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An Integrated Environmental Biotechnology for Enhanced Bioremediation of Crude Oil Contaminated Agricultural Land

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Abstract
An enhanced *in situ* bioremediation of crude oil contaminated soil samples was carried out by manipulating a natural bioremediation process. In this approach, the soluble microbial products (SMPs) released during the metabolism of the polluting crude oil by hydrocarbonoclastic bacteria exemplified by *Pseudomonas* sp; $NS_{50}C_{10}$, were assimilated as growth substrates by the surviving/compatible aerobic, free-living diazotrophs exemplified by *Azotobacter vinelandii*. The utilization of the SMPs by diazotrophs was coupled to growth, nitrogen fixation and cometabolism of solubilized petroleum hydrocarbons. The two species of mutualistic hydrocarbonoclastic and diazotrophic bacteria used as *consortium* for the bioremediation project, were isolated from the same previously crude oil – polluted soil. Bioremediation was deemed successfully completed based on about 85% germination and seedling development obtained for sorghum grains planted in the treated soil, relative to untreated controls. This degree of success in the bioremediation effort suggests a significant soil fertility recovery to warrant immediate cultivation.

Keywords: *Azotobacter, Pseudomonas,* cometabolism, nitrogen fixation, bioremediation, crude oil, sorghum, germination

Introduction

Biological systems have evolved in a way that cycling and self-remediation is natural functions of life. It is therefore imperative for man to examine closely the natural biological processes in an effort to learn sound remediation techniques with which to ameliorate environmental damage. Many microorganisms can adapt their catabolic machinery to utilise certain environmental pollutants as growth substrates, thereby bioremediating the environment. Some microorganisms in carrying out

their normal metabolic function may fortuitously degrade certain pollutants as well. This process termed cometabolism obviously requires adequate supply of growth substrates. Diazotrophs, such as *Azotobacter vinelandii*, beyond their ability to fix atmospheric nitrogen also have the capacity in some cases, to cometabolise petroleum hydrocarbons (Onwurah, 1999). Cometabolism is beneficial to the degradation of hydrocarbon pollutants both in natural aquatic and terrestrial environments, and in engineering biotreatment processes (Alexander, 1981; Arvin, 1991). This phenomenon was defined (Dalton and Stirling, 1982) as the transformation of a non-growth substrate in the obligate presence of a growth substrate or another transformable compound. Although this definition is not universal (Alexander, 1981; Hamer, 1992), it is generally accepted that a cometabolising organism does not derive energy for growth from direct oxidation of that (non-growth) substrate. For this reason, the population of cometabolising organism may not increase rapidly with time.

Some basic steps that may be necessary for any bioremediation project are as follows: compliance analysis, site characterization, method selection (feasibility studies), remediation proper and end of project analysis (Bonaventura *et al*, 1995). Compliance analysis requires examination of the contaminated site in the light of government regulation and the required action plan. Here environmental protection agencies (EPAs) such as Federal Environmental Protection Agency (FEPA) and Department of Petroleum Resources (DPR) of NNPC oversee such regulations in Nigeria. The second stage, which is site characterization is a very challenging and difficult aspect of a bioremediation project. It involves analysis of the physical characteristics and nature of the soil. Parameters such as cation exchange capacity, inorganic matter content, acidity (pH), aeration status, hydraulic properties, geologic and hydrogeologic factors, meteorological and climatological data of the soil may be evaluated (Middleton *et al.*, 1991; Sims *et al.*, 1990). Knowledge of these parameters requires the assistance of specialists in these areas. Feasibility study for possible bioremediation approach and bioassays for pollutant degradation are carried out under control laboratory conditions. The importance of bioassays is that they integrate the combined effects of the mixtures of all the chemical components of the crude oil and other chemicals present in the polluted soil, including their bioavailability (Wong *et al,* 1999). This confirms complete or near complete removal of the contaminants. The use of this type of biological test method is recommended for the evaluation of the effectiveness of remediation process (DECHAMA 1995).

The objective of this work is to evaluate the enhancement capacity of diazotrophic bacteria in bioremediation of crude oil polluted soil when applied in combination with compatible hydrocarbonoclastic bacteria. This enhancement capacity was evaluated by monitoring the germination and growth development of sorghum planted in the treated soil samples and controls. This may underline the importance of an integrated approach to biremediation of crude oil contaminated soil.

