Degradation of Crude Oil by Bacteria Isolated from Various Soil Plantation at Idanre, Nigeria

I. A. Onifade[1*], D. B. Orisadipe[1], N. D. Nkor[1], F. O. Ekundayo[1] and A. O. Arogunjo[1]

[1]*Department of Microbiology, School of Sciences, Federal University of Technology, PMB-704, Akure, Nigeria.*

Authors' contributions

This work was carried out in collaboration among all authors. Author FOE conceived, designed and supervised the study. Authors IAO, DBO and NDN performed the research work. Authors IAO and DBO wrote the manuscript, IAO, DBO and AOA performed the statistical analysis and managed the literature searches. All authors read and approved the final manuscript.

Original Research Article

ABSTRACT

Degradation of crude oil by bacteria isolated from three plantations soil at Idanre, Nigeria were comparatively investigated. Soil samples collected by hand trowel at 5cm rhizosphere of Cocoa, Orange, and Teak plantations from Idanre, Ondo State. The crude oil – forcados blend was obtained from Warri, Delta State, Nigeria. Gram negative bacteria were isolated from rhizosphere soil sample using standard microbiological methods. Preparation of Biomass was done by centrifuging nutrient broth repeatedly to wash cells. Harvested cells were obtained for the degradation of crude oil. Harvested cells were inoculated with crude oil and then incubated in a shaker. Degradation of crude oil was monitored by using spectrophotometer to read the OD at 540 nm. The bacteria isolated from rhizosphere of cocoa include: *Citrobacter freundii*, *Yersinia pestis*, *Edwardsiella tarda*,. *Serratia marcescens*. Rhizosphere of orange; *Providencia stuatii* while *Enterobacter agglumerans*, *Moellerella wisconsis* were isolated from rhizosphere of teak. The most effective hydrocarbon utilizing bacteria was *Enterobacter agglumerans*, which resulted in increase in population densities and reduction in hydrocarbon contents in the crude oil. Findings from this study shows the effectiveness of degrading hydrocarbon in liquid medium and further confirmed the potency of bacterial cells to degrade crude oil.

*Corresponding author: E-mail: isrealonifade@gmail.com;

1. INTRODUCTION

Crude oil occurs naturally in unrefined petroleum compound containing hydrocarbon deposits and additional organic matter [1]. Removal of crude oil impurities can generate usable substances such as gasoline, diesel and various forms of petrochemicals [2, 3]. It cannot be replaced naturally, making it a non-renewable resource [4]. Petroleum is contained numerous aliphatic, branched and aromatic hydrocarbons and other organic compounds (organometallic constituents inclusive) [5]. Many activities such as industrial and municipal runoffs, effluent release, offshore and onshore petroleum industry activities as well as accidental spills causes petroleum hydrocarbon pollution [6]. Most of them are toxic to humans, animals and vegetation. In long term, this pollution affects the environment [7].

Petroleum hydrocarbons influence biodiversity, distribution of microorganisms in the environment [6]. Biodegradation is a biological mediated catalysis which reduces complexity of chemical compound [8]. Several factors limit biodegradation of petroleum hydrocarbons in the environment. A cogent factor mitigating against biodegradation of polluted soils is low bioavailability and solubility of the hydrocarbon [9]. Crude oil is an important pollutant in the environment capable of causing colossus impairments to humans and the ecosystem. Prolonged exposure to high crude oil concentration may damage liver, kidney disease, bone marrow and increased risk of cancer [10]. The microorganisms used in the degradation of petroleum products has been established as efficient, economic, versatile and environmentally sound treatment [11].

Microbial degradation for cleaning untreated oil spills is slow, hence, exploration for effective and efficient methods of oil removal from contaminated sites has intensified in recent times [12]. Colonies of microorganisms, especially the inherent bacteria in the soil assist in the remediation of hydrocarbon-contaminated site [11]. These microorganisms can degrade extensive arrays of target constituents in oily slurry [13]. Many *Pseudomonas* strains capable of degrading polyclic aromatic hydrocarbons (PAHS) have been isolated from soil and aquifers. Other petroleum hydrocarbon-degraders include *Yokenella* spp., *Alcaligenes* spp., *Roseomonas* spp., *Stenotrophomonas* spp., *Acinetobacter* spp, *Flavobacter* spp, *Corynebacterium* spp, *Streptococcus* spp., *Providencia* spp., *Sphingobacterium* spp, *Capnocytophaga* spp, *Moraxella* spp, and *Bacillus* spp. The growth and proliferation of oil utilizing microorganisms in polluted soil is greatly influenced by the availability of nutrients and their hydrocarbonoclastic property [14].

