COMPARATIVE STUDY ON THE EFFECT OF SYMBIOTIC INTERACTION BETWEEN PLANTS AND NON-INDIGENOUS ISOLATES ON CRUDE OIL REMEDIATON

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Abstract. Effect of the symbiotic interaction between plants and non-indigenous isolates in remediation of crude oil contaminated soil was studied. Three organisms including *Bacillus subtilis, Pseudomonas putida* and *Candida albicans* obtained from Nigerian Institute of Medical Research (NIMR) were used. The plants used for this study were four annual indigenous crops including two annual forage leguminous crop, vegetable cowpea (*Vigna unguiculata* var *unguiculata*) and velvet bean *Mucuna pruriens*; a cereal- maize (*Zea mays*) and a vegetable crop- fluted pumpkin (*Telfaira occidentalis*). Gas chromatographic (GC) analysis revealed the total petroleum hydrocarbon (TPH) of sample comprising of sterilized soil seeded with *Bacillus subtilis*, sterilized soil with *Pseudomonas putida* and sterilized soil with *Candida albicans* to be 1.721 mg/kg, 5,791mg/kg and 4.987mg/kg respectively. Treated soil seeded with *B. subtilis* recorded the least value followed by treated soil with *C. albicans* and treated soil with *P. putida* in that order. However, for *Z. mays* sample that was coated with *B. subtilis* recorded the least value of 2,339mg/kg. By contrast though, amongst all the plant samples *V. unguiculata* coated with *C. albicans* recorded the lowest TPH value of 1,902mg/kg whereas *T. occidentalis* coated with *P. putida* had the lowest TPH value of 2.285mg/kg. Different alkane groups degraded during these remediation processes were also highlighted. C alkanes ranging from $C_8 - C_{12}$ were removed though some plants were not able to degrade C_8 and/or C_9 whereas C_{40} was generally degraded by all set ups. Statistical analysis depicting the effect of individual plant samples and non- indigenous microorganisms and different plants per individual non- indigenous microorganisms in degrading different concentration of crude oil at 5% significant difference and 95% confident limit was analysed using SPSS software. It showed that the performance of *B. subtilis* was more acceptable. Generally, the TPH values obtained from all the samples coated with non-indigenous isolates on comparative to the control samples with TPH of 9,487 mg/kg, 8376 mg/kg, 4517 mg/kg and 3828 mg/kg for *V. unguiculata, Z. mays, T. occidentalis* and *Mucuna pruriens* respectively had low TPH values.

Keywords: Bioremediation, crude oil, agricultural soil, crop plants, microorganisms, total petroleum hydrocarbon (TPH), Nigeria.

INTRODUCTION

The increasing dependence of humanity on fossil fuels, especially crude oil has led to the pollution of agricultural lands. The spillage of this oil usually occur during extraction and processing operations by many oil companies sited in oil producing countries in Nigeria. Such environmental impact is one of the major consequences of economic development [6, 14, 15, 17, and 22]. However, pollution of agricultural lands and groundwater can be as a result of not only industrial effluents/waste discharges, domestic and agricultural waste, but also as a result of oil spills which emanates from pipeline ruptures, oil well blowouts, seepages, tanker accidents and other diverse operations which contaminates the environment and negatively affects human health [2, 12, 14-16]. Furthermore, crude oil pollution on land also affects germination and growth of some plants, though the scale of impact depends on the quantity and type of oil spilled [18]. The effects of these pollutants to the environment and human health, has lead to quest for its reduction to at least environmentally safe levels [9].

The use of conventional remediation method in restoration of polluted sites has been reported to have adverse effects, expensive and disruptive to sites [20]. Soil excavation could lead to the generation of toxic air emissions, burning in burrow pits results in air pollution and landfills can lead to contamination of groundwater [10]. These are prohibitatively expensive when the amount of contaminants are large [23-24], and often results in cleanup delays while the contaminated soil continues to pollute the groundwater resources if on land and death of aquatic life if on water ways [1, 13, 19]. However, necessitating speedy removal of the contaminants is required; thus, quest for effective remediation of crude oil contaminated soil has made researchers to discover plants which in interaction with microorganisms within the rhizosphere, could be used for effective, inexpensive and less intrusive cleanup and restoration of contaminated soil [10, 21] .

This study is therefore, aimed at using the symbiotic interaction between plants and microorganisms to accomplish both effective detoxification and volume reduction, besides, this technology is believed to be non invasive and relatively cost effective.

MATERIALS AND METHODS

Sample Collection

The plant seed samples used for this study are seeds of four annual indigenous crops including two annual forage leguminous crop, vegetable cowpea (*Vigna unguiculata var unguiculata*) and velvet bean *Mucuna pruriens*; a cereal- maize (*Zea mays*) and a vegetable crop- fluted pumpkin (*Telfaira occidentalis*). These plant seeds were collected from different locations in the South Eastern part of Nigeria. The crude oil used was bonny light Crude Oil and was collected with sterile containers from Akiri in Oguta, Imo State Nigeria. Whereas the soil sample for microbial analysis was collected from an agricultural soil using sterile

containers, at the depth of 1-30cm, and taken to the laboratory for analysis within 1 hour of collection.

