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Capabilities of Pure Culture of Bacteria in the Biodegradation of Polycyclic Aromatic Hydrocarbons and Total Petroleum Hydrocarbons in Oilfield Wastewater

O. Aleruchi* and O. Obire

Department of Microbiology, Rivers State University, P.M.B 5080, Port Harcourt, Nigeria

**Corresponding author*

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A B S T R A C T

Polycyclic aromatic hydrocarbons (PAHs) and total petroleum hydrocarbons (TPHs) in oilfield wastewater are of environmental importance because of its negative impact in the environment where they are discharged. Therefore it is important to efficiently treat oilfield wastewater before its discharge into the environment. This study therefore investigated the capabilities of pure cultures of bacteria in the biodegradation of polycyclic aromatic hydrocarbons (PAHs) and total petroleum hydrocarbons (TPHs) in oilfield wastewater. Standard procedures where observed in the collection of oilfield wastewater samples and its investigations. The bacteria used for the study were isolated from soil enriched with oilfield wastewater. Four bacteria isolates were molecularly identified using 16S rRNA method as *Morganella morganii* (MN094330), *Pseudomonas xiamenensis* (MN094331), *Chryseobacterium cucumeris* (MN094332) and *Staphylococcus* sp (MN094333). Each biodegradation experimental 250 ml flask contained 125 ml of oilfield wastewater (ofww) and 6.25ml (5%) of the bacteria culture. The control contained only the ofww (125 ml). The set up were placed in a shaker incubator at 28^{o}C with 200 rpm for aeration. The experimental samples were periodically analyzed at day 1, 7 and 21 intervals for PAHs and TPHs using Gas chromatography (GC). The initial total amount of PAHs and TPHs in the oilfield wastewater on day 1 was 101.72992 mg/l and 342.89053 mg/l, respectively. At the end of the experiment (day 21), the treatment with *Pseudomonas xiamenensis* recorded the least remaining of 22.23959 mg/l of PAHs with 78.1% removal while the control recorded the highest remaining of 75.40663 mg/l of PAHs remaining with 25.9% removal. There was complete absence of Chrysene in the treatments with *Pseudomonas xiamenensis*, *Staphylococcus* sp and *Chryseobacterium cucumeris*. There were reductions in the peaks of the various PAHs on day 21 in all the treatments. The least and highest amount of TPHs remaining on day 21 was observed in the *Chryseobacterium cucumeris* (58.18741 mg/l) and control (240.74905 mg/l), respectively with percentage removal of 84.8% and 36.9%, respectively. The treatment with *Morganella morganii* on day 21 showed total clearance of C_{12}, Pr, C_{22} and C_{26}. After 21 days of treatment, *Pseudomonas xiamenensis* showed removal of C_{12}, C_{19}, C_{22} and C_{26}. *Staphylococcus* sp recorded removal of C_{12}, Pr, C_{19}, C_{20}, C_{22} and C_{26}. *Chryseobacterium cucumeris* completely removed C_{10}, C_{12}, Pr, C_{19}, C_{20}, C_{22}, C_{23} and C_{26}. At the end of the experiment, the ability of the individual bacterium to biodegrade PAHs and TPH were revealed by Gas chromatography (GC). However, some organisms' biodegraded PAHs faster than TPH and vis versa.

Introduction

Hydrocarbons are a ubiquitous family of several chemically related environmental importunate organic compounds of various structures and with different levels of toxicity. Oilfield wastewater generated by petrochemical industries are characterized by the presence of large quantity of polycyclic and aromatic hydrocarbons, phenols, metal derivatives, surface active substances, sulphides, naphthylenic acids and other chemicals (Aleruchi and Obire, 2018; Suleimanov, 1995). Due to the ineffectiveness of purification systems, wastewater may become dangerous, leading to the accumulation of toxic products in the soil or receiving water bodies with potentially serious consequences on the ecosystem (Bay *et al.*, 2003). Crude oil is a complex mixture of several polycyclic aromatic compounds and other hydrocarbons.

The major hydrocarbon classes found in crude oil are the normal alkanes which are easily degraded, branched alkanes and cycloalkanes, (difficult to identify), the isoprenoids (very resistant to biodegradation), the aromatics (fairly identified and much more soluble than other hydrocarbons), and finally the polar ones containing mainly sulphur, oxygen and/or nitrogen compounds. Non hydrocarbon compounds may also be found in crude oil and they include porphyrins and their derivatives (Callot and Ocampo, 2000). Bioremediation can be applied as green technologies which are environmentally friendly and cost effective response to oil pollution. In recent years, there has been increasing interest in developing cost effective in-situ technique for bioremediation of oil contaminated sites. Three main approaches of this technique: natural attenuation (reliance on natural biodegradation activities and rates), which is sometimes called intrinsic bioremediation; biostimulation (stimulation of natural activities by environmental modification such as fertilizer addition to increase rates of biodegradation); and bioaugmentation (addition of exogenous microorganisms to supplant the natural degradative capacity of the hydrocarbon-impacted ecosystem) (Kaplan and Kitts 2004; Prince and Atlas 2005; Chikere *et al.*, 2009a, b; Gertler *et al.*, 2009a). Microorganisms are the major agents in the degradation of petroleum hydrocarbons. The organisms include bacteria, yeast, filamentous fungi and algae (Prince, 1993; Atlas, 1981).