Materials and Methods

Hydrocarbonoclastic and diazotrophic bacteria, tentatively identified as *Pseudomonas sp,* designated $NS_{50}C_{10}$ by the Department of Microbiology, University of Nigeria, Nsukka and *Azotobacter vinelandii,* respectively were isolated from previously crude oil contaminated soil as described elsewhere (Onwurah 1999). The mineral media, procedure for isolation and multiplication to the required cell density were also described.

Table 1: Total PHC removal, specific growth rate of microbial population and cometabolism of PHC by *A vinelandii* in different contaminated soil samples.

Initial PHC in soil (mg PHC/g)	Total PHCs removed (mg PHC) at 4 days					Specific growth rates (μd^{-1})				
	Inoculation with autoclaved consortium		Pure $NS_{50}C_{10}$ inoculation with pure $NS_{50}C_{10}$ culture		Normal consortium inoculation with normal consortium	Inoculation with autoclaved consortium ($\mu_s d^{-1}$)		Pure $NS_{50}C_{10}$ inocula (μ_{pd-1})	Normal consortium inocula ($\mu_c d^{-1}$)	Cometabolism (μ_c-μ_p)
	$t_1 = 1$	t_2=4	$t_1 = 1$	t_2=4	$t_1 = 1$	t_2=4				
50	2.80 ± 0.14	6.50 ± 0.85	6.12 ± 0.39	18.10 ± 3.20	5.70 ± 0.30	41.20	0.281	0.361	0.659	0.298
100	2.73 ± 0.22	6.65 ± 0.10	13.09 ± 1.24	42.00 ± 3.65	13.26 ± 2.24	88.30	0.296	0.389	0.632	0.243
150	2.79 ± 0.018	6.67 ± 0.92	21.60 ± 3.46	73.35 ± 6.28	22.22 ± 3.74	142.60 ±10.69	0.291	0.408	0.620	0.212

(a) Consortium signifies the combination of $NS_{50}C_{10}$ and *A. vinelandii*

* Cometabolism was based on the difference in specific growth rates in soil sample inoculated with $NS_{50}C_{10}$/*A. vinelandii* and $NS_{50}C_{10}$ alone

Table 2: Evaluation of microbial nitrogen fixation in contaminated soil samples inoculated with a *Pseudomonas* sp ($NS_{50}C_{10}$) *A vinelandii* at 4 days of incubation.

Initial PHC in soil (mg PHC/g)	Total nitrogen content (%) in the soil samples					
	Inoculation with autoclaved $NS_{50}C_{10}$ and *A vinelandii*		Inoculation with Pure strain of $NS_{50}C_{10}$		Inoculation with normal consortium*	
	t_1 = 1 (d)	t_2 = 4 (d)	t_1 = 1 (d)	t_2 = 4 (d)	t_1 = 1 (d)	t_2 = 4 (d)
0	0.0704 ± 0.0024	0.0708 ± 0.002	0.07084 ± 0.002	0.068 ± 0.002	0.0703 ± 0.005	0.0715 ± 0.006
50	0.0703 ± 0.0041	0.071 ± 0.004	0.0704 ± 0.004	0.065 ± 0.004	0.0704 ± 0.003	0.0902 ± 0.008
100	0.0704 ± 0.006	0.0710 ± 0.006	0.0703 ± 0.003	0.066 ± 0.005	0.0704 ± 0.003	0.0914 ± 0.002
150	0.0703 ± 0.005	0.0712 ± 0.003	0.0704 ± 0.006	0.068 ± 0.003	0.0704 ± 0.003	0.0916 ± 0.007

* Consortium signifies combination of $NS_{50}C_{10}$ and *A. vinelandii*

Table 3: Effect of inoculating crude oil-polluted soil with hydrocarbonoclastic bacteria and adapted diazotroph on soil recovery (bioremediation) and hence germination and seedling development

(a)

Level of crude oil pollution (mg PHC/g soil)	Mean No of visible germination at 7 days of planting		
	Autoclaved inocula + resident microbial population	Pure $NS_{50}C_{10}$ incoula + resident microbial population	Normal $NS_{50}C_{10}$ and *A. vinelandii* inocula + resident microbial population
0	20 (100%)	20 (100%)	20 (66.35%)
50	7.38 ± 1.24 (36.9%)	15.35 ± 2.45 (76.75%)	13.27 ± 2.36 (66.35%)
100	2.54 ± 0.52 (12.7%)	16.64 ± 2.08 (83.20%)	12.14 ± 2.45 60.70%)
150	No visible germination (0%)	15.14 ± 2.34 (75.70%)	13.18 ± 2.18 (65.90%)

