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Petroleum Hydrocarbon Utilization by Some Molecularly Identified Filamentous Marine Fungi Isolated from a Polluted Area in the Mediterranean Sea

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THREE DIFFERENT newly isolated marine fungi were subjected to 18S rRNA gene partial sequence analysis. The resulting sequences showed that the isolates belong to *Fusarium solani, Trichoderma viride* and *Aspergillus niger*. The isolates have the ability to utilize some petroleum hydrocarbons such as hexane, motor oil and diesel oil. The tested fungi could grow up to 7 days, especially on medium supported with diesel oil. Although all of the tested fungi have the ability to utilize the increased concentrations of diesel oil, *Fusarium solani* EH has the highest ability, growing at a concentration of 4 ml/l. Static and shaking incubation conditions of *Fusarium solani* EH with 7.2 mg/100ml diesel oil as a sole carbon source, showed significant decrease in the hydrocarbon concentration (90.28% and 93.05%, respectively) within 7 days. Results of this study may provide a help in petroleum hydrocarbon spill clean-up.

Keywords: Marine, Fungi, 18S rDNA, Diesel oil.

Petroleum is a complex mixture of molecules called hydrocarbons. At high concentrations, hydrocarbon molecules are highly toxic to marine organisms, including diatoms, gastropods, mussels, crustaceans and fishes (Adekunle & Adebambo, 2007). These contaminants ultimately pose a serious threat to human health (Alexander, 1994 and Raghukumar *et al*., 2006). Biodegradation of hydrocarbon by natural populations of microorganisms represents one of the primary mechanisms by which petroleum and other hydrocarbon pollutants are eliminated from the environment (Leahy & Colwell, 1990). However, fungi have been found to be better degraders of petroleum than traditional bioremediation techniques including bacteria (Batelle, 2000).

Marine fungi is a collective term for a large group of fungi which are saprophytes or parasites in the marine environment (Hyde, 1989). Some marine fungi are able to grow using hydrocarbons as their sole source of organic carbon. Therefore, they are not only sources of nutrients in marine food webs, but also

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potential participants in degradation of spilled petroleum (Cooney *et al*., 1993). Floodgate (1984) has listed 27 genera of hydrocarbon-degrading fungi which have been isolated from the marine environment. Based on the published reports, the most important hydrocarbon-degrading/utilizing fungi from marine habitats are *Aureobasidium, Candida, Rhodotorula* and *Sporobolomy* spp. On the other hand, *Trichoderma* and *Mortierella* spp. are the most common soil isolates. Hydrocarbon-degrading *Aspergillus* and *Penicillium* spp. have been frequently isolated from both environments (Leahy & Colwell, 1990). Raghukumar *et al.* (2006) have isolated a marine fungus, *Flavodon flavus* NIOCC # 312, that has great potential in pollution clean-up, especially for the removal of polycyclic aromatic hydrocarbons. Although mixed fungal cultures were used for hydrocarbon removal, Okerentugba & Ezeronye (2003) demonstrated that *Penicillium, Aspergillus* and *Rhizopus* spp. were able to degrade hydrocarbons, especially when single cultures were used.

Conventional laboratory identification of fungi often relies on a variety of morphological and physiological tests. This process is tedious and time consuming (Fell, 1993; Sandhu *et al*., 1995; Kappe *et al.*, 1996; Shin *et al*., 1997 and Kim *et al*., 1999). On the other hand, PCR (Polymerase Chain Reaction) is a rapid and sensitive technique for specific amplification of a particular segment of DNA. As in other cells, ribosomal RNA (rRNA) genes in microbial cells are both highly conserved and present in multiple copies. Therefore, they are popular targets for gene amplification and molecular analysis (Lee & Taylor, 1990; Lewin, 1990; Sandhu *et al*., 1995 and Einsele *et al.*, 1997).

In this research, three newly isolated marine fungi which are capable of utilization of some hydrocarbons as sole carbon sources were successfully identified using fungi-specific primer pair (FF2/FR1) (Zhou *et al*., 2000). The primer pair was used to amplify and molecularly characterize approximately 400 bp of the 18S rRNA gene.

