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THE USE OF INDIGENOUS HYDROCARBON UTILIZING BACTERIA IN BIOREACTOR-BASED BIOREMEDIATION OF HYDROCARBON-POLLUTED SOIL OF AWOYE, ONDO STATE, NIGERIA

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ABSTRACT
Petroleum exploration in riverine areas of Ondo State have caused numerous problems by generating hazardous waste pollution that endangers plants, animals, and human lives. The aim of this study was to isolate indigenous hydrocarbon utilizing bacteria from oil polluted soil and use them in bioreactor-based bioremediation of hydrocarbon-polluted soil of Awoye, Ondo State, Nigeria. Soil samples were collected from Awoye community using standard techniques. The bacteria from the soil were identified using cultural and molecular methods. Proteus mirabilis, Escherichia fergusonii, Klebsiella pneumoniae and Clostridium sporogenes were identified. The four bacterial isolates were then tested in three bioremediation treatments of oil polluted soils inside 5 litres stirred tank bioreactors labeled Awoye soil on Natural attenuation (AWNA), Awoye soil on Bioaugmentation (AWBA) and Awoye soil whose contents have been heat killed (AWHK) over a 56 - day period. In the AWNA treatment, the Total Petroleum Hydrocarbon (TPH) decreased by 59.04 % while it was 78.33 % in the AWBA treatment and 3 % reduction in TPH content of the AWHK treatment. It was concluded that the oil polluted soils have inherent bacteria group that can utilize hydrocarbon as carbon source and can be used for bioremediation of oil polluted soil.

Keywords: Bioremediation, Crude oil, Pollution, Bacteria, Total Petroleum Hydrocarbons.

INTRODUCTION

Because of human scientific advancement in industry, agriculture, and urbanization, soil contamination caused by diverse anthropogenic activities is a growing problem. These activities damage the quality of life of both plants and animals that dwell in nature (Zerizghi *et al.*,2021). Pollutants from factories, the use of pesticides and inorganic fertilizers, agricultural waste, urbanization, deforestation, and inadequate waste management have all contributed to an increase in environmental health risks and pollution in many countries around the world, especially in developing countries. This is especially true in countries where waste management is inadequate (Lebea *et al.*, 2017; Yasser *et al.*, 2022; World Bank, 2022). There is a clear correlation between the degree of industrialization and the quantity of chemicals used hence the state of the environment will deteriorate if pollution control measures are not implemented. (Hanna-Attisha *et al*., 2016; Vijay and Yamunanagar, 2017; Jiming *et al.*, 2022).
Contamination of the environment (primarily terrestrial and aquatic) by crude oil is caused majorly by oil spillage (Ozturk *et al.*, 2021).

For several decade all industrialized countries around the world have faced the problem of land contamination caused by crude oil and its processed products. These pollutants enter the soil primarily from oil extraction and processing in refineries, as well as any flaws in fuel storage by the entire human race. (Azam *et al.,* 2018, Ahmad *et al.,* 2018; Ziarati *et al*., 2019; Arabian *et al.,* 2020; Ozturk *et al.,* 2021; Wojtowicz *et al.*, 2022). Although, oil pollution alters the chemical and physical properties of soil and erodes soil nutrients, oil exploration is something Nigeria cannot do without as it has been the backbone of her economy, it generates foreign exchange and provide energy for the country's various economic activities. Accidental spills, leaks from producing wells, storage tanks, gathering lines, pipelines, flow stations, refineries, and industrial dump sites have all contributed to increased pollution of the Niger Delta environment. (Osuji and Onojake, 2006; Usman *et al.,* 2022). It is well knowledge that the process of extracting crude oil has a detrimental effect on the soil, plant life, and aquatic systems of the communities where it is carried out. (Phil-Eze and Okoro, 2009; Aguilera *et al.,* 2010; Usman *et al.,* 2022; Bello and Nwaeke, 2023). Oil exploration results in pollution, which in turn leads to climate change. It also has a negative impact on public lands, which were originally designated for everyone's use. The long-term effects of toxic substances on living species, such as plants or animals, will eventually lead to the extinction of certain organisms within a community or to the extinction of the community as a whole, together with the habitats of the organisms. (Khlifi and Hamza, 2010).

