Monitoring of Metabolic Compounds from Degradation of Petrochemicals using Indigenous Consortium of *Pseudomonas* Strains

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Abstract: This study was performed to examine the potential of indigenous consortium of *Pseudomonas* strains by assessing the metabolic compounds from degradation of petrochemical contaminated soil. Native microorganisms were isolated using standard microbiological procedures and molecular identification technique. The physiochemical analysis was conducted using standard laboratory procedure of American Public Health Association (APHA). Bioremediation of Total Petroleum Hydrocarbon (TPH) and assessment of metabolic compounds from degradation of petrochemicals were by gravimetric technique. The identified bacteria were *Pseudomonas aeruginosa* strain PAER4 119, *Pseudomonas mendocina* strain NK-01 and *Pseudomonas putida* strain B6-2. Observations revealed that the physiochemical properties were affected due to high pollution level. Highest percentage reduction of the TPH was recorded at 74.5 9% by *P. putida*, 67.57 % by *P. aeruginosa*, 61.62 % by *P. mendocina*, and 80.81 % by consortium. Also highest percentage reduction of the metabolic compounds showed; 62%, 75 %, 68 %, and 81% for saturated hydrocarbon, 86.25 %, 87.50 %, 91 % and 92 % for phenolic compound, 94.06 %, 95.05 %, 96.53 %, and 97.03 % for asphaltene and polar compound, and 88.89 %, 94.44 %, 94.42%, and 97.22% for aromatic compound. The percentage reductions recorded were achieved by *P. mendocina, P. putida, P. aeruginosa,* and the consortium respectively. This study shows that the TPH and their metabolic compounds were highly degraded. These strains and the assessment technique can be employed in biodegradation of petrochemical contaminated environment and also in the monitoring of biodegradation studies

Keywords: Biodegradation, metabolic compounds, petrochemical contaminated soil, physicochemical parameters and *Pseudomonas* strains.

INTRODUCTION

Industrialization and modernization throughout history have led to growing disruption of the natural balance and the occurrence of different types of pollution. Ecological pollution with petroleum products has been recognized as a major concern (Alexander, 2000). Some sources of petrochemical contaminations include products, effluents which are released from petrochemical industries, road accidents, ship accidents or other anthropological processes. Soil contamination with these petroleum products pose serious public health hazards and organic pollution of ground water, which limits its use, poses economic loss, environment problem and decreases the agricultural productivity of soil (Thapa *et al.* 2012).

Petrochemicals are common products of crude oil distillation with very complex compositions. They consist mainly of low molecular weight alkanes and polycyclic aromatic hydrocarbons (PAHs) (Bona *et al.,* 2011). Though, the fate of the polycyclic aromatic hydrocarbons in nature may be of great public health importance, since PAHs have been considered toxic for plants and carcinogenic to people (Bona *et al.,* 2011; Alkio *et al.,* 2005; Reynoso-Cuevas *et al.* 2008). Petroleum compounds can decrease the availability of water, oxygen and nutrients in the soil, which in consequence, may decline the rate of plant growth (Nogueira *et al.* 2011).

Findings have indicated that global industrial and agricultural developments have released a large number of petrochemicals into the ecosystem (Rahman *et al.,* 2002). Petroleum hydrocarbons are highly complex in nature; it is complicated to comprehend the degradation mechanism. For years, many studies have adopted physicochemical methods which are found ineffective, difficult, non-ecofriendly and costly.

Numerous researchers studied on petroleum degradation by using bacteria at various parameters to determine optimal degradation conditions and also described the application of microbial consortia for hydrocarbons degradation throughout the world (Plaza *et al.,* 2008; Sathishkumar *et al.,* 2008; Bao *et al.,* 2012). Yet a lot of studies on degradation of petroleum hydrocarbons by employing indigenous bacterial consortia from this petro-chemically important geographical region are very limited (Das and Mukherjee, 2007).

Bioremediation is an option that offers the possibility to destroy or render harmless contaminants using natural biological activity. Microbial degradation of petroleum hydrocarbons is one of the major practices in natural decontamination process. Bioremediation of complex hydrocarbons mixture usually necessitate the cooperation of more than a single species or strain. Therefore, conglomerations of mixed species or strains equipped with broad enzymatic capacities are entailed to increase the rate and extent of petroleum biodegradation further (Calvo *et al.,* 2009; Joutey *et al.,* 2013). Bacteria are the most active agents in petroleum biodegradation and there is evidence of their fundamental role as primary degraders of spilled oil (Head *et al.,* 2006; Da Cruz *et al.,* 2011 and Oliveira *et al.,* 2012).

Pseudomonas is a genus of Gram-negative, rod-shaped bacteria. They are aerobic and non-sporulating with one or more polar flagella for motility (Özen and Ussery, 2012). The metabolic versatility of the genus allows *Pseudomonas* species to inhabit a wide variety of environments being able to degrade or utilize wide range of organic compounds (Yergeau *et al.,* 2012). *Psuedomonas* species offer potential for biodegradation technology. The degradation of hydrocarbons to simple molecules involves many chemical reactions in which catalytic proteins are involved, which have a central role in hydrocarbon degradation and are attractive indicators for monitoring various impacts on soil (Malia and Cloete, 2002).

This study examined the bioremediation potential of indigenous *Pseudomonas* strains. And also to assess the metabolic compounds from degradation of petrochemical contaminated soil through gravimetric analysis.

MATERIALS AND METHODS

Site description and sample collection

About 5 kg of petrochemical contaminated soil was collected aseptically from a depth of 0 – 30 cm at waste effluent disposing site. The site is a few meters away from the petrochemical plant of a company located at Umurolu along East-West Road, Eleme in Port Harcourt, Rivers State. A geographically similar virgin soil area located eighty-five meters away from the oil polluted area was used as control tagged normal soil sample. The soil samples were then put into polyethylene bags, labeled accordingly and stored at 4 ^{o}C until processed as described by Saadoun, (2000) at CESLAB GLOBAL SERVICES (RESEARCH) Analytical and Microbiological Laboratory, Umudike, Abia State.

