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SCREENING FOR HEAVY MOLECULAR WEIGHT HYDROCARBON UTILIZING BACTERIA FROM OIL IMPACTED, NON OIL IMPACTED SOIL AND NATURAL DEPOSITS

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ABSTRACT

Control and treatment of hazardous effects of heavy molecular weight oil (bitumen) pollution are essential in contaminated soil. This study involved the isolation and screening of microorganisms capable of utilizing heavy molecular weight hydrocarbon from oil impacted, non oil impacted soil and natural deposits of bitumen. Total heterotrophic bacterial counts in the samples ranged from 1.4×10^5 CFU/g to 2.0×10^6 CFU/g. Total oil utilizing bacterial counts varied from 1.5×10^4 CFU/g to 3.6×10^5 CFU/g. Isolates were identified using API 20E kit. They belong to the genera *Burkholderia, Pseudomonas,* and *Serratia.* Degradation efficiency of the isolates on Premium Motor Spirit (PMS), Dual Purpose Kerosene (DPK) and Low Pour Point Fuel Oil (LPFO) were carried out by a colorimetric rapid screen test using 2, 6-dichlorophenol indophenol (DCPIP) reduction test which was monitored by measuring absorbance at 600 nm at every 24 hrs for 120 hrs. Order of ability of the isolates to degrade PMS: *P. aeruginosa* > *P. mendocina* > *P. borbori* > *S. rubidae* > *P. cichorii* > *B. cepacia* while for DPK is *P. cichorii* > *P. borbori* > *S. rubidae* > *P. mendocina* > *B. cepacia* > *P. aeruginosa.* Ability to degrade LPFO: *P. cichorii* > *P. borbori* > *P. aeruginosa* > *P. mendocina* > *B. cepacia* > *S. rubidae*.

Keyword: Biodegradation, Bitumen, *Burkholderia, 2,6-Dichlorophenol Indophenol (*DCPIP), Hydrocarbon, *Pseudomonas*, *Serratia.*

INTRODUCTION

Until recently, conventional light crude oil has been abundantly available and has easily met world demand for this form of energy (Meyer *et al*., 2007). Conventional oil production has peaked and is now on a terminal, long-run global decline (Gordon, 2012). However, demand for crude oil worldwide has substantially increased which had resulted in straining the supply of conventional oil. This however, has led to consideration of alternative or insufficiently utilized energy source to supplement short and long-term needs (Meyer *et al*., 2007).

According to Gordon (2012), unconventional oils are heavy, complex, carbon laden, and locked up deep in the earth, tightly trapped between or bound to sand, tar, and rock. From extraction through final use, these oils require a greater amount of processes and treatment to produce than conventional oil. Consequently, as output drastically scales up to meet increasing global demand

for high-value petroleum products, unconventional oils are expected to deliver a higher volume of heavier hydrocarbons.
The world's oil reserves are dominated by heavy and super-heavy oils in the super-giant tar sands common in shallow reservoirs on the flanks of foreland basins in North and South America and elsewhere (Head *et al*., 2003). These oils also represent a significant fraction of the petroleum in conventional oil reserves and will be common among future oil discoveries likely to be made in deep-water areas of the world (for example, the Atlantic margin basins of Africa, South America, Canada and the Gulf of Mexico) (Head *et al*., 2003).

Total resources of heavy oil in known accumulations are 3,396 billion barrels of original oil in place, of which 30 billion barrels are included as prospective additional oil while the total natural bitumen resource in known accumulations amounts to 5,505 billion barrels of oil originally in place, which includes 993 billion barrels as prospective additional oil (Meyer *et al*., 2007). This resource is distributed in 192 basins containing heavy oil and 89 basins with natural bitumen (Meyer *et al*., 2007).

The advent of modern industrialization, development in transportation industry and the use of petroleum based energy products had increased water and soil pollution (Patil *et al*., 2012). Exploration and exploitation of the vast resources of heavy oil will eventually become a source of concern for the environment (Nwachukwu, 2003). Hence, the need to research possible ways of mitigating the effect of it on the environment.

The processes leading to the eventual removal of hydrocarbon pollutants from the environment has been extensively documented and involves the trio of physical, chemical and biological alternatives (Okoh and Trejo-Hernandez, 2006). However, bioremediation which is defined as any process that uses microorganisms or their enzymes to return the environment altered by contaminants to its original condition, is an attractive process owing to its cost effectiveness and the benefit of pollutant mineralization to CO_2 and H_2O (Okoh, 2006). Bioremediation is emerging as one of the most promising technologies for the removal of petroleum hydrocarbons from the environment (Varjani *et al*., 2013). For this, the screening of potential oil utilizing microorganisms is one of the key steps.