The principal bacteria and fungi genera responsible for oil degradation in both soils and aquatic environment have been identified as comprising mainly *Pseudomonas, Achrobacter, Bacillus, Micrococcus, Nocardia, Trichoderma, Penicillium, Aspergillus* and *Morteilla* (Atlas, 1981; Bossert and Bartha, 1984; Okpokwasili and Amanchukwu, 1988). This study therefore compares the potentials of individual isolates in the biodegradation of polycyclic aromatic hydrocarbons and total petroleum hydrocarbon in an oilfield wastewater.

Materials and Methods

Sample Collection and Isolation

Oilfield wastewaters were collected from Ogbugu flow station; an onshore oil production platform located in Ogba/Egbema/Ndoni local government Area (ONELGA) of Rivers State, Nigeria. The Oilfield wastewater samples were collected using 4 Litre capacity sterile sample bottles and stored in an ice packed cooler. The soil samples were collected 80 meters away from the discharge pond at a depth of 0 - 15 cm with a clean hand auger into sterile polythene bags and stored in an ice packed cooler. The collected oilfield wastewater and soil samples were immediately transported to the laboratory for analysis within 24 hours.

Bacteria were isolated from the soil (100g each) enriched with various concentrations (10%, 25%, 50%, 75%) of oilfield wastewater. The enriched soil sample was incubated in a rotary shaker incubator at 28^{o}C with 200 rpm for aeration and withdrawn seven (7) days interval for analyses.

Preparation of Enriched Soil Sample Inoculum

One gram (1g) of the enriched soil samples were serially diluted onto 9 ml of sterile normal saline in a test tube to give an initial dilution of 1:10 ml (10^{-1} dilution). Subsequent dilutions were done up to 10^{-3}dilution (Prescott *et al.,* 2005).

Isolation of Bacteria

Isolation of heterotrophic bacteria was done using nutrient agar by the spread plate technique as described by Prescott *et al.,* (2005). Aliquots (0.1ml) of serially diluted samples of 10^{-2}dilution were spread plated onto dried sterile nutrient agar plates in duplicates. The plates were incubated at 37^{0}C for 24 hours. Representative colonies were selected and sub-cultured to purify them into pure isolates for characterization. The purified colonies represented the bacteria isolated from the enriched soil samples. The individual isolate were labeled OA1, OA2, OA3 and OA4.

Molecular Identification of Bacterial Isolates

The 16S rRNA regions of the rRNA gene of the isolates (OA1- OA4) were amplified after extraction and quantification of the DNA using the 27F: 5'-AGAGTTTGAT CMTGGCTCAG-3' and 1492R: 5'-CGGTT ACCTTGTTACGACTT-3' primers on an ABI 9700 Applied Bio systems thermal cycler at a final volume of 40 microlitres for 35 cycles. The PCR mix included: the X2 Dream taq Master mix supplied by Inqaba, South Africa (taq polymerase, DNTPs, MgCl), the primers at a concentration of 0.5µM and the extracted DNA as template. The PCR conditions were as follows: Initial denaturation, 95ºC for 5 minutes; denaturation at 95 ºC for 30 seconds; annealing at 52ºC for 30 seconds; extension at 72 ºC for 30 seconds for 35 cycles and final extension at 72ºC for 5 minutes. The product was resolved on a 1% agarose gel at 130V for 30 minutes and visualized on a blue light transilluminator.

The sequences of the isolates were edited using the bioinformatics algorithm Trace edit; similar sequences were downloaded from the National Center for Biotechnology Information (NCBI) data base using BLASTN and was further aligned using ClustalX. The evolutionary history was inferred using the Neighbour-Joining method in MEGA 6.0 (Saitou and Nei, 1987). The bootstrap consensus tree inferred from 500 replicates (Felsenstein, 1985) was taken to represent the evolutionary history of the taxa analysed. The evolutionary distances were computed using the Jukes-Cantor method (Jukes and Cantor, 1969). The identified isolates were submitted to the Gene bank and were assigned accession numbers.

Biodegradation Experiment

The experiment was designed to analyze the potential of the selected organisms to biodegrade polycyclic aromatic hydrocarbons (PAHs) and total petroleum hydrocarbon in oilfield wastewater using Gas Chromatograph.

Preparation of Inoculum

The microbial inocula consisted of indigenous organisms (HUB) obtained earlier from the study of enrichment of various concentration of oilfield wastewater on soil microorganisms.