Petroleum-utilizing bacteria have emerged as a feasible technique of treating oil pollution in the environment due to their ability to scavenge hydrocarbons in the environment and use them as a source of food. This is despite the fact that it is difficult to remove oil contamination from soil and anything else in the near term (Barbara *et al.,* 2022). These microorganisms have the potential to be effective in the remediation of oil pollution. (Margesin *et al*., 2013; Ron and Rosenberg, 2014; Lea-Smith *et al.,* 2015). In recent years, the employment of bacteria as a method for dealing with environmental pollution has emerged as a potentially useful technique due to the fact that it is both inexpensive and kind to the environment. (Guerra *et al.,* 2018) The persistent growth and refinement of microbial remediation technology has also led to the creation of a novel approach for the remediation of petroleum hydrocarbon pollution, which has received a significant amount of attention as a result of its widespread application. (Dombrowski *et al.,* 2016; Dvořák *et al.,* 2017). The objective of the research is to use laboratory bioreactors to investigate the potential of indigenous microbial communities to biodegrade total petroleum hydrocarbon (TPH) in oil polluted soil and to establish the effect of mineral salt medium bioaugmentation on the degradation of TPH by the microbial communities.

MATERIALS AND METHODS

Sample Location

Soil samples used in this study were collected from two different locations at Awoye Community of Ilaje Local Government Area of Ondo State, Nigeria. The GPS location of each site were taken and recorded as Location A; N 05.91807°, E 004.99987° and Location B; N05.91805°, E 005.01677°. These areas show visible evidence of petroleum spills of both crude and refined oil products used by speed boat operators and other users. Oil Sheen test was carried out by using stick to disperse the sheen observed on the river, the petroleum sheen quickly try to reform after any disturbance confirming it to be petroleum sheen.

This indicates that the body of water is being routinely polluted by crude oil as seen in Figure 1. This oil-tainted water permeates the beach sediment from which we collected our soil samples.

The soil samples were collected from the river beach locations aseptically from 10 - 30 cm depth and transferred in an ice-chest to the Olusegun Agagu University of Science and Technology (OAUSTECH) laboratory for analysis within six hours of collection

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Figure 1: Sample location site at Awoye community

Isolation of bacteria.

1 gram of soil from each sample was dispensed into 10 milliliters of distilled water and used as the sample stock. With the aid of a sterile pipette, nine milliliters of distilled water were dispensed into various test tubes that would be used to transport one-to-ten serial dilutions. (Reynolds, 2005). Using the spread plate technique, 0.1 ml of the appropriate dilution factors 10^{-3} and 10^{-6}, as well as the stock used for each sample, were distributed on the surface of the medium in the petri dish using a sterile spreader rod. The Nutrient agar-containing dishes were incubated at 37 °C for 24 hours. Different culture characteristics were observed following incubation.

Characterization of bacterial isolates

The colonial morphology of the organisms that were isolated from the various plates was used to characterize the organisms. This morphology comprises the colony shape (such as circular, filamentous, or rhizoid), edge, elevation, color, and transparency. Standard procedures were utilized for the Gram staining, motility test, catalase test, oxidase test, indole test, Voges-Proskauer test, Methyl – red test, Nitrate reduction test, gelatin hydrolysis test, and sugar fermentation tests.

Molecular characterization of Bacterial Isolates

DNA Extraction

Genomic DNA extraction was carried out with column-based JENA Bioscience Bacteria DNA Preparation Kit following manufacturer's instructions. Bacteria cells were harvested from 500 µl aliquot of bacteria broth culture using a microcentrifuge at 10,000 g for 1min. The residual pellet was resuspended in 300 µl of Resuspension Buffer and 2 µl of Lysozyme Solution. The mixture was homogenized by inverting several times thereafter incubated at 37 °C for 1 hour.

Resuspended cells were recovered by centrifugation and lysed by adding 300 µl of Lysis Buffer after which 2 µl RNase A and 8 µl proteinase K solution were added; followed by incubation at 60°C for 10 mins. The tube was cooled on ice for 5 min. 300 µl binding buffer was added to the mixture and vortexed briefly; the mixture was cooled on ice for 5 min and thereafter centrifuged at 10,000 g for 5 min. The supernatant was transferred directly into the spin column and centrifuged at 10,000 g for 1min to trap the DNA. The trapped DNA was washed twice with washing buffer after which it was eluted with 50 µl elution buffer into a clean eppendorf tube. (Gupta, 2019).

