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Modelling Cometabolism of Petroleum Hydrocarbon Pollutants in Soil by *Azotobacter vinelandii* in the Obligate Presence of *Pseudomonas sp.*

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Pollution is the accumulation of matter or energy (as a result of man§s activity), which degrades both the biotic and abiotic environment. The problems of petroleum hydrocarbon pollution in particular, present a difficult challenge in terms of human/environmental health and economics. In Nigeria, the task of controlling petroleum hydrocarbon pollution is enormous. Best management practices (BMP) are put in place by the oil industry to minimise spills during operations. Instances where a large-scale pollution had occurred required bioremediation technology, which included mechanical mopping-up of floating oil. Bioremediation is the use of naturally occurring microorganisms, mostly bacteria to degrade harmful chemicals into less toxic or non-toxic forms. Bioremediation of a polluted coast after the Exxon Valdez oil spill was achieved through nutrient augmentation in the form of oleophilic fertilizer (Pritchard and Costa, 1991).

An important component of bioremediation process is cometabolism. Complete bioremediation of crude oil polluted environment is mediated through microbial consortium, some of them degrading the recalcitrant pollutant through the process of cometabolism. Dalton and Stirling (1982) defined cometabolism as the transformation of non-growth substrate in the obligate presence of a growth substrate. Cometabolism is beneficial to the degradation of hydrocarbon pollutants both in natural aquatic and terrestrial environments and in bioreactors (Arvin, 1991). Mathematical modelling of cometabolism has been quantified using competitive inhibition model (Chang *et al.*, 1992). In this approach, cometabolism was coupled to transformation of non-growth substrate during the consumption of a growth substrate for growth. In the present work, cometabolism of petroleum hydrocarbon (PHC) by *A. vinelandii* (a diazotroph) was coupled to specific growth rate difference between the consortium of *A. vinelandii* and a hydrocarbonoclastic bacterium *Pseudomonas* sp. and that of the *Pseudomonas* sp only when they were inoculated into petroleum hydrocarbon contaminated soil samples. The half-life model for the petroleum hydrocarbon in the soil samples was also used to quantify cometabolism when the consortium and the pure strain of the *Pseudomonas* sp. were separately applied as inocula.

MATERIALS AND METHODS

Hydrocarbonoclastic and diazotrophic bacteria, tentatively identified as *Pseudomonas sp.* and *Azotobacter vinelandii*, respectively were isolated from previously crude oil contaminated soil. The mineral media, procedure for isolation and multiplication to the required cell density, have been described (Onwurah, 1999)

The Bonny light crude oil used in this work was obtained from DPR, Port Harcourt. Its composition, as analysed by Ashland Petroleum Research and Development, has been reported (Onwurah, 1999). The fractional composition (%) of the crude oil gave 81.11% saturates, 7.20% aromatics, 2.48% asphaltenes and 9.21% residue (Amund and Akangbou, 1993).

The soil sample was taken from a depth of 0 -15 cm from the zoological garden, University of Nigeria, Nsukka. 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%

Oil spills were simulated by thoroughly mixing 50, 100 or 150 ml. fractions of Bonny light crude oil with 100 g batches of a composite soil sample in beakers. The mixing was done with a horizontal arm shaker adjusted to a speed of 120 rpm, for 30 minutes. The contaminated soil samples were inoculated with minimum optimal combinations (cell density) of *Pseudomonas sp.* and *A. vinelandii*. Water was added to the crude oil-contaminated soil samples (both inoculated and those not inoculated) to a total volume of 60 ml and then left to stand undisturbed for 4 days (ie, culture + distilled water). The *Pseudomonas sp.* was applied first, followed by *A. vinelandii,* 12 hours later. The relationships between the volumes (ml) of crude oil in the soil and cell densities of *Pseudomonas* sp. and *A. vinelandii* applied were correlated by using the method of least square with polynomial expressions of different orders (Wu *et al.,* 1993).

