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Original article

Detection of alkB and nahAc Genes in *Pseudomonas* sp. Isolated from Petroleum Contaminated Soil

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ABSTRACT

Petroleum and its byproducts are still the main sources of energy for many different types of businesses and daily living. However, because some products are acutely poisonous or have mutagenic, teratogenic, or carcinogenic qualities, their discharge into the environment is a global problem. Indiscriminate disposal of crude oil into the environment has no doubt increased hydrocarbon pollution in Nigeria. Microorganisms have been identified as major contributors in fighting pollution by utilizing hydrocarbons to enhance cell growth and energy needs. This study was aimed at detecting alkB and nahAc genes from *Pseudomonas* sp. isolated from petroleum contaminated soil of Kaduna Refining and Petrochemical Company (KRPC), Kaduna, Nigeria. Bacteria from the petroleum contaminated soil were isolated and confirmed using standard biochemical tests and molecular methods. The bacterial isolates were screened using soil enrichment technique. Out of the 18 bacteria isolated, *Pseudomonas* sp. having the highest hydrocarbon utilizing ability was selected for further studies. Polymerase chain reaction (PCR) experiments with specific primers confirmed the presence of alkB and nahAc genes in *Pseudomonas* sp. The presence of alkB and nahAc genes in *Pseudomonas* sp. from this study indicates that *Pseudomonas* sp. from the petroleum contaminated soil may have the potential to degrade petroleum and therefore be useful in the bioremediation of petroleum contaminated soil.

Keywords: Isolation, *Pseudomonas,* Detection, Genes, Petroleum

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INTRODUCTION

Given that petroleum and petroleum products are employed in practically all industrial processes as well as in many parts of daily life, they are a major cause of soil contamination. Environmental degradation and contamination on a big scale have been caused by industrialization and resource extraction [1]. Excessive volumes of hazardous waste have been scattered throughout thousands of polluted locations in the Niger Delta and other states with refineries, such as Kaduna, Nigeria. This poses a threat to both human and environmental health, and the combination of chemicals it contains is one of the main causes of the worldwide cancer and degenerative disease epidemics. Ecosystems in soil and water are at risk of petroleum contamination due to leaking above-ground and underground storage tanks, spills that occur during the transportation of petroleum products, abandoned manufactured gas sites, and a variety of industrial processes. Remediating these sources of contamination is costly. The introduction of hydrocarbons into certain habitats has been caused by human activity and, in rare instances, natural processes. This has resulted in significant alterations in microbial communities [2]. Thus, it is difficult to come up with creative and practical ways to clean up contaminated areas so that people can live there safely while still preserving the health of the ecosystems that sustain life [3].

The process of treating a contaminated environment using biological mechanisms is referred to as bioremediation [4]. At the moment, this biotechnological approach is the most affordable and effective at eliminating pollutants compared to traditional physicochemical approaches [5]. Growth and co-metabolism are the two processes that underpin this transformation. While growth substrate is used in co-metabolism, organic contaminants are the only source of energy and carbon during growth. The process leads to mineralization, which is a total degradation [6]. The primary and significant method of eliminating alkanes and polycyclic aromatic hydrocarbons (PAHs) from the environment is by microbial degradation, which is carried out by bacteria, yeasts, filamentous fungi, and algae [7]. Numerous bacteria that are able to metabolize PAHs have been discovered; these bacteria primarily come from the genera *Pseudomonas*, *Burkholderia*, *Mycobacteria*, *Rhodococcus*, *Alcaligenes*, and *Ralstonia* [8].

Potential benefits of treating polluted soil using microorganisms include full breakdown of the contaminants, reduced treatment costs, reduced soil disturbance, and increased safety [9]. The majority of the breakdown of PAHs and aliphatic compounds is accomplished by the mono- and dioxygenases that bacteria produce [10] which enables them use hydrocarbons as their exclusive source of carbon and are thought to have a selective advantage over other species. These genes, known as catabolic genes, are identified by or stimulate metabolic activity related to the disintegration of complex molecules (such proteins or lipids) and the organism's release of energy: associated with, typified by, or promoting catabolism [11]. These genes

can be isolated, purified, and engineered into other microorganisms with a shorter generation for effective degradation of hydrocarbons. The aim of this study was to detect catabolic genes with the potential of degrading hydrocarbon from hydrocarbon utilizing bacteria isolated from petroleum contaminated soil.

