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Assessment of Hydrocarbon-Utilizing Microorganisms from Spent Engine Oil-Polluted Soils from Mechanic Workshops in Awka Metropolis

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Abstract

The hydrocarbon utilizing potential of microorganisms from spent engine oil-polluted soils was evaluated. Samples were collected from spent engine oil-polluted soils from three mechanic workshops at Ifite, Arroma and Kwata in Awka metropolis, Anambra state Nigeria. The total heterotrophic Indigenous bacterial count was evaluated and oil polluted soil sample obtained from mechanic workshop in Arroma had the highest count with a colony count of 3.5×10^6cfu/g; soils obtained from Ifite and Kwata mechanic workshop had colony counts of 3.0×10^6cfu/g and 2.5×10^6cfu/g respectively. The Total Fungal count was also evaluated and soil samples from Kwata mechanic workshop showed the highest count with 3.9×10^5cfu/g; colony counts of soils obtained from Ifite and Arroma mechanic workshop were 2.0×10^5cfu/g and 2.5×10^5cfu/g respectively. The Hydrocarbonoclastic microbial count showed that soil samples from mechanic workshop in Arroma had the highest load of hydrocarbonoclastic bacteria with a colony count of 4.1×10^5cfu/g while soil samples from mechanic workshop in Kwata had the highest load of hydrocarbonoclastic fungi with a colony count of 3.3×10^5cfu/g. The morphological and biochemical characteristics of the Hydrocarbonoclastic organisms were examined and the following genera were identified and they include: *Pseudomonas* (25%), *Bacillus* (25%), *Acinetobacter* (12.5%), *Staphylococcus* (12.5%), *Micrococcus* (12.5%),) and *Enterobacter* (12.5%),); *Aspergillus* (33.3%), *Rhizopus* (16.6%), *Fusarium* (33.3%) and *Penicillium* (16.6%); *Candida* (66.7%) and *Trichosporon* (33.3%). This study showed that majority of the Indigenous organisms has the ability to utilize hydrocarbon as their sole carbon source hence are the major bioremediating agents in the sampling areas.

Keywords

Spent Engine Oil, Biostimulation, Hydrocarbonoclastic Organisms, Soil, Awka

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1. Introduction

Many indigenous microorganisms in water and soil are capable of degrading hydrocarbon contaminants. Bioremediation functions basically on biodegradation, which may refer to complete mineralization of organic contaminants into carbon dioxide, water, inorganic compounds, and cell protein or transformation of complex organic contaminants to other simpler organic compounds by biological agents like microorganisms [1].

Petroleum-based products are the major source of energy for industry and daily life. Leaks and accidental spills occur regularly during the exploration, production, refining, transport, and storage of petroleum and petroleum products.

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The amount of natural crude oil seepage was estimated to be 600,000 metric tons per year with a range of uncertainty of 200,000 metric tons per year [2]. Release of hydrocarbons into the environment whether accidentally or due to human activities is a main cause of water and soil pollution [3].

The process of bioremediation, defined as the use of microorganisms to detoxify or remove pollutants owing to their diverse metabolic capabilities is an evolving method for the removal and degradation of many environmental pollutants including the products of petroleum industry [4]. In addition, bioremediation technology is believed to be non-invasive and relatively cost-effective [5]. Biodegradation by natural populations of microorganisms represents one of the primary mechanisms by which petroleum and other hydrocarbon pollutants can be removed from the environment [1] and is cheaper than other remediation technologies [6].

There are the two main approaches to oil spill bioremediation: (a) bioaugmentation, in which known oil-degrading bacteria are added to supplement the existing microbial population, and (b) biostimulation, in which the growth of indigenous oil degraders is stimulated by the addition of nutrients or other growth-limiting co substrates.

The success of bioremediation efforts in the clean-up of the oil tanker Exxon Valdez oil spill of 1989 [7] in Prince William Sound and the Gulf of Alaska created tremendous interest in the potential of biodegradation and bioremediation technology. Most existing studies have concentrated on evaluating the factors affecting oil bioremediation or testing favoured products and methods through laboratory studies [8]. Only limited numbers of pilot scale and field trials have provided the most convincing demonstrations of this technology which have been reported in the peer-reviewed literature [9-12]. The scope of current understanding of oil bioremediation is also limited because the emphasis of most of these field studies and reviews has been given on the evaluation of bioremediation technology for dealing with large-scale oil spills on marine shorelines.

