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Oily sludge degrading potentials of single and consortium of autochthonous bacterial species

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Abstract

Oily sludge (OS) degrading potentials of single and consortium autochthonous bacterial population was carried out using standard analytical procedures. Three autochthonous bacterial species; *Pseudomonas aeruginosa*, *Bacillus cibi* and *Bacillus subtilis* were associated with the OS. The isolates exhibited varying OS utilizing and biosurfactant producing potentials. *P. aeruginosa* was the best OS utilizer while *B. subtilis* was the best biosurfactant producer (emulsification index of 15.6%). Degradation of the OS with single culture of the best OS utilizer (*P. aeruginosa*) and consortium of the best OS utilizer and best biosurfactant producer (*B. subtilis*) revealed that the consortium exhibited a remarkable potential to reduce the total petroleum concentration in the OS from 100.73 ppm to 41.39 ppm (58.91% degradation) as compared to 51.74 ppm (48.7% degradation) achieved by the single culture. The saturated fraction of the OS was the most susceptible to degradation followed by the aromatic faction while the NSO and asphaltene fraction were the least degraded. *P. aeruginosa* was able to reduce the saturated hydrocarbon content in the OS by 87.4% while a 95.5% reduction was recorded for the consortium. Similarly, the concentration of PAH in the OS was reduced from 27.94 ppm to 16.74 ppm by the single culture and 12.75 by the consortium. The potentials of these bacterial communities can be explored for broader use in remediating oily sludge contaminated soil as well as managing oily sludge waste in the oil and gas industry.

Keywords: Oily Sludge; Pseudomonas; Bacillus; Biosurfactant; Remediation

1. Introduction

Petroleum and its related products are the major sources of energy for both domestic and industrial activities and thus also on high demand. The various processes involved in the exploration and processing of petroleum results the accidental or intentional release of large amount of hydrocarbon into the environment mostly through spills from damaged pipes, accidents vandalization, etc [1]. The effect of petroleum in the environment are enormous and has been extensively reviewed by several authors [2,3,4]. Asides from spills, petroleum processing and equipment maintenance processes also results in the creation of considerable amount of hazardous solid waste such as oily sludge [5,6,7] which are usually disposed into the environment treated or untreated. These oily sludges are thick viscous, pasty semisolid mixture made of sand (a mixture of clay, silica and oxides), sediment, hydrocarbon, oil, water and chemicals used in petroleum processing and vessel or equipment cleaning [5,7]. The chemical composition of oily sludges is complex and depends on the source [8] with alkanes, aromatics, asphaltenes and resin [9] being the main constituents. Reports have shown that before now, oily sludge were improperly managed by dumping them into dykes, on ground or into ditches, trenches or casks for subsequent burial without any prior preparation of the area [6] and this led to the serious environmental problems especially in the Niger Delta Mangrove ecosystem [10, 11].

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Although contamination of the mangroves with oil has received very little scientific attention [12], several physical, chemical and biological recovery and remediation technologies have been developed [6,13]. Wide application of most of these techniques are limited by their cost and/or inability to completely remove the contaminant. Despite these limitations, bioremediation (biological treatment) appears to be the most effective and reliable. The technology is environmentally friendly and cost effective as it relies on the microorganisms to degrade, transform, detoxify or breakdown the contaminants. Microorganisms "the main force behind bioremediation processes" degrade or transform these pollutants as there carry out their normal metabolic activities under aerobic and anaerobic conditions provided their optimum growth conditions are provided [14]. This process of bioremediation can be enhanced by the addition of nutrients to stimulate metabolic activities (biostimulation) or microbes (autochthonous or allochthonous) with known potentials to utilize the target pollutant. This study is designed to evaluate the oily sludge degrading potentials of pure and mixed autochthonous bacterial species associated with contaminated Mangrove soil.

