Soil Microcosms for Bioaugmentation With Fungal Isolates to Remediate Petroleum Hydrocarbon-contaminated Soil

Dalel Daâssi (daleldaassi@yahoo.com)  
Jeddah University

Fatimah Qabil Almaghribi  
Jeddah University

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Abstract

The aim of this work was to isolate indigenous PAH degrading-fungi from petroleum contaminated soil and exogenous ligninolytic strains from decaying-wood, with the ability to secrete diverse enzyme activity. A total of ten ligninolytic fungal isolates and two native strains, has been successfully isolated, screened and identified. The phylogenetic analysis revealed that the indigenous fungi (KBR1 and KB8) belong to the genus *Aspergillus niger* and *tubingensis*. While the ligninolytic exogenous PAH-degrading strains namely KBR1-1, KB4, KB2 and LB3 were affiliated to different genera like *Syncephalastrum sp*, *Paecilomyces formosus*, *Fusarium chlamydosporum*, and *Coniochaeta sp.*, respectively. Basis on the taxonomic analysis, enzymatic activities and the hydrocarbons removal rates, single fungal culture employing the strain LB3, KB4, KBR1 and the mixed culture (LB3+KB4) were selected to be used in soil microcosms treatments. The Total petroleum hydrocarbons (TPH), fungal growth rates, BOD<sub>5</sub>/COD ratios and GC-MS analysis, were determined in all soil microcosmos treatments (SMT) and compared with those of the control (SMU). After 60 days of culture incubation, the highest rate of TPH degradation was recorded in SMT[KB4] by approximately 92±2.35% followed by SMT[KBR1] then SMT[LB3+KB4] with 86.66±1.83% and 85.14±2.21%, respectively.

Introduction

Over the last two decades, accelerated industrialization, and the massive use of aromatic compounds in explosives, dyestuffs, pesticides and pharmaceuticals has resulted in serious environmental contamination of soil, water, and air.

Oil spillage is a serious threat to all compartments of the ecosystem<sup>1</sup>. During extraction, transportation, storage and distribution operations, crude oil and its refined products are frequently exposed to accidental spillage causing soil pollution<sup>2</sup>.

Soils contaminated with persistent organic pollutants (POPs) associated with petroleum such as PAHs, have high potential health risk because its ability to enter food chain and its affinity for accumulation in living organisms<sup>3</sup>.

Soil matrix properties and functions are closely related to the different activities occurring on land and xenobiotic structures like PAHs- associated with petroleum. Owing to the chemical stability of PAHs, their hydrophobicity and recalcitrance to microbial degradation, spilled oil may damage the biological and physico-chemical properties of the petroleum-polluted soil.

Petroleum hydrocarbons cause alteration of soil biological properties, affecting the microbial diversity and the enzymatic activities as well as the physico-chemical characteristics<sup>4,5</sup>. Certain essential soil function may be lost due to the high toxicity of such persistent aromatic hydrocarbon structures<sup>6</sup>. Indeed, spilled oil may develop anaerobic conditions and asphyxia in soil pore with consequent impacts on the microbial activities<sup>7</sup>. In this regard, Klamerus-Iwan et al.<sup>8</sup> demonstrated a significative decline of the
microbial biomass and the enzymatic activities (urease and dehydrogenase) in soil polluted by chainsaw oil.

Further, Petroleum polluted-areas are characterized by a lower self-purification capacity results of the reduction on the indigenous microbes involved in soil-purification processes\textsuperscript{9}.

Remediation of petroleum polluted soils is important to remove pollutants from the environment and it can be done by several different methods involving removal/isolation or alteration of the contaminant.

In this regard, various physic-chemical treatment techniques for soil reclamation have been massively tested to remove or transform PAH pollutants.

Some of these remediation techniques are plasmas oxidation, photocatalytic degradation, vapor extraction, flotation, ultrasonication, electro kinetics remediation, thermal desorption, biochar adsorption\textsuperscript{10,11}. Whereas, those strategies are costly and most of them are not completely effective.

As an alternative, the biological treatment or bioremediation has become a promising approach for restoring petroleum-contaminated regions. Generally, biological method employs the natural potential of microbes including bacteria, yeasts, (bacterial remediation), algae (phycoremediation) and fungi (Mycoremediation), for the biodegradation of petroleum hydrocarbons pollutants. Bioremediation\textsuperscript{12} effectiveness is often in relationship with the microbial population and how it can be enriched and maintained in the polluted area\textsuperscript{12}.

The simplest bioremediation strategy is the natural attenuation. This method requires only the control of the natural degradation processes occurring by the native microbial population. But, this approach is not always successful and requires extensive long-term monitoring. It can be used for the restoration of areas with low level of contamination\textsuperscript{13}. Approximately 25% of all petroleum-contaminated land has been remediated using natural attenuation\textsuperscript{14}.

Conventionally, bioremediation processes can be accelerated by enhancement the intrinsic microbial population with pollutants-degrading potential microbes (either, indigenous or exogenous microorganisms). This approach is often used at high concentrations of spilled oil, where natural degrading microbes are absent or insufficient\textsuperscript{15}. Indeed, hydrocarbons compounds can delay or inhibit microbial proliferation and activities, so for an effective in situ biodegradation, bioaugmentation is important\textsuperscript{16}.

The employing of an indigenous microorganism consortium ensures that the organisms have a higher tolerance to the toxicity of aromatic hydrocarbon and are resistant to variations in the environment\textsuperscript{17,18}. Exogenous microbes are useful with more complex hydrocarbons structures, where the rates of intrinsic biodegradation will be the slower of hydrocarbons degradation.
Therefore, bioaugmentation approaches are necessary to enhance the performance of indigenous microbial population several fold through the introduction of microbes with specific metabolic activities for an effective in-situ remediation of polluted areas\textsuperscript{19}.

Typically, fungi are suited for bioremediation of crude oil in polluted sites owing of their diverse metabolic activities. They are able to secrete a board range of ligninolytic and non-ligninolytic enzymes to use petroleum hydrocarbons as a carbon and energy source and assimilate into fungal biomass\textsuperscript{20}. Moreover, the efficiency of fungal culture to remove or degrade PAHs from petroleum contaminated soil is related to various factors, among them pollutant bioavailability, survival of microorganism and their metabolic diversity are essential for bioaugmentation\textsuperscript{21}.

Previously, it was well demonstrated that Soil microcosms (SM) serve as test systems that may be adapted to various environmental conditions. Indeed, outcomes of microcosm studies are often used to develop remedial pilot process specifications\textsuperscript{22}.

The current study highlights the application of newly fungal isolates (indigenous from the investigated soil and exogenous from decay wood) in the PAHs-contaminated soil remediation processes. Individual and mixed fungal cultures are selected based on their taxonomy and metabolic diversity, to enhance the bioremediation performance in soil microcosmos systems.

**Materials And Methods**

**Chemicals**

The compounds used as laccase substrates were: 2,6- dimethoxyphenol (2,6-DMP), 2,2′- azinobis-(3-ethyl benzthiazoline- 6-sulphonate) (ABTS), guaiacol. The used chemicals were of analytical grade and were from Sigma-Aldrich (St. Louis, MO, USA).

**Soil Sampling, physico-chemical characteristics, and microbial population**

PAHs contaminated- soil samples were collected from spots around the oil well (7) located in Dammam city (Saudi Arabia). All samples of soils were taken at depth 5-10 cm from upper surface of topsoil. Samples were transferred to laboratory in nylon sterilized sac closed tightly and marked with relevant information (number, location specific characteristics and date). Before utilization in the treatment study, all soil samples were mixed and sieved to remove particulars greater than 1.25 cm and saved under 4 °C.