MATERIALS AND METHODS

Sample collection

Petroleum contaminated soil samples were collected from Kaduna refinery, Kaduna according to the method described by Moses *et al*. [12]. Using a sterile spatula, soil samples from three randomly selected locations, spaced approximately 1 m apart and reaching a depth of 4–6 cm were collected. The samples were then combined and put into sterile glass bottles from three distinct sample collecting locations, spaced 10 m apart. Bulked samples from the first, second and third points were labelled A, B and C respectively. All the samples were immediately transported to Microbiology Laboratory, Kaduna State University for further analysis.

Isolation of bacteria from petroleum contaminated soil

Soil enrichment technique was used for the isolation of bacteria from petroleum contaminated soil as described by Hesham *et al*. [10]. Ten grams (10 g) of petroleum contaminated soil sample was suspended in 90 mL mineral salt medium (MSM) containing (g/L) 1.0 $(NH_4)2SO_4$, 0.8 K_2HPO_4, 0.2 KH_2PO_4, 0.2 $MgSO_4 \cdot 7H_2O$, 0.1 $CaCl_2 \cdot 2H_2O$ and 0.005 $FeSO_4 \cdot 7H_2O$, pH 7.0 ± 0.2. The medium was supplemented with petroleum at concentration of 100 mg/L as a sole source of carbon. The flasks were incubated on an orbital shaker at 150 rpm at 30°C. After 7 days of incubation, an aliquot of 10% enriched cultures was transferred into another 250 mL conical flask containing 90 mL of fresh autoclaved mineral basal salt (MBS) medium supplemented with petroleum. This step was repeated five times to attain well-adapted hydrocarbon-degrading enriched bacterial consortia. Change in turbidity was taken as the measure of growth. Optical density readings at 600nm were taken for 7 days using cell density meter.

Screening of bacteria from petroleum contaminated soil

Bacterial colonies which appeared on mineral basal salt plates containing petroleum were picked randomly using a sterile glass rod and introduced into tubes containing 5 mL of fresh mineral basal salt broth and 0.5% petroleum as sole source of carbon and energy for screening. The tubes were incubated at 28°C in an orbital shaker at 120 rpm for 7 days. A control was prepared by excluding petroleum and kept under the same condition. Change in optical density readings was taken for 7 days. Optical density (OD) values between 0 and 0.25 were considered to have no growth, 0.26 and 0.50, minimal growth, 0.51 and 0.75, moderate growth and 0.76 and 1, maximum growth.

Characterization and identification of bacteria from petroleum contaminated soil

The bacterial isolates were characterized using colonial morphology, Gram staining and standard biochemical tests such as coagulase test, motility test, catalase test, methyl red test, Voges-Proskaeur test, indole test, citrate utilization test, urease test, carbohydrate utilization test and triple sugar iron (T.S.I) test [13] and identified by comparing their

characteristics with those of known taxa using Bergey's Manual of Systemic Bacteriology.

16S rRNA gene amplification of *Pseudomonas* sp. from petroleum contaminated soil

Genomic DNA from two strains of *Pseudomonas* sp. were isolated according to the method described by Hesham *et al*. [10]. Amplification was carried out using the following sets of universal primers: 27F (5-AGAGTTTGATCCTGGCTCAG-3) and 1492R (5-CGGCTACCTTGTTACGACTT-3) in a final volume of 20 µL of reaction mix containing 10 mM Tris-HCl (pH 9.0). 50 mM KCI, 2.5 mM $MgCl_2$, 200 µg/mL BSA, 200 µM each of dATP, dCTP, dGTP, dTTP, 0.2 µM each of primers and 2.5 µL of Taq polymerase. The reaction mixtures were overlaid with 100 µL mineral oil and boiled for 2 min prior to the addition of Taq polymerase. The samples were subjected to denaturation at 95^{o}C for 5 min, 35 cycles with 40s at 94^{o}C, 40s at 55^{o}C, 60s at 72^{o}C, and final elongation for 7min at 72^{o}C for the two genes alkB and nahAc. All PCR products were separated in 1.5% agarose gel, stained with ethidium bromide and viewed under UV light afterwhich it was photographed.

RESULTS

Cultural and biochemical characteristics of petroleum degrading bacteria

Eighteen (18) bacteria were isolated from the petroleum contaminated soil (Table 1). The microscopic and biochemical characteristics of bacteria observed from the petroleum contaminated soil showed species belonging to six genera - *Achromobacter* sp., *Bacillus* sp., *Providencia* sp., *Pseudomonas* sp., *Acinetobacter* sp. and *Alcaligenes* sp. The genus, *Pseudomonas* had the highest frequency of occurrence (28%), followed by *Bacillus* (22%), and *Providencia* (17%), while *Achromobacter, Acinetobacter,* and *Alcaligenes* were 11% each.

