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Research Article

Molecular identification of hydrocarbon-degrading bacteria isolated from alfisol-loam experimentally-contaminated with gasoline

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ABSTRACT: **Impacts of gasoline pollution on the soil environment, bacterial and weed population dynamics were investigated for several months. Known amounts of gasoline were added to 2kg of soil. In the first experiment the soil was immediately sampled for microbial load and presence of weeds. In the second experiment the soil was sampled after three months for microbial load and presence of weeds. In the third experiment the soil was sampled after 6 months for microbial load and presence of weeds. The soil was sampled weekly for twelve weeks in each of the three experiments. Weeds that grew on the soil were noted and identified. Bacteria isolated were identified using 16S rRNA sequencing. The identified bacteria include *Bacillus* spp., *Pseudomonas* sp, and *Ochrobactrum* sp. The bacteria were further tested for their ability to grow on gasoline as the only source of carbon and their growth measured by optical density, change in pH and total viable counts. Results showed ability to utilize gasoline as the only source of carbon and energy. The emulsification activities of the isolates were greater than 50% indicating higher potential to biodegrade gasoline. It is concluded that it is possible to isolate oil degrading bacteria capable of *in situ* biodegradation from the Niger Delta region of Nigeria. It is suggested that the identified weeds have some roles to play in the biodegradation of gasoline by bacteria.**

KEYWORDS: **Biodegradation, Bacteria, Gasoline, PCR, Weeds.**

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INTRODUCTION

Petroleum contamination is a recurring accident caused by the need for oil and gas in all segments of society and everyday life. Chemical degradation of these pollutants leaves cancer-causing compounds behind but microbial degradation does not. According to Jyothi *et al*. (2012), many microorganisms have the power to use hydrocarbons as sole sources of carbon and energy and these microorganisms are ubiquitous and widely spread in nature. Contaminated soils are threats to humans and the ecosystem. The majority of hydrocarbons are found in Diesel, Petrol, and Kerosene. The degradation rate is affected by several physical, chemical and biological parameters such as pH, temperature, nutrient and quantity of hydrocarbons (Santhini *et al*., 2009).

Petroleum production began in Nigeria in 1958 and since then, cases of petroleum and refined petroleum spills onto agricultural lands through petroleum production operations have been reported (Odu, 1977; Awobajo, 1981, Grevy, 1995, Moffat and Linden, 1995). The microbiological decontamination (bioremediation) of oil polluted soils is claimed to be an efficient, economic and versatile alternative to physico-chemical treatment (Atlas, 1991). Bioremediation involves the use of indigenous or introduced microorganisms to degrade environmental contaminants (Margesin and Schinner, 1997).

The soil environment is the most dynamic site of interactions in nature and it is also the region in which many of the biochemical reactions concerned in the decomposition of organic matter and nutrition of plants particularly agricultural crops occur (Torstensson *et al.,* 1998). Petrol is known as gasoline in North America. Outside North America it is referred to as petrol. Petrol contains low molecular weight compounds comprising high proportion of saturated hydrocarbons that are usually more toxic than long chained hydrocarbons. Some of the main components of gasoline are isooctane, butane, 3-ethyltoluene and the octane enhancer MTBE.

Exposure to gasoline in the workplace can occur by skin contact, eye contact swallowing it and breathing in vapors. The National Institute for Occupational Safety and Health (NIOSH) has designated gasoline as a carcinogen (CDC-NIOSH, 2016). The objectives of this work are to investigate the effect of gasoline on the germination of identified weeds, isolate bacteria present in the gasoline treated soil, and to observe the rate of natural biodegradation of the gasoline treated soil. The ultimate goal of this work is to use microorganisms to clean up oil contaminated soil and water for agricultural purposes.

