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Biodegradation of Pyrene by Bacterial Strain Isolated from Petroleum Sludge Collected from Barauni Oil Refinery, Begusari, Bihar (India)

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The purpose of present microcosmic study was to investigate the degradation of major PAHs of petroleum sludge i.e. Pyrene (4 aromatic rings) by petroleum hydrocarbon degrading bacteria isolated from petroleum sludge collected from Barauni Oil Refinery; Begusari, Bihar (India). Changes in pH, bacterial biomass and their protein amount were also observed during the degradation of selected Pyrene (100 ppm). The outcome of this microbiological work can be used for research on remediation of petroleum-contaminated environments.

Key words: Biodegradation, Barauni, Polycyclic aromatic hydrocarbon, Pyrene.

Many polycyclic aromatic hydrocarbons (PAHs) are known to be toxic and carcinogenic for humans, and their contamination of soils and aquifers is of great environmental concern. Petroleum oil and petrochemicals include significant amount of polycyclic aromatic hydrocarbons (PAHs), which are listed as priority pollutants by the US EPA [1]. Petroleum contaminated soil contains hydrocarbon mixtures, among which PAHs, are considered as a major environmental threat because of their potential for toxicity, mutagenicity and carcinogenicity. Due to large holding capacity for pollutant, soil acts as a long term sink and major repository of PAHs in the environment[2]. PAHs can also be emitted to the environment through anthropogenic sources mainly involving combustion of fossil fuels. PAHs can also be introduced to the environment through natural sources such as volcanic eruptions and forest fires. In addition, smoked food or weathering of petroleum can also result in the formation of hydrocarbons and other byproducts[3-4].

Petroleum is a naturally occurring flammable liquid processed by many oil refineries. Oily water from refining processes and surface water run-off (pretreated in oily separators) settles down by gravity to form oily sludge[5]. Sludge contains a complex mixture of petroleum hydrocarbons of various molecular weights. Crude oil could be classified according to respective distillation residues as paraffins -30%, napthelena-49% , aromatics-15%, asphaltics-6% (consist little paraffin wax and asphaltics residues. Hydrocarbons include two groups' i.e. aliphatic and aromatic compounds. The aromatic compounds are further classified into following:

Poly aromatic hydrocarbons (PAH)

They are non-polar compounds made up of 2 or more fussed aromatic rings, arranged in linear, angular or clustered structures. They are

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hydrophobic in nature with low water solubility. Due to their hydrophobic nature, most PAHs bind to particulates in soil and sediments and hence result in low bioavailability for biological uptake[6].

Low molecular weight PAHs

They contain more than 3 aromatic rings are high molecular aromatics. They are mutagenic and carcinogenic in nature, thus considered as genotoxic. For example: fluoranthene, phenanthrene, pyrene, benzo[a] pyrene.

Pyrene is a tetracyclic aromatic hydrocarbon with a symmetrical structure which is one of the top 129 pollutants as ranked by the U.S Environmental Protection Agency (USEPA). Pyrene is not a genotoxic compound by itself but it has four aromatic cores that are found in several PAH carcinogens, including benzopyrene, indeno (1,2,3-cd) pyrene and 1-nitropyrene[7].

The aim of our paper is to describe the ability of bacterial strain from petroleum sludge collected from Barauni Oil Refinery; Begusari, Bihar (India), to degrade Pyrene.

MATERIALS AND METHODS

Chemical

Aromatic hydrocarbons Pyrene were purchased from Sigma pharmaceutical Ltd., India.

Sample collection

To isolate Pyrene degrading bacteria, petroleum sludge was collected from Barauni Oil Refinery, Begusari, Bihar. The collected samples were poured into sterile polythene bags, stored at 4 °C and transferred to the laboratory within 24 h [8].