Values in parenthesis are percentage

(b)

Level of crude oil pollution (mg PHC/g soil)	Mean No of visible germination at 7 days of planting		
	Autoclaved inocula + resident microbial population	Pure $NS_{50}C_{10}$ incoula + resident microbial population	Normal $NS_{50}C_{10}$ and *A. vinelandii* inocula + resident microbial population
50	6.1 ± 2.3	8.4 ± 2.6	7.6 ± 1.4
100	4.5 ± 0.8	8.8 ± 2.9	6.3 ± 2.4
150	-	8.1 ± 2.8	5.8 ± 3.2

Crude oil spill of the soil was simulated by thoroughly mixing 50, 100 or 150 mg fraction of the crude oil with 100 g batches of a composite soil samples in beakers. The soil sample was taken from a depth of 0 – 15 cm from the zoological garden, University of Nigeria, Nsukka. The mixing was done with a horizontal arm shaker adjusted to a speed of 120 rpm, for 30 minutes. The contaminated soil samples, in beakers, were inoculated with minimum optimal combinations (cell density) of $NS_{50}C_{10}$ and *A. vinelandii*. Water was added to the crude oil-contaminated soil samples (both inoculated and those not inoculated to a saturation point but not in excess) and then left to stand

undisturbed for 4 days. $NS_{50}C_{10}$ was applied – first, followed by *A. vinelandii,* 12 hours later. The equation relating crude oil concentration in the soil and cell density of $NS_{50}C_{10}$ applied is given by the equation

$$[NS_{50}C_{10}]_{min} = -0.015[PHC]^2 + 0.3718[PHC] + 0.3905 \quad \text{..... (1)}$$

where $[NS_{50}C_{10}]_{min}$ is the minimum volume (ml) of the *Pseudomonas* sp. inocula at 10^8 cells/ml, (exponential growth phase), required in combination with *A. vinelandii* to remove 65 – 70% of PHCs from the contaminated soil samples at 4 days of incubation. [PHC] is the concentration of petroleum hydrocarbons (crude oil) in the soil samples. The corresponding inocular density of *A. vinelandii* required to achieve the above PHC removal is also governed by the following equation:

$$[NS_{50}C_{10}]_{min} = 0.0164\ [A.\ vinelandii]^2 + 0.4842\ [A.\ vinelandii] - 0.1082 \quad \text{.... .....(2)}$$

where [*A. vinelandii*] is the corresponding minimal volume (ml) of *Azotobacter* inocula at 10^8 cells/ml (exponential growth phase) required for optimum bioremediation.

At 24 h intervals, 1.0 g fractions of the soil samples were removed from the beakers and the remaining crude oil (petroleum hydrocarbons) extracted (double extraction) with 2 ml of ethanol – chloroform mixture (1:1). The optical density of the extracted PHC in the extraction mixture was read in a spectrophotometre at 520nm against a blank of the extraction mixture also run through uncontaminated soil sample. The crude oil remaining was quantified from a standard (calibration) curve of different percentage weight concentrations of the crude oil in the extraction mixture. Total PHC removed from each treated soil and untreated soil was quantified at $t_1 = 1$ day and $t_2 = 4$ days. The modified Kjedahl method (Jackson, 1964) was used for the determination of total nitrogen content of the contaminated soil samples at various time intervals of incubation.

At the seventh day of the soil treatment, 20 sorghum grains (previously soaked overnight in distilled water) were planted in each soil sample. The soil samples were thereafter irrigated to aid germination. Seven days after the planting of the sorghum grains, the soil from each beaker was carefully removed. The number of germinated seed per batch of soil sample was noted and the length of radicles measured and the mean length taken for each batch.

Results and Discussion

The characteristics of the hydrocarbonoclastic bacterium, *Pseudomonas* sp. (designated $NS_{50}C_{10}$) and the free-living aerobic, diazotroph, *Azotobacter vinelandii* have been reported (Onwurah, 1999). The *Pseudomonas* sp. grew well on agar plates containing a thin film of crude oil as the only carbon source while *A vinelandii* did not. However, the cell-free extract of *A vinelandii* fixed atmospheric nitrogen as ammonium ion (NH_4^+) under appropriate condition.

The characteristics of the soil which was contaminated with the crude oil include 16.72% clay, 2.0% silt, 51.3% fine sand and 29.98% coarse sand. Other parameters are organic carbon 1.4%; organic matter, 2.44%; total nitrogen, 0.07%; pH 6.8 (H_20) and 6.5 (KCl), while phosphorus content

was 0.29%.

