Isolation and Identification of Hydrocarbon-Degrading Bacteria from Diesel-Contaminated Soils

ABSTRACT

Aim:To Isolate, screen and identify bacterial species with hydrocarbon biodegradative potentials from diesel-polluted soils using redox indicator (2% v/v of 2,6 – dichlorophenol indophenols- DCPIP) and Turbidity measurements.

Place and duration of study: Microbiology laboratory in the Department of Environmental Management and Toxicology, Federal University of Petroleum Resources, Effurun, between 2022 and 2024.

Methodology: Soil samples from diesel-contaminated sitewere collected from diesel mechanic workshop, analysed using the spread plate isolation technique on Bushnell Haas mineral salt agar selective medium,were screened for Bacterial species with hydrocarbon degradative potential using the redox indicator (2% v/v of 2,6 – dichlorophenol indophenols- DCPIP) and Turbidity measurement. Selected Bacterial isolates were used for the hydrocarbon degradation studies, werecharacterized based on biochemical tests and identified with the aid of the Biolog database and 16S rRNA gene sequencing techniques.

Results: Eight diesel degrading Bacteria species were identified, belonging to the following genera;*Acinetobacter, Klebsiella,*and*Pseudomonas.* Among these, the four most potent degraders were identified as *Acinetobacter rudis (*M2712942.1), *Acinetobacter bereziniae* (MT111619.1), *Pseudomonas koreensis* (MW794239.1), and *Klebsiella aerogenes* (CP070520.1)and the % dieselreductionachieved in 15 dayswere; 70%, 48%, 47% and 45%, while the % Turbidity increase were 84%, 79%, 78% and 76% respectively. Consequently, the partial 16S rRNA gene sequences revealed the two most potent diesel degrading bacterial strains were *Acinetobacter rudis*and *Acinetobacter bereziniae*with 70% and and48% respectively.

Conclusion:These findings demonstrate the potential of indigenous bacteria for effective bioremediation of petroleum-contaminated environments. The study provides critical insights into sustainable solutions for mitigating hydrocarbon pollution, contributing to advancements in environmental microbiology and ecosystem restoration.

Keywords: Biodegradative potentials, Bacterial Species, Diesel contaminated soil,Screening, identification.

INTRODUCTION

Petroleum-derived products are of high importance and their demand nationally and internationally is greatly alarming. However, the anthropological activities of humans during the production and transportation of petroleum products have led to the contamination of the environments such as soil, air and water. Spillage of these hydrocarbon products affects the ecosystem's biotic and abiotic components (Kebede*et.al.,* 2021). In the Niger Delta's aquatic and terrestrial environments, oil spill incidents have occurred in various parts and at different times. Oil spillage may be due to carelessness during oil production, corrosion of pipes, oil tanker accidents and sabotage, resulting in long-term challenges and hazards to all forms of life (Asuka *et. al.,* 2021).

According to Katimu& Sani (2022), spilled crude oil causes significant changes in the physical, chemical and microbiological properties of the natural soil environment, it decreases water capacity and aeration propensity of the soil in addition to reduced magnesium, sodium, nitrogen, potassium, calcium, phosphorus and increase level of heavy metals and the bulk density of the polluted soil. This also results in the contamination of both terrestrial and aquatic ecological systems. Hydrocarbon-derived pollutant also causes in destruction of soil, farmland and crops, they are immune-toxicant, carcinogenic metabolism, hydrocarbon degradation and degradative enzyme activities. Research indicates that if these factors are monitored, optimized and manipulated, bacteria bioremediation may be more effective.

Hence this study was conducted to isolate, screen, select and identify bacteria species with hydrocarbon biodegradability potentials from hydrocarbon contaminated environments in the Niger Delta region of Nigeria.

2. 0 Material and Methods

2.1 Collection of Soil Samples

The diesel contaminated soil samples were collected randomly from a mechanic workshop situated at Osubi by Hulmas oil filling station along Osubi-Eku road, Warri, Delta State,Nigeria with a Global positioning system(GPS) of 5034`02.5" N 50450"4.2" E. A soil auger was used to collect the polluted soil at 0-15cm depth, stored at 4^0C in an icebox and transported to the laboratory for further analysis.

