

Rhizoremediation of hydrocarbon-contaminated soil by *Paspalum vaginatum* (Sw.) and its associated bacteria

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ABSTRACT

A rhizoremediation study was carried out on hydrocarbon-contaminated soil with *Paspalum vaginatum* (Sw.), a stoloniferous, perennial grass of the family Poaceae found mainly in the subtropics and tropical regions of the world. The contaminated soil analyses indicated a decrease in the level of hydrocarbons present after phytoremediation. There was equally, a significant reduction in growth parameters of the plant such as plant height, leaf number, tiller number and total dry weight, compared to the control. Anatomical studies of sections of the plants' stems did not reveal the presence of accumulated oil within the tissues but rather denatured internal parenchymal cells structure were observed. Bacteria capable of degrading hydrocarbons were isolated from the rhizosphere of the grass. The isolates include: *Arthrobacter* sp., *Bacillus pumilus*, *Bacillus sphaericus* and *Serratia marcescens*. Growth in mineral salts medium supplemented with 0.5% crude oil for 21 days resulted in 95.9%, 95.6%, 98.3% and 96.7% degradation of oil for *Arthrobacter* sp., *B. pumilus*, *S. marcescens* and *B. sphaericus* respectively. A soil microcosm set up with the consortium of the isolates resulted in 87.7% degradation of crude oil in 45 days. These results suggest that *P. vaginatum* and its associated microbes are good candidates for rhizoremediation of hydrocarbon polluted soils.

Keywords: rhizoremediation, hydrocarbon, soil, microbes, *Paspalum vaginatum*

INTRODUCTION

The contribution of rhizosphere microbial populations to bioremediation is known as rhizoremediation (Anderson et al., 1993; Schwab and Banks, 1994; Kuiper et al., 2004). Rhizoremediation involves the symbiosis between plants and the rhizosphere microbial community responsible for degrading contaminants in soils and groundwater. Exudates derived from plants can help to stimulate the survival and action of bacteria, which subsequently results in more efficient degradation of pollutants (Kuiper et al., 2004). The root system of plants can also help to spread bacteria in soil through layers that are otherwise impermeable. An important contribution to the degradation of oil pollution is ascribed to rhizosphere microorganisms of plants used during phytoremediation and certain plants that can adapt naturally in polluted sites (Bais et al., 2006).

Paspalum vaginatum (Sw.), a perennial stoloniferous grass, found in Southern Nigeria, has been shown to proliferate in crude oil contaminated sites (Yateem et al., 2007). It occurs in estuarine habitats and wetlands. It is tolerant of drought, salinity, a wide range of pH, extended period of low light intensity and flooding or extended wet period (Lakanmi and Okusanya, 1990). The plant has an extensive widely branched fibrous root system, resulting in large root surface area per unit volume of surface; therefore, providing a large surface for colonization by soil microorganisms than a tap root system (Paul et al., 2006).

This study was set out to determine the presence and effect of crude oil on *P. vaginatum* and to isolate hydrocarbon-degrading bacteria in the rhizosphere of *P. vaginatum* from pristine soil in the field and determine the

oil degradation rates by these organisms in liquid cultures and in microcosm studies.

MATERIALS AND METHODS

Sample collection

The plant sample, *P. vaginatum*, was collected from the shoreline of the University of Lagos lagoon front, Southwest Nigeria (6°27'11"N 3°23'45"E). It was taken to the laboratory for anatomical and microbiological analysis. The soil sample used for the study was collected from the rhizosphere of matured plants, approximately 3 m from the Faculty of Science, University of Lagos brackish water front (6°31'0"N 3°23'10"E). Bonny Light crude oil sample used for the degradation study was obtained from a flowstation, 12 km from Part-Harcourt Terminal of the Shell Petroleum Development Company of Nigeria.

