J. Gen. Appl. Microbiol., **61**, 185–192 (2015)
doi 10.2323/jgam.61.185
©2015 Applied Microbiology, Molecular and Cellular Biosciences Research Foundation

jGAM

Full Paper

Biodegradation of engine oil by fungi from mangrove habitat

(Received May 8, 2015; Accepted July 24, 2015)

Fuad Ameen,[1,*] Sarfaraz Hadi,[1] Mohamed Moslem,[1] Ahmed Al-Sabri,[1] and Mohamed A. Yassin[1,2]

[1] *Department of Botany & Microbiology, Faculty of Science, King Saud University, Riyadh 11451, Saudi Arabia*
[2] *Plant Pathology Research Institute, Agricultural Research Center, Giza, Egypt*

The pollution of land and water by petroleum compounds is a matter of growing concern necessitating the development of methodologies, including microbial biodegradation, to minimize the impending impacts. It has been extensively reported that fungi from polluted habitats have the potential to degrade pollutants, including petroleum compounds. The Red Sea is used extensively for the transport of oil and is substantially polluted, due to leaks, spills, and occasional accidents. Tidal water, floating debris, and soil sediment were collected from mangrove stands on three polluted sites along the Red Sea coast of Saudi Arabia and forty-five fungal isolates belonging to 13 genera were recovered from these samples. The isolates were identified on the basis of a sequence analysis of the 18S rRNA gene fragment. Nine of these isolates were found to be able to grow in association with engine oil, as the sole carbon source, under *in vitro* conditions. These selected isolates and their consortium accumulated greater biomass, liberated more CO_2, and produced higher levels of extracellular enzymes, during cultivation with engine oil as compared with the controls. These observations were authenticated by gas chromatography-mass spectrophotometry (GC-MS) analysis, which indicated that many high mass compounds present in the oil before treatment either disappeared or showed diminished levels.

Key Words: 18 rRNA gene; biodegradation; hydrocarbons; petroleum

Introduction

The processes of extraction, refining, transport, and the storage of petroleum and its derivatives, are inherently prone to leaks and spillages in differing volumes, resulting in corresponding magnitudes of environmental damage (Meyer et al., 2014). A broad range of petroleum products, including crude oil, engine oil, diesel fuel, creosote products and other fuel oil materials, contain complex mixtures of aliphatic and aromatic hydrocarbons, xenobiotics and other refractory organic compounds, some of which pose serious toxicity risks of variable levels to humans, plants and animals (Kumar et al., 2014; Van Hamme and Ward, 2001). The impact of these pollutants on life forms results in the potential of adversely impairing age-old balances within and in-between marine, freshwater and terrestrial ecosystems, and this feared process has started already (Xu et al., 2013). Therefore, various methodologies, including unconventional processes, such as the use of microbial degradation, need to be developed to neutralize or minimize the challenge posed by petroleum contaminants.

It is presumed that the long-standing state of pollution might have induced microbial communities to evolve physiological pathways to neutralize the deleterious effects of pollutants by breaking down the toxic molecules, and even to utilize some of the breakdown products as a source of carbon (Gagandeep and Malik, 2013).

It is now becoming increasingly evident that both fungi and bacteria can degrade petroleum-linked pollutants (Gadd, 2001; Okoh, 2003; Wu et al., 2010).

The Red Sea is one of the most extensively used sea routes for the transport of petroleum, and, consequently, has been subjected to substantial volumes of oil-linked pollutants by way of leaks, spillages, and occasional accidents (AL-Lihaibi and AL-Ghamdy, 1997). Like anywhere else, the inevitable menace of pollution has necessitated the investigation into various procedures, including biological means, for remedying the petroleum-affected soil and water reservoirs in Saudi Arabia; especially in view of the huge oil extraction activity in the country. In some

*Corresponding author: Fuad Ameen, Department of Botany & Microbiology, Faculty of Science, King Saud University, Riyadh 11451, Saudi Arabia.
Tel: +966501861181 E-mail: alfouad2004@hotmail.com

None of the authors of this manuscript has any financial or personal relationship with other people or organizations that could inappropriately influence their work.

initial studies, several fungal species including *Alternaria alternata*, *Aspergillus flavus*, *Curvularia lunata*, *Fusarium solani*, *Mucor racemosum*, *Penicillium notatum* and *Ulocladium atrum* isolated from petroleum-polluted soils have been found to show biodegradation activity on many petroleum compounds (Hashem, 2007). To date, reports on marine fungi from the Red Sea region, and their degenerative potential against pollutants of petroleum origin, are conspicuously lacking in the literature. To the best of our knowledge, the present study is the first attempt to assess the ability of fungal strains from mangrove ecosystems on the Red Sea coast of Saudi Arabia to breakdown hydrocarbons of engine oil. Biodegradation activity was detected by GC-MS analysis and the potential was evaluated in terms of biomass accumulation under cultivation with the substrate and the secretion of extracellular enzymes.

Materials and Methods

Collection of samples. Samples were collected from three discrete mangrove stands growing naturally in the coastal areas of Jeddah and Jazan cities along the Red Sea, and Farasan Island 40 km off the coast. These sites are considered to be heavily polluted with petroleum oils and municipal waste material.

Tidal water, floating debris, and sediment from around the plants and rhizophores were collected as the source of fungi. Samples from 3–4 points at each location were collected in sterile bottles and were transported to the lab in an ice box.

Procurement of engine oil. SAE 5W-30 non-synthetic engine oil produced and marketed by the Petromin Company was procured from the outlet of a leading supplier of petroleum products in Saudi Arabia (Al-Drees & Co.). The same brand and batch of oil was used for all experiments.