2. Material and methods

2.1. Sample collection

The oily sludge (OS) for this study was collected from Universal Energy Operation Site in Mbo Local Government Area of Akwa Ibom State. The site is situated in the Niger Delta oil rich region of Nigeria where most of the countries crude oil installations and export terminals are located. The area experiences heightened navigational activities and as such spills of petroleum hydrocarbons from both crude oil and refined products occur regularly. The area is characterized by a mean annual rainfall of 2369 mm and a mean maximum daily-temperature of 28^{o}C [15]. The source of the oily sludge sample was crude tank bottom sludge, product tank bottom sludge and American Petroleum Institute (API) separator unit.

2.2. Bacteriological Analysis

The bacterial community present in the oily sludge was determined using enrichment technique as described by Cerqueira *et al.,* [16]. Here, 1% of the oily sludge sample is added to a 250 mL Erlenmeyer flask containing 50 ml of sterile mineral medium [composition in g/L: KCl, 0.7; KH_2PO_4, 2.0; Na_2HPO_4, 3.0; NH_4NO_3, 1.0; micronutrients solution, 1 m/L ($MgSO_4$, 4.0; $FeSO_4$, 0.2; $MnCl_2$, 0.2; $CaCl_2$, 0.2)]. All the flask was incubated at 30 ^{o}C in a rotatory incubator (100 rpm) for five (5) days. After the fifth day, 1 ml of the aliquot was transferred into a new mineral medium and incubated under the same conditions. After 5 transferences, the aliquot was plated on a mineral salt agar (same composition as before) fortified with agar-agar (20 g/L) and incubated for 24 – 48 hours. Discrete colonies on the plate were counted, sub-cultured, purified and stored for further studies.

The potentials of the bacterial isolates to utilize oily sludge was determined using a modified hydrocarbon overlay method. Precisely 15 g of agar-agar was added to Mineral Salt Medium sterilized and allowed to set. The solidified plates were overlaid with 1% (v/v) sterile oily sludge, allowed for about 15 to 30 minutes then the test isolates were streaked on the surface of the plate. All inoculated plates were incubated at room temperature for 5-15 days with periodic observation. Colonies that eventually developed showing luxurious growth were selected and rated. The utilization was rated based on the diameter and luxurious nature of the developed colonies, i.e., '+', '++' or '+++' indicating the magnitude of the oil degrading potentials as described by Ekundayo and Obire [17].

2.3. Identification of Bacterial Isolates

Analysis of the 16SrRNA was used to identify the bacterial species associated with the oily sludge. The process was performed by picking a single colony of bacteria isolates from the nutrient agar medium using the tip of a sterile pipette and placing it in 100 µl of sterile distilled water in a 1.5 ml microcentrifuge tube. The tube was incubated at between 94 and 95 °C for 10 min using a digital dry bath (Bio Rad). A volume of 2 µl was used as a DNA template for the amplification reaction. The 16S rRNA region was amplified by PCR using the forward primer, 27F (5′-AGA GTT TGA TCC TGG CTC AG-3′) and reverse primer 1492R (5′-CGG CTA CCT TGT TAC GAC TT-3′) [18]. The amplification reaction was prepared using 10 µl of 2× PCR Master Mix (Thermo Scientific Phusion Flash High-Fidelity), 1 µl of each forward and reverse primer (10 µM), 2 µl of the DNA template and 6 µl of sterile distilled water resulting in a 20 µl reaction volume. The negative control was set up without genomic DNA. The amplification reaction was performed in a thermal cycler (Bio Rad T100™) as follows: one cycle at 98 °C for 10 s, followed by 34 cycles at 98 °C for one second, 53 °C for 1 min and 72 °C for 15 s. A final extension step at 72 °C for 1 min was performed for 1 cycle. The reaction was held at 4 °C until the amplicons were removed from the thermal cycler. The amplicons were then assessed by running 1% agarose gel electrophoresis and viewed in the Gel Doc imager (Bio Rad).