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

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

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 volume (ml) of petroleum hydrocarbons (crude oil) in the soil samples, while $[NS_{50}C_{10}]_{min}$ is the corresponding minimal inocula volume (ml) of *A. vinelandii* (at 10^8 cells/ml, exponential growth phase) required to achieve the above PHC removal.

At 24 hr intervals, 1.0 g fractions of triplicate soil samples were removed from the beakers and the remaining crude oil (petroleum hydrocarbons) extracted

exhaustively with 2 ml of ethanol - chloroform mixture (1:1). The optical density of the extracted PHC in the extraction mixture was read in a spectrophotometer at 520 nm against a blank of the extraction mixture, also run through uncontaminated soil sample (Snell and Snell, 1953). This is a semi-quantitative assay for "total petroleum hydrocarbons" dissolved in the extraction mixture, particularly for low molecular weight hydrocarbons in the crude oil, such as toluene, xylene, naphthalene, (DTSC, 1996). 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. Total nitrogen fixed at each of these two time intervals was evaluated by the Kjedahl method (Jackson, 1964).

The growth of the microbial population (indigenous and the inoculated) was assessed based on the specific growth rate calculated from the equation (Fukuzaki *et al.*, 1990):

$$\mu = \frac{In(M_2 / M_1)}{t_2 - t_1} \tag{3}$$

where μ (d^{-1}) is the specific growth rate of the microbial population per day, while M_1 and M_2 are the mean weights (g) (equivalent to the volumes applied to the soil samples) of PHCs degraded or transformed between time $t_1 = 1$ d and $t_2 = 4$ d respectively. Cometabolism of the PHC by *A. vinelandii* in the obligate presence of *Pseudomonas sp,* was equated to be equal to the specific growth rate of *A. vinelendii* μ_A (d^{-1}). This was evaluated as the difference in the specific growth rates of the consortium μ_C (d^{-1}) and that of the same cell density of the *Pseudomonas* only pure culture μ_p (d^{-1}). The relevant equation is given thus:

$$\text{Cometabolism } (\mu_A) = \mu_C - \mu_P \tag{4}$$

RESULTS AND DISCUSSION

The characteristics of the *Pseudomonas* sp. 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.

Total bacterial growth (inoculated and resident) 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). Table 1 shows the total PHC removed and the calculated specific growth rate values between day 1 and 4

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 (ml PHC/ g)	Total PHCs removed (ml PHC) between 1 - 4 days						Specific growth rates μ (d^{-1})			
	Inoculation with autoclaved [a]consortium		Inoculation with pure *Pseudomonas sp* culture only		Inoculation with normal [a]consortium		Inoculation with autoclaved [a]consortium	*Pseudomonas sp* inocula only	Normal [a]consortium inocula	*Comet-abolism
	Day 1	Day 4	Day 1	Day 4	Day 1	Day 4	μ_c (d^{-1})	μ_p (d^{-1})	μ_c (d^{-1})	(μ_c-μ_p)
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[b]	0.659[c]	0.298[d]
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[b]	0.632[c]	0.243[d]
150	2.79±.018	6.67±0.92	21.60±3.46	73.35±6.28	22.22±3.74	142.60	0.291	0.408[b]	0.620[c]	0.212[d]

[a]Consortium signifies the combination of *Pseudomonas sp* and *A. vinelandii* [b,c]Differences were significant only between corresponding pairs of (b) and (c) at $p < 0.05$ *Cometabolism was based on the difference in specific growth rates in soil sample inoculated with *Pseudomonas sp*/*A. vinelandii* and *Pseudomonas sp* alone. Differences amonst (d) were not significant ($P > 0.05$)

