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ORIGINAL ARTICLE

Isolation and Molecular Characterisation of Polycyclic Aromatic Hydrocarbons (PAHs) Degrading Bacteria from Petrochemical Contaminated Soil

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Abstract

Because of their ubiquitous nature, polycyclic aromatic hydrocarbons (PAHs) are widely dispersed in the environment as a result of both natural and human processes. One of the ways to deal with the harmful effects of these chemicals is through the use of microorganisms capable of degrading the pollutants. A petrochemical contaminated site was searched for these microbes. Eleven bacterial strains were obtained in this work using the culture enrichment technique on Bushnell Hass medium supplemented with (naphthalene, anthracene, and phenanthrene) as the only source of energy. Enumeration utilizing the spread-plate technique and liquid media were used to examine the PAH breakdown capacities of bacterial strains. The isolates were identified using standard methods of morphological and biochemical identifications. Furthermore, 16sRNA was utilized in order to classify the isolates at molecular level. The presence of PAHs degrading genes was also analysed in the isolates. Four isolates (G1, G2, G5, and G6) out of a total of eleven were able to tolerate and degrade the test PAH's up to 600 mg/l in liquid media. Isolate G1 showed the highest growth during screening followed by G6 while there were no differences between the other two isolates as demonstrated by an increase in their optical densities after 120 hours of incubation. Based on 16S rRNA gene sequences and molecular phylogenetic analysis, the isolate was identified as *Pseudomonas stutzeri, Stenetrophomonas sp, Pseudomonas lactis,* and *Achromobacterxylosoxidans* with the accession numbers OM039162, OM52851, OM52852, and OM52853 respectively. Fragments of 350 bp, 350 bp, and 867 bp for ring hydroxylating dioxygenase (RHD), hydratase-aldolase, and catechol 2, 3-dioxygenase were obtained from partial PCR amplification of catabolic genes, demonstrating the presence of a PAH degradation pathway in the organisms. These isolates have great potential for application in the bioremediation of PAHs-contaminated sites.

Keywords: Polycyclic aromatic hydrocarbons (PAHs); contaminated soil; bioremediation; catabolic genes

Introduction

Over 100 substances in the family of polycyclic aromatic hydrocarbons (PAHs) have two or more fused benzene rings. They make up a significant and varied category of important environmental contaminants (Ambade et al., 2022). Due to their occurrence in numerous anthropogenic activities like oil refining, incomplete combustion of fossil fuels, indiscriminate exploitation of mineral resources, destruction of forests by wildfire, processing of household waste, and others, PAHs are widely dispersed contaminants in soils, waters, and air (Shehu & Alias, 2019). According to reports, the range of PAH polluting concentrations in polluted areas is fairly broad and spans from 1 g/kg to over 300 g/kg. Low-molecular-weight (LMW) PAHs, which have two or three aromatic rings like naphthalene, anthracene, or phenanthrene, are acutely hazardous while high-molecular-weight (HMW) PAHs, which have four or more rings, are generally thought to be genotoxic (Tomasetig et al., 2020). Although it has already been shown that PAHs may covalently attach to proteins, RNA, and DNA, the degree of covalent contact between PAHs and DNA is what most closely ties to carcinogenicity (Šimečková et al., 2022). Additionally, some PAHs' transition products can have detrimental effects on cells and are more dangerous than the parent PAHs. PAHs enter cells due to their lipophilicity, which causes the genes for cytochrome P450 (CYP) enzymes to be produced. The CYPs break down PAHs into epoxides, some of which are extremely reactive (such "bay-region" diol epoxides) and are known as ultimate carcinogens because they may attach to DNA and trigger the transformation of normal cells into cancerous ones (Imam et al., 2022). In addition to being carcinogenic, PAHs also cause endocrine disruption, oxidative stress, immunotoxicity, and teratogenicity (Šimečková et al., 2022).

