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Biodegradation of kerosene: Study of growth optimization and metabolic fate of *P. janthinellum* SDX7

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Abstract

*Penicillium janthinellum* SDX7 was isolated from aged petroleum hydrocarbon-affected soil at the site of Anand, Gujarat, India, and was tested for different pH, temperature, agitation and concentrations for optimal growth of the isolate that was capable of degrading upto 95%, 63% and 58% of 1%, 3% and 5% kerosene, respectively, after a period of 16 days, at optimal growth conditions of pH 6.0, 30 °C and 180 rpm agitation. The GC/MS chromatograms revealed that *n*-alkane fractions are easily degraded; however, the rate might be lower for branched alkanes, *n*-alkylaromatics, cyclic alkanes and polynuclear aromatics. The test doses caused a concentration-dependent depletion of carbohydrates of *P. janthinellum* SDX7 by 3% to 80%, proteins by 4% to 81% and amino acids by 8% to 95% up to 16 days of treatment. The optimal concentration of 3% kerosene resulted in the least reduction of the metabolites of *P. janthinellum* such as carbohydrates, proteins and amino acids with optimal growth compared to 5% and 1% (v/v) kerosene doses on the 12th and 16th day of exposure. Phenols were found to be mounted by 43% to 66% at lower and higher concentrations during the experimental period. Fungal isolate *P. janthinellum* SDX7 was also tested for growth on various xenobiotic compounds.

Key words: *Penicillium janthinellum* SDX7, optimal growth, metabolites, kerosene degradation.

Introduction

Kerosene currently has several uses such as aircraft gas turbine and jet fuel for both commercial airlines and the military activities, as heating oil, and as a spray oil to combat insects on agricultural plants. Because of its availability compared to gasoline during wartime, commercial illuminating kerosene was the fuel chosen for the early jet engines. Consequently, the development of commercial jet aircraft following World War II focused primarily on the use of kerosene-type fuels (Irwin *et al.*, 1997). According to the US Coast Guard Emergency Response Notification System, kerosene is one of the most commonly spilled petroleum products containing paraffins (alkanes), cycloparaffins (cycloalkanes), aromatics, and olefins with carbon numbers between C9-C20 (Irwin *et al.*, 1997). Large amounts of spills and leaks of petroleum products such as gasoline, diesel, kerosene, and similar compounds have been refined and handled on land every year. Despite careful handling, there is a possibility of introduction into the soil environment. The penetration of hydrocarbons from the top of the soil into the subsoil leads to a direct risk of ground water contamination (Schinner and Margesin, 1997; Watkinson and Morgan, 1989). Although a significant proportion of the compounds in petroleum products are relatively innocuous, a number of lower molecular weight compounds are toxic or mutagenic and require remedial action to restrict environmental damage (Greer *et al.*, 2003)

Microorganisms are powerful alternatives to conventional methods in resolving environmental problems (Bento *et al.*, 2003). Bacteria, yeast and filamentous fungi have been reported as transforming agents due to their ability to degrade a wide range of pollutants because of their ubiqui-
tous nature. Various bacterial genera that have been reported to contain hydrocarbon degrading species include Pseudomonas, Vibrio, Arthrobacter and Bacillus (Zajic, 1972; Snape et al., 2001). Fungal degradation of defined single hydrocarbons as well as petroleum products has been performed by Penicillium, Candida, Fusarium, Aspergillus, Articulosporium (Sugiura et al., 1997; Mukherji et al., 2004). Kumar et al. (2013) performed biodegradation of high molecular weight PAHs such as benzo[a]pyrene and fluoranthene using cyanobacterial species - *Aulosira fertilissima* ghose. These organisms have been isolated in large numbers from many oil-polluted waters and soils but are found in lesser numbers in uncontaminated environments (Okoh, 2003). Biodegradation is considered to be a major mechanism for the removal of spilled petroleum hydrocarbons from the environment (Braddock and Lindstrom, 2002). Therefore, it is necessary to perform laboratory feasibility tests to determine the effectiveness of biodegradation of kerosene due to the significant effect of the inherent capabilities of the microorganisms, by their ability to overcome the bioavailability limitations in multi-phase environmental scenarios and by environmental factors such as pH, temperature, nutrients and electron acceptor availability (Mukherji et al., 2004; Trindade et al., 2005). Less is known about the biodegradability of petroleum commercial products such as kerosene (Gouda et al., 2005). However, less is known about the biodegradability of petroleum commercial products such as kerosene (Gouda et al., 2005). Shamiyan et al. (2013 a, b) investigated the interactions of soil nutrients with Total Petroleum Hydrocarbons (TPH), with isolation and characterization of different petroleum-degrading fungal isolates. Moreover, no attempt has been made in the proposed work to establish the differential effects of various concentrations of kerosene on biochemical constituents of the fungal isolate *Penicillium janthinellum* SDX7 in liquid media during the biodegradation process. Hence, in the present investigation, an attempt has been made to optimize the fungal isolate at different conditions and its biochemical response during the petroleum hydrocarbon biodegradation of kerosene.

Materials and Methods

Fungal Isolate, Media and Culture Conditions

A fungal strain *P. janthinellum* SDX7 was isolated and screened from aged petroleum hydrocarbon-infected soil at the site of Anand, Gujarat, India, supporting the data of the TPH in the contaminated soil compared to the uncontaminated soil (garden soil). The isolate was identified as *P. janthinellum* SDX7 based on morphological and molecular (18S rRNA) methods, and the sequences were submitted to the National Center for Biotechnology Information (NCBI) gene bank under accession no. KC545842-KC545843 (Shamiyan et al., 2013b). Two types of media were used for cultivation, Potato Dextrose Agar (PDA) media and for the optimization and degradation studies, Mineral Salt Medium (MSM) with the addition of various concentrations of kerosene as the sole carbon source. The liquid medium was sterilized at 121°C for 15 min before addition of the kerosene (Zajic and Supplisson, 1972).

Preparation of standardized inocula

Axenic spore suspensions of *P. janthinellum* SDX7 of approximately 105 spores/mL were grown by adding 15 mL of sterile distilled water to mature fungal colonies on PDA plates (4-5 days) to dislodge the spores from the mycelium. These suspensions were then used to inoculate 100 mL MSM containing 3% (w/v) glucose in 500 mL Erlenmeyer flasks (Kendrick and Ratledge, 1996). The cultures were incubated at 30 °C in an incubator shaker operating at 180 rpm for 48 h. The resultant active growing cultures were aseptically washed three times with 300 mL of sterile distilled water to remove remaining glucose fractions. This resulting culture was then used as the standard inoculum for further experiments.

Determination of optimal growth

The optimization study addresses different pH values, temperature, speed of agitation and kerosene concentration on *P. janthinellum* SDX7. A total of 10% (v/v) of standard inoculum was inoculated in each experiment and performed in triplicate. Biomass production (g/L) was used as an indicator for growth after 7 days of incubation. MSM medium with kerosene (without inoculation) was used as a control (Hamzah et al., 2012).

Growth parameters

pH

The influence of the pH of the initial medium on the fungal growth of *P. janthinellum* SDX7 was determined at pH 4.0, 5.0, 6.0, 7.0, 8.0 and 9.0. Ten percent (v/v) standard inoculum was inoculated in a 500 mL Erlenmeyer flask containing 100 mL of MSM with the addition of 3% (v/v) kerosene and incubated at 30 °C in an orbital shaker at 180 rpm for 7 days. The pH that promoted the highest biomass production (in terms of dry weight) was used for subsequent steps of the investigation.

Temperature

The effect of temperature on *P. janthinellum* SDX7 growth was studied at 20, 30 and 40 °C in the MSM medium with 3% (v/v) kerosene at the determined optimum pH and incubated in an orbital shaker at 180 rpm for 7 days. The temperature that promoted the highest biomass production was used for the subsequent steps of the investigation.

Speed of agitation

The effect of different agitation at 130, 180 and 230 rpm during incubation on growth of *P. janthinellum* SDX7 was performed in the MSM medium with 3% (v/v) kerosene at optimum pH using an orbital shaker. Incubation
was conducted at the determined optimum pH and temperature. The agitation speed that promoted the highest biomass production was used for the subsequent steps of the investigation.

Concentration of kerosene

The isolate of *P. janthinellum* SDX7 was grown in MSM prepared in accordance with the optimum nutrient parameters but supplemented with 1%, 3%, 5%, 10% and 20% (v/v) concentrations of kerosene and incubated at optimum pH, temperature and agitation speed for 7 days.

Dry weight measurement

The biomass of *P. janthinellum* SDX7 recovered by filtration using Whatman filter paper (No. 4) was washed with 100 mL chloroform to remove residual kerosene, then dried in the oven at 60 °C overnight, and cooled in a desiccators for 10-20 min prior to weighing.

Determination of biodegradation activity

The determination of the biodegradation activity of *Penicillium janthinellum* SDX7 was performed in 100 mL of the MSM medium treated by lower 1%, optimal 3% and higher 5% concentrations of kerosene in a 500 mL Erlenmeyer flask and incubated at 30 °C and agitated at 180 rpm for 16 days. The residual petroleum hydrocarbon was recovered by chloroform extraction at a ratio of 1:1 MSM medium: chloroform (Chailan et al., 2004). MSM without fungal inoculation was used as the control. Analysis of the fungal biodegradation activity was made using a computerized capillary gas chromatograph with flame ionization detector (GC/FID, Perkin Elmer-Auto System, SICART, V.V. Nagar) equipped with HP 3390A Integrator, split injector (split ratio 20/1) and flame ionization detector set at 300 °C. The carrier gas was nitrogen at a flow rate of 1.5 mL min⁻¹. The column was polydimethylsiloxane (length 30 m, internal diameter 0.32 mm, film thickness 0.25 μm). The temperature was programmed to increase from 60 to 320 °C at 4 °C min⁻¹. The total petroleum hydrocarbon (TPH) degradation by *P. janthinellum* SDX7 isolate was calculated according to the following equation:

\[
\%B = \frac{100 \text{ (TPHC} - \text{TPHI)}}{\text{TPHC}}
\]  

where B is biodegradation, TPHC is the total petroleum hydrocarbon in the abiotic control (without fungal inoculation) and TPHI is the total petroleum hydrocarbon with inoculation (in this case with *P. janthinellum* SDX7).

**GC/MS analysis**

GC/MS spectra were acquired in the electron ionization mode (70 eV, nominal) scanning from m/z 30 to 650 s⁻¹ and detected using an auto system XL GC apparatus (Perkin Elmer, SICART, V.V. Nagar). The column temperature was initially 80 °C, held for 5 min, then ramped from 80 °C- 290 °C at 10 °C min⁻¹. Helium (1.0 mL min⁻¹) was used as the carrier gas. Both line and injector temperatures were set at 250 °C. Each methanolic extract (1 μL) was injected in the split mode (1:40). MS conditions were EI + through a Perkin Elmer Turbo mass spectrometer as follows: ionization energy -70 eV, nominal; scan rate, 1.6 scans/s; inter-scan delay, 0.01 s; source temperature, 250 °C; mass range, 30 to 650 Daltons; solvent delay, 3.00 min. The gas chromatogram as reproduced by the mass spectrometer identified the mass spectrum scanned at each GC peak maximum. Data were thus obtained by comparing the mass spectra to those in the Wiley NIST/EPA/NIH Mass Spectral Library 2005.

**Determination of metabolites**

The variation in the metabolites was determined on the 4th, 8th, 12th and 16th days by cultivating the *P. janthinellum* SDX7 culture under optimal conditions such as pH 6, temperature of 30 °C, speed of agitation at 180 rpm and addition of optimal 3% (v/v) with higher 5% and lower 1% (v/v) dose of kerosene. Total carbohydrate release was determined spectrophotometrically by the anthrone method using glucose as the standard (Hedge and Hofreitte, 1991). The protein content of the crude cell-free extract was estimated (Lowry et al., 1951) using bovine serum albumin as the standard. An improved colorimetric determination of amino acids by the use of ninhydrin was performed (Lee and Takahashi, 1966). Phenol stress metabolites were estimated using Folin-Ciocalteu reagent (Malick and Singh, 1980). Each experiment was conducted in replicates of three and their ± SE values were calculated. Multivariate analysis was performed using KY plot (2.0 beta) to estimate the correlation between metabolites.

**Growth of *P. janthinellum* SDX7 on other xenobiotic compounds**

Fungal isolate *P. janthinellum* SDX7 was also tested for its growth on various xenobiotic compounds, namely, diesel fuel, gasoline fuel, toluene, benzene, xylene, naphthalene, pyrene and phenanthrene at a concentration of 0.1% [(w/v)/ (v/v)] as a sole carbon source in MSM and incubated under optimized conditions (at 30 °C temperature and shaking conditions of 180 rpm). The samples were withdrawn after 7 days, and the biomass was estimated in the form of the dry weight (Gojic et al., 2012).