2. MATERIALS AND METHODS

2.1 Sample Collection

Soil samples were collected at 5cm from the rhizosphere of cocoa, orange and teak from Idanre, Ondo State. Crude oil polluted soil samples were collected from Zion town in Ese Odo at Ilaje, Ondo State. Soil samples were collected in sterile polythene bags and tightly packed. They were then carefully transported to the laboratory for the analysis. Crude oil was collected from Warri in Nigeria.

2.2 Isolation of Microorganisms

Three-fold serial dilution was performed on the above soil samples. An aliquot (0.2 mL) of each diluent were plated on sterile Nutrient agar (NA) using the pour plate method. Incubation was done at 37 ± 2 °C for 24 hrs. Developed bacteria colonies were aseptically subcultured a number of times into fresh NA plates and incubated 37 ± 2 °C for 24 hrs until pure cultures of bacteria were obtained. Pure cultures were afterwards transferred onto McCartney bottles of doubled strength slant incubated at 37 ± 2 °C for 24hrs and stored at 4 °C in the refrigerator [15].

2.3 Identification of Isolates

The bacterial isolates were identified according to colonial morphology, surface, shape, size, margin and pigmentation on nutrient agar medium. The microscopic examination including Gram-staining, The biochemical tests including citrate utilization, starch hydrolysis, methyl red, Voges-Proskauer test (MR-VP), triple sugar iron test (TSI) for lactose, dextrose, sucrose, glucose and mannitol fermentation, carbohydrate fermentation, H2S production, indole production test, urease test, catalase test, citrate test, indole test and sugar fermentation test were carried out according to standard procedures and identified using Bergey's Manual of Systematic Bacteriology [16].

2.4 Preparation of Biomass

An inoculating loop was used to pick bacteria and inoculated on sterile nutrient broth and incubated at 37 ± 2 °C for 24hrs. For the harvested cells, the broth cultures were centrifuged at 5000 rpm for five minutes and suspended in 2 mL sterile distilled water. The content was centrifuged again at 5000 rpm for five minutes to wash the cells. Harvested cells were obtained after decanting the supernatant [17].

2.4.1 Test for degradation of crude oil

The enrichment procedure as described by Nwachukwu (2000) was used in the estimation of hydrocarbon utilizers. Liquid basal medium contained Na_2HPO_4 (2.0g), K_2SO_4 (0.17g), NH_4NO_3 (4.0 g), KH_2PO_4 (0.53 g), $MgSO_4 \cdot 7H_2O$ (0.2 g) and 1000ml of distilled water. Sterilization was done at 121 °C for 15 min. 3 ml of harvested cells was inoculated into 10 mL of the basal medium with 30mL crude oil in a conical flask. The conical flasks was plugged with sterile cotton wool wrapped with Aluminum foil so as to ensure maximum aeration and prevent cross-contamination. All the conical flasks were then incubated in a shaker for 37 °C for 20 days during which degradation of crude oil was monitored by reading the optical density at 540 nm at five days interval. The experiment was done in replicates while crude oil without inoculum served as control [18].

2.5 Statistical Analysis

All experiments were carried out in triplicates. Data obtained were analyzed by one way analysis of variance and means compared by Duncan's new multiple range test. Differences are considered significant at P<0.05.

3. RESULTS AND DISCUSSION

Seven bacteria were isolated from rhizosphere of the soil sample of orange, teak and cocoa and four bacterial species were isolated from crude oil polluted soil. They are *Serratia marcescens, Moellerella wisconsensis, Enterobacter agglomerans, Providencia stuartii, Citrobacter freundii, Escherichia coli, Yersinia pseudotuberculosis* and *Edwardsiella tarda*. Four bacterial species were rod and three bacterial species were cocci. All the bacterial species were gram negative and catalase positive and two were positive to methyl red (Table 1 and 2).

