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ANTIBIOTICS SUSCEPTIBILITY AND HYDROCARBON DEGRADATIVE ABILITY OF BACTERIA ISOLATED FROM DIESEL-CONTAMINATED SOIL

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ABSTRACT

Background: The most commonly used energy resources in the world are the hydrocarbons, which are major sources of environmental pollution.
Objectives: This study determined both antibiotics susceptibility pattern and hydrocarbon-degrading capability of bacterial isolates obtained from soil samples contaminated with used diesel.
Methods: Serial dilution technique was used to isolate bacterial samples from diesel-contaminated soil samples. Obtained isolates were characterized and identified using appropriate microbiological techniques and were subjected to antimicrobial susceptibility test using disc diffusion assay. The isolates were screened for their ability to utilize hydrocarbon products by streaking pure culture of the isolates on Bushnell Haas agar (BHA) incorporated with 0.5% crude oil. Isolates that showed visible growth within three days were used for degradation studies on hydrocarbon products. Degradation of hydrocarbon products was carried out for 21 days at room temperature while monitoring growth and degradation with appropriate techniques.
Results: Forty-one bacterial isolates were obtained from the diesel-contaminated soils, four of which showed visible growth when screened on BHA and these were identified as *Pseudomonas aeruginosa, Bacillus subtilis, Micrococcus luteus, and Arthrobacter sp.* All the isolates were resistant to Cefriaxome while all the Gram positive isolates were susceptible to Ciprofloxacin. The isolates utilized crude oil at different rates, with *Pseudomonas aeruginosa* having the highest growth and degradation rate followed by *Bacillus* sp. while *Micrococcus* sp. and *Arthrobacter* sp. showed moderate growth and degradation rate.
Conclusion: *Pseudomonas aeruginosa* obtained from diesel-contaminated soil sample in this study has shown the ability to degrade hydrocarbon products and is a promising candidate for remediating hydrocarbon-contaminated sites.

Keywords: Bushnell Haas medium, hydrocarbon degradation, gravimetric analysis, spectrophotometry, antibiotics susceptibility

INTRODUCTION

The increase in population and development worldwide has led to an increased dependence and usage of petroleum as a major source of energy. Human activities such as agriculture, and industrialization are major contributors to the spillage of hydrocarbons (Adipah, 2018). Hydrocarbons are the most simple organic compounds that contain only carbon (C) and hydrogen (H) (Elkelawy & Eldin, 2018). Hydrocarbons are major constituents of crude oil and petroleum. These hydrocarbons, especially petroleum hydrocarbons, have become a serious threat as they contribute to soil contamination, making it a prevalent issue around the world. Among the environmental elements connected to petroleum products that put human health at risk are spills and leaks. Petroleum hydrocarbons, polycyclic aromatic hydrocarbons (PAHs), pesticides, solvents, lead, and other heavy metals are among the chemicals involved (USEPA, 2012). Sources of soil contamination can be from diffused sources (mobilization and transportation by floodwaters) or point sources (households and industry), and these sources may have long-term effects on the quality of soils, human health, and quality of food

(DEFRA, 2009). The increase in soil contamination by petroleum hydrocarbons calls for drastic measures to remediate contaminated soils. Soil remediation techniques encompass mechanical, burying, evaporation, dispersion, and washing methods. However, these technologies can be costly and result in incomplete decomposition of contaminants (Das & Chandran, 2011). Therefore, the adoption of natural methods such as the use of hydrocarbon-degrading bacteria is necessary.

The metabolic capacity of microorganisms to change or mineralize organic pollutants into less dangerous, non-hazardous compounds that are subsequently incorporated into organic matter's natural biogeochemical cycles is known as biodegradation. Numerous complicated parameters, including nutrients, oxygen, pH level, composition, concentration, and bioavailability of the pollutants, as well as the chemical and physical properties of the soil, affect the biodegradability of petroleum hydrocarbons in soil. There is a generous distribution of hydrocarbon-degrading bacteria in aquatic and soil habitats. It is believed that the presence of several oil-degrading microorganisms in an oil-polluted environment indicates that these microbes are aggressive degraders of the surrounding environment (Okerentugba & Ezeronye, 2003). Several variables influence how quickly bacteria in soil and aquatic environments break down hydrocarbons through biodegradation, including the absence of vital minerals like calcium, phosphorus, and nitrogen (Ekanem & Ogunjobi, 2017).

