Comparative study on physicochemical parameters study of oil polluted sites and hydrocarbon degradation potentials of heterotrophic bacteria in sourthern Nigeria

ABSTRACT

In this study, comparative study on physicochemical parameters study of oil polluted sites and hydrocarbon degradation potentials of heterotrophic bacteria in sourthern Nigeria

hydrocarbon degradation potentials of heterotrophic bacteria isolated from oil-polluted soil were examined; Samples were collected from Sakpenwa, an oil producing community in Tai LGA of Rivers State, The gravimetric analysis showed the bacteria were capable of utilizing 96.9-99.7% the oil samples, The amount of hydrocarbon in the soil samples were determined using Gas Chromatography-Flame Ionization Detector, GC- FID. Statistical analysis was carried out using Statistical Package for Social Sciences (SPSS, version 20.0). Analysis of variance (ANOVA) carried out at 95% level of confidence software that the degree of hydrocarbon degradation varied amongst isolates, *Pseudomonas aeruginosa* and *Alcaligenes* sp. showed highest degrading activities while *Bacillus subtilis* sp. showed least activity. This study revealed that indigenous bacterial species possess the requisite gene necessary for hydrocarbon biodegradation. This concluded that degradation is most often the primary mechanism for contaminant destruction including petroleum contaminants.

KEYWORDS: physicochemical parameters, hydrocarbon degradation Gas Chromatography, Analysis of variance,

INTRODUCTION

One major substance causing environmental pollution in the South-South of Nigeria is Crude oil. Annually, a large amount of petroleum hydrocarbon is being released into the marine and estuarine environments, causing great harm to many organisms, while some (microbial degraders) use them as energy source[1]. A wide range of pollutants (e.g. PAHs) can be degraded by microorganisms found within the polluted environment [2]. However, environmental factors like soil pH, moisture, temperature and nutrients affect the rate of pollutants' degradation by microorganisms in the soil. Other factors that influence pollutants' degradation are toxicity of pollutant [3]. Bacteria are primary producer in the marine food web, where their role is to recycle nutrients and breaking down of hydrocarbon [4-6]. Various bacterial species isolated from soil have been proved to degrade PAHs [7]. Other hydrocarbon degraders include *Alcaligenes* sp., *Acinetobacter* sp., *Flavobacter* sp., *Cyanobacterium* sp., *Moraxella* sp. and *Bacillus* sp (Bhattacharya *et al.*, 2002).
Hydrocarbon utilizing bacterial genera include: *Pseudomonas*, *Arthrobacter*, and *Micrococcus* [8]. Previous studies have found the evolution of some obligate hydrocarbon degraders (also named as obligate hydrocarbonoclastic bacteria) of indigenous marine bacterial genera [9]. In summary, only few number of this organism can degrade hydrocarbon *in situ* [6]. *Alcanivorax* sp. is a good hydrocarbon degrader because it has been proved in many parts of the world including the United States to potential hydrocarbon degrader [10]. Microorganisms are active degraders of hydrocarbon in an environment if high numbers of oil degrading microbes are present in the environment [11]. Bacteria are the prominent degraders of petroleum in oil contaminated environment [12]. A lot of bacteria utilize hydrocarbons as their energy source [10]. Years ago, bacteria were used as the easiest agent for removing petroleum contaminants off the environment [13]. Utilization of n-alkanes ranging from C10–C40 by *Acinetobacter* sp. have been discovered [14]. Isolation of bacterial species from the genus, *Mycobacterium* capable of hydrocarbon degradation in the soil has been ascertained [15]. Hydrocarbon biodegradation usually requires the consortium of species. One of the most persistent groups of organic pollutant in the ecosystem is petroleum hydrocarbons [16]. They can disrupt the food chain, leading to ecological cycle destruction [17]. This study aimed to determine the physicochemical parameters and hydrocarbon degradation potentials of heterotrophic bacteria of oil polluted sites in sourthern Nigeria.

MATERIALS AND METHODS

2.1 Study area and sample collection

The study site was located at the oil polluted sites in Sakpenwa community in Ogoni land, Tai Local Government Area, Rivers State. This community covers an area of 159 km^2 and a population of 117,797 as of 2006 population count. Soil samples were collected 500m and 1000m away from the major spill sites. Fifty grams (50g) of the oil-polluted soil samples were collected from each of the sampling points using a soil sampler. The collected soil samples were transported in plastic nylon bags from the polluted sites to the Department of Microbiology, University of Port Harcourt laboratory for analysis within 24 hours.
The control soil was collected from low land and safe environment where residential buildings are located at Ikwere community in Obio/Akpor local Government Area of River state.

Figure 1: Oil polluted sites in Sakpenwa community in Ogoni land

A point was marked at the tip where the land was highly polluted at the centre and a tape was used to measured out, 500m and 1000m respectively where samples were collected.

2.2 Samples preparation

The soil samples collected were passed through a mesh sieve (2mm pore size) to remove large particles and were thoroughly mixed. Thereafter, 5g of each soil sample was suspended in 45 ml of distilled water. The suspended samples were mixed properly in a rotary shaker at 100 rpm at room temperature (28± 2^0C) for 1hour, 30 minutes to liberate the organisms into the liquid medium [18]. The pH of the samples was also taken.

2.3 Isolation and enumeration of total heterotrophic bacteria

The total culturable heterotrophic bacterial count for each degradation set-up was enumerated using the streak plate method Odokuma and Okpokwasili [19]. Serial dilutions of the samples were made and 0.1ml aliquot of the 10^{-1} to 10^{-4} dilutions of each sample were transferred onto well dried, sterile nutrient agar plates (in triplicate) and incubated at 37°C for 24. After incubation, the bacterial colonies that grew on the plates were counted and sub-cultured onto fresh nutrient agar plates using the streak-plate method in other to obtain pure cultures of each colony. Discrete colonies on the plates were then transferred into nutrient agar slants, properly labelled and stored at 4^0C as a stock culture for preservation and identification.