Isolation of pure cultures. For the isolation of fungi from the sediment, about one gram of soil was homogenized in one ml of sterile distilled water supplemented with 100 μl Tween-20, and 1 ml of 10^{-1} dilution was layered per plate of MF1 medium designed to simulate seawater conditions (Ameen et al., 2014). The composition of the medium per liter was: $MgSO_4$ 246.5 mg, $FeSO_4 \cdot 7H_2O$ 5.56 mg, $ZnSO_4 \cdot 7H_2O$ 0.29 mg, $MnSO_4 \cdot H_2O$ 0.34 mg, $CuSO_4 \cdot 5H_2O$ 0.025 mg, NH_4Cl 5.35 mg, KCl 7.46 mg, $CaCl_2 \cdot 2H_2O$ 1.47 mg, $COCl_2 \cdot 6H_2O$ 0.027 mg, KH_2PO_4 136 mg, Na_2MoO_4 24 mg, and NaCl 5.84 g with a pH adjusted to 5.5. The medium was gelled with 15 g/l agar. Dextrose (20 g/l) was added as a carbon source only at the isolation stage. To check the bacterial contamination, 500 mg l^{-1} streptomycin was added to the medium after sterilization. Similarly, 1 ml of 10^{-1} dilution of tidal water per plate was layered on the MF1 medium. For the isolation of fungi from debris, the solid material was surface sterilized by shaking in 1% sodium hypochlorite solution tinged with 2 μl of tween-20 for 2 minutes. After thoroughly rinsing with sterile distilled water, small pieces were laid on the MF1 medium. The plates were incubated at 30 ± 2°C for 5–7 days; and pure colonies were transferred to independent plates. Isolates were designated serially with the prefix "*FA*".

Preliminary screening of active isolates. During preliminary screening, 5 g of engine oil was suspended in 50 ml of MF1 broth. The suspension was separately inoculated with a single pre-weighed disc (6 mm dia.) of fungal mat of individual isolates. Flasks were incubated on a shaker (100 rpm) for 10 days, after which the suspension was passed through a Whatman No. 1 and the total dry fungal mass was weighed again. Active isolates were selected for experimentation on the basis of two criteria: (i) initiation of fungal growth around oil drops within 2 days, and (ii) 10%, or greater, gain in biomass during 10 days. These criteria were adopted after prolonged observation of the growth performance of all the fungal isolates. Nine isolates, namely, FA-16, FA-20, FA-21, FA-22, FA-23, FA-24, FA-41, FA-53, and FA-62 fulfilled the above criteria. Accordingly, these nine isolates and their consortium were used for further investigations.

Identification of active isolates. Active isolates used in the study were identified by sequencing an 18S rRNA gene fragment. The total genomic DNA of the isolates was extracted according to the method of Wiese et al. (2011). The 18S rRNA gene fragment was amplified by PCR using ITS1 and ITS4 primers as described by White et al. (1990). The PCR procedure was performed in a thermal cycler (BioRad C1000) using *Pfu* DNA polymerase and master mix (Solis Biodyne) containing buffer and standard components. The PCR program comprised 35 cycles of denaturation at 95°C for 30 seconds, annealing at 55°C for 50 seconds and extension at 72°C for 150 seconds. The PCR products were cleaned using a Mo-Bio Ultra Clean® PCR Cleanup Kit and sequencing was achieved with a Genetic Analyzer 3130 (Applied Biosystems). Sequences of the isolates showing biodegradation activity on engine oil were compared with 18S rRNA gene sequences available in the database of the National Center for Biotechnology Information (NCBI) using the BLAST program. A phylogenetic tree was constructed on the basis of the sequence analysis to depict the relatedness of the experimental isolates with their nearest matches.

Fungal growth and biomass accumulation under cultivation with engine oil. Nine selected isolates and their consortium were cultivated with engine oil to test their growth and biodegradation potential. MF1 broth with engine oil as the sole carbon source was used for the tests. For each isolate, 10 ml of engine oil was added to 100 ml of sterilized broth in a 150 ml conical flask, which was inoculated with a single pre-weighed disc (6 mm dia.) of the fungal mat. The flasks were incubated at 30 ± 2°C for 4 weeks in a rotary shaker gyrating at 100 rpm. Cultures growing under the same conditions, in MF1 broth without engine oil were retained as controls.

Biomass accumulation of an isolate growing in association with engine oil was estimated in comparison with its corresponding control after the 4-week incubation. The gain in biomass of the controls was evaluated on a dry weight basis as the difference between the initial weight of the inoculum (6 mm disc) and the final weight of the total fungal mass at the end of the treatment. The difference between the gain in the treatment and control was considered to be due to biodegradation activity of the fungus. The increase in biomass in the treatments was calculated as the difference of weight compared with the re-

spective controls in grams and in percent.

Enzyme assays. Nine fungal isolates, which showed good biomass accumulation under treatment, were cultivated with engine oil in replicates along with the controls as above. As a separate treatment, a consortium of all the selected nine isolates was also tested for enzyme activity. After four weeks of incubation, the enzyme level in the medium was assayed for controls and treatments.

Cultures were centrifuged at 10,000 rpm, 4°C; the pellet consisting of fungal cells was discarded and the enzyme level was determined in the extracellular fluids. Laccase (LAC) was estimated by the oxidation of 2,2-Azino-bis-3-ethyl-benzthiazoline-6-sulfonic acid (ABTS) according to Novotny et al. (1999) using 0.1 mM ABTS in the reaction buffer of 100 mM sodium tartrate (pH 4.5) with 50 μl of culture filtrate. One unit (U) of laccase activity was defined as the production of 1 μmol product per min at 30°C and pH 4.5. Manganese-dependent peroxidase (MnP) was estimated by using 0.01% phenol in the presence of 0.1 mM H_2O_2 and 1 mM $MnSO_4$ in 100 mM sodium tartrate (pH 4.5); while lignin peroxidase (LiP) was determined by the oxidation of 2 mM veratryl alcohol in 100 mM sodium tartrate (pH 4.5) with 0.4 mM H_2O_2 (Paszcymski et al., 1998). Catalase (CAT) activity was determined according to Aebi (1983) by measuring the decomposition of H_2O_2, and the decline in absorbance at 240 nm was followed for 3 min. The reaction mixture contained 50 mM of phosphate buffer (pH 7.0), 15 mM of H_2O_2, and 0.1 ml of enzyme extract in a 3 ml volume. All enzymes assayed in this study were expressed as Uml^{-1}.

