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Bioremedial potential of bacteria isolated from soil in automobile garages and fuel stations in Mbarara City, Southwestern Uganda

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ABSTRACT

The use of bacteria to clean up oil-contaminated soils is widely considered the most desirable bioremediation strategy. The majority of research has focused on contaminated soils in large oil-producing countries. However, accidental spills or poor handling and disposal of petroleum hydrocarbons (PHs) products, which happen at automobile maintenance garages and leakages at fuel stations contaminate soil. There is limited information on PH-degrading bacteria in soil patches around automobile garages and fuel stations for their possible bioremediation potential. We characterized bacteria with the alkane hydroxylase (*alkB*) gene isolated from soil around automobile repair garages in Mbarara City, Uganda, and assessed their efficiency and effectiveness to degrade PHs. The isolation and identification of cultured bacteria were performed using standard microbiology morphological and biochemical tests. Bacterial efficiency was determined by measuring their growth on used engine oil after 14 days using a turbidometer. The effectiveness of the bacteria was measured after 14 days by dichlorophenol-indophenol redox indicator method. Twelve different genera were identified in 45 bacterial isolates. *Enterobacter spp.* and *Aeromonas spp.* were the most effective and efficient bacteria in degrading the used diesel oil, respectively. The *alkB-3* gene was identified in 15 bacteria isolates. Two isolates of *Yersinia spp* and *Aeromonas spp* showed two genes of 157 bp and 250 bp. These findings are suggestive of the catabolic potential of the isolates to degrade alkanes and require further studies to establish the physicochemical requirements and conditions necessary to ensure the maintenance of an even microbial community composition and diversity needed for successful bioremediation approaches.

KEYWORDS

Alkane hydroxylase; bacterial isolates; contaminated sites; efficiency; gene; petroleum hydrocarbon-degrading bacteria; petroleum hydrocarbons; soil bioremediation

Introduction

Globally, petroleum and petroleum products are used as energy sources in industry and homes, making them a source of government revenue (Mahjoubi et al. 2018). Transportation fuel is one of the most common uses of petroleum and petroleum products. In many countries, automobiles are the dominant means of transport. It requires refueling stations, fuel storage tanks, vehicle repair services, which make accidental spills inevitable. Such spills contaminate soil and water (Hasani Moghaddam, Hashemi, and Ghadiri 2021). Used engine oil, like crude oil, has higher levels of total petroleum hydrocarbons (PH) than new oil (Ibrahim 2016). Although some high-income countries (HIC) have introduced the use of ethanol fuel, electric-powered cars, and trains for urban transportation to mitigate the challenges of petrogenic environmental pollution (Delavarrafiee and Frey 2018; Chen et al. 2019), these developments are not in the near future in many countries in sub-Saharan Africa. Bacteria utilize hydrocarbons as a source of carbon and energy and are thus able to thrive in restricting settings (Mansi, Akaranta, and Abu 2017). Petroleum hydrocarbon-degrading bacteria contain the alkane hydroxylase enzyme, AlkB, encoded by the *alkB* gene, which provides the catabolic capacity to use PHs as a carbon and energy source (Varjani 2017). It is for this reason that such bacteria are exploited for bioremediation in areas contaminated by petroleum oil. Most of the studies that have explored the bioremedial potential of bacteria to degrade PHs have

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focused on bacterial communities associated with large-scale oil leaks (Christova et al. 2019). However, small spills, like those that can occur at fuel stations, can seep through concrete and accumulatively contaminate nearby soil (Hilpert and Breysse 2014). The successful discovery of oil in Uganda in 2006 led to increased oil explorations around the large lakes in Kenya and Tanzania and along the Eastern Africa coastline down to Mozambique (Augé and Nakayi 2014). Consequently, there are concerns about environment protection because oil pollution can damage the ecosystem because crude oil is the major source of PHs (Umar et al. 2021; Polyak et al. 2018).

In the absence of any physicochemical limitation, the native populations of microorganisms determine the self-purification capacities of contaminated sites. The purpose of this study was, therefore, to screen bacteria in soils around garages and fuel stations in Mbarara City, Southwestern Uganda, for the presence of the *alkB* gene to explore the intrinsic bioremediation potential of these bacteria and to assess the efficiency of different bacterial communities in degrading oil-contaminated soils. The findings provide preliminary data for future studies that would inform a possible effective management strategy that can be used in bioremediation of oil-contaminated sites in the local context. It is expected that information on the available natural populations of petroleum-degrading bacteria in Uganda will be of interest for scientific and industrial applications.

Materials and methods

Study area

Mbarara is the largest city in southwestern Uganda and is located at 0°36'41.38"S, 30°39'17.77"E. The study area and sample collection sites were purposefully selected. Soil samples for identification of petroleum degrading bacteria were collected from two automobile garages (**S1** 0°36′49.512″S, 30°39′35.994″E; **S2** 0°36′4.236″S, 30°39′50.184″E), two fuel stations (**S3** 0°36′24.762″S, 30°39′48.372″E; **S4** 0°35′41.388″S, 30°39′55.908″E), and two control sites (**S5** 0°36′44.208″S, 30°39′31.662″E; and **S6** 0°35′40.470″S, 30°40′6.990″E); (Figure 1).

