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Identification and Characterization of Naphthalene Degrading Bacteria Isolated from Acid Mine Drainage Sample of Barjora Coal Mine, West Bengal, India

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Abstract: *Polycyclic aromatic hydrocarbons (PAHs) are a group of ubiquitous organic pollutants that are toxic as well as carcinogenic. Naphthalene is the well known PAH among recognized priority pollutants and some microorganisms use this PAH compound for bioremediation in the polluted environment. Three different types of mesophilic naphthalene degrading bacteria have been isolated from acid mine drainage soil of Barjora coal mine, West Bengal. They used naphthalene as a sole carbon source and they were able to grow in presence of pyrene, benzene and toluene. This result indicates that they are multi PAHs degrading bacteria. They all come under the genus Enterobacter sp. However, further research needs to be done on the identification of metabolites responsible for naphthalene degradation and metabolic pathway through GC-MS (Gas Chromatography- Mass Spectrometry), HPLC (High Performance Liquid Chromatography) analysis.*

Keyword: *Biodegradation, Naphthalene, Minimum Inhibitory Concentration (MIC), Enterobacter sp.*

I. Introduction

All substances originated into the environment either by biogenic or anthropogenic sources. Anthropogenic compounds describe synthetic compounds, and compound classes as well as elements and naturally occurring chemical entities which are mobilized by man's activities. These substances are released into the environment in amounts that are unnatural due to human activity. Anthropogenic inorganic and organic pollutants are dispersed throughout the atmosphere, hydrosphere and lithosphere, and they have tendency to transform into another compounds which may be toxic, less toxic and not toxic to flora and fauna. Polycyclic aromatic hydrocarbons(PAHs) are pollutant, often carcinogenic, mutagenic, or toxic, found in most terrestrial ecosystem that rise from industrial operations and from natural events such as forest fires[1,2].

Polycyclic aromatic hydrocarbons are ubiquitous contaminants in environments impacted by fossil fuels directly or by the combustion of fossil fuels. Due to their physical properties, namely low water solubility and high adsorption potential, they tend to accumulate in sediments. They have been identified in contaminated environments, their concentrations depending on distance from sources of industrial activity. They are of concern to human health because the four- and five-ringed compounds have been identified as genotoxicants in short-term mutagenicity assays and as carcinogens in long term rodent bioassays [7]. Sediments contaminated with polycyclic aromatic hydrocarbons (PAHs) are frequent consequences of industrialization in the Great Lake Regions, including Hamilton Harbour, Ontario [6], and the Grand Calumet River and Indiana Harbour, Indiana [9]. Due to the widespread distribution of PAHs in the Great Lakes, and their potential as a human health hazard, the remediation of PAH-contaminated sediments has assumed a high priority for regulatory agencies in the Great Lakes region. One of the most promising methods of removal of PAHs from contaminated environments is that of bioremediation [3].

Polycyclic Aromatic Hydrocarbons (PAHs) known as toxic contaminants of soil & aquifers and carcinogenic for humans are of great environmental concern. They are the group of chemical compounds, consists of numerous carbon atoms fused together to form multiple ring structure. These compounds are neutral non polar molecules, mainly found in fossil fuels. Most PAH compounds are insoluble in nature. The solubility of PAHs decreases as the molecular mass increases. For e.g. two and three ring PAHs are soluble in water so it is more available for biological uptake and degradation. In contrast four or five ring structure is insoluble in water and mainly present in solid form in the environment making it less accessible for biological uptake and degradation [4].

Coal represents a considerable portion of the total global fossil fuel reserve and continued demand for, and supply of this resource generates vast quantities of spoil and low grade waste. Large scale bioremediation technologies for the beneficiation of waste coal have unfortunately not yet been realized despite the many discoveries of microorganisms capable of lignite, lignin breakdown. Even so, solubilisation and de-polymerization of low grade coal appears to involve either ligninolytic enzyme action or the production of alkaline substances or both.