The method described by El-Borai *et al.*, (2016) was adopted. Bacteria were sub cultured on a sterile nutrient agar and incubated for 24 hours at 37 ^{o}C. A loopful of each bacterium isolates (OA1- OA4) were inoculated into 4 ml nutrient broth medium at 35 ^{o}C for activation of the organisms for biodegradation. The different strains from overnight cultures at the log phase of growth were transferred to 250 ml conical flasks each containing 50 ml of sterile defined mineral salts medium (MSM) for 24 hours at 35 ^{o}C in a shakers incubator. The bacterial suspension turbidity was adjusted to 0.5 McFarland standards (1.5×10^{8}).

Composition of Biodegradation Set up

The biodegradation experimental set up was made up of five conical flasks (250 ml) each, labeled A1 to A5 (Table 1). Each flask contained 125ml of oilfield wastewater (ofww) and 6.25 ml (5%) of the bacteria culture. The set up were placed in a shaker incubator at 28^{o}C with 200 rpm for aeration and were as presented on Table 1.

Results and Discussion

Figure 1 shows the phylogenetic tree and the evolutionary relationship of the individual isolates. The isolates labelled OA1 to OA4 showed 100% relatedness to their relatives in the gene bank and were assigned accession numbers. The isolates labelled OA1, OA2, OA3 and OA4 were identified as *Morganella morganii* (MN094330), *Pseudomonas xiamenensis* (MN094331), *Chryseobacterium cucumeris* (MN094332) and *Staphylococcus* sp (MN094333), respectively.

Figure 2 shows the initial concentration of the PAHs and the biodegradation by the single bacterium. On day 7 *Chryseobacterium cucumeris* recorded the least remaining while the control recorded the highest remaining. On day 21 *Pseudomonas xiamenensis* showed the least remaining.

The concentration of the PAHs on the initial was 101.72992 mg/l. The treatment option containing *Pseudomonas xiamenensis* had the least amount of 22.23959 mg/l remaining on day 21 with removal of 78.1%, which was followed by the treatment option with *Staphylococcus sp* which recorded remaining of 27.31228 mg/l with 73.2% removal, *Chryseobacterium cucumeris* had 34.98499 mg/l remaining with 65.6% removal and *Morganella morganii* recorded 35.50295 mg/l remaining with 65.1% removal. The control had the highest amount of 75.40663 mg/l remaining with 25.9% removal at the end of the experiment (Table 2).

The GC profile in Figure 3 (day 1) showed the presence of Naphthalene, Acenephthylene, Acenaphthene, Anthracene and Chrysene. The control and treatment with *Morganella morganii* did not show any clearance of the individual polycyclic aromatic hydrocarbons on day 21 (Figures 4 and 5). There was complete absence of Chrysene on day 21 in the treatments with *Pseudomonas xiamenensis*, *Staphylococcus* sp and *Chryseobacterium cucumeris* as shown in the GC (Figures 6, 7 and 8). There were reductions in the peaks of the various polycyclic aromatic hydrocarbons on day 21 in all the treatments.

The results in Figure 9 showed the concentration of the total petroleum hydrocarbon on day 1, 7 and 21. On day 7, the *Staphylococcus* sp, treated sample recorded the highest remaining while the least remaining was observed in the *Chryseobacterium cucumeris*. The highest and least remaining on day 21 was observed in control and *Chryseobacterium cucumeris*, respectively. Table 3 showed the initial, final concentration and the percentage removal of

the treatment options. The initial concentration on day 1 was 342.89053 mg/l. The final concentration recorded on day 21 for the control was 240.74905 mg/l with 36.9% percentage removal. *Morganella morganii* recorded remaining of 129.47221 mg/l with 66.1% removal. *Pseudomonas xiamenensis* on final day recorded 119.29648 mg/l and obtained percentage removal of 68.8%. Staphylococcus sp recorded 85.04915 mg/l on day 21 with 77.7% percentage removal. *Chrysebacterium cucumeris* recorded final concentration of 58.18741 mg/l with percentage removal of 84.8%.

The GC profiles of the various treatments are shown in Figure 9, 10, 11, 12, 13 and 14.

Figure 9 show the individual n-alkanes and their peaks on day 1. The n-alkanes recorded were C_8, C_9, C_{10}, C_{12}, C_{14}, C_{15}, Pr, C_{18}, C_{19}, C_{20}, C_{22}, C_{23} and C_{26}. Figure 10 showed the GC of the control on day 21, there was no clearance of n-alkanes as observed. Figure 11 showed the GC profile of the treatment with *Morganella morganii* on day 21, there was total clearance of C_{12}, Pr, C_{22} and C_{26}. Treatment with *Pseudomonas xiamenensis* on day 21 showed removal of C_{12}, C_{19}, C_{22} and C_{26} as shown in Figure 12. *Staphylococcus* sp treatment option on day 21 recorded removal of C_{12}, Pr, C_{19}, C_{20}, C_{22} and C_{26} as shown in Figure 13. Treatment option with *Chryseobacterium cucumeris* as shown in Figure 14 completely removed C_{10}, C_{12}, Pr, C_{19}, C_{20}, C_{22}, C_{23} and C_{26}. Generally there was reduction in the level of peaks.