Selection of study sites

Two out of 15 fuel stations and two out of the eight automobile repair garages in Mbarara Town were selected. Selection criteria were: automobile garages and fuel stations that had been in operation for more than 5 years to ensure that sites with long-term exposure to oil contamination are selected; the presence of soil within their working area or surrounding area; and those with the potential to pollute nearby resources either because of proximity or slope. The control sites were 300 m away from the oil-contaminated sites; one of them was on a golf course. From each study site, three locations for collecting soil

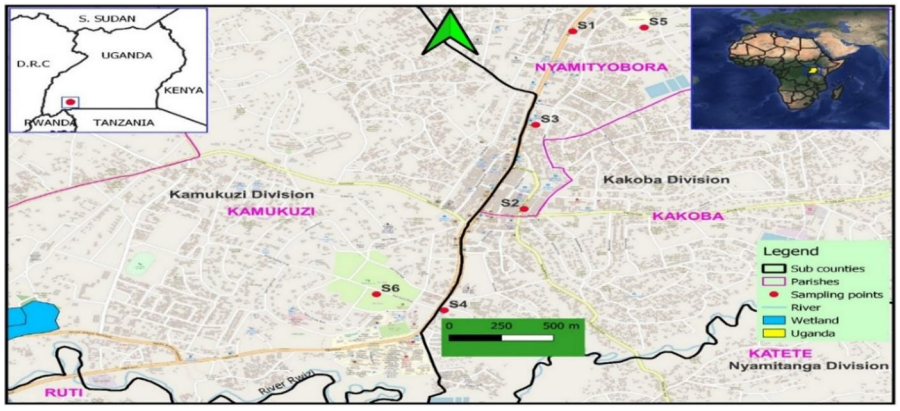


Figure 1. Map showing the location of sampling sites in Mbarara city, Uganda.
Sampling sites, S1-S6, where soil samples were collected. S1: study site 1 of automobile garage; S2: study site 2 of automobile garage; S3: study site 3 of fuel station; S4: study site 4 of fuel station; S5: study control site; S6: study control site in Mbarara Boma golf course.

samples were selected to increase homogeneity, reliability, and minimize bias in the results.

Collection of soil samples

Soil samples (250 g) were collected from three different depths: the top layer, between 1 and 5 cm deep below the surface, the middle layer, between 6 and 10 cm deep below the surface, and the bottom layer between 11 and 15 cm deep below the surface, using a sterilized shovel to avoid contamination. A total of nine soil samples from each site were collected in sterile universal bottles and transported on ice to the laboratory for analysis within an hour. Soil samples were homogenized by mixing them with sterilized distilled water to obtain a uniform sample as previously described by Rajaei et al. (2013) and stored at 4 °C until used.

Sample preparation

Bacterial isolation and culturing of bacteria

Isolation of petroleum hydrocarbon-degrading bacteria was carried out using the enrichment technique as described by Mwaura et al. (2018), with minor modifications (Mwaura et al. 2018). A soil sample (1 g) from each site was transferred into 10 mL of sterile Bushnell-Haas media (BHM) supplemented with 1% used engine oil as the sole carbon source and incubated at 37 °C for 7 days on a rotary shaker operating for 24 h at 40 revolutions per minute (rpm) (Xia et al. 2017). BHM composed of [$MgSO_4{\cdot}7H_2O$ (0.2 g/L), Na_2HPO_4 (1.0 g/L), KH_2PO_4 (1.0 g/L), $FeCl_3$ (0.05 g/L), NH_4SO_4 (1.0 g/L), $CaSO_4$ (0.02 g/L), pH 6.85 (Chaillan et al. 2004). Prepared Bushnell haas media (BHM) was sterilized using an autoclave (Tomy SX-700, High-Pressure Steam Sterilizer) at 121 °C for 15 min.

After 7 days of incubation, the 500 µL of inoculum was picked from each BHM falcon tubes, and streaked onto Luria bertani (LB) agar, and incubated overnight at 37 °C. The LB media was composed of the following in g/l: tryptone (10.0), yeast extract (5.0), NaCl (10.0), and agar (15.0) at a final pH of 7.2. Colonies were then picked from each plate using a sterilized wire loop and transferred into Falcon tubes containing LB broth. The tubes were incubated at 37 °C in a rotary shaker at 40 rpm for 24 h. Serial dilutions of up to 10^{-7} from each falcon tube were made, and an aliquot of 100 µl was pipetted and poured on LB agar plates and spread to obtain distinct colonies. The distinct colonies were then picked using sterile toothpicks, streaked on fresh LB agar plates, and incubated for 12 h. Purified colonies were then picked and added to LB broth for storage at −20 °C.

Identification of petroleum hydrocarbon-degrading bacteria

The bacterial isolates selected were identified by their morphological and biochemical characteristics.

Morphological characterization

Colony characteristics. All bacterial isolates were characterized by their morphological characteristics. The characterization was based on colony morphology on Luria bertani (LB) agar plates (Aldisi et al. 2016). The colony morphology of isolated strains was studied by streaking bacterial strains on LB agar plates in order to obtain pure isolated colonies. The colony form, cell shape, colony color, texture, and smoothness were recorded according to Xu et al. (2018).

