

## Isolation and Identification of Petroleum Hydrocarbon Degrading Bacteria from Polluted Top Soil Samples in Oxford, Pennsylvania

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### Abstract

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Petroleum hydrocarbon degrading bacteria were isolated from two polluted sites in Oxford, Pennsylvania by enrichment techniques using Bushnell Hass medium. No isolate in this study utilized gasoline; however, two isolates utilized engine oil and three, utilized kerosene. Identification by Gram stain, morphological, biochemical, deoxyribonucleic acid and fatty acid methyl ester tests showed that three isolates were *Pseudomonas plecoglossicida*, one was *Pseudomonas nitroreducens* and the last was *Achromobacter denitrificans*. This study corroborates that of other researchers that hydrocarbon degrading bacteria can be found in all types of contaminated and polluted environments; they can therefore be enriched and utilized for the remediation of oil polluted environments. We recommend that a culture bank for the collection and preservation of microorganisms specific for bioremediation be established to enable speedy access whenever cases of environmental pollution by oil products are reported.

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**Key words:** Isolation, identification, hydrocarbon degrading bacteria

### 1. Introduction

Crude oil and its products including gasoline, diesel and kerosene are major sources of energy for industrial and domestic purposes throughout the world. Spills and leakages of these products occur regularly during exploration, transportation, refining, conflicts, vandalism, theft and storage. For example, during the Gulf war of 1991, between 2 to 4 million barrels of crude oil was spilled into the Persian Gulf as Iraqi forces tried to block the landing of United States forces (Khordagui & Al-Ajmi, 1993). In 2010, there was a spill involving BP's Deepwater Horizon in which an estimated 3.19 million barrels of crude oil spilled into the Gulf of Mexico over a three-month period (Zengel et al., 2016). In 1979, two oil tankers collided resulting in the discharge of about 88.3 million gallons of crude oil into the Caribbean Sea (Jernelöv, 2010). In 1991, an oil tanker explosion expelled an estimated 51 million gallons of crude oil into the sea of Angola (Mohit, 2019). In 1989, an oil tanker, Exxon Valdez ran aground in Alaska discharging its contents onto the Alaskan coastline (Mohit, 2019). In Nigeria, over 20,000 oil spill incidents were reported in the Niger Delta area by the Nigerian National Petroleum Corporation (NNPC) between 2006 and 2013 resulting in the discharge of over 115,000 barrels of crude oil each year (Mohamadi, Liu & Xie, 2016).

Spillage of oil is usually accompanied by environmental catastrophes including the loss of marine life such as fish, marine mammals and birds as well as adverse effects on beaches, human settlements, wildlife habitat and mangrove forests. For example, frequent cases of oil spillage in the Niger Delta area of Nigeria have resulted in the migration of people who can no longer depend on fishing for their livelihood (Ipingbemi, 2009). It is estimated that oil spillage in the same area has resulted in the loss of five to ten percent of the mangrove ecosystem as well as loss of farmlands (Ipingbemi, 2009). After the oil spill in the Gulf of Mexico, residents experienced negative impacts such as anxiety, stress, loss of work and income, and diminished tourism as visitors cancelled their vacations (Graham et al., 2016; Ulrich, 2011). Species such as dolphins, whales and turtles showed elevated rates of stranding (Lichtveld et al., 2016). Bottlenose dolphins had 8% higher mortality rates and 63% lower reproductive rates (Lichtveld et al., 2016).

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There is also loss of property and investments when spills occur during transportation; and resources that could have been used in other sectors are diverted for cleaning, litigation and compensation. Researchers have also found elevated levels of phenanthrene in contaminated soils, defunct industrial sites and land based storm water runoff following spills of crude oil. Phenanthrene blocks the heart excitation-contraction coupling pathway (Brette et al., 2017). Since oil is one of the most abundant pollutants of oceans, various methods are employed to clean spills and remediate the ecosystem. One of the methods is oil boom which acts like a fence to prevent the spilled oil from floating away (Environmental Protection Agency, 1999a). Although this method is effective, it can only be employed immediately after the spill when the oil is still in one area. The second method is using skimmers to scoop the spilled oil into boats (Environmental Protection Agency, 1999a). A third method utilizes sorbents which soak up the oil before they are retrieved (Environmental Protection Agency, 1999a). Some people remove oil spills by burning; however, this method is effective only if the thickness of the oil spill is less than 3mm (Environmental Protection Agency, 1999b). Regardless, the toxic fumes produced by burning can create very hazardous environmental conditions (Environmental Protection Agency, 1999b). Dispersants have also been used to accelerate the rate of breakdown of oil, but they can create tar balls which can be carried to the shores (Environmental Protection Agency, 1999b). Also, dispersants have been found to adversely affect marine organisms (Environmental Protection Agency, 1999b). Hot water has been used for cleanup; however, released oil must be recovered immediately to prevent further contamination, and hot water can also kill organisms (Environmental Protection Agency, 1999c). Chemical stabilization using compounds like Elastol can gelatinize spilled oil, thus preventing it from spreading. Although very effective, the gelatin may entangle and suffocate aquatic animals (Kramer, 2018).