2.4 Biodegradation studies

The method proposed by Ekpo and Ekpo [20] was used. The biodegradation study of hydrocarbons in the polluted soil was carried out using the Bushnell-Haas broth. This medium consist $MgSO_4$ 0.02g; CaCl 0.2g; K_2HPO_4 100g; $KHPO_4$ 1g; NH_4NO_3 1g; F_2Cl 0.05g was autoclaved in 2 litres conical flasks. 99ml of the liquid medium (Bushnell-Haas broth) was dispensed into five (5) conical flasks into which I ml of sterile crude oil was added [20]. Precisely, 5ml of each of the bacterial isolates (in liquid broth) were inoculated into five (5) different conical flasks containing the liquid medium. The concentration of day zero was use as control to the other subsequent days. The bacterial cultures were incubated at ambient temperature (4°C) in an electric shaker of 100 strokes per minute for 30 minutes each day. Sampling period was set for every 5 days for 30 days [21]. Bacterial utilization of hydrocarbon was monitored using their optical density at 600nm wavelength [20]. The total petroleum hydrocarbon was measured

2.5 Determination of Crude oil Degradation rate in Soil

The crude oil degradation rate in the soil was determined by the solvent extraction method [22]. Five grams (5g) of soil sample was mixed with 100ml of normal hexane in a flask and corked. The mixture was shaken using a mechanical shaker for 1hr, and then allowed to settle. With the use of a sterile syringe, an aliquot of the oil extract in the solvent solution (20ml) was withdrawn and put in a previously weight evaporation dish. The dish and its content were evaporated to dryness in a rotary evaporator and the dish was reweighted to obtain the difference.

The percentage (%) of the degradation was shown as follows:

Weight of residue crude oil = weight of beaker containing extracted crude – weight of empty beaker

Amount of crude oil degraded= weight of crude oil added to the media – weight of residual crude oil.

$$\% \text{ Degradation} = \frac{\text{amount of crude oil degraded}}{\text{amount of crude oil added to the media}} \times 100$$

2.6 Sample preparation for Total Petroleum Hydrocarbon (TPH) analysis

Precisely, 2ml of sample was extracted with 20ml of dichloromethane. Separating funnel used to separate the sample and the dichloromethane layer was concentrated in a rotator evaporator. 1ml of acetonitrile was added into the concentration and transferred into vial ready for analysis [23].

Fixed setting: Generally, adjusting of gas flows to the columns was done, the inlets, the detectors and the split ratio. In addition, the injector and the detector temperature were also set. The detector was held at a high end of the oven temperature range to minimize the risk of analyte precipitation. All of these parameters were set to the correct values but double checking of all the instrument was done: Buck 550 gas chromatograph equipped with an on-column, automatic injector, flame ionization detectors, HP 88 capillary column(100mm×0.25µm film thickness,) CA,USA

Detector temperature A: 250^{o} C

Injector temperature 22^{o} C

Integrator chart speed: 2cm/min

The oven was set at a temperature of 180^{o}C which warms up the Gas Chromatogram, while its warming set was:

Table 1: Temperature condition for warming the oven.

Initial temp.	Hold time	Ramp time	Final temp
70^{o} C	5mins	10mins	220^{o} C
22° C	2mins	5mins	280^{o} C

Light will turn off and you begin your run your experiment, when the instrument is ready, then Inject 1 microliter sample onto column A, using proper injection technique.

2.7 Determination of changes in Total Petroleum Hydrocarbon content of soil

The extract was analyzed using the Buck 550 gas chromatography equipped with a Field Ionization Detector (FID), High Performance (HP) 88 capillary column (100mm × 0.25 µm) with a nominal film thickness of 0.25µm, while the volume of the injection was 0.8µL at 22°C. The carrier gas was Helium at (2cm/min) because hydrocarbon is a volatile compound. The holding capacity of the column is 35°C for 1.50 min.The temperature increases gradually from 22°C min^{-1}, to 280°C min^{-1} and held for 5mins. The total sum of the components present is equal to the sum of Total Petroleum Hydrocarbon (TPH) present in the GC capillary column between of 5 to 35 min retention time [24].

2.8 Statistical analysis

Statistical analysis was carried out using Statistical Package for Social Sciences (SPSS, Version 20.0). Analysis of variance (ANOVA), P- values test of significance, was carried out at 95% level of confidence, P - values was use to determine the significance levels between various treatments and data obtained during the study.

RESULTS AND DISCUSSION

The bacterial diversity present in the Control soil, Site A (500m) and Site B (1000m) of this study was as represented in Table 2.

Table 2: Culturable bacterial diversity presents in the various study sites

Control	Polluted site (500m) away	Polluted site (1000m) away
Acinetobacter sp.	*Pseudomonas* sp.	*Alcaligenes* sp.
Alcaligenes sp.	*Bacillus* sp.	*Citrobacter* sp.
Pseudomonas sp.	*Acinetobacter* sp.	*Bacillus* sp.
Serratia sp.		*Acinetobacter* sp.
Bacillus sp.		

Physicochemical Properties of the test soil

It is an established fact that pollutants (e.g. hydrocarbons) affect the physicochemical condition of perturbed soil [25]. Table 3 showed the level of changes in the physicochemical parameters of soil caused by the pollutant and shows how the contamination level increase, the amount of nitrate and phosphorous in the soil reduce. The control soil has a high and normal quantity of nitrate and phosphorous of 58.30mg/kg, and 10.20mg/kg respectively, after 500m away, from the polluted site the quantity of the nitrate and phosphorous were 13.90mg/kg and 1.50mg/kg and to further confirm the soil was polluted, a sample was taken from 100meter away from the polluted site for further study and this shows a value of 42.70mg/kg and 3.2mg/kg of nitrate and phosphorous respectively. Essential nutrients in the soil were reduced as soil is contaminated, the decrease in nitrate and phosphate level is attributed to the fact that they were been used in the metabolism of organism in building biomass. There is a positive correlation in the utilization of both nitrate and phosphate and this indicate their importance in cell metabolism. It was establish that the availability of nitrogen and phosphorus limit the microbial degradation of hydrocarbon [26,27] and the pH of the soil varies from 7.24 from the control soil sample to 5.08 and 6.47, 500 and 1000meters away from the polluted site respectively, which shows that the soil sample was acidic. This concord with the work of [28,29] which proofs that microbial utilization of hydrocarbons often leads to production of organic acids. Thus, the acids probably produced account for the reduction in pH levels.

