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Growth Patterns and Degradative Potentials of *Pseudomonas* sp. Isolated from Waste Dumpsite Soil in Crude Oil Supplemented Soil Extract and Mineral Salts Media

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Authors' contributions

This work was carried out in collaboration between all authors. Author OAO designed the study, performed the statistical analysis, wrote the protocol and wrote the first draft of the manuscript. Author JOO proofread the manuscript and gave technical inputs and author ATO managed the analyses of the study. Author ATO managed the literature searches while author EIA helped with interpretation of some of the data generated as well as proofreading the manuscript. All authors read and approved the final manuscript.

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ABSTRACT

Introduction: Pollution caused by crude oil and its products is the most prevalent and widespread problem in soil and water environments and is increasingly being implicated as being responsible for the global warming menace being battled worldwide
Aims: This study was carried out to evaluate the potential of *Pseudomonas* sp. from dumpsite soil to degrade crude oil hydrocarbons.
Place and Duration of Study: Department of Microbiology, Ekiti State University, Ado Ekiti, Nigeria, October 2013-May 2015.

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Methodology: Soil samples from different locations of Ekiti State Waste Management Board dumpsite in Ilasa, Ado-Ekiti were collected and screened for crude oil utilizing *Pseudomonas* sp. the isolates were screened using vapour transfer method and the growth kinetics of the selected best degrading species were monitored in mineral salt and soil extract media by measuring optical density. The rate of degradation was evaluated via gas chromatography and mass spectroscopy analysis. The effects of heavy metals on degradation was also evaluated as well as the presence of plasmids in the degrading species.
Results: Eighteen *Pseudomonas* spp. isolated from municipal waste dumpsite soil were recovered using standard microbiological methods. Five species *Pseudomonas* OWPSA. OWPS11, OWPS6, OWPS5 and OWPSJ showed enhanced ability to utilize crude oil hydrocarbons at concentrations up to 2.5% v/v with optimal utilization at 2.0%. All the *Pseudomonas* spp. showed tolerance to zinc and lead but were less tolerant to cobalt and mercury. The Gas Chromatography and Mass Spectroscopy analyses of the residual crude oil from the treatments revealed that *Pseudomonas* sp. OWPSJ exhibited the highest degradation efficiency in soil extract media resulting in loss of 18 peaks. There was marked reduction of hydrocarbon fractions by 75- 90% while the naphthalene fraction of the oil inoculated with *Pseudomonas* sp. OWPS11 increased. However, *Pseudomonas* sp. OWPS6 exhibited degradation of hydrocarbons fractions than strain *Pseudomonas* sp. OWPS11 with loss of 16 peaks. Plasmids were not observed to occur hence suggesting the possibility of the genes being located on the chromosome. Residual components of degradation were esters, methylated benzenes, alcohols and organic acids as well as shorter chain alkanes.
Conclusion: This study establishes the prospect of soil extract media as alternative media for evaluating the degradative ability of bacteria and also the potential of strain *Pseudomonas* sp. OWPSJ, isolated from a non-petroleum contaminated site as a good bioremediation agent for crude oil spills.

Keywords: Pseudomonas; bonny light; crude oil; heavy metals; soil extract media.

1. INTRODUCTION

Pollution caused by crude oil and its products is the most prevalent and widespread problem in soil and water environments and are increasingly being implicated as being responsible for the global warming menace being battled worldwide [1,2]. In 2011, over 40,000 barrels (1.68 million gallons) of Bonny-light crude oil was spilled in a Shell Company facility at Bonga, an oil creek in the Niger-Delta region of Nigeria, resulting in an oil slick 185km long off the Nigerian coast [3]. Nigeria's largest spill was an offshore well-blow out in January 1980 when an estimated (8.4million US gallons) spilled into the Atlantic Ocean from an oil industry facility, damaging 340 hectares of mangrove [4]. Oil spills are common events in Nigeria [5] resulting from corrosion, sabotage, ineffective oil production operations, engineering drills, inability to effectively control oil wells, failure of machines, and inadequate care in loading and unloading oil vessels [6]. Accidental release of hydrocarbons into the environment and its attendant detriments is not restricted to oil producing regions alone, but other areas which are also prone to the increasing risks and possibility of spills due to tanker accidents and leakage from ruptured pipelines networked across such areas [7]. These petroleum pollutants not only damage the environment but have health implications to human, microbial and aquatic life [8,9,10]. Also, these substances are recalcitrant in the environments, persisting for long in the ecosystem. Oil contamination in soil results in an imbalance in the carbon-nitrogen ratio at the spill site resulting in nitrogen deficiency in the oil - soaked soil thereby retards the growth of bacteria and the utilization of carbon sources [11] thereby limiting soil fertility and hence crop [12].

Oluwole et al. [13] and Umechuruba et al. [14] in their research findings showed statistically significant impact of such reckless disposal on plants, effects like height reduction, chlorophyll loss and protein level reduction. Oil pollution is also very damaging to aquatic ecosystem. Aquatic plants respond in various different ways to oil and many species may be killed off during oil pollution [15].

Conventional treatment technologies simply transfer the pollutants, creating a new waste such as incineration residues and do not eliminate the problem [16]. Being known to be hydrocarbonoclastic [17,18], microorganisms offer a promising solution to the problems of

clean up. The need for an alternative and additional response led to bioremediation that has emerged as one of the most promising secondary treatment options for oil removal since its successful application after the 1989 Exxon Valdez oil spill [19]. It is considered an environmentally acceptable way of eliminating oils and fuel because the majority of hydrocarbons in Bonny-light crude oils and refined products are biodegradable [20].

Bioremediation can offer a less ecologically damaging and cost effective alternative by taking advantage of the oil degrading microbes [21] and by establishing and maintaining the physical, chemical and biological conditions that favor enhanced oil biodegradation rates in the contaminated environment [19]. Microbes with the ability to degrade a wide range of oil components are ubiquitously distributed in soil and marine environment [22,23]. Many different aspects of these communities are studied including isolating and identifying the microorganisms present and determining what roles they play in the biodegradative processes [24]. *Pseudomonas* spp, among other species are versatile in surviving and metabolize the hydrocarbon contaminations. They are ubiquitous in soils and thrive both in organic rich and poor soils and hence posess capability to utilize hydrocarbons. Although a consortium of bacterial species degrades better than individual species, *Pseudomonas* species readily attack and degrade hydrocarbons and are able to degrade as much as a consortia do. This is evident in a research conducted by Al-Wasify and Hamed [25], who assessed the biodegrading efficiency of four local isolates and a consortium of these isolates to degrade Bonny-light crude oil. They found out that although the consortium was able to efficiently remove 88.5% of Egyptian Bonny-light crude oil after 28 days, *Pseudomonas aeruginosa* was able to degrade the oil with 77.8% efficiency.

Many studies have been conducted to isolate and characterize hydrocarbon degrading bacteria from soils but little or no work has been done on the bioremediation potential of bacterial isolates from waste dumpsites. This study therefore aims at evaluating the biodegradation potentials as well as understanding the growth patterns of biochemically characterized *Pseudomonas* species in Bonny-light crude oil supplemented media (mineral salts and soil extract media).

2. MATERIALS AND METHODS

2.1 Collection and Preparation of Soil Samples

Soil samples were collected from the Ekiti State Waste Management Board [EKWMB] dumpsite located at Irasa community along Iworoko road in Ado-Ekiti. The site is near the NESREA waste management plant and the Federal Radio Corporation of Nigeria (FRCN) broadcasting station both situated in that community. The wastes disposed are mainly domestic, market and household wastes. About 200 g each of soil samples from 5 different spots at the dumpsite was randomly collected using soil auger 10 cm below soil surface into sterile sampling bottles and transported immediately to the laboratory for analysis. The control samples were collected using the above procedure from an agricultural farmland located in Adehun area of Ado-Ekiti.

