



Biodegradation of Total Petroleum Hydrocarbon by Molecularly Identified Bacteria Isolated From an Oilfield Wastewater in Nigeria

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Abstract

Biodegradation of petroleum hydrocarbon is a complex process that depends on the nature and on the amount of the hydrocarbon present. Many microbial organisms have been shown to possess the capacity to biodegrade various components of hydrocarbon. Hence this study was aimed at assessing the potential of bacterial species isolated from oilfield wastewater to biodegrade total petroleum hydrocarbon in crude oil. Oilfield wastewater was collected from an onshore oil production platform. Standard procedures were observed during collection and microbiological analysis of wastewater samples. Bacteria isolated were identified using conventional and molecular methods. The biodegradation set up was done using six conical flasks containing basal medium of mineral salt broth and crude oil as the source of energy for growth. Bacteria isolated were identified as *Acinetobacter* species, *Enterobacter hormaechei*, *Myroides odoratimimus* and *Lysinibacillus* species. Flasks of experimental set ups were inoculated with 1ml of individual isolate and mixed culture except the control. The biodegradation of TPH were periodically monitored for 35 days using Gas Chromatography (GC). Total viable counts (CFU/ML) obtained during the experiment ranged from $0-1.8 \times 10^5$, $1.65 \times 10^5 - 3.2 \times 10^6$, $1.90 \times 10^6 - 1.28 \times 10^7$, $1.52 \times 10^6 - 8.9 \times 10^6$, $1.13 \times 10^6 - 1.48 \times 10^7$, $3.4 \times 10^6 - 2.19 \times 10^7$ in control, *Acinetobacter* species, *Enterobacter hormaechei*, *Myroides odoratimimus*, *Lysinibacillus* species and the mixed culture treatment options respectively. The initial concentration of TPH at day 1 was 3241.47mg/l. At the end of the experiment (day 35), control, *Acinetobacter* species, *Enterobacter hormaechei*, *Myroides odoratimimus*, *Lysinibacillus* species and the mixed culture treatment options recorded final concentration of 2823.33, 363.72, 383.44, 284.55, 472.70 and 212.21mg/l respectively. Highest percentage removal of 93.5% was observed in the mixed culture while the least of 12.9% was observed in the control; individual species showed significant reduction in TPH with different capability. Results showed that bacteria isolated from oilfield wastewater have the ability to degrade total petroleum hydrocarbon (TPH) and can be used in the clean-up of crude oil contaminated area.

Keywords: Biodegradation; Oilfield Wastewater; Total Petroleum Hydrocarbon; Bacteria

Abbreviations: TPH: Total Petroleum Hydrocarbon; TVBC: Total Viable Bacteria Counts.

Introduction

Biodegradation of petroleum hydrocarbon is a complex process that depends on the nature and on the amount of the hydrocarbon present. Petroleum hydrocarbon can be

divided into four classes: saturated, aromatics, asphaltenes (phenols, fatty acids, ketones, esters, and porphyrins), and the resins (pyridines, quinolines, carbazoles, sulfoxides, and amides) [1]. The potential of microorganisms, pointed out in literature as agents of degradation of several compounds indicates biological treatments as the most promising alternative to reduce the environmental impact caused by hydrocarbons. It is known that the main microorganisms

consuming petroleum hydrocarbon are bacteria and fungi [2,3]. Oilfield wastewater or produced water consists of formation and injection water that contains production chemicals generated during the production of crude oil and gas from onshore and offshore wells [4]; they also contain diverse populations of bacteria and fungi [4,5]. This study therefore investigated the potentials of bacteria isolated from oilfield wastewater to biodegrade total petroleum hydrocarbon in crude oil.

Materials and Methods

Collection of Oilfield Wastewater Samples

Oilfield wastewater samples were collected from an onshore oil production platforms located in Ogbia Local government Area of Bayelsa State, Nigeria. The oilfield wastewater samples were collected using 250ml sterile sampling bottles. Samples were collected according to the method described in Standard Methods for Water and Wastewater Analysis [6]. According to the above procedures, 250ml of the sterile sample bottles were filled from gentle stream of the wastewater. The sample bottles were appropriately labeled and stored in an ice packed cooler. The stored samples were immediately transported to the laboratory within 24 hours for processing and analysis.