Seed Preparation prior to Cultivation

All the seeds to be used for these analyses were surface sterilized by washing and shaking in 75% ethanol for 30seconds, rinsed three times with sterile water for 10 minutes each, after which they were washed with 5.25% sodium hypochlorite solution for 15minutes and then rinsed three times again with sterile water for 10 minutes per wash [25]. The seeds were allowed to germinate before planting. The germination was by incubating the seeds on wet sponges pre-sterilized by soaking them in a 2.5% sodium hypochlorite solution for 30 minutes, rinsed and autoclaved in a foil – covered beaker containing water making sure that the sponge was not submerged [26]. Following this, the surface sterilized seeds were placed on the sterile wet sponges and kept in a growth chamber with a light cycle consisting of 11h of darkness and 13h of light and with 65% humidity at 25 ^{0}C [26].

Microbial Inoculation of Germinated Seeds

After 3 – 7 days depending on the crop plant, the germinated seeds were inoculated with microbial culture by placing the seeds in an open Petri dish under a laminar flow hood. Followed by transfer of 10ml of an overnight bacterial culture grown in LB medium (Luria – Bertani Medium) into the seed – containing plate. This was allowed to dry under the hood for 4h before planting [26].

Exposure of the Plants to varying concentrations of crude oil

After 28 days of plant growth, 100ml of crude oil together with 50 ml of sterile water were added to the potted plants. Thereafter, no additional water was added during the remaining period of the experiment [26]. The same procedure was carried out for all the test plants.

Assay for Total Petroleum Hydrocarbon (TPH) level of crude oil using Gas Chromatography (GC).

The difference in the concentration of crude oil in polluted soils was determined to show the extent of degradation by the corresponding plants and isolates. This was determined using the gas chromatograph (GC), carried out by Technology Partners International Nigeria Limited (TPI), Port Harcourt.

RESULTS

The result of the interaction between plants and microorganisms (rhizoremediation) in remediating crude oil contaminated soil is as shown in Table 1. Generally, the TPH values of the samples in comparison to that of the control A anb B sample (Table 1) showed that there was degradation of alkanes from the crude oil sample and the various C-alkanes removed are as shown in Figures I – IV. However table 1 show that, for *Z. mays* samples coated with the organisms, that coated with *B. subtilis* recorded the least value of 2.339 mg/kg. By contrast though, amongst all the plant samples, *V. unguiculata* coated with *C. albicans* recorded the lowest TPH value of 1.902 mg/kg whereas *T. occidentalis* coated with *P. putida* and mixture of the three organisms had the lowest TPH values of 2.285mg/kg and 2.119mg/kg respectively. Generally, the TPH values obtained from all the samples coated with non-indigenous isolates on comparison to the control samples had low TPH values except Z. *mays* coated with *C. albicans* which had a higher TPH than control A but lower than control B (Table 1).

Interestingly, the individual carbon (C) chain removed from the crude oil as a result of hydrocarbon degradation shown in the figures depicts that aside C_6 which was originally absent in the crude oil sample used, the processes used were able to degrade $C_{7,10,11,12}$, and C_{40}. While some degraded only C_9, some did not. However, C_8 was not degraded by all the treatment processes employed in the study.

Statistical analysis on the use of the test plants seeded with the test isolates was also carried out (Fig. VI) to determine the effect of non-indigenous isolates and the study soil and the interactions between plants and microorganisms from different habitats in sustainable remediation of crude oil contaminated soil. Analysis of variance at 5% significant difference and 95% confidence limit depicts that there are obvious significant differences in the rate of degradation of crude oil by the test plants in interaction with the test organisms. The plants seeded with *B. subtilis* (A), reduced the crude oil concentration of the soil samples more than others. Comparing degradation effect per plant- per different organism – per concentration, rhizoremediation using *T. occidentalis* showed a better promise.

Table 1. TPH values for rhizoremediation studies using non indigenous isolates.

Plant Species	TPH values (mg/kg) after treatment with various microbial species					
	B. subtilis	*P. putida*	*C. albicans*	Mixture of ABC	Control A (sterile seed + sterile soil)	Control B (Sterile soil only without plant)
V.unguiculata var unguiculata	3383	3449	1902	4.324	9487	10,380
Z. mays	2339	4935	9405	3.147	8376	10,380
T. occidentalis	2928	2285	1285	2.119	4517	10,380
M. pruriens	2517	2958	2818	3.258	3826	10,380

Legend: A – *Bacillus subtilis*; B – *Pseudomonas putida;* C – *Candida albicans.*

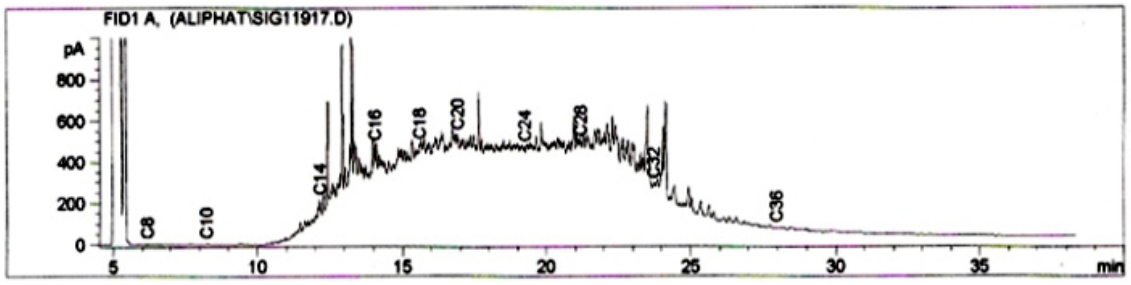

Figure Ia. Specific alkane group degraded during symbiotic interaction between *T. occidentalis* and *B. subtilis.*

