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EXPLORATION OF THE CATABOLIC PLASMID GENES PROFILE OF CRUDE OIL DEGRADING BACTERIA ISOLATED FROM AGED OIL CONTAMINATED SOILS OF ANAMBRA STATE

Ifediegwu, M. C., [1], Orji, M.U. [1], Onuora, S.C. [1] and Uba, B.O.[2*]
[1]*Department of Applied Microbiology and Brewing, NnamdiAzikiwe University, P.M.B. 5025Awka, Anambra State, Nigeria.*
[2]*Department of Microbiology, ChukwuemekaOdumegwuOjukwu University, P.M.B.02 Uli, Anambra State, Nigeria.*
*Correspondence: bo.uba@coou.edu.ng; +2348069693773

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ABSTRACT

This study was undertaken to explore the catabolic plasmid genes profile and molecular identification of crude oil degrading bacteria isolated from aged oil contaminated soils of Anambra State. Enrichment technique, turbidometric test, plasmid profiling and curing tests, biodegradation capability testing as well asmolecular identification method were carried out using standard procedures.A total of sixty (60) bacterial isolates were encountered in the four different soil samples. Of the total bacterial isolates, 22 strains demonstrated high crude oil degrading potentials (A600nm > 0.3). The plasmid DNA was present in 8 out of the 22 bacterial strains such that bacteria strains G_2, H_4, and K_4 had one plasmid each with molecular weight of 9416 base pairs while bacteria strains C_3, D_1, J_3 and L_2 had two plasmids each with molecular weight of 2111 base pairs. The bacterial strains C1 and J3 recorded the highest growths on the solid media after the plasmid removal. The isolates belong to various species which are Bacillus cereus C12 (100 % similarity), Pseudomonas aeruginosa KAVK01 (100% similarity), Bacillus licheniformis 126 (96 % similarity), Ochrobacteriumintermedium E85b (97% similarity), Bacillus subtillis SDDlas (100 % similarity), Bacillus subtillis LK4.5 (97 % similarity),Enterobacter cloacae GEBRI III (97 % similarity) and Bacillus cereus So24 (100 % similarity). Therefore, plasmid-borne Pseudomonas aeruginosa and Ochrobacteriumintermediumare recommended for use in bioremediation because of their resilience to the crude oil contaminant and degradation capacities.

Keywords: Anambra State**,** Bioremediation, Catabolic plasmid gene, Plasmid Profiling, Pollution.

INTRODUCTION

Many important processes influence the destination of hydrocarbons in the environment. Among these are sorption, volatilization, abiotic transformation (chemical or photochemical), and biotransformation. Biodegradation of oil contaminated soils, which exploits the ability of microorganisms to degrade and/or detoxify organic contamination, has been established as one of the efficient, economic, versatile and environmentally sound treatment (Akeredolu et al., 2017). The ability of these microorganisms to degrade an organic compound such as crude oil is as a result of its genetic makeup. Generally, the chemical reactions involved in metabolism are mediated

by enzymes. The range of enzymes which a bacterium has is a reflection of the specific genetic information in the cell. Genetic information in bacteria, as in all organisms, is stored in the form of DNA. The information is physically present in bacterial cells in two forms – the chromosome and the plasmids (Akpe et al., 2013).

Plasmids are defined as double stranded, extra chromosomal genetic elements that replicate independently of the host cell chromosome and are stably inherited. They are usually found in bacteria. Plasmids that have been found to harbour genes encoding the transformation of environmental pollutants are known as catabolic plasmids (Thavasi *et al*., 2007). The plasmid-encoded catabolic pathway has the special benefit of facilitating the horizontal transfer of the particular catabolic genes in the microbial population. These result in a rapid adaptation of microbial population to the presence of new aromatic pollutants in an ecosystem (Cao *et al.,* 2009).

Plasmid mediated degradation can be useful and effective during the remediation of crude oil contaminated sites. This process offers the advantage of low operating cost and safety among others. These advantages in bioremediation have served as motivation for developing different bioremediation processes and also optimizing the existing processes to restore and minimize harm caused by crude oil pollution (Fagbemi and Sansui, 2017).The plasmid pool of the soil bacteria and its significant contribution in oil degradation are poorly understood. This study investigated the role of bacterial plasmid in the remediation of hydrocarbon polluted soil which if unchecked will lead to reduced agricultural output and pose a serious threat to human and animal health. The plasmid-encoded gene has the special benefit of facilitating the horizontal transfer of the particular catabolic genes in the microbial population, thus results in rapid adaptation of microbial population to the presence of new aromatic pollutants in an ecosystem and biodegradation (Cao *et al*., 2009).

