Enhancement of microbial degradation of crude oil polluted soil using Mexican sunflower (*Tithonia diversifolia*) and wild groundnut (*Calapogonium mucunoides*)

Agbor R. B.^{1*} and Antai S. P.²

¹Department of Genetics and Biotechnology, University of Calabar, Calabar, Nigeria.

²Department of Microbiology, University of Calabar, Calabar, Nigeria.

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ABSTRACT

This study was driven by the quest for a cheaper and safer means of enhancing crude oil hydrocarbon degradation in the soil. Soil samples (0 to 20 cm) were polluted with crude oil at 300 ml, mixed thoroughly and allowed to stand for fourteen days. Thereafter, the agro-wastes: "*Calapogonium mucunoides* (CM) and *Tithonia diversifolia*" (TD), were applied using the following amendment levels: pristine control (0 g), crude oil control (0 g), 100 g CM, 150 g CM, 200 g CM, 100 g TD, 150 g TD, 200 g TD, 100 g CM + 100 g TD, 150 g CM + 150 g TD and 200 g CM + 200 g TD. The amended and unamended soils were thoroughly mixed and allowed to stand for 42 days. Microbial analysis was conducted for total heterotrophic bacteria and fungi, crude oil utilizing bacteria and fungi and total hydrocarbon contents. The microbial isolates identified were as follows: *Bacillus anthrax*, *Pseudomonas aeruginosa*, *Micrococcus luteus*, *Acinetobacter* sp., *Achromobacter* sp. and *Actinomyces* sp. as bacterial isolates. Fungi isolates identified were: *Aspergillus niger*, *Mucor indicus*, *Rhizopus* sp. and *Penicillium giabrum*. Among the various amended groups, 100 g TD had the highest bacterial count of 9.92×10^7 CFUg⁻¹, 200 g CM had a highest bacterial count of 2.25×10^8 CFUg⁻¹, 200 g TD had the highest fungal count of 9.62×10^5 CFUg⁻¹ and 200 g CM had the highest count of 8.46×10^6 CFUg⁻¹. However the combined amendment groups enhanced the microbial proliferation at 200 g CM + 200 g TD with highest bacterial count of 2.25×10^8 CFUg⁻¹ and 150 g CM + 150 g TD with highest fungal count of 8.83×10^6 CFUg⁻¹. The result showed that *Calapogonium mucunoides* and *Tithonia diversifolia* are effective in the enhancement of microbial degradation of crude oil-polluted soil.

Keywords: Degradation, crude oil, soil, microbial, polluted.

*Corresponding author. E-mail: agborreagan@yahoo.com.

INTRODUCTION

The growth of petroleum industries and the marketing of petroleum products in Nigeria have caused detrimental oil pollution in the environment especially in the Niger Delta region (Adewole and Bulu, 2012). Sources of petroleum pollution include oil wells blowout, tanker accidents, bunkering pipeline rupture, and sabotage; followed by subsequent discharge of crude oil into the environment. Crude oil spillage on the soil has distorted the original soil structure and also threatens human health and that of the organisms that are dependent on the soil for survival. It

also affects agricultural productivity, as most commercial farm plots are usually affected (Pezeshki et al., 2000).

Bioremediation is a technique that offers the possibility to destroy or render harmless, pollutants using natural biological activity. However, the bioremediation of crude oil pollution in the environment uses microorganisms to clean up oil spills. This is because certain microorganisms are advantageous through their abilities to transform hydrocarbon components into environmental friendly forms such as carbondioxide and water

(Akpoveta et al., 2011). But the potentiality of these microorganisms in breaking down hydrocarbon products is not reliable naturally, due to the fact that their breakdown or degradation activities are usually slow. Microorganisms play a significant role in the bioremediation of hydrocarbon products as several factors have also been recognized to be a limiting factor to this process. Many of such factors include nutrient availability, pH of soil, soil moisture content, oxygen content, soil structure and microbial populations (Osuji and Nwonye, 2007). Therefore, bioremediation also seeks to optimize these factors to enhance microbial degradation of the hydrocarbons.

In view of this, various suitable inorganic and organic materials have been applied to optimize these factors to enhance speedy biodegradation of the pollutants. Agro-wastes are organic materials developed from agricultural activities; and the use of agro-wastes appears to be a better option in ensuring an effective bioremediation of crude oil pollution. They are also recognized to be a cheaper means of bioremediation and proven to be more economically feasible and more versatile treatments. Agro-wastes are also the main source of nutrient supply to crude oil utilizing microbes (Swannell et al., 1996), which are largely dependent on these nutrients (nitrogen and phosphorus) for survival. The agro-wastes used in this study were "Mexican sunflower" (*Tithonia diversifolia*) and "wild groundnut" (*Calapogonium mucunoides*).

