
Screening for Hydrocarbon Degrading Bacteria Using Redox Indicator 2, 6-Dichlorophenol Indophenol

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Abstract: Contamination by petroleum products and its derivatives promotes serious environmental damage. Biodegradation capacity studies are important when deciding the correct bioremediation strategy to employ. The use of redox indicator 2, 6-Dichlorophenol Indophenol (DCPIP) is a rapid, simple and low cost model for evaluating capability of microorganisms to utilize and/or degrade petroleum hydrocarbons. This study involved isolation and screening of bacterial species capable of utilizing hydrocarbons from soil at two auto-mechanic workshops in Uyo, Akwa Ibom State. Results of the physicochemical analysis of the soil samples showed higher levels of properties (Moisture content, Organic Carbon content, Total Hydrocarbon Content) in the polluted soil samples when compared with unpolluted (control) soil sample. Total heterotrophic bacterial populations in polluted soil samples ranged between $4.4 \pm 1.90 \times 10^7$ and $6.0 \pm 32.0 \times 10^7$ CFU/g while hydrocarbon utilizing bacterial counts were between $3.2 \pm 0.05 \times 10^7$ and $5.2 \pm 25.2 \times 10^7$ CFU/g. Eight bacteria species capable of utilizing petroleum were isolated from these soils by enrichment technique. Isolated bacteria include: *Corynebacterium spp*, *Pseudomonas aeruginosa*, *Micrococcus agilis*, *Flavobacterium aquatile*, *Staphylococcus aureus*, *Micrococcus luteus*, *Serratia odorifera* and *Bacillus subtilis*. Screening of bacterial isolates for efficiency in hydrocarbon utilization using DCPIP, measured by using colorimetry, revealed *Bacillus subtilis*, *Pseudomonas aeruginosa*, *Micrococcus agilis*, *Flavobacterium aquatile* *Micrococcus luteus* and *Corynebacterium spp* to be able to rapidly utilize hydrocarbons from waste engine oil.

Keywords: Hydrocarbon, Bacteria, 2, 6-Dichlorophenol Indophenols

1. Introduction

Releases of persistent, bioaccumulative and toxic chemicals have a detrimental impact on human health and the environment. Petroleum hydrocarbon is one common example of chemicals [1]. The discharge of petroleum products into the environment from drilling, refinement, transportation, storage or distribution processes, accidentally or from sabotage and disruptive actions constitute one of the largest sources of environmental hazards [2].

Contamination of soil by petroleum hydrocarbons also stimulates indigenous microbial populations, which are capable of utilizing the petroleum hydrocarbons as their carbon and energy source thereby degrading the contaminants. The ability to degrade hydrocarbon substrates

is exhibited by a wide variety of bacteria genera [3]. The most important (based on frequency of isolation) genera of hydrocarbon utilizers are *Pseudomonas*, *Achromobacter*, *Arthrobacter*, *Micrococcus*, *Nocardia*, *Vibrio*, *Acinetobacter*, *Brevibacterium*, *Corynebacterium*, *Flavobacterium*, *Candida*, *Rhodotorula*, and *Sporobolomyces* [4], *Streptococcus sp.*, *Escherichia coli*, *Staphylococcus sp.*, *Klebsiella sp.*, *Bacillus sp.*, *Mycobacterium sp.*, *Enterobacter aerogenes*, *Salmonella sp.*, and *Micrococcus sp* [5]. A wide variety of literature on the mechanisms of microbial breakdown of petroleum hydrocarbons exist [3, 1, 6, 7].

To demonstrate that microorganisms are potentially useful for bioremediation, it is important to demonstrate the ability

of such microbes to utilize and/or biodegrade the hydrocarbon under controlled conditions. For practical reasons this cannot be easily accomplished *in situ* and thus must be accomplished in laboratory feasibility studies. The goal of a laboratory feasibility study is to identify biodegradative ability, limiting factors and recommend ways to mitigate these before application in the field [8].