2.4. Biosurfactant Producing Potentials of the Isolates

Biosurfactant producing potentials of the bacteria isolates was carried out using standard analytical procedures. Pure cultures of the bacterial isolates were subjected to hemolytic, emulsification capacity (% EC $_{24}$), drop collapse and oil spread tests. For the hemolytic activity determination, the test isolates were streaked on freshly blood agar plates and incubated at 37 °C for 48 hours as described by Youssef *et al.,* [19]. The plates were visually inspected for zone of clearance around the colonies, which was an indication of hemolytic activity and were reported as α-, β-, or γ- hemolysis. Emulsification capacity was determined using the Cooper and Goldenberg [20] method. The same volume of a 72-hour old test culture supernatant and kerosene in a ratio of 1:1 was mixed in a glass test tube (125 mm × 15 mm). Then, the mixture was vortexed for 2 min and left to stand for 24 h. The $\%EC_{24}$ is given as percentage yielded by dividing the height of the emulsified layer (mm) by the total height of the liquid in the glass test tube (mm), then multiplying by 100. The drop-collapse test was performed according to Plaza *et al.*, [21]. In this method, supernatant from each bacterial isolate was pipetted onto a microplate lid (12.7 × 8.6 cm^2) previously coated with Tapis crude oil. If the drop of the supernatant became flat 1 min after adding, the result was taken to be positive. If the drops remained beaded, the result was recorded as negative. For the oil spread assay, 10 µl of crude oil was added to the surface of 40 ml of distilled water in a petri dish to form a thin oil layer. Then, 10 µl of culture supernatant was gently placed on the center of the oil layer. If biosurfactant is present in the supernatant, the oil is displaced and a clearing zone is formed. The diameter of the clear zone is measured after 30 seconds and this diameter correlates to surfactant activity, also called oil displacement activity. The measurement is express in BS unit, known as biosurfactant unit.

2.5. Oily Sludge Degradation Study

2.5.1. Standardization of Isolates for Degradation

Prior to the oily sludge hydrocarbon utilization study, cultures of the best oily sludge utilizer (OSU) and biosurfactant producing bacterial (BPB) isolate were standardized. This was to determine the inoculum size to be used for the degradation study. Here, a loop full of the isolates was inoculated into separate test tubes containing 10 ml of sterile peptone broth. The inoculated test tubes were incubated at 27 ^oC for 24 hours. After 24 hours, 1 ml of the aliquot was aseptically transferred to a sterile Petri dish and about 15 ml of sterile Nutrient agar added. The plates were swirled and allowed to set. After which it was inverted and incubated at 27 ^oC for 24 hours. After 24 hours, discrete colonies that developed were counted and recorded as the initial Total viable cells per ml of the aliquot.

2.5.2. Oily Sludge Degradation Potentials of Pure and Consortium Bacterial Isolates

The OS degradation study was carried out using the modified method of Panda *et al,* [22] as described by Essien *et al.,* [23]. In this method, a 250 ml conical flask containing 150 ml of MSM and 2% OS was inoculated with 1ml of the standardized broth of *P. aeruginosa* from 2.4.1. Another conical flask containing the same constituent was augmented with 1 ml of the standardized broth culture of *B. subtilis* from 2.4.1. A control flask was set up containing only 150 ml of MSM and 2% oil sludge. All the conical flash were incubated at 28 °C in shaker incubator (100 rpm) for 30 days before analysis of degradation rate.

2.5.3. Estimation of Oily Sludge Degrading Potentials

The degree of the various hydrocarbon fraction of the oily sludge by the test bacterial isolates was measured in two ways. First, the determination of the bacterial growth through the estimation total viable bacterial count (TVBC), second by measuring the concentration of the residual hydrocarbon fractions in the culture medium after 30 days degradation study. The growth of the test isolates on oily sludge were determined by viable cell counts on Bacto nutrient agar using standard pour plate technique [24,25] at weekly interval. This was done by carrying out 10-fold serial dilution of the aliquot from the set up. One milliliter of the aliquot from 10^{-3} dilution was pour plated. The plates were inverted and incubated at 27 ^oC for 24 hours. After 24 hours discreet colonies on the plates were counted and recorded as the microbial load.