MATERIALS AND METHODS

Chemicals and Reagents

Nutrient agar, peptone water and nutrient broth were purchased from Oxoid, UK. Reagents such as KH_2PO_4, Na_2HPO_4, $MgSO_4.7H_2O$, KNO_3, $(NH_4)_2SO_4$ were of analytical grade and were purchased from Sigma, US. Nigeria imports gasoline, jet fuel and kerosene from Asia, Europe America, and Africa. A proportion of the gasoline and kerosene used locally is refined at the local refineries in Nigeria. It is not certain if jet fuel is sourced locally. The gasoline and kerosene used in this work was purchased from a filing station in Tanke, Ilorin. It was collected in sterile containers. The jet fuel used was obtained from the storage facility of one of the aircraft fueling companies in Lagos, Nigeria.

Experimental set up

Alfisol loam was collected in new polythene bags. The experimental set up comprised of eight treatment options including the control, each option had three replicates. Each pot contained 2kg of soil and was treated with eight different concentrations of gasoline; 0 (control), 7, 14, 21, 56, 112, 168 and 224 ml according to the method of Ekpo and Thomas (2007). The arrangement of the pots was randomised according to standard methods. Randomnisation here refers to scattered arrangement of the pots such that same concentration of gasoline in soil are not put side by side. The pots were perforated at the bottom and sides to allow for aeration and drainage of excess water. The set up was watered throughout the investigation period.

Isolation and identification of bacteria

The four bacteria used in this work were isolated from soil collected at the campus of the University of Ilorin, Nigeria and experimentally polluted with gasoline. For the identification of the isolates, colony PCR was performed using 16S rRNA-gene primers: Bact 27F (5' AGAGTTTGATCMTGGCTCAG-3') and U1492R (5'-TACGGYTACCTTGTTACGACTT-3') as described by Spears *et al.* (2005). Amplified PCR products were cleaned using ExoSAP-IT and sequenced at the DNA core facility, Oklahoma State University, Stillwater, Oklahoma. Amplified 16S rRNA gene sequences were analyzed using the National Center for Biotechnology Information (NCBI) database - BLASTn. The organisms were identified based on the percentage similarity of their sequences with those in the NCBI database.

Growth of bacterial isolates on gasoline substrate

Time course of degradation of the gasoline was performed using mineral salts medium described by Adetitun *et al.,* (2014) and Vecchioli *et al*., (1990). Growth of the bacterial

Table 1. Isolated and identified bacterium, closest relative, phylum, sequence similarity and sequence length (nt).

Bacterium	Closest Relative	Phylum	Sequence similarity (%)	Sequence length (nt)
Pseudomonas sp.	*Pseudomonas aeruginosa* strain	*Proteobacteria*	95	1185
Bacillus sp.	*Bacillus subtilis strain MR3*	*Firmicute*	96	1157
Ochrobactrum sp.	*Ochrobactrum sp. VH-19*	*Proteobacteria*	97	1145
Bacillus sp.	*Bacillus subtilis* strain	*Firmicute*	95	1106

species on hydrocarbon substrates was carried out by the inoculation of each of the bacterial cultures into a 250 ml Erlenmeyer flask containing 99 ml of mineral salts medium (MSM) as described before (Adetitun *et al.* 2014); Oboh *et al.* 2006). The composition of the MSM (g/L): 0.5 KH_2PO_4; 1.4 Na_2HPO_4; 0.2 $MgSO_4.7H_2O$; 0.3 KNO_3; 1.0 $(NH_4)_2SO_4$. The pH of the medium was adjusted to 7.0. Each flask was supplemented with 1ml of gasoline added to the medium as the only carbon source. Control flasks with no added gasoline were setup similarly. The culture flasks were incubated for 168 hours at 37^oC with continuous agitation in a rotatory incubator shaker. The optical density (OD 600nm), colony forming unit (CFU) and pH of the culture fluids were monitored every 24 hours as indicators of biodegradation.

