Characterization of Autochthonous Bacterial Isolates with The Potentiality to Degrade Spent Engine Oil from Contaminated Soil Area Enriched with *Glycine max*

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ABSTRACT: This study was conducted to identify and characterize bacteria capable of degrading spent oil contaminated soil. The physicochemical parameters of used engine oil were analyzed according to existing standards, while the total heterotrophic plate counts (HBC) and hydrocarbon utilizing bacteria counts were ascertained with the pour plate methods using nutrient agar and minimal salt agar (MSA) media respectively. The results indicated a mean total HBC ranging from 2.86 ± 0.08 to $5.76 \log_{10}$ CFU/g and mean hydrocarbon utilizing bacterial counts from 1.32 ± 0.09 to $3.82 \pm 0.25 \log_{10}$ CFU/g, with samples enriched with *Glycine max* (Soybean) recorded to have the highest bacterial counts. The phenotypic identification of the hydrocarbon utilizing bacteria as sources of carbon and energy showed the presence of two primary bacterial genera: *Bacillus* and *Micrococcus*. However, from the overall 50 counted colonies, the frequency of occurrence for *Bacillus* was 41 (82 %) whereas, the *Micrococcus* was (9) 18%. The obtained data, confirmed the breakdown capacity of autochthonous (indigenous) organisms notably; *Bacillus* in the reduction of pollutants linked with oil spillage. This provides for reliable and promising approach of ameliorating crude oil pollutants and its inherent threats.

Spent engine oil or used motor oil abounds in oil mechanic workshops and industrial sources that may occur as hydraulic oil, turbine, process and brake oils and consists of large number of long-chain saturated hydrocarbons. The crude oil is a mixture of hydrocarbon and hydrocarbon-like, polycyclic aromatic hydrocarbons (PAHs) and heavy metals (Cappello *et al*., 2007). The contamination and eventual pollution of the environment have raised an issue of global interest due to their widespread distribution and multiple threats on the ecosystem especially, their toxicological impacts on human beings, plants and other organisms (Ohenhen *et al*., 2006). The abilities of certain microorganisms such as *Bacillus*, *Pseudomonas, Acinobacter* and *Flavobacillus* to breakdown crude oil and its products had been previously reported (Omoni and Abu, 2004). Subsequently, these degrading organisms possess specific enzyme systems that enable them to utilize hydrocarbons as their carbon and energy sources. Nevertheless, there is a concerted effort by the scientific communities in reducing pollutants from crude oil spill through the cost-effective and promising approach known as bioremediation (Barathi and Vasudevan, 2001). This is the use of microorganisms to get rid of harmful substances from the environment by transforming them into less toxic, harmless and useful products (Emetola, 2018).

The aim of this study is to determine bacterial isolates capable of utilizing spent oil contaminated soil enriched with *Glycine max* as source of carbon and energy.

MATERIALS AND METHODS

Sample Collections: One kilogram of soil sample contaminated with spent engine oil was obtained from a motor mechanic workshop along Mission Road, in Okada, Ovia North East Local Government Area, Edo State. Ten grams (10 g) of the contaminated soil sample from the sampling site was blended with soybean, measured into 2 polyethene bags, label A and B. Another 10 g of the contaminated spent engine oil was taken into 2 other polyethene bags as samples C and D, while an uncontaminated soil sample was collected from the Igbinedion University, Okada botanical garden. The 5 samples were transported to

the Department of Microbiology Laboratory, Igbinedion University for microbiological analysis.

Physicochemical analysis of soil samples: Ten grams (10 g) each of samples A, B, C, D and E as measured above were also collected for the determination of physicochemical analysis. After eliminating the debris and other hard particles therein, the 5 samples were stored in McCartney bottles and were immediately transported to Nigeria Institute for oil-palm Research (NIFOR) for analysis. The parameters measured include pH, moisture content, turbidity, electrical conductivity, total organic carbon (TOC); total nitrogen, polycyclic aromatic hydrocarbons (toluene, naphthalene, phenaphthalene) heavy metals (lead and iron) and were determined using standard methods (APHA, 2015).

