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Assessment of Diesel Degrading Potential of Fungal Isolates from Sludge Contaminated Soil of Petroleum Refinery, Haryana

[1]Smita Chaudhry, [1]Jyoti Luhach, [1]Vandana Sharma and [2]Chetan Sharma
[1]Institute of Environmental Studies, Kurukshetra University, Kurukshetra, Haryana, India
[2]Department of Microbiology, Kurukshetra University, Kurukshetra, Haryana, India

Corresponding Author: Smita Chaudhry, Institute of Environmental Studies, Kurukshetra University, Kurukshetra-136119, Haryana, India

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. In the present study, soil samples from five different petroleum sludge contaminated sites were studied for assessment of their diesel degrading potential. The average heterogeneous fungal count in different soil samples ranged from 35.67±5.69 to 51.33±7.64 and the average count of diesel utilizing fungi ranged from 3.33±1.15 to 26.00±4.00. Total heterogeneous fungal count and diesel utilizing fungal count varied significantly in sludge production and disposal site, as compared to control soil. Thirteen native fungi species of six fungal genera were isolated from different soil samples. The identified fungal genera included *Aspergillus, Fusarium, Cladosporium, Penicillium, Rhizopus* and *Alternaria*. 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., *Alternaria* sp., *Penicillium* sp. and *Fusarium* sp. displayed highest capability for biodegradation of diesel. Hence, these fungal species can be used for bioremediation of sludge and diesel contaminated sites.

Key words: Indigenous fungi, diesel, bioremediation, petroleum sludge, biodegradation

INTRODUCTION

Petroleum products continue to be used as the principle source of energy; however, despite its important usage, petroleum hydrocarbons also act as a globally environmental pollutant. Since, the petroleum hydrocarbons are used widely, oil spills are inevitable even in virtually inhabited areas like Antarctica. Hydrocarbons are biopersistent, bioaccumulative and can cause deleterious effects to aquatic fauna and flora as well as to humans (Benson *et al*., 2007). However, not all hydrocarbon contamination is anthropogenic. Approximately five million tonnes of crude oil and refined oil enter the environment each year as a result of anthropogenic sources such as oil spill (Hinchee and Kitte, 1995). Extensive changes in marine, as well as terrestrial ecosystem resulting from the grounding of Exxon Valdez, 1989; Erica spill, 1999 and Prestige spill, 2002 have recently increased the attention of environmentalist, chemists, biotechnologists and engineers.

Polycyclic Aromatic Hydrocarbons (PAHs) are organic molecules with two or more benzene rings in which the number and arrangement of the rings result in diverse physical and chemical

properties (Leahy and Colwell, 1990). Petroleum industries are responsible for the generation of large amounts of organic residues causing pollution of soil, sea and rivers. In the petrochemical sector, as on 01-10-2009, India has 20 refineries (17 in Public sector and 3 in Private sector) throughout the country with a total refining capacity of 177.956 MMTPA at present and by expansion of existing refineries there will be an addition of 17.146 MMTPA to the refining capacity. At present, total sludge generated from all refineries is 28,200 tons per annum. This amount will increase further by proposal of expansion and new refineries (Bhattacharya and Shekdar, 2003).

Petroleum hydrocarbon compounds bind to different soil components and these are difficult to remove or degrade (Erdogan and Karaca, 2011). Bioremediation process is an attractive approach for better management of this huge amount of sludge and cleaning up of hydrocarbons from environment because it is simple technique, easy to maintain, applicable over large areas, cost-effective and leads to the complete destruction of the contaminant (Bento *et al*., 2005; Achal *et al*., 2011). Main reason for this concept is that the majority of the molecules in the crude oil and refined product are biodegradable. Biodegradation of petroleum and other hydrocarbons in the environment is a complex process who's quantitative and qualitative aspects depend on the nature and amount of oil or hydrocarbon present, the environmental conditions and the composition of the autochthonous microbial community (Ekpenyong *et al*., 2007). Successful application of bioremediation methodology to contaminated systems is affected by several physical and biological parameters (Al-Turki, 2009) like characteristics of the site and the parameters that affect the microbial biodegradation of pollutants (Jain *et al*., 2011; Sabate *et al*., 2004). Many physical, chemical and environmental factors like temperature, nutrients, oxygen, biodegradability, photo-oxidation, bio-availability, soil moisture, soil acidity and alkalinity etc. affects the process of biodegradation of hydrocarbons (Rahman *et al*., 2003; Venosa and Zhu, 2003; Delille *et al*., 2004; Pelletier *et al*., 2004; Maki *et al*., 2001; Trindade *et al*., 2005).