Estimation of CO_2 evolution. Fungal isolates were cultivated with engine oil as above and the quantity of CO_2 evolved during incubation of 4 weeks was estimated by volumetric and gravimetric methods using the Sturm test. Three flasks were maintained for each isolate together with corresponding control flasks inoculated with the fungus but devoid of engine oil.

For gravimetric analysis sterile air was sequentially passed through 1M KOH solution to remove atmospheric CO_2 and then through the flasks under assessment. The bubbling air provided aeration for the fungal activity and, at the same time, allowed any CO_2 evolving from the fungal activity to dissolve readily in the broth. The test was performed at room temperature (26 ± 2°C). The amount of CO_2 dissolved in the broth was estimated by adding 100 ml of 0.1 M $BaCl_2$ which formed a precipitate of barium carbonate, and the CO_2 released was gravimetrically calculated by measuring the weight of the precipitate. The difference in the values obtained between the control and test bottles were recorded.

For volumetric analysis, the dissolved carbon dioxide present in the medium was estimated by titration. The broth was filtered to remove fungal mass from the engine oil and 25 ml of filtrate was taken in a conical flask to which 0.05 ml of 0.1N thiosulphate solution was added. After the addition of 2 drops of methyl orange indicator, the solution was titrated against 0.02 M sodium hydroxide solution. The end point appeared as a change in color from orange-red to yellow. After this, two drops of phenolphthalein indicator was added and titration was continued until a pink color was observed. Volumes of the titrant used were noted and the amount of CO_2 evolved was calculated using the formula: $[A \times B \times 50 \times 1000] \div V$; where A = volume of NaOH titrant in ml, B = normality of NaOH, and V = volume of sample in ml. The values were converted to grams per liter for the sake of uniformity.

The increase in CO_2 evolution in the treatments was calculated as the difference in weight compared with the respective controls, in grams per liter and in percent.

GC-MS analysis of engine oil. Engine oil treated with fungal mass in broth was separated by partitioning in diethyl ether. The mixture was shaken well and 5 ml of the sample was transferred into a 50 ml separatory funnel; and 5 ml of diethyl ether was added. The separatory funnel was shaken vigorously for about 2 minutes with periodic venting to release vapor. The organic layer was allowed to separate for 10 minutes and was recovered into a 50 ml beaker. The aqueous layer was re-extracted twice with 2 ml of diethyl ether; and the combined extract was dried by passing through a funnel containing anhydrous sodium sulfate. The dried extract was concentrated with a stream of nitrogen gas.

GC-MS analysis was carried out with a PerkinElmer Mass Spectrometer with a HP-5MS column according to the method of Wu et al. (2010) with a minor modification. For analysis, a fuse-silica capillary column (30 m × 0.25 mm column - 0.25 μm particle size) (HP-5MS, HP Inc.) was used. The column temperature program was set as follows: 100°C hold for 1 min, 15°C/min to 160°C, at 5°C/min to 300°C, and a hold for 7 min. The GC injector was held isothermally at 280°C with a splitless period of 3 min. The solvent delay time was set at 5 min. Helium was used as the carrier gas, at a flow rate of 1 ml min^{-1} by using electronic pressure control. The GC/MS interface temperature was maintained at 280°C. The analysis was carried out in full scan mode to generate total ion chromatograms. The MS was operated in an electron impact (EI) ionization mode with an electron energy of 70 eV and scan ranged from 50 to 500 amu (atom to mass unit) to determine the appropriate masses for selected ion monitoring (SIM). The MS ion source and mass filter (quad) temperatures were held at 230°C and 150°C, respectively. To minimize the baseline shifting after a derivatizing reagent peak, the signal was turned off as soon as the derivatizing reagent appeared and turned on again after the derivatizing reagent had eluted. To increase the sensitivity, SIM mode was used to quantitatively analyze the peak, and the molecular ion was detected and quantified with the ion loss of the methyl group (mass 190 for lactone); the dwell time was 0.1 s and the scan cycle was 4.26/s. All samples under analysis were run in triplicates.

Statistical analysis. In each experiment, at least three replicates of treatments and corresponding controls were maintained, and the data were analyzed using an SAS Test. The significance of the difference between the treatments and the respective controls was determined on the basis of LSD at $P \leq 0.05$.

Results

Isolation and identification of fungi

Forty-five fungal isolates were obtained from the sam-

Table 1. Sequence-based identification of active fungal isolates.

Sl. No.	Isolate No.	Accession numbers	Closely related fungal sequence	Homology (%)	Coverage
1	FA-16	KP033199	*A. nomius* DQ370001.1	96	426/446
2	FA-20	KP033200	*P. alba* HM116755.1	100	516/516
3	FA-21	KM979604	*P. variotii* EU037050.1	99	507/513
4	FA-22	KP033201	*P. vinaceum* NR121242.1	100	335/337
5	FA-23	KP033202	*A. terreus* KM116156.1	99	365/370
6	FA-24	KP033203	*A. alternata* KJ947119.1	100	488/488
7	FA-41	KP033204	*G. pallida* KF808303.1	100	496/496
8	FA-53	KM979605	*C. sphaerospermum* KC311475.1	100	504/504
9	FA-62	KM979606	*E. hirayamae* JN626096.1	99	481/482