Determination of kerosene, gasoline and jet fuel utilization by isolated bacteria

Kerosene, gasoline and jet fuel utilizing bacteria were noted by streaking the organisms on mineral salts medium (containing 1.5% agar for solidification) described by Adetitun *et al.,* (2014) and Vecchioli *et al.*, (1990). The composition of the MSM (g/L): 0.5 KH_2PO_4; 1.4 Na_2HPO_4; 0.2 $MgSO_4.7H_2O$; 0.3 KNO_3; 1.0 $(NH_4)_2SO_4$. The pH of the medium was adjusted to 7.0. Control flasks with no added gasoline were setup similarly. Each flask was supplemented with 1% of gasoline as the only carbon source. Other set ups contained kerosene and jet fuel as the sole carbon and energy sources. Growth on the agar plates was recorded after 24 to 48 hours.

Emulsification measurement

Emulsification activity was measured according to the method of Dhail and Jasupa (2012). To 4 ml of culture supernatant or biosurfactant crude extract (0.5%, w/v), 4 ml of gasoline were added and vortexed at high speed for 2 min. The mixture was allowed to stand for 10 min prior to measurement. The emulsification activity is defined as the height of the emulsion layer divided by the total height and expressed as percentage.

$$\text{Emulsification Activity} = \frac{\text{Height of emulsion layer}}{\text{Total height}} \times 100$$

Statistical analyses

One-way analysis of variance (ANOVA) test was used to determine whether time course of hydrocarbon degradation of hydrocarbons and other measured parameters differed significantly according to type of inocula. P value of less than 0.05 was considered to indicate statistical significance.

RESULTS AND DISCUSSION

At the start of the experiment (0, 3 and 6 months after contamination) the soil had no weeds. But as watering was done on the experimental set up in the field and the experiment proceeded weeds were observed on the gasoline treated soil and they were noted as indicated in table 2, 3 and 4. The results of the blasted sequences showed that the organisms belong to various genera including *Bacillus, Pseudomonas,* and *Ochrobactrum* (Table 1).

The results obtained in this work shows that ageing improves the quality and performance of soil in terms of ability to sustain plants (weeds). It also shows that ageing helps the biodegradation process of contaminated soils. It was observed that soil left for 3 and 6 months did better than soil that was left for 0 month. Soil left for 6 months did better than those left for 3 months. Thirty weeds grew on the soil treated with gasoline and left for 6 months while eighteen plants were identified in the soil treated with gasoline and left for three months. Seventeen weeds were obtained in the soil treated with gasoline and analysed after 0 month. Lerch *et al.* (2009) reported that ageing processes and soil microbial community affects the biodegradation of nonextractable residues (NER) of pesticides in soil. Njoku *et al.* (2008) reported that the performance of *Glycine max* L. (Merrill) grown in oil polluted soil got better with ageing suggesting that the toxicity of petroleum to the plant became lower with age.

Table 2: Occurrence of identified weed plants in the control and gasoline treated soils *(0 month post contamination).*

	Identified Weeds	Gasoline Concentration							
		0ml	7ml	14ml	21ml	56ml	112 ml	168 ml	224 ml
1	*Eragrostis tenella*	-	-	-	-	+	-	-	+
2	*Desmodium tortuosum*	-	+	-	-	-	-	+	+
3	*Oplismenus bumanni*	+	-	+	-	+	-	-	-
4	*Phyllanthus amarus*	+	+	+	+	+	-	-	-
5	*Gomphrena celosidoides*	+	-	-	-	-	+	-	+
6	*Dactyloctenium aegyptium*	-	-	-	+	+	-	-	-
7	*Borrena spp*	-	-	-	+	+	-	-	-
8	*Paspalum conjugatum*	-	-	-	+	+	-	+	-
9	*Euphorhia hita*	-	+	-	-	+	+	-	-
10	*Tridax procumbens*	+	+	-	-	+	-	-	-
11	*Oldenlandia corymbosa*	-	-	+	+	-	-	+	-
12	*Gomphrena celosiodes*	-	+	+	-	-	+	-	-
13	*Eleucine indica*	-	-	+	-	+	-	-	-
14	*Digitaria horizontalis*	-	-	+	-	+	-	-	-
15	*Cyperrus differmis*	-	-	+	+	-	-	-	-
16	*Oldentandiia herbacea*	+	-	-	-	+	+	+	+
17	*Axonopus compressus*	+	+	+	-	-	+	-	-