Whenever, PAHs are unavailable to biological systems, then toxicity is reduced but process of biodegradation is inhibited because they are partially unavailable for microbial degradation (Al-Turki, 2009). It is known that greater degradation of oil pollutants is carried out in situ by a consortium of microorganisms and more than 200 species of bacteria, fungi and even algae can biodegrade hydrocarbons (Onifade and Abubakar, 2007). Many native strains including ligninolytic fungi have great potential for remediation of pentachlorophenol (PCP) and polycyclic aromatic hydrocarbon from diesel-contaminated soils in oil refinery sites (Low *et al*., 2008). The advantages associated with fungal bioremediation lay primarily in the versatility of the technology and its cost efficiency compared to other remediation technologies. The use of fungi is expected to be relatively economical because they can be grown on inexpensive agricultural or forest wastes such as corncobs and sawdust. In one such study, Davies and Westlake (1979) examined 60 fungal isolates for their ability to grow on n-tetradecane, toluene, naphthalene and seven crude oils of various compositions. Forty cultures, including 28 soil isolates, could grow on the crude oils. In another study, *Fusarium* sp. F092 was found degradation capacity for aliphatic fraction in crude oil under saline conditions (Hidayat and Tachibana, 2012).

Keeping better degradation ability of native strains of fungi in mind and to find an appropriate cost effective solution to the above mentioned problems by bioremediation, the objectives of this study were therefore, to isolate and identify indigenous fungal flora from contaminated soils of petroleum refinery and to evaluate the diesel degrading potential of the potent isolates.

MATERIALS AND METHODS

Sampling site: The soil samples for present study were collected from different locations of the refinery of Indian Oil Corporation Limited, Panipat. Panipat Refinery set up in 1998, is the seventh

refinery of the Indian Oil. It is located in the state of Haryana and is about 23 km away from Panipat city. Its refining capacity is 15 MMTPA (Million Metric Tonnes Per Annum). Soil samples were taken from nearby of sludge processing and disposal areas contaminated with sludge and oil waste.

Soil sampling: Soil samples from surface soil (0-5 cm depth) were collected from different sites (sample 1-uncontaminated soil from Effluent Treatment Plant (ETP) (control soil), sample 2- sludge contaminated soil from sludge production site in ETP, sample 3-contaminated soil from sludge drying pits in ETP, sample 4-contaminated soil from final disposal of sludge at nearby site and sample 5-composite sample of first four samples. Soil samples from four different sites were taken consecutively after tilling with a sterile scoop and transferred into sterile polythene bags for microbiological determination. Then, small parts of soil from all collected samples from the four sites were mixed to form a composite sample or sample 5 for biodegradation experiments. The sample was transported to the laboratory and kept in a refrigerator (in order to keep the organisms viable and free from any contaminant) before analysis.

