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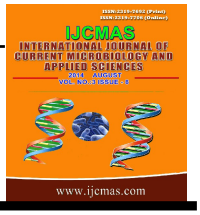

Original Research Article

Isolation and assessment of biodegradation potential of fungal species isolated from sludge contaminated soil

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Keywords

Bio-remediation, *Aspergillus niger, Mucor sp. and Penicillium sp.*

A B S T R A C T

Bioremediation is considered as one of the safer, cleaner, cost effective and environmental friendly technology for decontaminating sites which are contaminated with wide range of pollutants. In present study, soil samples from different petroleum sludge contaminated sites were studied for assessment of their hydrocarbon degradation potential. The average heterogeneous fungal count ranged from 15.5±1.6 to 44±5.8 and hydrocarbon degrading fungal count ranged from 3±1.7 to 11.8±3.67. A total of 13 species were isolated out of five genera. Out of which Aspergillus niger, Mucor sp. and Penicillium sp. had shown the best potential of degradation depending upon the changes that they bring in the Bacto-Bushnell Hass media. So, these sp. can be used for bioremediation of sludge and sludge contaminated soil. Key Words: Bioremediation, sludge, Poly-cyclic aromatic hydrocarbons (PAHs), Biodegradation.

Introduction

Industrialization is an important requirement for an economy. The environment is facing a huge payment for this in the form of increasing effluents, pollution, increasing levels of CO_2, global warming, depleting ozone layer etc. In the last years a large number of ecosystems have been changed by the growing influence of industries and human activities. As a result many people have become aware of the need to protect ecosystem as well as to evaluate the damage caused by contamination. The World demand for oil in 2010 was 94.3 million barrels per day and expected to be 101.6 million barrel per day in 2015 (Medjor *et al*., 2012). However, since the petroleum hydrocarbons are used widely, uncontrolled release of petroleum will give negative impact to our soil resources and become toxic (Wang *et al*., 2008). Oil refineries generate large quantities of oily sludge, the safe disposal of which is major problem. At present, total sludge generated from all 21

private and public sector refineries in India is 28,200 tons per annum (Bhattacharya and Sedhkar, 2003). The sludge is generated during cleaning of storage tanks, cleaning and desilting of oil separator basins, distillation column residues, exchanger tube bundle sludge and sludge generated from effluent treatment plant (ETP).

Oil sludge is a thick, viscous mixture of sediments, water, oil and high hydrocarbon concentration (Ubani *et al*., 2013). Oil sludge is mainly composed of alkanes (40-70%), aromatics (15-30%) and heavy fraction like NSO (nitrogen, sulphur and oxygen fraction) asphaltenes & resin (5-15%) (Mandal *et al*., 2012). Oil sludge has been classified by the United States Environmental Protection Agency (US EPA) as a hazardous organic complex (US EPA, 1997; Liu *et al*., 2010). This contaminant enters the environment as a result of human activities, which includes deliberate dumping, improper treatments and management, storage, transportation and landfill disposal. This calls for concern because many of the oil sludge components have been found to be cytotoxic, mutagenic and potentially carcinogenic (Bojes and Pope, 2007). In severe cases, Polycyclic Aromatic Hydrocarbon metabolism in human body produces epoxide compounds with mutagenic and carcinogenic properties that affects the skin, blood, immune system, liver, spleen, kidney, lungs, developing foetus (TERA, 2008; Sidney, 2008; Bayoumi, 2009). The hydrocarbons spill impacts soil by affecting soil physical structure by coating soil aggregates, affecting soil water holding capacity, reducing and diverting water infiltration into the soil, reducing cation/anion exchange on soil aggregates and it also results in an imbalance in C/N ratio in soil which cause a nitrogen deficiency which retards the growth of microbes (Dindar *et al*., 2013).

The physical and chemical methods employed for sludge processing like Pyrolysis, Lime stabilisation, Solvent extraction, Incineration are expensive, high energy demanding and also generate end products that are more harmful than sludge (Selivanovskaya *et al*., 2013). The hydrocarbons present in sludge posses biodegradable property. The hydrocarbons present at contaminated site can be used by microbes as substrate to full-fill their basic need of energy and carbon source, and these can be used to degrade or remediate environmental hazard (Sharma, 2012). The microbes have the ability synthesize enzymes that can catalyse the reaction in which these contaminants are degraded to simpler, lower molecular chains and less toxic compounds (CO_2 and H_2O), through obtaining the nutrients and energy necessary for their survival in the process (Ho and Rashid, 2008).

