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Isolation and Characterization of Naphthalene and Pyrene Bio-remediating Soil Fungi

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A B S T R A C T

Microbes play crucial role in bioremediation of hydrocarbon contaminated soil and solid waste. In this study, poly-aromatic hydrocarbons resistant fungi were isolated from soil samples of different areas including Delhi-NCR and Meerut city (Uttar Pradesh), the bioremediation of hydrocarbons like naphthalene and pyrene by these isolates were characterized to evaluate their applicability for poly-aromatic hydrocarbon remediation from contaminated soil. The optimum pH and temperature conditions for growth and hydrocarbon degradation were determined for isolates. Out of twenty seven fungal isolates only twelve showed potentials for degradation of poly-aromatic hydrocarbon. The selected fungal isolates were further characterized on the basis of their degrading capacity in medium inoculated with 1% naphthalene and pyrene with different carbon sources, nitrogen sources, temperature, salinity and hydrogen ion concentration.

Introduction

Poly aromatic hydrocarbons (PAHs) are hydrophobic organic compounds that can enter terrestrial and aquatic ecosystems in a number of ways. These methods of introduction include incomplete combustion of fossil fuel or other organic matter; coal gasification and liquefaction; spilling of hydrocarbons and oils; and oil seepage and surface run-offs resulting from forest fires and other natural geological processes (Wilson and Jones 1993; Yuan *et al.,* 2000). The soil contaminated by poly-aromatic hydrocarbon requires a tough practice to overcome from that condition. Industrialization and urbanization has caused hazard to soil (Jarerat and Tokiwa, 2000), in such a manner that it loose its fertility, to re fertile such land needs a lot of expenses, but using microorganisms it could be economical.

The cleaning of poly-aromatic hydrocarbon from water bodies and solid waste is done by various microbial species (Atlas, 1981). There are several factors which contribute to or affect the rate of degradation of hydrocarbons in a soil including temperature, physical and chemical nature of the pollutant, composition of the hydrocarbons, bioavailability of the substrates and microbial population with their types (Ogbo *et al.,* 2006). Although pesticides are hydrocarbon pollutants of the soil, the main source of hydrocarbon pollution are the spills and leak of petroleum products(Potter, 1993).

Various pollutants or hazardous compounds including, polycyclic aromatic hydrocarbon (PAH), pentachloro-phenol's (PCP), polychlorinated biphenyls (PCB), benzenes, toluene and ethyl benzene are persistent in the environment and are well known to have carcinogenic or mutagenic effects (Allard and Nelson, 1997). White-rot fungi such as *Phanerochaete chrysosporium, Pleurotus ostreatus, P. tubes-regium, P. Pulmonarius, Lentinus* and *Aspergillus,* have been used in the bioremediation of polluted soil and bioaccumulation of heavy metals (Watanabe *et al.,* 2001). Their biochemical persistence in the environment arises from dense clouds of π-electrons on both sides of the ring structures, making them resistant to nucleophilic attack (Jonsen *et al.,* 2005). They are ubiquitous contaminants of aquatic and terrestrial ecosystems whose presence is attributed to a number of petrogenic and pyrogenic sources, which had increased since the end of the Second World War (Jonsen *et al.,* 2005).

Previously, studied using animals have shown the specific carcinogenic, mutagenic and teratogenic effects of some PAHs (Autrup, 1990). Even though higher molecular weight PAHs such as those containing benzene rings are considered to be responsible for the majority of the potential hazards of these compounds to the environment and human health (EPA, 1984). Despite of this some physical process such as volatilization, leaching, chemical and photo oxidation are often effective in reducing the environmental level of PAHs (Heitkamp *et al.,* 1988), biodegradation using microorganisms is usually the preferred and major route of PAH removal from contaminated environments because of its cost effectiveness and complete cleanup (Pothuluri and Cerniglia, 1994).