Many physical and chemical techniques, including landfilling, combustion, ultrasonic decomposition, and photo-oxidation, have been employed over the years to eliminate PAHs from the environment (Adeola & Forbes, 2021). However, these techniques are expensive, time-consuming, and unfriendly to the environment because they produce other toxic metabolites (Haneef et al., 2020). Bioremediation is a biological strategy that, in contrast to the non-biological approach, uses microbial species with the ability to consume contaminants and create harmless molecules out of them. Due to its success in removing contaminants from polluted locations, this technology has grown in importance over time (Gou et al., 2020).

Depending on whether the procedure calls for oxygen, bacteria can break down PAHs in one of two ways. The aerobic degradation process is the main oxygen-involved mechanism in surface layer soils, and it is reliant on gene products (enzymes) (Acer et al., 2021). Bacterial breakdown of PAH is often accomplished by the actions of enzymes such as oxygenases, peroxidases, and hydratases (Ghosh & Mukherji, 2021). An essential enzyme in the bioremediation of PAHs and other harmful pollutants is ring-hydroxylating dioxygenases (PAH-RHDs) (Liang et al., 2019). According, RHDs are multicomponent enzymes that have an active site with a component made of either α_n, β_n, or α_n. These multicomponent enzymes are assumed to represent the rate-limiting step in the PAH degradation process of different aromatic hydrocarbons, since they catalyze the initial step in the oxidation of PAH by adding di-oxygen to aromatic ring structures (Liang, et al., 2019). The hydratase-aldolase enzyme, which catalyzes the fifth step of the aerobic degradation of PAH by converting analogs of trans-o-hydroxybenzylidene-pyruvate (tHBPA) into aldehydes and pyruvic acid, is another essential enzyme for the microbial breakdown of PAHs. This process is critical in PAH degradation since it is at this time when degrading bacteria begin to genuinely receive energy from PAH breakdown (Liang et al., 2019). The aldehyde product is further oxidized to create salicylic aldehyde. Salicylate is further oxidized in two ways: viasalicylate-1-monooxygenase to produce catechol or with the participation of salicylate- 5-hydroxylase to produce gentisic acid. To properly mineralize the PAH, they must be able to breakdown these intermediates (catechol and gentisic acid) by the reaction of either catechol 1,2-dioxygenase or catechol 2,3-dioxygenase to CO_2 (Rodríguez et al., 2022).

Even though several microorganisms have previously been discovered that can degrade PAHs. It is crucial, even for adaptation purposes, to search a microbial community for new strains that can be applied in that same environment for bioremediation purposes. The goal of this research was to isolate, characterize, and identify PAH-degrading bacteria from petrochemical polluted soil in Kano state, Nigeria.

Materials and Methods

Media and Chemicals

All chemicals employed in this study were of high purity analytical grade and obtained commercially (Solarbio ®, Life Sciences, Co., Ltd and Sigma-Aldrich, Fisher Scientific Pvt. Ltd, and HiMedia). Sigma Aldrich, Bellefonte, USA, provided several PAHs such as naphthalene (Nap) and anthracene (Ant). Phenanthrene (phe) was obtained from Solarbio ®, Life Sciences and Technology Co., Ltd in Beijing, China. Fisher Scientific Pvt. Ltd., Mumbai, India, supplied the solvents (acetone) utilized in the investigation. HiMedia Laboratories Pvt. Ltd., Mumbai, India, provided more chemicals. Individual PAH stock solutions of 1000mgL-1 were produced in acetone and stored at 4 °C in brown bottles.

Sample Collection

A soil sample was taken from Kwakwachi Mechanic village in Kano State's DawakinTofa LGA. The sample was taken from the soil's surface to a depth of 15 cm, evenly mixed, and carefully placed in a sterile polyethylene bag held at room temperature before being promptly transferred to the Department of Microbiology Laboratory, Bayero University Kano for bacteria isolation.

