Biodegradation of petroleum oil by mangrove fungi from Saudi Red Sea Coast

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Abstract
Mangrove sediments were collected from different localities on the south Red Sea Coast of Saudi Arabia. Forty five isolates belonging to 13 genera were recovered and three of these isolates were found to be able to grow in association with petroleum oil as sole carbon source under in vitro conditions. These isolates were identified as Cladosporium sphaerospermum, Eupenicillium hirayamae and Paecilomyces variotii using 18S rDNA gene analysis. The three isolates have high potential to degrade petroleum hydrocarbons by co-cultivation under aeration on a rotary shaker. As compared to the controls, these fungi accumulated significantly higher biomass, produced extracellular enzymes and liberated larger volumes of CO_2. These observations with Gas chromatography data confirm that these strains could be interesting to use in future field tests.

Keywords: Biodegradation, Petrol substrate, Mangrove,Farasan Island, 18 rDNA.

Introduction

The technologies commonly used for soil remediation of petroleum hydrocarbons include mechanical burying, evaporation, dispersion and washing. These remedial measures are not only cost intensive and time consuming but also not very effective. On the other hand, bioremediation leads to complete mineralization of organic compounds into CO_2 and water by indigenous microorganisms and hence a preferred choice being eco-friendly and cost-effective[1]. The process of biodegradation defined as the use of microorganisms to detoxify or remove pollutants owing to their diverse metabolic capabilities is an evolving method for the removal and degradation of many environmental pollutants including the products of petroleum industry[2].

Numerous works were devoted to research on biodegradation with the use of fungi[3,4]. Mycoremediation as a process focuses on the degradation of organic compounds by fungi. Many studies concluded that most filamentus fungi species are excellent hydrocarbons degraders[5, 6]. It is practically established that fungi (mostly white rot fungi) are capable of using their mycelia to bioremediate hydrocarbon products due to their high production of organic acids, chelators, oxidative enzymes and extracellular enzymes that enable them to utilize the hydrocarbon products faster[7,8] .

Some prior researchers reported that some fungal species are resistant to petroleum-pollution and they are capable to remove soil pollution. The results of Ulfig et al[9] indicated that keratinolytic fungi, especially *Trichophyton ajelloi*, is a potential tool for assessment of soil petroleum hydrocarbon contamination and associated bioremediation progress. The fungal strains *Alternaria alternata, Aspergillus flavus, Curvularia lunata, Fusarium solani, Mucor racemosum, Penicillium notatum* and *Ulocladium atrum* were isolated from the soils in the petroleum-polluted areas in Saudi Arabia[10]. Eggen and Majcherczykb[11]showed that the white rot fungus, *Pleurotus ostreatus*, was able to remove polycyclic aromatic hydrocarbons (PAHs) from contaminated soil. However, little attention has been paid to the role of fungal species in the environmental biotechnology and bioremediation of petroleum pollution, especially in Middle Eastern region[10,12].

Some fungal strains including *Alternaria alternata, Aspergillus flavus, Curvularia lunata, Fusarium solani, Mucor racemosum, Penicillium notatum and Ulocladium atrum* were isolated from the soils in the petroleum-polluted areas in Iran[13]. Fourteen marine fungi species have been reported from Saudi Arabia[14-17]. This paper aims to explore the degradative capability of three fungal strains *Cladosporium sphaerospermum, Eupenicillium hirayamae* and *Paecilomyces variotii* isolated from mangrove sediments using Petroleum oil as the sole carbon source and to compare fast degradation among strains and their consortium.

Material and Methods

Collection of sample: Samples were collected from three discreet 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. The debris consisted of dead fallen leaves, pieces of pneumatophores, bark and wood surface soil sediments. Subsurface samples at 2 cm depth from the same stands were collected. The samples were brought to lab for isolation of fungi. The petroleum oil was obtained from Saudi Aramco, Riyadh City, Saudi Arabia.

