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Growth Dynamics of Bacteria Isolated from Spent Engine Oil Contaminated Tropical Soil

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Abstract: Sites contaminated with spent oil in Canaan land Ota Nigeria were screened for the presence of spent oil degrading bacteria. The method of continual enrichment on the spent oil yielded five pure cultures that were selected for further physiological studies. From the morphological and biochemical characterization and comparison with respect to the standard references, the isolates were most likely the members of the genera *Acinetobacter*, *Arthrobacter*, *Bacillus*, *Mycobacterium* and *Pseudomonas* species. Time course studies of these organisms on the spent oil monitored by OD and pH fluxes resulted in exponential increases in the turbidity and steady declines in the pH. The mean OD obtained was 0.147±0.052-1.591±0.320 with pH 7.31±0.01-6.30±0.03. From the study, it was evident that bacterial species in tropical ecosystem can be explored for the recovery of sites polluted with spent engine oil.

Keywords: Biostimulation, enrichment, ecosystem, ecosystem, optical density, spent engine oil

INTRODUCTION

Although several research advances have been made in the isolation of bacterial species from environments polluted with spent oil, in particular from temperate ecosystem, bacterial species from the tropical ecosystem have been less explored. Spent oil or waste-lubricating oil is oil that has been used and obtained after servicing and draining from industrial machines and automobiles (Adesodun and Mbagwu, 2008). It is noteworthy, that spent oil is similar to unused oil, except that additional chemicals and metals such as lead, manganese, iron, tin and silicon have been added to the spent oil, due to high temperature and pressure of the operating engines where the oil serves as engine lubricant (Abdulsalam *et al*., 2012). In addition, these added chemicals impurities contribute significantly to chronic hazards because of their solubility in soil surface and groundwater (Abdulsalam *et al*., 2012; Blodgette, 2001). Some of the health hazards associated with individuals exposed to environments where the spent oil has been disposed off include anemia and tremor with consequent deaths.

Spent engine oil also called used motor oil abound in oil change workshops, garages and industry sources. The spent oil may occur as hydraulics oil, turbine oil, process oil and metal working fluids (Speight, 1991). According to Koma *et al*. (2003), spent oil consists of large amounts of long-chain saturated hydrocarbons (80 - 90%) (base oil) and additives 10-20%. Due to the paucity of knowledge about the environmental consequences of the spent oil, artisans indiscriminately dispose-off the waste oil into gutters, water drains, open vacant plots and farmlands.

In most developing countries, particularly in Africa, governments' drive towards the monitoring, control and regulation of these activities of has achieved little or no goal (Anoliefo and Vwioko, 1995). This is due to the lack of baseline data on spills and the absence of information on the incidences of poor disposal. With the disadvantages associated with spent oil spills notwithstanding, the oil can be recycled or used for purposes of generation of electricity or as industrial burners. The oil can be mixed with asphalts for paving space waters in automotive bays. These benefits, albeit, the challenges posed by spills in developing countries, far outweigh their potential uses (Mishra *et al*., 2001).

Spent oil contains substrates and metabolites that could provide an environment for the development of complex microbial community (Butler and Mason, 1997). With current efforts, by the scientific communities in reducing environmental pollution via the decay of pollutants or attack by enzymes, bioremediation is being explored. Bioremediation involves the use of organisms to get rid of substances from their environment via growth and metabolism of such substances. It has been noted that bacteria, protista and fungi could degrade complex molecules and incorporate the products into their biomass.

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In recent years, several organisms have been isolated that are capable of degrading spent oil (Mandri and Lin, 2007). Owing to the need to provide and document different bacteria species capable of utilizing spent engine oil from the tropical soils, this study was carried out to investigate the growth dynamics of bacteria isolated from spent oil contaminated ecosystem.

MATERIAL AND METHODS

Chemicals and reagents: The reagents $(NH_4)_2SO_4$, $MgSO_4.7H_2O$, $Ca(NO_3)_2.4H_2O$, K_2HPO_4, KH_2PO_4 and NaCl of analytical grades were obtained from the Merck, Germany. Nutrient agar and nutrient broth were obtained from Micro Master India.Urease base agar, starch agar, methyl red and voges proskauer medium and peptone water were procured from the Microbiology Laboratory, Covenant University.Spent oil was sourced from the mechanic workshop in Canaanland Ota, Ogun State Nigeria. The spent oil had low viscosity with filth carbon.

