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Study on Characterization of Plasmids from Bacteria that use Polycyclic Aromatic Hydrocarbons

Mr. Amit Khasat[1]*, Dr. Sachin Singh[2]

[1] Research Scholar, Shri Krishna University, Chhatarpur M.P.

[2] Professor, Shri Krishna University, Chhatarpur M.P.

Abstract - Polycyclic aromatic hydrocarbons (PAH) are carcinogenic to humans, animals, and plants. Polycyclic aromatic hydrocarbon concentrations have been steadily growing in the environment, including water, soil, air, sediments, marine water, and vegetables. A sustainable method will be the formation of consortia of efficient polycyclic aromatic hydrocarbons degraders for the total removal of polycyclic aromatic hydrocarbons from polluted locations. The hydrocarbon polluted site soil sample and subsurface soil sample were utilised for bacterial species separation by enrichment.The 2,6-Dichlorophenol indophenol (2,6-DCPIP) test was used to screen for efficient polycyclic aromatic hydrocarbon degraders, and potential polycyclic aromatic hydrocarbon degraders were found using 16S-rDNA sequencing. The detected polycyclic aromatic hydrocarbons degrader was chosen for bioremediation research based on the catabolic route employed for catabolism of high molecular weight-polycyclic aromatic hydrocarbons such as pyrene and chrysene, as well as the examination of catabolic plasmid properties. On the basis of highest hydrocarbon degradability, consortia of high molecular weight-polycyclic aromatic hydrocarbons degrading bacteria were formed.The effective polycyclic aromatic hydrocarbon degraders were found to be Paracoccus denitrificans C5, Bacillus cereus C7, Rhodococcus pyridinivorans A1 and Pseudomonas stutzeri G11. Among the various bacterial consortia developed, the consortia generated from Paracoccus denitrificans C5 and Rhodococcus pyridinivorans C7 show the most promise. Hence it is concluded that the consortia formed by efficient polycyclic aromatic hydrocarbons digesting bacteria with new plasmids is effective for polycyclic aromatic hydrocarbon bioremediation.

Keywords - Polycyclic aromatic hydrocarbon, Bioremediation, DCPIP, Consortia

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INTRODUCTION

Non-polar chemical compounds containing two or more fused benzene rings organised linearly, angularly, or in clusters are referred to as "polycyclic aromatic hydrocarbons" (PAHs). They are exceedingly persistent in the environment and are found naturally in coal, crude oil, and gasoline. Furthermore, PAHs are found in fossil fuel-derived products such as coal tar pitch, creosote, and asphalts. The aqueous solubility of PAHs decreases logarithmically as molecular mass increases, and the majority of PAHs are not soluble in water, limiting their environmental solubility (Bayoumi, 2009). PAHs have been linked to stomach, bladder, skin, lung, and liver cancer in well-established animal model studies.PAH exposure has also been linked to heart issues and poor prenatal development. Many PAHs are teratogenic to humans and animals, as well as mutagenic, genotoxic, and carcinogenic (Marston et al., 2001). The number of aromatic rings in PAHs influences their mutagenicity. Since the US-EPA (United States Environmental Protection Agency) listed 16 PAHs as priority pollutants, some of which are regarded to be potential human carcinogens, the dispersion of these pollutants in the environment and human exposure to them have been of special concern (Harvey, 1996).

Pollutants such as PAHs are mostly caused by human activity. Some of the industrial processes that generate and disperse PAHs are the production of aluminium, iron, and steel, coal gasification, tar distillation, hale oil extraction, production of coke, creosote, carbon blades, calcium carbide, and asphalt, production of rubber tyres, production of or use of metalworking fluids, and coal activity in natural gas power plants. PAH concentrations have continuously grown since the turn of the century due to the expansion of human sources, atmospheric deposition from natural sources, and the persistence of PAHs.

Some of the physical and chemical strategies used to remove PAHs from the environment include volatilization, photo-oxidation, chemical oxidation, and adsorption. These technologies, however, are costly and risk incomplete pollutant destruction. As a result of abiotic breakdown in the atmosphere and

higher layers of surface water, nitrogenated, halogenated, hydroxylated, and oxygenated PAHs can be formed. Some of these compounds are more hazardous, water soluble, and mobile than their parent PAHs. Efforts to remove considerable amounts of PAHs from contaminated areas generated interest in in situ bioremediation research.Depending on specific microbiological, chemical, and hydro-geological constraints, in situ PAHs bioremediation by microorganisms is a cheaper, environmentally sound, and cost-effective remediation approach than non-biologic remediation. PAHs are largely metabolised and destroyed biologically in the environment by microorganisms (Ahn et al., 2005).