The concentration of the residual hydrocarbon fraction (saturate, aromatics, NSO compounds and asphaltene) was determined using gravimetrically using EPA method 3540C. The gravimetric method was carried out by modifying the method described by Joseph [26]. The culture aliquot was mixed with anhydrous sodium sulphate and consecutively soxhlet- extracted with n-hexane, dichloro methane and chloroform (l00 ml each) in an extraction thimble. All the extracts were pooled and evaporated in a rotary vacuum evaporator to about 2 ml. The distilling head was removed, and dried in vacuum, cooled, and weighed. The concentration of TPH in the original sample was calculated as.

$$TPH\ (mgkg^{-1}\ dry\ weight) = \frac{Gain\ weight\ of\ the\ flask\ (mg)}{weight\ of\ solid\ (g)}\ x\ 1000$$

After the gravimetric quantification, the residual TPH was fractionated into alkane, aromatic, asphaltenes and NSO fractions on a silica gel column [27]. The TPH (300 mg) was dissolved in n-pentane and separated into soluble and insoluble fractions (Asphaltenes). The weight of asphaltenes was determined gravimetrically while the soluble fraction was loaded on a silica gel (activated at 110 °C) column. The alkane fraction was eluted with 100 ml of hexane, aromatic traction was eluted with 100 ml benzene, and finally NSO fraction was eluted with methanol and chloroform (100 ml each). The methanol and chloroform fractions were combined, evaporated and weighed to get the weight of NSO compounds. Analysis of the hydrocarbon constituents in the hexane and benzene faction was also carried out using gas chromatography (GC) coupled with a Flame ionization detector (FID). Here, the factions were fed into a Varian 3800 gas chromatograph equipped with an FID, split injector (Split ratio was 100:1) and an open tabular column 100 m X 0.25 mm ID, fused silica coated with 0.5-micron bonded methyl silicone (Petrocol (TM)DH). Helium carrier gas linear flow was 48 cm/s. Injector temperatures was 300 °C and FID temperature was 300 °C, Hydrogen fuel was used at the rate of 29-30 cc/min and zero air @ 300 cc/min. The column oven temperature was programmed as 35 °C held for 15 minutes initially, and further raised by l °C /min to 60 °C and held for 20 minutes at 60 °C followed by, 2 °C/min rise up to 200 °C to a total run time of 130 minutes. Injection volume was I µL. FID signal was recorded and processed on Star work station software for Detailed Hydrocarbon Analysis (DHA) of compounds up to carbon number 15.

Analysis of the hydrocarbon constituent of the oily sludge was carried out using a gas chromatography (Agilent Technologies, USA) coupled with mass spectroscopy (GC-MS) (Agilent Technologies, USA) for the identification of the components above C_{15}. Analyses were performed using a MS 1200 L Single Quadrupole bench top mass spectrograph attached to a Varian 3800 gas chromatograph. The GC was equipped with a split injector and a 30 m X 0.25 mm ID, Low Bleed 5 % Phenyl, 95 % dimethylpolysiloxane open tabular column 0.25 pm film thickness, helium carrier linear gas flow was 40 cm/s. Injector temperature was 280 °C and split ratio was 100:1. Transfer line temperature was 279.6 °C. MS source temperature was 279.7 °C. The column oven temperature programme was initial temperature 65 °C, ramp 10°C /min to 300°C hold for 5 min. The MS was operated in centroid scan, mass range 40-800, with unit mass resolution.

3. Results and discussion

The culturable bacterial community associated with the oily sludge sample were isolated, characterized and identified using standard microbiological technique. The analysis revealed the present of three bacterial species; OS_1, OS_2 and OS_3 with varying oily sludge utilizing potentials (Table 1). All the isolates exhibited the potentials to utilize oily sludge as a carbon and energy source for growth by independently growing in oily sludge supplemented medium. Of the three, isolates OS_1 had the highest OS utilizing potential. Morphological and biochemical characterization of the isolates revealed the identities of OS_1, OS_2 and OS_3 to be *Pseudomonas aeruginosa, Bacillus cibi* and *Bacillus subtilis* respectively (Table 1). The identities of the isolates were confirmed using 16sRNA analysis. The result revealed that the isolates had a very close (99 – 100%) similarly with those in the database. The summary and the accession numbers of the isolates from the 16sRNA analysis are as presented on Table 2.