Enrichment and Isolation of PAH-Degrading Bacteria

Hydrocarbon-degrading bacteria were enhanced in Bushnell-Haas (BH) liquid medium with 1000 mg/L PAHs (naphthalene, anthracene, and phenanthrene) separately and incubated aerobically at 37°C as per a modified approach by (Patel et al., 2018). The medium contains 100 ml of distilled water, 0.2 g of MgSO4, 1 g of KH2PO4, 1 g of K2HPO4, 1 g of NH4NO3, 0.02 g of CaCl2, and 0.05 g of FeCl3. 15 g of agar-agar (a Cole-Palmer® product) were added to Bushnell Hass Agar and sterilized in an Astell® autoclave. in the case of Bushnell-Hass Agar. At the start of the enrichment, 10 g of the sediment sample was suspended in a 250 ml Erlenmeyer flask containing 100 mL of BH broth and 500 mgL-1 of each specific PAH as the only carbon and energy source. The culture was cultured for 7 days at 37 °C and 120 rpm in a Wise Cube Shaking incubator.10 g of the sediment sample was suspended in a 250 ml Erlenmeyer flask containing 100 mL of BH broth that contained 500 mgL-1 of each particular PAH as the only carbon and energy source at the beginning of the enrichment.

The culture was cultured for 7 days at 37 °C and 120 rpm in a Wise Cube Shaking incubator. The three times this method was done led to the isolation of the bacterium strain. The traditional spray plat approach was employed for the isolation and purification procedure. This involved dissolving 100 mgL-1 of each in acetone, spraying it on the surface of the BHA as the only source of carbon and energy, and incubation at 370C for 72 hours to see the bacteria grow. Selective purification was done on colonies of various morphotypes. The isolated isolates were preserved for identification and characterization after being repeatedly sub-cultured to remove contamination.

Screening Of Isolated Strains for PAH Utilization

Screening of the Isolated Strains on Solid Media

A confirmatory spray-plate method was used to assess the effectiveness of isolated bacterial strains grown on Bushnell-Haas agar (BHA) treated with certain PAHs (Bibi et al., 2018). After the medium had solidified, each PAH dissolved in acetone was aseptically pipetted and homogeneously dispersed over the agar surface of the pre-dried plates containing the bacteria culture, leaving the PAH on the surface to be digested. The medium was incubated at 37°C for 72 hours. This method was performed with different PAH concentrations to assess bacterial tolerance.

Screening of the Isolated Strains on Liquid Media

As the last step in the selection procedure, the pure isolates were grown on Bushnell-Haas (BH) medium to cross-validate their ability to use the test PAHs. Bushnell-Haas (BH) medium that has been supplemented with 600 mg L-1 of each PAH was used to grow 2% (v/v) of each isolate in 250 ml flasks. Thereafter, the flasks were incubated for seven days at a temperature of 37 °C. An increase in the medium's turbidity, which was measured using a spectrophotometer (Spectrum-Lab-7525) at an optical density (OD) of 600 nm, was a sign that each isolate could use naphthalene, anthracene, or phenanthrene. This increase in turbidity is a measure of the growth of the isolates in each PAHs enrichment medium.

Characterization and Identification of Isolates

Morphological and Biochemical Identification of the Isolates

Based on Bergey's manual of determinative Bacteriological studies, pure colonies of the most efficient PAH-degrading bacteria were identified and characterized using gram reaction and biochemical tests such as urease test, citrate utilization, indole test, catalase test, oxidase test, and nitrate reduction tests (Bergey, 1994).

Molecular Characterization

A loopful of each isolate was inoculated in 5ml Laurie-Bertani (LB) medium and incubated at 37°C for 24 h. The bacteria suspension formed was centrifuged at 10,000 rpm for 5 min. This was then extracted according to the kit's instruction. The region of 16S rRNA was amplified with KAPATaq DNA polymerase using genomic DNA and the primers presented in Table1.

The Total reaction volume was 25 µL. The reaction mixture comprises 2 µL each of the genomic DNA, 2.5 µL of 10 TaqA Buffer, ~0.4 M (0.85 µL) of each forward, and reverses primer 1.25mM (1.5 µL) of $MgCl_2$, 0.25 mM (0.2 µL) of dNTP mixes and 0.2 µL of Taq DNA polymerase, in ddH_2O. Condition used for amplification of 16S RNA segment was; an initial 5 min at 95 °C of denaturation is followed by 35 cycles of 30 s at 94 °C (denaturation), 30 s at 60 °C (primer annealing), and 1 min at 72 °C (extension), and finally 10 min of final extension at 72 °C.