Polymerase Chain Reaction

Each PCR reaction mixture consisted of 12.5 µl mastermix (2 x JENA Ruby hot start mastermix), 1 µl (10 pmol) each of forward primer 27F 5'AGA GTT TGA TCM TGG CTC AG3' and reverse primer 1492R-5' TAC GGY TAC CTT GTT ACG ACT T 3', 1 µl DNA template and 9.5 µl sterile nuclease free water to make up a total reaction volume of 25 µl. PCR amplification was carried out in an Applied Biosystem 2720 Thermocycler. The mixture was subjected to an initial denaturation at 94 °C for 3 min; followed by 35 cycles of denaturation at 94 °C for 45 sec , annealing at 55 °C for 60 sec and extension at 72 °C for 60 seconds; and a final extension at 72 °C for 10mins (Gupta, 2019).

Gel electrophoresis:

PCR products were visualized on a 2 % agarose gel containing ethidium bromide in 0.5x Tris borate buffer (pH 8.0) using blue led transilluminator. A molecular ladder marker (Jena Bioscience, 200 bp) was run simultaneously to determine the size of the amplicons.

Sequencing

PCR products were purified and sequenced by Sanger sequencing method using AB1 3730 XL sequencer and done by Inqaba biotec, Pretoria, South Africa.

RESULTS

The raw sequences for each isolate is edited and subjected to BLAST and the screenshot of the results are used to produce phylogenetic trees of each organism to scale.

Determination of Total Petroleum Hydrocarbons

Total petroleum hydrocarbon (TPH) was analysed using Agilent 7890B Gas chromatograph equipped with a flame ionization detector (FID), fitted with a HP-5 capillary column coated with 5 % Phenyl Methyl Siloxane (30 m length x 0.32 mm diameter x 0.25 µm film thickness) (Agilent Technologies). 1 µL of the samples were injected in spitless mode at an injection temperature of 220 °C, at a pressure of 14.861 psi and a total flow of 21.364 mL/min. Purge flow to split vent was set at 15 mL/min at 0.75 min. Oven was initially programmed at 60 °C (1 min) then ramped at 7.5 °C/min to 300 °C (9 min). FID temperature was 300 °C with Hydrogen: Air flow at 30 mL/min: 30 0mL/min, Nitrogen was used as makeup gas at a flow of 18mL/min. After calibration, the samples were analyzed, and corresponding concentrations calculated.

Screening of Bacteria Degrading Hydrocarbon

The detected bacterial isolates were grown on a medium consisting of Agar - Agar medium that had 5 % petroleum added to it as the only carbon source. This allowed the bacteria to be tested for their capacity to breakdown petroleum. The Agar-Agar medium does not include any minerals; rather, they are added to it to help solidify the petroleum, which is necessary for bacterial growth. The temperature for the incubation was 30 °C.

It may be concluded that isolates that were able to grow on this medium could grow using crude oil as their only supply of carbon, and as a result, they have the potential to breakdown hydrocarbons.

Bioremediation in Bioreactors

One (1) kilogram each of oil polluted soil samples from Awoye community was treated in three (3) 5L stirred tank bioreactors designated AWNA, AWBA and AWHK respectively over a 56-day period. In AWNA treatment, bioremediation was by natural attenuation that relies on the indigenous microbial population in the impacted soil, to degrade and remove TPH contaminants from that soil while AWBA treatment rely on indigenous microbial population with augmented nutrient addition (Mineral Salt Medium – MSM – One (1) liter of MSM contains 0.5 grams of CaCO3; 2.5 grams of NH3NO2; 1 gram of $Na_2HPO_47H_2O$; 0.5 grams of KH_2PO_4; 0.5 grams of $MgSO_3.7H_2O$; and 0.2 grams of $MnCl_2.7H_2O$). In AWHK however, the microbial population are heat killed to serve as control. The bioreactors were continuously stirred at 120 rpm throughout the 56-day experimental period at room temperature (30^oC) and pH 7.5. (Chioma *et al.,* 2012).

Statistical Analysis

The results obtained were entered into IBM Statistical Package for Social Sciences version 23 and analyzed. Descriptive statistics including frequency distribution, mean, standard deviation and range of the various parameters were determined.