2.2 Culture media.

The media used for the isolation and purification of isolates are Nutrient agar (NA) and Mineral salt media (MSM), a modification of Bushnell Haas (BH) medium for the isolation of heterotrophic and hydrocarbon-utilizing bacteria respectively. The composition (g/L) of (BH) medium as stated below: K_2HPO_4 (0.1g), KH_2PO_4 (0.1g),

NH_4NO_3 (0.1g), $MgSO_4•7H_2O$ (0.02g), $FeCl_3•6H_2O$ (0.005g), $CaCl_2•2H_2O$ (0.002g), was used as enrichment medium with filter sterilize diesel, 1% (v/v) added as the sole carbon source. The nutrient agar and BH media were autoclaved at 120^oC for fifteen (15) minutes and allowed to cool before carbon source were added(Tudararo-Aherobo&Mesogboriwon, 2020) and (Shettima*et al.,* 2021).

2.3Enrichment of the Diesel contaminated soil and isolation of hydrocarbon-degrading Bacteria

Diesel Contaminated soil from the mechanic workshop (10g) was enriched with 90ml of Sterile MSM (Broth) in the Erlenmeyer culture flask. It was then incubated for seven (7) days at 30^0C. After incubation, serial dilution was done by transferring aseptically (using sterile syringes) 1ml of the mixture into 9ml of sterilized normal saline(0.85%Nacl). Spread plating technique was used for plating the inoculum, using 10^{-3} and 10^{-5} dilutions in duplicate in MSA. Following the solidification of the medium, the vapour phase method was adopted where sterile filter paper (Whatman no. 1) was saturated with filter sterilized diesel. The filter paper saturated with diesel served as a sole source of carbon. It was placed inside the cover of the petri dish, covered and inverted, and the culture was incubated for 28 ± $2^{0o}C$ for 6 days. Discrete bacteria colonies were purification by streakingon MSA and the pure cultures were identified culturally and biochemically as

described in Bergey's Manual for Determinative Bacteriology (Ataikiru*et. al*., 2017) and (Bekele *et. al.,* 2022).

2.4 Screening of diesel degrading Bacteria isolates

The modified method of Ataikiru*et al.* (2017) was adopted for the screening procedure. A loop full of 24 hour of each pure bacterium culture (12 isolates) was used to inoculate 200mls of sterile Bushnell Haas broth containing 2mls of sterile diesel and 1ml of REDOX indicator (2% v/v of 2,6 – dichlorophenol indophenols- DCPIP) separately.The reaction mixture was left in room temperature at 25- 30^0C using the orbital shaker (120 rpm) the degradative ability of the isolates was observed by recording a discoloration of the medium from blue to colorless. A control was set up which contains only the MSM media (200ml) and 2mls diesel without any isolate or bacteria culture making a total of 13 bottles. The

medium was observed for decolourization of the blue colour of the indicator. The screening analysis was set up for 15 days. Four sets of 13 transparent bottles were used, and a set each was taken for physicochemical analysis for pH, Turbidity and Total Petroleum Hydrocarbon (TPH) on days 0, 5, 10 and the 15. The test microcosm were prepared in three replicates.

2.5 Biochemical identification of hydrocarbon-degrading bacteria

Pure cultures of the isolates with high Turbidity and biodegradative potentials of the indicator solution, were identified based on morphological and biochemical characteristics according to the method of Prescott and Harley, (2002) and Bergey's Manual for Determinative Bacteriology (Ataikiru*et. al*., 2017).

