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Research Article

BIODEGRADING FUNGAL FLORA ISOLATED FROM PETROLEUM CONTAMINATED SOIL IN RURAL AREA OF JAIPUR DISTICT, RAJASTHAN (INDIA)

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ABSTRACT

Various fungi were isolated from petroleum hydrocarbon contaminated soil of various sites of Jaipur, Rajasthan (India). Isolation of fungi from soil samples were carried out by serial dilution plate technique followed by their identification through microscopic and macroscopic method. Potato Dextrose Agar media was used for the growth of fungi. In our findings, following fungi were isolated and identified which include: *Aspergillus niger, Aspergillus flavus, Aspergillus terreus, Penicillium* sp. and various other species of *Aspergillius* and *Penicillium.* Among all these isolated fungi, *Aspergillus niger* was more dominant and shows the fast and highest growth pattern at temperature 28^0C and pH 5.6. These fungi play important role in the degradation of petroleum hydrocarbon. Bioremediation processes are nature-based, safe, and less costly as compare to physical, chemical and thermal processes. These fungi isolated in our study are useful in the bioremediation of soil contaminated with petroleum hydrocarbon**.**

Key Words*: Bioremediation, Petroleum Hydrocarbon, Aspergillus sp., Contamination*

INTRODUCTION

With continuous increase in the industrial development there occurs a serious environmental pollution. Environmental contamination by petrol derivatives has been a subject of study over the past four decades. The leakage of these derivative oils, such as lubricant oils, is capable of harming the environment in many ways (Atlas, 1995). Thus, in order to prevent the hazardous effects of oil pollution control and treatment strategies are required. However, conventional physical-chemical treatments are very costly when the contamination is high and can produce toxic residues to the environment. Adapting "bioremediation" process which is highly efficient, less costly, and easy way of regaining the contaminated site as compare to other physiochemical processes (Bhupathiraju *et al.,* 2002). Bioremediation is a modern method in which the natural ability of microorganisms is employed for the reduction of the concentration and/or toxicity of various chemical substances, such as petroleum derivatives, aliphatiderivatives, aliphatic and aromatic hydrocarbons, industrial solvents, etc. A number of microbial agents (bacteria and fungi) which are capable of biodegrading petroleum and its derivatives have been identified (Atlas and Bartha, 1972; 1993). Fungi are an important component of the soil micro biota. (Ainsworth, 1995). The role of fungi in the soil is an extremely complex one and is fundamental to the soil ecosystem (Diana, 1994). There is at present a worldwide search for suitable fungi which are capable of degrading petroleum products. In addition, some research is known to have been carried out to investigate the possibilities for large-scale production of these fungi (Mahro *et al.,* 1994; Field *et al.,* 1996; Anon, 1998). The present study was therefore undertaken with a view to isolate and identify fungal flora from petroleum hydrocarbon contaminated soil.

MATERIALS AND METHODS

Collection of soil samples

Oil contaminated soil samples were collected from five different sites of Jaipur, Rajasthan (India) includes- Pushapraj petrol pump (Bhakrota), HP petrol pump (Bagru), soil collected near generator site (Jharna), Hindustan Petroleum depot (Chitrolli), Bharat petrol pump (Jaipur). One sample collected from each site at about 3- 5 cm depth with the help of hand-driven auger in pre- sterilized plastic bags. There were total five soil samples collected in a plastic bag. Each plastic bag was labeled indicating the date and site of collection. These samples were then tightly closed to maintain the original moisture and brought to research lab for further processing.

Media for isolation of fungi

Potato dextrose agar (PDA) was used for isolation and enumeration of total heterotrophic fungi. The composition of the medium was- potato, 200 g; distilled water, 500 mL; glucose – D, 15 g and agar No. 1, 20 g (Harrigan and McCance, 1990; Paul and Clark, 1988; Walker and Colwell, 1976). The pH of media was maintained at 5.6. Autoclaving of media has been done at 15 lb pressure for 15 min and the medium was allowed to cool to 45 °C under aseptic conditions, mixed thoroughly and then dispensed into sterile Petri dishes to set.

Isolation of fungi

1.0g of homogenized, 2mm sieved soil sample was aseptically transferred, using a flame- sterilized steel spatula, into a sterile test tube containing 9ml sterile distilled water. This gave 10^{-1} dilution. Subsequently, ten-fold serial dilutions in the range of $10^{-1} – 10^{-9}$ were prepared using sterile distilled water (Atlas and Bartha, 1972). 0.1 ml aliquot of each soil sample dilution was aseptically removed with a sterile pipette and separately spread plated with flame-sterilized glass spreader on well-dried PDA plates. The cultured plates were incubated at room temperature for 5 to 7 days. After incubation, discrete colonies were sub-cultured onto PDA plates until pure cultures were obtained. The colonies developed were then identified.