Material and Methods

Marine fungi isolation and maintenance

Sea water samples were collected from a hydrocarbon-polluted area in the Mediterranean Sea, El- Max, Alexandria, Egypt, during spring 2009. Marine fungi were isolated from collected samples in a solidified medium according to Cooke (1963), with a slight modification (using sea water instead of dist. water). The medium composition (g/l) is: glucose 10, peptone 5, Rose Bengal 0.033 and agar 15. Medium components were then dissolved in one liter of sea water and sterilized at 121°C for 15 min. After pouring and solidification in Petri-dishes, the plates were inoculated with 1 ml of the diluted samples, by spreading over the medium surface. Fungal cultures were incubated at 28 °C for 5 days. Colonies were then picked up, purified and maintained in malt extract agar medium.

Hydrocarbon utilization

Hydrocarbon utilization screening plates were composed of mineral salt medium (MSM) proposed by Cunha *et al.* (2001), supplemented with 0.24, 0.4, 0.8, 1.6, 2.4, 3.3 or 4% (v/v) petroleum derivative as a sole carbon source (hexane, diesel or motor oil), in addition to 0.5% glucose for growth enhancement. The MSM composition in g/l is: KH_2PO_4 0.4, MgSO4.7H_2O 0.2, NaCl 0.1, $CaCl_2.2H_2O$ 0.025, $MnSO_4.H_2O$ 0.003, $NH_4NO_3.2H_2O$ 0.5 and agar 15. The isolated fungi were used to centrally inoculate the surface of Petri plates (5 mm) containing hydrocarbon-based MSM. Three replicates were used for each fungus with a certain concentration of a sole hydrocarbon source. The plates were incubated for 7 days at 28°C and examined periodically for changes in colony diameter.

For measuring of the total hydrocarbons, mycelia were allowed to grow in diesel oil containing liquid MSM under shaking and static conditions for 7 days at 28°C. Cultures were then centrifuged and supernatants were treated according to Parsons *et al.* (1984). Total hydrocarbons were quantified using Flurometer 450, USA.

Standard deviations for colony diameter values were obtained using SPSS program.

Electron transmission microscopy (ETM)

For fungal examination by ETM, mycelia were dehydrated serially throughout increased alcohol concentrations and treated by standard procedures (Reynolds, 1963). Finally, samples were scanned by ETM (JEOL 100CX, Japan) at the Electron Microscope Unit- Faculty of Science- Alexandria University.

DNA extraction, purification and amplification

200 mg of fungal mycelia were collected and vigorously squashed. DNA was extracted and purified using EZNA Fungal DNA kit, Omega Bio-Tek. Approximately 400bp of the fungal 18S rRNA gene were amplified using a fungal-specific primer pair FF2/FR1 (Zhou *et al*., 2000). The forward primer FF2 is: 5'- GGTTCTATTTTGTTGGTTTCTA-3', and the reverse is: 5'-CTCTCAATCTGTCAATCCTTATT-3'. Two µl (100 ng) of DNA were amplified with each primer (10 pmol) and 2X PCR Master Mix, ready- to – use PCR mixture containing 0.05 U/µl Taq polymerase, PCR buffer, 4mM $MgCl_2$ and 0.4mM dNTPs (MBI Fermentas, Germany). The 50µl PCR mixture-containing tube was placed in the DNA thermocycler, Gene Cycler™ BIO-RAD, USA. The conditions for PCR were as follows: initial denaturation of DNA at 95°C for 3 min and then 35 cycles of three-step PCR amplifications consisting of denaturation at 94°C for 1 min, primer reannealing at 52°C for 1 min and extension at 72°C for 2 min. Samples were subjected to an additional extension at 72°C for 10 min at the end of the amplification cycles (Zhou *et al*., 2000).

Gel electrophoresis

Ten µl of PCR products, mixed with loading buffer, were loaded on a 2% w/v agarose gel and electrophoresed with 1X TEA (Tris EDTA Acetate) buffer. DNA was visualized by UV transillumination after staining with ethidium bromide (0.5 µg/ml). The molecular sizes of the amplified DNA fragments were estimated using DNA ladder of 100 bp.

DNA sequencing

PCR products were purified to remove excess primers using QIA quick PCR purification reagents (Qiagen, USA) and then sequenced with the BigDye Terminater cycle sequencing kit (Applied Biosystems, USA) in ABI Prism 3730 sequencer (Perkin Elmer, Applied Biosystem, USA). Sequences were deposited in the GenBank and the accession numbers are listed in Table 5.