2.6 Molecular characterizationof hydrocarbon-degrading bacteria

Pure cultures with high biodegradative potentials were also characterized molecularly. For the molecular characterization, genomic Deoxyribonucleic Acid (DNA) was extracted by DNA was extracted using Zymo Quick-DNA Fungal/Bacterial Miniprep (Zymo Research, USA) following manufacturer protocols. The universal primers are called 27F and 907R, used to amply the 16s target with target sequence 5'in 3', the 16S rDNA sequence (AGAGTTTGATCMTGGCTCAG and CCGTCAATTCCTTTRAGTTT) described previously by Muyzer*et al*. [19]. After the collection of DNA sequencing reaction, the sequencing products were purified using the gel filtration method with Sephadex. After the purification process, the DNA sequencing process was started. This was performed on the ABI 3130XL device using the capillary electrophoresis method (Abdulhakeem, *et.al*., 2023).

2.7 Statistical analysis

Data were analyzed using the SPSS 25.0 version. The prevalence of the Turbidity, TPH was expressed in simple descriptive statistics such as mean and standard deviation. One-way analysis of variance (ANOVA) was used, where the level of significance was set at $P<0.05$ to compare the means of the Turbidity and TPH between the different Bacteria Isolates.

3.0. RESULTS AND DISCUSSION

3.1 Baseline physicochemicaland microbiologicalcharacteristics oftest sample (Diesel contaminated soil and Diesel).

The physicochemical parameters of the uncontaminated soil (US), diesel-contaminated soil (DCS) and diesel are presented in Table 1. The pH values of US were 9.31and DCS was 7.65. Both values were all alkaline, while that of the diesel (5.16) was acidic (Fig 1). This agreed with the work of Amajuoyi and Wemedo (2015). The total heterotrophic bacteria count (THB) and hydrocarbon utilizing bacteria count (HUB) counts recorded for US (2.65 $\times 10^5$ and 3.00$\times$ 10^3) CFU/g, DCS (2.55 $\times 10^5$ and 0.75$\times$ 10^3) CFU/g and Diesel HUB(3.50 $\times 10^3$) CFU/g respectively (Table1).The THB count in uncontaminated soil is higher than that of diesel contaminated soil, this is due to the fact that some microbes cannot degrade hydrocarbon present in polluted soils.

A gas chromatography-flame ionization detector (GC-FID) was employed to ascertain Total petroleum hydrocarbon (TPH) and Poly AliphaticHydrocarbon (PAH) concentrations. The TPH and PAH values of the samples are; US (<0.001mg/Kg and<0.001mg/Kg). DCS (209.03mg/Kg and 24.851mg/Kg), Diesel (22618.294mg/Kg and 1565.764mg/Kg) respectively(Table1). Diesel had the highest TPH and PAH, followed by DCS, the US had no TPH and PAH. As usual, the concentration of TPH and PAH in the diesel-contaminatedsoil (DCS) is higher than that of the uncontaminated soil (US) which had the lowest value as presented in Table 1. This is due to the high rate of hydrocarbon contamination which is toxic to the ecosystem. This is in line with the research study of Tudararo-Aherobo*et al*., (2017).

Table 1:Baseline physicochemicaland microbiologicalcharacteristics ofUncontaminated soil, Diesel contaminated soil and Diesel

S/n	Parameters	Uncontaminated soil	Diesel contaminated soil	Diesel
1	pH.	9.31	7.65	5.16
2	Electrical Conductivity (µS/m)	98	193	NIL
3	Salinity as Cl^- (mg/Kg)	15.81	34.49	NIL
4	Alkalinity (mg/Kg)	110.00	92.00	NIL
5	N03-N (mg/Kg)	0.477	0.088	NIL
6	Available Phosphorous (mg/Kg)	23.037	20.688	NIL
7	$SO4^{2-}$ (mg/Kg)	9.469	5.904	NIL
8	Chromium, (mg/Kg)	1.106	0.851	NIL
9	Cupper, (mg/Kg)	2.638	5.106	NIL
10	Nickel, (mg/Kg)	8.929	22.702	NIL
11	Lead, (mg/Kg)	13.862	31.629	NIL
12	Zinc, (mg/Kg)	19.999	106.463	NIL
13	PAH (mg/Kg)	<0.001	24.851	1567
14	TPH (mg/Kg)	<0.001	209.03	22618
15	THB (10×10^5 CFU/g)	2.65	2.55	NIL
16	THF (10×10^3 CFU/g)	4.00	4.70	NIL
17	HUB (10×10^3 CFU/g)	3.0	7.50	3.5
18	THB (10×10^2 CFU/g)	6.0	7.50	NIL