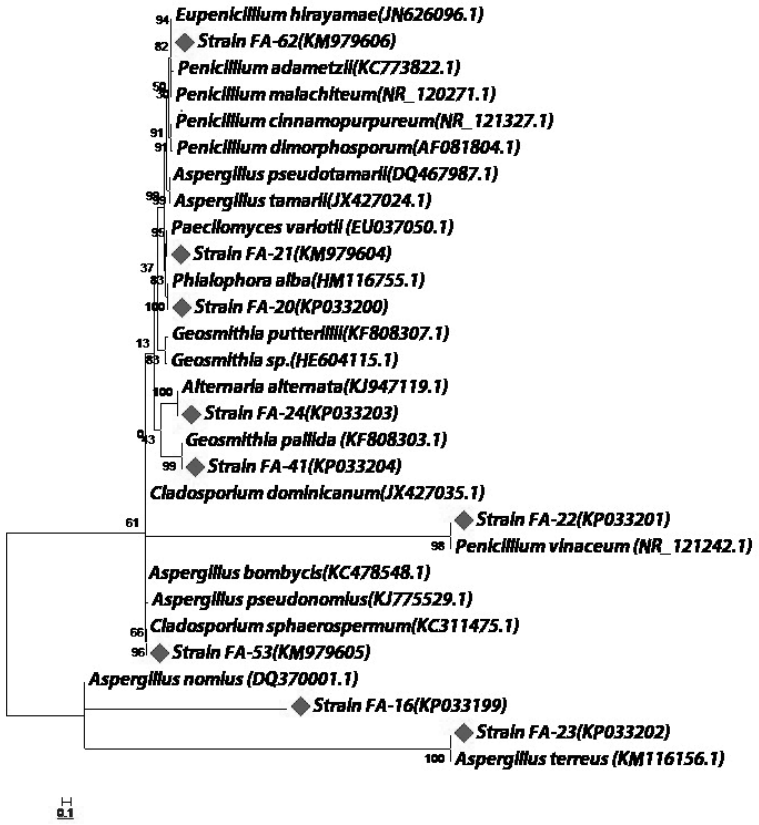

Fig. 1. Phylogenetic tree based on the ITS sequences of active isolates showing relatedness.

The tree was constructed by a neighbor-joining algorithm using a maximum composite likelihood model. Bootstrap percentages from 100 replicates are shown.

ples, and were identified by the sequence analysis of the 18S rRNA gene fragment. The isolates included eight species each of *Aspergillus* and *Penicillium*, three species of *Candida*, while the remaining nine were individual species of *Acremonium*, *Alternaria*, *Emericella*, *Eurotium*, *Exophiala*, *Geosmithia*, *Paecilomyces*, *Pichia*, *Phialophora* and *Cladosporium*.

The sequences of the partial 18S rRNA gene fragments from the active strains were compared with the sequence data available in the GeneBank by an online alignment search (Table 1). The results indicated that the sequences of isolates FA-20, FA-22, FA-24, FA-41 and FA-53 were 100% identical to those of *P. alba*, *A. alternata*, *G. pallida* and *C. sphaerospermum*, respectively (Accession No. HM116755.1, KJ947119.1, KF808303.1 and KC311475.1); while the sequences of isolates FA-21, FA-23 and FA-62 were 99% identical to those of *P. variotii*, *P. vinaceum*, *A. terreus* and *Eupenicillium hirayamae*, respectively (Accession No. EU037050.1, NR121242.1, KM116156.1 and JN626096.1). Isolate FA-16 was 96% identical to *A. nomius* (Accession No. DQ370001.1). Comparison of the experimental sequences with the database is depicted in a phylogenetic tree (Fig. 1). The sequences were submitted to the NCBI Genebank and accession numbers were obtained.

Biomass accumulation by fungi during cultivation with engine oil

During the fungal treatment of engine oil as a substrate, inocula of nine isolates as well as their consortium gradually developed association with the oil droplets and within 15–20 days grew to form colonies of variable size and

Table 2. Biomass accumulation by fungal isolates during cultivation with engine oil.

Fungal isolate	Gain in dry weight after 4 weeks			
	Controls (g)	Treatments		
		Actual (g)	Increase over controls	
			Weight (g)	Percent (%)
A. alternata	0.642	0.826	0.184*	28.6
A. nomius	0.462	0.579	0.117*	25.3
A. terreus	0.625	0.845	0.220*	35.2
C. sphaerospermum	0.599	0.846	0.247*	41.2
E. hirayamae	0.591	0.858	0.267*	45.0
G. pallida	0.382	0.425	0.043	11.2
P. variotii	0.594	0.795	0.201*	33.8
P. vinaceum	0.555	0.647	0.092*	16.5
P. alba	0.524	0.661	0.137*	26.0
Consortium of above nine fungi	0.662	1.030	0.368*	55.5

*Significantly different from the control at $P \leq 0.05$.

Table 3. Enzyme activity of fungal isolates during co-cultivation with engine oil as the sole carbon source.

Fungal isolate	LAC (U/ml^{-1})		MnP (U/ml^{-1})		LiP (U/ml^{-1})		CAT (U/ml^{-1})	
	Control	Treatment	Control	Treatment	Control	Treatment	Control	Treatment
A. alternata	2.6	4.87	6.15	7.44	ND	ND	ND	11.27*
A. nomius	ND	2.18*	9.98	10.57	2.65	4.24	ND	2.66*
A. terreus	1.6	3.62	1.51	2.43	1.56	3.44	1.95	15.73*
C. sphaerospermum	2.34	5.240	ND	ND	1.87	4.08	1.80	20.25*
E. hirayamae	ND	5.34*	2.56	8.27*	2.38	12.12*	1.88	28.81*
G. pallida	ND	ND	1.95	1.72	0.94	1.83	0.93	2.45
P. variotii	ND	3.85*	1.78	6.63*	ND	ND	1.37	17.82*
P. vinaceum	2.4	3.19	ND	1.58*	0.74	2.31	ND	2.55*
P. alba	2.4	4.84	3.92	5.11	ND	ND	ND	4.67*
Consortium	9.8	12.69	20.27	20.76	6.04	11.38*	6.33	36.93*

*Significantly different from the control at $P \leq 0.05$.

appearance. In particular, profuse growth of *P. variotii*, *C. sphaerospermum* and *E. hirayamae*, respectively, was noticed around the engine oil droplets in the broth. All isolates, except *G. pallida*, showed a greater accumulation of biomass as compared with their corresponding controls (Table 2).