**Results and Discussion**

**Optimization of growth parameters**

*P. janthinellum* SDX7 grew better under acidic conditions, showing optimal growth (0.38 g/L) at pH 6. Previous studies also reported that several fungal isolates such as *Fusarium solani*, *F. oxysporum*, *Trichoderma viride* (Verdin et al., 2004) and *Aspergillus niger* (Srivastava and Thakur, 2006) cultured in acidic MSM medium provided good growth. Although its growth was highest under acidic
conditions, isolate *P. janthinellum* SDX7 was able to grow sparsely in a relatively wide range of pH values from 4.0 to 9.0, suggesting that this isolate could degrade kerosene under not only acidic but also under neutral and alkaline conditions (Figure 1a). Among the parameters that could affect biomass production, temperature was generally considered to be the most important and limiting factor (Delille, 2004). The common incubation temperature for the growth of fungi such as *A. niger*, *Fusarium sp.*, *Penicillium sp.*, and *Graphium sp.* is taken to be 30 °C (Santos and Linardi, 2004). Moreover, in this study, *P. janthinellum* SDX7 cultured at different temperatures produced maximum biomass (0.38 g/L) when incubated at 30 °C compared to 20 °C and 40 °C, temperatures that resulted in the production of a maximum of 0.31 g/L and 0.33 g/L biomass, respectively (Figure 1b). This difference may result from a greater production of enzymes and optimal growth conditions of the isolate for its kerosene degradation (Rohilla et al., 2012). This range of temperatures makes this isolate suitable for use in bioremediation in tropical climates. *P. janthinellum* SDX7 showed an increase of biomass as the rate of agitation increased up to 180 rpm; biomass production was then reduced when the speed of agitation accelerated to 230 rpm (Figure 1c). Agitation influenced the tested fungi to absorb more nutrients by not only increasing the surface area of the microorganism for degradation of the kerosene oil hydrocarbons (Lee et al., 1996) but also by booming the amount of dissolved oxygen in the cultivation medium (Purwanto et al., 2009). Agitation speed has also been proven to be a critical factor influencing mycelial biomass (Hamzah et al., 2012). *P. janthinellum* was found to grow in a wide range of kerosene concentrations. The production of biomass rose to 0.38 g/L in the presence of 3% (v/v) of kerosene on the 5th day. However, the production of biomass declined when the concentration of kerosene escalated beyond 3% (v/v) (Figure 1d). Notably, minimal growth was found when the medium contained 10% and 20% kerosene doses.

**Biodegradation of kerosene**

To determine the ability of *P. janthinellum* SDX7 to degrade kerosene, the total petroleum hydrocarbons were estimated at the end of the 4th and the 16th day of treatment at 1%, 3% and 5% kerosene treatments. Where 1% v/v kerosene showed the highest degradation at 85% and 95%, followed by 3% kerosene treatment at 32% and 63% degradation and a 5% kerosene dose by 28% and 58% reduction on 4th and 16th day, respectively (Figure 2). The biodegradation of kerosene was confirmed by the reduction in the

![Figure 1](image_url) - Optimal biomass production of *P. janthinellum* SDX7 after 7 days of incubation.
The area under the hydrocarbon peaks of the chromatograms when compared to that of the abiotic control (without organism), suggesting that the removal of kerosene hydrocarbon components ranged from 8 to 18 carbon atoms. In the chromatographic images of 1%, 3% and 5% (v/v) kerosene doses in a liquid medium, the sharp and highest peak stands for the n-alkanes, and the peaks between them comprise the naphthenes and aromatics. \textit{P. janthinellum} SDX7 showed a reduction in the area under the hydrocarbon peaks corresponding to the carbon atoms (C8 to C18) when compared to the abiotic control in the case of the 1%, 3% and 5% kerosene, where the lowest concentration of 1% displayed the highest reduction (Figure 3) followed by 3% (Figure 4) and 5% (Figure 5). The chromatograms showed that the n-alkane fractions are easily degraded by the tested fungal isolate as the days progress. However, the rate might be lower for branched alkanes followed by n-alkylaromatics, cyclic alkanes and polynuclear aromatics. The present results are in agreement with the findings obtained by Wang \textit{et al.} (1998), who studied the comparison of crude oil composition changes due to biodegradation and physical weathering using different fungal and bacterial isolates. Moreover, Nocentini \textit{et al.} (2000) studied the biotreatability and feasibility of a bioremediation process by a bacterial species for biodegradation of kerosene.

\textbf{Figure 2} - Degradation of different concentrations of kerosene after 4th and 16th days by \textit{P. janthinellum} SDX7.

\textbf{Figure 3} - GC/MS chromatograms of 1% (v/v) kerosene in MSM medium. (a) Abiotic control, (b) After 4 days of incubation with \textit{P. janthinellum} SDX7 strain, and (c) After 16 days incubation with \textit{P. janthinellum} SDX7 strain. The sharp and highest peaks stand for the n-alkanes, and the peaks between them comprise the naphthenes and aromatics.
a kerosene-contaminated soil. The results indicated that the P. janthinellum SDX7 isolate in this study is extremely efficient in degrading kerosene hydrocarbons. Our results also agreed very well with the findings of Mancera-López et al. (2008), who reported that the Penicillium genus was one of the major hydrocarbon-degrading groups.

**Metabolites**

Based on the inhibitory effects and growth arrest, the release of certain metabolic products such as carbohydrates, amino acids and proteins was affected at the earlier stage of kerosene applications, a result that is in agreement with the study of Kumar et al. (2013) who studied the impact of PAH exposure on certain cyanobacterial species. The level of total carbohydrates decreased after 12 to 16 days in all the concentration of kerosene-treated isolate. After the 12th day of exposure, carbohydrate content ranged from 0.6 mgmL^{-15} to 2.24 mgmL^{-15} and was significantly reduced by 50%, 11% and 76%, whereas a higher level of reduction was encountered after the 16th day by 59%, 24% and 80% at 1%, 3% and 5% kerosene exposures to P. janthinellum SDX7, respectively (Figure 6a). A similar reduction in the carbohydrate content was recorded by Kumar et al. (2008) in nitrogen-fixing cyanobacteria when treated with endosulfan. Protein content fluctuated from 1.44 mgmL^{-15} to 3.78 mgmL^{-15} and was inhibited by 46%, 13% and 67% on the 12th day (Figure 6b) and 70%, 48% and 81% after the 16th day, significantly at 1%, 3% and 5%, respectively, of kerosene-treated P. janthinellum SDX7 isolate as shown by Babu et al. (2001) and Laxmi et al. (2007) in response to lindane and organophosphorus on certain cyanobacterial species. At the end of the experiment after the 16th day, the highest reduction of amino acids in P. janthinellum SDX7 (by 95%) was observed when treated with 5% kerosene relative to the control, which ranged from 0.05 mgmL^{-15} to 0.4 mgmL^{-15} (Figure 6c). The optimal concentration of 3% kerosene showed less impact on the metabolite reduction. However, the greatest reduction observed at the higher concentration of 5% followed by the lower concentration of 1% kerosene showed results quite well correlated with the findings of Standyk et al. (1971), who depicted concentration-dependent inhibition of amino acids.
acids and proteins at an earlier stage based on inhibitory effects and growth arrest in fresh water algae in response to pesticide treatments.

A more pronounced effect of kerosene on phenol content was observed on the tested fungal isolate. The release of the stress metabolite phenol was significantly stimulated after 4 days of exposure, varying from 0.4 mgmL$^{-15}$ to 0.64 mgmL$^{-15}$ and higher in all treated cultures compared to untreated isolate (Figure 6d). The highest level of phenol was observed in 5% treated isolates, showing 61%, 63%, 65% and 66% increase on the 4th, 8th, 12th and 16th days, respectively. Elevated levels of phenols might be due to the liberation of the phenolic compounds during gradual degradation of kerosene by hydrolysis or oxidation processes and release under stress conditions because of the catabolic activity of primary metabolites (Mostafa and Helling, 2002). Kumar and Kumar (2002) also suggested that the release of phenols due to applications of different concentrations of fungicide was higher than untreated cultures, possibly due to the accumulation of phenolic compounds from the larger polycyclic aromatic compounds.

A significant positive correlation between carbohydrates, proteins and amino acids ($r = 0.88$ to 0.94) was encountered, whereas a highly negative correlation was registered with phenols ($r = -0.48$ to -0.72) after 16 days of exposure to kerosene (Table 1).

**Table 1 - Correlation matrix for P. janthinellum SDX7 after 16 days of exposure to kerosene.**

<table>
<thead>
<tr>
<th></th>
<th>Carbohydrates</th>
<th>Proteins</th>
<th>Amino acids</th>
<th>Phenols</th>
</tr>
</thead>
<tbody>
<tr>
<td>Carbohydrates</td>
<td>1</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Proteins</td>
<td>0.88</td>
<td>1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Amino acids</td>
<td>0.88</td>
<td>0.94</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>Phenols</td>
<td>-0.72</td>
<td>-0.56</td>
<td>-0.48</td>
<td>1</td>
</tr>
</tbody>
</table>
Growth of *P. janthinellum* SDX7 on various xenobiotic compounds

The efficiency of the fungal isolate in utilizing other xenobiotic compounds as the sole source of carbon is represented in Table 2. *P. janthinellum* SDX7 displayed a very high growth in the presence of kerosene, diesel and gasoline fuels. However, a moderate growth was encountered in case of naphthalene and poor growth was registered for phenanthrene, toluene, benzene and phenol. The isolate was not able to utilize the high molecular weight PAHs such as pyrene. Our results have been further substantiated by earlier studies of Okerentugba and Ezeronye (2003), revealing the degradation of various xenobiotic compounds by *P. janthinellum* SDX7 based on the ability of utilizing these xenobiotic compounds as the sole source of carbon.

The present investigation suggests that the fungal isolate *P. janthinellum* SDX7 employed in this study is extremely efficient in degrading kerosene, displaying a maximum degradation of 95% after 16 days under optimal growth conditions based on its ability to utilize kerosene. The optimal kerosene concentration of 3% showed the least reduction of the metabolites- carbohydrates, proteins and amino acids compared to the 1% and 5% (v/v) kerosene doses on the 12th and 16th days of exposure. Stress metabolite phenol was found to rise at lower and higher concentrations due to the inhibitory effect and growth retardation of the test organism in response to kerosene treatments. The highly efficient degradative ability of *P. janthinellum* SDX7 proved to be suitable for mycoremediation of kerosene-contaminated soil environments.

### Table 2 - Growth of kerosene-degrading *P. janthinellum* SDX7 in the presence of different xenobiotic compounds (0.1% [(v/v)/(w/v)].

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Growth profile</th>
</tr>
</thead>
<tbody>
<tr>
<td>Kerosene</td>
<td>+++</td>
</tr>
<tr>
<td>Diesel Fuel</td>
<td>+++</td>
</tr>
<tr>
<td>Gasoline Fuel</td>
<td>+++</td>
</tr>
<tr>
<td>Naphthalene</td>
<td>++</td>
</tr>
<tr>
<td>Phenanthrene</td>
<td>+</td>
</tr>
<tr>
<td>Pyrene</td>
<td>-</td>
</tr>
<tr>
<td>Benzene</td>
<td>+</td>
</tr>
<tr>
<td>Phenol</td>
<td>+</td>
</tr>
<tr>
<td>Toluene</td>
<td>+</td>
</tr>
<tr>
<td>Xylene</td>
<td>-</td>
</tr>
</tbody>
</table>

+++ Very high growth, ++ moderate growth, + low growth, - no growth.

**Growth of *P. janthinellum* SDX7 on various xenobiotic compounds**

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Enzymatic Evaluation During Biodegradation of Kerosene and Diesel by Locally Isolated Fungi from Petroleum-Contaminated Soils of Western India

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Enzymatic Evaluation During Biodegradation of Kerosene and Diesel by Locally Isolated Fungi from Petroleum-Contaminated Soils of Western India

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The current study suggests that the fungal isolates 

P. decumbens PDX7, P. janthinellum SDX7, and A. terreus PKX4 degraded kerosene by 95%, 96%, and 75% and diesel by 79%, 75%, and 70% after 16 days based on the ability of utilizing these compounds as sole carbon sources. GC-MS chromatograms revealed that n-alkane fractions are easily degraded; however, the rate is lower for branched alkanes, n-alkyl aromatics, cyclic alkanes, and polynuclear aromatics displaying delayed and lower degradation. The ratio of aromatic/aliphatic hydrocarbons >0.8 indicates the efficiency of these fungi in removing the aromatic hydrocarbons of the petroleum products. All of the treated fungal strains exhibited higher MnP, laccase, and dehydrogenase activities on the twelfth and sixteenth days as compared to the initial fourth and eighth days. In addition, P. decumbens PDX7 and P. janthinellum SDX7 displayed higher enzymatic activities as compared to A. terreus PKX4. Fungal isolates were also tested for their growth on various xenobiotic compounds as sole carbon sources.

Keywords Fungal isolates, kerosene and diesel degradation, enzyme activity, correlation

Introduction

Petroleum-based products are the major source of energy for industry and daily life. Leaks and accidental spills occur regularly during the exploration, production, refining, transport, and storage of petroleum and petroleum products. The amount of natural crude oil seepage was estimated to be 600,000 metric tons per year (Kvenvolden and Cooper, 2003). The penetration of hydrocarbons into subsoil leads to a direct risk of groundwater contamination (Morgan and Watkinson, 1989; Margesin and Schinner, 1997).

Although a significant proportion of the compounds in petroleum products are relatively innocuous, a number of lower-molecular-weight compounds are toxic or mutagenic.
and require remedial action to restrict environmental damage (Greer et al., 2003). Petroleum and petroleum products are complex mixtures consisting of thousands of compounds that are usually grouped into four fractions: aliphatics, aromatics, nitrogen–oxygen–sulphur (NSO) compounds, and asphaltenes. Asphaltenes are generally solvent-insoluble and resistant to biodegradation. Aliphatic hydrocarbons consist of normal alkanes (n-alkanes), branched alkanes (isoalkanes), and cyclic alkanes (naphthenes); isoalkanes, naphthenes, and aromatics are much less biodegradable than n-alkanes.