2.2 Physicochemical Analysis of Soil Samples

Physicochemical analysis of soil from the sampling site was carried out. With the exception of moisture content analysis, the respective soil samples were placed on large wooden trays and air-dried for 72 hours. Lumps of moist soil samples were broken by hand prior to air drying of the samples. The air dried samples were also sieved using a 2 mm mesh. The soil samples from the different location of the dumpsite were thoroughly mixed to ensure proper mixing so as to obtain homogeneity prior to analysis. Parameters evaluated included pH, electrical conductivity was done at the Laboratory of Ekiti State University, Ado-Ekiti. The pH and Electrical conductivity was read using digital pH and E.C. meter (HANNA H19113) using the method described in AOAC [26]. The Total organic matter, Total organic carbon, Potassium, Phosphorus, Nitrogen, Exchangeable Calcium and Magnesium and water holding capacity was determined at the Central Laboratory of Federal University of Technology, Akure [27,28].

2.3 Isolation Procedure

The total viable count of bacterial population was determined using the pour plate technique and growth estimated on plate count agar (PCA). Serial dilutions of 10^{-1} to 10^{-8} were prepared aseptically by weighing 1g of the soil sample into 9 ml of sterile distilled water and mixed thoroughly before being diluted through. About 0.1 ml aliquots from the dilutions 10^{-5} and 10^{-6}

were inoculated into or on sterile Petri dishes with already prepared PCA plates and incubated at a temperature of 35^{o}C for 24 hours. Also, 0.1ml aliquot was added to *Pseudomonas* Agar Base (PAB) supplemented with Glycerol as Carbon source for recovery of *Pseudomonas* species. Distinct green colonies appearing on the PAB were purified on Nutrient agar and transferred into slants.

2.4 Characterization of Isolates

Phenotypic characterization of the isolates recovered was done using morphological and biochemical characterization described by Holt et al. [29] and Vos et al. [30].

2.5 Screening and Degradation Studies on *Pseudomonas* Species in Enrichment Media

The *Pseudomonas* isolates were screened for their degradative ability first on mineral salt agar with 0.5% Bonny-light crude oil as sole carbon source using the vapor transfer method of Abu and Ogiji, [31]. The isolates that showed luxuriant growth on the agar plate were selected for degradative ability. The effect of varying concentrations of Bonny-light crude oil on the isolates ability to utilize the carbon source was evaluated using (0.5%, 1.0%, 1.5%. 2.0% and 2.5% v/v). Bonny-light crude oil concentrations and was monitored for 7days. The absorbance reading (OD_{600}nm) was taken after 7 days of the setup and isolates with the highest absorbance readings were selected for the degradation study.

The degradative potentials of the best hydrocarbon utilizing isolates at 2% oil to media concentration was determined in mineral salt and soil extract media for 14 days. An absorbance reading (OD_{600}nm) was used as a measure of growth and utilization of substrate. The readings were taken at time intervals using a UV spectrophotometer (SpectrumLab 752s) [32]. The soil extract media was prepared using a modified method of Provasoli et al. [33] which involves mixing 100 grammes of soil with 1000 ml of de-ionized water, followed by repeated (3 times) filtration using Whatman filter No.1 and autoclaving to eliminate all life forms while the mineral salt media was prepared using a modified Mineral Salt Medium of Kastner et al. [34] containing (0.2 g of $MgSO_4.7H_2O$, 0.02 g of $CaCl_2$, 1 g of KH_2PO_4, 1 g of K_2HPO_4, 1 g of NH_4NO_3 and 2 drops of 60% $FeCl_3$) in 1000 ml of sterile distilled water. Extraction and the analysis of the residual oil after the degradation studies was done using Solvent Extraction and GC/MS method of Adebusoye et al. [35].

2.6 Effect of Heavy Metals on Degrading Microbes

The tolerance of the degrading isolates to heavy metals was investigated using heavy metal salt solutions of Cobalt (Cobalt Chloride), Mercury [Mercury (II) oxide], Zinc (Zinc Sulphate) and Lead (Lead nitrate) by a modified method of Hemalatha and Veeramanikandan [36]. Filter paper disks (7mm) were soaked in heavy metal solutions prepared in 1, 3 and 5mM concentrations overnight. The disks were then allowed to dry and subsequently sterilized. Each plate was spread with overnight cultures of crude oil degrading test isolates. Distilled water was used as control. These plates were incubated at 28℃ for 48 hours, at which time the zones of inhibition were measured. A zone size greater than 1 mm was recorded as sensitive [37].

2.7 Plasmid Profiling

The presence of plasmids in the degrading species was also investigated adopting the method described by Sambrook and Russel [38]. After electrophoresis on a 0.7% horizontal agarose gel at 50 V for 3 h, the gels was stained with ethidium bromide and band visualized with a UV transmilluminator [39].

3. RESULTS AND DISCUSSION

3.1 Estimation of Microbial Population of Soil Samples

The total heterotrophic bacteria (THB) and total heterotrophic fungal count (THF) from the dumpsite were estimated. The values of the total heterotrophic bacteria count ranged from 1.1×10^6 CFU/g to 7.0×10^6 CFU/g while the values of the total heterotrophic fungal count ranged from 0.1×10^4 to 1.4×10^4 CFU/g. Also, the mean values of the bacteria and fungi count were 3.57×10^6 CFU/g and 0.69×10^4 CFU/g respectively (Table 1).

3.2 Phenotypic Identity of Oil Degrading Bacterial Isolates

The morphological and biochemical characterization of the isolates revealed that they were gram negative, non-spore forming, catalase, oxidase, Indole, citrate and urease

positive while it was oxidase negative and did not ferment lactose, sucrose, glucose neither did they produce H_2S hence a tentative characterization as *Pseudomonas* spp.

3.3 Screening for the Efficient Oil Degrading Bacteria

Eighteen *Pseudomonas* isolates were screened for their ability to utilize hydrocarbons (0.5% v/v Bonny-light crude oil) in mineral salt medium using the agar overlay method and it was observed that nine of the isolates gave luxuriant growth after 72 hours. The nine isolates were then observed for their growth in increasing concentrations of Bonny-light crude oil. The result showed that the isolates could grow at the various concentrations tested (0.5, 1.0, 1.5, 2.0, 2.5% concentrations of Bonny-light crude oil) but showed the highest OD_{600} at 2% Bonny-light crude oil concentration as shown in Fig. 1. Five isolates codenamed (OWPSA. OWPS11, OWPS6, OWPS5 and OWPSJ) with highest OD_{600} readings were selected for the degradation studies in mineral salts and soil extract media *in-vitro* for 14 days.

3.4 Heavy Metal Tolerance Spectrum of Selected Crude Oil Degrading Isolates

The tolerance spectrum of the nine selected *Pseudomonas* spp. in varying concentrations of heavy metals (zinc lead, mercury and cobalt) was studied using Kirby Bauer disk diffusion method and the results are presented in Table 2. All the isolates were tolerant to zinc and lead at all concentrations used. Eight of the isolates showed tolerance to Cobalt at 1 mM concentration but sensitive to higher concentrations of cobalt, only one isolate OWPs11 showed tolerance to all concentrations of cobalt, lead and zinc but was sensitive to the concentrations of mercury. Isolates OWPs1 and OWPs6 showed tolerance to 1 mM concentration of mercury while other isolates were inhibited by the concentration although mercury concentrations 2 mM and 5 mM inhibited the growth of all the isolates at varying degree as shown in Table 2. The zone of inhibition was measured in millimetres.