A number of techniques have evolved for screening of hydrocarbon degrading microorganisms. These include use of liquid medium with hydrocarbons [9], oil-containing mineral agar plates [30], measurement of turbidity in microtiter plates, oxygen consumption, most probable techniques and shoen screen technique [10]. However, all these methods are either laborious, time consuming, expensive or not reliable [11].

2, 6-Dichlorophenol Indophenol (DCPIP) as originally used and reported by Vernon and Zaugg, [12] is a qualitative and quantitative redox indicator. Its principle is based on oxidation of carbon source in which electrons are transferred to electron acceptors such as oxygen, nitrate and sulfate [13]. By incorporating an electron acceptor such as DCPIP to the culture medium, it is possible to ascertain the ability of the microorganism to utilize the substrate by observing the color change of DCPIP from blue (oxidized) to colorless (reduced). The strains, which decolorize the DCPIP in the quickest time, are chosen as the best oil degraders. The absorbance values in 600 nm could also be used to precisely collect data regarding the biodegradation process through time. The colorimetric screening technology was first reported to be applied in oil biodegradation by Hanson *et al.* [14]. The DCPIP technique is a rapid, simple and low cost procedure for evaluating capability of different microorganisms to degrade different oil [15], and has been successfully employed in several studies [16, 17].

This study surveyed physicochemical parameter and bacteria present in hydrocarbon contaminated soil and determined qualitatively and quantitatively the utilization of hydrocarbons by these isolates growing in waste engine oil-supplemented medium.

2. Materials and Methods

2.1. Sample Collection

Composite waste engine oil-contaminated soil samples were collected from two mechanic workshops in Uyo, Akwa Ibom State. Samples were taken at the depth of 10-20 cm

using surface sterilized soil auger. Unpolluted soil samples (control samples) were collected from unpolluted zones of the same environment.

2.2. Microbiological and Physicochemical Analysis of Samples

Microbiological analysis of samples was done using the method of Cheesbrough, [18] and Holt *et al.*, [19]. Physicochemical analysis of samples was done using the method of AOAC [20].

3. Results

The results of the physicochemical analysis of the soil samples as shown in Table revealed higher levels of properties in the polluted soil samples when compared with the unpolluted soil sample. Soil pH was 6.79 ± 0.24 in unpolluted soil, 5.98 ± 0.13 in MW1 and 5.78 ± 0.03 in MW2. Moisture content was 32.25 ± 9.30 in unpolluted soil, 43.09 ± 0.65 in MW1 and 45.13 ± 7.02 in MW2. Organic carbon content was 3.7 ± 2.43 in unpolluted soil, 5.32 ± 2.65 in MW1 and 9.79 ± 0.51 in MW2. THC was 39.97 ± 13.49 in unpolluted soil, 2933.76 ± 404.27 in MW1 and 3202.61 ± 675.07 in MW2. Total heterotrophic bacterial count of soil samples ranged from $1.9 \pm 2.00 \times 10^6$ for the control soil, $4.4 \pm 1.90 \times 10^7$ to $6.0 \pm 3.20 \times 10^7$ for impacted soil. The HUB ranged from $6.5 \pm 0.04 \times 10^5$ in control soil, $3.2 \pm 0.05 \times 10^7$ and $5.2 \pm 2.52 \times 10^7$ in impacted soils (Table 2). Eight bacterial isolates were obtained from the impacted soil samples. They include: *Corynebacterium* spp, *Pseudomonas aeruginosa*, *Micrococcus agilis*, *Flavobacterium aquatile*, *Staphylococcus aureus*, *Micrococcus luteus*, *Serratia odorifera* and *Bacillus subtilis*. *Corynebacterium* spp, *Bacillus subtilis*, *Pseudomonas aeruginosa*, *Micrococcus agilis*, and *Micrococcus luteus* had high utilization of hydrocarbons as revealed by their absorbance within 120 hours of incubation (Figure 2). *Bacillus subtilis* showed the highest utilization ability; absorbance readings were not determinable after 96 hours of incubation at 600 nm. The DCPIP indicator was also rapidly decolorized in cultures containing these isolates 72 hours of incubation (Figure 1). DCPIP indicator was not decolorized within culture containing *Flavobacterium aquatile* and *Serratia odorifera*. Decolourization only occurred in cultures of *Staphylococcus aureus* after 96 hours incubation.