Stock solutions and media: All the enrichment and degradation experiments were performed using chloride-free minimal salts (MS) medium as described by Kim and Picardal (2000), Nwinyi *et al*. (2008) and Nwinyi (2010, 2011) with slight modification. The medium consists of 0.5 g $(NH_4)_2SO_4$, 0.1 g $MgSO_4 \cdot 7H_2O$, 0.076 g Ca $(NO_3)_2$. $4H_2O$ and 40mM phosphate buffer (pH 7.25). Solid MS medium was made by the addition of 1.8% Bacto-agar (Difco Laboratories, Detriot, MI USA). MS medium was supplemented with the spent oil.

Enrichment of bacterial species: Soil samples were collected from Canaanland Mass Transit workshop (CLMT) Ota, Nigeria. Soil samples were obtained from specific locations around the workshop with heavy spillages of spent oil. The location had no plants growing on it. The soil samples had humus fused with clayey texture that was dark in appearance.

Spent oil degrading bacteria were initially isolated by traditional enrichment culture methods: For this 5.0 g of the soil samples were mixed with MS medium contained in a conical flask (200 mL). The medium was amended with spent oil (0.2% v/v) as the primary carbon source. The flasks were incubated on a shaker (Model H2Q-X 300) at 65 rpm at 30°C for 14 days. Subsequent transfers from these enrichments were made fortnightly by using the same methods and conditions. After about one month, the enrichment cultures were transferred to a fresh medium using a 12% inoculum and continued cultivation under the same conditions. Subsequent transfers were carried out using 2% inoculum and the procedure was repeated for five successive times.

Determination of physicochemical parameters of soil: The soil samples were collected randomly at the site of study using a clean container. After eliminating the stones by sieving, the soil samples were thoroughly mixed and stored in 20 mL McCartney bottles. The sample were analyzed for PAH, heavy metals and pH.

Isolation, purification and characterization of Spent-engine oil degrading bacterial species: Pure cultures from the spent oil-enriched media were isolated by plating 1.0 mL of the enriched cultures onto Minimal Salt (MS) agar, sprayed with spent-engine oil on the surface. This was incubated in dark at 28-32°C for five days. Colonies were periodically transferred to MS agar to obtain the pure culture. Using the morphological examinations, bacterial species were isolated and sub-cultured onto separate agar plates. The pure cultures were incubated at 37°C for 18-24 h. These were named as ES1, ES2, ES3, ES4 and ES5and classified using the standard cultural and morphological techniques and comparison with standard reference organisms (Cowan, 1985; Olutiola *et al*., 1991). The following tests were carried out: Gram stain, morphology, catalase, oxidase, colony motility, methyl red, voges proskauer, indole, nitrate reduction, gelatin hydrolysis, spore test, starch hydrolysis, coagulase citrate and sugar utilization.

Inoculum preparation: Cultures were incubated on a freshly prepared sterile nutrient broth in cotton wool stoppered balch tubes and incubated at 30°C for 120 h. The cells were harvested by centrifugation at 35×100 rpm for 50 min, washed two times in phosphate buffer saline at pH of 7.30, transferred into a sterile 2.0 mL eppendorf tubes and re-suspended in MS medium as previously described by Nwinyi *et al*. (2008) and Nwinyi (2011) to an optical density of 0.4 at 600 nm. The inocula were used for the growth and degradation studies.

Degradation/growth of bacterial species in spent oil: Pure cultures were tested for their ability to degrade the spent oil. The tests were performed in balch tubes containing MS medium (9 mL) supplemented with the spent oil, which were inoculated by the pure cultures at 10^5 cells/mL. Tubes were incubated in shaker (Model H2Q-X 300) at 65 rpm at 30°C for 15 days. An abiotic control was set up with the inoculated control (MS medium and spent oildevoid of organism) incubated at the same conditions. Measurements of the Optical Density (OD) and pH were carried out at 1, 72, 144, 216 and 288. Optical density was measured at 600 nm using Genesys 10 UVS Spectrophotometer.

Statistical analysis: Statistical tests (mean and standard deviation) were performed using the Graph pad prism 4.0 computer software programme.