Biodegradation is considered a major mechanism for the removal of spilled petroleum hydrocarbons from the environment (Lindstrom and Braddock, 2002). Bacteria, yeast, and filamentous fungi have been reported as transforming agents due to their ability to degrade a wide range of pollutants (Wongsa et al., 2004; Gouda et al., 2007; Saratale et al., 2007; Laila and Nour, 2008). Although hydrocarbon degraders may be expected to be readily isolated from a petroleum oil-associated environment, the same degree of expectation may be anticipated for microorganisms isolated from a totally unrelated environment (Ojo, 2005). Information on the fungal degradation of a defined single hydrocarbon is available in the literature (Sugiura et al., 1997), but less is known about the biodegradability of commercial petroleum products such as kerosene and diesel (Gouda et al., 2007). In addition, Shamiyan et al. (2013a; 2013b) stated that fungal isolates from aged petroleum-product-contaminated sites were capable of degrading commercial petroleum products utilizing them as sole carbon sources.

Fragmentary studies have shown the presence of certain fungi species after oil spills, which indicates that the fungi develop an enzymatic system after long contact with hydrocarbons (Pinholt et al., 1979). Little is known of the enzymatic oxidation of petroleum hydrocarbons during their degradation by fungal organisms. Faber (1997) discussed biocatalytic oxidation reactions of alkanes, alkenes, and aromatics by dehydrogenase, laccase, and mangenese peroxidise. Laccase from the white rot fungus Trametes hirsuta is employed for the oxidation of alkenes (Niku-Paavola and Viikari, 2000). Enzymatic oxidation can be more efficient in biodegradation of petroleum hydrocarbons when compared to chemical catalysis. Therefore, it is necessary to perform laboratory feasibility experiments to determine the effectiveness of biodegradation of kerosene and diesel due to the significant effect of the inherent capabilities of the fungi (Mukherji et al., 2004; Trindade et al., 2005). In the present study, a correlation was established between the degradative enzyme activities laccase, mangenese peroxidise, and dehydrogenase during the process of degradation of kerosene and diesel by the selected fungal isolates in vitro.

Materials and Methods

Fungal Isolate, Media, and Culture Condition

Fungal strains P. decumbens PDX7, P. janthinellum SDX7, and A. terreus PKX4 were isolated and screened for kerosene and diesel degradation from a petroleum-contaminated soil site in Anand, Gujarat, India. Fungal isolates were identified based on morphological and molecular (18S rRNA) methods and the sequences were submitted to a NCBI gene bank (Shamiyan et al., 2013b; 2013c) (Table 1).

Preparation of Standardized Inoculums

Axenic spore suspensions of the fungal isolates of about $10^5$ spores/mL were grown by adding 15 mL of sterile distilled water to mature fungal colonies on PDA plates.
(4–5 days) to dislodge the spores from the mycelium. These suspensions were then used to inoculate 100 mL MSM (Minimal Salt Medium) containing 3% (w/v) glucose in 500 mL Erlenmeyer flasks (Kendrick and Ratledge, 1996). The cultures were incubated at 30°C in an incubator shaker operating at 180 rpm for 48 h. The resultant active growing cultures were aseptically washed three times with 300 mL of sterile distilled water to remove remaining glucose fractions. This resulting culture was then used as standard inoculum for further experiments.

Determination of Biodegradation Activity

The determination of the biodegradation activity of the three fungal isolates was carried out in 100 mL MSM medium (Mancera-Lopez et al., 2007) treated with 10,000 mg of TPH L⁻¹ concentration of kerosene and diesel in a 500 mL Erlenmeyer flask, incubated at 30°C, and agitated at 180 rpm for 16 days. The residual petroleum hydrocarbon was recovered by chloroform extraction at a ratio of 1:1 MSM medium:chloroform (Chaillan et al., 2004). MSM without fungal inoculation was used as the control. Analysis of the fungal biodegradation activity was made using a computerized capillary gas chromatography with flame ionized detector (GC-FID, Perkin Elmer-Auto System) equipped with an HP 3390A Integrator, split injector (split ratio 20/1), and flame ionization detector set at 300°C. The carrier gas was nitrogen at a flow rate of 1.5 mL/min. The column used was polydimethylsiloxane (length 30 m, internal diameter 0.32 mm, film thickness 0.25 μm) and the temperature was programmed to increase from 60 to 320°C at 4°C min⁻¹. The total petroleum hydrocarbon (TPH) degradation by the fungal isolates was calculated according to the following equation:

\[ \%B = \frac{100(TPHC - TPHI)}{TPHC} \]

where B is biodegradation, TPHC is the total petroleum hydrocarbon in the abiotic control (without fungal inoculation), and TPHI is the total petroleum hydrocarbon with inoculation in this case with the three fungal isolates.

GC-MS Analysis

GC-MS analyses spectra were observed in the electron impact mode (70 eV) scanning from m/z 30 to 650 s⁻¹and detected using an auto system XL GC apparatus (Perkin Elmer, Clarus 560 S GC/MS 120/230 V). The column temperature was initially 80°C, held for 5 min, then ramped from 80°C–290°C at 10°C/min. Helium (1.0 ml/min) was used as the carrier gas. Both line and inject or temperatures were set at 250°C. 1 μl of
each chloroform extract prepared was injected in the split mode (1: 40). MS conditions were run in EI + through a Perkin Elmer Turbo Mass spectrometer as follows: ionization energy –70 eV; scan rate 1.6 scans/sec; inter scan delay 0.01 sec; source temperature 250°C; mass range 30 to 650 m/z; solvent delay 3.00 min. The chromatograms obtained by mass spectrophotometer were identified and scanned at each GC peak maximum. Data were compared with Wiley NIH Mass Spectral Library 2005.

**Growth of Fungal Isolates on other Xenobiotic Compounds**

Fungal isolates were also tested for growth on various xenobiotic compounds, namely gasoline fuel, toluene, benzene, xylene, naphthalene, pyrene, and phenanthrene, at a concentration of 1000 mg of TPH L⁻¹ [(w/v)/(v/v)] as a sole carbon source in MSM and incubated under optimized conditions (at 30°C temperature and shaking condition of 180 rpm). The samples were withdrawn after seven days and the biomass was estimated in the form of the dry weight (Gojic-Cvijovic et al., 2012).

**Enzymatic Evaluation**

Manganese peroxidase, laccase, and dehydrogenase activities were assayed spectrophotometrically in extracellular fluid of culture supernatants by the methods of Wariishi et al. (1992), Andrea et al. (2001), and Mehrasbi et al. (2003), respectively, on the fourth, eighth, twelfth, and sixteenth days during degradation of kerosene and diesel. All enzyme activities were expressed in units per liter, with 1 μmol of substrate oxidized per minute.

**Statistical Analysis**

Multivariate analysis was carried out to correlate between enzyme activities and degradation of kerosene and diesel by fungal isolates using KY plot (2.0 beta) by KyensLab Inc.

**Results and Discussion**

**Kerosene Degradation**

All three isolates grew in a mineral salt medium with 10,000 mg of TPH L⁻¹ kerosene, of which *P. decumbens* PDX7 showed the highest degradation of aliphatic hydrocarbons and aromatic hydrocarbons up to 97% and 90%, followed by 94% and 85% by *P. janthinellum* SDX7 and 84% and 70% for *A. terreus* PKX4, respectively (Table 2). The ratio of aro-

<table>
<thead>
<tr>
<th>Fungal isolate</th>
<th>Aliphatic hydrocarbons (%)</th>
<th>Aromatic hydrocarbons (%)</th>
<th>Aromatic/aliphatic hydrocarbons (%)</th>
<th>TPHs</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>P. decumbens</em> PDX7</td>
<td>97 ± 2</td>
<td>90 ± 4</td>
<td>0.92</td>
<td>96 ± 1</td>
</tr>
<tr>
<td><em>P. janthinellum</em> SDX7</td>
<td>94 ± 3</td>
<td>85 ± 5</td>
<td>0.90</td>
<td>95 ± 2</td>
</tr>
<tr>
<td><em>A. terreus</em> PKX4</td>
<td>84 ± 4</td>
<td>70 ± 4</td>
<td>0.83</td>
<td>75 ± 4</td>
</tr>
</tbody>
</table>
matic/aliphatic hydrocarbons >0.8 indicates the efficiency of these isolates in removing the aromatic hydrocarbons, which are more complex to break down. The maximum TPH reduction, up to 400 mg/L, was displayed by *P. decumbens* at the end of the sixteenth day, whereas *A. terreus* demonstrated minimum reduction corresponding to 2500 mg/L (Figure 1). The biodegradation of kerosene was confirmed by the reduction in the area under the aliphatic and aromatic hydrocarbon peaks of the chromatograms when compared to that of the abiotic control (without organism), suggesting the removal of kerosene hydrocarbon (Figures 2 a–d). In the chromatographic images, it has been recorded that the sharp and highest peaks stand for the n-alkanes and the peaks between them comprise the naphthenes and aromatics corresponding to carbon atoms (C8 to C18). The chromatograms showed that n-alkane fractions are easily degraded as the days progress by the tested fungal isolate; however, the rate might be lower for branched alkanes, followed by n-alkyl aromatics, cyclic alkanes, and polynuclear aromatics. These results are in agreement with the findings obtained by Wang *et al.* (1998), who studied the comparison of crude oil composition changes due to biodegradation and physical weathering using different fungal and bacterial isolates. Moreover, Nocentini *et al.* (2000) emphasized the bioremediation of soils contaminated by hydrocarbon mixtures that displayed a similar trend of degradation of kerosene by certain bacterial species. These results indicate that the three isolates employed in this study are extremely efficient in kerosene degradation. *P. decumbens* PDX7 showed the greatest capacity to remove 10,000 mg of TPH L$^{-1}$ due to adaption of these fungi to hydrocarbon composition, as well as the enzyme activities (Mancera *et al.*, 2008).

**Diesel Degradation**

Fungal isolates grown in mineral salt medium with 10,000 mg of TPH L$^{-1}$ of diesel showed the highest degradation of aliphatic hydrocarbons and aromatic hydrocarbons, up to 80% and 67% by *P. decumbens* PDX7, 76% and 64% by *P. janthinellum* SDX7, and 71% and 60% by *A. terreus* PKX4, respectively (Table 3). The percent of aromatic/aliphatic hydrocarbons >0.8 indicates the efficiency of these fungi in removing the aromatic hydrocarbons of diesel, which are more complex to break down. *P. decumbens* displayed maximum TPH reduction, up to 2,100 mg/L, whereas the minimum reduction of

![Figure 1. Kerosene degradation by the three fungal isolates as a function of time.](image-url)
3,000 mg/L TPH was exhibited by *A. terreus* at the end of the sixteenth day (Figure 3). The chromatographic images have shown the smaller peaks of naphthenes and aromatics, corresponding to carbon atoms (C10 to C22) between the sharp and tallest peaks of the n-alkanes. The biodegradation of diesel was confirmed by the reduction in the area under

**Figure 2.** GC-MS chromatograms displaying (a) abiotic control; (b) *P. decumbens* PDX7; (c) *P. janthinellum* SDX7; and (d) *A. terreus* PKX4 degrading kerosene after 16 days.
the aliphatic and aromatic hydrocarbon peaks of the chromatograms when compared to that of the abiotic control, suggesting the removal of diesel hydrocarbon (Figures 4 a–d). The chromatograms showed that the rate of degradation is lower for branched alkanes followed by n-alkyl aromatics, cyclic alkanes, and polynuclear aromatics by the tested fungal isolates. The n-alkane fractions of diesel, ranging from C10 to C15, are degraded completely by the fungal isolates as the days progress. These results were in agreement with the findings of Dussan and Numpaque (2012), who stated that the degradation of diesel fuel by certain bacterial species is in the order n-alkanes > branched alkanes > n-alkyl aromatics > cyclic alkanes > polynuclear aromatics. The study revealed that the three fungal isolates were extremely efficient at degrading diesel, with *P. decumbens* PDX7 demonstrating the greatest degradation efficiency. The results also agree with the findings of Mancera et al. (2008), who reported *Penicillum* sp. and *Aspergillus* sp. were the major hydrocarbon-degrading groups.

### Growth of Fungal Isolates on Various Xenobiotic Compounds

The efficiency of the fungal isolates to utilize other xenobiotic compounds as a sole source of carbon is represented in Table 4. Besides being able to utilize naphthalene, the isolates were also able to utilize other PAH, such as phenanthrene; however, they showed moderate growth in phenanthrene from 0.41 to 0.44 g/L by

<table>
<thead>
<tr>
<th>Fungal isolate</th>
<th>Aliphatic hydrocarbons (%)</th>
<th>Aromatic hydrocarbons (%)</th>
<th>Aromatic/aliphatic hydrocarbons (%)</th>
<th>TPHs</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>P. decumbens</em> PDX7</td>
<td>80 ± 2</td>
<td>67 ± 4</td>
<td>0.83</td>
<td>79 ± 2</td>
</tr>
<tr>
<td><em>P. janthinellum</em> SDX7</td>
<td>76 ± 3</td>
<td>64 ± 5</td>
<td>0.84</td>
<td>75 ± 2</td>
</tr>
<tr>
<td><em>A. terreus</em> PKX4</td>
<td>71 ± 4</td>
<td>60 ± 4</td>
<td>0.84</td>
<td>70 ± 3</td>
</tr>
</tbody>
</table>

Table 3. Diesel hydrocarbon degradation after 16 days

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Figure 3. Diesel degradation by the three fungal isolates as a function of time.
A. terreus and P. decumbens, respectively. P. decumbens was the only isolate which was able to utilize the high-molecular-weight PAH, like pyrene, displaying moderate growth of 0.23 g/L, which was supported by the findings of Verdin et al. (2004), who reported the lower growth and degradation capacity of fungal isolates.
in the presence of high-molecular-weight PAH compounds causing toxic effects on the isolates. P. decumbens PDX7, P. janthinellum SDX7, and A. terreus PKX4 displayed very high growth, ranging from 1.09 to 1.56 g/L in the presence of kerosene, diesel, and gasoline fuels; however, a moderate growth was encountered in the case of toluene, benzene, and phenol. Xylene did not support the growth of P. janthinellum SDX7 and A. terreus PKX4, whereas P. decumbens PDX7 showed moderate growth, up to 0.12 g/L. Okerentugba and Ezeronye (2003) also suggested various xenobiotic compounds used as sole carbon sources by Penicillium and Aspergillus species during degradation.

