Annals of Biology **38** (2) : 172-176, 2022

Impact Score : 0.32 (Scopus)

Development of Phenanthrene (PHE) Degrading Bacterial Consortium from the Petrochemical Contaminated Soil Samples

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(Received : January 05, 2022; Accepted : January 20, 2022)

ABSTRACT

Phenanthrene is a persistent low molecular weight polycyclic aromatic hydrocarbon, and its elevated concentration due to anthropogenic activities shifts the diversity of microbes at contaminated sites and threatens the ecosystem's normal functioning. In this investigation, a phenanthrene degrading consortium 'NS-PAH-2015-PNP-5' was developed from the petrochemical contaminated sites. Using the Illumina-based 16S rRNA metagenomic approach, the bacterial diversity of NS-PAH-2015-PNP-5 was identified. The results showed that Burkholderiaceae (34.17%), Pseudomonadaceae (32.91%), Sphingomonadaceae (12.28%), Xanthomonadaceae (7.32%) and Rhodanobacteraceae (4.1%) were the leading taxa of family-level diversity. Whereas at genus level diversity Pseudomonas (32.91%), Castellaniella (22.62%), Sphingobium (11.63%), Achromobacter (9.56%), Stenotrophomonas (6.86%) and Dokdonella (4.1%) were the dominating taxa. The result of GC analysis showed NS-PAH-2015-PNP-5 degraded 90% of 500 ppm of phenanthrene after seven days of incubation. Also, this study advocated using the mixed naturally enriched diversity of bacteria for safe and viable bioremediation strategies.

Key words : Phenanthrene, consortium, biodegradation, polycyclic aromatic hydrocarbon

INTRODUCTION

Polycyclic aromatic hydrocarbons, commonly abbreviated as 'PAHs,' are compounds having two or more fused aromatic rings and aggregating to form different linear (anthracene), angular (phenanthrene) and cluster (pyrene) shapes (Abdel-Shafy and Mansour, 2016; Dat and Chang, 2017). These are produced due to various natural and anthropogenic activities where the incomplete burning of carbon-containing material takes place (Rose *et al.*, 2015). These are toxic, teratogenic, mutagenic compounds, and 16 PAHs are described as priority pollutants by the US Environmental Protection Agency (Dat and Chang, 2017; Hussain *et al.*, 2018). Also, these are classified as light molecular weight (LMW) PAHs upto three rings and high molecular weight (HMW) PAHs above three rings (Abdel-Shafy and Mansour, 2016). Due to their recalcitrant nature, their long-term persistence is a major concern for the environmentalist (Urana *et al.*, 2019, 2021). The long-term exposure of PAHs through the intake of contaminated food and air shows adverse effects on living's health (Domingo *et al.*, 2020; Marquès *et al.*, 2020). The PAH decontamination strategy depends on contamination level and environmental conditions (Kuppusamy *et al.*, 2017). The bioremediation approach using various microbes is affected by biotic and abiotic factors (Patel *et al.*, 2020).

PHE ($C_{14}H_{10}$) is a three benzene ring bearing LMW PAH and has the peculiar K and bay-region (Fig. 1; Ghosal *et al.*, 2016). Therefore, it is widely used as a model PAH for different research purposes (Fanesi *et al.*, 2018). PHE is a prominent and persistent pollutant around acidic and non-acidic contaminated environmental sites (Liu *et al.*, 2019). Various novel bacterial strains with PAH catabolic efficiency and the ability to tolerate extreme conditions have been isolated (Liu *et al.*, 2019). Decontaminating the sites polluted with PAHs through bacterial-mediated biodegradation is a safe, effective and affordable strategy (Mnif *et al.*, 2017; Gong *et al.*, 2018). Genetical modified, bioengineered microorganisms are configured to faster the process of soil health restoration by decontaminating the soil (Rebello *et al.*, 2021). Although, risks and precautions are post-release impacts of GMO's release. Therefore, an efficient PHE (model PAH for the study) degrading consortium was

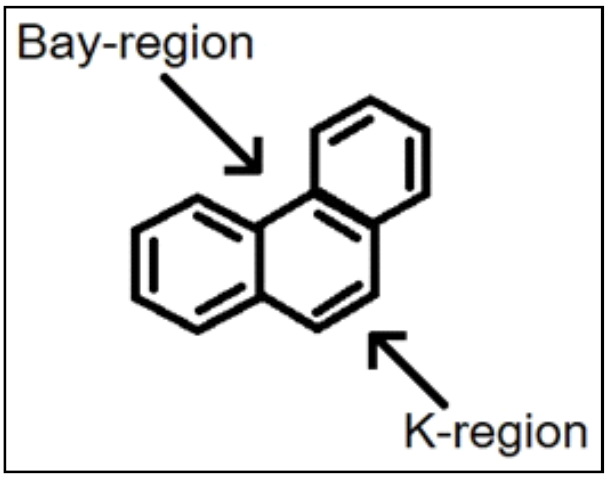

Fig. 1. K-region and Bay-region in phenanthrene.

developed from the contaminated petrochemical sites of the Panipat (29.39°N, 76.96°E) region (Panchal *et al.*, 2022). Large diversity, better adaptability, co-metabolism, and biosurfactant production by the constituenting bacterial species make the consortia an efficient scavenger of PAHs (Patowary *et al.,* 2016; Muangchinda *et al.*, 2018; Zhang *et al.*, 2019).

