ASSESSMENT OF PETROLEUM HYDROCARBON DEGRADATION FROM SOIL AND TARBALL BY FUNGI

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ABSTRACT

Four fungi strains viz. *Aspergillus niger, Aspergillus terreus, Rhizopus sp* and *Penicillium sp* were isolated from soil and tarball samples collected from mangrove forest of Alibaug and Akshi coastal area, Maharashtra, India. These strains were assessed for their degradation capability of petroleum hydrocarbons measuring growth diameter in Potato Dextrose Agar (PDA) solid media for different concentrations of kerosene (5%- 20% (v/v)). *Rhizopus sp* showed the highest growth diameter in 5% kerosene and *Aspergillus niger* showed the highest growth diameter in 20% kerosene while, *penicillium sp* showed the lowest growth diameter at all the concentrations of kerosene as compared to other three strains. The bioremediation of 20% oil contaminated soil by different fungi strains was found in the order *Aspergillus niger> Rhizopus sp> Aspergillus terreus* > *Penicillium sp.* In order to determine the effect of mixed fungal culture in contrast with single one, studies were carried out in 10% (v/v) oil contaminated PDA media. It was observed that a mix culture consisting of *penicillium sp*, *Rhizopus sp* and *Aspergillus terreus* showed highest growth diameter.

Key words: Bioremediation**,** fungi species, mangrove, petroleum hydrocarbon, *soil, tarball*

INTRODUCTION

Environmental pollution due to oil spill is one of the most common problems along the coastal region. Crude oil is naturally composed of complex mixture of hydrocarbon and non-hydrocarbon compounds which are harmful to organisms (Nelson and Smith, 1973). The impacts of petroleum product and its derivatives on the environment depend on their composition and concentration and the time of spill. High contamination immediately shows adverse effects on plant and animal life (Baker, 1970; Steinhart and Steinhart, 1972; Rowell, 1977; Fagbami *et al.,* 1988).

However, various species of microbes and their susceptibility varies with stages of life (Nelson and Smith, 1973). Besides petroleum contamination effects on microbial population are various (Ahearn and Meyers, 1976). The effects of petroleum pollution depend on chemical composition of oil product and the species of microorganism. Certain microbes show increase in population due to use of petroleum hydrocarbons as nutrients (Westlake *et al.,* 1974). Such species are commonly being used for remediation of contaminated site. These organisms are directly involved in biogeochemical cycles of the degradation of many carbon sources, including petroleum hydrocarbons (Santos *et al.,* 2011). The inputs of various condition related to indigenous microbial communities at contaminated sites are also required for their use in bioremediation approaches (Desai *et al.,* 2010). After oil spill on 17th August of 2010 in Mumbai coast, oil was found accumulating in mangrove forest of Navi Mumbai, Uran and Alibaug. Tarballs were also found on shore along the Raigad coastline. Remediation action was done using OILZAPPER by TERI. Oilzapper is the first product developed by assembling five naturally occurring bacterial species, which can biodegrade all the fractions of crude oil and oily sludge. Potential of remediation depends on physical, chemical and biotic characteristics of soil. The success of this remediation depends on pH, water holding capacity and level of nutrients in soil.

The present work deals with the isolation and identification of fungi from soil and tarball samples and their role in remediation of oil spill.

MATERIALS AND METHODS

The soil samples were collected from Alibaug mangrove forest and tarball samples were collected from Akshi coastal area for fungi isolation. The average relative humidity is over 80% during the South West monsoon season and in the rest of year the relative humidity is between 65% and 75%. The average annual rainfall is between 2000 to 2200 mm.

Isolation and Identification of fungi

1g previously dried grounded soil samples was mixed with 10 ml sterile distilled water and diluted 10 to 10,000 times using sterile distilled water. 200 µl of each diluted sample was distributed on solid medium of Potato Dextrose Agar. Fungal colonies were selected and sub-cultured on the PDA plates and incubated at room temperature (28^{o}C - 31^{o}C) for 7 days until pure colonies were obtained. After 7 days four strains were isolated and used for degradation assays in triplicate (Fig.1).

