *Fusarium oxysporum* C61 between the tested fungi, and resulted in 1.68±0.04 g/100 dry biomass over that resulted in the other genera.

Table (1): Biodegrading abilities of the twenty-six identified fungal strains.

No.	Fungal isolates	No. of strains	Mean dry weight (g/100ml)± SD*
1	Absidia corymblfra	1	0.75±0.01
2	*Aspergillus candidus*	2	0.97±0.01
3	*Aspergillus flavus*	4	0.85±0.01
4	*Aspergillus flavipes*	1	0.92±0.02
5	*Aspergillus fumigatus*	4	0.91±0.01
6	*Aspergillus niger*	4	0.88±0.02
7	*Aspergillus ochraceus*	4	0.96±0.01
8	*Aspergillus oryzae*	1	0.94±0.01
9	*Aspergillus tamari*	2	1.43±0.03
10	*Aspergillus terreus*	5	0.91±0.01
11	*Aspergillus versicolor*	2	1.32±0.02
12	*Cunninghamella elegans*	1	0.96±0.01
13	*Eupenicillium alutaceum*	1	0.90±0.01
14	*Fusarium oxysporum*	1	1.68±0.04
15	*Nectria haematococca*	1	1.51±0.01
16	*Penicillium chrysogenum*	1	0.67±0.01
17	*Penicillium citrinum*	1	0.91±0.02
18	*Penicillium canescens*	2	0.82±0.01
19	*Penicillium duclauxii*	2	0.91±0.01
20	*Penicillium funiculosum*	2	0.92±0.02
21	*Penicillium glabrum*	1	0.90±0.01
22	*Penicillium islandicum*	1	0.82±0.02
23	*Penicillium janczewskii*	2	0.95±0.01
24	*Penicillium variabile*	2	1.01±0.04
25	*Phoma glomerata*	1	0.88±0.01
26	*Syncephalastrum racemosum*	1	0.76±0.01

* P>0.0000; F. value, 2553.28; $LSD_{0.03}$ = 0.016.

After an extensive identification of fungal genera and species isolated from polluted soil in Upper Egypt (Assuit), fifty fungal isolates were observed, belonged to twenty-six fungal genera, which identified and /or characterized as follows: ten species of *Aspergillus*, nine species of *Penicillium* and one species of *Absidia*, *Cunninghamella*, *Eupenicillium*, *Fusarium*, *Nectria*, *Phoma* and *Syncephallastrum*. The genera *Aspergillus* and *Penicillium* were the most dominant. Our results agree with (Nyns *et al.*, 1968; Al-Gounaim and Diab, 1998; April *et al.*, 2000; Diab, 2000; Bayoumi and Abdallh, 2004; Chaillan *et al.*, 2004; Eshafie *et al*., 2007).

Snellman *et al.*, (1988) isolated a number of fungi from tar balls including Aspergillus and Penicillium species, which were also isolated from petroleum-contaminated soil in Saudi Arabia (Bokhary and Parvez,1993; Hesham, 1995). The presence of oil degrading microorganisms such as bacteria and fungi is not restricted to a particular ecosystem and has been found in the Arctic, Antarctic and temperature regions but little work has been reported in high temperature ecosystems (Prince, 1993). Several fungi were found to exhibit greater hydrocarbon biodegradation than bacteria (Cerninglia and Perry,1973) and thus differ in their abilities to utilize n-alkane and crude oil (Snellman *et al*., 1988; Bakhary and Parvez, 1993). Nevertheless, very few studies have been conducted on the ability of filamentous fungi to utilize pure aliphatic hydrocarbons.

Davis and Westlake (1979) reported that 28 out of 34 fungi they studied were capable of growing on a variety of crude oil. Compared with isolates of other fungi, Aspergillus and Penicillium isolates were reported to be rich in hydrocarbon assimilatory strains were capable of crude oil degradation (Fedorack *et al*., 1984;Hashim,1995). In this study *Aspergillus tamari*, *Aspergillus versicolor*, *Fusarium oxysporum* and *Nectria haemotococca* (Table 1) and *Fusarium oxysporum* was selected as best one for more detailed studies. Fungi show tremendous diversity and adaptability in utilization of different organic molecules as a carbon source however, their abilities to degrade a specific hydrocarbon as a source of energy and / or biomass may differ. The chemical composition of a crude oil may also be a factor in determining the type of fungi, which may grow on it (Davies and Westlake,1979). Chaillan *et al*., (2004) isolated of different fungal genera from petroleum-polluted soils belonging to *Aspergillus*, *Penicillium*, *Fusarium*, *Amorphoteca*, *Neosartorya*, *Pacilomyces*, *Talaromyces* and *Graphium* in synthetic liquid media with crude oil as sole carbon and energy source.

