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# Assessment of Spent Engine Oil Degrading Potentials of Bacteria Isolated from the Air

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## ABSTRACT

Hydrocarbon contamination from oil related activities is increasingly becoming a global problem. This study is aimed at isolating airborne bacteria with potentials to degrade hydrocarbon, measure their degrading process by Gas chromatography, and identify the isolates using molecular characterization and sequencing. Six bacteria isolates were isolated from the air and were collected at the diesel contaminated sites by the generator house behind School of Science and Technology building, Yaba College of Technology. Physico-chemical parameters of the spent engine oil were determined with Gas chromatography (Agilent 7890A Gs system 5975C VLMSD). Upon exposure of agar plate to the air; two of the isolates showed the capabilities to degrade spent (used) engine oil as their carbon source. The airborne bacteria isolates were cultured in 10ml MSM broth devoid of carbon source enriched with 0.1ml spent engine oil for seven days at $35^0$C in a shaker incubator. Using 16s rRNA sequencing of specific primers, the two bacteria isolates that showed growth when streaked on nutrient agar plates were identified. Results showed *Enterobacter xiangfangensis* and *Bacillus zhangzhouensis* as air dwelling bacteria with high potential for degrading hydrocarbon. The molecular characterization identified the particular strains of the *Enterobacter xiangfangensis* and *Bacillus zhangzhouensis* with tendency to degrade engine oil. The 16s rRNA genes result with Accession number showed 99.79% and 100% homology to those of *Enterobacter xiangfangensis* and *Bacillus zhangzhouensis* respectively.

**Keywords:** Spent engine oil; Aerobic bacteria; Petrochemical industry; Gas Chromatography; 16s rRNA sequencing

## INTRODUCTION

Crude oil contamination and its products has become a huge environmental concern globally. At least 0.08 to 0.4% of the internationally produced oil has been estimated to be spilled in the marine ecosystem as pollutants (1,2). Spent engine oil is one that has undergone destructive changes in property when exposed to conditions such as; oxygen, combustion gases and high temperature (3). Engine oil that is disposed-off improperly contains potentially toxic substances which can seep into the water tables and cause ground water contamination (4). Spent engine oil has been reportedly implicated by (5,6) as a pollutant of concern, discharging large volumes into aquatic ecosystems via water runoff as a result of rainfall.

(7) stated that biological methods are the most sensitive indicator of changes in the environment, used to assess the negative impacts caused by environmental contaminants. Microorganisms are capable of degrading many complex molecules by adaptation of their enzymatic action (8,9). The aerobic bacteria are ubiquitous prokaryotic microorganisms that are found everywhere due to presence of oxygen ($O_2$), the availability of energy source and carbon as carbon dioxide ($CO_2$) present in the air**.** (10) reported that bacteria are the most dynamic agents in oil degradation, and they work as primary degraders of leaked oil in environment. (11), investigated an experiment on Biodegradation of Fresh and Used Engine oils by *Pseudomonas aeruginosa* strain LP5, findings showed a notable potential for use in breakdown of both fresh and used engine oils. Most researches carried out in the above stated reports, have been on soil microorganisms and marine microbes as biodegraders. The paucity of information regarding air bacteria as oil degraders therefore necessitated the present study to complement the available data in existence. The study, therefore, aims on the degradation of used engine oil using bacteria isolated from the air.

## METHODOLOGY

### Study Site

The study site chosen for this research is diesel spilled environment around the generator house, behind School of Science and Technology building, Yaba College of Technology, Yaba,

Lagos State. The sample site location with coordinates (6° 31'N and 3°22'E) was specified using the Global Positioning Application on a mobile phone.

**Sample Collection**

Spent engine oil was aseptically obtained from an auto mechanic shop in Shomolu area of Lagos. It was transferred into a sterile container and stored in a cool, dry place prior to the time of analysis. The spent engine oil sample was screened for its sterility following method of (12) by using a sterile cotton swab to streak neatly on a prepared sterile nutrient agar plate and incubated at $35^0$C for 24 hours. The absence of growth on the nutrient agar plate after 24 hours indicated that the sample was sterile and appropriate for the experiment.

