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Isolation and Identification of Nah-B Gene from Bacteria Isolated from Effluents & Testing its Hydrocarbon Degrading Efficiency

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ABSTRACT

One of the major environmental problems today is hydrocarbon contamination resulting from the activities related to the petrochemical industry. Accidental releases of petroleum products are of particular concern in the environment. Hydrocarbon components have been known to belong to the family of carcinogens and neurotoxin organic pollutants. Bioremediation is the promising technology for the treatment of these contaminated sites since it is cost-effective and will lead to complete mineralization. In this project hydrocarbon degrading bacteria isolated from hydrocarbon contaminated soil by using nutrient agar medium and mineral salt agar medium supplemented with liquid petrol and diesel which was used as the sole carbon source for bacterial growth was performed. The bacteria was identified by using Bergey's manual of bacterial classification. The nah gene which responsible for the hydrocarbon degradation was amplified. The sequence has similarity about 90% to nahB gene.

INTRODUCTION

Petroleum-based products are the major source of energy for industry and daily life. Leaks and accidental spills occur regularly during the exploration, production, refining, transport, and storage of petroleum and petroleum products. The amount of natural crude oil seepage was estimated to be 600,000 metric tons per year with a range of uncertainty of 200,000 metric tons per year. Since accumulation of pollutants in animals and plant tissue may cause death or mutations.

Hydrocarbons are chemical compounds consisting entirely of carbon and hydrogen. They are a subset of organic compounds. Hydrocarbons are one of the Earth's most important energy resources. As carbon-carbon bonds are the strongest in all of chemistry, long chains with carbon backbones are extremely durable, and seem to have a practically unlimited extent.

Hydrocarbons are referred to as consisting of a backbone or skeleton composed entirely of carbon and hydrogen and other bonded compounds, and have a functional group that facilitates combustion. Hydrocarbons come in a variety of forms. They may be gases (methane and propane), liquids (hexane and benzene), waxes (paraffin wax), or polymers (polyethylene and polystyrene). Hydrocarbons can be processed to create plastics. The primary source of hydrocarbons here on Earth is through fossil fuels – coal, oil, and natural gas.

Oil refineries are one way hydrocarbons are processed for use. Crude is processed in several stages to form desired hydrocarbons, used as fuel and in other products.

Extracted hydrocarbons in a liquid form are referred to as petroleum (literally "rock oil") or mineral oil, whereas hydrocarbons in a gaseous form are referred to as natural gas. Petroleum and natural gas are found in the Earth's subsurface with the tools of petroleum geology and are a significant source of fuel and raw materials for the production of organic chemicals.

Oil reserves in sedimentary rocks are the source of hydrocarbons for the energy, transport and petrochemical industry.

Hydrocarbons are economically important because major fuels such as coal, petroleum and natural gas, and its derivatives such as plastics, paraffin, waxes, solvents and oils are hydrocarbons.

Like most fuels, diesel is a mixture of hydrocarbons, and the components have different freezing points. A heavy petroleum fraction used as fuel in diesel engines.

Diesel fuel is about 18% heavier than gasoline and consists mainly of hydrocarbons that range from C10 to C24, meaning 10 to 24 carbon atoms with various configurations of hydrogenations attached to the carbon atoms.

Naphthalene is an organic compound with formula $C_{10}H_8$. It is the simplest polycyclic aromatic hydrocarbon, and is a white crystalline solid with a characteristic odour that is detectable at concentrations as low as 0.08 ppm by mass. As an aromatic hydrocarbon, naphthalene's structure consists of a fused pair of benzene rings. It is best known as the main ingredient of traditional mothballs.

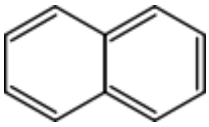

Figure: 1

XENOBIOTICS IN THE ENVIRONMENT

A xenobiotic is a chemical which is found inan organism but which is not normally produced or expected to be present in it**.**

Xenobiotic substances are becoming an increasingly large problem in Sewage Treatment systems, since they are relatively new substances and are very difficult to categorize. Some xenobiotics are resistant to degradation. For example, they may be synthetic organochlorides such as plastics and pesticides, or naturally occurring organic chemicals such as polyaromatic hydrocarbons (PAHs) and some fractions of crude oil and coal. However, it is believed that microorganisms are capable of degrading almost all the different complex and resistant xenobiotics found on the earth. Many xenobiotics produce a variety of biological effects, which is used when they are characterized using bioassay.

MATERIALS & METHODS

Sample Collection: The study includes three types of samples to isolate the hydrocarbon degrading bacteria. Soil sample extending from the ground surface to a depth of 10–20 cm were collected from petroleum- contaminated areas near refining areas, and water samples such as chlorine water from swimming pool, wastewater .Samples were then transported to laboratory under sterile conditions.

The bacteria were isolated from the collected samples by spreading the sample on nutrient agar medium. From the numerous colonies obtained on the NAM Plates, they were screened for the hydrocarbon degrading bacteria.

Isolation of Hydrocarbon Degrading Bacteria:

The bacteria were isolated by inoculating the soil and water samples on enrichment medium that contains the autoclaved mineral salt medium (MSM) supplemented with single hydrocarbon compound as sole carbon source (1% liquid petrol and diesel). The medium contains K2HPO4 (1.8 g/L); NH4Cl (4 g/L);
MgSO4.7H2O (0.2 g/L); NaCl (0.1 g/L);
Na2SO4.7H2O (0.01 g/L); agar (20 g/L); carbon source (1% petrol , diesel); and distilled water (1L) with pH 7.2. The medium without hydrocarbons was sterilized by autoclaving at 121°C for 15 min. The medium was supplemented with 1% (v/v) filter sterilized hydrocarbons (petrol, diesel) to serve as the only source of carbon and energy . The medium was incubated at 37^0C for 5-10 days. After the incubation period the bacterial colonies that were grown on the medium were identified by Gram's staining and biochemical characterization according to Bergy's manual.