DISCUSSION

The ability of the bacteria in the petroleum hydrocarbon contaminated site of Awoye to utilize hydrocarbons as sole carbon source was confirmed when they survived on Agar – Agar media infused with 5% crude oil as the only carbon source. This agrees with the works of earlier researchers who reported that hydrocarbon degrading bacteria can survive on crude oil as the only carbon source. (Bento *et al*., 2005; Gkorezis *et al.*, 2016). Soil from petroleum oil contaminated sites could be a potential source for the isolation of hydrocarbon degrading bacteria, which is why indigenous bacteria isolated from oil polluted soil in Awoye were tested for their ability to degrade petroleum oil. Table 1 shows the results of morphological and biochemical characteristics of the bacteria isolated from the soil. Phylogenetic trees of isolated strains are as shown in Figures 2, 3, 4 and 5 for *Clostridium sporogenes*, *Escherichia fergusonii*, *Proteus mirabilis* and *Klebsiella pneumoniae* respectively. In this study, microbial population in oil polluted soils are in consonant with a similar study which posited that microorganisms with potentials for oil degradation are widespread in nature and that they can be isolated from oil and oil contaminated sites for bioremediation purposes (Barbara *et.al.,* 2022). Other researchers also posited that there are several indigenous bacteria in oil polluted environment with inherent ability to degrade hydrocarbons (Jayashree *et al.,* 2012; Balogun *et al*., 2013; Vinothini *et al.,* 2015).

Table 1: Isolation and Characterization of bacterial isolates from Oil Polluted soil of Awoye

Isolate	Gr test	Form	Motility	Cat	Cit	Ure	Oxi	Indole	VP test	MR	GH	Suspected Organism
1	-	Rod	+	+	+	+	-	-	-	+	+	*Proteus mirabilis*
2	-	Rod	+	+	-	-	-	+	-	+	-	*Escherichia fergusonii*
3	-	Rod	-	+	+	+	-	-	+	-	-	*Klebsiella pneumoniae*
4	+	Rod	+	-	+	+	-	-	-	+	+	*Clostridium sporogenes*

Keys: Gr test – Gram staining test, Form- shape under microscopic view, Cat – Catalase test, Cit – Citrate test, Ure- Urease test, Oxi- Oxidase, VP - Voges–Proskauer, MR – Methy Red, GH = Gelatin Hydrolysis

Fig. 2: *Clostridium sporogenes*

Evolutionary analysis by Maximum Likelihood method

Evolutionary history was inferred by using the Maximum Likelihood method and Kimura 2-parameter model (Kimura, 1980). The bootstrap consensus tree inferred from 500 replicates (Felsenstein, 1985) is taken to represent the evolutionary history of the taxa analyzed. (Felsenstein, 1985). Branches corresponding to partitions reproduced in less than 50 % bootstrap replicates are collapsed. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (500 replicates) are shown next to the branches. Initial tree(s) for the heuristic search were obtained automatically by applying Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using the Maximum Composite Likelihood (MCL) approach, and then selecting the topology with superior log likelihood value. This analysis involved 26 nucleotide sequences. All positions with less than 80 % site coverage were eliminated, i.e., fewer than 20 % alignment gaps, missing data, and ambiguous bases were allowed at any position (partial deletion option). There were a total of 868 positions in the final dataset. Evolutionary analyses were conducted in MEGA X (Kumar *et.al*., 2018).

