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MOLECULAR CHARACTERISATION OF BACTERIA MONOOXYGENASE AND DEHYDROGENASE GENES INVOLVED IN PAHs REMEDIATION

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ABSTRACT

Bacterial catabolic genes (*alkB*, *alkH*, *C12O*, and *C23O*) are a good biomarker for selecting the choice of the organism for polycyclic aromatic hydrocarbon (PAH) degradation. Low molecular weight (LMW) and high molecular weight (HMW) PAHs metabolism can be made possible by monooxygenase and dehydrogenase enzymes which code for the catabolic genes. In this study, the monooxygenase and dehydrogenase genes were characterized from the bacterial population isolated from motor mechanic workshop soils and landfill soil artificially polluted with waste engine oil (WEO). Standard microbiological methods were followed for the isolation and characterization of the bacterial population. The PCR cycling for *alkB* and *alkH* followed initial denaturation at 94°C for 5 minutes, followed by 35 cycles of denaturation at 95°C for 1 minute, annealing at the correct temperature (*alkB* 49°C, *alkH* 72°C). PCR cycling for *C12O* and *C23O* genes followed initial denaturation at 95°C for 5 minutes, 35 cycles of denaturation at 94°C for 20 s, annealing at 63°C for 30 s, extension at 60°C for 45 s, with final extension for 5 minutes at 72°C. Final elongation step for all the catabolic genes at 72°C for 10 minutes and holding temperature at 10°C forever. Amplified fragments were visualized on safe view-stained 1.5% agarose gel electrophoresis. The result of the characterization revealed base pair sizes of the genes; *alkB* (100 to 300 bp), *alkH* (< 700 bp), *C12O* (>250 bp), and *C23O* (<80 pb). All the bacterial populations invested in this study expressed the monooxygenase and dehydrogenase genes. Monooxygenase and dehydrogenase genes are coding for the enzymes responsible for hydroxylation and intradiol or extradiol ring-cleaving of PAHs.

Keywords: *Catabolic genes, alkB, alkH, C12O, C23O, Monoxygenase, Dehydrogenase.*

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INTRODUCTION

Petroleum products are derived from crude oil and petroleum fossils which are formed underneath the ground from the remains of animals, plants, and diatoms that thrived millions of years in marine environments. The major petroleum products are crude oil, bitumen, asphalt, and natural gas. These products are sourced through exploration and exploitation activities of oil and gas industries as crude oil and other products. Crude oil today accounts for one-third of the world's energy demand as a result of global demand for premium motor spirits (PMS) which are used to drive the majority of engines produced in the 21st century (Jones, 2018; Maniruzzaman, 2005; Wu *et al.*, 2021). The activities in refineries/petrochemical industries result in an accidental and intentional release of these products into the soil, water, and air in the form of spills, explosions, fires, and leaks. One major environmental pollutant generated by petroleum products is polycyclic aromatic hydrocarbons (PAHs). PAHs are compounds with multiple benzene rings fused in a linear, angular, and cluster arrangement (Abbasian *et al.*, 2016; Alfarhani & Hammza, 2022; Law *et al.*, 2021). PAHs are formed when petroleum products are exposed to high temperatures of about 650-900 °C in a process called pyrolysis or pyro-synthesis (Krzyszczak *et al.*, 2021; Ye *et al.*, 2022). PAHs have decolourised electrons in fused aromatic rings owing to their uncharged and non-polar molecules. The United State Environmental Protection Agency (EPA) 1979 identified sixteen (16) PAHs as priority pollutants (da Silva Junior *et al.*, 2021; Hong *et al.*, 2020; Song *et al.*, 2021; Younis *et al.*, 2018); because of their potential for human exposure, toxicity, an occurrence at hazardous waste sites, and awareness about them. PAHs are considered persistent organic pollutants (POPs) because they are recalcitrant and can accumulate in the food chain. Low molecular weight (LMW) PAHs with <3 benzene ring cleavage is acutely toxic while high molecular weight (HMW) PAHs (<4 benzene ring) are genotoxic (Ali & Wang, 2021; Kweon *et al.*, 2019; Nzila, 2018; Sivaram *et al.*, 2019; Zhou *et al.*, 2020). LMW PAHs (naphthalene, anthracene, and phenanthrene) are more volatile, soluble in water, and susceptible to bioconversion/biodegradation, unlike the HMW PAHs which serve as signature compounds to detect PAH contamination in the environment.