Isolation of fungi: Fungi from solid debris were isolated by splitting the specimen into smaller pieces and directly laying on a synthetic medium 'MF1' formulated to simulate seawater conditions based on the compositions[18-20]. Composition of the medium per liter was: $MgSO_4$ 246.5mg, $FeSO_4.7H_2O$ 5.56mg, $ZnSO_4.7H_2O$ 0.29mg, $MnSO_4.H_2O$ 0.34mg, $CuSO_4.5H_2O$ 0.025mg, NH_4Cl 5.35g, KCl

7.46mg, $CaCl_2.2H_2O$ 1.47mg, NaCl 5.84mg, $COCl_2.6H_2O$ 0.027mg, KH_2PO_4 136mg, Na_2MoO_4 24mg with pH adjusted to 5.5. The medium was gelled with 15g agar and 15g dextrose per liter. For the sake of convenience, the medium was tentatively designated as 'MF1'. Isolation of fungi from water accompanying the specimen was done by serial dilution. Ten-fold dilutions were laid on 'MF1' medium. For isolation from sediments, dilution technique was used. One gram of sediment was suspended in 10 ml of sterilized distilled water and tenfold dilution thereof was spread on the same medium. The plates were incubated at 30 ± 2º C for 5-7 days.

Identification of fungal isolates: Pure fungal isolates were established from the initial plates using standard procedures of fungal cultivation. Fungal genera were examined under the microscope and were identified according to morphology characters and classified according to taxonomy keys in many literature[21-24]. Species were identified by using DNA sequence method. A suitable mass of inoculum of fungal isolate was prepared carefully removing the upper surface of the isolate without agar medium, the DNA extraction technique was used to remove inhibitory materials i.e. polysaccharides, proteins, mineral salts etc. which limit the sensitivity of the different reactions in which isolated DNA is applied[25]. Genomic DNA was extracted from fungal isolates using a Mo-Bio Power Soil DNA extraction kit following manufacturer's protocol (Mo- Bio, Carlsbad CA, USA). Fungal 18S rDNA genes were PCR amplified using ITS1 [5'-TCCGTAGGTGAACCTGCGG-3'] and ITS4 [5'-TCCTCCGCTTATTGATATGC-3'].

Reactions were performed in 20 µl volumes containing 4 µl 1X master mix, 0.6 µl of each primer, 5 ng templates DNA and 9.8 µl deionized water. Thermo cycling conditions consisted of an initial denaturation stage of 95°C for 5 minutes followed by 30 cycles of 95°C for 45 seconds, 55°C for 60 seconds and 72°C for 90 seconds and a final stage of 72°C for 10 minutes. Amplicons were cleaned using a Mo-Bio Ultra Clean® PCR Cleanup Kit and sent for sequencing to Macrogen Company, South Korea. The basic local alignment search tool-BLAST was used to classify and identify closely related fungal sequences.

Fungal growth and biomass accumulation on Petrol substrate: Isolates derived from different types of samples were co-cultivated with petrol substrate to test bioremediation activity. 'MF1' broth devoid of carbon source was used for the tests. 10% of petrol was transferred to 150 ml conical flasks containing 100 ml sterilized broth. Each flask was inoculated with a single 6 mm disc of an individual mature isolate. The flasks were incubated at 30 ± 2º C for 4 weeks in a rotary shaker gyrating at 100 rpm. Three replicate flasks were maintained for each fungal isolate. For each treatment, three flasks with corresponding inoculum but without petrol substrate were retained as controls. Biomass accumulation of those isolates which showed growth in association with petrol was estimated in comparison with the controls after four-week incubation. Gain in biomass under each treatment and the corresponding control was recorded by subtracting the initial dry weights and difference between gain in treatment and control was considered to be due biodegradation activity of the fungus.

