Integral Research (Peer-reviewed, Open Access & Indexed Multidisciplinary Journal) ISSN: 3048-5991
Journal home page: https://integralresearch.in/, Vol. 02, No. 08, August. 2025

Screening of Hydrocarbon-degrading Bacteria Isolated from Petroleum-contaminated Soil and their Potential for Bioremediation of Lead (Pb) Contaminated Soil

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Abstract

Petroleum-contaminated soil poses significant environmental challenges, necessitating effective bioremediation strategies. This study was aimed at the isolation and screening of hydrocarbon- degrading bacteria from four petroleum-contaminated sites, Including Sokoto Old Garage (SOG), Illela Garage (ILLG), Buzaye (BZY), and Trailers Garage (TG) and study their potential to remediate Lead-contaminated soil. Mineral salt media was used for screening of hydrocarbon- degrading bacteria after isolation with Nutrient agar. The physicochemical analysis of the petroleum-contaminated soil was done according to standard methods. The lead bioremediation potential of the bacterial isolate was determined using Atomic absorption spectroscopy (AAS). The physicochemical

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Received 22 July. 2025; Accepted 17 August. 2025. Available online: 30 August. 2025.
Published by SAFE. (Society for Academic Facilitation and Extension)

analysis of the soil revealed pH for Illela garage (ILLG) 6.57±0.466b while Sokoto Old Garage (SOG) had 7.085±0.021a. Electrical conductivity for Trailers Garage (TG) 149.35±1.626c while Illela garage (ILLG) had 938.5±192a. Cation exchange capacity for Trailers garage (TG), was 5.715±0.049d while Sokoto old garage (SOG) had 16.625±0.304a. all were indicating site-specific contamination profiles. Bacterial colony counts for Illela garage (ILLG) was 2.00×106±0.028c CFU/g) while Sokoto Old Garage (SOG) had 51.59×106±0.113a CFU/g. In this study eight (8) Bacterial species were identified, namely Pseudomonas putida, Bacillus alvei, Proteus vulgaris, Klebsiella pneumonia, Staphylococcus aureus, Actinomyces viscosus, Bacillus licheniformis, Bacillus subtilis. Pseudomonas putida that exhibited the highest potential in hydrocarbon degradation was identified using molecular techniques. Spectrophotometric analysis at 600 nm showed that Pseudomonas putida and Bacillus subtilis demonstrated the highest hydrocarbon degradation rates over a three-day period, with Pseudomonas putida achieved 2.194±0.257 absorbance and Bacillus subtilis achieved 1.887±0.090. Lead bioremediation potential was assessed using Atomic Absorption Spectrophotometry. Pseudomonas putida reduced lead concentrations from 300 mg/L to 185±1.41mg/L over 72 hours, while Bacillus subtilis achieved a reduction to 227.5±0.70 mg/L. This study demonstrated that Pseudomonas putida isolated from petroleum-contaminated soil had the highest potential for lead bioremediation which can be used to remediate lead contaminated sites.

Keywords: Bacteria, Hydrocarbon, Lead, Contamination, Degradation, Bioremediation Molecular.

Introduction

Petroleum contamination referred to as a threat to environment and human health when release a various of hydrocarbons and heavy metals into the soil (Nemati et al., 2024). Hydrocarbon-degrading bacteria have considered as a biological agent that have ability in bioremediation of hydrocarbon and heavy metal contaminated sites. The hydrocarbon-degrading bacteria can degrade hydrocarbons and heavy metals due to their metabolic capability by making the contaminated sites less toxic (Mekonnen et al., 2024). Lead (pb) is an element that can be found in earths. The lead can be toxic, is a threat, can cause a large number of consequences to humans and animals (Kumar et al., 2020). The lead toxicity comes from anthropogenic activities by using prolong use of leaded gasoline, industrial facilities. The lead and lead compounds are using in a wide different products that found in or around our home including paint, ceramics, pipes, plumbing materials, solders, batteries, gasoline, ammunition and cosmetics (Bełcik et al., 2024).

Lead from the industrial sources and contaminated sites e.g former lead smelters can be emitted into the environment. While the natural levels of lead in soil ranged between 50 and 400 parts per million (PPM), smelting, mining and refining activities resulted in increase in lead levels in the environment more especially close to smelting and mining sites (Bełcik et al., 2024).

Lead can leach into ground water from soil depending on the form of lead and the soil qualities (Lighty et al., 2000). The occurrence of lead in soil is a matter of great concern because of its stability and toxicity and it can pose danger for terrestrial and aquatic organisms as well as human population because of their bioaccumulation in the food chain (Onyena et al., 2024). Consequently, conventional remediation approaches to lead contamination through excavation and chemical applications are costly and cause immense environmental disruption. Therefore, alternative and environmentally friendly approaches need to be sought to solve the issue (Ashkanani et al., 2024). Hydrocarbon-degrading-Bacteria might be a strategic solution for handling both petroleum and lead contamination due to the capacity to consume hydrocarbons and address heavy metal contamination (Al-Mailem et al., 2010). The metabolic adaptation by these bacteria for the provision of energy and carbon, as well as reaction with the organic carbon source, can utilize the abilities of human beings. Therefore, metabolic flexibility might be a feasible method not only to degrade petroleum hydrocarbons but to address the lead contamination problem (Maqsood et al., 2023).

The isolation technique and molecular identification of bacteria are the best approaches that can be used to characterize the bacterial isolates with the potential in hydrocarbon-degradation and bioremediation of lead contaminated sites (Muhammad et al., 2024). The soil can change the living ecosystem that forms a habitat for many of living organisms, including plants, microorganisms and animals (Bhattacharyya et al., 2015). Soil is a reservoir of organic and inorganic nutrients that requires for growth and development of organisms and also it can provides the essential needs like fuel, foods and fibers (Bhattacharyya et al., 2015). Microorganisms found in soil plays a pivotal role in carrying out several biochemical processes such as phosphate solubilization, nitrogen fixation, mineralization, denitrification, nitrification and ammonification and leads to help in growth of plant (Kaur and Vyas, 2024). Soil functioning can be altered due to the present of harmful chemical compounds and that can affects the existing living organisms and can decrease plant productivity (Kaur and Vyas, 2024). The development of urbanization and industrialization has brought social and economic growth but also increased environmental degradation due to anthropogenic activities (Bhatti et al., 2024).

Petroleum or crude oil consists of mixtures of cycloalkanes, alkanes, aromatic hydrocarbons, resins and asphaltenes it also naturally occurring flammable fossil fuels that can be found beneath the earth's crust. (Speight, 2019). The daily life of human individuals is dependent on petroleum hydrocarbons (Ite et al., 2013). The hydrocarbons are used for petrochemicals, fuels,

polymers and precursors to chemical synthesis and to meet other daily requirements (Ekejiuba, 2018).

1.2 Statement of the Research Problem

Soil contamination with lead in mechanical workshops can occur due to disposal of used lubricants, lead acid batteries, paints and metal scraps containing lead (pb). Spillage and leaks from the engine oils, grease and welding activities can also contribute to lead accumulation. Offshore and onshore of crude oil exploration and drilling lead contamination can influence lead contamination due to drilling fluids, pipe coatings and also the use of lead base additives in lubricants. The improper disposal of drilling mud leakage from stored tanks and emmissions from flaring and machinery can introduce lead in the soil and can lead to prolong environmental hazard (Sarkingobir et al., 2023). Heavy metals when released into the environment through mining, industrial processes, mining activities and improper disposal of petroleum products have environmental consequences (Khalef et al., 2022). Heavy metals like leads (pb) can accumulate in soil, water and if it is not removed can have severe health effects especially in children, developing fetus and pregnant women. This can affect the nervous system, cognitive function, growth and development body organs and also posing risks to ecosystems and human health (Jaiswal et al., 2018).