Data analysis and construction of the phylogenetic tree

After obtaining the sequences, homology search was performed against DDBJ (DNA Data Base Japan), using Blast program to find the sequences producing significant alignment with the obtained sequences. Similarity percentages among the sequences were obtained using Biology WorkBench software version 3.2. Multisequence alignment and molecular phylogeny were performed using ClustalW (a distance-based analysis program at DDBJ) program. The tree topology was evaluated using the neighbor-joining method (Saitou & Nei, 1987) based on 1000 resamplings.

Results

Three marine fungi were isolated recently from a hydrocarbon-polluted sea spot in the Mediterranean Sea and they are as follows: *Aspergillus niger* EH, *Trichoderma viride* EH and *Fusarium solani* EH. The growth of each fungus on the mineral salt medium (MSM) containing one hydrocarbon (0.24v/v) as a sole carbon source was generally screened in Table 1. It shows that all of the isolates can utilize diesel oil as an organic carbon source. Motor oil cannot be utilized properly by them. Only *Fusarium* and *Trichoderma* showed promising growth in a MSM containing hexane. However, growth was found to be more vigorous in case of *Fusarium* grown on diesel oil. Accordingly, diesel oil was subjected to more study throughout different time intervals at a concentration of 0.24 (v/v), using the three isolates. Data in Table 2 shows that diesel oil is utilized increasingly with time as indicated by colony diameter. After 7 days of incubation, the maximum colony diameter was detected for each fungus, especially for *Fusarium* colonies which reached a diameter of approximately 20 mm. There is almost no growth after 7 days of incubation.

TABLE 1. General screening for some hydrocarbon utilization by the isolated fungi after 7 days of incubation at 28°C.

Fungi	Hydrocarbon (0.24% v/v)		
	Motor oil	Hexane	Diesel oil
Fusarium solani	-	+	++
Trichoderma viride	-	+	+
Aspergillus niger	-	-	+

++, heavy growth; +, moderate growth and -, weak growth

TABLE 2. Average colony diameters (mm) ± standard deviations for growth on diesel oil (0.24% v/v) during different incubation periods.

Fungi	Time (days)		
	2	4	7
Fusarium solani	13±0.36	16±0.25	20±0.25
Trichoderma viride	11±0.32	13±0.35	18±0.21
Aspergillus niger	10 ±0.35	13 ±0.31	18 ±0.22

- No diesel oil or alternate carbon source.

Within a fixed time, 7 days, diesel oil utilization starting from an elevated levels of its concentration (0.4-4 v/v) was detected for each fungus (Table 3). Generally, colony diameter increased in parallel with the elevated diesel oil levels. *Fusarium*, *Trichoderma* and *Aspergillus*, respectively showed the most vigorous growth at a concentration of 4% (v/v) of diesel oil. Again, the maximum colony diameter, 35 mm, was obtained in case of *Fusarium*. Accordingly, *Fusarium* was selected for further analysis to measure the total hydrocarbon content remaining after 7 days of shaking and stagnant conditions in the presence of diesel oil- containing MSM. Results in Table 4 show that around 90.28% of the diesel oil was utilized by *Fusarium* under static incubation and about 93.05% were utilized under shaking. Controls with no fungus showed almost no diesel loss by evaporation within 7 days.

TABLE 3. Colony diameter (mm) of *Fusarium*, *Trichoderma* and *Aspergillus* grown on different concentrations of diesel oil after 7 days .

Fungi	Diesel oil concentrations % (v/v)						
	0.0*	0.4	0.8	1.6	2.4	3.3	4
Fusarium solani	2.2±0.04	22±0.22	26±0.17	29±0.25	32±0.24	34±0.18	35±0.20
Trichoderma viride	2±0.03	20±0.31	24±0.18	27±0.22	29±0.32	31±0.26	31±0.35
Aspergillus niger	1.6±0.04	21±0.24	24±0.22	27±0.32	30±0.33	32±0.22	33±0.30

TABLE 4. Total hydrocarbon content remained after diesel oil utilization by *Fusarium*.

Total hydrocarbon content (mg/100ml)			
Incubation conditions	**Zero time (control)**	**2 days**	**7 days**
Static	7.2	3.6	0.7
Shaking	7.2	3.3	0.5
Static, non- inoculated	7.2	7.2	7.12
Shaking, non- inoculated	7.2	7.2	7.13

Fusarium from control and diesel cultures were examined using electron transmission microscopy (Fig. 1). Figures 1A and 1B show normal conidial and hyphal cells with their cytoplasmic organelles, while growing in a malt extract medium. *Fusarium* cells still have the ability to divide in the presence of 4% (v/v) of diesel oil as a sole source of organic carbon (Fig.1C). Distortion of cell organelles, presence of large vacuoles and appearance of inclusions are obvious (Fig. 1D and 1E), when cells subjected to hydrocarbon stress.