MATERIALS AND METHODS

The soil samples were collected from the different petrochemical contaminated sites near the Indian Oil Corporation Limited (IOCL) refinery, Panipat, India (Fig. 2). All the soil samples were mixed in equal amounts and enriched over the minimal salt (MS) media supplied with phenanthrene as the sole energy carbon source to enrich the population of PHE degrading bacteria. The developed consortium NS-PAH-2015-PNP-5 was revived frequently every seven days of interval for four months for further use.

NS-PAH-2015-PNP-5 was enriched and maintained at MS media having 500 ppm of phenanthrene with a per liter composition of Na_2HPO_4 8.5 g, KH_2PO_4 3.0 g, NaCl 0.5 g, NH_4Cl 1.0 g, $MgSO_4.7H_2O$ 0.5 g, $CaCl_2.2H_2O$ 14.7 g, and trace elements (KI 1.0 mg, $CuSO_4$ 0.4 mg, KI 1.0 mg, $MnSO_4.H_2O$ 4.0 mg, H_3BO_3 5.0 mg, $ZnSO_4.7H_2O$ 4.0 mg, $Na_2MoO_4.2H_2O$ 1.6 mg, $FeCl_3$ 2.0 mg i. e. modified Zhao, 2009 media (Panchal *et al.*, 2022).

Stock solutions of phenanthrene (Mol. wt. 178.2, HiMedia with ≤98% purity) in hexane and trace elements in distilled water were prepared. The aqueous solutions of Na_2HPO_4,

Fig. 2. Petrochemical contaminated sites for the development of NS-PAH-2015-PNP-5 (A) 29.4581°N,76.9003°E; (B) 29.4973°N, 76.8763°E and (C and D) 29.3750°N and 76.9762°E).

KH_2PO_4 and rest MS media with trace elements were autoclaved (Autoclave, York, Scientific Industries Pvt. Ltd., India) separately at 121°C and 15 psi for 15 min, cooled and mixed in laminar airflow (Atlantis, New Delhi, India). Phenanthrene stock was added, after the evaporation of hexane. Media was sonicated (Ultrasonic BathCPX2800, Branson Ultrasonics Corporation, USA) to make the phenanthrene miscible.

Genomic DNA of consortium 'NS-PAH-2015-PNP-5' was extracted using the C-TAB and phenol : chloroform method. 2 µl of which was run on 0.8% agarose gel for 60 min at 120V. Moreover, using the Nano Drop, the quality was determined at A_{260}/A_{280}. After the quality pass, V_3-V_4 region-specific genes were targeted using the forward (5'-GCCTACGGGNGGCWGCAG-3') and reverse (5'-ACTACHVGGGTATCTAATCC-3') primers designed and synthesized at Eurofins Genomics Laboratory. The amplicon libraries were prepared using the Nextera XT Index kit (Illumina Inc.) by following the 16S metagenome library preparation protocol (Part # 15044223). The PCR product was resolved on 1.2% agarose gel at 120V for 1 h (Fig. 3). Following the standard Illumina protocol, the amplicons with Illumina adapters were amplified using the i5 and i7 primers to add the multiplexing index sequences and common adapters for the cluster generation

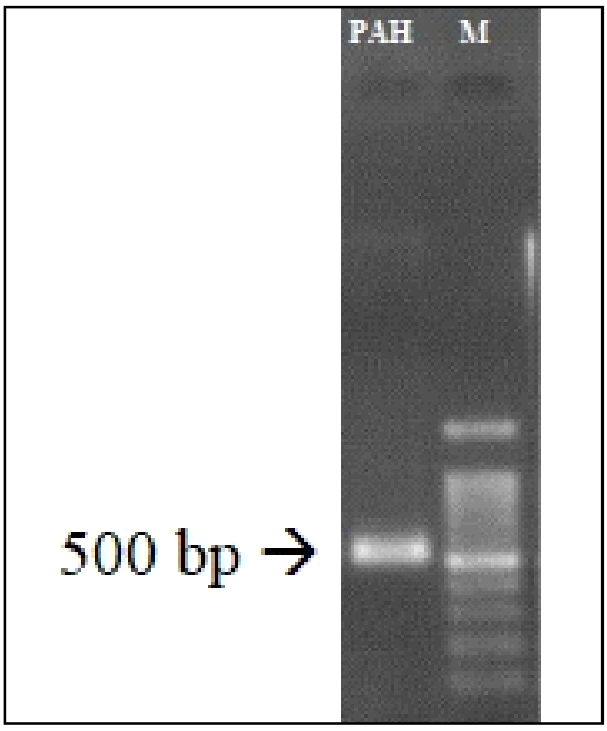

Fig. 3. Quality analysis of amplicon on 1.2% agarose gel.