The identification of bacteria at the genomic level will help to understand the evolutionary and phylogenetic perspectives of the bacteria, documentation of such microorganisms will lead to conservation of indigenous bacteria, which are of economic and environmental importance. In other words, the result of this research can be exploited in designing site-specific and contamination-specific bioremediation protocols for efficient and effective reclamation of contaminated sites. Thus, for proper exploitation of the native microorganisms in various treatment methods, the study of roles of bacterial catabolic plasmid gene becomes necessary. With new biotechnological innovations in pollution control, the documentation of bacteria with high bioremediation capabilities will help in formulating novel strategies. The phylogenetic study of the diverse catabolic genes gives an insight of the possible adaptation mechanisms of bacterial strains thriving in such extreme conditions. This study was undertaken to explore the catabolic plasmid genes profile and molecular identification of crude oil degrading bacteria isolated from aged oil contaminated soils of Anambra State. Therefore, this research will be of high importance to investors in oil industry, biodegradation researchers and consultants in decision making process.

MATERIALS AND METHODS

Materials

Procurement of Bonny light crude oil

Bonny light crude oil (API gravity=32.15) was acquired from the Port Harcourt Refinery of the Nigerian National Petroleum Corporation (NNPC) at Alesa-Eleme, Rivers State, Nigeria.

Sampling Site

Samples of soil were taken from four locations in Anambra State—Awka, Aguleri, Onitsha, and Ekwulobia—where hydrocarbon contamination dates back fifteen years (Table

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1). The Awka sampling site, which is situated near Aroma Junction in the Awka South Local Government Area of Anambra State, is situated between latitude N06.22677^{o} and longitude E007.07602^{o}. Its mean elevation is 133 meters above sea level. The Onitsha sample site is located at the Upper Iweka Axis of the Onitsha South Local Government of Anambra State and is latitude N6.13378^{o} and longitude E6.79393^{o}, with a mean elevation of 43 meters above sea level. The Ekwulobia area is located at latitude N5.99053^{o} and longitude E7.17018^{o}, with a mean elevation of 88 meters. The sample site is Ekwulobia Motor Park in the Aguata Local Government Area of Anambra State.

Table 1: Geographical location of soil samples

Sampling Sites	Location	LGA	No of Sample	Georeference
Automobile Workshop	Aguleri	Anambra East	19	N6.33231°, E6.87444°
Automobile Workshop	Awka	Awka South	16	N6.22677^{o} ,E7.07602^{o}
Automobile Workshop	Onitsha	Onitsha North	15	N6.13378^{o} , E6.79393^{o}
Automobile Workshop	Ekwulobia	Aguata	25	N5.99053^{o} , E7.17018^{o}
Total			**75**	

Sampling Method

The georeferenced point in the center of the site and two additional points eight meters distant were used to gather soil samples at depths ranging from 0 to 10 cm. Additionally, three subsamples, spaced six meters apart, were taken from each point (Nakamura et al., 2014). From the four sample locations, a total of 75 composite samples were gathered. With the aid of a soil auger, the sample was taken and placed in a plastic bag. The composite sample's polyethylene bag was kept cold in a refrigerator for microbiological and chemical analysis after being placed in a plastic bucket filled with dry ice. It was then promptly transferred to the Microbiology Laboratory of NnamdiAzikiwe University in Awka, Anambra State.

Soil Sample Processing

Composite soil samples were bulked together in the laboratory, crushed to break the large soil aggregates and air dried under room temperature. The samples were sieved through a 2 mm sieve to take out big grains, debris and stones. The sieved soil was ground to pass through a 0.5 mm sieve and kept for the physico-chemical analysis (Ekpo and Thomas, 2007)

Isolation of Hydrocarbon Degrading Bacteria

According to Gayathri et al. (2014), the enrichment culture approach was utilized to isolate bacterial strains that could use crude oil as their only source of carbon and energy. When the media were about to harden (between 45 and 50 ^{o}C) different quantities of crude oil (1%, 2%, 5%, and 10%) were carefully mixed with 100 mL of media (Luria Bertani, Mineral Salt and Nutrient broth). This process produced crude oil mixed media. After being suspended for 24 hr at 27 ±°C temperature in 9 mL of distilled water, 1 g of each soil sample was used. The following day, 250 µL of the supernatant was applied to crude oil-containing Nutrient agar, Luria Bertani and Mineral Salt Petri dishes in order to isolate bacteria capable of breaking down hydrocarbons. The inoculated plates were incubated at 27 ±°C temperature. The following day and up to the fourth day, the Petri dishes were checked for the development of bacterial colonies (Hyina et al., 2003). Seven transfers in a series were used to randomly select the cultures. In preparation for

additional examination, the purified bacterial strains were refrigerated at 4 °C on agar slants.