Determination of Bacterial Biodegradative Activity by Turbidometry:

Turbidometry is to determine the bacterial growth by utilizing the hydrocarbons (1% petrol and diesel given as carbon source in MSM broth. this shows whether the bacterium possess the degrading activity of hydrocarbons like phenol, petrol and diesel. The degrading activities of each isolates were obtained by using Mineral salt broth (MSB) in which 1% of each hydrocarbon (petrol and diesel) was added and incubated at room temperature for 15 days. The growth of the bacterium was measured by taking the O.D readings at 595nm from 0hrs- 15 days at regular intervals of 2 days against mineral salt medium as blank.

Isolation of genomic DNA from bacteria:

DNA was extracted from 1ml of bacterial culture. The culture was pelleted by centrifuging at 12,000rpm for 2 min. the pellet was treated with lysis solution and proteinase k and incubated at 60 0C for 30min. Nucleic acids were precipitated with isopropanol by centrifuging at 10,000 rpm for 10 min, washed with 1 ml of a 70% (v/v) ethanol solution and dissolved in 0.1 ml of a TE buffer.

The purity and quantity of DNA were examined by recording its UV absorption spectrum and running on 1% agarose gel electrophoresis.

Amplification of nah-B gene

The bacteria were screened for the presence of Nah-B gene that involved in hydrocarbon degradation.

PCR primer design: PCR primers for amplifying Nah-B gene were constructed based upon conserved nucleotide regions. Primers were synthesized at the Micelles Lifesxiences Pvt Limited, India. PCR conditions were optimized using lab net thermal cycler. The PCR temperature program began with an initial 5-min denaturation step at 94°C; 35 cycles of 94°C for 45sec , 55°C for 1 min, and 72°C for 1 min; and a final 10-min extension step at 72°C. All reaction mixtures were held at 4°C until analyzed.

The PCR reaction mixture contains 10XPCR buffer, 25 mM, Magnesium chloride, 2.5mM dNTP's , 10pm/µl primer concentrations template DNA tests were done for the isolated thirteen bacteria, one of them is found to be Gram-negative and the rest of them were Gram-positive. Different Amplification of the PCR products of expected size was confirmed by electrophoresis, 1.5% (w/v) agarose gel in a TAE buffer.

Sequence determination of PCR amplified product:

The sequence of the PCR amplified product was Determined with a Dye terminator sequencing kit (Applied Biosystems), and the product was analyzed with an ABI Prism DNA sequence (Applied Biosystems 3500).

RESULTS AND DISCUSSION

The bacteria were isolated from three different types of samples on nutrient agar medium. Further the samples were screened for the presence of hydrocarbon degrading bacteria on mineral salt medium with 1% of the hydrocarbons as the sole carbon source namely petrol and diesel individually.

Hydrocarbons are needed as a carbon source but it can be toxic to microorganisms due to the solvent effects of diesel and petrol that could destroy bacterial cell membrane. Many biodegradation studies were reported on diesel are carried out using lesser diesel concentrations ranging from 1.0% to 3.0%. O.D readings of biodegrading activity of each isolates on hydrocarbons (petrol and diesel). The O.D readings based on the turbidity of MSM degradative activity on hydrocarbons by bacteria. The results demonstrated that Pseudomonas have the greatest ability to degrade petrol while .*Corynebacterium kutscheri* and demonstrated the greatest ability to degrade diesel. The graphs based on the O.D readings at various time intervals of incubation period on the degrading activity of the oil-degrading bacteria. Our results showed that all the organisms maximally utilized all the hydrocarbon substrates (petrol and diesel) when supplied as the sole source

of carbon and energy although, the level of utilization differs from one microbe to another (due to differences in Their growth) and from one hydrocarbon substrate to the other, due to the obvious differences in their Molecular sizes.

The ability of an organism to degrade a specific substrate is clear evidence that its genome harbors the relevant degrading gene. The previous studies on hydrocarbon degradation by bacteria reveal that nah B gene is responsible for hydrocarbon degradation. The presence of this gene in these identified hydrocarbon degrading bacteria identified by amplifying the gene coding the enzyme using nah B specific primers the primers used, gave the 216bp PCR product in three of the isolates.

There is no amplification in the rest of the isolates

However, it is true that the presence of a single gene does not ensure that the entire catabolic pathway will be present or that these genes will be expressed. The amplified product was sequenced and the aligned sequence gave 97% similarity with the known nah B gene sequence of

Pseudomonas species

CONCLUSION

Bioremediation is one of the most rapidly growing areas of environmental biotechnology, which has been used for the cleaning up of pollutants. This is because of its low costs and its public acceptability. Degradation of hydrocarbons by environmental microflorae involves microorganisms having specialized metabolic capacities. In polluted environments, specialized microorganisms are abundant because of the adaptation of the microflorae to pollutant. It is evident from this study that, hydrocarbon degrading organisms are ubiquitous in environment and they can be isolated from hydrocarbon polluted sites and waste water.

It has also been shown that four bacterial strains isolated from waste water and seven bacterial strains from contaminated soil can be good petrol and diesel degraders.

This study can focus on more cost effective applications of native bacterial strains for petrol and diesel degradation at large scale in industries, where it pose an alarming problem due to its detrimental health effects on different organisms and human beings. The degrading ability demonstrated by the microorganisms is a clear indication that they possess a gene that is used in hydrocarbon degradation. This study revealed that nahB gene was present in bacterial species identified hydrocarbon degrading bacteria.

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