Many heterotrophic bacteria in soil use PAH as a major source of carbon and energy. Because bacteria have an extensive enzyme system for degrading PAHs encoded by chromosomal and extrachromosomal (plasmid) DNA, prokaryotes play a significant role in PAH degradation, and bacteria account for the bulk of PAH degraders isolated from soil and sediments. Bacteria and fungi are well recognised for degrading LMW-PAHs. The ring number of the PAHs molecule has an inverse association with the biodegradation processes.Higher molecular weight PAHs with four or more fused rings degrade at a slower rate than lower molecular weight PAHs (Mrozik et al., 2003). The bulk of monoaromatic and low-molecular-weight PAHs can be easily broken down by microorganisms; however, the high-molecular-weight PAHs-degrading microbial community is less suitable, and its rate of PAHs degradation in nature is limited (Yamada et al., 2003). High molecular weight PAHs are frequently incompatible with microbial digestion.

Decontamination of PAH-polluted locations is critical since many PAH chemicals are poisonous, mutagenic, or carcinogenic. The United States Environmental Protection Agency (EPA) has designated 16 PAH chemicals as priority pollutants based on their prevalence and toxicity (Isiodu et.al., 2016).Because it potentially offers benefits like the complete destruction of the pollutants, lower treatment cost, greater safety, and less environmental disruption, biological treatment of soils contaminated with PAH should be an efficient, economical, and versatile alternative to physicochemical treatment. Therefore, the biodegradation of PAHs has received a lot of attention from researchers. It was discovered that microorganisms either destroy PAHs by metabolism or cometabolism. The breakdown of PAH combinations and high-molecular-weight PAHs depends on cometabolism (Habe& Omori, 2014).The US EPA has identified the chemical structures of 16 priority PAH compounds which can be observed in the following figure (I. Keuper,2010):

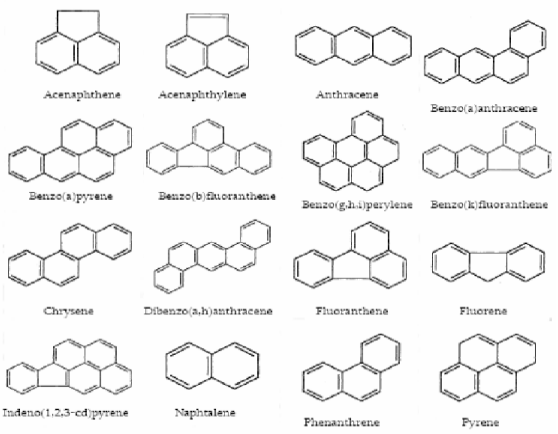

Figure 1: priority PAH compounds

The components of hydrocarbons, particularly polycyclic aromatic hydrocarbons (PAHs), are very toxic, mutagenic, and carcinogenic, posing a significant risk to both human and environmental health. To lessen the dangers caused by PAHs, several clean-up approaches, both physical and chemical, have been developed. Although the majority of physico-chemical remediation procedures are effective in removing a range of PAH pollutants, they are extremely expensive and can be hazardous to the structure and texture of the soil. Because of its cost, efficacy, and environmental friendliness, biodegradation or bioremediation has emerged as a promising option for the treatment of hydrocarbon-contaminated soil.The capacity of microorganisms to convert organic pollutants like PAHs into non-toxic by-products like water and carbon dioxide is known as biodegradation (Alrumman et.al., 2015).

On the fields of biodegradation and bioremediation, proteomics and metabolomics have a significant impact (Keum et al., 2008). Finding proteins and their roles in the biodegradation of aromatics using proteomics is a useful tool (Kim et al., 2007) While metabolomic may be utilised to characterise primary metabolites produced in response to PAH exposures as well as the breakdown products of PAHs. The enzymes mono- and dioxygenase are crucial for the bacterial breakdown of PAHs. In order to catalyse the first hydroxylation of unsubstituted PAHs, bacteria need molecular oxygen. The bacteria that break down PAHs first oxygenate the compounds to create catechol and dihydrodiol, which can then be further oxidised to make further dihydrooxygenated intermediates. To produce ring fission products, these intermediates are cleaved.