In recent years, there has been an increasing amount of literature on the removal of PAHs. One of the most widely used and promising technology to break down the pollutants is bioremediation, which uses selected microorganisms such as fungi, yeast, and bacteria. For the destruction of PAH contaminants, bioremediation is considered a better process than chemical or physical remediation processes because the technique is economical and efficient. Bacteria have been studied extensively, and there is information regarding their ability to degrade xenobitics, including PAHs. PAHs could be degraded by bacteria under aerobic conditions through the initial oxidation of the aromatic ring, which is catalyzed by the dioxygenase enzyme [5]. PAH resistance to oxidation, reduction, and vaporization increases with increasing molecular weight, whereas the aqueous solubility of these compounds decreases. As a result, PAHs differ in their behaviour, distribution in the environment, and their effects on biological systems. PAHs can be divided into two groups based on their physical, chemical, and biological characteristics. The lower molecular weight PAHs (e.g., 2 to 3 ring group of PAHs such as naphthalenes, fluorenes, phenanthrenes, and anthracenes) have significant acute toxicity to aquatic organisms, whereas the high molecular weight PAHs, 4 to 7 ring (from chrysenes to coronenes) do not. However, several members of the high molecular weight PAHs have been known to be carcinogenic [8].

Naphthalene (C10H8; MW = 128.16 g), formed from two benzene rings fused together, has the lowest molecular weight of all PAHs. The environmentally significant PAHs are those molecules which contain two (e.g., naphthalene) to seven benzene rings (e.g., coronene with a chemical formula C24H12; MW = 300.36 g). In this range, there are a large number of PAHs which differ in number of aromatic rings, position at which aromatic rings are fused to one another, and number, chemistry, and position of substituents on the basic ring system [6].

Here, investigation was carried out for isolation of few naphthalene degrading bacteria present in the open cast mining area of Barjora coal mine from Birbhum district, West Bengal, India. General morphological and biochemical characterization was carried out and growth curve was prepared. Molecular characterization was also undertaken. Their ability for other Polycyclic Aromatic Hydrocarbon (PAH) degradation was determined and for the identification of probable compounds responsible for these kinds of degrading activities.

II. Materials and Methods

1. Sample collection

Water samples were collected from Barjora Coal mine (Latitude: 23.887115 and Longitude: 87.579129), which is situated at Birbhum district of West Bengal. pH was checked of the soil samples.

2. Enrichment and Isolation of bacteria

Isolation of naphthalene degrading bacteria from soil sample of Barjora Coal Mine was carried out as followed by the method given in the paper of Roy *et al.,* 2012 with slightly modifications. Mineral salt medium [10] containing 10 mM naphthalene was prepared for enrichment and isolation. 1 ml of coal mine drainage water sample was inoculated in 25 ml of MSM broth supplemented with 10 mM naphthalene as the sole carbon and energy source, and incubated at 28˚C on a rotary shaker (180 rpm and) for 5 days. Then isolation of naphthalene degrading bacteria was subjected by serial dilution and spread plate followed streak plate method in MSM agar (2% agar w/v) medium. Naphthalene was added in the lid and was dissolved by adding Di-ethyl ether on the lid. Growth was checked after 48 hours incubation at 28˚C. Finally, three strains capable of growing in the presence of naphthalene as the sole carbon source (all are *Enterobacter sp*.) were selected for further analysis.

3. Morphological and biochemical characterization of bacteria

Morphological features of three *Enterobacter sp*. were studied by phase-contrast microscopy (). Conventional biochemical tests were performed using standard methods [5, 8, 12]. The 16S rRNA gene was amplified using universal bacteria-specific primers 27f and 1492r (Johnson, 1994). Levels of 16S rRNA gene sequence similarity were determined using BLAST version 2.2.12 of the National Centre for Biotechnology Information [3].

4. MIC (Minimal inhibitory concentration) determination

Minimal inhibitory concentration (MIC) also was checked of the isolates after getting growth in naphthalene at 10 mM concentration and it is the minimal concentration of naphthalene at which the growth of the micro organisms is inhibited. The naphthalene degrading isolates were grown in different concentrations of Naphthalene. MSM broth was prepared in 5 different conical and naphthalene is added in it, in five different concentrations such as 5 mM, 7 mM, 10 mM, 15 mM and 20 mM respectively. These broths were inoculated with the Naphthalene degrading isolates and incubated.

5. Growth in the presence of pyrene, benzene and toluene

The isolates were grown in the presence of other PAHs like pyrene, benzene, and toluene. MSM media was prepared and divided in separate conicals and marked according to the PAH added in it. 100µl of benzene and phenol added in respective conicals and pyrene was added in 0.5 mM concentration. The medium was inoculated with the naphthalene degrading isolates.