1.3 Justification for the study

Petroleum-contamination of soil has become a global issue that causes environmental degradation because of oil spills, leaks and improper disposal of petroleum products in the environment (Nuhu et al., 2022). The occurrence of hydrocarbon- contamination in soil can not only cause risks to ecosystem health but also is a threat to human populations through the ability groundwater contamination and bioaccumulation of toxic compound e.g. Lead in the food chain (Abdulai et al., 2024).

Microbial bioremediation is considered as an alternative for soil remediation, it’s friendly to the environment, cost effective and capable of decontaminating toxic compounds, like heavy metals, petroleum-hydrocarbon by its metabolic activity (Yessentayeva et al., 2024). To mitigate the environmental impact of heavy metals (Lead) regulatory measures so that to control discharges, emission. Implementing technologies that can be used to prevent pollution in a wide range of contamination of heavy metals and promoting sustainable practice in industries, management of waste systems and human activities (Mekonnen et al., 2024).

1.3 Aim and Objectives of the Research

The aim of this research is to isolate and screen hydrocarbon-degrading-Bacteria from petroleum contaminated soil and assess their potential for Lead bioremediation. The specific objectives are:

i. To determine the physical and chemical parameters of hydrocarbon-contaminated soil

ii.To isolate, enumerate, characterize and screen hydrocarbon-degrading bacterial isolates from petroleum-contaminated soil

iii. To evaluate the potentials of the bacterial isolates for clean up the Lead-polluted soil

iv. To confirm the hydrocarbon-degrading bacteria with high potential for lead bioremediation using molecular techniques

Methodology

3.1 Study Area

Sokoto State is located in the extreme North-West of Nigeria, sharing borders with Zamfara State to the East, Niger Republic to the North, Kebbi State to the South-East, and Benin Republic to the West. Sokoto Metropolis is the capital city of Sokoto state, Nigeria (Lamidi et al., 2024). As of the last available data, Sokoto State had a population of over 5 million people. However, Population figures may have changed since then due to factors such as births, deaths, and migration (Buonomo and Della, 2024).

3.2 Collection of Samples

Two hundred grams (200g) of each hydrocarbon contaminated soil were collected in clean polyethylene bags. The samples of soil collected from four different hydrocarbon-polluted soils in Sokoto, the samples' locations namely, Illela Garage with 13.055° Latitude (N), 5.217° Longitude (E), Sokoto, Old Garage with 13.019° (N), 5.234° (E), Buzaye (J. Allen), with 13.045° (N), 5.232° (E), Traillers Garage, with 13.019° (N), 5.240° (E) respectively. The hydrocarbon-contaminated soil samples were collected at a depth of 10 cm. The fifth sample was collected from non-hydrocarbon contaminated soil that served as control (Ali et al., 2024).

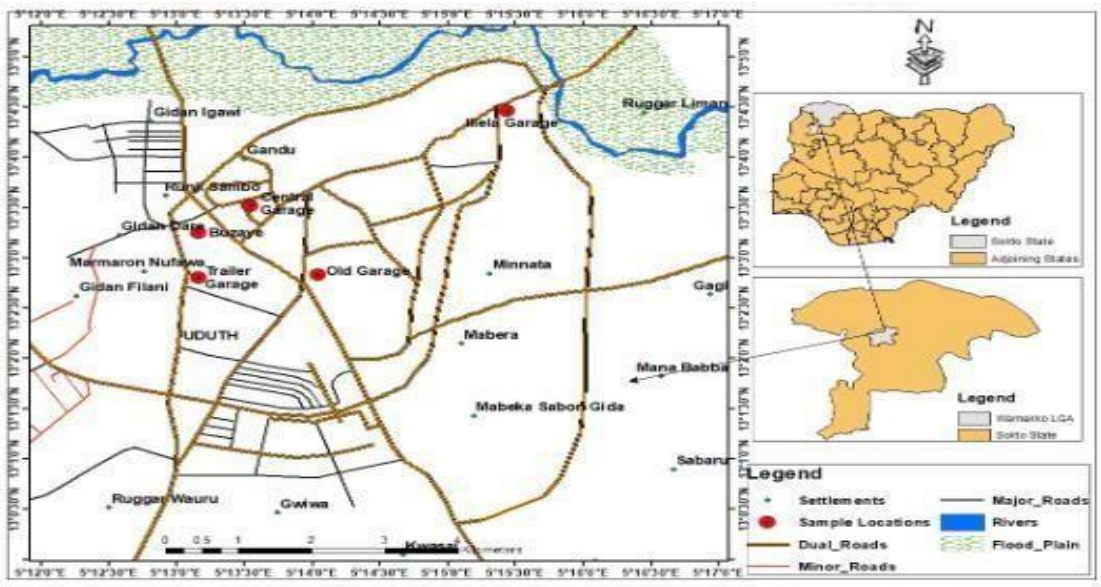

Figure 1. Map of the Study Area showing Sampling Locations Source: (GIS Lab, UDUS).

Analysis of Hydrocarbon-contaminated Soil Using Conventional Approach

Determination of Organic Carbon

The hydrocarbon-contaminated soil sample was air-dried, sieved and the large debris were removed. One (1) gram of soil sample was weighed. The Walkley-Black method in a carbon analyzer was used. The organic matter was oxidized using a potassium dichromate solution in an acidic medium and also the unreacted dichromate was titrated to calculate organic carbon content. The sample obtained was burned in a furnace and also the release of CO2 was measured using infrared detectors (Amin et al., 2024).

Determination of Soil pH

Hydrocarbon-contaminated soil samples were collected using a clean and non-reactive tool that ensured less disturbance. The samples were dried at room temperature and sieved to remove debris. ten gram (10g) was weighed and mixed with 25ml of distilled water in a 1:2.5 soil to water ratio. The mixture was stirred, and it was allowed to settle for 30 minutes. The pH was recorded, and electrode was clean to avoid cross-contamination (Vasquez et al., 2024).

3.3 .6 Cation Exchange Capacity (CEC)

An ammonium acetate (NH4OAc) at a pH 7.0 was applied into 5 gram of soil. The exchangeable cations in the particles of soil were shifted by this solution. Then, a leaching solution was to recover the displaced cations after the excessive ammonium eliminated. Then,

the cations were measured often using flame photometry. The number of cations in which the soil exchanged and retained was used for computing the CEC (Aprile & Lorandi, 2012).

3.3.7 Determination of Soil Nutrient

Soil sample was first allowed to air-dry before being sieved. To break down the organic debris and liberate the nutrients, the sample was subsequently digested using a combination of concentrated acids, including perchloric acid (HClO4) and nitric acid (HNO3). The extract was examined for several nutrients, such as potassium, nitrogen, phosphorus, calcium, magnesium, and trace elements, after the sample had been digested and filtered. Atomic absorption spectrophotometry was among the methods used to do this. The nutrient availability in the polluted soil was then evaluated by quantifying the nutrient concentrations and comparing the findings to standard values (Moursy et al., 2022).