Aerobic bioconversion/degradation of PAHs is made possible by oxygenase-mediated metabolism (monooxygenase or dioxygenase enzymes). The first step in the aerobic bacterial degradation of PAHs in the environment is the hydroxylation of an aromatic benzene ring through a dioxygenase, forming cis-dihydrodiol that is rearomatized to a diol intermediate by the action of a dehydrogenase (Elyamine *et al.*, 2021; Okolafor & Ekhaise, 2022; Srivastava & Kumar, 2019). Secondly, the diol intermediates are immediately cleaved by intradiol or extradiol ring-cleaving dioxygenases through either an ortho-cleavage or meta-cleavage pathway, resulting in intermediates of catechols which are converted to TCA cycle intermediates (Pérez-Pantoja *et al.*, 2019; Phale *et al.*, 2020). The genes coding for these degrading enzymes is expressed in all bacteria populations capable of attaching to the Bay-region and K-region (Kotoky *et al.*, 2022; Wang *et al.*, 2018). The most widely reported PAH genes expressed by most hydrocarbon-degrading bacterial populations for the degradation LMW and HMW PAHs are aldehyde dehydrogenase (*alkB*) (Williams *et al.*, 2022), alkane hydroxylase gene (*alkH*) (Abbasian *et al.*, 2016; Pacwa-Płociniczak *et al.*, 2019), naphthalene dioxygenase (*nahAcR*) (Mawad *et al.*, 2020), PAH ring hydroxylating dioxygenase (*PAH-RHDα*) (Imam et al., 2022; Obi et al., 2020), catechol 1,2-dioxygenase (*C12O*) (Aravind *et al.*, 2021) and catechol 2,3-dioxygenase (*C23O*) (Mawad *et al.*, 2020). The bacteria gene markers for the aerobic bioconversion/degradation of 16 priority

PAHs pollutants are the *alkB*, *alkH*, *C12O*, and *C23O*. This study was focused on the characterization of biomarker genes from bacterial isolates from waste engine oil (WEO) polluted soil.

MATERIALS AND METHODS

Isolation and characterization of bacteria genes

Soil samples from the motor mechanic workshop polluted with waste engine oil (WEO) and soil samples from landfill soil polluted with WEO (1 gram) were introduced into a test tube containing 10 ml of Sterile Bushnell Haas (BH) broth. The mouth of the test tube was covered with sterile cotton wool and the mixture was allowed to stand for up to 1 month to allow for the growth of hydrocarbon-degrading bacteria. Serial dilution of the suspension was carried out up to 10^{-8} and plated on BH agar medium and incubated at 48 h to 72 h. Discrete colonies were randomly counted, subcultured, and characterized. The representative hydrocarbon (HC) degrading bacterial isolates were further characterised by molecular methods described in our previous study (Okolafor *et al.*, 2021).

DNA Extraction

The DNA extraction was carried out initially, using QuickDNA™ Fungal/Bacterial MiniPrep Kit from Zymo Research, according to the manufacturer's protocol.

Polymerase Chain Reaction (PCR) amplification

The PCR amplification was carried out using 5x Firepol® Master Mix from Solis BioDyne, the 16S rRNA gene was amplified in 20 µl reactions containing; Master mix (5x): 4 µl, Forward primer: 0.6 µl, Reverse primer: 0.6 µl, Template: 2 µl and nuclease-free water: 12.8 µl. The PCR cycling condition followed denaturation at 95 ^{o}C for 3 min, 25 cycles of denaturation at 95 ^{o}C for 30 s, annealing at 54 ^{o}C for 30 s, and extension at 72 ^{o}C for 30 s, with a final extension at 72 ^{o}C for 10 min. The primer sequence for the characterization of bacterial isolates was 27F: AGAGTTTGATCMTGGCTCAG, 1525R: AAGGAGGTGWTCCARCCGCA.

