INTERNATIONAL JOURNAL OF ENVIRONMENT
Volume-9, Issue-2, 2019/20 ISSN 2091-2854
Received: 13 February 2020 *Revised: 19 July 2020* *Accepted: 6 October 2020*

ASSESSMENT OF CRUDE OIL DEGRADATION BY MIXED CULTURE OF *Bacillus subtilis* and *Pseudomonas aeruginosa* AT DIFFERENT CONCENTRATIONS

[1]*Faggo, A.A.; [2]Kawo, A.H.; [3]Gulumbe, B.H. and [4]Ijah, U.J.J.
[1]Bauchi State University, Faculty of Science, Department of Microbiology, Bauchi, Nigeria
[2]Bayero University, Faculty of Life Science, Department of Microbiology, Kano, Nigeria
[3]Federal University Birnin Kebbi, Faculty of Science, Department of Microbiology, Nigeria
[4]Federal University of Technology Minna, Department of Microbiology, Nigeria
*Corresponding author: abdullahiadamufaggo@gmail.com

Abstract

Petroleum hydrocarbon (PHCs) contamination of soil, freshwater and air is of global concern. The aim of this study was to assess the extent of crude oil degradation by mixed bacterial culture of different crude oil concentrations using gas chromatography-mass spectrometry (GC-MS). Seven oil samples were collected from petroleum-contaminated fields in Kano state, Nigeria, and screened for crude oil utilizing bacteria. A control sample of soil from an ecological garden (control soil) was also analyzed. Crude oil-degrading bacteria were isolated, enumerated and identified using cultural, morphological and biochemical characteristics, and screened for their ability to utilize Bonny Light Oil as a source of carbon and energy. Bacteria with the highest potential to utilize crude oil were selected and subjected to bioremediation studies at three different pollution levels (5%, 10% and 15%) for 56 days. The residual crude oil was assessed using GC-MS. The results revealed that the mixed culture completely degraded eighteen components ranging from C10 to C25 at 5% crude oil concentration while only C8 to C11 and C8 to C9 were degraded at 10 and 15% respectively. The results of this study indicated the potential of *B. subtilis* and *P. aeruginosa* in bioremediation of crude oil contaminated soil.

Keywords: Biodegradation, crude oil degrading bacteria, concentrations, GC-MS.

DOI: http://dx.doi.org/10.3126/ije.v9i2.32511

1. Introduction

Petroleum hydrocarbons (PHCs) contamination of the soil, freshwater and air is of global concern and has attracted public attention due to many aliphatic and polycyclic aromatic hydrocarbons being toxic, mutagenic and carcinogenic (Bao et al., 2012). Even though a number of physical, chemical and biological methods exist for the remediation of contaminants (Hu et al., 2010), bioremediation (biological approach) is considered the best strategy, as it is more efficient, eco-friendly and cost-effective than other methods (Ismail et al., 2013).

The role of microbial activity in the biodegradation of hydrocarbons has been well recognized for more than a century (Ojo, 2006). The indigenous bacterial populations in crude oil contaminated soil have the ability to degrade hydrocarbons (Head et al., 2006). The crude oil degrading bacterial genera; *Pseudomonas, Acinetobacter, Burkholderia, Paraburkholderia*, *Luteibacter* and *Flavobacterium* have been reported to play a significant role in the degradation of petroleum hydrocarbons (Cui *et al*., 2020). Bioremediation of a complex hydrocarbon mixture usually necessitates the cooperation of more than a single species, because an individual microorganism can generally metabolize only a limited range of hydrocarbon substrates. Therefore, conglomerations of mixed populations, equipped with broad enzymatic capacities, are required to increase the rate and extent of petroleum biodegradation further (Patowary *et al*., 2017). Another factor that influences the biodegradation of crude oil is the crude oil concentration, which is underreported. Petroleum hydrocarbon concentrations affect microbial activity directly. Consequently, if the concentrations are too high, the hydrocarbons may have deleterious effects on the bacteria in the soil. The degradation of crude oil decreased with increasing concentration, perhaps due to the toxicity of the crude oil in the medium (Farag et al., 2018). Therefore, this study aimed at assessing the extent of crude oil degradation by the mixed bacterial culture at different crude oil concentrations using gas chromatography-mass spectrometry (GC-MS).

2. Materials and Methods

2.1 Collection of soil samples

Soil samples were collected from seven different locations (B, C, D, E, F, G and H) at the depth of 10cm using a ditch auger, from petroleum-contaminated area of the Nigerian National Petroleum Corporation (NNPC), depot in Hotoro, Kano State, North-Western Nigeria. Samples were stored in dark, clean polythene bags and kept in a flask containing ice. Similarly, a control (non-contaminated) soil sample was collected from the ecological garden of the Department of Biological Sciences, Bayero University, Kano (location A) and transported to the Microbiology Laboratory, Department of Microbiology of the same institution for further analysis in accordance with the method of Romanus et al. (2015). All samples were collected in triplicates for seven days at 9:00 am when the weather was conducive (Kulkarni, 2014; Kawo and Kabiru, 2016).

2.2 Isolation and identification of crude oil degrading bacteria

The isolation of crude oil-degrading bacteria was carried out using the method described by Patowry *etal.* (2017). One gram of each hydrocarbon contaminated soil sample was inoculated into mineral salt medium (MSM) containing 2% (v/v) Bonny Light Oil as carbon source for enrichment. The conical flasks were then incubated at 35°C at 150 rpm for a week. Subsequently, 1ml inoculum was introduced again to 100 ml of fresh Bushnell Hass medium and incubated similarly under the same conditions for another week to reduce the number of unwanted microbes. The following week, 1ml of the original grown culture was used for serial dilution. 100 µl from the 10^{-6} diluted sample was then spread onto nutrient agar plates which were incubated at 35°C for 24 hours.