Hydrocarbon degrading microorganisms are widely distributed in marine, freshwater, and soil habitats. The ability to isolate high numbers of certain oil-degrading microorganisms from an environment is commonly taken as evidence that those organisms are active degraders in that environment (Okerentugba and Ezeronye, 2003).

Microorganisms have been known to utilize hydrocarbons but there is limited data on the organisms that can utilize heavy molecular weight hydrocarbon. Hence, this study was designed to isolate, characterize and screen for isolates that are able to utilize heavy molecular weight hydrocarbon.

MATERIALS AND METHODS

Collection of samples

Soil impacted with refined bitumen were collected randomly from Odeda, Osiele and Isolu, all in Odeda Local Government of Ogun State, while the natural bitumen used was obtained from Agbabu bitumen deposit in Odigbo Local Government of Ondo State. The unimpacted soil samples (control samples) were collected from the campus of

Federal University of Agriculture, Abeokuta (FUNAAB) Ogun State. Collection of soil samples was carried out as described by Balogun and Fagade (2008). Samples were collected in sterile aluminum foil and stored at 4 ^{0}C until used. All samples were collected in triplicates.

Total Heterotrophic Bacterial Count (THBC)

Total heterotrophic bacterial count was determined using the method of Rahman *et al*. (2002). One gram of each of the samples were serially diluted up to 10^{-5} in sterile distilled water. The 1ml of the diluents was aseptically dispensed into sterile Petri-dishes. Using the pour plate method, Plate Count Agar (Lab M, UK) prepared according to manufacturer's instructions was poured aseptically on inoculated plates. The plates were then incubated at 28 ^{0}C for 24 h.

Total Oil Utilizing Bacterial Count (TOUBC)

Oil utilizing bacterial count was carried out on Mineral Salt Medium (MSM) agar on which Dual Purpose Kerosene (DPK) was used as the sole carbon source. Prior to use, DPK was filtered using a Whatman filter paper No1 (Jyothi *et al*., 2012). Agar (2%) was added to solidify the medium. The MSM composition as described by Balogun and Fagade (2010) was made up of Basal Salt Medium (BSM) and Trace element solution. The BSM contained (g/L): K_2HPO_4, 1.8; KH_2PO_4, 1.2; NH_4CL, 4.0; $MgSO_4.7H_2O$, 0.2; NaCl, 0.1; yeast extract, 0.1 and $FeCl_2.4H_2O$, 0.05. Trace elements solution contained: H_3BO_3, 0.1; $ZnSO_4.7H_2O$, 0.1; $CuSO_4.5H_2O$, 0.05 and $MnSO_4.H_2O$, 0.04 with the pH of 6.5. The basal salt medium and the trace elements solution were sterilized separately. Exactly 10 ml of the trace elements solution was added aseptically to the sterilized basal salt medium to make it up to a litre. One millilitre of the serially diluted samples was aseptically dispensed into sterile Petri-dishes using the pour plate method. The medium was allowed to solidify and the plates incubated at 28 ^{0}C for 5 days.

Confirmatory screening of hydrocarbon degrading bacteria by 2,6- dichlorophenol indophenol oxidation test

The hydrocarbon degradation abilities of the isolates were tested on PMS, DPK and LPFO. The method of Bidoia *et al*. (2010) was employed. One hundred microlitres of 24 h pre-cultured isolates in peptone water, with optical density of 0.5, was inoculated into 7.5 ml of MSM incorporated with 50 µL of the hydrocarbon substrate. Then, 400 µL of 2, 6-dichlorophenol indophenol (DCPIP) was added and incubated at 28 ^{0}C for 5 days. The absorbance of the medium at 600 nm was measured at intervals of 24 h for 120 h using a colorimeter (Jenway, UK). The DCPIP was used as the blank and 5 ml of the sample was used to determine the colorimetric readings using a glass cuvette. The control experiment was set up in similar manner but without seeding with the isolates. Absorbance which has a direct relationship with colour intensity of DCPIP indicator was used as a means of measuring hydrocarbon degradation.

Characterization of bacterial isolates

Biochemical characterization of the isolates was done using API 20E identification kit (Biomérieux, France). API 20E kit was used according to the manufacturer's instruction.