Previous studies have focused extensively on the distribution, fates and biodegradation of PAHs in the terrestrial and marine environments. Several different bacteria have been identified as PAH –degraders, including species of *Pseudomonas* (Ahn *et al.,* 1998; Chen and Aitken 1999), *Alcaligenes* (Weisenfels *et al.,* 1990), *Rhodococcus* (Walter *et al.,* 1991), *Burkholderia* (Juhasz *et al.,* 1997), *Sphingomonas* (Ye *et al.,* 1996; Ho *et al.,* 2000). Even though most fungi cannot utilize PAHs as their sole carbon and energy source, they can co-metabolically transform PAHs into detosified metabolites (Sutherland 1992). The representative PAH-degrading white rot fungi are species of *Phanerochaete* (Barclay *et al.,* 1995), *Pleurotus* (Bezalel *et al.,* 1996) and *Trametes* (Collins *et al.,* 1996).

Fungal species like *Trametes versicolar* (Khadrani *et al.,* 1992), *Pleurotus ostreatus* (Sasek *et al.,* 1998), *Aspergillus* (Fields *et al.,* 1999), *Ganoderma lucidium* (Ting and Yuan, 2011) have been implicated in hydrocarbon remediation. The advantages associated with fungal bioremediation technologies (such as incineration, thermal desorption, extraction) (Ishii *et al.,* 2007). The use of fungi is expected to be relatively economical as they can be grown on a number of inexpensive agricultural or forest wastes such as corncobs and sawdust (Cook *et al.,* 1981). Moreover, their utilization is a gentle non-aggressive approach. The application of bioremediation

capabilities of indigenous organisms to clean up pollutants is viable and has economic values (Bikiaris *et al.,* 2006).

The application of bioremediation capabilities of indigenous organisms to clean the pollutants is viable and has economic values (Walter *et al.,* 1991). The utilization of myco remediation is gentle economic and non-violent approach. The objective of this study were therefore to isolate some of the fungal flora from poly aromatic hydrocarbon (PAH) contaminated sites and evaluation of their bioremediation properties as well as to optimize the degradation conditions for better degradation (Lamaire *et al.,*1992).

Materials and Methods

Sources of soil sample

The seven PAH contaminated solid waste samples were collected for isolation from different sites located at Ghazipur (GZ), Noida industrial area (NIA), Ghaziabad industrial area (GIA), Meerut (ME), Okhla industrial area (OIA), Modinagar sugar mill (MSM) and Modinagar effluents (M) for the isolation of fungi, samples from each sites were collected in sterile poly bags and blue cap bottles 100 ml, the collected samples were kept in refrigerator at 4˚C until isolation and processing.

Isolation of the poly-aromatic hydrocarbon degrading fungi

1 g of collected soil samples were homogenously mixed with 100 ml of distilled water and carefully stored at room temperature for 24 h, to settle the debris in the pellet. After 24 h the supernatant was filtered through 2.5 mm sieve filter for the further removal of debris. The moisture content of filtered soil was removed by using hot air 50˚C for 40 min. 1gram of each soil sample was mixed in 100 ml of distilled water for the serial dilution of each sample upto five times, then from third dilution, 1 ml of each sample was spread on streptomycin (10µg/ml) containing SDA plates for isolation of fungi for 5 days at 28˚C in an incubator. The fungal colonies were separated and sub cultured aseptically by sterilized 2mm cork borer. The pure cultures were then transferred on SDA slants at 4˚C for further studies.

Primary screening of poly-aromatic hydrocarbon degrading fungi

For the screening of PAH degrading fungi from all fungal isolates the method described by Sundman and Nase (1971) was used. Isolated fungi for polyphenol oxidase activity, for this the fungal isolates were inoculated on Czapek dox agar contained 10% tannic acid and incubated for 15 days at 25˚C. The positive activity indicated brown colour of medium around the fungal colony (Kleeberg *et al.,* 2005).

Secondary screening of poly-aromatic hydrocarbon degrading fungi

For the screening of hydrocarbon degrading fungi, the polymers naphthalene and pyrene were used as referenced PAH. 1g each referenced PAH was mixed in 100 ml chloroform and benzene separately. This mixture was then poured into 1000 ml of distilled water containing $CaCL_2.12H_2O$ (0.01g), $NaHPO_4$ (3 g), $MgSO_4$ (0.15g), KH_2PO_4 (1.5g) and pH 6.5 as test broth control broth did not have naphthalene and pyrene.