### Enzymatic Evaluation During Degradation

All of the three fungal isolates exhibited laccase, MnP, and dehydrogenase activities up to a certain extent and degradation after treating them with kerosene and diesel. The enzyme activities varied with growth and also the carbon source. P. decumbens PDX7 displayed the highest degradation of kerosene and diesel further showed the highest MnP unit activities L⁻¹ of 27.65 and 19.65, respectively, after 16 days’ duration, whereas A. terreus PKX4 exhibited the lowest 1.06 and 0.36 MnP activities after the fourth day in the presence of kerosene and diesel, respectively. Laccase and dehydrogenase enzymes also encountered the highest activities in the case of P. decumbens PDX7 of 1.85 and 11.52 unit L⁻¹ during kerosene degradation (Figure 5). Laccase is well documented with a mediator 2, 2’-azinobis (3-ethylbenzothiazoline-6-sulfonic acid) (ABTS), to oxidize certain aromatic compounds, called mediated oxidation. The observations recorded were similar to the findings of Andrea et al. (2001), who found that the fungus produces manganese dependent peroxidase (MnP) and laccase, which are able to oxidize the aromatic compounds during the degradation of various PAHs in vivo by P. laevis. Mehrasbi et al. (2003) explored the degradation of petroleum products and reported

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### Table 4

Growth of fungal isolates in presence of different xenobiotic compounds (0.1% [(v/v)/(w/v)]

<table>
<thead>
<tr>
<th>Substrate</th>
<th>P. decumbens PDX7 (g/L)</th>
<th>P. janthinellum SDX7 (g/L)</th>
<th>A. terreus PKX4 (g/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Kerosene</td>
<td>1.54 ±0.4</td>
<td>1.49 ±0.3</td>
<td>1.50 ±0.4</td>
</tr>
<tr>
<td>Diesel fuel</td>
<td>1.2 ±0.2</td>
<td>1.15 ±0.2</td>
<td>1.09 ±0.3</td>
</tr>
<tr>
<td>Gasoline fuel</td>
<td>1.56 ±0.2</td>
<td>1.53 ±0.4</td>
<td>1.54 ±0.2</td>
</tr>
<tr>
<td>Naphthalene</td>
<td>0.64 ±0.02</td>
<td>0.59 ±0.03</td>
<td>0.56 ±0.02</td>
</tr>
<tr>
<td>Phenanthrene</td>
<td>0.44 ±0.01</td>
<td>0.43 ±0.02</td>
<td>0.41 ±0.04</td>
</tr>
<tr>
<td>Pyrene</td>
<td>0.23 ±0.04</td>
<td>0.21 ±0.02</td>
<td>0.1 ±0.01</td>
</tr>
<tr>
<td>Benzene</td>
<td>0.35 ±0.01</td>
<td>0.33 ±0.02</td>
<td>0.32 ±0.01</td>
</tr>
<tr>
<td>Phenol</td>
<td>0.14 ±0.02</td>
<td>0.12 ±0.02</td>
<td>0.13 ±0.03</td>
</tr>
<tr>
<td>Toluene</td>
<td>0.16 ±0.03</td>
<td>0.13 ±0.04</td>
<td>0.11 ±0.05</td>
</tr>
<tr>
<td>Xylene</td>
<td>0.12 ±0.03</td>
<td>0.06 ±0.01</td>
<td>0.04 ±0.02</td>
</tr>
</tbody>
</table>
that dehydrogenase is involved in the electron transport system to remove the oxidative substrate, leading to degradation of petroleum products. A high positive correlation obtained between enzyme activities (r = 0.93–1) and degradation (r = 0.78–1) of the kerosene and diesel by the fungal isolates revealed the role of these enzymes involved in the oxidation of the petroleum products, leading to efficient degradation (Table 5).

The current study suggests that the fungal isolates *P. decumbens* PDX7, *P. janthinellum* SDX7, and *A. terreus* PKX4 are extremely competent for kerosene and diesel degradation in the order *P. decumbens* PDX7 > *P. janthinellum* SDX7 > *A. terreus* PKX4, as well as utilizing the aliphatic and aromatic fractions of the fuels and other xenobiotic compounds as sole carbon sources. The oxidative enzyme activities showed positive correlation with kerosene and diesel degradation by the fungal isolates. The highly potential degradative ability of *P. decumbens* PDX7 makes it exceedingly suitable for mycoremediation of kerosene- and diesel-contaminated environments.

**Table 5**

<table>
<thead>
<tr>
<th></th>
<th>Laccase</th>
<th>MnP</th>
<th>Dehydrogenase</th>
<th>Degradation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Laccase</td>
<td>1</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MnP</td>
<td>0.93</td>
<td>1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Dehydrogenase</td>
<td>0.94</td>
<td>0.96</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>Degradation</td>
<td>0.78</td>
<td>0.79</td>
<td>0.82</td>
<td>1</td>
</tr>
</tbody>
</table>

*Figure 5.* Activity of MnP, laccase, and dehydrogenase by the three fungal isolates during kerosene and diesel degradation.
Funding

One of the authors, Ms. Shamiyan Rahat Khan, is thankful to the University Grants Commission (UGC) for financial support from the Maulan Azad Fellowship. The authors are also thankful to Sophisticated Instrumentation Centre for Advanced Research and Testing (SICART) for analysis of samples using GC.

References


Ex-Situ Studies on Biodegradation of Artificially Enriched Kerosene and Diesel Soils by Fungal isolates

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Shamiyan R. Khan*, Nirmal Kumar J.I., Mansi Banker* & Rita N. Kumar

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ABSTRACT

To demonstrate the potential of biodegradation of soils enriched with kerosene and diesel, an ex-situ study with the objective of evaluating and comparing the effects of three different fungal isolates *P. janthenillum*, *P. decumbens* and *A. terreus* was performed. The study dealt with the biodegradation of artificially enriched kerosene and diesel soils by 5%, 10% and 15% (w/w). The experiment was performed by ex-situ large scale tray method using 24 plastic trays 6' X 3' X 1' in each containing 60 kg enriched soil. After 8 weeks of
Physicochemical properties, heavy metal content and fungal characterization of an old gasoline-contaminated soil site in Anand, Gujarat, India

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²Department of Biological & Environmental Sciences, N.V. Patel College of Pure & Applied Sciences, Vallabh Vidya Nagar, Gujarat, India

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Abstract

The current investigation was aimed to study physicochemical properties, heavy metal concentration and fungal communities of top soil samples taken from an old gasoline-contaminated site and from uncontaminated garden site in Anand, Gujarat, India. The total petroleum hydrocarbon concentrations were 11 500 and 142.65 mg kg⁻¹ for gasoline contaminated and uncontaminated soils, respectively. The contamination was associated with increased soil organic carbon, nitrogen concentration and clay content (2.77, 0.58 and 35.70%, compared to 1.50, 0.15 and 32.4% in the garden soil, respectively), i.e., increased organic and inorganic nutrient contents. Increased concentration of heavy metals like cadmium, copper, iron, zinc and lead in contaminated soil was evident. Nine native fungal species belonging to a total of six genera, included Aspergillus terreus, Aspergillus versicolor, Aspergillus niger, Fusarium oxysporum, Penicillium janthinellum from garden soil, and in addition Aspergillus niger, Fusarium oxysporum, Cladosporium bruhnei and Galactomyces geotrichum from the aged gasoline-contaminated soil were identified based on 18S rRNA. The changes created by gasoline contamination resulted in change of intensity of some physicochemical properties, soil conductivity, pH, fungal growth and soil fertility indices.

Key words: 18S rRNA, fungal growth, gasoline contamination, heavy metals, soil physicochemical analysis, total petroleum hydrocarbons.

Introduction

Gasoline, like all fossil fuels, primarily consists of a complex mixture of molecules called hydrocarbons, including straight chain, branched, cyclic hydrocarbons, polycyclic aromatic hydrocarbons and inorganic substances. In large concentrations, petroleum products are highly toxic to many organisms, including humans (Alexander 1994). The dominance of petroleum products in the world economy creates conditions for distributing large amounts of these toxins into populated areas and ecosystems around the globe (Ojumu 2004). With continued utilization, greater quantities of gasoline are being transported over long distances. Therefore, gasoline can enter into the environment through spillage, may occur as a result of oil pipe corrosion, pipeline/flow leakage, rupture of tanks, effluents from sabotage and human errors (Chikere, Chijioke–Osuji 2006). Since petroleum contains some gaseous components, these fractions will volatilize from oil polluted soil leaving the non-volatile components as residues (Odu 1977). It has been demonstrated that oil spillage affects the physical and chemical nature of soils (Minai-Tehran, Herfatmanesh 2007). The economic and environmental impacts of oil pollution on soil are enormous, causing serious damage to vegetation, soil fertility (Nwachukwu, Ugorji 1995) and soil-borne microorganisms; the toxicity varies depending on the type of oil and additives used during refining and also on the biota of spillage (Reddy 2001).

Bioremediation of such soils involves intentional release of microorganisms to the contaminated site for clearance of the pollutants. Fungi plays an important role in removing hazardous compounds from water and soil. Sediment particles contaminated with petroleum products from spills is one of the ecological niches for fungi, which use carbon from hydrocarbons in polluted sediment particles, leading to their biodegradation. Fungi have been found to be better degraders of petroleum than those used in traditional bioremediation techniques, such as bacteria (Al-Nasrawi 2012). The size of the microbial biomass is generally considered to be important in bioremediation. The microbial biomass itself represents a considerable pool of nutrients, which is continuously diverted into growth cycles of micro- and macrophytes.

Consequently, soils that maintain a high level of
microbial biomass are capable of storing more nutrients, as well as cycling more nutrients through the ecosystem (Torstensson et al. 1998). For optimization of effective gasoine bioremediation processes, it is essential to consider environmental and biological factors affecting the process. The environmental factors include availability of nutrients, pH, soil texture and extent of gasoline contamination in the polluted soil, whereas the biological factors encompass the bacterial, fungal or algal species that are responsible for bioremediation of gasoline in the contaminated soils (Bahuguna et al. 2011). The present study was carried out to study the effect of refined petroleum hydrocarbons on physicochemical properties, heavy metal enrichment and fungal load of aged gasoline-contaminated soil as compared with garden soil. The obtained results can be further useful for standardization of in situ bioremediation as well as establishment of biodegradation protocols.

Materials and methods

Collection of soil samples
Aged gasoline-contaminated soil samples were collected from a subsurface automobile garage located in Vallabh Vidyanagar, Anand, Gujarat, India. The soil in this sample area was chronically polluted with gasoline for at least fifteen years. Uncontaminated garden soil was used as control. At both sites, 10 top and sub-soil samples were collected randomly within a 0.5-m plot and mixed into a composite representative sample with a sterile spatula into a sterile polyethylene bag and taken to the laboratory immediately. Further, stones were removed from the samples and soils were homogenized through a 2 mm sieve. The soil samples were designated as gasoline and garden, and were stored in dark bottles until further analysis. All parameters were measured in triplicate and the mean value of three measurements was used for data analysis.

Determination of soil physicochemical properties
Soil samples were analysed for particle size by the international pipette method (Gee, Bauder 1986), bulk density by metal core sampler method (Blake, Harte 1986), and porosity and moisture content according to Maiti (2003). The pH and electric conductivity of the soil samples were determined in soil/water (1:1, v/v) suspension by a pH meter and a conductivity meter (Sension 5, HACH, USA). Total organic carbon (TOC) concentration was determined with the titration method of Walkey and Black (1934). Total organic matter (TOM) concentration was quantified by multiplying TOC with 1.724 (Osuji, Nwoye 2007). Total nitrogen was measured by Kjeldahl digestion (Gerhardt, Turbotherm, Germany) and steam distillation method (Black 1965), sodium and potassium were determined using a flame photometer. Total salinity, sulphate, phosphate, magnesium and calcium were estimated using procedures of Maiti (2003). Soil fertility indices were calculated considering the N, P, K, TOC and TOM (Osuji, Nwoye 2007).

Analysis of heavy metals
For estimation of heavy metal (Cd, Pb, Cu, Zn, Co, and Fe) concentrations, 0.5 g of dried soil was digested with concentrated HNO₃, H₂SO₄, and H₂O₂ (2:6:6) as prescribed by Nirmal Kumar et al. (2008). The blanks were run in a set, and samples were analysed with an Inductive Coupled Plasma Analyzer (Optima 3300 RL, Perkin-Elmer, USA) at Sophisticated Instrumentation Centre for Applied Research and Testing, Vallabh Vidya Nagar, Gujarat, India. The concentrations of heavy metals were expressed as mg kg⁻¹. The enrichment factor was calculated to derive the degree of soil contamination and heavy metal accumulations in soil from the contaminated site with respect to uncontaminated soil (Kisku et al. 2000).

Determination of total petroleum hydrocarbons
The soil samples were cleaned of roots, thoroughly mixed, and 2 g of each soil sample was weighed into a clean extraction container. Then 10 mL of extraction solvent (chloroform/dihloromethane, 1:1, v/v) was added into each sample, mixed thoroughly and allowed to settle. The mixtures were carefully filtered into a clean solvent extraction vessel using filter paper fitted into buchner funnels. The extracts were concentrated to 2 mL and then transferred for cleanup/separation. The concentrated aliphatic fractions were transferred into labelled vials with teflon caps for gas chromatograph analysis. The amount of TPH was measured following the USEPA Method 8015B for GC analysis with a FID detector and a HP-5 column, at the Sophisticated Instrumentation Centre for Applied Research and Testing. External calibration was conducted with original gasoline fuel.