Microorganisms obtained from hydrocarbon polluted environment have been known to be efficient in using hydrocarbons as carbon and energy sources (Obire *et al.*, 2020; Aleruchi and Abu, 2015; Cui *et al.*, 2008). The results clearly showed that the bacteria isolates were capable of growing in hydrocarbon polluted environment as they were isolated from soil enriched with oilfield wastewater. Bacteria isolate showed 100% relatedness to their relatives in the gene bank. Microorganisms capable of utilizing hydrocarbon are widely distributed in nature and have been found in areas not directly contaminated with hydrocarbon (Yousseff *et al.*, 2010).

The biodegradative capabilities of the single isolates to biodegrade polycyclic aromatic and total petroleum hydrocarbons were compared. For polycyclic aromatic hydrocarbon, *Chryseobacterium cucumeris* recorded the least remaining on day 7, this was followed by *Staphylococcus* sp, *Morganella morganii*, *Pseudomonas xiamenensis* and control. *Pseudomonas xiamenensis* on day 21 had the least remaining and the highest percentage removal, followed by Staphylococcus sp, *Chryseobacterium cucumeris*, *Morganella morganii* and the control. The results indicate that some organisms have different strategy they use to attack polycyclic aromatic hydrocarbon, while some may attack faster, some slowly. This was seen in the case of treatment with *Pseudomonas xiamenensis* which had highest remaining concentration of polycyclic aromatic hydrocarbon among the treatment options on day 7 but on day 21 it recorded the least remaining, *Chryseobacterium cucumeris* and *Morganella morganii* reduced in its degradation rate after day 7. Staphylococcus sp maintained its degradation rate. The control recorded the highest remaining concentration of polycyclic aromatic hydrocarbons on day 21. The Gas Chromatography on day 21 showed total clearance of chrysene by *Pseudomonas xiamenensis*, *Staphylococcus* sp and *Chyrseobacterium cucumeris*.

Table.1 Biodegradation Set up

Set up	Content
A1	Control (ofww only)
A2	*Morganella morganii* + ofww
A3	*Pseudomonas xiamenensis* + ofww
A4	*Staphylococcus* sp + ofww
A5	*Chryseobacterium cucumeris* + ofww

Table.2 Biodegradation of PAHs by Single Isolates (Bacterium)

Treatments	Initial (Day 1)(mg/l)	Final (Day 21)(mg/l)	% Removal
Control (ofww)	101.72992	75.40663	25.9
***Morganella morganii* + ofww**	101.72992	35.50295	65.1
***Pseudomonas xiamenensis* + ofww**	101.72992	22.23959	78.1
***Staphylococcus* sp + ofww**	101.72992	27.31228	73.2
***Chryseobacterium cucumeris* + ofww**	101.72992	34.98499	65.6

KEY: offw= oilfield wastewater

Fig.1 Phylogenetic tree showing the evolutionary distance between the bacterial Isolates

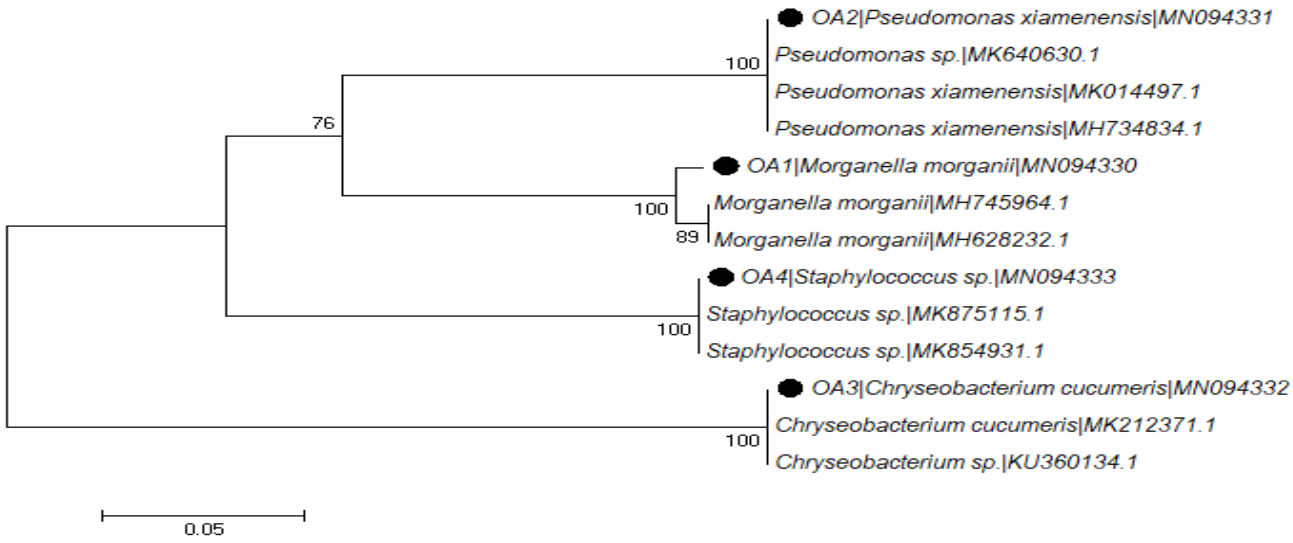


Table.3 Biodegradation of TPHs by Single Isolates (Bacterium)