Estimation of CO_2 evolution: Fungal isolates and petrol substrate were co-cultivated as above and quantity of CO_2 evolved was estimated for an incubation period of 4 weeks. Three flaks were maintained for each isolate together with corresponding control flasks inoculated with the fungus but devoid of petrol. Volumetric and gravimetric estimation of CO_2 evolved during 4-week incubation was performed using sturm test. For gravimetric analysis sterile air was sequentially passed through1M 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). Amount of CO_2 dissolved in the broth was estimated by adding 100 ml of 0.1 M $BaCl_2$ that formed precipitate of barium carbonate and CO_2 released was gravimetrically calculated by measuring the weight of the precipitate. Difference in the values obtained between control and test bottles was recorded.

For volumetric analysis, the dissolved carbon dioxide present in the medium was estimated by titration. The broth was filtered to remove fungal mass and the petrol substrate and 25 ml filtrate was taken in a conical flask to which 0.05ml of 0.1N thiosulphate solution was added. After the addition of 2 drops of methyl orange indicator, solution was titrated against 0.02M sodium hydroxide solution. End point appeared as 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.

Enzyme activity assays: Three fungal isolate which showed biomass accumulation under treatment were co-cultivated with petroleum substrate in replicates along with the controls as above. As a separate treatment, consortium of the three above isolates was also tested for enzyme activity. After four weeks of incubation, 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 enzyme level was determined in the extracellular fluids. Catalase activity

was determined according to Aebi[26] 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 phosphate buffer (pH 7.0), 15 mM H_2O_2 and 0.1 ml of enzyme extract was used which started the reaction in 3 ml.

Laccase was estimated by the oxidation of 2,2-Azino-bis-3-ethyl-benzthiazoline-6-sulfonic acid (ABTS) according to Novotny et al[27] using 0.1 mM ABTS in the reaction buffer of 100 mM sodium tartrate (pH 4.5) with 50 µl 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[28,29]. All enzymes assayed in this study were expressed as U/ml.

Gas chromatography for petroleum products: Perkin Elmer Mass Spectrometer with a HP-5MS column was used for analysis using the method of Wu et al[18] with minor modification. For analysis, 30 m × 0.25 mm I.D. × 0.25 µm HP-5MS fuse-silica capillary column (from HP Inc.) was employed. The column temperature program was set as follows: 100º C hold for 1 min, 15º C/min to 160º C, at 5º C/min to 300º C hold for 7 min. The GC injector was held isothermally at 280º 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 280ºC. The MS was operated in electron impact (EI) ionization mode with electron energy of 70 eV and scan ranged from 50 to 500 amu (atom to mass unit) to determine appropriate masses for selected ion monitoring.

The MS ion source and mass filter (quad) temperatures were held at 230º C and 150ºC 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 was eluted. To increase sensitivity, selected ion monitoring (SIM) mode was used to quantitatively analyze the peak and the molecular ion was detected and quantified with ion loss of methyl group (mass 190 for lactone), the dwell time was 0.1 s and the scan cycle was 4.26/s. All samples in the present study were in triplicate.

Results and Discussion

Isolation and identification of Petrol degrading fungal isolates: Forty five isolates were derived from the samples. These 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*. All the isolates were tested for activity against Petrol substrate. Isolates *FA-1, FA-2* and *FA-3* as well as their consortium were selected among all the isolated strains for their faster growth rate and relatively higher petrol substrate degradation ability. The sequences of the partial 18S rDNA gene fragments cloned from these strains were compared with similar information available at the GenBank by an online alignment search. The phylogenetic tree based on a comparison of the sequences is shown in fig. 1.

The results indicated that the partial 18S rDNA sequences of both *FA-1*, *FA-3* were 100% identical to that of *Cladosporium sphaerospermum* (Accession No. KC311475.1), *Eupenicillium hirayamae* (Accession No. JN626096.1) respectively and *FA-2* strain were 99% identical to that of *Paecilomyces variotii* (Accession No. EU037050.1). Molecular analysis of fungal rDNA at the sequence level provides a powerful technique for assessing fungal diversity at the genus level. PCR primers play a crucial role in the molecular assessment of environmental microbes. The specificity of the primer pairs is vital in this context to allow selective or enriching amplifications of fungal rRNA genes from environmental DNA[30].