The occurrence of these microorganisms in the rhizosphere of all the soil samples examined varied. [19] reported that the quality of root exudates can promote differential recruitment of microorganisms present in the soil. The rhizosphere soil samples used in this study harbored microorganisms that utilize hydrocarbon as source of nutrients. These include *Serratia* sp, *Enterobacter* sp, *Citrobacter* sp, *Providencia* sp, *Edwardsiella* sp, *Yersinia* sp, and *Moellerella* sp. The flora reflects the diverse heterotrophic bacteria present in rhizosphere soil

Table 1. Biochemical and cultural characteristics of bacterial isolates

H₂S	MrVP	Suc	Glu	Lac	Ure	Gas	Cat	Ind	Cit	Sta	G.S	Probable Identity
-	+	-	+	-	+	AG	+	+	+	-	-	*Serratia marcescens*
-	+	-	+	-	+	-	+	-	+	+	-	*Enterobacter* sp
-	VP-	-	-	-	-	-	+	-	+	-	-	*Providencia stuatii*
-	VP-	+	+	-	-	AG	+	-	-	-	-	*Citrobacter freundii*
-	VP-	+	+	-	+	-	+	-	-	-	-	*Yersinia* sp
+	VP-	+	+	-	-	AG	+	+	-	+	-	*Edwardsiella tarda*
-	VP-	+	+	-	-	-	+	-	-	-	-	*Moellerella wisconsen*

Key: AG = Acid and Gas production; VP = Voges-Proskauer; GS = Gram staining; +, - = Positive and Negative

Table 2. Morphological description of bacterial isolates

Isolate code	O3a	T3a	O3b	C2a	C2b	C4b	T2b
Shape	irregular	rhizoid	circular	rhizoid	irregular	filament	rhizoid
Colour	pink	cream	cream	Transparent	cream	Cream	cream
Elevation	flat	flat	flat	flat	flat	Flat	flat
Texture	moist	mucoid	moist	mucoid	moist	mucoid	dry
Surface	smooth	rough	smooth	smooth	rough	smooth	rough
Margin	undulate	filiform	entire	filiform	filiform	filiform	filiform
Cell enlargement	rod	cocci	rod	cocci	cocci	Rod	rod

Key: O3a: Serratia marcescens isolated from orange; T3a Enterobacter agglumerans isolated from teak; O3b Providencia stuatii isolated from orange; C2a Citrobacter freundii isolated from cocoa; C2b Yersinia pseudotuberculosis isolated from cocoa; C4b Edwardsiella tarda isolated from cocoa; T2b Moellerella wisconsens isolated from cocoa

Table 3. Degradation of crude oil by bacteria cells

	Optical density at different days in litres (mole×cm)						
Selected isolates	**1st day**	**5th day**	**10th day**	**15th day**	**20th day**	**25th day**	**30th day**
Control	2.26±0.03[a]	2.26±.00[a]	2.26±0.00[a]	2.27±0.00[a]	2.26±0.00[a]	2.26±0.00[a]	2.27±0.00[a]
*Moelerell*asp	2.27±0.01[a]	2.27±0.01[a]	2.26±0.00[b]	2.26±0.01[b]	2.25±0.00[a]	2.24±0.00[a]	2.23±0.06[a]
Providencia sp	2.26±0.01[a]	2.26±0.01[a]	2.25±0.00[a]	2.25±0.00[a]	2.25±0.00[a]	2.25±0.00[a]	2.25±0.00[a]
Serratia sp	2.26±0.00[c]	2.26±0.00[b]	2.26±0.00[c]	2.26±0.00[c]	2.25±0.00[b]	2.25±0.00[a]	2.25±0.00[a]
Enterobacter sp	2.26±0.00[e]	2.26±0.00[e]	2.26±0.00[d]	2.25±0.00[d]	2.24±0.00[b]	2.23±0.00[b]	2.20±0.00[a]
Yersinia sp	2.27±0.01[b]	2.27±0.01[b]	2.27±0.00[b]	2.26±0.03[a]	2.26±0.00[a]	2.25±.00[a]	2.26±0.00[a]
Edwardsiella sp	2.27±0.00[c]	2.26±0.00[c]	2.27±0.00[b]	2.26±0.00[b]	2.25±0.00[b]	2.25±0.00[a]	2.25±0.00[a]
Citrobacter sp	2.27±0.00[b]	2.27±0.00[b]	2.27±0.00[b]	2.27±0.00[a]	2.26±0.00[a]	2.26±0.01[a]	2.26±.01[a]