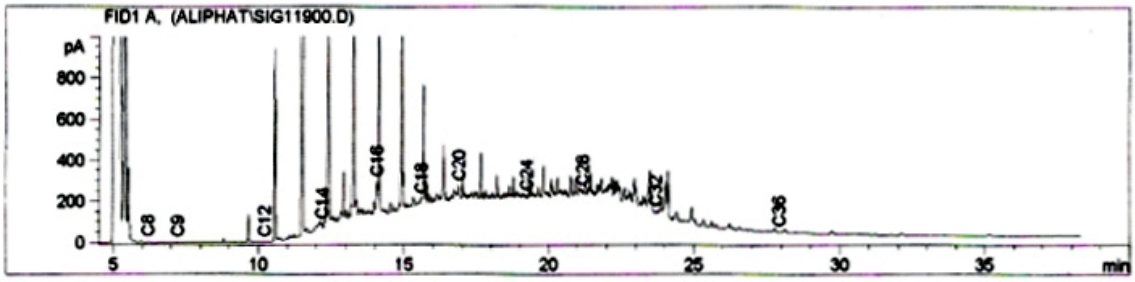

Figure Ib. Specific alkane group degraded during symbiotic interaction between *T. occidentalis* and *P. putida.*

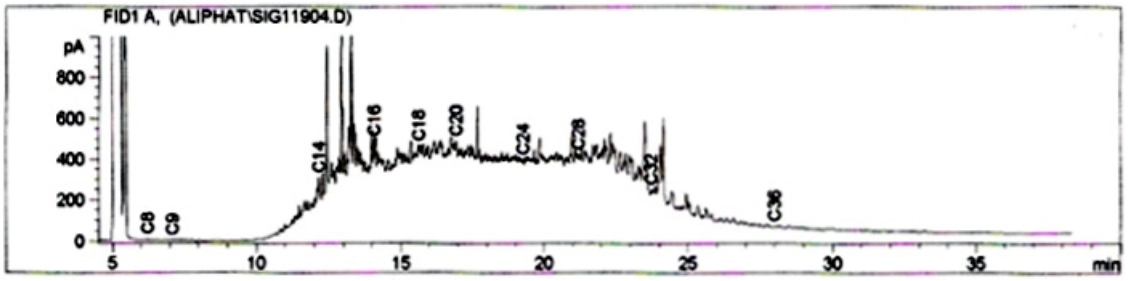

Figure Ic. Specific alkane group degraded during symbiotic interaction between *T. occidentalis* and *C. albican.*

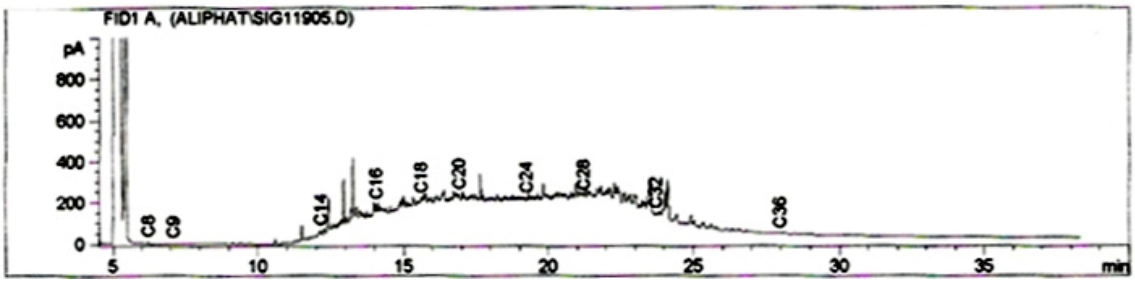

Figure Id. Specific alkane group degraded during symbiotic interaction between *T. occidentalis* and mixture of the three organisms (*Pseudomonas putida, Bacillus subtilis* and *Candida albicans*).

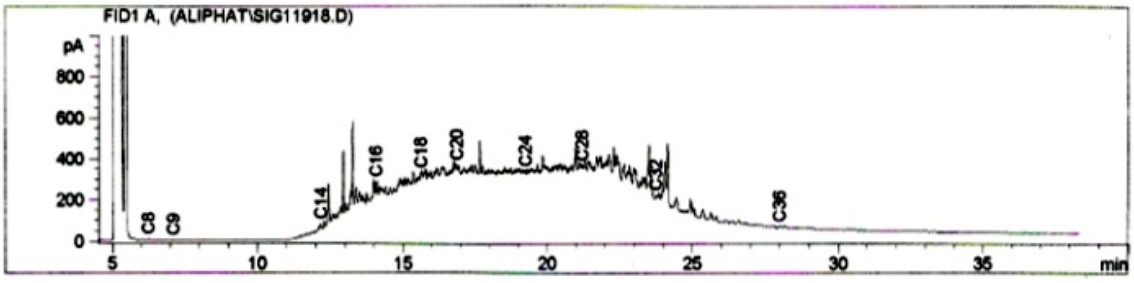

Figure IIa. Specific alkane group degraded during symbiotic interaction between *V. unguiculata* and *B. subtilis.*