RESULTS

From the physicochemical analysis, the concentrations of the hydrocarbons and heavy metals in the contaminated soil as shown in Table 1 included fluorene 2.84 ppm, Indeno (1, 2, 3-cd) pyrene 31.26 ppm, Lead 0.14 mg/kg. Acenapthalene, fluoranthene and phenanthrene were not detected. The pH of the contaminated soil was 6.34.

The isolates ES2, ES3 and ES4 were catalase positive, gram positive rod while only isolates ES1, ES2 and ES3 were capable of fermenting glucose. The isolates ES1and ES5 were catalase positive and gram negative rods. The data obtained from the morphology and biochemical characterization Table 2, suggested the organisms ES1, ES2, ES3, ES4 and ES5 are similar to the members of the genus *Acinetobacter*, *Arthrobacter*, *Bacillus*, *Mycobacterium* and *Pseudomonas* species respectively (Fig. 1).

Table 1: Hydrocarbons detected at the selected site

PAH Hydrocarbons	Selected soil
Physical appearance	Dark and hardened
Acenapthalene	Not detected
Acenaphthene	Not detected
Fluorene	2.84 ppm
Phenanthrene	Not detected
Indeno (1,2,3-cd) pyrene	31.26 ppm
Lead	0.14 mg/kg

Fig. 1: Colonies of *Pseudomonas* on mineral salt agar amended with spent oil

Table 2: Cultural and biochemical characteristics of pure bacterial species capable of degrading spent oil

	Isolates				
Tests	ES1	ES2	ES3	ES4	ES5
Gram stain	-	+	+	+	-
Colony morphology	Cocco-bacillary	rods	rods	rods	Uniform rods
Catalase	+	+	+	+	+
Indole	+	+	-	+	-
Motility	_	_	_	_	_
Starch hydrolysis	-	+	+	+	-
Sucrose	-	_	-	-	+
Glucose	+	+	+	-	+
Lactose	+	-	_	_	-
Spore stain	-	-	+	-	-
Acid fast stain	_	_	_	+	_
Growth at 35°C	‡	‡	+	+	+
Growth at pH 6.0	‡	‡	+	+	+
Growth in 5% of NaCl	‡	‡	+	+	+

Most probable organism *Acinetobacter*, *Arthrobacter*, *Bacillus*, *Mycobacterium*, *Pseudomonas*; +: Positive Reaction, -: Negative Reaction, ‡: inconclusive

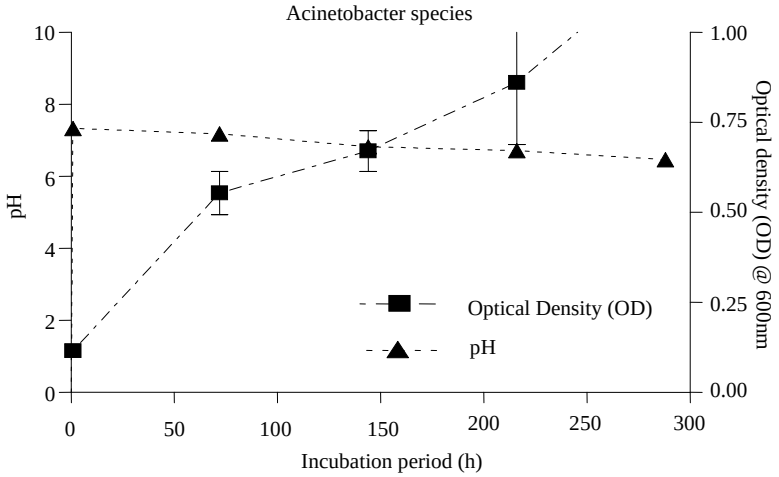

Fig. 2: Spent engine oil -dependent growth (OD) and pH fluxes of *Acinetobacter* species after 288 h-incubation. Data represent the mean of triplicate tubes for initial time represented as shown and the final time (288 h). The error bars were due to differential responses of the cells in the triplicate tubes. The x-axis value range was chosen as such to allow for even spread of the pH and OD dynamics