Preparation of soil and plant cuttings

Humus soil of 21 kg was contaminated with crude oil at concentrations of 21.0 kg L⁻¹ (1000 ml), 14.0 kg L⁻¹ (1500 ml), 10.5 kg L⁻¹ (2000 ml), 8.4 kg L⁻¹ (2500 ml) and 7.0 kg L⁻¹ (3000 ml) of oil. Each bag was subsequently divided into 32 smaller nursery bags representing 8 replica bags for 4 harvest time. *Paspalum vaginatum* culms were raised in a nursery and after 2 weeks, uniform tillers were removed from the culms and transplanted to nursery bags containing treated soils and untreated soil as control. Each bag received a tiller. Watering was done with tap water daily for 6 weeks. At harvest, measurements of morphological features such as the plant height, leaf number, tiller number and total dry weights were determined.

Anatomical investigation of the stem

Fresh stems from the control and the treated plants were sectioned using a razor and the best thin segments were stained with Sudan III and viewed under the microscope.

Isolation of hydrocarbon degrading bacteria

Hydrocarbon-degrading bacteria were isolated by standard plate culture technique on Mineral Salts (MS) medium described by Kästner et al. (1994) and solidified with purified agar (1.5%). The medium contains per litre Na₂HPO₄, 2.13 g; KH₂PO₄, 1.30 g; NH₄Cl, 0.50 g and MgSO₄·7H₂O, 0.20 g. Trace elements solution (1 ml l⁻¹) of Bauchop and Elsden (1960) was added to the medium. The pH was adjusted to 7.2. Crude oil (0.5%) served as sole source of carbon and energy, which was supplied through vapour phase transfer (Amund et al., 1987). Plates were incubated at room temperature (28°C) for 24

- 48 h. Pure cultures were isolated from discrete colonies by subculturing on Luria Bertani (LB) agar and incubated at room temperature for 24 - 48 h. Pure cultures were maintained in LB slants at 4 °C. The isolates obtained were identified by Gram staining and various biochemical tests.

Screening of bacterial isolates for ability to utilize crude oil

The ability of the bacteria isolates to degrade crude oil was confirmed by inoculating each isolate (24 h) into 250 ml Erlenmeyer flasks containing 50 ml MS medium as previously described (Kastner et al., 1994). The medium was supplemented with 0.5% (v v⁻¹) crude oil as sole carbon and energy source. This was cultivated at room temperature for 7 days. Non-inoculated media flasks served as control. The isolates with good degradative ability were selected for further studies. The optical density (OD_{600 nm}), total viable count (TVC) and pH of the culture media were monitored every 3 days for 21 days as biodegradation indices and the residual hydrocarbon concentration was determined using Gas chromatography. The isolates were maintained on LB slants at 4 °C and subcultured every 2 weeks to maintain purity and viability.

Soil microcosm experiment

Transplanting

Biodegradation of crude oil by a consortium of the axenic bacterial isolates was assessed in pristine soil. *Paspalum vaginatum* of total vine length average (apex leaf to roots) between 30- 40 cm was transplanted into 11 kg of pristine soil in medium sized bags (20 cm by 15 cm) and weekly watered with lagoon water. Ten days after establishment of the grass, confirmed by the emergence of tillers, the pristine soil was artificially contaminated with 111 kg L⁻¹ (100 ml) of crude oil. This was left to stand for 3 days to allow the volatile gases to evaporate and the indigenous microorganisms adapt to the contaminated soil.

Cultivation

The isolates were grown in MS medium supplemented with 0.5% crude oil as sole carbon and energy source. This was monitored until an optical density (OD) of 0.5 was achieved. The bacterial consortium was prepared by mixing equal volumes of the standardized suspensions of each isolate.

Inoculation

The crude oil artificially contaminated soil was prepared

Table 1. Analyses of total hydrocarbon content in *Paspalum vaginatum* (Sw.) and oil treated soils

Oil concentrations (kg/L)	Total hydrocarbon content (mg/kg)			
	Soil			Plant
	T ₀ (mg/kg)	T ₄ (mg/kg)	% Detected	T ₄ (mg/kg)
Control (0)	Not detected	Not detected	None	Not detected
21.0 (1000 ml)	296128.30	109123.20	36.2	Not detected
14.0 (1500 ml)	543519.50	128707.52	23.7	600.50
10.5 (2000 ml)	580256.60	128964.10	77.8	9800.27
8.4 (2500 ml)	725320.80	228760.70	31.5	18015.03
7.0 (3000 ml)	754333.60	288964.90	38.3	15890.04

T₀, beginning of experiment; T₄, end of experiment after 4 weeks.

as described by Okoh (2003). A set of bags artificially contaminated with crude oil and with *P. vaginatum* plant growing in them, was inoculated with a consortium of isolates. Another set of artificially contaminated bags with the plant growing in them, were not inoculated with the consortium and served as control. They were incubated at ambient temperature for 45 days. The bags were watered with sterilized lagoon water and harvested for residual hydrocarbon estimation on days 0, 15, 30 and 45.

