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Isolation and Molecular Identification of Hydrocarbon Degrading Bacteria from Contaminated Soil in Mechanic Village Dutse, Jigawa State

Ringim Mahi Saidu[*], Gumel Ahmad Mohammed, Shiaka Gimba Peter

Department of Microbiology and Biotechnology, Faculty of science, Federal University Dutse, Dutse City, Jigawa State, Nigeria

Email address:
Mahiringim007@gmail.com (R. M. Saidu), dr.gumel@fud.edu.ng (G. A. Mohammed), gimpet1971@gmail.com (S. G. Peter)
[*]Corresponding author

Abstract: The spillage of oil and other petroleum products have been a major source of environmental pollution globally. Rampant use of chemical remediation methods were shown to be noxious with detrimental consequences. Exploration of microbiodata for probable candidate to be utilized for degradation of oil spillage or contamination is of great interest. In this study, soil samples from 10 different mechanic workshops in mechanic village Dutse were collected as well as control sample from unpolluted hydrocarbon free soil at same site, bacterial species were isolated, identified using biochemical and molecular 16S rRNA gene sequencing and post sequencing analyses. Species enumerated were *Bacillus cereaus, Bacillus subtilis, Bacillus licheniformis* and *Paenibacillus alvei*, The sequences were compared to closest relative species in the GenBank data base of National Centre for Biotechnology Information. However, the substrate used was engine oil and all the isolates were found to utilized the test substrate. Biodegradation studies have been carried out through Gas Chromatography Mass Spectrometry (GC-MS). Of all the isolates, *Bacillus subtilis* might be the most hydrocarbon degrading bacterial species due to high average relative abundance concentration of degraded hydrocarbon compound.

Keywords: Bacteria, Bioremediation, Hydrocarbon, Oil Spill, Petroleum, Pollution, Polymerase Chain Reaction (PCR)

1. Introduction

The growth of petroleum industry in Nigeria and the demand of petroleum products have made oil pollution a serious environmental problem. In mechanic workshops, there are accidental or deliberate releases or discharge of petrol, diesel, solvents, grease and lubricants on soil.

Contamination of the environment may result from seepage from natural deposits, transportation and storage of petroleum products or from accidental events [3]. Whatever the origin of the contamination, there is possibility that some may enter the soil environment and this may present a direct risk of ground water contamination, lakes or water-course that serve as stable water supply for domestic and industrial use. Although a significant proportion of the compounds in crude oil are relatively harmless, a number, especially the aromatic hydrocarbons are toxic or mutagenic and require remedial action

to ameliorate environmental damage following a spill. Certain diseases namely cancer, bone marrow damage and kidney and liver diseases have been associated with the high concentration of petroleum spills compounds [17].

Along this line, natural ecosystems are contaminated with petroleum hydrocarbons, and microbial species are capable of degrading the spilled hydrocarbons [25]. Thus one of the best approaches to restoring contaminated soils is to make use of microorganisms capable of degrading those toxic compounds a process known as bioremediation [9]. Hydrocarbon degrading bacteria and fungi are mainly responsible for the mineralization of oil pollutants and are distributed in diverse ecosystems [13].

Mechanical and chemical methods for remediation of hydrocarbon-polluted environments are often expensive, technologically complex and lack public acceptance [24]. Thus bioremediation remains the method of choice for effective

removal of hydrocarbon pollutant in the environment [16].

The speed and efficiency of bioremediation of a soil contaminated with petroleum and petroleum products depends on the number of hydrocarbon degrading microorganisms in the soil. The most important factors for population growth are temperature, oxygen, pH, hydrocarbon class and their effective concentration. Moreover, the degree and rate of biodegradation are influenced by the type of soil in which the process occurs [10].

Automobile contaminated sites contain a variety of aliphatic, aromatic and polycyclic aromatic hydrocarbons (PAHs). Among them, polycyclic aromatic hydrocarbons pose more threat to environment and human health [11]. It has been reported that biological degradation appears to be the main process responsible for the removal of polycyclic aromatic hydrocarbons in soil [20].