+ = Present: -= Absent

About 94–98% loss of petroleum product has been reported to occur in the upper 5 cm layer within 2 days in enclosures such as we have used in our set up. This is attributed to physicochemical processes, i.e. a combination of leaching deeper into the sediment and evaporation (Jarsjö *et al.* (1994). In this study soil samples were taken from a depth of 5-10cm.

One unit emulsifying activity is the amount of emulsifier that gave a percent emulsification of 20. Emulsification activity greater than 50% is taken as a higher propensity to biodegrade gasoline and hence clean up polluted sites. This observation indicates that the bacterial isolates therefore have the potential to initiate biodegradation of hydrocarbons and specifically in this case gasoline.

P*seudomonas* sp. occurred throughout the period of investigation and especially were isolated from the pots with higher pollution, starting from 168 ml to 224 ml. *Pseudomonas putida* have been reported in the biodegradation of chlorpyrifos (Liu *et al*., 2016). In a recent *study, Bacillus* sp was able to degrade cypermethrin (Pankaj *et al*., 2016). *Pseudomonas aeruginosa* and *Bacillus subtilis* have clearly been involved in biodegradation of petrol and crude oil as reported by Das and Mukherjee (2006); Darsa and Thatheyus (2014). *Ochrobactrum* sp have been reported as an agent of biodegradation of BTEX hydrocarbons by Eraky *et al*. (2015). Ochrobactrum sp had been reported to be instrumental in the biodegradation thifensulfuron-methyl (Zhao *et al*., 2015).

Table 3: Occurrence of identified weed plants in the control and gasoline treated soils *(3 months post contamination).*

	Identified Weeds	Gasoline Concentration							
		0ml	7ml	14ml	21ml	56ml	112ml	168ml	224ml
1	*Eragrostis tenella*	+	-	-	+	+	-	-	+
2	*Desmodium tortuosum*	+	+	-	+	+	+	-	+
3	*Oplismenus bumanni*	+	-	+	-	-	+	-	-
4	*Phyllanthus amarus*	+	+	+	+	+	+	+	+
5	*Gomphrena celosidoides*	+	-	-	+	-	-	+	-
6	*Dactyloctenium aegyptium*	+	-	+	-	-	-	-	-
7	*Borrena spp*	+	-	-	-	-	-	-	-
8	*Paspalum conjugatum*	-	+	+	-	-	-	+	-
9	*Euphorhia hita*	-	+	+	+	+	+	-	-
10	*Tridax procumbens*	-	+	-	-	+	-	-	-
11	*Oldenlandia corymbosa*	-	+	+	+	-	-	+	-
12	*Gomphrena celosiodes*	-	-	+	-	-	+	-	-
13	*Eleucine indica*	-	-	+	-	-	-	-	-
14	*Digitaria horizontalis*	-	-	+	-	-	-	-	-
15	*Cyperrus differmis*	-	-	-	+	-	-	-	-
16	*Oldentandiia herbacea*	-	-	-	+	+	+	+	+
17	*Axonopus compressus*	-	-	-	-	-	+	-	-
18	*Kyllinga squamlata*	-	-	+	-	-	-	-	-

+ = Present: -= Absent

The changes in optical densities, total viable counts and pH of the experimental flasks containing the culture fluids during the period of incubation is an indication of ongoing biological and chemical reactions of the gasoline substrate. (Oboh *et al.,* 2006; Atlas and Bartha, 1972). These microbes have been shown to be hydrocarbon degraders by researchers such as Oboh *et al.* (2006), Atlas and Bartha (1972), Nwanchukwu (2001). This can be attributed to genetic makeup due to the constitutive expression of hydrocarbon catalysis enzymes or physiological owing to exposure to exogenous hydrocarbons present in the gasoline.