Preparation of Oilfield Wastewater Sample Inoculum

One milliliter (1ml) of oilfield wastewater samples were serially diluted using one milliliter pipette by aseptically introducing 1ml of the oilfield wastewater into 9ml of sterile normal saline in a test tube to give an initial dilution of 1:10ml (10^{-1} dilution). Subsequent dilutions were done up to 10^{-3} dilution [7].

Isolation of bacteria from Wastewater Samples

This involved isolation of heterotrophic bacteria using nutrient agar by the spread plate technique as described by Prescott, et al. [7]. Aliquots (0.1ml) of serially diluted samples of 10^{-2} dilution were spread plated onto dried sterile nutrient agar plates in duplicates. The plates were incubated at 37°C for 24 hours. Representative colonies were selected and sub-cultured to purify them into pure isolates for characterization. The purified colonies represented the bacteria isolated from the oilfield wastewater samples.

Molecular Identification of Bacterial Isolates

Purified bacterial isolates were subjected to preliminary identification techniques using their colonial and morphological characteristics. Further identification

employing the 16S rRNA gene was carried out. The 16S rRNA regions of the rRNA gene of the isolates were amplified after extraction and quantification of the DNA using the 27F: 5'-AGAGTTTGATCMTGGCTCAG-3' and 1492R: 5'-CGGTTACCTTGTTACGACTT-3' primers on an ABI 9700 Applied Bio systems thermal cycler at a final volume of 40 microlitres for 35 cycles. The PCR mix included: the X2 Dream taq Master mix supplied by Inqaba, South Africa (taq polymerase, DNTPs, MgCl), the primers at a concentration of 0.5 μ M and the extracted DNA as template. The PCR conditions were as follows: Initial denaturation, 95°C for 5 minutes; denaturation at 95°C for 30 seconds; annealing at 52°C for 30 seconds; extension at 72°C for 30 seconds for 35 cycles and final extension at 72°C for 5 minutes. The product was resolved on a 1% agarose gel at 130V for 30 minutes and visualized on a blue light trans illuminator.

The sequences of the isolates were edited using the bioinformatics algorithm Trace edit; similar sequences were downloaded from the National Center for Biotechnology Information (NCBI) data base using BLASTN and was further aligned using Clustal X. The evolutionary history was inferred using the Neighbour-Joining method in MEGA 6.0 [8]. The bootstrap consensus tree inferred from 500 replicates [9] was taken to represent the evolutionary history of the taxa analysed. The evolutionary distances were computed using the Jukes-Cantor method [10].

Biodegradation Experiment

The experiment was designed to determine the potential of some bacteria isolated from the oilfield wastewater to degrade total petroleum hydrocarbon (TPH) in crude oil.

Preparation of Bacterial Inoculum

Nutrient broth was prepared according to manufacturer's specifications after which the bacterial species were inoculated into it and incubated at 37°C for 24 hours to allow bacterial growth. Serial dilutions were made for each bacterial culture to determine the number of cells/millimeter aliquot. Aliquot 0.1ml of appropriate dilution was spread plated out and used in the set-up designated as A, B, C, D, E and F treatment options.

Composition of Biodegradation Set up

Six conical flasks were used for the degradation experiment and were properly labeled. Each flask contained 1000ml of mineral salt broth, 1ml of the each bacterial species, and 5ml of crude oil sterilized by filtration method using Whatman filter paper previously sterilized in the autoclave at 121°C for 15 minutes. Mixed cultures of the 4 bacterial species were used for the last set up (F). The

experimental set ups were presented in Table 1.