All the biodegradation parameters in the present study were carried out at 30ºC. Temperature plays very important roles in biodegradation of petroleum hydrocarbons, firstly by its direct effect on the chemistry of the pollutants and secondly on its effect on the physiology and diversity of the microbial milieu. Ambient temperature of an environment affects both the properties of spilled oil and the activity or population of microorganisms (Venosa and Zhu,2003). At low temperature, the viscosity of the oil increases, while the volatility of toxic low molecular weight hydrocarbons is reduced delaying the onset of biodegradation. Temperature also variously affects the solubility of hydrocarbons. Although hydrocarbon biodegradation can occur over a wide range of temperature, the rate of

biodegradation generally decreases with decreasing temperature. Highest degradation rates generally occur in the range of 30-40ºC in soil environments,20-30ºC in some fresh water environments (Cooney,1984). Temperature plays a significant role in controlling the nature and extent of microbial hydrocarbon metabolism (Nedwell,1999).Temperature affects the rate of biodegradation, as well as the physical nature and chemical composition of hydrocarbons (Rowland *et al.*, 2000). A temperature increase leads to a diffusion rate of organic compounds notably a decrease of their viscosity.

Successful application of bioremediation technology to contaminated systems requires knowledge of the parameters that affect the microbial biodegradation of pollutants (Sabate *et al*., 2004). In figure(1), the effect of pH on crude oil biodegradation was clarified by *Fusarium oxysporum* C61. It was found that, pH 8 achieved maximum biodegrading ability. Figures (2-5), exhibit the effect of different parameters on the degradation rate of crude oil. It was noted that, the addition of sodium nitrate, ammonium phosphate, phenylalanine and vitamin B1, enhanced biomass development in *F. oxysporium* C61. The highest biomasses (mean dry weight, g/100 ml) were 0.91±0.01, 0.93±0.02, 0.98±0.01, and 0.89±0.02 in case of, nitrogen source, phosphorous source, amino acid, and vitamin, respectively. Significant differences were observed in crude oil degradation among all treatments. The most important factor affecting biodegradation of petroleum hydrocarbons include pH. The pH of seawater is generally stable and slightly alkaline (Bossert and Bartha,1984). In contrast, the pH of freshwater and soil environments can vary widely. Organic soils in wetlands are often acidic, while mineral soils have more neutral and alkaline conditions. Most heterotrophic bacteria and fungi favor a neutral pH, with fungi being more tolerant of acidic conditions. Studies have shown that degradation of oil increases with increasing pH and that optimum degradation occur under slightly alkaline conditions.

Nutrients are very important ingredients for successful biodegradation of hydrocarbon pollutants, especially nitrogen, phosphorous and in some cases iron (Cooney,1984). Depending on the nature of the impacted environment, some of these nutrients could become limiting thus affecting the biodegradation processes. When a major oil spill occurs in marine and freshwater environments, the supply of carbon in dramatically increased and the availability of nitrogen and phosphorus generally becomes the limiting factor for oil degradation. The effect of nutrients amendment was much more pronounced on the assimilation of linear and branched alkanes than on cyclic alkanes indicating that nutrient amendment stimulates preferentially the microorganisms that assimilate paraffin hydrocarbons.

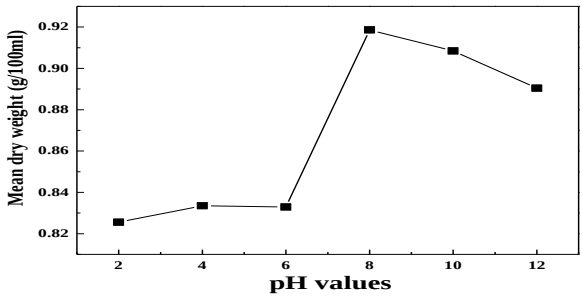

Fig.(1): Effect of different pH values to the biodegradation power of crude oil by *Fusarium oxysporum* C61. P> 0.0000; F. value, 148.36; $LSD_{0.05}$= 0.02.

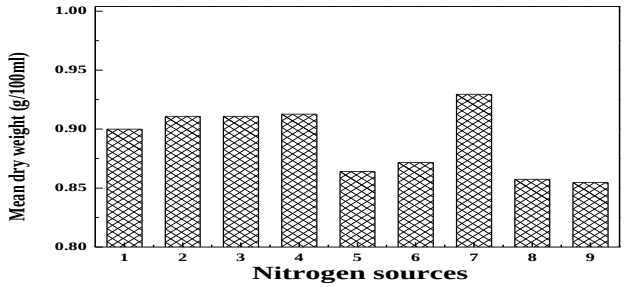

Fig.(2): Effect of different of nitrogen sources to the biodegradation of crude oil by *F. oxysporum* C61. 1, calcium nitrate; 2, ammonium chloride; 3, ammonium nitrate; 4, potassium nitrate; 5, silver nitrate; 6, ammonium sulphate; 7, sodium nitrate; 8, ammonium persulphate and 9, ammonium peroxide sulphate. P>0.0000; F. value, 81.73; $LSD_{0.05}$= 0.017.