**Laboratory study**

The bench work for this research was done in Microbiology laboratory, Department of Biological Science, Yaba College of Technology, Yaba, Lagos. The physicochemical and gas chromatography analysis of the spent engine oil was determined at University of Lagos Central laboratory, Akoka, Lagos. The molecular characterization and sequencing was carried out at the FOWN Biotechnology laboratory, Jibowu, Yaba, Lagos.



**Laboratory investigation**

**Culturing**

Seven grams (7g) Nutrient agar (Oxoid) was prepared following the manufacturer's instructions carefully and 2.5ml of the spent engine oil sample was aspirated using sterile syringe to enrich the media at temperature of about $55^0$C and vigorously, yet carefully stirred to obtain a more homogenized solution. At about 40-$45^0$C it was aseptically transferred into sterile Petri dishes (duplicate) and the lids were secured. The plates were allowed to solidify and were exposed for 30 minutes at the study site before the lids were secured and transferred into the incubator and incubated at $37^0$C for 24 hours to obtain mixed colonies of airborne bacteria. The colonies present were differentiated by morphology, colour, colony size, among other characteristics.

**Cultural/morphological characterization**

After about 24 hours, the differentiated bacterial colonies were isolated aseptically (using a sterile inoculating loop) from the mixed culture and streaked aseptically on new plates of nutrient agar and incubated at $37^0$C for 24 hours to obtain pure cultures. The morphological characterization of the bacterial isolates isolated from the mixed culture was carried out and were grouped among shape, size, margin, elevation, texture, pigmentation and appearance.

**Identification of bacteria isolates and screening**

The isolates were further Gram stained to ascertain their Gram reaction and grouping. The purified cultures of the bacterial isolates in the McCartney bottles were introduced, a loopful into test tubes containing 10ml of the sterilized Mineral Salt Broth and 0.1ml of spent engine oil and each were incubated in a shaker incubator alongside their control, containing minimal salt broth and 0.1ml of spent engine oil but without the isolate. All the test tubes were incubated with shaking at 150rpm and 35°C for 7 days. Samples to be extracted were taken into a separating funnel and 20ml of Dichloromethane was added to it. The process was repeated 3 times, and then the extract collected was treated with anhydrous sodium sulphate and silica gel. The extract was then concentrated by using Solute Phase Extractor (SPE) for sample treatment.



**Spent engine oil degrading capability of separate isolates**

The six isolates were individually cultured in 10 ml mineral salt broth (pH 7) for 7 days at $35^0$C in a shaker incubator at 150 rpm with 1% (v/v) (0.1ml) spent engine oil. The bacteria exhibited variable degrees of growth. The 7 days old cultures were taken with sterile inoculating loops and transferred into nutrient agar plates and incubated at $37^0$c for 24 hours under strict aseptic techniques. Only two of the six isolates showed the ability to degrade the diesel as evidenced by the growth on the nutrient agar plate after 24 hours incubation at $37^0$C. They were thereafter labeled C1o and S3.

**Gas Chromatography Analysis**

The extract was put in a vial bottle, then run in Gas Chromatography-Mass Spectrometry instrument (Agilent 7890A Gs system 5975C VLMSD; column length 30m Internal diameter 0.320 mm, initial temperature 40°C, maximum temperature 320°C). An aliquot of 1µl was

injected using a 5µl-volume Agilent total syringe. The major hydrocarbon compounds of the engine oil were established based on their mass spectrum and by comparison to those of analytical standards. The biodegradation loss of individual compounds was calculated as degradative loss % from the equation:

$$\frac{100 - (\text{conc.}) \text{ sample} \times 100}{(\text{conc.}) \text{ control}}$$

**DNA extraction**

Overnight cultures of pure culture of bacterial isolates on Tryptone Soy Broth (TSB) were used for genomic DNA extraction by using the Jena Bacteria DNA extraction kit according to the instructions of the manufacturer