Preliminary Screening of Bacterial Isolate for Hydrocarbon Degradation

Inoculum preparation

The inoculum was prepared following the procedure described by Nwanyanwu et al. (2016). For 48 hrs, the test isolates were cultured in nutrient broth medium in 100 mL Erlenmeyer flasks at room temperature. The cells were then extracted using centrifugation for 10 min at 6000 rpm, followed by a rinse in sterile deionized water. The cell suspensions were utilized throughout the investigation, unless otherwise noted, and were standardized by adjusting the turbidity to optical density of 0.1 at absorbance of 540 nm.

Turbidometric test

The ability of the bacterial isolates to use the hydrocarbon fraction (4% v/v) was also evaluated using the MS medium. In this test, 50 mL of the MS medium were added to a 100 mL conical flask, which was then sterilized to make the medium. Each of the 60 bacterial isolates were added separately to a flask. For seven days, the flasks were incubated at room temperature at 120 rpm on an orbital shaker. Crude oil degradation capability was measured by cell growth as measured by optical density at 600 nm (OD_{600}) (Nwanyanwu et al., 2016). Bacteria that break down crude oil and have a high potential for degradation were chosen and were used for future studies.

Plasmid Extraction, Electrophoresis and Curing

In this experiment, plasmids were assayed in 22 bacterial isolates that showed rapid growth on the modified mineral salt media. Consequently, the isolates were subjected to plasmid isolation using the procedure described by Isiodu et al. (2016) and Uba (2019a). Plasmid profiling was carried out usingagarose gel electrophoresis. The DNA bands were matched with those for lamda DNA Hind III digest kb molecular weight marker. The approximate molecular weight of each plasmid was obtained by extrapolation on graphical plots of molecular weight of marker against the distance travelled by the respective plasmid band (Ajao, 2013). Eight out of the twenty-two bacterial isolates that underwent plasmid curing after being screened for the presence or absence of plasmid were discovered to be plasmid-borne. Nutrient broth (NB) was used to grow bacterial cells overnight. A 0.1 mg/mL acridine orange supplement was added to five (5) milliliters of nutritional broth. Zero point one milliliter (0.1 mL) of newly made culture suspension of the test strains from a 10^{-5} dilution was added to nutrient broth that contained acridine orange. The mixture was then incubated in the dark for 4 days at 37 ºC and pH 7.6 before being plated out on Nutrient Agar. Acridine orange was not added to control when they were prepared in nutrient broth. The bacterial isolates regarded as cured were colonies that could grow on nutrient agar but not on modified solid mineral salt medium.

Confirmation Tests for Isolates Cured of Plasmids

Tests carried out to confirm the loss of plasmid after plasmid curing of the eight plasmids - borne isolates include the followings.

Plasmid re-profiling test

Plasmid re-profiling was done usingagarose gel electrophoresis as previously described in plasmid profiling test described by Ajao (2013).

Biodegradation capability test of plasmid cured isolates in solid media

Eight bacterial isolates were used in a biodegradation ability test to verify the plasmid curing's efficacy. Nutrient broth was used to grow both the control and isolate plasmid-borne cells (plasmid free cells). 250 mL Erlenmeyer flasks containing 100 mL of sterile mineral salt medium supplemented with 2 mL (2 % v/v) of Bonny-light crude oil were filled with one milliliter (1.0 mL) of the cell suspensions of each of the plasmid free cells and plasmid borne cells. After that, the flasks

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were incubated for three days at 30 °C and 120 rpm in a shaker. As a backup, a flask with 2 % crude oil and sterile mineral salt medium but no test organisms was used. The three-day-old cultures were plated at a 10^{-5} dilution on MS media after being serially diluted (tenfold dilutions). As a proof of biodegradation, the isolates' growths in the media were qualitatively categorized as good (+++), moderate (++), poor (+) and no growth (-).

Molecular Identification of Crude Oil Degrading Organism

Molecular studies were done for the eight bacterial isolates (B_1, C_3, D_1, H_4, I_6, J_3, K_4, and L_2) that had catabolic plasmid genes following DNA extraction, PCR amplification of the bacterial 16S rRNA genes and gel electrophoresis of the isolate were done at FOWM Biotechnology LTD, Jibowu, Yaba, Lagos and the Molecular Biology Laboratory of National Institute for Medical Research (NIMR), Yaba, Lagos, Nigeria. The PCR product was sent to Epoch Life Science, Texas, USA where the Sanger Sequencing was carried and blasting of the generated sequences was done at National Centre for Biotechnology Information (NCBI) websites where organisms of similar origin were identified from their closest relatives in the GenBank as described by Uba (2018) and(2019b).