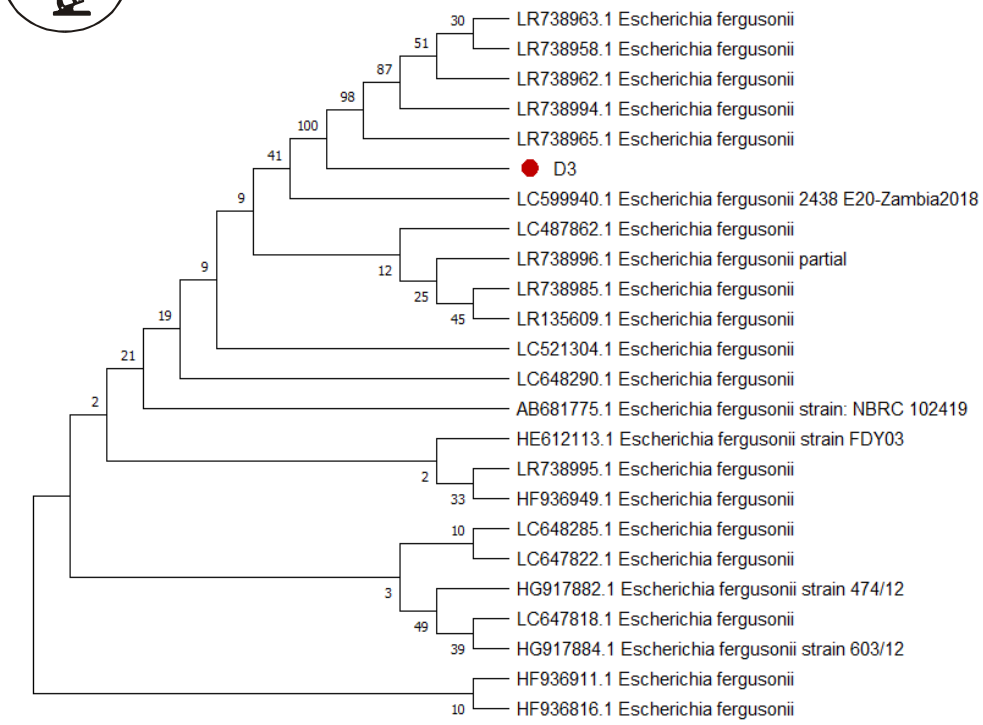

Fig. 3: ***Escherichia fergusonii***

Evolutionary analysis by Maximum Likelihood method

The evolutionary history was inferred by using the Maximum Likelihood method and Tamura 3-parameter model (Tamura, 1992). The bootstrap consensus tree inferred from 500 replicates (Felsenstein, 1985) is taken to represent the evolutionary history of the taxa analyzed (Felsenstein, 1985). Branches corresponding to partitions reproduced in less than 50 % bootstrap replicates are collapsed. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (500 replicates) are shown next to the branches. Initial tree(s) for the heuristic search were obtained.automatically by applying Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using the Tamura 3 parameter model, and then selecting the topology with superior log likelihood value. This analysis involved 24 nucleotide sequences. All positions with less than 80 % site coverage were eliminated, i.e., fewer than 20 % alignment gaps, missing data, and ambiguous bases were allowed at any position (partial deletion option). There were a total of 723 positions in the final dataset. Evolutionary analyses were conducted in MEGA X (Kumar, 2018).