MATERIALS AND METHODS

The experiment was carried out in the Department of Genetics and Biotechnology, University of Calabar, Nigeria.

Microbial analysis was carried out in the Department of Microbiology, and total hydrocarbon content (THC) analysis was carried out in the Department of Chemistry, University of Calabar.

The crude oil (Bonny light) was obtained from Nigerian Agip Oil Company, Port Harcourt, Rivers State, while *Calapogonium mucunoides* and *Tithonia diversifolia* were collected from hedges around farms/households, along roadsides and in waste lands in Calabar.

The collected agro-wastes (*C. mucunoides* and *T. diversifolia*) were dried and then pulverized into powder form using electric blender. The pulverized samples were thereafter sieved through a 2mm sieve and stored in labeled containers.

Top soil (0 to 20 cm depth) samples were randomly collected from three points using a dutch auger, bulked to form a composite soil sample, weighed and transferred into 45 labeled plastic buckets with drainage holes at the base. The plastic buckets were arranged in triplicates in Completely Randomized Design (CRD).

The soil samples contained in the various plastic buckets except the pristine control group were polluted with 300 ml of crude oil. The plastic buckets containing the crude oil were mixed thoroughly and allowed to stand for fourteen days (this was to allow indigenous micro-organisms to be acclimatized with the new soil condition).

Soil treatment

After fourteen (14) days of soil acclimatization, the following amendments were applied:

1. *C. mucunoides* was applied based on the following concentrations: pristine (0 g), crude oil control (0 g), 100 g CM, 150 g CM and 200 g CM
2. The *T. diversifolia* was applied based on the following concentrations: Pristine (0 g), crude oil control (0 g), 100 g TD, 150 g TD and 200 g TD.
3. The combined treatments were applied on the following concentrations: pristine (0 g), crude oil control (0 g), 100 g CM + 100 g TD, 150 g CM + 150 g TD and 200 g CM + 200 g TD in the ratio of 1:1.

The forty five (45) perforated plastic buckets containing the amended and the unamended soil samples were watered every 2 days interval to keep the soil moist for microbial degradation of the hydrocarbon.

Soil samples were taken to the Department of Microbiology for determination of the bacterial and fungal population in the soil using surface plating method.

The total heterotrophic bacterial (THB) count was determined using the spread plate method on nutrient agar (NA) according to American Public Health Association (APHA) (1998). Soil suspensions were prepared by 10-fold serial dilutions with 1 g of soil, and 0.1 ml of 10^{-6} and 10^{-7} dilutions was spread on the plates in triplicates. The colony forming units (CFU) of the bacteria were counted after incubation at 28°C for 24 h.

The method of Sexton and Atlas (1977) was used. In this method the total number of fungi present in the soil was enumerated using surface spreading techniques. Serial dilutions of the soil samples were prepared from 10^{-6} and 10^{-7} , and 0.1 ml of 10^{-7} dilution was plated out on Sabouraud dextrose agar (SDA) plates. The plates were prepared in triplicate as for fungi and was incubated at 37°C for 72 h before the colonies were counted and expressed as colony forming units per gram of the soil samples (CFUg⁻¹).

The procedure of Hamamura et al. (2006) was used. Crude oil utilizing bacteria in the soil samples were enumerated by the viable count. Method using the surface spreading technique and the mineral salts medium of Zajic and Supplisson (1972). The mineral salts medium was solidified by the addition of 1.5% agar. Soil suspensions were prepared by 10-fold serial dilutions with 1g of soil and 0.1 ml of 10^{-4} and 10^{-6} dilution were spread on the plates in triplicates. After inoculation of the agar plates with the sample, a sterile filter paper (Whatman No. 1), saturated with crude oil, were aseptically placed onto the inside of the slide petri dishes. The filter paper saturated with crude oil served as a sole carbon and energy sources for growth of the organisms on the surface through vapour phase transfer (Okpokwasili and Amanchukwu, 1988). The plates were incubated in an inverted position at room temperature for 7 days, after which the average counts from triplicate plates were counted and recorded.