Table 1. Total bacterial and fungal counts of the waste dumpsite soil

Sampling sites	Bacterial count ($\times 10^6$ CFU/g)	Fungal count ($\times 10^4$CFU/g)
OW1	2.0	0.1
OW2	4.6	0.9
OW3	1.1	1.4
OW4	7.0	0.35
OW5	3.15	0.69
Mean value	3.57	0.69

Legend: OW = Organic waste dumpsite soil

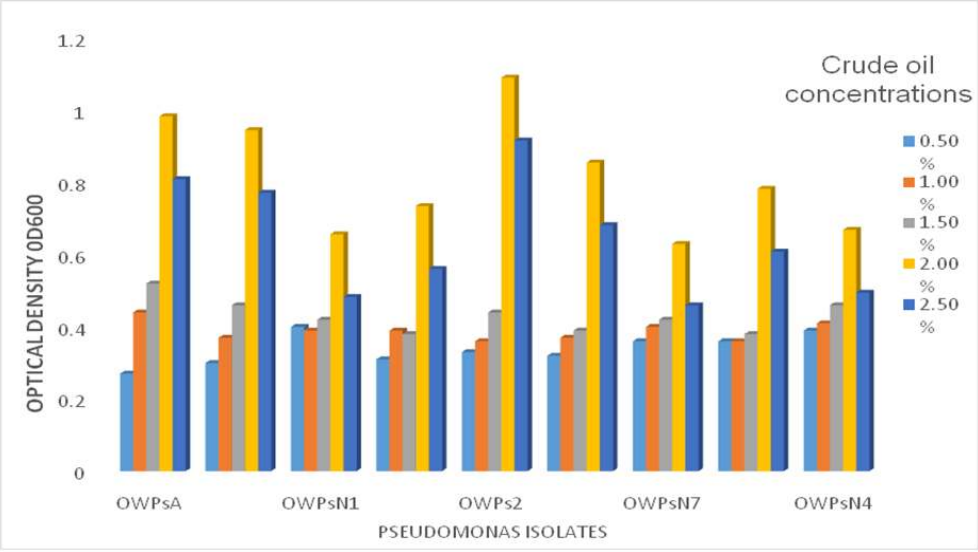

Fig. 1. The utilization potential of *Pseudomonas* isolates in varying concentrations of Bonny-light crude oil in mineral salt medium after 7 days

Table 2. Tolerance in millimetres of potential hydrocarbon degrading *Pseudomonas* spp. to varying concentrations of some heavy metals

Heavy metal sources	Conc. mM	OW PS1	OW PS2	OW PS4	OW PS5	OW PS6	OW PS7	OW PS11	OW PSA	OW PSJ
Cobalt	1	8	0	0	0	0	0	0	0	0
(Cobalt	3	10	7	10	9	11	8	0	11	10
chloride)	5	12	10	17	13	14	11	0	14	11
Mercury	1	0	4	8	4	0	4	0	4	4
(Mercury II	3	6	4	9	6	4	4	2	7	6
oxide)	5	8	8	10	7	6	8	6	10	9
Lead	1	0	0	0	0	0	0	0	0	0
(Lead	3	0	0	0	0	0	0	0	0	0
nitrate)	5	0	0	0	0	0	0	0	0	0
Zinc	1	0	0	0	0	0	0	0	0	0
(Zinc	3	0	0	0	0	0	0	0	0	0
sulphate)	5	0	0	0	0	0	0	0	0	0

Key: OWPS: Organic waste dumpsite soil Pseudomonas spp.

3.5 Growth Patterns of Hydrocarbon Utilizing *Pseudomonas* spp. in Different Media

The growth patterns of the selected five *Pseudomonas* species in mineral salts medium supplemented with 2% Bonny-light crude oil was monitored by measuring the optical density OD_{600} for 14 days. The treatments were observed to increase in turbidity over the 14 days with evident fluorescing in the medium indicating activity of the *Pseudomonas* isolates. Most of the isolates' OD_{600} readings peaked on day 7 with similar patterns except isolate OWPS6 which peaked on day 5 with value 1.392. Isolates OWPS11 and OWPS6 had the best growth patterns after 12 days and were selected for gas chromatographic analysis for the determination of residual oil content of their degradation after 14 days (Fig. 2).

Their growth pattern was also monitored in Bonny-light crude oil supplemented soil extract broth. Soil extract broth is a nutritionally weak medium that mimics the nutrient availability in soil. It was observed that the optical density values (OD_{600}) of the media were not as high when compared to those observed in the mineral salt medium although there was fluorescent pigment production into the medium. *Pseudomonas* isolates OWPS11 and OWPSJ had the highest OD_{600} readings indicative of Bonny-light crude oil utilization after day 9 with values of 0.635 and 0.631 respectively while OWPSJ had the highest optical density value on the 14^{th} day of degradation studies but OWPS2 dropped. Isolate OWPS1 had the lowest OD reading value after day 9 and 14 respectively Hydrocarbon degradation profile was analyzed using GCMS (Fig. 3).

3.6 Analysis of Biodegradation Data

The chemical analysis of basic organic contents in the control (untreated sample) by GC-MS revealed a very complex mixture of hydrocarbons. The analysis detected about 46 compounds with carbon range C_8-C_{31} in the mixture with retention time ranging from 4 – 28 minutes. Little amounts of unsaturated compounds was detected, primarily C18 and C19 (mainly octadecenoic acid and their alkylated derivatives) compounds. Compounds of carbon numbers of less than 10 were lost during the incubation due to evaporation. The gas chromatographic profile of the residual oil of selected treatments in both mineral salt and soil extract media showed that there was reduction in the components as compared to the control treatments (Fig. 4a-4e). Comparing the treatments with the control's chromatogram, some compounds were completely degraded by the isolates while some of the compounds were transformed into other compounds (Table 3). Many peaks disappeared because of biodegradation, some peaks were markedly reduced and new peaks appeared at different retention times, this is an indication of the transformation of original fractions.

Isolate *Pseudomonas* OWPSJ was the most efficient, degrading most of the crude oil fractions with complete loss of 18 peaks as compared to the control chromatogram. Also, fractions with

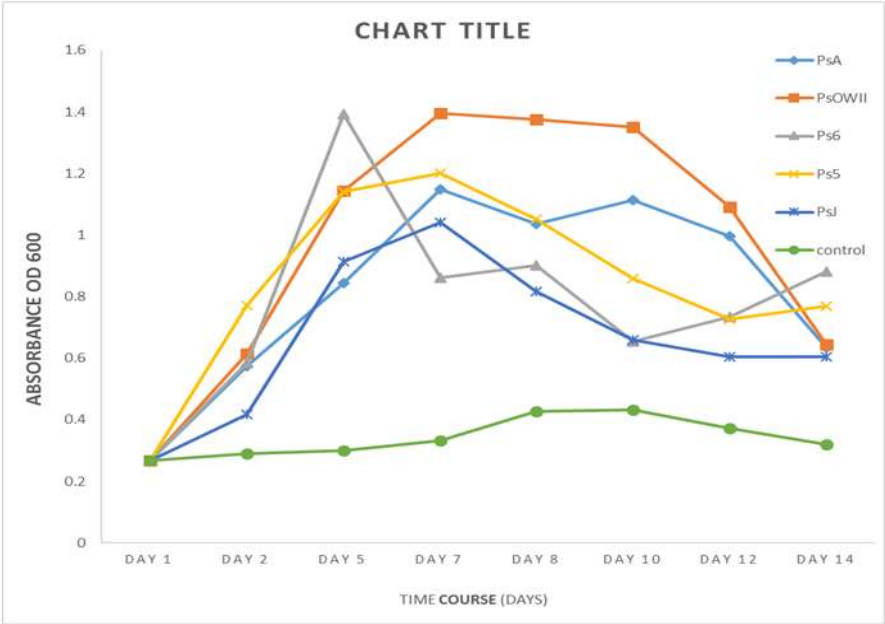

Fig. 2. Time course and growth pattern of *Pseudomonas* species in mineral salt medium supplemented with 2%w/v Bonny-light crude oil

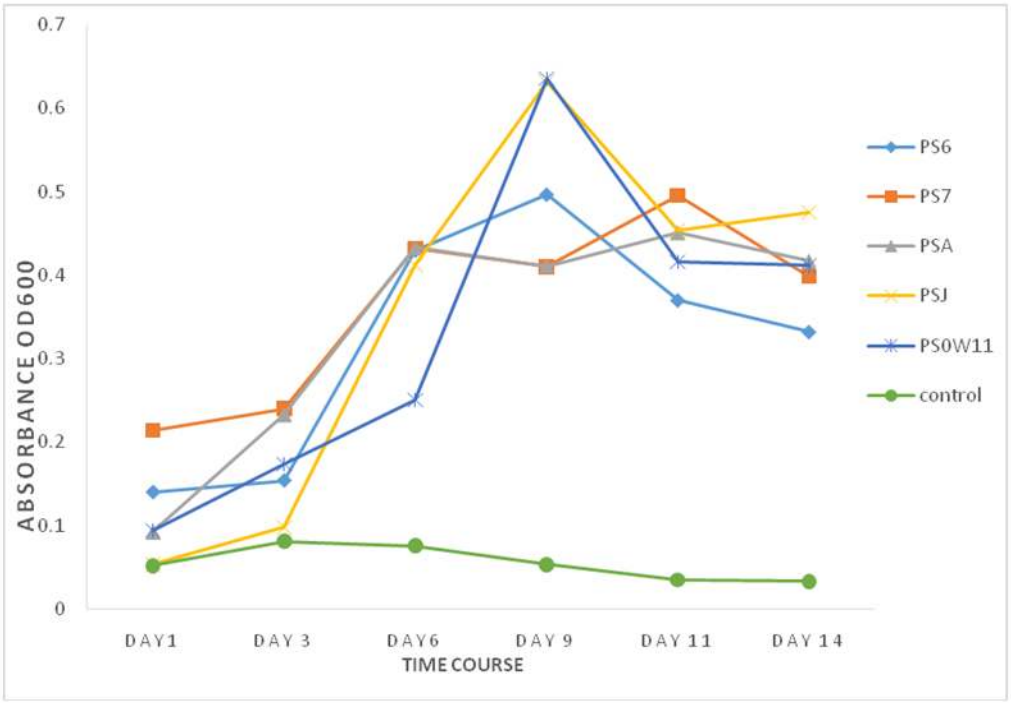

Fig. 3. Time course and growth pattern of *Pseudomonas* species in soil extract medium supplemented with 2% v/v Bonny-light crude oil