Isolation of pyrene-degrading bacteria

Pyrene utilizing bacteria were isolated by following serial dilution method using NA (nutrient agar 1L): 10g peptone 10g beef extract, 5g sodium chloride and 12g agar(pH=7.3) plate[9]. Enrichment and isolation of oil-degrading bacterial cultures were done by using mineral salts medium(0.1% KH_2PO_4, 0.05%$MgSO_4$, 0.05%NH_4Cl, 0.05%KNO_3, containing 0.1%C_OCl_2, 0.01%H_3BO_3, 0.02% folic acid, 0.1% pyridoxine-HCL, 0.05% thiamine HCL, 0.05% riboflavin, 0.05% nicotinic acid, 0.05% pentothenic acid, 0.01% cyanobalamine) with a Pyrene as sole carbon source[10]. The isolated bacterial strains coded BB and further bio-chemically characterized and tested for the Pyrene degradation.

Identification of pyrene-degrading bacteria

The strain was tested for some cultural, morphological and biochemical properties and the results were compared with Bergey's Manual of Systematic Bacteriology classification[11].

Pyrene degradation assay

250ml Erlenmeyer flasks containing 100ml of mineral-salts medium with (100ppm) Pyrene was taken for performing the experiment. The experimental flasks were inoculated with 2% inoculum (1×10^5 CFU/ml) and incubated in a shaker at 200 rpm at 30 C for 30 days. This experimental study continues for 30 days.

Bacterial growth analysis

For bacterial growth analysis at every 1 week intervals 1ml of sample from flask was used for the enumeration of the microbial population by pour plate technique on plate-count agar.

Analysis of remaining pyrene from media

One ml of MSM broth with 10ml of Hexane was shaken by orbital shaker at 180rpm for 18 h. Hexane extract sample was collected after centrifugation at 10,000g for 10 min. Extracts were evaporated under a gentle nitrogen hood and residue was dissolved in acetronitrile. Prepared sample was analyzed with HPLC (ultimate 30000) using C18 (25 cm×4.6 cm×5µm) applying linear gradient of acetronitrle(50-85% in 35 min) in HPLC grade water. Flow was maintained at 1.5 ml min^{-1}. Pyrene was analyzed at 240nm using PDA detector (200-400nm). Generally HPLC was done twice in the whole study, once in the 1st week and another in the 4th week.

pH Analysis

pH of the samples was measured by pH meter (Orion EA940), it was calibrated by using standard buffer solutions of pH=7.0,4.0 and 10.01.

Protein analysis

For protein estimation Cells were harvested. Samples were prepared by centrifugation of 1 ml of sample: water (1:10) at 13000rpm for 10 min. Supernatant was discarded and pellet was redissolved in 1N NaOH. It was boiled for 3 min H_3PO_4 was added after samples normal temperature. Sample thus prepared was used for protein estimation. Protein was determined by using the standard method of Lowry *et al.,*[12].

RESULTS

The bacteria strain isolated from the petroleum sludge of Barauni Oil Refinery after enrichment in MSM with Pyrene was coded as BB. The strain could utilize Pyrene as a sole of carbon and energy. BB was found to posses various characters mention below:

Colony Morphology of BB on Agar Plate

Size(mm)	Shape	Elevation	Surface	Cell morphology
3	circular	convex	smooth	bacillus

Physical Test of BB

Gram Reaction	Cell Size(μm)	Oxygen use	Glucose Use	Motility
Negative	3	obligate	no	yes

Biochemical Test of BB

Urease test	Oxidase test	Nitrate Reduction Test	Indol Production Test	Methyl Red Test	Triple sugar iron test	Citrate Utilization Test	Voges Proskauer test	Catalse test	Gelatin test
Negative	Positive	Positive	Negative	Negative	Negative	Positive	Negative	Positive	Positive

The results of degradation study indicate that degradation of Pyrene increased with the incubation period. It was observed that in 4th week of incubation Pyrene was degraded by 82%.