The species isolated and used were *Aspergillus trreus* and *Rhizopus sp* from the tarball and *Penicillium sp* and *Aspergillus niger* from the soil (Fig.2). The identification of isolated strains was done as per the guidelines of Gilman and Watanabe (Gilman, 1998; Watanabe, 2002) and general principles of fungal classification.

Determination of the Fungi Hydrocarbon Growth Capability

Qualitative determinations of the fungi growing on hydrocarbon incorporated PDA media were determined by culturing of fungi. Four concentrations of oil-contaminated PDA media (5%, 10%, 15% and 20% (v/v) of kerosene) and one control PDA media (non-oil-contaminated) were prepared in twenty petri dishes and isolated strains were inoculated in each. All the petri dishes were then incubated at room temperature (28^{o}C - 31^{o}C) for 10 days. After 10 days, the control and radially growing colony were examined for the growth diameter. Similarly mixers of all the four species were used for the assay.

Confirmatory Test for Hydrocarbon Utilization Potentials of the Isolated Fungi

200 g non-polluted soil was sterilized by autoclaving. 50 g kerosene was added to this soil (20% w/w). Five glass bottles were sterilized and above contaminated soil was added equally to these bottles. An equal part of four isolated strains were separated and inoculated in each bottle. One without fungi was selected as control. The bottles were incubated at room temperature (28^{o}C - 31^{o}C) for 60 days. It is to be noted that the percentage of nitrogen and water holding capacity (WHC) of soil were 0.013 and 23.2 respectively.

Measurement of petroleum hydrocarbons in soil

After 60 days percentage of total oil and grease (TOG) in soil samples was measured according Environmental Protection Agency (EPA 9071A, 1994) and (EPA 3550B, 1996) using following formula.

$CR = IC - FC$; $PR = ((CR/ IC)*100)$ (1)

Bioremediation Rate (%) = $PR_{Fungi} - PR_{Control}$ (2)

Where *CR*=Concentration of Remediate TOG (%), *IC*=Initial Concentration of TOG (%), *FC*=Final Concentration of TOG (%), *PR*=Percentage of Remediate TOG.

Statistical Analysis

For interpretation of data, analysis of variance (one-way ANOVA test) was carried out using statistical software.

RESULTS AND DISCUSSION

The growth rate of each fungus shows that *Rhizopus sp.* had the highest growth diameter in 5% kerosene contaminated PDA media culture and *Aspergillus niger* had the highest growth diameter in 20% kerosene while *Penicillium sp* had the lowest growth rate at all the concentrations.

The similar observation reported by Adekunle and Adebambo, (2007) in which the isolated *Rhizopus* species from the seed of *Detarium Senegalense* (J. F Gmelin) showed the highest ability to degradation of kerosene amongst *Aspergillus flavus*, *Aspergillus niger*, *Mucor* and *Talaromyces.*

Four isolated strains were capable to grow in polluted PDA media and utilized kerosene as sole carbon source.

Table 1: Average growth diameter of fungi strains in Oil- contaminated PDA media culture after 7 days.