Table 1. Physicochemical parameters of soil sample.

Parameter	Unpolluted	Polluted	
		MW1	MW2
pH	6.79 ± 0.24	5.98 ± 0.13	5.78 ± 0.03
Moisture content (%)	32.25 ± 9.30	43.09 ± 0.65	45.13 ± 7.02
Organic carbon content (%)	3.7 ± 2.43	5.32 ± 2.65	9.79 ± 0.51
THC (mg/kg)	39.97 ± 13.49	2933.76 ± 404.27	3202.61 ± 675.07

Key: MW1-Mechanic workshop 1
MW2-Mechanic workshop 2

Table 2. Total bacterial counts of soil samples.

Bacterial count	Unpolluted	Polluted	
		MW1	MW2
THB (CFU/g)	1.9±2.00x10 ⁶	4.4±1.90x10 ⁷	6.0±3.20x10 ⁷
HUB (CFU/g)	6.5±0.04x10 ⁵	3.2±0.05x10 ⁷	5.2±2.52x10 ⁷

Key: MW1-Mechanic workshop 1
MW2-Mechanic workshop 2

Table 3. Morphological and biochemical characteristics of bacterial isolates from soil samples.

Isolate number	Cell shape	Gram stain	Spore	Catalase	Motility	Oxidase	Litmus reaction	Urease	Gelatin
MW1e, MW2d, MW3d	Chain rods	+	+	+	+	+	+	-	+
MW1g	Rods	-	-	+	+	-	-	+	+
MW1b, MW2a, MW3c	Rods	-	-	+	+	-	-	-	+
MW1a	Short rods	+	-	+	-	-	-	-	-
MW3b, MW1c	Cocci	+	-	+	-	+	-	+	-
MW1d	Short rods	-	-	+	-	+	-	-	-
MW3a, MW2b	Cocci	+	-	+	+	-	+	-	-
MW1f, MW3e	Cocci	+	-	+	-	+	-	-	-

Table 3. Continued.

Isolate number	Citrate	Glucose	Maltose	Manitol	Lactose	Sucrose	Arabinose	Probable Organism
MW1e, MW2d, MW3d	+	AO	AO	AO	-	AO	AO	<i>Bacillus subtilis</i>
MW1g	+	AO	AO	AO	AO	AO	AO	<i>Serratia odorifera</i>
MW1b, MW2a, MW3c	-	-	AO	AO	AO	-	AO	<i>Pseudomonas aeruginosa</i>
MW1a	+	AO	AO	AO	-	-	-	<i>Corynebacterium</i> sp
MW3b, MW1c	-	AO	-	AO	-	-	AG	<i>Micrococcus agilis</i>
MW1d	-	AO	AO	-	AO	AO	-	<i>Flavobacterium Aquatile</i>
MW3a, MW2b	-	-	-	-	-	AO	-	<i>Staphylococcus aureus</i>
MW1f, MW3e	-	-	-	AO	-	AO	-	<i>Micrococcus luteus</i>