Isolation of the indigenous fungal population
For isolation of the indigenous fungal population, 1 g of each sieved soil sample was homogenously mixed with 1 drop (approximately 0.1 mL) of Tween 80. About 1.0 g of homogenized soil sample was aseptically transferred using a flame-sterilized steel spatula into a sterile test tube containing 9.0 mL of sterile distilled water. This gave a 10⁻¹ dilution, and subsequently, three-fold (10⁻³) serial solutions were prepared from the 10⁻¹ dilution. Diluted samples (1 mL) was poured on Potato Dextrose Agar plates and Sabouraud Dextrose Agar plates. Streptomycin (500 mg L⁻¹) as an antibiotic to inhibit bacterial growth was added to media after sterilization (Harrigan, McCance 1990). Then, the plates were incubated at temperature 28 to 31 °C for 48 h or more depending on the rate of fungal growth. To obtain pure cultures of the fungal isolates, fungal cultures were aseptically subcultured into fresh plates and incubated until the fungus began to sporulate, followed by subsequent subculturing to obtain pure cultures consisting of only one
type of fungal isolate. A part of the pure culture was then aseptically transferred into sterile agar slants which had previously been prepared in sterile sugar tubes. The sugar tubes were then incubated till full growth of the fungus and they then served as stock cultures.

**Estimation of total fungal population**

Both the contaminated and uncontaminated soils were assessed for total fungal population using the colony forming unit (CFU) method (Lily et al. 2009). For that, 1 g of each soil sample was suspended into 10 mL of sterile distilled water and was aseptically serially diluted further up to 10-7 dilution. An aliquot of 0.1 mL from each diluted soil suspension was poured onto Potato Dextrose agar plates using the spread plate technique. Plates were incubated for 3 to 5 days at 30 °C. Results were recorded as CFU per gram of soil.

**Identification of fungal isolates**

Fungal genera were identified according to morphological characters and classified according to taxonomical keys published in the literature (Nelson-Smith 1973; Malloch 1997). The inoculated plates were identified on the basis of cultural (colour and colonial appearance of the fungal colony) and morphological characteristics.

Species were identified by DNA seqination method. A suitable mass of inoculum of fungal isolate was prepared carefully by removing the upper surface of the isolate, without agar medium. DNA was extracted following the technique of Al-Nasrawi (2012). Genomic DNA was isolated from fungal samples using the Chromous fungal genomic DNA isolation kit following manufacturer’s protocol (Chromous Biotech, Banglore, India). Mechanical lysis was enhanced using a Talboys High Throughput Homogenizer (Troemner, Horofare, NJ, USA) at 1600 rpm for 3 min.

DNA extracts were assessed using a Nano-drop ND-1000 Spectrophotometer (Thermo Scientific, Wilminton, DE, USA). Fungal 18S rRNA genes were PCR-amplified using 5’-GTAGTCATAGCTTGTCTC-3’ and 5’-GAAACCTTGTTACGACTT-3’ primers. Reactions were performed in 100 μL volume containing 4 μL dNTPs, 400 ng of each primer, 10X Taq DNA Polymerase Assay Buffer 10 μL, 1 μL of Taq DNA Polymerase enzyme, and 1 μL template DNA. Thermocycling conditions consisted of an initial denaturation stage of 94 °C for 5 min followed by 35 cycles of 94 °C for 30 s, 55 °C for 30 s, and 72 °C for 1 min, and a final stage of 72 °C for 5 min. PCR products obtained were Gel eluted using Chromous Gel extraction kit and sent for sequencing on an ABI 3500 XL Genetic Analyzer (Applied Biosystems Inc., Foster City CA, USA). The basic local alignment search tool-BLAST was used to classify and identify closely related fungal sequences (Al-Nasrawi 2012).

**Nucleotide sequence accession numbers**

The nucleotide sequences reported in this paper have been submitted to the NCBI, GenBank database under the accession numbers KC545846-KC545855, and KC545868-KC545875.

**Results and discussion**

**Physicochemical properties of gasoline-contaminated and uncontaminated soil**

The total volume of petroleum products used today surpasses all other chemicals of environmental and health concern. Due to the number of facilities, individuals and processes and the various ways the products are stored and handled, environmental contamination by petroleum products is potentially widespread. In this study the total petroleum hydrocarbon (TPH) concentration found in gasoline-contaminated soil was 11 500 mg kg⁻¹, in comparison to 143 mg kg⁻¹ in soil of uncontaminated garden site (Table 1). The high TPH is a result of soil contamination with gasoline through the operation of the automobile garage on the site. These concentrations of TPH create soil conditions unsatisfactory for plants and microbial growth (Dejong 1980). The negative effect of contamination can be increased by the presence of other toxic materials, such as creosol, phenols, and chlorine which might inhibit growth of hydrocarbon-oxidizing microorganisms (Ujowundu et al. 2011).

Soil texture as an important physical parameter and plays

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Uncontaminated garden soil</th>
<th>Gasoline-contaminated soil</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sand (%)</td>
<td>42.4</td>
<td>37.9</td>
</tr>
<tr>
<td>Silt (%)</td>
<td>25.2</td>
<td>26.4</td>
</tr>
<tr>
<td>Clay (%)</td>
<td>32.4</td>
<td>35.7</td>
</tr>
<tr>
<td>Texture class</td>
<td>Clay loam</td>
<td>Clay loam</td>
</tr>
<tr>
<td>Moisture content (%)</td>
<td>18.09</td>
<td>13.49</td>
</tr>
<tr>
<td>Bulk density (g mL⁻¹)</td>
<td>0.68</td>
<td>0.90</td>
</tr>
<tr>
<td>Porosity (%)</td>
<td>69.95</td>
<td>66.30</td>
</tr>
<tr>
<td>pH</td>
<td>7.2</td>
<td>5.9</td>
</tr>
<tr>
<td>Electrical conductivity (µS cm⁻¹)</td>
<td>428</td>
<td>212</td>
</tr>
<tr>
<td>Salinity (%)</td>
<td>1.4</td>
<td>0.9</td>
</tr>
<tr>
<td>Na (mg kg⁻¹)</td>
<td>90.3</td>
<td>58.0</td>
</tr>
<tr>
<td>Ca (mg kg⁻¹)</td>
<td>33.7</td>
<td>13.6</td>
</tr>
<tr>
<td>Mg (mg kg⁻¹)</td>
<td>141.3</td>
<td>74.7</td>
</tr>
<tr>
<td>Sulphate (mg kg⁻¹)</td>
<td>879</td>
<td>1781</td>
</tr>
<tr>
<td>Total petroleum hydrocarbons (mg kg⁻¹)</td>
<td>143</td>
<td>11 500</td>
</tr>
</tbody>
</table>
a very important role in establishment and development of microorganisms and plants. Gasoline-contaminated soil had a slightly higher clay and silt content, in comparison to uncontaminated garden soil (Table 1). Both soils were classified as clay-loam. The higher percentage of clay and silt in the gasoline-contaminated soil caused lower soil aeration and porosity, as compared to uncontaminated garden soil, which could affect crop productivity, yield and growth (Sztompka 1999). Also, moisture content was lower for contaminated soil. The low moisture content of contaminated soils could be due to the presence of hydrocarbons and polycyclic aromatic hydrocarbons, which can cause an increase in soil hydrophobicity, leading to a decrease in the moisture holding capacity of soil (Balks et al. 2002).

A review of existing data on the Niger Delta by Osuji (2001) showed that extremely high hydrocarbon levels in soil affect both above- and belowground flora and fauna, which are essential factors in the biogeochemical cycle, as they affect availability of plant nutrients. Among soil fertility indices, the concentrations of macronutrients N, P and K in both contaminated and uncontaminated soils were low (Fig. 1), as compared to acceptable ranges of 15 000, 2 000 and 10 000 mg kg\(^{-1}\) for N, P and K respectively, as recommended for agricultural soils (HSEENV 2004). The concentrations of extractable macronutrients P and K, as well as Ca and Mg in the gasoline-impacted soil were significantly lower than in uncontaminated garden soil. This could be due to utilization of the nutrients by resident microflora. Osuji and Nwoye (2007) suggested that it is unlikely that the oil release is directly responsible for the loss of macronutrients from soil. However, higher concentration of sulphate and nitrogen (5.8 mg kg\(^{-1}\)) in gasoline-contaminated soil, in comparison to uncontaminated soil (1.5 mg kg\(^{-1}\)), supports the findings of Ujowundu et al. (2011), who studied the biochemical and physical properties of diesel-contaminated soil in southeastern Nigeria. The increase of soil extractable nitrogen could be due to the nitrogen content of refined gasoline fuel (Slavica et al. 2003). In addition, the high amount of organic carbon and organic matter in the contaminated soil samples (Fig. 1) could be due to gasoline fuel, which is composed of hydrocarbon and polycyclic aromatic hydrocarbons (Atlas 1981).

![Image](https://via.placeholder.com/150)

**Fig. 1.** Soil fertility indices in uncontaminated garden soil and gasoline-contaminated soil of Anand, Gujarat, India. TOC, total organic carbon; TOM, total organic matter. Bars indicate SE.

The contamination resulted in the soil pH (5.9) as compared to pH 7.2 in the uncontaminated soil (Table 1). The low pH may have affected fungal growth in the contaminated soil, which was observed to be low. It has been shown that optimal activity for microbial degradation occurs at pH 7.4 while considerable inhibition can be seen both at pH 4.5 and 8.5 (Verstrate et al. 1975). Similarly, the high content of TPH may have also caused the reduced fungal biomass observed in contaminated soil.

Soil electrical conductivity is a measure of soluble salt content in the soil and is used as an overall indicator of the level of macro- and micronutrients in the soil. Conductivity was estimated as 212 μS cm\(^{-1}\) in the gasoline-contaminated soil, compared to 428 μS cm\(^{-1}\) in the uncontaminated soil (Table 1). This indicates that the gasoline contaminant affected soil structure and modified its physicochemical properties (Hawrot, Nowak 2006). The reduction in concentrations of sodium, calcium, potassium and magnesium (Table 1), which are suitable terminal electron acceptors affecting the indigenous microbial growth and

<table>
<thead>
<tr>
<th>Heavy metal</th>
<th>Permissible Limits</th>
<th>Uncontaminated soil</th>
<th>Contaminated soil</th>
<th>Enrichment factor</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>WHO/APHA</td>
<td>Indian standards</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cadmium</td>
<td>0.3</td>
<td>3–6</td>
<td>BDL</td>
<td>1.3</td>
</tr>
<tr>
<td>Cobalt</td>
<td>N/A</td>
<td>N/A</td>
<td>12.92</td>
<td>24.14</td>
</tr>
<tr>
<td>Copper</td>
<td>150</td>
<td>135–270</td>
<td>79.08</td>
<td>935.22</td>
</tr>
<tr>
<td>Iron</td>
<td>N/A</td>
<td>N/A</td>
<td>1604.4</td>
<td>2545.6</td>
</tr>
<tr>
<td>Zinc</td>
<td>500</td>
<td>300–600</td>
<td>79.08</td>
<td>241.66</td>
</tr>
<tr>
<td>Lead</td>
<td>40</td>
<td>250–500</td>
<td>BDL</td>
<td>466</td>
</tr>
</tbody>
</table>

Table 2. Concentration of heavy metals (mg kg\(^{-1}\)) in gasoline-contaminated and uncontaminated garden soil in comparison to permissible limits. BDL, below detectable limit; N/A, not applicable.
This could be the reason for the lower fungal growth in contaminated soil samples.

### Heavy metals

The results of the heavy metal analysis are presented in Table 2. Gasoline-contaminated soil contained significantly higher concentrations for all analyzed heavy metals (Cd, Co, Cu, Fe, Zn, Pb) in comparison to uncontaminated garden soil. The concentration of Cd in the gasoline-contaminated soil was above the permissible limit. Fe had elevated concentration in both contaminated and uncontaminated soils. Although iron occurs naturally in groundwater, the higher concentration of iron has negative impact for human and animals. Pb concentration found in the contaminated soil also exceeded the limit. However, Cd, Cu, Zn and Pd concentrations in the uncontaminated garden soil were within the permissible limits of WHO and APHA. According to Indian standards (Awasthi 2000; Sharma et al. 2006; Gupta et al. 2008) all the heavy metals, except Cu, in the gasoline-contaminated soil were within the permissible limits.

There was a significant difference in enrichment factor values between different heavy metals. Among the six metals estimated, the maximal enrichment was found in the case of Cu, and minimum in the case of Cd) and Pb (Table 2). An enrichment factor values above 1 indicates higher availability and distribution of metals in contaminated soil, thereby increasing the metal accumulation in plants species grown in contaminated soil (Kisku et al. 2000; Gupta et al. 2008).

### Fungal characterization

In total, nine fungal strains belonging to six genera were isolated from the two different soil samples in the present investigation. Five strains, including *Aspergillus terreus*, *Aspergillus versicolor*, *Aspergillus niger*, *Fusarium oxysporum* and *Penicillium janthinellum* were found in the uncontaminated garden soil (Table 3). From the gasoline-contaminated soil, *Aspergillus niger*, *Fusarium oxysporum*, *Cladosporium bruhnei* and *Galactomyce geotrichum* were isolated.

