

# **BIODEGRADATION OF CRUDE OIL BY BACTERIA ISOLATED FROM THREE PLANTATIONS SOIL AT IDANRE, NIGERIA**

## **ABSTRACT**

The comparative studies of biodegradation of crude oil by bacteria isolated from three plantations soil at Idanre, Nigeria were investigated. Soil samples were collected with hand trowel at 5cm from the rhizosphere of Cocoa, Orange, and Timber from Idanre, Ondo State. Forcados blend Crude oil was collected from Warri, Delta State in Nigeria. Gram negative bacteria were isolated from the above rhizosphere soil sample using standard microbiological methods. Preparation of Biomass was done by centrifuging nutrient broth at 5000rpm and suspended in 2ml sterile distilled water. The process was repeated for another 5min to wash the cells. Harvested cells were obtained for the degradation of crude oil. 3 ml of harvested cells was inoculated into 10 mL of the basal medium with 30mL crude oil in a conical flask, the conical flask were plugged with sterile cotton wool wrapped with Aluminium 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 30 days during which degradation of crude oil was monitored by using spectrophotometer to read the OD at 540 nm at five days interval. Also, crude oil without inoculum served as control. The bacteria isolated from rhizosphere were *Citrobacter freundii*, *Yersinia pestis*, *Edwardsiella tarda*, were isolated from rhizosphere of cocoa. *Serratia marcescens*, *Providencia stuartii* were isolated from rhizosphere of orange while *Enterobacter agglomerans*, *Moellerella wisconsinis* were isolated from rhizosphere of timber. The most effective hydrocarbon utilizing bacteria was *Enterobacter agglomerans*, 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.

**Keywords:** Crude oil, biodegradation, bacteria, hydrocarbon,

## **1 Introduction**

Crude oil is a naturally occurring, unrefined petroleum product composed of hydrocarbon deposits and other organic materials [1]. A type of fossil fuel, crude oil can be refined to produce usable products such as gasoline, diesel and various forms of petrochemicals. It is a nonrenewable resource, which means that it can't be replaced naturally at the rate we consume it and is therefore a limited resource. Petroleum is composed of hundreds or thousands of aliphatic, branched and aromatic hydrocarbons and other organic compounds including some organometallic constituents. Many activities such as industrial and municipal runoffs, effluent release, offshore and onshore petroleum industry activities as well as

accidental spills cause petroleum hydrocarbon pollution. Most of them are toxic to humans, animals and vegetation. In long term, this pollution affects the environment [2].

Presence of Petroleum hydrocarbons has been reported to influence the biodiversity, distribution and pollution of microorganisms in an environment. Biodegradation is defined as the biologically catalyzed reduction in complexity of chemical compound [3]. Biodegradation of petroleum hydrocarbons in the environment may be limited by a large number of factors. An important limiting factor in the biodegradation polluted soils is often the low bioavailability and solubility of the hydrocarbon [4]. Crude oil is one of the most significant pollutants in the environment as it is capable of causing serious damages to humans and the ecosystem. Prolonged exposure and high oil concentration may cause the development of liver or kidney disease, possible damage to the bone marrow and an increased risk of cancer. The microorganisms in the degradation of petroleum and its products has been established as an efficient, economic, versatile and environmentally sound treatment [5].

The search for effective and efficient methods of oil removal from contaminated sites has intensified in recent years, because microbial degradation for cleaning untreated oil spills is slow. Microbial remediation of a hydrocarbon-contaminated site is accomplished with the help of a diverse group of microorganisms, particularly the indigenous bacteria present in soil [5]. These microorganisms can degrade a wide range of target constituents present in oily sludge. A large number of *Pseudomonas* strains capable of degrading polycyclic 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. Microorganisms have enzyme systems to degrade and utilize diesel oil as a source of carbon and energy. The growth and proliferation of oil utilizing microorganisms in polluted soil is greatly influenced by the availability of nutrients and their hydrocarbonoclastic property [6].

Some microorganisms have the astonishing, naturally occurring, microbial catabolic diversity to degrade, transform or accumulate a huge range of compounds including hydrocarbons. Bioremediation process can be divided into three phases or levels [7]. First, through natural attenuation, contaminants are reduced by native microorganisms without any human augmentation. Second, bio-stimulation is employed where nutrients and oxygen are applied to the systems to improve their effectiveness and to accelerate biodegradation [8].