PCR Product clean up

Amplicons were cleaned up for sequencing using DNA clean and Concentrator™-5 from Zymo Research. The method was according to the manufacturer's protocol. Cleaned amplicons were eluted with 30 µl nuclease-free water. The cleaned products were checked on 1.5% agarose for purity.

Sequencing

Quality check of Plasmid/PCR products by gel electrophoresis was carried out and was followed by DNA Sequencing with specific universal primers using Sanger Sequencing chemistry. After sequencing, Q20 Read Length up to 800 bases with ABI or SCF, FASTA File with Quality Control reports were obtained.

Sequence Identification

The ABI Sequence Result was aligned as a FASTA file using Bioedit Software. Sequence Identification was carried out on NCBI Website (Http://Blast.Ncbi.Nlm.Gov) using the gene bank Basic Alignment Search Tool (BLAST) (Hesham *et al.,* 2012). To Succinctly categorize the bacteria sequences, sequences were aligned with muscle as

implemented in genome-wide pairwise identities using the Sequence Demarcation Tool (SDT) (Martin *et al.*, 2015). The sequences were assigned a unique accession number and deposited in the NCBI database.

PCR Amplification of Catabolic Genes

Primer amplification and Optimization

Primer amplification was carried out using the method described earlier, while the primer was optimized following the method of Lorenz (2012) Table 1. Partial *alkB, alkH, C120* and *C23O* genes were amplified using the forward and reverse primer.

Table 1: Primers used for the detection of catabolic genes

Target gene	Primer sequence (5 to 3)	Fragment size (bp)	Annealing temperature (^{0}C)	Reference
Aldehyde dehydrogenase alk-H1-F alk-H3-R	5′-CIGIICACGAIITIGGICACAAGAAGG-3′ 5′-IGCITGITGATCIIIGTGICGCTGIAG-3′	549	60	Phillips *et al.* (2008)
Alkane hydroxylase alkB-F alkB-R	5′-AAC TAC MTC GAR CAY TAC GG-3′ 5′-TGA MGA TGT GGT YRC TGTTCC-3′	100	49	Phillips *et al.* (2008)
Catechol 1,2-dioxygenase C12O-F C12O-R	5′-GCCAACGTCGACGTCTGGCAGCA-3′ 5′CGCCTTCAAAGTTGATCTGCGTGGTTG GT-3′	350	63	Sei *et al.* (1999)
Catechol 2,3-dioxygenase C23O-F C23O-R	5′-AAGAGGCATGGGGGCACCGGTTC GA-3′ 5′-TCACCAGCAAACACCTCGTTGCGG TTGCC-3′	900	63	Sei *et al.* (1999)

PCR detection of catabolic gene

The PCR cocktail mix contained 2.5 µl of 10x PCR buffer,1 µl of 25 mM $MgCl_2$, 1 µl each of forward and reverse primer, 1 µl of DMSO, 2 µl of 2.5 mM dNTPs, 0.1 µl of 5 µ/µl Taq DNA polymerase, and 3 µl of 10 ng/µl DNA. The total reaction volume was made up to 25 ul using 13.4 µl nuclease-free water. The PCR cycling for *alkB* and *alkH* followed initial denaturation at 94^{o}C for 5 minutes, followed by 35 cycles of denaturation at 95^{o}C for 1 minute, annealing at the correct temperature (Table 1) for the genes (*alkB* 49^{o}C, *alkH* 72^{o}C). The PCR cycling for *C12O* and *C23O* genes followed initial denaturation at 95^{o}C for 5 minutes, 35 cycles of denaturation at 94^{o}C for 20 s, annealing at 63^{o}C for 30 s, extension at 60^{o}C for 45 s, with final extension for 5 minutes at 72^{o}C. Final elongation steps for all the catabolic genes at 72^{o}C for 10 minutes and holding temperature at 10^{0}C forever. Amplified fragments were visualized on safe view-stained 1.5% agarose electrophoresis gels. The size of the amplicon was 50 bp and the DNA ladder used was Hyperladder from Bioline.