The presence of these bacterial species in the oily sludge agrees with several reports. Nkwelang *et al.,* [28] in their study reported that *Pseudomonas, Bacillus, Serratia* and *Acinetobacter* were the most abundant bacterial species in tropical soil contaminated with oily sludge. Analysis of the biosurfactant producing potentials of the isolates revealed that the bacterial isolates also exhibited varying biosurfactant producing potentials. From the results, it was apparent that *B. subtilis* demonstrated the highest emulsification capacity (15.2 %). This was followed by *P. aeruginosa* with an emulsification capacity of (14.6 %). The isolate with the least biosurfactant producing potential was *B. cibi* with an emulsification capacity of 7.2 % (Table 3).

The 12 weeks degradation study showed that the degradation of the TPH components of the OS by the best OS utilizing bacteria (*P. aeruginosa*) was faster or enhanced with culture of the best biosurfactant producer (*B. subtilis*) than when carried out alone. Single culture of *P. aeruginosa* was able to reduce the total petroleum hydrocarbon constituent (TPH) of the OS from 100.73 ppm to 51.74 ppm (48.7 % degradation) but when the culture was enhanced with culture of *B. subtilis,* the TPH was reduced to 41.39 ppm (58.91 % degradation). In general, the saturated fraction of the OS was the most susceptible to degradation. (Figure 1). This was followed by the aromatic faction. The least utilized faction of the oily sludge was the NSO compounds and the asphaltene faction.

Table 1 OS utilizing potential, Morphological and Biochemical Characteristics of Bacterial Isolates Associated with the Sample

Characteristic	**OS_1**	**OS_2**	**OS_3**
OS utilizing potential	+++	++	++
Gram's stain	Gram negative	Gram positive	Gram positive
Morphology	Rods	Rods	Rods
Endospore	-	Central	Central
Oxidase	+		+
Catalase	+	-	+
Motility	+	+	+
Indole production	-	-	-
Citrate utilization	+	-	+
Gelatin hydrolysis	+	-	+
Urease	+	-	-
Starch hydrolysis	-	+	+
Methyl red test	-	-	-
Voges Proskauer test	-	-	+
Nitrate reduction	+	-	+
Acid production from glucose	-	-	+
Lactose	-	+	+
Mannitol	-		+
Trealose	-	+	+
DNAse	-	-	+
Tentative Organism	*Pseudomonas aeruginosa*	*Bacillus cibi*	*Bacillus subtilis*

Key: + = Positive; - = Negative;

Table 2 Molecular Identities of the Isolates

	Isolates	**Identity**	**E. value**	**Percent Identity**	**Accession number**
1	*Pseudomonas aeruginosa*	*Pseudomonas aeruginosa*	0.0	99	CP008861.1
2	*Bacillus subtilis*	*Bacillus subtilis*	0.0	99	EU679368
3	*Bacillus cibi*	*Bacillus cibi*	0.0	99	DSM 16189 (T)

Table 3 Biosurfactant Producing Potentials of the Bacterial Isolates

Isolates Code	**Biosurfactant Producing Potentials**			
	Hemolytic Activity	**Emulsification Capacity (%)**	**Drop collapse**	**Oil spread**
Pseudomonas aeruginosa	+ (β)	14.2	+	+
Bacillus subtilis	+ (β)	15.6	+	+
Bacillus cibi	+	7.2	+	+