This work is aimed at assessing the hydrocarbon utilizing potential of microorganisms isolated from spent engine oil-polluted soils from mechanic workshops in Awka metropolis

2. Materials and Method

2.1. Study Site

The study was carried out in Awka, Anambra state Eastern Nigeria. Indiscriminate disposal of used engine oil is the major source of oil pollution in this locality as transportation is one of the main sources of income. Three mechanic workshops at Ifite, Arroma and Kwata contaminated with used engine oil were randomly selected for this study.

2.2. Sample Collection

Oil contaminated soils were collected around three selected mechanic workshops at Ifite, Arroma and Kwata at a depth of 10cm after removing the top soil using a sterile cutlass and a hand auger. Samples were labelled and immediately taken to the Microbiology laboratory at Nnamdi Azikiwe University for analysis.

2.3. Culture Media

The analytical media employed in the course of this research included: Nutrient agar (NA), Sabouraud dextrose agar (SDA), Mineral Salt Agar (MSA). All media were prepared according to the manufacturer's instruction and sterilized at 121°C for 15 minutes at 15psi.

2.4. Sample Preparation

Ten (10 g) of each sample was weighed out, added to 90 ml of sterile deionized water and vigorously shaken for 1 minute. Ten-fold serial dilution of the samples was prepared.

2.4.1. Total Heterotrophic Bacteria (THB) and Total Fungi Count (TFC) of Indigenous Organisms

The counts of total heterotrophic bacteria each sample were determined by the spread plate technique using nutrient agar (NA).

2.4.2. Isolation, Purification and Maintenance of Pure Microbial Isolates

Distinct or representative colonies from the culture plates were selected for characterization. Bacterial colonies were transferred to freshly prepared nutrient agar plates by the streak-plate method and allowed to grow for 24 hours before stocking. Similarly, distinct fungal colonies were sub cultured by point inoculation and streak-plate method for yeast on freshly prepared Sabouraud's dextrose agar plates for 72 hours before stocking.

2.5. Identification and Characterization of Bacterial Isolates

2.5.1. Morphological Characteristics

Representative colonies of bacteria isolates were evaluated using morphological characteristics on media such as shape, colour, margin and elevation.

2.5.2. Biochemical Tests

The bacteria isolates were also identified based on biochemical characteristics by carrying out the following biochemical test:

Gram-staining Test, Catalase Test, Citrate utilization Test, Coagulase Test, Indole Test, Methyl red Test, Voges Proskauer Test, Oxidase Test, Motility Test, Sugar fermentation Test

2.6. Hydrocarbonoclastic Bacterial and Fungal Count

The counts of crude oil utilizing bacteria were enumerated by spread and pour plate techniques (Mills *et al*., 1978; Obire *et al*., 2008) using vapour phase transfer technique on Mineral Salts Agar (MSA). The number of colonies formed was used to estimate the hydrocarbon utilizing Bacteria and Fungi.

$$\text{Colony forming unit per gram } \left(\frac{\text{cfu}}{\text{g}}\right) = \frac{\text{Average number of colonies}}{\text{Volume of sample} \times \text{ Dilution value}}$$

2.6.1. Isolation, Purification and Maintenance of Pure Hydrocarbonoclastic Microbial Isolates

Distinct or representative colonies from the culture plates were selected for characterization. Bacterial colonies were transferred to freshly prepared nutrient agar plates by the streak-plate method and allowed to grow for 24 hours before stocking. Similarly, distinct fungal colonies (Mold) were sub cultured by point inoculation and streak-plate method for yeast on freshly prepared Sabouraud dextrose agar plates for 72 hours before stocking.

2.6.2. Biochemical Characteristics of Hydrocarbonoclastic Bacterial isolates

The Hydrocarbonolastic bacterial isolates were also identified based on biochemical characteristics according to the tests carried out on the Indigenous bacteria as stated above.