6. Growth curve in the presence of Naphthalene

Each isolates were inoculated in MSM media containing same concentrations of naphthalene in which they gave MIC. The growth of the bacteria in liquid medium was observed by the increasing turbidity of the medium. Growth was measured by taking the absorbance at 620 nm of the medium through UV-visible spectrophotometer. In the first day, reading was taken in every one hour interval after incubation up to 6 hours and from the next day readings were taken after every 24 hours i.e. 24, 48,72 and 96 hours. This absorbance was plotted on a graph paper against time to obtain a growth curve. Growth curve was done for each isolates in presence of naphthalene. These experiments were performed in triplicate and results were expressed as average values.

7. Indole test

Several enzymes, such as monooxygenases, dioxygenases, and cytochrome P450, were characterized for indigo production. For the indole test of the Naphthalene degrading bacteria replica plating was prepared. MSM media was prepared and plating was done in two petri plates, one with the presence of indole and the other with the absence of indole. All of the strains were streaked onto the plates by dividing the plate into two compartments .The plates were then sealed with parafilm and kept in a incubator at 30ºC for three days and observed by taking indole crystals on the lid of the petri plates for the formation of the colour indigo.

III. Results

Isolation and characterization of three bacteria

Using an enrichment culture technique, three naphthalene degrading bacteria were isolated from the drainage water sample of Barjora coal mine. Cells of all three strains were Gram-negative rods, small in size and formed milky white colonies on Luria-Bertani agar plates. *Enterobacter* is a genus of Gram negative, facultative anaerobic, rod shaped, non spore forming bacteria of the family *Enterobacteriaceae*. All strains were mesophilic as the temperature limits for growth were 20-40ºC with an optimum between 30-35ºC. The 16S rRNA gene sequences of the three isolates were determined and they all showed the highest similarity with *Enterobacter sp.*

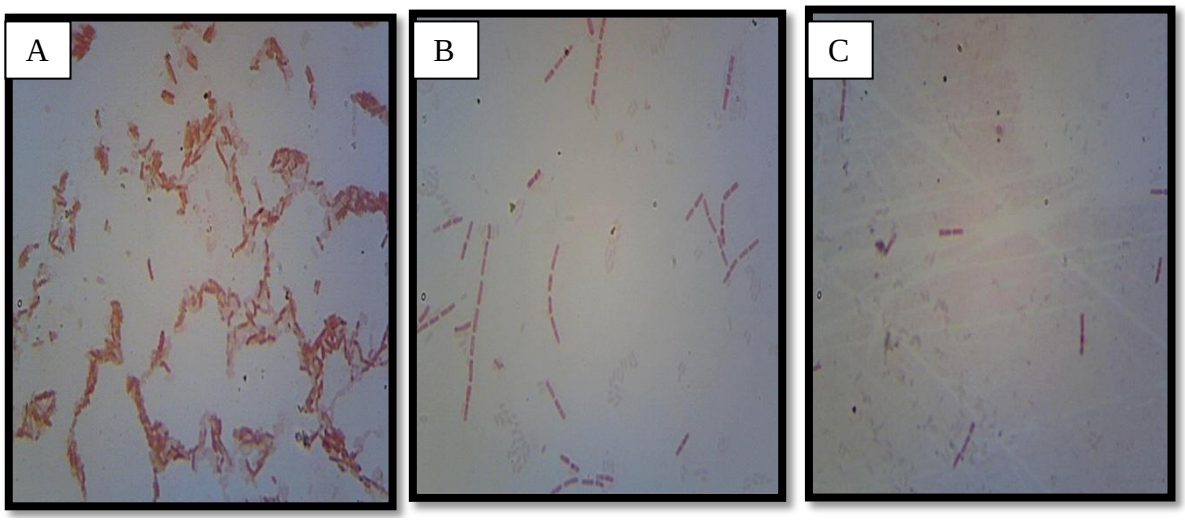

Fig.1. A- Gram staining showing gram negative and rod shaped bacterium in cluster under microscope (NP1). B- Gram staining showing gram negative and straight rod shaped bacterium under microscope (NP2). C- Gram staining showing gram negative and straight rod shaped bacterium under microscope (NP3).