Natural organisms, either indigenous or extraneous (introduced), are the prime agents used for bioremediation (Prescott *et al.,* 2002) A mixture of different bacterial species that can degrade a broad range of the hydrocarbon constituents such as present in oil sludge would show more potential (Oteyza *et al.,* 2006). Liu *et al* (2010) suggested that indigenous microorganisms isolated from a contaminated site will assist in overcoming this problem, as the microorganisms can degrade the components and have a higher tolerance to toxicity that may wipe off other introduced species.

Keeping in mind better degradation of these hydrocarbons by native strains and to find cost effective method to the above mentioned problem, the aim of this study was to isolate and assess the hydrocarbon degradation potential of native fungal species.

Materials and Methods

Sampling Site: The soil samples for present study were collected from different sites from Panipat refinery, Panipat, Haryana. It was set up in 1988. It is the seventh largest refinery in India with the refining capacity of 15 MMT/yr.

Soil Sampling: The soil samples were collected from subsurface soil (0-5 cm) from different site near sludge disposal area. The soil samples were taken consecutively after tilling with a sterile scoop and transferred into sterile polythene bags for microbiological determination. The samples were then transported to the laboratory and kept in refrigerator to keep the organisms viable.

Media and Chemicals: The media used for isolation of fungi was Potato Dextrose Agar (PDA) containing potato (250.0 g/L); Dextrose (20.0g/L); Agar (15.0 g/L) in Distilled Water (1 Lit.). For isolation of hydrocarbon utilizing fungi, oil agar media was used. This media was prepared by adding 1% diesel (v/v) to Mineral Salt Media (MSM) that was prepared according to modified Mills *et al.* (1978). For testing degradation potential of indigenous fungal isolates, Bacto Bushnell-Haas broth containing $MgSO_4$ (0.2 g/L); $CaCl_2$ (0.02g/L); KH_2PO_4 (1g/L); $FeCl_2$ (0.05g/L) and NH_4NO_8 (1g/L) was used. Tween 80 (0.1%), redox reagent (2%) and Diesel (1%) were all incorporated into the broth.

Isolation, Enumeration and culturing of heterotrophic indigenous fungi in the soil sample

The soil samples were homogeneously mixed and sieved using 2.0 mm sieve to remove unwanted soil debris. Isolation and enumeration was done by serial dilution agar plating method. Potato Dextrose Agar (PDA) culture media was used for isolation purpose. Then, 0.1 mL aliquot of 10^{-3} dilution was aseptically removed with a sterile pipette and spread with sterilized glass spreader on PDA plates in triplicates. The cultured plates were incubated at 27^0C for 5-7 days. After incubation, the colonies were sub-cultured to get pure culture.

Isolation and enumeration of hydrocarbon utilizing indigenous fungi in the soil samples

For isolation and preliminary identification of hydrocarbon utilizing capability of fungi, oil agar media was used. Oil agar plates were inoculated in triplicate with 0.1 mL aliquots of each soil sample and incubated at 27^0C for 7 days. Colonies appeared on oil agar plates were counted after a week and recorded as substantial growth of hydrocarbon utilizing molds for different soil samples. The colonies counted were expressed as Colony Forming Unit (CFU) per gram soil.

Identification of indigenous fungal isolates

For the purification of fungal isolates, the grown cultures of heterotrophic fungi and hydrocarbon utilizing fungi were further carefully and aseptically sub-cultured on PDA. 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-Hass broth was used, which is a modified method used by Desai *et al* (1993). Two agar plugs (1cm^2 each) of a pure growth of each isolate were inoculated into broth (50ml/250 Erlenmeyer flask) incorporated with sterile diesel (1%). Control flask does not contain any organisms. Incubation was done at room temperature (27^{o}C) with constant shaking at 180rev/min for 7 days. Daily 5ml of aliquots were collected from each flask and the absorbance was noted on spectrophotometer at 600nm.

Final step for confirming biodegradation potentials of fungal isolates

For confirming biodegradation ability of indigenous fungal isolates, Mineral salt media was used. Two agar plugs (1cm^2 each) of a pure growth of each isolate were inoculated into broth (50ml/250 Erlenmeyer flask) incorporated with sterile diesel (1%). Control flask does not contain any organisms. Incubation was done at room temperature (27^{o}C) with constant shaking at 180rev/min for 7 days. Daily 5ml of aliquots were collected from each flask and change in pH, total protein content was observed.

Results and Discussion

According to results of enumeration of indigenous fungi, the average count of hetrogeneous fungi on PDA plates and the average counts of hydrocarbon utilizing fungi in the media were expresses as (X 10^3 CFU /G soil). The counts of total heterogeneous fungi ranged from 28-31 with an average count of 29.5±3.42 for sample 1; 42 to 46 with average count of 44±5.8 for sample 2; 14-17 with average of 15.5±1.6 for sample 3; 31 to 37 with average count of 34±5.2 for sample 4; 29-35 with average count of 32±3.1 for sample 5. While the count for hydrocarbon utilizing fungal count was from 2-6 with average of 3±1.7 for sample 1; 9-13 with average of 11±1.8 for sample 2; 6-9 with average count of 7.5±1.5 for sample 3; 5-9 with average count of 7±1.8 for sample 4; 12-16 with average count of 11.8±3.67 for sample 5 (fig. 1). When hydrocarbon utilizing fungal counts were expressed as % of total fungal counts for each sample, then percentage varied as 10, 25, 48, 20 and 36 %, respectively (Figure 1).