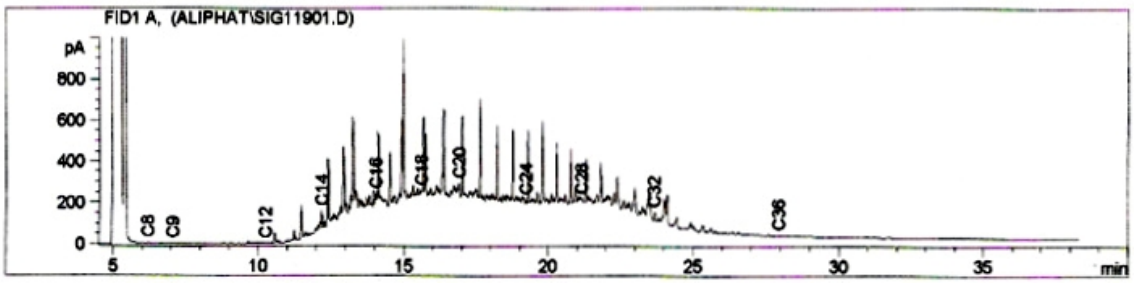

Figure IIb. Specific alkane group degraded during symbiotic interaction between *V.unguiculata* and *P. putida.*

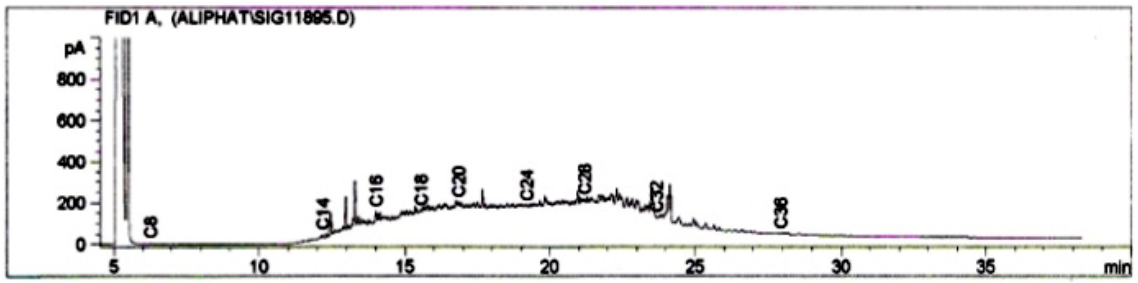

Figure IIc. Specific alkane group degraded during symbiotic interaction between *V.unguiculata* and *C. albicans*

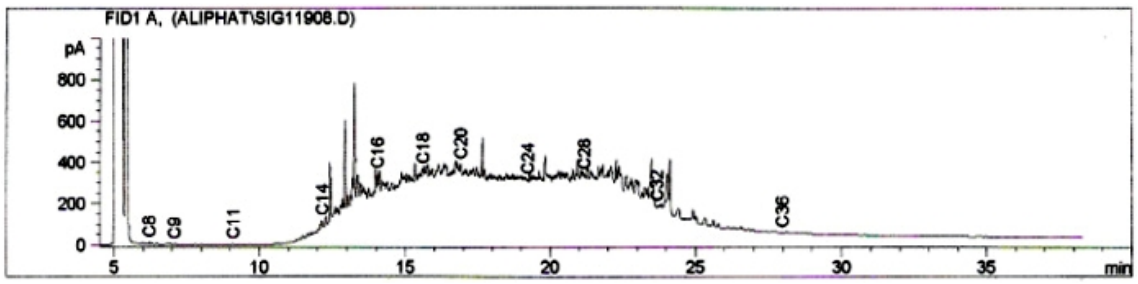

Figure IId. Specific alkane group degraded during symbiotic interaction between *V. unguiculata* and mixture of the three organisms (*Pseudomonas putida , Bacillus subtilis* and *Candida albicans*).

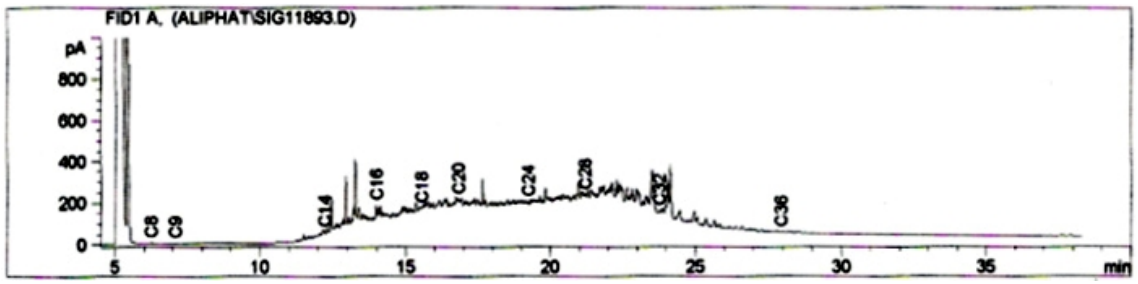

Figure IIIa. Specific alkane group degraded during symbiotic interaction between *Z mays* and *Bacillus subtilis.*

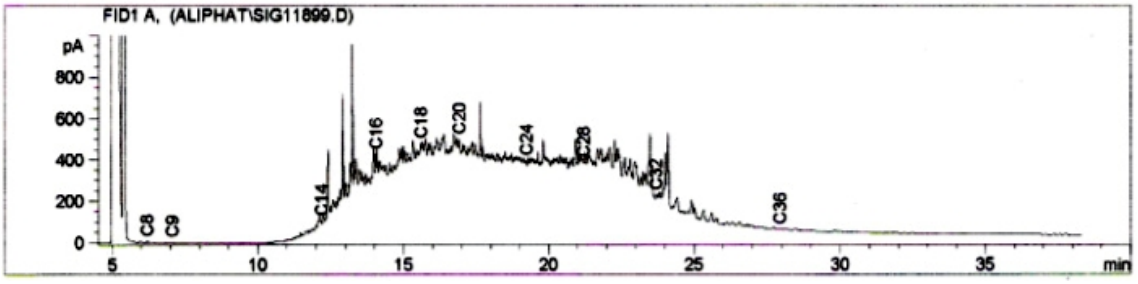

Figure IIIb. Specific alkane group degraded during symbiotic interaction between maize (*Zea mays*) and *Pseudomonas putda.*