Optimization of cultural conditions of selected fungal isolates for better degradation

Best PAH degrading fungi were further optimized for different parameters such as

tested different carbon sources, nitrogen sources (organic and inorganic), different temperature, pH and NaCl concentration against the culture condition of the degradation media.

The five carbon sources including glucose, fructose, sucrose, maltose and dextrose were used for the optimization of culture conditions. There were two types of nitrogen sources were included in this study which were organic (beef extract, yeast extract and peptone) and inorganic (Ammonium sulphate, ammonium nitrate and ammonium chloride). Temperatures used were 4^0C, 28^0C, 37^0C and 50^0C, whereas optimized pH was 4.0, 6.5, 7.0 and 9.0. Tested NaCl concentrations were 0.5g ml^{-1}, 1.0g ml^{-1}, 1.5g ml^{-1}, 2.0g ml^{-1}.

1g each different carbon sources were mixed in 100 ml of emulsified test broth as well as in emulsified control broth separately, whereas the other constituents were remained same in both tested and control broth. The broth containing flasks were then incubated for 7 days at 28˚C in an incubator, after incubation the spectrophotometery was done at OD_{600}against distilled water for observation of PAH degrading capacity of fungal isolates.

For the optimization of broth, 1g each nitrogen source was mixed separately in the standard broth as well as in control, other constituents were remained same. The broth was then incubated for 7 days at 28^0C in an incubator. Then specrtophotometery was done at OD_{600}against distilled water for observation of PAH degrading capacity of fungal isolates.

For the optimization of broth conditions different pH and NaCl concentrations were set accordingly. The nitrogen source used for the optimization was organic nitrogen including beef extract, peptone and yeast extract while for inorganic nitrogen source ammonium nitrate, ammonium sulphate and ammonium chloride. The combined carbon and nitrogen sources for optimization of fungal isolates. The pH variations from acidic to alkaline used for optimization of fungal isolates.

The salt concentration also effect the degrading capacity of fungal isolates for optimization the NaCl concentration was varied from 0.5%, 1.5%, 1% and 2%. The temperature optimization of fungal isolates was done by keeping the broth flask containing the above parameters were kept at different temperatures varying from 4˚C, 37˚C, 50˚C. The % of biodegradation was measured by amount of control and treatment difference and fraction by control.

% Biodegradation

$$= \frac{(O.D.control - O.D.treatment) \times 100}{O.D.control}$$

Results and Discussion

The total twelve isolates were initially obtained from twenty seven cultures out of which the two potentially active isolates were SWME1 and SWGZ1were best poly-aromatic hydrocarbon degraders.

The ability of these isolates to degrade the hydrocarbon was detected by spectrophotometer and the turbidity of broth in control and test were measured and compared for the degradation of hydrocarbon after inoculation of fungal isolates, this resulted the two best degraders which showed high change in optical density of broth the hydrocarbon presence make broth turbid after inoculation the flasks were incubated at 28˚C for 7 days, the optical density was measured before inoculation and after inoculation at 600 nm. The decrease in optical density showed degradation of poly-aromatic hydrocarbons naphthalene and pyrene separately.

Fungal growth and degradation of polyaromatic hydrocarbon

In the degradation of polymers by fungus, optimization of medium component and environmental factors is vital because they can significantly affect polymer degradation. The degradation of polymer was detected by the clear zone and spectroscopic methods. The presence of polymer decomposing microorganisms was confirmed by the formation of clear zones

Surrounding the colonies of such organisms on the turbid agar plates on which they were grown (Jarerat and Tokiwa, 2003). This happens when the polymer-degrading microorganisms excrete extracellular enzymes which diffuse through the agar and degrade the polymer to water soluble materials (Fields *et al.*, 1974). Nishida and Tokiwa (1993) pointed out that the clear zone technique is a powerful method forecological investigation of plastic degradation. In addition, the procedure has the advantage that any inoculum, from the soil suspension to a pure culture (Atagana, 2004; 2006) can be tested for its ability to degrade any PAH that can be ground into a fine powder and incorporated into an agar matrix. The degradation of PAH was detected by the spectroscopic methods in the liquid media through spectrophotometer.