Mixed bacterial colonies from the overnight culture were also selected and grown on nutrient agar plates to obtain a pure culture. The pure isolates were stored on nutrient agar slants at 4°C for further studies. The samples were characterized using cultural descriptors (shape, color, odor, pigmentation) (Chessbrough, 2006), in addition to morphological (Gram's reaction) (Chessbrough, 2006) and biochemical test (indole, catalase, coagulase, Methyl Red/Voges-Proskauer, spore stain, motitlity, oxidase and citrate) tests carried out using standard microbiological methods (Chessbrough, 2006).

2.3 Screening of crude-degrading bacteria for their potential to utilize Bonny Light Oil

The crude oil-degrading ability of the isolates was measured using turbidity as described by Okpokwasili and Okorie (1988). Eleven bacterial isolates,i.e., *Bacillus subtilis* (3 isolates), *Bacillus cereus* (2 isolates), *Micrococcus leteus* (2 isolates), *Pseudomonas aeruginosa* (2 isolates) and *Staphylococcus aureus* (2 isolates) were grown separately in nutrient broth and incubated at 37°C for 24 hours. Following this, 1ml (1.5×10^8 cfu/ml) of the nutrient broth-grown culture of each isolate was introduced into seperate test tubes containing 5ml of crude oil broth (1.7g K_2HPO_4, 1.32g KH_2PO_4, 1.26g NH_4Cl, 0.01g $MgCl_2$.6H2O, 0.02g $CaCl_2$, 100ml distilled water and 1ml of filtered sterilized crude oil). The inoculated tubes were incubated at 37^{o}C for 7 days. After the incubation, the extent of oil utilization by the isolates was assessed by visual observation of the turbidity of the medium. The extent of bacterial growth was represented as either maximal growth (+++), moderate growth (++), minimal growth (+) or no growth (-).

Standardization of the Inoculum

McFarland standard turbidity solution was prepared according to the method described by Andrew (2009) and used for standardization of bacterial inoculum. 0.6 ml of 1% barium chloride solution was added to 99.4ml of 1% sulfuric acid solution and thoroughly mixed before being kept for inoculums' standardization. Eleven bacterial isolates were taken using a sterile wire loop and transferred into sterile physiological saline (0.85% w/v NaCl) and shaken to obtain a uniform suspension. The turbidity of the resulting suspension was then matched with that of the McFarland standard to achieve 1.5×10^8 cfu/ml of bacterial suspension of each isolate

2.4 Biodegradation Studies

The bacterial isolates that have shown highest degradation potential in screening, i.e., *Bacillus subtilis* B004 and *Pseudomonas aeruginosa* B008, were chosen for this study. Residues from the soil were removed and the soil was sieved using 2mm mesh. Two kilograms of soil were placed in plastic containers and treated with 5%, 10% and 15% of sterile Bonny Light Oil and mixed with *Bacillus subtilis* and *Pseudomonas aeruginosa* cultures. No bacterial cultures were added to the control soil; only the sterile crude oil. Following inoculation, the experiments were set up in triplicate and incubated at 35°C for eight weeks. Residual crude oil from both the control and test soil samples were analyzed using GC-MS for assessment of biodegradation and thus comparison between samples. The residual crude oil was extracted from soil samples using n-hexane using with the Soxhlet extraction method (Deepika and Rajalakshmi, 2013) and analyzed by GC-MS (Riskuwa-Shehu and Ijah, 2016).

2.5 Gas chromatography-mass spectrometry

The crude oil extracts were subjected to GC-MS analysis. Briefly, 1 µl of the extractable crude oil was diluted with 1 µl of n-hexane and analyzed on a 25-m Cpsip CB capillary column installed in a capillary gas chromatograph (Packard instruments, Delft. The Netherlands) equipped with a flame ionization detector (FID). A Split injector was used with helium as the carrier gas. The column oven temperature was initially set at 45°C for 2 min and increased at a rate of 10°C /min to 280°C(Riskuwa-Shehu and Ijah, 2016).

2.6 Statistical analysis

All experiments conducted were carried out in triplicate. The results obtained represent the mean ± standard deviation. One-way analysis of variance (ANOVA) with the least significant difference (LSD) test was carried out to identify any significant differences in the hydrocarbon degradation potential of co-cultures at various crude oil concentrations over different time periods. Statistical analyses were conducted using SPSS version 20 (Chicago,IL).

3. Results and Discussion

3.1Screening, isolation, identification and of crude oil degrading bacteria

A total of eleven bacterial isolates,i.e., *Bacillus subtilis* (3 isolates), *Bacillus cereus* (2 isolates), *Micrococcus leteus* (2 isolates), *Pseudomonas aeruginosa* (2 isolates)and *Staphylococcus aureus* (2 isolates) were identified in this study (Table 1). The colonial, morphological and biochemical characteristics of the isolates are shown in Table 1. Testing the isolates for their potential to utilize Bonny Light Oil as a source of carbon and energy revealed that 8 isolates (72.7%) were able to utilize the oil to varying extent (Table 2). Two isolates (18.2%), *Bacillus subtilis* and *Pseudomonas aeruginosa* utilized crude oil at a maximum rate and were able to grow in the crude oil medium after 7 days of incubation, while 4 isolates (36.4%)utilized the crude oil moderately and 2 isolates

(18.2%) minimally while other 3 isolates (27.3%) showed no growth at all (Table 2). The growth of these organisms on Bushnell Hass medium supplemented with Bonny Light Oil indicated their potential to utilize crude oil as the only carbon source. The crude oil-degrading bacteria were identified using cultural characteristics, cell morphology and responses to biochemical tests (Table 1). The results of this study indicate that *Bacillus* species (62.25 %) had the highest number of occurrences. This might be attributed to the ability of members of the genus *Bacilli* to survive harsh environmental conditions by producing endospores, which rapidly reproduce upon the return of favorable conditions (Abdel Rahman, 2011). The prevalence of *Bacillus* species in petroleum-contaminated sites had also been observed by other researchers, especially in tropical regions. This corroborates the findings of Ijah and Antai (2003) as well as Yakubu (2007) who reported that *Bacillus* species were the predominant isolate in petroleum-contaminated soils. Similarly, the isolation of *P. aeruginosa* from petroleum-contaminated soil (Table 1) in this study was not uncommon, since this organism was widely reported as an effective and commonly isolated crude oil degrading bacteria in contaminated soil (Das and Mukherejee, 2007).