Key: + = Positive; - = Negative

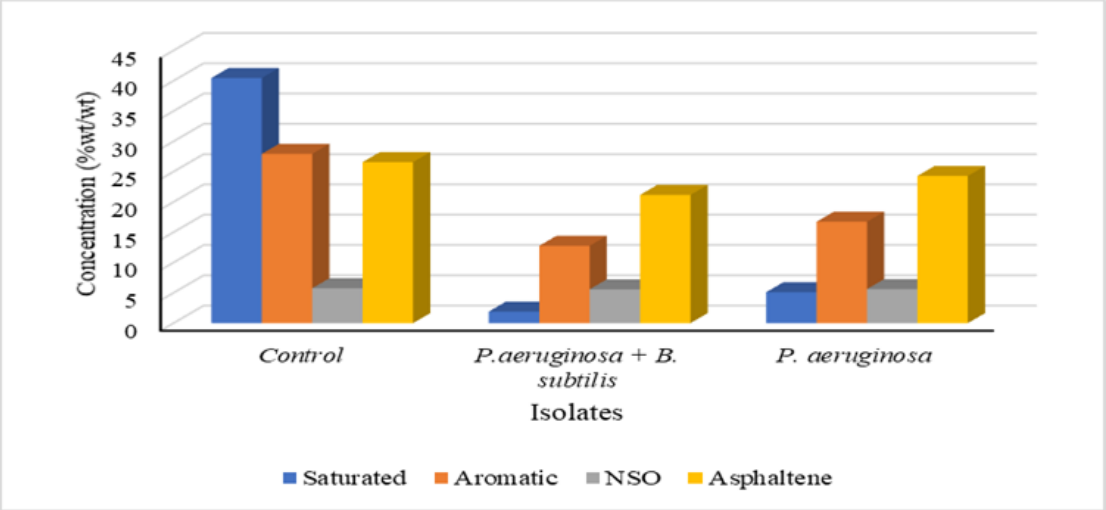

Figure 1 Concentrations of the Hydrocarbon Fraction after 30 days degradation

P. aeruginosa alone was able to reduce the concentration of the saturated hydrocarbon in the oily sludge supplemented medium by 87.4 %. On the other hand, the concentration of the saturated hydrocarbons in the microcosm containing the consortium was reduced by 95.2 %. A summary of the degradation rate of the saturated hydrocarbons is as presented on Figure 2. Similarly, analysis of polycyclic Aromatic hydrocarbon (PAHs) suites in the microcosms revealed that the consortium reduced the total PAHs levels in the OS more than the single bacterial population. The total residual PAH concentration of the samples after 30 days of degradation was 16.74 ppm for the single bacterial population and 12.75 ppm for the consortium. Analysis of the individual PAHs suits revealed competitive degradation of the 17 PAH suites (Naphthalene, 2-methylnapthalene, Acenaphthene, Acenaphthylene, Fluorene, Phenanthrene, Anthracene, Fluoranthene, Pyrene, Benz(a)anthracene, Benzo(b)fluoranthene, Chrysene, Benzo(k)fluoranthene, Benzo(a)pyrene, Dibenz(a,h)anthracene, Benzo(g,h,i)perylene, and Indeno(1,2,3-cd)pyrene) with the consortium demonstration and enhanced degradation.