(P5 and P7). Further, purified through 1X AMpureXP beads, quantified by Qubit fluorometer, and analyzed in 4200 Tape Station (Agilent Technologies) using the D1000 Screen tape (Chakrawarti *et al.*, 2020). Using the MiSeq 2x300 bp chemistry, the libraries were sequenced and analyzed through the bioinformatics tools such as QIIME to identify the diversity of bacteria in consortium NS-PAH-2015-PNP-5.

To determine the rate of phenanthrene biodegradation efficiency at the initial study stage, 100 ml of MS media having 500 ppm phenanthrene in 250 ml Erlenmeyer conical flask was inoculated with NS-PAH-2015-PNP-5 at 37°C with an agitation speed of 100 rpm. After seven days of incubation, the inoculated media was extracted with toluene (Panchal *et al.*, 2022). The extracted samples were analyzed by Gas Chromatograph (Varian 450-GC, equipped with the FID detector) to estimate the concentration of residual phenanthrene. The rate of phenanthrene biodegradation (degradation efficiency %) was calculated using the formula given below :

$$\text{Degradation efficiency \%} = \frac{\text{(Initial conc. of phenanthrene - Residual conc. of phenanthrene after degradation)}}{\text{Initial concentration of phenanthrene}} \times 100$$

RESULTS AND DISCUSSION

The results of bioinformatics tools to analyze the composition of NS-PAH-2015-PNP-5 showed that Burkholderiaceae (34.17%), Pseudomonadaceae (32.91%), Sphingomonadaceae (12.28%), Xanthomonadaceae (7.32%), Rhodanobacteraceae (4.1%), Rhizobiaceae (3.49%), Alcanivoracaceae (2.56%), Paracaedibacteraceae (1.75%) and Sphingobacteriaceae (0.5%) were the major families at the phylum level diversity of NS-PAH-2015-PNP-5. Fig. 4 depicts the abundance of different genus i. e. Pseudomonas (32.91%), Castellaniella (22.62%), Sphingobium (11.63%), Achromobacter (9.56%), Stenotrophomonas (6.86%), Dokdonella (4.1%), Ochrobactrum (2.68%), Alcanivorax (2.56%), unclassified genus of Burkholderiaceae (1.91%), Candidatus Paracaedibacter (1.75%), Sphingopyxis (0.65%) and Olivibacter (0.5%) were major genus in NS-PAH-2015-PNP-5.