RESULTS

Isolation Profile of Hydrocarbon Utilizing Bacteria

Table 2 showed the location of soil samples, number of isolates per location and designation of isolates. A total of sixty (60) bacterial isolates shown in Table 2 were isolated from the soil samples. Soil samples collected from Aguleri had the highest number of bacterial isolates (18) while thesoil samples obtained from Ekwulobia had the least number (12) of isolates. The number of isolates in the soil samples collected from Onitsha and Awka were 13 and 17, respectively.

Table 2: Locations of soil samples, number of isolates per location and designation of isolates

Sample codes	Location	Isolates number	Designation of Isolates
A	Aguleri	18	A_1,A_2,A_3,B_1,B_2,B_3,B_4,C_1,C_2,C_3,D_1 D_2, D_3, E_1, E_2, E_3, E_4, E_5.
B	Awka	13	F_1, F_2, F_3, F_4, F_5, G1, G2, G_3, H_1, H_2 H_3, H_4 H_5
C	Ekwulobia	12	I_1, I_2, I_3, I_4, I_5, I_6, J_1, J_2, J3, J_3, J_4, J_5
D	Onitsha	17	K_1, K_2, K_3, K_4, K_5, L_1, L_2, L_3, L_4, M_1, M_2, M_3, M_4, N_1, N_2, N_3, N_4,
Total		60	

Preliminary Screening of Isolates for Hydrocarbon Degradation

Table 3 showed that the optical densities (A600 nm) of the bacteria isolates variedfrom 0.10 to 0.42 in the growth medium containing crude oil, as indicated by the preliminary screening test results. Twenty-two isolates designated with the strain codes A1, A2, B1, C1, C3, D1, E1, E3, F2, F5, G2, H2, H4, I3, I5, I6, J3, K1, K4, L2, M1, and N4, were chosen for further studies after exhibiting noticeable growths (OD600 nm > 0.30), as shown in Table 3. With an optical density of 0.42, isolate C3 had the fastest growth, while isolate K5, which had an optical density of 0.10, had the slowest growth.

Table 3: Preliminary screening of isolates for hydrocarbon degradation

Isolate code	OD600	Isolate code	OD600	Isolate code	OD600	Isolate code	OD600
A1	**0.35**	**E1**	**0.31**	G4	0.18	J2	0.12
A2	**0.37**	E2	0.28	G5	0.11	**J3**	**0.40**
A3	0.28	**E3**	**0.35**	H1	0.15	J4	0.28
B1	**0.35**	E4	0.18	**H2**	**0.34**	J5	0.20
B2	0.27	E5	0.19	H3	0.12	**K1**	**0.33**
B3	0.29	F1	0.31	**H4**	**0.31**	K2	0.12
B4	0.19	**F2**	**0.36**	I1	0.25	K3	0.14
C1	**0.31**	F3	0.29	I2	0.11	**K4**	**0.30**
C2	0.27	F4	0.15	**I3**	**0.30**	K5	0.10
C3	**0.42**	**F5**	**0.32**	I4	0.17	L1	0.13
D1	**0.38**	G1	0.16	I5	**0.31**	**L2**	**0.37**
D2	0.22	**G2**	**0.37**	**I_6**	**0.36**	L3	0.15
D3	0.24	G3	0.14	J1	0.10	L4	0.14
M1	**0.35**	M2	0.21	M3	0.17	M4	0.23
N1	0.28	N2	0.19	N3	0.15	**N4**	**0.32**

Screening of Isolate for Presence of Plasmid

Table 4 showed the plasmid profiles of the isolates.Twenty-two isolates which showed significant growths during the preliminary screening for growth potentials in the presence of crude oil were tested for presence or absence of the plasmid DNA. Plasmid DNA was present in eight bacterial isolates as shown in Table 4. The plasmids were found in the isolates designated as C_3, D_1, G_2, H_4, I_6, J_3, K_4 and L_2 .In addition, the bacterial isolates exhibited similarities in its plasmid profiles. Isolates G_2, H_4, and K_4 had one plasmid each with molecular weight of 9416 base pairs while isolates C_3, D_1, J_3 and L_2 had two plasmids each with molecular weight of 2111 base pairs.

Table 4: Plasmid Profile and molecular weight of bacterial isolates

Code of Isolate	No. of plasmid	Mol. weight of plasmid (bp)
C_3	2	2011
D_1	2	2011
G_2	1	9416
H_4	1	9416
I_6	1	6557
J_3	2	2011
K_4	1	9416
L_2	22011	

Confirmation Tests for Isolates Cured of Plasmids

Plasmid re-profiling test

Agarose gel electrophoregrams of pre-cured and post curedplasmid DNA of the isolates were shown in Figures 1A – B and 2A - B.Plasmid re-profiling indicated that the plasmids were successfully cured with the evidence in thedisappearance of the bands in post plasmid curing asshown in Figures 1B and 2B .Agarose gel electrophoregram of pre-curedplasmid DNA (Figure 1) showed double plasmid bands of isolates in lanes C, D, J and L with M as DNA ladder.However, these plasmid bands in Figure 1A were conspicuously absent as shown in post cured agarose gel electrophoregram of plasmid DNA (Figure 1B). In addition, agarose gel electrophoregram of plasmid DNA in Figure 2A

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indicated single bands of plasmid in lanes I, H, K and G while there were absence of plasmid bands in lanes A, B, E and F in Figure 2B indicating absence of plasmid genes in isolates designated as A, B, E and F.