All the bacterial isolates have been demonstrated to grow on MSM with gasoline as the sole source of carbon. Normally, microbial growth in fuel can drive the pH up or down. Most commonly, microbially produced organic acids cause the pH to fall over time. However, alkaline metabolites such as carbon dioxide and some polypeptides may also accumulate and is reflected through the increase in pH. All these were observed in this study (Figure 1 – 4). These variations however do not hinder the net degradation process (Passman *et al,* 1997). The observations recorded in this study conform to earlier findings by Oboh *et al*., (2006) and Moneke and Nwangwu, (2011); where increase in total viable counts, rise in optical densities and a drop in pH was observed. The pH changes observed and recorded here are characteristic part of deterioration process as reported by these workers. The implication of changes in pH in monitoring biodegradation is not clear. This is because pH can rise or fall depending on the products or metabolites that predominate in the reaction mixture. The observed increase in optical densities indicates ability to use as carbon source and hence degrade the oil.

Adetitun *et al.,* (2014; 2016) observed that kerosene contamination affected soil physicochemical properties in addition to pH, plant and bacterial biomass which are important indicators for assessing soil quality, fertility and productivity. The same can be said here. Nevertheless, the ability of the weeds to grow on the contaminated soil is a pointer to the fact that they can be useful in phytoremediation studies. In actual oil pollution clean-ups, a combination of technologies is employed with the ultimate goal of cleaning up the polluted site. Wheat inoculation has been demonstrated to promote the biodegradation of alkane and polycyclic aromatic hydrocarbon (Ingrid *et al*., 2016).

Table 4: Occurrence of identified weed plants in the control and gasoline treated soils *(6 months post contamination).*

	Identified weeds	Gasoline Concentration							
		0ml	7ml	14ml	21ml	56ml	112ml	168ml	224ml
1	*Eragrostis tenella*	-	-	-	-	-	-	+	-
2	*Desmodium tortuosum*	-	-	-	-	-	-	-	-
3	*Oplismenus bumanni*	-	-	-	-	-	-	+	-
4	*Phyllanthus amarus*	-	-	+	+	+	-	-	-
5	*Gomphrena celosidoides*	-	-	-	-	-	-	-	-
6	*Dactyloctenium aegyptium*	-	-	-	-	-	-	-	-
7	*Borrena spp*	-	-	-	-	-	-	-	-
8	*Paspalum conjugatum*	-	+	-	-	-	-	-	-
9	*Euphorhia hita*	-	-	+	-	-	-	+	-
10	*Tridax procumbens*	+	+	-	+	+	-	+	+
11	*Oldenlandia corymbosa*	+	-	-	-	-	-	-	-
12	*Gomphrena celosiodes*	-	-	-	-	-	-	-	-
13	*Eleucine indica*	-	-	-	-	-	-	-	-
14	*Digitaria horizontalis*	-	-	-	-	-	-	-	-
15	*Cyperrus differmis*	+	-	-	-	-	-	-	-
16	*Oldentandia herbacea*	+	-	-	-	+	+	-	-
17	*Axonopus compressus*	-	-	-	-	-	-	-	-
18	*Kyllinga squamlata*	-	-	-	-	-	-	-	-
19	*Ageratum conyzoides*	+	+	+	+	+	+	-	+
20	*Mariscus alternifolius*	+	-	+	+	-	+	+	+
21	*Mariscus flabelliformis*	-	+	+	-	+	-	-	-
22	*Brachiaria lata*	-	+	-	-	+	-	-	-
23	*Brachiaria deflexa*	-	-	-	-	+	-	-	-
24	*Cyperus esculentum*	-	+	-	-	-	-	-	-
25	*Euphorbia hyssopifolia*	-	-	-	+	-	-	-	-
26	*Crotolaria retusa*	-	-	-	+	-	-	-	-
27	*Spigellia anthelmia*	-	-	-	-	-	+	-	-
28	*Fimbristilis littoralis*	-	-	-	-	-	+	-	-
29	*Elitrophorus spicatus*	-	-	-	-	-	+	-	-
30	*Emilia coccinea*	-	-	-	-	-	-	-	+