Biomass accumulation by fungi under co-cultivation with Petrol substrate: During the fungal treatment of substrate of petroleum as a hydrocarbon waste, inocula of three isolates as well as their consortium gradually developed association with the substrate and within 15-20 days grew to form colonies of variable size and appearance (Fig. 2). Profuse growth of *Paecilomyces variotii*, *Cladosporium sphaerospermum* and *Eupenicillium hirayamae* respectively was noticed around the petroleum substrate. These isolates showed greater accumulation of biomass as compared to their corresponding controls (Table 1). *Eupenicillium hirayamae* gained the maximum weight of (21.9%) followed by C*ladosporium sphaerospermum* (19.6%)) whereas minimum weight gain (17.5%) was recorded in *Paecilomyces variotii*.

Several other studies which claimed to demonstrate biodegradation potential of different fungi, have also reported biomass accumulation under similar conditions. Hasan[31] has reported a significant gain in fresh weight of *Aspergillus niger* and *Rhizopus stolinifer* on 10% kerosene pollution (with 0.530 gm dry weight of mycelia after 7 days growth) and the dry weight of *R.stolinifer* reached to 0.522 gm. A study by Lotfinasabasl et al[32] recorded that the isolate *Aspergillus niger* showed the highest growth diameter in 20% kerosene amongst *Aspergillus terreus, Rhizopus sp.* and *Penicillium sp.*

CO_2 evolution due to fungal activity on Petroleum substrate: No significant difference was observed between CO_2 evolution estimates taken by volumetric and gravimetric methods; therefore, data was merged as means of the two procedures for each fungal isolate (Table 2). *Eupenicillium hirayamae* showed maximum enhancement

of CO_2 emission (45.7%) followed by C*ladosporium sphaerospermum* (42%) and *Paecilomyces variotii* (37%). Liberation of carbon dioxide during the degradation of petrol can be used as an indication for the activity of fungi in the growth medium. Vanishree et al[8] studied the amount of CO_2 released during biodegradation of petrol substrate as indicator for activity of *Aspergillus sp.* Balba et al[38] stated that mineralization studies involving measurements of total CO_2 production can provide excellent information on the biodegradability potential of hydrocarbons in contaminated soils.

The biodegradation of petroleum oil by the fungal isolates was studied by CO_2 evolution test also known as Sturm test[33]. A lot of modified forms of CO_2 evolution test are reported in literature but the basic purpose was to study the complete assimilation of polymeric carbon[34-37]. Liberation of carbon dioxide during the degradation of petrol can be used as an indication for the activity of fungi in the growth medium.

Enzyme activity of fungal isolates: Production of laccase, MnP, LiP and catalase enzymes was recorded in most of the control and treatment cultures of the three tested fungi as well as their consortium (Table 3). Consortium of the three tested fungi showed remarkably higher level of enzymes as compared to the individual fungi. This may be due to synergistic effect of the pooled genotypes. *Eupenicillium hirayamae* produced high level of Catalase (12.58± 1.71 U ml^{-1}) followed by C*ladosporium sphaerospermum* with Catalase (8.57±0.84 U ml^{-1}) which corroborates well with biomass accumulation in the same order (Table 1). This trend closely matched with the trends of biomass accumulation and CO_2 emission suggesting that variable degree of biodegradation of petroleum substrate has taken place by fungal activity reflected in corroborative levels of enzyme production, biomass accumulation and CO_2 liberation.