Table 1: Colonial, Morphological and Biochemical Characteristics of Bacterial Isolates.

S/N	COLONIAL MORPHOLOGY	MICROSCOPIC MORPHOLOGY	BIOCHEMICAL CHARACTERISTICS											No. of Isolates	PRESUMED ISOLATES
			Grm	Sp	Ca	Co	Ox	Mr	VP	Ind	Cit	Ur	Mo		
1.	Circular, convex yellow	Cocci in buntchess	+	-	+	+	NA	-	+	NA	+	+	-	*2*	*Staphylococcus aureus*
2.	Small, round, entire, yellow	Cooci in pairs and treatrads	+	-	+	NA	NA	-	+	NA	-	+	-	*2*	*Micrococcus leteus*
3.	Creamish white, dry undulate irregular	Rod-shape	+	+	+	NA	NA	-	+	-	+	-	+	3	*Bacillus subtilis*
4.	Circular, entire convex raised with green-blue pigmentation	Rod	-	-	NA	NA	+	-	-	-	+	-	+	*2*	*Pseudomonas aeruginosa*

Key: NA= not applicable; - = Negative, + = Positive. Gram's stain (Grm), Spore stain (Sp), Catalase (Ca), Oxidase test (Ox), Methyl red test (Mr), Voges proskauer test (VP), Indole (Ind), Citrate (Cit), Urea (Ur), Motility (mo).

Table 2: Crude Oil-Degrading Potentials of the Isolates in Crude Oil Media.

Bacterial Isolates	Growth in Crude Oil Medium
Bacillus cereus B001	+
Bacillus cereus B002	++
Bacillus subtilis B003	++
Bacillus subtilis B004	+++
Bacillus subtilis B005	++
Micrococcus leteus B006	-
Micrococcus leteus B007	-
Pseudomonas aeruginosa B008	+++
Pseudomonas aeruginosa B009	++
Staphylococcus aureus B010	+
Staphylococcus aureus B011	-

Key: +++: Maximum growth, ++: Moderate growth, +: Minimal growth, -: No growth

3.2 Effect of crude oil concentration on biodegradation of Bonny Light Oil

The mixed culture of *B. subtilis* and *P. aeruginosa* was tested for biodegradation of Bonny Light Oil at three different concentrations; 5%, 10% and 15%. The result shows that maximum degradation was observed at 5% (1.05×10^6 cfu/mL), followed by 10% (6.76×10^5 cfu/mL) and the least was recorded at15% (4.13×10^5 cfu/mL) crude oil concentrations (Fig. 1). The least bacterial counts found in 15% could possibly be attributed to the toxic components and high concentration of crude oil used. This is in line with the findings of Farag *et al.* (2018) who investigated the crude oil degradation potential of *Pseudomonas* species (sp2, sp48 and sp50) at crude oil concentrations of 0.2%, 0.5%, 1%, and 1.5%. The study revealed that the maximum percentage of oil removal was found at 0.5% (v/v) of crude oil in the MSM medium with *Pseudomonas* sp48 having the highest potential. In a different study, Chen *et al.* (2017) observed that the degradation efficiency of both free and immobilized bacterial consortia at the lower crude oil concentration of 2% (*w/v*) attained maximum values of 58.6% and 63.9%, respectively. However, the degradation efficiency of immobilized bacteria began to reduce drastically as the concentration of crude oil increased from 4% to 10% (*w/v*) and further to 20%. Degradation decreased with increasing concentration perhaps due to the toxicity of the crude oil in the medium (Farag *et al*., 2018). A similar finding was also reported by Kachieng and Momba (2017). Dibble and Bartha (1979) reported increases in biodegradation as indicated by increases in CO_2 evolution in soil polluted with 5% crude oil. However, no increase was observed at 10% pollution level while the degradation rates declined at 15% level. The decrease in biodegradation activity at high oil loading concentrations was attributed to the inhibition of microbial activity by toxic compounds in the oil sludge. Additionally, Lawniczak *et al.* (2020) have also linked slow rate of crude oil biodegradation to complex nature of contaminated soil matrix which reduced the bioavailability crude oil components. Additionally, AI-Blehed (1999) found that increasing the oil concentration adversely influenced the biodegradation of Arab crude oil, possibly due to presence of components

such as toluene and ethylbenzene that have been reported to inhibit biodegradation at high concentrations.