During fungal isolation, a total of 13 fungal isolates were initially obtained from sludge contaminated soil. Of these total, five known genera were recorded (*Aspergillus*-6 isolates, *Rhizopus*-1 isolate, *Penicillium*-3 isolates, *Mucor*-2 isolates, *Altenaria*-1 isolate). The isolated species are given in Table 1.

During primary step for confirming biodegradation potential of fungal isolates, these isolated produced a colour change in the Bacto Bushnell- Haas broth medium (from blue to pink and finally colourless). The absorbance of broth decreased according to degradation extent in each flask (Figure 2). Among the 13 isolates only 4 isolates *i.e. Penicillium sp., Mucor sp., Aspergillus sp.* and *Aspergillus niger* had maximum potential for hydrocarbon degradation. The more is the change in colour the more is the degradation potential.

For final confirmation of degradation potential the change in the pH and protein content of MNM was observed. The pH decrease was maximum for *Aspergillus niger, Aspergillus flavous* and *Penicillium*

sp. (Figure 3) and the protein content was maximum for *Aspergillus niger*, *Penicillium sp*. and *Mucor sp*. (Figure 4).

The results of heterogeneous fungi and hydrocarbon utilizing fungi in all soil samples suggest that the diesel utilizing fungi were adapted to the higher quantity of hydrocarbons in the environment, hence the increase in the counts of petroleum-utilizing fungi in heavy sludge polluted areas. These findings are similar to the related study conducted on bacteria by Obire and Nwaubeta (2001, 2002). The percentage of 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).

During primary confirming of biodegradation potentials of fungal isolates, the ability of isolates produces a colour change in Bacto Bushnell-Hass broth medium due to reduction of the indicator by the oxidized products of hydrocarbon degradation. Better performing 4 isolates produced total colour change showing the fastest onset colour change and hence, highest capability of biodegradation. These isolates were *Penicillium sp*., *Aspergillus sp*. *Mucor sp*., *Aspergillus niger*. The higher rate of hydrocarbon degradation by fungi can emanate from their massive growth and enzyme production response during growth phase. This could be supported by the findings of Bogan and Lamar (1996).

During Final confirmation, constant reduction in the pH was observed. This can be attributed to the microbial degradation of hydrocarbons which often leads to production of organic acids and other metabolic products (by products), thus the organic acids probably caused the reduction in pH. More the increase in protein concentration more will be the adaptability of bacterial isolates for contaminants in soil, hence, more will be degradation potential. It was observed that isolate No. *Aspergillus niger*., *Mucor sp*. and *Penicillium sp*. had maximum total protein content. Increasing of protein content during the incubation period reveals that isolates can use crude oil as the source of carbon and energy.

Table.1 Identification of native fungal Isolates

Fungal Isolate	Identification	Fungal Isolate	Identification
Control	No Fungi	F-7	*Aspergillus flavus*
F-1	*Aspergillus fumigates*	F-8	*Penicillium sp*
F-2	*Aspergillus sp.*	F-9	*Mucor sp.*
F-3	*Rhizopus arhizus*	F-10	*Aspergillus niger*
F-4	*Penicillium sp.*	F-11	*Aspergillus flavus*
F-5	*Mucor sp.*	F-12	*Altenaria sp*
F-6	*Penicillium sp.*	F-13	*Aspergillus niger*

Fig.1 Average count of total heterogeneous and hydrocarbon utilizing fungi in soil samples