Results showed that the activity of the enzymes was increased considerably as compared to controls. Consortium of the three tested fungi showed remarkably higher level of enzymes as compared to the individual fungi. This may be due to synergistic effect of the pooled genotypes. Biodegradation of a high mass polymer and hydrocarbons has to be facilitated by extracellular enzymes released by the acting microorganisms[39-42]. Hence, elevated enzyme levels in our treatment broths are direct indication of the degenerative activity. Wu et al[18] while investigating biodegradation potential of *Fusarium solani* strains against anthracene (ANT) and benz[a]anthracene (BAA) recorded laccase to be the only active enzyme and found no traces of MnP and LiP. In general, level of catalase was highest among the four enzymes followed by laccase and LiP. Level of the four enzymes was conspicuously higher in cultures growing in association with petrol substrate as compared to the controls; probably because, here the enzymes were in greater demand for hydrocarbons breakdown reactions. The activity of three enzymes, including catalase (CAT), was determined by Mohsenzadeh et al[43] in the fungal strains *Acromonium sp., Alternaria sp., Aspergillus terreus* and *Penicillium sp.,* during the growth in the media with different concentrations of petroleum pollution. High activity of catalase was also reported in the soil microorganisms in petroleum-polluted soils[44,45].

Luo et al[46] reported that the dominant benzo[a]pyrene-degrading bacteria from a marine enrichment were isolated and faster degradation was seen when the three strains (*Ochrabactrum, Stenotrophomonas* and *Pseudomonas spp*.) were combined than when tested individually. To the wood-decaying fungi, the average rates of polycyclic aromatic hydrocarbons conversion have been correlated with average activities of ligninolytic enzymes[47]. Ali et al[48] indicated that the *Aspergillus terreus* isolated from Orman Garden soil and *Penicillium chrysogenum* isolated from Wadi Degla protectorate soil, exhibited lignin peroxidase and manganese peroxidase activities during biodegradation study of some PAHs that is in accordance with our results.

Gas Chromatographic determination of biodegradation of Petrol substrate by fungal isolates: The gas chromatogram of petrol substrate extracted from the inoculated medium at 0 and 30 days is shown in figs.3A-D. It was found that the fungal strains as well as their consortium were efficient petroleum degrader. They completely degraded the following main hydrocarbons [Tritetracontane, 1,2-Benzenedicarboxylicacid,mono(2-ethylhexyl) ester and Tetrapentacontane, 1,54-dibromo] present in petroleum oil and other short chain compounds were synthesized. The consortium of the three tested fungi showed remarkably higher level of enzymes as compared to the individual isolates. This may be due to synergistic effect of the pooled genotypes. Luo et al[46] reported that the dominant benzo[a]pyrene-degrading bacteria from a marine enrichment were isolated and faster degradation was seen when the three strains (*Ochrabactrum, Stenotrophomonas* and *Pseudomonas spp*.) were combined than when tested individually.

A decrease in the area of the substrate peaks completely degrades main compounds and the appearance of new peaks (assumed metabolites) associated with the live mycelia cultures, but not in the controls, was indicator of the ability of the fungus to degrade multiple hydrocarbon compounds. All three fungal isolates were found to be good candidates for PAH bioremediation showing good degradation of the low molecular weight PAHs. This can be adduced to the usage of these fractions contained in the petroleum oil by each of the fungus for their metabolic processes. Oboh et al[49] have reported the abilities of bacterial species such as *Pseudomonas, Bacillus, Alcaligenes, Citrobacter* and fungi such as *Aspergillus sp., Penicillum, Rhizopus* and *Rhodotorula* species to grow on crude petroleum as the sole carbon and energy source when screened for hydrocarbon utilization. At some point in this

study, it was observed that there was growth and extension of the hyphae forming mycelium in the medium.

Conclusion

In this study, it was observed that there was growth and extension of the hyphae forming mycelium in the medium. It would be interesting to remark that more biomass, liberation of CO_2 and enzyme production lead to higher petroleum degradation values confirming that these strains could be interesting to use in future field tests.

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Table 1
Biomass accumulation by fungal isolates during co-cultivation with petrol as sole carbon source.