Isolation and identification of *Pseudomonas* strains from the petrochemical polluted soil

Isolation was performed by serial dilution where 1 g of polluted soil sample was diluted serially with 9 ml of saline water. King's B medium was used for isolation of *Pseudomonas* strains. Inoculum of 1 ml of suspension from dilutions (10^{-4} and 10^{-6}) was added in sterilized Petri plates containing 20 ml of sterilized King's B medium (10g proteose peptone, 1.5 g anhydrous K_2HPO_4, 15 g glycerol and 5 ml $MgSO_4$ [1M; sterile]) by pour –plate technique. The plates were incubated at 30 ^{0}C for 48 hours. Prominent bacterial colonies with yellow green and blue white pigments were selected. Streak plate method was used to subculture the bacterial colonies on nutrient agar and incubated at 28±2 ^{0}C for 48 hours. Pure cultures were obtained and maintained on nutrient agar (NA) slants at 4 ^{0}C for further studies (Garrity*et, et al.,* 2005).

Morphological and biochemical characterization of bacterial strains

Subcultures of the colonies were examined for their colony morphology, fluorescence and cell shape (Garrity*et al*., 2005). Microscopic observations in oil imersion were recorded on the basis of their shape, size, colour, opacity and mucosity. Each bacterial isolates was streaked on the medium plate and incubated at 30 °C for 4 to 5 days to record colony characteristics.

The Gram stain test was used to confirm the negativity of the colonies for this stain (Gram-), to this group belongs the *Pseudomonas* species. Catalase and oxidase tests were conducted to differentiate *Pseudomonas* spp. (oxidase +) from other species of *Pseudomonas* and other Gram-negative bacilli (oxidase -). Species characterization and identification were performed by other biochemical tests such as, Citrate, Indole, Gelatin liquefaction, Manitol, Lactose, Maltose, Sucrose, Starch hydrolysis etc for probable identification adopting Bergey's Manual (Holt, *et al.,* 1998).

Molecular Identification of *Pseudomonas* strains

Confirmation of bacterial strains by molecular identification technique was performed at International Institute of Tropical Agriculture (IITA), Ibadan, Nigeria. The procedures involved DNA extraction, agarose gel electrophoresis and amplification by polymerase chain reaction (PCR), and full length sequencing of the 16S rRNA gene. The full-length sequences obtained were matched with previously published sequences available in NCBI using BLAST (Sambrook and Russell, 2001).

Determination of physicochemical properties of petrochemical contaminated soil

Soil temperature was determined *in situ* with Mercury thermometer (Glass Soil-Thermometer, Huryana, India) while the pH was measured with the aid of a HACH Mini Digital Pen Type pH Meter (Santa Fe, NM 87505, USA) in a 1:25 (w/v) soil water ratio. Particle size distribution and soil texture were measured with Soitest 152H hydrometer (Bouyocous Scale hydrometer, USA) using the Bouyocous hydrometer method (Kotler *et al.* 2001). Organic carbon was performed using the Degtjareff method (Walkey and Black, 1995). Also the Water Holding Capacity was measured as described by APHA, (2004) while porosity was performed by FAO/IIASA, (2008). Conductivity was determined using Systronics Digital Conductivity Meter (PW 9504 Philips, USA). Other chemical analyses of the soil were performed using standard methods by APHA, (2004) which include Bray-1 spectrophotometric method for phosphorus, Kjeldah method for soil nitrogen, ammonium acetate extraction for total exchangeable bases (Ca, Mg, K and Na) and subsequent flame photometry with Photoelectric Colorimeter AE-IID (Ocean Med. China). All measurements were performed using MEDIFIELD Electric Balance (Equipment & Scientific Ltd, India).

Sampling Layout

Biodegradation study was carried out using mineral salt medium (MSM). Inoculum preparations in mineral broth (overnight cultures) were prepared for each of the test organisms and their consortium. Soil samples were sterilized by autoclaving thrice at 121°C for 15 minutes prior to study. Five uniform 3 cm X 3 cm sterile plastic boxes, each containing 100 g contaminated soil and another plastic box (3 cm X 3 cm) with 100 g soil as control were used. Three of these boxes were treated with each indigenous *Pseudomonas* inoculum strains and the fourth set was treated with their consortium. There was no treatment for the control which is the fifth box. Each containing plastic box was labeled $P_{1A,}$ $P_{2B,}$ $P_{3C,}$ and P_{CD} (control). Incubation period was for 28 days at 37 °C under designated periods tagged (T_0 – T_4) at 7 days intermittent sub-sampling. During each sub-sampling, 1 g of soil sample was collected from each of the test boxes and analyzed. Analysis for TPH and assessment of the metabolic compounds from biodegradation of petrochemicals were performed by gravimetric technique.

Analysis of Total Petroleum Hydrocarbons (TPH) in contaminated soil

The gravimetric method described by Lin and Mendelssohn, (2009) was employed to determine the percentage reduction of TPH. Soil sample (5 g) and 100 ml of n-hexane were mixed and transferred in a glass centrifuge tube in order to extract the TPHs ultrasonically for 1 hr (Osuji, 2006). After extraction, the samples were centrifuged for 10 minutes at 3000 rpm. Then, the extracts were transferred into an Erlenmeyer flask, dried to a constant weight, and bathed under 65 ^{o}C to evaporate volatile chloroform. After evaporation of the solvent, the amount of residual TPHs was recovered and calculated (Lin and Mendelssohn, 2009).This was carried out at various stages of study; prior to biodegradation and for period of 28 days at 7 days' interval of intermittent sub-sampling.

Assessment of metabolic compounds from biodegradation of petrochemical polluted soil

The metabolic compounds such as saturated hydrocarbon, aromatic hydrocarbon, asphaltene and polar compound, and phenolic compound were assessed. The procedure was performed at room temperature during biodegradation at 7 days' interval of intermittent sub-sampling for a period of 28 days. Each metabolic compound was extracted and quantified after separation using aluminum oxides from the contaminated soil.