Set up	Content
A	Control (Crude oil + Mineral Salt Medium)
B	<i>Acinetobacter</i> species (1ml of 1.8×10^6) + crude oil + Mineral salt medium
C	<i>Enterobacter hormaechei</i> (1ml of 2.6×10^6) + crude oil + Mineral salt medium
D	<i>Myroides odoratimimus</i> (1ml of 2.5×10^6) + crude oil + Mineral salt medium
E	<i>Lysinibacillus</i> species (1ml of 2.1×10^6) + crude oil + Mineral salt medium
F	<i>Acinetobacter</i> species + <i>Enterobacter hormaechei</i> + <i>Myroides odoratimimus</i> + <i>Lysinibacillus</i> species (0.25ml each) + crude oil + Mineral salt medium

Table 1: Experimental Set Ups.

Bioremediation Procedure

Each of the bioremediation experimental set-ups was incubated at room temperature. The set-ups were agitated manually three times daily to aid aeration and even distribution of crude oil. Content of the experimental set ups was analyzed for total viable bacteria counts (TVBC) and total petroleum hydrocarbon (TPH) at weekly intervals for 35 days. TVBCs for each set-up were enumerated using spread

Day(s)	Control	<i>Acinetobacter</i> species	<i>Enterobacter hormaechei</i>	<i>Myroides odoratimimus</i>	<i>Lysinibacillus</i> species	Mixed culture of 4 species
1	0	1.65×10^5	2.69×10^6	1.90×10^6	2.75×10^6	3.44×10^6
7	0	2.35×10^6	1.90×10^6	1.52×10^6	1.89×10^6	1.42×10^7
21	1.0×10^5	3.22×10^6	2.66×10^6	2.05×10^6	1.13×10^6	1.50×10^7
35	1.8×10^5	1.52×10^6	1.28×10^7	8.88×10^6	1.48×10^7	2.19×10^7

Table 2: Total Viable Bacterial Counts (CFU/ML) of the Experimental Set-up.

Molecularly Identified Bacterial Isolates

The molecularly identified bacterial isolates were shown to be *Acinetobacter* species, *Enterobacter hormaechei*, *Myroides ordaratimimus* and *Lysinibacillus* species. These bacterial species were used for the biodegradation of total petroleum hydrocarbon.

Degradation of Total Petroleum Hydrocarbon

Table 3 shows the initial concentration of the total petroleum hydrocarbon and the degradation by individual and mixed culture of bacteria. Concentration of total petroleum hydrocarbon on the initial day was 3241.47081mg/l. The A

plate method on nutrient agar and incubated at 37°C for 24 hours. After which, colonies that developed on the nutrient agar plates were counted and recorded as numbers of total viable bacteria obtained. TPH values were determined using Gas Chromatography method.

Results

Population Changes of Viable Bacteria During Remediation of TPH

The total viable bacterial counts are shown in Table 2. The A set up (control) which contained only crude oil recorded zero total viable bacterial counts on day 1 and day 7 while day 21 and day 35 had total viable bacterial counts of 1.0×10^5 CFU/ml and 1.8×10^5 CFU/ml respectively. The B set up that contained *Acinetobacter* species recorded 1.65×10^5 CFU/ml on day 1 with progressive increase in the total viable bacterial counts towards the end of experiment. Experimental set ups C and D containing *Enterobacter hormaechei* and *Myroides ordaratimimus* obtained counts of 2.69×10^6 CFU/ml and 1.90×10^6 CFU/ml respectively on day 1; on day 7 the total viable bacterial counts decreased in both set ups and increased again on day 21 and day 35. *Lysinibacillus* species (E set up) counts on day 1 were 2.75×10^6 CFU/ml, which decreased on day 7 and day 21, and increased again on day 35. The mixed culture set up (F) increased progressively from the beginning to the end of experiment.

(control) option had the highest amount of 2823.32534mg/l remaining on day 35 with removal of 12.9%. The least amount of 212.21130mg/l remaining was observed in the F treatment option that had the mixed culture with percentage removal of 93.5%. This was followed by D set up with *Myroides odoratimimus* which had remaining TPH of 284.54994mg/l and 91.2% removal. *Acinetobacter* species (B set up) had remaining TPH of 363.71647mg/l with 88.8% removal. *Enterobacter hormaechei* (C set up) had remaining TPH of 383.44001mg/l with 88.2% removal. *Lysinibacillus* species (E set up) recorded 472.69664mg/l amount of TPH remaining with 85.4% removal. There was reduction in peak and its number in the various treatment options on day 35 as shown in Figure 1-6.