Media and chemicals: The media used for isolation of fungi was Potato Dextrose Agar (PDA) containing potato (200 g), glucose (30 g), distilled water (1 L) and agar (20 g). For isolation of diesel utilizing fungi, oil agar media was used. The media was prepared by adding 1% diesel (v/v) sterilized with 0.22 μm pore size Millipore filter paper (Moslein France) to the mineral salt medium (MSM) that was prepared according to modified Mills et al. (1978). The composition of the medium was NaCl (10.0 g), $MgSO_4.7H_2O$ (0.42 g), KCl (0.29 g), KH_2PO_4 (0.83 g), Na_2HPO_4 (1.25 g), $NaNO_3$ (0.42 g), agar (20 g), distilled water (1 L) and pH of 7.2. For testing degradation capability of indigenous fungal isolates, Bacto Bushnell-Haas broth containing $MgSO_4$ (0.2 g L^{-1}), $CaCl_2$ (0.02 g L^{-1}), KH_2PO_4 (1 g L^{-1}), K_2HPO_4 (1 g L^{-1}), $FeCl_2$ (0.05 g L^{-1}) and NH_4NO_3 (1 g L^{-1}) was used. Tween 80 (0.1%), redox reagent (2%) and diesel (1%) were all incorporated into the broth.

Culturing, isolation and enumeration of heterotrophic indigenous fungi in soil samples: Collected sample was homogeneously mixed and carefully sorted to remove stones and other unwanted soil debris using 2.0 mm sieve. Isolation and enumeration of heterotrophic fungi was done by serial dilution agar plating method. This method is based on the principle that when material containing microorganism is cultured, each viable microorganism will develop into a colony, hence colonies appearing on the plates represent the living organisms present in the sample (Aneja, 2005). Potato Dextrose Agar (PDA) culture media was used to isolate the fungal species that were present in all soil samples. Sterile saline, i.e., 0.85% (w/v) sodium chloride was used as diluent for inoculum preparation. 1.0 g of homogenized, soil sample was aseptically transferred into a sterile test tube containing 9.0 mL of the diluent. This gave 10^{-1} dilution. Subsequently 10^{-8} serial solutions were prepared from the 10^{-1} dilution. Then, 0.1 mL aliquot of 10^{-8} dilution of each soil sample was aseptically removed with a sterile pipette and separately spread plated with flame-sterilized glass spreader on PDA plates in triplicates. The cultured plates were incubated at 27°C for 5-7 days. After incubation, the colonies appeared on PDA plates were recorded as counts of total viable heterotrophic fungi for all five soil samples.

Isolation and enumeration of diesel utilizing indigenous fungi in soil samples: For isolation and preliminary identification of diesel utilizing capability of fungi, oil agar media was

used. Oil agar plates were inoculated in triplicate with 0.1 mL aliquots of 10^{-3} dilutions of each soil sample and incubated at 27°C for 7 days. Colonies appeared on oil agar plates were counted after a week and recorded as substantial growth of diesel utilizing molds for different soil samples. The colonies counted were expressed as Colony Forming Unit (CFU) per gram soil. The counts of diesel degraders was further calculated and expressed as a percentage of the total heterotrophic diesel degrader's population of fungi.

Identification of indigenous fungal isolates: For the purification of fungal isolates, the grown cultures of heterotrophic fungi and diesel utilizing fungi were further carefully and aseptically sub-cultured on same culture media (PDA), which were stored on potato dextrose agar slants for subsequent characterization and identification tests. The inoculated plates were identified on the basis of cultural (colour and colonial appearance of fungal colony) and morphological characteristics in lacto-phenol cotton blue wet mount by compound microscope and the software Honstech-TVR and VT Size-5 were used to identify the different fungal species. Observed characteristics were recorded and compared with the established identification key (Nelson-Smith, 1973; Malloch, 1997; Aneja, 2005).

Primary step for confirming biodegradation potentials of fungal isolates: For confirming biodegradation ability of indigenous fungal isolates, Bacto Bushnell-Haas broth was used, which is a modified method used by Desai *et al.* (1993). Two agar plugs (1 cm^2 each) of a pure growth of each isolate were inoculated into Bacto Bushnell-Haas broth (50 mL/250 Erlenmeyer flask) incorporated with sterile diesel (1% v/v). During this set up the control flask had no organism. Incubation was done at room temperature (27°C) with constant shaking at 180 revolution/min for 7 days. The aliquots in the flasks were monitored daily for colour change from deep blue to pink (initially) and then colourless (finally). On a daily basis, 5 mL of the aliquots were collected from each flask and the absorbance was noted on spectrophotometer at 600 nm wavelength.