Most of the methods highlighted above are either not available or affordable in many poor communities, or are hazardous to the environment and the workers who apply them. Bioremediation is therefore another option that is affordable, environmentally friendly, works on even tiny oil droplets not usually eliminated by other methods, and safe to use in shoreline and soil. Bioremediation is the utilization of naturally occurring microorganisms like bacteria to degrade hazardous substances in the environment to non-toxic or less hazardous substances (Rounald, 1995). In the case of oil or petroleum products, these organisms will remedy the environment by breaking down oil or petroleum products into simpler non-toxic substances. Bioremediation is a very cost effective way of cleaning up oil spillages, it is easy to maintain, it can be applied to large areas and it leads to complete removal of the pollutant (Tanzadeh & Ghasemi, 2016). It can be used to complement other methods for cleaning oil spillage. Bioremediation was extensively used in the cleanup of the Exxon Valdez oil spill, and fertilizer was added to speed up the rate of biodegradation (Atlas & Hazen, 2011). In 2001 and 2003, National Oceanic and Atmospheric Administration (NOAA) determined that 97.8% of the oil had been biodegraded, a decline of about 22% per year from 1991 (Atlas & Hazen, 2011).

Some studies of biodegradation in marine sediments have identified *Arthrobacter*, *Burholderia*, *Mycobacterium*, *Pseudomonas*, *Sphingomonas* and *Rhodococcus* as the microorganisms responsible (Das & Chandran, 2011). Other studies in Nigeria have also isolated *Pseudomonas fluorescens*, *Pseudomonas aeruginosa*, *Bacillus subtilis* and others from crude oil polluted streams in Nigeria (Adebusoye et al., 2006). Studies on the isolation and characterization of hydrocarbon-degrading bacteria from the top soil of contaminated mechanic workshops have also been carried out in Nigeria (Ebakota et al., 2017). This study was therefore initiated to isolate and characterize hydrocarbon degrading bacteria from top soil of two dump sites for old farm tractors in Oxford, Pennsylvania. We hypothesized that hydrocarbon degrading bacteria would be present in the soil where seepage of petroleum products from leaking engines had occurred.

## 2. Methods and materials

### 2.1 Sample collection and preparation of Bushnell Hass medium

Soil samples were collected in Oxford, Pennsylvania from two separate dump sites for old farm tractors. Samples were collected from areas showing seepage of petroleum products. Kerosene, gasoline, and engine oil were used as sources of carbon, and purchased from a local gas station in Oxford, Pennsylvania.

Bushnell Hass Mineral Salts (BHMS) medium was prepared as prescribed by Panda, Kar & Panda, 2013. Each liter of BHMS contained 0.2g of  $MgSO_4 \cdot 7H_2O$ , 0.02g of  $CaCl_2$ , 1g of  $KH_2PO_4$ , 1g of  $K_2HPO_4$ , 1g of  $NH_4NO_3$ , 0.05g of  $FeCl_3$ . The pH was adjusted to 7.0. The medium was dispensed in 30ml amounts into 150ml Erlenmeyer flasks before autoclaving at 121°C for 20 minutes. Before use, 7.5 ml of the petroleum products (kerosene; gasoline; engine oil) was added to the medium.

## 2.2 Enrichment procedure for hydrocarbon degrading bacteria

Two grams of soil sample from the two sites (A and B) were added respectively to each of the flasks with the petroleum products. The six flasks (KER-A1; KER-B1; ENG-A1; ENG-B1; GAS-A1; GAS-B1) were placed into a shaking incubator at 25°C and agitated at 150 rpm for seven days.