Isolation and Enumeration of bacteria: Isolation of bacteria from spent engine oil contaminated soil samples was performed according to methods established (Atlas, 2004; Udeani *et al*., 2009). The total heterotrophic bacterial count (HBC) was determined by the pour plate technique on nutrient agar. One gram of soil sample was aseptically introduced onto 9 ml of sterile peptone water, thoroughly shaken and serially diluted. Aliquot (0.1 ml) of 10^{-1} to 10^{-4} dilution factors were inoculated on sterile plates containing nutrient agar. The plates were incubated at room temperature, while the distinct bacterial colonies developed in the nutrient agar plates were counted and used to deduce the heterotrophic bacteria load.

Identification and characterization: Individual bacterial colonies were picked based on different colony morphology and color and further sub-cultured by pouring onto fresh nutrient agar slants and incubated at room temperature for 48 hr. The colonies were repeatedly poured on agar slants until pure cultures were obtained and identified. Gram staining was carried out to differentiate the bacterial isolates into gram positive and negative bacteria according to standardized protocol (Atlas, 2004; Omoni and Abu, 2014). The following biochemical tests were performed to characterize and to further identify the isolated organisms; catalase, citrate, indole, lactose, methyl red and oxidase (APHA, 2015).

Enumeration of total hydrocarbon utilizing bacteria: The total hydrocarbon utilizing bacteria was cultured on minimal salt agar (MSA) using the pour plate method as prescribed by Atlas (2004) and modified by Udeani *et al*., 2009. Ten-fold serial dilutions were performed on the spent engine oil and 1 ml of each dilution was mixed with 20 ml of sterile MSA in a plate, incubated at 30 °C for 72 hr. The bacterial colonies on agar plates were then counted and subsequently expressed as hydrocarbon utilizing bacteria per ml of spent oil. Thereafter, the colonies were randomly picked and streaked onto sterile nutrient agar slants in McCartney bottles. The pure isolates were then stored in the refrigerator at -4 °C for further analysis.

Identification and characterization of hydrocarbon utilizing bacteria: The isolates were identified and characterized using morphological and biochemical tests respectively (Mandigan *et al*., 2017).

RESULTS AND DISCUSSION

This represents the counts of total heterotrophic bacteria (THC) in the soil samples. The mean THC were respectively estimated at 2.86 ± 0.08 $\log_{10}$ CFU/g, 5.18 ± 0.11 $\log_{10}$ CFU/g and 5.76 ± 0.26 $\log_{10}$ CFU/g for the uncontaminated soil, engine oil-contaminated soil and engine oil-contaminated soil enriched with *Glycine max*. The count of hydrocarbon-utilizing fraction of the total heterotrophic bacteria in the soil samples are presented in Table 3. Using spent engine oil as the sole carbon source, the hydrocarbon-utilizing bacteria counts were respectively 1.32 ± 0.09 $\log_{10}$ CFU/g, 3.31 ± 0.12 $\log_{10}$ CFU/g and 3.82 ± 0.25 $\log_{10}$ CFU/g for uncontaminated soil, engine oil-contaminated soil and engine oil-contaminated soil enriched with *Glycine m*

Table 1: Physicochemical analysis of soil samples contaminated with spent oil.

Samples	Parameters											
	PA	pH	MC	TUR	OM	TN	TOC	Fe	PB	NAP	TOL	AC
SA	LM	4.71	2.10	ND	2.17	35.5	10.2	ND	ND	ND	ND	ND
SB	LM	4.92	2.15	ND	3.0	30.0	15.0	ND	ND	ND	ND	ND
SC	LH	5.70	1.75	10.0	3.55	2.16	35.0	0.06	0.29	ND	20.0	0.03
SD	LH	5.03	2.0	6.81	3.26	2.01	40.0	0.02	0.20	ND	13.9	0.01
SE	LH	6.90	1.64	0.05	4.45	1.50	29.0	ND	ND	ND	ND	0.01

S: sample; PA: physical appearance; MC: moisture content; TUR: turbidity; OM: organic moisture; TN: total nitrogen; TOC: total organic carbon; Fe: iron; Pb: lead; NAP: naphthalene; TOL: toluene; AC: acenapthalene.