carbon atoms C8- C13 completely removed while there was substantial disappearance in most of the heavier fractions (C_{18} -C_{31}) (Table 2). Also, it was able to utilize the aromatic fractions (o-xylene, 1, 2, 4-trimethyl benzene completely while naphthalene fraction was greatly reduced

as observed by the peak abundance) (Fig. 4b). However, fractions 2, 6, 10, 15- tetra-methyl-heptadecane and 2, 6, 10- trimethyl pentadecane were not effectively utilized as its percentage increased after degradation suggesting it was a residual product of degradation by the isolate (Table 3).

Also, treatment with isolate *Pseudomonas* OWPS11 in soil extract media revealed degradation of aliphatic fractions but reduced utilization of the aromatic fractions as shown in Table 4 and Fig. 4c. It degraded completely the short chain alkanes effectively but it was observed that the degradation of this strain was limited (Fig. 4c and Table 4). Also, in the mineral salt media treatments with isolates *Pseudomonas* sp. OWPS11 and OWPS6, the chromatography profile showed that the OWPS6 degraded more efficiently than the OWPS11 as shown in Fig. 4d and 4e respectively.

Pseudomonas sp. OWPS6 degraded the short chain aromatic and alkane fractions completely as revealed in the mass spectra profile (Fig. 4b) but did not effectively metabolize the middle chain fractions that included the methylated naphthalenes, and the substituted alkanes (C12-19) which is usually resistant to microbial actions. However, it degraded the heavier fractions into esters and alcohols (Table 6). It was observed that there was an increase in the abundance of 2,6- dimethyl heptadecane than what was observed in the control (Table 4), this could be as a result of the compound being an intermediate end product that could not be degraded further. Also some new compounds were formed as a result of the activity of the isolate (C28 compound octacosane and 4-bromo-n-butyl 2-Piperidione) (Table 5).

Pseudomonas sp. OWPS11 could only utilize the short chain hydrocarbons and transformation of the long chain alkane and aliphatic compounds into newer compounds which might be undegradable for the strain. This was unexpected given the high optical density reading recorded over the 14 days of investigation (Fig. 2). This suggests that optical density readings might not reflect accurately the degradation of crude oil in crude oil supplemented media. It was observed that more compounds were detected in the analysis of treatment with *Pseudomonas* OWPS11 in the soil extract media than what obtained in the control while *Pseudomonas* OWPSJ had less compounds detected after the degradation process (Table 4). However, all the isolates except *Pseudomonas* OWPS11 in the soil extract media could degrade polycyclic aromatic hydrocarbon (simple PAH) fractions of the crude oil. It was also discovered that when comparing the degradation in the two media setups, the treatments in soil extract media yielded higher degradation that in the conventional mineral salt media as revealed by the gas chromatography profile.

The three isolates *Pseudomonas* OWPS6, OWPSJ and OWPS11 capable of degrading Bonny light crude oil were analysed for the presence of plasmids which often serve as carriers of degradative genes. It was observed that all the three isolates did not possess plasmids as revealed on agarose gel electrophoresis (Fig. 5).

3.7 Discussion

The ability of *Pseudomonas* species to degrade crude oil hydrocarbons was evaluated in this study. The total bacterial count ranged from 1.1×10^6 to 7.0×10^6 CFU/g which was significantly lower than what Adekanle et al. [40] estimated (11.2×10^6 to 18.7×10^6 CFU/g) but higher than the findings of Oviasogie et al. [41]. The physicochemical analysis of the dumpsite soil showed that the mean pH was slightly acidic to neutral (6.81). Adejumo [42] examined five dumpsites and found their pH to range from 5.25 to 6.75. Incidentally, the pH value range indicates the soil suitability as landfill for wastes rich in heavy metal since their mobility would not be supported by pH range as most of them will be in insoluble form and hence they are unavailable to the environment [43]. The % organic matter ranged from 2.77- 3.75 with a mean value of 3.20 while the “control soil” was 2.67%. Badmus et al. [43] reported a similar level of organic matter in dumpsite soil with 2 of the four locations having (2.39% and 3.22%) while two other locations had (8.09% and 9.14%). This higher values could be due to decomposition of the wastes present in the site which included food wastes, animal dungs and market waste.

The total organic carbon of the site ranged from 1.68-1.93% and was similar to the findings of Osazee et al. [44] who evaluated dumpsites in Benin City, Nigeria and reported values ranging from 1.03-1.56% and also Oviasogie and Oviasogie, [45] who found the TOC% of 2 different dumpsites as 1.28 and 1.41% respectively. Essien and Hanson [46] indicated that a very high level of organic carbon TOC (≥5.00%) is indicative of high level of

biodegraded materials in the soil which could be as a result of microbial activity or burning. The soil electrical conductivity values ranged from 180-417 with a mean value of 265 µs/cm which is lower than that reported by Badmus et al. [43] with mean value of 608 µs/cm. Salinity, soil texture, cation exchange capacity influence the conductivity of soil and higher organic matter result in enhanced conductivity (Badmus et al. [43]. The available phosphorus was lower than what was detected by Amos-Tautua et al. [47] which ranged from 68.22-84.20 mg/kg in contrast to 32.48-41.93 mg/kg found in this study, however the% nitrogen found in this studies sample site was higher than that obtained by Amos-Tautua et al. [47] who reported a range 0.06-0.24 against 0.57-0.65 recorded in this study.

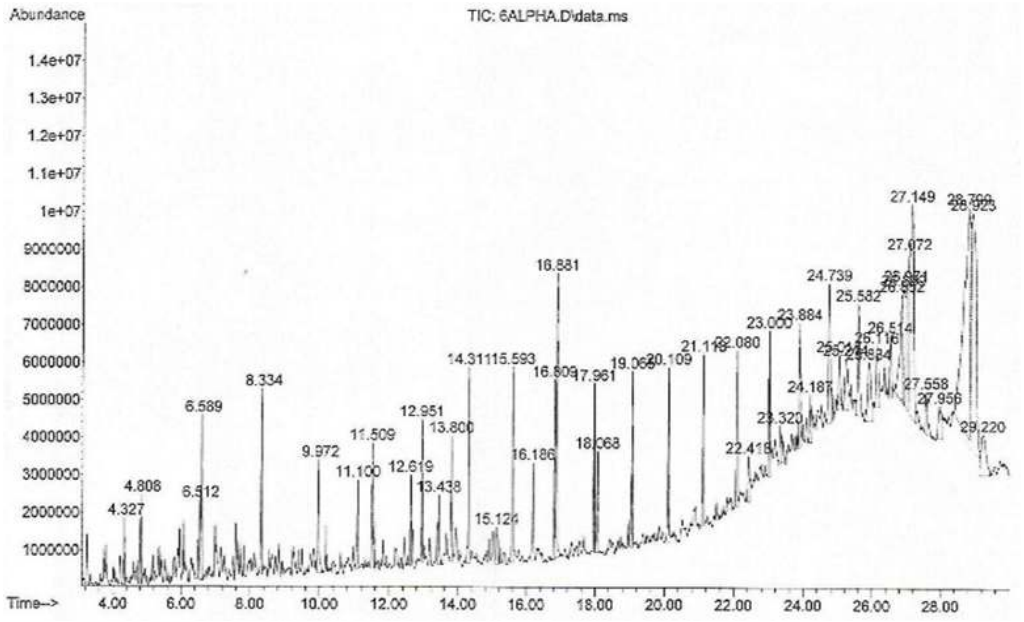

Fig. 4a. Mass spectra of untreated Bonny light crude oil sample (control)