Concentration of Kerosene / *Microorganism*	*Growth Diameter/cm*				
	Control	**5%**	**10%**	**15%**	**20%**
	4.20	2.50	2.00	1.80	1.50
Rhizopus sp	4.80	3.50	2.70	2.20	0.90
	3.80	3.50	2.80	2.20	0.90
Mean	**4.27**	**3.17**	**2.50**	**2.07**	**1.10**
SD	**0.50**	**0.58**	**0.44**	**0.23**	**0.35**
	4.60	2.60	3.40	3.70	3.70
Aspergillus niger	3.90	2.20	3.00	3.80	3.00
	3.50	2.80	2.60	3.90	3.80
Mean	**4.00**	**2.53**	**3.00**	**3.80**	**3.50**
SD	**0.56**	**0.31**	**0.40**	**0.10**	**0.44**
	2.10	2.00	3.00	1.60	0.30
Aspergillus terreus	2.70	1.50	1.50	1.60	0.60
	2.70	2.70	1.50	0.20	0.60
Mean	**2.50**	**2.07**	**2.00**	**1.13**	**0.50**
SD	**0.35**	**0.60**	**0.87**	**0.81**	**0.17**
	1.00	1.00	0.50	0.20	0.00
Penicillium sp	1.10	0.90	0.80	0.50	0.40
	0.90	1.10	0.20	0.70	0.00
SD	**1.00**	**1.00**	**0.50**	**0.47**	**0.13**
Mean	**0.10**	**0.10**	**0.30**	**0.25**	**0.23**

A study by (*Wemedo et al.,* 2002) also recorded that the genera of fungi such as *Penicillium*, *Aspergillus* and *Rhizopus* are associated with kerosene-polluted soil. Oboh *et al*., (2006) reported all of these isolates were able to grow on crude petroleum as the sole source of carbon and energy when screened for hydrocarbon utilization.

As can be seen from Fig. 3 the growth diameter of fungus decreased with increasing kerosene concentration except for *Aspergillus niger* in which the growth diameter of colony was increased by raising kerosene concentration. *Rhizopus sp* shows gradual decrease in growth diameter as compared to control while other species show slight changes in the diameter of colony growth, indicating higher degradation capacity (Fig. 4). Table 1 shows that at the low concentration of kerosene contamination the highest bioremediation activity belongs to *Rhizopus sp* and at the high concentration of kerosene contamination it belongs to *Aspergillus niger*.

The mean values and standard deviation for the concentration of remediated TOG for each fungi are presented in (Table 2).The statistical result of ANOVA test shows that at the 99% confidence level, the concentrations of remediate TOG for each four strain are significantly different from other fungus and control too (p value=0.00 & <0.01). As can be seen from Table 3 the mix culture consisting of *penicillium sp*, *Rhizopus sp* and *Aspergillus terreus* shows highest growth diameter and that consisting of *Penicillium sp*, *Aspergillus niger* and *Aspergillus terreus* Shows lowest growth diameter. In all petri dishes the highest growth diameter was around the *Rhizopus* colonies indicating good ability to degrade kerosene among the other isolated strains. The growth diameters of fungi in mixed status were less than single status (Fig. 5). Okerentugba and Ezeronye (2003) in related study on single and mixed culture fungi have reported similar finding. Bioremediation with mixed culture showed better result due to favourable factors such as oxygen, nutrient, pH, temperature and water for successful bioremediation in accordance with that of Obire and Putheti, 2009).

Table 2: Bioremediation Rate of Kerosene by Fungi.

Concentration Of TOG / *Microorganism*	*Initial concentration of TOG (IC) (%)*	*Final concentration of TOG (FC) (%)*	*Concentration of Remediated TOG(CR) (%)*	*percentage of Remediated TOG (PR)*	*Bioremediation Rate (%)*
		4.70			
control	20	4.59			
		4.35	15.45	77.25	0
Mean		4.55			
SD		0.18			
		0.40			
Aspergillus niger	20	0.44			
		0.49	19.56	97.8	20.55
Mean		0.44			
SD		0.05			
		0.55			
Rhizopus sp	20	0.56			
		0.56	19.44	97.20	19.95
Mean		0.56			
SD		0.01			
		0.93			
Aspergillus terreus	20	1.00			
		1.35	18.91	94.55	17.30
Mean		1.09			
SD		0.23			
		1.00			
Penicillium sp	20	1.29			
		1.50	18.74	93.70	16.45
Mean		1.26			
SD		0.25			

Table 3: Average growth diameter of mixed fungi strains in Oil- contaminated (10%) PDA media culturer after 7 days.