The crude oil used, Bonny light (B – 111, Nigerian) obtained from DPR, Port Harcourt was analysed by Ashland Petroleum Research and Development and the result showed that the sample contained the following: carbon residue 0.92%; iron, 1.00 wt, ppm; vanadium, 2.00 wt, ppm; nickel, 4.00 wt, ppm, sulphur, 0.10%; other characteristics include API gravity, 35.10; specific gravity, 0.8493; acid number, 0.39; pour point, 1.35^{0}F; salt content, (LBS/1000 BBL), 77.90; reid vapour pressure (PSI), 4.90 and organic chloride, 8.00 ppm. The crude oil, as analysed showed no value for nitrogen, thus suggesting no detectable or traceable nitrogen.

Growth of the bacterial inocula, $NS_{50}C_{10}$ and *A. vinelandii* and of the resident microbial populations in the soil samples were assessed on the basis of the reduction in the PHC at time t = 1 and t = 4 (d), using the specific growth rate model (Fukuzaki *et al.*, 1990):

$$\mu = \ln (M_2/M_1)/t_2 - t_1) \quad \ldots\ldots (3)$$

where μd^{-1} is the specific growth rate per day of the microbial population in each soil sample (including those that were inoculated). M_1 and M_2 are the mean concentrations of PHC (crude oil) degraded between time $t_1 = 1$ (d) and $t_2 = 4$ (d). Table 1 shows the total PHCs removed at day 1 and day 4 of incubation and the calculated specific growth rate values. The specific growth rate values in contaminated soil samples inoculated with both normal $NS_{50}C_{10}$ and *A vinelandii* (consortium) were highest in all cases. The values for 50, 100 and 150 mg PHC/g soil contamination were respectively 0.659, 0.632 and 0.620 d^{-1}. For soil samples inoculated with only $NS_{50}C_{10}$, the specific growth rate in 50mg PHC/g crude oil contamination was 0.361 d^{-1}, while the normal soil (inoculated with autoclaved $NS_{50}C_{10}$ and *A. vinelandii*) had a specific growth rate of 0.281d^{-1}. The improvement in bioremediation in soil inoculated with the hydrocarbonoclastic bacterium, $NS_{50}C_{10}$ over the control with autoclaved inocula ranged from 28.47 to 40.21%. By adding an aerobic, free-living diazotroph *A. vinelandii* with the *Pseudomonas sp.* ($NS_{50}C_{10}$), an improvement on bioremediation over that of the pure $NS_{50}C_{10}$ alone was achieved to the order of 51.96 to 82.55%. It should however be noted that the efficiency in the effect of inoculating with *A vinelandii* decreased with increased concentration of crude oil in the soil. This goes to underline the toxic effect of the crude oil on *A. vinelandii*, especially at high concentrations as explained elsewhere (Onwurah and Eze, 2000). The contribution of *A vinelandii* in the faster bioremediation of the contaminated soil, through cometabolism is also shown in Table 1. The concept/importance of cometabolism in bioremediation process have been documented (Alexander 1981., Dalton and Stirling 1982., Hamer 1992., Onwurah 1999). In this work, cometabolism was evaluated as the difference between specific growth rate values in soil samples inoculated with both $NS_{50}C_{10}$ and *A vinelandii* and those inoculated with only $NS_{50}C_{10}$, at corresponding cell densities. It is apparent also that cometabolism decreased in soil samples containing higher amounts of crude oil. This effect may be corrected by allowing a longer space of time between inoculation with the crude oil degrader and the compatible diazotroph or by slightly increasing the concentration of *A vinelandii* inocula above the calculated value. This was considered necessary because soil nitrification is affected by the concentration of polycyclic aromatic compounds present in a given crude oil sample (Sverdrup *et al.* 2002), and this may affect the nitrogen fixation potential of *A. vinelandii.*