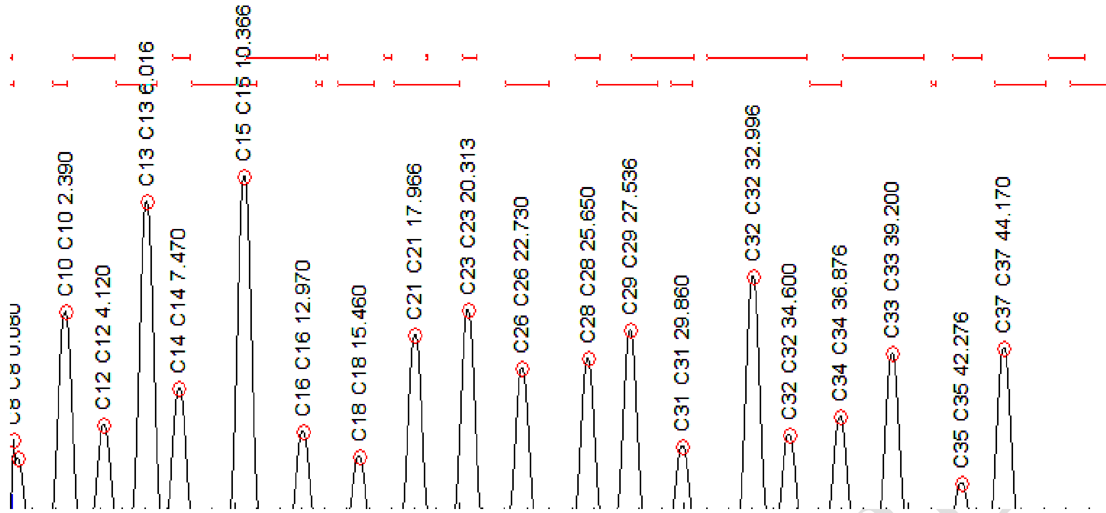
Table 3: Physicochemical parameters of the soil samples

S/N	Parameters	Control Soil	Polluted Soil 500m Away	Polluted Soil 1000m Away
1	pH	7.24	5.08	6.47
2	Conductivity (µs/cm)	126.00	750.00	1097.00
3	Sulphate (So_4) (mg/kg)	10.00	95.00	90.00
4	Moisture content (%)	0.20	2.20	0.04
5	Ammonia, (No_3) (mg/kg)	0.00	0.08	0.13
6	AmmoniaNitrogen (NH_3N) (mg/kg)	0.00	0.07	0.11
7	Nitrate (NO_3) (mg/kg)	56.30	13.90	42.70
8	Nitrate-Nitrogen (NO_3-N) (mg/kg)	13.20	3.10	9.70
9	Calcium (Ca) (mg/kg)	0.20	0.24	0.00
10	Magnesium (Mg) (mg/kg)	0.14	0.28	0.33
11	Sodium (Na) (mg/kg)	5.00	34.00	22.00
12	Potassium (K) (mg/kg)	1.30	8.00	5.08
13	Nickel (Ni) (mg/kg)	0.00	0.00	0.00
14	Mercury (Hg) (mg/kg)	0.00	0.00	0.00
15	Lead (Pb) (mg/kg)	0.31	0.21	0.29
16	Copper (Cu) (mg/kg)	0.05	0.00	0.00
17	Iron (Fe) (mg/kg)	4.13	3.93	4.33
18	Zinc (Zn) (mg/kg)	5.24	0.49	0.01
19	CEC (mg/kg)	0.56	1.70	1.17
20	Total Organic Carbon (%)	31.60	33.00	34.70
21	Phosphate (P_2O_5) (mg/kg)	31.40	4.60	9.70
22	Phosphorous (P) (mg/kg)	10.20	1.50	3.20
23	Phosphate (PO_4^{3-}) (mg/kg)	23.40	3.50	7.20
24	Ash content (%)	85.00	85.00	90.82
25	TPH, /kg	16.00	12984.00	184.80
26	Total Organic Matter	63.20	66.00	69.40

Figure 2 - Figure 5 below showed the Chromatographic profile of hydrocarbon degradation of the various hydrocarbon utilizing bacteria at the initial day, day (0) and the final day for degradation study, day 30 respectively. Thus showing the result of how the hydrocarbon was reduced by each bacterium

The hydrocarbon-utilizing bacterial genera isolated from the oil contaminated soil were *Pseudomonas, Bacillus, Acinetobacter*, *Alcaligenes and Citrobacter*. Okpokwasili and Okorie [30] isolated similar hydrocarbon utilizing bacteria from Niger Delta aquatic systems. Chikere and Okpokwasili [31] also made similar findings on petroleum effluents. It has also been observed that some microorganisms are more abundant in areas of high concentration of hydrocarbons. These micro floras are actively oxidizing the hydrocarbons and this is considered as another source of carbon for use in the ecosystem.