Biochemical characterization

Strain NP1 and NP2 gave positive result in citrate utilization test, catalase test and urease test where as strain NP2 showed positive result in indole test, voges-proskauer test, citrate utilization test, starch hydrolysis, oxidase test, urease test and Test for H_2S production.

Sl. No	Biochemical tests	NP1	NP2	NP3
1.	Indole test	Negative	Positive	Positive
2.	Methyl red test	Negative	Negative	Negative
3.	Voges-proskauer test	Negative	Positive	Negative
4.	Citrate utilization test	Positive	Positive	Positive
5.	Starch hydrolysis	Negative	Positive	Negative
6.	Catalase test	Positive	Negative	Positive
7.	Oxidase test	Negative	Positive	Negative
8.	Urease test	Positive	Positive	Positive
9.	Test for H_2S production	Negative	Positive	Negative
10.	Fermentation test			
	Glucose	Acid	Acid	Acid
	Fructose	Acid	Acid	Acid
	Sucrose	Acid	Acid	Acid

Table.1. Results of biochemical characterization has shown in the above table.

All three strains were able to grow in presence of pyrene, benzene and toluene. Every isolates showed turbid growth in MSM media containing naphthalene. Strain NP1 indicated MIC at 0.5 mM concentration of pyrene and MIC of pyrene of NP2 and NP3 was 0.75 mM as they showed turbid growth at 0.75 mM concentration of pyrene. This result indicates that all three Naphthalene degrading bacteria can also degrade others PAHS like pyrene, benzene and toluene.

MIC of naphthalene

MIC of naphthalene was determined in case of all three strains in five different concentrations. The Minimum Inhibitory Concentration (MIC) of NP1 and NP2 in presence of naphthalene was 15mM whereas; MIC of NP3 in presence of Naphthalene was 10mM.

Growth curve

Growth curve of naphthalene of three strains were prepared at the same concentration of naphthalene in which the isolates gave MIC. Here positive control was kept where growth was observed in Luria-Bertani medium without naphthalene. This is a comparative study of the growth of three strains in presence of naphthalene with respect to growth in Luria-Bertani medium. Different strain took different time to reach exponential phase. NP3 showed maximum growth in comparison with other strains. NP1 showed minimum growth. Lag phase remains for almost 8 hours. After 8 hours the optical density increased that mean the growth shift toward log/exponential phase. It almost took near about 48 hours to reach stationary phase.

The growth curve is given in the following graph (fig.3).

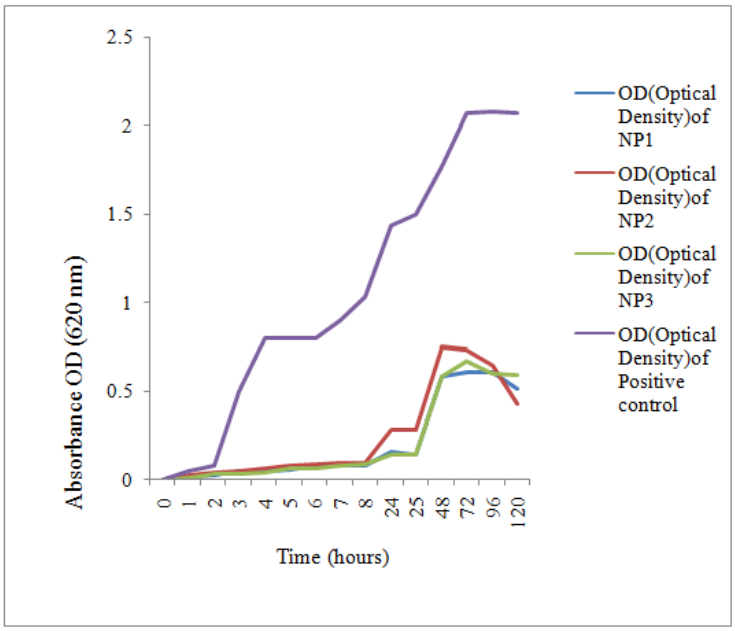

Fig.3. Growth curve was plotted of all three strains in presence of naphthalene (concentrations of naphthalene was the MIC values of the strains) at different time interval. Here, X axis is indicating time (hours) and Y axis is showing readings of OD (Optical Density) at 620 nm.