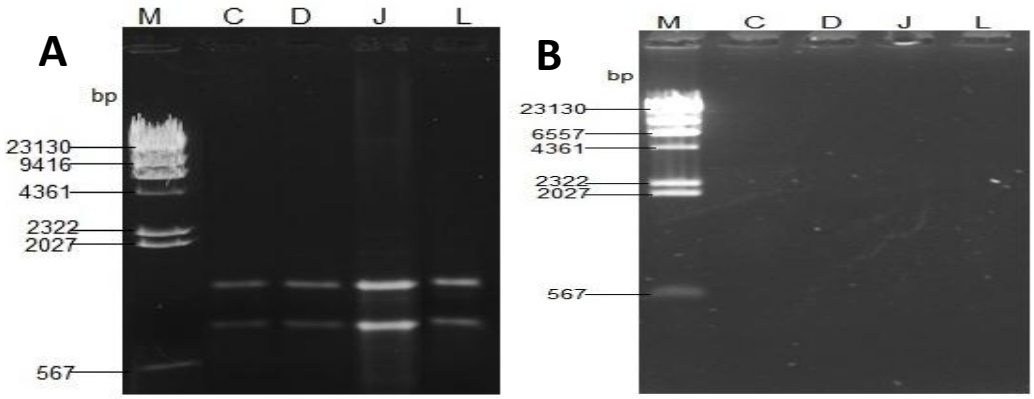

Figure 1A: Agarose gel electrophoregram showing presence of plasmid DNA.

Key:

Lane M = DNA Ladder	Lane M = DNA Ladder
Lane C = Plasmid borne isolate C	Lane C = Plasmid cured isolate C
Lane D = Plasmid borne isolate D	Lane D = Plasmid cured isolate D
Lane J = Plasmid borne isolate J	Lane J = Plasmid cured isolate J
Lane L =Plasmid borne isolate L	Lane L =Plasmid cured isolate L

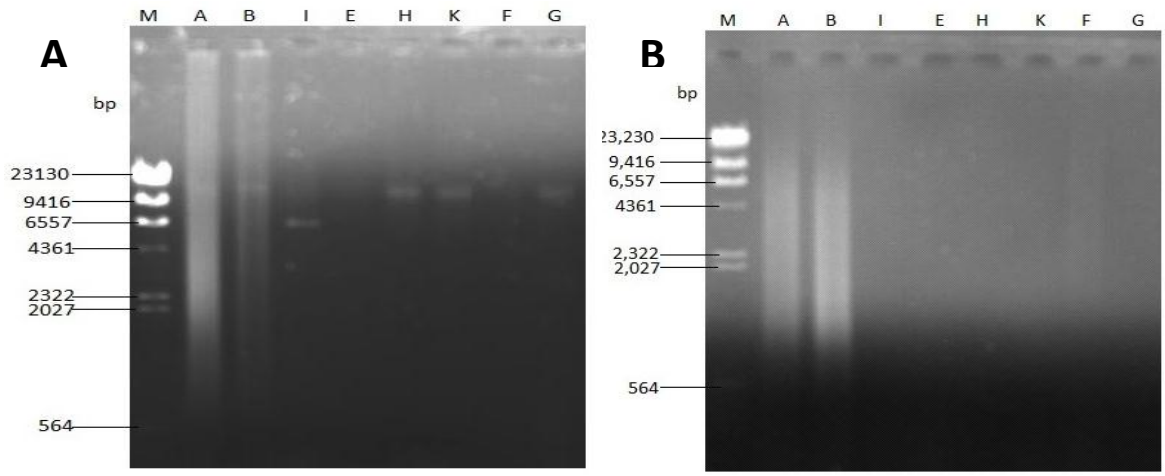

Figure 2A: Agarose gel electrophoregrampresence of Plasmid DNA. 2B: Agarose gel electrophoregramshowing absence of plasmid DNA

KEY:

M = DNA ladder	M = DNA ladder
Lane A = Non-plasmid borne isolate A	Lane A = Non-plasmid borne isolate A
Lane B = Non-plasmid borne isolate B	Lane B = Non-plasmid borne isolate B
Lane I = Plasmid borne isolate I	Lane I = Plasmid cured isolate I
Lane E= Non-plasmid borne isolate E	Lane E= Non-plasmid cured isolate E
Lane H =Plasmid borne isolate H	Lane H =Plasmid cured isolate H
Lane K= Plasmid borne isolate K	Lane K= Plasmid cured isolate K
Lane F = Non-plasmid borne isolate F	Lane F = Non-plasmid borne isolate F
Lane G =Plasmid borne isolate G	Lane G =Plasmid cured isolate G

Biodegradation capability test for plasmid cured isolate in solid media

Table 5qualitatively showed the growth of eight (8) bacterial isolates in the solid8 bacteria in the solid media before and after the removal of the plasmid DNA. However; the growths of the isolates were not the same after the removal of their plasmids. Isolates designated as C_1, D_1, L_2 and J_3 showed various degrees of growths after curing of their plasmids whereas isolates G_2, H_4, K_4 and I_6 were unable to grow after plasmid removal. In addition, isolates C1 and J3 recorded the highest growths on the solid media after the plasmid removal.

Table 5: Biodegradation capability of plasmid cured isolates in solid media

Isolate designates	Before curing	After curing
C_3	+ + +	+ +
D_1	+ + +	+
G_2	+ +	-
H_4	+ +	-
I_6	+ +	-
J_3	+ + +	+ +
K_4	+ +	-
L_2	+ + +	+

Key:
+ + + = Heavy growth; + + = Moderate growth; + = Minimal growth; - = No growth

Identification of Crude Oil Degrading Organisms

The identities of the isolates were further confirmed by molecular ribotyping based on the partial gene sequence of 16S rRNA gene which were amplified and sequenced as explained in materials and methods. The size of the amplicon was found to be 1500bp (Figure 3). Table 6 showed the closest relative of the isolates in Gen Bank and specific area of isolations.It was observed that 2 bacterial isolates (C_3 and D_1) were isolated from soil sample collected from Awka, 3 bacterial isolates (G_2, K_4 and I_6) were isolated from Aguleri while Ekwulobia and Onitsha samples had one organism each (Table 6).

Table 6: Closest genera and percentage sequence homology of the 16S rRNA of the Isolates

Isolate code	Closest relative in Gen Bank	Max score	Total score	Query cover	Per. identity	Accession number	Isolation site
D1	*Bacillus cereus* C12	1768	2999	94%	100%	MF800952	Awka
C3	*Pseudomonas aeruginosa* KAVK01	2745	2745	100%	100%	KC119195	Awka
L2	*Bacillus subtilis* SSDlas	1866	1866	100%	100%	HQ262546	Onitsha
J3	*Ochrobacterium intermedium* E85b	1291	1291	90%	96.79%	KM894187	Ekwulobia
H4	*Bacillus licheniformis* 129	2641	2641	100%	99.93%	KU922363	Onitsha
G2	*Bacillus cereus* So24	1829	1829	100%	100%	MG00925	Aguleri
K4	*Bacillus subtilis* LK4.5	1602	1602	95%	96.89%	KY083700	Aguleri
I6	*Enterobacter cloacae* GEBRI III	1555	1555	94%	97.20%	MH473593	Aguleri

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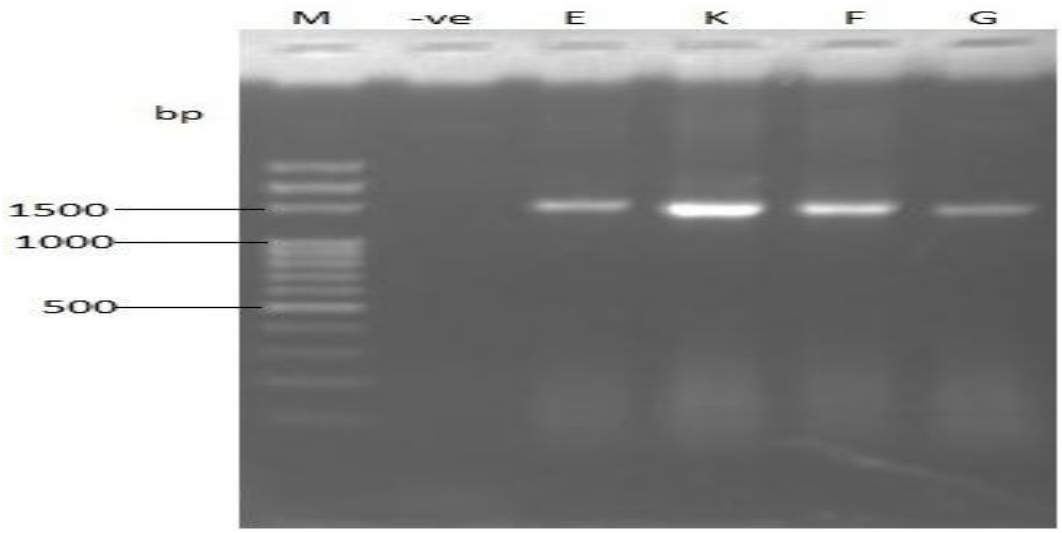