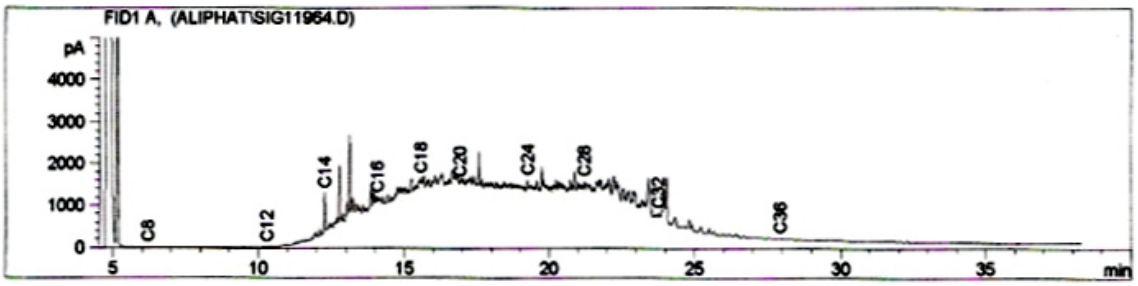

Figure IIIc. Specific alkane group degraded during symbiotic interaction between *Z mays* and *Candida albicans.*

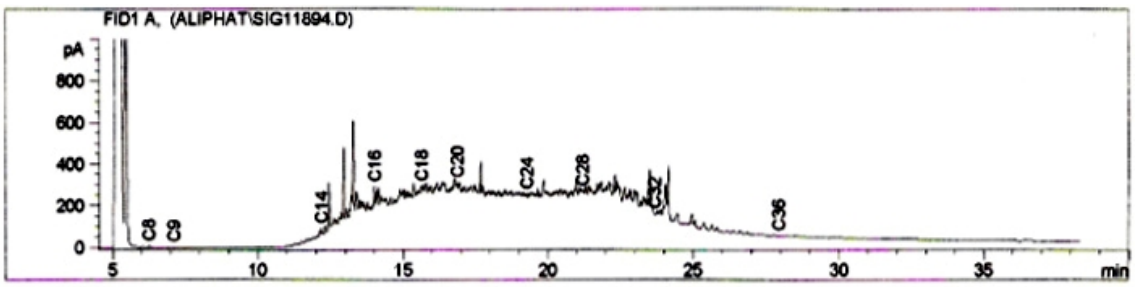

Figure IIId. Specific alkane group degraded during symbiotic interaction between *Z mays* and mixture of the three organisms (*Pseudomonas putida , Bacillus subtilis* and *Candida albicans*).

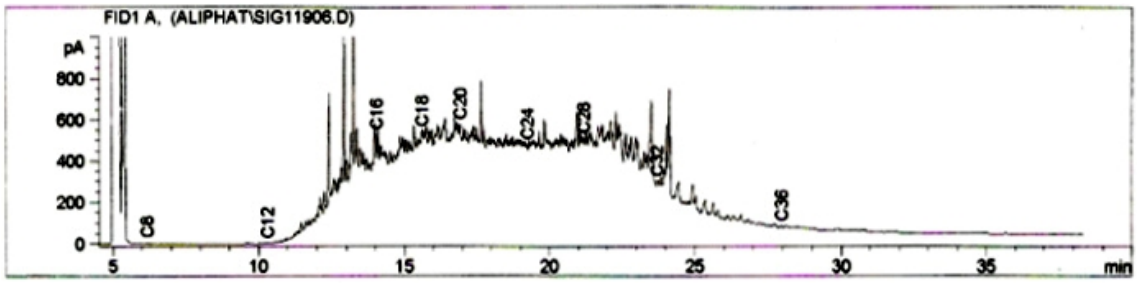

Figure IVa. Specific alkane group degraded during symbiotic interaction between *M. pruriens* and *B. subtilis*.

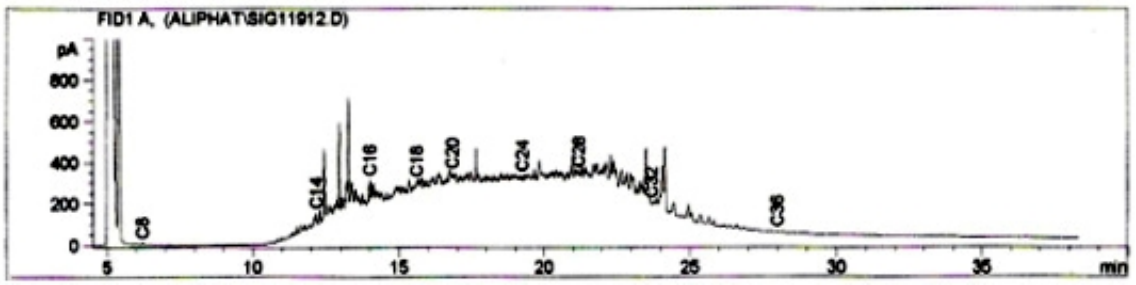

Figure IVb. Specific alkane group degraded during symbiotic interaction between *M. pruriens* and *P. putida.*