Screening of bacteria from petroleum contaminated soil

The extent of crude oil utilization by the bacterial isolates is reported in Table 2. It shows the mean value of the optical density (600nm) of the tubes taken and the inference. Out of the eighteen bacteria isolated from the petroleum contaminated soil, Isolates A_4 and B_3 representing 11 % of the isolates showed maximum growth in the mineral salt broth with OD values of 0.87 and 0.97 respectively. On the other hand, A_3 and B_6 showed moderate growth representing 11 % of the isolates with corresponding OD values of 0.63 and 0.61. All other isolates showed minimal growth in the mineral salt broth representing 78 % of the entire isolates. The control sample however showed no growth with an OD value of 0.2. *Pseudomonas* sp. having the highest OD reading was selected for further studies.

Table 1: Cultural and Biochemical Characteristics of Petroleum Utilizing Bacterial Isolates Obtained from Soil

Isolate code	Shape	Gram Rxn	Citrate	Catalase	Indole	MR	VP	H_2S production	Oxidase	Glucose	Lactose	Mannitol	Sucrose	Probable organism
A_1	Rod	+ve	+	+	-	-	+	-	-	AG	A	A	A	*Achromobacter* sp.
A_2	Rod	+ve	+	+	-	-	+	-	-	A	A	A	-	*Bacillus* sp.
A_3	Rod	-ve	+	+	+	+	-	-	-	A	A	A	A/G	*Providencia* sp.
A_4	Rod	-ve	-	+	-	-	-	-	+	A/G	A	-	A;	*Pseudomonas* sp.
A_5	Rod	-ve	-	+	-	+	-	-	+	A	A	A	A	*Alcaligenes* sp.
B_1	Rod	-ve	+	+	-	+	-	-	+	A	-	-	A	*Acinetobacter* sp.
B_2	Rod	+ve	+	+	-	-	+	-	-	A	A	A	-	*Bacillus* sp.
B_3	Rod	-ve	+	+	-	+	-	-	+	A	-	-	A	*Pseudomonas* sp.
B_4	Rod	-ve	-	+	-	-	-	-	+	A/G	A	-	A;	*Acinetobacter* sp.
B_5	Rod	+ve	+	+	-	-	+	-	-	AG	A	A	A	*Achromobacter* sp.
B_6	Rod	-ve	+	+	+	+	-	-	-	A	A	A	A/G	*Providencia* sp.
B_7	Rod	+ve	+	+	-	-	+	-	-	A	A	A	-	*Bacillus* sp.
C_1	Rod	-ve	+	+	+	+	-	-	-	A	A	A	A/G	*Providencia* sp.
C_2	Rod	-ve	-	+	-	-	-	-	+	A/G	A	-	A;	*Pseudomonas* sp.
C_3	Rod	-ve	-	+	-	-	-	-	+	A/G	A	-	A;	*Pseudomonas* sp.
C_4	Rod	+ve	+	+	-	-	+	-	-	A	A	A	-	*Bacillus* sp.
C_5	Rod	-ve	-	+	-	+	-	-	+	A	A	A	A	*Alcaligenes* sp.
C_6	Rod	-ve	-	+	-	-	-	-	+	A/G	A	-	A;	*Pseudomonas* sp.

Key A = Acid and G = gas

Table 2: Bacterial Growth in Petroleum Medium and their Mean Optical Density (OD)

Coded Isolates	Mean OD_{600nm}	Inference
A_1	0.41	Minimal growth
A_2	0.45	Minimal growth
A_3	0.63	Moderate growth
A_4	0.87	Maximum growth
A_5	0.38	Minimal growth
B_1	0.42	Minimal growth
B_2	0.39	Minimal growth
B_3	0.97	Maximum growth
B_4	0.43	Minimal growth
B_5	0.37	Minimal growth
B_6	0.61	Moderate growth
B_7	0.44	Minimal growth
C_1	0.42	Minimal growth
C_2	0.38	Minimal growth
C_3	0.43	Minimal growth
C_4	0.45	Minimal growth
C_5	0.44	Minimal growth
C_6	0.36	Minimal growth
Control	0.2	No growth

16S rRNA gene amplification of Pseudomonas sp. from petroleum contaminated soil

The gel electrophoresis of the two bacteria with highest OD from the petroleum contaminated soil is represented in Figure 1. The gel electrophoregram revealed the presence of *Pseudomonas* sp. (B_3 and A_4). Alkane monooxygenase (alkB) and naphthalene dioxygenase (nahAc) genes were detected for the most effective petroleum degrader (*Pseudomonas* sp. B_3) isolated in this study as shown in Figure 2. The sizes of PCR products for alkB and nahAc were approximately 100 bp and 487 bp respectively. Finally, no signals for alkB1 gene detected.