Identification of fungi

Isolated fungal floras were identified on the basis of macroscopic and microscopic features. For fungal examination, a small portion of the fungal growth was picked up with the help of a flame sterilized loop, mounted on a slide under covered glass containing a drop of lacto phenol blue staining solution, and examined under a microscope for the identification of the fungi. Whenever essential, photomicrographs were also taken to get a clear picture about the microorganism. The measurements, shape, arrangements of spores and other structures were also taken. The color, texture, pigmentation on reverse side of colony and colony characters were recorded for fungal identification.

RESULTS AND DISCUSSION

Out of five soil samples examined for isolating fungi from petroleum hydrocarbon contaminated site in different sites of Jaipur, Rajasthan, by serial dilution plate technique were identified as *Aspergillus* and *Penicillium* species. Species isolated and identified as: *Aspergillus flavus, Aspergillus niger, Aspergillus terreus, Penicillium* sp. and various other species of genus *Aspergillus* and *penicillium*. Out of all these isolated fungi, *Aspergillus niger* showed highest and fastest growth pattern at temperature 28^0C and pH 5.6.

Studies on the isolation of filamentous fungi in environments containing oil or its subproducts found a very similar diversity of genera to that found in our study, such as: *Aspergillus* and *Penicillium* (Cerniglia, 1997; Bento *et al.,*, 2005; Chaillan *et al.,*, 2004). Recently, it was recorded that the genera of fungi such as *Penicillium*, *Aspergillus*, *Fusarium*, *Rhizopus* and *Mucor* are associated with petroleum hydrocarbon contaminated soil. Our results were supported by Akpoveta *et al.*, (2011); in their studies they isolated *Penicillium* and *Aspergillus* sp. From hydrocarbon contaminated soil and identified as hydrocarbon degrading fungi along with *Tricodema, Fusarium, Rhizopus* sp. The similar results of our

study were also obtained by Obire and Anyanwu, (2009), in their studies on impact of various concentrations of crude oil on fungal populations of soil. They isolated fourteen fungal genera from soil. These include *Alternaria, Aspergillus, Candida, Cephalosporium, Cladosporium, Fusarium, Geotrichum,*

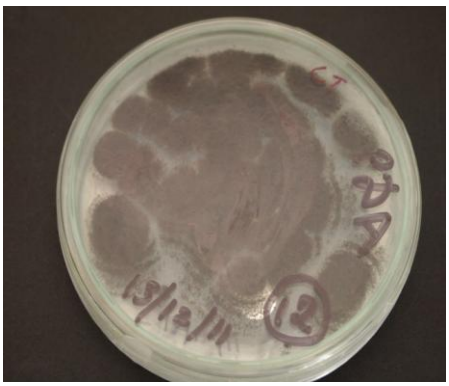
Table1: Physical characterization of various soil samples

	Bharat Petrol pump (Jaipur)	**HP Petrol Pump (Bagru)**	**Pushapraj Petrol Pump (Bhakrota)**	**Generator site (Jharna)**	**HP depot (Chittrolli)**
Temperature	25^0C	31^0C	25.7^0C	31^0C	32^0C
Humidity	28%	36%	30%	34%	57%
Pressure	989mb	749mb	950mb	750mb	1018mb
pH	8.26	6.7	8.07	6.5	7.4
Water holding Capacity	32.1%	30.6%	38.4%	27.5%	21.8%
Soil moisture content	10.3%	5%	10.0%	4%	1.046%

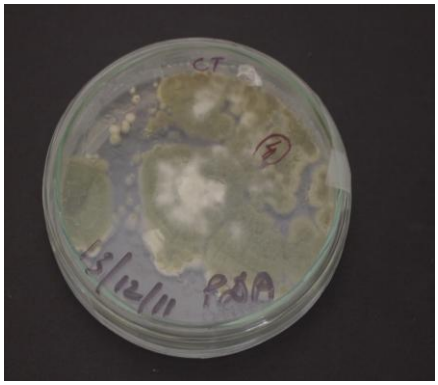
Table 2: Identification of fungal colonies based on their macroscopic and microscopic morphologies