Gram test. Bacterial isolates were characterized by Gram's staining test to differentiate between gram-positive and gram-negative bacteria. An optical microscope (Olympus CX 21) was used to examine the morphological properties of the isolated colonies after incubation for 24–48 h on an LB agar slant.

Samples were heat-fixed onto a clean, grease-free slide. A crystal violet solution was added in drops to the sample and then allowed to stand for one minute. Then the stain was washed under running tap water. Iodine solution (Gram's iodine) (2 drops) was then added, allowed to stand for one minute, and then washed in tap water. The sample was rinsed with 2 ml of ethanol for 30 s and then gently washed in running water. Exactly 2mls of carbol fuchsin was added to the slide, allowed to stand for one minute, and washed. The slide was allowed to air dry and mounted on the microscope to be viewed under

a total magnification of ×1000 (eye piece X10 and objective lens ×100).

Biochemical characterization

Various biochemical tests used to identify bacterial species isolated from the study sites were performed in triplicate.

Starch hydrolysis test. A pure bacterial colony from each isolate was streaked in a straight line on a mineral salt agar plate containing 2% starch and incubated at 37°C for 48 h. A pure bacterial colony from each isolate was streaked in a straight line on a mineral salt agar (MSA) plate containing 2% soluble starch and incubated at 37 °C for 48 h. Mineral salt media composition in g/L was: $(NH_4)_2SO_4$ (1.0), $MgSO_4{\cdot}7H_2O$ (0.1), KH_2PO_4 (1.0), K_2HPO_4 (0.76), agar (15.0) supplemented with 1 ml trace element solution (mg/L); $ZnSO_4$ (100), H_3BO_3 (300), $CaCl_2{\cdot}2H_2O$ (134.2), $FeSO_4{\cdot}7H_2O$, Cu $Cl_2{\cdot}2H_2O$ (10), $NaMoO_4{\cdot}2H_2O$ (30), $NiCl_2{\cdot}6H_2O$ (20), and $MnCl_2{\cdot}4H_2O$ (30). The plates were then flooded with Gram's iodine solution to produce a dark blue-colored starch-iodine complex. Gram's iodine is composed of 250 mg iodine crystals, 2.5 g potassium iodide, and 125 ml water. The formation of a clear zone around bacterial streaks indicated starch hydrolysis as a result of amylase enzyme activity, as described by Mwaura et al. (2018), Kumari et al. (2013), and Vignesh et al. (2016).

Catalase test. Two drops of 5% hydrogen peroxide were placed on a glass slide, and a loopful of pure bacterial cells was added. Catalase enzyme activity was indicated by the formation of air bubbles, as described by Kumari, Khare, and Dange (2014) and Vignesh et al. (2016).

Carbohydrate fermentation in triple sugar iron agar test. Glucose, lactose, and sucrose fermentation tests were carried out using triple sugar iron agar (TSI). The agar was boiled and sterilized in an autoclave for 15 min at 121 °C. The TSI agar (CM0277, OXOID) was prepared by dissolving 65.0 g of TSI in 1000 ml of distilled water, and then 5 ml of the sterilized medium were poured into 15 ml Falcon tubes. Observations were made on color changes and gas production, as indicated by the presence of bubbles and cracks trapped within the media after 24 h of incubation.

Citrate utilization test. Citrate utilization by bacterial isolates of the family Enterobacteriaceae was carried out using a fresh, pure culture. A single isolated colony was streaked on the surface of a slant. The observations were made on color changes after 24 h of incubation.

Urease test. The urease test was used to identify bacterial isolates of the family Enterobacteriaceae that are capable of hydrolyzing urea to produce ammonia and carbon dioxide. A heavy inoculum from pure culture was streaked on the entire slant surface of the tube. The butt of the tube was not stabbed with the inoculum to work as a color control. The tubes with inoculated media were then incubated with loosened caps at 35 °C for 24 h. The medium was prepared by adding 0.9 g of urea broth base (CM0071, OXOID) to 95 ml of distilled water and sterilized by autoclaving for 15 min at 121 °C. The resultant solution was cooled to 55 °C and aseptically added to one ampoule of sterile urea solution (SR20). The solution was mixed well, and 10 ml of the sterilized medium was poured into 15 ml Falcon tubes using a 25 ml Falcon pipette. Urea broth base was composed of g/l peptone (1.0), glucose (1.0), disodium hydrogen phosphate (1.2), potassium dihydrogen phosphate (0.8), sodium chloride (5.0), and phenol red (0.004). The observations were made on color changes after 24 h of incubation.

Indole test. The test was used to identify bacterial isolates of the family Enterobacteriaceae, which can produce indole from tryptophan amino acid. The tubes of tryptone broth (prepared following the manufacturer's instructions) were inoculated with a small amount of fresh, pure culture. The tubes were incubated at 35 °C for 24 h. Five drops of Kovac's reagent were then added directly into the tube. A positive indole test was indicated by the formation of a pink to red color in the reagent layer on top of the medium within seconds of adding the reagent. The medium was prepared by suspending 36.52 g of Motility-

Indole-Lysine (MIL) in 1000 ml distilled water. The mixture was boiled to dissolve the medium completely. The medium was dispensed into the tubes in 5 ml amounts and sterilized by autoclaving at 121 °C for 15 min. The tubes were allowed to cool before inoculation. The MIL was composed of peptone (10 g/L), tryptone (10 g/L), yeast extract (3 g/l), L-lysine hydrochloride (10 g/L), dextrose (1 g/l), ferric ammonium citrate (0.5), bromocresol purple (0.02 g/L), and agar (2 g/L); pH at 25 °C was 6.6 ± 0.2.