This was done using the surface spreading technique. The same procedures as described in the enumeration of crude oil degrading bacteria were used. But in this case, 0.1 ml of 10^{-6} dilution was plated onto mineral salt agar medium. After inoculation of the agar plates with the samples, sterile filter papers (Whatman No. 1), saturated with crude oil were placed onto the inside cover of the petri dishes to serve as a source of carbon and energy for the growth of fungi through vapor phase transfer (Okpokwasili and Amanchukwu, 1988). All plates were prepared in triplicates, inverted and incubated at 28°C for 7 days before the colonies were counted and expressed as colony forming units per gram of the soil sample (CFUg⁻¹). These isolates were sub-cultured onto freshly prepared Sabouraud dextrose agar (SDA) plates.

The bacterial isolates that were obtained from mineral salt medium were purified by repeated sub-culturing. The isolates were subjected to series of transfers unto fresh medium. The bacterial isolates were transferred onto fresh nutrient agar medium and incubated at 37°C for 24 h. The colonies of bacteria were maintained on slopes of nutrient agar (NA) and stored in a

refrigerator at 8°C. They were brought out when needed for studies.

Standard inocula was prepared from the preserved stock culture by taking a loopful of the isolates and aseptically inoculating onto sterile nutrient agar (NA) plates. The plates were incubated at 37°C for 24 h. The characterization of the isolates was performed, by employing gram staining reaction, oxidase test, catalase, test, citrate test, urease test, coagulase test, TSI (triple sugar iron agar) test, MIO (Motility, Indole, Ornithine) test and methyl red and voges proskauer test as described in Bergey's manual of determinative bacteriology 9th edition (1994). The fungal isolates were examined macroscopically and then microscopically using the wet mount method (cotton-blue-in-lactophenol). Fungal identification followed the scheme of Hunter and Bennett (1973).

The amount of crude oil in the soil samples were determined using air dried soil that was sieved through 1 mm mesh. One gram of the soil samples was suspended in 10 ml of hexane and shaken for ten minutes using a mechanical shaker. The solution was filtered using Whatman filter paper and the filtrate diluted by taking 1 ml of the extract into 50 ml of hexane (Akpoveta et al., 2011). The absorbance of this solution was read at 43 mm with Jenway model 6404 UV/VIS spectrophotometer using n-hexane as blank.

RESULTS AND DISCUSSION

The use of microorganisms in the bioremediation of crude oil pollution has become widely acceptable since certain microbes have been known to break down hydrocarbon compounds in order to derive their sole source of carbon and energy (Mehrasbi et al., 2003). In this study, microorganisms capable of degrading crude oil hydrocarbons were isolated from crude oil polluted soils amended with the agro-wastes: *Calapogonium mucunoides* (CM) and *Tithonia diversifolia* (TD) in both single and combined forms. Microbial populations were assessed in each amended soil samples and total hydrocarbon contents of the amended and unamended soil samples were also analyzed. The microbial analysis shows the degradation percentage of the crude oil hydrocarbons under 21 and 42 days respectively. Total heterotrophic bacterial count (THBC) of the soil amended with CM shows a degradation percentage of 14.82 and 15.39% under 21 and 42 days respectively and has highest bacterial count of 2.28×10^8 CFUg⁻¹ at 200 g CM concentration level (Figure 1). Soil amended with TD shows a degradation percentage of 11.07 and 11.87% under 21 and 42 days respectively and shows highest bacterial count of 9.92×10^7 CFUg⁻¹ at 100g TD concentration level while the combined (TD + CM) amended soil shows a degradation percentage of 14.23 and 14.47% under 21 and 42 days respectively, and has highest bacterial count of 2.25×10^8 CFUg⁻¹ at 200 g CM + 200 g TD concentration level. For the total heterotrophic fungi (THFC), soil amended with CM shows a degradation percentage of 4.73 and 5.91% under 21 and 42 days respectively and has highest fungal count of 8.46×10^6 CFUg⁻¹ at 200 g CM, soil amended with TD shows a degradation of 5.53 and 6.97% under 21 and 42 days respectively and has highest fungal count of 9.62×10^6 CFUg⁻¹ at 200 g TD while the combined amendment (TD + CM) shows a degradation percentage of 6.27 and