3.4 Preparation of Media 3.1 Mineral Salt Medium (MSM)

According to method of Muhammad et al. (2024). The mineral salt medium and 3% diesel was used as a carbon source. Stock solution was sterilized using autoclave and it was added to the Mineral Salt Medium. The pH 7.2 was used as the adjusted pH of the medium and the medium was sterilized at 121°C for 15 min.

3.2 Nutrient Agar (NA)

The powder of nutrient agar (2.8g) was used and dissolved into 1000mls of water that was distilled. The prepared powder was heated using hot plate and dissolved completely. Cotton wool was used and placed on top of the conical flask, and it was wrapped with aluminum foil. The nutrient agar solution was autoclaved at 121°C for 15 minutes and it was allowed to cool and dispensed in a cleaned petri dish (Namasivayam et al., 2024).

3.5 Microbiological Analysis of Soil Samples

The cleaned test tubes containing dilution were labeled. A soil sample was diluted with 9 ml of sterilized distilled water. The contents of the tubes were mixed to ensure homogeneity. A 1 ml of diluted solution was transferred from the first test tube to the second tube labeled with the next dilution factor. Step 3 and 4 were repeated until a needed dilution was achieved. The contents of each tube were mixed after dilution. 1 ml of suspension from 10-3, 10-4 and 10-5 dilutions were aseptically plated on a prepared nutrient agar (NA) and incubated at 30°C for 24 hrs (Agu, 2015).

The result was determined by multiplying the number of counts with the used dilution and expressed as colony forming units per gram (cfu/g) of soil and the colonies were sub-cultured in nutrient agar to obtained pure culture of isolates (Liu et al., 2024).

3.6 Bacterial Screening of Hydrocarbon-degradation

The method of Muhammad et al. (2024) was adopted, the bacteria isolated from different hydrocarbon-contaminated soil were screened based on their ability to degrade diesel oil supplemented in the Mineral Salt medium (MSM). The bacterial isolates were grown in Mineral Salt Medium (MSM) with 1% diesel oil (v/v) as the only carbon source and incubated for 9 days at 37°C. Degradation was monitored after 3 days interval using a spectrophotometer at 600 nm. The absorbance was measured and recorded after the spectrophotometer had been calibrated to zero with formulations identical to those used in the treatment.

3.7 Biochemical Characterization of Hydrocarbon-degrading-bacterial Isolates

3.7.1 Catalase Test

A portion of bacterial colony was added on a slide containing a drop of hydrogen peroxide. If the bacteria produced catalase the bubbles of oxygen gas were observed. The formation of bubbles indicates the presence of catalase and considered positive catalase. But the absence of bubble gas is considered negative catalase (Hadwan et al., 2024).

3.7.2 Oxidase Test

A piece of filter paper was impregnated with an oxidase reagent (tetramethyl-p-phenylenediamine dihydrochloride) was used. Bacterial colonies smeared on the filter paper. Bacterial colony will produce cytochrome c oxidase reagent; the oxidase reagent was turning blue purple color. The development of blue purple color was observed within 10-30 seconds which indicates the presence of oxidase and considered positive. Some bacteria developed no color changes within 10-30 seconds and that indicates the absence of oxidase (Hadwan et al., 2024).
(Thind et al., 2024).

3.7.3 Triple Sugar Ion Test (TSI)

The surface of a TSI agar slant was inoculated by streaking it with a sterile loop, and the butt of the agar was stabbed deeply to introduce the bacteria. The tube was incubated at 35–37°C for 18–24 hours. Observations were made for color changes: yellow indicated acid production

(sugar fermentation), red indicated an alkaline reaction (no fermentation), and black precipitate indicated H S production. Gas production was evidenced by cracks or bubbles in the medium (Jiang et al., 2024).

3.7.7 Gram Staining

The procedure began by preparing a heat-fixed smear of the bacterial sample on a glass slide. The smear was flooded with crystal violet dye for about one minute, then rinsed gently with water. Next, iodine solution, a mordant, was applied for one minute to form a crystal violet-iodine complex. The slide was then rinsed again and decolorized with alcohol or acetone for a few seconds, which removed the dye from Gram-negative bacteria but not from Gram-positive ones. After immediate rinsing to stop decolorization, the slide was counterstained with safranin for one minute and rinsed again. Finally, the slide was blotted dry and observed under a microscope. Gram-positive bacteria appeared purple due to retained crystal violet, while Gram-negative bacteria appeared pink from the safranin counterstain (Lainjong, 2024).

3.7.8 Spore Staining Test

The method of Begum et al. (2024) was adopted by smearing the bacteria on a slide, allowed to air-dry, and then the slide was heat-fixed, placed over a steaming water bath, covered with a piece of paper towel, and saturated with malachite green stain. The slide was then left to cool, rinsed thoroughly with water to remove excess stain, counterstained with safranin for 1-2 minutes, rinsed again with water, and blotted dry. Under a microscope, endospores appeared green, while vegetative cells appeared pink.

3.8. Lead Bioremediation Assay

Two grams (2g) of sterilized soil was artificially contaminated with lead. The lead was also sterilized, and concentrations of lead were varied in 100mg/L, 200mg/L and 300mg/L. Contaminated medium with the bacterial isolates were inoculated and tested. Control samples without bacterial inoculation were also included. The samples incubated at 37°C under conditions conducive to bacterial growth. The medium was sampled and measured the changes in lead concentration started from 00 hours, 24 hours, 48 hours and 72 hours. Atomic absorption spectroscopy (AAS) was used for lead quantification (Sardans et al., 2011).

3.8.1 Assessment of Lead Bioremediation Efficiency

The percentage of lead removed or reduced was calculated by the bacterial strains and compared to control samples. Additional characterization of the bioremediation products and by-products were also performed. Bacterial strains showing higher lead removal efficiency were considered more effective in lead bioremediation. Both lead resistance and lead bioremediation assays are valuable tools for understanding the capabilities of microorganisms in dealing with lead contamination, whether by resisting its toxicity or actively participating in its removal from the environment (Kirillova et al., 2017).

3.9 Molecular Identification of the Bacterial Isolate

3.9.1 Extraction of DNA

Phenol-chloroform-isoamyl alcohol mixture were added to the lysate. Then, was also mixed thoroughly and centrifuged to separate the aqueous (DNA-containing) phase from the organic (protein and lipid-containing) phase to remove protein. The aqueous phase was transferred to a new tube and precipitate DNA by adding cold ethanol or isopropanol. Centrifuge to pellet the DNA. The DNA pellet was washed off with ethanol to remove residual salts and contaminants. Air-dry was used to remove excess ethanol, then Extracted DNA was quantified using spectrophotometry and stored appropriately for downstream applications (Janabi et al., 2016).

3.9.2 Polymerase Chain Reaction (PCR)

The hydrocarbon-degrading bacterial isolate was purified using two procedures: ammonium sulfate precipitation method and ZnCl2 precipitation method. In the ammonium sulfate precipitation method, it involved four steps: ammonium sulfate fractionation, chilled acetone treatment, hexane treatment, and silica gel column chromatography. In the ZnCl2 precipitation method, 10 ml of the culture supernatant was concentrated with ZnCl2 to a final concentration of 75 mM. The precipitated material was dissolved in 10 ml of sodium phosphate buffer (pH 6.5), extracted twice with equal volumes of diethyl ether. The pooled organic phase was evaporated to dryness, and the pellets were dissolved in 100 µL of methanol. Further purification was achieved by preparative TLC (Godheja et al., 2014).