The same condition was applied for segment of PAH-RHD gene except the annealing temperature which was 62 °C and 63°C for segment of hydratase aldolase (PahE1) and catechol 2,3-dioxygenase (C23O).

Table 1. List of Primers used for 16S rRNA and catabolic gene detection of PAH-degrading bacteria by PCR

Primers		Sequence (5′→3)	Expected product size (bp)	Reference
16SrRNA	**F**	*TGGAGAGTTTGATCCTGGCTCAG*	**1.5kb**	(Kumar et al., 2018)
	R	*TACCGCGGCTGCTGG CAC*		
PAH-RHD-396	**F**	*ATA GGT GTC TCC AAC GAA GTT*	**320**	(Ding et al., 2010)
	R	*ATT GCG CTT ACC ATG GTT GG*		
PahE1α	**F**	*TGC GGC GGG TGT CAA TGG CAT*	**350**	(Liang et al, 2019)
	R	*CCT GAG GAA TCT CGG ACA TCT GTG CCC AAA A*		
C23O	**F**	*GAC GGC GTC GAC GGA ATC AT*	**877**	(Singh & Tiwary, 2017)
	R	*TCA GGG TTG TCG TAG ATC A*		

F=Forward R=Reverse

Agarose gel purification and Sequencing of amplified 16S rRNA gene

On a 1.5 percent agarose gel (CSL-AG500, Cleaver Scientific Ltd.), the integrity of the PCR amplicons was examined using the Syngene Gel Documentation System of Ingenius, England (IG31459). The existence of a product in the anticipated sizes was a successful outcome. Partial 16S rRNA PCR products were sent to Inqaba Biotechnological Industries for purification and sequencing. The organism and closest species were then determined by searching the sequences in the nucleotide databases of the National Center for Biotechnology Information (NCBI) using the blast function. The sequences were deposited in the NCBI GenBank under the accession numbers OM039162, OM52851, OM52852, and OM52853 respectively.

Phylogenetic analysis of amplified 16S rRNA gene

The development of the phylogeny and evolutionary history of the isolates using the Neighbor-Joining technique was done after multiple sequence alignments using CLUSTALW. The proportion of replicate trees that have clustered related taxa is shown next to the branches. The evolutionary relationship was calculated using the Maximum Composite Likelihood method in Molecular Evolutionary Genetics Analysis (MEGA 11.0) for Windows (Tamura et al., 2021).

Results and Discussion

Isolation and screening

Table 1 presents the result of bacterial screening on Bushnell-Haas media that was supplemented with naphthalene, phenanthrene and anthracene at various concentrations. G_1 and G_6 grow fully at all the concentrations while G_2 and G_5 showed moderate growth at all concentrations. Others have started to show grow at lower concentrations but failed to show any grow at higher concentrations.

Table 2. Screening of bacteria isolates on Bushnell –Haas solid media supplemented with various PAH

S/N	Isolate	Polycyclic Aromatic Hydrocarbons (Naphthalene, Anthracene, and Phenanthrene) concentrations								
		200 mg/L	400 mg/L	600 mg/l	200 mg/L	400 mg/L	600 mg/l	200 mg/L	400 mg/L	600 mg/l
1.	G_1	+++	+++	+++	+++	+++	++	+++	+++	++
2.	G_2	+++	++	+	++	++	+	++	++	+
3.	G_3	++	+	+	+	-		+	-	
4.	G_4	+	-		+	-		-	-	
5.	G_5	+++	++	++	++	++	++	++	++	+
6.	G_6	+++	+++	+++	++	++	++	++	++	++
7.	G_7	+	-		-	-		-	-	
8.	G_8	++	-		+	-		+	-	
9.	G_9	+	+		-	-		-	-	
10.	G_{10}	++	+	+	-	-		-	-	
11.	G_{11}	+	+	-	-	-		-	-	

Key: +++ = Rich growth ++ = Medium growth + = Less growth
(-) = No growth

Figure 1 presents the result for the growth of G_1, G_2, G_5 and G_6 at 400mg/L benchmark concentration of individual PAHs as measured using optical density (OD) at 600nm. G_1 showed the highest growth in all individual PAHs followed by G_6.