Several bacteria species were known to feed exclusively on hydrocarbons as such generate their energy on hydrocarbons [26]. *Acinetobacter species* are found to be capable of utilizing n-alkanes of chain length C_{10}-C_{40} as a sole source of carbon [22]. Bacterial genera namely, Gordonia, Mycobacterium, Brevibacterium, Aeromicrobium, Dietzia, Burkholderia and Pseudomonas isolated from petroleum contaminated soil proved to be the potential organisms for hydrocarbon degradation [6].

In this study, oil contaminated soil samples from mechanic sites in Dutse metropolis were used to isolate petroleum hydrocarbon degrading bacteria. The study is aimed at providing an insight to the utilization of microbial diversity in solving one of the Nigeria's eminent pollution problem.

2. Materials and Methods

2.1. Study Area

Mechanic village is located in metropolitan Dutse Local Government Area of Jigawa State, Nigeria. It is located in the eastern part of the city. The geographical location of the state is between latitudes 11.00°N to 13.00°N and longitudes 8.00°E to 10.15°E (Dutse Capital Development Authority, 2012).
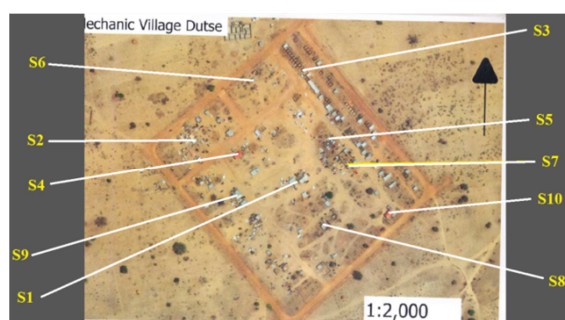
Source: Dutse Capital Development Authority (DCDA).

Figure 1. Satellite pictogram of Mechanic village. Scale: 1:2,000cm (sampling sites S1 to S10).

2.2. Sample Collection

Soil samples were collected from ten different mechanic garages situated at different locations within the Mechanic village (Figure 1). Soil sample which serve as control was also collected from unpolluted and hydrocarbon free soil at same site. Soil samples were collected by the use of sterile dark polythene bag and sterile spatula at the depth of 10cm [12] and transported to Microbiology Laboratory, Federal University Dutse for further processing.

2.3. Determination of Physicochemical Parameters of the Soil

Physicochemical analyses were performed in the Microbiology Laboratory, Federal University Dutse. Soil pH was determined using digital pH meter. Temperature of the soil was measured at the point of sample collection using mercury in glass thermometer. Organic matter/organic carbon content of the soil was estimated using method deployed by Ritesh [18]. The moisture content of the soil was estimated using the procedure described by NCTEL [15].

2.4. Isolation of Hydrocarbon Degrading Bacteria

Hydrocarbon degrading bacteria were isolated from oil contaminated soil. Media used was Trypicase Soy Agar (TSA). Pour plate inoculation method was carried out, before pouring the medium peteridishes contained 0.05g drop of petroleum supplement (engine oil), with 1ml from sample 10^{-1} to 10^{-8} range of serial dilutions, which consist of mineral salts composed of 2.0g of Na_2HPO_4. $2H_2O$, 0.8g of KH_2PO_4, 0.2g of $MgSO_4$ and 1.8g of $(NH_4)_2SO_4$ in 1L of distilled water [21]. Four prepared dishes for each sample (making 44 dishes including control sample) were incubated at 37°C for 48 hrs after which colonies were counted and expressed in colony-forming unit (cfu/g of soil) [4]. Isolates were identified on basis of colony morphology, gram staining and biochemical characteristics after incubation [8].

2.5. Molecular Identification of Hydrocarbon Degrading Bacteria

DNA extraction and PCR amplification of 16S rRNA were carried out at DNA LABS Nigeria Ltd, Kaduna. The total DNA of five selected isolates were extracted using DNA extraction kit. The extracted DNA was stored at -20°C before subjected to PCR.