A feasible remedial technology requires microorganisms being capable of quick adaptation and efficient uses of pollutants of interest in a particular case in a reasonable period of time [9].

## **2 Materials and Methods**

### **2.1 Sample Collection**

Soil samples were collected at 5cm from the rhizosphere of cocoa, orange and timber 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  $37 \pm 2$  °C for 24hrs. Developed bacteria colonies were aseptically subcultured a number of times into fresh NA plates and incubated  $37 \pm 2$  °C for 24hrs until pure cultures of bacteria were obtained. Pure cultures were afterwards transferred onto McCartney bottles of doubled strength slant incubated at  $37 \pm 2$  °C for 24hrs and stored at 4 °C in the refrigerator [10].

## **2.3 Identification of isolates**

The bacteria 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, H<sub>2</sub>S 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 [11].

## **2.4 Preparation of biomass**

An inoculating loop was used to pick bacteria and inoculated on sterile nutrient broth and incubated at  $37 \pm 2$  °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 [12].

## **2.5 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<sub>2</sub>HPO<sub>4</sub> (2.0g), K<sub>2</sub>SO<sub>4</sub> (0.17g), NH<sub>4</sub>NO<sub>3</sub> (4.0 g), KH<sub>2</sub>PO<sub>4</sub> (0.53 g), MgSO<sub>4</sub>•7H<sub>2</sub>O (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 (13).

## 2.6 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 species were isolated from rhizosphere of the soil sample of orange, timber 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).

**Table 1 Biochemical and cultural characteristics of bacterial isolates**

H <sub>2</sub> S	MrVP	Suc	Glu	Lac	Ure	Gas	Cat	Ind	Cit	Sta	G.S	Probable Identity
-	+	-	+	-	+	AG	+	+	+	-	-	<i>Serratia marcescens</i>
-	+	-	+	-	+	-	+	-	+	+	-	<i>Enterobacter sp</i>
-	VP-	-	-	-	-	-	+	-	+	-	-	<i>Providencia stuartii</i>
-	VP-	+	+	-	-	AG	+	-	-	-	-	<i>Citrobacter freundii</i>
-	VP-	+	+	-	+	-	+	-	-	-	-	<i>Yersinia sp</i>
+	VP-	+	+	-	-	AG	+	+	-	+	-	<i>Edwardsiella tarda</i>
-	VP-	+	+	-	-	-	+	-	-	-	-	<i>Moellerella wisconsen</i>

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 agglomerans* isolated from timber

O3b *Providencia stuartii* 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

The occurrence of these microorganisms in the rhizosphere of all the soil samples examined varied. [14] 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 hydrocarbon utilizers which include *Serratia* sp, *Enterobacter* sp, *Citrobacter* sp, *Providencia* sp, *Edwardsiella* sp, *Yersinia* sp, *Moellerella* sp. The flora reflects the diverse heterotrophic bacteria present in rhizosphere soil and the diversity could be as a result of the varied sources of the refuse, dumped at the sites. Several hydrocarbon degrading organisms have been isolated from diverse environments; soil and aquatic sources which are the two major environments affected by hydrocarbon pollution [15] and their isolation is not restricted to hydrocarbon-bearing environments. The table below revealed the absorbance reading for *Moellerella* 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.

**Table 3: Degradation of crude oil by bacteria cells**

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

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)

In this study we focused 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. And 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 [16] 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 [17]. [18] 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 [19]. Because of the enormous quantities of crude and refined oils that are transported over long distances and consumed in large amounts, hydrocarbons have now become a very important class of substrates for microbial

oxidation [20]. 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.

#### 4 Conclusion

The bacteria isolated from rhizosphere were *Citrobacter freundii*, *Yersinia pestis*, *Edwardsiella tarda*, were isolated from rhizosphere of cocoa. *Serratia marcescens*, *Providencia stuartii* were isolated from rhizosphere of orange while *Enterobacter agglomerans*, *Moellerella wisconsinis* were isolated from rhizosphere of timber. The most effective hydrocarbon utilizing bacteria was *Enterobacter agglomerans*, 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.

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