After seven days of incubation, a new set of BHMS medium was prepared as previously described; however, each flask contained 15 ml of the petroleum product and no soil sample. Each of the six new flasks also contained 15ml of the culture from the first week of enrichment (KER-A2; KER-B2; ENG-A2; ENG-B2; GAS-A2; GAS-B2). The new flasks were put back into the shaking incubator at 25°C for seven days at an agitation of 150 rpm. This procedure was repeated for five weeks. At the end of the five-week enrichment process, one loopful of sample was collected from each flask and plated on Brain Heart Infusion agar. After incubation at room temperature for 48 hours, representative colonies were sub-cultured on Brain Heart Infusion agar slants for further tests.

## 2.3 Screening for bacteria with highest hydrocarbon utilization

Thirty milliliters (30 ml) of Bushnell Hass medium was mixed with each of the petroleum products (1.33 ml) to achieve a concentration of 4%. Five milliliters of each medium was dispensed into test tubes. Cultures of representative colonies on the Brain Heart Infusion agar slants were suspended in sterile normal saline to a concentration of 0.5 McFarland; thereafter, five loopful of the suspension was added to each of the test tubes containing Bushnell Hass medium. The test tubes were placed into a shaking incubator at 25°C and 150rpm for seven days. After seven days of incubation, the optical density of each specimen was determined at a wavelength of 550nm. Specimens with highest optical density were selected for further screening.

## 2.4 Final confirmatory test for degradation of petroleum product

To ensure that the selected samples of bacteria will consistently degrade petroleum products, they were reintroduced into the Bushnell Hass media. For this test, 30ml of Bushnell Hass medium containing 4% of the petroleum product was prepared in 150ml Erlenmeyer flask. Five loopful of selected bacterial samples equilibrated to 0.5 McFarland in normal saline was added to each flask and incubated in a shaking incubator at 25°C and 150rpm. A control flask with medium and no bacteria sample was also placed in the incubator. The optical density and viable plate count was measured at 0, 7, 14, and 21 days. For plate count, serial dilutions of the samples were plated on Tryptic Soy Agar and colony forming units were counted after 48 hours.

## 2.5 Cultural and biochemical identification of isolates

Bacterial isolates that efficiently utilized the petroleum products, based on the optical density and colony forming units (CFU/ml) in the final confirmatory test, were selected for additional tests including Gram stain, catalase, indole production, motility, citrate utilization, oxidase, methyl-red and Voges-Proskauer. They were also tested for their ability to ferment glucose, galactose, mannitol and lactose.

## 2.6 Identification of isolates by DNA and Fatty Acid Methyl Esther (FAME)

DNA and FAME identification of isolates was carried out by MIDI Labs Inc., Newark, Delaware. DNA identification was based on 16S rRNA gene sequence similarity. Sequence analysis was performed using Sherlock® DNA microbial analysis software and database. The 16S rRNA gene was PCR amplified from genomic DNA isolated from pure bacterial colonies. Primers used are universal 16S primers that correspond to positions 0005F and 0531R for a 500bp sequence, and 0005F and 1513R for the 1500bp sequence. Amplification products were purified from excess primers and dNTPs, and checked for quality and quantity. Cycle sequencing of the 16S rRNA amplification products was carried out using DNA polymerase and dye terminator chemistry. Excess dye-labeled terminators were then removed from the sequencing reactions. The samples were electrophoresed on a 3130xl Genetic Analyzer.

Identification of the isolates by FAME was based on Similarity Index in the MIDI Sherlock Microbial Identification System (MIS) which expresses how closely the fatty acid composition of an unknown sample compares with the mean fatty acid composition of the strains used to create the library entry listed as its match.