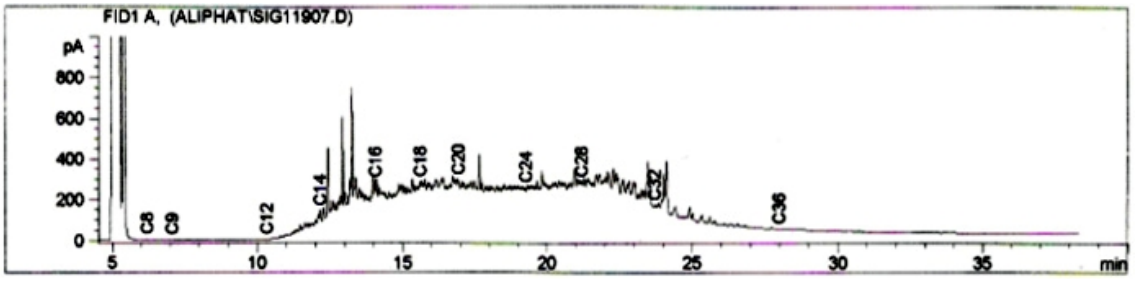

Figure IVc. Specific alkane group degraded during symbiotic interaction between *M. pruriens* and *C. albicans.*

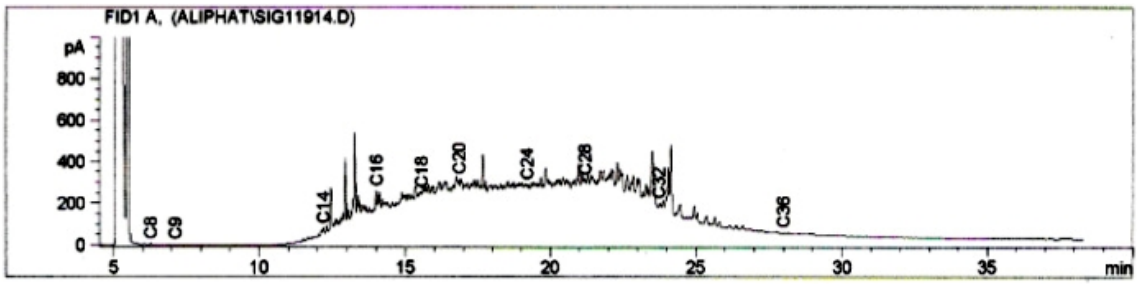

Figure IVd. Specific alkane group degraded during symbiotic interaction between *M. pruriens* and mixture of the three organisms (*Pseudomonas putida , Bacillus subtilis* and *Candida albicans*).

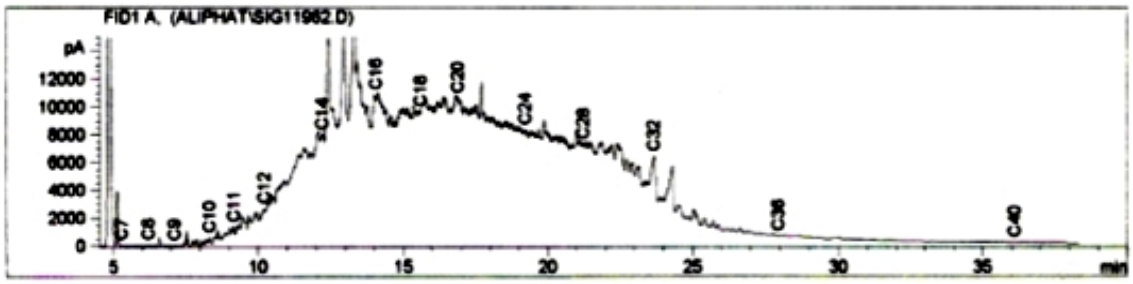

Figure V. Specific alkane groups originally present in the crude oil used in polluting sterile soil only (control).

DISCUSSIONS

The result obtained from the use of the known bacterial and fungal isolates (which were non indigenous to the soil under study) vis-à-vis *Pseudomonas putida, Bacillus subtilis* and *Candida albicans* obtained from NIMR, in coating the germinated seeds prior to planting as observed in this study showed varying degree of reduction in the concentration of TPH. This however, agrees with the findings of Yee *et al*. [26] who removed trichlorotethylene (TCE) from the soil using wheat coated with a recombinant *Pseudomonas fluorescens* that expresses the genes for taking up this pollutant. It also lend more weight to the observation made by Jussila, [8] who reported that coating of seed with bacteria is often used to apply beneficial microbes in a bioinoculant.

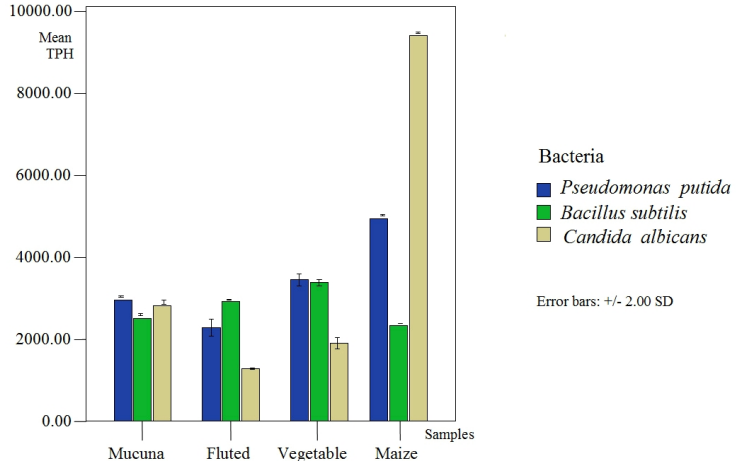

Figure VIa. Statistical analysis depicting the effect of individual plant samples and non- indigenous microorganisms in degrading different concentration of crude oil (during Rhizoremediation and Bioaugumentation) at 5% significant difference and 95% confident limit.