S. no.	**Fungi**	**Media**	**Macroscopic Morphology**	**Microscopic morphology**
1.	*Aspergillus flavus,*	PDA	Light green Colony. Cream colour from reverse side of plate. Cottony texture.	Septate and hyaline hyphae. uncoloured and coarsely rough Conidiophores. smooth Conidia
2.	*Aspergillus niger*	PDA	Black colour colony from front side of plate, pale yellow from reverse side. fast growing	Septate and hyaline hyphae. Long, smooth and hyaline Conidiophores. black Conidia
3.	*Penicillium* sp.	PDA	Colonies are woolly or cottony in texture. Colonies are initially white and become blue green. Fast growing, reverse side shows yellow colour.	Septate hyaline hyphae. Simple or branched Conidiophores. Round shaped, unbranched chains conidia. Phialides form brush like clusters.
4.	*Aspergillus terreus*	PDA	Brown colour colony from front side and Yellow colour pigmentation produced from reversed side. Colony shows fast growth rate.	Septate and haline hyphae. Smooth-walled and hyaline Conidiophores. Conidia are short and smooth.

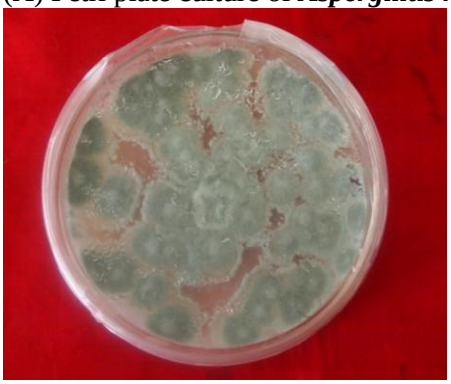
Mucor, *Penicillium*, *Rhizopus*, *Rhodotolura*, *Saccharomyces*, *Torulopsis* and *Trichoderma*. Oboh *et al.*, (2006) reported that the fungal isolates obtained in their study were mainly *Aspergillus* species, while others were *Trichoderma*, *Penicillum*, *Rhizopus* and *Rhodotorula* species which were all able to utilise hydrocarbon as carbon source. Araujo and Lemos (2005) found that soils contaminated by 5 % oil were able to isolate several species from the *Aspergillus, Penicillium, Paecilomyces* and *Fusarium* genera which are in their majority, were able to degrade petroleum hydrocarbons. Reiche and Lemos (2006), isolated several filamentous fungi from soil, which were able to degrade crude oil. *Fusarium* and *Aspergillus* sp. were again isolated by Akpoveta *et al.*,, (2011), they isolated and identified hydrocarbon degrading fungi from hydrocarbon contaminated soil. *Aspergillus* species were isolated form soil polluted by petroleum products in cross River University of Technology, Calabar, Nigeria by Eja *et al.*, (2006). In our present work, *Aspergillus* and *Penicillum species* were present in dominant numbers. Our finding coincides with the work of Elisane *et al.*, (2008), who also isolated four strains from the contaminated soil. They were identified as *Aspergillus* sp. According to Chaillan *et al.*, (2004), *Aspergillus* are the most commonly encountered genera of hydrocarbon degraders in oil contaminated tropical soils, which are in agreement with the present work. Our result was also supported by Mancera-López *et al.*, (2007), who isolated three fungi from total hydrocarbon contaminated soil and identified by microscopy as *Penicillium*, A*spergillus* and *Rhizopus* sp. The different result from our findings were obtained by Ravelet *et al.*, (2000), who also isolated many fungal species that were able to degrade poly-cyclic aromatic hydrocarbons. The species isolated were *Coniothyrium fuckelii, Gliocadium virens*, *Phialophora alba, Phialophora hoffmannii, Scopulariopsis brumptii* and *Trichoderma hazianum* along with genera *Penicillium*, which were similar to our finding.



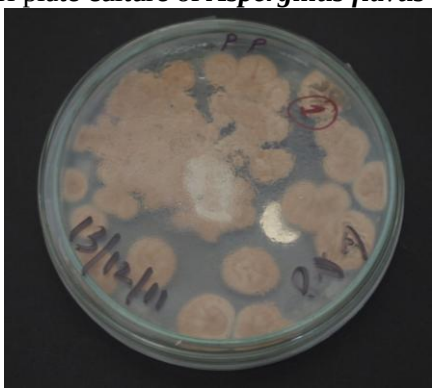
(A) Petri plate culture of ***Aspergillus niger***