## 3. Results

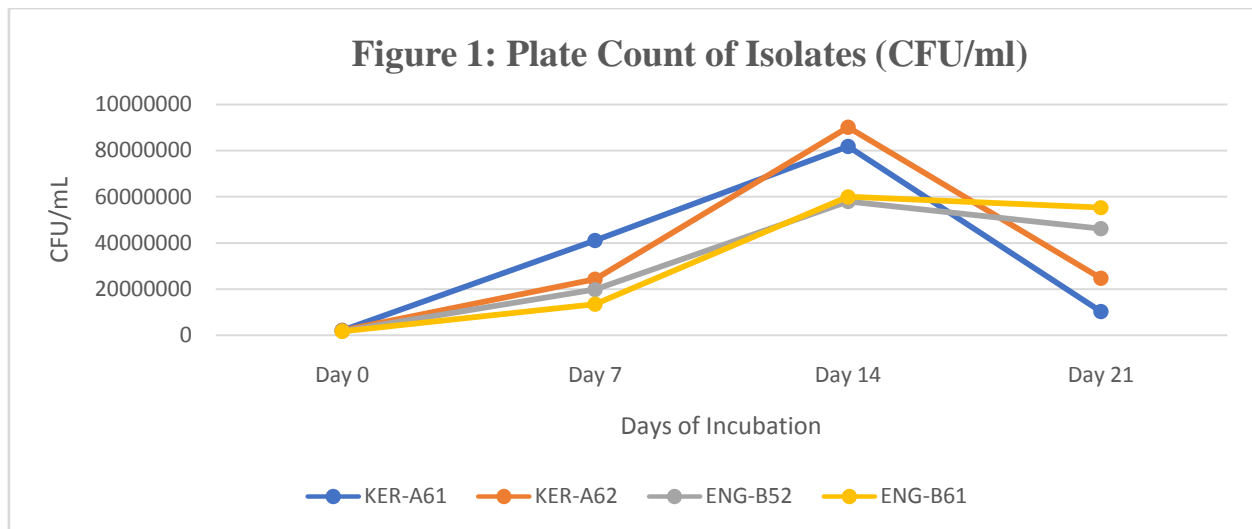
After the initial enrichment process and subculture on Brain Heart Infusion Agar, nine colonies were selected from kerosene samples, five colonies were selected from gasoline samples and six colonies were selected from engine oil samples. When each of these colonies was inoculated into Bushnell Hass medium and incubated for 7 days, the optical density readings showed that none of the colonies from the gasoline medium produced any significant

turbidity (Table 1). Two of the colonies showed significant turbidity on the kerosene medium (KER-A6<sub>1</sub>; KER-A6<sub>2</sub>) while four colonies of the engine oil medium showed turbidity (ENG-A6; ENG-B5<sub>2</sub>; ENG-B6<sub>1</sub>; ENG-B6<sub>2</sub>).

Based on these results, two isolates from the kerosene medium (KER-A6<sub>1</sub>; KER-A6<sub>2</sub>) and two from the engine oil medium (ENG-B5<sub>2</sub>; ENG-B6<sub>1</sub>) were selected for further testing on Bushnell Hass medium. After 21 days incubation on Bushnell Hass medium, the two isolates from the kerosene medium produced more growth than the isolates from engine oil medium as measured by the optical density readings and plate counts (Table 2; Figure 1). More growth was recorded after 14 days than after 21 days indicating that after 14 days, the growth phase had moved from exponential to death phase. It was also observed that the kerosene utilizing bacteria were only isolated from site A, and none from site B. On the other hand, isolates from site B grew on both engine oil and kerosene. The isolates also grew faster with kerosene than with engine oil; however, isolates on engine oil had sustained growth after 21 days than those on kerosene.

**Table 1: Optical density of isolates at 550nm after 7 day-incubation**

Kerosene	OD 550nm	Gasoline	OD 550nm	Engine oil	OD 550nm
KER-A5 <sub>1</sub>	0.018	GAS-A5 <sub>1</sub>	0.002	ENG-A5	0.155
KER-A5 <sub>2</sub>	0.041	GAS-A5 <sub>2</sub>	0.003	ENG-B5 <sub>1</sub>	0.066
KER-B5 <sub>1</sub>	0.064	GAS-B5	0.023	ENG-B5 <sub>2</sub>	0.336
KER-B5 <sub>2</sub>	0.002	GAS-A6	0.071	ENG-A6	0.168
KER-A6 <sub>1</sub>	0.642	GAS-B6	0.003	ENG-B6 <sub>1</sub>	0.179
KER-A6 <sub>2</sub>	0.275			ENG-B6 <sub>2</sub>	0.164
KER-B6 <sub>1</sub>	0.018				
KER-B6 <sub>2</sub>	0.003				
KER-B6 <sub>3</sub>	0.072				