A diverse community of microorganisms exhibits the degradative and utilization abilities of hydrocarbons (Leahy & Colwell, 1990). It has been documented that more than 75 bacterial species break down oil and other hydrocarbons in a variety of environmental conditions and some of these microbial species can be recovered from the soil (Ayotamuno *et al*., 2006; Adoki, 2007; Prince, 2010; Ae *et al*., 2013). These microorganisms will degrade a wide variety of desired constituents present in hydrocarbon-contaminated soil (Aguilar *et al*., 2018). Hydrocarbon-degrading bacteria include strains of *Acinetobacter* spp, *Alcaligenes* spp , *Bacillus* spp, *Corynebacterium* spp, *Capnocytophage* spp, *Flavobacter* spp,*Pseudomonas*, *Yokenella* spp, , *Rosemonas* spp, *Stenotrophomonas* spp, *Streptococcus* spp, *Povidencia* spp, *Sphingobacterium* spp and *Moraxwlla* spp (Kazem & Hussein, 2021). Aliphatic saturated hydrocarbons and aliphatic unsaturated hydrocarbons, cyclic hydrocarbons are amongst the hydrocarbons that bacteria can degrade through either anaerobic or aerobic processes (Das & Chandran, 2011).

The antibiotic susceptibility of the bacterial isolates must be determined to determine the role of these microorganisms in the breakdown of hydrocarbon-contaminated soils. This lessens the likelihood that genes for antibiotic resistance will proliferate in such an environment (Tega, 2020). The past several decades have seen an increase in bacterial strains resistant to antibiotics that produce illnesses that are hard to cure and occasionally deadly (Calero-Cáceres *et al*., 2014). Antibiotics and the factors that cause resistance to them are actually, a natural occurrence that has long existed in the ecosystem (Foster & Grundmann, 2006). Many reservoirs of resistance determinants, such as resistance genes and the mobile genetic elements that serve as their vectors, may be found in naturally in the ecosystem. Although antibiotic resistance has caught the interest of the medical, environmental, and general populations, its impact on health and economic activities remains largely unidentified (Tega, 2020). This study was therefore aimed at determining the antibiotics susceptibility pattern and hydrocarbon degrading ability of bacterial species isolated from soil samples contaminated with used diesel.

Materials and Methods

Sample collection

Diesel-contaminated soil samples were collected from four locations within Ado metropolis; two samples were collected around two generator houses (SGHA and SGHB) in Afe Babalola University and two samples were collected in a tipper repair garage (SMWA) and an auto-mechanic workshop (SMWB) respectively. Agricultural soil with no prior exposure to diesel contamination was collected from Afe Babalola University and this served as the control.

Physicochemical analysis of soil sample

Diesel-contaminated soil samples were analysed for texture, total petroleum hydrocarbon, moisture content, and pH.

A pH meter (Crison micro pH 2000 model) was used to measure the pH. For every soil sample, 2.5g was suspended in 20 milliliters of distilled water and well mixed. A phosphate buffer solution was used to calibrate the pH meter before the pH was measured twice (Anuoluwa and Ogunjobi, 2020). Gravimetric technique was employed in determining the total petroleum hydrocarbon as described by Villalobos *et al.* (2008).

Isolation of Bacterial isolates

Determination of total viable bacteria in the contaminated soil samples was carried out by adapting the pour plate methods described by Olutiola *et al*. (2000) and Anuoluwa and Ogunjobi (2020) while adjusting the incubation temperature to 37 ^{o}C. The streaking technique was used to subculture morphologically distinct bacterial colonies till pure cultures were obtained. The isolates were subjected to different biochemical tests such as oxidase test, catalase test, motility test, MR-VP test, starch hydrolysis and ability to ferment various sugars etc to aid their identification. These were stored in agar slants and kept at 4 ^{o}C for further studies.