+ = Present; - = Absent

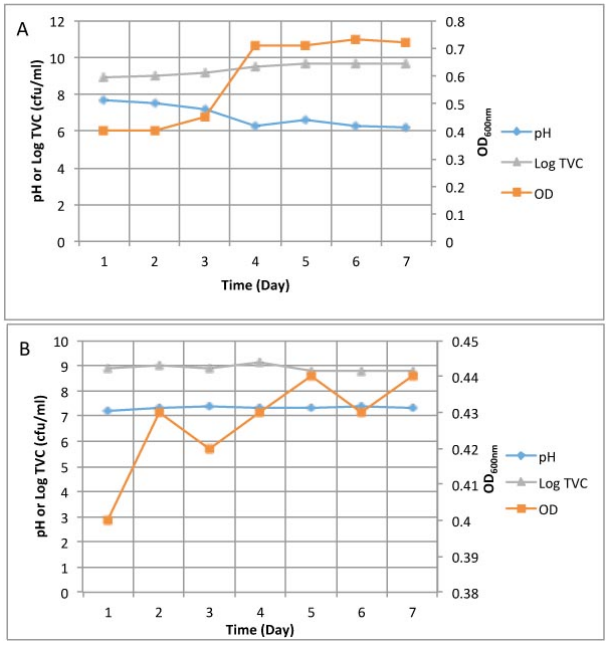

Figure 1: Growth configuration of *Bacillus* sp (Closest relative - *Bacillus subtilis* strain MR3) on gasoline. (A) with gasoline; (B) without gasoline.

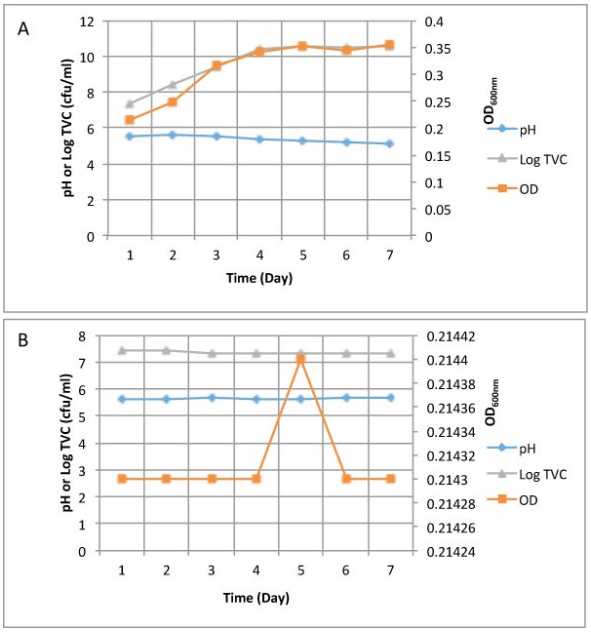

Figure 3: Growth configuration of *Bacillus* sp (Closest relative - *Bacillus subtilis* strain) on gasoline. (A) with gasoline; (B) without gasoline.