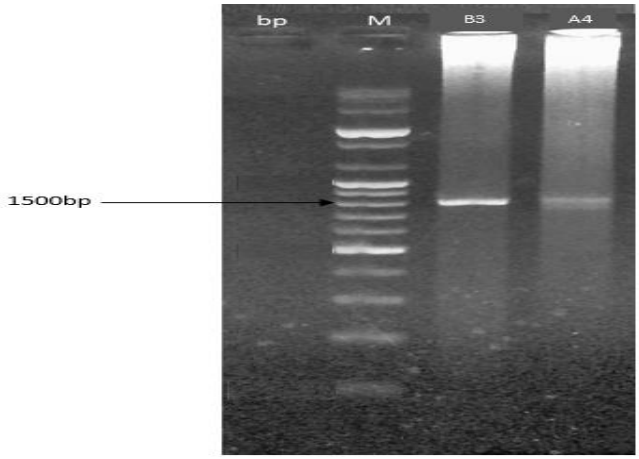

Figure 1: Gel electrophoregram for amplified 16S rRNA of isolates (*Pseudomonas* sp. B_3 and *Pseudomonas* sp. A_4)

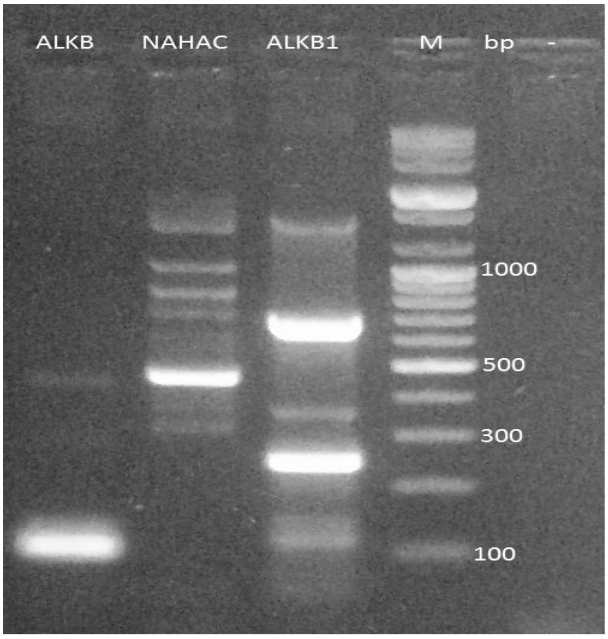

Figure 2: PCR products based on primers specific for the catabolic genes from the left to the right, AlkB and nahAc detected in *Pseudomonas* sp. (B1) with 1000 bp DNA ladder (M).

DISCUSSION

Petroleum hydrocarbons will always remain the most important energy and chemical source as well as the most challenging organic pollutants in future [15]. Microbial strains isolated from

specific contaminated environments have shown bioremediation potentials thus offering a green alternative approach to clean up these hazardous environmental pollutants [16].

In this study, *Pseudomonas* had the highest frequency of occurrence, followed by *Bacillus*, *Providencia*, *Achromobacter, Acinetobacter* and *Alcaligenes.* The bacteria isolated in this study were similar to the report of other researchers with few differences. The differences in organisms could be attributed to the soil type, different hydrocarbon exposed to soil and extent of hydrocarbon pollution of the contaminated soil. *Pseudomonas* sp. being the predominant bacterial isolate from hydrocarbon contaminated soil in this study is contrary to the work of Ijah and Antai [17] who reported *Bacillus* sp. as being the predominant bacteria isolated from hydrocarbon contaminated soil. According to Sharma *et al*. [18], *Pseudomonas* sp. has been identified as the most predominant group in the metabolism of hydrocarbons. This bacterium possesses a broad array of physiological and metabolic properties as well as a complex enzymatic system that enables it to utilize a wide range of aliphatic aromatic compounds as their sole carbon source. Sharma *et al*. [18] worked with *Pseudomonas aeruginosa* DSVP20 and suggested that the ability of *Pseudomonas* sp. to take up alkanes was due to their production of a biosurfactant called rhamnolipids. Degradative plasmids such as OCT (octane), ALK (alkanes), TOL (toluene), XYL (xylene) and NAH (naphthalene) found in *Pseudomonas* makes it metabolically versatile [19]. *Acinetobacter* sp. as a hydrocarbon degrader has been reported also by Ugoma *et al*. [20]. In a research by Ho *et al*. [21], *Acinetobacter calcoaceticus* CA16, isolated from Canadian soil was able to degrade 82 to 92% of aliphatic alkane hydrocarbons (CnHn + 2; where n = 12–18) in 28 days. Several diesel-degrading genes (such as alkM and xcpR) that are present in other microbes were also found to be activated in CA16.