The greatest accumulation of biomass (55.5%) was recorded when the engine oil substrate was treated with the consortium of all nine fungal isolates. Individually, *E. hirayamae* gained the maximum weight of 45% followed by *C. sphaerospermum* (41%); whereas the minimum weight gain (11.2%) was noticed in *G. pallida*, which was not significantly different from its corresponding control. At some point, it was observed that there was growth and extension of the hyphae forming mycelium in the medium.

Enzyme activity of fungal isolates

Production of catalase, laccase, manganese-dependent peroxidase MnP, lignin peroxidase LiP, and cellulase enzymes was recorded in most of the control and treatment cultures of the nine tested fungi, as well as their consortium (Table 3). The level of catalase was found to be significantly elevated as compared with the corresponding controls in most of the treatments. In the case of enzyme activity, the highest enhancement was again recorded with the consortium of the nine fungi: catalase (36.93 U/ml), laccase (12.69 U/ml), and LiP (11.38 U/ml). In general, laccase and lignin peroxidase showed only a modest increase over the controls, while the level of manganese-dependent peroxidase did not show a significant change except in the case of *E. hirayamae* and *P. variotii*. Among the nine isolates tested, *E. hirayamae* appeared to have produced the highest level of all four enzymes assayed: catalase (28.81 U/ml), LiP (12.12 U/ml) MnP (8.27 U/ml) and laccase (5.34 U/ml); followed by *C. sphaerospermum*: catalase (20.25 U/ml), LiP (4.08 U/ml), and laccase (5.24 U/ml).

CO_2 evolution during the treatment of engine oil with fungal isolates

No significant difference was observed between CO_2 evolution estimates taken by volumetric and gravimetric methods; therefore, the data was merged as the means of the two procedures for each fungal isolate (Table 4). The greatest differential of CO_2 production over the corresponding control (88.0%) was recorded for the consortium of the nine fungi. Among the individual isolates, *E. hirayamae* showed the maximum enhancement of CO_2 emission (75.6%), followed by *C. sphaerospermum* (71.7%) and *A. terreus* (66.6%). This trend closely matched the trends of biomass accumulation and enzyme activity suggesting that a variable degree of bioremediation of

Table 4. CO_2 evolved during cultivation of fungal isolates with engine oil as the sole carbon source.

Fungal isolate	CO_2 emission in 4 weeks			
	Controls (g/l)	Treatments		
		Actual (g/l)	Increase over controls	
			Weight (g/l)	Percent (%)
A. alternata	0.460	0.667	0.207*	45.0
A. nomius	0.397	0.506	0.109*	27.4
A. terreus	0.435	0.725	0.290*	66.6
C. sphaerospermum	0.432	0.742	0.310*	71.7
E. hirayamae	0.448	0.787	0.339*	75.6
G. pallida	0.399	0.453	0.054	13.5
P. variotii	0.445	0.734	0.289*	64.9
P. vinaceum	0.452	0.531	0.079	17.4
P. alba	0.466	0.598	0.132*	28.3
Consortium	0.493	0.927	0.434*	88.0

*Significantly different from the control at $P \leq 0.05$.