Final confirmation for biodegradation potential of fungal isolates: Fungal isolates showing better performance (fastest colour change) in primary step were further tested by vapour phase transfer diesel utilization test (Thijsse and van der Linden, 1961). This test was carried out for the confirmatory identification of actual diesel-utilizing moulds. The composition and preparation of the diesel utilization test medium was the same as that of oil agar medium except that diesel was made available via vapour phase transfer. Putative diesel-utilizing fungal isolates in first step of confirmation were streaked on plates of agar medium (one isolate per plate). Inner side of Petri dish was covered with a sterile filter paper (Whatman No. 1) saturated with filter-sterilized diesel. The main aim was to supply diesel (source of hydrocarbons) as sole source of carbon and energy for the growth of the microorganisms on the mineral salts agar medium surface through vapour phase transfer. All the plates were inverted and incubated at 27°C for 7-14 days (Okpokwasili and Amanchukwu, 1988). Colonial growth of different fungi which appeared on the mineral salts agar medium was noted as confirmed diesel-utilizers.

RESULTS

According to results of the enumeration of indigenous fungi, the average counts of total heterogeneous fungi on PDA plates and the average counts of diesel utilizing fungi in the oil agar media were expressed as ($\times 10^3$ CFU g^{-1} soil). The counts of total heterogeneous fungi ranged

Table 1: Identification of native fungal isolates from all soil samples

Fungal isolates	Identification	Fungal isolates	Identification
Control	Control	F-11	*Aspergillus fumigatus*
F-1	*Aspergillus flavus*	F-12	*Aspergillus niger*
F-2	*Fusarium* sp.	F-14	*Penicillium* sp.
F-3	*Aspergillus flavus*	F-15	*Aspergillus flavus*
F-5	*Aspergillus niger*	F-17	*Rhizopus* sp.
F-6	*Cladosporium* sp.	F-19	*Alternaria* sp.
F-7	*Fusarium* sp.		
F-8	*Penicillium* sp.		

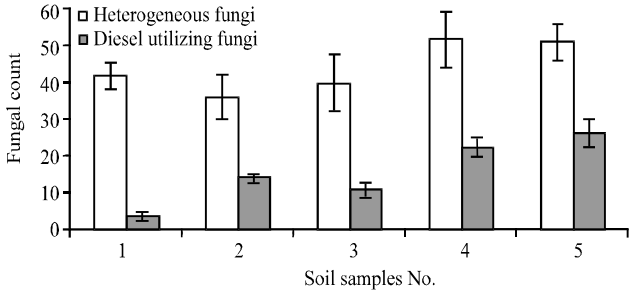

Fig. 1: Average count ($\times 10^8$ CFU/g soil) of total heterogeneous fungi and diesel utilizing fungi in soil samples

38 to 45 with an average count of 41.33±3.51 for sample 1; 31 to 42 with an average count of 35.67±5.69 for sample 2; 31 to 46 with an average count of 39.67±7.77 for sample 3; 43 to 53 with an average count of 51.33±7.64 for sample 4 and 46 to 56 with an average count of 50.67±5.03 for sample 5. While the counts of diesel-utilizing fungi in the soil samples ranged from 2 to 4 with an average of 3.33±1.15 for sample 1; 13 to 15 with an average count of 13.67±1.15 for sample 2; 8 to 12 with an average count of 10.33±2.08 for sample 3; 20 to 25 with an average count of 22±2.65 for sample 4 and from 22 to 30 with an average count of 26±4.00 CFU g^{-1}, respectively (Fig. 1). When diesel-utilizing fungal counts were expressed as percentage (%) of the corresponding total fungal counts in the soil samples, then for sample 1,2, 3, 4 and 5 the percentage varied as 8, 38, 26, 42 and 51%, respectively.