Preparation of bacterial glycerol stock

Glycerol stocks of pure bacterial isolates were prepared by mixing an overnight culture with 50% sterile glycerol, and mixed briefly before storage at -20^0C (Mwaura et al. 2018). Glycerol stocks were used to store bacterial cultures for at least four months so that bacterial cells were made structurally stable and safe. Storage was done by mixing 500 µl of an overnight cultured bacterial cell in LB broth with 500 µl of 50% sterilized glycerol in 2 ml Eppendorf tubes. A 50% glycerol solution was prepared by diluting pure glycerol with an equal volume of distilled water and then sterilized after shaking to ensure that the liquids were evenly mixed.

Determination of efficiency and effectiveness of petroleum hydrocarbon-degrading bacteria

Efficiency of petroleum hydrocarbon degrading bacteria using densitometer

Bacterial isolates were identified for their ability to degrade petroleum hydrocarbons (used engine oil). Mineral salt media supplemented with used engine oil as the sole carbon source was used. Bacterial cells were washed twice with 0.85% NaCl solution before being suspended in the same solution. An aliquot of 100 µl of bacterial cells cultured overnight in LB media was transferred to falcon tubes containing 5 ml mineral salt media following autoclaving for 15 min at 121 °C. Used engine oil (0.5 ml, 1%) was added, and the mixture was incubated at 30 °C in a rotary shaker with a speed of 40 rpm for 14 days (Xia et al. 2017). The observations were made after following the procedures as described in Appendix 6 A. The experiment was carried out in triplicate, and two controls, of negative and positive, were prepared by excluding the substrate in one and the inoculum in the other, and the two were kept under the same conditions. Change in turbidity was taken as the measure of growth after 14 days at the wavelength of 550 nm using a densitometer (Biomerieux) (Busse, Calhoun, and Sedgwick 1993) based on McFarland standards of 0.5, 1.0, 2.0, 3.0, 4.0, 5.0, and 6.0. Following the laboratory protocol for interpretation of McFarland standards, the corresponding numeric values of the approximate bacterial suspension concentration at 550 nm optical density were estimated. These standards corresponded to 1.5×10^8 cells/ml, 3×10^8 cells/ml, 6×10^8 cells/ml, 9×10^8 cells/ml, 12×10^8 cells/ml, 15×10^8 cells/ml, and 18×10^8 cells/ml, respectively (Guzmã et al. 2018).

Effectiveness of petroleum-hydrocarbon bacteria using DCPIP redox indicator

The bacteria's ability as single isolates to degrade different hydrocarbons was verified using the method based on the redox indicator 2, 6-dichlorophenol indophenol (DCPIP) (Ubani et al. 2016). Biodegradation of used engine oil by bacteria isolates was verified using the redox indicator-DCPIP test through blue color disappearance observation (Lima et al. 2020). The absorbance was measured at 600 nm using the ultra violet-visible (UV–VIS) spectrophotometer (JENWAY 6305) (Obi, Atagana, and Adeleke 2016). The percentage of biodegradation was estimated as follows:

$$\%\ \text{of degradation} = 1 - \frac{Absorbance\ of\ treated\ sample}{Absorbance\ of\ control} \times 100$$

(Ahirwar and Dehariya 2013)

Each bacterial culture was inoculated into 100 ml of nutrient broth medium and incubated at 37 °C for 24 h at 40 rpm. The cultures were used as inoculum for hydrocarbon degradation. Exactly 100 ml of Bushnell-Has media (BHM) were prepared with 1% diesel engine oil as the sole carbon source. To the medium was added 20 mg/L of 2,6-dichlorophenol indophenol (DCPIP), and then

it was sterilized at 121 °C for 15 min. To each medium was added 1 ml of each inoculum and incubated at 35 °C at 40 rpm for 14 days. The negative control experiment was set in a medium without inoculum of bacterial cultures. The absorbance of the mixture after 14 days was measured at 12-, 24-, and 48-h' time intervals.

Molecular identification of bacteria with alkB gene

Genomic DNA extraction

The bacterial strains were sub-cultured on nutrient agar and incubated overnight. The colonies were inspected after 24 h and used as the source of genomic DNA.