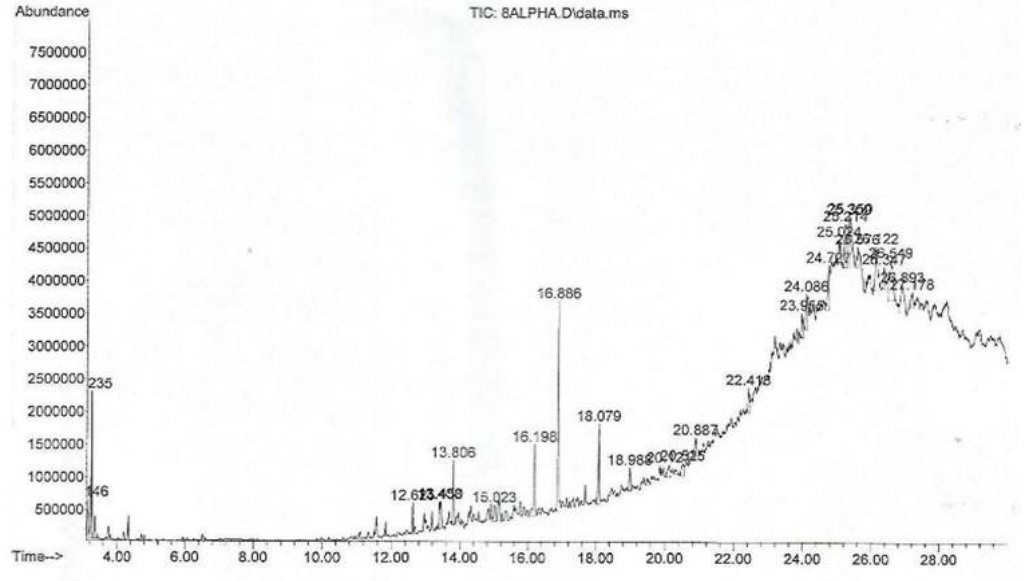

Fig. 4b. Mass spectra of treatment with *Pseudomonas* OWPSJ in soil extract medium

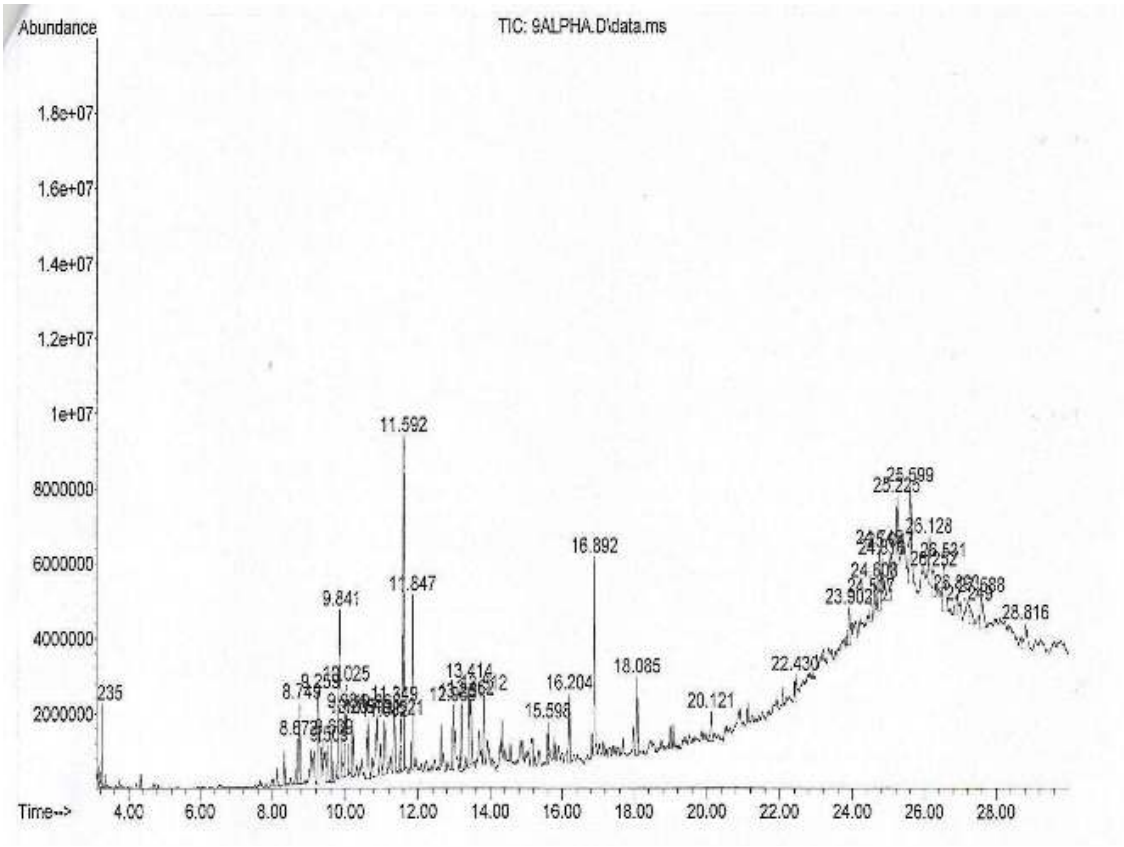

Fig. 4c. Mass spectra of treatment with *Pseudomonas* OWPS 11 in soil extract medium

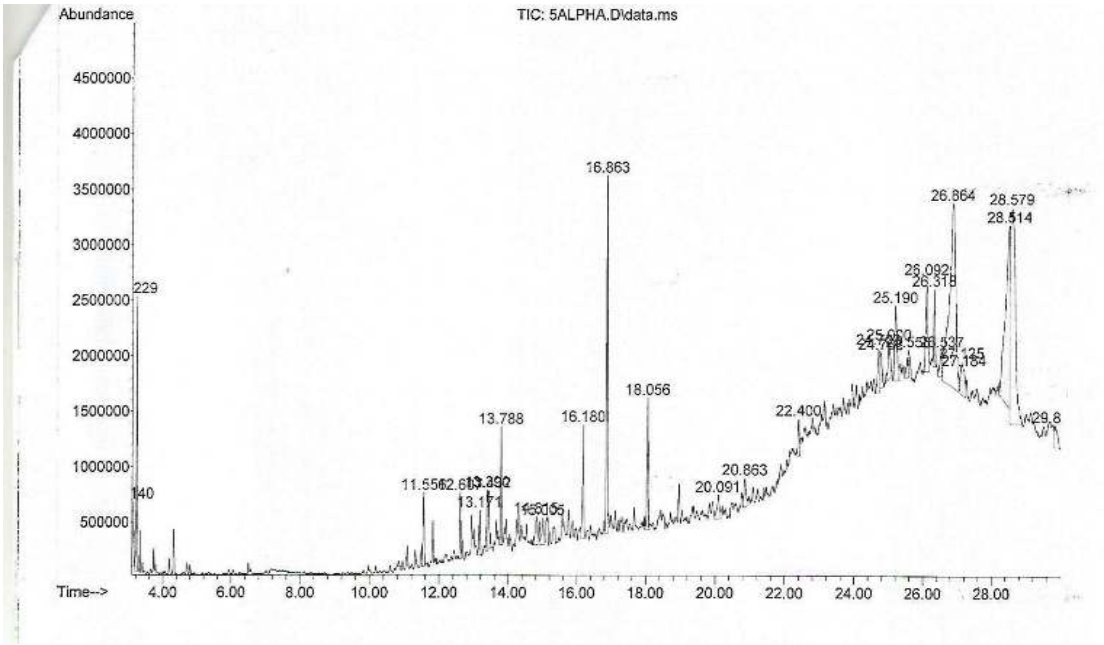

Fig. 4d. Mass spectra of treatment with *Pseudomonas* OWPS6 in mineral salt medium