of incubation. The specific growth rate values in 50, 100 or 150ml PHC/g. contaminated soil samples inoculated with both the *Pseudomonas sp.* and *A. vinelandii* were respectively 0.659, 0.632 and 0.620 d^{-1}. For soil samples inoculated with only *Pseudomonas sp.* the specific growth rate in 50 ml PHC/g crude oil contamination soil was 0.361 d^{-1} . Specific growth rate of 0.281 d^{-1} was recorded in normal soil inoculated with autoclaved consortium. The improvement in bioremediation of soil inoculated with the *Pseudomonas sp.* alone over the control with autoclaved inocula ranged from 28% to 40%. By adding an aerobic, free-living diazotroph, *A. vinelandii* with the *Pseudomonas sp.* an improvement in bioremediation over that of the only pure *Pseudomonas sp.* was achieved to the order of 52% to 83%. The contribution of *A. vinelandii* in the faster bioremediation of the contaminated soil is also shown in Table 1. It should however be noted that the efficiency achieved by inoculating with *A. vinelandii* decreased with increased concentration of petroleum hydrocarbon in the soil. This goes to underline the toxic effect of the crude oil on *A. vinelandii*, especially at high concentrations (Onwurah and Eze, 2000). This may be alleviated by allowing a longer time lag between inoculation with the oil degrader and the compatible diazotroph or by slightly increasing the concentration of *A. vinelandii* inocula above the calculated value.

The major idea behind *Azotobacter* inoculation is to constantly supply fixed nitrogen compound for the growth of *Pseudomonas sp.* Pure cultures of *Azotobacter* spp do not degrade or show any significant growth in medium containing crude oil as the only carbon source. However, when the medium was supplemented with mannitol as alternative carbon source, growth was established, as well as degradation of some components of the crude oil (Onwurah, 1998). The above suggests cometabolism. Metabolic intermediates of crude oil degradation by hydrocarbonoclastic bacteria can support the growth of adapted *Azotobacter* (Gottshalk, 1999; Walker and Harris, 1970). Figure 1 depicts the relationship between *Pseudommas* sp and A*zotobacter* sp under crude oil polluted environment, while Table 2 shows nitrogen compound supplying- potential of the latter. The consortium of *Pseudomonas sp/A. vinelandii* (under limiting level of inorganic N- compound) produced higher degradation rate of Bonny light crude oil when compared with equal cell density of the same *Pseudomonas sp* only culture amended with adequate level of inorganic N-compound. However, the pure culture of *Pseudomonas sp* with limiting N-compound showed minimal degradation and growth rates (Onwurah, 1999; Onwurah and Nwuke, 2004). The above cases suggest that *A. vinelandii* not only performs cometabolic activity but provides the fixed N- compound needed for the growth and proliferation of *Pseudomonas sp.* The scenario where the *Pseudomonas sp* produces soluble microbial products (SMPs), exopolysaccharides (EPS), 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.

The rates of PHC removal shown in Table 3 are not true constants. They are variables that decline with time (Loehr *et al.*, 1992). The half-life values of