Fungal Isolate	Comparison of CO_2 emission after 4-weeks Co-Cultivation			
	Controls (g/l)*	**Treatments (g/l)**	**Weight (g/l)**	**%**
Cladosporium sphaerospermum	0.432±0.018	0.614±0.031	0.182	42%
Eupenicillium hirayamae	0.448±0.045	0.653±0.027	0.205	45.7%
Paecilomyces variotii	0.445 ± 0.004	0.610±0.095	0.165	37%
Consortium	0.493±0.043	0.745±0.047	0.252	51%

*Data represents mean of three replicates ±Standard Deviation

Table 2
CO_2 evolved during co-cultivation of fungal isolates with petrol as sole carbon source.

Fungal Isolate	Biomass (Dry Weight) comparison after 4-weeks Co-Cultivation			
	Controls* (g/ l)	**Treatments (g /l)**	**Weight (g /l)**	**%**
Cladosporium sphaerospermum	0.599±0.048	0.717±0.031	0.118	19.6%
Eupenicillium hirayamae	0.591±0.049	0.721±0.038	0.130	21.9%
Paecilomyces variotii	0.594 ± 0.038	0.698±0.075	0.104	17.5%
Consortium	0.662±0.027	0.824±0.075	0.162	24.5%

* Data represents means of the estimates via volumetric and gravimetric methods

Table 3
Enzyme activity of fungal isolates during co-cultivation with petrol as sole carbon source

Fungal Isolate	Laccase (U/ml)		Mnp (U/ml)		Lip (U/ml)		Catalase (U/ml)	
	Controls*	Treatments	Controls	Treatments	Controls	Treatments	Controls	Treatments
Cladosporium sphaerospermum	2.34±1.20	3.22±1.18	ND ND ND	ND ND ND	1.87±1.08	4.81±1.53	1.80±0.66	8.57±0.84
Eupenicillium hirayamae	ND ND ND	ND ND ND	2.56±0.53	3.88±0.25	2.38±0.75	2.68±0.60	1.88±0.87	12.58±1.71
Paecilomyces variotii	ND ND ND	2.19±1.02	1.78±0.63	4.36±0.72	ND ND ND	ND ND ND	1.37±0.65	6.05±0.75
Consortium	9.8±2.48	11.22±0.75	20.27±2.9	21.59±1.44	6.04±1.95	8.18±2.004	6.33±2.18	22.54±2.07