2.6.3. Identification and Characterization of Hydrocarbonolastic Fungal Isolates

Colony Morphology, Fungi (Mold and yeast) Identification using lactophenol cotton-blue staining technique, Gram-staining Test, Sugar fermentation Test were carried out.

2.7. Screening Test for Hydrocarbon Utilization

The test is carried out to ascertain that the isolated organisms have the ability to grow in the presence of a known hydrocarbon source. The inoculum was prepared by transferring cultures from Nutrient agar and Sabourauds dextrose agar slants into freshly prepared Nutrient agar and Sabourauds dextrose agar media for 24hours at 37°C. Cultures were then inoculated into test tubes containing Mineral salt Broth (Which was prepared without the gelling agent-Agar), along with crude oil ($1\%^{v}/_{v}$). All tubes were incubated at 37°C for 7-10 days. Control tubes were also provided. The growth rate after 10 days was recorded based on degree of turbidity in individual test tubes.

3. Result

The total heterotrophic Indigenous bacterial count was evaluated and oil-polluted soil sample gotten from mechanic workshop in Arroma had the highest count with a colony count of 3.5×10^{6}cfu/g; soils obtained from Ifite and Kwata mechanic workshop had colony counts of 3.0×10^{6}cfu/g and 2.5×10^{6}cfu/g respectively. The Total Fungi count was also evaluated and soil samples from Kwata mechanic workshop showed the highest count with 3.9×10^{5}cfu/g; colony counts of soils obtained from Ifite and Arroma mechanic workshop were 2.0×10^{5}cfu/g and 2.5×10^{5}cfu/g respectively.

The morphological and biochemical characteristics of the Indigenous organisms examined showed the following genera: *Pseudomonas* (27.2%), *Bacillus* (18.2%), *Proteus* (9.1%), *Acinetobacter* (9.1%), *Staphylococcus* (18.2%), *Micrococcus* (9.1%),) and *Enterobacter* (9.1%),); *Aspergillus* (33.3%), *Rhizopus* (22.2%), *Fusarium* (22.2%) and *Penicillium* (22.2%); *Saccharomyces* (40%), *Candida* (40%) and *Trichosporon* (20%). *Pseudomonas* spp was found to be the most dominant Indigenous bacteria. Its prevalence could be attributed to the fact that members of the genus demonstrate a great deal of metabolic diversity and consequently able to colonize a wide range of niches. *Aspergillus* spp was found to be the most dominant Indigenous fungi (mold). This could be as a fact that they produce resistant spores that thrive in harsh environment making them ubiquitous. [14] reported the isolation of *Aspergillus*, *Fusarium* and *Rhizopus* among other molds from soil. *Candida* and *Saccharomyces* spp were the most dominant Indigenous yeast species.