Determination of saturated hydrocarbon.

Five grams of soil samples was extracted ultrasonically with mixture of methanol, di-chloromethane (DCM) and water (1:5:4), centrifuged for 15 minutes. The mixture was filtered through Whatman No.1 filter paper and filtrates collected with conical flask. The extracts were transferred quantitatively into a previously weighed evaporation dish for drying. The dish was put in a drought cupboard containing charged desiccators to maintain a moisture free atmosphere. The dish was weighed at intervals of 1 hour until no diminution was seen on the weight (i.e. a constant weight is attained). By difference, the weight of the extracted saturated hydrocarbons (residual oil) was recorded (Bento *et al.,* 2003).

$$\frac{W_2 - W_1}{W} \times 100 = \%\ \text{Saturated HC extract}$$

W = Weight of sample (soil)

W_1 = Weight of empty evaporation dish

W_2 = Weight of dish and saturated hydrocarbon (HC) extract

Determination of aromatic hydrocarbon

Soil sample (1 g) was mixed with 20 ml of ethane and ethylene chloride in 1:1 (v/v) mixture ratio. The mixture was transferred in a centrifuge tube in order to extract the aromatic hydrocarbon and centrifuged at 5000 rpm for 5 minutes. The supernatant was read at 600 nm wavelength spectrophotometrically. Then, extracts were transferred into an Erlenmeyer flask, dried to a constant weight, and bathed under 75 ^{o}C to evaporate volatile solvents. Quantification of the residual extract was employed to assess the biodegradation of the extract (Manal, 2011).

Determination of asphaltene and polar compound.

Asphaltene and polar compound in the soil sample (1 g) was extracted with 50 ml of equal volumes of DCM and petroleum ether. The extract was passed through anhydrous sodium sulfate and evaporated under vacuum using a rotary evaporator (Lin *et al.*, 2005). Quantification was carried by gravimetric technique (Lin and Mendelssohn, 2009).

Determination of phenolic compound

Soil sample (0.5 g) oil was extracted with 20 ml of pure methanol by shaking for 30 minutes at room temperature. Thereafter, the mixture was filtered. An aliquot of the filtrate, 2 ml, was treated with 1 ml of Folin-ciocaltena reagent in a test tube (Rui *et al.* 2012). The resulting blue colouration was measured spectrophotometrically at 510 nm wavelength. Absorbance measurement was done in relation to standard phenol solution and the formula below was used to quantify the phenols.

$$\text{Phenol mg/kg} = \frac{1000}{W} \times \frac{au}{as} \times C \times \frac{V_f}{V_a} \times D$$

W = Weight of soil analyzed
au = absorbance of sample
as = absorbance of standard phenol solution
C = concentration of standard phenol solution (mg/kg)
V_f = Total volume of methanolic extract
V_a = Volume of extract analyzed
D = Dilution factor where applicable.

RESULTS

Characterization and Identification of Bacterial Isolates.

The three strains were isolated from the petrochemical contaminated soil using pour-plate technique. All the three isolates of *Pseudomonas* species were inoculated on nutrient agar and incubated at 28±2 ^{0}C for 48 hours to study various morphological characters. The growth in all the isolates initiated after 48 hours of incubation and isolate P_1 showed smooth, slimy, and blue to red pigment. Isolate P_2 showed circular, smooth, slimy yellow pigment and isolate P_3 showed smooth, slimy green pigment. Gram staining showed that these isolates were Gram negative bacteria. Microscopic observation showed that the three isolates were rods and motile (Table 1).

The biochemical characterization of isolates was performed and they were identified as *Pseudomonas aeruginosa* (P_1), *Pseudomonas mendocina* (P_2) *and Pseudomonas putida* (P_3) as shown in Table 1. The isolates were positive to Catalase, Oxidase, Citrate, Arginine dihydrolase, and Glucose tests while negative results were observed for Indole, Maltose, Sucrose and Starch hydrolysis tests. Other biochemical tests such as gluconate, gelatin hydrolysis, casein, urease utilization, lipase, manitol, sucrose tests served as differential tests for the three isolates (Table 1)

Molecular identification technique was employed for confirmation of the bacterial isolates. Phylogenetic studies revealed that the 16S rRNA of the strain *Pseudomonas mendocina* NK-01 had 100% similarity with the nearest match in the (Accession Number: NZ CP017290.1) Genebank. *Pseudomonas putida* B6-2 had 99% similarity with the nearest match in the (Accession Number: NZ CP015202.1) Genbank. Then, *Pseudomonas aeruginosa* PAER4 119 had 96% similarity with the nearest match in the (Accession Number: NZ CP013113.1) Genebank (Table 2, 3 and 4).

Table 1. The morphological and biochemical identification of *Pseudomonas* isolates

S/N	Morphological and biochemical tests	*Isolate P_1*	*Isolate P_2*	*Isolate P_3*
1	Colony character	Blue to red pigment	Yellowish pigment	Yellow to brown, green pigment
2	Cell type	Rod	Rod	Rod
3	Motility	Motile	Motile	Motile
4	Gram reaction	-	-	-
5	Gluconate	+	-	-
6	Gelatin hydrolysis	+	-	-
7	Oxidase	+	+	+
8	Casein	+	-	V
9	Urease utilization	+	-	-
10	Arginine dihydrolase	+	+	+
11	Indole production	-	-	-
12	Glycerol	+	-	+
13	Catalase	+	+	+
14	Ammonuim salts "sugars" Ethanol	+	-	+
15	Glucose	+	+	+
16	Maltose	-	-	-
17	Manitol	+	-	-
18	Citrate	+	+	+
19	Sucrose	-	-	- or V
20	Starch hydrolysis	-	-	-