(B) Petri plate culture of ***Aspergillus flavus***



(C) Petri plate culture of ***Penicillium* sp.**



(D) Petri plate culture of ***Aspergillus terreus***

Figure 1: Macroscopic culture of few isolates from Petroleum Hydrocarbon contaminated Soil

Macromorphological features which are considered include conidial and mycelial colour, colony diameter, colony reverse colour, production of exudates and soluble pigments, presence of sclerotia and cleistothecia. Micromorphology characterization is mainly dependent on seriation, shape and size of vesicle, conidia and stipe morphology, presence of Hülle cells, and morphology of cleistothecia and ascospores (Klich, 2002). Furthermore, all these morphological features have to be determined under standardized laboratory conditions by trained mycologists, in order to obtain an accurate identification (Okuda *et al.,* 2000). With the serial dilution plate technique, the richest genera in terms of the number of species were *Aspergillus* and *Penicillium,* and the most common ones in these two were *Aspergillus niger*. In our study, *Aspergillus niger* shows dominant growth characteristics as compared to other isolated fungi. As with fungi in general, *Aspergillus* taxonomy is complex and ever evolving. The genus is easily identified by its characteristic conidiophore, but species identification and differentiation is complex, for it is traditionally based on a range of morphological features. The macroscopic cultures of the isolated fungal species were recorded. Fig 1 (A) showed the macroscopic culture of *Aspergillus niger*, fig (B) showed the macroscopic culture of *Aspergillus flavus*, fig (C) showed the macroscopic culture of *Penicillium* sp. and fig (D) showed the macroscopic culture of *Aspergillus terreus*. Fungal morphology on PDA media at room temperature is summarized in table 2. Several *Aspergillus* taxonomic keys and guides are available (Klich, 2002; Raper and Fennell, 1965). In our observation, the macroscopic morphology of *Aspergillus niger* on potato dextrose agar showed white colour colony at temperature 28^0C and soon became black. On the reverse side of the plate, pale yellow colour was observed. In microscopic morphology, septate and hyaline hyphae were observed. Conidiophores were long, smooth, hyaline and darker at the apex. Conidia were brown to black. The entire vesicle was covered by metulae and phialides. The same microscopic results of *Aspergillus niger* were obtained by de Hoog *et al.,* (2000) and Sutton *et al.,* (1998). The macroscopic morphology of *Aspergillus flavus* showed olive to lime green colour colonies on PDA at 28^0C temperature with a cream colour on the reverse side of plate. Texture of the culture was woolly to cottony to somewhat granular. About microscopic morphology, hyphae were septate and hyaline. Conidiophores were uncoloured and coarsely roughened. Conidia were smooth. The similar macroscopic and microscopic features of *Aspergillus flavus* were also obtained by de Hoog *et al.,* (2000); Klich and Pitt, (1988); Raper and Fennell, (1965); Sutton *et al.,* (1998). The macroscopic features of colonies of *Penicillium* sp. Grown on PDA at temperature 28^0C were initially appeared as white and changed to blue green in time. The reverse side of the plate showed pale to yellowish pigmentation. The microscopic features of *penicillium* sp. Showed septate hyaline hyphae, conidiophores were simple or branched, the arrangement of phialides at the tip of the conidiophores formed a brush like structure. Conidia were present at the tip of the phialides as a round, unicellular, and unbranched chain. The same macroscopic and microscopic features were observed by de Hoog *et al.,* (2000); Larone (1995); St-Germain and Summerbell (1996); Sutton *et al.,,* (1998). *Aspergillus terreus* was also isolated from petroleum contaminated soil and identified macroscopically and microscopically. Colonies observed on PDA were brown in colour from the front side and shows fast spreading growth. From the reverse side, yellow colour was observed. Colonies observed were finely granular with conidial production. Under microscope the hyphae observed were septate and haline. Conidia are smooth, small in size. Conidiophores were smooth-walled, hyaline and end in mostly globose vesicles. Observations of our microscopic and macroscopic identification of *Aspergillus terreus* were supported by de Hoog *et al.,,* (2000) and Sutton *et al.,* (1998). They observed the similar features as of ours.

Conclusion

It was concluded from our studies that oil degrading fungal flora could be isolated from Petroleum hydrocarbon contaminated soils without need for time consuming enrichment media. This study proofs that the fungal flora isolated from petroleum contaminated soils have potential application in the bioremediation of sites polluted with petroleum hydrocarbon. Among all the fungi isolated from

petroleum contaminated soil collected from various sites of Jaipur, Rajasthan (India), the *Aspergillus niger* was more dominant and shows more fast and frequent growth in contaminated soil.

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