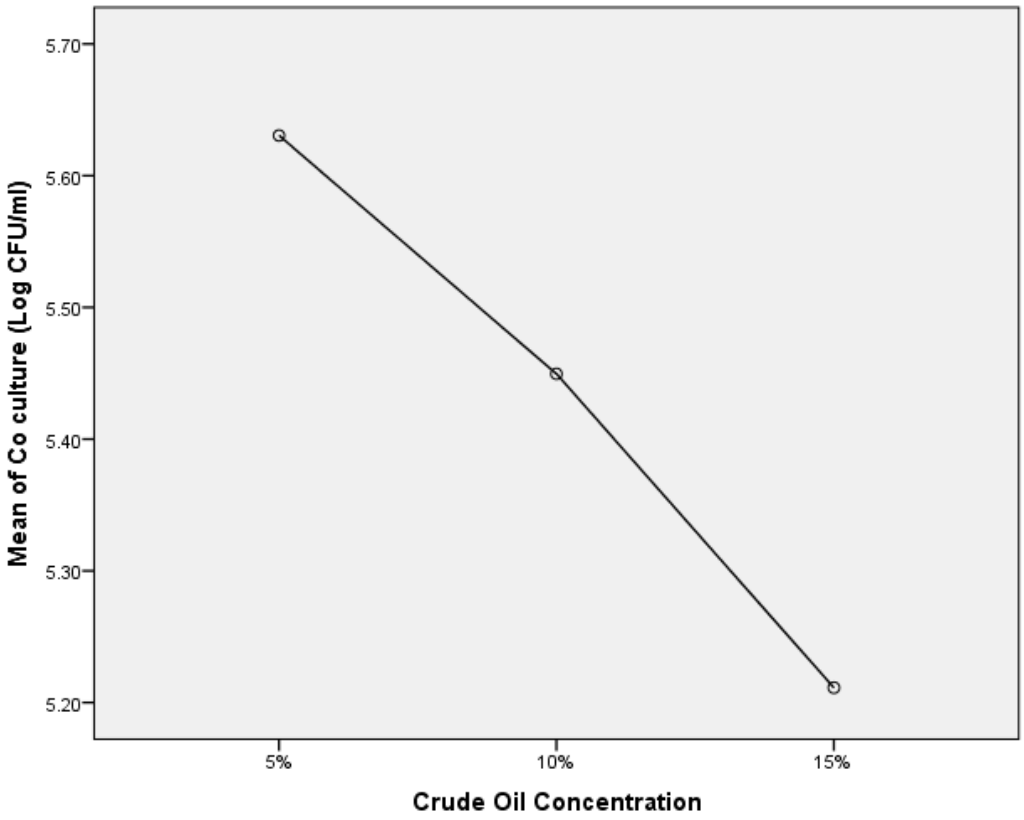

Fig.1. Effect of crude oil concentration on the biodegradation of Bonny Light Oil using co-culture of *B. subtilis* and *P. aeruginosa* (1:1)

3.3 Effect of incubation time on biodegradation of crude oil

The microbial counts expressed in log of cfu/ml were used for determining crude oil degradation. The results revealed that the total crude oil degrading bacterial counts on zero to day 42 were ($1.15.20 \times 10^5$ - 1.05×10^6 cfu/mL), (9.28×10^4 - 6.76×10^5 cfu/mL) and (5.30×10^4 - 4.13×10^5 cfu/mL) for the co-culture at 5%, 10% and 15% respectively. The crude oil degradation pattern of the co-culture of *B. subtilis* and *P. aeruginosa* showed that there was an increasing trend in degradation of the Bonny Light Oil for each successive day up to the day 42 (Figure 2). The gradual increase of the bacterial cell number observed might be attributed to the adaptation and subsequent utilization of the crude oil by the co-culture. The difference in microbial counts at different incubation periods was statistically significant ($P<0.05$); however, there was no significant increase in degradation after day 42. Similar finding were reported by Patowry *et al*. (2017) on the degradation pattern of *P. aeruginosa* strain PG. It was observed that the degradation pattern of the organism significantly increased ($P<0.05$) from first to the fifth week of incubation, with a

significant reduction being recorded after week 5. Ukpaka *et al.* (2020) found that crude oil-degrading bacteria progressively increased from 14 to 56 days across all treatments.

However, contrary to the present study, Chen *et al.*(2017) observed that the degradation of crude oil by free bacterial consortium was maximum in the first 7 days while immobilized bacterial consortium in the first 5 days exhibited degradation efficiencies of 57.3%, and 65.9%, respectively. These differences might be a result of the lower concentrations (2% (w/v) of crude oil used in their study.

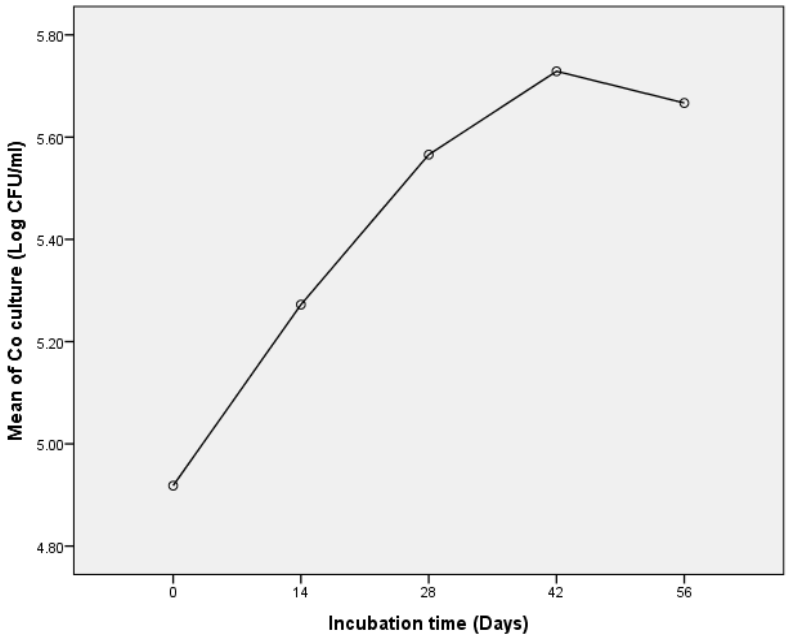

Fig.2. Effect of incubation time on the biodegradation of crude oil using co-culture of *B. subtilis* and *P. aeruginosa* (1:1).

3.4 Hydrocarbons generated during biodegradation

Comparison of the GC/MS chromatograms of the control sample (untreated crude oil; Table 3) and the test samples (treated with co-culture at different crude oil concentrations; Tables 4-6) demonstrate the degradation process. The co-culture of *B. subtilis* and *P. aeruginosa* was tested for biodegradation of Bonny Light Oil at three different crude oil concentrations; 5%, 10% and 15%. The results show that the maximum degradation was observed at 5% followed by 10% and 15% respectively (Tables 4-6). The GC-MS results validated the growth utilization of microbes in the media, indicating that out of the 28 different compounds detected in the control sample at 5% crude oil concentration, the co-culture was able to completely degrade 18 components ranging from C_{10} to C_{25} (Table 4). This is in line with the findings of Farag *et al.*(2018), who investigated the crude oil degradation potential of *Pseudomonas* species (sp2, sp48 and sp50) at 0.2%, 0.5%,