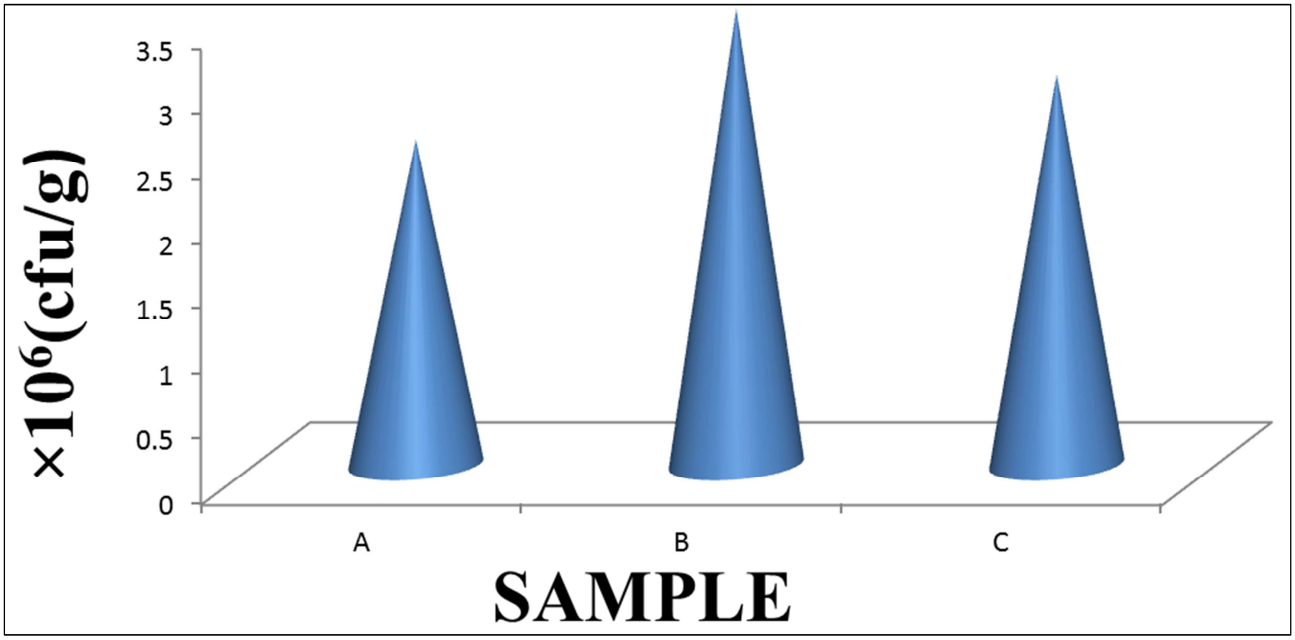

Figure 1. Heterotrophic Bacterial count of the indigenous Bacteria from the soil Samples.

Key:
Sample A: Oil-contaminated soil from mechanic shop in Kwata
Sample B: Oil-contaminated soil from mechanic shop in Arroma
Sample C: Oil-contaminated soil from mechanic shop in Ifite

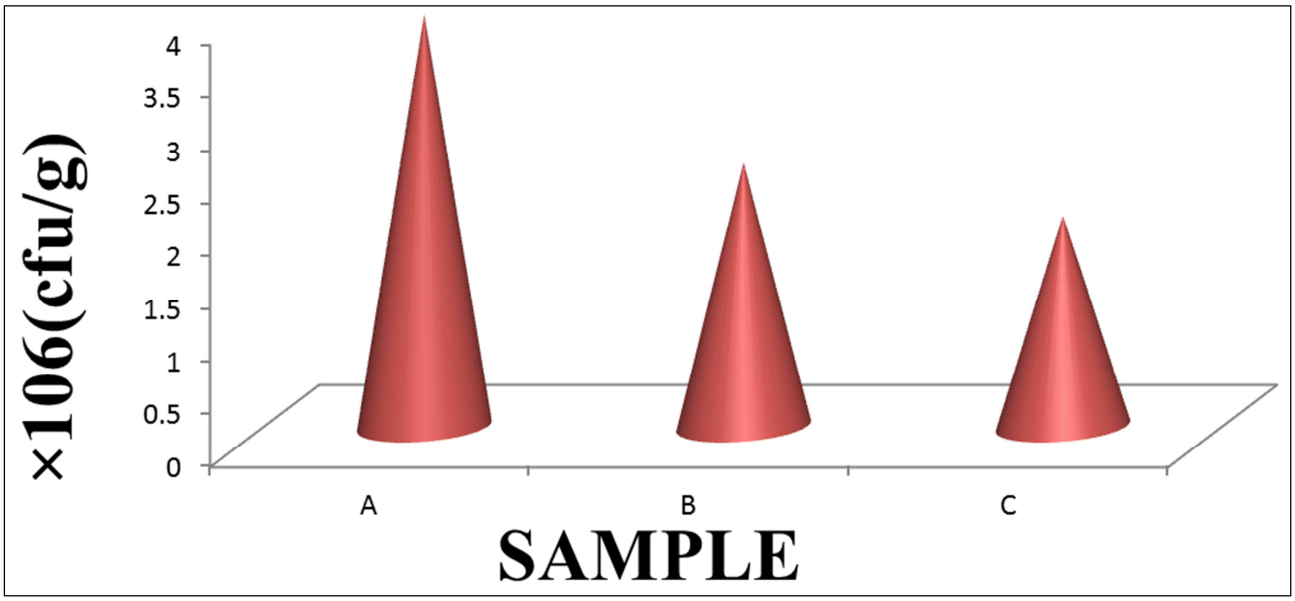

Figure 2. Heterotrophic Fungal count of the indigenous Fungi from the soil Samples.