Amongst the *Zea mays* samples, plant samples coated with *Bacillus subtilis* had the lowest value of TPH concentration. This showed that the exudates produced by the root of the *Zea mays* stimulated the growth of *Bacillus subtilis* which possibly encouraged the biological interactions between the roots and the organism, and induced the metabolic and enzymatic activities of the organisms as reported by Lines-Kelly [11] and Juhanson *et al.* [7]. By contrast, however, samples containing *V. unguiculata* coated with *Candida albicans* and *T. occidentalis* coated with *P. putida* and mixture of the three isolates (multispecies) was lower than that of the other plant samples coated with similar organisms. This however, shows that *V. unguiculata* supported the activities of *Candida albicans* more than *T. occidentalis* while the vegetable

crop *T. occidentalis* enhanced *P. putida* proliferation more than *V. unguiculata* and the non-leguminous plant as observed in this study. This possibly could be due to the composition of the exudates which, according to Lines-kelly [11], depends on the plant stages of growth and plant type, which forms the basis of attracting or repelling nature of root exudates towards particular microbial species and populations. This also supports the findings of Aprill and Sims [3] that legumes such as alfafa and cowpea posses highly branched roots that habour microorganisms and promote their survival and ecological interactions in the rhizosphere; and lends more weight to the findings of Jussila [8] that used goat's rue plant, *Galega orientalis*, a leguminous plant which was seeded with *Rhizobium galegae, Bacillus, Rhodobacter, Arthrobacter, Pseudomonas putida* and Gram negative *Pseudomonas* sp., respectively to remove crude oil from contaminated soil. From this study, therefore, it is obvious that *C. albicans* and *P. putida*, performed better on interaction with roots of legumes whereas *B. subtilis* was better on non-leguminous plants which was significant in the result obtained in this study. By contrast though, high TPH value on comparison with control A obtained in sample with *Z. mays* coated with *C. albicansis* indicates that the presence of the organism in question affected the performance of the plant hence an ammensalistic relationship ensued. Nevertheless, performance of only the plant without *C. albicans* but with *B. subtilis and P. putida* seems better and preferable.

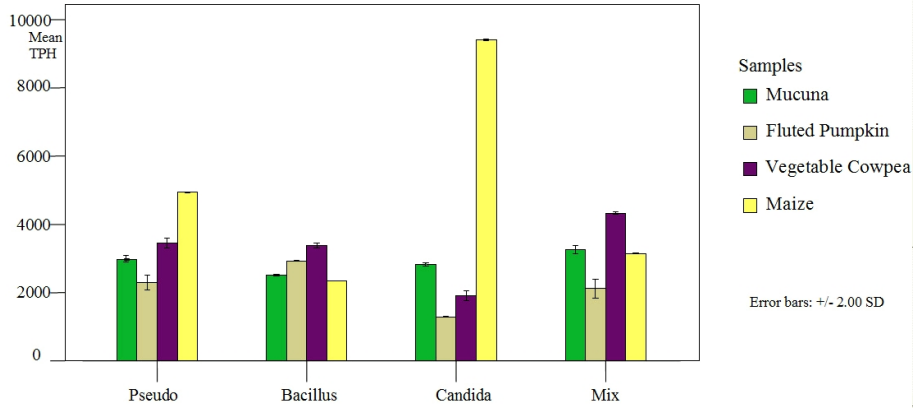

Figure VIb. Statistical analysis depicting the effect of different plants per individual non- indigenous microorganisms in degrading different concentration of crude oil (during Rhizoremediation) at 5% significant difference and 95% confident limit.

The lowest TPH value observed in *T. occidentalis* coated with a mixture of the three organisms could also be as a result of synergistic effort of the bacteria in the mixture whose proliferation was supported by the said plant. This also is in consonance with the findings of Jussila [8].

Gas chromatographic analysis carried out in this study indicated that $C_{7,10,\ 11,12}$,and C_{40} were degraded during the process. Nonetheless, biodegradation of n-alkanes with molecular chain lengths up to n-C_{44} have been demonstrated which according to Atlas [4] normally proceeds by monoterminal attack resulting in the formation of a primary alcohol, an aldehyde and a monocarboxylic acid. These attacks have also been reported elsewhere [5] to be initiated by monogenase enzymes produced by microorganisms such as *Corynebacterium* sp. Similar enzymes have also been detected in other bacteria and yeasts such as *Acinetobacter calcoaceticus, Pseudomonas putida* and *Candida tropicalis, Candida rugosa* and *Candida lipolytica* respectively [4-5]. This, however, is in line with the findings of this study.

REFERENCES

[1] Abed, R.M.M., Safi, N.M.D., Koster, J., de Beer, D., El-Nahhal, Y., Rullkolter, J., Garcia-Pichel, F., (2002): Microbial diversity of a heavily polluted microbial mat and its community change following degradation of Petroleum compound. Applied and Environmental Microbiology, 68(4): 1674-1683.