Data are represented as Mean±S.E(n=3). Values with the same superscript letter(s) along the same column are not significantly different (p<0.05)

and the diversity could be as a result of the varied sources of the refuse dumped at the sites [20]. Several hydrocarbon degrading organisms have been isolated from diverse environments; soil and aquatic sources which are the two major environments affected by hydrocarbon pollution [21] and their isolation are not restricted to hydrocarbon-bearing environments. The table below revealed the absorbance reading for *Moelerrella* sp, *Providencia* sp, *Serratia* sp, *Enterobacter* sp, *Yersinia* sp, *Edwardsiella* sp, *Citrobacter* sp and control on day 1 to be between 2.26 and 2.27. On day 5 the absorbance level shows no significance difference for all organisms. While on day 30 *Enterobacter* sp absorbance reading drop to 2.20, this clearly shows that *Enterobacter* sp effectively degraded hydrocarbon.

This study focus on hydrocarbon utilizing isolates obtained, which had varied degree of degradation. However, *Enterobacter* sp, *Edwardsiella* sp, appeared to be the fastest growing species in crude oil followed by *Serratia* sp, *Providencia* sp, *Moellerella* sp *Yersinia* sp *Citrobacter* sp. Also, *Enterobacter* sp, *Moellerella* sp, *Yersinia* sp, appeared to be the fastest growing species in polluted soil, followed by *Serratia* sp, *Providencia* sp, *Edwardsiella* sp, *Citrobacter* sp which are the slow degraders. While for nanoparticles bioremediation, *Enterobacter* sp, appeared to be the fastest on polluted soil followed by *Citrobacter* sp, *Providencia* sp, and *Serratia* sp, *Yersinia* sp. However, several other workers also reported on *Micrococcus varians, Bacillus badius, Corynebacterium ulcerans* and *Corynebacterium amycolatum* appeared to be the fastest growing species in crude oil. The study of [22] identified the above genera among hydrocarbon degrading microorganisms. The results clearly showed that the values of consumed hydrocarbons decrease gradually with increasing incubation period. Microorganisms are capable of utilizing oil and oil products as a sole source of carbon and energy occur practically everywhere in air, water and soil [23]. [24] reported that bacteria, as primary degraders of spilled oil in the environment are the most active agents in petroleum degradation. Earlier studies have shown that a bacterial consortium comprising hydrocarbon degrading *Pseudomonas aeruginosa* and *Bacillus* spp. could effectively biodegrade crude oil petroleum in liquid cultures as well as in polluted soil and sand [25]. The enormous quantities of crude and refined oils are transported over long distances and consumed in large amounts, hydrocarbons have now become a very important class of substrates for microbial oxidation [26]. The result clearly shows that *Enterobacter* sp utilized hydrocarbon more than the other organisms, this could be as a result of the organism able to utilize hydrocarbon as a sole source of energy although, the result did not show much significant difference in degradation from day 1 to day 30 which actually be as a result of the bacteria needing more time for the degradation because degradation processes takes longer time frame [6].

4. CONCLUSION

The bacteria isolated from rhizosphere were *Citrobacter freundii, Yersinia pestis, Edwardsiella tarda*, were isolated from rhizosphere of cocoa. *Serratia marcescens, Providencia stuatii* were isolated from rhizosphere of orange while *Enterobacter agglumerans, Moellerella wisconsis* were isolated from rhizosphere of teak. The most effective hydrocarbon utilizing bacteria was *Enterobacter agglumerans*, which resulted in increase in population densities and reduction in hydrocarbon contents in the crude oil. The results of this work revealed the degradation of hydrocarbon in liquid medium and further confirmed the effectiveness of bacterial cells to biodegrade crude oil.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

REFERENCES

1. Mandri T, Lin J. African Journal of Biotechnology. 2006;12:6-22.
2. James G, Speight. The Chemistry and Technology of Petroleum (Fourth ed.). CRC Press; 2006.
3. Gary JH, Handwerk GE. Petroleum Refining Technology and Economics (2nd ed.). Marcel Dekker, Inc;1984.
4. America's climate choices: panel on advancing the science of climate change; national research council. Advancing the Science of Climate Change. Washington, D.C.: The National Academies Press; 2010.
5. Rajasekar A. Biodegradation of petroleum hydrocarbon and its influence on corrosion with special reference to petroleum industry. In: Heimann K, karthikeyan O, Muthu S. (eds) biodegradation and

bioconversion of hydrocarbons. Environmental Footprints and Eco-design of Products and Processes. Springer, Singapore; 2017.