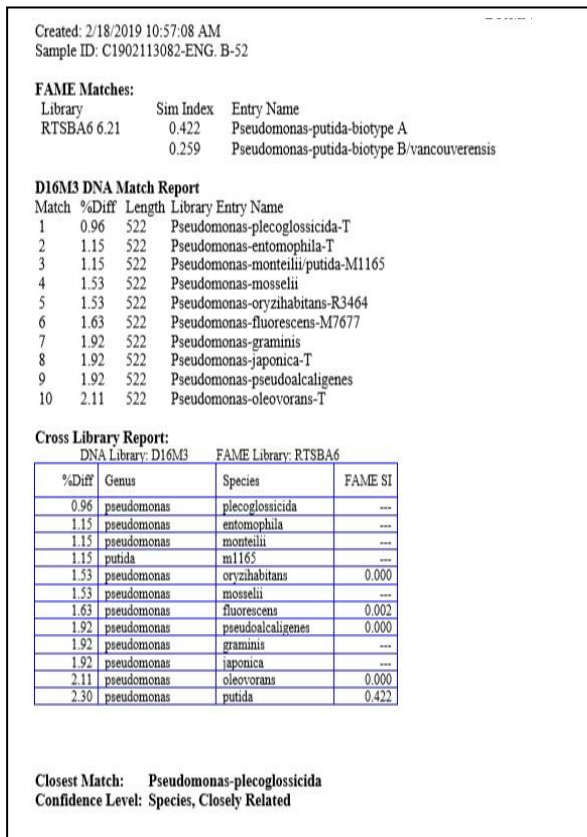
**Table 2: Optical density of isolates at 550nm after 7, 14 and 21-day incubation**

Isolates	Day 0	Day 7	Day 14	Day 21
KER-A6 <sub>1</sub>	0.010	0.20	0.401	0.050
KER-A6 <sub>2</sub>	0.010	0.119	0.442	0.121
ENG-B5 <sub>2</sub>	0.009	0.097	0.284	0.226
ENG-B6 <sub>1</sub>	0.008	0.066	0.294	0.271

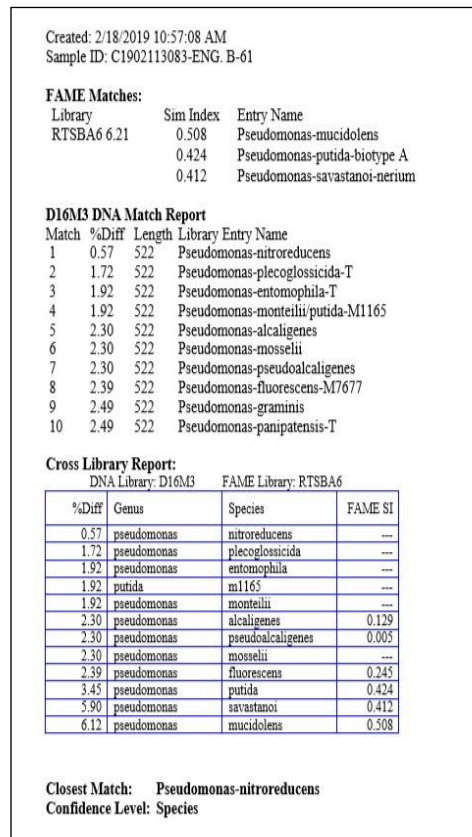
**Table 3: Colonial Morphology and Biochemical Reaction of Isolates**

Tests	KER-A6 <sub>1</sub>	KER-A6 <sub>2S</sub>	KER-A6 <sub>2B</sub>	ENG-B5 <sub>2</sub>	ENG-B6 <sub>1</sub>
Gram	Negative	Negative	Negative	Negative	Negative
Shape	Bacillus	Bacillus	Bacillus	Bacillus	Bacillus
Color/texture	Cream/orange	Cream/mucoid	Cream/mucoid	Cream/mucoid	Brown/shiny
Size (mm)	2-3	1.5-2	2-3	2-3	2-3
Motility	Positive	Positive	Positive	Positive	Positive
Catalase	Positive	Positive	Positive	Positive	Positive
Oxidase	Positive	Positive	Positive	Positive	Positive
Citrate	Positive	Positive	Positive	Positive	Positive
Indole	Negative	Negative	Negative	Negative	Negative
MR	Negative	Negative	Negative	Negative	Negative
VP	Negative	Negative	Negative	Negative	Negative
D-Glucose	Acid/no gas	Acid/no gas	Acid/no gas	Acid/no gas	Acid/no gas
D-Galactose	Negative	Negative	Negative	Negative	Negative
Lactose	Negative	Negative	Negative	Negative	Negative
Mannitol	Negative	Negative	Negative	Negative	Negative