Key:
Sample A: Oil-contaminated soil from mechanic shop in Kwata
Sample B: Oil-contaminated soil from mechanic shop in Arroma
Sample C: Oil-contaminated soil from mechanic shop in Ifite.

Table 1. Biochemical test results of Indigenous bacterial Isolates.

Sample	Isolate	Catalase	Citrate	Coagulase	Gram staining	Indole	MR	Oxidase	VP
	FAS 1	+	+	-	-	-	-	+	-
A	FAS 2	+	+	-	-	-	-	+	-
	FAS 3	+	+	-	+	-	-	-	+
B	EXD 1	+	+	-	-	-	+	-	-

Sample	Isolate	Catalase	Citrate	Coagulase	Gram staining	Indole	MR	Oxidase	VP
	EXD 2	+	-	+	-	-	-	-	-
	EXD 3	+	+	-	-	-	-	+	-
	EXD 4	+	+	+	+	-	+	-	+
C	IDP 1	+	-	-	+	-	-	+	+
	IDP 2	+	+	-	+	-	-	-	+
	IDP 3	+	+	+	+	-	+	-	+
	IDP 4	+	+	-	-	-	-	-	+

Table 1. Continued.

Sample	Isolate	Motility	Shape	Sugar fermentation				Probable Organism
				Glu	Mal	Lac	Suc	
	FAS 1	+	Rod	-	-	-	-	*Pseudomonas* spp
A	FAS 2	+	Rod	-	-	-	-	*Pseudomonas* spp
	FAS 3	+	Rod	A+	A+	-	A+	*Bacillus* spp
	EXD 1	+	Rod	AG	-	-	-	*Proteus* spp
B	EXD 2	-	Rod	-	-	-	-	*Acinetobacter* spp
	EXD 3	+	Rod	-	-	-	-	*Pseudomonas* spp
	EXD 4	-	Cocci (in cluters)	A+	A+	A+	A+	*Staphylococcus* spp
C	IDP 1	-	Cocci	A+	A+	-	-	*Micrococcus* spp
	IDP 2	+	Rod	A+	A+	-	A+	*Bacillus* spp
	IDP 3	-	Cocci (in cluters)	A+	A+	A+	A+	*Staphylococcus* spp
	IDP 4	+	Rod	AG	A+	-	A+	*Enterobacter* spp

Key:
Sample A: Oil-contaminated soil from mechanic shop in Kwata

Sample B: Oil-contaminated soil from mechanic shop in Arroma

Sample C: Oil-contaminated soil from mechanic shop in Ifite

Abbreviations
VP: Voges Proskauer
Lac: Lactose
MR: Methyl red
Suc: Sucrose
Glu: Glucose
A+: Positive with Acid production only
+: Positive
Mal: Maltose
AG: Positive with Acid and Gas production
-: Negative

Table 2. Wet mount/Lactophenol cotton blue stain results of Indigenous Fungi (Mold).

Sample	Isolate	Hyphae (Septate or Aseptate)	Type of Asexual spore	Presence of special structures (Rhizoid/Stolon)	Probable Organism
A	AWK 1	Septate	Conidiospores	Stolon	*Aspergillus* spp
	AWK 2	Septate	Conidiospores	Stolon	*Penicillium* spp
	TFI 1	Septate	Conidiospores	Stolon	*Aspergillus* spp
B	TFI 2	Septate	Conidiospores	-	*Fusarium* spp
	TFI 3	Aseptate	Sporangiospores	Stolon and Rhizoid	*Rhizopus* spp
	TFI 4	Aseptate	Sporangiospores	Stolon and Rhizoid	*Rhizopus* spp
	RRA 1	Septate	Conidiospores	Stolon	*Aspergillus* spp
C	RRA 2	Septate	Conidiospores	Stolon	*Penicillium* spp
	RRA 3	Septate	Conidiospores	-	*Fusarium* spp