Content	Initial (mg/l)	Final (mg/l)	% Removal
Control (Crude oil + Mineral Salt Medium)	3241.47081	2823.32534	12.9
<i>Acinetobacter</i> species + crude oil + Mineral salt medium	3241.47081	363.71647	88.8
<i>Enterobacter hormaechei</i> + crude oil + Mineral salt medium	3241.47081	383.44001	88.2
<i>Myroides odoratimimus</i> + crude oil + Mineral salt medium	3241.47081	284.54994	91.2
<i>Lysinibacillus</i> species + crude oil + Mineral salt medium	3241.47081	472.69664	85.4
<i>Acinetobacter</i> species + <i>Enterobacter hormaechei</i> + <i>Myroides odoratimimus</i> + <i>Lysinibacillus</i> species + crude oil + Mineral salt medium	3241.47081	212.2113	93.5

Table 3: Bioremediation of Total Petroleum Hydrocarbon.

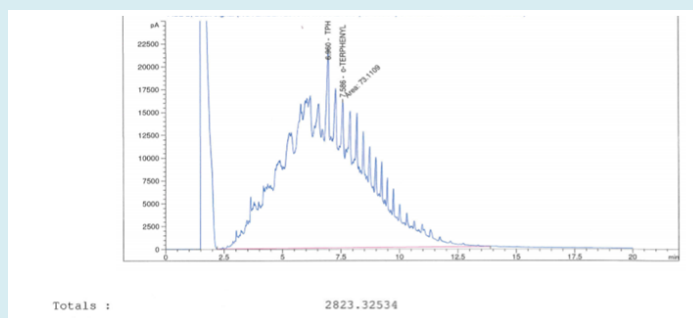


Figure 1: GC Profile Showing the Remediation of Total Petroleum Hydrocarbon (TPH) In the Control on Day 35.

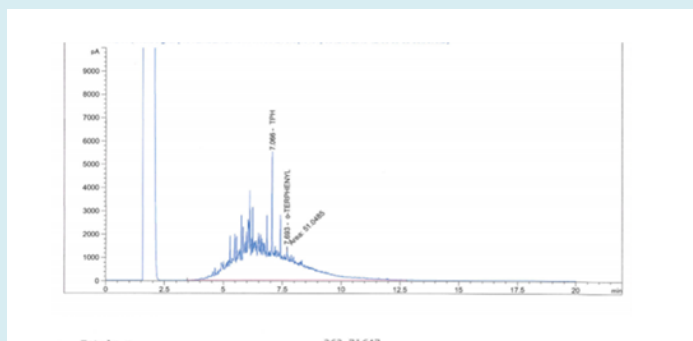


Figure 2: GC Profile Showing the Remediation of Total Petroleum Hydrocarbon (TPH) By *Acinetobacter* Species on Day 35

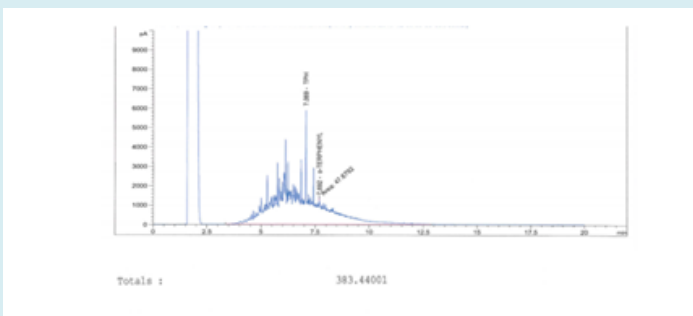


Figure 3: GC Profile Showing the Remediation of Total Petroleum Hydrocarbon (TPH) By *Enterobacter Hormaechei* on Day 35.