6.92% under 21 and 42 days respectively and has highest fungal count of 8.83×10^6 CFUg⁻¹ at 150 g CM + 150 g TD (Figure 2). The results reveal that the different amendment stimulates microbial proliferation at different rates and these microbial proliferations reflect on the rate of biodegradation of the amended soil samples. Microbial proliferation in this study is attributed to be a response to the nutrient contents of the agro-wastes (*Calapogonium* and *Tithonia*). This however suggests that the agro-wastes, are rich in nutrient such as nitrogen and phosphorus required for microorganisms to grow in the soil. For the crude oil utilizing bacteria, soil amended with CM shows a degradation percentage of 8.71 and 10.4% under 21 and 42 days respectively, and has the highest bacterial count of 6.15×10^6 cfug⁻¹ at 100 g CM. Soil amended with TD shows a degradation percentage of 16.96 and 15.95% under 21 and 42 days respectively and has the highest bacterial count of 2.61×10^7 CFUg⁻¹ at TD 200 g. The combined treatment (TD + CM) shows a degradation percentage of 13.86 and 15.61% under 21 and 42 days respectively and has the highest bacterial count of 2.43×10^7 CFUg⁻¹ at 200 g CM + 200 g TD. Soil amended with CM shows a degradation percentage of 4.46% and 6.0% under 21 and 42 days respectively, and has the highest fungal count of 7.78×10^5 CFUg⁻¹ at 200 g CM level. TD shows a degradation percentage of 5.76 and 6.23% under 21 and 42 days respectively and has the highest fungal count of 8.98×10^5 CFUg⁻¹ at TD 200g level. The combined treatment (TD + CM) shows a degradation percentage of 5.79 and 7.83% under 21 and 42 days respectively and had the highest fungal count of 9.83×10^5 CFUg⁻¹ at 150 g CM + 150 g TD level. The result also shows that crude oil utilizing bacteria and fungi were more in soils amended with 100 g CM (6.15×10^6 CFUg⁻¹) and 150 g CM + 150 g TD (9.83×10^5 CFUg⁻¹) respectively. Also, Eneje et al. (2012) recommended *C. mucunoides* (CM) for effective bioremediation of crude oil polluted soil when combined with pig dung and Nkereuwem et al. (2010) reported that *T. diversifolia* (TD) has effective crude oil remediation potential when combined with organo-mineral fertilizer (OMF). These reports also reflect on the results of this study that the combined form (CM + TD) enhances higher biodegradation of the crude oil hydrocarbons in the soil more than the single amendments which although are also effective. In this study, Mexican sunflower (*T. diversifolia*) and wild groundnut (*C. mucunoides*) were used as amendments to observe their enhancement potentials in microbial degradation of crude oil hydrocarbons in the soil. From the result obtained, it can be summarized that *C. mucunoides* (CM) and *T. diversifolia* (TD) possess a great potential in enhancing microbial degradation of crude oil hydrocarbons. Generally, these amendments significantly reduced the total hydrocarbon content (THC) and significantly increased the population of crude oil utilizing bacteria (CUB) and fungi (CUF) respectively in the amended soils (Figure 3 and 4). The microbial isolates identified were as

Figure 1. Proliferations of heterotrophic bacteria in amended soil.

Figure 2. Proliferations of heterotrophic fungi in amended soil.

follows: *Bacillus anthrax*, *Pseudomonas aeruginosa*, *Micrococcus luteus*, *Acinetobacter sp*, *Achromobacter sp* and *Actinomyces sp* as bacterial isolates (Table 1). Fungi isolates identified were: *Aspergillus niger*, *Mucor indicus*,

Rhizopus sp and *Penicillium giabrum*. However, *Pseudomonas sp.*, *Bacillus sp.*, and *Aspergillus sp* have been reported by several authors to possess specific abilities in the degradation of hydrocarbon products in the

Figure 3. Proliferations of crude oil utilizing bacteria in amended soil.

Figure 4. Proliferations of crude oil utilizing fungi in amended soil.

Table 1. Cultural characteristics cell and biochemical characterization of bacteria isolates.