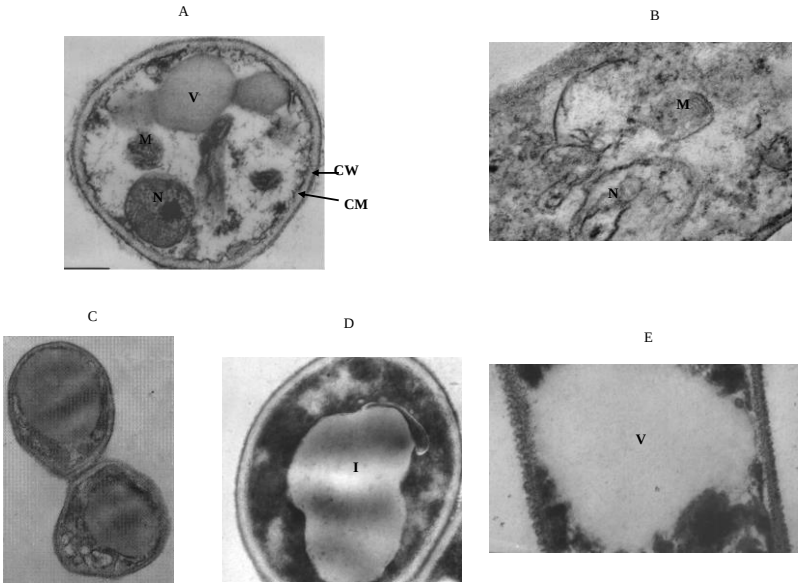

Fig. 1. Transmission Electron Microscopy of *Fusarium solani* growing in malt extract medium (A and B) and diesel oil containing mineral salt medium (C, D and E). Conidia are shown in A, C & D and fungal hyphae are shown in B and E. N, nucleus; M, mitochondria; V, vacuole; I, inclusion; CM, cell membrane and CW, cell wall.

Fungal specific primer pair, FF2/FR1 (Zhou *et al*., 2000), was used in this study to amplify around 400 bp of the 18S rRNA genes from the newly isolated fungi (Fig. 2). FF2 and FR1 were also used for partial sequencing of the 18S rRNA gene. Most of the amplified DNA fragment has a highly conserved DNA sequences and the rest of it has a variable regions (Fig. 3). Accordingly, sequencing of this DNA fragment has proven useful for fungal identification (Zhou *et al*., 2000). The DNA sequences revealed that the isolated fungi are almost certainly *Aspergillus niger*, *Fusarium solani* and *Trichoderma viride* with similarity percentages of 100, 98 and 99, respectively. The sequences were deposited in the GenBank and accession numbers are given in Table 5. The phylogenetic analysis of the fungal sequences is summarized in Fig. 4. From the tree topology, it can be deduced that there are three fungal groups to which the new isolated strains belong. The *Fusarium* clade shows high homology with the target strain, *F. solani* EH, and *F. solani* strain E17084 (98% similarity). *Aspergillus* clade exhibits strong homology between the present new strain, *A. niger* EH and *A. niger* strain contig An03c0110 (100% similarity). Finally, the *Trichoderma* best matched *T. viride* strain RIB40 (99% similarity).

TABLE 5. Accession numbers of the newly isolated fungal strains.

Fungus	Accession number
Aspergillus niger EH	GU733609
Fusarium solani EH	GU733608
Trichoderma viride EH	GU733610

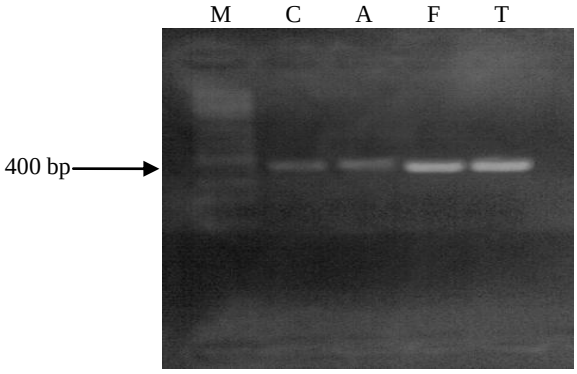

Fig. 2. Gel electrophoresis of approx. 400 bp of the 18S rRNA gene. M, 100 bp genetic marker; C, control (*Aspegillus niger* ATCC 16404); A, *Aspergillus niger* EH; F, *Fusarium solani* EH and T, *Trichoderma viride* EH.