1%, and 1.5% concentrations. Their study revealed that the maximum percentage of degradation was found at 0.5% (v/v) of crude oil in the mineral salt medium, with *Pseudomonas* sp48 having the highest potential. Degradation decreased with increasing concentration, perhaps due to the toxicity of the crude oil in the medium (Farag *et al*., 2018). A similar finding was also reported by Kachieng and Momba (2017). However, in present study, some components including 2,5-dimethylhexane, nonane, 2,2,5-trimethyl-3,4-hexanedione, 1-(2-carboxyphenyl)-3,3-dimethyltriazene ($C_9H_{11}N_3O_2$), 2,8-dimethylundecane ($C_{13}H_{28}$), decanoic acid ($C_{11}H_{22}O_2$), docosanoic acid ($C_{23}H_{46}O_2$) oxirane ($C_{17}H_{34}O_2$), 9,12-q21octadecadienal ($C_{18}H_{32}O$), and (E)-2-nonenal ($C_9H_{16}O$) were not degraded. This could be due to the low bioavailability of the crude oil components in the medium for bacterial degradation (Liu *et al*., 2020) and crude oil composition (Lofthusa *et al*., 2020). Pasumarthi and Mutnuri (2013) reported that cultures of *P. aeruginosa* and *E. fergusonii* isolated from the coast of Goa degraded alkanes mostly ranging from C_{12} to C_{33} rather than *n*-C_{34}. Similar findings was also reported by Liu *et al*. (2020), in that, it was observed that *P. aeruginosa* XJ16 degraded 96.3%–99.7% of C17–C32 respectively while least degradation was recorded (60.2%) for C35 after 90 days of incubation. Interestingly, Liang *et al.* (2018) reported that a mixed culture of *Pseudomonas* sp. ZS1 and *Alcaligenes* sp. CT10 was found to degrade 22.5% of the total crude oil after 36 days of incubation while a single culture of *Alcaligenes* sp. CT10 only degraded selected hydrocarbons including C_9, C_{10}, C_{11}, and C_{28}, suggesting a complex mechanism of hydrocarbon degradation by various microorganisms in the environment.

Table 3: Total components of crude oil present in the control treatment.

Peak	Retention Time	Molecular Weight	Chemical Formulae	Compounds
1.	3.776	112	C_8H_{16}	Cyclohexane
2.	3.992	114	C_8H_{18}	Octane
3.	4.467	112	C_8H_{16}	Ethylcyclohexane
4.	5.008	106	C_8H_{10}	1,4-Dimethylbenzene
5	5.250	142	$C_{18}H_{22}$	n-Decane
6.	5.700	126	C_9H_{18}	Propylcyvlohexane
7.	6.483	156	$C_{11}H_{24}$	n-Undecane
8.	7.683	184	$C_{13}H_{28}$	Tridecane
9.	9.233	184	$C_{13}H_{28}$	Dimethylundecane
10.	10.725	212	$C_{15}H_{32}$	Dodecane
11.	11.308	184	$C_{13}H_{28}$	Tridecane
12.	12.725	192	C_8H_{17}	2-Bromooctane
13.	13.150	252	$C_{17}H_{32}O$	(6E)-5-Butyl-2,2-dimethyl-6-undecen-3-one
14	14.150	226	$C_{16}H_{34}$	n-Hexadecane
15.	14.742	226	$C_{16}H_{34}$	Hexadecane
16.	16.142	192	$C_8H_{17}Br$	3-Bromooctane
17.	16.742	282	C_21H_{44}	2-methylicosane
18.	17.392	184	$C_{13}H_{28}$	Tridecane
19.	17.433	254	$C_{18}H_{38}$	Pentadecane
20.	18.533	282	$C_{20}H_{42}$	Eicosane
21.	18.617	128	C_9H_{20}	2,3,4-Trimethylhexane
22.	19.592	224	$C_{25}H_{50}O_3S$	Sulfurous acid, cyclohexylmethyl octadecyl ester
23.	20.650	282	$C_{20}H_{42}$	Eicosane
24.	21.875	226	$C_{16}H_{34}$	Hexadecane
25.	23.383	254	$C_9H_{19}I$	1-Idononae
26.	24.650	182	$C_{13}H_{26}$	Cyclopentane
27.	25.650	184	$C_{13}H_{28}$	5,7-dimethylundecane
28.	26.492	140	$C_{10}H_{20}$	3,7-Dimethyloct-1-ene

In contrast to high degradation recorded at low concentration (5%) (Table 4), Table 5 shows that the co-culture was only able to degrade C_8 to C_{11} at 10% crude oil concentration. Similarly, C_{12}-C_{21} components were not degraded in the sample. Santisi *et al.* (2015) found that long chain alkanes (C_{19}-C_{30}) were not degraded due their solid nature and low solubility which affect bacterial degradation. Moreover, it was observed that some intermediates hydrocarbons in the form of esters and acids which initially were not present or completely absent in the control were found in the sample after degradation. These include butanoic acid ($C_{14}H_{26}O_2$), pentanoic acid ($C_{16}H_30O_2$), phthalic acid ($C_{10}H_{10}O_4$), nonadecanoic acid ($C_{19}H_{38}O_2$), 6-octadecenoic acid ($C_{19}H_{36}O_2$), hexadecanoic acid ($C_{19}H_{38}O_4$), 9,12-octadecadienal ($_{C18}H_{32}O$), nonadecanol ($C_{19}H_{40}O$), and heptacosane ($C_{27}H_{56}$) (Table 5). The sudden appearance of these new compounds might result either from the degradation of the compounds or from the synthesis of new metabolites and intermediates in the degradation process (Seo *et al*., 2009; Singh *et al*., 2012). This concurs with the findings of Patowary *et al*. (2017), in that 12 different degradation intermediates, including cyclohexylmethyl oxalic acid; 10-chlorodecyl formic acid ester; carbonic acid, 2-biphenyl ester; octanoic acid 2-pentadecyl ester; 9-10-Dimethylanthracene; hexa-decanoic acid, methyl ester; octacosanoic acid methyl ester; 3,4-dihydroxy-phenanthrene diol; 2,4,5 trifluorobenzyl alcohol; propanedioic acid, dipropyl, dimethyl ester; phthalic acid ester; and oxalic acid, cyclohexylmethyl-tridecyl ester, were detected in the treated sample. Conversely, least degradation was observed at 15% crude oil concentration than at 5% and 10%. These results reveal that the co-culture degraded only four components ranging from C_8 to C_9 (Table 6). Similarly, C_{10} (n-decane) to C_{15} (pentadecane) were partially degraded while C_{16} (hexadecane) to C_{20} (eicosane) were not degraded (table 6). Obafemi *et al.* (2018) found that Bacillus sp. (SB4), Pseudomonas sp. (SC8), Serratia sp. (SC11) and Acinetobacter sp. (SC12) degraded varying percentages of C9-C20 but all the isolates were unable to degrade nonadecane (C19). Higher degradation occurred in samples treated with 5% (Table 4), followed by 10% (Table 5) while the least degradation was recorded in samples treated with 15%. This might be due to the high concentration of the crude oil in the soil sample. This coincides with the findings of Ijah and Antai (2003) who determined the effect of various concentrations (10, 20, 30 and 40% v/w) of Nigerian Light Oil (Transniger pipeline crude, TNP) on the indigenous bacterial population of bacteria in the soil. It was observed that C_{14} to C_{32} hydrocarbon components were extensively degraded at lower concentrations (10% and 20%) of crude oil, but conversely, the least degradation was recorded at 30% and 40% crude oil concentrations. This is likely due to high concentration of the crude oil in the soil sample (Ijah and Antai, 2003). It has often been reported that at high concentrations of crude oil cause failure to thrive, or even death, of both lower and higher organisms alike (Ali *et al.*, 2020)