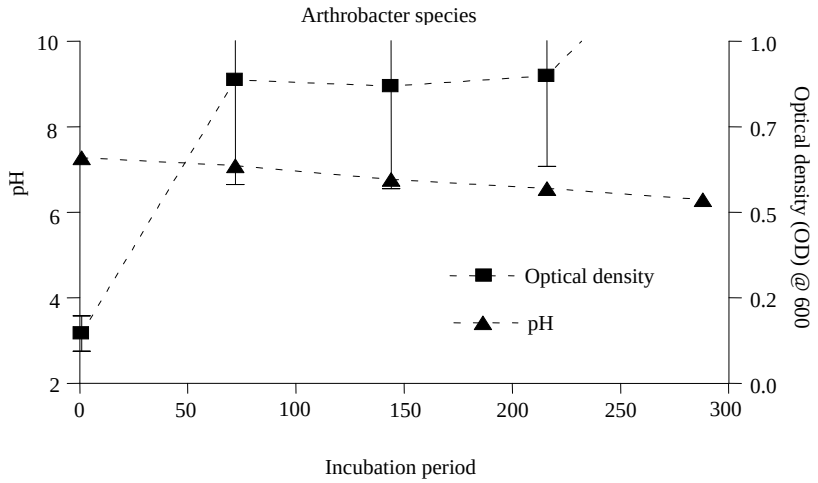

Fig. 3: Spent engine oil -dependent growth (OD) and pH fluxes of *Arthrobacter* species after 288 h-incubation. Data represent the mean of triplicate tubes for initial time represented as shown and the final time (288 h). The error bars were due to differential responses of the cells in the triplicate tubes. The x-axis value range was chosen as such to allow for even spread of the pH and OD dynamics

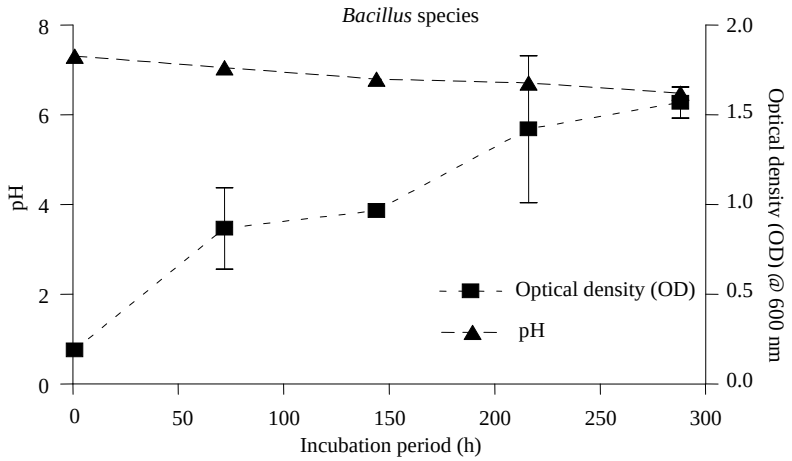

Fig. 4: Spent engine oil -dependent growth (OD) and pH fluxes of *Bacillus* species after 288 h-incubation. Data represent the mean of triplicate tubes for initial time represented as shown and the final time (288 h). The error bars were due to differential responses of the cells in the triplicate tubes. The x-axis value range was chosen as such to allow for even spread of the pH and OD dynamics

In this study, increase in the turbidity, measured by the optical density fluxes and the decrease in pH and of at least one order of magnitude when compared to the values of abiotic and biotic controls may be regarded as degradation/utilization of the spent oil as carbon and energy sources by the bacteria species. The initial and final values obtained for the abiotic and biotic controls had no significant difference with the values obtained at the initial stage for the test samples. Additionally, the dark colored spent oil resulted in high optical density by the control and the test samples at the inception of this investigation. Equally, the measure of degradation or utilization of the spent oil was indicated by increase in dark color of the test samples which was equivalent to increase in optical density. Thus, bacteria species that gave the highest optical density were adopted as the good degrader of the spent oil in the MS medium. Hydrocarbon degradation often result in the production of acidic products that lower the pH of the medium, thus in this study, decreases in pH may therefore be regarded that degradation had occurred.