The fungal growth rate expressed as the number of colony forming units was $5.4 \times 10^6$ CFU g$^{-1}$ in the uncontaminated and $3.5 \times 10^6$ CFU g$^{-1}$ in gasoline-contaminated soil, i.e. 35.18% lower in contaminated soil. The lower microbial population in the contaminated soil could be a direct or indirect effect of the gasoline. Considering direct effect, it was demonstrated that the presence of C$_5$ – C$_{10}$ homologues in the petroleum fraction is inhibitory to the majority of the hydrocarbon-degrading microorganisms (Okoh 2006). These solvents tend to disrupt membrane lipid structures of microorganisms. In addition, polycyclic aromatic hydrocarbons are highly toxic to microbial cell membranes, having both carcinogenic and mutagenic activity (Amellal 2001). Indirectly, an extremely high level of TPH in contaminated soil can result in impairment of gaseous metabolism (Ujowundu 2011). This could be the reason for the lower fungal growth in contaminated soil samples.

### Table 3. Fungal taxa isolated from gasoline-contaminated (GC) and uncontaminated (UC) garden soils, identified by the length of 18S rRNA sequences by BLAST analysis

<table>
<thead>
<tr>
<th>Soil ID</th>
<th>Length</th>
<th>TOP BLAST</th>
<th>Similarity (%)</th>
<th>Class</th>
<th>Order</th>
<th>Family</th>
<th>Forward</th>
<th>Reverse</th>
</tr>
</thead>
<tbody>
<tr>
<td>UC SGX8</td>
<td>2713</td>
<td><em>Penicillium janthinellum</em></td>
<td>99%</td>
<td>Eurotiomycetes</td>
<td>Eurotiales</td>
<td>Trichocomaceae</td>
<td>KC545846</td>
<td>KC545847</td>
</tr>
<tr>
<td>PGX9</td>
<td>1687</td>
<td><em>Aspergillus niger</em> strain HKS11</td>
<td>99%</td>
<td>Eurotiomycetes</td>
<td>Eurotiales</td>
<td>Trichocomaceae</td>
<td>KC545848</td>
<td>KC545849</td>
</tr>
<tr>
<td>SGX9</td>
<td>1714</td>
<td><em>Fusarium oxysporum</em></td>
<td>100%</td>
<td>Sordariomycetes</td>
<td>Hypocreales</td>
<td>Nectriaceae</td>
<td>KC545850</td>
<td>KC545851</td>
</tr>
<tr>
<td>PGX10</td>
<td>1733</td>
<td><em>Aspergillus terreus</em></td>
<td>99%</td>
<td>Eurotiomycetes</td>
<td>Eurotiales</td>
<td>Trichocomaceae</td>
<td>KC545852</td>
<td>KC545853</td>
</tr>
<tr>
<td>PGX11</td>
<td>1733</td>
<td><em>Aspergillus versicolor</em></td>
<td>99%</td>
<td>Eurotiomycetes</td>
<td>Eurotiales</td>
<td>Trichocomaceae</td>
<td>KC545854</td>
<td>KC545855</td>
</tr>
<tr>
<td>GC PPX1</td>
<td>1687</td>
<td><em>Aspergillus niger</em> strain HKS11</td>
<td>99%</td>
<td>Eurotiomycetes</td>
<td>Eurotiales</td>
<td>Trichocomaceae</td>
<td>KC545868</td>
<td>KC545869</td>
</tr>
<tr>
<td>PXX2</td>
<td>1747</td>
<td><em>Cladosporium bruhnei</em> strain USN 11</td>
<td>100%</td>
<td>Dothideomycetes</td>
<td>Capnodiales</td>
<td>Davidiellaceae</td>
<td>KC545870</td>
<td>KC545871</td>
</tr>
<tr>
<td>SPX3</td>
<td>1767</td>
<td><em>Fusarium oxysporum</em></td>
<td>100%</td>
<td>Sordariomycetes</td>
<td>Hypocreales</td>
<td>Nectriaceae</td>
<td>KC545872</td>
<td>KC545873</td>
</tr>
<tr>
<td>SPX4</td>
<td>1668</td>
<td><em>Galactomyce geotrichum</em> strain SK15</td>
<td>100%</td>
<td>Saccharomycetes</td>
<td>Saccharomycetales</td>
<td>Dipodascaceae</td>
<td>KC545874</td>
<td>KC545875</td>
</tr>
</tbody>
</table>
exchange and retention of soil carbon dioxide (Ujowundu 2011). These conditions in the present study might have resulted in increased acidity and decreased porosity of the contaminated soil.

In conclusion, it was shown that the aged gasoline-contaminated soil has extremely high concentration of petroleum hydrocarbons, which affected soil physicochemical properties, the fungal population and caused heavy metal enrichment. High organic carbon and total nitrogen concentration, a low soil fertility index, low pH and low moisture probably decreased fungal growth in the contaminated soil. These adverse changes can affect nutrient cycling, impede nutrient uptake by plant roots and subsequently lead to reduction in crop yield. The findings of the study could be utilized for the standardization of bioremediation protocols. Growth and activity of microorganisms in such sites could be enhanced by increasing moisture content and incorporating surfactants in the soil, which may further increase bioavailability of petroleum hydrocarbons in the soil for microbial degradation.

Acknowledgements

One of the authors (Ms. Shamiyan Rahat Khan) is highly thankful to University Grants Commission (UGC) for financial support by receiving Maulana Azad Fellowship. Authors are also thankful to Sophisticated Instrumentation Centre for Advanced Research and Testing (SICART) for analysis of the samples.

References

Characterization of aged gasoline-contaminated soil site in Anand, Gujarat, India


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In Vitro Study on Assessment of Petrol, Kerosene and Diesel Degrading Potential of Indigenous Fungal Isolates From Different Petroleum Product Effected Soils

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Abstract- Application of micro organisms for effective removal of hydrocarbon contamination from soil has been considered by several workers since decontamination of polluted soil by other methods leads to production of toxic compounds and these techniques are non-economic also. Therefore, in the present study, soil samples from three highly aged petroleum contaminated sites were studied for assessment of their petrol, kerosene and diesel degrading potential by fungal isolates. Twenty native fungi species belonging to eight fungal genera were isolated from different petroleum contaminated soil samples. The identified fungal genera included Aspergillus sp., Fusarium sp., Penicillium sp., Rhizopus sp., Candida sp., Cladosporium sp., Galactomyce sp. and Sympodiomyces sp. based on 18S rRNA identification. The closely related sequences were aligned to construct a phylogenetic tree for these fungal isolates and were deposited in NCBI, GenBank under different accession numbers. Biodegradation ability of all isolates was confirmed by shake flask culture and vapour phase transfer method. The results showed that indigenous fungal isolates Aspergillus sp., Penicillium sp. and Rhizopus sp. displayed highest capability of biodegradation of the petroleum products. Hence, these fungal species can be effectively utilized for the degradation of petrol, kerosene and diesel.

Key words- Petroleum contaminated soils, Fungal isolates, 18S rRNA, Biodegradation ability, Phylogenetic analysis.

I. INTRODUCTION

Petroleum like all fossil fuels primarily consists of a complex mixture of molecules called hydrocarbons. In large concentrations, the hydrocarbon molecules that make up crude oil and petroleum products are highly toxic to many organisms, including humans [1]. The dominance of petroleum products in the world economy creates the conditions for distributing large amounts of these toxins into populated areas and ecosystems around the globe [2]. The most rational way of decontamination of the environment loaded with petroleum derivatives is an application of methods based mainly on metabolic activity of microorganisms [3]. However, single cultures of fungi have been found to be better than mixed cultures [4] and more recently, fungi have been found to be better degraders of petroleum than traditional bioremediation techniques including bacteria [5].

Although, hydrocarbon degraders may be expected to be readily isolated from a petroleum oil-associated environment, the same degree of expectation may be anticipated for microorganisms isolated from a totally unrelated environment [6]. The source of fungi for the efficient biodegradation of petroleum products has become the major concern however, the source of fungi is selected from polluted soils or waters (their natural environments), rhizoplanes, food or crops and root tubers contaminated by petroleum products.

Recently, many researchers studied the role of fungi in biodegradation process of petroleum products and the most common fungi which have been recorded as biodegraders belongs to following genera: Alternaria, Aspergillus, Candida, Cephalosporium, Cladosporium, Fusarium, Geotrichum, Gliocladium, Mucor, Paecilomyces, Penicillium, Pleurotus,
Polyporus, Rhizopus, Rhodotolura, Saccharomyces, Talaromyces, Torulopsis etc [7-9]. Therefore, in the present investigation an assessment of petrol, kerosene and diesel degrading potential of indigenous fungal isolates from different petroleum product contaminated soil sites has been carried out.

II. MATERIALS AND METHODS

Surface soil samples at 0-15 cm depth, weight of 400 g were collected from three different sites which were contaminated with petrol, kerosene and diesel separately along with non-contaminated garden soil. Samples were made from 3-4 random locations per site, mixed and transferred into sterile bottles using sterile spatula for microbiological quality determination and stored in ice box to avoid contamination. In the lab, stones and other unwanted soil debris were removed by using 2.5 mm sieve, one gram of each sorted soil sample was homogenously mixed with 1 drop (0.1 ml) of Tween 80. 1.0 g of homogenized, 2 mm sieved soil sample was aseptically transferred, using a flame-sterilized steel spatula, into a sterile test tube containing 9.0 mL of sterile distilled water. This gave $10^{-1}$ dilution and subsequently, three-fold ($10^{3}$) serial solutions were prepared from the $10^{-1}$ dilution. 1 ml of dilution was poured on Potato Dextrose Agar (PDA) plates and Soubraurd Dextrose Agar (SDA) plates, Streptomycin (500 mg/l) as antibiotic inhibit bacterial growth was added to the media after sterilization process [10-11]. Afterwards, the plates were incubated at a temperature of 28 – 31°C for 48hours or more depending on the rate of growth. To obtain pure cultures of the fungal isolates, fungal cultures were aseptically subcultured into fresh PDA and SDA plates and incubated until the fungus begins to sporulate followed by subsequent sub culturing to get pure cultures consisting of only one type of fungus isolates. A part of the pure culture was then aseptically transferred into sterile agar slants which had previously been prepared in sterile sugar tubes. The sugar tubes are then incubated till full growth of the fungus and they then serve as stock cultures.

Bushnell-Haas broth medium was used for the primary screening test which composed of: MgSO$_4$ (0.2 g/l), CaCl$_2$ (0.02 g/l), KH$_2$PO$_4$ (1 g/l), K$_2$HPO$_4$ (1 g/l), FeCl$_2$ (0.05 g/l) and NH$_4$NO$_3$ (1 g/l). Tween 80 (0.1%), redox reagent (2% 2, 6-dichlorophenol indophenols) and petroleum products (1% of each Kerosene, Petrol, Diesel) were administered into the broth [12-13]. For vapour phase transfer method, mineral salt medium (MSM) was prepared according to modified Mills et al. (1978). The composition of the medium was NaCl (10.0 g), MgSO$_4$.7H$_2$O (0.42 g), KCl (0.29 g), KH$_2$PO$_4$ (0.83 g), Na$_2$HPO$_4$ (1.25 g), NaNO$_3$ (0.42 g), agar (20 g), distilled water (1 L) and pH of 7.2.

III. IDENTIFICATION OF FUNGAL ISOLATES

Fungal genera were identified according to morphological characters [14]. The inoculated plates were identified on the basis of cultural (colour and colonial appearance of fungal colony) and morphological characteristics. Species were identified by using DNA sequence method. A suitable mass of inoculum of fungal isolate was prepared with carefully removing the upper surface of the isolate without agar medium. The DNA extraction technique 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 [12], [15]. Genomic DNA was isolated from the fungal sample using Chromous fungal genomic DNA isolation kit following manufacturer’s protocol (Chromous Biotech., Bangalore, India). Approximately 100 mg of fungal hyphae were scraped off from petridishes and transferred to bead tubes provided in the kit. Mechanical lysis was enhanced using a Talboys High Throughput Homogenizer (Troemner, Thorofare, NJ, USA) at 1600 rpm for 3 minutes. DNA extracts were assessed using a Nano-drop ND-1000 Spectrophotometer (Thermo Scientific, Wilmington, DE, USA). Fungal 18S rRNA genes were PCR amplified using 5’- GTAGTCAATGTGTTGTC - 3’ and 5’- GAAACCTTGTTACGACTT -3’ primers. Reactions were performed in 100 µl volumes containing 4 µl dNTPs, 400ng of each primer, 10X Taq DNA Polymerase Assay Buffer 10 µl, 1 µl of Taq DNA Polymerase enzyme, and 1 µl template DNA. Thermo cycling conditions consisted of an initial denaturation stage of 94°C for 5 minutes followed by 35...
cycles of 94°C for 30 seconds, 55°C for 30 seconds, and 72°C for 1 min, and a final stage of 72°C for 5 minutes.

PCR products obtained were Gel eluted using Chromous Gel extraction kit and sent for sequencing on an ABI 3500 XL Genetic Analyzer (Applied Biosystems Inc., Foster City CA, USA). The basic local alignment search tool-BLAST was used to classify and identify closely related fungal sequences [12].

Nucleotide Sequence Accession Numbers

The nucleotide sequences reported in this paper have been submitted to the NCBI, GenBank database under the following accession numbers KC545836 to KC545875 (Table I).

Primary step for confirming biodegradation potential of fungal isolates (Shaking flask method).