Screening of Bacterial isolates for ability to utilize hydrocarbon

Obtained bacterial isolates were screened for their hydrocarbon degrading ability by subculturing them on Bushnell Haas agar ((M349) by HIMEDIA) which was prepared and sterilized according to manufacturers. It was allowed to cool to 40-45 ^{o}C and incorporated with 0.5% of crude oil as described by Osuji *et al.* (2023). The plates were incubated at 37 ^{o}C. The isolates that showed visible growth within three days were used for further degradation studies.

Antimicrobial Susceptibility Testing

Using the Kirby-Bauer disk diffusion test on Muller-Hinton agar (Oxoid CM0337 Basingstoke, England) technique, an antibiotic susceptibility test was performed on the isolates obtained from the samples following the Clinical and Laboratory Standards Institute (CSLI) as described by Osibote *et al.* (2017) and compared to the standard ranges by CSLI (CSLI, 2020).

Degradation of Petroleum products

Preparation of minimal salt broth (MSB)

Minimal salt broth used for the degradation exercise was prepared following the methods described by Balogun *et al.* (2013) and Mohammed *et al* (2023) with slight modifications. The broth was prepared by weighing the following elements (g/L): KH_2PO_4 1.0; $MgSO4.7H_2O$ 1.2; NaCl 0.1; $Cacl_2$ 0.2; KNO_3 0.5; $FeCl_3$ 0.1; Na_2HPO_4 1.25 and $NaNO_3$ 0.32. Trace elements solution contained (mg/L): H_3BO_3 0.1; $ZnSO_4.7H_2O$ 0.1; $CuSO_4.5H_2O$ 0.05 and $MnSO_4.H_2O$ 0.04 and the pH was adjusted to 6.5 and 50ml was dispensed into each Erlenmeyer flask and autoclaved at 121^{o}C for 15mins.

Degradation exercise

Bacterial isolates which showed a positive tendency in their ability to degrade crude oil when grown on BHA were grown overnight at 37 ^{o}C in peptone water. Each conical flask containing sterilized MSB was inoculated with 1 ml of the bacterial isolates and this was supplemented with sterilized 0.5 ml of each hydrocarbon product (petroleum, kerosene, engine oil, and diesel). This was incubated at room temperature for 21 days and growth of the bacterial isolates in the medium was monitored every seven days by measuring the absorbance using UV/Visible Spectrophotometer (UNICO Model 1200) at 660 nm while the degradation of the hydrocarbon products was monitored using gravimetric analysis, following the method described by Almutairi (2022) in which the hydrocarbon product was extracted after degradation, using an acetone: hexane (1:1 v/v) mixture. An uninoculated minimal salt medium served as the control.

Results

The contaminated soil samples collected were observed to be a bit dark in colour and have an oily appearance in addition to the characteristic smell of diesel. The result of the physicochemical analysis is shown in Table 1. The total bacteria counts (TBC) of the soil samples were 7.6×10^4; 3.6×10^4; 5.8×10^4 and 1.5×10^4 cfu/g from SGHA, SGHB, SMWA, and SMWB respectively. Forty-one bacterial isolates were obtained from the diesel-contaminated soils and they were observed to belong to seven different genera namely: *Pseudomonas aeruginosa* (8)*, Bacillus sp* (11)*, Micrococcus luteus* (4)*, Proteus mirabilis* (6)*, Arthrobacter* sp (5)*, Escherichia coli* (3) and *Alcaligenes faecalis* (4). Twenty (48.78%) of the isolates were Gram positive while the remaining twenty-one (51.22%) were

Gram negative. Figure 1 shows the frequency of occurrence of the bacterial isolates. The screening of the isolates on BHA revealed that only four of the forty-one isolates showed a positive tendency for utilizing hydrocarbon. The isolates were identified as *Bacillus subtilis, Micrococcus luteus, Arthrobacter* sp and *Pseudomonas aeruginosa.*

Antibiotic susceptibility testing revealed that most of the Gram negative bacterial isolates were resistant to most of the antibiotics with the highest resistance observed in Cefriaxome (CTX) (100%) followed by Ofloxacin (OFX) (95.24%) and then Amoxycillin/Clavulanate (AUG) (90.48%). The least resisted antibiotics among (ERY) (19.05%) followed by Ceftazidime (CAZ) (23.61) as shown in Table 2.