Genomic DNA nucleic acid was extracted using a combination of cetyl trimethyl ammonium bromide (CTAB)-chloroform/alcohol and the heat-lysis method. Briefly, a loopful of the colonies were picked using sterile plastic loops, emulsified in 500 µl of Tris-EDTA (TE) buffer in 1.5 mL Eppendorf tubes, and centrifuged at 10,000 rpm for 5 min. The supernatant was discarded, and the sediment was re-suspended in a further 200 µL of TE buffer, vortexed for 10 s, and incubated at 95 °C for 15 min. The high temperature denatures the proteins in the bacterial cell membrane, which results in pores forming in the bacterial cell thus allowing the external solution to enter the cell, causing bursting of the cell, and releasing cellular components, including DNA, into the solution. Exactly 200 µl of 5 M Sodium Chloride (NaCl) solution was pipetted in a 1.5 ml Eppendorf tube, followed by 200 µl of 10% CTAB/NaCl solution at room temperature. The tubes were inverted several times and incubated for 1 h at 65 °C. An equal volume of freshly prepared chloroform/isoamyl alcohol (24:1) was added. The tubes were further inverted several times and centrifuged for 10 min at 10,000 rpm. About ¾ of the aqueous phase was transferred to a new 1.5 ml tube, and 1 ml of absolute ethanol was added to precipitate the DNA. The tubes were mixed gently by inverting several times and incubated at room temperature for 15 min, after which they were kept at −20 °C for 1 h. After the incubation at −20 °C, the tubes were removed and centrifuged at 10,000 rpm for 3 min. The supernatant was decanted, being careful not to dislodge the pellet at the bottom of the tube. The pellet was washed in 1 ml of 70% ethanol for 5 min at 10000 rpm. The ethanol was decanted, and the tubes were left to air dry at room temperature for 10 min. Following the air drying of the sediment, 60 µl of nuclease-free water (PCR water) was added into the tubes and then incubated at 65 °C for 1 h to completely dissolve the DNA. The DNA was quantified and assessed for quality and purity using the Nanodrop Lite spectrophotometer (Thermo Scientific) at 260/280 nm absorbance. The quality was considered satisfactory if the ratio of absorbance at 260/280 nm was between 1.8 and 2.0. The DNA was stored at −20 °C until use.

Alkane hydroxylase gene amplification

The presence of the AlkB-III gene (AlkB-3) was established by PCR amplification of genomic DNA using the following set of primers: *AlkB*-3F (5′-CCATTGAAGTCTGGCGAGC-3′) and *AlkB*-3R (5′-CGATGCGAGAAAACATTGACCG-3′). The total reaction mix used was 25 µL, consisting of 1.5 µL of each of the forward and reverse primers (25 µM), 12.5 µL of 2X master mix (M0486S; New England Biolabs) containing standard buffer, deoxynucleoside triphosphates, and Taq polymerase (New England Biolabs), 4.5 µL of nuclease-free water, and 5 µL of the template DNA. *Brucella abortus* with a 157 bp fragment of the alkB-3 gene was used as a positive control (Doust et al. 2007).

Amplification was carried out in a Multi-Gene Optimax thermocycler (Multi-Gene Optimax Labnet, USA) under the following PCR conditions: initial denaturation at 94 °C for 5 min followed by 40 cycles at 94 °C for 1 min, annealing at 54 °C for 1 min and extension at 72 °C for 1 min with a final extension of 72 °C for 5 min (Doust et al. 2007). Gel electrophoresis was performed using a 1.5% agarose gel containing Safe View DNA stain (Safe View Classic cat. No. G108, ABM, USA), 6x loading dye (Thermo Scientific #R0611), and a 100 bp molecular weight marker (NEB-Biolabs) for 45 min at 120 V. PCR amplicons were visualized using the Gene-Flash Trans-illuminator/Dark Reader (ClareChemical.com, DR-46B).

Data analysis

All analyses were carried out in triplicate, and the experimental data was analyzed using one-way univariate analysis of variance (ANOVA) to analyze the difference in efficiency among the means of all the populations and one-way repeated measure analysis to compare the effectiveness of petroleum hydrocarbon-degrading bacteria using Statistical Package for Social Sciences (SPSS), version 20.0. Differences among mean values for treatments at $p < 0.05$ were evaluated using the post hoc test (Bonferroni test). The presence of the AlkB gene in bacterial isolates was analyzed by looking at the band on electrophorograms, and the distribution of the gene among isolates was expressed as a percentage of the DNA amplified. The diversity of petroleum hydrocarbon-degrading bacteria identified was presented as percentages.

Ethical considerations

The proposal was presented to and approved by the department of biochemistry at Mbarara University of Science and Technology (MUST) before being submitted to the Faculty of Medicine Research Committee (FRC) for scientific review and clearance. Final ethics approval was obtained from MUST Research Ethics Committee prior commencement of the study (study number 01/05-20). Administrative clearance to conduct the study in automobile garages and around fuel stations in Mbarara town was obtained from the respective managers. The handling of the bacteria, culturing, and disposal followed the standard operation procedures in the MUST Microbiology laboratory. At the end of the study, all culture media with or without visibly grown bacteria were destroyed by autoclaving before disposal.

Results

Bacteria identification and characterization

Twelve genera were identified in bacterial isolates from oil-contaminated sites. They were all bacilli or rods in shape; their distribution in soil per soil sampling depth was different. The most abundant PH-degrading bacteria in contaminated sites were *Bacillus spp.* (23.2%) and *E. coli* (16.1%). The most abundant bacteria in non-contaminated sites was *Klebsiella spp.* (23.3%) (Table 1).