The physicochemical analysis was performed by the Arabia Life Sciences Division-Environmental Saudi Arabia (ALS) which is diversified testing services organization.

The analytical procedures used by the ALS have been developed from established internationally recognized procedures such as those published by the USEPA. APHA AS. NEPM. FDA/BAM. AOAC. ISO etc.
The pH, moisture content, biological oxygen demand (BOD), total petroleum hydrocarbon (TPH) semi-volatile and volatile organic compounds- BTEX (benzene, toluene, ethylbenzene and xylene) of the petroleum-contaminated soil before and after fungal treatments were determined (Table 3).

For the estimation of the total Petroleum hydrocarbon (TPH) (semi-volatile), the USEPA 8015B method gas chromatography/flame ionization detection (GC/FID) was conducted. Sample extracts were analyzed by Capillary GC/FID and quantified against alkane standards over the range C10 - C40.

TPH Volatiles / BTEX were determined using the method EPA 8260 Purge and Trap- gas chromatography-mass spectrometry (GC/MS). Extracts are analyzed by Purge and Trap. Capillary GC/MS. Methanol Extraction of Soils for Purge and Trap was performed by the method (USEPA SW 846 - 5030A)

The soil pH was determined by digital pH meter in a soil water suspension (1:2.5) as described by Jackson.

The moisture contents of soil were determined gravimetrically, based on weight loss over 12 hours drying period at 103-105 °C. This method is compliant with NEPM (2013).

Enumeration of bacteria from contaminated soil was performed by using serial dilution and plating technique. The microbial populations were counted in terms of Colony forming units (CFUs).

Isolation of decay-wood decomposing fungi and indigenous soil fungi

Decay-wood samples were collected in sterilized and labeled plastic bag from different biotopes of the region of Barzah and Rahat from Khulais-Jeddah city, during March 2020. Malt Extract Agar (MEA) (30.0 g/L, pH 5.5.) supplemented with antibiotics (0.01% of ampicillin and streptomycin) was used as selective media for the isolation of fungal strains. The isolation of decay-wood decomposing fungi was carried out by direct plat method as suggested by Daâssi et al. The purity of the fungal strain was proofed by microscopic observation.

Both soil-plates and soil-suspensions methods were used for the isolation of soil fungi. Plates were prepared by transferring 0.05 to 0.015 g of the contaminated-soil to be examined into a sterilized Petri dish. Cooled medium of MEA was added and the soil particles dispersed throughout the agar by gentle shaking of plates before the agar solidifies. Soil-suspension solution was prepared from 10 g of the dried contaminated soil dissolved in 100 mL of sterile physiological water (NaCl 9 g/L) and maintained for 20 min under agitation on a reciprocating shaker at 120 rpm. After shaken, serial dilutions and plating technique were performed according to Agrawal et al. All plates were incubated at 30 °C during 5 to 7 days. Fungal colony was sub-cultured on fresh MEA supplemented with 0.01% of ampicillin and streptomycin until getting a pure strain. Preliminary identification of the fungal isolates was performed through macroscopic and microscopic observation.

Selection of Hydrocarbon-degrading fungal isolates
Preliminary screening of oil-degrading fungal isolates (both ligninolytic and native fungal isolates) was performed by agar well diffusion method. As sole source of carbon and energy, 5% of Diesel fuel (Saudi Aramco, defined according to Daâssi et al.\textsuperscript{52} has been spread on the surface of the MEA plates with glass rod. Fungal strains suspensions (in sterile water) was prepared and downloaded in MEA plate wells. The culture plates were incubated at 30 °C, and the appearance of substantial growth was daily monitored during 5 days. Also, the culture plates with and without addition of Diesel fuel were examined for growth.

Then the selected fungal strains on the agar well diffusion method, were checked for its ability to grow and mineralize the petroleum hydrocarbons in the contaminated soil. Cultures were carried out on Mineral medium (MM).

Mineral Medium (MM) used for the selection of oil-degrading fungi of the following composition per liter: KH\textsubscript{2}PO\textsubscript{4},1.0 g; KCl, 0.5 g; MgSO\textsubscript{4} \cdot 7H\textsubscript{2}O, 1.0 g; 1 mL of oligo-element solution. This solution was used to improve mycelial growth according to Daâssi et al.\textsuperscript{52}.

The composition of the oligo-element solution was as below per liter: B\textsubscript{2}O\textsubscript{7}Na\textsubscript{2} \cdot 10H\textsubscript{2}O, 0.1 g; CuSO\textsubscript{4} \cdot 5H\textsubscript{2}O, 0.01 g; FeSO\textsubscript{4} \cdot 7H\textsubscript{2}O, 0.05 g; MnSO\textsubscript{4} \cdot 7H\textsubscript{2}O, 0.01 g; ZnSO\textsubscript{4} \cdot 7H\textsubscript{2}O, 0.07 g; (NH\textsubscript{4})\textsubscript{6}Mo\textsubscript{7}O\textsubscript{24} \cdot 4H\textsubscript{2}O, 0.01 g. Before autoclaving, the pH of the solution was adjusted.

5 g of the soil sample was mixed with 20 mL of MM and inoculated with 1% of spore suspension of each isolate. Then the 250-mL Erlenmeyer flasks were incubated in stationary-phase for 14 days at 30 °C.

\textbf{Plate-agar tests for the investigation of the enzymatic activities}

Plates containing selective media (supplemented with the suit enzyme substrate) were used as a qualitative test to detect the enzymatic activities in the fungal collection. Under aseptic conditions, mycelial fraction was taking from each pure strain, and placing on the surface of the selective agar media. After incubation at 30 °C, fungal strains were recorded as positive or negative based on the appearance of degraded halo surrounding mycelium growth.

\textbf{Laccase activity}

To detect laccase-producing fungi, strains were grown on selective solid medium MEA supplemented with 150 µM copper sulfate (as Lac inducer) and 5 mM of 2,6-DMP/or 0.2 mM of ABTS (Lac substrates) then, incubated at 30 °C. Fungal isolates which showed red-brown (with 2,6-DMP) and green halos with ABTS), were selected as Lac positive (Lac (+)) strains and transferred into 250 mL flasks of MEB added with 150 µM CuSO\textsubscript{4}, as inducer, for further characterization.

\textbf{Proteolytic activity}

Sterile milk (250 mL L\textsuperscript{-1} (v/v)) was incorporated as fungal protease substrate in the nutrient agar medium (pH 5.5) containing 5 g/L of peptone and 3 g/L of yeast extract after sterilization and semi-cooling of the media. The presence of the degraded milk halo is evidence of the proteolytic activity\textsuperscript{53}.
CMCase activity

Modified Mandels and Reese medium was used as screening medium to select CMCase positive (CMCase+) species\textsuperscript{54}. Fungi were grown on the agar medium and incubated for 7 days at 30 °C. After fungal growth, CMC agar plates were stained with 1\% (w/v) Congo-Red solution for 15 min and discolored with NaCl (1 M) for 15 min. CMCase activity is detected by the presence of a halo around the isolate\textsuperscript{55}.

Lipase activity

For the detection of lipase activity, a selective medium of MEA amended by 1\% olive oil and 0.001\% rhodamine B was used. The fungal isolates were incubated at 30 °C during 7 days then revealed using 365 nm light. The positive strains showed fluorescence under the UV- light\textsuperscript{50}.

Amylase activity

For the α -amylase activity detection, starch agar plate method was performed. The fungal isolates were inoculated onto a starch plate and incubated at 30 °C until growth is seen. The petri dish is then flooded with an iodine solution to visualize the degraded halo. Amylase positive (Amyl+) species presents a clearing halo around the mycelial growth\textsuperscript{56}.