*Data represents mean of three replicates ±Standard Deviation; ND: Not detected

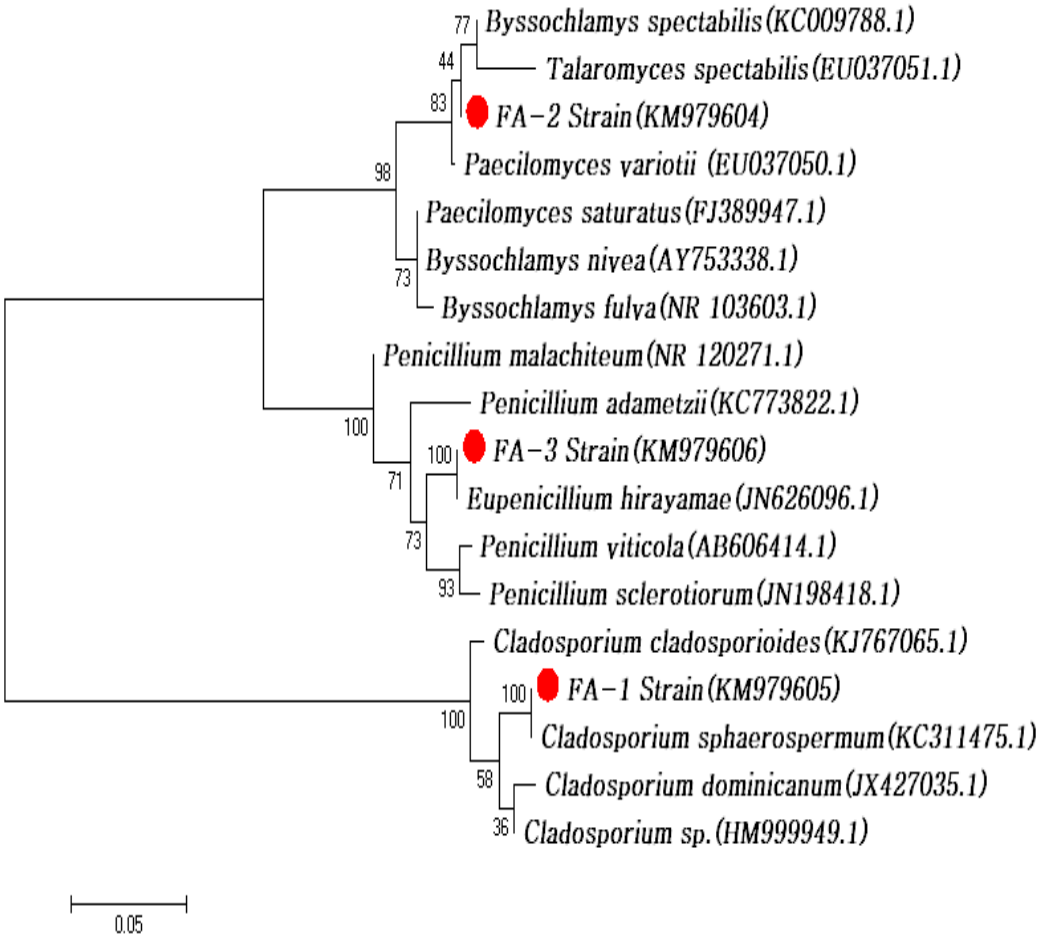

Fig. 1: Phylogenetic tree based on 18S rDNA sequence with maximum likelihood method by using program MEGA 6. Branch support was assessed using 100 bootstrap replicates

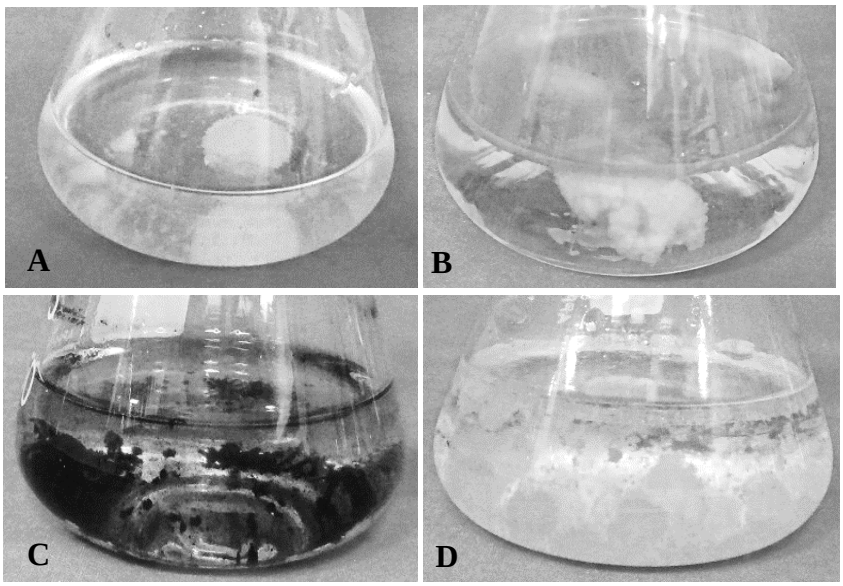

Fig. 2: Co-cultivation of Fungi with petrol substrate.
(A) Control (Without petrol): *Eupenicillium hirayamae,* showing meager growth of fungus in the absence of petrol substrate.
(B-D) Treatments (with petrol): *Paecilomyces variotii, Cladosporium sphaerospermum* and *Eupenicillium hirayamae* respectively, showing profuse growth of fungus in association with petrol substrate.