Sample code	Isolate No	Cultural characterization of bacteria isolates	Cell or morphological shapes	Gram reaction	MIO test			MRVP test			Carbohydrate utilization test using TSI			Probable organism					
					Motility	Indole	Ornithine	Methyl red	Voges proskauer	Citrate	Catalase	Oxidase	Coagulase		Lactose	Sucrose	Glucose	Gas	Hydrogen sulphide
CM	1.	Smooth, round and greenish pigment colony	Slightly curved rod	-	+	-	-	+	-	+	+	+	-	-	-	-	-	-	<i>Pseudomonas aeruginosa</i>
	2.	Large, irregular, flat colony	Bacilli rod	+	+	-	+	-	+	+	+	+	-	-	-	+	-	-	<i>Anthrax bacillus</i>
	3.	Red colony, mucoid and spread	Rod in pairs	-	-	-	-	-	+	+	+	-	-	+	+	-	+	+	<i>Acinetobacter</i> spp.
TD	1.	Pale yellow, smooth glistening colour	Bacilli rod	-	+	-	-	-	-	+	+	+	-	+	-	+	-	-	<i>Achromobacter</i> spp.
	2.	Circular waxy yellow pigmented	Branched rod	+	-	-	-	-	+	+	-	-	-	-	-	-	-	+	<i>Actinomyces</i> spp.
	3.	Bright yellow convex and raised colony	Cocci in clusters	+	-	-	+	+	-	+	+	+	-	-	-	-	-	+	<i>Microbial luteus</i>
CM + TD	1.	Smooth, round and greenish pigmented	Curved rod	-	+	-	-	+	-	+	+	+	-	-	-	-	-	-	<i>Pseudomonas aeruginosa</i>
	2.	Large irregular and flat colony	Bacilli rod	+	+	-	+	-	+	+	+	+	-	-	-	+	-	+	<i>Bacillus anthrax</i>
	3.	Pale yellow, smooth glistening colour	Bacilli rod	-	+	-	-	-	-	+	+	+	-	+	-	-	+	-	<i>Achromobacter</i> spp.
	3.	Smooth, round and greenish pigment colony	Slightly curved rod	-	+	-	-	+	-	+	+	+	-	-	-	-	-	-	<i>Pseudomonas aeruginosa</i>

soil and water ecosystem. The presence of these microbes and the rate at which the hydrocarbon products were degraded in the soil confirms earlier reports.

CONCLUSION

The result obtained showed that *C. mucunoides* and *T. diversifolia* enhanced the microbial proliferation and biodegradation of hydrocarbons in crude oil polluted soils. It was concluded that *Calapogonium* and *Tithonia* should be considered as an effective material for bioremediation of crude oil-polluted soils.

REFERENCES

- Adewole MB, Bulu YI, 2012.** Influence of different organic-based fertilizers on the phytoremediating potential of *Calapogonium mucunoides* from crude oil polluted soils. J Bioremediat Biodegrad, 3: 144.
- Akpoveta OV, Egharevba F, Medjor OW, Osaro KI, Enyemike ED, 2011.** Microbial degradation and its kinetics on crude oil polluted soil. Res J Chem Sci, 1(6): 8-14.
- APHA, 1998.** Standard Methods for the Examination of Water and Wastewater. 20th edition. American Public Health Association, Washington, D.C.
- Eneje RC, Nwagbara C, Uwumarongie-Ilori EG, 2012.** Amelioration of chemical properties of crude oil contaminated soil using compost from *Calapogonium mucunoides* and poultry manure. Int Res J Agric Sci Soil Sci, 2(6): 246-251.
- Hamamura N, Olson SH, Ward DM, Inskeep WP, 2006.** Microbial population dynamics associated with crude oil biodegradation in diverse soils. J Appl Environ Microbiol, 72(9): 6316-6324.
- Mehrasbi MR, Haghighi B, Shariat M, Naseri S, Kaddafi K, 2003.** Biodegradation of petroleum hydrocarbons in soil. Iran J Public Health, 32: 28-32.
- Nkereuwem ME, Edem ID, Fagbola O, 2010.** Bioremediation of oil polluted soils with organomineral fertilizer (OMF) and Mexican sunflower (*Tithonia diversifolia*). Nig J Agric Food Environ, 6(1 & 2): 13-20.
- Okpokwasili GC, Amanchukwu SC, 1988.** Petroleum hydrocarbon degradation candida species. Int J Environ, 14: 243-247.
- Osuji CL, Nwonye I, 2007.** An appraisal of the impact of petroleum hydrocarbon on soil fertility: the Owaza experience. Afr J Agric Res, 2(7): 318-324.
- Pezechki SR, Hester MW, Lin Q, Nyman JA, 2000.** The effects of oil spill and clean up on dominant US Gulf coast marsh macrophytes: A review. Environ Pollut, 108: 129-139.
- Swannell RPJ, Lee K, McDonagh M, 1996.** Field evaluations

of marine oil spill bioremediation. Microbiol Rev, 60: 342-365.
Zajic E, Supplisson B, 1972. Emulsification and degradation of "bunker C" fuel oil by microorganisms. J Biotechnol Bioeng, 14: 331-343.

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