Table 4: Degradation of Crude Oil (5%) by Co-culture of *B. subtilis* and *Pseudomonas aeruginosa* (1:1)

Peak	Retention Time	Molecular Weight	Chemical Formulae	Compounds
1.	4.458	114	C_8H_{18}	2,5-Dimethylhexane
2.	5.717	128	C_9H_{20}	Nonane
3.	6.950	156	$C_9H_{16}O_2$	2,2,5-Trimethyl-3,4-hexanedione
4.	15.842	193	$C_9H_{11}N_3O_2$	1-(2-Carboxyphenyl)-3,3-dimethyltriazene
5	16.783	184	$C_{13}H_{28}$	2,8-Dimethylundecane
6.	17.150	186	$C_{11}H_{22}O_2$	Decanoic acid
7.	19.317	354	$C_{23}H_{46}O_2$	Docosanoic acid
8.	24.217	270	$C_{17}H_{34}O_2$	Oxirane
9.	25.867	264	$C_{18}H_{32}O$	9,12-Octadecadienal
10.	26.042	140	$C_9H_{16}O$	(E)-2-Nonenal

Table 5: Degradation of Crude Oil (10%) by Co-culture of *B. subtilis* and *Pseudomonas aeruginosa* (1:1)

Peak	Retention Time	Molecular Weight	Chemical Formulae	Compounds
1.	13.800	152	$C_{11}H_{20}$	4,8-Dimethyl-1,7-nonadiene
2.	15.833	194	$C_{10}H_{10}O_4$	Phthalic acid, monoethyl ester
3.	16.783	212	$C_{15}H_{32}$	n-Pentadecane
4.	17.975	282	C_20H_{42}	Eicosane
5	18.850	184	$C_{13}H_{28}$	3,7-Dimethylundecane
6.	19.31	354	$C_{23}H_{46}O_2$	Docosanoic acid
7.	20.042	298	$C_{19}H_{38}O_2$	Nonadecanoic acid
8.	21.400	294	$C_{19}H_{34}O_2$	9,12-Octadecadienoic acid, methyl ester
9.	22.600	266	$C_{18}H_{34}O$	9-Octadecenal
10.	24.217	330	$C_{19}H_{38}O_4$	Hexadecanoic acid
11.	25.467	298	$C_{18}H_{31}ClO$	9,12-Octadecadienoyl chloride
12.	25.883	266	$C_{18}H_{34}O$	9-Octadecenal
13.	26.042	284	$C_{19}H_{40}O$	1-Nonadecanol
14	26.300	316	$C_{18}H_{36}O_4$	Pentadecanoic acid

Table 6: Degradation of Crude Oil (15%) by Co-culture of *B. subtilis* and *Pseudomonas aeruginosa* (1:1)

Peak	Retention Time	Molecular Weight	Chemical Formulae	Compounds
1.	5.808	142	$C_{10}H_{22}$	n-Decane
2.	7.008	142	$C_{10}H_{22}$	n-Decane
3.	8.583	130	$C_6H_{10}O_3$	Propanoic acid,
4.	8.825	142	$C_{10}H_{22}$	3-Ethyl-2,5-dimethylhexane
5	10.075	170	$C_{12}H_{26}$	2,6-Dimethyldecane
6.	10.683	198	$C_{14}H_{30}$	Tridecane (2-Methyltridecane)
7.	12.075	142	$C_{10}H_{22}$	3-Ethyl-5-methylheptane
8.	12.517	184	$C_{13}H_{28}$	Tridecane
9.	13.500	226	$C_{16}H_{34}$	n-Hexadecane
10.	14.108	268	$C_{19}H_{40}$	2-Methyloctadecane
11.	15.500	268	$C_{10}H_{21}I$	1-Iodo-2-methylnonane
12.	16.100	184	$C_{13}H_{28}$	5-Ethylundecane
13.	16.750	226	$C_{16}H_{34}$	n-Hexadecane
14	17.892	254	$C_{18}H_{38}$	n-Octadecane
15.	18.958	212	$C_{15}H_{32}$	n-Pentadecane
16.	20.008	282	$C_{20}H_{42}$	Eicosane
17.	21.258	158	$C_{10}H_{22}O$	4-Decanol
18.	22.758	304	$C_{15}H_{29}BrO$	14-Bromo-2-methyltetradec-1-en-3-ol
19.	24.025	282	$C_{20}H_{42}$	Eicosane
20.	24.217	312	$C_{20}H_{40}O_2$	Acetic acid,
21.	25.017	226	$C_{16}H_{34}$	n-Hexadecane
22.	25.875	264	$C_{18}H_{32}O$	9,12-Octadecadienal
23.	26.042	160	$C_{10}H_{21}F$	1-Fluorodecane
24.	26.592	282	$C_{20}H_{42}$	Eicosane

4. Conclusion

It can be concluded from this study that the efficient crude oil degrading bacteria consisting of B. subtilis and P. aeruginosa can be isolated from hydrocarbon-contaminated soil. These isolated strains can be used to develop a mixed culture for the study of biodegradation process. Maximum degradation of Bonny Light Oil occurred at 5% concentration while the least degradation occurred at 15% concentration. This study further affirms that incubation time modulates the biodegradation of crude oil.