Figure 3: PCR amplicon of 16S rRNA gene obtained from isolates

DISCUSSION

The presence of plasmids in bacteria has been widely reported. The plasmids that bear genes encoding enzymes capable of hydrocarbon degradation are considered important. These plasmids, known as catabolic plasmids can give the organisms harbouring themthe ability to degrade certain compounds (Esmaeili et al., 2020).In this study, a laboratory scale experiment was conducted in order to explore the catabolic plasmid genes profile and molecular identification of crude oil degrading bacteria isolated from aged oil contaminated soils of Anambra State.

Also, sixty hydrocarbon utilizing bacteria were isolated from crude oil supplemented mineral salt agar medium (Table 3). Since all the bacterial isolates encountered in this study were isolated from a petroleum contaminated soil sample, they survived in the oil-supplemented media very easily as also reported by Rahman et al. (2003). Table 3 indicated that 22 of the isolates were observed to have high pollutant degrading potentials (A_{600}nm > 0.3) due to their crude oil utilization ability.Absorbance (A_{600nm}) of the cells grown in a medium with petroleum as a sole carbon source has been used as an index of PHC biodegradation potential (Binazadeh et al., 2009; Celik et al., 2008; Ciric et al., 2010; Husain et al., 2011). The other isolates showed variations in their growth on the substrate and the isolate with least growth has optical density of 0.11. Ciric et al. (2010) had differentiated growth of the alkane degraders based on A_{600nm} using the following criteria: No growth = A_{600nm} 0.00–0.019; +, minimal growth = A_{600nm} 0.02–0.099; ++, moderate growth = A_{600nm} 0.1–0.2; +++, maximum growth = A_{600nm}>0.2. Nwanyanwu et al. (2016)isolated*Micrococcus*sp. RS38 which showed impressive level of growth during screening in crude oil and other petroleum products where the organisms grew at equal optical densities of > 0.2 within 14 days of incubation.Vinothini et al. (2015) reported optical density of 0.55 by *Pseudomonas putida* which crude oil degradation ability was screened based on the growth efficiency on 2 % crude oil at the 7th day of incubation period.

Table 4 showed that 8 out of 22 bacterial isolates had plasmid DNA and their sizes varied from 2011 to 9416 bp.Four isolates had double plasmid DNA whereas 4 had single plasmid DNA. Ohtani et al. (2008) reported the presence of a 65kb conjugal plasmid pLS20 in *Bacillus subtilis*. Esumeh et al. (2009) isolated *Klebsiellapneumoniae, Enterobacter cloacaeand Pseudomonas aeruginosa*from pawpaw *(Carica Papaya)* and sweet orange *(Citrus sinensis)*. All these

isolates had one plasmid of 23.1kbp each except *Enterobacter cloacae*, which possessed an additional plasmid of 12.0 kbp. It was interesting to note the presence of approximately 22 kb mega plasmid in *Geobacillus stearothermophilus*PS11. Earlier, mega plasmid was detected in anthracene degrading *Geobacillus stearothermophilus* AAP7919 (Kumar et al., 2012) and PAH degrading *Sphingomonas* sp. strain KS14 (Cho et al., 2001). The result in Table 3 indicated that 75 % (6 out of 8) of plasmid-borne bacteria belong to the genera *Bacillus*. This corroborates the report of Ndubuisi-Nnaji et al. (2014) which stated that the identification of plasmid-carrying isolates revealed approximately 75 % of them to be Gram positive rods particularly of the *Bacillus* genera. Several species of Gram positive bacteria carry multiple plasmids which serve as adaptive mechanism especially those belonging to the genera *Staphylococcus, Streptococcus, Lactobacillus, Bacillus and Corynebacterium* (Kunninalaiyan et al., 2001, Igwilo-Ezikpe et al., 2010).

Figures 1A–B and 2A – B indicated that the plasmids cured with acridine orange were successfully done with the evidence in thedisappearance of the bands in post plasmid cured cells.Buckner et al. (2018) reported that the DNA intercalating agents such as acridine orange, ethidium bromide and acriflavine have plasmid curing properties. Soliman et al. (2009) reported that plasmids are eliminated by these agents because of interference with their replication, DNA intercalating dyes (acridine orange) and by alterations of their membrane attachment sites. Plasmid elimination *in vitro* is a method of isolating plasmid free bacteria for biotechnology without any risk of inducing mutations (Spengler et al., 2006).Acridine orange cured *E. coli* (Keyhaniet al., 2006; Zaman et al., 2010), *Vibrio parahaemolyticus* (Letchumananet al., 2015) and *Lactobacillus plantarum* (Adeyemo and Onilude, 2015).Dam et al. (2012) reported that bacterial plasmid was cured with acridine orange in sub-lethal doses in bacterial populations that led to the elimination of plasmid DNA without harming the bacterial chromosome and maintained the ability to reproduce and generate off spring.