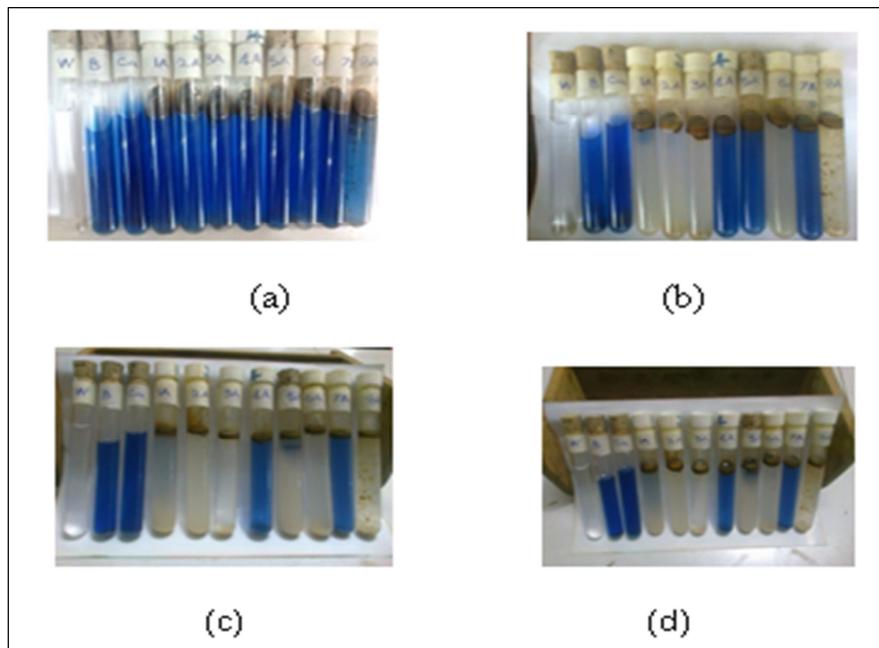


Figure 1. (a-d): colorimetric screening of bacterial isolates for hydrocarbon utilization with DCPIP indicator, after incubation at (a) 48 hours, (b) 72 hours, (c) 96 hours and (d) 120 hours

Keys: W= Water only; B = DCPIP blank; CO = DCPIP + MSM only
1A= *Corynebacterium* sp; 2A =*Pseudomonas aeruginosa*; 3A =*Micrococcus agilis*; 4A = *Flavobacterium aquatile*; 5A = *Staphylococcus aureus*; 6A =*Micrococcus luteus*; 7A = *Serratia odorifera*; 8A =*Bacillus subtilis*

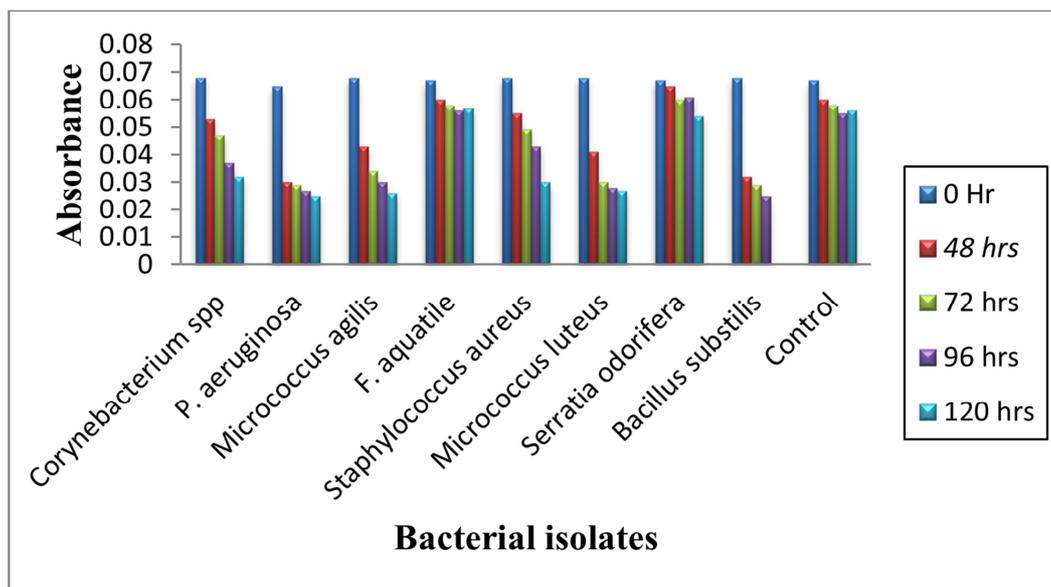


Figure 2. Absorbance (OD_{600}) of bacterial growth in hydrocarbon.