RESULTS

Total heterotrophic bacterial count (THBC) and total oil utilizing bacterial count (TOUBC) from bitumen impacted and unimpacted soil (Table 1) showed that the THBC range from 1.4×10^5 to 2.0×10^6 CFU/g with Odeda having the highest bacterial counts. The sample from Odeda had the highest total oil utilizing bacterial count (TOUBC) of 3.6×10^5 CFU/g while the lowest TOUBC of 1.5×10^4 CFU/g was recorded at FUNAAB.

Table 1. Heterotrophic and oil utilizing bacterial counts from various sampling points

Sample	Location	Coordinates		THBC 104 CFU/g)	TOUBC 104 CFU/g)
1	Odeda	7.23119 N	3.52687 E	205.0±7.1b	36.0±5.7b
2	Osiele	7.23278 N	3.52294 E	23.50±2.1a	4.5±0.7a
3	Isolu	7.20425 N	3.44136 E	19.3±1.1a	7.0±1.4a
4	Agbabu	6.35253 N	4.49547 E	16.8±1.8a	7.5±0.7a
5	FUNAAB	7.22844 N	3.43618 E	16.0±2.8a	1.5±0.7a

Key:
THBC: Total Heterotrophic Bacterial Count
TOUBC: Total Oil Utilizing Bacterial Count
Values are Mean ± Standard Error of Means
Mean values with same letter within a column are not significantly different at $P<0.05$

Biochemical profile of the isolates

All the isolates were Gram negative rods, catalase positive and grew on MacConkey agar (Table 2). All the isolates were able to utilize glucose except *Pseudomonas borbori*. All the isolates were able to liquefy gelatin except *Pseudomonas cichorii*. All were able to utilize saccharose except *Burkholderia cepacia*. However, all the isolates were Voges-Proskauer negative, do not produce ornithine decarboxylase and lysine decarboxylase. Only *B. cepacia* was able to utilize sorbitol.

Absorbance which correlates to the ability of the isolates to utilize PMS was determined colorimetrically using DCPIP as indicator (Table 3). *Pseudomonas aeruginosa* had the lowest absorbance value of 0.39 after 5 days of incubation followed by *P. mendocina* with the value of 0.55. *Burkholderia cepacia* had the highest absorbance value of 0.65 after 5 days.

Table 2: Biochemical characterization of isolates using API 20E Kit

Name of biochemical test	Isolates					
	SD3	SD4	SI11	SI13	SI17	SA18
Gram reaction	-	-	-	-	-	-
Shape	Rod	Rod	Rod	Rod	Rod	Rod
ONPG: β-galactosidase	-	-	-	-	-	+
Arginine Dihydrolase	-	-	-	+	+	-
Lysine Decarboxylase	-	-	-	-	-	-
Ornithine Decarboxylase	-	-	-	-	-	-
Citrate Utilization	-	-	+	+	+	+
Hydrogen sulphide production	-	-	+	-	-	-
Urease production	-	-	+	-	-	-
Tryptophan Deaminase	-	-	+	-	-	-
Indole production	-	-	+	-	-	-
Voges–Proskauer	-	-	-	-	-	-
Gelatin test	-	+	+	+	+	+
Glucose oxidation	+	-	+	+	+	+
Mannitol oxidation	-	-	+	-	+	+
Inositol oxidation	-	-	-	-	+	+
Sorbitol oxidation	-	-	-	-	+	-
Rhamnose oxidation	-	-	+	-	+	-
Saccharose oxidation	+	+	+	+	-	+
Melibiose oxidation	-	-	-	+	-	-
Amygdalin oxidation	-	+	+	+	-	-
Arabinose oxidation	-	-	-	-	-	-
Oxidase test	-	+	+	+	+	-
Growth on MacConkey Agar	+	+	+	+	+	+
Catalase test	+	+	+	+	+	+
Probable Organism	Pseudomonas cichorii	Pseudomonas borbori	Pseudomonas aeruginosa	Pseudomonas mendocina	Burkholderia cepacia	Serratia rubidae

Table 3. Oxidation of DCPIP (Indicator) by isolates as a measure of PMS degradation

	Absorbance at 600 nm				
Isolate	24 h	48 h	72 h	96 h	120 h
Control	0.90	0.90	0.86	0.85	0.79
P. cichorii	0.84	0.84	0.78	0.69	0.61
P. borbori	0.81	0.76	0.64	0.60	0.58
P. aeruginosa	0.64	0.60	0.51	0.45	0.39
P. mendocina	0.81	0.75	0.70	0.64	0.55
B. cepacia	0.84	0.80	0.80	0.74	0.65
S. rubidae	0.77	0.70	0.63	0.59	0.59

Figure 1 shows the utilization of DPK by the isolates. *P. cichorii* had the lowest colour intensity as revealed by the absorbance value of 0.06 followed by *P. borbori* with absorbance of 0.09 while *P. aeruginosa* had the highest at 0.73 after 120 hours.