Key: (+) means positive, (-) means negative and (v) means variable

Table 2. Molecular identification of *Pseudomonas aeruginosa* PAER4 119

Sample No.	Sequences Obtained	% Similarity	Genbank Accession No.	BLAST ID
P_1	CAGCAGCCGCGGTAATACGAA AGGGTGCAAGCGTAATCCGGA ATTACTGAGACGTCAAAGCGC GCGCAGGTGGTTTCAGCAAGT CTGATGTGAAATCCCCGTGCTC AACCTGGGGAGGTCATCCAAA ACTACTGAGCTAGAGTACGGT AGAGGGGTGGTGGAATTTCCT GTGTAGCGGTGAAATGCGTAG ATATATGAAGGAACACCAGTG GCGAAGGCGACTCACCTGGTC TGATACTGACACTGAGGTGCG AAAGCGTGGGGAGCAAACAG GATTAGATACCCTGGTAGTCC ACGCCGTAAACGATGTCGACT AGCCGTTGGGATCCTTGAGAT CTTAGTGGCGCAGCTAACGCG ATAAGTCGACCGCCTGGGGAG TACGGCCGCAAGGTTAAAACT	96%	NZ CP013113.1	*Pseudomonas aeruginosa* PAER4 119

Table 3. Molecular identification of the *Pseudomonas mendocina* NK-01

Sample No.	Sequences Obtained	% Similarity	Genbank Accession No.	BLAST ID
P_2	GTCAACGAGAAGTATCTGAGC CGTTTGCTCGAGTTGCTCGGTG AACGTGCAGGCGGTCTGGTTC CCGCGCTTTCCCTATTAATAGG CAGCAAGCGTGCCGCCACTGC TCGGGTTGCACCCACTGCCCCC CTGCCCGCGGCTGCGCGCGCT GCTCAGGTTCAGGCTGCCGCT GCAGTGAGCAAGCCGCTTCCT CCGCCCGTGCAGGAGGAACCT TCGCGAGCCAGCTTCGATTCG ATGGCCGGTTCGGCGCCGCAA CCGCCAACCCCTG	100%	NZ CP017290.1	*Pseudomonas mendocina* NK-01

Table 4. Molecular identification of *Pseudomonas putida* B6-2

Sample No.	Sequences Obtained	% Similarity	Genbank Accession No.	BLAST ID
P_3	GGCAATGTTTTGGGGGGGCGG TCCTATAATGCAGTCGAGCGG ATGACGGGAGCTTGCTCCTCG ATTCAGCGGCGGACGGGTGAG TAATACCTAGGAATCTGCCTG GTAGTGGGGGACAACGTTTCG AAAGGAACGCTAATACCGCAT ACGTCCTACGGGAGAAAGCAG GGGACCTTTGGGCCTTGCGCT ATCAGATGAGCCTAGGTCGGA TTAGCTAGTTGGTGGGGTAAT GGCTCACCCAGGCGACGATCC GTAACTGGTCTGAGAGGATGA TCAGTCGCAATGGAACTGAGA CACGGTCCACACCCCTACGGG AGGCGGCAGTGGGGAATGTTG	99%	NZ CP015202.1	*Pseudomonas putida* B6-2

Physicochemical Analysis of Petrochemical Contaminated Soil and Normal Soil.

The physiochemical analysis was conducted and results compared the physiochemical properties of the contaminated soil and normal soil (control) as shown in Table 5.

Based on observation, both the contaminated soil sample and normal soil were classified as Sandy Loam soil. There was no significant difference in temperature (30 ^oC) for both test soil and normal soil. Bulk density (2.19 and 1.48 g/cm^3) and electrical conductivity (0.43 and 0.31 µs/cm) showed to be higher in contaminated soil than normal soil. However, the results also revealed that other parameters such as porosity (56.60 and 64.50 %), pH (7.2 and 7.78), water holding capacity (43.25 and 61.43 %), were higher in normal soil than the contaminated (Table 5).

Most of the results obtained from the physicochemical analysis of the contaminated soil showed less values compared to the normal soil. These results were seen in parameters like calcium, sodium, potassium, magnesium, effective cation exchangeable capacity, exchangeable acids, phosphorous and nitrogen (Table 5). It was observed that TPH and organic carbon content were higher in the test soil than in normal soil (7.40 and 0.00 %) and (0.43 and 0.31 µs/cm) respectively.

Table 5. Physicochemical analysis of contaminated soil sample and normal soil

S/N	Parameter	Unit	Contaminated soil	Normal Soil
1.	Textural Class		Sandy loam soil	Sandy loam soil
2	Temperature	(^{o}C)	30	30
3	Bulk Density	(g/cm^3)	2.19	1.48
4	Porosity	(%)	56.60	64.50
5	Soil pH	(%)	7.2	7.78
6	Electrical Conductivity	(µs/cm)	0.43	0.31
7	Water holding capacity	(%)	43.25	61.43
8	Organic carbon	(%)	2.38	0.83
9	Total Nitrogen	(%)	0.24	1.92
10	TPH	(%)	7.40	0.00
11	Total Phosphorus (P)	(ppm)	11.28	13.31
12	Magnesium (M)	(cmokg^{-1})	0.46	1.98
13	Potassium (K)	(cmokg^{-1})	0.16	1.26
14	Calcium (Ca)	(cmokg^{-1})	1.26	5.20
15	Sodium (Na)	(cmokg^{-1})	0.44	1.41
16	EA	(cmokg^{-1})	0.49	1.40
17	ECEC	(cmokg^{-1})	2.81	11.85

Key: EA=exchangeable acid; ECEC=effective cation exchangeable capacity

Analysis of Total Petroleum Hydrocarbon (TPH)

Biodegradation of Total Petroleum Hydrocarbon (TPH) was studied. The percentage reduction of TPH in the petrochemical contaminated soil was achieved after the treatment with each indigenous *Pseudomonas* strains and their consortium (Fig. 1).