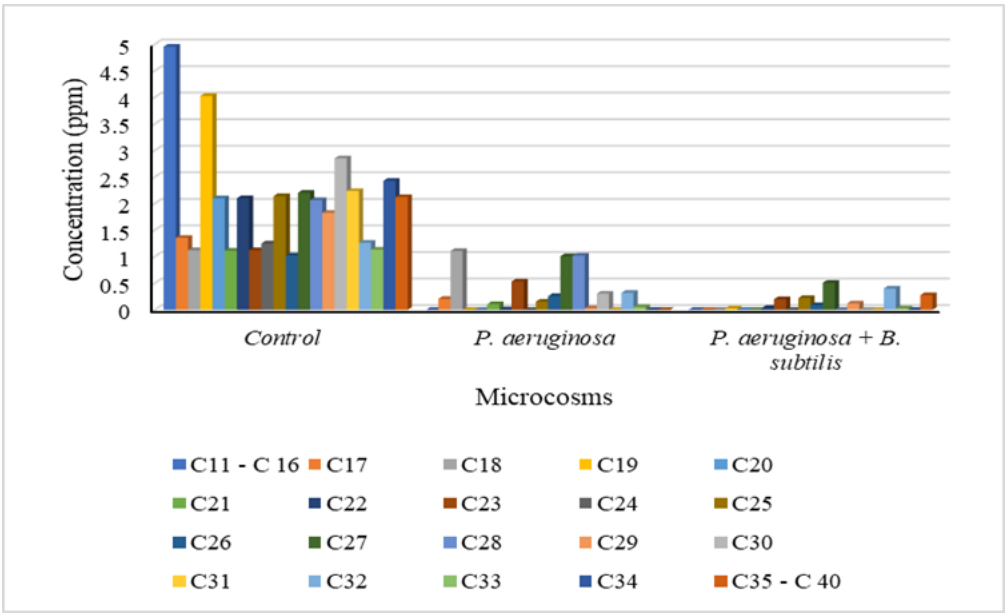

Figure 2 Concentrations of the Saturated Fractions of after 30 days degradation

The OS degradation potentials demonstrated by these bacterial species may be attributed to several factors, one of such is their ability to produce biosurfactant. Microbial surfactants have been reported to reduce the surface and interfacial tension between the hydrophobic crude oil and bacterial cells surface via its amphiphilic property [29]. Previous studies have revealed that biosurfactants play a crucial role in enhancing the degradation of crude oil both in laboratory and field trials [30, 31, 32, 33, 34]. The finding of this study is also in line with several studies which have reported that

biodegradation is enhanced by surfactants due to increased bioavailability of pollutants [35]. Nwaogu *et al* [36] reported that the amphiphilic property of biosurfactants enhances crude oil degradation by reducing the surface tension between the crude oil and the test organisms. Cameotra and Singh [37] in their study on enhanced bioremediation of oil sludge using biosurfactants and microbial consortium consisting of two isolates of *Pseudomonas aeruginosa* and one isolate *Rhodococcus erythropolis* from soil contaminated with oily sludge reported a 90% degradation of hydrocarbons in 6 weeks in liquid culture.

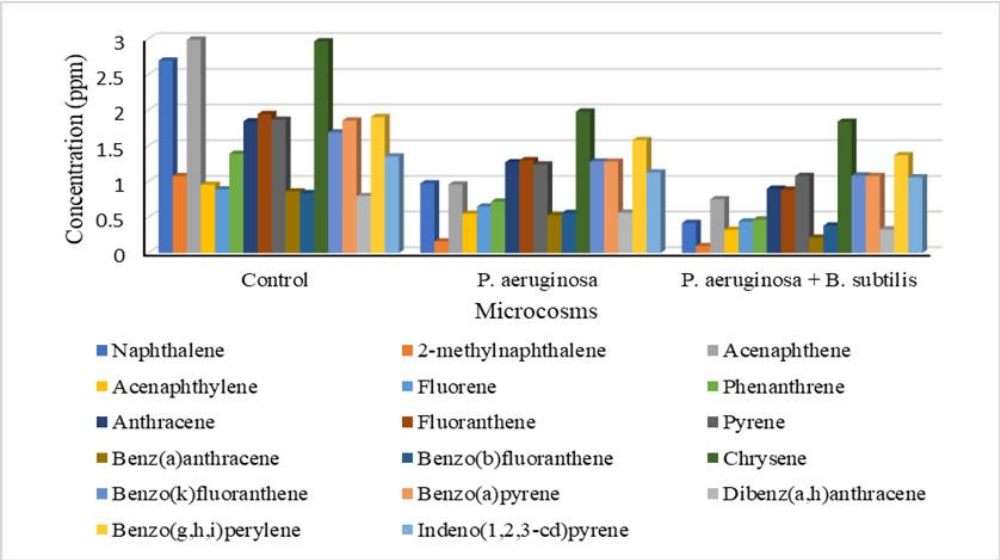

Figure 3 Concentrations of the PAH Fractions of after 30 days degradation

4. Conclusion

The result of this study has revealed OS degrading potentials of pure and consortium autochthonous bacterial species. It is suggestive that the biosurfactant produces by the bacterium notable enhanced the degradation of the saturated and aromatic fraction of the oily sludge by about 20%. Although the bacterial species did not demonstrate high degrading potential for the NSO and asphaltene fraction of the OS, the saturated and aromatic fraction were remarkable reduced? The potentials of theses bacterial species can be explored in the remediation of oily sludge contaminated soils as well as constitute an effective management technique for oily sludge waste in the oil and gas sector.

Compliance with ethical standards

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Disclosure of conflict of interest

The authors have declared that there is no conflict of interest.

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