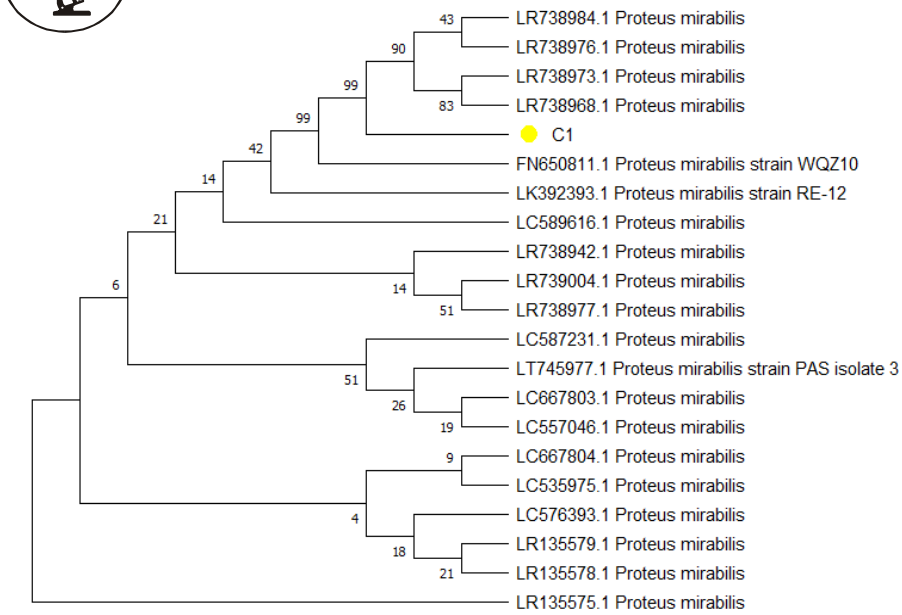

Fig. 4: ***Proteus mirabilis***

Evolutionary analysis by Maximum Likelihood method

The evolutionary history was inferred by using the Maximum Likelihood method and Kimura 2-parameter model (Kimura, 1980). The bootstrap consensus tree inferred from 500 replicates is taken to represent the evolutionary history of the taxa analyzed (Felsenstein, 1985). Branches corresponding to partitions reproduced in less than 50 % bootstrap replicates are collapsed. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (500 replicates) are shown next to the branches (Felsenstein, 1985**)**. Initial tree(s) for the heuristic search were obtained automatically by applying Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using the Maximum Composite Likelihood (MCL) approach, and then selecting the topology with superior log likelihood value. This analysis involved 21 nucleotide sequences. Codon positions included were 1st+2nd+3rd+Noncoding.All positions with less than 80 % site coverage were eliminated, i.e., fewer than 20% alignment gaps, missing data, and ambiguous bases were allowed at any position (partial deletion option). There were a total of 539 positions in the final dataset. Evolutionary analyses were conducted in MEGA X (Kumar, 2018).

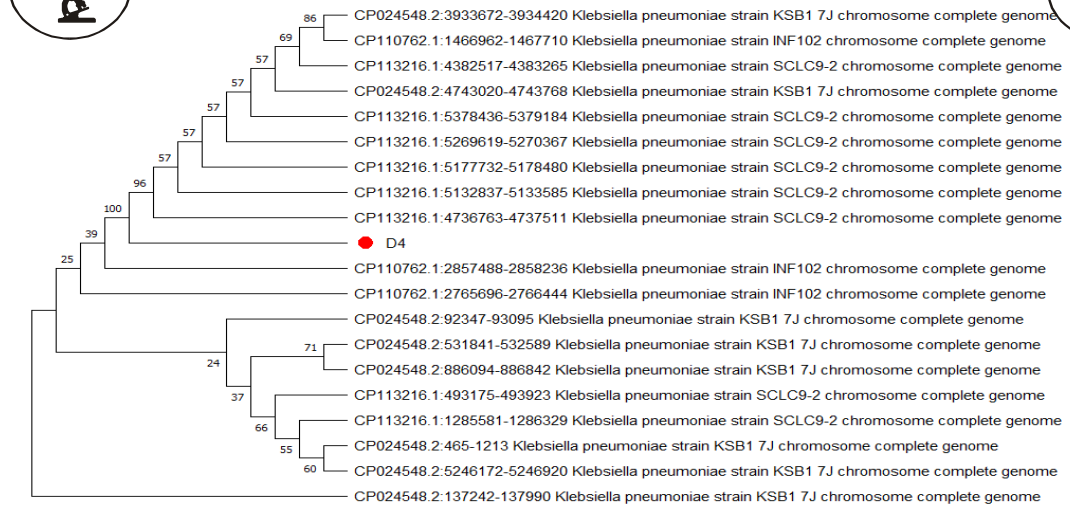

Figure 5: *Klebsiella pneumonia*

Evolutionary analysis by Maximum Likelihood method

The evolutionary history was inferred by using the Maximum Likelihood method and Jukes-Cantor model (Jukes and Cantor, 1969). The bootstrap consensus tree inferred from 500 replicates (Felsenstein, 1985) is taken to represent the evolutionary history of the taxa analyzed (Felsenstein 1985) Branches corresponding to partitions reproduced in less than 50 % bootstrap replicates are collapsed. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (500 replicates) are shown next to the branches [3]. Initial tree(s) for the heuristic search were obtained automatically by applying Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using the Jukes-Cantor model, and then selecting the topology with superior log likelihood value. This analysis involved 21 nucleotide sequences. All positions with less than 95% site coverage were eliminated, i.e., fewer than 5 % alignment gaps, missing data, and ambiguous bases were allowed at any position (partial deletion option). There were a total of 38 positions in the final dataset. Evolutionary analyses were conducted in MEGA X (Kumar et al., 2018)

When bioremediation is compared to other remediation techniques, it is an economical and sustainable method for removal of petroleum-based pollutants by microbial cells' metabolic process. Certain bacteria can metabolize many of the petroleum-based pollutants all the way to CO_2, H_2O and CH_4 (Rahman and Alam, 2021). A great number of studies have shown that the most effective bacteria in petroleum biodegradation were isolated from oil contamination sites (Hamzah *et al.*, 2010).