The results obtained showed that percentage reduction of TPH ranged between 40.54 % and 80.81 % for the remediation period of 28 days (Fig. 1). Maximum percentage reduction that was recorded by the consortium was 80.81 % at 28th day. However, it was observed that each indigenous *Pseudomonas* strains showed maximum percentage reduction of TPH at 74.59 % (*P. putida* B6-2), *67.57 %* (*P. aeruginosa* PAER4 119), and 61.62 % (*P. mendocina* NK-01). Also, the result revealed the minimum percentage reduction of TPH by the *Pseudomonas* isolates. The results were recorded at 40.54 % by *P. mendocina* NK-01, 45.06 % by *P. aeruginosa* PAER4 119, 52.25 % by *P. putida* B6-2 and 60.27 % by consortium on day 7. Generally, the results showed that the percentage reduction of TPH increased as number of days increased. The rate of TPH degradation was more rapid during the initial period as observed between day 0 and day 7. There was no significant difference in the percentage reduction of TPH as recorded on day 14 for *P. mendocina* and *P. aeruginosa* (Fig. 1).

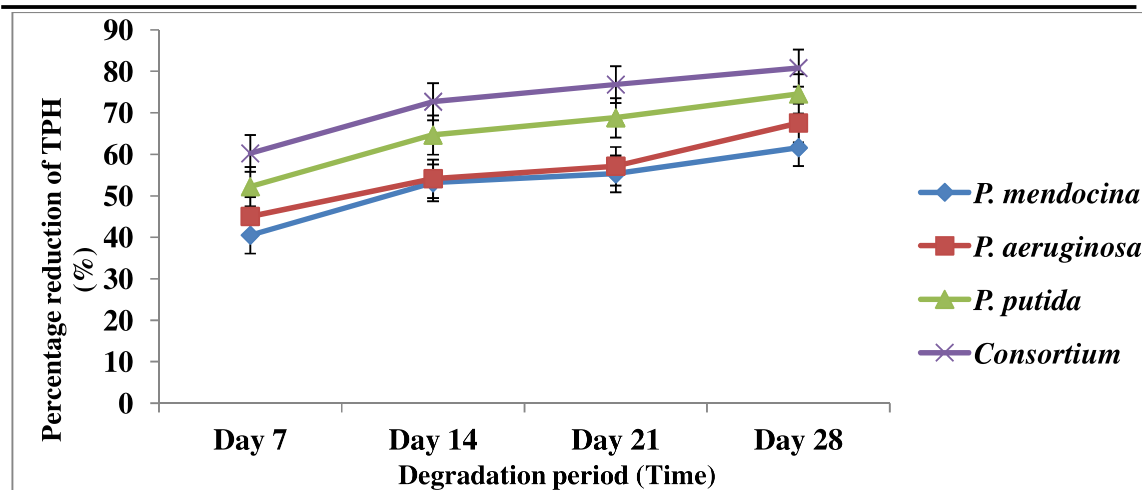

Fig. 1 Percentage reduction of TPH by *Pseudomonas* strains and consortium

Assessment of metabolic compounds from degradation of petrochemical

In this present study, assessment of metabolic compounds from degradation of petrochemical was analyzed. The result showed that saturated hydrocarbon, aromatic hydrocarbon, phenolic compound, and asphaltene and polar compound were identified as shown in Fig. 2, 3, 4 and 5. Generally, the rate of the metabolic compounds degradation was more rapid during the initial period as observed between day 0 and day 7 (Fig. 2, 3, 4 and 5).

In the study for degradation of saturated hydrocarbon, the reduction in concentration ranged from 5.00 mg/kg (32 %) to 1.42 mg/kg (81 %). There was a progressive reduction in concentration of saturated hydrocarbon with time (Fig. 2). The control slightly remained same throughout the degradation period. Maximum reduction in concentration of saturated hydrocarbon was achieved by consortium at 1.42mg/kg (81 %). The result also revealed the maximum reduction of the individual *Pseudomonas* strains. The result showed *P. mendocina* at 2.84 mg/kg (62 %), *P. aeruginosa at* 2.40 mg/kg (68 %), *P. putida at* 1.88 mg/kg (75 %). It was also observed that *P. mendocina* and *P. aeruginosa* had similar potential rate of degradation as shown on day 7 and day 21 therefore not significantly difference but with slight difference on day 14 (Fig. 2).

The reduction in concentration of aromatic compound ranged from 0.18 mg/kg (50 %) to 0.01 mg/kg (97.22 %) as shown in Fig. 3. The maximum reduction of aromatic compound was recorded by consortium at 0.01 mg/kg (97.22%) on the 28th day. However, high reductions in concentration of the aromatic compound were observed by *P. mendocina* 0.04 mg/kg (88.89 %), *P. aeruginosa* 0.02 mg/kg (94.42 %) and *P. putida* 0.024 mg/kg (94.44 %). Reduction in concentration of aromatic compound increased with time. There was no significant difference in the control for the whole period of degradation. There was no significant difference for the rate of degradation by *P. mendocina* and *P. aeruginosa* as recorded on day 14. Equal rate of degradation was also observed by *P. putida* and *P. aeruginosa* on day 21 thus not significantly different (Fig. 3).

The result showed that phenolic compound was largely degraded (Fig. 4). The reduction in concentration of phenolic compound ranged between 0.46 mg/kg (71.25 %) and 0.13 mg/kg (92 %). Maximum reduction in concentration were observed by *P. mendocina* at 0.22 mg/kg (86.25%), *P. putida* at 0.20 mg/kg (87.50 %), *P. aeruginosa* at 0.14 mg/kg (91 %), and consortium at 0.13 mg/kg (92 %). Generally, the *Pseudomonas* strains had different potential rate of degradation as observed. The reduction in concentration increased with time. There was no significant difference in concentration level for the control as shown.

Similar reduction in the concentration of the phenolic compound was observed on day 7 as a result of catabolic activities of *P. putida* and *P. aeruginosa.* There was no significant difference noticed on day 14 for *P. putida* and *P. mendocina.* Similar observation was noticed on day 21 for *P. aeruginosa* and *P. mendocina. P. putida* and *P. mendocina* recorded similar result on day 28 (Fig. 4).