Extracted DNA was amplified using universal forward and reverse primers (Ribos 1 and Ribos 2). Each PCR mixture contained 1μl of each primer. PCR ramping amplification temperatures were as follows: Pre-Denaturation at 95°C for 5 minutes, Denaturation at 94°C for 1 minute, annealing at 52°C for 1 minute. Extension was observed at 72°C for 1 minute. Final Extension at 72°C for 7 minutes. The PCR amplification process was observed at 25 cycles. After the completion of the PCR, the DNA amplicons were separated by electrophoresis using 2% agarose gel [8].

2.6. Sequencing of 16S rRNA and Phylogenetic Analysis

The purified PCR product was sequenced using Dye

terminator sequencing quick start kit. Sequences were then compared to those in Gen Bank database of the National Center for Biotechnology Information (NCBI) using website for BLAST (www.ncbi.nlm.nih.go). The sequences were aligned and the phylogenetic tree was constructed [8].

2.7. Biodegradation Studies

The biodegradation activities of each isolate was obtained using Gas chromatography tandem mass spectrometry (GC-MS) carried out at Chemistry Laboratory, ABU Zaria. The GC-MS method deployed is as follows: 1µl of the methanolyzed sample mixture consisted of colonies of the test organisms and gel agar around petroleum supplement (prepared at Microbiology/Biotechnology Lab, FUD) was injected into the GC-MS at a split ratio of 1:10 and Helium gas was used as a carrier gas at a flow rate of 48 ml/min and 0.40 bar. The GC injector temperature was set at 120°C, while the oven and column ramping temperature was set as

follows: 45°C hold for 2min, rise to 120°C at 5°C /min and hold for 3min, rise to 150°C at 10ºC/min hold for 2min, rise to 200°C at 20°C /min and hold for 5min. This interface temperature was set at 200ºC. Mass Spectra was collected at 1250 scan rate using ion source temperature of 280°C and 70eV. National Institute of Standard and Testing, USA data base was used for similarity parsing to established the identity of the degradation product [19].

3. Results and Discussion

3.1. Physicochemical Parameters of the Contaminated and Control Soil Samples

Physicochemical parameters were the most dominant factor of bacterial community distribution and abundance. Soil sampling properties are presented in Table 1.

Table 1. Mean Physicochemical characteristics of the contaminated and uncontaminated soil samples.

| Parameters | Contaminate Soil Sample | | | | | | | | | | Control Soil Sample |
	S1	S2	S3	S4	S5	S6	S7	S8	S9	S10	CS
Temperature (°C)	41	40	35	36	39	42	38	45	44	45	42
pH	7.5	7.6	7.9	8.1	8.2	7.2	7.9	6.9	7.3	6.1	5.5
Moisture (%)	3.1	2.8	3.7	0.8	3.1	2	4.8	3.7	5.1	2	8.6
Organic Matter (%)	7.3	7.4	7.5	8.6	8	8.6	7.3	8.9	8.7	6.7	1.3

In this research work the temperature of the obtained contaminated soil samples are in range of 35°C to 45°C, while control soil sample had 42°C. Previous study reported similar temperature values (10°C – 40°C) required for effective bioremediation [12].

The pH values ranged from 6.9 to 8.2 for contaminated soil sample while control soil sample had 5.5. It has been reported that the pH value of soil contaminated with petroleum products lie within the limit of pH 6 to 8 as most appropriate range of bioremediation [23, 24]. Our findings strongly agreed with that of Less and Senior, (1995), that pH value required for effective bioremediation is 5.5 to 8.5.

The moisture contents of the contamination site ranged from 0.8% to 5.1%, which is lower than the value obtained from uncontaminated site 8.6%. The low moisture contents observed in the contaminated site is attributed to the presence of oil in the soil, which result in the blocking of the pores; hence, the low water retention [14].