It is feasible to determine the extensive spectrum of proteins and genes involved in PAH degradation by analysing the proteome and genome of the microorganism that degrades HMW-PAH. A vast range of naturally occurring organic and xenobiotic compounds are destroyed by structural genes carried by catabolic plasmids. The first plasmids discovered were circular plasmids (Hardy, 1981), but later linear plasmid topologies were identified, and some of them were implicated in the

degradation of xenobiotics and refractory pollutants (Broker et al., 2004).

Plasmids, a distinct characteristic of PAH-degrading bacteria, are in charge of PAH breakdown. Consortia's genetic make-up, including certain plasmids, influences how successfully they digest PAHs. These plasmid-bearing bacteria are used not just to build consortia, but also to develop superbugs with improved PAH biodegradation capacity. Because these plasmids feature unique genes like nidA and phnAc, as well as unique sizes, restriction sites, genes, and gene sequences. Plasmids from efficient high molecular weight PAHs-degrading bacteria can be obtained for characterization studies.Because each organism has a distinct genetic composition, this strategy has been used in the current study to build consortia of effective PAH degraders, allowing them to profit from the degrading process and even live in a competitive environment. In bioremediation investigations, the following features must be considered: The copy number of the plasmid, the size of the plasmid, the availability of a restriction site for an array of restriction enzymes, which is necessary for the creation of a superbug for the bioremediation process, the mapping and identification of PAH degradative genes, the generation of a probe for monitoring the biodegradation process at PAH contaminated sites, and the understanding of evolution in metabolic process among PAH degraders are all important.

The present work is primarily concerned with isolating PAH contaminated areas from PAH degraders. The degradability of isolates that may break down PAHs may be identified when PAH degraders are tested utilising the DCPIP assay. The putative PAH degrader was found further utilising the molecular biology technique 16S-rDNA sequencing, and its efficacy in PAH degradation was evaluated by assessing residual PAH, biomass production during growth, extracellular protein synthesis, medium pH change, and metabolic intermediate. Studies on bioremediation that can be acquired by isolating and curing plasmids necessitate plasmid isolation and characterization. In extreme environmental conditions with varied media compositions, the PAH degradation capabilities of monoculture and consortia were proved to achieve the maximum PAH degradation.
In 1952, American molecular researcher Joshua Lederberg used the term "plasmid" to refer to any extra chromosomal hereditary component. Plasmids have sizes ranging from 1 to 400 kb. Depending on the size of the plasmid, there might be one to several hundred copies of the same plasmid in a single cell. The larger the plasmid, the fewer plasmids there are altogether. Plasmids are generally circular, double-stranded DNA molecules that are smaller than nuclear chromosomes. It can multiply itself by using already available cellular enzymes. Plasmid genes, on the other hand, control the commencement of replication and the distribution of plasmid copies into daughter cells. Many plasmids include genes that control the organism's phenotypic expression.

There have been reports of many plasmid types, and it is possible for plasmids of different types to coexist in a single cell, albeit related plasmids are typically incompatible, hence plasmids may be grouped into the compatibility group. Plasmids found in bacteria include episomes, F-plasmids, R-plasmids, heavy metal resistance plasmids, Col-plasmids, virulence plasmids, cryptic plasmids, and degradative plasmids.

OBJECTIVES OF STUDY

- Isolation of PAH degrader from PAH contaminated sites.
- Screening of PAH degrader by using DCPIP assay.
- Identify the potential PAH degrader.
- Isolation and characterization of plasmid.
- Composition of media for optimizing highest PAH degradation.
- Characteristics feature of isolated plasmid from these bacterial species.

MATERIAL & METHODS

The materials utilised in the current research are as follows:

- The five PAHs-contaminated soil samples were collected from the coal and ash deposited site of a thermal power plant in Parali Vaijanath (Maharashtra), transformer oil-contaminated soil in Dhanora (B.K.), gasoline-contaminated soil near a gasoline pump, and S.T. Garage effluent-contaminated soil in Nanded. Air dried soil samples were sieved (to a depth of 2 mm) and put in sterile plastic bags.
- The orbital shaking incubator (REMI-24 BL), cooling centrifuge (REMI), bacteriological colony counter (Hi-Media), UV-VIS Spectrophotometer (Shimadzu), HPLC (Shimadzu Corp., Japan), and GC-MS (Shimadzu Corp., Japan) were all used in the investigation (Shimadzu QP5050).