Optical microscopy

Optical microscopy images of the suspended mycelium were taken after a seven-days old MEA-plate fungal cultures using an optical VHX-5000 digital microscope (Keyence).

Identification and phylogenetic tree of fungal isolates

The selected hydrocarbon-degrading fungal strains were cultivated in a 150 mL flask containing 50 mL of liquid Malt Extract Broth medium (MEB) for 5 days. Then mycelium was harvested by filtration and successive washings with sterile Milli-Q water. The genomic DNA was extracted from the fungal cells using a DNeasy Plant Mini Kit (QIAGEN). The purity and the quantity of DNA samples were estimated by the optical density ratio A260/A280. The molecular identification was carried out with the protocol suggested by Daâssi et al.\textsuperscript{57}. The primers used for the amplification were ITS1 (5\textsuperscript{\textendash}TCCGTAGGTGAACCTGCGG-3) and (3\textendash TCCTCCGCTTATTGATATGC-5)\textsuperscript{58}. Blastn analysis was used for the resulting sequences (\textit{www.ncbi.nlm.nih.gov/BlastN}). The organisms were identified based on the subjected sequences in the databases showing the highest identity.

Multiple sequence alignment was achieved using ClustalW between the selected subjected sequences and the query ITS sequences of the isolated strains\textsuperscript{59}.

Phylogenetic tree was inferred using the neighbor-joining method (NJ)\textsuperscript{60} in the MEGA11 program with bootstrap values based on 1000 replicates\textsuperscript{61}. Sequences have been deposited in GenBank.
Biodegradation of Petroleum hydrocarbon-contaminated soil

Fungal inoculum preparation

Mycelial suspension from the selected hydrocarbon-degrading fungi exogenous ligninolytic (LB3; RB4) and indigenous (BKR1) was prepared as described by Potin et al.\textsuperscript{62}. Seven-days old MEA-plate fungal cultures of the 3 selected isolates was washed with 5 mL of sterile physiological water to obtain the fungal suspension which further was filtrated through sterile glass wool to separate mycelia from spores. The collected spore suspensions were estimated by Thoma cell counting chamber.

25 mL MEB amendment was added to each microcosm in order to induce spore germination in the microcosms. Spores were added to the medium in calculated volumes to give a final total spore concentration of $10^4$ spores g soil$^{-1}$.

Soil microcosms assays for bioaugmentation studies

Soil microcosms were used in this study for the mycoremediation of the PHC-contaminated soil. Each microcosm contained 50 g of 6 mm sieved soil mixed with 125 mL of MEB inside 400 mL glass bottles sealed with rubber caps and aluminum seals and incubated at room temperature for 60 days.

Control soil Microcosms (zero day/untreated/without fungal inoculation) and test soil microcosm (treated) for each fungal culture (mono or co-culture) were set up during 60 days of treatment in order to evaluate the biotic vs abiotic degradation of the PHC contaminated soil. All the microcosm experiments were conducted in triplicate.

Soil microcosm treatments (SM) were designed:

(SMU-sterile): control microcosms conducted by air-dried untreated contaminated soil (sterile soil) to assess abiotic losses of hydrocarbons.

(SMU-Not sterile): control microcosms formed by untreated not sterile soil to assess biotic degradation.

(SMT[KBR1]): bioaugmentation with indigenous fungal biomass; consisting of soil inoculated with the selected native fungus (KBR1).

(SMT[LB3]): bioaugmentation with exogenous monoculture; consisting of soil inoculated with the selected ligninolytic hydrocarbon-degrading fungi (LB3).

(SMT[KB4]): bioaugmentation with exogenous monoculture; consisting of soil inoculated with the selected ligninolytic hydrocarbon-degrading fungi (KB4).

(SMT[LB3+KB4]): bioaugmentation with exogenous mixed culture; consisting of soil inoculated with the co-culture (LB3+KB4).
All the microcosm treatments were inoculated with an initial concentration of $10^4$ spores per gram of soil. Soil amendment was maintained regularly at 2.5 mL g$^{-1}$ (MEB/ soil).

Soil samples from each microcosm were collected manually with clean and sterilized (ethanol 70%) stainless steel spatulas on days 15, 30 and 60 for the analytic analysis to assess hydrocarbons degradation in the soil microcosms.

**Assessment of Petroleum-contaminated soil degradation by fungal isolates**

**Biomass estimation**

The variation in the rate/biomass of fungal cultures (mono or co-cultures) was determined gravimetrically. The weight of flask was taken before and after incubation period.

**Biological Oxygen Demand (BOD) and Chemical Oxygen Demand (COD)**

The rate of degradation and the efficiency of the fungal isolates in the treatment of the PAHs-contaminated soil, were evaluated by the BOD and COD analysis performed by standard methods$^{63}$ (APHA, 2001 and IS-3025)

**Total Petroleum Hydrocarbon (% TPH) and Gas chromatography-Mass Spectrometry (GC-MS) analysis**

The extraction of the residual petroleum hydrocarbons from the contaminated soil was carried out by mechanical shaking as described by Siddique et al.$^{64}$ with some modifications.

For each culture incubation period (15, 30 and 60 days), the remaining petroleum hydrocarbons (PHCs) was extracted from each soil microcosms (untreated/treated) using of 30 mL of Dichloromethane (DCM). 10g of soil sample were put in glass bottle added with anhydrous sodium sulphate ($\text{Na}_2\text{SO}_4$) to remove moisture. The mixture was acidified with of concentrated HCl (12 N) to avoid further degradation and shaken on a reciprocating shaker at 120 rpm for 3 hours. Then, the he reactional mixture was separated by centrifugation (10 min, 8000, at 4 °C). The supernatant was transferred in separating funnel (250 mL) to remove the aqueous layer and sequentially extracted twice with 2 volumes of dichloromethane, respectively. Finally using a rotator evaporator, the dichloromethane was evaporated in 55 °C and the extracts were concentrated near dryness then re-dissolved in 1 mL dichloromethane solution. The samples were kept at -20 C until being analyzed. The residual PHC concentration of the untreated and the treated by the mono or the co-fungal cultures was determined.

The percentage of Total Petroleum Hydrocarbon (TPH) by gravimetrically and results were expressed as percentages of respective controls$^{51}$.

Biodegradation efficiency (%) was calculated by the formula given by Bishnoi et al.$^{65}$. Biodegradation efficiency (%) = \((C_0-C_e)/C_0\)
where $C_0$ initial concentration of PHC (mg/L); $C_e$ equilibrium concentration of PHC (mg/L).

The treated/extracted petroleum hydrocarbons were analyzed by gravimetric analysis and gas chromatography (GC) by Agilent GC-MSD (6890N-5973) with pole temperature kept at 80 °C for 4 min, then increased at a rate of 5 °C·min$^{-1}$ to 250 °C and maintained at 250 °C for 20 min.

**Results And Discussion**

**Isolation of decay-wood decomposing fungi and soil native fungi**

Pieces of decaying wood samples used in this study were collected from different biotopes of western Saudi Arabia (Khulais-Jeddah). Barzah and Rahat are two natural habitats from the Khulais, selected for the decaying wood sampling during March 2020.

Primary identification of the ligninolytic fungal isolates was based on plate morphology while the purity of the isolates was proofed using microscopic parameters (Fig. 1a-f).

From the decay wood samples, a total of ten pure wood-decomposing fungal isolates were obtained and proofed by microscopic observation.

Ten morphologically different fungal strains were isolated successfully, two abundant strains from the polluted soil and ten from the decay-wood, then were maintained as pure cultures in MEA.