Bacterial growth was found to be growing with the incubation period. It became maximum during the 2nd week and it became highest in the 3rd week .After 3rd week of incubation period the growth rate gradually declined.

Growth of bacterial biomass in relation to incubation time (Cfu/ml)

0(Initial)	1st week	2nd week	3rd week	4th week
1×10^5	1.55×10^6	2.32×10^6	4.7×10^6	2.11×10^6

The pH of media was found to be alkaline throughout the incubation period. pH of the media at the time of inoculation was found to be 6.6. But after during the incubation the pH starts decreasing.

The variation in pH values with the incubation time

0(Initial)	1st week	2nd week	3rd week	4th week
6.6	7.2	7.3	7.6	7.6

The initial protein content of the bacterial strain was found in the range of 10.275µg at the time of inoculation. The protein content was augmented up to 2nd and 3rd week then started decreasing.

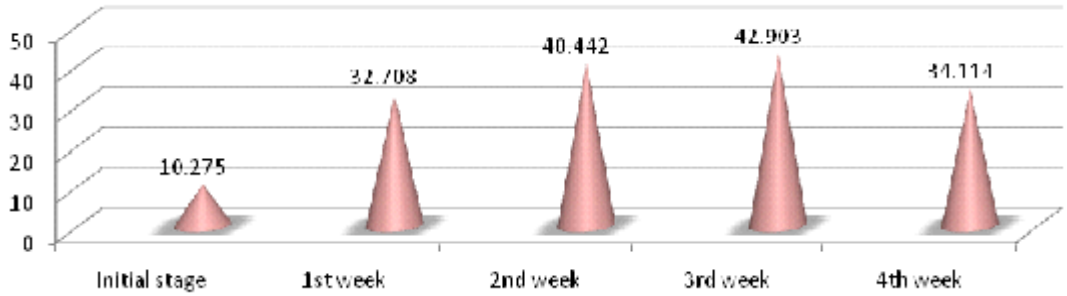

Fig. 1. Protein amount (µg) of bacterial stain calculated in respect to incubation time

DISCUSSION

In our microcosmic study, Pyrene being the major components of the petroleum sludge was considered as targeted compounds for degradation. Bacterial strain was isolated from petroleum sludge of Barauni oil refinery and used in degradation of Pyrene. The isolated bacteria BB belong to *Pseudomonas* spp as per the, morphological and biochemical testing. The BB was found to degrade 82% of 100ppm Pyrene, respectively in 4 weeks of incubation period. This investigation clearly indicated that the strains were capable of degrading Pyrene. The degradation process was facilitated by over expression of protein synthesis activity by increasing the protein amount from 10.275µg to 42.903µg. This results in increase of bacterial biomass from 1×10^5 to 4.7×10^6 and decrease of pH from 6.6 to 7.6. Thus, these bacterial strains were found to have high potential to be utilized in the microbial technology for recovery of oil contaminated sites.

CONCLUSION

Petroleum, being a major source of energy, supports the modern society and serves as a source for serious environmental pollutants leads to contamination of most parts of the ecosphere. In May 2010, 42,000 gallons of oil was spilled at Port Arthur, Texas .Another oil spill was reported in January, 2010 at Gulf of Mexico. In addition to the accidents of oil spillage, there are 20 oil refineries in India producing 28000 tons of sludge every year[13]. Disposed of Petroleum sludge posses' serious environmental threat. Petroleum contaminated soil contains hydrocarbons mixtures, PAHs are considered as a major environmental threat because of their potential for toxicity, mutagenicity, and carcinogenicity. Bacteria play an important role in the bioremediation of petroleum hydrocarbon contaminated area they utilize a wide range of components within the oils as nutrient sources. The use of micro biota for bioremediation of contaminated soil is of great interest, as these microorganisms are more adapted to the particular soil environment than non-indigenous commercial microbial inocula. Therefore isolation of some new PAH degrading microbes could add further advantage for bioremediation of petroleum contaminated soils in tropical countries.

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