RESULTS AND DISCUSSION

The maximum likelihood consensus phylogenetic tree of bacterial isolates (Figure 1) showed the evolutionary relationship of HC degrading bacteria isolated from motor mechanic workshop soil polluted with WEO and landfill soils polluted with WEO. Four HC degraders which were not reported widely in literature were identified as *Carnobacterium gallinarum*, *Bacillus albus*, *Ochrobactrum intermedium,* and *Providencia vermicola* which showed evolutionary variance compared to other HC degrading bacterial isolates in this study. *Enterococcus faecalis* (MT345789) isolated from landfill soil polluted with WEO revealed 75% confidence similarity with those isolated from motor mechanic workshop soil, which is an indication of their evolutionary relationship.

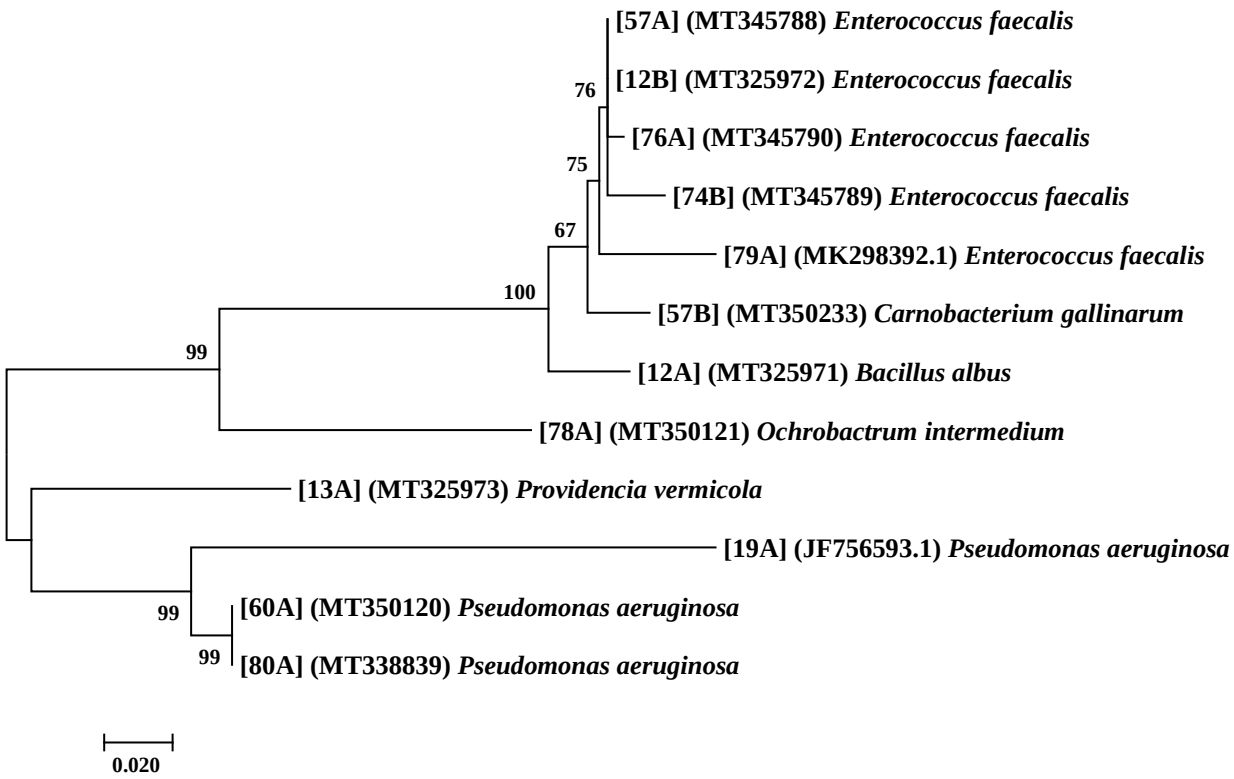

Figure 1: Phylogenetic tree of bacterial isolates showing the evolutionary relationship of the bacteria isolated from waste engine oil (WEO) polluted