Nitrogen fixation by *Azotobacter* provides fixed nitrogen for the growth and proliferation of the hydrocarbonoclastic bacterium which degrades the crude oil while the former derives its growth substrates from the products of crude of metabolism. A class of secondary substrates that support growth is formed and released as part of microbial metabolism of primary substrates (Bouwer, 1992). Hence organic compounds in crude oil (Whittle, 1982) could be metabolised to release secondary substrates that support the growth and cell proliferation of *A. vinelandii.* This has been demonstrated in a liquid culture containing *Azotobacter* and *Pseudomonas* sp. (Onwurah, 1999). The soluble microbial products (SMP) released during metabolism of the primary substrates (Namkung and Ritman, 1987; Ritman *et al.*, 1987) are formed partly as a result of substrate utilization and partly through basal metabolism and decay of active biomass (Gaudy and Blachly,1984). This shows the possibility of cross-feeding and nutrient recycling between these two bacteria. Table 2 illustrates the capability of *A. vinelandii* in maintaining or supplying adequate fixed nitrogen for $NS_{50}C_{10}$. The scenario where hydrocarbonoclastic bacteria produce SMPs, exopolysaccharides (EPS), biosurfactants which may serve as energy-providing substrates for aerobic or anaerobic free-living diazotrophs in the contaminated sites is an application for an optimized bioremediation for today and the future. The primary limitation to the widespread use of many bioremediation approaches is often judged to be the extent to which the pollutant is available to the microbial population. Development of methods that make pollutants more bioavailable to current systems is clearly needed.

The damaging of soil by crude oil and salt water is of common occurrence in the oil mineral producing areas and any practical means of evaluating the degrees of success in any bioremediation effort must be obvious. One desirable method is the mass-balance analyses, but this is rarely obtained. However, the use of some acute and chronic bioassays to determine the ecological risk and biremediation efficiency of oil polluted soils have been described (van Gestel *et al.* 2001)These bioassays involve the use of bacteria (*Bacillus sp.),* algae *(Raphidocelis subcapitata)* and plants *(Lactuca sativa)*. The durability and stability of bioremediation technology used in this work was tested by a bioassay method, which involve the germination and growth development of sorghum grains. The percentage germination and radicle development for the sorghum grains planted in the crude oil polluted soil, after bioremediative technology applied in this work, was used as an index of success. Table 3 shows the soil fertility recovery in terms of percentage germination and radicle development of sorghum grains planted in the soil after treatment, relative to soil inoculated with autoclaved bacteria (control). In soil contaminated with 150mg PHC/g, there was no visible germination and radicle development, but for the same soil treated with pure $NS_{50}C_{10}$ inocula, there was 65.90% germination and radicle development. Against this was a further improvement for the same soil treated with $NS_{50}C_{10}$ and *A. vinelandii* (75.70% germination). This goes a long way to show the importance of diazotrophs in the ecosystem. The relative importance of *Azotobacter* in some ecosystems has been demonstrated (Mulder, 1975, Jensen, 1941, 1954, Dicker and Smith, 1980). Apart from cometabolising PHC, the diazotroph enriches the soil with fixed nitrogen, thereby contributing to the quick restoration of the damaged agricultural soil. The effect of crude oil on soil fertility remain for several years, depending on the quantity spilled (Plice, 1948, Klokk, 1948). Okpokwasili and Odokuma (1994) reported that increasing amounts of crude oil could retard nitrification process. Conversely, it has been observed that considerable nitrogen fixation took place

when several nitrogen-free organic materials were added to crude oil-polluted soil (Plice, 1948), as a result of the lowered redox potential, which favours nitrogen fixation. It then follows that if *Azotobacter* could survive under such redox state, there will be an enhancement in nitrogen fixation. Instances of nitrogen fixation and organic matter formation in soil, in large quantity, by microorganisms utilising hydrocarbon materials as their energy source occur quite often about leaks in natural gas lines (Plice, 1948). Apart from laying credence to an earlier report on the role of diazotrophic bacteria in bioremediation of crude oil – polluted soil (Onwurah, 1999), the present investigation provided a mathematical model for working out the minimum cell density combination of the hydrocarbonoclastic bacterium and the compatibile diazotroph, which may produce 65 – 70% bioremediation within a very short time.

Crude oil spill is deleterious to the environment and may cause agricultural soil to remain "sterile" for several years, if no bioremediation effort was made. Inoculating crude oil contaminated soil with adequate concentration of hydrocarbonoclastic bacteria would hasten the bioremediation process, but this process may become much better and faster if the crude oil degrader is co-inoculated with a compatible aerobic, free-living diazotroph, such as *A vinelandii*, which may not only supply fixed nitrogen but also performs some cometabolisation role. Hydrocarbonoclastic bacteria in concert with "adapted" *Azotobacter* and coupled to land farming is therefore a promising biotechnology for enhanced recovery of crude oil – polluted agricultural lands.

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