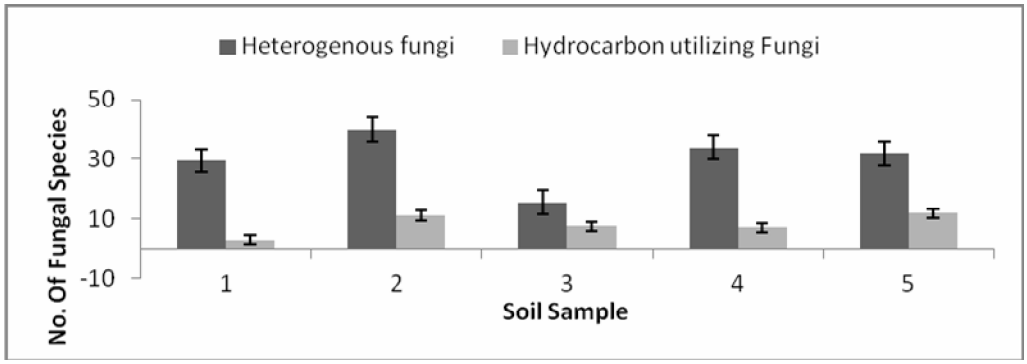


Fig.2 Variation in the absorbance of BH broth after fungal inoculation

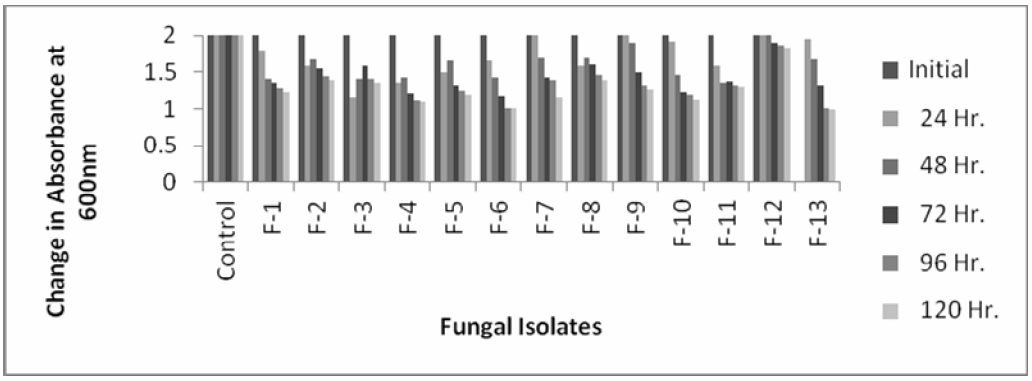


Fig.3 Variation in the pH of MNM after fungal inoculation

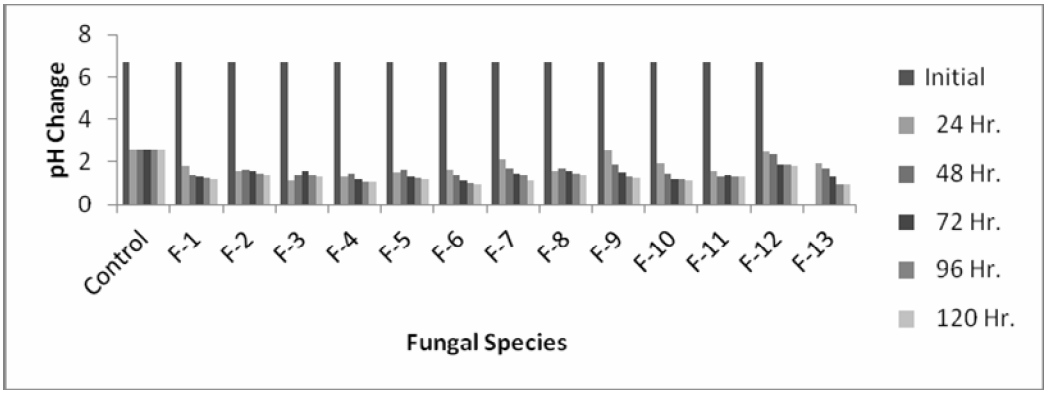
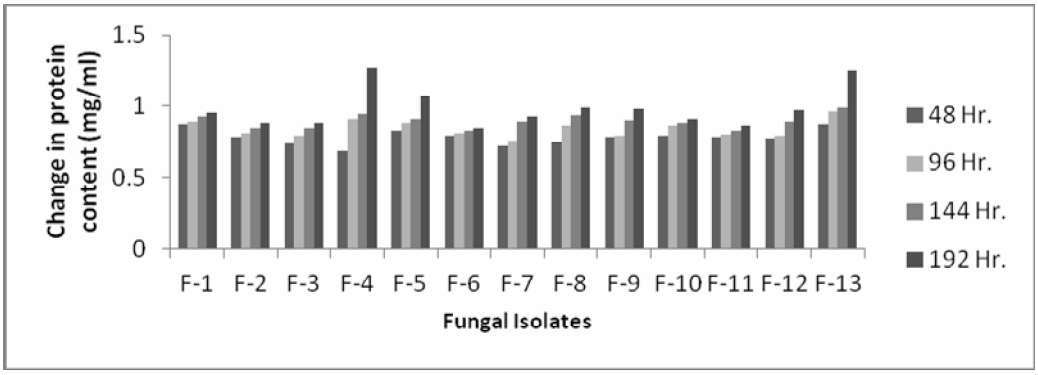


Fig.4 Variation in the Protein content (mg/ml) of MNM after fungal inoculation



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