Asphaltene and polar compound were highly degraded. The result showed that there was a progressive decrease in the concentration of the residual metabolic compound with time (Fig. 5). Maximum reduction in concentration of asphaltene and polar compounds was observed to range between 0.30 mg/kg (85 %) and 0.06 mg/kg (97.03 %). The results showed that the control remained partially same through the degradation period. The maximum reduction in concentration of the metabolic compound was recorded at 0.06 mg/kg (97.03 %) by consortium. Other maximum reductions by the strains were at 0.07 mg/kg (96.53 %) by *P. aeruginosa*, *P. putida* at 0.10 mg/kg (95.05 %.), and *P. mendocina* at 0.12mg/kg (94.06%) respectively. The potential rate of degradation of the asphaltene and polar compound was similar among the *Pseudomonas* strains. Equal reduction in the concentration of the metabolic compound was recorded on day 7 as a result of catabolic activities of *P. mendocina* and *P. aeruginosa.* Also, there was no significant difference as observed on day 14 and 21 for *P. aeruginosa* and *P. putida.* Also similar results were recorded for *P. putida* and *P. mendocina* on day 28 (Fig. 5).

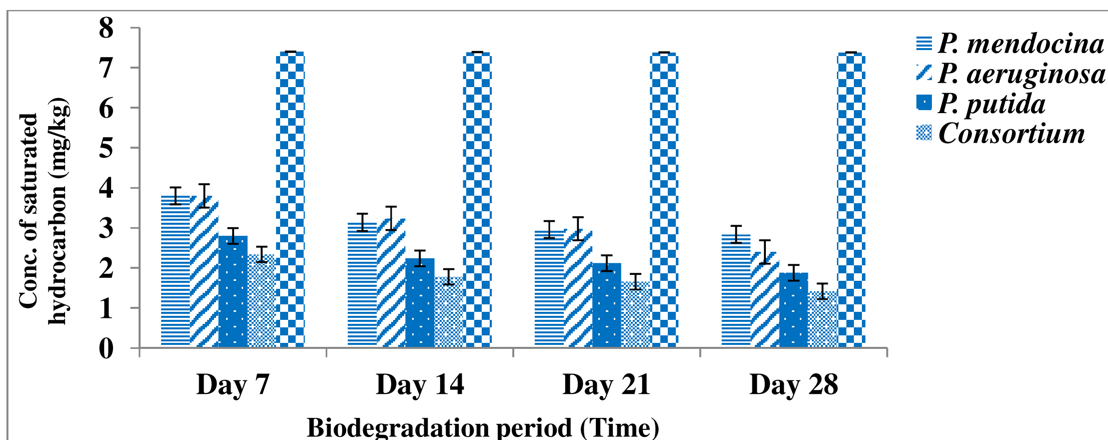

Fig. 2. Degradation of saturated hydrocarbon by *Pseudomonas* strains and consortium