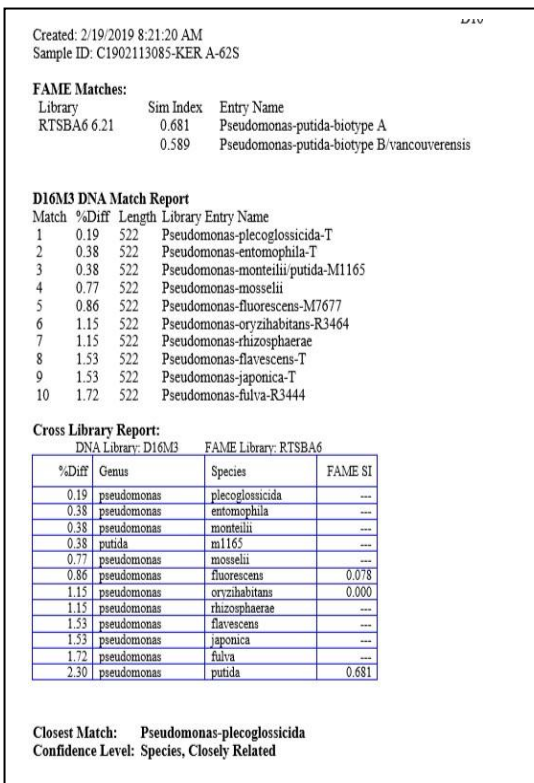
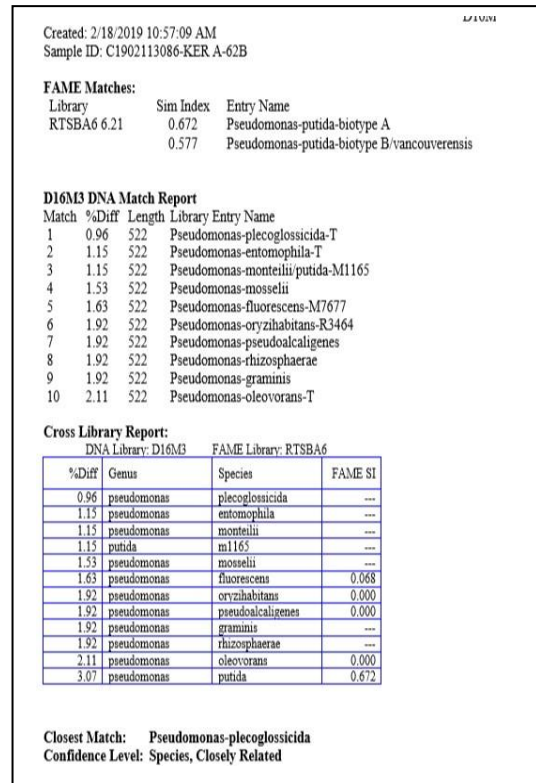
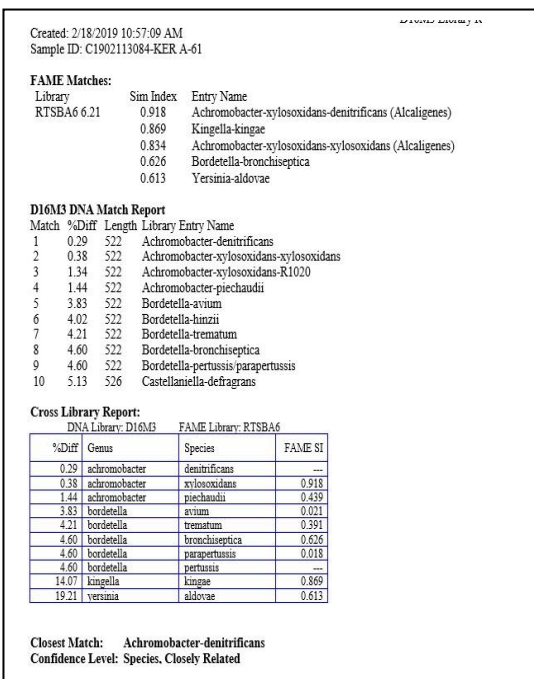
Table 3 shows the results of the colonial morphology and biochemical reactions of the isolates. It would appear from the results that the five isolates exhibited similar characteristics except for color and texture as well as size.