Indole test

Indole test was performed to check the presence of di-oxygenase or mono-oxygenase enzymes in the bacteria which degrade PAH compounds. NP2 bacterium gave blue colour which means blue coloured colonies were formed in the petri plate after incubating with indole. This result indicates that the di-oxygenase enzyme is present in NP2 bacterium. This enzyme degrades indole and gives blue colour. The other two strains i.e. NP1 and NP3 did not give blue colour which confirms that di-oxygenase enzyme is not present to these two bacteria and they have mono-oxygenase.

IV. Discussion

It can be concluded that three different naphthalene degrading bacteria have been successfully isolated from acid mine drainage soil of Barjora coal mine, West Bengal, which is considered a suitable source of PAH degrading bacteria. All strains were mesophilic and non spore forming bacteria. Based on almost complete sequence of the 16S rRNA gene, strain NP1, formed phylogenetic cluster with *Enterobacter hormaechai* and *Enterobacter cancerogenus* with 94% similarities. NP2 formed phylogenetic cluster with *Enterobacter sp* and *Kocuria rosea* and 92% similarities were observed with these two species. Strain NP3 formed phylogenetic cluster with *Enterobacter hormaechai* and *Enterobacter sp.* which showed 96% similarities. It was shown that all three strains utilizes naphthalene in MSM as the sole of carbon and energy, as established from its growth yield and complete substrate (naphthalene) removal. However, further research needs to be done on the identification of metabolites responsible for naphthalene degradation and metabolic pathway through GC-MS (Gas Chromatography- Mass Spectrometry), HPLC (High Performance Liquid Chromatography) analysis.

V. Conclusion

Three different types of mesophilic naphthalene degrading bacteria have been isolated from acid mine drainage soil of Barjora coal mine, West Bengal. They used naphthalene as a sole carbon source and they were able to grow in presence of pyrene, benzene and toluene.

Reference

[1] Mrozik, Z. P. (2003). Bacterial Degradation and Bioremediation of Polycyclic Aromatic Hydrocarbons. *Polish Journal of Environmental Studies*, 15-25.

[2] Haritash, C. K. (2009). Biodegradation Aspects of Polycylic Aromatic Hydrocarbons (PAHs): A review. *Journal of hazardous materials*, 1-15.

[3] Altschul, S. F., Gish, W., Miller, W., Myers, E. W. & Lipman, D. J. (1990). Basic local alignment search tool. J Mol Biol 215, 403–410.

[4] Cerniglia, C. E. (1992). Biodegradation of polycyclic aromatic hydrocarbons. Biodegradation 3, 351–368.

[5] Diane, D. Murphy, S. M.-Y. (2000). Synucleins Are Developmentally Expressed, and α-Synuclein Regulates the Size of the Presynaptic Vesicular Pool in Primary Hippocampal Neurons. *The Journal of Neuroscience*, 20(9): 3214-3220.

[6] DK., N. (2000) Advances in the hydrogeochemistry and microbiology of acid mine waters. *Int. Geol. Rev.* 42, 499–515.

[7] HARAYAMA, R. A. (2000). Biodegradation of High-Molecular-Weight Polycyclic Aromatic hydrocabons by bacteria. *Journal of bacteriology*, p. 2059–2067.

[8] John Menzies, R. J. (2006). Accuracy of Neural Network and Regression Leaf Area Estimators for the Amazon Basin . *GIScience & Remote Sensing*, 43(4)1-11.

[9] Roy, M., Khara, P. (2012). meta-Cleavage of hydroxynaphthoic acids in the degradation of phenanthrene by Sphingobium sp. strain PNB. *Microbiology*, 158, 685–695.

[10] Mallick, S., Chatterjee, S. & Dutta, T. K. (2007). A novel degradation pathway in the assimilation of phenanthrene by Staphylococcus sp. strain PN/Y via meta-cleavage of 2-hydroxy-1-naphthoic acid: formation of trans-2,3-dioxo-5-(29-hydroxyphenyl)-pent-4-enoic acid. Microbiology 153, 2104–2115.

[11] Michael, A., Heitkamp, W. F. (1988). Microbial Metabolism of Polycyclic Aromatic Hydrocarbons: Isolation and Characterization of a Pyrene-Degrading Bacterium. *Applied and Enviornmental Microbiology* , 2549-2555.

[12] Yingfei, M., L. W. (2006). Pseudomonas. *Envi* , 23.