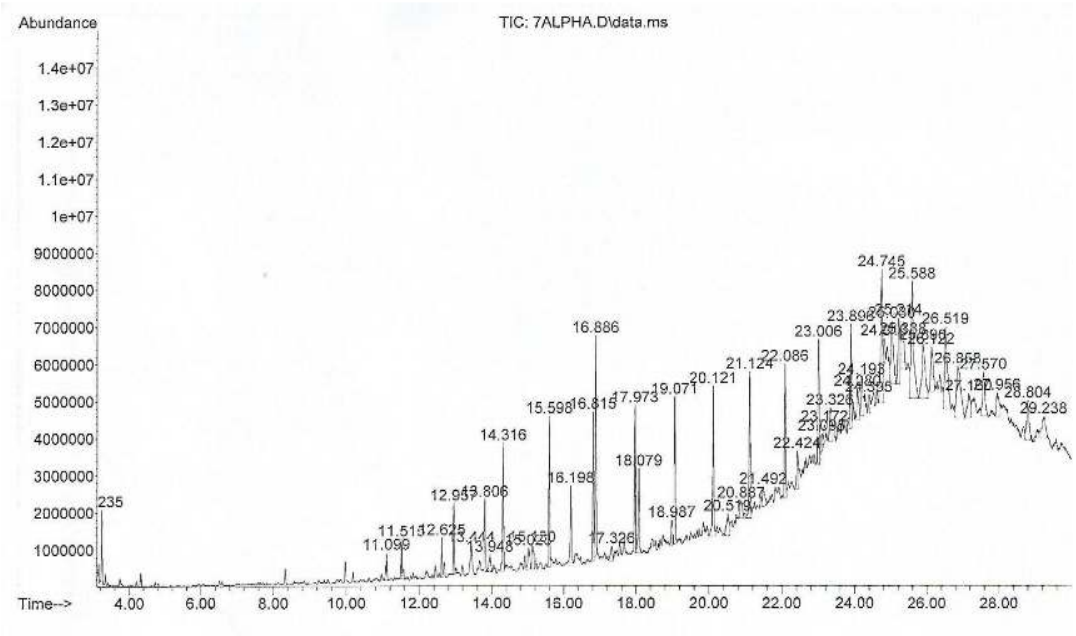

Fig. 4e. Mass spectra of treatment with *Pseudomonas* OWPS 11 in mineral salt medium

It was observed that the isolates grew optimally on 2% v/v crude oil. Several reports have evaluated the effects of varying concentrations of Bonny-light crude oil on the growth of bacteria in culture medium. Darvishi et al. [48] in their investigation using concentrations (0.25, 0.5, 1.0, 2.5, 5, 7.5 and 10% w/v) of heavy Bonny-light crude oil reported that 0.5% concentrations gave highest values for Optical density at 600 nm. However, Kumari et al. [49] observed that a *Pseudomonas* strain BP10 had maximum growth measured by OD600 nm at concentration of 2% when they supplemented MSM with different concentrations of oil (1%, 2%, 5% and 10% v/v). They found out that could survive in 10% of Bonny-light crude oil but their growth was found maximum in 2%. Their findings correlate with the observation in this current study were maximum OD600 was observed at 2% Bonny-light crude oil concentration. They also found out that the growth peaked on the 6th day with optical density of 1.8. Bouchez-Naitali et al. [50] and Kumari et al*.* [49] suggested that the success of any bioremediation process of oil spills not only depends on ability of the strains, but also on physical, chemical and biological conditions in the polluted environment.

One factor affecting the bioremediation of Bonny-light crude oil is the presence of stressors such as heavy metals that halt the biodegradative potentials of the indigenous microbiota resulting in prolonged bioremediation and the accumulation of toxic hydrocarbons in the environment [51]. Heavy metals impact the physiology of biodegrading organisms with the impact depending on the metal concentrations, the test organisms, the time of exposure and the test organisms [52]. The heavy metals tolerance spectrum of the nine crude oil utilizing *Pseudomonas* sp. was studied and it was observed that all the isolates were able to tolerate up to 5 µg/ml concentrations of lead and zinc. However, concentrations of 3µg/ml and 5µg/ml of cobalt and mercury inhibited their growth. A similar investigation by Hemalatha and Veeramanikandan [36] on the tolerance of poly-aromatic hydrocarbons degrading *Pseudomonas* sp. to varying concentrations of heavy metals observed the same pattern of tolerance to zinc and lead as well as a similar pattern to cobalt and mercury. They therefore concluded that the presence of heavy metals such as cobalt and mercury even in 1 mM concentration was highly toxic to the bacteria isolated from oil contaminated sites and they will pose serious threat to their metabolism in the environment. Heavy metal tolerance is often indicative of antibiotics resistance and usually found to be plasmid encoded [53]. Tiku et al. [54] in their investigation observed that the sensitivity exhibited by bacteria isolates including *Pseudomonas* spp. from both the petroleum polluted and pristine soil samples were

proportional to the concentration of the heavy metals utilized. They also observed that *Pseudomonas* species (AMT4), they isolated was able to tolerate high concentration (300µg/ml) of chromium, copper and lead, and did not show multiple antibiotic resistance to the antibiotics tested against, hence they could be useful in the bioremediation of hydrocarbon-heavy metal contaminated environment.

Several reports have estimated the degradative potentials of *Pseudomonas* spp. [55,56,57,58,59] most of the investigations have been on species isolated indigenous to contaminated soils [60,61]. Vecchiolli et al. [62] established that in addition to autochthonous hydrocarbon degrading bacteria in soils, exogenous microbial inoculation is able to speed up the biodegradation whenever conditions are suitable. *Pseudomonas a* have been established to be able to metabolize a large number of hydrocarbons [63,64,65]. Five *Pseudomonas* spp. with the best growth measured by optical density readings at 600nm were selected for degradation studies by investigating their growth pattern in 2% v/v Bonny-light crude oil supplemented minimal and soil extract medium. Turbidity in the treatment culture flasks was observed and O.D values in mineral salt medium over the 14days of investigation were measured. The investigation revealed that the top degraders(s) recorded optimum OD values with evident turbidity of the media over time as well as visible fluorescent pigment production reflective of active metabolism of the oil as carbon source. Darsa and Thatheyus [66] investigated the potential of *Pseudomonas aeruginosa* to grow in different concentrations of petrol. They observed that the isolate was able to grow optimally at 7.5% petrol concentration. They also found out that the peaks observed in the control were missing but several new peaks with different retention time were appearing which indicated the degradation of petrol into several intermediates. Also, Silva et al. [67] in their investigation observed that *P. aeruginosa* utilized 81% of the crude oil fractions with the aliphatic compounds being degraded first before the mono and poly aromatics. Garapati [68] also mentioned that within 30 days, *P. aeruginosa* degraded 90% of light crude oil and 76% of heavy crude oil. Linda and Bouziane [69] also reported that the biodegradation rate of petrol by *P. aeruginosa* was 88.5% however, elevating the concentration caused to decrease the biodegradation rate. Vinothini et al. [70] observer that *Pseudomonas putida* recovered in their investigation showed the highest degradation of crude oil with up to 70% degradation after 7 days of incubation hence establishing the suitability of species of *Pseudomonas* as excellent degrades.

The gas chromatography fingerprint of the treatments confirmed that there was utilization of Bonny-light crude oil by the all the selected *Pseudomonas* isolates both in the mineral salt and soil extract media. There is a dearth in information reporting the use of soil extract media for degradation studies although it has been reportedly used for the cultivation of soil bacteria that are unculturable on conventional media [71]. The results obtained in this investigation using soil extract broth revealed that it can be used as an alternative to conventional mineral salt media as it is naturally optimized for the growth of soil bacteria by provides all the essential nutrients required for growth of soil microorganisms with the Bonny-light crude oil serving as the carbon source [72].

Isolate OWPSJ exhibited the highest degradation as shown in Fig. 4b when compared with the control profile (Fig. 4a). There was marked reduction in the abundance of the most of Bonny-light crude oil hydrocarbon contents as well as total removal of some of the compounds. Also, isolate OWPS11 and OWPS6 degraded large fractions of the Bonny-light crude oil with OWPS11 utilizing the heavier fractions as shown by the GC/MS profile' reduction in peak of compounds with retention time from 18.4 to 28 minutes.