3.2 Screening of Bacteria isolated from diesel contaminated soil

Hydrocarbon degradative potential of the bacterial isolates screened showed some to have positive, weak and negative potentials (Table 2). Results obtained for the parameters monitored during the screening analysis(pH, Turbidity),on day 0, 5,10 and 15,are stated inFig. 1 and2respectivey) The four most efficient bacteria isolatesrecorded% Turbidityvalues as follows: SEO 1 (84%), SEO 2 (79%),SEO 3 (78%) and SEO 5 (76%)(Table 2).The four isolates also recorded the highest % TPH reduction as follows: SEO1(70%), SEO2(48%), SEO3(475) and SEO5(45%)(Table 2).The control setup had the lowest percentage increase in turbidity (6%) and lowest hydrocarbon percentage reduction(7%).Ataikiru*et. al.,(*2017) in their study confirmed that bacterial species are resistant to hydrocarbon content and were able to utilize them as a source of energy as observed in this study.

Table 2: Bacterial isolates screening result

S/N	SAMPLE ID	DEGRADATIVE ABILITY	%REDUCTION IN TPH	% INCREASE IN TURBIDITY
1	SEO 8	W	26%	15%
2	SEO 3	P	47%	78%
3	SEO 11	N	3%	4%
4	SEO 9	N	28%	11%
5	SEO 10	N	30%	15%
6	SEO 12	N	6%	19%
7	SEO 2	P	48%	79%
8	SEO 6	W	35%	30%
9	SEO 5	P	45%	76%
10	SEO 4	W	12%	16%
11	SEO 1	P	70%	84%
12	SEO 7	W	22%	26%
13	CONTROL	CONTROL	7%	6%

Legend:

P=positive degraderofhydrocarbon N= Negative degrader of hydrocarbon

W = weak degrader of hydrocarbon

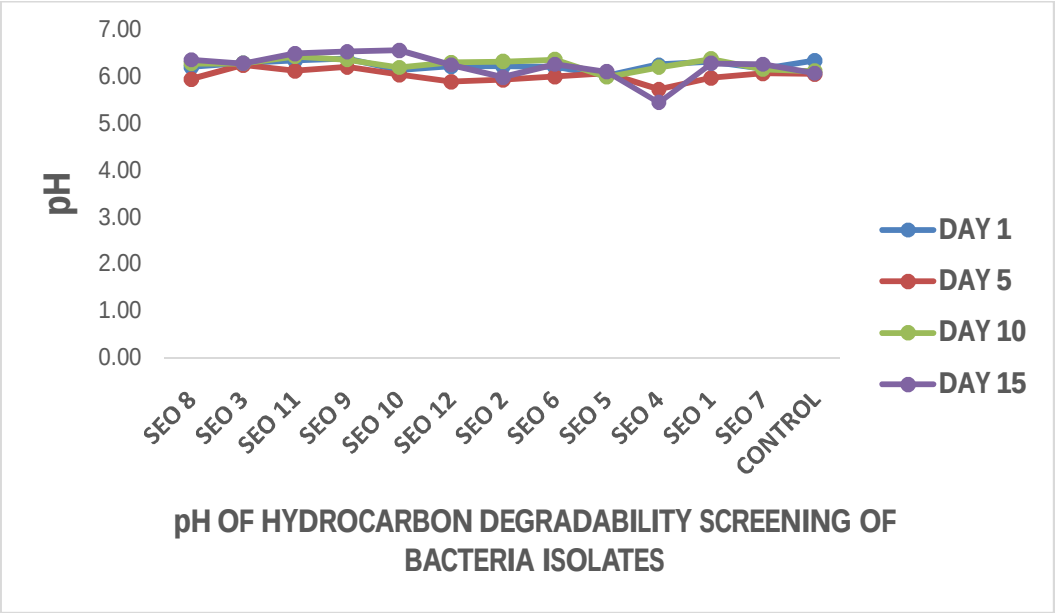

Fig. 1: pH of Bacterial cultures during screening for hydrocarbon degradation.