During present investigation, thirteen heterotrophic fungal species belonging to a total of six genera were isolated from all five soil samples. These include *Aspergillus (A. flavus, A. niger, A. fumigatus)*; *Fusarium* sp.; *Alternaria* sp.; *Cladosporium* sp.; *Penicillium* sp. and *Rhizopus* sp. (Table 1). Of these total six genera, five genera viz.; *Aspergillus* sp.; *Fusarium* sp.; *Penicillium* sp.; *Alternaria* sp. and *Rhizopus* sp. were found diesel-utilizers.

During primary step for confirming biodegradation potentials of fungal isolates, these isolates produced a colour change in the Bacto Bushnell-Haas broth medium. The absorbance of broth medium changed according to degradation extent in each flask. 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 nine isolates that produced total colour change, *Aspergillus flavus, Alternaria* spp., *Penicillium* spp. and *Fusarium* spp. displayed the fastest onset colour change (decrease in absorbance of broth medium) and hence, highest capability of biodegradation (Fig. 2). This figure shows the decrease in the absorbance of the Bacto Bushnell Haas broth medium after

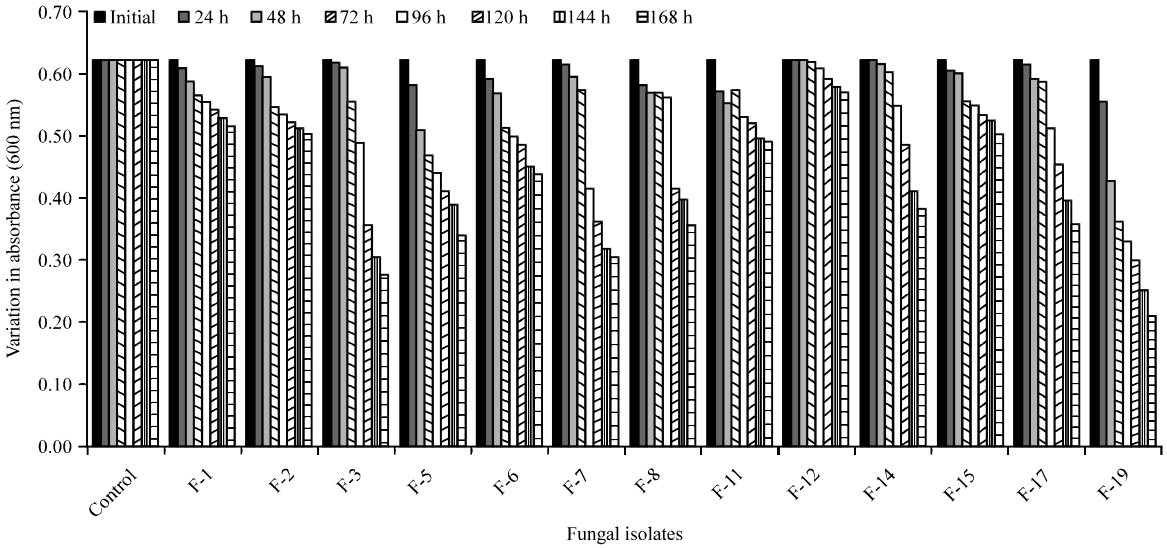

Fig. 2: Variation in the absorbance of BH broth media by different fungal isolates

fungal treatment. Control here refers the media without inoculation. Almost all fungal isolates showed change in color from blue to pink, indicate that these native cultures have ability to grow and to degrade diesel contaminants. More the change in color of broth more will be the degradation ability of fungal isolates.

For confirmatory identification of actual diesel-utilizing moulds, all fungal isolates which were taken in primary step were further tested by vapour phase transfer diesel utilization test. After 10 days of incubation, the six fungal isolates i.e., *Aspergillus flavus*, *Aspergillus niger, Fusarium* sp, *Alternaria* sp., *Penicillium* sp. and *Rhizopus* sp. showed better growth during diesel treatment by vapour phase transfer method. This further confirms diesel biodegradation potentials of these fungal isolates.