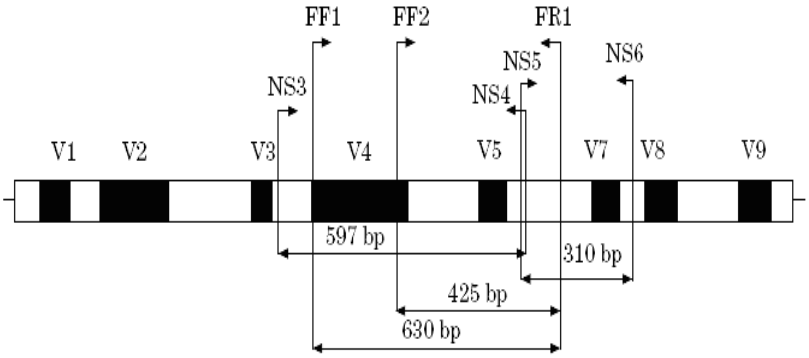

Fig. 3. Map of the 18S rRNA gene in fungi (adapted from Kappe *et al.*, 1996) with highly conserved regions (□) and variable regions, V1 to V9 (▬). The positions of primers and the sizes of amplified fragments are indicated.

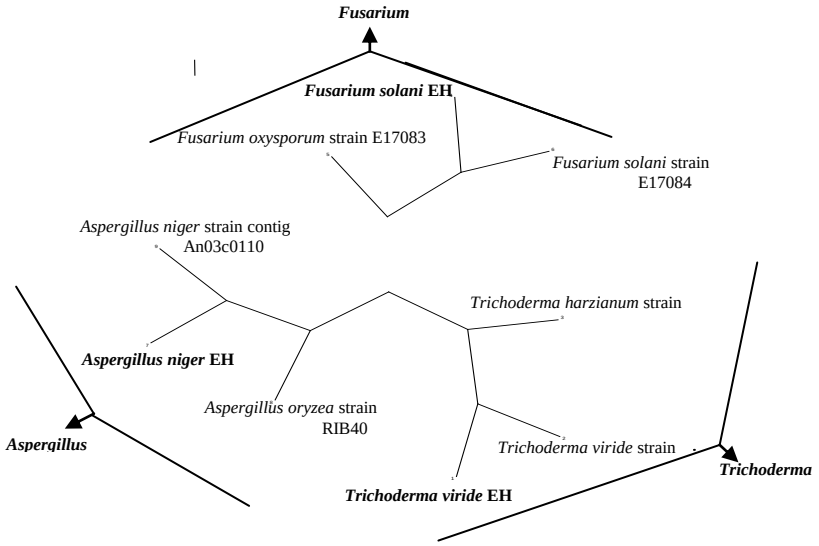

Fig. 4. Phylogenetic tree based on partial sequences (approx. 400 pb) of the fungal rRNA gene. The tree constructed by neighbor-joining method using ClustalW software.

Discussion

Results of the current research indicate that some marine fungi, namely *F. solani, A. niger* and *T. viride* are capable of degrading some petroleum hydrocarbons. Bartha & Atlas (1997) listed 14 genera of fungi which had been demonstrated to contain members with the ability to utilize petroleum hydrocarbons. All of the 14 genera had been isolated from aquatic environment. Also, Okerentugba & Ezeronye (2003) have demonstrated that *Penicillium* spp., *Aspergillus* spp. and *Rhizopus* spp. were capable of degrading hydrocarbons especially, when single cultures were used. Batelle (2000) showed that fungi were better degraders of hydrocarbons than bacteria. Results of the current work demonstrate that utilization of different hydrocarbons, namely, motor oil, hexane and diesel oil, varies among the isolated fungi. This may also account for the difference in growth rates among them Adekunle & Adebambo, (2007). Colombo *et al*. (1996) who had studied hydrocarbon utilization by *Aspergillus terreus*, *Penicillium chrysogenum* and *Fusarium solani*, verified that the degradation capability varied according to the fungus. However, the fungi under study showed weak growth on motor oil, and *Aspergillus* exhibited significant growth (Table 1) in only one hydrocarbon, diesel oil. On the other hand, *Fusarium* was found to be the most promising among the tested isolates, especially when grown in diesel oil.