**Multiplex PCR amplification of 16s rDNA gene, functional genes and sequencing**

A multiplex PCR reaction for the amplification of 16S rDNA and functional genes from genomic DNA was carried out using Eppendorf Vapo protect thermal cycler (Nexus Series). For PCR amplification, 20 µl reactions was used containing 4µl of 5X PCR Master Mix (Solis Biodyne 5X HOT FIREPol), 2.0 µl of template DNA, 0.2 µl of both forward and reverse primers (BIOMERS, Germany) and 13.6 µl of nuclease-free water (Thermo Fisher Scientific, Germany). PCR conditions with an initial denaturation step at $95^{o}$ C for 5 min followed by 35 amplification cycles at $95^{o}$C for 30 seconds; hybridization at $58^{o}$C for 1 min and elongation at $72^{0}$C for 30 Seconds. This was followed by a final extension step at 72°C for 10 min. The amplification product was separated on a 1.5% agarose gel and electrophoresis was carried out at 80V for 1 hour 30 minutes and DNA bands were visualized by ethidium bromide staining. 100bp DNA ladder was used as DNA molecular weight standard. The 16S rRNA gene purified amplicons were sequenced using the same set of primers used for the PCR at Epoch Life Science (USA) for Sanger sequencing. The corresponding sequences were identified using the online blast search at http://blast.ncbi.nlm.nih.gov/Blast.cgi

## Table 1: Primers and their sequences

| S/N | Genes | Primer Name | Sequence ($5^1 – 3^1$) |
|---|---|---|---|
| **1** | 16s RNA gene | 27F | AGAGTTTGATCCTGGCT CAG |
| | | 1492R | GGTTACCTTGTTACGACTT |
| **2** | Functional gene | 23CAT-F | CGACCTGATCTCCATGCCGA |
| | | 23CAT-R | TCAGGTCAGCACGGTCA |
| **3** | Functional gene | alkB-lF | AAYACNGCNCAYGARCTNGGNCAYAA |
| | | alkB-lR | GCRTGRTGRTCNGARTGNCGYTG |

## RESULTS

Six different bacterial colonies were isolated from the air to test for their spent engine oil degradation capability using nutrient broth. The ability to utilize spent engine oil as carbon source was ascertained by observing growth in mineral salt broth with merely spent engine oil. The six isolates were labeled as A1, A2, B1, B2, C1 and C2. These bacteria, upon purification were screened for their colonial and morphological appearance on the nutrient agar plate and the results are shown in the Table 2 below:

**Table 2: Colony morphology of eight isolates in Nutrient agar**

| Isolate designation | Shape | Margin | Elevation | Size | Texture | Pigmentation | Appearance |
|---|---|---|---|---|---|---|---|
| **A1** | Circular | Undulate | Umbonate | Large | Textured | White | Dull |
| **A2** | Circular | Entire | Raised | Small | Smooth | Yellow | Shinny |
| **B1** | Circular | Entire | Raised | Small | Smooth | Yellow | Shinny |
| **B2** | Circular | Undulate | Umbonate | Large | Textured | White | Dull |
| **C1** | Circular | Entire | Convex | Small | Smooth | White | Shinny |
| **C2** | Circular | Irregular | Umbonate | Large | Textured | White | Dull |

**Spent engine oil degrading ability of different isolates**

The result of the study, revealed only two of the six isolates showed the ability to degrade the diesel as indicated by the growth on the nutrient agar plate after 24 hours incubation at $37^0$C. They were thereafter labeled C1o and S3 (Table 4).

**Physico-chemical Parameters of Spent Engine oil**

The result of the physico-chemical parameters of the spent engine oil analysis (Table 3) showed the concentrations of pH to be 6.04, temperature to be 29$^o$C, sulphur to be 0.97 (mg/kg), and iron to be 26.92 (mg/kg) respectively. Other parameters determined were moisture content (13.92 %), density (0.94 g/cm$^3$), viscosity (24.00) and flash point (122.00).

**Table 3: Physico-chemical properties of spent engine oil used for contamination of the soil.**

| S/N | Parameters | Results |
|---|---|---|
| **1** | pH | 6.04 |
| **2** | Moisture % | 13.92 |
| **3** | Temperature $^0$C | 29.80 |
| **4** | Density (g/cm$^3$) | 0.94 |
| **5** | Viscosity | 24.00 |
| **6** | Flash point | 122.00 |
| **7** | Sulphur(mg/kg) | 0.97 |
| **8** | Iron (mg/kg) | 26.92 |



**Molecular analysis**

The bacteria isolates obtained in this study, showing spent engine oil degrading ability were 16s rRNA sequenced using specific primers as shown in Fig.1 below and Table 1 respectively.