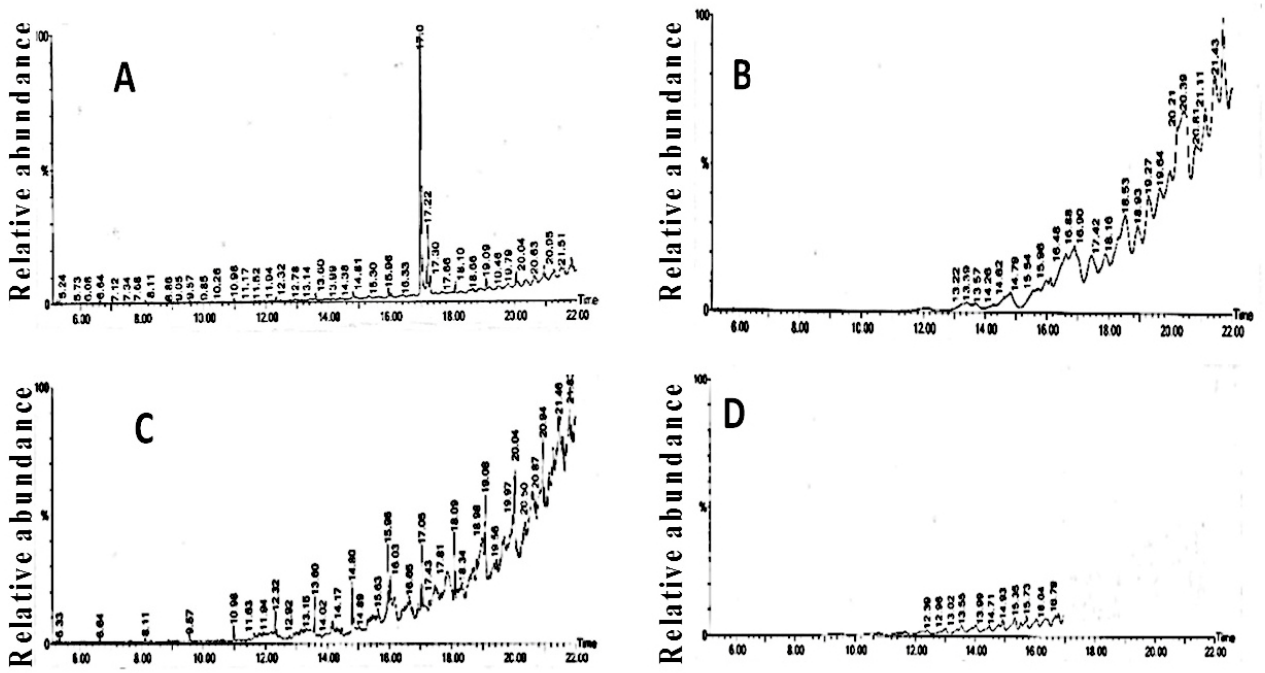

Fig. 2. GC-MS chromatograms of engine oil before and after treatments with fungal isolates: (A) Control, 1,2-benzenedicarboxylic acid, mono(2-ethylhexyl) ester as the major hydrocarbon; (B) Substrate treated with *E. hirayamae*, showing 5-Methyl-Z-5-docosene (RT:17.83 min); (C) Substrate treated with *C. sphaerospermum*; and (D) treated with the consortium of fungi; both showing Dodecane, 1-fluoro (RT: 16.12 min) as a major compound among other short chain molecules.

engine oil hydrocarbons took place by fungal activity, as reflected in the corroborative levels of enzyme production, biomass accumulation, and CO_2 liberation.

GC-Mass analysis

The total ion chromatograms of engine oil extracted from the inoculated medium at 0 and 30 days are presented in Fig. 2 showing the relative abundance of different sections of the chromatogram on the ordinate against their masses represented by peak height. The relative abundance of the consecutive chromatograms showed that the fungal strains, as well as their consortium, were efficient oil degraders. They completely degraded the main hydrocarbons of the engine oil, namely, tritetracontane, 1,2-benzenedicarboxylic acid, mono (2-ethylhexyl) ester, and Bis(2-ethylhexyl) phthalate at retention times (RT) 17.00 and 17.22, respectively. As a result of the breakdown, several low mass molecules, such as 5-Methyl-Z-5-docosene and Dodecane, 1-fluoro appeared predominantly (Figs. 2B, C, and D).

Discussion

The genotype of the nine active fungal isolates was confirmed by the sequencing of 18S rRNA gene fragment and phylogenetic analyses. Sequencing of conserved segments within internal transcribed sequences is considered one of the most authentic tools for the screening and identifi-

cation of environmental microbes (Pang and Mitchell, 2005). Genotypic diversity within a comparatively small set of nine isolates is remarkable, since, except for two species of *Aspergillus*, all the other isolates belong to different genera.

All the tested isolates showed a greater accumulation of biomass as compared with their corresponding controls when cultivated with engine oil as the sole source of carbon, indicating that the fungi were able to feed upon the engine oil. In whatever form, native or broken down, the fungi have consumed the substrate, implying the removal of oil from the cultivation system; although it is established that fungi can degrade complex molecules to simpler forms for uptake as food (Calmon et al., 2000; Chandra and Rustgi, 1998; Zee et al., 1994). These findings have demonstrated the oil scavenging potential of the fungal isolates originating from the polluted mangrove ecosystem as a continuum of the Red Sea.

Several other studies on the biodegradation potential of fungi have also reported biomass accumulation under similar conditions. Hasan (2014) reported a significant gain in the fresh weight of *A. niger* and *Rhizopus stolinifer* on 10% kerosene in the culture broth. Another study by Lotfinasabasl et al. (2012) showed that an *A. niger* isolate recorded better growth in 20% kerosene as compared with *A. terreus*, *Rhizopus* sp. and *Penicillium* sp.

The continued growth of fungi driven by biodegradation of engine oil was confirmed by Sturm test (Sturm, 1973) for the evolution of the CO_2. Several modified forms of the CO_2 evolution test have been reported in the literature, but the basic purpose remains to study the complete assimilation of polymeric carbon (Calmon et al., 2000; Chandra and Rustgi, 1998; Muller et al., 1992; Zee et al., 1994). Liberation of carbon dioxide during the cultivation of fungi with engine oil is direct evidence of fungal growth and an indirect indication of degradation activity. Vanishree et al. (2014) also evaluated the amount of CO_2 released during the biodegradation of petrol substrate as an indicator of the activity of *Aspergillus* sp. Balba et al. (1998) have emphasized that mineralization studies involving measurements of total CO_2 production can provide authentic information about the biodegradability of hydrocarbons in contaminated soils.

In the present study, the levels of the enzymes LAC, LiP, and CAT were significantly elevated as compared with the corresponding controls, during the cultivation of fungi with engine oil, probably because, here, the enzymes were in greater demand for hydrocarbon breakdown reactions. Catalase was the most abundant enzyme with a significant increase over the controls in all the treatments. Among the isolates, *Eupenicillium* showed a remarkably higher level of catalase indicating its strong degradation capability. It was the only fungus showing a higher production of all the enzymes assayed. Again, the fungal consortium showed the highest production of enzymes confirming a synergistic relationship. An elevated activity of three enzymes, including catalase (CAT), was noticed by Mohsenzadeh et al. (2012), also in the fungal strains of *Acromonium* sp., *Alternaria* sp., *A. terreus* and *Penicillium* sp., during the growth in media with different concentrations of petroleum pollutants. A high activity of catalase was also recorded in the soil microbial consortia in petroleum-polluted soils (Ugochukwu et al., 2008).

Biodegradation of a high mass polymer and hydrocarbons has to be facilitated by extracellular enzymes released by the acting microorganism (Mohan and Srivastava, 2010; Zheng et al., 2005). Hence, elevated enzyme levels in our treatment broths are a direct indication of biodegradation activity. Wu et al. (2010) investigated the biodegradation potential of *F. solani* strains against anthracene (ANT) and benz[a]anthracene (BAA) and recorded laccase to be the only active enzyme. They found no traces of MnP and LiP. In the case of wood-decaying fungi, the average rates of polycyclic aromatic hydrocarbon biodegradation have been correlated with the average activities of ligninolytic enzymes (Eibes et al., 2006).