Conflict of interest

The authors declare that there is no conflict of interest.

Authors contribution statement

Kawo, A.H. and Ijah, U.J.J: Conceived and designed the experiments; Faggo, A.A: Conducted the experiments; Faggo, A.A, Kawo, A.H, Ijah, U..J..J and Gulumbe, B.H: Wrote the paper; Gulumbe, B.H and Faggo, A.A: Analyzed and interpreted the data.

Acknowledgments

The authors sincerely acknowledge Bayero University, Kano and Bauchi State University, Gadau for their technical support in the course of this research. We also appreciate Dr Rebecca Dewey of Nottingham University for her major contributions.

References

Abdel-Alim, H.E.,and AI-Blehed, M.S.,1999. Bacterial Isolate from Arabian Gulf Coast Soils in Saudi Arabia Able to Degrade Arab Crude Oil. Journal of King Saud University - Engineering Sciences,10, (2): 163-181.DOI: https://doi.org/10.1016/S1018-3639(18)31001-8

Abdel Rahman, M.E., 2011. Biodegradation of Crude Oil by Bacteria Isolated from Produced Formation Water of an Onshore Oil Field in Sudan. Thesis Submitted to the Graduate College, Department of Botany, Faculty of Science, University of Khartoum pp. 01-140. https://pdfs.semanticscholar.org/3a1c/0cd301b7d8ff6e5cf4232292123a72d4d31a.pdf (accessed on: 18 March, 2017).

Ali, N, Dashti, N, Khanafer, M, Al-Awadhi , H & Radwan , S., 2020. Bioremediation of soils saturated with spilled crude oil. Scientific Report, 10:1116.DOI: https://doi.org/10.1038/s41598-019-57224-x

Andrew, J.M., 2009. British Society for Antimicrobial Chemotherapy: Standardized disc susceptibility testing method (version 8). Journal of Antimicrobial Chemotherapy, 64, 454-489. DOI: https://doi.org/10.1093/jac/dkp244

Bao, M.T, Wang, L.N, Sun, P.Y, Cao, L.X, Zou, J. and L.i, Y.M., 2012. Biodegradation of crude oil using an efficient microbial consortium in a simulated marine environment. Marine Pollution Bulletin, 64(6):1177-85.DOI: https://doi.org/10.1016/j.marpolbul.2012.03.020 PMID: 22498316

Chen., Q, Li., J, Liu., M, Sun., H. and Bao, M., 2017. Study on the biodegradation of crude oil byfree and immobilized bacterial consortium in marine environment. PLoS ONE, 12(3): e0174445. DOI: https://doi.org/10.1371/journal.pone.0174445

Chessbrough, M., 2006. District Laboratory Practice in Tropical Countries: Part 2 Second edition, Cambridge University Press, New York, pp.35-70.

Cui, J, Chen, H. · Sun, M and Wen, J., 2020.Comparison of bacterial community structure and function under different petroleum hydrocarbon degradation conditions Bioprocess and Biosystems Engineering, 1 to asddya https://doi.org/10.1007/s00449-019-02227-1

Das, K, and Mukherjee, A. K., 2007. Crude petroleum-oil biodegradation efficiency of Bacillus subtilis and Pseudomonas aeruginosa strains isolated from a petroleum-oil contaminated soil from North-East India. Bioresource Technology, 98, 1339–1345. https://doi.org/10.1016/j.biortech.2006.05.032

Deepika, P.M. and Rajalakshmi, G., 2013. Biodegradation of Petroleum Hydrocarbons Pollutants in Soil Using Microbial Consortium. International Journal of Plant, Animal and Environmental Sciences, 3(3):174-176. http://www.ijpaes.com/admin/php/uploads/372_pdf.pdf (accessed on: 16 August, 2016).

Dibble, J. T. and Bartha, R., 1979. Effect of environmental parameters on the biodegradation of oil sludge. Applied Environmental Microbiology, 37(4):729-739. https://doi.org/10.1128/aem.37.4.729-739.1979

Farag, S, Soliman, N.A and Abdel-Fattah, Y.R., 2018. Statistical optimization of crude oil bio-degradation by a local marinebacterium isolate Pseudomonas sp. sp48. Journal of Genetic Engineering and Biotechnology,16:409-420.DOI: https://doi.org/10.1016/j.jgeb.2018.01.001

Godleads, O.A, Prekeyi, T.F., Samson, F.O and Igelenyah, E., 2015. Bioremediation, Biostimulation and Bioaugmention: A Review. International Journal of Environmental Bioremediation & Biodegradation, 3 (1): 28-39. DOI: 10.12691/ijebb-3-1-5.

Head, I.M, Jones, D.M, Roling WF., 2006. Marine microorganisms makes a meal of oil. Nature Reviews Microbiology, 4:173–182. DOI:https://doi.org/10.1038/nrmicro1348.

Hu, L, Wu, X, Liu, Y, Meegoda, J. N, and Gao, S., 2010. Physical modeling of air flow during air sparging remediation. Environmental Science & Technology, 44, 3883–3888. DOI: https//doi.org/10.1021/es903853v.