[2] Anderson, B., (1996): The Petroleum Industry and the Nigerian Environment: Environmental Issues and Management Strategies. A key note address at the International seminar on environment, The Shell Petroleum Development Company (SPDC). Port Harcourt, pp. 1-14.

[3] Aprill, W., Sims, R.C., (1990): Evaluation of the use of prairie grasses for stimulating polycyclic aromatic hydrocarbon treatment in soil. Chemosphere, 20: 253-265.

[4] Atlas, R.M., (1981): Microbial degradation of petroleum hydrocarbons: An environmental perspective. Microbiological Reviews, 45: 180-209.

[5] Higgins, I.J., Gilbert, P.D., (1989): The Biodegradation of Hydrocarbons. Cambridge University press, Cambridge, UK., pp 80- 106.

[6] Ifeadi, C.N., Nwankwo, J.N., (1989): Oil spill incident in Nigeria Petroleum Industry: A critical analysis. Napetcor, 8: 11-45.

[7] Juhanson, J., Truu, J., Heinaru, E., Heinaru, A., (2007): Temporal dynamics of microbial community in soil during phytoremediation field experiment. Journal of Environmental Engineering and Landscape management, 4(15): 213-220.

[8] Jussila, M.M., (2006): Molecular Biomonitoring during Rhizoremediation of oil- contaminated soil. Dissertationes bioscientiarum molecularium Universitatis Helsingiensis, Vikki, pp. 13- 66

[9] Khan, A.G., Kuek, C., Chaudhny, T.M., Khoo, C.S., Hayes, W.J., (2000): Role of Plants mycorrhizae and

phytochelators in heavy metal contaminated land remediation. Chemospgere, 41: 197-207.
[10] Kuiper, I., Lagendijk, E.L., Bloemberg, G.O., Lugtenberg, B.J.J., (2004): Rhizoremediation. A beneficial plant-microbe interaction. Molecular Plant Microbe Interactions, 17(1): 6-15.
[11] Lines-Kelly, R., (2005): The rhizosphere: soil biology basics. Profitable and sustainable primary industries, www.dpi.nsw.gov.au
[12] Mentzer, E., Ebere, D., (1996): Remediation of hydrocarbon contaminated sites. Proceedings of the International Seminar on Petroleum Industry and the Nigerian Environment, Nigerian National Petroleum Corporation (NNPC), pp. 51-59.
[13] Mishra, S., Jyot, J., Kuhad, R.C., Lal, B., (2001): Evaluation of inoculum addition to stimulate *in situ* bioremediation of oily sludge-contaminated soil. Applied and Environmental Microbiology, 67(4): 1675-1681.
[14] NNPC, (2004a): Use of sophisticated alarm systems will deter pipeline vandalization. NNPC News – Official Monthly Newsletter of the Nigerian National Petroleum Corporation, 25(6): 3.
[15] NNPC, (2004b): PPMC loses N34 billion to pipeline vandalization. NNPC News – Official Monthly Newsletter of the Nigerian National Petroleum Corporation, 25(8): 1-2.
[16] Okecha, S.A., (2000): Pollution and conservation of Nigeria's environment. T'Afrique International Associates, Owerri, Nigeria pp. 33-42.
[17] Olagbinde, O.T., Ede, G.O., Inyang, L.E.D., Gundlach, E.S., Page, D.S., (1999): Scientific and clean up response to the Idoho-Qua Iboe Terminal oil spill, Nigeria. Environmental Technology, 20: 213-222.
[18] Onwurah, I.N.E., (2000): Bioremediation technologies for oily wastes. A Perspective of Industrial and Environmental Biotechnology, Snaap Press Ltd., Enugu, Nigeria, pp. 53-71.
[19] Page, C.A., Bonner, J.S., McDonald, T.J., Autenrieth, R.L., (2002): Behaviour of chemically dispersed oil in a wetland environment. Water Research, 36: 3821-3833.
[20] Pilon-Smits, E., (2005): Phytoremediation. Annual Review of Plant Biology. 56: 15-39.
[21] Schnoor, J.L., Licht., L.A., Mc Cutcheon, S.C., Wolfe, N.L., Carreira, L.H., (1995): Phytoremediation of organic and nutrient contaminants. Environmental Science and Technology, 29(7): 318-323.
[22] SVMS, (2001): SPDC people and environment annual report. Shell Virtual Media Services (SVMS) of the Shell Petroleum Development Company of Nigeria, pp. 36-37.
[23] United States Environmental Protection Agency (USEPA), (1996): A Citizen's Guide to Natural Attenuation Technology fact sheet. Office of Solid Waste and Emergency Response. EPA 542-F-96-015.
[24] United States Environmental Protection Agency (USEPA), (1999): Use of monitored natural attenuation at superfund, RCRA Corrective action and underground storage tank sites.
[25] Wu, C.H., Wood, T.K., Mulchandani, A., Chen, W., (2006): Engineering plant- microbe symbiosis for rhizoremediation of heavy metals. Applied and Environmental Microbiology, 72(2): 1129-1134.
[26] Yee, D.C., Maynard, J.A., Wood, T.K., (1998): Rhizoremediation of trichloroethylene by a recombinant root–colonizing *Pseudomonas fluorescens* strain expressing toluene ortho-monoxygenase constitutively. Applied and Environmental Microbiology, 64(1): 112-118.

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