**Figure 2: DNA/FAME: ENG-B5<sub>2</sub>**



**Figure 3:DNA/FAME: ENG-B6<sub>1</sub>**

Figure 4:DNA/FAME: KER-A6<sub>2</sub>SFigure 5:DNA/FAME: KER-A6<sub>2</sub>BFigure 6: DNA/FAME: KER-A6<sub>1</sub>

In order to perform DNA and FAME identification, the isolates were again sub-cultured on Brain Heart Infusion Agar to ensure that the colonies were morphologically identical. It was observed that one of the kerosene isolates (KER-A6<sub>2</sub>) had large and small colonies. The larger one was therefore labelled KER-A6<sub>2</sub>B and the smaller one labelled KER-A6<sub>2</sub>S before they were sent to MIDI Labs Inc. for identification. Results show that four of the isolates were *Pseudomonas* spp., while one was *Achromobacter* spp. The two morphologically different colonies of KER-A6<sub>2</sub>B and KER-A6<sub>2</sub>S were eventually identified as the same species, *Pseudomonas plecoglossicida*.

Overall, three isolates were identified as *Pseudomonas plecoglossicida*, one was *Pseudomonas nitroreducens* and the last was *Achromobacterdenitrificans* (Figures 2 to 6).

#### 4. Discussion

The results of this study clearly indicate that it is possible to isolate hydrocarbon utilizing bacteria from contaminated soil samples by enrichment process. The soil samples taken from two different areas generated three types of bacteria namely: *Pseudomonas plecoglossicida*, *Pseudomonas nitroreducens* and *Achromobacterdenitrificans*. While *Pseudomonas plecoglossicida* was isolated from both soil samples, *Pseudomonas nitroreducens* was isolated from B site and *Achromobacterdenitrificans* from the A site. The results also show that of the three bacterial isolates, only *Pseudomonas plecoglossicida* utilized both kerosene and engine oil for growth.

*Pseudomonas plecoglossicida* has been implicated as the causative agent of bacterial hemorrhagic ascites of ayu, *Plecoglossusaltivelis*. (Nishimori, Kita-Tsukamoto & Wakabayashi, 2000). Only few studies have associated *Pseudomonas plecoglossicida* with the degradation of hydrocarbons. In one of the studies, this bacterium was used in the biodegradation of polycyclic aromatic hydrocarbons (Amini, Tahmourespour & Abdollahi, 2017).

*Pseudomonas nitroreducens* has also been identified in few studies involving the degradation of hydrocarbons. In one such study, this bacterium was isolated from oil contaminated soil in China (Yao et al., 1999). The researchers reported that the bacterium synthesized polyhydroxybutyrate homo polymer from medium-chain length fatty acids including hexanoate and octanoate.

There are more references of the association of *Achromobacterdenitrificans* with the degradation of hydrocarbons. One study found that the bacterium degraded diesel bilge water in Cordoba, Columbia (Mezquida, Oviedo, & Lara, 2015). Another study showed that *Achromobacterdenitrificans* could be used for the degradation of low density polythene (Ambika, Lakshmi & Hemalatha, 2015). The orange-red pigment of this bacterium has also been purified from di-(2-ethylhexyl) phthalate for the production of prodigiosin analog, an anti-cancer, immunosuppressive, anti-diabetic, anti-rheumatic, anti-fungal and anti-parasitic metabolite (Pradeep et al., 2014).

Finally, our results suggest that since these hydrocarbon degrading bacteria can be found in all sorts of contaminated and polluted environments, they can be enriched and utilized for the remediation of oil polluted environments. We also recommend that a culture bank for the collection of microorganisms specific for bioremediation be established to enable speedy access whenever cases of environmental pollution by oil products are reported.

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This work was supported by resources from Lincoln's Excellent Academic Program in Science – Transformation (LEAPS-T) which is funded by the National Science Foundation's Historically Black Colleges and University-Undergraduate Program (HBCU-UP)