Table 5 showed that plasmid cured mutants of *Pseudomonas aeruginosa* KAVK01, *Bacillus cereus* C12, *Ochrobacterium intermedium* E85b and *Bacillus subtilis* SDDlas were able to grow in a crude oil medium while plasmid cured strains of *Bacillus cereus* S024, *Bacillus subtillis* LK 4-5, *Enterobacter cloacae* GEBRI and *Bacillus licheniformis* 129 were unable to grow in crude oil supplemented medium. Table 5 also demonstrated that the non-plasmid cured isolates had greater growths than plasmid free ones. Unlike the findings of Kalaivani et al. (2012) which all the three plasmid cured isolates (*Pseudomonas aeruginosa, Pseudomonas putida and Bacillus subtilis*) lost the ability to grow in a crude oil medium,4 out of 8 bacteria cured of plasmids in this study retained their ability to degrade crude oil although non-cured isolates recorded higher growths. Dam et al. (2012) reported that the plasmid cured culture of *Geobacillus stearothermophilus* PS11 strain could not grow in presence of crude oil or any of the solvents. The inability to grow in the presence of crude oil or other solvents might be due to the removal or inactivation of gene(s) responsible for petroleum hydrocarbon degradation. Similarly, Kumar et al. (2012) also reported the absence of growth by plasmid cured colony of *Geobacillus stearothermophilus* strain AAP7919 which was grown on nutrient broth with anthracene as carbonsource. The inability of the isolates to grow might be due to the removal or inactivationof gene(s) responsible for anthracene degradation from *Geobacillus stearothermophilus* AAP7919**.** Coral and Karagoz (2005) also reported that catabolic pathways, which encode different aromatic hydrocarbon degradation routes, are frequently located on plasmids, although degradative genes can be located on either chromosome or plasmid.

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The result in Table 6 of the blasted sequences indicated that isolates belonged to various species which are *Bacillus cereus* C12 (100 % sequence similarity), *Pseudomonas aeruginosa* KAVK01 (100% sequence similarity), *Bacillus licheniformis* 126 (96 % sequence similarity), *Ochrobacterium intermedium* E85b (97% sequence similarity), *Bacillus subtillis* SDDlas (100 % sequence similarity), *Bacillus subtillis* LK4.5 (97 % sequence similarity), *Enterobacter cloacae* GEBRI III (97 % sequence similarity) and *Bacillus cereus* So24 (100 % sequence similarity). Primers 27F and 1492R were used for forward and reverse reactions, respectively. The two primers were top on the list of bacterial universal primers (Macrogen, 2013). Frank et al. (2008) evaluated these 2 primers critically for bacterial 16S rRNA gene and discovered that 27F and 1492R formulations was simple .Seven distinct primer sequences were compared and there was minimal loss of overall amplification efficiency and specificity.Rajesh. (2017) isolated *Pseudomonas* sp. Strain IR1 from vehicle service station site of Chandigarh, India and cultured it in BSM broth supplemented with 0.1% w/v naphthalene as substrate. Erakyet al. (2015) isolated *Ochrobactrum* sp. which was reported as an agent of biodegradation of BTEX hydrocarbons. Chai et al. (2015) reported that environmental isolate of *Ochrobactrum intermediumwas* able to degrade crude oil. Ikuesan et al. (2016) identified crude oil degrading bacteria belonging to both the Gram positive and Gram negative groups, although, the Gram negative bacteria predominate in all the samples. This research finding corroborates the report of Salam et al. (2011) that both Gram negative and positive bacteria have been implicated in the mineralization of hydrocarbon pollutants.

CONCLUSION

The study revealed the studied areas in Anambra State, Nigeria harbour a lot of crude oil hydrocarbon degrading bacterial isolates. Eight bacterial strains were found to possess catabolic plasmid genes with varying plasmid number and sizes. The plasmid profiling revealed that the plasmid bands were lost after plasmid curing by acridine orange which was further confirmed by loss in degradation of the crude oil by the bacterial strains. The identification of these bacterial strains revealed the significant role of catabolic plasmids in the degradation of organic compounds. The plasmids of *Pseudomonas aeroginosa* KAVK01 *and Ochrobacterium intermedium* E85b can also be used in recombinant DNA technology to develop bacterial cell with the potential to effectively degrade crude oil.

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