Legends	Taxonomy	Abundance
	k__Bacteria;p__Proteobacteria;c__Gammaproteobacteria;o__Pseudomonadales;f__Pseudomonadaceae;g__Pseudomonas	32.91%
	k__Bacteria;p__Proteobacteria;c__Gammaproteobacteria;o__Betaproteobacteriales;f__Burkholderiaceae;g__Castellaniella	22.62%
	k__Bacteria;p__Proteobacteria;c__Alphaproteobacteria;o__Sphingomonadales;f__Sphingomonadaceae;g__Sphingobium	11.63%
	k__Bacteria;p__Proteobacteria;c__Gammaproteobacteria;o__Betaproteobacteriales;f__Burkholderiaceae;g__Achromobacter	9.56%
	k__Bacteria;p__Proteobacteria;c__Gammaproteobacteria;o__Xanthomonadales;f__Xanthomonadaceae;g__Stenotrophomonas	6.86%
	k__Bacteria;p__Proteobacteria;c__Gammaproteobacteria;o__Xanthomonadales;f__Rhodanobacteraceae;g__Dokdonella	4.1%
	k__Bacteria;p__Proteobacteria;c__Alphaproteobacteria;o__Rhizobiales;f__Rhizobiaceae;g__Ochrobactrum	2.68%
	k__Bacteria;p__Proteobacteria;c__Gammaproteobacteria;o__Oceanospirillales;f__Alcanivoracaceae;g__Alcanivorax	2.56%
	k__Bacteria;p__Proteobacteria;c__Gammaproteobacteria;o__Betaproteobacteriales;f__Burkholderiaceae;Other	1.91%
	k__Bacteria;p__Proteobacteria;c__Alphaproteobacteria;o__Paracaedibacterales;f__Paracaedibacteraceae;g__Candidatus Paracaedibacter	1.75%
	k__Bacteria;p__Proteobacteria;c__Alphaproteobacteria;o__Sphingomonadales;f__Sphingomonadaceae;g__Sphingopyxis	0.65%
	k__Bacteria;p__Bacteroidetes;c__Bacteroidia;o__Sphingobacteriales;f__Sphingobacteriaceae;g__Olivibacter	0.5%
	k__Bacteria;p__Proteobacteria;c__Gammaproteobacteria;o__Xanthomonadales;f__Xanthomonadaceae;g__Pseudoxanthomonas	0.46%
	k__Bacteria;p__Proteobacteria;c__Alphaproteobacteria;o__Rhizobiales;f__Rhizobiaceae;g__Brucella	0.42%
	k__Bacteria;p__Proteobacteria;c__Alphaproteobacteria;o__Rhizobiales;f__Rhizobiaceae;g__Shinella	0.26%
	k__Bacteria;p__Proteobacteria;c__Alphaproteobacteria;o__Rhodovibrionales;f__Fodinicurvataceae;g__uncultured	0.23%
	k__Bacteria;p__Proteobacteria;c__Alphaproteobacteria;o__Parvibaculales;f__Parvibaculaceae;g__Parvibaculum	0.23%
	k__Bacteria;p__Bacteroidetes;c__Bacteroidia;o__Cytophagales;f__Amoebophilaceae;g__Candidatus Amoebophilus	0.11%
	k__Bacteria;p__Proteobacteria;c__Alphaproteobacteria;o__Caulobacterales;f__Caulobacteraceae;g__Brevundimonas	0.1%
	Others	0.48%

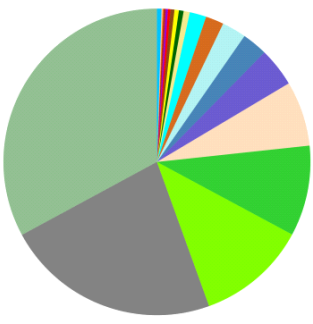

Fig. 4. The pie chart shows the abundance of the different genus in the phenanthrene degrading consortium NS-PAH-2015-PNP-5.

Similar diversity of different taxa of phylum Proteobacteria was observed in previous PAH degradation (Patel *et al.*, 2021). Phylum Proteobacteria was a dedicated hydrocarbon-degrading group of bacteria and inhabitat in hydrocarbon-contaminated sites (Devi *et al.,* 2021), a foremost population of these bacteria in the NS-PAH-2015-PNP-5 illustrateed its phenanthrene degrading potential.

The peak of phenanthrene was observed at 21.71 (min) in the GC chromatogram (Panchal *et al.*, 2022). The result of GC analysis showed that the consortium NS-PAH-2015-PNP-5 degraded 90% of 500 ppm of phenanthrene after seven days of incubation. Other studies showed a similar rate of phenanthrene biodegradation by using the bacterial consortium (Zafra *et al.*, 2017). Mawad and others showed that consortium (mixed population of bacteria) had a higher phenanthrene biodegradation efficiency than the individual pure strains (Mawad *et al.*, 2021). A similar study by Mnif showed a halo tolerant bacterial consortium biodegraded the PHE through the protocatechuate pathway, and intermediates such as naphthalenol and phthalic acid were detected (Mnif *et al.*, 2017). This study showed that NS-PAH-2015-PNP-5 utilized the PHE from the MS media for the energy and carbon source.

CONCLUSION

PAHs are persistent hydrocarbons due to their recalcitrant nature. Although, a large diversity of bacterial species can efficiently degrade different PAHs. This study developed a mixed population of bacteria i. e. consortium 'NS-PAH-2015-PNP-5' from the petrochemical contaminated sites. Using the phenanthrene as the model PAH, it was concluded that NS-PAH-2015-PNP-5 had a rich bacterial diversity, which can efficiently eliminate a high concentration of phenanthrene. However, optimizing the physico-chemical condition can be advantageous to get the optimum biodegradation rate. Moreover, implementing natural, enriched bacterial diversity for the *in situ* project was more feasible than genetically altered microbes. Therefore, the biodegradation approach using the natural inhabiting population of the petrochemical contaminated sites was safe and economical.

ACKNOWLEDGEMENTS

The authors are grateful to BIF-DBT, DST-FIST, DST Indo-Ukraine Project, DST-PURSE, HSCST (Panchkula), and UGC-SAP for financial support in the form of the project. Metagenomic data related to NS-PAH-2015-PNP-5 are available on NCBI under the bioproject : PRJNA587569.

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