The biodegradability of isolated fungi was verified using the modified technique based on the redox indicator 2, 6-dichlorophenol indophenol (DCPIP) [13], [16]. Two plugs from 7 days old fungal isolates (1 cm$$^2$$ for each plug) were picked from the peripheral area of petri-dish and transferred carefully into 50 ml Bacto Bushnell-Haas broth medium using 250 ml conical flask. 0.1% (v/v) Tween 80 and 1% (v/v) petrol, kerosene and diesel and 0.008 mg/50 ml of redox indicator as a powder were added to the Bacto Bushnell-Haas broth medium. All flasks incubated in room temperature using a shaker with 180 rpm for seven days. Five mL of the aliquots were collected daily from each flask and the absorbance was noted on spectrophotometer at 600 nm wavelength. Change in color of inoculated media in the flasks from deep blue to colorless indicates the ability of fungi to biodegrade petroleum products like petrol, kerosene and diesel.

Final confirmation for biodegradation potential of fungal isolates (Vapor phase transfer method)

Fungal isolates showing better performance (fastest colour change) in primary step were further tested by vapor phase transfer petroleum products utilization test [13], [17]. This test was carried out for the confirmatory identification of actual petroleum products-utilizing moulds. For petroleum product utilization test, mineral salt medium (MSM) was prepared according to modified media of [18] however, petroleum products were made available to the inoculants via vapor phase transfer instead of media. Putative -utilizing fungal isolates in first step of confirmation were streaked on plates of agar medium (one isolate per plate). Inner side of petridish was covered with a sterile filter paper (Whatman No. 1) saturated with filter-sterilized petrol, kerosene and diesel. The main aim was to supply petroleum products (source of hydrocarbons) as sole source of carbon and energy for the growth of the fungi on the mineral salts agar medium surface through vapor phase transfer. All the plates were inverted and incubated at 27°C for 7-14 days [19]. Colonial development of different fungi appearing on the mineral salts agar medium was confirmed as petroleum products utilisers.

IV. RESULTS

Results revealed that 20 fungal strains belonging to a total of eight genera were isolated from the four different soils during the present investigation. These include Aspergillus (A. terreus, A. versicolor, A. niger, A. fumigatus); Fusarium oxysporum; Cladosporium bruhnei, Penicillium (P. janthinellum, P. decumbens), Candida tropicalis, Galactomyces geotrichum., Sympodiomycopsis paphiopedili and Rhizopus oryzae (Table I).

Biodegradation potential of Fungal isolates

During primary step for confirming biodegradation potentials of fungal isolates, out of the total identified eight genera, three genera such as Aspergillus sp.; Candida sp.; and Cladosporium sp. were found Kerosene-utilizers. Two genera viz.: Fusarium sp. and Galactomyces sp.; were effective petrol degraders and four genera viz., Rhizopus sp.; Aspergillus sp.; Penicillium sp.; and Sympodiomycopsis sp. showed efficiency for diesel degradation (Fig. 1). These isolates produced a colour change in the Bacto
Bushnell-Haas broth medium. The absorbance of broth medium changed according to degradation capacity rate and extent in each flask. Almost total colour change (blue to colourless) was also observed in some flasks while in other flasks colour changes up to some extent. Among the better performing seven isolates, *Penicillium janthinellum*, *Aspergillus terreus*, *Rhizopus oryzae*, and *Penicillium decumbens* displayed the fastest onset colour change (decrease in absorbance of broth medium) and hence, highest capability of biodegradation (Fig. 2). However, there is no colour change in control which refers the media without fungal inoculums. These figures show the decrease in the absorbance of the Bacto Bushnell Haas broth medium after the fungal inoculation.

For confirmatory identification of actual petroleum products-utilizing moulds, all fungal isolates which were taken in primary step were further tested by vapour phase transfer petrol, kerosene and diesel utilization test. After 10 days of incubation, the six fungal isolates i.e., *Aspergillus terreus*, *Fusarium oxysporum*, *Rhizopus oryzae*, *Aspergillus niger*, *Penicillium decumbens*, *Penicillium janthinellum* showed better growth during petroleum products treatment by vapour phase transfer method. These further prove petrol, kerosene and diesel biodegradation potentials by these fungal isolates (Fig. 3).

![Graphs showing absorbance changes in different media](image-url)

Fig. 1 Variation in the absorbance of Bacto Bushnell Hass broth medium by different fungal isolates (abbreviations in this fig. expanded in Table I)
V. DISCUSSION

The partial 18S rRNA gene sequence described in this paper revealed a high level of conservation and relatively late onset of divergence in the fungal isolates from contaminated and non-contaminated soils. It appears that the morphological functional and petroleum product adaption of the fungal isolates is not mirrored by the molecular evolution of these organisms which is supported by the findings of [20] while studying rRNA sequence and evolutionary relationships among toxic and nontoxic cyanobacteria.

During primary step for confirming biodegradation potentials of fungal isolates, the ability of these isolates to produce a colour change in the Bacto Bushnell-Haas broth medium is presumably due to the reduction of the indicator by the oxidized products of hydrocarbon degradation. The total colour change (blue to colourless) supports the fact that the isolates are potential hydrocarbon oxidizers. Among better performing seven isolates which produced significant colour change species Penicillium janthinellum, Aspergillus terreus, Rhizopus oryzae and Penicillium decumbens displayed the fastest onset color disappearance (decrease in absorbance of broth medium) and hence, highest capability of biodegradation. The high rate of petroleum products (hydrocarbon) degradation by the three fungi could emanate from their massive growth and enzyme production responses during their growth phases. This could be supported by the findings of [21], who suggested that extracellular ligninolytic enzymes of white rot fungi are produced in response to their growth phases. Besides, the utilization of 0.1% of Tween 80 during the assay and the implication of these three organisms in hydrocarbon degradation from our results is similar to the findings of [22]. In vapour phase transfer petroleum product utilization test after 10 days of incubation for confirmatory
identification of actual petroleum products-utilizing moulds, the six fungal isolates i.e., *Aspergillus terreus*, *Fusarium oxysporum*, *Rhizopus oryzae*, *Aspergillus niger*, *Penicillium decumbens* and *Penicillium janthinellum* showed better growth during petroleum products treatment by vapour phase transfer method, which further confirming petrol, kerosene and diesel utilization and biodegradation potentials of these fungal isolates, and corroborated with the findings of [13] while studying the diesel degrading potential of fungal isolates from sludge contaminated soils of petroleum refinery, Haryana, India.

An interesting findings generated in this work show that an increase in rates of fungal growth in the media containing petroleum products as compared with media without petroleum products, this might be due to the fact that the fungi use petroleum products as a substrate for their survival growth and using extra cellular enzymes to break down the recalcitrant hydrocarbon molecules, by dismantling the long chains of hydrogen and carbon, thereby, converting petroleum into simpler forms or products that can be absorbed for the growth, development and nutrition of the fungi.

VI. CONCLUSION

In this study, we observed that higher biodegradation efficiency was encountered by *Penicillium janthinellum*, *Aspergillus terreus*, *Rhizopus oryzae* and *Penicillium decumbens*, providing these fungi to be better petroleum product degraders. Thus, they can be effectively utilized for the degradation of petrol, kerosene and diesel for biodegradation of oil polluted or contaminated soils especially those located within the vicinity of the petroleum processing and disposal sites.

VII. ACKNOWLEDGEMENTS

One of the author’s Ms. Shamiyan Rahat Khan is highly thankful to University Grants Commission (UGC) for financial support. Authors are also thankful to Sophisticated Instrumentation Centre for Advanced Research and Testing (SICART) for providing the facilities.

VIII. REFERENCES


Table I
Fungal taxa isolated from different petroleum product contaminated and non-contaminated soils identified by length of DNA sequences by BLAST analysis

<table>
<thead>
<tr>
<th>Soil ID</th>
<th>Sequence ID</th>
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<th>% Similarity</th>
<th>Class</th>
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Abstract - The potential of biodegradation of soils contaminated with diesel was evaluated ex-situ with the objective of comparing the effects of three different fungal isolates *P. janthinellum*, *P. decumbens* and *A. terreus*. The study dealt with the biodegradation of artificially contaminated diesel soils by 5%, 10% and 15% (w/w). The experiment was performed by *ex-situ* large scale tray method using 12 plastic trays 6” X 3” X 1” in each containing 60 kg contaminated soil. After 8 weeks of inoculation of the fungal isolates, *P. janthinellum* found to be potential compared to the other two and displayed the highest diesel degradative capacity resulting 82%, 70% and 62% degradation at 5, 10 and 15% diesel contaminated soils after 60 days. Moreover, the total fungal population declined during contamination whereas increased as a function of time during degradation. A first-order kinetic model equation showed that the specific biodegradation rate constant (*k*) value was 0.0285 day⁻¹ for 5% diesel contamination by *P. janthinellum* treatment strategy which was comparatively higher than the values for other two organisms tested. Thus, the degree of effectiveness of these bioremediation strategies in the soils contaminated with diesel is in the following order: *P. janthinellum* > *P. decumbens* > *A. terreus*.

Keywords— Diesel, *Ex-situ* large scale tray method, first-order kinetic, fungal isolates.

I. INTRODUCTION

Petroleum and associated products have a high worldwide demand for as a source of energy, which has resulted in increased oil exploration, production, and refining, and has consequently led to a high level of environmental pollution. Oil spills due to blow outs, leakage from underground storage tanks, tanker accidents, sabotage and accidental rupture of pipelines as well as dumping of waste petroleum products introduce nonorganic, carcinogenic and growth-inhibiting chemicals present in the crude oil and their toxicity to microorganism and man is well known [1]. Also, it results in significant decline in the quality of soil and makes it unfit for use [2] as well as affects plants and animal [3]. Crude oil is an extremely complex mixture of aliphatic and aromatic hydrocarbons, including volatile components of gasoline, petrol, kerosene, diesel, lubricant oil, and solid asphaltene residues; however, the kerosene and diesel fractions pose the greatest pollution threats and problems owing to their excessive use [4].

Bioremediation technology is gaining prominence due to its simplicity, environmental friendliness, higher efficiency, and cost-
effectiveness in comparison to other several remediation technologies available for petroleum hydrocarbons, removal from the soil and groundwater, technologies [5]. The actual mechanism that breaks down these petroleum products is biodegradation mediated by microorganisms [6]. Crude oil as well as other commercial hydrocarbons could be sparsely/ biodegradable in soils [2]; however, differences in the extent of biodegradation depending on soil and hydrocarbon source type, concentration of total hydrocarbons, and oxygen and nutrient availability have been reported by Bento et al. [7]. Studies have been conducted on petroleum hydrocarbons degradation by microbial activities [8], but much work has not been emphasized on the biodegradation of some commercial petroleum products such as kerosene and diesel. Shamiyan et al. [9, 10] carried out soil characterization and isolated certain petroleum degrading fungal strains from aged petroleum affected natural soils. However, there is little or no information available in the literature on the bioremediation of artificially contaminated diesel soils Ex-situ. Therefore in the present investigation an attempt has been made to apply the first-order kinetic model equation during Ex-situ studies on degradation of kerosene and diesel contaminated soils [11].

II. MATERIALS AND METHODOLOGY

A) Fungal Isolates

Fungal strains P. decumbens PDX7, P. janthinellum SDX7 and A. terreus PKX4 were isolated and screened for potential diesel degradation from aged petroleum hydrocarbon affected soil sites of Anand, Gujarat, India and identified based on morphological, molecular (18S rRNA) methods and the sequences were submitted to NCBI gene bank (Table 1) [9, 10].

<table>
<thead>
<tr>
<th>Sequence ID</th>
<th>TOP BLAST</th>
<th>Similarity</th>
<th>NCBI GenBank accessions numbers</th>
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<td>Aspergillus terreus</td>
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<td>KC545840, KC545841</td>
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<tr>
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<td>Penicillium janthinellum</td>
<td>100</td>
<td>KC545842, KC545843</td>
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</tbody>
</table>

B) Preparation of carrier based inoculums.

Hundred milliliters of axenic and exponentially growing dense cultures (10⁷ Colony Forming Unit fungal isolates mL⁻¹) were prepared in sterile Mineral Salt Media (MSM) and inoculated on 1.0 kg of broken autoclaved rice grains in autoclavable plastic bags under aseptic conditions. The fungal isolates were allowed separately to sporulate on autoclaved broken rice grains in dark at 30±2°C for ten days. This carrier based inoculum was used for the Ex-situ studies on degradation of kerosene and diesel contaminated soils [11].

C) Ex-situ Experimental set up

Soil sample was randomly collected with a Dutch auger at a depth of 15 cm from an uncontaminated agricultural field in Anand, Gujarat, India, brought to the laboratory and were homogenized, dried, sieved, passed through a 2-mm mesh screen, and stored in a polythene bag at room temperature. Soil samples were analyzed three times i.e. initial execution of the work, after contamination with petroleum products and at the end of the experiment for particle size by the international pipette method [12], bulk density by metal core sampler method [13], porosity and moisture content according to Maiti [14]. The pH and electric conductivity of the soil samples were determined in soil/water (1:1, v/v) suspension by pH meter and conductivity meter (Sension 5, HACH, USA). Total organic carbon (TOC) concentration was determined with the titration method [15]. Total nitrogen was measured by...
Kjeldahl digestion (Gerhardt, Turbotherm, Germany) and steam distillation method [16].

The soil of 800 kg was taken and divided into 4 heaps of 200 Kg each and were artificially contaminated with 5, 10 and 15% of diesel separately and remaining heap were used as control. The soils were thrice contaminated with diesel at an interval of 3 days up to 9 days. They were then divided into 12 plastic trays each containing 60 Kg of treated soils. One Kilogram of carrier based fungal inoculum was added in each tray and six control trays were maintained simultaneously without the carrier based inoculums. The water (moisture) content of soil in each tray was adjusted every week by addition of sterile distilled water to a moisture holding capacity of 50%. In order to avoid anaerobic conditions, contents of the trays were aerated by tailings every 3 days. Samples were excavated every fifteen days and analyzed for residual diesel and fungal population. The whole experiments were carried out in triplicates [17].