DNA amplification using the polymerase chain reaction (PCR) involved a series of steps to amplify a target DNA sequence. First, DNA was extracted from the sample and added to a reaction mix containing DNA primers specific to the target sequence, nucleotides (dNTPs), a buffer, and Taq DNA polymerase. The PCR procedure began with an initial denaturation step at 94-95°C for 2-5 minutes to separate the DNA strands. This was followed by 25-35 cycles of

denaturation (94-95°C for 30 seconds), annealing (50-65°C for 30 seconds), and extension (72°C for 1 minute per kilobase of DNA). A final extension at 72°C for 5-10 minutes ensured complete synthesis of DNA strands. The amplified DNA, called the PCR product or amplicon, was then analyzed using gel electrophoresis or other detection methods. The resulting amplified DNA was then analyzed or used for further experiments, such as sequencing, sequence analysis and phylogenetic analysis. Primers used for PCR were short, single-stranded oligonucleotides designed to be complementary to the flanking regions of the target DNA sequence. These primers guided DNA polymerase to initiate replication. Two types of primers were used in PCR.

Forward Primer. This primer bound to the complementary sequence on the 3' end of the sense strand of DNA and extended in the 5' to 3' direction during amplification.

Reverse Primer. This primer bound to the complementary sequence on the 3' end of the antisense strand and also extended in the 5' to 3' direction.

Primers were typically 18 to 25 nucleotides long and designed with a melting temperature (Tm) of around 50-65°C. They were carefully synthesized to minimize self-complementarity and avoid the formation of secondary structures or primer dimers. An example procedure might involve selecting primers specific to a bacterial 16S rRNA gene, such as:

Forward Primer: 5'-AGAGTTTGATCCTGGCTCAG-3'

Reverse Primer: 5'-GGTTACCTTGTTACGACTT-3' (Godheja et al., 2014).

3.9.3 DNA Sequencing

The bacterial isolate was sub-cultured and sent to Inqabah Biotech, Ibadan, Oyo state, Nigeria. After DNA was extracted and Amplified, the DNA Sequecing was doned Using the ABI DNA 3730 XL sequencing sequencer (Applied Biosystems), the 16S rRNA purified PCR product was submitted. Sequencing of the bacterial isolate 16S rRNA gene was carried out in both directions.

The bacterial species was determined with the obtained sequence, which was searched for using BLAST. The sequences were submitted to the NCBI GenBank after sequence matching percentages and accession numbers were obtained (Grada and Weinbrecht, 2013).

3.9.4 Sequence Analysis

Homology search was used to identify similar sequences in databases using algorithms like BLAST (Basic Local Alignment Search Tool) or HMMER. This helped in finding related sequences that shared evolutionary ancestry or functional similarity. Motif and domain analysis

were used to identify conserved regions or motifs within sequences that were indicative of functional or structural importance. MEME (Multiple EM for Motif Elicitation) or Pfam were used for motif and domain discovery. Sequence databases (e.g., GenBank, UniProt) were searched to find similar sequences or annotate newly obtained sequences. BLAST was used for database searches. (Rather et al., 2023).

Phylogenetic Analysis

Phylogenetic analysis began with the collection of Nucleotide or DNA sequence after Sequence was blast in NCBI using mega 11 software, neighbor joining method to construct a phylogenic tree and also the evolutionary relationship of the molecularly identified isolate was identified (Li et al., 2024).

Data Analysis

All experiments were carried out, and almost all results in this study were expressed in the form of ANNOVA, mean and standard deviation. Descriptive statistical methods were employed in this research using Excel's inbuilt statistical tools at a significant level of p=0.05. Bioinformatic tools such as sequence BLAST (NCBI), Mega 11 were used to analyze the data obtained and construction of phylogenetic tree (Morales-Ávila et al., 2024).

Results

Table 1 presented the results of analysis of hydrocarbon-contaminated soil using conventional approach. Hydocarbon-contaminated soil from four different soil samples collected from the sampling sites namely, Illela Garage (ILLG), mechanical workshop, Traillers Garage (TG) mechanical workshop, Buzaye (BZY), mechanical workshop, Sokoto old Garage (SOG), mechanical workshop and the Control site (C) respectively. The result obtained shows that there are small differences that found in the pH values among the values from the contaminated sites (SOG, ILLG, BZY and TG) ranged from slightly acidic to neutral (6.57-7.085). The control site that has an acidic pH (6.6) determined the sample was influenced by buffering or petroleum contamination.

Integral Research (Peer-reviewed, Open Access & Indexed Multidisciplinary Journal) ISSN: 3048-5991
Journal home page: https://integralresearch.in/, Vol. 02, No. 08, August. 2025

Table 1. Analysis of Hydrocarbon Contaminated-soil Using Conventional Approach

S/No	Parameters	SOG	ILLG	BZY	TG	Control
		Mean ± S.D	Mean ± S.D	Mean ± S.D	Mean ± S.D	Mean ± S.D
1	Ph	7.085±0.021^{a}	6.57±0.466^{b}	7.035±0.035^{a}	7.02± 0.028^{a}	6.6±0.084^{b}
2	EC µs/cm	832.5±3.535^{a}	938.5±9.192^{a}	269±1.414^{b}	149.35±1.626^{c}	23.65±0.070^{d}
3	Ca cmol/kg	0.72±0.042 ^{b}	1.135±0.021^{a}	1.295±0.063^{a}	0.35±0.212	^{c} 0.24±0.014 ^{c}
4	Mg cmol/kg	2.73±0.028^{c}	3.005±0.007^{b}	355±0.205^{a}	3.24±0.014^{b}	0.15±0.070^{d}
5	Na cmol/kg	1.33±0.007 ^{a}	1.11±0.141^{ab}	0.86±0.056 ^{b}	0.435±0.021^{c}	0.195±0.091^{c}
6	K cmol/kg	12.2±0.282 ^{a}	6.965±0.049^{b}	8.65±0.636^{b}	1.5±0.070^{c}	0.155±0.035^{d}
7	CEC cmol/kg	16.625±0.304^{a}	12.33±0.042^{b}	8.65±0.636 ^{c}	5.715±0.049^{d}	0.825±0.091^{e}
8	%N	0.17±0.070 ^{a}	0.72±0.028^{b}	0.065±0.021^{a}	0.035±0.021 ^{a}	0.066±0.033^{a}
9	%O.C	0.065±0.007 ^{a}	0.065±0.007^{a}	0.195±0.077^{b}	0.115±0.021^{c}	0.35±0.070 ^{d}
10	%Sand	94.7±0.707 ^{a}	93.2±0.141^{b}	94.65±1.909^{a}	89.85±0.636 ^{c}	96.9±0.424 ^{d}
11	%Silt	4.625±0.035 ^{a}	2.5±0.141 ^{b}	3.5±0.141^{c}	4.6±0.141^{a}	2.2±0.565^{b}
12	%Clay	0.205±0.007 ^{a}	4.05±0.070 ^{b}	4.05±0.070 ^{b}	5.55±0.777 ^{c}	1.65±0.070 ^{d}
13	PO_4 mg/kg	0.14±0.014 ^{a}	0.125±0.007^{a}	0.19±0.070 ^{b}	0.185±0.063^{b}	0.165±0.091^{b}

Key: SOG = Sokoto Old Garage, ILLG = Illela Garage, BZY = Buzaye and TG = Traillers Garage.
Means with different superscripts in the same column are significantly different

Bacteriological Counts of Soil Samples

Table 2 presents an enumeration of viable aerobic heterotrophic bacteria from hydrocarbon contaminated-soil. Buzaye (BZY), Trailers Garage (TG), Illela Garage (ILLG), Sokoto Old Garage (SOG), and a control site (C) are amongs the sampling sites in which the mean of bacterial colony counts (Colony forming Units) were determined and also the result shows differences. Sokoto Old Garage had the highest mean of bacterial count of (51.59 × 106 CFU ± 0.113) which was the higher than the other sites were the Control site had the lowest Colony Count of (1.85 × 106 CFU ± 0.275).