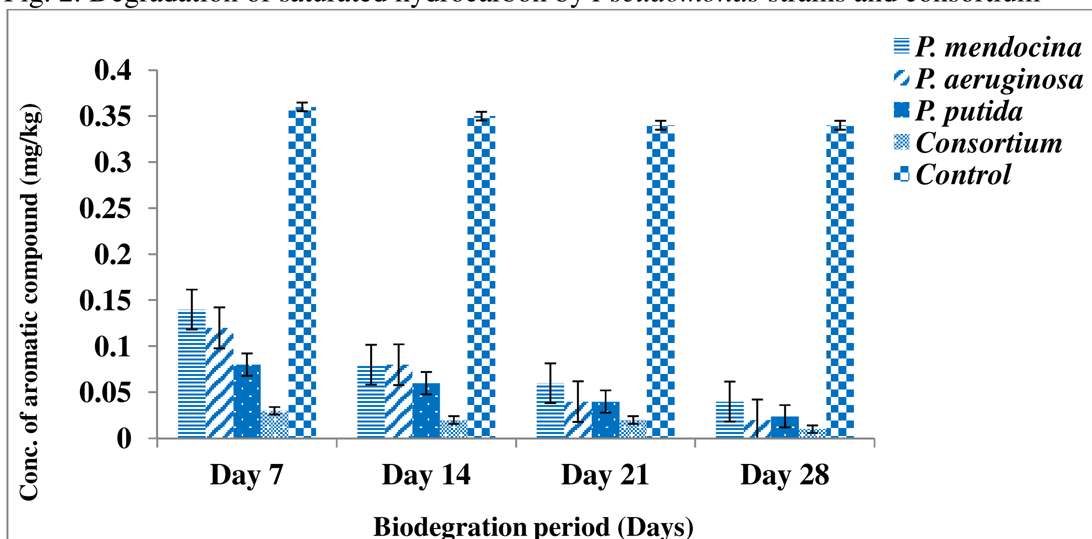

Fig. 3 Degradation of aromatic compound by *Pseudomonas* strains and consortium

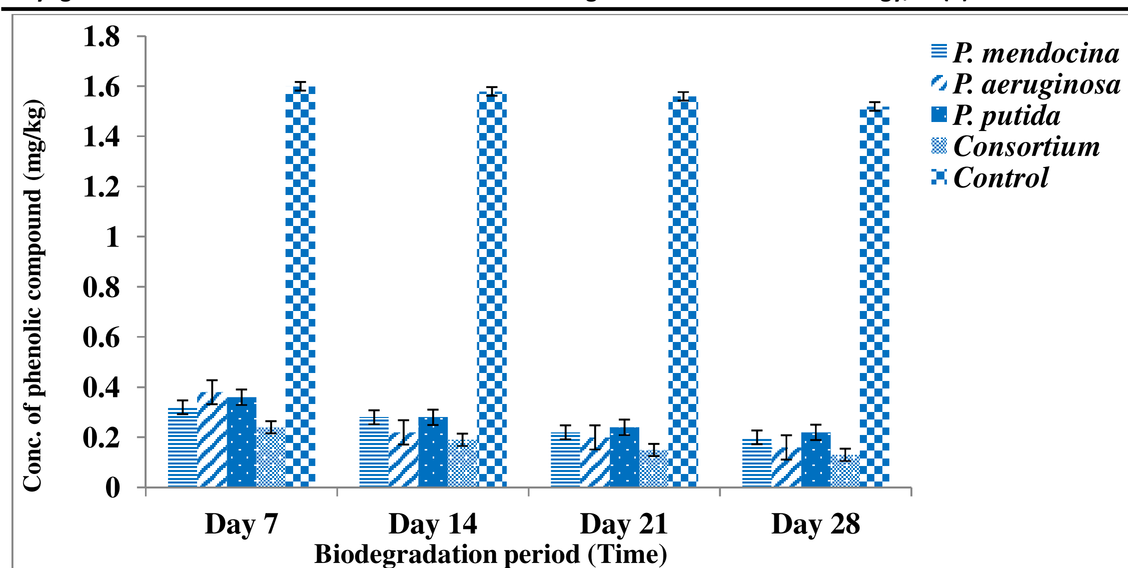

Figure 4: Degradation of phenolic compound by *Pseudomonas* strains and consortium.

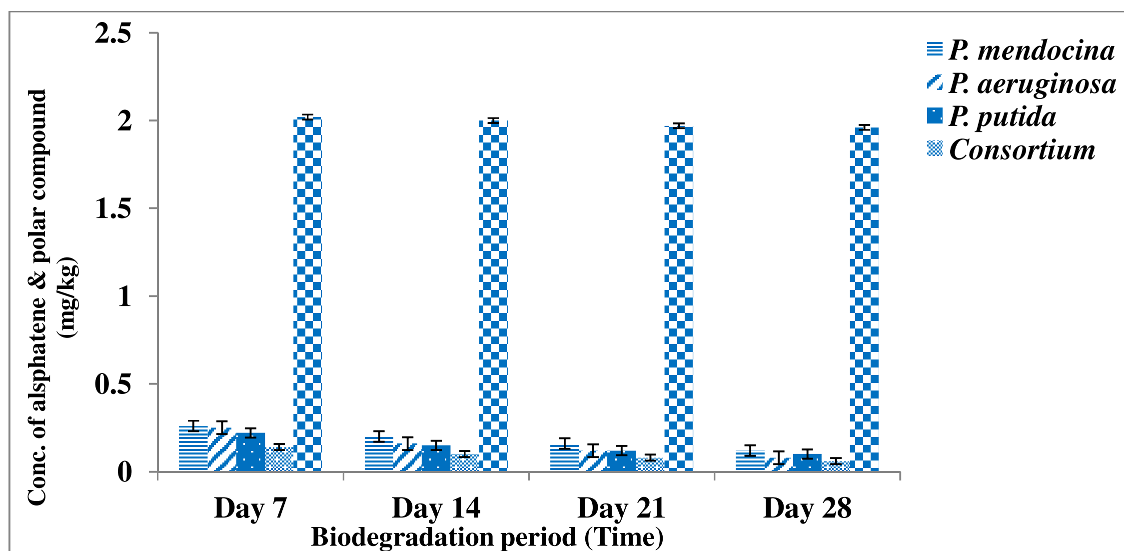

Fig. 5 Degradation of alsphatene & polar compound by *Pseudomonas* strains and consortium

DISCUSSION

In bioremediation study, the approach of using appropriate medium that has the ability to support the growth of the targeted organism(s) in petroleum contaminated environment is very important. The King B medium supported the isolation, differentiation and growth of the targeted organisms and the report conforms to the work of Garrity*et, et al.* (2005).

In the present study of morphological characterization, the bacterial cells retained pink safranin colour and they are gram negative. It is due to the fact that in gram-negative bacteria, the thin peptidoglycan layer in the periplasm does not retain the dark stain, and the pink safranin counter stain stains the peptidoglycan layer. Motility is due to the presence of flagella (Holt *et al.,* 1998; Oberhofer, *et al.,* 1999). A variation in the colony colour could be attributed to the production of different pigments metabolites. Different species of *Pseudomonas* are known to produce various kinds of pigments, the allocation of which in the genus is uncertain (Holt *et al.,* 1994).

The indication of green to blue colour during citrate test showed the production of enzyme citritase. Enzyme citritase breaks down citrate to oxaloacetate and acetate. Oxaloacetate is further broken down to pyruvate and carbon dioxide (CO_2). Production of sodium bicarbonate ($NaHCO_3$) as well as ammonia (NH_3) from the use of sodium citrate and ammonium salts results in alkaline pH (Holt *et al.,* 1994; Oberhofer, *et al.,* 1999). Yellow color in the surface alcohol layer of the bacterial during indole test is due to lack of production of intracellular enzymes *tryptophanase* that split indole from the amino acid tryptophan. The observed bubbling is due to the evolution of Oxygen (O_2) gas during catalase test which shows the ability of the organism to produce enzyme catalase that breaks hydrogen peroxide (H_2O_2) into water (H_2O) and O_2. In oxidase test, a colour change to blue is due to the oxidation of a reducing agent, chromogenic present in oxidase reagent and the participation of Cytochrome oxidase in transferring electrons from a donor molecule to oxygen. Also during arginine test, the alkalinity pH indicated by pH indicator phenol red, that turns a dark pink color under oil is due the production of an enzyme, arginine dihydrolase which releases ammonium from arginine (Holt *et al.,* 1994). This shows that the probable organisms belong to *Pseudomonas* species. No clear zone around the bacterial cells during starch hydrolysis which shows the inability of the organism to produce certain exoenzymes; α-amylase or oligo-1,6-glucosidase, that can hydrolyze starch (Holt *et al.,* 1994; Oberhofer, *et al.,* 1999). The taxonomic characteristics of these identified *Pseudomonas* species are similar to the reports of Sathishkumar, *et al.,* (2008). Based on the phenotypic characters, it was noted that all these strains belong to the genus *Pseudomonas* (Public Health England, 2015).