Results from the Gas Chromatography analyses from figure 1 to 4 shows that during the first (5) days, Total Petroleum Hydrocarbon (TPH) reduction was high due to evaporation which shows that some components of the hydrocarbon were volatile. The reductions in both the number and sizes of the peaks and height from the profiles corroborate with the report by Okpokwasili and Okorie [30] which states that they are hydrocarbon degraders which utilize the breakdown products of hydrocarbon. *Aciligenes* growth increased simultaneously with decrease in TPH throughout the 30days period monitored leading to nutrient limitation in the soil.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C23 C23 20.313
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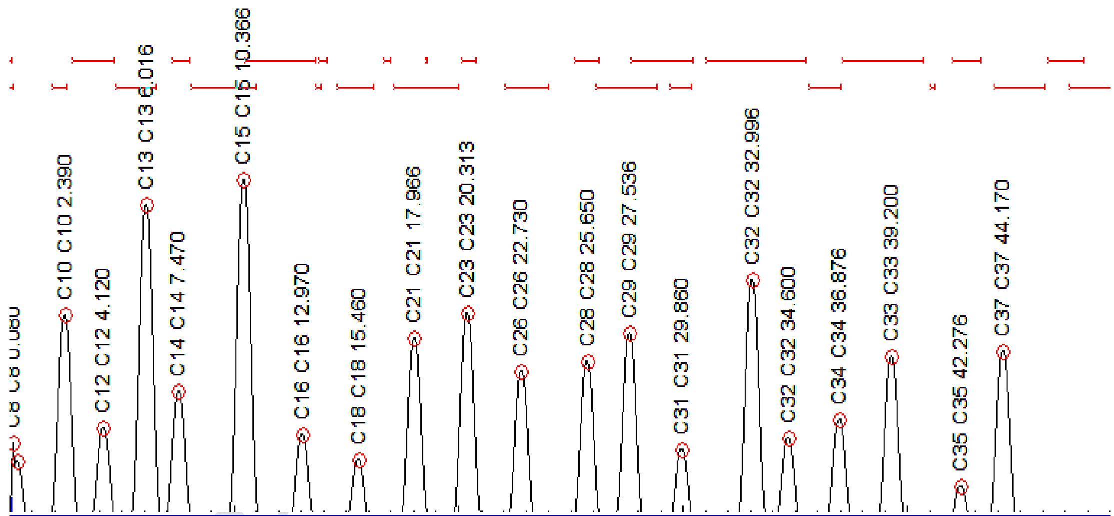
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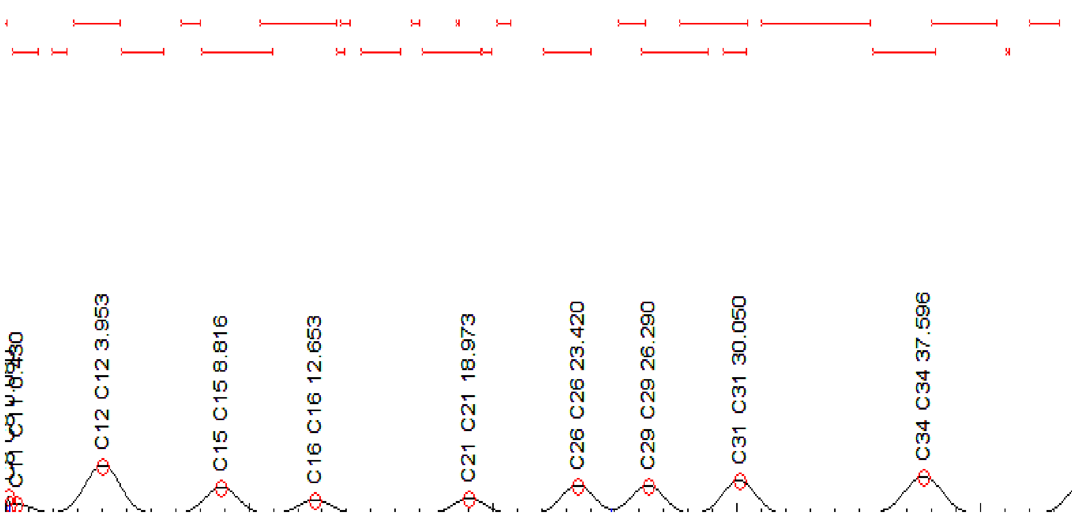
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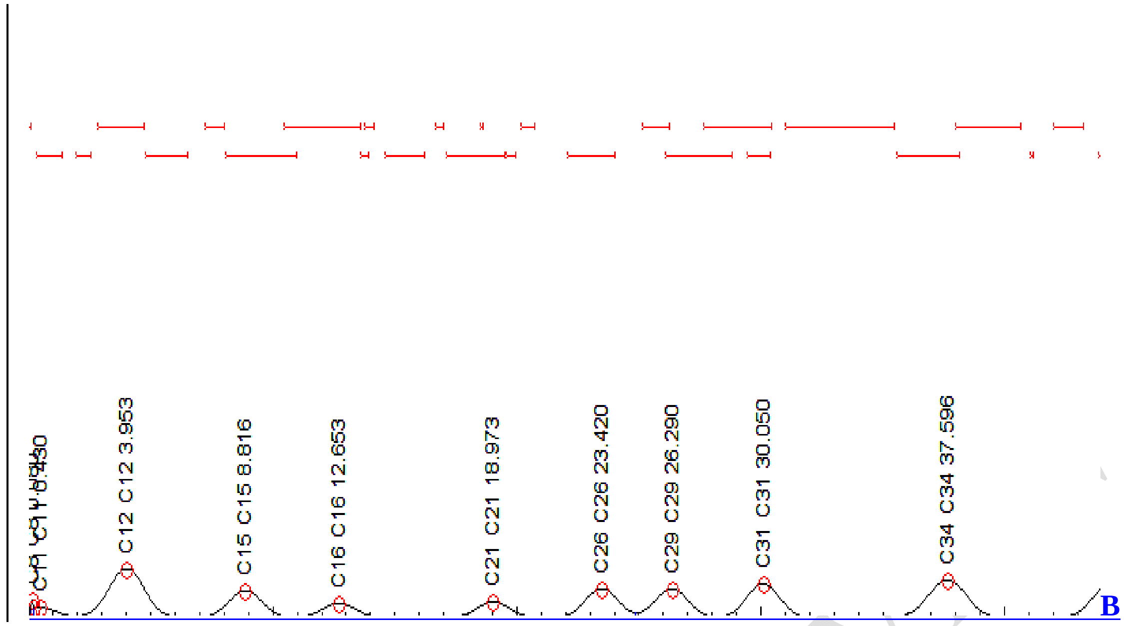
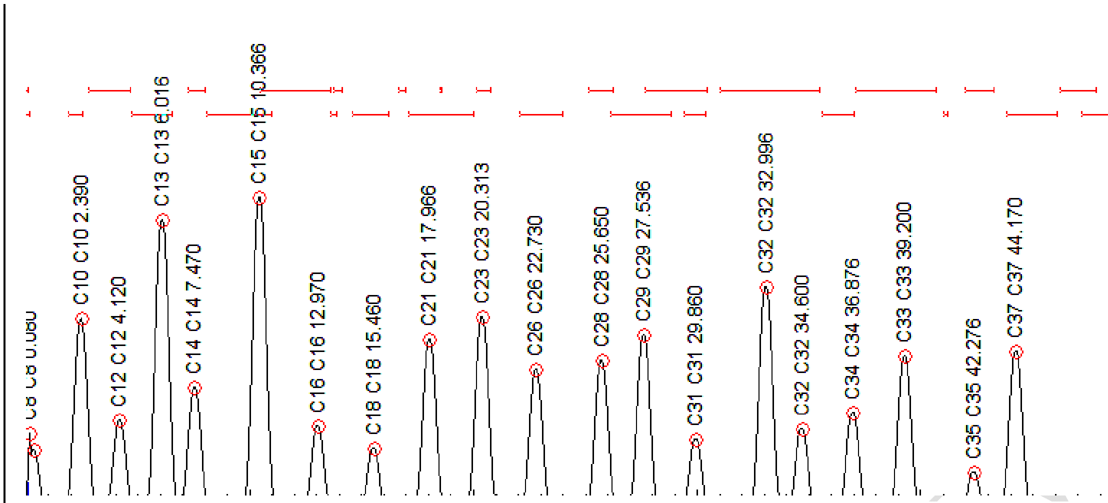