Table 3 shows the result for the antibiotic susceptibility test of the Gram positive isolates. It was observed that the isolates were all resistant to Cefuroxime and Cefriaxome (100%) while showing 70% and 60% resistance to Nitrofurantoin and Ofloxacin respectively. The least resisted antibiotics was Gentamicin (30%). All the isolates showed 100% susceptibility to Ciprofloxacin while showing the least susceptibility to Amoxycillin/Clavulanate (25%) as shown in Table 2.

Table 4 shows the spectrophotometric reading used in monitoring the growth of bacterial isolates in the MSM used for the degradation exercise. It was observed that all the isolates grew better in the MSM which was supplemented with diesel though *Pseudomonas aeruginosa* had the highest growth indices at the end of 21 days. It was observed that on day 21 which marked the end of the degradation exercise, *Pseudomonas aeruginosa* was able to degrade all the hydrocarbon products achieving a percentage degradation of 85, 78, 67, and 80 in Kerosene, Diesel, Engine oil, and Petroleum respectively. The lowest percentages of degradation on the hydrocarbon products as shown in Figure 2 were observed in *Arthrobacter* sp which had a percentage degradation of 35 and 45 on Kerosine and Petroleum respectively and *Micrococcus luteus* which had a percentage degradation of 30 and 44 on Diesel and Engine oil respectively.

Discussion

The bacterial count obtained in this study was within the limits that have been reported by earlier investigations (Obire and Nwaubeta, 2002). In this study, the bacterial species isolated from the diesel-contaminated soil samples were *Alcaligenes faecalis, Arthrobacter* sp, *Bacillus* sp*, Escherichia coli, Pseudomonas aeruginosa, Micrococcus luteus* and *Proteus mirabilis,* this supports the findings of Tesar *et al*. (2002) who observed that a wide variety of bacteria across phylogenetic ranges, including species and strains of *Norcadia, Arthrobacter, Achromobacter, Acidovorax, Alcaligenes, Bacillus, Corynebacterium, Flavobacterium, Mycobacterium, Micrococcus, Pseudomonas, Sphinogomonas, Rhodococcus* and *Xanthomonas* have been identified in the breakdown of hydrocarbons. Subathra *et al.* (2013) revealed that bacterial isolates involved in hydrocarbon degradation in decreasing order of prevalence belong to the genera *Pseudomonas, Achromobacter, Flavobacterium, Nocardia, Arthrobacter, Bacillus, Micrococcus and Acinetobacter.* Four bacterial isolates namely *Pseudomonas aeruginosa, Arthrobacter* sp, *Micrococcus luteus,* and *Bacillus subtilis* exhibited a high tendency for utilizing hydrocarbon as a carbon source when screened using BHA supplemented with crude oil. This is similar to previous observations by Balogun *et al.* (2013) and Osuji *et al.* (2023). The majority of the bacterial isolates obtained in this study were observed to be resistant to most of the conventional antibiotics tested against them. This is of great importance to public health. Multidrug resistance microorganism is a threat to public health and having a vast number of such organisms in the soil environment may be a cause for concern as it can encourage the proliferation of drug resistance even among clinical isolates through gene transfer in the environment. This is in line with the findings of Vemuri *et al.* (2014) and Ughala and Osaro- Matthew (2023) in which they observed diesel and engine oil-contaminated soil samples harbour antibiotic-resistant bacterial isolates. The spectrophotometric result obtained for the growth monitoring of the bacterial isolates used in the degradation exercise showed that they were all able to grow under the experimental conditions. However, the growth does not directly correspond to the percentage degradation ability of the isolates in agreement with the findings of Al-Kaabi *et al.* (2020). This could be a result of the different metabolic pathways used by isolates. *Pseudomonas aeruginosa* was observed to achieve the highest percentage degradation on all the hydrocarbon products; this agrees

Table 1: Physicochemical analysis of contaminated soil samples

Parameters	SGHA	SGHB	SMWA	SMWB
TPH (%)	2.3	1.92	0.56	0.46
Moisture Content (%)	2.26	1.83	1.57	0.62
pH	7.16	7.05	7.10	7.18
Texture	Sandy loamy	Sandy loamy	Sandy loamy	Sandy loamy