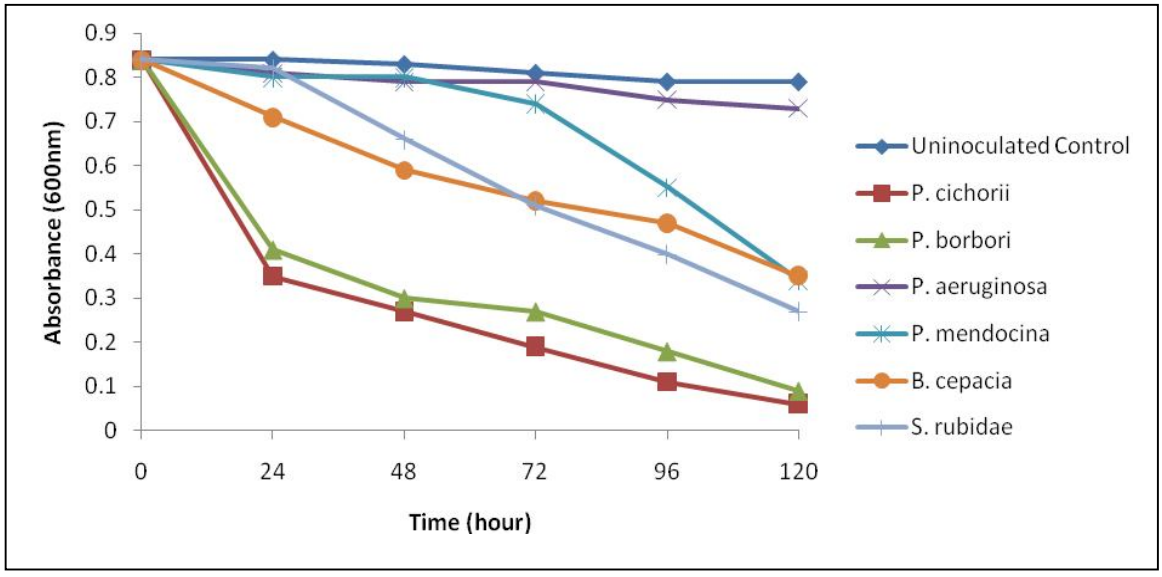

Figure 1. Oxidation of DCPIP by isolates as a measure of DPK degradation

Figure 2 represents the absorbance at 600 nm of the isolates growing on LPFO as the only carbon source. *S. rubidae* had the highest colour intensity as shown by the absorbance value of 0.56 at 120 hours while *P. cichorii* had the lowest colour intensity of 0.12.