Table 2: Counts of total heterotrophic bacteria in the soil samples

	Counts of total heterotrophic bacteria (THC)					
	Uncontaminated soil		Contaminated soil		Contaminated soil + GM	
Sample	Count N = 1 Log₁₀ CFU/g	Mean ± SE count N=5 Log₁₀ CFU/g	Count N = 1 Log₁₀ CFU/g	Mean ± SE count N = 5 Log₁₀ CFU/g	Count N = 1 Log₁₀ CFU/g	Mean ± SE count N = 5 Log₁₀ CFU/g
1	2.60		5.19		5.45	
2	2.85		4.92		5.59	
3	3.02	2.86 ± 0.08	5.51	5.18 ± 0.11	6.78	5.76 ± 0.26
4	2.83		5.30		5.51	
5	2.99		5.00		5.48	

Key: GM: *Glycine max; N: total number of samples examined; SE: standard error of mean; CFU/g: coliform forming unit per gram*

Table 3: Counts of hydrocarbon-utilizing bacteria in the soil sample

	Counts of total heterotrophic bacteria (THC)					
	Uncontaminated soil		Contaminated soil		Contaminated soil + GM	
Sample	Count N = 1 Log₁₀ CFU/g	Mean ± SE count N=5 Log₁₀ CFU/g	Count N = 1 Log₁₀ CFU/g	Mean ± SE count N = 5 Log₁₀ CFU/g	Count N = 1 Log₁₀ CFU/g	Mean ± SE count N = 5 Log₁₀ CFU/g
1	1.12		3.13		3.63	
2	1.18		2.95		3.50	
3	1.40	1.32 ± 0.09	3.62	3.31 ± 0.12	4.81	3.82 ± 0.25
4	1.29		3.51		3.59	
5	1.62		3.33		3.56	

Key: GM: *Glycine max; N: total number of samples examined; SE: standard error of mean; coliform forming unit per gram.*

Table 4: Phenotypic characterization of hydrocarbon-utilizing bacterial colonies obtained from the contaminated soil samples

Carbon Source	Representative Isolates	Colony and Morphological characteristics		Biochemical Characteristics						Identified isolates	Relative occurrence of the hydrocarbon-utilizing bacteria	
		Colony characteristics	Gram staining	Co	Ca	Ox	Mr	Vp	Ht		F	%
Engine Oil	1	Dry colonies	Positive rods	-	+	-	-	+	β	*Bacillus*	41/50	82.00
	2	Mucoid colonies	Positive cocci	-	+	+	+	-	β	*Micrococcus*	9/50	18.00

Key: *Co: coagulase; Ca: catalase; Ox: oxidase; Mr: methyl red; Vp: Voges Proskauer; Ht: haemolysis tests. F: relative occurrence; β (beta): haemolytic bacterial colony.*

The phenotypic characterization of hydrocarbon-utilizing bacterial colonies isolated from the contaminated soil samples is presented in Table 4. The identified hydrocarbon-utilizing bacteria were *Bacillus* and *Micrococcus* species. *Bacillus* species were the hydrocarbon-utilizing bacteria that most frequently occurred in the contaminated soil samples. The analysis of the physicochemical parameters revealed the average pH values from 4.71 to 6.90 and signified that all soil samples were acidic, with SA being the most acidic (4.71) (Table 1). However, soil acidity could be attributed to the carbonic acid formed when the carbon fraction of the hydrocarbon might have been hydrolyzed resulting in bicarbonate ions (HCO3-) in soil samples. More so, increased acidity and total nitrogen depend on the activities of nitrifying bacteria in soil (Ewetola, 2018). This is influenced by the presence of dissolved oxygen in an enzymatic reaction during nitrification process to produce nitric acid that tend to increase the pH of the soil. Low pH causes metals to precipitate as carbonate and hydroxyl ions that can lead to irritation of the skin and eye when in contact with used oil in polluted sites. The concentrations of iron (Fe) were detected in SC (0.06 mg/g) and SD (0.02 mg/g) (Table 1). Contact and accumulation of iron in body result to a disease condition referred to as hemochromatosis (Iron overload disease) cirrhosis, nausea, vomiting and surface stains. Additionally, the concentrations of lead (Pb) were recorded in SC (0.29 mg/g) SD (0.20 mg/g) and exceeded the recommended threshold (0.01 mg/g). Excess lead has been linked to cancer, kidney and liver malfunction, low blood haemoglobin and high blood pressure (Kvenovolden and Cooper, 2019). The results of PAHs analyses (Table 1) revealed that naphthalene was not detected in any of the soils, C and D recorded high concentrations of toluene 0.29 and 0.20 mg/g respectively and acenaphthalene concentrations ranged from 0.01 to 0.03 mg/g in C, D and E. This indicated that, toluene and acenaphthalene were always present in spent oil contaminated soils (C and D). It has been reported that toluene is irritating to the eye, skin and respiratory tract, causes systemic toxicity by ingestion or inhalation and could damage