Effectiveness and efficiency of petroleum hydrocarbon-degrading bacteria

The ability of bacteria strains to degrade PHs was highest in *Enterobacter spp.* (4.91×10^8) and *Citrobacter spp.* (4.44×10^8) from the automobile repair garages; *Salmonella spp.* (3.87×10^8) from the fuel stations; and it was lowest with *Yersinia spp* (3.1×10^7) from the fuel stations (Table 2).

The highest transmittance was seen at 48 h following 14 days of incubation for the bacteria isolate (Figure 2). The biodegradation rates of diesel engine oil ranged from 9.8% to 75.6%. Eight out of 45 of the isolated strains showed a high growth rate on used diesel engine oil as per

Table 1. Types of bacteria species isolated from contaminated and control sites.

	Number of isolates, $N = 56$		
	Contaminated sites		Control sites
Bacterial species (spp.)	Automobile repair garages, *n* (%)	Fuel stations, *n* (%)	Control sites, *n* (%)
Citrobacter spp.	2 (3.6)	1 (1.8)	0
Enterobacter spp.	4 (7.14)	0	4 (7.14)
Klebsiella spp.	3 (5.4)	3 (5.4)	7 (12.5)
E. coli	4 (7.14)	5 (8.93)	0
Yersinia spp.	3 (5.4)	2 (3.6)	0
Salmonella spp.	0	1 (1.8)	0
Proteus spp.	0	3 (5.4)	0
Providencia spp.	0	1 (1.8)	2 (3.6)
Aeromonas spp.	0	1 (1.8)	0
Acinetobacter spp.	0	0	1 (1.8)
Pseudomonas spp.	0	0	1 (1.8)
Bacillus spp.	7 (12.5)	5 (8.9)	1 (1.8)
Corynebacterium spp.	1 (1.8)	0	0
Clostridium spp.	0	1 (1.8)	0

Table 2. Bacterial cells from each sampling site.

	CFU per site $\times 10^8$					
Bacterial species	S1	S2	S3	S4	S5	S6
Enterobacter spp.	2.00	4.91	–	–	2.05	0.46
Klebsiella spp.	2.48	–	0.76	1.42	2.95	1.05
E. coli	0.73	3.59	–	1.41	–	
Bacillus spp.	1.33	0.64	–	–	–	–
Citrobacter spp.	1.42	–	4.44	–	–	–
Yersinia spp.	–	2.35	0.31	2.11	–	–
Corynebacterium spp.	–	0.59	–	–	–	–
Proteus spp.	–	–	1.59	–	–	–
Salmonella spp.	–	–	3.87	–	–	–
Providencia spp.	–	–	–	1.89	2.45	0.43
Acinetobacter spp.	–	–	–	–	1.45	0.80
Pseudomonas spp.	–	–	–	–	–	0.63

Abbreviations: CFU: (colony forming unit) is a measure of the number of bacteria cells in a sample that are viable enough to proliferate and form small colonies under controlled conditions. S1: study site 1 of automobile garage; S2: study site 2 of automobile garage; S3: study site 3 of fuel station; S4: study site 4 of fuel station; S5: study control site; S6: study control site in the Mbarara Boma golf course.

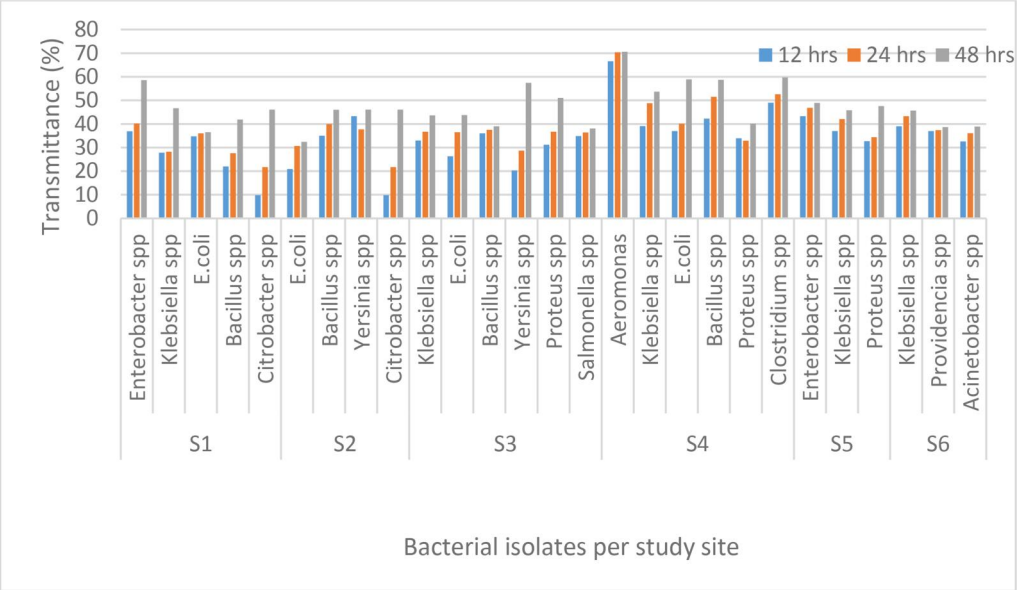


Figure 2. Utilization of used diesel engine oil by bacterial isolates.
The figure shows the change in turbidity of the bacteria growth medium containing mineral salt and used engine oil, indicated as transmittance, measured at different time points following incubation of bacterial isolates for 14 days.

percent transmittance. *Aeromonas spp.* showed the highest utilization of diesel engine oil (75.6%) after 48 h while *Citrobacter spp.* had the lowest value of 9.8% (Figure 2).