Analytical procedure

Gas chromatography (GC) was used to determine the residual hydrocarbons present in the samples. The liquid culture sample was extracted with hexane (1:1) and was concentrated to 1 ml, while the hydrocarbon was extracted from the soil samples with a mixture of hexane:dichloromethane (DCM) (70:30). A standard profile of the GC was first obtained by injecting 1 ml of the hydrocarbon standard into the GC and a chromatogram was generated to serve as a calibration window with which the test sample was analyzed. After generating the standard profile, 1 µl of the test sample was injected into the GC and an equivalent chromatogram was generated. The peak areas of the standard and the test sample chromatogram were compared with respect to the concentration of standard of the sample (Odebunmi et al., 2002).

RESULTS

Analyses of total hydrocarbon content in *Paspalum vaginatum* (Sw.) plant and oil treated soils

The soil analyses indicated a decrease in the level of THC (Table 1). The soil treated with 10.5 kg L⁻¹ (2000 ml) recorded the highest reduction of THC (77.8%). The level

of THC detected in 21.0 kg L⁻¹(1000 ml), 14.0 kg L⁻¹ (1500 ml), 8.4 kg L⁻¹ (2500 ml) and 7.0 kg L⁻¹ (3000 ml) of oil treated soil were 36%, 23.7%, 31.5%, 38.3% respectively. Analyses of the oil treated plants also showed the presence of THC in the tissues (Table 1).

Anatomical study of the stem

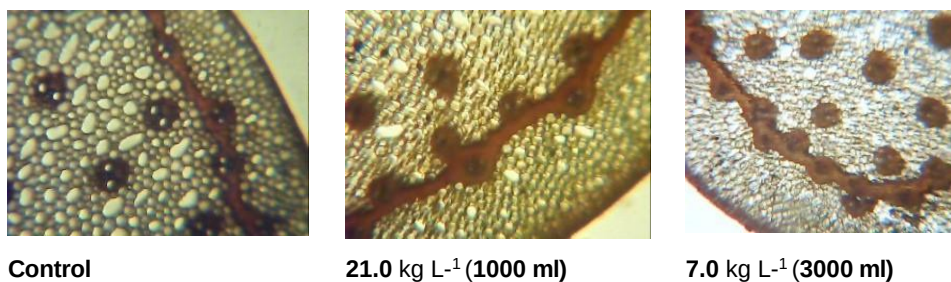
The presence of oil was not observed in anatomical structures of the treated *Paspalum vaginatum* stems. However, the parenchymal tissues were structurally disrupted, while cells in the stem of control plants remained unchanged (Figure 1).

Screening of hydrocarbon degrading bacteria on crude oil as a sole carbon source

The bacterial isolates obtained from the *P. vaginatum* and its rhizosphere soil were identified. Seven bacterial isolates were obtained from the rhizosphere soil. These include: *Serratia marcescens*, *Bacillus sphaericus*, *Bacillus pumilus*, *Arthrobacter* sp., *Pseudomonas aeruginosa* and *Micrococcus* spp. Four of the bacterial isolates which exhibited good growth during screening were selected for further studies. The isolates selected were *Bacillus pumilus*, *Serratia marcescens*, *Bacillus sphaericus* and *Arthrobacter* sp.