2. Bacteriological Counts of Soil Samples from Sampling Location

2. Bacteriological Counts of Soil Samples from Sampling Location

Samping Sites	Mean Bacterial Colony Count (CFU) ± S.D
Buzaye (BZY)	$2.025 \times 10^6 \pm 0.035^{c}$
Trailers Garage (TG)	$3.545 \times 10^6 \pm 0.190^{b}$
Illela Garage (ILLG)	$2.00 \times 10^6 \pm 0.028^{c}$
Sokoto Old Garage (SOG)	$51.59 \times 10^6 \pm 0.113^{a}$
Control (C)	$1.825 \times 10^6 \pm 0.275^{bc}$

In each column, means followed by different letter (s) are significantly different

Biochemical Characterization of Bacterial Isolates

Table 3 shows a biochemical identification of bacteria isolated from different locations of hydrocarbon-contaminated soil highlighted their morphological and metabolic characteristics. The identified isolates include Pseudomonas putida, Bacillus subtilis, Bacillus alvei, Proteus vulgaris, Klebsiella pneumonia, Bacillus licheniformis, Staphylococcus aureus and Actinomyces viscious. This shows that all the identified isolates are capable in metabolizing hydrocarbon with the potential role in bioremediation.

3.Biochemical Characterization of Bacterial Isolate

Biochemical Characterization of Bacterial Isolate

Code	Shape	Spore	Gra Rn	Catalase	Oxidase	M-R	V-P	Indole	Glucose	Lactose	Sucrose	H_2S	Gas	Identified Organisms
TGB2	Rods	-	-	+	+	-	-	-	+	-	-	-	-	*Pseudomonas putida*
TGB1	Rods	+	+	+	-	-	+	-	+	-	+	-	-	*Bacillus subtilis*
ILLGB	Rods	+	+	+	-	-	+	-	+	+		+	-	*Bacillus alvei*
ILLGA1	Rods	-	-	+	-	+	-	+	+	-	+ -	+	+	*Proteus vulgaris*

ILLGA2	Bacillus	-	-	+	-	+	-	-	+	+	+	-	-	*Klebsiella pneumonae*
BZYA1	Rods	-	-	+	+	-	-	-	+	-	-	-	-	*Pseudomonas putida*
BZYA2	Rods	-	-	+	+	-	-	-	+	-	-	-	-	*Pseudomonas putida*
TGA1	Rods	+	+	+	-	+	-	+	+	-	-	-	-	*Bacillus licheniformis*
TGA2	Cocci	-	+	+	-	+	-	-	+	+	+	-	-	*S. aureus*
BZYB	Rods	-	+	+	+	-	-	-	+	+	+	+	-	*Actinomyces viscosus*
SOGA	Rods	-	-	+	-	+	-	+	+	-	-	+	+	*Proteus vulgaris*
SOGB1	Rods	-	-	+	+	-	-	-	+	-	-	-	-	*Pseudomonas putida*
SOGB2	Rods	+	+	+	-	+	-	+	+	-	-	-	-	*Bacillus licheniformis*

Key: + = Positive, - = Negative, M-R = Methyl Red, V-P = Voges Proskauer test and H_2S = Hydrogen Peroxide

Frequency of Occurrence of the Identified Bacterial Isolates

Table 4.4 shows the Bacteria Isolated from samples collected are Pseudomonas putida which had 30.77% occurrence and identified 4 times out of 13. While proteus vulgaris and Staphylococcus aureus 15.4% with two isolates. Other isolates were Bacillus alvei, Klebsiella pneumonia, Actinomyces viscosus, Bacillus licheniformis and Bacillus subtilis were all had 7.7% from the total isolates.

4. Frequency of Occurrence of the Identified Bacterial Isolate from Hydrocarbon-contaminated Soil

3. Frequency of Occurrence of the Identified Bacterial Isolate from Hydrocarbon-contaminated Soil

S/No	Identified Islates	Frequency of Occurrence	Percentage Frequency of Occurrence (%)
1	*Pseudomonas putida*	4	30.77
2	*Bacillus alvei*	1	7.7
3	*Proteus vulgaris*	2	15.4
4	*Klebsiella pneumonia*	1	7.7
5	*Staphylococcus aureus*	2	15.4
6	*Actinomyces viscosus*	1	7.7
7	*Bacillus licheniformis*	1	7.7
8	*Bacillus subtilis*	1	7.7
	Total	13	**100**

Screening of Bacterial Isolates for Hydrocarbon-degradation

Table 4.5 shows how the bacterial isolates were screened for hydrocarbon-degradation. The spectrophotometer at 600 nm was use at day (00) to screened the initial degradation of hydrocarbon by the bacterial isolates, then, after 3 days, 6 days and 9 days interval the record of degradation was obtained and also the turbidity was determined the bacterial growth in the medium which contained waste engine oil as carbon source, bacterial isolates (Colony) and Mineral Salt Media. TG B2 and BZY A2 Have the highest recorded for hydrocarbon-degradation.

5. Screening of Bacterial Isolates for Hydrocarbon-degradation

5. Screening of Bacterial Isolates for Hydrocarbon-degradation

S/NO	Isolates	Day 00	Day 3	Day 6	Day 9
1	BZY B	0.678 ± 0.032	0.789 ± 0.032	1.322 ± 0.156	0.719 ± 0.085
2	TG A^1	0.611 ± 0.061	0.809 ± 0.120	0.893 ± 0.125	0.77 ± 0.070
3	SOG B^2	0.584 ± 0.067	1.067 ± 0.064	1.285 ± 0.077	0.867 ± 0.050
4	ILLG B	0.858 ± 0.061	0.865 ± 0.072	1.191 ± 0.015	0.696 ± 0.065
5	ILLG A^1	0.583 ± 0.071	0.856 ± 0.065	1.21 ± 0.140	0.543 ± 0.159
6	SOG A	0.577 ± 0.045	0.758 ± 0.079	1.203 ± 0.144	0.530 ± 0.197
7	TG B^2	0.853 ± 0.068	0.848 ± 0.091	1.816 ± 0.135	0.739 ± 0.122
8	TG B^1	0.772 ± 0.210	1.887 ± 0.090	1.734 ± 0.041	0.706 ± 0.136
9	TG A^2	0.873 ± 0.154	1.511 ± 0.078	1.445 ± 0.192	0.407 ± 0.239
10	BZY A^1	0.879 ± 0.078	1.291 ± 0.072	1.477 ± 0.037	0.46 ± 0.167
11	ILLG A^2	0.792 ± 0.088	1.149 ± 0.488	1.74 ± 0.152	0.454 ± 0.207
12	SOG B^1	0.566 ± 0.088	1.318 ± 0.117	1.331 ± 0.056	0.473 ± 0.041
13	BZY A^2	0.718 ± 0.118	2.194 ± 0.257	1.524 ± 0.140	0.712 ± 0.043