**Multiplex PCR amplification of 16s RNA gene and functional genes**

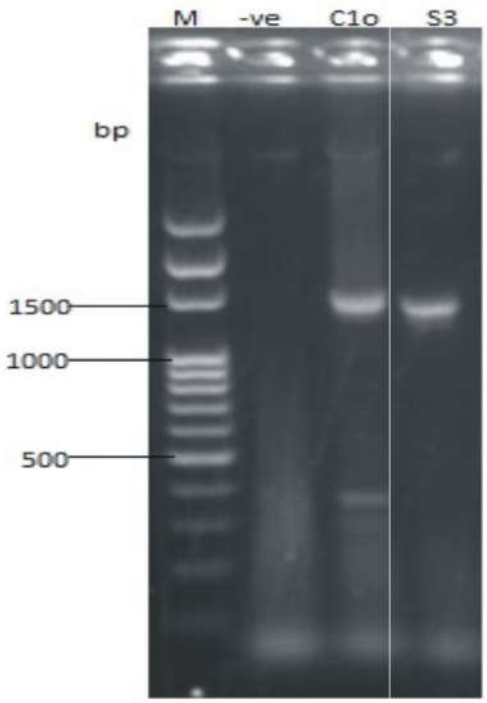
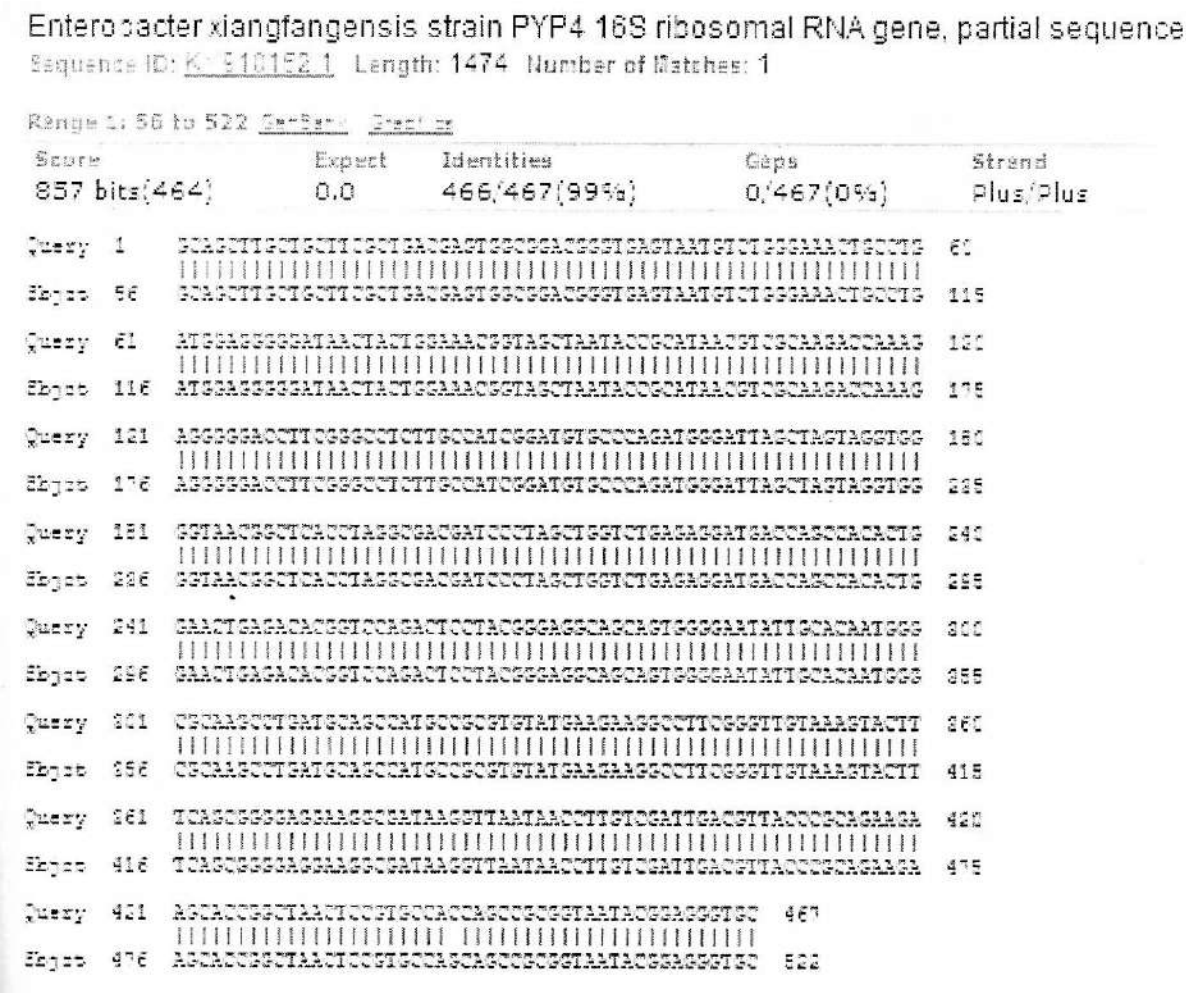