Growth profiles and degradation kinetics of bacterial isolates in liquid mineral salts medium

The population density of *B. pumilus* increased from 1.00 x 10² (day 0) to 3.55 x 10⁶ cfu ml⁻¹ (day15) and then there was a decline to 2.95 x 10⁵ cfu ml⁻¹ by day 21 (Figure 2). The growth rate constant was 0.0398 µd⁻¹ and the mean generation time was 17.39 d (Table 2). The percentage hydrocarbon degraded in 21 days was 95.6% (Table 3). *Serratia marcescens* and *Bacillus sphaericus* degraded about 98.3% and 96.7% hydrocarbon in the



Control

21.0 kg L⁻¹ (1000 ml)7.0 kg L⁻¹ (3000 ml)

Figure 1. Anatomical structures of sections of the stems of *Paspalum vaginatum* in hydrocarbon polluted soils.

Table 2. Growth kinetics of bacteria from the rhizosphere of *Paspalum vaginatum* in mineral salts medium with crude oil as sole carbon and energy source

Bacterial isolate	Growth rate constant (μd^{-1})	Mean generation time (d)
<i>Bacillus pumilus</i>	0.0398	17.39
<i>Serratia marcescens</i>	0.0467	14.85
<i>Arthrobacter</i> sp.	0.0725	9.567
<i>Bacillus sphaericus</i>	0.0272	25.53
Bioaugmented (soil with bacterial consortium)	0.0067	103.50
Control (soil without bacterial consortium)	0.0071	98.02

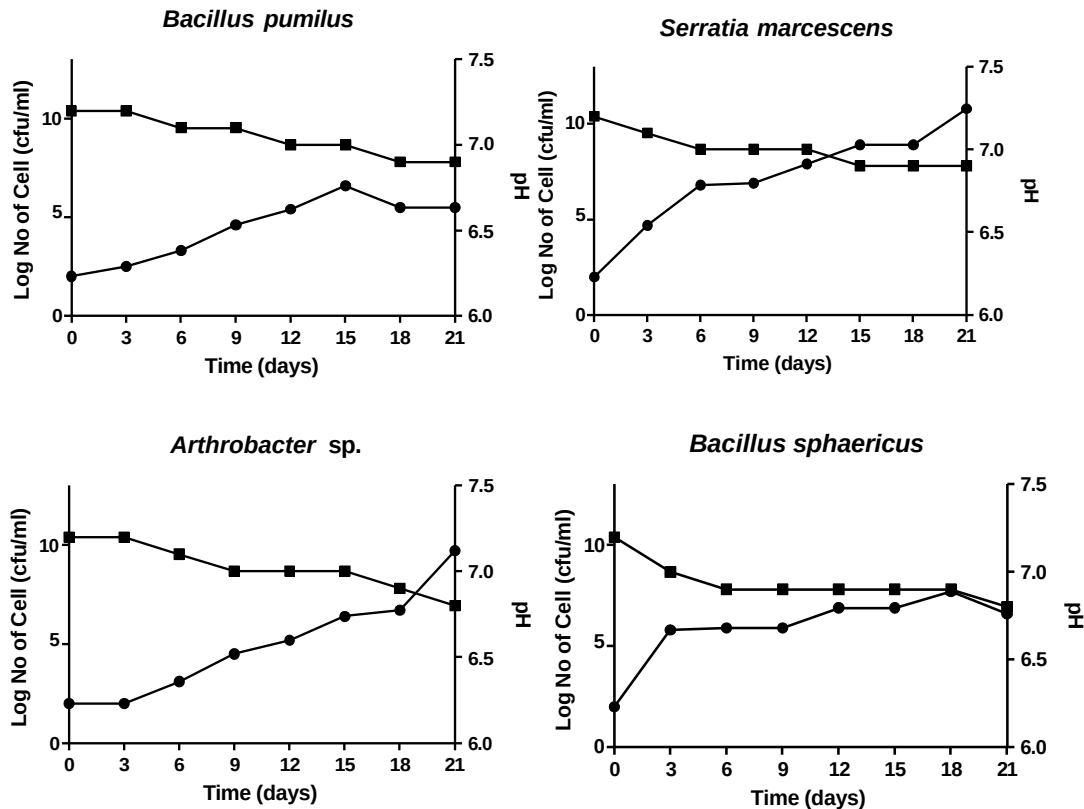
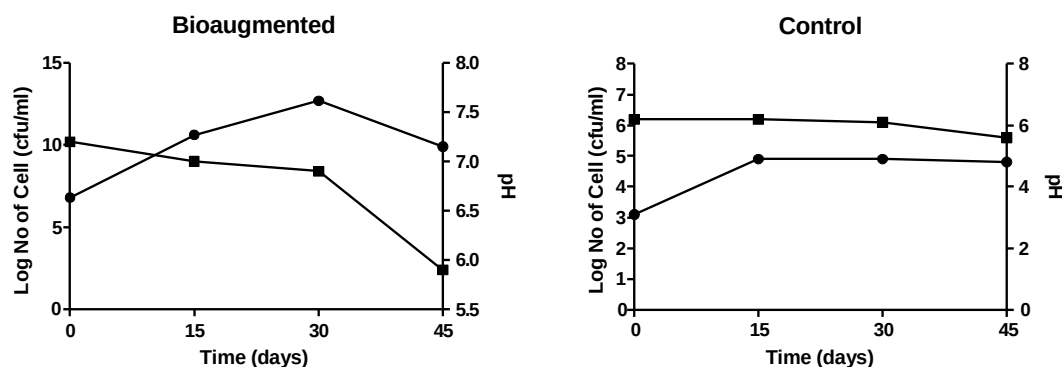
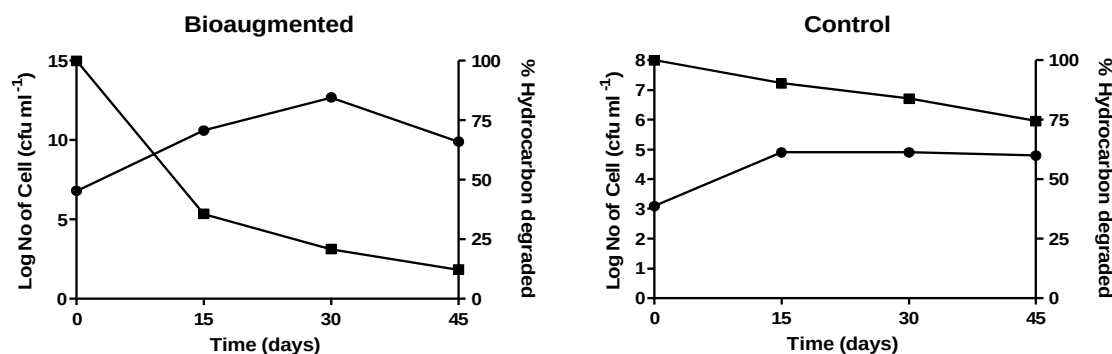