Treatments	Initial (Day 1)(mg/l)	Final (Day 21)(mg/l)	% Removal
Control (ofww)	342.89053	240.74905	36.9
***Morganella morganii* + ofww**	342.89053	129.47221	66.1
***Pseudomonas xiamenensis* + ofww**	342.89053	119.29648	68.8
***Staphylococcus* sp + ofww**	342.89053	85.04915	77.7
***Chryseobacterium cucumeris* + ofww**	342.89053	58.18741	84.8

KEY: offw= oilfield wastewater

Fig.2 Biodegradation of PAH by single bacterium (*Morganella morganii*, *Pseudomonas xiamenensis*, *Staphylococcus* sp and *Chryseobacterium cucumeris*)

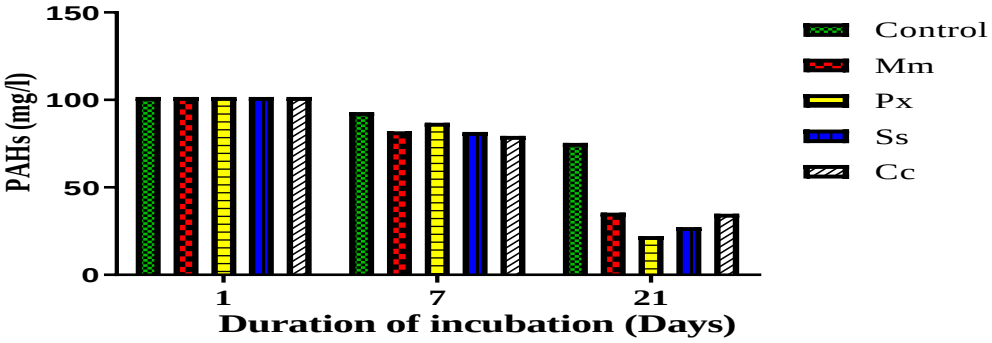


Fig.3 GC profile showing the polycyclic aromatic hydrocarbon (PAH) on Day 1

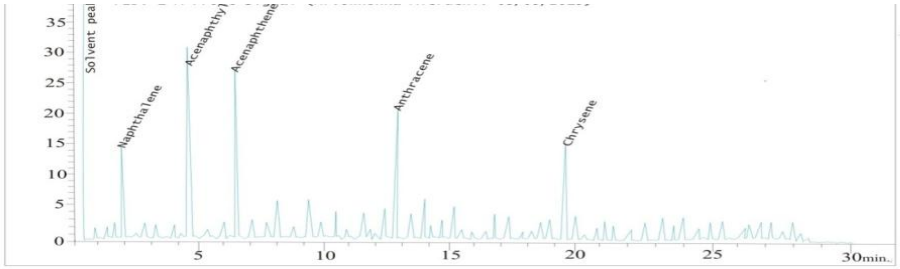


Fig.4 GC profile showing the biodegradation of polycyclic aromatic hydrocarbon (PAH) by the control on day 21

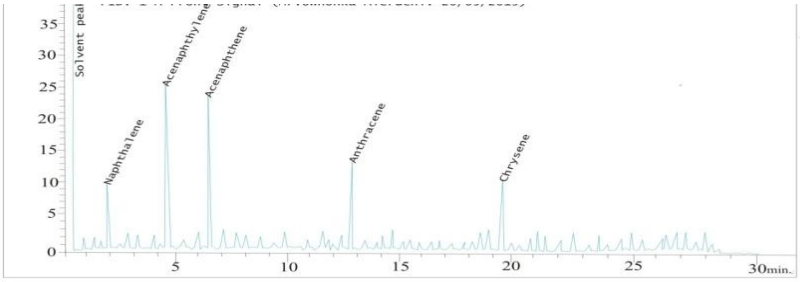


Fig.5 GC profile showing the biodegradation of PAHs by *Morganella morganii* on day 21

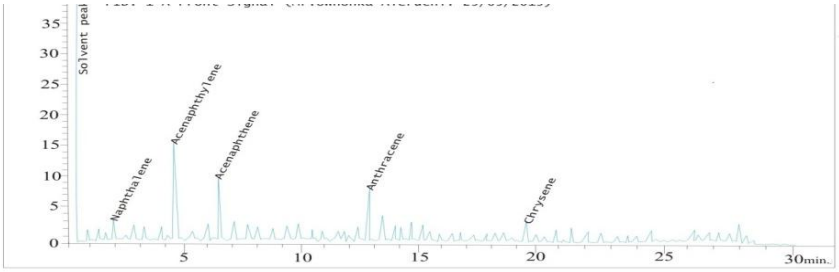


Fig.6 GC profile showing the biodegradation of PAH by *Pseudomonas xiamenensis* on day 21