Figure 2: Profile of hydrocarbon degradation by *Alcaligenes* sp on day zero (A, top) and after 30 days (B, below).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C37 C37 44.170

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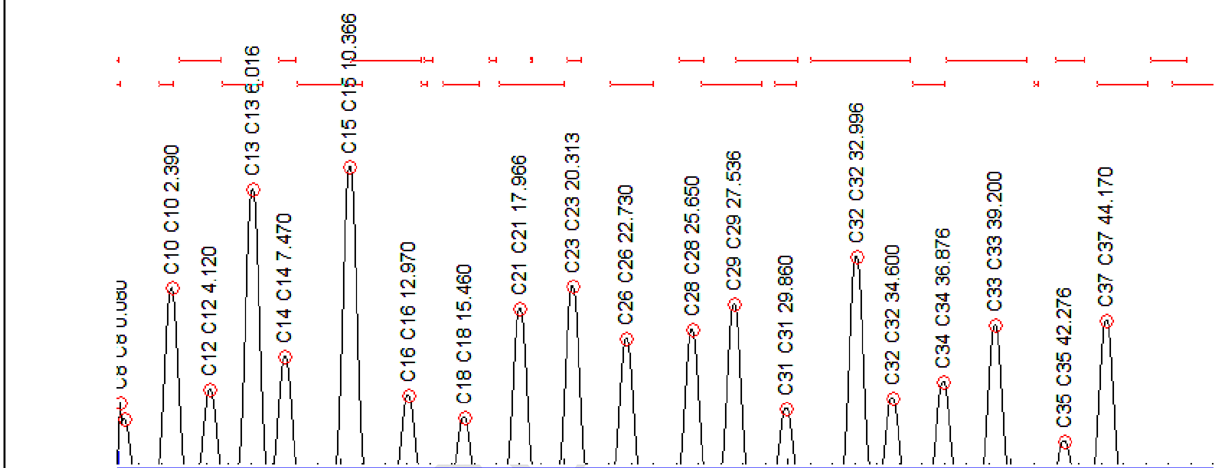
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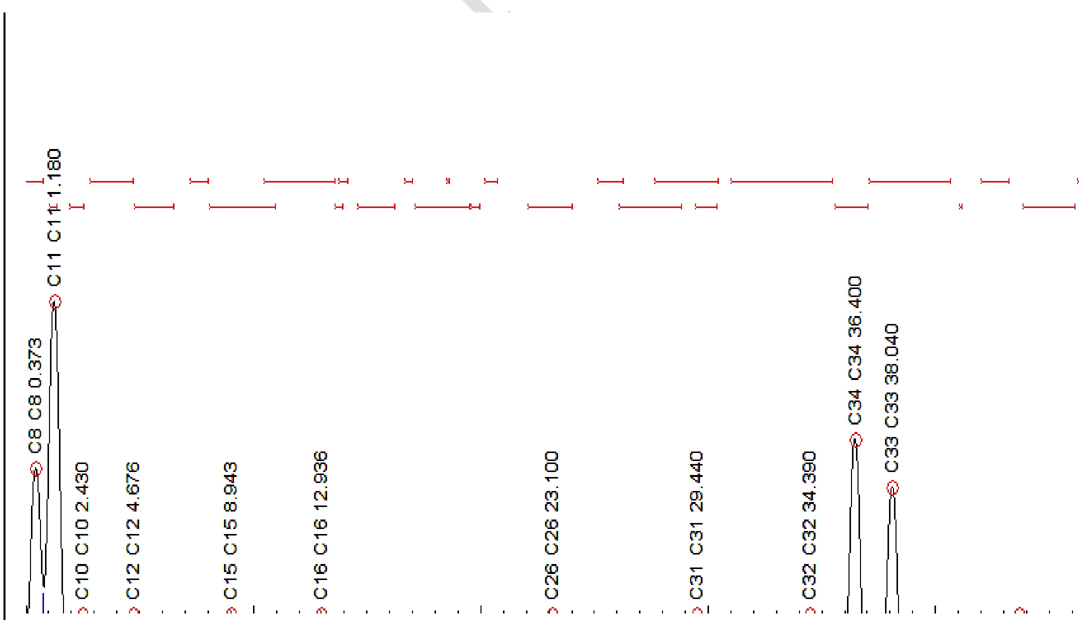
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B