Both soil-plate and soil-suspension methods were used for the isolation of soil native fungi.$^{23}$ Two fungal strains presented the highest abundance and growth ability in the soil sample were examined by the soil plate and soil suspension isolation method.

These native fungi designed by KBR8 and KBR1 were chosen and identified by morphological characters and taxonomical keys. Based on the morphological aspects and microscopic observation, the strains KBR1 and KBR8 demonstrated the general characteristics of the genus *Aspergillus*.

**Selection of hydrocarbon-degrading fungal isolates**

The indigenous fungal isolates and the ligninolytic fungi were tested for their ability to degrade petroleum hydrocarbons. Primary screening was conducted on culture plates basis on the agar well diffusion method (Fig. 2).

Out of the twelve isolated strains investigated in the culture plate experiment, six were recorded as petroleum hydrocarbon-degrading via a degraded halo surrounding mycelium growth. The diameter of the halo demonstrates the ability of fungus to utilize petroleum hydrocarbons.

In all petri dishes the highest growth diameter was around the mycelia of KBR1, KBR8 (native soil isolates) and KB4, LB3 (ligninolytic isolates) indicating good ability to degrade diesel hydrocarbons among the other isolated strains. However, KBR1-1 and KB2 showed a tight halo during fungal growth.
In addition, the mix culture consisting of LB3+KB4 showed the highest growth diameter.

Lotinasabasli et al.\textsuperscript{24} in related study on fungal isolated strains from soil hydrocarbon-polluted samples indicated that isolated fungi can be used in hydrocarbon bioremediation processes however, their efficiency varied within the species and the metabolic diversity according to the fungi.

Later, a confirmatory assay for hydrocarbon degradation, potentials of the isolated fungi was conducted on Erlenmeyer flasks with the investigated soil. The gravimetric determination of the residual hydrocarbon after biodegradation was performed by weighing the quantity of the petroleum hydrocarbons against the control.

The estimated crude oil degradation efficiency after 14 days demonstrated that the ligninolytic isolate KB4 showed maximum ability to utilize crude oil, giving the highest percent degradation of 29.32%, followed by the native isolates KBR1 and KBR8 indicating 22.68 and 20.34% degradation, respectively (Fig. 3).

These results are in line with those recorded by Lotinasabasli et al.\textsuperscript{24} who reported a highest remediation rate belongs to \textit{Aspergillus niger} (20.55%) and the lowest rate belongs to \textit{Penicillium sp} (16.453%).

Additionally, the mycelial growth and the biomass gain profiles indicated that all the selected strains previously tested on the culture plate were able to grow and use the petroleum hydrocarbons as carbon source.

Figure 3 shows an increase in rates of fungal growth in the media containing petroleum contaminated soil compared with inoculated media (MM) with not polluted soil. This result demonstrates the ability of the fungal strains to assimilate petroleum hydrocarbons molecules using diverse extra cellular enzymes for their growth.

In this regard, Oboh et al.\textsuperscript{25} reported the ability of \textit{Aspergillus sp., Penicillium, Rhizopus} and \textit{Rhodotorula} species to grow on crude petroleum as the sole source of carbon and energy.

Fungal culture on petroleum polluted soil proofed the potent of the selected fungi in the degradation of TPH from the contaminated soil and were thus selected for further study.

The degradation ability of different genera from different habitats makes their catabolic potential even more versatile to transform persistent organic compounds into inert and non-toxic molecules\textsuperscript{26}.

Among the Twelve isolated fungi, the most interesting fungal strains in term of crude oil degradation (LB3, KBR1, KBR8, KB2, KBR1-1) were cultivated and run for molecular identification based on the analysis of the amplified nucleotide sequences of the nuclear ribosomal ITS1-5.8-ITS4 region.

\textbf{Molecular identification of the selected petroleum hydrocarbon- degrading fungi}
The molecular identification of the strains was performed by BLAST alignment tool of the National Center for Biotechnology Information (NCBI) database. Closely related sequences were obtained from the GenBank database with similarity greater than 95% (Table 1).

The ITS regions of the fungal strains were sequenced at Macrogen (Republic of Korea) and submitted to GenBank database with accession numbers; MZ817958, MW699896, MW699897, MZ817959, MW699895 and MW699893.

Based on the percentage of similarity (Table 1) all fungal isolates were attributed to the genus or even the species level according to Rossello Mora and Amman.

The isolated wood-decomposing fungi designed as KBR1-1, KB4, KB2 and LB3 were affiliated to different genus like *Syncephalastrum* sp, *Paecilomyces formosus*, *Fusarium chlamydosporum*, and *Coniochaeta* sp. Respectively. While the indigenous petroleum degrading fungi (KBR1 and KB8) belong to the genus *Aspergillus niger* and *tubingensis* based on the Blast analysis.

Based on the multiple alignment of the ITS sequences provided by Clustal Omega program, the phylogenetic analysis was run to find the evolutionary relationships of the newly fungal isolated to previously characterized species (Fig. 4).

A phylogenetic tree was created by Neighbor Joining Method (NJ) based on alignment of the ITS sequences of exogenous fungal strains (KBR1-1, KB4, KB2 and LB3) and indigenous strains (KBR1 and KB8) with their homologue sequences obtained from NCBI database.

The dendrogram obtained shows that the isolate LB3 (Accession no. MW699893 clustered closely with *Coniochaetaceae* and *Sordariomycetes* sp. (99.68% identity) and with *Lecythophora* sp. (98.64% identity).

Analysis of 18S rRNA genes of the genus *Coniochaeta* revealed that the taxon appears as a monophyletic group related to teleomorphs of the genus *Lecythophora*.

According to Lopez et al., *Lecythophora (Coniochaeta)* is a filamentous ascomycetous which belongs to the *Coniochaetaceae* family and *Sordariales* order.

The strain KB4 (Accession no. MW699897) showed 98.39% ITS identifies with *Paecilomyces formosus*, *Thermoascaceae sp.* and *Penicillium sp.* and closed to the genus *Byssochlamys spectabilis* (98.12%). The morphological traits of the fungus were determined to affiliate the isolate to the genus *Paecilomyces formosus* (yellow septate hyphae, conidia unicellular).

The genus *Paecilomyces* was first described by Bainier as a genus closely related to Penicillium and comprising only one species, *P. variotii* Bainier.

Accordingly, previous study of Moreno-Gavíra et al. reported that the genus *Paecilomyces* has yellowish septate hyphae, with irregularly branched conidiophores and smooth walls. The conidia are unicellular, in
chains; and the youngest conidium is at the basal end.

The indigenous isolates (KBR8 and KBR1) were affiliated to the genus *Aspergillus* based on BLAST analysis of the ITS sequences. In addition, the phylogenetic analysis showed that the two indigenous isolates clustered in a clade comprising exclusively Aspergillus species, with high bootstrap values for each branch. ITS sequences of the fungal isolates were deposited at GenBank under accession numbers MW699895 and MW699896 for KBR8 and KBR1 respectively.

The KBR1-1 isolate showed homology with close strains including, *Sordariomycetes sp.* (Accession no. KR231683) (99.58% identity), *Syncephalastrum racemosum* (Accession no. KP764903.1) (98.87% identity).

It can be inferred from the phylogenetic tree that the strain closest to isolate KBR1-1 is the species *Syncephalastrum racemosum*. The related sequence, corresponding to strain KBR1-1 was deposited under the accession no. MZ817958.

The KB2 isolate (Accession no. MZ817957) showed 98.84% ITS identities with *Fusarium chlamydosporum*.