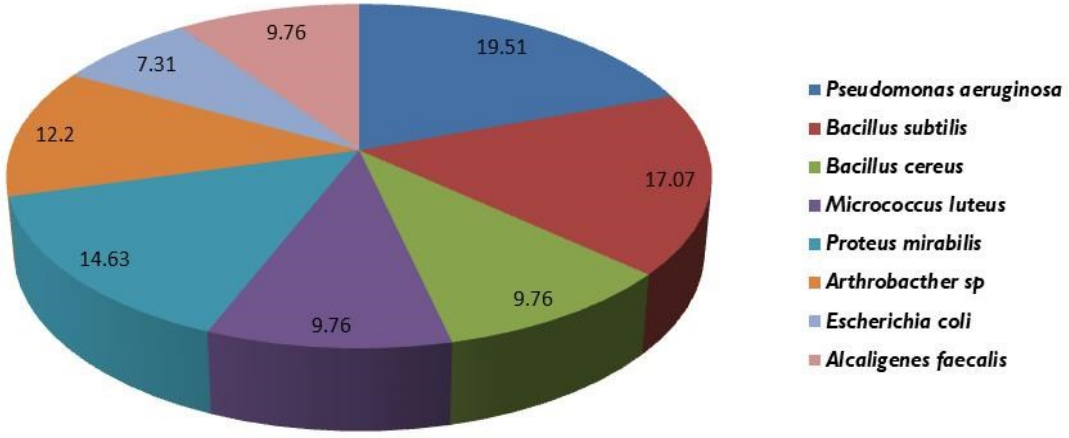

Figure 1: Frequency of occurrence of bacteria isolated from diesel-contaminated soil

Table 2: Antibiotic susceptibility of Gram negative isolates

S/N	ORGANISM	Antibiotics discs used							
		GEN (10µg)	CAZ (30µg)	CRX (30µg)	CTR (30µg)	ERY (5µg)	CXC (5µg)	OFL (5µg)	AUG (30µg)
1	*Alcaligenes faecalis* (SGHAI)	S	S	R	R	S	R	R	R
2	*E. coli* (SMWA1)	S	S	R	R	S	R	R	S
3	*Proteus mirabilis* (SGHA3)	R	S	R	R	S	R	R	R
4	*Pseudomonas aeruginosa* (SGHB2)	S	S	R	R	S	S	R	R
5	*E. coli* (SMWA4)	S	S	R	R	R	R	R	S
6	*Proteus mirabilis* (SMWB2)	R	R	R	R	S	R	R	R
7	*Alcaligenes faecalis* (SMWB3)	S	R	R	R	S	R	R	R
8	*Proteus mirabilis* (SGHB5)	R	S	S	R	S	R	R	R
9	*Alcaligenes faecalis* (SMWB5)	S	R	S	R	S	R	R	R
10	*E. coli* (SMWB6)	S	S	R	R	S	R	R	R
11	*Pseudomonas aeruginosa* (SMWA6)	S	R	R	R	S	S	R	R
12	*Proteus mirabilis* (SGHA4)	R	S	R	R	R	R	R	R
13	*Pseudomonas aeruginosa* (SMWB9)	R	S	S	R	S	S	R	R
14	*Alcaligenes faecalis* (SMWA7)	S	S	S	R	S	R	R	R
15	*Pseudomonas aeruginosa* (SGHB6)	S	S	R	R	S	S	R	R
16	*Proteus mirabilis* (SMWB10)	R	S	R	R	S	R	R	R
17	*Pseudomonas aeruginosa* (SGHA6)	S	S	R	R	R	S	R	R
18	*Pseudomonas aeruginosa* (SGHB7)	S	R	R	R	S	S	R	R
19	*Proteus mirabilis* (SGHB10)	R	S	R	R	S	R	S	R
20	*Pseudomonas aeruginosa* (SMWA9)	S	S	R	R	R	S	R	R
21	*Proteus mirabilis* (SGHA11)	S	S	R	R	S	R	R	R
% Susceptibility		66.7	76.19	19.08	0	80.95	33.33	4.76	9.52
% Resistance		33.3	23.81	80.92	100	19.05	66.7	95.24	90.48