The sizes of PCR products of catabolic genes, alkane hydroxylase gene (*alkB*), and aldehyde dehydrogenase gene (*alkH*) revealed base pair (bp) sizes of the product for *Enterococcus faecalis* (MK298392.1) as 100 and 650 bp, *Bacillus albus* (MT325971) as 100 and 600 bp, *Enterococcus faecalis* (MT325972) as 300 and 400 bp. *Providencia vermicola* revealed size of the gene (MT325973) as 100 and 300 bp, *Enterococcus faecalis* (MT345788) as 100 and 150 bp, *Carnobacterium gallinarum* (MT350233) as 250 and 450 bp (Figure 2 and 3). *Pseudomonas aeruginosa* (JF756593.1) showed PCR product of *alkH* gene as 650 bp whereas *Pseudomonas aeruginosa* (MT350120) as 200 and 700 bp, *Enterococcus faecalis* (MT345789) as 100 and 200 bp,

Enterococcus faecalis (MT345790) as 100 and 250 bp. The PCR product for the catabolic gene for *Ochrobactrum intermedium* (MT350121) revealed size as 100 and 100 bp, *Pseudomonas aeruginosa* (MT338839) as 100 and 100 bp. All the bacterial isolates investigated expressed *alkB* and *alkH* genes whereas *Pseudomonas aeruginosa* (JF756593.1) did not express *alkB* gene (Table 2).