Monooxygenase (alkB) and dioxygenase (nahAc) genes were detected in this study. The existence of monooxygenase (alkB) and dioxygenase (nahAc) genes was confirmed and this is in agreement with other researchers who detected similar catabolic genes. Gurav *et al*. [22] detected alkB and nahAc genes which is in agreement to the genes detected in this present study. Hesham *et al*. [23] in addition detected $C_{12}O$ and $C_{23}O$ dioxygenases genes. The difference in catabolic genes detected could be due to the difference in organism isolated and also the difference in the hydrocarbon used can contribute. Monooxygenase (alkB) and dioxygenase (nahAc) genes are conserved among different Gram-negative bacteria and this could be the reason why they were detected in this study. However, no signals for AlkB1 gene was detected since it is conserved among Gram-positive bacteria [24]. The results in this study demonstrated that the two genes were present in *Pseudomonasv* sp., suggesting that this bacterium can play an active role in the degradation of aliphatic and PAHs and hence be recommended for bioremediation of petroleum compounds in the environment.

CONCLUSION

This study reports the isolation, identification, and characterization of a very efficient bacterium species - *Pseudomonas* sp. from petroleum

contaminated soil. The existence of catabolic genes including monooxygenase (alkB) and dioxygenase (nahAc) genes responsible for aliphatic and polyaromatic hydrocarbons degradation were confirmed. The detection of these genes shows that *Pseudomonas* sp. may possess the potential to degrade petroleum.

Authors contribution

DD, AOP and MFM conceptualized and designed the study. DD participated in fieldwork. DD, AOP, MFM, MBE and YJB interpreted the data. DD, MBE and YJB prepared the first draft of the manuscript, reviewed by AOP and MFM. All authors contributed to the development of the final manuscript and approved its submission.

Disclosure of conflict of interest

None

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REFERENCES

1. Ashraf, M. A., Maah, M. J. and Yusoff, I. (2014). Soil contamination, risk assessment and remediation. *Environmental Risk Assessment of Soil Contamination*, *1*, 3-56.

2. Kebede, G., Tafese, T., Abda, E. M., Kamaraj, M. and Assefa, F. (2021). Factors influencing the bacterial bioremediation of hydrocarbon contaminants in the soil: mechanisms and impacts. *Journal of Chemistry*, *2021*, 1-17.

3. Yakubu, O. H. (2017). Particle (soot) pollution in Port Harcourt Rivers State, Nigeria—double air pollution burden? Understanding and tackling potential environmental public health impacts. *Environments*, *5*(1), 2.

4. Adam, F. V., Niyomugabo, A. and Sylvester, O. P. (2017). Bioremediation of Crude Oil Contaminated Soil Using Agricultural Wastes. *Procedia Manufacturing,* (7), 459-464.

5. Razzak, S. A., Faruque, M. O., Alsheikh, Z., Alsheikhmohamad, L., Alkuroud, D., Alfayez, A., ... and Hossain, M. M. (2022). A comprehensive review on conventional and biological-driven heavy metals removal from industrial wastewater. *Environmental Advances*, *7*, 100168.

6. Gao, L. and Gu, J. D. (2021). A new unified conceptual framework involving maintenance energy, metabolism and toxicity for research on degradation of organic pollutants. *International Biodeterioration and Biodegradation*, *162*, 105253.

7. Thacharodi, A., Hassan, S., Singh, T., Mandal, R., Khan, H. A., Hussain, M. A. and Pugazhendhi, A. (2023). Bioremediation of polycyclic aromatic hydrocarbons: An updated microbiological review. *Chemosphere*, 138498.

8. Kour, D., Khan, S. S., Kour, H., Kaur, T., Devi, R., Rai, P. K.,... and Yadav, A. N. (2022). Microbe-mediated bioremediation: Current research and future challenges. *Journal of Applied Biology and Biotechnology*, *10*(2), 6-24.