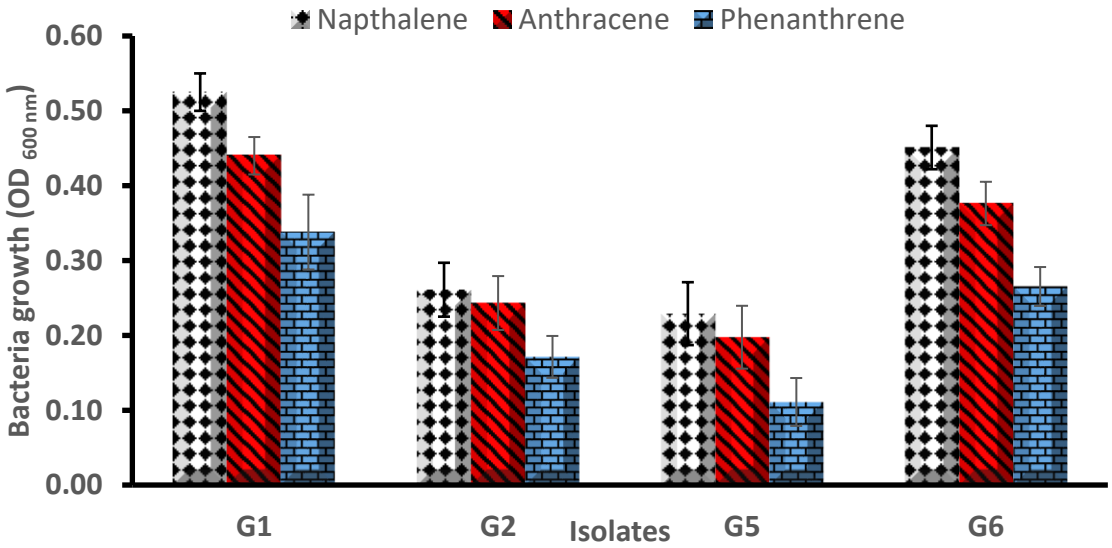

Figure 1. UV-spectrophotometric analysis of microbial growth (OD $_{600\ nm}$) after 120 h incubation in standardized Bushnell-Haas (BH) broth enriched with 400 mg/L of PAH. Each bar represents the mean of triplicate data ± Standard deviation.

Table 3 present the morphological and biochemical characteristics of G_1, G_2 G_5, and G_6. All the organisms are gram negative and rod shaped and respond differently to various biochemical tests.

Table 3. Morphological and Biochemical Characteristics of the recovered PAH-Degrading isolates

Parameters	Isolate			
	G_1	G2	G5	G6
Gram Reaction	-	-	-	-
Cellular Morphology	Rod	Rod	Rod	Rod
Catalase	+	+	+	+
Oxidase	+	-	+	+
Methyl Red	-	+	-	-
Voges-Proskauer	-	_	-	-
Nitrate Reduction	+	+	+	+
Indole Production	+	_	+	+
Citrate Utilization	+	-	+	+
Starch Hydrolysis	+	+	+	+
Urease	+	-	+	+

- **KEY: + : Positive : -= Negative**

Figure 2 presents the evolutionary relationship between G_1, G_2 G_5, and G_6. While G_1 and G_5 belongs to *Pseudomonas* species, G_6 belongs to *Achromobacter* specie and G_2 belongs *stenotrophomas* specie.