DISCUSSION

The results of heterogeneous fungi and diesel utilizing fungi in all five soil samples suggest that the diesel utilizing fungi were adapted to the quantity of hydrocarbons in the environment; hence the increase in the counts of petroleum-utilizing fungi in heavy sludge polluted areas. Obire and Nwaubeta (2001, 2002) in a related study on bacteria have reported similar findings. In all five samples, the reduction in heterogeneous fungi population in sample of sludge production and drying site was because of toxicity of hydrocarbons. But in sample of sludge disposal site, the fungal count increased because in disposal area toxic effects of sludge got diluted. The toxicity of crude oil or petroleum products varies widely and the scale of pollution depends on the quantity of oil and the damage done to the environment (Colwell *et al*., 1977). Almost same trend was observed in case of diesel utilizing fungi, where highest average numbers of diesel utilizing fungal isolates were observed in composite soil sample followed by soil samples of sludge disposal site, indicating the adaptability of native fungal isolates in contaminated environment. The percentage of diesel (hydrocarbon) utilizers in a particular environment appears to be an index of the presence of hydrocarbons in that environment and environmental exposure to petroleum hydrocarbons. These results agree with the reports of Mulkins-Phillips and Stewart (1974).

The occurrence of a variety of fungal genera (i.e., fungal diversity) of both heterotrophic fungi and diesel degrading fungi in the sludge disposal site and composite soil samples was found to be higher. The addition of sludge to the soils resulted in selective increases and decreases in the numbers of fungal populations and enrichment of various fungal genera. Some of these organisms have earlier been reported as hydrocarbon bio-degraders by April *et al*. (2000).

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. Better performing nine isolates which produced total colour change, *Aspergillus flavus, Alternaria* spp., *Penicillium* spp. and *Fusarium* spp. displayed the fastest onset colour change and hence, highest capability of biodegradation. The high rate of diesel (hydrocarbon) degradation by the four fungi could emanate from their massive growth and enzyme production responses during their growth phases. This could be supported by the findings of Bogan and Lamar (1996), which showed that extracellular ligninolytic enzymes of white rot fungi are produced in response to their growth phases. 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 April *et al*. (2000).

In vapour phase transfer diesel utilization test after 10 days of incubation for confirmatory identification of actual diesel-utilizing moulds, the six fungal isolates i.e., *Aspergillus flavus*, *Aspergillus niger, Fusarium* sp, *Alternaria* sp., *Penicillium* sp. and *Rhizopus* sp. showed better growth, further confirming diesel biodegradation potentials of these fungal isolates.

CONCLUSION

In this study, we observed that higher biodegradation efficiency was exhibited by *Aspergillus flavus, Alternaria* sp., *Penicillium* sp. and *Fusarium* sp, providing these fungi to be better hydrocarbon degraders. Thus, they can be effectively utilized for the degradation of sludge and for bioremediation of oil polluted farm lands especially those located within the vicinity of the petroleum processing and disposal sites. Since microorganisms play an essential role in biogeochemical cycling, interference with microbial metabolic activities by pollutants in the environment can have far reaching ecological consequences. During field applications for bioremediation of sludge contaminated sites, after large scale production of the potent fungal organisms, adequate carriers or extenders like tween 80 can be used for enhancement of degradation process. Inert stickers or adhesives like molasses, corn syrup, skim milk, casein and latexes may be incorporated into the formulation in order to prevent run offs. A good sticker combined with charcoal can serve as a protectant for reducing the effects of ultra-violet light, desiccation and other detrimental environmental factors. During application, the sludge and oil contaminated sites should first be tilled to loosen the soil. Thereafter, the loosened soil should be enriched with adequate nutrients necessary for the growth of the organism before applying the microbial inoculants which must be properly mixed with the moist soil.

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