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Research article

Role of nutrients and illuminance in predicting the fate of fungal mediated petroleum hydrocarbon degradation and biomass production

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ABSTRACT

Biodegradation and biomass production are affected by numerous environmental factors including pH, oxygen availability and presence of pollutants. The present study, for the first time, elucidated the effects of nutrients and light on mycodegradation of petroleum hydrocarbons in diesel oil. Seven fungal strains (*Aspergillus terreus* FA3, *Aspergillus niger* FA5, *Aspergillus terreus* FA6, *Penicillium chrysogenum* FP4, *Aspergillus terreus* FP6, *Aspergillus flavus* FP10, and *Candida* sp. FG1) were used for hydrocarbon degradation under static conditions, in four combinations of nutrient media and illuminance for 45 days. Highest degradation was achieved by *Aspergillus terreus* FA6 and *Candida* sp. FG1 under both conditions of light and dark, with nutrient deprived HAF (Hydrocarbon adopted fungi) broth. Under HAF/Dark diesel oil degradation by FA6 and FG1 was 87.3% and 84.3% respectively, while under HAF/Light both FA6 and FG1 performed 84.3% biodegradation. The highest biomass was produced by *Aspergillus flavus* FP10 in PDB (Potato dextrose broth)/Dark (109.3 mg). Fungal degradation of petroleum hydrocarbons was negatively affected by the presence of other simpler-to-degrade carbon sources in the medium. The biomass production was enhanced by improved nutrient availability and diminished by illuminance.
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1. Introduction

The presence of petro-chemicals in environment poses serious hazards to human health due to their toxic, mutagenic, carcinogenic effects and capability of accumulation in food chain (Adeniyi and Afolabi, 2002; Baehr and Corapcioglu, 1987; Obuekwe et al., 2009). Biodegradation, i.e. the use of microorganisms proficient in degrading noxious compounds, have become an attractive technology as compared to many available physico-chemical methods in terms of energy efficiency, financial expenses, labour requirements and use of chemical reagents for petroleum degradation (Hamdi et al., 2007; Maila and Cloete, 2004).

Fungi possess vast potential for biodegradation of petroleum hydrocarbons (Harms et al., 2011). The potential of fungi to remove organic pollutants is extended to hazardous metals, metalloids and radionuclides (Atlas, 1981; Harms et al., 2011; Pinedo-Rivilla et al., 2009). They are known to perform chemical modification and subsequently enhanced bioavailability of pollutants. Other merits of using fungi in biodegradation include mycelia growth and extension, catabolic enzymes with low substrate specificity, and less dependence on pollutants to be utilized as sole growth substrate. In addition, fungi possess different non-specific enzymes that enhance degradation because of their substrate non-specificity. Different enzymes, including cytochrome P450 mono-oxygenases, laccases, miscellaneous transferases, nitroreductase and peroxidases (Cerniglia and Sutherland, 2010; Harms et al., 2011), can be used as key indicators of fungal activities in contaminated soil. Fungi can also be used in consortia with bacteria, other fungi and with plants as endo-or-ectomycorrhizal associations for biodegradation of hydrocarbons (Harms et al., 2011).

Biodegradation of hydrocarbons is mainly affected by nutrient availability, temperature, pH, aeration, light flux and potent microbial population. These factors influence the number,

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composition and activity of hydrocarbon degrading microbial community in the environment (Briggs, 2014; Vasudevan and Rajaram, 2001). The amendment of nutrients in soil to improve degradation of petroleum hydrocarbons by microorganism has been assessed (Chaineau et al., 2005; Das and Chandran, 2010; Tyagi et al., 2011; Xu and Obbard, 2004), but there is a lack of research work in determining the effect of light in combination with nutrients on hydrocarbon degradation and biomass production. In the present study effect of light flux (illuminance) and type of nutrient media on fungal mediated hydrocarbon degradation, fungal biomass production, and the correlation between biodegradation and biomass production were investigated. For this purpose diesel oil was selected, as it offers a complex mixture of hydrocarbons containing cycloparaffins, paraffins, aromatic and olefinic hydrocarbons with carbon numbers predominantly in the range of C9 to C25 (IARC, 1988). To the best of our knowledge the role of light along with nutrients in mediating the fungal biodegradation of hydrocarbons is yet to be explored, and there is a wide open area for investigation of fungal photo-physiology.

2. Materials and methods

2.1. Soil sample collection and isolation of fungi

Soil samples for fungal isolations were collected from 3 sites, lubricant industry located in Hattar industrial estate Haripur, bioremediation site of oil refinery located in Rawalpindi and automobile garage located in Islamabad, Pakistan. The surface soil samples were taken at the depth of 5–15 cm with the help of sterile spatula, packed in polyethylene bags, immediately stored in ice box, just after excavation form sampling site to avoid any deterioration in the microhabitat, brought to Environmental Microbiology and Bioremediation laboratory, Quaid-i-Azam University, Islamabad and were stored at 4 °C for further analysis.