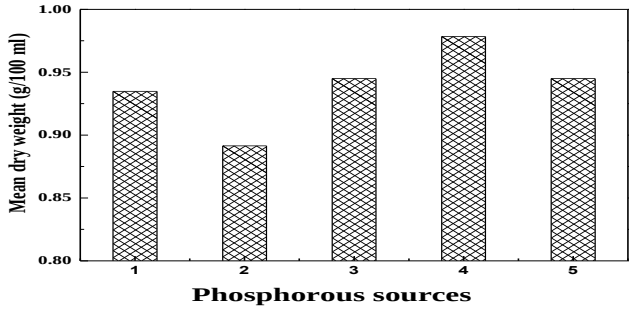

Fig.(3): Effect of different phosphorous sources to biodegradation of crude oil by *F. oxysporum* C61. 1, sodium monohydrogen phosphate; 2, sodium dihydrogen phosphate; 3, potassium monohydrogen phosphate; 4, ammonium phosphate and 5, potassium dihydrogen phosphate. P>0.0001; F. value, 43.89; $LSD_{0.03}$= 0.02.

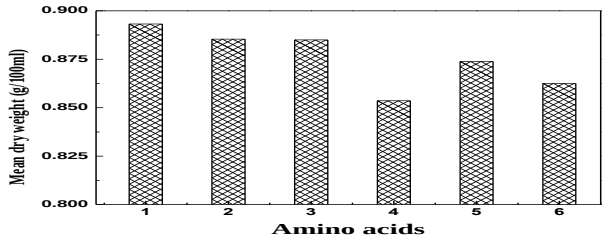

Fig.(4): Effect of different amino acids to the biodegradation of crude oil by *F. oxysporum* C61. 1, phenylalanine; 2, asparagine; 3, methionine; 4, aspartic acid; 5, lysine and 6, isoleucine. P>0.0000; F. value, 13.07; $LSD_{0.05}$= 0.02.

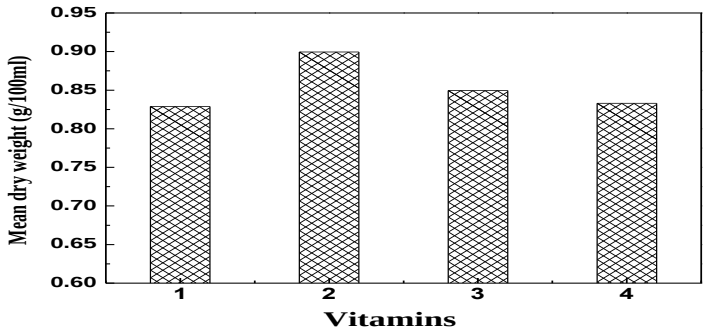

Fig.(5): Effect of different vitamins to the biodegradation of crude oil by *F. oxysporum* C61.1, vitamin B6; 2, vitamin B1; 3, folic acid; and 4, riboflavin. P>0.0001; F. value, 67.27 $LSD_{0.05}$= 0.02.

Hydrocarbon Analysis:

Results recorded in tables (2&3) and represented graphically in figures (6&7), shows a typical GC-MS profile of crude oil components, with the following retention time (min): decane, 27.7; dodecane, 30.6; tridecane, 33.3; pentadecane, 35.9; hexadecane, 38.5; heptadecane, 40.9; octadecane, 43.2; eicosane, 45.4; and docosane, 47.7.

The use of GC-MS showed the presence of fungal reaction products, with the following retention time (min): tridecane, 33.2; pentadecane, 35.9; hexadecane, 38.4; and docosane, 47.6, which brought about greater reduction in peak of crude oil components (Fig.7). This agree with Bartha,(1986); Jakson and Pardue,(1999); Chang and Weaver,(1997); Bayoumi and Abdallh, (2004).

Most fungi utilize crude oil as a source of carbon and energy and the hydrocarbons molecules are metabolized to CO_2 and biomass. The highest biomass was obtained by *Aspergillus tamari*, *Aspergillus versicolor*, *Fusarium oxysporum* and *Nectria haematococca* (Table1), which is highly correlated with highest oil utilization. In this study, *Fusarium oxysporum* was found to utilize different some hydrocarbons (Table 2&3). This could be explained by the fact that the ability to utilize oil as a sole carbon source in not a stable physiological characteristic present in all members of the same genus or isolated fungal genera. The chemical composition of a crude oil determines the type of fungi, which grow on it and some fungi may grow on some crude oil and not others. These result agreement with Lowery et al., (1968) who noted great diversity in the abilities of fungi were noted on hydrocarbons. Varying responses of fungi were noted on hydrocarbons. Prince (1993) reported that microorganisms show a distinct preference for some hydrocarbons over others. Some hydrocarbons were reported to be lethal to some fungi and others were not as toxic (Nynes *et al*., 1968).