4. Discussion

The results of the physicochemical analysis of the soil samples as shown in Table 1 revealed higher levels of properties in the polluted soil samples when compared with the unpolluted soil sample. The results agree with the results of Chikere, [21] and Chikere and Ekwuabu, [22] which reported high physicochemical parameters in polluted soil samples compared to unpolluted samples determined and indicated previous exposure of the polluted samples to hydrocarbon contamination with traces of other organic and inorganic contaminants. The parameters determined indicated that the samples had been exposed to hydrocarbon contamination [23, 21]. The contamination may have resulted in the low pH of 5.98 ± 0.13 for MW1 and 5.78 ± 0.03 observed in polluted soils as compared to 6.79 ± 0.24 in the pristine (control) soil which is neutral to slightly alkaline. Previous studies have demonstrated that the pH range optimal for biodegradation of hydrocarbons is 6-7 [24, 25].

Determination of the total bacterial populations of the oil-polluted soil shows that a significant proportion of the total heterotrophic bacterial populations are hydrocarbon utilizers. The high bacterial counts recorded in polluted soil samples $4.4 \pm 1.90 \times 10^7$ in MW1 and $6.0 \pm 3.20 \times 10^7$ in MW2 compared with $1.9 \pm 2.00 \times 10^7$ in the unpolluted control samples could be attributed to the myriad of nutrients, high organic matter concentration and other ecological factors that influence the survival of soil microorganisms that play important roles in the decomposition and recycling of nutrients [26].

The difference between THB and HUB counts was observed to be insignificant suggesting that most of the micro-organisms present in the various polluted sample sites are hydrocarbonoclastic. Continuous input of petroleum-based pollutants usually results in an enriched microbial community capable of surviving toxic contamination [22].

Isolation and screening of microorganisms for their

efficiency in utilization of hydrocarbons before field trials is important in the bioremediation process. A total of seven bacterial genera; *Bacillus*, *Pseudomonas*, *Micrococcus*, *Serratia*, *Staphylococcus*, *Flavobacterium*, *Corynebacterium* and as presented in Table 3 were isolated from polluted soil. The prevalence of most of these isolates; *Flavobacterium*, *Enterobacter*, *Nocardia* and *Acinetobacter* [27], *Micrococcus*, *Bacillus*, *Corynebacterium*, *Vibrio*, *Pseudomonas* and *Flavobacterium* [28], *Serratia*, *Pseudomonas*, *Proteus*, *Klebsiella*, *Micrococcus* and *Staphylococcus* [29] in hydrocarbon polluted has been reported. These isolates have also been demonstrated by other researchers to be hydrocarbon degraders [30, 31].

To ascertain microbial ability to utilize hydrocarbon substrates by simply observing the color change of DCPIP, in which the quickest decolorization time represents the best oil degradation, is a major breakthrough in biodegradation studies. *Bacillus subtilis*, *Serratia odorifera*, *Pseudomonas aeruginosa*, *Micrococcus agilis*, *Flavobacterium aquatile* and *Micrococcus luteus* and were able to utilize hydrocarbons in waste engine oil as shown by change in the colour of the DCPIP indicator over time. This is due to reduction of the indicator by oxidized products of hydrocarbon degradation which supports the facts that the isolates are potential hydrocarbon oxidizers [32]. *Bacillus* and *Pseudomonas* species have been reported to be among the most frequently isolated bacteria from hydrocarbon-polluted sites [33, 34]. The oxidation of DCPIP supports the facts that the isolates were potential hydrocarbon degraders [16].

The control assays (C0 and C1) maintained the blue color, although with slight reductions in absorbance readings. 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.* [35].

Based on rapid decolourisation and absorbance readings,

Bacillus subtilis was observed to be the most active hydrocarbon utilizer. This agrees with previous observations identifying the *Bacillus* genus as an efficient hydrocarbon degrading microorganism [36, 37]. This suggests high adaptability within this environment with potential for application in hydrocarbon degradation.

5. Conclusion

This study reveals a quantitative and qualitative approach towards the determination of potentials of hydrocarbonoclastic bacteria, as well as giving a measureable ability of these groups of bacteria for possible use in hydrocarbon impacted soil remediation. Using the Redox indicator 2, 6-dichlorophenol indophenols approach of determination of potential hydrocarbonoclastic bacteria proofs to be a better technique and this approach is therefore recommended for determination of potential hydrocarbonoclastic bacteria.

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