Key:
Sample A: Oil-contaminated soil from mechanic shop in Kwata
Sample B: Oil-contaminated soil from mechanic shop in Arroma
Sample C: Oil-contaminated soil from mechanic shop in Ifite

The morphological and biochemical characteristics of the Hydrocarbonoclastic organisms were examined and the following genera were identified and they include: *Pseudomonas* (25%), *Bacillus* (25%), *Acinetobacter* (12.5%), *Staphylococcus* (12.5%), *Micrococcus* (12.5%),) and *Enterobacter* (12.5%),); *Aspergillus* (33.3%), *Rhizopus* (16.6%), *Fusarium* (33.3%) and *Penicillium* (16.6%); *Candida* (66.7%) and *Trichosporon* (33.3%). *Pseudomonas* spp and *Bacillus* spp were found to be the most dominant Hydrocarbonoclastic bacteria. Its prevalence could be attributed to the fact that members of the genus *Pseudomonas* demonstrate a great deal of metabolic diversity and consequently able to colonize a wide range of niches. Members of the genus *Bacillus*, produce resistant spore that have the ability to survive in harsh environmental conditions which could be a reason of their prevalence.

Table 3. Biochemical test results of Hydrocarbonoclastic bacterial Isolates.

Sample	Isolate	Catalase	Citrate	Coagulase	Gram staining	Indole	MR	Oxidase	VP
A	KDA 1	+	+	-	-	-	-	+	-
	KDA 2	+	+	-	+	-	-	-	+
	KDA 3	+	+	-	+	-	-	-	+
B	RJP 1	+	+	+	+	-	+	-	+
	RJP 2	+	-	+	-	-	-	-	-
	RJP 3	+	+	-	-	-	-	+	-
C	KFC 1	+	+	-	-	-	-	-	+
	KFC 2	+	-	-	+	-	-	+	+

Table 3. Continued.

Sample	Isolate	Motility	Shape	Sugar fermentation				Probable Organism
				Glu	Mal	Lac	Suc	
A	KDA 1	+	Rod	-	-	-	-	*Pseudomonas* spp
	KDA 2	+	Rod	A+	A+	-	A+	*Bacillus* spp
	KDA 3	+	Rod	A+	A+	-	A+	*Bacillus* spp
B	RJP 1	-	Cocci (in clusters)	A+	A+	A+	A+	*Staphylococcus* spp
	RJP 2	-	Rod	-	-	-	-	*Acinetobacter* spp
	RJP 3	+	Rod	-	-	-	-	*Pseudomonas* spp
C	KFC 1	+	Rod	AG	A+	-	A+	*Enterobacter* spp
	KFC 2	-	Cocci	A+	A+	-	-	*Micrococcus* spp

Key:
Sample A: Oil-contaminated soil from mechanic shop in Kwata

Sample B: Oil-contaminated soil from mechanic shop in Arroma

Sample C: Oil-contaminated soil from mechanic shop in Ifite

Abbreviations
A+: Positive with Acid production only
+: Positive
AG: Positive with Acid and Gas production
-: Negative
VP: Voges Proskauer
lac: lactose
MR: Methyl red
Suc: sucrose
Glu: Glucose

It was observed that 86% of the Indigenous bacteria had the ability to degrade Crude oil which implies that these bacteria are the chief bioremediating agents in their various environments. All the Indigenous Molds were found to degrade crude oil with *Aspergillus* and *Fusarium* spp being the predominant ones.

Table 4. Biochemical test results of Hydrocarbonoclastic Fungi (Yeast).