The ability to isolate high numbers of certain oil-degrading microorganisms from oil-polluted environment is evidence that these microorganisms are the active degraders of that environment. All the bacterial isolates namely *Proteus mirabilis, Escherichia fergusonii, Klebsiella pneumoniae* and *Clostridium sporogenes* were able to utilize the crude oil as carbon source It is then concluded that *Proteus mirabilis, Escherichia fergusonii, Klebsiella pneumoniae* and *Clostridium sporogenes* have high potential in biodegradation of petroleum. This study reveals that 75 % of the isolated bacteria are Gram negative and this is contrary to study by Prakash et al. (2014) who reported that there are more Gram-positive bacteria from both crude oil and drilling fluid than Gram negative bacteria because Gram positive bacteria can better adapt to adverse environmental conditions such as high temperature and osmotic pressure easily with the contribution of their strong cell walls.

However, some studies showed that Gram-negative as well as Gram-positive bacteria can predominate in various petroleum and petroleum products (Benka and Olumagin, 1996).

Table 2: Removal Efficiency of Total Petroleum Treatments during Bioremediation

Sample	TAH	PAH	TPH (PPM, Day 0)	TPH (PPM, Day 56)	% Reduction of TPH
AWNA	84.632	18.301	102.933	42.164	59.04
AWBA	75.622	9.475	85.097	18.437	78.33
AWHK	82.751	14.333	97.084	93.417	3.78

Key: TAH – Total Aliphatic Hydrocarbon, PAH – Polycyclic Aromatic Hydrocarbon
TPH – Total Petroleum Hydrocarbon TPH = TAH + PAH.

Table 2 above revealed the gas chromatographic analysis of Total Aliphatic Hydrocarbon (TAHs) as well as the Polycyclic Aromatic Hydrocarbons (PAHs) in the soil samples both of which when added gave the sum value of Total Petroleum Hydrocarbon (TPH). From the result, it was evident that the isolated indigenous bacteria are acclimatized to hydrocarbon and are actively using it as carbon source since there were losses in the TPH in the 56 days bioremediation process. This similar trend has been reported by earlier researchers (Chioma *et al*., 2012; Margesin *et al.,* 2013). In the AWNA treatment that relies on the indigenous microbial population in the soil sample to degrade and remove TPH contaminants from the polluted soil , the (TPH) decreased by 59.04 % while the AWBA treatment that rely on indigenous microbial population with augmented nutrient addition (Mineral Salt Medium – MSM) has a much higher TPH reduction efficiency of 78.33 %. This high reduction efficiency may be due to enhanced performance by the addition of the MSM as earlier reported that addition of conditioner could significantly improve the soil conditions and offer microorganism enough N, P, and K, which would promote microbial growth and played a key role on bioremediation of oil-contaminated soil (Bilen and Seyis, 2017; Ke *et al*., 2021). However, there was only a 3 % reduction in TPH content of the AWHK treatment because the microbial community has been destroyed by heat hence the 3 % reduction may possibly be due to influence of temperature and other abiotic factors which may evaporate some components of TPH such as Volatile petroleum hydrocarbons at room temperature. Bilen and Seyis (2018) reported the ability of *Clostridium sporogenes* to survive for over three years in tested crude oil fields confirming its ability to make use of crude oil as a carbon source while Primadani *et al*. (2020) also confirmed that *Thiobacillus* sp. and *Clostridium* sp achieved hydrocarbon compounds with a removal efficiency of 65 %.

CONCLUSION

Bioremediation is a relatively recent and environmentally friendly method that can either happen by itself or be helped along by the introduction of nutrients or bacteria that are able to break down toxins. The effectiveness of indigenous bacteria isolated from oil-polluted soil in natural attenuation and bioaugmentation with nutrients was evaluated for total petroleum hydrocarbons (TPH) reduction. This was done with the help of natural attenuation and bioaugmentation. The natural attenuation treatment (AWNA) had a lower efficiency compared to the bioaugmentation treatment (AWBA) which had the highest TPH decrease and the best results overall.

The heat killing treatment (AWHK) had the least efficiency overall. It is therefore concluded that there exist indigenous bacteria in oil contaminated soil, and that these bacteria can be enhanced with relevant nutrients for successful bioremediation of the oil polluted soil, and that the utilization of these bacteria can contribute to advanced research of bioremediation.

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Data Availability

The datasets generated during and/or analyzed during the current study are available from the corresponding author on reasonable request

Conflict of Interest

The authors have no competing interests to declare that are relevant to the content of this article**.**

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