In the present work, *Aspergillus niger* (KBR1), *Lecythophora (Coniochaeta)* (LB3), *Paecilomyces formosus* (KB4), *Syncephalastrum racemosum* (KBR1-1), *Aspergillus tubingensis* (KBR8), and *Fusarium chlamydosporum* (KB2) were the perfect fungal isolates demonstrated efficiency to biodegrade petroleum hydrocarbons. Our results agree with results of Gesinde et al.\(^{31}\) who reported that *Aspergillus niger* have very active degradation capability of Nigerian and Arabian Crude Oils. Furthermore, in the same study, the genera, *Aspergillus, Penicillium* and *Fusarium* species were demonstrated as the most efficient metabolizers of hydrocarbons in comparison with other isolates.

**Screening of the enzyme activity for the newly isolates**

The capacity of the fungal isolates to produce several enzymatic activities such as lipases, proteases, amylases, cellulases, and laccases enzymes was investigated on selective solid media.

Out of the twelve tested isolated strains, seven strains were recorded to secrete laccases, 10 strains were cellulases positive, 4 strains were amylases positive, 21 strains were found to produce proteases and 4 strains were able to produce lipases (Table 2).

Among all the species, the *Aspergillus sp.*, attributed to the indigenous isolates KBR1 and KBR8, exhibited high amylolytic activity. The KBR1 strain (*Aspergillus niger*) showed the presence of laccase activity. Maximum cellulolytic activity was recorded for *Syncephalastrum racemosum* (KBR1-1). The fungal species *Paecilomyces formosus* (KB4) and *Fusarium chlamydosporum* (KB2) exhibited maximum lipase activity. The species *Lecythophora (Coniochaeta)* (LB3) showed the presence of ligninolytic activity.
Hence, according to Lopez et al.\textsuperscript{28} the ascomycete \textit{Coniochaeta ligniaria NRRL} was able to produce lignocellulose-degrading enzymes including cellulase, xylanase and two lignin peroxidases (manganese peroxidase, MnP and lignin peroxidase, LiP), but no laccase activity was recorded.

Due to the complexity of the lignin-cellulosic materials, ligninolytic fungi are involved in the cycling of nutrients versatile metabolic activities such as (hydrolases, oxidoreductases and esterases) for the degradation of the complex organic molecules into simplest\textsuperscript{32,33}.

Recent researches reported the role of lipase activity in the petroleum hydrocarbons degradation. Similarly, Ramdass and Rampersad\textsuperscript{34} demonstrated the presence of lipase activity in five newly isolates from crude oil polluted soil.

Our results of the enzyme activity screening demonstrate a high metabolic diversity of the isolated fungal strains makes their catabolic potential in the PAHs remediation processes. Basis on the taxonomic analysis and the metabolic diversity, the isolates KBR1, LB3, KB4 and the mixed culture of LB3+LB4 were selected to be used for the PAH-contaminated soil remediation in microcosm systems.

**Microcosms for petroleum-contaminated soil remediation**

The petroleum-contaminated soil samples were collected from the oil well (7) located in Dammam city (Saudi Arabia), and maintained in plastic containers.

The soil samples were collected from the surface layer (5-10 cm) at different spots around petroleum pipe line spillage. The soil samples were mixed together forming a composite sample which will used to represent areas of contamination in this study. The composite sample was sieved with a 6 mm grid before soil microcosm treatments and 2 mm grid for soil characterization.

The primary physical properties of the soil were the dark color, the pH of 6.8, and the average moisture content of 1.6 ± 0.2\%. (Table 3).

The initial total petroleum hydrocarbons (TPH) (C10 - C40 Fraction) content in the soil was approximately 23500 mg kg\textsuperscript{-1}. Hydrocarbons of the chain length C15-C28 were prevailed with 57.02\%, followed by the fraction C29-C36 (31.36\%) and C37-C40 (11.57\%).

Similar high content of TPH was shown in the study of Torres et al.\textsuperscript{35} who reported a range of 51550 to 192130 mg/kg of TPH in soil samples collected from production zone and oil exploration of Tabasco State of Mexico. The toxicity of TPH in contaminated soil has been established at concentration range greater than 1000.00 mg kg\textsuperscript{-1}\textsuperscript{36}.

The soil sample contained an initial total aerobic, heterotrophic bacteria population of $2.5 \times 10^5$ colony forming units per gram of soil. The investigated soils showed high values of BOD (57±1.34 mg/L) and COD (168 ± 2.1 mg/L).
Soil microcosms batch were conducted in several treatment systems (Table 4), to assess the potential of single or mixed fungal strains in the remediation of petroleum-polluted soil. The investigated soil was not sterilized to preserve its microbial indigenous flora as well as its physico-chemical properties. Indeed, the indigenous soil flora constitute an important heterogenous microbial population for the enhancement of the biodegradation process.

Soil microcosms were treated with selected fungal strain shown ability to degrade petroleum hydrocarbons and with broad enzymatic capacities.

**Profile of the TPH in soil microcosms**

To study the petroleum hydrocarbon removal ability of the fungal strains, the TPH in each SM system was followed at different periods during the treatment (Fig. 5).

Total petroleum hydrocarbons decreased by a 41.22 ±1.59% in SMT[KBR1], while a rapid decrease of 44.30 ±1.8% recorded in SMT[LB3+KB4], after 15 days of incubation, contrast to a 11.4 ±1.23% in SMT[LB3], and just a 1.69 ±0.15% in the control (SMU) (Fig. 6).

After 60 days of culture incubation, the highest rate of TPH degradation was recorded in SMT[KB4] by approximately 92±2.35% followed by SMT[KBR1] then SMT[LB3+KB4] with 86.66±1.83% and 85.14±2.21%, respectively.

The soil microcosm augmented with the indigenous strain KBR1 showed a good degradation rate (approximately 86.66±1.83%) which agree with previous similar studies.

The kinetic of TPH during the soil microcosms treatments (Fig.5) demonstrated a rapid decrease of the hydrocarbons in the SMT[LB3+KB4] compared with others SM inoculated by single strain. This result highlights the essential role of the co-occurrence of different microbial species to enhance the biodegradation yields. While, previous findings obtained by Okerentugba and Ezeronye, demonstrated that single fungal culture found to be better than mixed cultures.

Allover results highlight the improvement of the biodegradation yields by bio-augmenting soil microcosms by indigenous or exogenous fungi.

**BOD5 and COD in soil microcosms**

For the evaluation and the monitoring of the degradation process, BOD and COD were estimated at different period of the soil microcosm treatments. The results of organic removal are shown in Fig. 7. BOD\textsubscript{5} and COD of the control microcosm (SMU) were 57 and 145 mg/L, respectively as seen in Table 3. The average BOD\textsubscript{5} and COD percent removal efficiency in this study was approximately 86.5% and 57.8%, respectively.
The BOD$_5$/COD ratio can give indication on the biodegradability of the petroleum contaminated soil. Polluted samples may be biodegradable when the BOD$_5$/COD ratio value was between 0.4 and 0.8. The BOD$_5$/COD ratio within the same time interval was found as 0.28.

The ratio of BOD$_5$ to COD decreased from 0.39±1.24 in the SMU control system to 0.15; 0.17; 0.27 and 0.18 in SMT[KBR1], SMT[KB4]; SMT[LB3] and SMT[LB3+KB4] respectively, indicating most biological degradable materials were decomposed in the bioaugmented microcosms. Despite the increase in the biodegradation potential after fungal treatment (Figure 7), the samples can still not be considered as highly biodegradable (BOD$_5$/COD > 0.4).

**GC-MS analysis for soil microcosms**

The remained petroleum hydrocarbons (PHCs) were extracted and characterized by GC-MS for each soil microcosm (SM) treatment system after 60 days of cultures incubation. Fig. 8 illustrated the superposed profiles of PHCs in the degrading soil microcosm by GC-MS analysis.