Key: GEN –Gentamicin, CAZ- Ceftazidime, CRX- Cefuroxime, CTR- Cefriaxone, ERY- Erythromycin, CXC- Cloxacillin, OFL- Ofloxacin and AUG- Amoxycillin/Clavulanate
R- Resistant, I- Intermediate, S- Susceptible

Table 3: Antibiotic susceptibility of Gram positive isolate

S/N	ORGANISM	Antibiotics discs used							
		GEN (10µg)	CAZ (30µg)	CRX (30µg)	CTR (30µg)	CIP (5µg)	NIT (200 µg)	OFL (5µg)	AUG (30µg)
1	*Bacillus subtilis (*SMWA2*)*	S	R	R	R	S	R	S	R
2	*Bacillus cereus (*SGHA2)	S	S	R	R	S	R	R	S
3	*Micrococcus luteus (*SMWA3*)*	R	S	R	R	S	R	R	R
4	*Arthrobacter sp (*SMWB1*)*	S	S	R	R	S	S	R	R
5	*Bacillus cereus (*SGHA5*)*	R	S	R	R	S	R	R	S
6	*Bacillus subtilis (*SGHB1*)*	S	S	R	R	S	R	S	R
7	*Bacillus subtilis (*SMWB4*)*	S	R	R	R	S	R	S	R
8	*Micrococcus luteus (*SGHA7*)*	R	S	R	R	S	S	R	R
9	*Bacillus cereus (*SGHB3*)*	S	R	R	R	S	R	R	S
10	*Arthrobacter sp (*SMWA5*)*	R	S	R	R	S	S	R	R
11	*Bacillus subtilis (*SGHA8*)*	S	R	R	R	S	R	S	R
12	*Arthrobacter sp (*SMWB7*)*	S	R	R	R	S	S	R	R
13	*Micrococcus luteus (*SGHB4*)*	R	S	R	R	S	S	R	R
14	*Bacillus subtilis (*SGHB8)	S	S	R	R	S	R	S	R
15	*Micrococcus luteus (*SGHA9)	R	S	R	R	S	R	R	S
16	*Bacillus cereus (*SMWB8*)*	S	S	R	R	S	R	S	S
17	*Bacillus subtilis (*SGHB10*)*	S	R	R	R	S	R	S	R
18	*Arthrobacter sp (*SGHA10)	S	S	R	R	S	R	R	R
19	*Arthrobacter sp (*SMWA8)	S	R	R	R	S	S	R	R
20	*Bacillus subtilis (*SMWB11)	S	R	R	R	S	R	S	R
% Susceptibility		70	60	0	0	100	30	40	25
% Resistance		30	40	100	100	0	70	60	75

Key: GEN –Gentamicin, CAZ- Ceftazidime, CRX- Cefuroxime, CTR- Cefriaxome, CIP- Ciprofloxacin, NIT- Nitrofurantoin, OFL- Ofloxacin and AUG- Amoxycillin/Clavulanate; R- Resistant, I- Intermediate, S- Susceptible

Table 4: Spectrophotometric measurement of bacterial growth in MSM used for biodegradation of hydrocarbon product

ORGANISM	DAY 7				DAY 14				DAY 21			
	PETROLEUM	ENGINE OIL	KEROSINE	DIESEL	PETROLEUM	ENGINE OIL	KEROSINE	DIESEL	PETROLEUM	ENGINE OIL	KEROSINE	DIESEL
Pseudomonas aeruginosa	0.04± 0.01^{a}	0.13± 0.08^{c}	0.14± 0.03^{d}	0.24± 0.04^{f}	0.12± 0.06^{h}	0.12± 0.04^{i,j}	0.22± 0.27^{k}	0.60± 0.04^{l}	0.13± 0.06^{n,o}	0.15± 0.03^{p}	0.62± 0.03^{q}	1.25± 0.10^{s}
Bacillus subtilis	0.07± 0.02^{a}	0.62± 0.48^{b}	0.11± 0.01^{d,e}	0.14± 0.02^{f}	0.08± 0.00^{h}	0.11± 0.01^{i,j}	0.14± 0.03^{k}	0.24± 0.04^{m}	0.07± 0.00^{o}	0.24± 0.05^{p}	0.15± 0.04^{r}	0.73± 0.28^{t}
Micrococcus luteus	0.06± 0.00^{a}	0.02± 0.00^{c}	0.09± 0.01^{e}	0.35± 0.42^{f}	0.48± 0.36^{g}	0.06± 0.05^{j}	0.13± 0.03^{k}	0.21± 0.15^{m}	0.36± 0.25^{n}	0.11± 0.07^{p}	0.15± 0.04^{r}	0.74± 0.26^{t}
Arthrobacter sp	0.04± 0.03^{a}	0.15± 0.01^{c}	0.09± 0.00^{e}	0.15± 0.04^{f}	0.05± 0.03^{h}	0.18± 0.02^{i}	0.19± 0.03^{k}	0.19± 0.03^{m}	0.08± 0.02^{o}	0.15± 0.10^{p}	0.25± 0.09^{r}	0.73± 0.28^{t}
Control	0	0	0	0	0	0	0	0	0	0	0	0