**Figure 1:** DNA bands visualized by ethidium bromide staining and100bp DNA ladder used as DNA molecular weight standard.

**Blast sequences and identification of spent engine oil degrading isolates**



Enterobacter xiangfangensis strain PYP4 16S ribosomal RNA gene, partial sequence

Sequence ID: KC810152.1 Length: 1474 Number of Matches: 1

Range 1: 56 to 522 GenBank Graphics

| Score | Expect | Identities | Gaps | Strand |
|---|---|---|---|---|
| 857 bits(464) | 0.0 | 466/467(99%) | 0/467(0%) | Plus/Plus |

```
Query  1    GCAGCTTGCTGCTTCGCTGACGAGTGGCGGACGGGTGAGTAATGTCTGGGAAACTGCCTG  60
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  56   GCAGCTTGCTGCTTCGCTGACGAGTGGCGGACGGGTGAGTAATGTCTGGGAAACTGCCTG  115

Query  61   ATGGAGGGGGATAACTACTGGAAACGGTAGCTAATACCGCATAACGTCGCAAGACCAAAG  120
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  116  ATGGAGGGGGATAACTACTGGAAACGGTAGCTAATACCGCATAACGTCGCAAGACCAAAG  175

Query  121  AGGGGGACCTTCGGGCCTCTTGCCATCGGATGTGCCCAGATGGGATTAGCTAGTAGGTGG  180
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  176  AGGGGGACCTTCGGGCCTCTTGCCATCGGATGTGCCCAGATGGGATTAGCTAGTAGGTGG  235

Query  181  GGTAACGGCTCACCTAGGCGACGATCCCTAGCTGGTCTGAGAGGATGACCAGCCACACTG  240
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  236  GGTAACGGCTCACCTAGGCGACGATCCCTAGCTGGTCTGAGAGGATGACCAGCCACACTG  295

Query  241  GAACTGAGACACGGTCCAGACTCCTACGGGAGGCAGCAGTGGGGAATATTGCACAATGGG  300
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  296  GAACTGAGACACGGTCCAGACTCCTACGGGAGGCAGCAGTGGGGAATATTGCACAATGGG  355

Query  301  CGCAAGCCTGATGCAGCCATGCCGCGTGTATGAAGAAGGCCTTCGGGTTGTAAAGTACTT  360
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  356  CGCAAGCCTGATGCAGCCATGCCGCGTGTATGAAGAAGGCCTTCGGGTTGTAAAGTACTT  415

Query  361  TCAGCGGGGAGGAAGGCGATAAGGTTAATAACCTTGTCGATTGACGTTACCCGCAGAAGA  420
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  416  TCAGCGGGGAGGAAGGCGATAAGGTTAATAACCTTGTCGATTGACGTTACCCGCAGAAGA  475

Query  421  AGCACCGGCTAACTCCGTGCCAGCAGCCGCGGTAATACGGAGGGTGC  467
            |||||||||||||||||||| ||||||||||||||||||||||||||
Sbjct  476  AGCACCGGCTAACTCCGTGCCAGCAGCCGCGGTAATACGGAGGGTGC  522
```

**Figure 2:** Blast sequence showing spent engine oil degrading isolate labeled as C1 to be *Enterobacter xiangfangensis* strain PYP4.