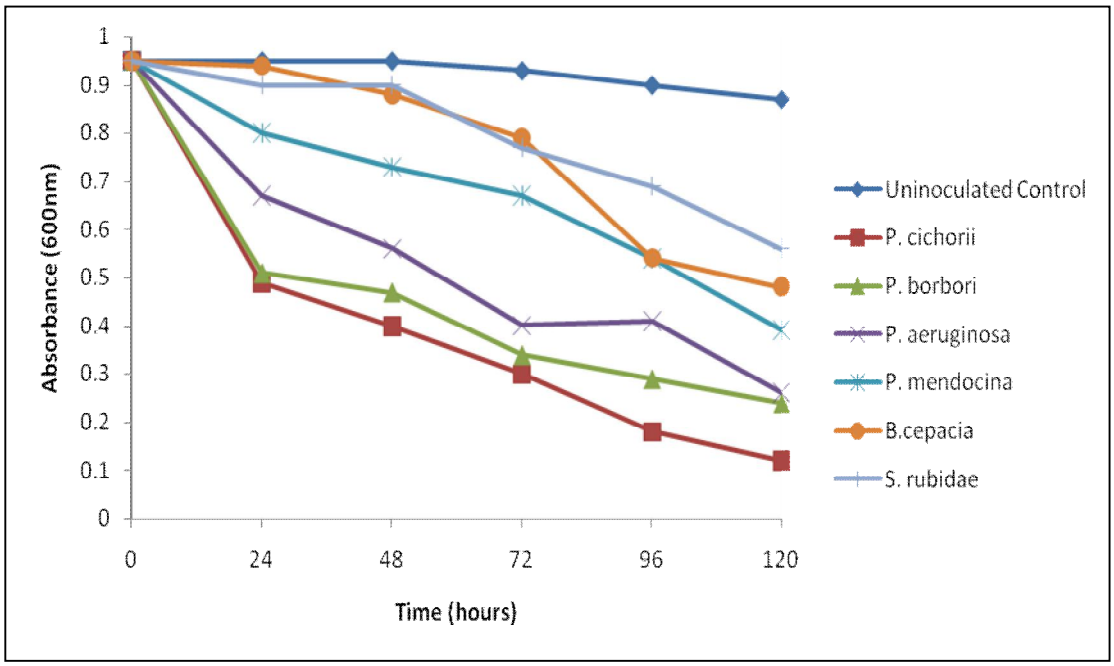

Figure 2. Oxidation of DCPIP by isolates as a measure of LPFO degradation

DISCUSSION

This study showed high bacteria counts in all the samples. Obayori *et al*. (2012) and Wang *et al*. (2012) have reported higher bacterial counts from hydrocarbon-polluted site as observed in this study. The range of THBC recorded in this study was consistent with the findings of Jennings and Tanner (2000). The high THBC recorded in unimpacted soil was due to the high level of organic matter usually present in fallow uncultivated soil which supports the growth of microorganisms.

The enumeration of TOUBC is an important criterion for the determination of potential for microbial degradation of oil contaminated environments, and to assess the amount of oil pollution that has occurred (Varjani *et al*., 2013). The distribution of bacterial isolates obtained from various sampling sites indicated common occurrence of metabolically active strains contaminated with hydrocarbon suggesting the ability of these microorganisms to utilize these hydrocarbons as a carbon and energy source. The ability to utilize hydrocarbon substrate is exhibited by a wide variety of bacteria genera (Dally *et al*., 1997; Bogan *et al*., 2003) that are widely distributed in oil polluted as well as pristine soils (Cappello *et al*., 2007; Van Beilen and Funhoff, 2007). Some general trends have indicated that Gram negative *Proteobacteria* group are major hydrocarbon utilizers (Kaplan and Kitts, 2004; Chikere *et al* 2009). These groups were usually associated with the fast degradation and their abundance was positively correlated to hydrocarbon utilization (Chikere *et al* 2009). The dominant hydrocarbon utilizing bacteria from this study belongs to the genus *Pseudomonas*. Others belong to the genera *Burkholderia* and *Serratia* affirming the earlier claim of Gram negative *Proteobacteria*

(Chikere *et al* 2009).

Several *Pseudomonas* species have been found by several authors to utilize hydrocarbon through oxidation of DCPIP (Roy *et al* 2002; Joshi and Pandey, 2011; Patil *et al* 2013). The oxidation of DCPIP supports the facts that the isolates were potential hydrocarbon degraders. Absorbance at a wavelength of 600 nm was monitored for the organisms because a peak in absorbance was observed at 600 nm as reported by Yoshida *et al*. (2001).

Each organism has a preference for a particular hydrocarbon as individual strain is usually characterized by ability to utilize only a few kinds of hydrocarbons (Adebusoye *et al*., 2007). *P. cichorii* was able to utilize DPK better than LPFO and PMS as shown by the lowest absorbance value over a period of five days. *P. aeruginosa* had preference for LPFO and PMS compared to DPK. *P. cichorii* showed a better potential in utilizing the entire hydrocarbon tested over other strains. The *Pseudomonas* species have high hydrocarbon degrading potential over the strains of *S. rubidae* and *B. cepacia.* The decrease observed even in the control assays without bacteria showed overall pattern of natural disintegration of DCPIP chemical structure through time as reported by Bidoia *et al*. (2010). In general DPK was utilized faster than LPFO and PMS. The rate and extent of colour change of DCPIP from blue (oxidized) to colourless (reduced) with time indicated that the isolates can utilize the hydrocarbon tested as a carbon source Patil *et al*., 2012).

CONCLUSIONS

This study showed that population of microorganisms found in these sites have different abilities to utilize premium motor spirit, dual purpose kerosene and low pour point fuel oil at different rates and that the colorimetric technique is a simple and rapid methodology that can be applied to determine the potentials of hydrocarbon degrading microorganisms.

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