PHCs remained in the soil microcosms, showed decrease in the area of major peaks compared to control SMU, suggesting degradation of the main compounds; while the appearance of new peaks in these samples indicated the breakdown of products or presumed metabolites. As seen in the Fig. 9, chromatograms revealed a significant reduction in the intensity of PHC peaks after SM treatment by fungal bioaugmentation (Fig. 9b-d) compared with the control (Fig. 9a).

GC-MS analysis performed after biodegradation showed that the biodegradation patterns of petroleum hydrocarbon fractions in SM treated by single strain and SM treated by mixed species, were markedly different throughout time, compared to the control microcosm.

GC-Ms profiles demonstrated the efficiency of the newly isolates fungal strains to remediate petroleum contaminated soil in the microcosm system.

Further quantitative and qualitative identification of the main compounds in the extracted PHCs were conducted at different SM treatment systems (Table 5). The control system (SMU) (Not sterile soil) represented the biotic effect of the native microbial comities in the contaminated soil, was incubated in same experimental conditions as the treated SM.

The best degradation efficiency was obtained in the SMT[KB4] followed by SMT[KBR1] then SMT[LB3+KB4] and SMT[LB3]. These findings demonstrated that Bioaugmentation by indigenous or exogenous fungal strains is a promising strategy to enhance bioremediation efficiency in soil microcosms systems. In the same context, significant role of native microbial flora (bacteria and fungi) has been reported in biodegradation/bioremediation process.

For instance, Reyes-César et al. studied the role of *Aspergillus terreus*, *Talaromyces spectabilis*, and *Fusarium sp.* in the remediation of 2000 mg kg$^{-1}$ of a mixture of phenanthrene and pyrene soil in a solid-
state microcosm system during 2 weeks.

Data presented in table 5, showed that the abundant hydrocarbon fractions identified in the extracted PHCs from the control system (SMU) were C15-C28, followed by C29-C36 then C37-C40 hydrocarbon chains length.

Comparing the control system SMU with all the SM treatment systems, it is clearly showed that the fractions C29-C36 and C37-C40 were completely removed in the treated system SMT[KB4+LB3] constituted of mixed culture *Coniochaeta sp.* (LB3) and *Paecilomyces formosus* (KB4). Whereas, despite its decreasing, those fractions still persistent in the others SMT systems augmented by the monoculture of the same strains. The fungal co-cultrue [KB4+LB3] containing lipolytic, cellulosic and ligninolytic enzymes, is a potential consolidated bioprocessing approach for PAHs-contaminated soil degradation. Therefore, assemblages of mixed populations with broad enzymatic activities are required to reach the maximum rate and make the petroleum hydrocarbon degradation processes much faster.

In this regard, many scientists reported that mixed populations with overall broad enzymatic capacities are required to degrade complex structures of hydrocarbons. Okerentugba and Ezeronye in related study on single and mixed culture fungi have reported similar finding.

Furthermore, Atlas and Cerniglia, suggested that although the fungi are able to metabolize some hydrocarbons, they do not have the enzymes required for transforming the co-oxidation products.

Additionally, the system SMT[KBR1] employing the indigenous strain (*Aspergillus sp.*) was able to fully degrade the fraction C37-C40, and highly remove C29-C36 (few amounts were recorded) after 60 days of culture incubation. These findings demonstrated that the native fungal isolated KBR1 plays an effective role as biological agents for cleanup of oil spills.

This result is consistent with that of Ezekoye et al. who attributed the enhancement of the bioremediation to the effective role of indigenous microorganisms. Thus, the employing of the native microbial population ensures their higher tolerance to the toxicity of the PAHs and their resistant to the environmental conditions.

Allover results highlight the potential of the ligninolytic fungi as well as soil indigenous fungi in the biodegradation or removal of petroleum hydrocarbons.

**Conclusion**

The Present study revealed that, the fungal ligninolytic isolates and the indigenous fungi collected from petroleum contaminated soil samples holds promise for the effective PAHs-bioremediation. Further, to enhance the biodegradation efficiency, bio-stimulation, the properties of biosurfactant and enzymes and mechanism of degradation is necessary.
Declarations

Conflict of interest

the authors declare that they have no conflicts of interest.

Compliance with ethical standards

Conflict of interest the authors declare that they have no conflicts of interest.

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**Tables**

**Table 1** Molecular identification of Petroleum hydrocarbon degrading fungal isolates
| Isolates ID | Max identity (%) | Strains of closed match (accession Number) | Identification | Accession Number | Phylum       |
|-------------|------------------|------------------------------------------|----------------|-----------------|--------------|
| KBR1-1      | 99.58%           | *Syncephalastrum sp.* strain EVGF23 (MN913763.1) | *Syncephalastrum sp* | MZ817958        | Zygomyctota  |
| KBR1        | 95.54%           | *Aspergillus niger* strain MNM003 (MW578514.1) | *Aspergillus niger* | MW699896        | Ascomycota   |
| KB4         | 98.39%           | *Paecilomyces formosus* strain CBS 628.66 (FJ389927.1) | *Paecilomyces formosus* | MW699897       | Ascomycota   |
| KB2         | 98.84%           | *Fusarium chlamydosporum* isolate KJCC1179 (MF038146.1) | *Fusarium chlamydosporum* | MZ817957       | Ascomycota   |
| KBR8        | 98.88%           | *Aspergillus niger* isolate (MW142509.1) | *Aspergillus tubingensis* | MW699895       | Ascomycota   |
| LB3         | 99.68%           | *Coniochaetaceae sp.* MAE-2017 (MG519607.1) | *Coniochaetaceae sp* | MW699893       | Ascomycota   |

**Table 2** Enzymatic activities detected on selective solid media for the fungal isolates.
| N° | Isolates ID | Lac<sup>a</sup> | CMCase<sup>b</sup> | Amyl<sup>c</sup> | Prot<sup>d</sup> | Lip<sup>e</sup> |
|----|-------------|----------------|-------------------|----------------|----------------|----------------|
| 1  | KBR1-1      | ++             | ++++              | -              | ++            | -              |
| 2  | KBR1        | +              | -                 | +++            | -             | ++            |
| 3  | KB4         | +              | +                 | -              | +             | +++           |
| 4  | KB2         | +              | -                 | -              | +             | ++            |
| 5  | KBR8        | +              | -                 | +++            | -             | ++            |
| 6  | LB3         | +++            | ++                | -              | ++            | -             |
| 7  | KB2         | -              | +                 | ++             | +             | -             |
| 8  | KB14        | -              | ++                | -              | -             | ++            |
| 9  | KB12        | -              | ++                | ++             | +             | -             |
| 10 | KB17        | -              | -                 | +              | +             | -             |
| 11 | W4          | +              | ++                | -              | -             | -             |
| 12 | D11         | -              | +                 | +              | ++            | -             |

Lac, laccase activity; CMCase, cellulase activity; Prot, protease activity; Amyl, amylase activity; Lip, lipase activity. (+) Diameter of the halo surrounding mycelium growth 0-15 mm, (++) halo diameter 15-25 mm, (+++) halo diameter up to 25 mm.