Mixed Culture	*Growth Diameter / cm*				
	R+At+An	*P+An+At*	*P+R+At*	*P+R+An*	*P+R+An*
culture 1	0	0.25	1.5	0.5	1
culture 2	0	0.2	1	0.5	1.5
culture 3	0	0.3	2.00	0.5	0.50
Mean	0	0.25	1.5	0.5	1
SD	0	0.05	0.5	0	0.5

An. *Aspergillus niger*, **At**. *Aspergillus terreus*, **P**. *Penicillium sp*, **R**. *Rhizopus sp*

These results indicate potential application of native isolated fungi for hydrocarbon bioremediation activity. The ability of four isolated fungi for bioremediation of petroleum hydrocarbons has been previously reported by various research workers (April *et al.,* 2000; Chaillan *et al.,* 2004; George-Okafor *et al.,* 2009; Al-Ghamdi, 2011; chaudary *et al.,* 2012).

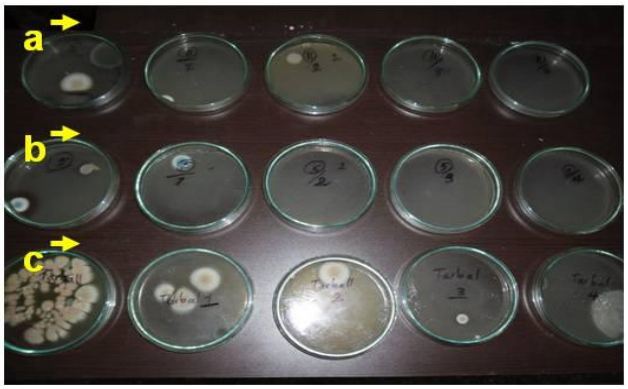

Fig 1: Isolation of fungi from soil and tarball samples **a**. *Aspergillus niger*, **b**. *Penicillium sp*, **c**. *Aspergillus terreus* & *Rhizopus sp*

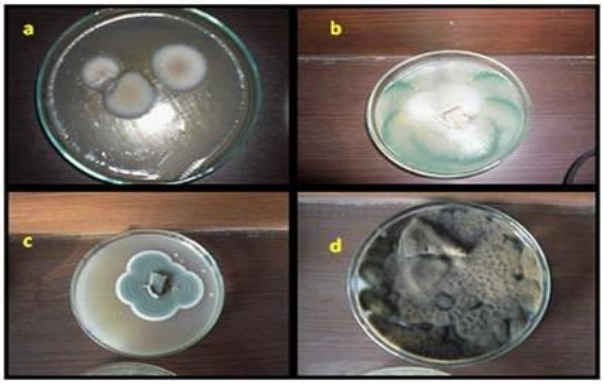

Fig 2: Pure colony of fungi from soil and tarball samples ***a***. *Aspergillus terreu,* ***b***. *Rhizopus sp,* ***c***. *Penicillium sp,* ***d***. *Aspergillus niger*

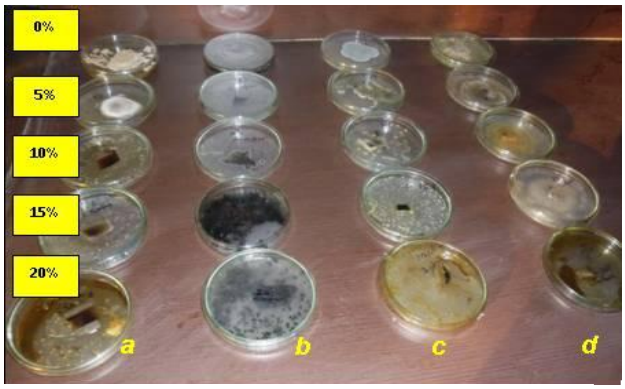

Fig 3: Growth determination of the fungi hydrocarbon growth capability in different concentration of kerosene a. *Aspergillus terreus*, b. *Aspergillus niger*, c. *Penicillium sp*, d. *Rhizopus sp*