Alkanes in the C10 to C26 range are considered the most readily and frequently utilized hydrocarbons Venosa and Zhu [73] and this is similar to the findings in this study with the rapid utilization of alkanes fractions by all the isolates screened and isolate *Pseudomonas* OWPSJ degrading most of the alkane fractions in the Bonny-light crude oil treatment. Branched alkanes are less readily degraded in comparison to *n*-alkanes and this was also observed in this experiment in all the treatments with moderate to high residual branches of alkane fractions. Although the aromatics are generally more resistant to biodegradation, some low molecular-weight aromatics such as naphthalene may actually be oxidized before many saturates. Alkylated aromatics are degraded less rapidly than their parent compounds; the more highly alkylated groups are degraded less rapidly than less alkylated ones. Ptacek et al. [74] reported

that many of the petroleum degrading bacteria produce extracellular emulsifying agents. *P. aeruginosa* produce rhamonlipids during growth on hydrocarbons which is necessary to emulsify aromatic and aliphatic hydrocarbons. Thavasi et al. [75] also observed that biosurfactant produced by *P. aeruginosa* was able to emulsify xylene, naphthalene and anthracene hence its ability to degrade these crude oil hydrocarbons.

It was observed that the degradation by this isolates measured by the reduction in the peak which correlates to loss of hydrocarbon components of the Bonny-light crude oil in soil extract medium was striking than in the mineral salt medium that is commonly used for degradation studies given that the optical density readings wasn't as high as what obtained in the treatments of the mineral salt media. The high optical density readings recorded in the mineral salt medium could have been influenced by undissolved salts used to formulate the medium. The soil used for the preparation of the media was from the site where the organisms were isolated suggesting that the organisms were well adapted to the growth conditions that the soil provided. Slater et al. [76] suggested that carbon sources that are more labile than PAHs are often present in the natural soil environment and microorganisms may preferentially utilize natural organic matter (NOM) resulting in the persistence of contaminants however reports from this study doesn't agree with that finding because the rate of utilizing Bonny-light crude oil fractions was established using the gas chromatography (GC-MS) profile.

Traditionally, most genes that are responsible for the degradation of hydrocarbons are expected to be located on the Chromosomal DNA [77]. Bacterial species with either chromosomal or plasmid-borne genes capable of crude oil hydrocarbon metabolism are well documented. The most extensively characterized gene is encoded by the *Pseudomonas putida Gpo1* [78]. The role of plasmid is also well documented in the bacteria communities, especially in the *Pseudomonads*. *Pseudomonas* spp. are the most common aerobic soil bacteria which possess diverse aliphatic and aromatic hydrocarbon degradative genes most of which have been shown to be plasmid-borne [79,80,81]. The investigation in this study on the likely presence of plasmids in conferring the ability to degrade Bonny-light crude oil revealed that no plasmids were present in the isolates screened. Catabolic genes that encodes different aromatic hydrocarbon degradation pathways, are frequently located on plasmids, although degradative genes can be located on either chromosome or plasmid [39]. Most studies have reported that hydrocarbon degrading species harbors plasmids which confers the ability to biodegrade xenobiotic compounds especially Bonny-light crude oil hydrocarbons [82,75,83]. Presence of catabolic genes responsible for the degradation of naphthalene in plasmid of *Pseudomonas putida* was reported by Park et al. [84]. However, Amund et al. [85] in their study on the utilization of alicyclic compounds observed that the ability wasn't conferred by plasmids and was probably catalysed by chromosomally encoded enzymes.

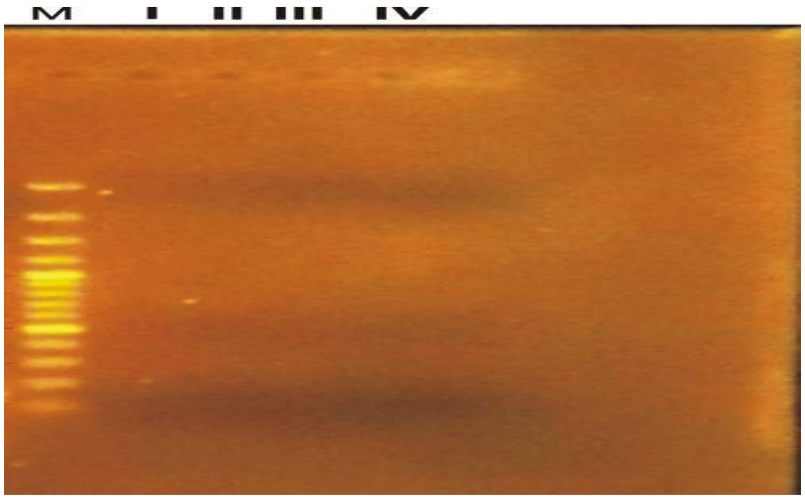

Fig. 5. Agarose gel electrophoresis of plasmids

Table 3. Compounds (peaks) completely (100%) removed from the treatments by *Pseudomonas* spp. activities on Bonny light crude oil after 14 days

S/N	***Pseudomonas*** **OWPSJ (SEM)**	***Pseudomonas*** **OWPS11 (SEM)**	***Pseudomonas*** **OWPS6 (MSM)**	***Pseudomonas*** **OWPS11 (MSM)**
1.	O- xylene	O- xylene	O- xylene	O-xylene
2.	Nonane	Nonane	Nonane	Nonane
3.	1,2,4 trimethyl benzene	1,2,4-trimethyl-Benzene	1,2,4-trimethyl-Benzene	1,2,4-trimethyl- Benzene
4.	Decane	Decane	Decane	Decane
5.	Undecane	Undecane	Undecane	Undecane
6.	Dodecane	Hexadecane	Dodecane	Dodecane
7.	4,6, dimethyl dodecane	2, 6, 11, 15-tetramethyl hexadecane	4,6-dimethyl-Dodecane	Lanosta -8, 24-diene-3-ol, acetate, 3.beta.)-
8.	Tridecane	Eicosane	Nonadecane	urs-12-en-24-oic acid, 3-oxo-, methyl ester, (+)-
9.	Nonadecane	2, 6, 10, 15-tetramethyl heptadecane	2,6,10,15-tetramethyl-heptadecane	
10.	Tetradecane	Heptadecane	Tetradecane	
11.	Hexadecane	Heneicosane	2,3,6-trimethyl-Naphthalene	
12.	2,6,10,15-tetramethyl heptadecane	Nonadecane	Hexadecane	
13.	Heptadecane	2-(octadecyloxy) Ethanol	Tetracosane	
14.	Heneicosane	11-decyl-Heneicosane	Nonadecane	
15.	Tricosane		2,6,10,15-tetramethyl-heptadecane	
16.	Lanosta -8, 24-diene-3-ol,acetate,3.beta.)-		Hexadecane	
17.	Eicosane		Heptadecane	
18.	2-Octadecyloxy- ethanol		Heneicosane	
19.	11 Decyl-heneicosane		1-Formyl-2-, 2-dimethyl-3-trans-(3-methyl-but-2-enyl) -6-methylidene-cyclohexane	
20.			Lanosta- 8, 24-dien-3-ol, acetate, 3.beta.)	

SEM: Soil extract medium treatment; MSM: Mineral salts medium treatment

Table 4. Residual degraded compounds in treatments and their percentages in relation to the compounds in undegraded crude oil

S/N	Control (untreated sample) 100%	*Pseudomonas* OWPSJ in soil extract media	*Pseudomonas* OWPS11 in soil extract media	*Pseudomonas* OWPS6 in mineral salt media	*Pseudomonas* OWPS11 in mineral salt media
1.	1-hexacosene	-	-	230%	445%
2.	2-(octadecyloxy) ethanol	-	-	-	29.23%
3.	2, 6, 10-trimethyl dodecane	436.7%	205%	249.4%	159.5%
4.	2, 6,10-trimethyl pentadecane	403%	178.2%	198.3%	153%
5.	2, 6-dimethyl-naphthalene	-	233.3%		
6.	Dodecane	-		-	263.8%
7.	Eicosane	-	88.9%	120%	177%
8.	Heptadecane	-			322.3%
9.	Hexadecane	-	23.2%		228.5%
10.	Lanosta-8,24-diene-3-01, acetate, 3.beta.)-	-	-	218.3%	-
11.	Nonadecane		20.3%	30.2%	81.3%
12.	Tetracosane	-	22.3%	-	141.5%
13.	Tricosane		-	140.8%	417.8%
14.	Tridecane	-	120%	-	64%
15.	Urs-12-en-24-oic acid, 3-oxo-, methyl ester, (+)-	-	-	55.2%	-

Table 5. Comparison of compounds found in analyzed untreated oil samples and residual fractions of treatments with *Pseudomonas* spp.