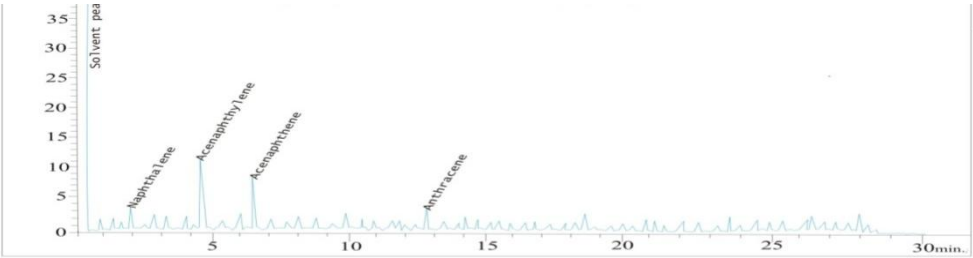


Fig.7 GC profile showing the biodegradation of *Staphylococcus* sp PAHs by on day 21

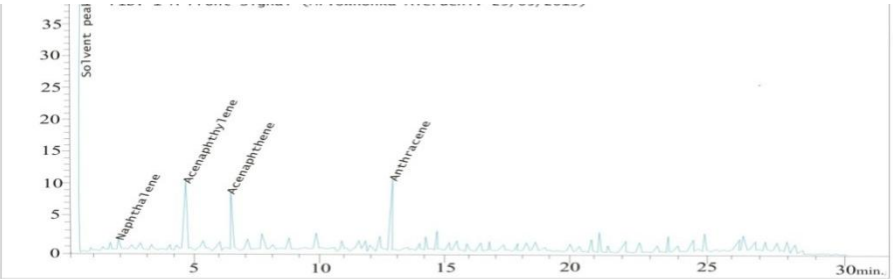


Fig.8 GC profile showing the biodegradation of PAHs by *Chryseobacterium cucumeris* on day 21

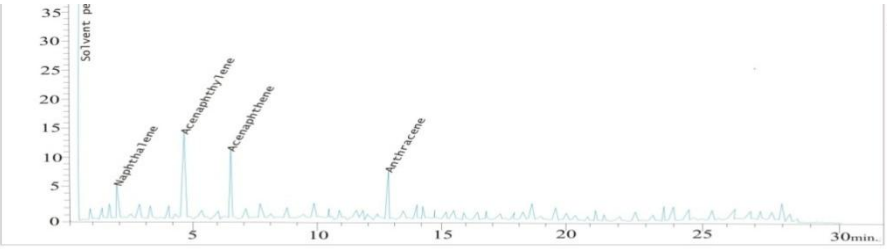


Fig.9 Biodegradation of TPH by single bacterium (*Morganella morganii*, *Pseudomonas xiamenensis*, *Staphylococcus* sp and *Chryseobacterium cucumeris*)

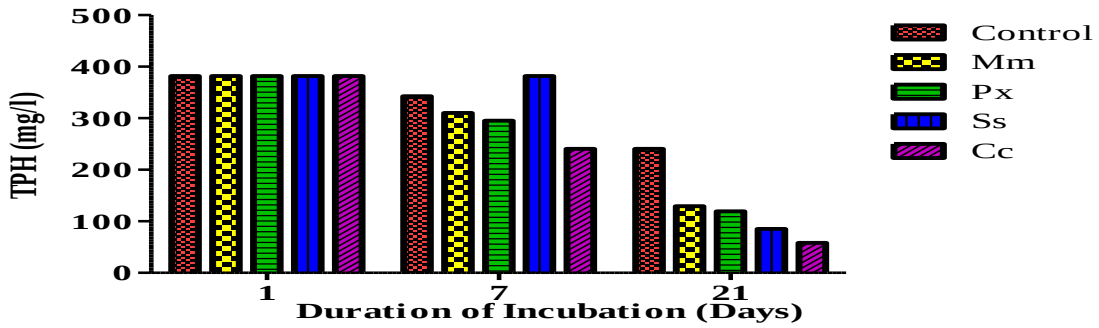


Fig.10 GC profile showing the total petroleum hydrocarbon (TPH) of the control on day 1

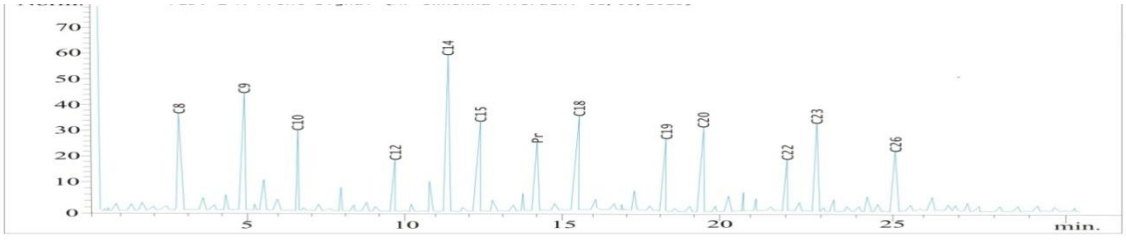


Fig.11 GC profile showing the biodegradation of total petroleum hydrocarbon (TPH) by the control on day 21