Table 2. GC-MS chromatogram of hydrocarbons components of crude oil before fungal treatment.

Serial no.	Hydrocarbon compound	Height A/H(sec) (Area %)	Retention Time(minutes)
1	Decane	11.347	27.775
2	Ddodecane	12.173	30.641
3	Tridecane	12.724	33.375
4	Pentadecane	13.391	35.992
5	Hexadecane	13.017	38.500
6	Heptadecane	13.039	40.905
7	Octadecane	13.198	43.218
8	Eicosane	13.099	45.439
9	Docosane	12.775	47.785

Table 3. GC-MS chromatogram showing the presence of hydrocarbons compounds after fungal biodegradation.

Serial no.	Hydrocarbon compound	Height A/H(sec) (Area %).	Retention Time(minutes)
1	Tridecane	12.70	33.29
2	Pentadecane	13.33	35.90
3	Hexadecane	13.17	38.41
4	Docosane	12.25	47.67

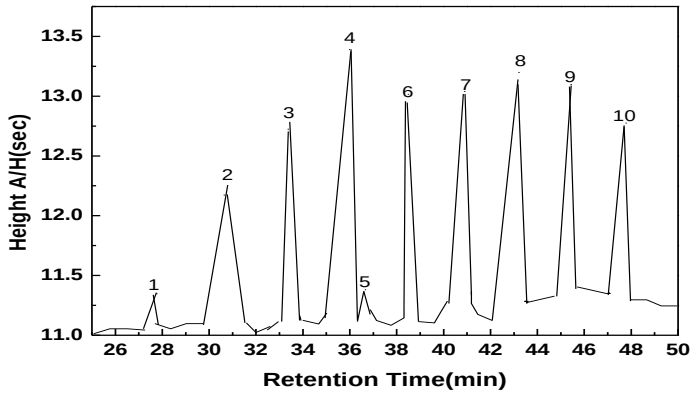

Fig. (6): GC-MS chromatogram of hydrocarbons components of crude oil before fungal treatment. 1. decane, 2. dodecane, 3. tridecane , 4. pentadecane, , 6. hexadecane, 7. heptadecane, 8. octadecane, 9. eicosane, 10. docosane.

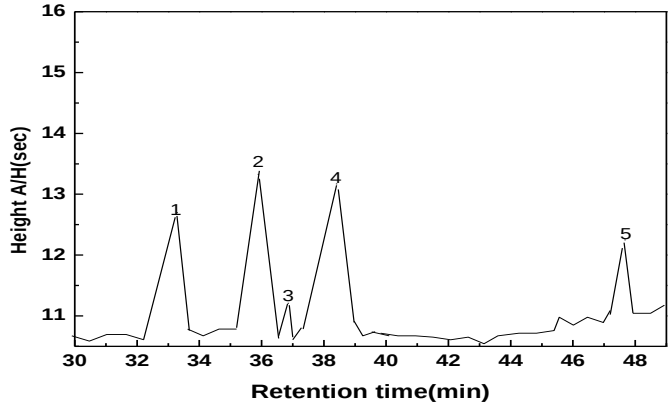

Fig. (7): GC-MS chromatogram showing the presence of hydrocarbons components of fungal reaction products. 1. tridecane, 2. pentadecane, 4. hexadecane and 5. docosane.

Recently, attention has been given to the use of microbial consortia of PAHs degraders consisting of bacteria and fungi. Compared to pure cultures, these consortia have been more effective in the degradation of these compounds, due the capacity to use a larger number of PAHs and higher degradation and mineralization rate *In Vitro* and in soil (Boonchan *et al.,* 2000; Kohlmeier *et al.,* 2005; Wick *et al.,* 2007; Xiaojun *et al.,* 2008).

Conclusions

The results obtained in the present study confirmed that the inoculation of *Fusarium oxysporium* C61 to degrade in oil-contaminated Egyptian sues gulf could be considered as a potential method. However, more than fifty percent of hydrocarbons components were removed. Therefore, it was an acceptable method to use the present fungal strain to biodegrade crude oil from contaminated Egyptian Suez Gulf area. Finally, with the understanding of the variation of fungal hydrocarbon degradation in the laboratory experiments, it would the possible to develop strategies for using fungi for the removal of hydrocarbons from contaminated sea water before they develop into tar balls that might pollute the Red Sea and Suez Gulf beaches in Egypt.

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