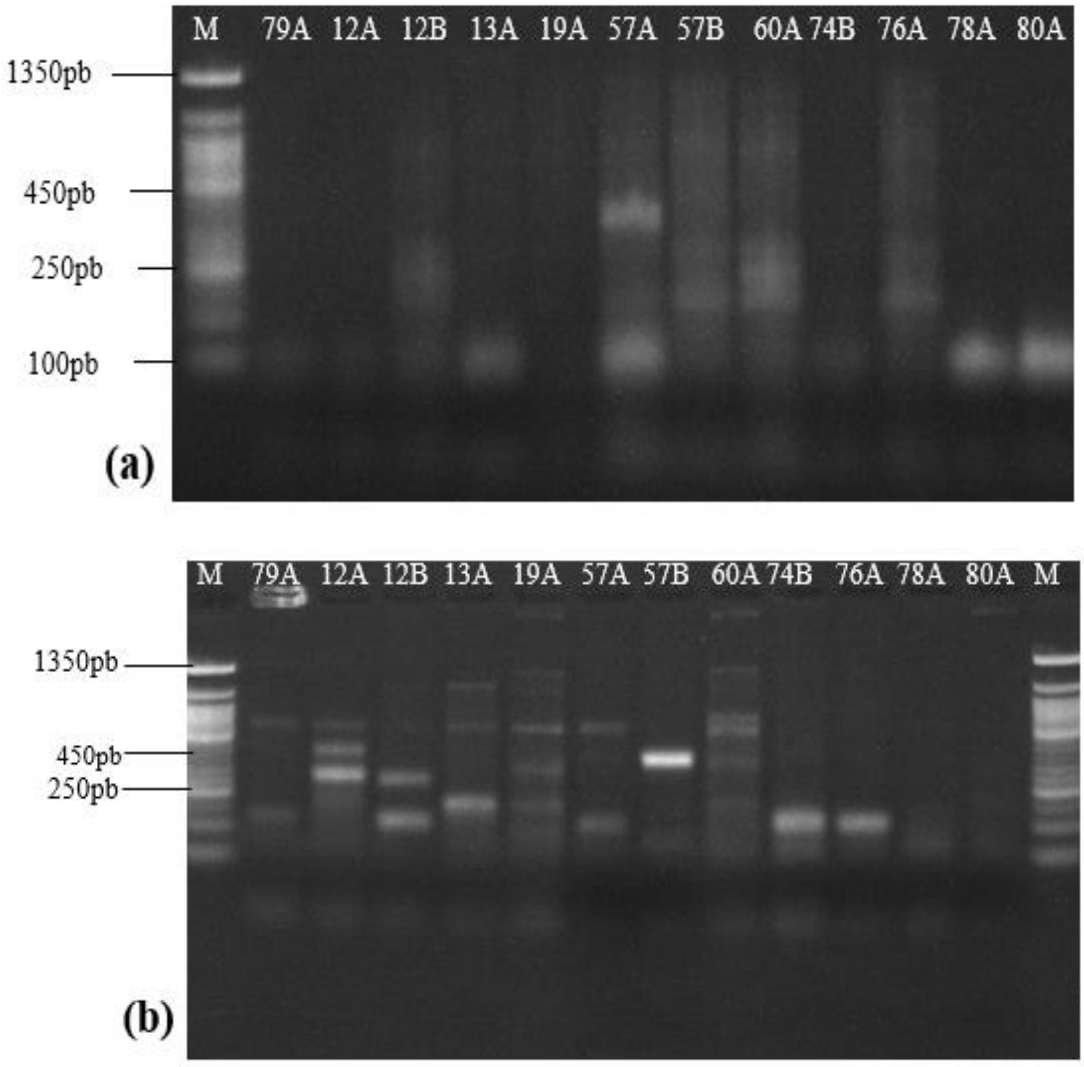

Figure 2: PCR product of targeted catabolic genes of bacterial isolates, (a): *alkB* genes, (b): *alkH* genes

Lane M: marker at varied bp size, lane 2 to 12 indicate corresponding bacterial isolates; **12A**: *Bacillus albus*, **12B**: *Enterococcus faecalis*, **13A**: *Providencia vermicola*, **19A**: *Pseudomonas aeruginosa*, **57A**: *Enterococcus faecalis*, **57B**: *Carnobacterium gallinarum* , **60A**: *Pseudomonas aeruginosa*, **74B**: *Enterococcus faecalis*, **76A**: *Enterococcus faecalis*, **78A**: *Ochrobactrum intermedium*, **79A**: *Enterococcus faecalis*, **80A**: *Pseudomonas aeruginosa*

Table 2: PAHs catabolic gene detected from bacterial isolates

Sample Codes	Bacterial Isolates	Genes		Expected Bands (bp)	
		alkB	***alkH***	***alkB***	***alkH***
79A	*Enterococcus faecalis*	+	+	100	650
12A	*Bacillus albus*	+	+	100	600
12B	*Enterococcus faecalis*	+	+	300	400
13A	*Providencia vermicola*	+	+	100	300
19A	*Pseudomonas aeruginosa*	-	+	-	650
57A	*Enterococcus faecalis*	+	+	100	150
57B	*Carnobacterium gallinarum*	+	+	250	450
60A	*Pseudomonas aeruginosa*	+	+	200	700
74B	*Enterococcus faecalis*	+	+	100	200
76A	*Enterococcus faecalis*	+	+	100	250
78A	*Ochrobactrum intermedium*	+	+	100	100
80A	*Pseudomonas aeruginosa*	+	+	100	100

+: present, -: absent, *alkB*: alkane hydroxylase gene, *alkH*: aldehyde dehydrogenase gene, A: isolated from motor mechanic workshop soil, B: isolated from landfill soil polluted with WEO

C12O and *C23O* genes were expressed at different bp sizes as presented in Table 3. The PCR products of the catabolic gene coding for catabolic enzyme *C12O* (75 to 800 bp) and *C23O* (< 80 bp) revealed bp sizes of the PCR product for *Enterococcus faecalis* (MK298392.1), *Bacillus albus* (MT325971), *Providencia vermicola* (MT325973), *Pseudomonas aeruginosa* (JF756593.1), *Enterococcus faecalis* (MT345788), *Carnobacterium gallinarum* (MT350233), *Pseudomonas aeruginosa* (MT350120), *Enterococcus faecalis* (MT345789), *Enterococcus faecalis* (MT345790), *Ochrobactrum intermedium* (MT350121) and *Pseudomonas aeruginosa* (MT338839).