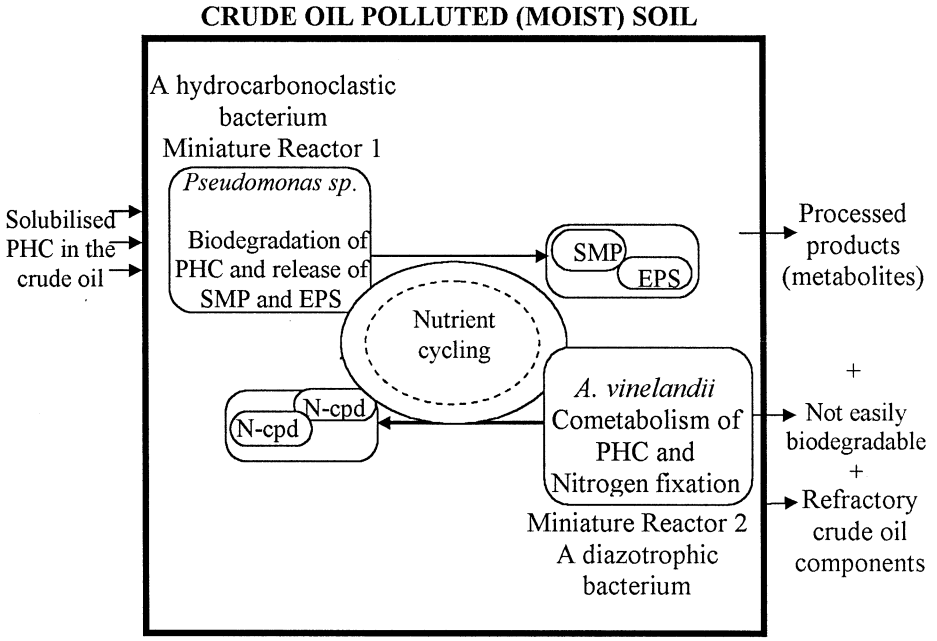

Figure 1. Simplified bioremediation conceptual model of *Pseudomonas sp.* and *A. vinelandii* operating as a unit of two miniature sequencing bioreactors, in situ
SMP: Soluble microbial products; N-cpd: Fixed nitrogen compounds;
EPS: Exopolysaccharide; PHC: Petroleum hydrocarbons

Table 2. Evaluation of microbial nitrogen fixation in contaminated soil samples inoculated with a *Pseudomonas* sp. *and A. vinelandii* at 4 days of incubation.

Initial PHC in soil (ml PHC/g)	Total nitrogen content (ppm) in the soil samples					
	Inoculation with autoclaved consortium*		Inoculation with pure strain of *Pseudomonas sp.*		Inoculation with normal consortium*	
	Day 1	Day 4	Day 1	Day 4	Day 1	Day 4
0	704±24	708±20	708.4±20	680±20	708±50	715±60
50	703±41	710±40	704±40	650±40	704±30	902±80
100	704±60	710±60	703±30	660±50	704±30	914±20
150	703±50	712±20	704±60	680±30	704±30	916±70

* Consortium signifies combination of *Pseudomonas sp.* and *A. vinelandii*

Table 3. Half-life periods and loss rates of PHCs in the crude oil contaminated soil samples at minimal optimum cell density combinations of *Pseudomonas sp.* and *A. vinelandii* and *Pseudomonas sp.* alone to give 65 - 70% removal of PHCs, at 96hr of incubation.

Crude oil concentrations in soil samples (ml PHC/g)	*Pseudomonas sp.* inocula only		*Pseudomonas sp.* and *A. vinelandii* inocula	
	Loss rate constant (k) (d^{-1})	Half-life periods, $t_{1/2}$ (d)	Loss rate constant (k) (d^{-1})	Half-life periods, $t_{1/2}$ (d)
50	0.0846	8.1880	0.206	3.358
100	0.1589	4.3607	0.280	2.473
150	0.1618	4.2815	0.262	2.473

crude oil in contaminated soil samples inoculated with the consortium were lower than for the samples inoculated with only *Pseudomonas sp.* However, there seems to be some variations in the rate of PHC removal by *Pseudomonas sp.* alone and the optimal minimum combinations of the consortium for the various oil contaminated soil samples. This may be due to non-uniform mixing of the crude oil with the soil samples, in spite of the uniform application and mixing procedure. The range of data therefore indicates the variations that may be expected for each treatment. In a similar experiment performed in a liquid nutrient medium (Onwurah, 1999), the removal of PHCs from the incubation medium shows a first-order reaction model. Many factors, such as temperature, soil pH, relative toxicity, nutrient and type/number of organisms in the soil may affect rate of removal of PHC. Nevertheless, first-order loss rates are commonly adapted for organic losses in soils (Loehr *et al.*, 1992). Therefore, it will not be out of place if a first order mathematical model is assumed for this work. The resulting evaluation of the rate of PHCs removal from various oil-contaminated soil samples and the respective half-lives show an enhanced bioremediation with *Pseudomonas sp./A. vinelandii* within the period of incubation. The correlation coefficients that were obtained (from 1 – 4 days of incubation) for the data in Table 3 indicated that a first-order relationship was a reasonable representation of the bioremediation model. This then suggests that eventually there will be a complete removal of the constituent PHCs of the crude oil in the soil.

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