Figure 2 to 6 illustrates the mean changes in the pH and optical density of spent- oil degradation study of the bacterial species. The phosphate buffer washed cells of *Acinetobacter* species were able to utilize the spent oil as shown in Fig. 2. From the optical density OD at 600 nm, it showed that the species showed a logarithmic increase in the cell numbers/biomass that continued until the end of the experiment. The OD ranged from 0.115±0.019-1.201±0.010, while the pH

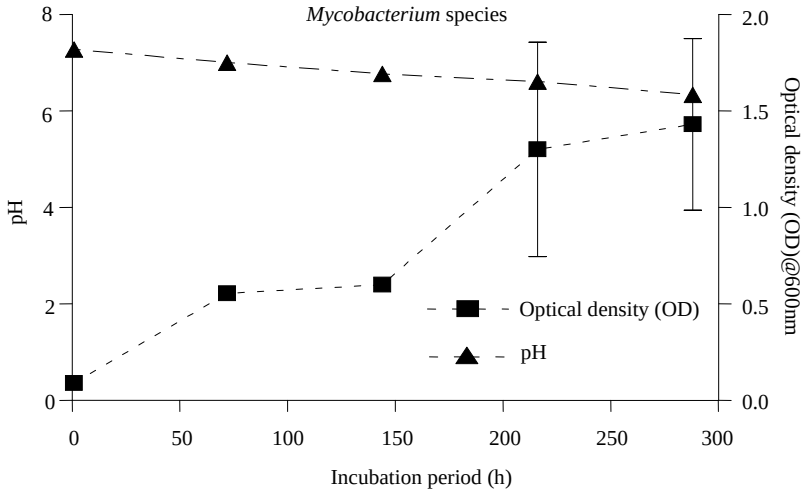

Fig. 5: Spent engine oil-dependent growth (OD) and pH fluxes of *Mycobacterium* species after 288 h-incubation. Data represent the mean of triplicate tubes for initial time represented as shown and the final time (288 h). The error bars were due to differential responses of the cells in the triplicate tubes. The x-axis value range was chosen as such to allow for even spread of the pH and OD dynamics

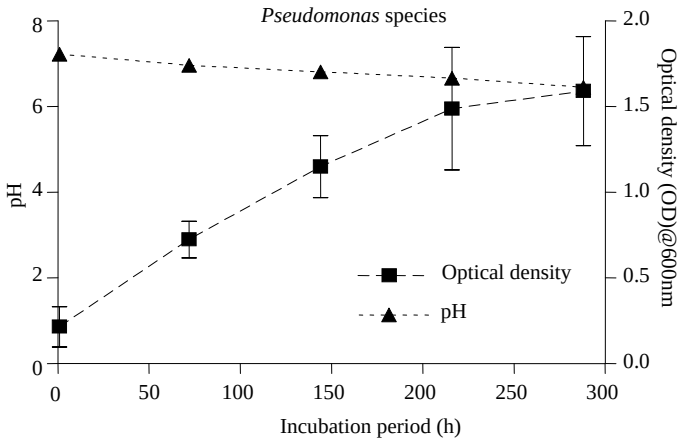

Fig. 6: Spent engine oil-dependent growth (OD) and pH fluxes of *Pseudomonas* species after 288 h-incubation. Data represent the mean of triplicate tubes for initial time represented as shown and the final time (288h). The error bars were due to differential responses of the cells in the triplicate tubes. The x-axis value range was chosen as such to allow for even spread of the pH and OD dynamics

showed a significant decrease from the 7.33±0.01-6.46±0.06.

In Fig. 3 the optical density curve showed that the *Arthrobacter* species had a logarithmic increase in cell numbers/biomass within the first 72 h of incubation. Consequently, the organisms entered a stationary phase that lasted till the end of the experiment. Possibly the organisms' enzymes were less tolerable to the hydrocarbons and other contaminants present within the spent oil. The pH changes showed a progressive decline until the end of the study. The OD ranged 0.147±0.052-1.356±0.09, while the pH decreased from 7.28±0.03-6.30±0.03.

The ability of *Bacillus* species to degrade spent oil was also examined using the phosphate washed cells in Fig. 4. The optical density showed a stepwise increase after 72 h of incubation. The pH showed a steady decline. The OD ranged 0.189±0.001-1.569±0.087, while the pH decreased from 7.31±0.03-6.49±0.04.

In Fig. 5, the *Mycobacterium* species was able to show steady increase in turbidity over the period of the experiment with a steady decrease in the pH. The OD ranged 0.089±0.015-1.430±0.445, while the pH decreased from 7.28±0.03-6.34±0.02.