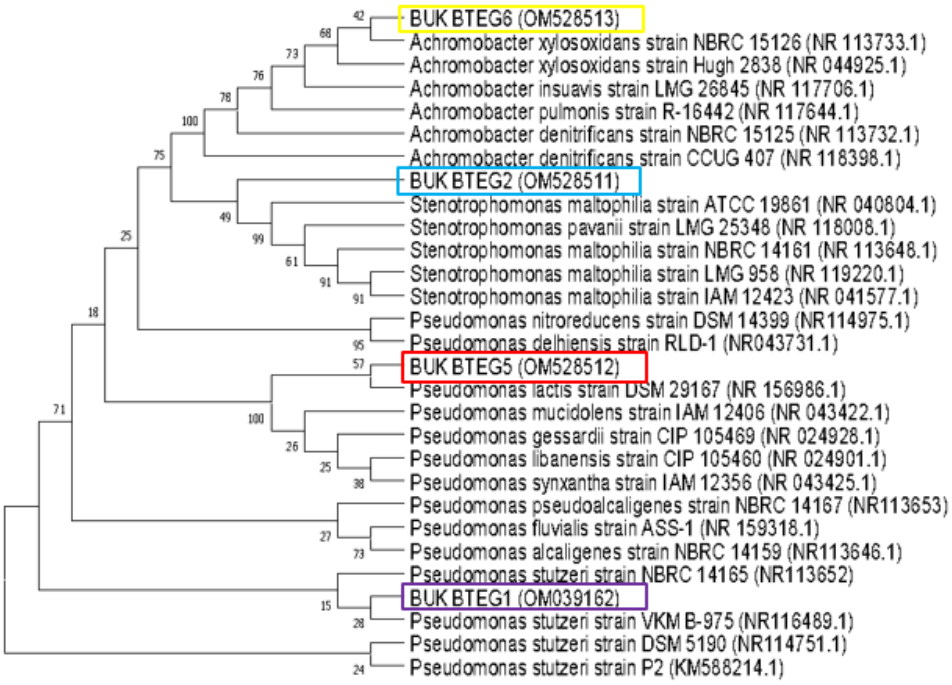

Figure 2. Phylogenetic and Evolutionary relationships of taxa of bacteria strain BUK-BTE (G1, G2, G5, and G6). A rectangular box displayed information about the strains' evolutionary position. With the use of the neighbor-joining method, the evolutionary history was deduced. The accession numbers are listed alongside each species' name.

Figure 3 presents the result of PCR amplification of various PAH degrading genes in G_1, G_2 G_5, and G_6.