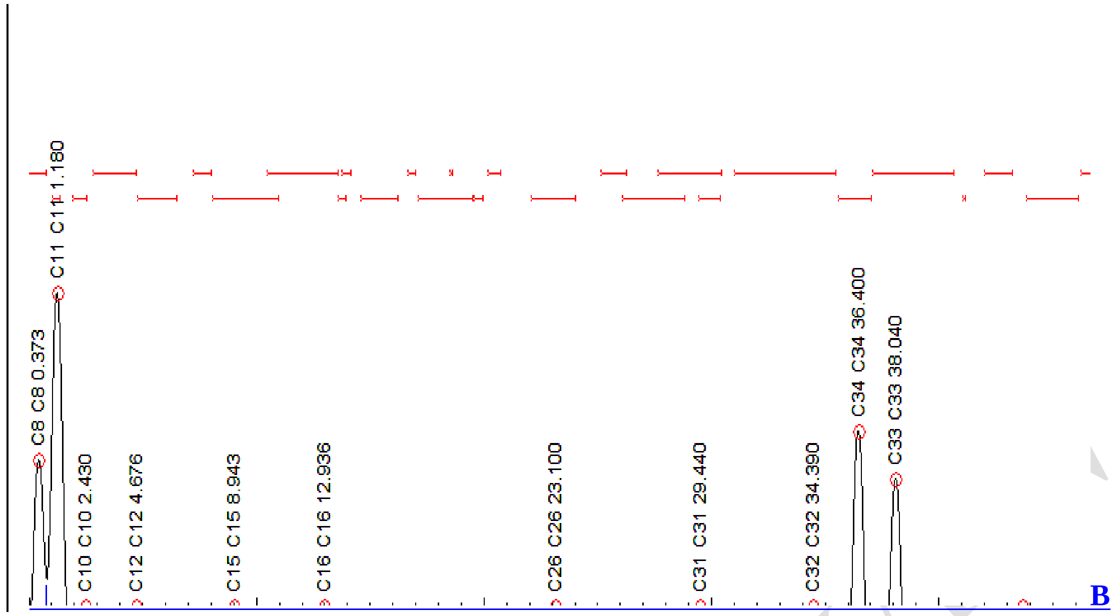
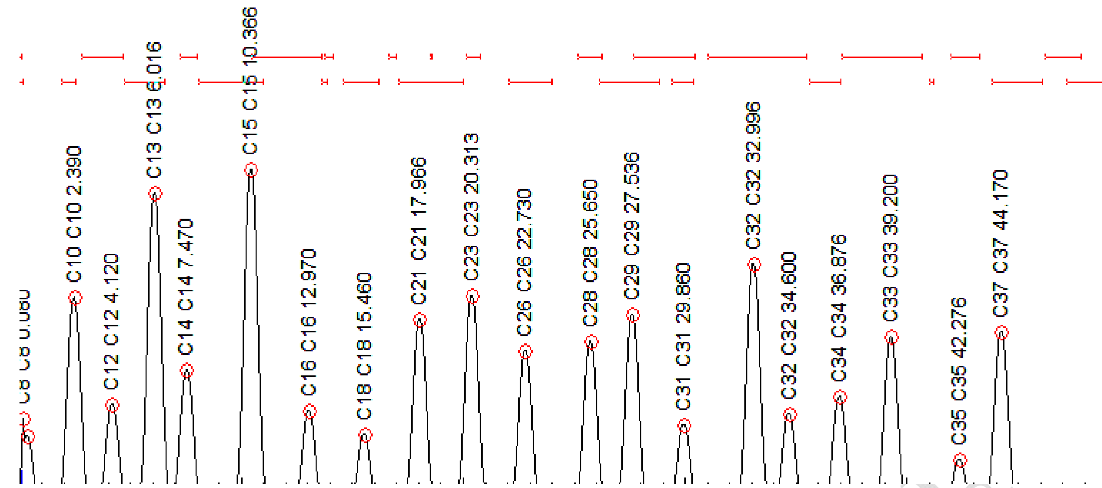


Figure. 3: Profile of hydrocarbon degradation by *Acinetobacter* sp on day zero (A, top) and after 30 days (B, below).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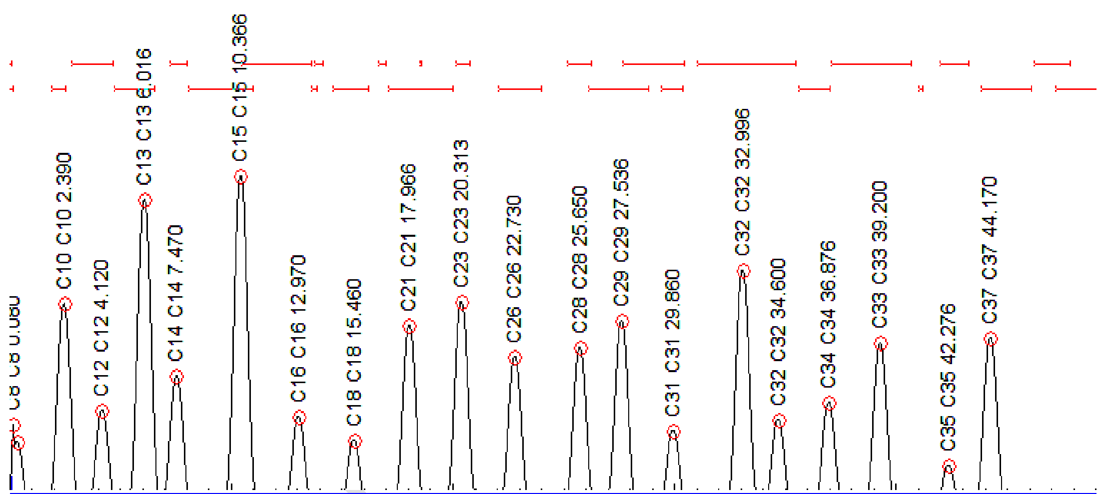
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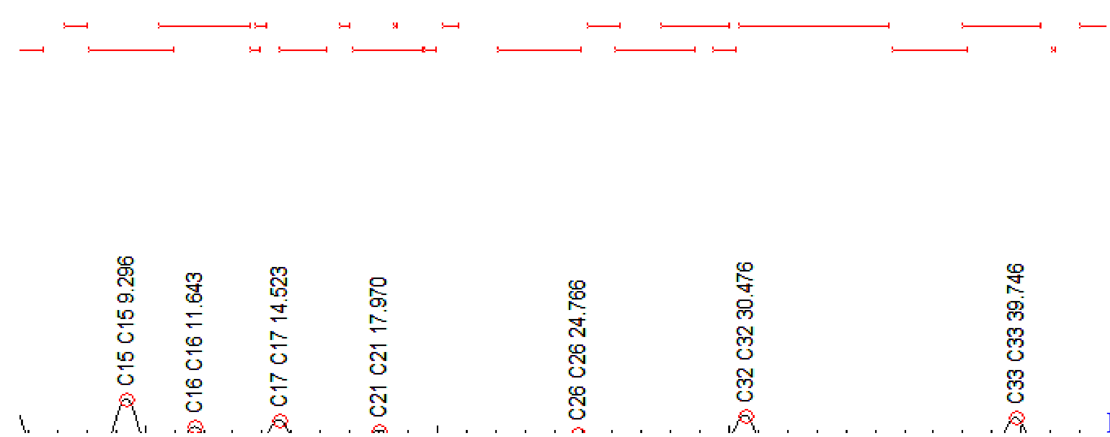
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B