D) Biodegradation activity and Total Petroleum Hydrocarbons (TPH)

The determination of the bio-degradation activity of the three fungal isolates was carried out by collecting 2 gm of soil samples at four places in each tray and combined to form a composite sample for further analysis. The residual petroleum hydrocarbon was recovered by 1:1 of Chloroform: Dichloromethane [18]. Analysis of the biodegradation activity was made using a computerized capillary gas chromatography with flame ionized detector (GC-FID, Perkin Elmer-Auto System) equipped with HP 3390A Integrator, split injector (split ratio 20/1) and flame ionization detector set at 300°C. The carrier gas was nitrogen at flow rate of 1.5 mL/min. The column used was polydimethylsiloxane (length 30 m, internal diameter 0.32 mm, film thickness 0.25 μm) and the temperature was programmed to increase from 60 to 320°C at 4°C min⁻¹. The total petroleum hydrocarbon (TPH) degradation by the fungal isolates was calculated according to the following equation.

\[ \% B = \left( \frac{100 \times (TPHC - TPHS)}{TPHC} \right) \]

Where B is biodegradation, TPHC: Total petroleum hydrocarbon of Control TPHS: Total petroleum hydrocarbon of Sample.

E) Statistical analysis

Student’s t-test was used to study the significant differences between treatments for aromatic and aliphatic hydrocarbon degradation using KY plot (2.0 beta).

F) Kinetics of Degradation

The rate of petroleum hydrocarbon biodegradation was measured by the application of first-order kinetic model equation (Equation 1) to the biodegradation data, which has generally been used for biodegradation of petroleum hydrocarbons in soil [19, 20]. Applying the biodegradation data obtained for each soil treatment to first-order kinetic model using the linear regression routine of MATLAB 7.0 software package made possible further evaluation and comparison of the applicability of the various biodegradation treatment strategies.

\[ S = S_o e^{-kt} \]

Taking the natural logarithm of Equation 1

\[ \ln(S/S_o) = -kt \]

Where \( S, S_o, k, \) and \( t \) are the initial petroleum hydrocarbon concentration, final petroleum hydrocarbon concentration, specific degradation rate constant, and time, respectively.

G) Estimation of total fungal population

Both the control and contaminated soils were assessed for total fungal population using the colony forming unit (CFU) method [21]. For that, 1 g of each soil sample was suspended into
10 mL of sterile distilled water and was aseptically serially diluted further up to $10^{-7}$ dilution. An aliquot of 0.1 mL from each diluted soil suspension was poured onto Potato Dextrose Agar plates using the spread plate technique. Plates were incubated for 3 to 5 days at 30 °C and the results were recorded as Log CFU.

III. RESULTS AND DISCUSSION

A) Soil Physico-chemical characterization.

The soil physico-chemical characterization of uncontaminated, post contaminated and post degraded diesel displayed low porosity and high bulk density. Soil exhibited maximum bulk density 0.93 g mL$^{-1}$ due to the presence of high concentration of hydrocarbons which cause an increase in soil hydrophobicity and therefore leading to decrease in the moisture holding capacity of soil [22].

Our results showed highest value 7.78% of total nitrogen in diesel contaminated soil and lowest 2.45% in uncontaminated soils which corroborated with the findings of Ujowundu et al. [23], who studied the biochemical and physical characterization of diesel contaminated soil in south-eastern Nigeria. The increase could be derived from the nitrogen content of the refined petroleum fuels [24]. High amount of organic carbon 5.19% in diesel contaminated soil samples could be due to the high carbon content in the petroleum product. The artificial contamination resulted in the acidic pH values which were found to restore to alkaline pH during the passage of the degradation process. The low pH may have caused a reduction in the fungal population growth in the contaminated soils. A study by Verstrate et al. [25] was conducted on optimal activity for microbial degradation at a pH of 7.4 and considerable inhibition at pH 4.5 and above 8.5. A reduction of nutrient content of the contaminated soils might have caused lowest conductivity value 217 µS cm$^{-1}$ followed by 235 µS cm$^{-1}$ in diesel and kerosene, respectively where the values heaved up as the degradation progressed as a function of time (Table 2). According to Hawrot and Nowak [26] the change in the physicochemical properties of soils could be due to the contamination of diesel.

Table 2: Physicochemical properties of uncontaminated, Post contamination and post degradation of kerosene and diesel soil by the fungal isolates.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Uncontaminated soil</th>
<th>Post Contamination Diesel</th>
<th>Post Degradation Diesel</th>
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<td>Sand (%)</td>
<td>42.4±1.6</td>
<td>40.4±1.8</td>
<td>41.8±1.1</td>
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<td>Silt (%)</td>
<td>25.2±0.8</td>
<td>26.2±1.2</td>
<td>25.2±0.9</td>
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<td>Clay (%)</td>
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<td>Porosity (%)</td>
<td>69.95±2</td>
<td>62.2±2.8</td>
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<td>Electrical conductivity (µS cm$^{-1}$)</td>
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<td>Salinity (%)</td>
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<td>Total Organic Carbon (%)</td>
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<td>Sulphate (mg kg$^{-1}$)</td>
<td>879±6</td>
<td>1350±30</td>
<td>920±14</td>
</tr>
</tbody>
</table>
B) Diesel Degradation

The soils contaminated by 5%, 10% and 15% diesel contained hydrocarbons ranging from 11,305 mg/kg, 19,950 mg/kg and 45,058 mg/kg respectively. The highest diesel degradative capacity of *P. janthinellum* SDX7 exhibiting 82% for 5%, followed by 70% and 62% for 10 and 15% diesel contaminated soils respectively after 60 days. Similarly about 75%, 61% and 54% degradation of hydrocarbons by *P. decumbens* PDX7 was encountered at 5, 10 and 15% diesel contaminated soils. However, *A. terreus* PKX4 degraded diesel hydrocarbons by 68%, 57% and 49% for the three concentrations (Figure 1). The percent of Aromatic/ Aliphatic hydrocarbons >0.8 indicate the efficiency in removing the aromatic hydrocarbons of diesel by *P. janthinellum* SDX7 is more as compared to the other two fungal isolates (Table 3). The chromatographic images depict the smaller peaks of naphthenes and aromatics corresponding to carbon atoms (C10 to C22) between the sharp and tallest peaks of the n-alkanes. *P. janthinellum* SDX7 degraded a maximum of 69% and 65% of aliphatic and aromatic fraction of 5% diesel (t-test, P≥0.05) where the results are very well substantiated with the findings of Manee et al. [27] who proved the higher efficiency of the fungal isolates to degrade the aliphatic fractions compared to the aromatic fractions during crude oil degradation. The biodegradation of diesel was confirmed by the reduction in the peak area under the aliphatic and aromatic hydrocarbon of the chromatograms when compared to that of the abiotic control which reveals the removal of these diesel hydrocarbons (Figure 2a,b,c,d). The chromatograms showed n-alkane fractions are easily degraded as a function of time and completely eliminating C10 to C15 which is corroborated with the findings of Dussan and Numpaque [28] who stated the degradation of diesel fuel by certain bacterial species. However, the rate of degradation was lower for branched alkanes followed by n-alkyl aromatics, cyclic alkanes and polynuclear aromatics by the tested fungal isolates.

The results indicate that the ‘k’ value was maximum 0.0285 day⁻¹ for *P. janthinellum* SDX7, while the the lowest ‘k’ value (0.0112 day⁻¹) was registered for *A. terreus* PKX4 during diesel degradation (Table 4). Therefore, value of the kinetic parameter showed that the degree of effectiveness of these bioremediation strategies in the cleanup of soil contaminated with diesel using fungal isolates is in the following order: *P. janthinellum* SDX7> *P. decumbens* PDX7> *A. terreus* PKX. Similar results were reported by Dadrasnia and Agamuthu [29] who studied the first order kinetics to compare 5% diesel degradation by using different organic wastes, where use of soy cakes were proved to be the best degradation strategy. The study revealed *P. janthinellum* SDX7 was found to be more efficient than the other two species. Mancera et al. [30] also reported hydrocarbon degradation by *Penicillium* sp. and *Aspergillus* sp.

Figure 1: Diesel degradation by the three fungal isolates as a function of time (SDX7-5, SDX7-10 and SDX7-15% - *P. janthinellum* treated with 5, 10 and 15% diesel; PDX7-5, PDX7-10 and PDX7-15% - *P. decumbens* treated with 5, 10 and 15% diesel and PKX4-5, PKX4-10 and PKX4-15% - *A. terreus* treated with 5, 10 and 15% diesel).
Figure 2: GC-FID chromatograms of Diesel degradation by most potential fungal isolate *P. janthinellum* SDX7 after 60 days (a- Control, b- 5% diesel enrichment, c- 10% diesel enrichment and d- 15% diesel enrichment).

Table 3: Diesel hydrocarbon degradation after 60 days by the three fungal isolates

<table>
<thead>
<tr>
<th>Fungal isolate</th>
<th>Aliphatic hydrocarbons (%)</th>
<th>Aromatic hydrocarbons (%)</th>
<th>Aromatic/Aliphatic hydrocarbons (%)</th>
<th>Student ‘t’ test</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>5%</td>
<td>10%</td>
<td>15%</td>
<td>5%</td>
</tr>
<tr>
<td><em>P. janthinellum</em> SDX7</td>
<td>68±2</td>
<td>60±1</td>
<td>52±2</td>
<td>65±4</td>
</tr>
<tr>
<td><em>P. decumbens</em> PDX7</td>
<td>65±3</td>
<td>52±1.5</td>
<td>40±2</td>
<td>52±5</td>
</tr>
<tr>
<td><em>A. terreus</em> PKX4</td>
<td>55±4</td>
<td>45±2</td>
<td>32±4</td>
<td>40±4</td>
</tr>
</tbody>
</table>

S- Significant (P≤0.05); N.S- Non Significant (P≥0.05)

Table 4: Specific Degradation Rate Constant (k) and Correlation Coefficient (R²) during Diesel Degradation.

<table>
<thead>
<tr>
<th>Soil treatments</th>
<th>Diesel Degradation</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>k (day⁻¹)</td>
<td>R²</td>
<td></td>
</tr>
<tr>
<td>SDX7-5%</td>
<td>0.0285</td>
<td>0.9184</td>
<td></td>
</tr>
<tr>
<td>SDX7-10%</td>
<td>0.0200</td>
<td>0.907</td>
<td></td>
</tr>
<tr>
<td>SDX7-15%</td>
<td>0.0161</td>
<td>0.8875</td>
<td></td>
</tr>
<tr>
<td>PDX7-5%</td>
<td>0.0231</td>
<td>0.8833</td>
<td></td>
</tr>
<tr>
<td>PDX7-10%</td>
<td>0.0156</td>
<td>0.894</td>
<td></td>
</tr>
<tr>
<td>PDX7-15%</td>
<td>0.0129</td>
<td>0.8961</td>
<td></td>
</tr>
<tr>
<td>PKX4-5%</td>
<td>0.0189</td>
<td>0.8778</td>
<td></td>
</tr>
<tr>
<td>PKX4-10%</td>
<td>0.0140</td>
<td>0.8848</td>
<td></td>
</tr>
<tr>
<td>PKX4-15%</td>
<td>0.0112</td>
<td>0.8979</td>
<td></td>
</tr>
</tbody>
</table>

(SDX7-5, SDX7-10 and SDX7-15% - *P. janthinellum* treated with 5, 10 and 15% diesel; PDX7-5, PDX7-10 and PDX7-15% - *P. decumbens* treated with 5, 10 and 15% diesel and PKX4-5, PKX4-10 and PKX4-15% - *A. terreus* treated with 5, 10 and 15% diesel)

C) Fungal Population Study

The fungal growth rate expressed in colony forming units reviled that there was a sharp depletion in the fungal population at the time of the contamination of the soil with diesel. The diesel contaminated soil encountered 1.1X 10⁵ CFU/g in 15 % contamination. Moreover Okoh...
(2006) demonstrated the presence of C5 – C10 in the petroleum fraction shown to be inhibitory to the majority of hydrocarbon degraders which tend to disrupt phospholipids and lipoprotein membrane structures of microorganisms. The lower microbial population in the contaminated soils could be due to the effect of the petroleum hydrocarbons which lead to impairment of gaseous exchange and retention of soil carbon dioxide [23]. However, the Log CFU/g values were found to increase along with the degradation of diesel displaying highest values upto 6.6X 10^6 CFU/g for the treatments after 15 days (Figure 3). These results were in agreement with the findings of Mulkins-Philip and Stewart [32] and Obire [33] who emphasized an increase in the microbial population due to the decline in the xenobiotic compounds and attenuation of the indigenous species.

Figure 5: Effect of diesel degradation on fungal population. (SDX7-5, SDX7-10 and SDX7-15% - *P. janthellum* treated with 5, 10 and 15% diesel; PDX7-5, PDX7-10 and PDX7-15% - *P. decumbens* treated with 5, 10 and 15% diesel and PKX4-5, PKX4-10 and PKX4-15% - *A. terreus* treated with 5, 10 and 15% diesel)

IV. CONCLUSION

In present study revealed the rate of biodegradation of diesel in artificially contaminated soils could be enhanced by the addition of carrier based inoculum of fungal isolates by *Ex-situ* tray methods. The three fungal isolates used in the study exhibited high degree of degrading potential in the order *P. janthellum* SDX7 > *P. decumbens* PDX7 > *A. terreus* PKX4. Thus, the use of fungal isolate *P. janthellum* SDX7 for the degradation of petroleum hydrocarbons in diesel contaminated soils may be recommended for *Ex Situ* bioremediation strategies for environmental cleanup.

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V. REFERENCES