The organic matter content ranged from 6.7 to 8.9% for contaminated soil sample while 1.3% for control sample. This showed that isolates are capable of utilizing the petroleum products found in the soil environments, as their source of carbon and hydrogen. Bacteria utilized the carbon obtains from used motor oil for growth in the soil [1]. In a similar research work carried out revealed that, the organic matter content in the automobile contaminated site is higher than that of uncontaminated site, this indicated that bacteria utilized the carbon obtain from used motor oil for growth in the soil [5]. This also confirms the report that certain bacteria do utilized petroleum hydrocarbons [2].

Furthermore, additional research revealed that the total hydrocarbon content of the soil sample is greater than the intervention value cited by Dutch standard (40mg PAH/kg soil and 0.1 mg volatile hydrocarbon /kg soil) [8]. So, the soil sample are highly polluted by hydrocarbon. Comparative study of oil biodegradation is also defending on the type of soil which shows that, the microorganism activity is related to the soil water content [19].

3.2. Bacterial Enumeration of Contaminated and Control Soil Samples

The bacterial culture was observed and counted as shown in Table 2 below.

Table 2. Mean Bacterial count and cultural characteristics from contaminated and uncontaminated sites.

Contaminated Soil Samples	Cultural Characteristics	Bacterial load (cfu/ml)
S1	Creamy Yellow	230×10^{-5}
S2	Creamy White	196×10^{-3}
S3	Creamy White	148×10^{-3}
S4	Creamy Yellow	292×10^{-4}
S5	Creamy Yellow	128×10^{-4}
S6	Creamy Yellow	108×10^{-3}
S7	Yellow	188×10^{-2}
S8	Creamy White	220×10^{-2}
S9	Creamy Yellow	76×10^{-5}
S10	Creamy Yellow	22×10^{-6}
CS	Yellow	TNTC

CS: control sample; TNTC: Too numerous to count.

The bacterial colony counts of soil samples from both contaminated and uncontaminated sites as presented in Table 2 showed that the uncontaminated site had the highest counts. This indicates that the petroleum products in the contaminated sites had adversely affect the growth of the organisms [7]. In a similar study carried out shows that, automobile uncontaminated site had the highest bacterial count when compared with bacterial count of automobile contaminated site. This indicated that the petroleum products in the contaminated sites have adversely affect the growth of the organisms [5].

3.3. Morphological and Biochemical Characteristics of Bacterial Isolates

These microscopic and biochemical characteristics of bacterial isolates in contaminated soil samples are presented in Table 3.

Table 3. Cultural and biochemical characteristics of bacterial isolates from contaminated soil.

Samples	GR	Shape	MP- TSA	Cat	Coa	Ur	Cit	Ind	MR	VP	Oxi	Lip
S1	+	Cocci in Clusters	Creamy Yellow	+	+	-	+	-	-	+	-	+
S2	+	Bacilli short chain & singles	Creamy White	+	-	-	+	-	-	+	-	+
S3	+	Bacilli short chain & singles	Creamy White	+	-	-	+	-	-	+	-	+
S4	+	Bacilli long chain	Creamy Yellow	+	-	-	+	-	-	+	-	+
S5	+	Cocci in Clusters	Creamy Yellow	+	+	-	+	-	-	+	-	+
S6	+	Bacilli short chain & pairs	Creamy Yellow	+	-	-	+	+	-	+	+	+
S7	+	Cocci long chain	Yellow	+	-	-	+	-	-	+	-	+
S8	+	Bacilli single	Creamy White	+	-	-	+	-	-	+	-	+
S9	+	Cocci Clusters	Creamy Yellow	+	-	-	+	-	-	+	-	+
S10	+	Cocci Clusters	Creamy Yellow	+	-	-	+	-	-	+	-	+

GR: Gram staining; MP-TSA: Morphology on TSA; Cat: Catalase; Coa: Coagulase; Ur: Urase; Cit: Citrate; Ind: Indole; MR: Methayl red; VP: Voges proskeur; Oxi: Oxidase; Lip: Lipase.

The colonies were rod shape and circular. Additional biochemical tests were carried out to identify the isolates strain as shown in table 3 above.

3.4. Biodegradation Studies of the Isolates from Contaminated Soil Sample

Table 4 showed various biodegrading compounds and relative abundance of the isolates through GC-MS.