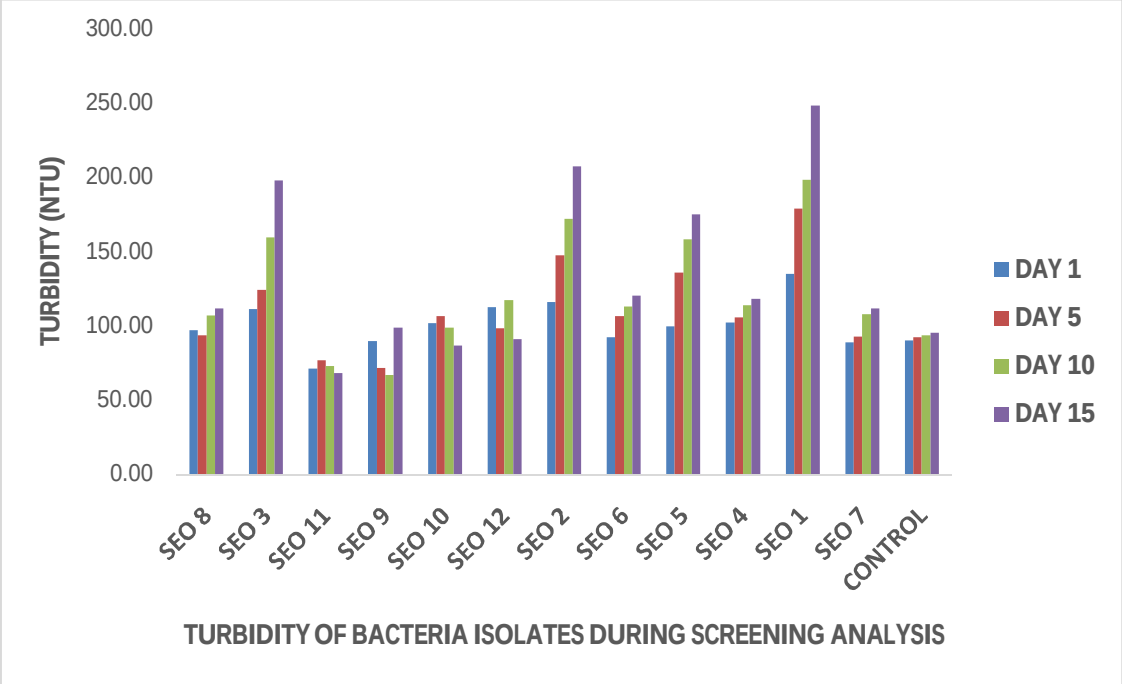

Fig. 2:Turbidity of Bacterial cultures during screening for hydrocarbon degradation.