**Table 3** Physicochemical Analysis of the PAHs-contaminated soil.
| Compound             | Cas#     | Technique/ Method Reference | a\_LOR | Unit   | Soil samples |
|----------------------|----------|-----------------------------|--------|--------|--------------|
| Moisture Content     | —        | —                           | 1.0    | %      | 1.6          |
| pH                   | —        | —                           | —      | —      | 6.8          |
| C6 - C9 Fraction     | —        | USEPA 5030/8260             | 10     | mg kg\(^{-1}\) | <10          |
| C10 - C14 Fraction   | —        | USEPA 3510/8015 GC/FID      | 50     | mg kg\(^{-1}\) | <50          |
| C15 - C28 Fraction   | —        | —                           | 100    | mg kg\(^{-1}\) | 13400        |
| C29 - C36 Fraction   | —        | —                           | 100    | mg kg\(^{-1}\) | 7370         |
| C37 - C40 Fraction   | —        | —                           | 50     | mg kg\(^{-1}\) | 2720         |
| ^ C10 - C40 Fraction (sum) | —       | —                           | 100    | mg kg\(^{-1}\) | 23500        |
| Benzene              | 71-43-2  | USEPA 5030/8260             | 0.2    | mg kg\(^{-1}\) | 0.2          |
| Ethylbenzene         | 100-41-4 | —                           | 0.5    | mg kg\(^{-1}\) | 0.5          |
| meta- & para-Xylene  | 108-38-3 106-42-3 | — | 0.5 | mg kg\(^{-1}\) | 0.5 |
| ortho-Xylene         | 95-47-6  | —                           | 0.5    | mg kg\(^{-1}\) | 0.5          |
| Toluene              | 108-88-3 | —                           | 0.5    | mg kg\(^{-1}\) | 0.5          |

**Notes:**
- ^ Moisture Content (dried @ 103°C)
- pH
- Total Petroleum Hydrocarbon (TPH)
- C6 - C9 Fraction
- C10 - C14 Fraction
- C15 - C28 Fraction
- C29 - C36 Fraction
- C37 - C40 Fraction
- Volatile Organic Compounds - BTEX
- Benzene
- Ethylbenzene
- meta- & para-Xylene
- ortho-Xylene
- Toluene
- Volatile Organic Compounds - Surrogates
- 1,2-Dichloroethane-D4
Toluene-D8 2037-26-5 0.2 % 99.3

4-Bromofluorobenzene 460-00-4 0.2 % 95.9

DESCRIPTIVE RESULTS

Chemical oxygen demand —— S-MISC 2 mg L\(^{-1}\) 145

Biochemical oxygen demand —— S-MISC 2 mg L\(^{-1}\) 57

\(^a\) LOR= Limit of reporting

| Surrogate Control Limits | Recovery Limits (%) |
|-------------------------|---------------------|
| Compound                | Cas# | Low | High   |
| VOLATILE ORGANIC COMPOUNDS - SURROGATES |      |     |       |
| C COMPOUNDS - SURROGATES | 17060-07-0 | 53   | 134    |
| 1.2-Dichloroethane-D4   |      |     |       |
| 4-Bromofluorobenzene    | 460-00-4 | 59   | 127    |
| Toluene-D8              | 2037-26-5 | 60   | 131    |

Table 4 Treatment systems used in the biodegradation tests.

| Treatment systems | Assay name | Microorganisms                  |
|-------------------|------------|---------------------------------|
| Controls/ untreated | SMU        | Without inocula                 |
| Test / treated    | SMT[LB3]   | *Lecythophora (Coniochaeta)* LB3|
| Test / treated    | SMT[KBR1]  | *Aspergillus niger* KBR1        |
| Test/ treated     | SMT[KB4]   | *Paecilomyces formosus* KB4     |
| Test / treated    | SMT[LB3+KB4] | LB3+KB4                        |

Table 5 Main compounds identified in the dichloromethane extracted PHCs in the different soil microcosm treatment systems.
| RT   | Area% | Height% | Compounds            | Molecular formula | Fractions |
|------|-------|---------|----------------------|-------------------|-----------|
| 11.735 | 5.34 | 1.81    | Heptadecane          | C17H36            | C15-C28   |
| 12.995 | 7.64 | 1.98    | Heptadecane          | C17H36            |           |
| 14.191 | 8.89 | 1.93    | Heptadecane          | C17H36            |           |
| 15.326 | 9.2  | 1.82    | Eicosane             | C20H42            |           |
| 15.436 | 3.43 | 2.34    | Eicosane             | C20H42            |           |
| 16.407 | 8.75 | 1.81    | Eicosane             | C20H42            |           |
| 17.15  | 1.15 | 5.02    | Heptadecane. 2-methyl-|                  |           |
| 17.439 | 8.5  | 2.07    | Eicosane             | C20H42            |           |
| 18.142 | 0.83 | 5.11    | 2-methylhexacosane   | C27H56            |           |
| 18.423 | 7.84 | 1.88    | Eicosane             | C20H42            |           |
| 19.366 | 6.7  | 1.87    | Eicosane             | C20H42            |           |
| 20.268 | 5.93 | 1.89    | Eicosane             | C20H42            |           |
| 21.136 | 5.46 | 1.9     | Tetracosane          | C24H50            |           |
| 21.969 | 4.44 | 1.83    | Hexatriacontane      | C36H74            | C29-C36   |
| 22.771 | 3.88 | 2.01    | Hexatriacontane      | C36H74            |           |
| 23.544 | 3.16 | 1.82    | Hexatriacontane      | C36H74            |           |
| 24.289 | 2.68 | 1.89    | Hexatriacontane      | C36H74            |           |
| 25.011 | 2.4  | 1.96    | Hexatriacontane      | C36H74            |           |
| 25.76  | 2.08 | 2.18    | Tetrapentacontane    | C54H110           | C37-C40   |
| 27.558 | 1.7  | 3.27    | 2-methylhexacosane   | C27H56            | C15-C28   |

**SMT[LB3]**

| RT   | Area% | Height% | Compounds     | Molecular formula | Fractions |
|------|-------|---------|---------------|-------------------|-----------|
| 11.733 | 4.12 | 1.71    | Heptadecane   | C17H36            | C15-C28   |
| 12.991 | 6.07 | 1.85    | Heptadecane   | C17H36            |           |
| 14.184 | 7.16 | 1.76    | Heptadecane   | C17H36            |           |
| 15.32  | 7.13 | 1.81    | Heneicosane   | C21H44            |           |
| 15.432 | 2.54 | 2.3     | Eicosane      | C20H42            |           |
| 16.401 | 7.28 | 1.78    | Heneicosane   | C21H44            |           |
|                  |        |      |            |           |
|------------------|--------|------|------------|-----------|
| 17.432           | 7.2    | 1.8  | Eicosane   | C20H42    |
| 18.417           | 6.71   | 1.77 | Eicosane   | C20H42    |
| 19.359           | 6.18   | 1.79 | Eicosane   | C20H42    |
| 20.263           | 5.69   | 1.75 | Eicosane   | C20H42    |
| 21.131           | 5.3    | 1.89 | Eicosane   | C20H42    |
| 21.329           | 3.07   | 1.94 | Cyclononasipoxane.octadecamethyl- | C18H54O9Si9 |
| 21.964           | 4.31   | 1.86 | Eicosane   | C20H42    |
| 22.492           | 4.03   | 1.94 | Cyclononasipoxane.octadecamethyl- | C18H54O9Si9 |
| 22.766           | 4.4    | 1.8  | Hexatriacontane | C36H74   |
| 23.539           | 3.37   | 1.89 | Tetracontane | C40H82  |
| 23.584           | 4.65   | 1.9  | Cyclononasipoxane.octadecamethyl- | C18H54O9Si9 |
| 24.6             | 4.17   | 1.97 | Cyclononasipoxane.octadecamethyl- | C18H54O9Si9 |
| 25.582           | 3.19   | 2.25 | Cyclodecasiloxane. eicosamethyl- | C20H60O10Si10 |
| 26.696           | 3.43   | 2.5  | Cyclononasipoxane.octadecamethyl- | C18H54O9Si9 |