Detection of alkane hydroxylase 3 gene in bacterial isolates

DNA was extracted from 45 isolates, and after being amplified, 14 (31.1%) bacterial isolates from all study sites had the *AlkB-3* gene. Of the 14 isolates, 3 were gram-positive and 11 were gram-negative; two isolates from fuel stations S3 and S4 harbored two catabolic genes of 157 bp and 250 bp (Figure 3).

Discussion

Bioremediation is considered a sustainable method for the removal of hydrocarbon contaminants from polluted environments. There is a growing interest in the scientific community to identify bacteria with the potential to degrade petroleum hydrocarbons. In oil-producing countries, efforts have been made to identify petroleum hydrocarbon-degrading enzymes given the accidental spills during exploration, storage, and transportation (Verasoundarapandian et al. 2021). We described for the first time in this study the bioremediation potential bacteria of *Citrobacter, Enterobacter, Klebsiella, E. coli, Bacillus, Yersinia,*

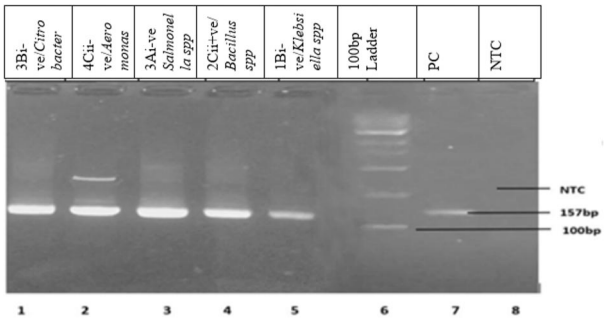


Figure 3. Electrophoresis profile of the amplified products using alkB-3 primer.
The figure shows gel electrophoresis results of the PCR amplification of *AlkB* genes. Lanes 1-5 bacterial isolates; 6-ladder (molecular marker (100 bp); 7- PC- positive control (*Brucella abortus*-vaccine strain); Lane 8 NTC- negative control. Isolates in lanes 1-5 are positive for 157 bp fragment; lane 2 has both 157 bp and 250 bp sized amplicons. 1Bi-ve: gram negative isolate from depth B of the S1 at depth of 1-5 cm; 2Cii + ve: gram positive isolate from depth C of the S2 at depth of 6-10 cm; 3Ai-ve: gram negative isolate from depth A of S3 at depth of 1-5 cm; 3Bi-ve: gram negative isolate from depth B of S3 at depth of 1-5 cm; 4Cii-ve: gram negative isolate from depth C of S4 at depth of 6-10 cm.

Corynebacterium, Salmonella, Proteus, Providencia, and *Clostridium* from Mbarara City, Uganda.

Soil from automobile repair garages and fuel station sites had more bacterial isolates, probably because of the presence of hydrocarbon, which are used as a source of carbon for energy. Interestingly, even soil from the control sites in the ever-green Mbarara golf course presumed not to be contaminated with oil or PHs also had bacterial isolates. This corroborates what has been reported elsewhere: that natural ecosystems have petroleum hydrocarbon-degrading bacteria even if they are not exposed to oil pollutants or their products (Allamin 2014). The relative higher number of isolates from contaminated sites supports the concept of substrate-induced bacterial gene expression (Meier, Paterson, and Lambert 2015). More bacterial isolates indicate a potentially higher microbial diversity in these environments influenced by contamination (Bidja Abena et al. 2020). Automobile repair garages and fuel stations often introduce a mix of organic pollutants and oils into the soil, creating unique niches that support diverse microbial communities (Patowary, Devi, and Mukherjee 2023). Diverse hydrocarbon pollutants into the soil create selective pressures that favor a wide range of microorganisms capable of metabolizing or tolerating these contaminants (Pandolfo, Barra Caracciolo, and Rolando 2023). Higher microbial diversity could also indicate that these environments are subjected to higher levels of environmental stress (Shu and Huang 2022) due to contamination, which may lead to the emergence of specialized strains with unique metabolic capabilities that is, adaptation and diversification to stress.

In the current study, we noted more Gram-negative isolates than Gram-positive. This is probably because of the presence of a highly impermeable outer membrane with a lipopolysaccharide component, which produces biosurfactants and biofilms that could trap PHs and increase their bioavailability to bacteria, ultimately stimulating the biodegradation process (Smits et al. 2002). Indeed, gram-negative bacteria have been reported to be more tolerant to hydrocarbons than gram-positive bacteria (Bailey 2020). *Citribacter, Enterobacter, Klebsiella,* and *E. coli* have been studied for their ability to degrade organic pollutants such as petroleum-derived compounds through hydrocarbon degradation and bio-surfactant production (Poddar et al. 2023; Adetunji et al. 2021). *Bacillus spp.,* which are gram-positive, are widely distributed in soil. Several studies have recognized *Bacillus* strains for their ability to degrade hydrocarbons because they produce lipase and protease enzymes that contribute to pollutant breakdown (Abubakar et al. 2024; Bhandari et al. 2021).