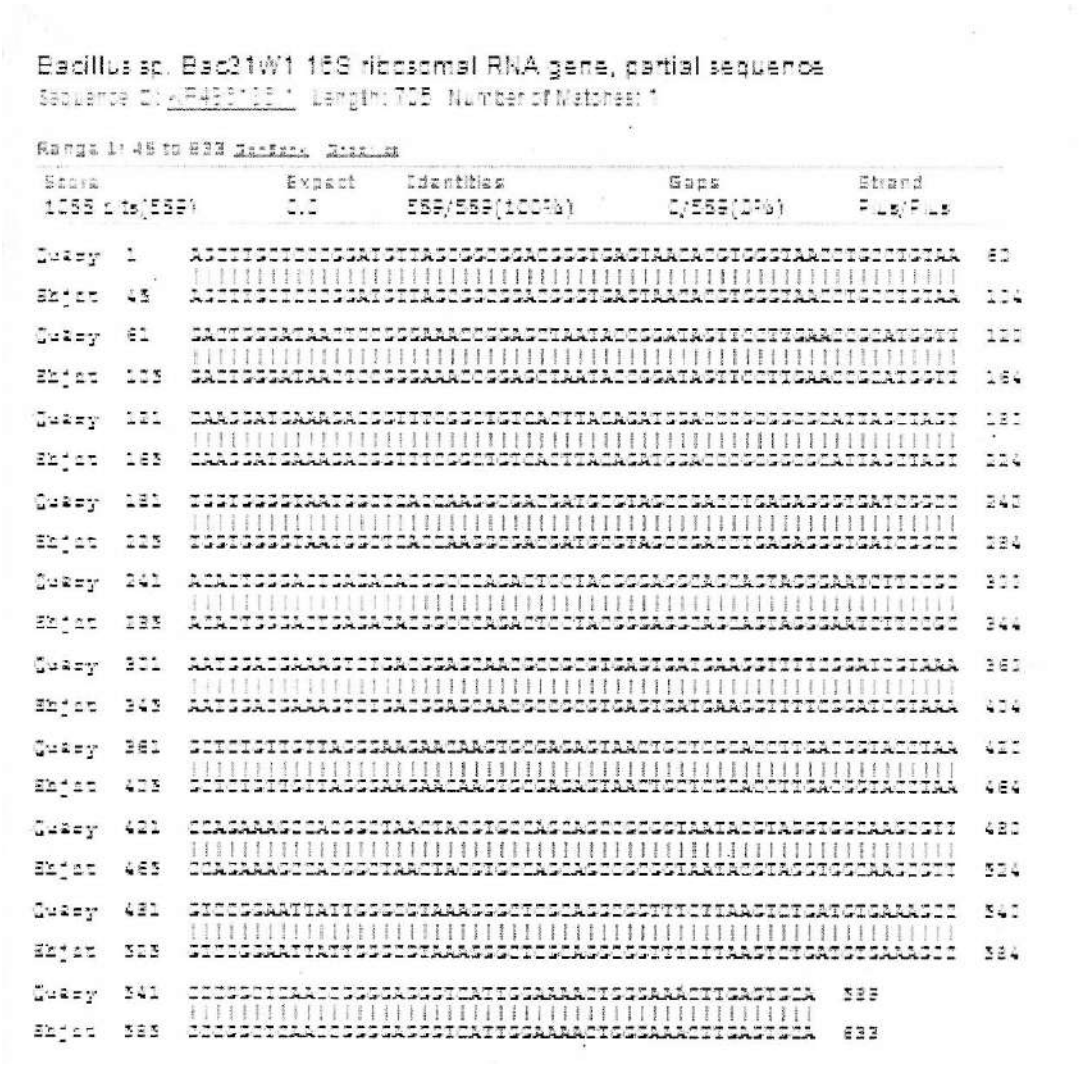



**Figure 3:** Blast sequence showing the engine oil degrading isolate labeled as S3 to be *Bacillus zhangzhouensis* strain 168

**Table4: Blast sequence data of the purified isolates**

| Sample | Closest relative | % Identity | Accession number |
|---|---|---|---|
| **C1o** | *Enterobacter xiangfangensis* | 99.79 | HF679035 |
| **S3** | *Bacillus zhangzhouensis* | 100 | JOTP01000061 |