The methods employed in the study are as follows:

- **Isolation of pyrene utilizing bacteria:** On the well-isolated colonies, cultural and morphological characterisation were done. The bacterial isolates were stored on LB slants for further use.
- **Screening of pyrene utilizing bacterial isolates by DCPIP assay:** Using the 2, 6-dichlorophenol indophenol (2, 6-DCPIP) test, bacterial isolates were screened for their capacity to utilise pyrene. The optical density (OD) at 600 nm was pre-cultivated in 5 mL of LB broth at 30 °C and 150 rpm until it reached 1.0. After centrifuging at 5000 rpm for 5 minutes and washing the cell pellet with 0.9 percent saline, the cell density was adjusted to 1.0 at 600 nm.The

medium's transformation from blue to colourless served as evidence that the isolates could break down pyrene (Kubota et al., 2008).

- **Biochemical identification of bacterial isolates:** Bergey's Manual of Systematic Bacteriology assays were used to identify the 30 pyrene-producing bacterial isolates
- **DCPIP test to screen pyrene using bacteria for use with chrysene:**After five minutes of centrifugation at 5,000 rpm with a 1,0 cell density, the pellet was washed with 0,9% saline and the adjustment made to 600 nm. 240 l of bacterial cell suspension and 25 l of sterilised chrysene were mixed with 2250 l of Fe-free W medium, 150 l of FeCl3.6H2O solution (150 g/mL), and 150 l of 2, 6-DCPIP solution (50 g/mL), and incubated at 32 A shift in the medium's hue from blue to colourless proved that the isolates could break down chrysene.
- **Isolation of plasmids from pyrene and chrysene utilizing bacteria:** The plasmid profile of both pyrene and chrysene degrading bacteria was examined using a well-isolated colony of seven pyrene and chrysene degrading bacteria.
- **Analysis and identification of pyrene and chrysene degradation metabolites:**In *Paracoccus denitrificans, Bacillus cereus, Rhodococcus pyridinivorans*, and *Pseudomonas stutzeri*, which shown more efficacy toward both pyrene and chrysene degradation than other bacteria, the metabolites formed during the degradation were examined.
- **Characterization of plasmids from *Paracoccus denitrificans, Bacillus cereus, Rhodococcus pyridinivorans* and *Pseudomonas stutzeri*:**As a representative PAH for plasmid characterisation, pyrene was selected. Seven different bacterial species' plasmids were isolated using the Alkaline lysis technique. The molecular weight of plasmids from *Paracoccus denitrificans, Bacillus cereus, Rhodococcus pyridinivorans*, and *Pseudomonas stutzeri* were determined using the molecular weight marker. The restriction digestion pattern of isolated plasmids was examined using six restriction enzymes. The obtained plasmids from the various isolates' dioxygenase genes were sequenced.
- **Plasmid curing from pyrene degrading bacteria:**Utilizing an overnight culture of four different bacterial species, plasmid curing was performed. The four bacterial species were injected in LB medium and cultured at 32oC for 48 hours to reach a cell density of 108 cfu/ml. Serial dilutions were produced and plated on LB agar plates, and the greatest concentration of acridine orange was used to plate on those plates. All of the plates were incubated at 32°C for 24 hours.
- **Consortia preparation for pyrene degradation:** Ten (10) distinct consortia from *Paracoccus denitrificans, Bacillus cereus, Rhodococcus pyridinivorans, and Pseudomonas stutzeri* were produced using diverse combinations. 50 mL of BHB with 1000 mg/L pyrene was used as the only carbon and energy source to inoculate a 1 mL active inoculum. At 5-day to 20-day intervals, the pyrene growth and % degradation for each consortium were determined.