Figure 2. Growth profile of rhizosphere bacteria from *Paspalum vaginatum* in mineral salts medium with crude oil as sole carbon and energy source. ●, Log No of cell; ■, pH.

Table 3. Degradation of crude oil by rhizosphere bacteria of *Paspalum vaginatum* in mineral salts medium

Isolates	Residual hydrocarbon content (mg l ⁻¹)		
	Beginning (Day 0)	After (21 days)	% degraded
Control	4603.01	3596.31	21.9
<i>Bacillus pumilus</i>	4603.01	203.95	73.7
<i>Serratia marcescens</i>	4603.01	79.24	76.4
<i>Bacillus sphaericus</i>	4603.01	151.55	74.8
<i>Arthrobacter</i> sp.	4603.01	189.73	74.0

**Figure 3.** Growth profile of bioaugmented hydrocarbon polluted soil microcosm study of *Paspalum vaginatum*. ●, Log No of Cell (cfu ml⁻¹); ■, pH.**Figure 4.** Residual hydrocarbon degraded in polluted bioaugmented soil used in the cultivation of *Paspalum vaginatum*. ●, Log No of Cell (cfu ml⁻¹); ■, %hydrocarbon degraded

medium respectively in 21 days (Figure 2 and Table 3). Their population densities increased from 1.01×10^2 and 1.10×10^2 cfu ml⁻¹ (day 0) to 5.72×10^{10} and 5.13×10^7 cfu ml⁻¹ (day 21) respectively. Unlike the other isolates, a lag phase of nearly 3 days was observed in the growth of *Arthrobacter* sp., thereafter, there was a slow progression of growth. The population density of the isolate increased from 1.10×10^2 to 5.43×10^9 cfu ml⁻¹ in 21 days. It had a mean generation time of 9.56 d and about 95.9% of the hydrocarbon supplied as carbon and energy source was utilized in 21 days (Figure 2, Table 2 and Table 3). The

pH of the culture media of all the isolates reduced from 7.2 to 6.9 (Figure 2).