Ali et al. (2012) indicated that the *A. terreus* isolated from Orman Garden soil and *P. chrysogenum* isolated from Wadi Degla protectorate soil, exhibited higher lignin peroxidase and manganese peroxidase activities during a biodegradation study on some PAHs, which is in agreement with our findings.

It is noteworthy that, in the present study, isolates, such as *E. hirayamae*, *C. sphaerospermum*, *A. terreus*, and *P. variotii*, which have accumulated a greater biomass in descending order, have shown corroborative levels of CO_2 and enzyme production. This consistent performance of these genotypes enables them to be ranked in terms of their biodegradation potential.

Consortium of the nine tested isolates was found to be more efficient in biomass accumulation, CO_2 liberation and enzyme production as compared with any of the fungi tested individually. This superior activity of the consortium could be due to a synergistic effect of the pooled genotypes. Silva et al. (2015) reported that a microbial consortium consisting of bacteria and yeasts from a polluted environment showed a high ability to degrade diesel oil constituents, and the maintenance of appropriate conditions leads to the transformation of this oily source into less toxic compounds. Luo et al. (2009) have also shown that dominant benzo[a]pyrene-degrading bacteria isolated from a marine enrichment showed a faster degradation when the three strains (*Ochrabactrum*, *Stenotrophomonas* and *Pseudomonas* spp.) were combined than when tested individually.

Chromatograms of engine oil treated with fungal cultures showed the disappearance of major engine oil peaks present initially, which indicated the degradation of these molecules. Disappearance of major peaks (Fig. 2A) and the appearance of several peaks representing low mass ions (Figs. 2B, C, and D) indicate that the main hydrocarbon of the engine oil has been broken down into many residues, which the fungi might have utilized for its growth. The number and intensity of the ions has been further diminished in treatments containing a consortium of all the isolates, which verifies that these fungi have acted synergistically in a community culture. Absence of this change in the controls was a strong indicator of the ability of fungi to degrade multiple hydrocarbon compounds. All the nine tested fungal isolates may prove to be good candidates for PAH biodegradation as they carried out degradation of high molecular weight PAHs of the engine oil.

Oboh et al. (2006) have reported the ability of some bacterial species, such as *Pseudomonas*, *Bacillus*, *Alcaligenes*, *Citrobacter*, and some fungi, such as *Aspergillus* sp., *Penicillum* sp., *Rhizopus* sp., and *Rhodotorula* sp., to grow on crude petroleum as the sole carbon and energy source when screened for hydrocarbon utilization.

It is significant to note that the greater biomass accumulation, CO_2 liberation, and enzyme production have demonstrated the potential of the tested isolates for the biodegradation of engine oil, suggesting that these strains could be further tested in field tests. The present study has demonstrated, for the first time, the potential of mangrove fungi from Red Sea coastal areas to degrade engine oil. Also, it is the first report describing the ability of *E. hirayamae* to degrade petroleum compounds. This study may be extended to more isolates from this habitat and to more pollutants of a diverse nature.

Acknowledgments

This study was supported by King Saud University, Deanship of Scientific Research, College of Science Research Center.

References

Aebi, H. (1983) Catalase in Methods of Enzymatic Analysis, 3rd ed., Verlag Chemie, H.U. Bergmeyer, Weinheim, pp. 273–286.

AL-Lihaibi, S. and AL-Ghamdy, T. (1997) Petroleum hydrocarbons in Saudi Red Sea Coastal Waters. *JKAU: Mar. Sci*., **8**, 83–89.

Ali, M., Khalil, N. M., and Abd El-Ghany, M. (2012) Biodegradation of some polycyclic aromatic hydrocarbons by *A. terreus*. *Afr. J. Microbiol. Res*., **6**, 3783–3790.

Ameen, F., Moslem, M. A., Hadi, S., and Al-Sabri, A. (2014) Biodegradation of cellulosic materials by marine fungi isolated from South Corniche of Jeddah, Saudi Arabia. *J. Pure Appl. Microbiol*., **8**, 3617–3626.

Balba, M. T., Al-Avadhi, N., and Daher, R. A. L. (1998) Bioremediation of oil—Contaminated soil microbiological methods for feasibility assessment and field evaluation. *J. Microbial. Methods*, **32**, 15–16.

Calmon, A. L., Bresson, D., Maurel, V. B., Feuilloley, P., and Silvestre, F. (2000) An automated test for measuring polymer biodegradation. *Chemosphere*, **41**, 645–651.

Chandra, R. and Rustgi, R. (1998) Biodegradable polymers. *Prog. Polym. Sci*., **23**, 1273–1335.

Eibes, G., Cajthaml, T., Moreira, M., Feijoo, G., and Lema, J. (2006) Enzymatic degradation of anthracene, dibenzothiophene and pyrene by manganese peroxidase in media containing acetone. *Chemosphere*, **64**, 408–414.

Gadd, G. M. (2001) Fungi in Bioremediation, 1st ed., Cambridge University Press, London, 472 pp.

Gagandeep, S. and Malik, D. K. (2013) Utilization of 2T engine oil by *Pseudomonas* sp. isolated from automobile workshop contaminated soil. *Int. J. Chem. Anal. Sci*., doi:10.1016/j.ijcas.2013.05.002.

Hasan, I. (2014) Biodegradation of Kerosene by *A. niger* and *Rhizopus stolinifer*. *Appl. Environ. Microbiol*., **2**, 31–36.

Hashem, A. R. (2007) Bioremediation of petroleum contaminated soils in the Persian Gulf region: a review. *J. Kuwait Sci*., **19**, 81–91.