RESULTS

- **Isolation of pyrene utilizing bacteria:** After spreading 0.1 mL of enhanced BHB medium, the BH agar medium with pyrene as the only carbon and energy source displayed well-isolated colonies. The isolated colonies were found to be unique after 48 hours of incubation in terms of their size, shape, colour, margin, elevation, surface, opacity, consistency, Gram's nature, and motility.
- **Screening of pyrene utilizing bacteria:**The 2,6-DCPIP dye reduction test was used to assess the ability of pyrene consumption. Out of the 112 bacterial isolates that were screened, the 30 pyrene-using bacteria were shown to be more effective at removing 2,6-DCPIP's colour.
- **Screening of pyrene utilizing bacterial species for chrysene utilization:**Utilizing the DCPIP test, it was determined if bacteria were using chrysene as a more resistant PAH. Thirty bacterial species were present, however only 15 of them used media's chrysene effectively.
- **Isolation of plasmid from PAH degrading bacteria:** Adenovirus *Paracoccus Rhodococcus pyridinivorans* strain A1, *Pseudomonas stutzeri* strain G11, *Bacillus cereus* strain C5 and strain C7 all demonstrated the presence of plasmid, which was identified using the alkaline lysis technique. By measuring the absorption ratio at 260/280 and using a Nano-Drop spectrophotometer, the purity of the plasmid DNA was validated. Similar to this, 0.8% agarose gel electrophoresis was used to confirm the presence of plasmids in various bacteria that used pyrene and chrysene
- **Analysis and identification of pyrene and chrysene degradation metabolites:** The degradation metabolites of *Paracoccus denitrificans* C5, *Bacillus cereus* C7, *Rhodococcus pyridinivorans* A1, and *Pseudomonas stutzeri* G11 during pyrene and chrysene metabolism were revealed by extracting degradation intermediates and analysing through TLC, HPLC, and GC-MS. Pyrene and chrysene provide carbon and energy source through varying intermediates.

- **Characterization of plasmids from *P. denitrificans, B. cereus, R. pyridinivorans* and *P. stutzeri*:** Agarose gel electrophoresis was used to separate the plasmids from the bacteria *Paracoccus denitrificans, Bacillus cereus, Rhodococcus pyridinivorans,* and *Pseudomonas stutzeri* that had been isolated and purified. *Bacillus cereus, Rhodococcus pyridinivorans,* and *Pseudomonas stutzeri* all had larger plasmid sizes than *Paracoccus denitrificans* (11641 bp, 11271 bp, and 10914 bp, respectively).These plasmids were subjected to restriction digestion experiments using the restriction enzymes *SmaI, StuI, PvuII, EcoRI, PstI,* and*BglII*, which revealed a variety of restriction sites on the plasmids. The *StuI, PstI, EcoRI,* and*BglII* restriction sites are present on the plasmids derived from *Paracoccus denitrificans*, whereas the *SmaI, StuI, PvuII, EcoRI,* and*PstI* restriction sites are present on the plasmids obtained from Bacillus cereus. While Pseudomonas stutzeri plasmid possesses restriction sites for *SmaI, StuI, PvuII, EcoRI, BglII,* and*PstI, Rhodococcus pyridinivorans* plasmid was cleaved by *PvuII, EcoRI, PstI,* and*BglII*

CONCLUSION

It is hereby concluded that the capacity to use refractory polycyclic aromatic hydrocarbon as a source of carbon and energy is acquired by the bacterial species through adaptation and the acquisition of genes crucial for PAH consumption from the bacterial population. The results suggest that there is a diversity of pyrene-using bacterial species in the hydrocarbon-contaminated soil sample because the pyrene-using bacterial species have been isolated on solid media containing pyrene as carbon and energy source. These bacterial isolates also exhibited distinctive cultural and morphological characteristics.The capacity of the pyrene-using bacterial isolates to reduce or decolorize the dye dichlorophenol indophenol (DCPIP) varied. The major cause of DCPIP's decolorization is dye reduction, which is brought on by the catabolic activity of pyrene-using bacteria. On the basis of the DCPIP decolourization experiment, the pyrene-using bacterial species of the genera *Bacillus, Pseudomonas, Paracoccus, Rhodococcus, Acetobacter, and Azotobacter* were shown to be more effective at using chrysene, whereas *Proteus and Citrobacter* are unable to do so. Due to synergistic or cooperative metabolic activity, the efficient bacterial species in consortium forms digest a larger percentage of pyrene. The combination of *Paracoccus denitrificans C5 and Rhodococcus pyridinivorans* A1 was the most effective in degrading pyrene, destroying 95.20 percent of it in 20 days of incubation.

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Corresponding Author

Mr. Amit Khasat*

Research Scholar, Shri Krishna University, Chhatarpur M.P.