S/N	Control (untreated sample)	*Pseudomonas* OWPSJ in soil extract media	*Pseudomonas* OWPS11 in soil extract media	*Pseudomonas* OWPS6 in mineral salt media	*Pseudomonas* OWPS11 in mineral salt media
1.	1, 2, 4-trimethyl-benzene	N/D	N/D	-	-
2.	11-decyl- octacosane	N/D	N/D	-	-
3.	1-formyl-2-,2-dimethyl-3-trans-(3-methyl-but-2-enyl)-6-methylidene-cyclohexane	-	-	-	-
4.	1-hexacosene	-	-	D	D
5.	1-iodo hexadecane	-	-	-	-
6.	2-(octadecyloxy) ethanol	-	-	-	D
7.	2-(octadecyloxy) ethanol,	N/D	N/D	-	-
8.	2, 3, 6-trimethyl naphtalene	-	-	-	-
9.	2, 6, 10, 15-tetramethyl heptadecane	N/D	-	-	-
10.	2, 6, 10, 15-tetramethyl heptadecane	N/D	N/D	-	-
11.	2, 6, 10, 15-tetramethyl heptadecane	-	-	-	-
12.	2, 6, 10-trimethyl dodecane	D	D	D	D
13.	2, 6, 10-trimethyl pentadecane	D	D	D	D
14.	2, 6, 11, 15-tetramethyl hexadecane	-	N/D	-	-
15.	2, 6-dimetyl-naphthalene	-	D	-	-
16.	4,6-dimethyl dodecane	N/D	-	-	-
17.	Decane	N/D	N/D	-	-
18.	Dodecane	N/D	-	-	D
19.	Eicosane	N/D	N/D	D	D
20.	Eicosane	N/D	D	-	D
21.	Heneicosane	N/D	N/D	-	-
22.	Heptacosyl acetate	-	-	-	-
23.	Heptadecane	-	N/D		D
24.	Hexadecane	N/D	N/D		D
25.	Hexadecane	N/D	D		D
26.	Hexadecane	-	-	-	D
27.	Lanosta-8, 24-dien-3-01, acetate, 3.beta.)	-	-	-	-
28.	Lanosta-8, 24-diene-3-01, acetate, 3.beta.)-	N/D	-	D	-
29.	Nonadecane	N/D	N/D	-	D

S/N	Control (untreated sample)	*Pseudomonas* OWPSJ in soil extract media	*Pseudomonas* OWPS11 in soil extract media	*Pseudomonas* OWPS6 in mineral salt media	*Pseudomonas* OWPS11 in mineral salt media
30.	Nonadecane	-	D	-	-
31.	Nonadecane	-	-	D	D
32.	Nonane	N/D	N/D	-	-
33.	Noneicosane	-	-	-	-
34.	Octacosane	-	-	-	-
35.	O-xylene	N/D	N/D	-	-
36.	Pyridine-3-caboxamide, oxime, n-(2-trifluoromethylphenyl)	-	-	-	-
37.	Tetracosane	-	D	-	D
38.	Tetracosane	-	-	-	D
39.	Tricosane	N/D	-	D	D
40.	Tridecane	-	D	-	D
41.	Undecane	N/D	N/D	-	-
42.	Urs-12-en-24-oic acid, 3-oxo-, methyl ester, (+)-	-	-	D	-
43.	Urs-12-en-24-oic acid, 3-oxo-, methyl ester, (+)-	-	-	-	-

Key: ND: Not detected (peak removed), D: compounds found in the control and in the respective treatment

Table 6. New compounds formed after degradation by the selected *Pseudomonas* isolates

S/N	*Pseudomonas* OWPSJ in soil extract media	*Pseudomonas* OWPS11 in soil extract media	*Pseudomonas* OWPS6 in mineral salt media	*Pseudomonas* OWPS11 in mineral salt media
1.	1-ethyl,2-methyl cyclo-Pentane	3-ethyl-Hexane	1-ethyl 2-methyl cyclo-Pentane	Octane
2.	Octane	1,2,3,5-tetramethyl Benzene	Octane	2,6-dimethyl- Octane
3.	3,7-dimethyl Nonane	1,2,4,5-tetramethyl-Benzene	1-methyl-naphthalene	2,3-dimethyl-Naphthalene
4.	2,3-dimethyl Naphthalene	2,4-dimethylstyrene	2,7-dimethyl-naphthalene	Decahydro-4,4,8,9,10-pentamethyl-Naphthalene
5.	1,6- dimethyl Naphthalene	3-methyl-undecane	2,7-dimethyl-naphthalene	17-pentatriacontene
6.	Tritetracontane	1-methylpropyl-1-methyl-4-Benzene	1,4,5-trimethyl-naphthalene	4-bromo-n-butyl 2-Piperidione
7.	1-bromo heptadecane	Naphthalene	3,6-dimethyl-decane	3,8-dimethyl-decane
8.	Hexatriacontyl pentaflouropropionate	Pentamethyl-Benzene	4-bromo-n-butyl 2-Piperidione	(1-hexyltetradecyl)-cyclohexene
9.	Dotriacontyl pentaflouropropionate	Pentamethyl-Benzene	9-hexacosene	1-bromo-Octadecane
10.	2-chloropropionic acid, octadecyl ester	1-ethyl-1-propenyl-Benzene	Octatriacontyl pentaflouropropionate	Octatriacontyl pentaflouropropionate
11.	Octatriacontyl pentaflouropropionate	4,7-dimethyl-2,3-dihydro-1H-indene	Hexatriacontyl pentaflouropropionate	Hexatriacontyl pentaflouropropionate
12.	Octatriacontyl triflouroacetate	4,7-dimethyl-2,3-dihydro-1H-indene	(-)- Globulol	z-14-Nonacosane
13.	Octatriacontyl pentaflouropropionate	Pentamethyl-Benzene	Trichlorooctadecyl- Silane	Z-12-Pentacosene
14.	Tricontyl acetate	2-methyl-Naphthalene		Octatriacontyl pentaflouropropionate
15.	z-14-nonacosane	5-phenyl-bicyclo- (2.2.1 heptene)		Tetratriacontyl triflouroacetate
16.	Octacosyl triflouroacetate	2,3-dimethyl-Naphthalene		Octatriacontyl triflouroacetate
17.	z-14-nonacosane	Triacontyl acetate		1-iodo octadecane
18.	Cyclotriacontane	octatriacontyl pentafluoropropionate		Octatriacontyl pentaflouropropionate
19.	Hexatriacontyl pentaflouropropionate	z-12-pentacosene		
20.	Octatriacontyl pentaflouropropionate	Triacontyl acetate		
21.	Tetratriacontyl heptaflourobutyrate	Octatriacontyl pentafluoropropionate		
22.		Dotriacontyl pentafluoropropionate		

The ability of these isolates to utilize Bonny-light crude oil even without prior exposure to petroleum contaminants is remarkable given that they were isolated from organic waste dumpsite although such sites may contain some amounts of hydrocarbons. Methane gas is the most prominent of hydrocarbons in dumpsite environments due to the anaerobic degradation of organic matter. Amuda and Adelowo-Imeokparia [86] found traces of polycyclic aromatic hydrocarbons in ashes of waste dumpsites in Lagos metropolis. Also, Ogunyemi et al. [87] analysed dumpsite soils in Lagos metropolis and found the total hydrocarbon content to range from 0.18 to 0.65 mg/kg.

4. CONCLUSION

The ability of *Pseudomonas* spp. for bioremediation has been well documented and affirmed by this current study. These bacterial strains were able to degrade Bonny-light crude oil fractions with Pseudomonas spp. OWPSJ being the most efficient of the isolates screened after 14 days removing various fractions and reducing more recalcitrant components of Bonny-light crude oil. These suggest the strain may be effectively used for bioremediation of oil contaminated sites. Further research into the use of soil extract media for the screening of biodegrading species should be conducted giving the findings obtained from its use in this study.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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