***values with the same letters on each column are not significantly different from each other

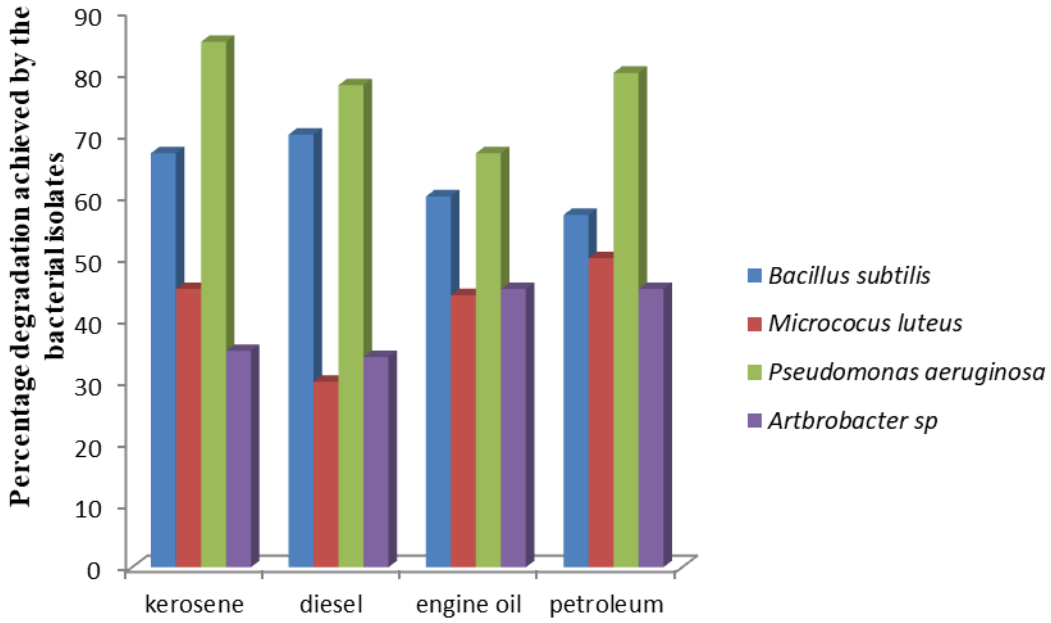

Figure 2: Degradation of various hydrocarbon products by bacterial isolates at day 21

with the result obtained in some earlier studies (Vignesh *et al*., 2016; Kumar and Manjunatha, 2017; Veerapagu *et al* 2019). In agreement with Veerapagu *et al* (2019), previous exposure of bacterial isolates to hydrocarbon contamination can play a pivotal role in their ability to degrade hydrocarbon.

Conclusion

Pseudomonas aeruginosa obtained from diesel-contaminated soil sample in this study has shown the ability to degrade hydrocarbon products such as kerosene, diesel, engine oil, and petroleum under experimental conditions. This isolate shows great promise as an environmentally acceptable and economically viable remediation solution for hydrocarbon-contaminated sites. It is recommended that further studies focusing on the genes responsible for this ability be carried out and also studies focusing on the various pathways employed by the organism in degrading the hydrocarbon products be encouraged.

Conflict of interest: The authors declare that there is no conflict of interest.

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