Growth profile and degradation kinetics of bacterial consortium in rhizospheric soil

The exponential phase for the bioaugmented soil with bacterial consortium occurred between day 0 and 30 with the highest population density of 4.51×10^{12} cfu g⁻¹ on day 30 at pH 6.85 (Figure 3). The bacterial population had a growth rate constant of $0.0067 \mu\text{d}^{-1}$ and mean

generation time of 103.50 d. The pH decreased to 5.86 with population density of 8.2×10^9 cfu g⁻¹ by day 45. Also, 87.7% of the hydrocarbon content present in the rhizosphere soil was degraded by day 45 (Figure 4). The population density of the non-inoculated control soil increased from day 0 to day 15 and remained relatively constant until the experiment was terminated on day 45. The hydrocarbon content also reduced by 25.5% (Figure 4).

DISCUSSION

Paspalum vaginatum grew remarkably well in hydrocarbon polluted soil used in the microcosm study despite the known toxicity of hydrocarbons. Analysis of the polluted soil at the end of the experiment showed a significant reduction in the level of total hydrocarbon content (THC). The presence of hydrocarbons in plant extracts indicates that the plant was mopping up the oil in its surrounding and was able to phytoremediate the soil. The plant was able to reduce the level of hydrocarbon in the contaminated soil possibly using a combination of phytoextraction as indicated by the uptake of hydrocarbon and rhizoremediation.

Although the presence of oil was not visible from anatomical studies but the damage to the cell membranes indicates that the hydrocarbon readily penetrated the plant and disrupted the cell membrane making it less permeable to water and thus affecting plant growth. Damage to cell membranes, leakages of cell contents, reduction in the rate of transpiration and photosynthesis and death of plants as a result of the penetration and accumulation of oil has been reported in several studies (DeLaune et al., 2003; Olusola and Anslem, 2010; Al-Hawas et al., 2012).

All the hydrocarbon degrading bacteria isolated in the study were aerobic. Many aerobic bacteria have the ability to utilize most hydrocarbons, mostly the *n*-alkane homologues (Frick et al., 1999). The tolerance of the *Bacillus* spp isolated in this study to high levels of hydrocarbons in soil supports the growing evidence that isolates belonging to the *Bacillus* sp. could be effective in clearing oil spills (Ghazali et al., 2004). The reason for their survival in this environment may be due to their resistant endospores. *Serratia marcescens* was shown to possess the ability to degrade a wide spectrum of hydrocarbons by Wongsu et al. (2004). *Paspalum vaginatum* supported the growth of a diverse assemblage of Hydrocarbon-degrading microorganisms including nitrogen-fixing bacteria such as *Arthrobacter* spp, which are known as an important group of plant growth-promoting rhizospheric microorganisms or PGPRs (Yateem et al., 2007).

There are many known consortia of microorganisms which are able to degrade mineral oil hydrocarbons under laboratory or field conditions (Popp et al., 2006).

However, a single bacterium usually has only a relatively small degradation range and thus, not all fractions of the mineral oil hydrocarbons can be degraded by a single species (Mishra et al., 2001). In this study, re-inoculation of the contaminated soil with the rhizosphere bacteria consortia showed a higher degradation potential for use in the remediation of oil-polluted sites.

P. vaginatum harbours in its leaves, stems and rhizosphere crude oil degrading bacteria. Therefore, its potential as a rhizoremediation tool is a cost-effective, environmental friendly approach. The use of native bacterial consortium with petroleum hydrocarbon utilizing capabilities as seed unto oil-impacted environment could prove a more efficient process which would on the long run enhance sustainable development rather than the use of exotic bacterial strains and chemicals.

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