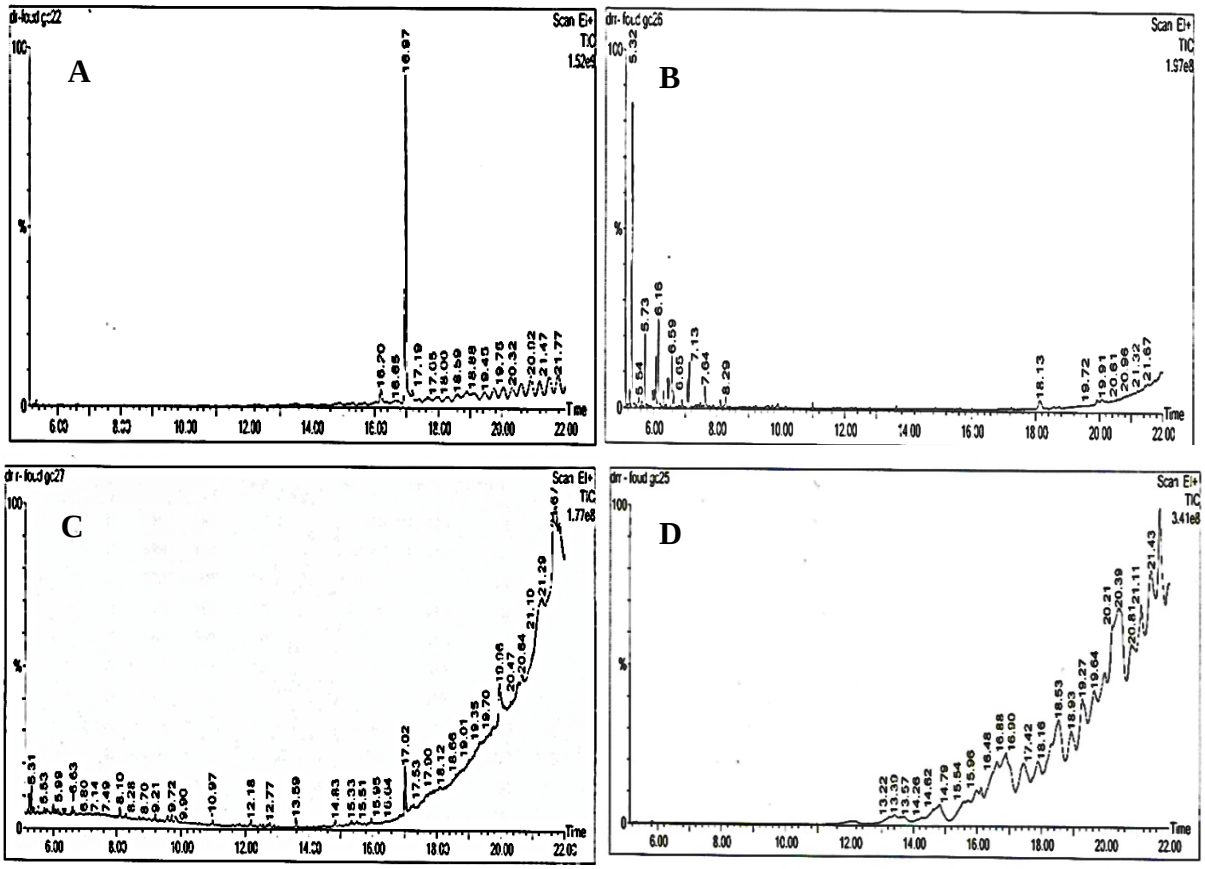

Fig. 3: Gas chromatogram of petroleum oil before and after treatment with fungal isolates a) before bioremediation b) after 30 days incubated with *Eupenicillium hirayamae; c)* after 30 days treatment with *Cladosporium sphaerospermum* and d) after 30 days incubated with fungal consortium

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