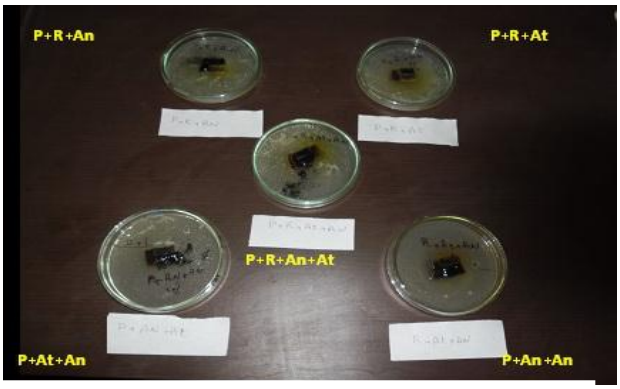

Fig 5: Effect of fungi mixed culture on kerosene biodegradation
An. *Aspergillus niger*, **At**. *Aspergillus terreus*, **P**. *Penicillium sp*, **R**. *Rhizopus sp*

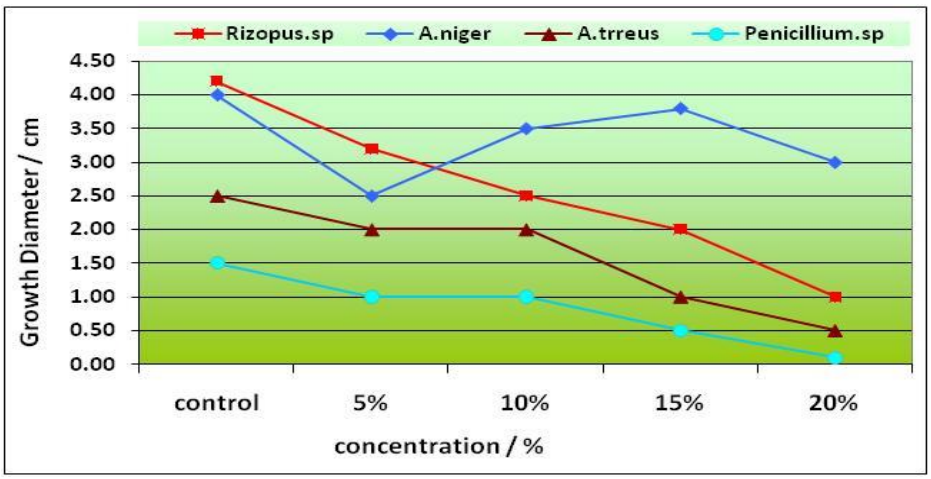

Fig 4: The Growth pattern of fungi in oil-polluted PDA media culture

CONCLUSION

All the strains isolated from the soil and tarball were capable of consuming kerosene as a sole carbon source. The highest remediation rate belongs to *Aspergillus niger* (20.55%) and the lowest rate belongs to *Penicillium sp* (16.453%). The results show that all the four isolated strains are capable to grow in PDA media that were contaminated with kerosene and utilize that as a sole carbon source.

The growth diameter of fungus decreases with increasing kerosene concentration except *Aspergillus niger* in which the growth diameter of colonies were increased with increasing kerosene concentration. In case of pollution with 5% concentration of kerosene the best bioremediation activity was found in *Rhizopus sp* and in case of 20% concentration due to *Aspergillus niger*. All isolated fungi can be used in hydrocarbon bioremediation activity but the potential of the fungal strains varied within the species that indicates the degradation capability alter according to the fungi. The mix culture consisting of *penicillium sp*, *Rhizopus sp* and *Aspergillus terreus* showed highest growth diameter.

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