Petroleum-derived diesel is composed of about 75% saturated hydrocarbons (primarily paraffins including n, iso, and cycloparaffins), and 25% aromatic hydrocarbons (including naphthalenes and alkylbenzenes) (ATSDR, 1995). An interesting observation generated in this study was that *Fusarium* has increased colony diameter with time upon using diesel oil as a sole organic carbon source, at least to a concentration of 4% (v/v). Moreover, the total hydrocarbon content decreased 90.28% and 93.05% after 7 days of static and shaking incubation conditions, respectively. This fungus was able to use the hydrocarbon as a substrate for growth, presumably by releasing extra cellular enzymes and acids which are capable of breaking down the hydrocarbon molecules, thus dismantling the long chains of hydrogen and carbon into simpler forms that can be absorbed for growth and nutrition (Adekunle & Adebambo, 2007). Previous studies have demonstrated utilization of many petroleum hydrocarbons by filamentous fungi (Santos *et al*., 2008 and Hadibarata & Tachibana, 2009) and by *Fusarium* (Flippin *et al*., 1964; Michalcewicz, 1995; Yagafarova *et al*., 2001 and Chulalaksananukul *et al*., 2006).

The cell envelope of microorganisms basically consists of cell wall and 1 or 2 lipid membranes (Beveridge & Graham, 1991). The cytoplasmic membrane has a low permeability for polar and charged molecules. Apolar compounds such as cyclic hydrocarbons, can easily penetrate the lipid bilayer. The transfer of such molecules across the membrane is most probably a diffusion process (Sikkema *et al*., 1995). Witholt *et al.* (1990) postulated that outer membrane lipopolysaccharides (LPS) are released and encapsulate hydrocarbon droplets. This may lead to the presence of inclusions (Fig. 1D) of unmodified hydrocarbons (Witholt *et al*., 1990).

Besides, cell organelles are seriously affected by the presence of hydrocarbons (Sikkema *et al*., 1995), organelles distortion can be seen in Fig. 1D and 1E. For instance, cyclic hydrocarbons have serious effect on mitochondria, starting with the disappearance of the respiratory control and deenergization of the organelle, followed by respiration inhibition (Sikkema *et al*., 1995).

In contrast to phenotyping, which means classification based on the appearance or the reaction of the fungus, genotypic classification relies directly on analysis of DNA sequences. In general, the genotypic procedures are considered to be technically more complex, but the reproducibility and discriminatory power provides better results (Busch & Nitschko, 1999). FF2/FR1 is a fungus-specific pair of PCR primers (Zhou *et al*., 2000) which was successfully used in this study to amplify approximately 400 bp of the 18S rRNA genes. The phylogenetic analysis along with the similarity percentages, based on utilization of theses 18S rRNA gene partial sequences, revealed that the present isolated marine fungi are, or very closely related to *A. niger*, *T. viride* and *F. solani* (Fig. 3).

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استخدام الهيدروكربونات البترولية بواسطة بعض الفطريات البحرية المعرفة جزيئيا والمعزولة من احدى المناطق الملوثة فى البحر الأبيض المتوسط

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باستخدام التتابع الجزئى لجين ال ١٨ S الريبوزومى، تم تعريف ثلاثة سلالات جديدة ومختلفة من الفطريات البحرية حديثة العزل، وهى كالتالى: *فيوزاريوم سولانى، تريكوديرما فيريدى وأسبيرجيلاس نايجر*. وللفطريات المعزولة القدرة على استخدام بعض الهيدروكربونات البترولية مثل الهكسان، زيت السيارات وزيت الديزل. بالاضافة لهذا، أظهرت الفطريات تحت الدراسة القدرة على النمو لمدة سبعة أيام فى وجود الهيدروكربونات وخاصة زيت الديزل كمصدر وحيد للكربون. ولفطر *الفيوزاريوم* القدرة على النمو فى وجود تركيز يصل إلى٤ مل/ لتر من زيت الديزل. كما أن له القدرة على استخدام ٢, ٧ مج/١٠٠ مل من ذات الزيت كمصدر وحيد للكربون فى ظروف نمو ساكنة ومتحركة بنسبة تكسير تصل إلى٩٠,٣ و ٩٣٪ على الترتيب خلال ٧ أيام.

وعلى هذا توصى الدراسة الحالية باستخدام فطر *الفيوزاريوم سولانى* الحالى فى التخلص من الهيدروكربونات البترولية التى قد تتسرب إلى مياة البحار.