## DISCUSSION

The bioremediation of petroleum pollutants is a slow process, that generally involves bio-stimulation and bio-augmentation, it often requires many months to degrade the bulk of the oil, it is relatively cheap and seemingly innocuous around the environment (13,14). The morphological characterization revealed *Enterobacter xiangfangensis* and *Bacillus zhangzhouensis* have the tendency to degrade the engine oil as the same as they exhibited in almost the same appearance both macroscopically and microscopically (Table 2). The result of this work, showed that *Enterobacter xiangfangensis* and *Bacillus zhangzhouensis* (Figs 2 and 3) isolated from the air have the ability to degrade engine oil, using the carbon as its source of energy. The bacteria isolates used could therefore be relevant in abatement of ecosystems that may be contaminated with hydrocarbons as the Minimal Salt Medium (MSM) was deprived of carbon, a source of energy. The blast sequence data result of the purified isolates (Table 4) comprising of the Samples, Closest relative, % Identity and the Accession number showed 99.79% and 100% homology to those of *Enterobacter xiangfangensis* and *Bacillus zhangzhouensis* respectively.

The Physico-chemical parameters of the spent engine oil determined in this work found the concentrations of pH to be 6.04, temperature to be 29$^{o}$C, sulphur to be 0.97 (mg/kg), and iron to be 26.92 (mg/kg) respectively (table 3). The value obtained for iron metal supports the finding of (15) to constitute a potential hazard to man, faunas and plants at large. It also support the experiment by (16) who carried out the crude oil spills test in the environment, effects and some innovative clean-up biotechnologies. (6) stated that temperature factor often determines the degradation rates of a pollutant together with the composition of the microbial community tasked with microbial degradation. In a similar report, (17,18) suggested that temperature strongly

affected biodegradation efficiency. pH similar to temperature also plays a role in the determination of the ability of microorganisms to grow or flourish in a particular environment. Most typically, microorganisms mainly bacteria grow optimally within a narrow pH range of between 6.7 and 7.5. Metabolic activities of microorganisms in a system can often be directly connected to the pH (acidity or alkalinity) of the system that is under investigation. Several research studies have revealed that microorganisms have got the capability of changing the pH of their environment through the production of metabolic waste products that can be either acidic or basic (19)**.** The study also, through the colony count of hydrocarbon degrading bacteria from the study site corroborates the report by (20) that all natural ecosystems contain hydrocarbon degrading bacteria that can metabolize some components of oil even if those ecosystems have never been exposed to oil or its products

The growth of microbial biotechnology and high-throughput sequencing technology, such as microfluidic techniques (21,22, 23), is advantageous for screening and identifying functional microorganisms from petroleum hydrocarbon-contaminated environments. Consequently, the molecular characterization of the results obtained in this work (Figs. 2 and 3) however, showed that, not only was the study able to identify the species of the organism isolated, it went as far as identifying the particular strains of the *Enterobacter xiangfangensis* and *Bacillus zhangzhouensis*. Therefore, the blast sequence results obtained in Figs. 2, 3 and table 4 showed the relevance of molecular biotechnology in degradation potential, as dependency on biochemical and morphological characterization of organisms tends to bring about a bias in result and conclusion of study of this nature. Furthermore, (24,25) demonstrated the biodegradation of used engine oil and diesel oil under shake flask conditions with an efficient bacterial consortium A2457: using bacterial strains of *Stenotrophomonas maltophilia*, *Bacilluscereus,* and *Bacillus pumilus*. They ascribed the biodegradation ability of these organisms to effectively use diesel oil and used engine oil as sole source of carbon and energy. The evaluation of the biodegrading potentials of microbes isolated from the air using spent engine oil as demonstrated in this study is therefore significant in bioremediation process and also forms the basis for the growth of efficient techniques which serves as tool for bioremediation of hydrocarbon polluted areas.

## CONCLUSION

Physico-chemical characteristics of the spent engine oil was determined in this study, the results obtained corroborated with the findings of other investigators, who reported that pH and temperature strongly affected biodegradation efficiency. Two bacteria isolates showing spent engine oil degrading ability were 16s rRNA sequenced using specific primers and were identified as *Bacillus zhangzhouensis* and *Enterobacter xiangfangensis*. The identified diesel degrading bacteria were then tested for their potential for engine oil biodegradation using gas chromatography. The 16s rRNA genes result showed 99.79% and 100% homology to those of *Enterobacter xiangfangensis* and *Bacillus zhangzhouensis* respectively.

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