The cell counts and growth rates correlate with the effectiveness of the isolate in utilizing the diesel oil as the sole carbon source (Aldisi et al. 2016). The higher the value of bacterial cells, the faster the rate of growth, implying that bacteria have metabolic pathways that degrade PHs as a source of carbon for energy to attain a larger population. Thus, the population of bacteria steadily increased because of sufficient nutrients to facilitate their reproduction process. *Enterobacter spp.* from the automobile garage was reported to have the highest bacterial cell counts, indicating a faster growth rate and hence being most effective in degrading PH. *Enterobacter* spp. are known for their metabolic versatility, and some strains within this genus have been

reported to possess hydrocarbon-degrading capabilities (Muneeswari et al. 2021). The higher cell counts observed in the current study may indicate a large population of metabolically active *Enterobacter* spp., contributing to PH degradation. Lima and others reported that *Bacillus spp.* was the most effective in degrading n-alkane (C10-C27) from diesel after 7 days of cultivation in the mineral medium (Lima et al. 2020). Although bacteria growth rate is affected by physical-chemical factors like pH, temperature, and oil chemical composition (Dasgupta and Brahmaprakash 2021), this is unlikely to be the reason for the low growth rate in some bacteria given that the culture conditions were the same. Our findings show that bacteria growth was highest in organisms from oil-contaminated sites of automobile garages and fuel stations. Microorganisms that inhabit locations contaminated with hydrocarbons often contain enzymes that can degrade oil into harmless products.

Aeromonas spp. were the most efficient isolated microorganisms for degrading hydrocarbons. These Gram-negative bacteria produce biosurfactants that enable them to break down complex organic compounds of petroleum products into simpler and nontoxic substances, hence producing electrons and hydrogen ions (Djahnit et al. 2019) that oxidize and reduce DCPIP, hence lowering its absorbance. The bacteria are adapted to such mechanisms so that they can utilize PHs as a source of carbon and energy. The isolates with a high percentage of diesel oil degradation had low absorbance, hence more efficient in the degradation of diesel oil. The percentage of diesel oil degradation was highest at 48 h, implying that the efficiency of the bacteria in breaking down diesel oil increases with time. In another study by Abou-Shanab and others, the biodegradation rates ranged from 10% to 93% (Abou-Shanab et al. 2016).

In the current study, 15 isolates harbored the *alkB-3* gene, which is indicative of their potential to degrade alkane fractions of petroleum hydrocarbons. The alkane hydroxylase gene was observed mostly in Gram-negative bacteria rather than Gram-positive bacteria. Our finding is in agreement with Liang et al. (2017), who reported that more Gram-negative bacteria had the alkane hydroxylase gene than Gram-positive bacteria (Liang et al. 2017). *AlkB*-3 encodes a third group of alkane hydroxylase that catalyzes long-chain alkanes, unlike short-chain alkanes, which are degraded faster and earlier (VAN Beilen and Funhoff 2007).

The presence of two catabolic genes in two isolates suggests that the degradation of some petroleum hydrocarbons in soil from fuel stations involves different metabolic pathways. The expression of these genes would yield different proteins or enzymes in the bacteria. These bacteria strains could be used in industrial and petroleum-contaminating environments for bioremediation.

These findings can be of importance to the Ugandan government in developing strategies for cleaning up oil-contaminated areas, even those that are usually considered insignificant, such as automobile repair garages and fuel stations. Additionally, these findings provide the basis for future studies to establish the optimal conditions for the growth and maintenance of microbial communities to enhance their diversity and capacity to biodegrade petroleum hydrocarbons (biostimulation).

Conclusion

This study identified 45 bacterial isolates in soil obtained from oil-contaminated sites. These bacteria included the genera *Citrobacter spp., E. coli, Yersinia spp., Proteus spp., Aeromonas spp., Bacillus spp., Corynebacterium spp., Clostridium spp.,* and *Salmonella spp. E. coli* and *Aeromonas spp.* were the most effective bacteria in degrading used diesel oil. The alkane hydroxylase *alkB-3* gene was identified in fifteen bacteria isolates, indicating the catabolic potential of isolates to degrade alkanes. while two isolates of *Yersinia spp.* and *Aeromonas spp.* had two genes of 157 bp and 250 bp. We concluded that these strains are highly tolerant to oil. It is important that the bacterial degradation of PHs be optimized. Thus, future research in the region ought to focus on establishing the soil physicochemical parameters favoring bacterial degradation of PHs and their optimization.

Additionally, molecular characterization of bacterial 16S rRNA genes followed by sequence blast with what is in the NCBI database should be conducted for full taxonomy of petroleum hydrocarbon degrading bacteria as a prerequisite for a bioremediation strategy of petroleum contaminated soils in Uganda. Though this study identified and characterized pure strains through culture, there is a need to do metagenomics to identify the petroleum degrading bacteria that cannot grow in laboratory settings.

Disclosure statement

No potential conflict of interest was reported by the author(s).

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