3.3 Morphological, biochemical, and molecular identification of bacterial Isolates

The eight (8) most effective hydrocarbon degraders were taken for biochemical and molecular identification. Most were rods and coccobacilli in shape(Table 3). For Molecular characterization (Table 4), the 16S rRNA gene was amplified, and then identified by BLASTN study.The sequencesweredepositedinGenbankandaccessionnumberswereassignedfor each ofthem. FourBacteriaspecies wereusedinthisstudieswhich were identifiedasIsolate(SEO18)*Acinetobacterrudis*witha n 84.16% increaseinturbidityand70.34%reductioninTPH(Table 2), Isolate(SEO12)*Acinetobacterbereziniae*with 79.10%increaseinturbidityand48.30%reduction inTPH, Isolate(SEO3) *Pseudomonaskoreensis*with77.9%increaseinturbidityand47.21%reductioninTPHandIsol ate(SEO14)*Klebsiella aerogenes* with 75.57% increase in turbidity and 45.17% reduction in TPH(Table 2). Bacteria growth wasdetermined by turbidity, Isolate (SEO1) *Acinetobacterrudis* recorded the highest Turbidity value of 84% (Table.2),asaresult,itwasregardedasthemostefficienthydrocarbon degrader. This observation aligns with the investigation of Tudararo-Aherobo*et al.,* (2017)whichrecordedthat*Acinetobacters*pshowedthehighestpercentage of dieselbiodegradation (81.34%)forthe10%experimentalset-up.Subsequently,Dixit*et al.,* 2018 reported that bacteria were isolated from diesel-contaminated soil and identified as *Acinetobacter baumannii* by the 16S rRNA gene. However, according to Bekele *et al.* (2022), six diesel degraders were identified and belong to,*Achromobacter*, *Providencia*, *Roseomonas*, *Pseudomonas Stenotrophomonas*, and *Bacillus.* Consequently, the partial 16S rRNA gene sequences revealed that the two most potent bacterial strains (AAUW23 and AAUG11) were *Pseudomonas aeruginosa*, while AAUG36 was *Bacillus subtilis*.

Table 3: Morphological and Biochemical characteristics of the total hydrocarbon utilizing bacteria isolates

S/n	Isolates Codes	Morphological Characteristics	Gram's reaction	Catalase	Indole	Citrate	Oxidase	Glucose fermentation	Lactose Fermentation	Sucrose fermentation	Hydrogen sulphide Production	Nitrate Reduction	Methyl Red	Urease	Motility	Voges–Proskauer	Acid/Gas Production	Probable isolate
1	SEO 1	Coccobacilli	-	+	-	+	-	+	+	-	-	-	-	-	-	-	+	*Acinetobacter* species
2	SEO 4	Rods	-	+	-	+	-	+	+	+	-	+	-	+	+	+	+	*Klebsiella* species
3	SEO 3	Rods	-	+	-	+	+	+	-	-	-	-	-	+	+	-	+	*Pseudomonas species*
4	SEO 5	Rods	-	+	-	+	-	+	+	+	-	+	-	+	-	+	+	*Klebsiella* species
5	SEO 2	Coccobacilli	-	+	-	+	-	+	+	-	-	-	-	-	-	-	+	*Acinetobacter* species
6	SEO 7	Rods	-	+	-	+	-	+	+	+	-	+	-	+	+	+	+	*Klebsiella* species
7	SEO 6	Coccobacilli	-	+	-	+	-	+	+	-	-	-	-	-	-	-	+	*Acinetobacter* species
8	SEO 8	Rods	-	+	-	+	-	+	+	+	-	+	-	+	+	+	+	*Klebsiella* species

TABLE 4: Molecularidentificationofbacteria isolates

S/N	SAMPLE ID	BLAST PREDICTION	% RELATEDNESS	GENBANK ACCESSION NUMBER
I	SEO 1	*Acinetobacter rudis*	85.29%	M2712942.1
2	SEO 2	*Acinetobacter bereziniae*	87.20%	MT111619.1
3	SEO 3	*Pseudomonas koreensis*	74.04%	MW794239.1
4	SEO 4	*Klebsiella pneumoniae*	87.61%	KU321273.1
5	SEO 5	*Klebsiella aerogenes*	95.15%	CP070520.1
6	SEO 6	*Acinetobacter bereziniae*	84.82%	NR_117625.1
7	SEO 7	*Klebsiella pneumoniae*	94.20%	MW132403.1
8	SEO 8	*Klebsiella pneumoniae*	97.39%	CPO31795.1

CONCLUSION

Four viable bacteria withthe highest percentage increases in turbidity were isolated and identified from diesel-contaminated soil collected from a diesel mechanic workshop in Osubi, Delta State. Among the bacterial isolates, *Acinetobacter rudis* showed the

highest hydrocarbon degradation capacity, with an 84.16% increase in turbidity. This was followed by *Acinetobacter bereziniae*, which exhibited a 79.10% increase, *Pseudomonas koreensis* with a 77.9% increase, and *Klebsiella aerogenes* with a 75.57% increase. These hydrocarbon-degrading bacteria have the potential for degradation of hydrocarbons, hence could be used for the decontamination of petroleum-polluted sites.