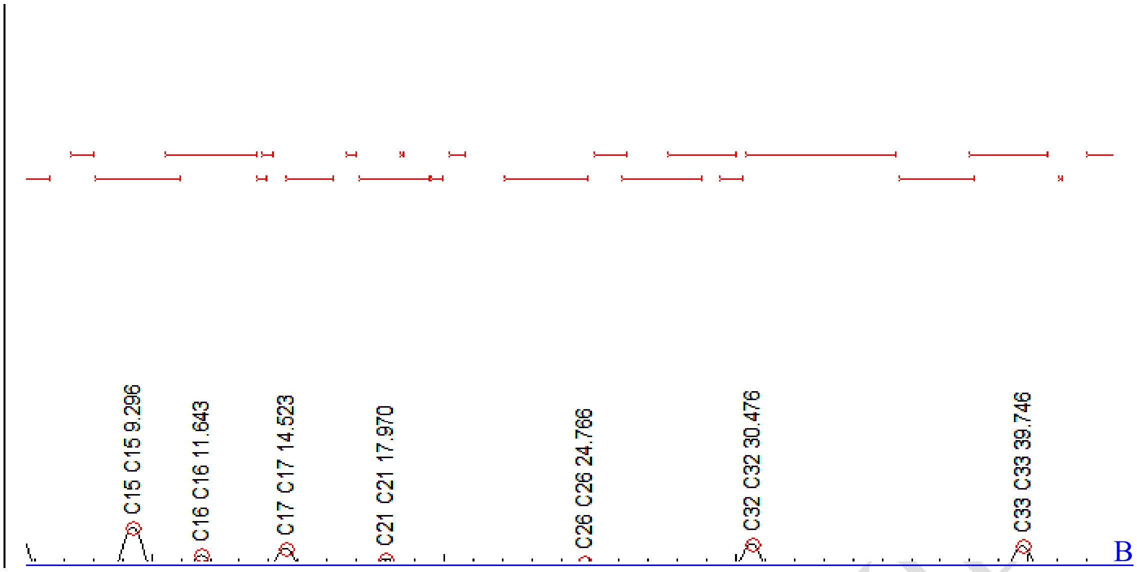
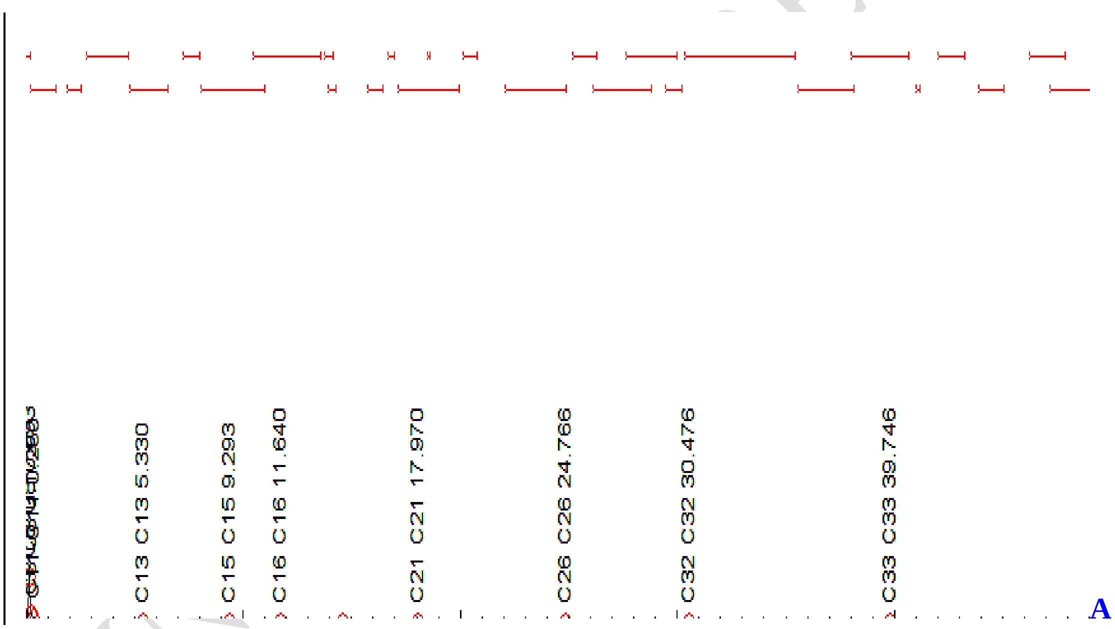


Figure 4: Profile of hydrocarbon degradation by *Bacillus subtilis* on day zero (A, top) and after 30 days (B, below)