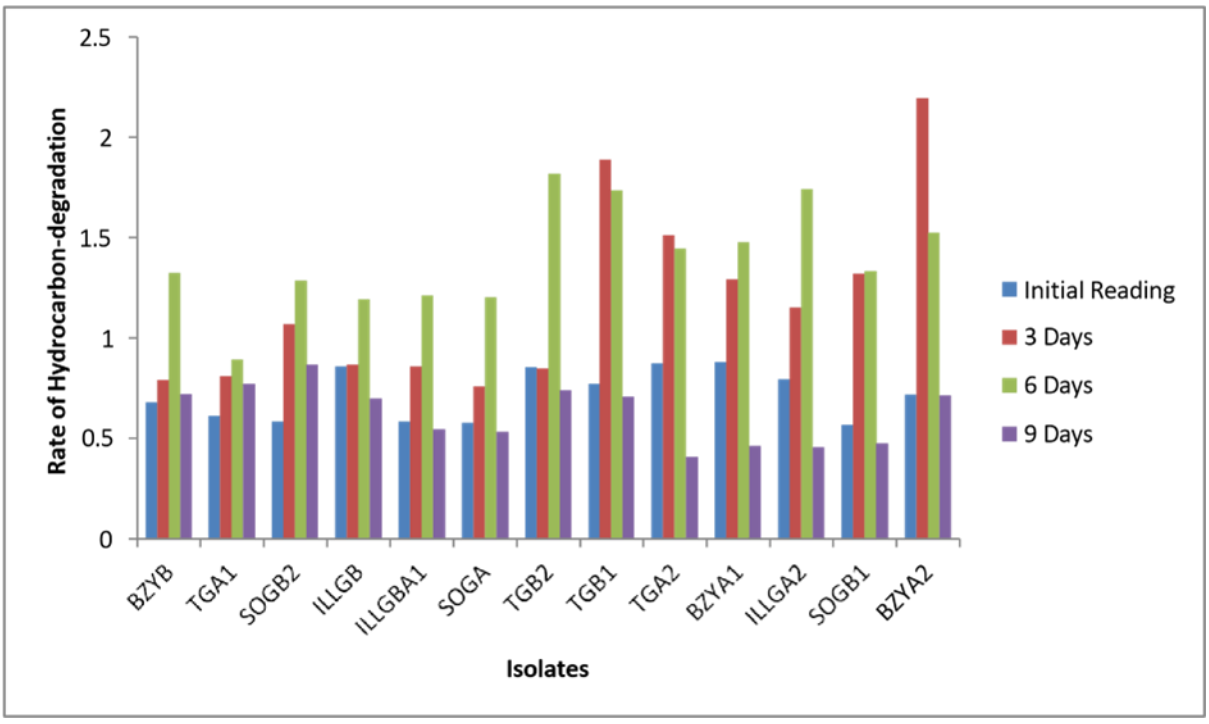

Figure 2. Growth Profile of Hydrocarbon-degrading Bacterial Isolates

Evaluation of Lead Bioremediation by Bacterial Isolates

This Bacillus subtilis and Pseudomonas putida was obtained. The lead acetate was varied in (100 mg/L, 200 mg/L and 300 mg/L). The result was obtained before the inoculation of the isolate (00 hour), in a medium that contained Mineral Salt Media. The record of lead

bioremediations were obtained at 00 hour, 24 hours, 48 hours and 72 hours where Pseudomonas putida found with the high potent for bioremediation of Lead-contaminated soil.

5. Lead Concentration before and after bioremediation by baterial Isolates

5. Lead Concentration before and after bioremediation by baterial Isolates

Isolates	Time	Lead Concentration 100mg/L	Lead Concentration 200mg/L	Lead Concentration 300mg/L
Pseudomonas putida	00	100 ± 0.00	200 ± 0.00	300 ± 0.00
	24	80 ± 1.41	166 ± 2.82	276 ± 24
	48	57 ± 1.41	142 ± 1.41	212 ± 2.12
	72	45.5 ± 2.12	105.5 ± 4.94	185 ± 1.41
Bacillus subtilis	00	100 ± 0.00	200 ± 0.00	300 ± 0.00
	24	87 ± 2.82	171 ± 1.41	283 ± 1.41
	48	65.5 ± 2.12	152 ± 1.41	233 ± 24
	72	56 ± 1.41	139 ± 1.41	277.5 ± 0.70
Control	00	100 ± 0.00	200 ± 0.00	300 ± 0.00
	24	100 ± 0.00	199 ± 1.41	297.5 ± 0.70
	48	99 ± 1.41	198.5 ± 2.12	290.5 ± 0.70
	72	99 ± 1.41	197.5 ± 0.70	299 ± 1.41

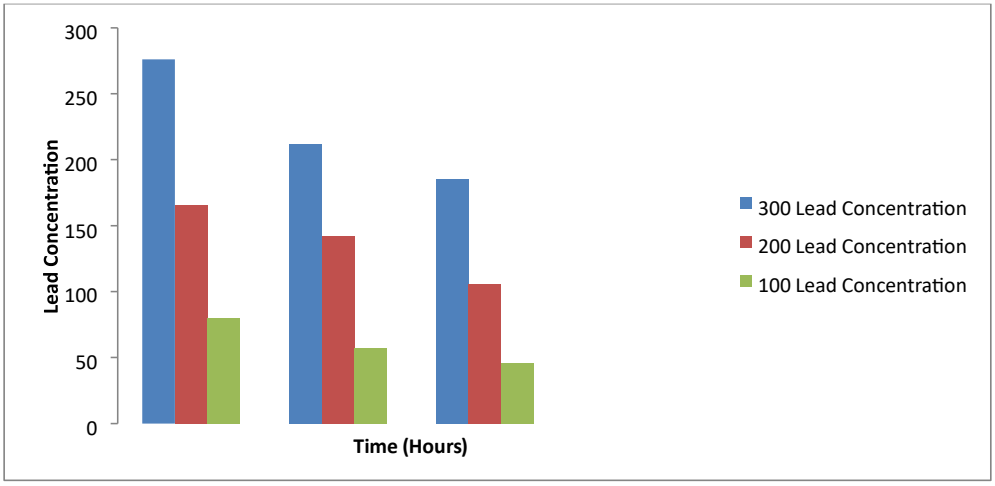

Figure 3. The Rates of lead Bioremediation by Pseudomonas putida

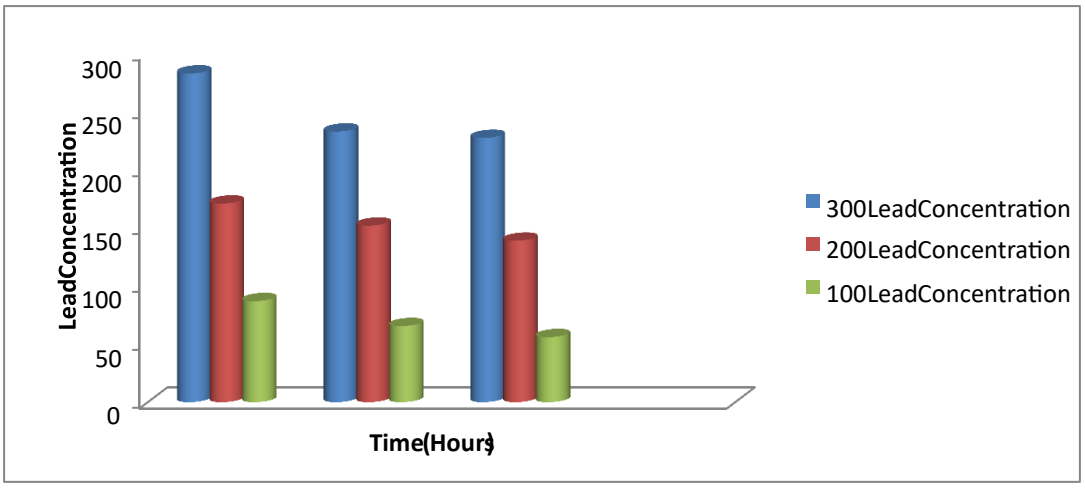

Figure 4. The Rates of lead Bioremediation by Bacillus subtilis

Molecular Analysis Polymerase Chain Reaction (PCR) Amplification

PCR amplification of Pseudomonas putida with sequence length of 1414 base pairs (bp), plate 1 shows that was an amplification of the predicted and expected DNA region of the gene of interest that can be used for downstream application, gene analysis and phylogenetic analysis.