Table 4. Biodegradation abilities of bacterial isolates from contaminated soil samples through GC-MS.

Samples	Compound Name	Compound Formular	Retention Time	Average relative abundance (conc.μg/μl)
S2	Allyl methallyl ether	$C_7H_{12}O$	15: 48sec	2395.77
S3	Ethanamine, N-ethyl-	$C_4H_{11}N$	14: 57sec	549.85
S4	Oxalic acid, allylpendadecyl ester	$C_{20}H_{36}O_4$	27: 49sec	194.28
S5	Formaldehyde, dimethylhydrazone	$C_3H_8N_2$	14: 30sec	24.98

Biodegradation capabilities through GC-MS of the isolates presented in Table 4 above, showed different kinds of hydrocarbon compounds specifically utilized by the isolates in the process of obtaining their nutrient using hydrocarbon as a source of energy. It is showed that *Bacillus subtilis* might have more degradation capabilities over the identified organisms with average relative abundance of 2395.77μg/μl.

3.5. Molecular Identification of Test Organisms from Contaminated Soil Sample

Table 5 presented the test organisms identified at molecular level from corresponding five samples through DNA extraction, PCR and gene sequencing.

Table 5. Identified organisms from contaminated soil samples using molecular techniques.

Samples	Identified Organisms
S1	*Bacillus cereus*
S2	*Bacillus subtilis*
S3	*Bacillus licheniformis*
S4	*Paenibacillus alvei*
S5	*Bacillus licheniformis*