**SMT[KB4]**

|                  |        |      |            |           |
|------------------|--------|------|------------|-----------|
| 6.858            | 4.67   | 2.02 | Cyclopentasiloxane. decamethyl- | C10H30O5Si5 |
| 12.99            | 4.12   | 1.73 | Hexadecane | C16H34    |
| 14.184           | 5.61   | 1.78 | Heptadecane | C17H36   |
| 15.184           | 7.13   | 1.74 | Methyl 13-methyltetradecanoate | C16H32O2   |
| 15.318           | 6.85   | 1.83 | Heneicosane | C21H44   |
| 16.4             | 7.51   | 1.73 | Heneicosane | C21H44   |
| 16.669           | 4.14   | 1.86 | Hexadecanoic acid. methyl ester | C17H34O2 |
| 17.431           | 7.44   | 1.88 | Eicosane   | C20H42    |
| 18.417           | 6.84   | 1.89 | Eicosane   | C20H42    |
| 18.667           | 1.69   | 2.89 | Methyl stearate | C19H38O2 |
| 19.359           | 6.9    | 1.74 | Eicosane   | C20H42    |
| 20.262           | 6.15   | 1.89 | Eicosane   | C20H42    |
| 21.13            | 5.67   | 1.93 | Eicosane   | C20H42    |
| 21.962           | 4.66   | 1.95 | Eicosane   | C20H42    |
| Mass  | dD   | Isotope  | Name                                           | Molecular Formula | CAS Number |
|-------|------|----------|-----------------------------------------------|-------------------|------------|
| 22.558| 2.31 | 5.05     | Bumetrizole                                   | C17H18ClN3O       | 17285-00-4 |
| 22.765| 4.8  | 1.84     | Eicosane                                      | C20H42            | 7155-82-0  |
| 23.539| 3.56 | 1.71     | Tetrapentacontane                             | C54H110           | 5565-65-0  |
| 24.284| 4.34 | 1.6      | 1-Eicosanol. 2-hexadecyl-                     | C36H74O           | 1368-16-7  |
| 25.005| 2.79 | 2.02     | Hexatriacontane                               | C36H74            | 1170-97-6  |
| 25.753| 2.82 | 1.96     | Hexatriacontane                               | C36H74            | 1170-97-6  |
|       |      |          | SMT[KB4+LB3]                                 |                   |            |
| 5.758 | 6.34 | 2.1      | Cyclotetrasiloxane. octamethyl-               | C8H24O4Si4        | 3516-96-4  |
| 17.869| 6.65 | 1.51     | Cyclononasiloxane. octadecamethyl-           | C18H54O9Si9       | 4521-73-5  |
| 18.826| 42.13| 1.77     | 1-benzylindole                                | C15H13N           | 3620-42-2  |
| 19.108| 7.45 | 2.56     | Cyclodecasiloxane. eicosamethyl-             | C20H60O10Si10     | 7580-59-8  |
| 19.727| 8.31 | 2.36     | Cyclononasiloxane. octadecamethyl-           | C18H54O9Si9       | 4521-73-5  |
| 20.23 | 5.38 | 2.17     | 13.17.21-Trimethylheptatriacontane            | C7H14             | 3580-15-3  |
| 20.408| 6.45 | 1.94     | Cyclodecasiloxane. eicosamethyl-             | C20H60O10Si10     | 7580-59-8  |
| 21.209| 6.1  | 2.8      | Tetracosamethyl-cyclododecasiloxane          | C24H72O12Si       | 5282-28-7  |
| 22.178| 6.44 | 2.94     | Tetracosamethyl-cyclododecasiloxane          | C24H72O12Si       | 5282-28-7  |
| 23.394| 4.75 | 3.62     | Tetracosamethyl-cyclododecasiloxane          | C24H72O12Si       | 5282-28-7  |
|       |      |          | SMT[KBR1]                                     |                   |            |
| 20.8  | 1.39 | 5.44     | Silane. diethylheptyloxyoctadecyloxy-        | C29H62O2Si        | 2599-51-9  |
| 22.489| 2.59 | 2        | Cyclodecasiloxane. eicosamethyl-             | C20H60O10Si10     | 7580-59-8  |
| 23.581| 3.21 | 2.08     | Tetracosamethyl-cyclododecasiloxane          | C24H72O12Si       | 5282-28-7  |
| 23.966| 58.04| 2.78     | 1.3-Benzenedicarboxylic acid. bis(2-ethylhexyl) ester | C24H38O₂·        | 613-81-7  |
| 24.598| 3.15 | 2.04     | Tetracosamethyl-cyclododecasiloxane          | C24H72O12Si       | 5282-28-7  |
| 25.581| 2.58 | 2.18     | Tetracosamethyl-cyclododecasiloxane          | C24H72O12Si       | 5282-28-7  |
| 26.163| 22.36| 7.93     | Propanoic acid. 3.3'-thiobis-. didodecyl ester | C30H58O4S        | 5398-45-7  |
|     |     |     | **Tetracosamethyl-cyclododecasiloxane** | C24H72O12Si |
|-----|-----|-----|----------------------------------------|-------------|
| 26.688 | 2.56 | 2.88 |                                        |             |
| 28.015 | 2.51 | 3.53 |                                        |             |
| 29.654 | 1.61 | 4.06 |                                        |             |

**Figures**
Figure 1

Microphotographs of light microscopy showing morphological characteristics of the PAHs-degrading fungal isolates: (a) Aspergillus niger [KBR1] [MW699896] (b) Paecilomyces formosus [KB4] [MW699897] (c) Aspergillus tubingensis [KBR8] [MW699895] (d) Fusarium chlamydosporum [KB2] [MZ817957] (e) Coniochaetaceae sp. [LB3] [MW699893] (f) Syncephalastrum sp. [KBR1-1][MZ817958].
**Figure 2**

Screening of crude oil-degrading fungal isolates by Agar well diffusion method on MEA supplemented with 5% Diesel.

**Figure 3**

Percentage of petroleum degradation and biomass gain (g) of the tested isolated strains after 14 days on 5g of the investigated soil with 30 mL MM. (data are mean of three replicates; error bars represent s.d.)
Figure 4

Phylogenetic tree for the hydrocarbon-degrading isolated fungi (exo and indigenous isolates) and related sequences based on the BLAST alignment of ITS sequences. The ClustalW program was used to generate the phylogenetic trees using the NJ method with bootstrap replicates.
Figure 5

Gravimetric analysis of PHCs (mg/kg) among different soil microcosm

| Fungal cultures | 60 days | 30 days | 15 days |
|-----------------|---------|---------|---------|
| LB3             | 67.86842| 54.34393| 11.404  |
| KB4             | 92.46706| 76.97519| 32.01645346|
| KBR1            | 86.66347| 60.65458| 41.22858563|
| KB4+LB3         | 85.14347| 59.66464| 44.30683  |
Figure 6
Degradation efficiency (%) in soil microcosm for each treatment against control microcosm.

Figure 7
BOD5/COD ratios during the soil microcosm treatment of petroleum-contaminated soil
Figure 8

GC-MS superposed chromatograms of the residual hydrocarbons extracted from soil microcosms treatment systems; (blue) control SMU (orange) SMT[LB3] (grey) SMT[KB4] (yellow) SMT[KBR1] (Green) SMT[KB4+LB3].
Figure 9

GC-MS chromatograms of the residual hydrocarbons extracted from soil microcosms treatment systems; (a) SMU (b) SMT[LB3] (c) SMT[KB4] (d) SMT[KBR1] (d) SMT[KB4+LB3].

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