6. Nilanjana Das, Preethy Chandran. Microbial degradation of petroleum hydrocarbon contaminants: an overview. Biotechnology Research International; 2011;13.
Article ID 941810

7. Atlas R, Bragg J. Bioremediation of marine oil spills: when and when not-the Exxon Valdez experience. Microbial Biotechnology.2009;2(2):213-221.

8. Grangemard IJ, Wallach R, Marget-Dana F, Peypous. Applied Biochemistry Biotechnology. 2001;90-199.

9. Lloyd CA, Cackette TA. Air and waste management association. 2001;51: 805.

10. Patrick-Iwuanyanwu KC, Onyemaenu CC, Wegwu MO, Ayalogu EO. Hepatotoxic and Nephrotoxic Effects of Kerosene and Petrol-Contaminated Diets in Wistar Albino Rats. Research Journal of Environmental Toxicology. 5(9):49-57.

11. Bhattacharya D, Sarma PM, Krishnan S, Mishra S, Lal B. Applied Environmental. Microbiology. 2002;5(69):1435.

12. Rathi V, Yadav V. Oil Degradation Taking Microbial Help and Bioremediation: A Review. J Bioremediat Biodegrad. 2019;10:460.

13. Van Hamme JD, Singh A, Ward OP. Recent advances in petroleum microbiology. Microbiol Mol Biol Rev. 2003;67(4):503-549.

14. Nwaogu LA, Onyeze GOC, Nwabueze RN. African Journal of Biotechnology. 2008;20:754-1939.

15. Fawole MO, Oso BA. Laboratory manual on microbiology. Spectrum Books Limited, Ibadan, Nigeria. 2007;127.

16. Kumar CG, Mamidyala SK. Extracellular synthesis of silver nanoparticles using culture supernatant of *Pseudomonas aeruginosa* Colloids and Surfaces B: Biointerfaces. 2012;84:462-466.

17. Latha R, Kalaivani R. Bacterial degradation of crude oil by gravimetric analysis. Advances in Applied Science Research. 2012;3(5):2789-2795.

18. Wolfe BE, Klironomos JN. Breaking new ground: Soil communities and exotic plant invasion. Bio Science. 2005;55(6):477-487.

19. Mittal Y, Chisti and Banerjee U. Synthesis of metallic nanoparticles using plant extracts. Biotechnology Advances. 2013; 31:346-356.

20. Barathi S, Vasudevan N. Utilization of petroleum hydrocarbons by Pseudomonas fluorescence isolated from a petroleum-contaminated soil. Environ. Int. 2001;26:413-416.

21. Rastogi M, Nandal M, Khosla B. Microbes as vital additives for solid waste composting. Heliyon. 2020;6(2):e03343. Published 2020

22. Ollivier B, Magot M. Petroleum microbiology. 1st ed. ASM Press: Washington, DC. 2005;365.

23. Brooijmans W, Pastink M, Siezen J. Hydrocarbon-degrading bacteria: the oil-spill clean-up crew. Microbial Biotechnology. 2009; 2(6):587–594.

24. Salleh AB, Ghazali FM, Zaliha RN, Basri MM. Biodegradation of crude oil polluted soil. Indian Journal of Biotechnology.2003;2:411-425.

25. Rosenberg E, Ron EZ. Bioremediation of petroleum contamination in Crawford RL and Crawford DL" (eds.) Cambridge: Cambridge University Press. Bioremediation: Principles and Application. 1996;100-124.

26. Salleh AB, Ghazali FM, Zaliha RN, Basri MM. Biodegradation of crude oil polluted soil. Indian Journal of Biotechnology. 2003;2:411-425.

_____