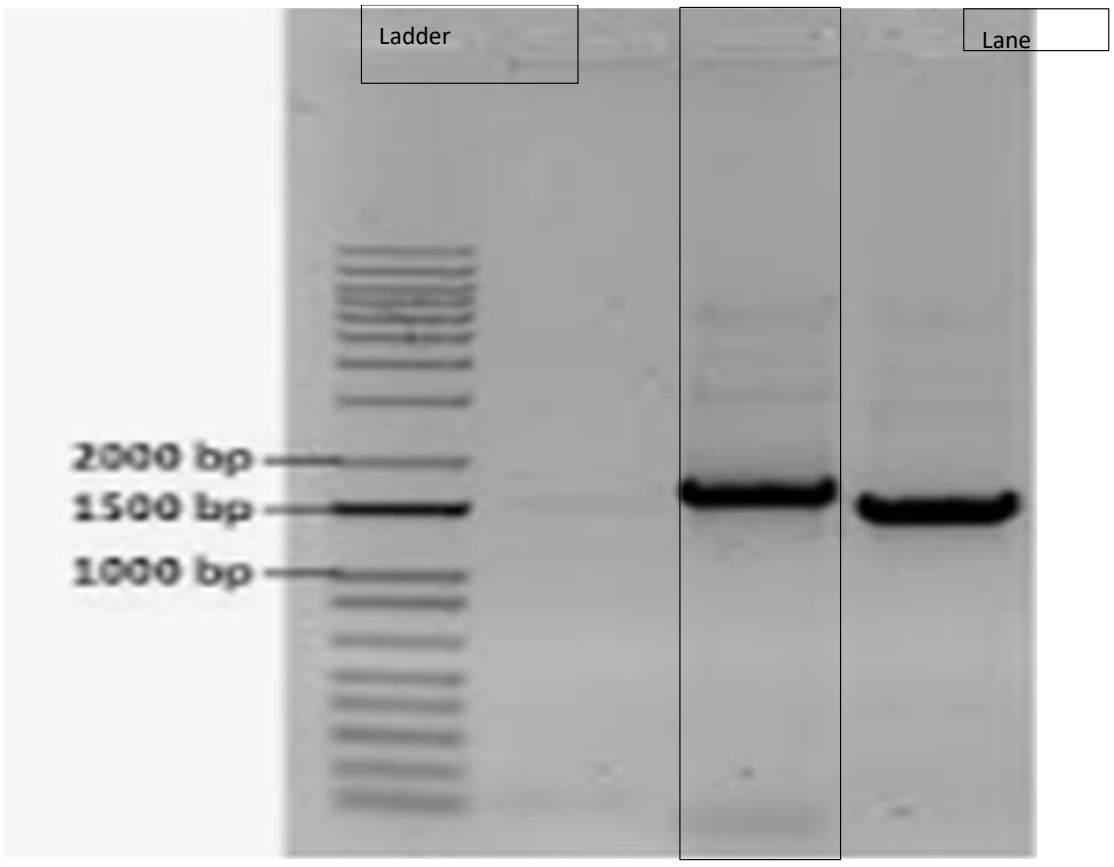

Plate 1: Agarose Gel ladder Image Indicating the Amplified of the Targeted 16S Region of the Isolate Pseudomonas putida

Phylogenetic Analysis of the Bacterial Isolate

Table 4.8 and Figure 4.6 illustrated the evolutionary relationships derived from phylogenetic analysis, with the optimal tree displayed alongside the branches. The phylogenetic tree for the isolate studied, identified as Pseudomonas putida (AM411067.1:53-1481) and designated with the code BZYA2, was constructed using nine closely related sequences identified through a BLAST search in the NCBI database. These sequences included Pseudomonas putida strain MX- 2 (CP046872.1:162544-163972,CP046872.1:528998-530426, and CP046872.1:877023-878451), Pseudomonas kurunegalensis strain NIOT.M2S7W8HA9 (PP516280.1:38-1466), Pseudomonas putida strain PP2323 (CP047148.1:519996-521424 and CP047148.1:692868-694298), Pseudomonas putida strain LPK411 (MF455219.1:54-1482), and additional Pseudomonas putida strain MX-2 sequences (CP046872.1:4602801-4604228 and CP046872.1:5272210-5273638). All these organisms shared a common evolutionary ancestor, which was Pseudomonas putida.

Table 6. BLAST Results Sample ID Predicted Percentage Accession Organism Similarity Number