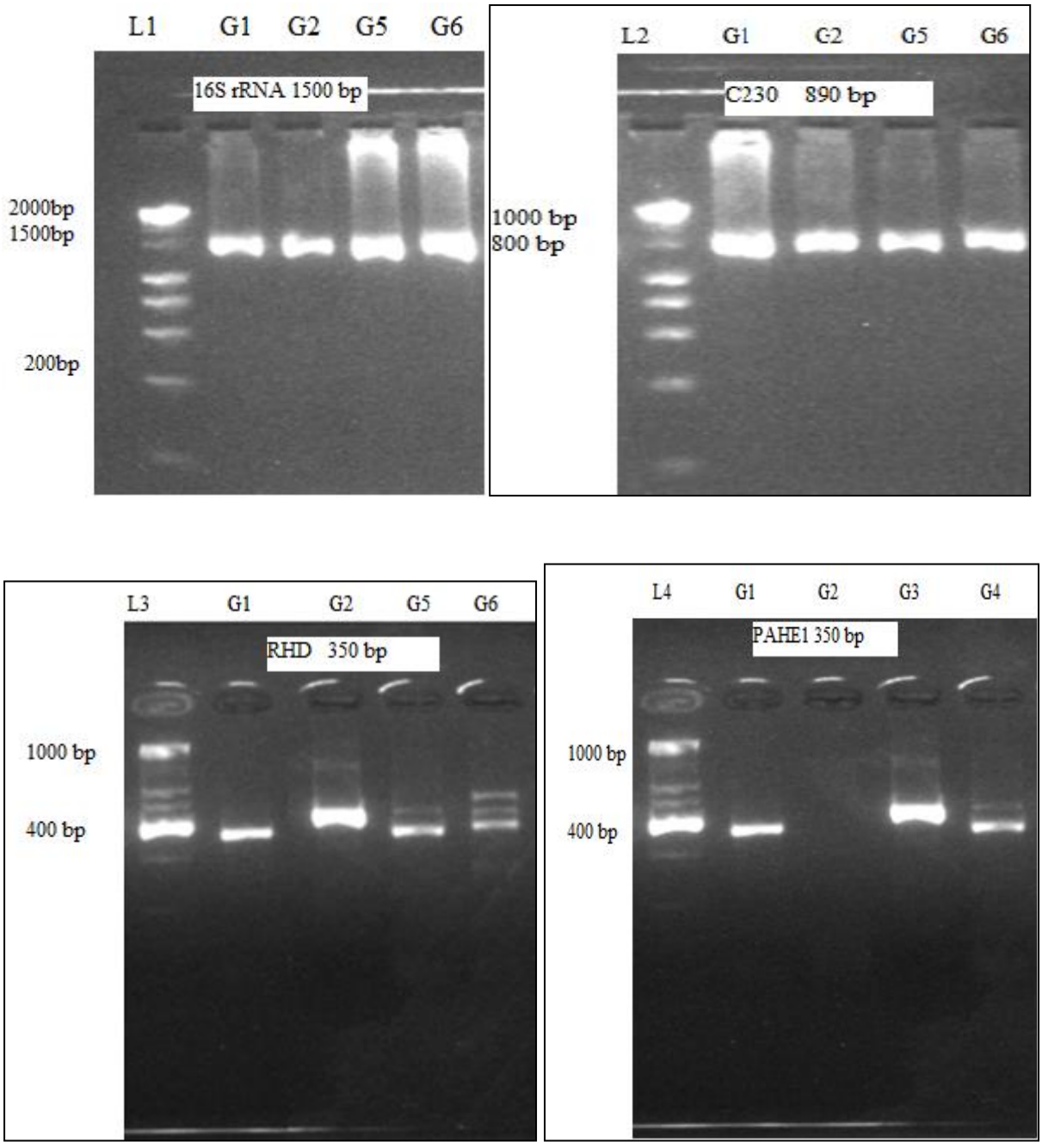

Figure 3. Visualization of PCR amplification products of the (A) 16S rRNA and PAHs degrading genes (B) Pah-RHDα (C) PahE1 and (D) C23O from the isolates.
*L=molecular size standard (L1=2000 bp, L2, L3, and L4 =1000 bp ladder).

In this study, polycyclic aromatic hydrocarbons (PAHs); anthracene, phenanthrene and naphthalene degradation was carried out using the isolate obtained from the petrochemical-contaminated soil. A soil sample was taken from the location for isolation and screening for

efficient bacteria capable of degrading PAHs. PAH-degrading bacteria were isolated from contaminated soil samples using enrichment procedures on a Bushnell-Haas medium containing naphthalene, anthracene, or phenanthrene as the sole source of carbon and energy. This medium produced a rigid environment that encouraged the development of bacteria with the metabolic machinery needed to thrive in such an environment (Bibi et al., 2018).

Numerous studies have shown ample evidence that bacteria can biodegrade PAHs, and these bacteria have been identified (Muhammed et al.; Xu et al., 2021). Upon plating on BH solid media under aerobic conditions, a total of eleven isolates were identified (Table 2). However, out of the eleven isolates, only four showed better tolerance and utilization capability of up to 600 mg L-1 of PAHs as the exclusive carbon source (Figure 1). Isolate G_1 achieved the highest optical density (OD) which was indicated by the development of turbidity as an indicator of the biomass formed followed by isolate G_6. However, there was no much difference in terms of growth and degradation concerning isolating G_2 and G_5 in each respective PAH utilization within five days (120 h) of incubation.