Fungi were isolated on Potato dextrose agar (PDA) containing (g/L) of potato infusion, 4; D (±) glucose, 20; and agar, 15, amended with 1% filter-sterilized diesel as a carbon source. PDA also contained streptomycin 0.03 g/L to avoid bacterial growth. Soil suspensions were serially diluted up to 10^{-4}. Fungal colonies on each plate were selected randomly (based on colonial morphology) and transferred to PDA amended with 1% (v/v) filter-sterilized diesel, and were incubated at 30 °C for 7 days. These fungal isolates were then transferred to hydrocarbon-adoptive fungi (HAF) agar, containing (g/L) KCl, 0.25; NH_4NO_3, 1; NaH_2PO_4, 0.1; agar, 15; final pH near 7; amended with 5 ml/L of filter sterilized diesel, specific for the growth of hydrocarbon-adapted fungi and incubated at 30 °C for 24 days (Elshafie et al., 2007; Oudot et al., 1993).

2.2. Screening for diesel degradation potential

After preliminary screening, strains were inoculated in HAF broth, containing 10 ml/L diesel as sole carbon source, to test their diesel degradation potential. HAF broth containing (g/L) KCl, 0.25; NH_4NO_3, 1; NaH_2PO_4, 0.1; final pH near 7, was used (Elshafie et al., 2007; Oudot et al., 1993). An agar plug of 2 mm diameter from each PDA fungal cultivated plate, was transferred to HAF broth and incubated at 30 °C for 15 days, in triplicates. The visible growth of each strain was monitored and noted at scale from 0 to 3 (0 means no growth and 3 for best growth). Strains showing best growth were identified initially through microscopic examination (Elshafie et al., 2007; Fedorak et al., 1984). Microscopically identified fungal strains were counter checked using ITS sequence amplification via PCR (Pancher et al., 2012; White et al., 1990). PCR amplification of ITS sequences was done using primers ITS1 and ITS4, the sequences of primers were as follow, ITS1 5′-TCCGTAGGTGAACCTGCGG-3′ and ITS4 5′-TCCTCCGCTTATTGATATGC-3′ (Pancher et al., 2012; White et al., 1990). For PCR, total genomic DNA was extracted through protocol described by Saitoh et al. (2006). DNA amplification was done using following scheme: denaturing at 94 °C for 2 min; 30 rounds of 94 °C for 30 s, 57 °C for 30 s, 72 °C for 1 min; and a final extension at 72 °C for 7 min (Saitoh et al., 2006). Amplified DNA was sequenced using BigDye Terminator v3.1. BLASTN comparison was performed on online database, using National Center for Biotechnology Information DNA sequence database (Wheeler et al., 2007). Best Hit in NCBI database were at least 98% for all of the fungal sequences, where possible the isolates were discriminated to specie level, while where ITS sequences were non-discriminating; identification was done to genus level. Strains identified by ITS sequence analysis were *Aspergillus terreus* FA3, *Aspergillus niger* FA5, *Aspergillus terreus* FA6, *Penicillium chrysogenum* FP4, *Aspergillus terreus* FP6, *Aspergillus flavus* FP10, and *Candida* sp. FG1.

2.3. Hydrocarbon degradation experiment

Seven fungal strains (*Aspergillus terreus* FA3, *Aspergillus niger* FA5, *Aspergillus terreus* FA6, *Penicillium chrysogenum* FP4, *Aspergillus terreus* FP6, *Aspergillus flavus* FP10, and *Candida* sp. FG1) were inoculated in potato dextrose broth (PDB) amended with 1% diesel oil for hydrocarbon degradation experiment. Inoculated liquid cultures were incubated in static conditions at 30 °C for 15 days. After visible growth each of the strains were washed three times with 0.9% N saline. Few drops of tween 20 were added to cultures, centrifuged at 3500 rpm for 15 min, and cells were retained. Washed cells were re-suspended in 0.9% N saline. After final washing, cell suspensions' concentrations were maintained to 10^6 cells ml^{-1} (Wiegand et al., 2008). Each strain was subjected to four conditions 1) nutrient rich (PDB) with illuminance (in light), 2) nutrient rich without illuminance (in dark), 3) nutrient deprived (HAF) with illuminance, and 4) nutrient deprived without illuminance. A negative control containing all ingredients without inoculum was also made for each of the conditions, to check the effect of abiotic factors alone on degradation. For each treatment 10 ml broth was dispensed in test tubes and 100 mg diesel (equivalent to 135 µl) was added. Each test tube was inoculated with 100 µl cell suspension containing 10^6 cells per ml (Wiegand et al., 2008), excluding negative controls. In each of the four conditions, 8 treatments (7 strains +1 negative control) were made in triplicate. After fungal inoculation test tubes were distributed in two parts, one for incubation in light, while other for incubation in dark. All test tubes for dark environment incubation were packed and sealed to prevent light contact. Fig. 1 portrays the experimental set up used for light experiment. For light conditions, two light sources (100 W incandescent bulbs) were used. Light flux was maintained to 500 lux on both sides where the test tubes were placed. From both sources a collective lux of 1000 ±38 was maintained and monitored with the help of Extech light meter 401,025. Both test tubes for dark and light environment were incubated at 30 °C for 45 days.

2.4. Residual hydrocarbon extraction and biomass production

Residual hydrocarbons were extracted by washing media with dichloromethane (DCM) at 1:1 ratio. Washing of media was done thrice with DCM, and separated with the help of a separatory funnel. Fungal biomass was calculated by filtering the solution from separatory funnel through pre-weighed filter paper. The wet filter papers were dried at 80 °C, till constant weight. Pre-weighed screw bottles were used for calculation of residual diesel oil. These bottles were placed under hood till constant weight and degraded diesel oil was calculated (Elshafie et al., 2007).

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