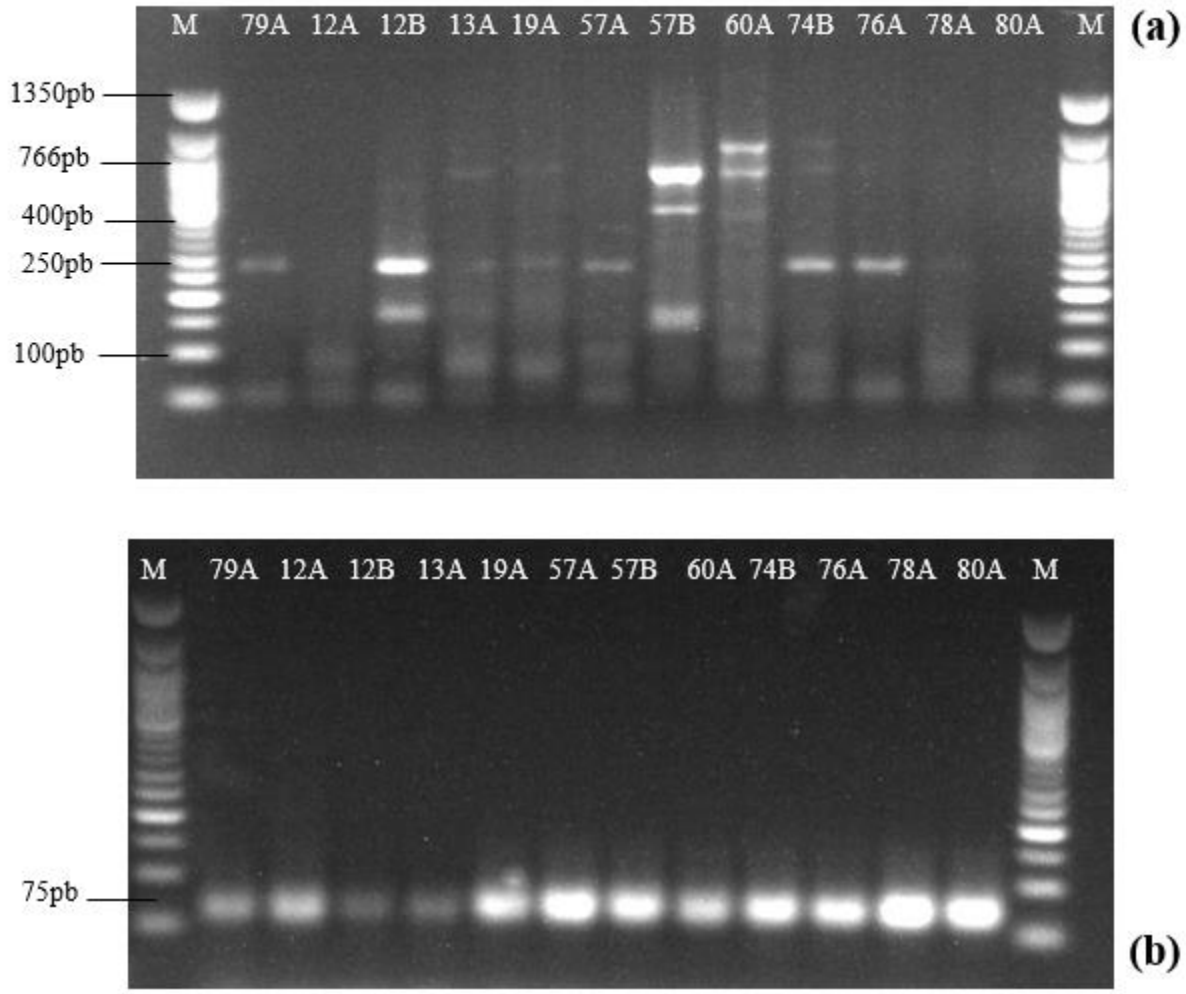

Figure 3: PCR product of targeted catabolic genes coding for degrading enzymes of bacterial isolates, (a): *C12O*, (b): *C23O* genes