Disclaimer (Artificial intelligence)

Option 1:

Author(s) hereby declare that NO generative AI technologies such as Large Language Models (ChatGPT, COPILOT, etc.) and text-to-image generators have been used during the writing or editing of this manuscript.

References

Abdulhakeem, M.A., Alreshidi, M., Bardakci, F., Hamadou, W.S., De Feo, V., Noumi, E., &
Snoussi, M. (2023). Molecular Identification of Bacteria Isolated from Marketed Sparus aurata and Penaeus indicus Sea Products: Antibiotic Resistance Profiling and Evaluation
of Biofilm Formation. Life, 13, 548. https://doi.org/10.3390/life13020548

Asuka, E., Hyginus, O., &Chinago, A. (2021) Crude Oil Polluted Niger Delta Environment:
Conservation Techniques for Survival. *Scholars Bulletin*, 7(9), 252-263.

Ataikiru, T.L., Okorhi-Damisa, B.F. &Akpaiboh, J.I. (2017). Microbial communitystructure of an oil polluted site in Effurun,Nigeria. *International Research Journal of Public and Environmentak Health*, 4(3), 41-47.

Bekele, G. K., Gebrie, S.A., Mekonen, E., Fida, T.T., Woldesemayat, A.A., Abda, E. M., Tafesse, M., &Assefal, F. (2022). Isolation and Characterization of Diesel-Degrading

Bacteria from Hydrocarbon-Contaminated Sites, Flower Farms, and Soda Lakes. *International Journal of Microbiology,* 2022, 12p

Harley, J.P, and Prescott, L.M. (2002. Laboratory Exercises in Microbiology. 5th ed. McGraw-
Hill Higher Education; 496 p.

Kebede, G., Tafese, T., Abda, E. M., Kamaraj, M., & Assefa, F. (2021). Review Article Factors
Influencing the Bacterial Bioremediation of Hydrocarbon Contaminants in the Soil: Mechanisms and Impacts. Journal of Chemistry, 2021: 17p

Katimu, Y., & Sani, Y. (2022). Biostimulation of Hydrocarbon-Utilizing Bacteria in Soil Amended with Spent Engine Oil Using Citrullus lanatus and Citrus sinensis Peels Agro-Wastes. *Nigerian Journal of Microbiology,* 36(1): - 5961 – 5970

Louca, S., Parfrey, L. W., &Doebeli, M. (2016). Decoupling function and taxonomy in the global ocean microbiome. *Science*, 353(6305), 1272–1277

Meena, T., Neelam, D.K., Gupta, V., Devki & Rahi, R. (2021). Bioremediation-An Overview International. *Journal of Recent Scientific Research*, 12 (03): 41125-41133

Muyzer, G., Teske, A., Wirsen, C.O. &Jannasch, H.W. (1995). Phylogenetic relationships of
Thiomicrospira species and their identification in deep-sea hydrothermal vent samples by denaturing gradient gel electrophoresis of 16S rDNA fragments. *Arch. Microbiol*. 64, 165–172

Shettima, H., Allamin, I. A., Halima, N., Ismail, H.Y., & Musa, Y. (2021). Isolation and Characterization of Hydrocarbon-degrading Bacteria in Soils of Mechanical Workshops in Maiduguri, Borno State. *Journal Of Environmental Bioremediation and Toxicology* (JEBAT), 4(2):35-38

Tudararo-Aherobo, L., &Mesogboriwon, S. (2020). Bioremediation of Spent Engine Oil
Contaminated Soils using Indigenous fungi species. *International Journal of Scientific Research in Science and Technology* (IJSRST), 7 (2): 2395-6011

Varjani, S., &Gnansounou, E. (2017). Microbial dynamics in petroleum oilfields and their relationship with physiological properties of petroleum oil reservoirs. *Bioresource Technology*, 245, 1258–1265.