Kumar, A. G., Vijayakumar, L., Joshi, G., Peter, D. M., Dharani, G. et al. (2014) Biodegradation of complex hydrocarbons in spent engine oil by novel bacterial consortium isolated from deep sea sediment. *Bioresour. Technol*., doi:10.1016/j.biortech.2014.08.008.

Lotfinasabasl, S., Gunale, V. R., and Rajurkar, N. S. (2012) Assessment of petroleum hydrocarbon degradation from soil and tarball by fungi. *Biosci. Disc*., **3**, 186–192.

Luo, Y. R., Tian, Y., Huang, X., Yan, C. L., Hong, H. S. et al. (2009) Analysis of community structure of a microbial consortium capable of degrading benzo[a]pyrene by DGGE. *Mar. Pollut. Bull*., doi:10.1016/j.marpolbul.2009.03.024.

Meyer, D. D., Beker, S. A., Bücker, F., Peralba, M. D., Frazzon, A. P. et al. (2014) Bioremediation strategies for diesel and biodiesel in oxisol from southern Brazil. *Int. Biodeter. Biodegr*., doi:10.1016/j.ibiod.2014.01.026.

Mohan, S. K. and Srivastava, T. (2010) Microbial deterioration and degradation of polymeric materials. *J. Biochem. Technol*., **2**, 210–215.

Mohsenzadeh, F., Chehregani Rad, A., and Akbari, M. (2012) Evaluation of oil removal efficiency and enzymatic activity in some fungal strains for bioremediation of petroleum-polluted soils. *Iranian J. Environ. Health Sci. Eng*., 9:26.

Muller, R. J., Augusta, J., and Pantke, M. (1992) An inter laboratory investigation into biodegradation of plastics; Part I: A modified Sturm-test. *Mater. Org*., **27**, 179–189.

Novotny, C., Erbanova, P., Sasek, V., Kubatova, A., Cajthaml, T. et al. (1999) Extracellular oxidative enzyme production and PAH removal in soil by exploratory mycelium of white rot fungi. *Biodegradation*, doi:10.1023/A:1008324111558.

Oboh, B. O., Ilori, M. O., Akinyemi, J. O., and Adebusoye, S. A. (2006) Hydrocarbon degrading potentials of bacteria isolated from a Nigerian Bitumen (Tarsand) Deposit. *Nature. Sci*., **4**, 51–57.

Okoh, A. I. (2003) Biodegradation of bonny light crude oil in soil microcosm by some bacterial strains isolated from crude oil flow stations saver pits in Nigeria. *Afr. J. Biotechnol*., doi:10.5897/AJB2003.000-1021.

Pang, K. L. and Mitchell, J. I. (2005) Molecular approaches for assessing fungal diversity in marine substrata. *Bot. Mar*., doi:10.1515/bot.2005.046.

Paszcymski, A., Crawford, R., and Huynh, V. B. (1998) Manganese peroxidase of *Phanerochaete chrysosporium*: purification. *Meth. Enzymol*., doi:10.1016/0076-6879(88)61028-7.

Silva, D. P., Cavalcanti, D. D., de Melo, E. V., dos Santos, P. N. F., da Luz, E. L. P. et al. (2015) Bio-removal of diesel oil through a microbial consortium isolated from a polluted environment. *Int. Biodeter. Biodegr*., doi:10.1016/j.ibiod.2014.09.021.

Sturm, R. N. J. (1973) Biodegradability of nonionic surfactants: screening test for predicting rate and ultimate biodegradation. *J. Oil. Chem. Soc*., **50**, 159.

Ugochukwu, K. C., Agha, N. C., and Ogbulie, J. N. (2008) Lipase activities of microbial isolates from soil contaminated with crude oil after bioremediation. *Afr. J. Biotechnol*., doi:10.5897/AJB08.093.

Van Hamme, J. D. and Ward, O. P. (2001) Volatile hydrocarbon biodegradation by a mixed-bacterial culture during growth on crude oil. *J. Microbiol. Biotechnol*., doi:10.1038/sj.jim.7000145.

Vanishree, M., Thatheyus, A. J., and Ramya, D. (2014) Biodegradation of petrol using *Aspergillus* sp. *Ann. Res. Rev. Biol*., **6**, 914–923.

White, T. J., Bruns, T., Lee, S., and Taylor, J. (1990) Amplification and direct sequencing of fungal ribosomal RNA genes for Phylogenetics. *In* PCR Protocols: A Guide to Methods and Applications, ed. by Innis, M. A., Gelfand, D. H., Sninsky, J. J., and White, T. J., Academic Press, San Diego, CA, pp. 315–322.

Wiese, J., Ohlendorf, B., Blümel, M., Schmaljohann, R., and Imhoff, J. F. (2011) Phylogenetic identification of fungi isolated from the marine sponge *Tethya aurantium* and identification of their secondary metabolites. *Mar. Drugs*, doi:10.3390/md9040561.

Wu, Y., Luo, Z., and Vrijmoed, L. (2010) Biodegradation of anthracene and benz[a]anthracene by two *F. solani* strains isolated from mangrove sediments. *Bioresour. Technol*., doi:10.1016/j.biortech.2010.07.049.

Xu, N., Bao, M., Sun, P., and Li, Y. (2013) Study on bioadsorption and biodegradation of petroleum hydrocarbons by a microbial consortium. *Bioresour. Technol*., doi:10.1016/j.biortech.2013.09.024.

Zee, M. V. D., Sutsma, L., Tan, G. B., Tournois, H., and De Wit, D. (1994) Assessment of biodegradation of water insoluble polymeric materials in aerobic and anaerobic aquatic environments. *Chemosphere*, **28**, 1757–1771.

Zheng, Y., Yanful, K. E., and Bassi, A. S. (2005) A review of plastic waste biodegradation. *Crit. Rev. Biotechnol*., **25**, 243–250.