Lane M: marker at varied bp size, lane 2 to 12 indicate corresponding bacterial isolates; **12A**: *Bacillus albus,* **12B**: *Enterococcus faecalis,* **13A**: *Providencia vermicola*, **19A**: *Pseudomonas aeruginosa*, **57A**: *Enterococcus faecalis,* **57B**: *Carnobacterium gallinarum* , **60A**: *Pseudomonas aeruginosa*, **74B**: *Enterococcus faecalis,* **76A**: *Enterococcus faecalis,* **78A**: *Ochrobactrum intermedium,* **79A**: *Enterococcus faecalis,* **80A**: *Pseudomonas aeruginosa*

Table 3: PAHs catabolic enzymes (*C12O* and *C23O*) detected from bacterial isolates

Sample Codes	Bacterial Isolates	Genes		Expected Bands (bp)	
		C12O	***C23O***	***C12O***	***C23O***
79A	*Enterococcus faecalis*	+	+	200	75
12A	*Bacillus albus*	+	+	100	75
12B	*Enterococcus faecalis*	+	+	250	75
13A	*Providencia vermicola*	+	+	100	75
19A	*Pseudomonas aeruginosa*	+	+	100	80
57A	*Enterococcus faecalis*	+	+	250	85
57B	*Carnobacterium gallinarum*	+	+	700	85
60A	*Pseudomonas aeruginosa*	+	+	800	80
74B	*Enterococcus faecalis*	+	+	250	80
76A	*Enterococcus faecalis*	+	+	250	80
78A	*Ochrobactrum intermedium*	+	+	75	80
80A	*Pseudomonas aeruginosa*	+	+	75	75

A: isolated from motor mechanic workshop soil, B: isolated from landfill soil polluted with WEO

Phylogenetic trees may show evolutionary relationships and evidence for the acquisition of specific genes by bacteria general (Coleman *et al.*, 2021; Filée *et al.*, 2008). *Carnobacterium gallinarum*, *Bacillus albus,* and *Ochrobactrum intermedium* may have evolved through genetic changes from *Enterococcus faecalis* since they shared a common ancestral origin. *Providencia vermicola* is closely related to *Pseudomonas aeruginosa* with the same ancestral origin. The catabolic gene alkane hydroxylase (*alkB*) and aldehyde dehydrogenase (*alkH*) is used to trace the degrading capacity of hydrocarbon utilizing bacterial isolates (Hesham *et al.*, 2014). The PCR product by *alkB* genes revealed low bp size (100 to 300 bp) indicating their ability to travel freely from the cathode (negative) end to the anode (positive) on the DNA ladder. The *alkH* genes were expressed at large bp size (< 700 bp), indicating slow movement. The weight of the PCR product of *alkH* genes may be attributed to their confinement to the cell wall whereas the *alkB* genes are hydrophobic in nature which can make them move from the cell wall to the cell membrane. The *alkB* and *alkH* exists as monooxygenase and dioxygenase which are majorly conserved among the Gram negative HC degrading bacteria (Cébron *et al.*, 2008). The *C12O* and *C23O* genes are used by bacteria to initiate the auto and meta-cleavage activities of carbon to carbon bonds in PAHs ring. *C12O* genes (>250 bp) were observed to be higher compared to *C23O* genes (<80 pb). PAHs ring biotransformation via cleavage pathway is dependent on the bacterial species and substrate for growth as reported by Haleyur *et al.* (2018) which was the case for this study.

CONCLUSION

The complex interplay in the degradation of PAHs by bacterial populations can only be understood if we get a better understanding of hydrocarbon-degrading genes possessed by these organisms. This study examined four important PAH degrading genes (*alkB*, *alkH*, *C12O*, and *C23O*) from bacteria populations capable of degrading LMW and HMW PAHs. These genes are coding for the enzymes responsible for hydroxylation and intradiol or extradiol ring-cleaving activities. It was observed from this study that the bacterial population from the artificially polluted landfill soil possessed these genes, which is an indication that genes are released during adaptation to the surrounding environmental conditions.

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CONFLICT OF INTEREST

The authors declare that there was no conflict of interest during the cause of the study.

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