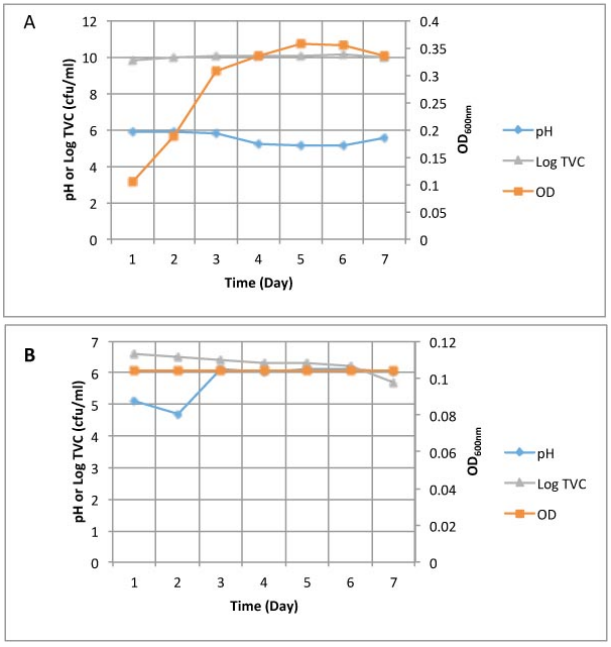

Figure 2: Growth configuration of *Pseudomonas* sp (Closest relative - *Pseudomonas aeruginosa* strain) on gasoline. (A) with gasoline; (B) without gasoline.

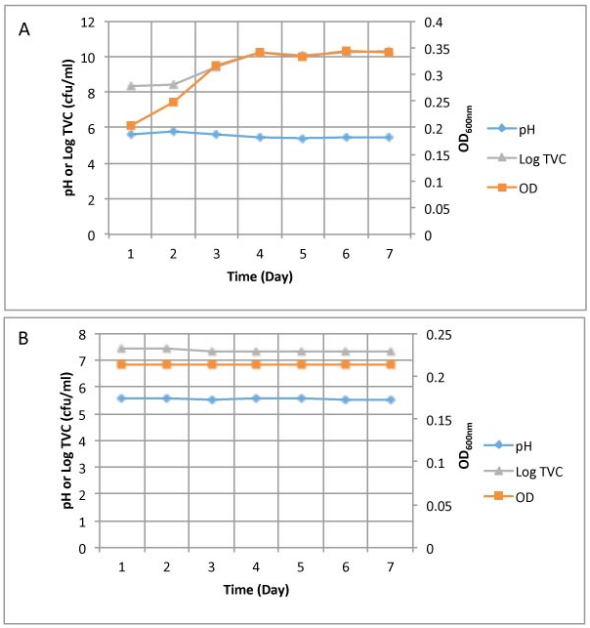

Figure 4: Growth configuration of *Ochrobactrum* sp (Closest relative - *Ochrobactrum* sp. VH-19) on gasoline. (A) with gasoline; (B) without gasoline.

Table 5: Emulsification activity, kerosene utilization and jet fuel utilization of the isolates obtained from gasoline contaminated soil.

Organisms	Emulsification Activity (%)	Kerosene Utilisation	Jet Fuel Utilisation
Bacillus sp.	52.08±0.02[b]	+	+
Bacillus sp.	50.00±0.03[a]	+	+
Pseudomonas sp.	54.90±0.04[c]	+	+
Ochrobactrum sp.	50.00±0.04[a]	+	+

Values are presented as Mean ± SD (n=3). Each value is a mean of three determinations ± standard deviation. Different superscripts are significantly different (P<0.05).

The identified weeds in Tables 2, 3 and 4 may have some roles to play in the biodegradation of gasoline. As gasoline has more than 50% of alkanes, the identified weeds may have roles to play in the biodegradation of the alkanes present in gasoline.

The outcome of this study point out the need to give attention to the interrelated variables that influence bacterial community structure in complex habitats like soil. This investigation represents a successful attempt to check natural attenuation of gasoline soil. The main conclusion of this work is that a natural population readily able to degrade gasoline is present in the soil used for this experiment. This is quite interesting as the soil had not previously been exposed to any form of oil spill. This brings high hopes of getting oil degrading bacteria capable of *in situ* biodegradation from the Niger Delta region of Nigeria and other polluted environments in the world.

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