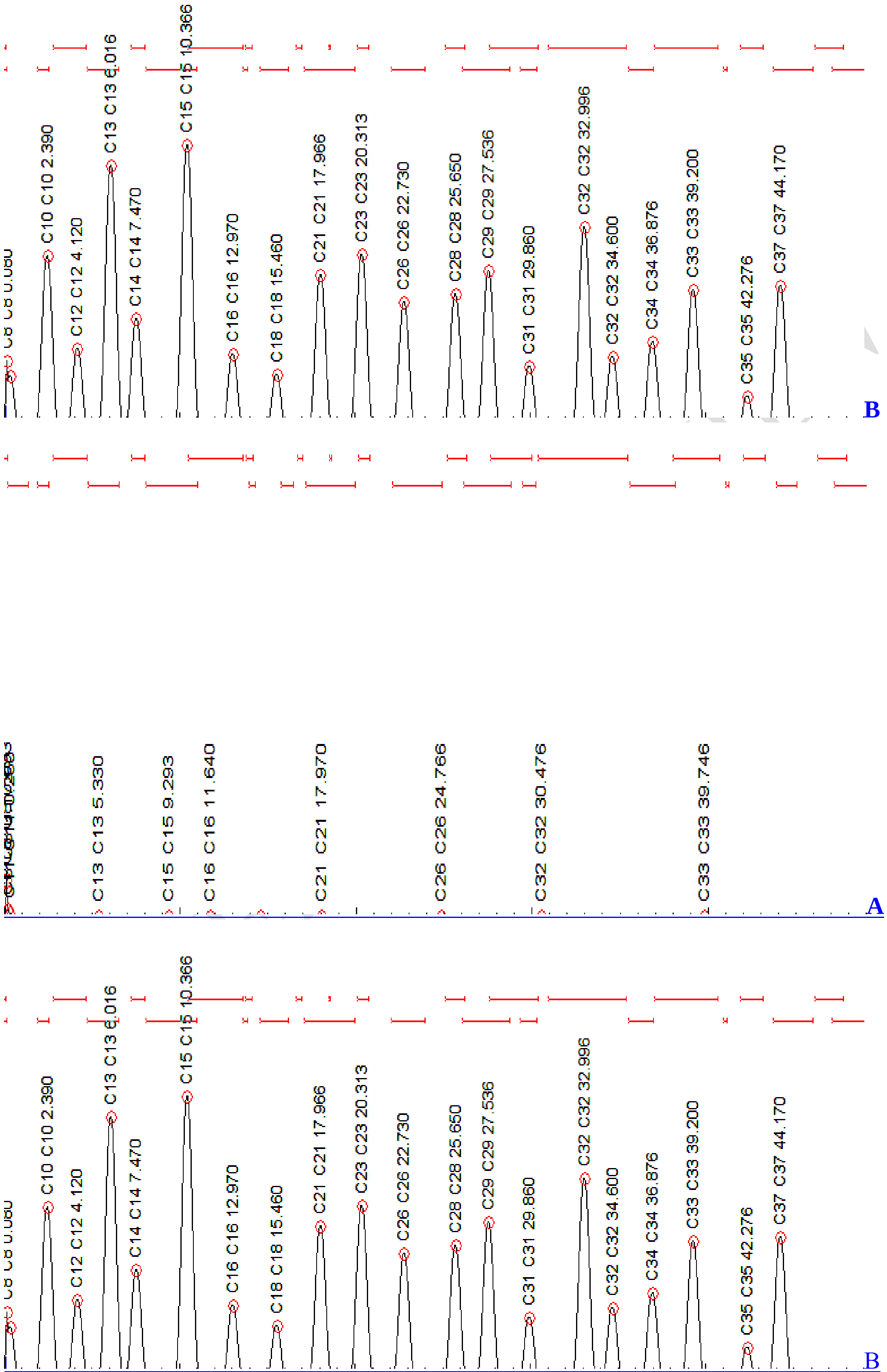


Figure 5: Profile of hydrocarbon degradation by *Pseudomonas aeruginosa* on day zero (A, top) and after 30 days (B, below)

The TPH present were C-8,C-10, C-11, C-12, C-13, C-14, C-15, C-16, C-18, C-21, C-23, C-26, C- 28, C-29, C-31, C-32, C-33, C-34, C-35 and C-37 for the treatment on day 0-30 days as showed in table 3.. The total amount of TPH after contamination of soil with crude oil showed that the treatment had TPH of (13729.70mg/ml) for the first day and reduced significantly after 30 days by each of the bacterium as follows: *Bacillus* sp (426.15mg/ml) which was able to degrade the Hydrocarbon by 96.90%, *Pseudomonas* sp. (58.68mg/ml), which was able to degrade the Hydrocarbon by 99.60% *Alcaligenes* sp.(111.07mg/ml) which was able to degrade the Hydrocarbon by 99.20% and *Acinetobacter* sp. (38.37mg/ml) which was able to degrade the Hydrocarbon by 99.70%

This concord with the work of Quatrini *et al.* [32] which demonstrated that *Actinobacter* sp. play important role during petroleum hydrocarbon degradation.

C-8, C-11, C-15, and C-26, were recalcitrant to degradation while C-10, C-14, C-18, C-23, C-28, C-29, and C-35 were all degraded in the degradation study.

Statistical analysis of the result shows that there is significant difference between various hydrocarbon and soil samples.

Table 3: The extent of degradation of the hydrocarbons by selected bacterial degraders after 30days

Hydrocarbons	*Bacillus* sp.	*Pseudomonas* sp.	*Alcaligenes* sp.	*Acinetobacter* sp.
C_8	✓	✓	✓	✓
C_{10}	-	-	-	-
C_{11}	✓	✓	✓	✓
C_{12}	-	-	✓	✓
C_{13}	✓	✓	-	-
C_{14}	-	-	-	-
C_{15}	✓	✓	✓	✓
C_{16}	-	✓	✓	✓
C_{18}	-	-	-	-
C_{21}	✓	✓	-	-
C_{23}	-	-	-	-
C_{26}	✓	✓	✓	✓
C_{28}	-	-	-	-
C_{29}	-	-	-	-
C_{31}	-	-	✓	✓
C_{32}	✓	✓	-	✓
C_{33}	✓	✓	-	-
C_{34}	-	-	✓	✓
C_{35}	-	-	-	-
C_{37}	-	-	✓	-
Hydrocarbon remaining	426.15mg/ml	58.68mg/ml	111.07mg/ml	38.37mg/ml
Hydrocarbon degraded	96.90%	99.60%	99.20%	99.70%

Key: Present (remaining) = ✓ ; Complete removal/degradation = -

CONCLUSION

This study revealed that the location Sakpenwa Community, Tai L.G.A, Rivers State, is one of the oil exploration zone of southern Nigeria, harboring a large number of microbial hydrocarbon degraders which are able of utilizing Bonny light crude oil as carbon source when monitored. The increasing values obtained indicate that both test organisms: *Bacillus* sp. and *Pseudomonas* sp. can grow and utilizing Bonny light as carbon source but *Aciligenes* sp grow at a slower rate as the days of incubation increase further. The results of the chromatographic analyses of the total petroleum hydrocarbons (TPHs) also showed that *Acinetobacter* sp. can degrade Bonny light crude oil more efficient and this evidence is shown from the reductions of both the number, sizes of the peaks and in the percentage of degradation. Therefore, it appeared that *Acinetobacter* sp+. a bacterium could be more useful in the degradation study contaminated with Bonny light crude oil and is recommended for controlling oil polluted site. This study revealed that indigenous bacterial species possess the requisite hydrocarbon degradation gene necessary for hydrocarbon degradation we concluded from the findings that this study revealed that indigenous bacterial species possess the requisite gene necessary for hydrocarbon biodegradation. This concluded that biodegradation is most often the primary mechanism for contaminant destruction including petroleum contaminants.

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