9. Aparicio, J. D., Raimondo, E. E., Saez, J. M., Costa-Gutierrez, S. B., Alvarez, A., Benimeli, C. S. and Polti, M. A. (2022). The current approach to soil

remediation: A review of physicochemical and biological technologies, and the potential of their strategic combination. *Journal of Environmental Chemical Engineering*, *10*(2), 107141.

10. Hesham, A., Wang, Z. Y., Zhang, Y., Zhang, J. and Yang, M. (2006). Isolation and identification of a yeast strain capable of degrading four and five ring aromatic hydrocarbons, *Annals of Microbiology*, vol. 56, pp. 109–112.

11. Scholtes, C. and Giguère, V. (2022). Transcriptional control of energy metabolism by nuclear receptors. *Nature Reviews Molecular Cell Biology*, *23*(11), 750-770.

12. Moses, B. E., Egwim, C. E., Abdulkadir, A., Liman, M. L., Alex, V. A. and Manya, Z. (2020). Production, Partial Purification and Characterization of Lipase Enzyme Expressed by *Klebsiella pneumoniae* of Vegetable Oil Contaminated Soil. *International Journal of Biochemistry and Biophysics,* 8(2): 30 – 39.

13. Cheesbrough, Monica. *District laboratory practice in tropical countries, part 2*. Cambridge university press, 2005.

14. Laurie, A.D. and Jones, G. (2000). "Quantification of phnAc and nahAc in contaminated New Zealand soils by competitive PCR," *Applied and Environmental Microbiology*, *66*(5) pp. 1814–1817.

15. Kuppusamy, S., Maddela, N. R., Megharaj, M. and Venkateswarlu, K. (2020). Total petroleum hydrocarbons. *Environ. Fate Toxic. Remediat*.

16. Jia, Z., Deng, J., Chen, N., Shi, W., Tang, X. and Xu, H. (2017). Bioremediation of cadmium-dichlorophen co-contaminated soil by spent Lentinus edodes substrate and its effects on microbial activity and biochemical properties of soil. *Journal of Soils and Sediments*, *17*, 315-325.

17. Ijah, U.J.J. and Antai, S. P. (2003). The potential use of chicken-drop micro-organisms for oil spill remediation. *The Environmentalist, 23*(1), 89-95.

18. Sharma, D., Ansari, M. J., Al-Ghamdi, A., Adgaba, N., Khan, K. A., Pruthi, V. and AlWaili, N. (2015). Biosurfactant production by Pseudomonas aeruginosa DSVP20 isolated from petroleum hydrocarbon-contaminated soil and its physicochemical characterization. *Environmental Science and Pollution Research. 22*(22), 17636-17643.

19. Bhatt, P., Bhandari, G., Bhatt, K., Maithani, D., Mishra, S., Gangola, S., and Chen, S. (2021). Plasmid-mediated catabolism for the removal of xenobiotics from the environment. *Journal of Hazardous Materials*, *420*, 126618.

20. Ugoma, F. E., Ijah, U. J. J., Abioye, O. P. and Musa, I. O. (2023). Remediation of crude oil contaminated soil with locally formulated bioremediation agent. *Science World Journal*, *18*(4), 575-583.

21. Ho, M. T., Li, M. S., McDowell, T., MacDonald, J. and Yuan, Z. C. (2020). Characterization and genomic analysis of a diesel-degrading bacterium,

Acinetobacter calcoaceticus CA16, isolated from Canadian soil. *BMC biotechnology*, *20*(1), 1-15.

22. Gurav, R., Lyu, H., Ma, J., Tang, J., Liu, Q. and Zhang, H. (2017). Degradation of n-alkanes and PAHs from the heavy crude oil using salt-tolerant bacterial consortia and analysis of their catabolic genes. *Environmental Science and Pollution Research*, *24*, 11392-11403.

23. Hesham, A. E. L., Mawad, A. M., Mostafa, Y. M. and Shoreit, A. (2014). Biodegradation ability and catabolic genes of petroleum-degrading Sphingomonas koreensis strain ASU-06 isolated from Egyptian oily soil. *BioMed research international*, *2014*.

24. Liu, X., Liu, M., Zhou, L., Hou, L., Yang, Y., Wu, D.,… and Gu, J. (2021). Occurrence and distribution of PAHs and microbial communities in nearshore sediments of the Knysna Estuary, South Africa. *Environmental Pollution*, *270*, 116083.