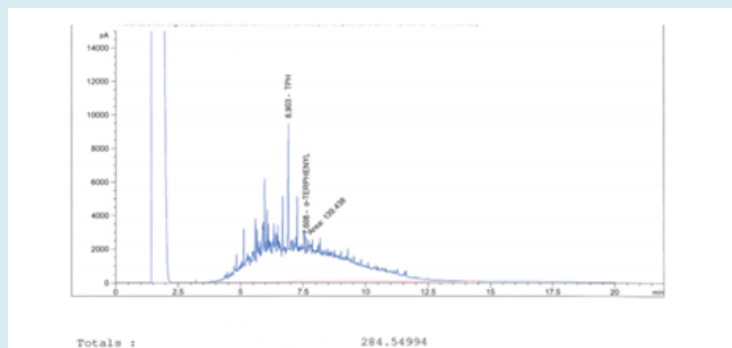


Figure 4: GC profile showing the remediation of total petroleum hydrocarbon (TPH) by *Myroides odoratimimus* on day 35.

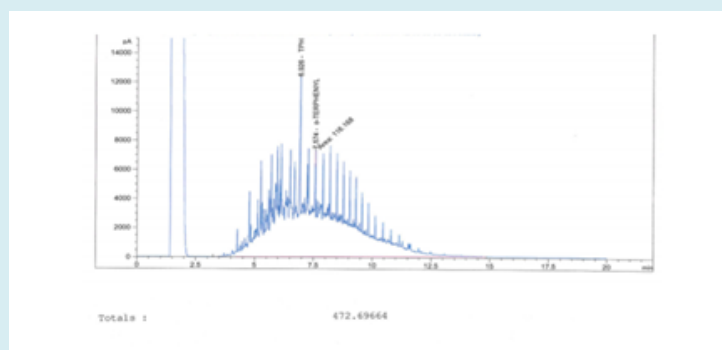


Figure 5: GC Profile Showing the Remediation of Total Petroleum Hydrocarbon (TPH) By *Lysinibacillus* Species on Day 35.

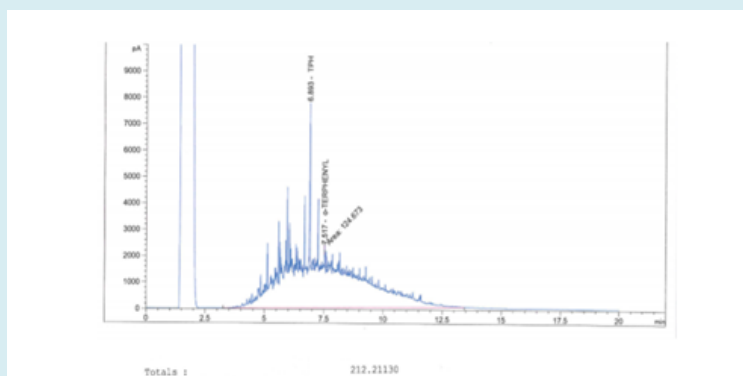


Figure 6: GC Profile Showing the Remediation of Total Petroleum Hydrocarbon (TPH) By *Acinetobacter* Species + *Enterobacter Hormaechei* + *Myroides Odoratimimus* + *Lysinibacillus* Species on Day 35.

Discussion

Counts of total viable bacteria fluctuated throughout the period of the experiment in course of remediation of total petroleum hydrocarbon. The control samples recorded zero count on day 1 and day 7, but had viable counts on day 21 and day 35 respectively. Total viable bacterial counts for *Acinetobacter* species and the mixed culture increased progressively from day 1 to day 35. This could

be attributed to the gradual utilization of hydrocarbon by the microbes. *Enterobacter hormaechei* and *Myroides odoratimimus* total viable bacterial counts reduced during day 1 and day 7 and increased thereafter on day 21 and day 35. *Lysinibacillus* species reduced in counts from day 1 to day 21 but increased on day 35. The decrease in counts after day 1 for *Enterobacter hormaechei* and decrease on day 7 and day 21 for *Lysinibacillus* species counts could be attributed to toxic effects of the hydrocarbon contents on

the organisms. It is well known that hydrocarbon degrading bacteria occur in a wide variety of environments particularly in areas with presence of petroleum production activity. The use of native or indigenous microbiota for bioremediation is of great interest since it is more useful and beneficial than commercial inoculants [11].