Genomic DNA was isolated from all isolates, a PCR products of 789bp of the five isolates were analysed on agarose gel electrophoresis (Figure 2). Based on the 16S rRNA gene sequencing and phylogenetic analysis (Figure 3), the isolates (*Bacillus cereus, Bacillus subtilis, Bacillus licheniformis* and *Paenibacillus alvei)* were identified compared to the closet relative species in the GenBank data base.
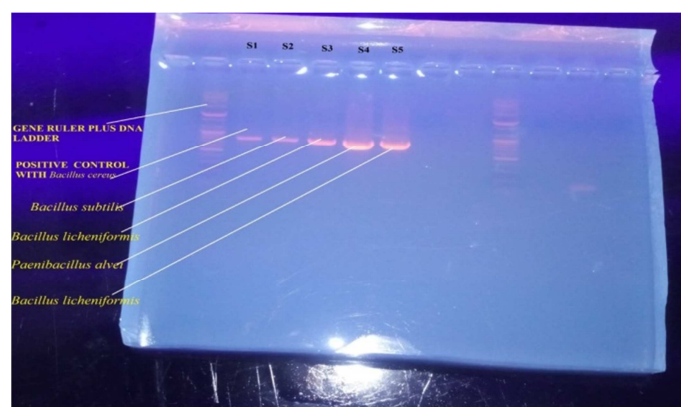
Figure 2. *Agarose gel electrophoresis of PCR amplification product of bacterial isolates using primers: Gene ruler plus DNA ladder, the lane showed positive control with all the 5 isolates with 789bp.*
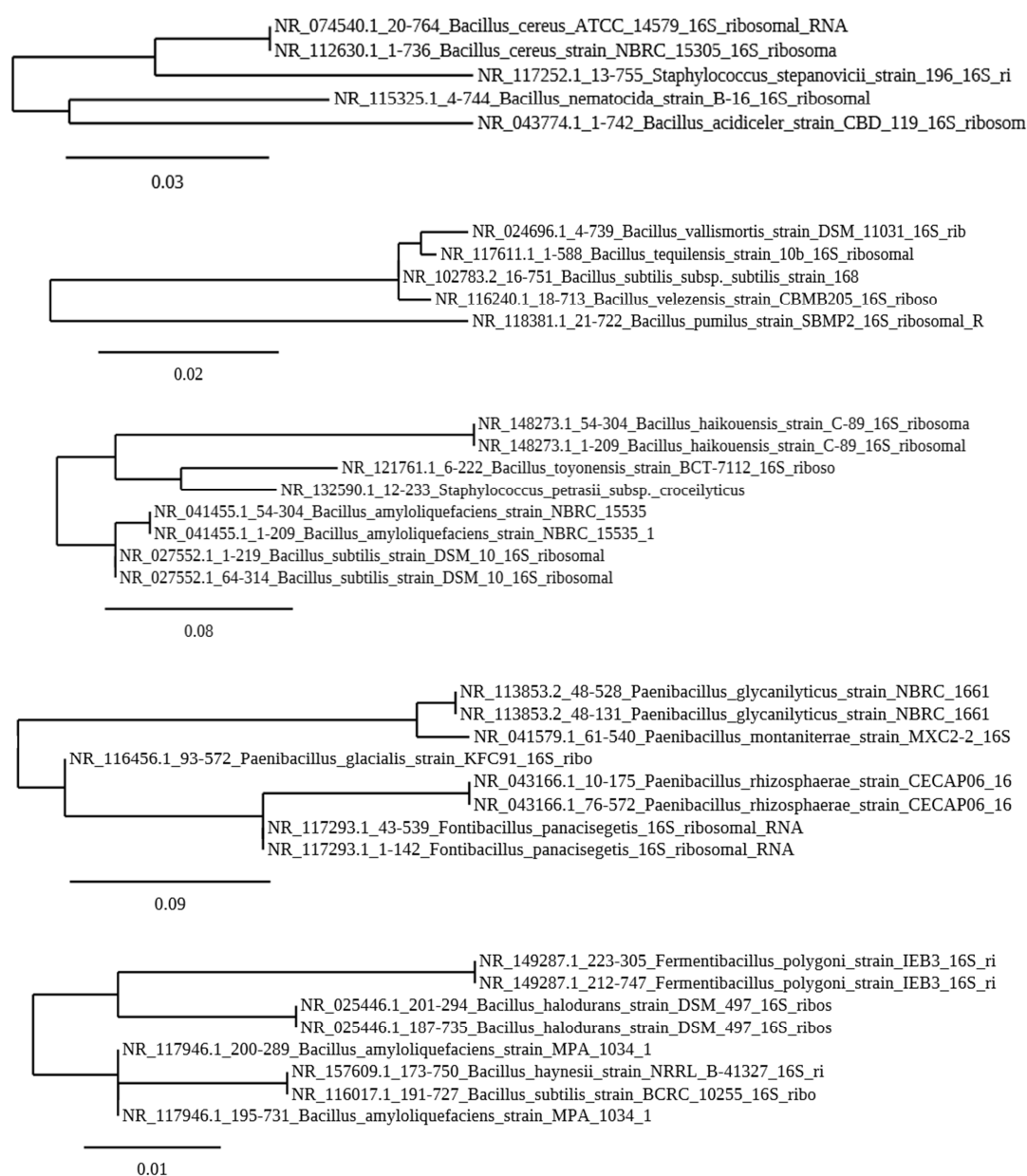

Figure 3. *Phylogenetic tree based on the partial 16S rRNA gene sequences of the five isolates (Bacillus cereus, Bacillus subtilis, Bacillus licheniformis, Paenibacillus alvei and Bacillus licheniformis respectively) and related species found by a BLASTn database search.*

4. Conclusion

Bacteria capable of utilizing and degrading used petroleum products (petrol and engine oil) exist in contaminated soil with these products at Mechanic village Dutse. In this current study, five bacterial strains were isolated from ten different oil contaminated garages in mechanic site. These strains have significant ability to utilize petrol and waste engine oil as a sole source of carbon and energy. On the basis of morphological, biochemical and molecular identification, *Bacillus cereus, Bacillus subtilis, Bacillus licheniformis, Paenibacillus alvei* and *Bacillus licheniformis* were identified. This study reported that *Bacillus subtilis* might had the highest hydrocarbon degradation ability. The ability of these strains to degrade hydrocarbon in oil polluted site suggested that they could be used for the treatment of other oil wastes in soil and ground water. Additional works will be suggested in our future research for the application of bioremediation using isolated strains.

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