the kidney, liver, lung and impaired immune function (Mandigan *et al.*, 2017). The levels of adaptation of microbial communities to and transformation of hydrocarbons associated with oil polluted environments have been reported to be on increase in many studies (Barathi and Vasudevan, 2001). This finding is in diadem with earlier remarks which indicated the wide distribution of existence of *Pseudomonas* and *Bacillus* species in contaminated sites. Among the endowed metabolic properties enabling them to degrade these compounds include; tolerance to high concentrations of solvents, embodiment of complimentary metabolic capacity through rapid alteration and the exchange of genetic material, possession of specific enzyme system for oxidation of organic compounds by which molecular oxygen is incorporated by dioxygenases to form cis-dihydrodiols. The intermediates of dihydrodiol dehydrogenation are metabolized into carbon dioxide and water through the catechols by the action of catechol dioxydrogenases and other enzymes (Chikere *et al.*, 2011; Akpoka *et al.*, 2020). Consequently, from the pool of the total heterotrophic bacteria count, the HBC mean ranged from $2.86 + 0.08$ $\log_{10}$ CFU/g to $5.76+ 0.26$ $\log_{10}$ CFU/g with the highest HBC count ($5.76+ 0.26$ $\log_{10}$ CFU/g) recorded from contaminated soil enriched with *Glycine max* and lowest count was obtained from the uncontaminated soil (Table 2). Subsequently, the mean hydrocarbon utilizing bacteria counts ranged from $1.32 + 0.09$ to $3.82 + 0.25$ $\log_{10}$ CFU/g, with the highest count also recorded from the contaminated soil blended with G. max. According to Ewetola (2018), the increase in bacteria load of the contaminated soil blended with *G. max* could be adduced to the rich composition of proteins, B-group vitamins (cobolamin and tocopherol) minerals and plant compound such as isoflavones that can enhance the growth of hydrocarbon breaking organisms.

These potential genera (*Bacillus* and *Micrococcus*) were selected based on their abilities to utilize crude oil as their carbon source, identified and characterized by biochemical tests. The screening results showed that *Bacillus* genus was the predominated hydrocarbon degrader in spent engine oil environment with relative prevalence of 41 from total of 50 samples surveyed, indicating 82 %, compared to the *Micrococcus* with frequency of 9 (18 %) (Table 4). The *Bacillus* has been implicated in abscesses, septicaemia, osteomylitis, wound and burn infections, while infections caused by *Micrococcuus* include; bacteremia, pneumonia, systemic arthritis and peritonitis (Udeani *et al.*, 2009; Mandigan *et al.*, 2017). The strain *Lysinbacillus sphaericus* A-3-10 in bioremediation, has been identified to possess the potential to reversibly bind heavy metals such as lead, copper, cadmium and uranium due to the presence of the proteinaceous surface overlying the bacteria cell, called the S-layer (Cappello *et al.*, 2007).

Conclusion: The ability of autochthonous active bacteria in spent oil to utilize hydrocarbon as their sole source of carbon enhances healthy environment and economical process for curbing crude oil contamination and necessary for the recovery of sites polluted with hydrocarbon contaminants of the affected ecosystems.

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