Ijah, U.J.J. and Antai, S.P., 2003. Removal of Nigerian light crude oil in soil over a 12-month period. International Journal of Biodeterioration & Biodegradation, 51(2): 93-99. DOI:https//doi.org/10.1016/S0964-8305(01)00131-17.

Ismail, W, Al-Rowaihi, I. S, Al-Humam, A. A, Hamza, R. Y., El Nayal, A. M. and Bououdina, M., 2013. Characterization of a lipopeptide biosurfactant produced by a crude-oil-emulsifying Bacillus sp. I-15. International Biodeterioration and Biodegradation, 84, 168–178. DOI: https://doi.org/10.1016/j.ibiod.2012.04.017.

Kachieng, L. and Momba, M.N.B., 2017. Kinetics of petroleum oil biodegradation by a consortium of three protozoan isolates (Aspidisca sp., Trachelophyllum sp. and Peranema sp.). Biotechnology Reports, 15:125–131. DOI: https://doi.org/10.1016/j.btre.2017.07.001

Kawo, A.H. and Kabiru, M.R., 2016. Biodegradation Potentials of Bacteria Isolated from Textile Dye-Contaminated Soil in Kano, Nigeria. Biological and Environmental Science Journal for the Tropics 13(1): 145-151.

Kulkarni, S.V., 2014. Bioremediation of Petroleum Hydrocarbon Polluted Sites for the Conservation of Soil Microbial Diversity. Thesis submitted to University of Pune, 411008, India, pp. 9-143. http://dspace.ncl.res.in:8080/xmlui/bitstream/handle/20.500.12252/2215/TH2029.pdf?sequence=1 (accessed on: 21 March, 2017).

Liang, J, Cheng, T, Huang, Y. and Liu, J., 2018. Petroleum degradation by Pseudomonassp. ZS1 is impeded in the presence of antagonist Alcaligenes sp. CT10. AMB Express, 8, 88. DOI: https://doi.org/10.1186/s13568-018-0620-5

Liu, Y, Wan, Y.Y, Wang, C, Ma, Z, Liu, X. and Li, S., 2020. Biodegradation of n-alkanes in crude oil by three identified bacterial strains. Fuel 275, 117897 DOI: https://doi.org/10.1016/j.fuel.2020.117897.

Lofthusa, S, Bakkea, I, Tremblay, J, Greer, C.W Brakstad, O.C., 2020. Biodegradation of weathered crude oil in seawater with frazil ice. Marine Pollution Bulletin, 154, 111090 DOI: https://doi.org/10.1016/j.marpolbul.2020.111090

Lawniczak, L, Wozniak-Karczewska, M, Loibner, A.P. Heipieper, H.J, and Chrzanowski, L., 2020. Microbial degradation of hydrocarbons-basic principles for bioremediation: a review. Molecule, 25(4): 856. DOI: 10.3390/molecules25040856.

Obafemi, Y.D, Taiwo, O.S, Omodara, O.J. Dahunsi, O.S, Oranusi, S., 2018.Biodegradation of crude petroleum by bacterial consortia from oil-contaminated soils in Ota, Ogun State, South-Western, Nigeria. Environmental Technology & Innovation 12 (2018) 230–242. DOI: https://doi.org/10.1016/j.eti.2018.09.006

Okpokwasili, G.C. and Okorie, B.B., 1988. Biodeterioration potentials of microorganisms isolated from car engine lubricating oil. Tribology International 21(4): 215-220. DOI: https//doi.org/10.1016/0301-679X(88)90020-5.

Ojo, O.A., 2006. Petroleum hydrocarbon utilization by nativebacterial population from a wastewater canal Southwest Nigeria. African Journal Biotechnology 5(4):333–337. https://www.ajol.info/index.php/ajb/article/view/137800 (accessed on: 1 January, 2020).

Pasumarthi, R. and Mutnuri, S., 2013. Biodegradatio of crude oil by Pseudomonas aeruginosa and Escherichia fergusonii isolated from the Goan cost. Marine Pollution Bulletin, 76(1-2): 276-282. DOI:https//doi.org/10.1016/j.marpolbul.2013.08.026.

Patowary, K, Patowary, R, Kalita M.C. and Deka, S., 2017. Characterization of Biosurfactant Produced during Degradation of Hydrocarbons Using Crude Oil As Sole Source of Carbon. Front. Microbiol. 8:279. DOI: https//:doi.org/10.3389/fmicb.2017.00279.

Riskuwa-Shehu, M. and Ijah, U.J.J., 2016. Enhanced Removal of Crude Oil in Soil by Mixed Culture of Bacillus Megaterium UL05 and Pseudomonas Aeruginosa UL07. International Journal of Environmental Bioremediation & Biodegradation 4(1):8-12. https//doi.org/10.12691/ijebb-4-1-2.

Romanus, A. A., Ekundayo, A. O., Aigere, S.P. and Okwu, G.I., 2015. Bacterial Degradation of Petroleum Hydrocarbons in Crude Oil Polluted Soil Amended with Cassava Peels. American Journal of Research Communication 3(7): 99-118. https://pdfs.semanticscholar.org/5d7f/52a8f2c9f771b3bd314edde4a6df73ca558f.pdf (accessed on: 16 August, 2016).

Santisi, S, Cappello, S, Catalfamo, M, Mancini, G, Hassanshahian, M, Genovese, L, Giuliano, L and Yakimov, M.M., 2015. Biodegradation of crude oil by individual bacterial strains and a mixed bacterial consortium. Brazilian Journal of Microbiology, 46(2): 377-387. DOI: 10.1590/S1517-838246120131276.

Ukpaka, C. P. Lezorghia, S. B. and Nwosu, H., 2020, Crude oil degradation in loamy soil using Neem root extracts: An experimental study. Chemistry International, 6(3),160-167. DOI: https://doi.org/10.5281/zenodo.3645478

Yakubu, M. B., 2007. Biodegradation of Lagoma crude oil using pig dung. African Journal of Biotechnology, 6(24), 2821-2825. DOI: https//dx.doi.org/10.5897/AJB2007.000-2450.