Sample	Isolate	Gram Staining	Sugar Fermentation				Probable Organism
			Glu	Mal	Lac	Suc	
A	DEA 1	+	+	+	-	-	*Candida* spp
B	CDA 1	+	+	+	-	-	*Candida* spp
C	IFF 1	+	+	+	+	+	*Trichosporon* spp

Key
Sample A: Oil-contaminated soil from mechanic shop in Kwata
Sample B: Oil-contaminated soil from mechanic shop in Ifite
Sample C: Oil-contaminated soil from mechanic shop in Arroma
Abbreviations:
Glu: Glucose
Mal: Maltose
Lac: Lactose
Suc: Sucrose
+: Positive
-: Negative

All isolates gave a positive result to the hydrocarbon utilization screening test with varying growth rates with *Pseudomonas* spp, *Bacilus* spp, *Aspergillus* spp, *Candida* spp showing heavy growths; *Fusarium* spp, *Staphylococcus* spp showing moderate growths; *Acinetobacter* spp, *Enterobacter* spp, *Micrococcus* spp, *Rhizopus* spp, *Penicillium* spp and *Trichosporon* spp showed low growths.

Table 5. Screening test results for Hydrocarbon utilization.

Bacteria			Fungi					
			Mold			Yeast		
Sample	Isolate	Growth	Sample	Isolate	Growth	Sample	Isolate	Growth
A	KDA 1	+++	A	ABB 1	+++	A	DEA 1	+++
	KDA 2	++		ABB 2	++	B	IFF 1	++
	KDA 3	++	B	DIV 1	++	C	CDA	+++
B	RJP 1	+		DIV 2	+			
	RJP 2	+	C	KEO 1	+			
	RJP 3	+++		KEO 2	+++			
C	KFC 1	+						
	KFC 2	++						

Key:
Sample A: Oil-contaminated soil from mechanic shop in Kwata
Sample B: Oil-contaminated soil from mechanic shop in Arroma
Sample C: Oil-contaminated soil from mechanic shop in Ifite

+++ = Heavy growth
++= Moderate growth
+= Low growth

4. Discussion

The assessment of the Hydrocarbon utilizing potential of microorganisms isolated from spent engine oil-polluted soil in this work was not in agreement with earlier report by [13]. The morphological and biochemical characteristics of the Indigenous organisms examined showed the following genera: *Pseudomonas* (27.2%), *Bacillus* (18.2%), *Proteus* (9.1%), *Acinetobacter* (9.1%), *Staphylococcus* (18.2%), *Micrococcus* (9.1%),) and *Enterobacter* (9.1%),); *Aspergillus* (33.3%), *Rhizopus* (22.2%), *Fusarium* (22.2%) and *Penicillium* (22.2%); *Saccharomyces* (40%), *Candida* (40%) and *Trichosporon* (20%). *Pseudomonas* spp was found to be the most dominant Indigenous bacteria. Its prevalence could be attributed to the fact that members of the genus demonstrate a great deal of metabolic diversity and consequently able to colonize a wide range of niches. *Aspergillus* spp was found to be the most dominant Indigenous fungi (mold). This could be as a fact that they produce resistant spores that thrive in harsh environment making them ubiquitous. [14] reported the isolation of *Aspergillus*, *Fusarium* and *Rhizopus* among other molds from soil. *Candida* and *Saccharomyces* spp were the most dominant Indigenous yeast species. It was observed that 86% of the Indigenous bacteria had the ability to degrade Crude oil which implies that these bacteria are the chief bioremediating agents in their various environments. All the Indigenous Molds were found to degrade crude oil with *Aspergillus* and *Fusarium* spp being the predominant ones. [17] reported the involvement of inherent microorganisms in the cleanup of xenobiotics in their environments. *Candida* spp was the predominant Hydrocarbonoclastic yeast. The Hydrocarbonoclastic microbial count evaluation according to [15] and [16] showed that soil samples from mechanic workshop in Arroma had the highest load of hydrocarbonoclastic bacteria with a colony count of 4.1×10^5cfu/g; soil samples from mechanic worshops in Ifite and Kwata had colony counts of 3.04×10^5cfu/g and 1.62×10^5cfu/g while soil samples from mechanic workshop in Kwata had the highest load of hydrocarbonoclastic fungi with a colony count of 3.3×10^5cfu/g.

5. Conclusion

The ability of microorganisms to utilize hydrocarbons is of utmost importance. The results of this work showed that hydrocarbon utilizing microoganisms can be readily isolated from soil samples from mechanic workshops in Kwata, Arroma and Ifite and as such, these Organisms can be used in the clean-up of soils polluted by diesel polluted soils.

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