All the four (4) bacterial isolates labelled G_1, G_2 G_5, and G_6 were subjected to morphological and biochemical identification tests. They were found to be gram-negative, rod-shaped, catalase, starch hydrolysis, and nitrate reduction positive. Except for isolate G2, all isolates were positive for the enzyme's urease, oxidase, citrate consumption, and indole synthesis. Each isolate tested negative for Voges-Proskauer. While other isolates tested negative for methyl red, isolate G2 was positive.

For bacterial identification, it has been discovered that 16S rRNA gene sequencing maintains a respectable level of accuracy and dependability (Hong and Farrence, 2015). Consequently, a partial section of the isolate's 16S rRNA gene was amplified by PCR to identify the isolate. When run on 1.5 percent agarose gel electrophoresis, the isolate's amplification result showed roughly 1500 bp (Figure 3). The amplicons were successfully sequenced and blasted using NCBI. The Blast analysis of 16S rRNA sequences of the isolate G_1 and G_5, revealed homology to genus *Pseudomonas (*99%), while G_2 and G_6 *Stenetrophomonas* (98%), and *Achromobacter* in the existing database of the National Center of Bioinformatics, respectively (NCBI). Molecular phylogenetic studies using the neighbor-joining method linked the identity of the obtained bacteria sequences to *Pseudomonas stutzeri, Stenetrophomonas sp, Pseudomonas lactis,* and *Achromobacterxylosoxidans*. Thus, these bacteria were tentatively assigned as strain BCH_BTE (G1, G2, G5, and G6). Detailed molecular phylogenetic studies are presented in Figure 2. In other studies, several investigations on the identification and taxonomy of bacteria isolated from contaminated soil with PAH degrading abilities have been reported for bacteria belonging to the genus *Pseudomonas, Stenetrophomonas and Achromobacter* (Elufisan et al., 2020; Singh & Tiwary, 2017).

Furthermore, numerous publications have observed that the genes encoding aromatics-degrading enzymes are situated on the chromosomes (Sun et al., 2019). Such gene clusters are frequently discovered from effective PAH-Degraders, and examples include the *Nah*-like gene family and the *Pah*-like gene family. These gene clusters are essential for PAH biodegradation due to their broad substrate specificities and ability to degrade the condensed polycyclic aromatic ring, which is a crucial and rate-limiting stage in the process. The ability of these bacterial isolates to successfully grow on naphthalene, anthracene, and phenanthrene suggests the presence of an active enzyme system unique to the substrate (Sun et al., 2019).

Also in the current work, the main catabolic genes (PAH-RHD, C23O, and PahE1) responsible for the breakdown of PAHs were analyzed utilizing primers and extracted chromosomal DNA as a template. The results from the PCR amplification products of PAH-RHD, hydratase aldolase, and catechol 2, 3-dioxygenase show that these bacteria have these genes. However, there was no amplicon generated from *Stenetrophomonas sp* for the (PahE1) hydratase aldolase gene segment, this may be due to the absence of the gene segment in DNA or non-primer compatibility **(**Figure 2**).** Similar studies have shown that these catabolic genes' existence

may be a sign of their capacity to break down PAHs. The fact that the bacterial isolates had the genes for ring hydroxylating dioxygenase, catechol-2, 3-dioxygenase, and hydrates-aldolase suggests that they are able to break down Naphthalene, Anthracene, and Phenanthrene utilizing them as a sole source of carbon and energy.

Conclusion

This research revealed that the petrochemical polluted soil hosted bacteria capable of degrading PAHs. *Pseudomonas stutzeri, Stenetrophomonas sp, Pseudomonas lactis,* and *Achromobacterxylosoxidans* were isolated and identified from contaminated soil through 16S rRNA. Furthermore, catabolic genes for biodegradations of PAHs were also identified using specific primers. These isolates demonstrate the potential to utilize and biodegrade naphthalene, anthracene, and phenanthrene as the only carbon and energy source efficiently under agar and broth medium. These bacteria may offer an environmentally acceptable means of addressing the toxicity and environmental damage brought on by these PAHs.

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