The bacteria isolated from oilfield wastewater samples were identified as *Acinetobacter* species, *Enterobacter hormaechei*, *Myroides odoratimimus* and *Lysinibacillus* species. These microorganisms were used for the biodegradation of total petroleum hydrocarbon in this study. The ability of the organisms to degrade total petroleum hydrocarbon as single and mixed culture was examined, and varied with the species. Degradation of total petroleum hydrocarbon by *Acinetobacter* species showed percentage reduction of 88.8% at the end of the experiment. *Enterobacter hormaechei* had reduction of 88.2%, *Myroides odoratimimus* 91.2% and *Lysinibacillus* species 85.4% at the end of the experiment. The mixed culture had reduction of 93.5% at the end of the experiment. The highest percentage reduction of total petroleum hydrocarbon was in the mixed culture which was followed by the treatment with *Myroides odoratimimus*. The least reduction in TPH was observed in the control which recorded reduction of 12.9%. This represents natural attenuation of crude oil which has been shown to be generally slow. The individual isolates demonstrated high level reduction of total petroleum hydrocarbon as shown in the percentage reduction obtained and reduction in the graphs of Gas Chromatography. *Acinetobacter* species have been found to play vital role in petroleum hydrocarbon degradation [12]. *Enterobacter hormaechei* is a coilform bacteria and its ability to degrade total petroleum hydrocarbon have also been reported by several researchers [13,14]. Divya, et al. [15] reported the degradation of diesel hydrocarbon by *Myroides odoratimimus*. Ashikodi and Abu, et al. [16] also reported the degradative potential of *Lysinibacillus* species. Results of degradation of TPH clearly showed that mixed culture of bacteria had the highest percentage removal of total petroleum and was proved to be most efficient. This could be attributed to the collective effort of the individual bacteria that formed the mixed culture. The higher degradation rate and microbial activity observed in the mixed culture may be related to shift in importance of one metabolic pathway over another possibly as a result of microbial synergism. This agrees with Friello, et al. [17] who reported that a wide variety of metabolic and physiological factors are required for the degradation of different compounds in diesel oil. Similarly Adebuseye, et al. [18] demonstrated that mixed culture of microbial community is required to complete biodegradation of crude oil pollutants because the hydrocarbon mixtures differs markedly in volatility, solubility and susceptibility to degradation and the necessary enzymes needed cannot be found in a single organisms. This coincides with Al-Saleh,

et al. [19] who reported that individual microorganisms metabolize only a limited range of hydrocarbon substrates and crude oil is made up of mixture of compounds, such that biodegradation of it requires mixture of different bacterial groups or consortia functioning to degrade a wide range of the hydrocarbon components. Several reports [20,3] have confirmed microbial consortia as better degraders than individual pure isolates. In a culture, some species utilize intermediates of degradation of the original hydrocarbon produced by other members of the culture leading to a complete degradation of the oil [21]. Thus, a mixed culture is a better inoculum for bioremediation. Degradation of petroleum involves progressive or sequential reactions, in which certain organisms may carry out the initial attack on petroleum constituents. This produces intermediate compounds that are subsequently utilized by a different group of organisms in the process that results in further degradation [22]. Generally speaking the individual species in this study proved effective in degradation of TPH as all of them had between 85% and 91% removal of total petroleum hydrocarbon.

Conclusion and Recommendation

The potentials of some bacteria isolated from oilfield wastewater in this study to remediate total petroleum hydrocarbon were demonstrated and the organisms were found to have such ability. The microbial species obtained effectively utilized the total petroleum hydrocarbon during the degradation experiment. The study showed that the individual species possess high capability to degrade total petroleum hydrocarbon, however mixed culture of the individual isolates was more efficient in the remediation experiment. These bacteria can be harnessed and used for cleanup of crude oil contaminated environment.

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