BZYA2	*Pseudomonas putida*	90.01%	AM411067.1

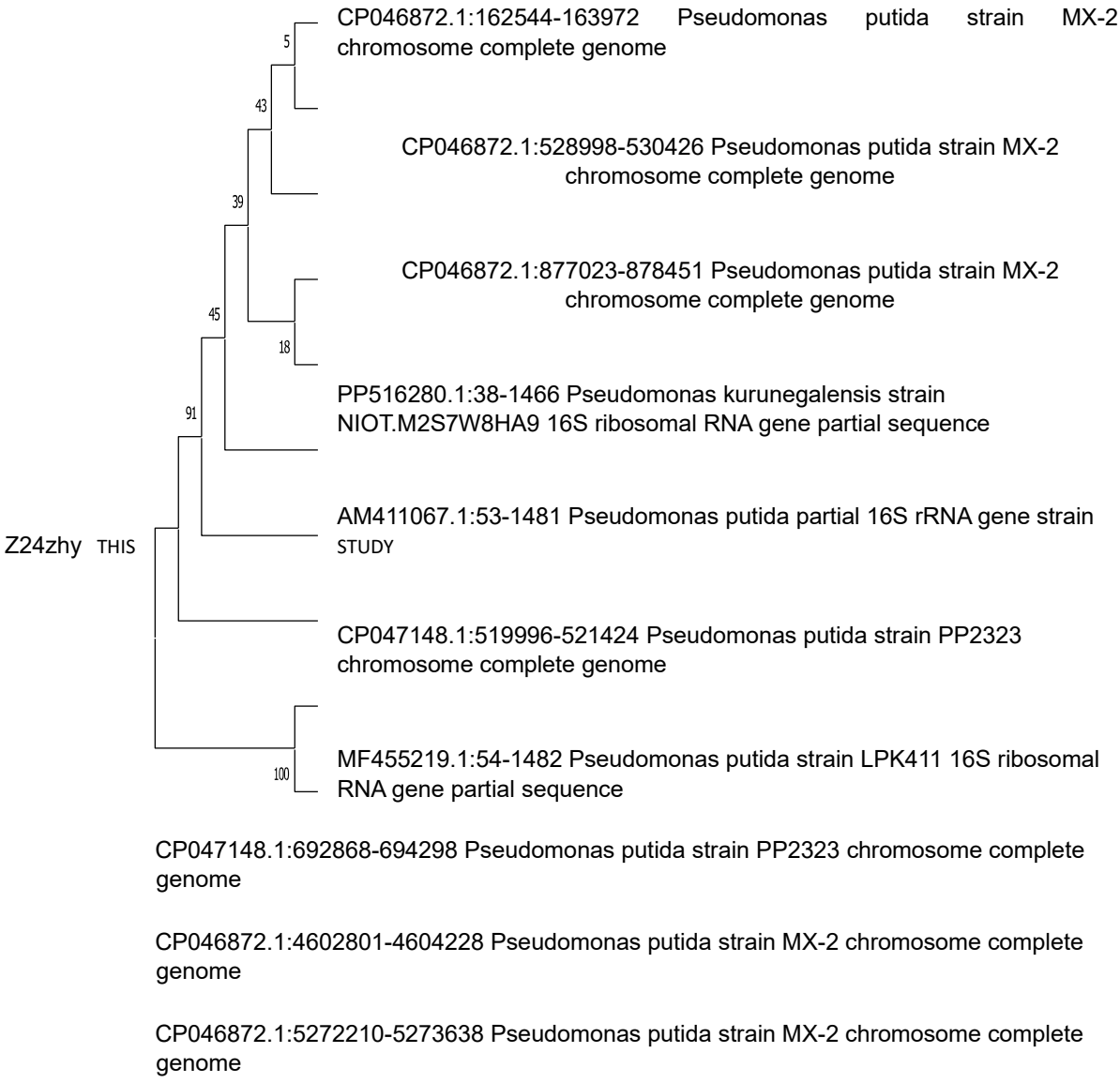

Figure 5. Phylogenetic Tree based 16S Rrna Sequence using Neighbor Joining Method

Discussion

The hydrocarbon-contaminated soil samples that analyzed had a pH value ranged from 6.57 and 7.08 which indicates that the soil condition is slightly acidic. This determined that the conditions are favourable for microbial activity and nutrient availability and has not changed by the contamination. The pH result of this present research was relatively like the findings of Akpe et al. (2015), who worked on Bacterial degradation of petroleum hydrocarbons In crude

oil polluted soil amended with cassava peels and had a pH range of 6.54 to 7.35. The result is almost similar to the findings of Onojake and Osuji (2012) who worked on the Assessment of the physico-chemical properties of hydrocarbon contaminated soil and had a pH value of 6.4. The notable variation in the numbers of aerobic heterotrophic bacteria identified in the hydrocarbon-contaminated soil samples ranged from 2.025×106 to 51.59×106 CFU/g (colony forming units per gram of soil). The colony counts of the currents study disagreed to the findings of Eze et al. (2014) who worked on Microbiological and Physicochemical Characteristics of Soil Contaminated with Used Petroleum Products in Umuahia, Abia State, Nigeria due to sampling sites differences.

In this study the following bacteria identified from hydrocarbon-contaminated soil Pseudomos putida, Bacillus subtilis, Bacillus licheniformis, Proteus vulgaris, Actinomyces viscosus, Klebsiella pneumonia, Staphylococcus aureus. The bacterial isolates identified determined that have survived in the hydrocarbon-contaminated soil due to their metabolic, enzymatic and genomic characteristics. The current study of biochemical characterization related to the findings of Al-Sharidah et al. (2000) were isolated Pseudomonas putida and Bacillus subtilis from oil contaminated soil. The worked shows the similar finding was Isolation and characterization of two hydrocarbon-degrading Bacillus subtilis strains from oil contaminated soil of Kuwait. The two bacterial isolates, Pseudomonas putida and Bacillus subtilis have found that are the isolates with the highest potential for hydrocarbon-degradation amongst the bacterial isolates. Hydrocarbon-degradation by Bacillus subtilis recorded a 0.77 at initial reading and rose to 1.88 while Pseudomonas putida was 0.71 to 2.19 Optical density (OD) after 3 days interval. This shows that Pseudomonas putida had the highest potential of hydrocarbon-degradation compared to Bacillus subtilis. The current result of total hydrocarbon degradation by Pseudomonas putida and Bacillus subtills is similar to the findings of Titah et al. (2021) who worked on Biodegradation of crude oil spill using Bacillus subtilis and Pseudomonas putida in sequencing method. The result also like the findings of Ghorbannezhad et al. (2022) who worked on Biodegradation of high molecular weight hydrocarbons under saline condition by halotolerant Bacillus subtilis and its mixed cultures with Pseudomonas species.

The removal of Lead (pb) by Pseudomonas putida and Bacillus subtilis was quantified using Atomic Absorption Spectroscopy (AAS), where found that Pseudomonas putida had the highest removal of Lead (pb) at overall concentration of 300 mg/L were reduced to 276 mg/L at 24 hours while Bacillus subtilis had 283. The potential of Pseudomonas putida to bioremediate lead was reported by several authors including Okpara-Elom et al. (2024) who worked on Bioremediation of heavy metal polluted soil using plant growth promoting bacteria:

an assessment of response., Saidu et al. (2019) also reported Pseudomonas putida as promising bacterial isolate for lead bioremediation who worked on Diesel-Degrading Potential of Pseudomonas putida Isolated from Effluent of a Petroleum Refinery in Nigeria.

In This study Pseudomonas putida had found that was the most potent isolate with the highest bioremediation of Lead-contaminated soil compare to Bacillus subtilis. The identification of Pseudomonas putida was confirmed using molecular technique to know the genomic region. The base pair of the molecularly identified Pseudomonas putida was 1414 bp that indicated the successful obtained targeted genomic. The NCBI was used for sequence analysis and phylogenetic tree construction to detect the evolutionary relationship, and the Pseudomonas putida shared a relationship with other Pseudomonas putida strain with MX-2. Identification of lead bioremediation by Pseudomonas putida using molecular techniques was reported by several authors including Pal et al. (2024) who worked on Molecular and eco-physiological responses of soil-borne Lead (Pb2+)-resistant bacteria for bioremediation and plant growth promotion under Lead Stress. Tasleem et al. (2023) also reported Pseudomonas putida as the potential bacterial isolate identified in lead bioremediation using molecular techniques, who worked on Pseudomonas putida Metallothionein: Structural Analysis and Implications of Sustainable Heavy Metal Detoxification in Madinah.

Conclusion

The findings of this study show the impact of hydrocarbon-contamination on the physicochemical properties of the soil bacteria and their potential for hydrocarbon-degradation. Hydrocarbon-contamination soil contained elevated electrical conductivity, cation exchange capacity and ionic content compared to the control that reflects a soil modification. The bacterial colony counts varied across sampling sites Sokoto Old Garage (SOG) showing the highest colony count of (51.59 × 106) due to accumulation of organic waste and anthropogenic activity. Numerous bacterial communities including hydrocarbon-degrading species includes Pseudomonas putida and Bacillus spy were identified and shows their metabolic versatility. Hydrocarbon-degradation revealed a varying bacterial isolates performance, like BZYA2 and TG B1 showing high initial hydrocarbon-degradation potential. Phylogenetic analysis confirmed the identification of Pseudomonas putida as the most potential hydrocarbon-degrader and scored for bioremediation applications. This study demonstrated that Pseudomonas putida isolated from petroleum-contaminated soil had the highest potential for lead bioremediation which can be used to remediate Lead-contaminated soil.

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