The phosphate buffer washed cells of *pseudomonas* species were able to utilize the spent oil. From the OD, it showed that the species showed a logarithmic increase in population that continued until the end of the experiment. The pH values were also reduced. The OD ranged 0.216±0.117-1.591±0.320, while the pH decreased from 7.23±0.03-6.45±0.05.

DISCUSSION

The use of metabolic capabilities of different microorganisms in sustainable remediation involves breaking the contaminant pathway between the source and receptor. This has continuously been exploited in hydrocarbon transformation. Soil heterotrophic microbial communities are the primary mediators of key biological processes in soil. These include degradation and mineralization (Li *et al*., 2009).

One of the best ways of isolating bacterial strains with specific metabolic capabilities is by enrichment with the target compound. In this approach, the target compound serves as potential carbon source. The prior exposure to the target compounds may augment the organism's metabolic capabilities Conversely, bacteria species with degradation ability may fail to function when inoculated into the natural environment. This strongly indicates that the environmental conditions including physical and chemical conditions of the contaminated sites play a crucial role in the degradation of the contaminated site.

In this present study, the microbial communities from the spent oil- contaminated soil were enriched on spent oil. Thirteen isolates were obtained and was screened down to five promising isolates based on preliminary studies. The five pure bacteria species include: *Acinetobacter*, *Arthrobacter*, *Pseudomonas*, *Mycobacterium* and *Bacillus*. Results obtained showed that all the bacteria species increased in their cell numbers/biomass when grown in the spent oil. The physicochemical analyses revealed the presence of polyaromatic hydrocarbons and trace metal as shown in Table 1. The hydrocarbons detected include: fluorene, indeno (1, 2, 3-cd) pyrene and the trace metal (lead). Of significance are the abilities of *Pseudomonas* and *Acinetobacter* sp. These organisms have been previously demonstrated as species noted to utilize hydrocarbons (Rusansky *et al*., 1987; Briganti *et al*., 1997; Nwinyi, 2010, 2011). It has been noted also that *Acinetobacter* sp. are wide spread in nature and can remove or degrade a wide range of organic such as phenol, toluene and heavy metals (Mandri and Lin, 2007).

Significant growth/utilization of the spent oil was observed from the OD data as shown in Fig. 2 to 6. The *Pseudomonas* and *Acinetobacter* species were the best degrader as evident by their significant growth when compared to other isolates. This ability might have been due to their production of biosurfactants. The biosurfactants effectively reduces the water tension between the spent oil and the organisms; hence more of the hydrocarbons were available for microbial utilization (Beal and Betts, 2000; Chang *et al*., 2009; Henry and Abazinge, 2009). This phenomenon may account for the increases in OD data obtained for these organisms.

Few researchers have reported about the roles of *Bacillus* sp. in hydrocarbon bioremediation particularly at extreme environments. Ijah and Antai (2003) reported *Bacillus* sp. as one of the notable isolates of crude oil utilizing bacteria from highly polluted soil samples (30 and 40% crude oil). Ijah and Antai (2003) proposed that *Bacillus* sp. were more tolerant to high levels of hydrocarbons in soil due to their resistant endospores. This report corroborates with our findings on the growth dynamics of *Bacillus* and *Mycobacterium* species that are spore formers. In addition, Peng *et al*. (2008) documented of several *Mycobacterium* species with the ability to degrade wide range of PAHs. They noted that *Mycobacterium* sp. strain AP1 could grow with pyrene as its source of carbon and energy but could not remove the substrate completely due to assimilation of the carbon source. This may probably be reason for the slow but step wise increase in OD for our obtained *Mycobacterium* species.

From this study it is apparent that periodic enrichment provided exotic bacterial species that abound in the tropical ecosystem. In conclusion, the results of this study have shown that *Acinetobacter*, *Arthrobacter*, *Bacillus*, *Mycobacterium* and *Pseudomonas* species might have the potential of utilizing spent oil as carbon and energy sources. This study is, by no means, extensive as other possible mechanisms for assessment of biodegradation indices were not explored. Future research work will be focused on in-situ studies on bacterial community dynamics on spent oil polluted sites, with the view of gaining better understanding of the roles played by microbes in the remediation of spent oil.

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