Fig.1

Treatment of different carbon source on degradation of pyrene & naphthalene

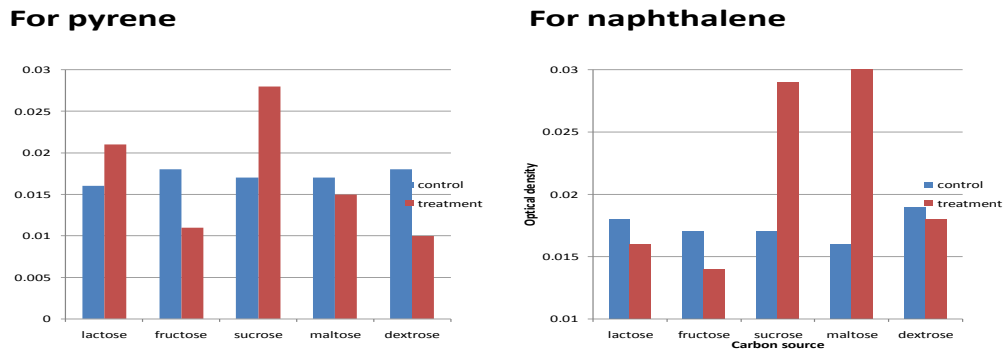


Fig.2

Treatment of different nitrogen source on degradation of pyrene and naphthalene

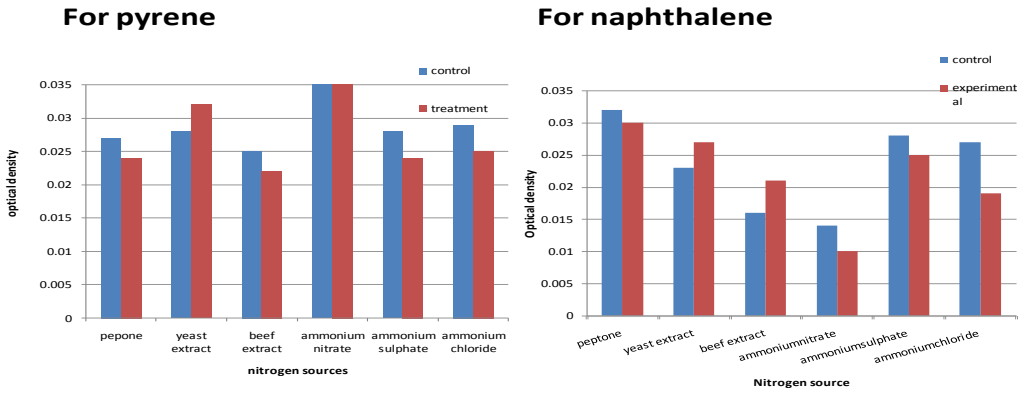


Fig.3

Treatment of different pH on degradation of pyrene and naphthalene

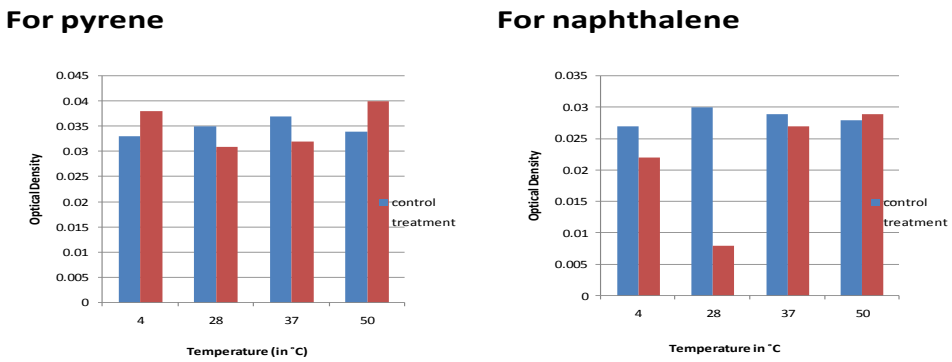


Fig.4

Treatment of different NaCl con. On degradation of pyrene and naphthalene

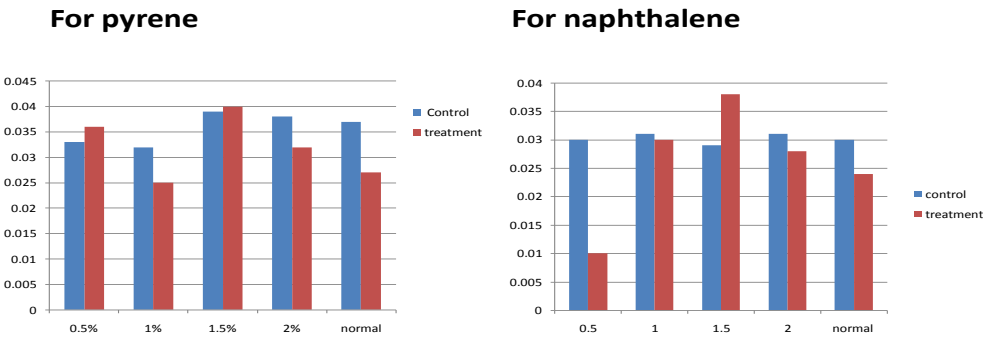
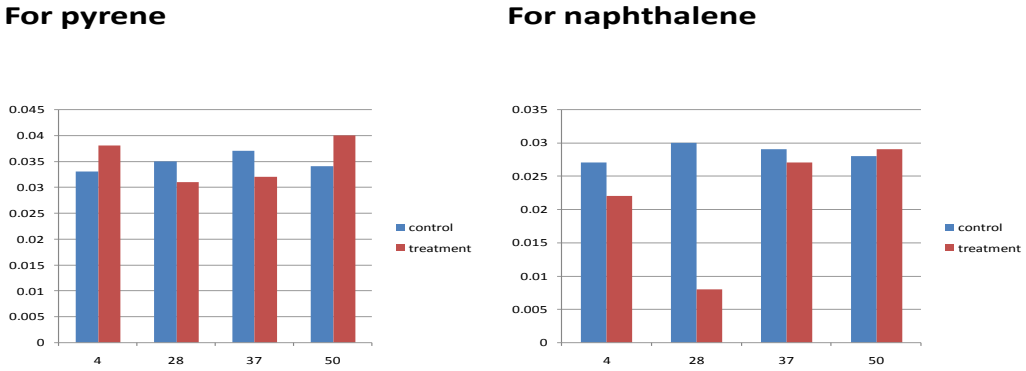


Fig.5

Treatment of different temperature on degradation of pyrene and naphthalene



Effect of different carbon sources on degradation of naphthalene and pyrene

The different carbon source was used out of which the best carbon source for naphthalene degradation was sucrose which shows 64.71% degradation in SWME1. For pyrenedegradation dextrose was found to be the best carbon source which showed 44% degradation in SWGZ1 (Fig. 1).

Effect of different nitrogen sources on degradation of naphthalene and pyrene

The different nitrogen sources were used out of which the best nitrogen source for naphthalene degradation was ammonium chloride which shows 30% degradation in SWME1. For pyrene degradation ammonium nitrate was found to be the best nitrogen source which showed 59% degradation in SWGZ1 (Fig. 2).

Effect of different carbon and nitrogen sources on degradation of naphthalene and pyrene

The different carbon and nitrogen source were used out of which the best carbon and nitrogen source for naphthalene degradation was dextrose and ammonium chloride which shows 43% degradation in SWME1. For pyrene degradation fructose and ammonium nitrate was found to be the best nitrogen source which showed 34% degradation in SWGZ1.

Effect of different pH on degradation of naphthalene and pyrene

The different pH was used out of which the best pH for naphthalene degradation was 6.5 which show 67 % degradation in SWME1. For pyrene degradation 4.5 was found to be the best pH which showed 27% degradation in SWGZ1 (Fig. 3).

Effect of different salinity on degradation of naphthalene and pyrene

The different salinity was used out of which the best salinity for naphthalene degradation was 0.5% NaCl which shows 67% degradation in SWME1. For pyrene degradation normal salinity was found to be the best salinity which showed 35% degradation in SWGZ1 (Fig. 4).

Effect of different temperature on degradation of naphthalene and pyrene

The different temperatures were used out of which the best temperature for napthalene degradation was 28^0C which showed 73% degradation in SWME1. For pyrene degradation 27^0C -30˚C was found to be the best temperature which showed14% degradation in SWGZ1 (Fig. 5).

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