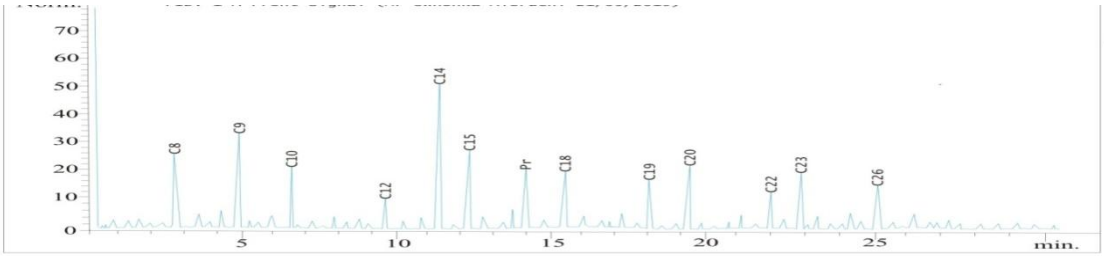


Fig.12 GC profile showing the biodegradation of TPH by *Morganella morganii* on day 21

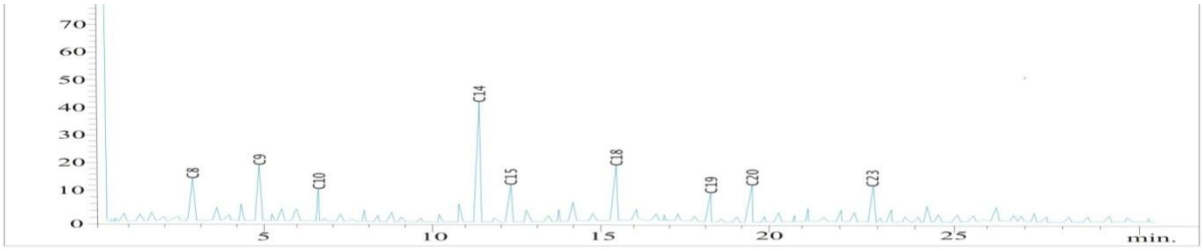


Fig.13 GC profile showing the biodegradation of TPH by *Pseudomonas xiamenensis* on day 21

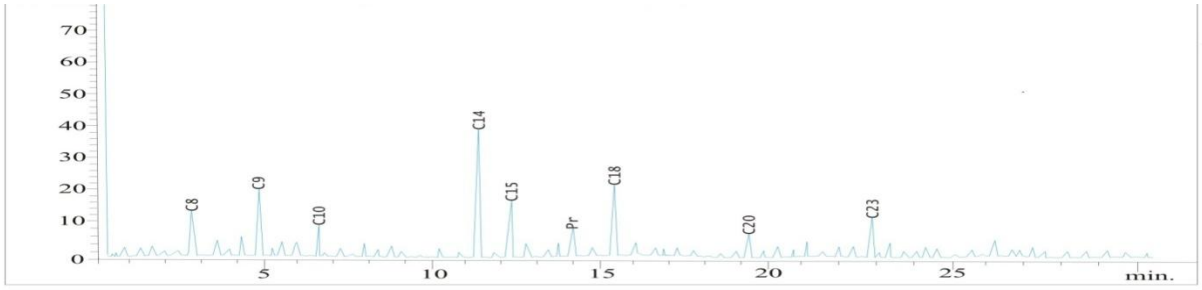


Fig.14 GC profile showing the biodegradation of TPH by *Staphylococcus* sp on day 21

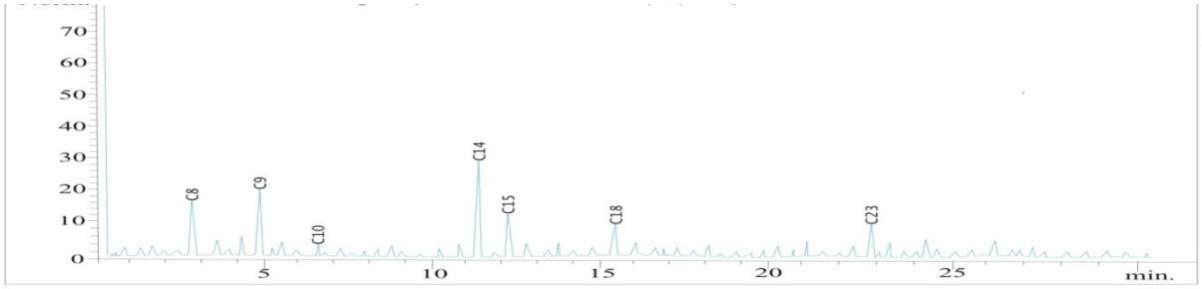
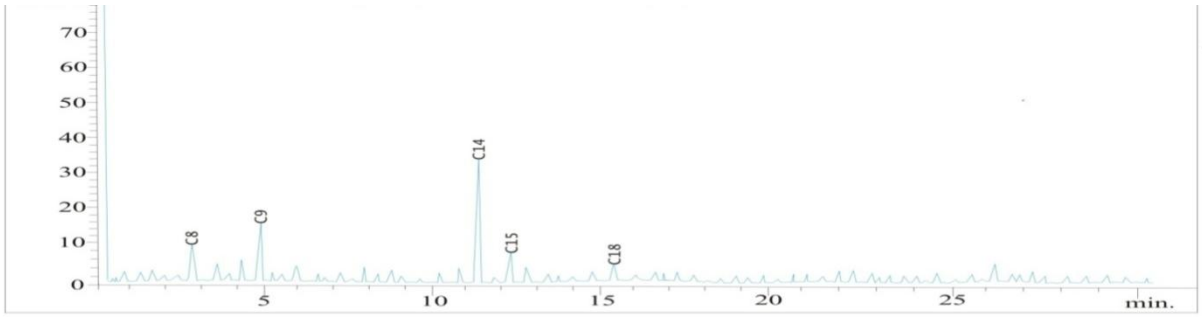


Fig.15 GC profile showing the biodegradation of TPH by *Chryseobacterium cucumeris* on day 21



On day 7, the biodegradation of total petroleum hydrocarbon recorded least remaining in the treatment with *Chyrseobacterium cucumeris* which was followed by *Pseudomonas xiamenensis*, *Morganella morganii*, control and *Staphylococcus sp*. *Staphylococcus sp* did not reduce the total petroleum hydrocarbon on day 7 however it recorded the second best in the reduction of total petroleum hydrocarbon on day 21, while the best or least reduction was observed in the treatment with *Chyrseobacterium cucumeris*. The control on day 21 did not show any clearance of the individual n alkane but removal most n alkanes were observed in other treatment options. *Chrseobacterium cucumeris* showed more clearance of the n alkane which was followed by Staphylococcus sp. Comparing the degradative capabilities of the individual isolates to biodegrade polycyclic aromatic hydrocarbon and total petroleum hydrocarbon.

It was observed that *Chryseobacterium cucumeris* biodegraded polycyclic aromatic hydrocarbon and total petroleum hydrocarbon simultaneously on day 7, the degradation was more on total petroleum hydrocarbon but slowed in the polycyclic aromatic hydrocarbon. *Staphylococcus* sp on day 7 did not attack the total petroleum hydrocarbon but degraded polycyclic aromatic hydrocarbon.

On day 21, *Staphylococcus* sp recorded the second best in the degradation of both polycyclic aromatic hydrocarbon and total petroleum hydrocarbon.

Pseudomonas xiamenensis on day 7 degraded total petroleum hydrocarbon better than polycyclic aromatic hydrocarbon but degraded polycyclic aromatic hydrocarbon better than total petroleum hydrocarbon on day 21. *Morganella morganii* also degraded both polycyclic aromatic hydrocarbons and total petroleum hydrocarbon but was not recorded among the first or second best degraders. Biodegradation of PAHs and TPH by single bacterium showed significant reduction in the amount of PAHs and TPH remaining at the end of the experiment. The significant reduction could be as a result of the enzymes, degradation gene they possess or their abilities to absorb them into their cytoplasmic membranes. This is in agreement with the reports of Alexander (1999) and Mandri and Lin (2007) that microorganisms degrade hydrocarbon compounds by using enzymes in their metabolisms. It is interesting to note that most of the bacterium used in this study has little or no report on biodegradation as compared to well documented hydrocarbon degraders. There has been wide report on the biodegradation ability of *Pseudomonads* (El-borai *et al.*, 2016; Rahman *et al.*, 2003; Cameotra and Singh, 2008).

However the *Pseudomonas xiamenensis* in this study has not been reported. Staphylococcus sp generally has been reported as strong primary utilizers and has potential for biodegradation ability (Unell, 2008). Little is known about the biodegradation potential of *Chryseobacterium cucumeris*, this study shows their ability to biodegrade PAHs and TPH. Biodegradation ability of *Morganella morganii* has been reported by Arunagiri and Sangeetha (2015).

This study demonstrated the potentials of single bacterium cultures, in the biodegradation of PAHs and TPH. The results showed their individual capabilities which could be attributed to the degradative gene or enzymes they produce.

Microorganisms isolated from soil enriched with oilfield wastewater could be useful in determining the level of pollution in such an environment as shown in this study and thus, can be employed in the biodegradation of polycyclic aromatic hydrocarbon and total petroleum hydrocarbon in the environment.

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