The molecular results revealed the similarities of the strains of *Pseudomonas mendocina*, *Pseudomonas putida*, and *Pseudomonas aeruginosa* with their nearest matches in the Genbank. Similar result was reported by Yergeau *et al.,* (2012), which also showed the relative abundance of *Pseudomonas* species in petroleum polluted area. The *Pseudomonas* species are among the best petroleum degraders. *Pseudomonas* is termed as Oil Eating Bug in petroleum industry (Kilbane, *et al.,* 2000).. An interesting and useful characteristic of many Pseudomonads are their metabolic versatilities to use wide variety of carbon sources as nutrients. As well as their ability to adapt to adverse environmental conditions (Kilbane, *et al.,* 2000).

The physicochemical properties of petrochemical contaminated soil and normal soil were evaluated in this study. The result revealed that the levels of organic carbon and TPH were higher when compared with the control. This may be attributed to the presence and the high content of carbon in the hydrocarbons from the petrochemical (Pathak *et al.*, 2011). Ekundayo and Obuekwe, (1997), also reported similar work where he noted increases in organic carbon content of oil polluted soils in Southern Nigeria. The increase may also be related to the slow decomposition rate of the amendment by soil organisms since contamination of soil with crude oil might have resulted in poor soil aeration.

The total nitrogen level of the polluted soil was observed to be less than the control. The report of Akubugwo *et al.,* (2009) did not support the recorded results, where total nitrogen level was higher in the impacted soil, compared with the control. The activities of nitrogen-fixing bacteria and other microbes associated with decomposition of organic matters might be inactivated in the polluted area. This could be the reason for the increase in total nitrogen level in the control. The pH of the contaminated soil was more acidic than the control. Similar result was reported by Nwaogu and Onyeze, (2010). The acidity could be due to the petrochemical contamination which can affect the metabolism of living things within an ecosystem.

The electric conductivity was higher in normal soil when compared to the contaminated soil sample which may be attributed to presence of metal or other ions from exchangeable acids. An increase in crude oil concentration increased the soils ionic strength thereby increasing nutrient available in the soils (Luepromchai *et al.*, 2007). However, there was no appreciable change in temperature for both soils (Pathak *et al.*, 2011). The authors (Pathak *et al.*, 2011); Luepromchai *et al.,* (2007), also observed high carbon content in the soil sample as compared to uncontaminated soil. And subsequently, a decrease in the total N and P content as seen in the PHC polluted soils.

The concentration of some mineral nutrients (Ca, Na, K, Mg), showed that effective cation exchangeable capacity (ECEC), exchangeable acids (EA), nitrogen and phosphorous in the polluted soil were less than that of normal soil (control) except that Ca^{2+} and Mg^{2+}. Similar results were recorded by Akubugwo *et al.,* (2009) and Onyeike *et al.,* (2000), who reported such increase in Ca^{2+} and Mg^{2+} from refined petroleum and crude oil polluted soils. The high concentration of exchangeable Ca^{2+} and Mg^{2+} in soil could be attributed to rapid decay and mineralization of organic and mineral materials in the soils. These processes lead to the release of cations and trace elements (Nnaji *et al.,* 2005). Reduction in K^{+} and Na^{+} may be due to nutrient immobilization consequent on the formation of complexes in the soil after degradation and uptake. Therefore, it has been observed that the growth of microorganisms in petrochemical polluted environments is limited due to these factors (Rahman *et al.*, 2002).

Soil texture and bulk density have also been considered to be very important for bioremediation study. Several factors affecting the degradation process like soil aeration, movement of nutrients through soil pores, water holding capacity etc. (Luepromchai *et al.,* 2007). The bulk density was higher than the normal soil due to depth of soil sample (0-30 cm). Porosity and water holding capacity properties of the soil sample were less than normal soil. These factors determine the extent of water retention and aeration in the soil. An increase in the soil hydrophobicity and reduction in the water holding capacity of the soil is based on presence and level of petrochemical in the soil (Osuji and Nwoye, 2007). Bundy *et al.,* (2002) reported that nutrient balance (C and N), pH, organic matter and moisture content of soil are usually affected as a result of contamination by hydrocarbons. The altered physico-chemical properties of petrochemical contaminated soil affects soil conditions, microorganisms and plants (Uche *et al.*, 2011). It also leads to deterioration of soil structure, loss of organic matter contents, loss of soil mineral nutrients such as potassium, sodium, calcium, magnesium, nitrogen and phosphate (Akubugwo *et al.*, 2009).

In this study, the consortium recorded maximum percentage reduction of TPH in petrochemical contaminated soil. Similar findings of Haramaya *et al.,* (1997) reported that a microbial consortium of *Pseudomonas* spp exhibited higher activity than an axenic culture of *Acinetobacter* for the biodegradation of light and heavy crude oils. The use of a consortium shows to be more efficient because of the diversity of catabolic enzymes that each microorganism used (Ghazali *et al*., 2004). There was also a high level of percentage reduction of TPH by the individual *Pseudomonas* isolates. Earlier studies demonstrated that bacterial isolates and consortium comprising hydrocarbon degrading *Pseudomonas* strains could effectively biodegrade crude oil. This could be achieved individually and collectively in liquid cultures as well as in polluted soil and sand (Salleh *et al.,* 2003). *Pseudomonas putida* exhibited maximum percentage reduction among the *Pseudomonas* isolates (Jirasripongpun, 2002). The decrease in hydrocarbon concentration means that the strains were able to use petroleum hydrocarbon as the unique carbon and energy source (Jirasripongpun, 2002)

The rate of TPH biodegradation was more rapid during the first 7 days. It might be due to the high potential of *Pseudomonas* species and their consortium in the test soil having efficiency in utilizing the residual petroleum hydrocarbon as a source of carbon and energy (Antai, 1990). Generally, it was observed that the rate of biodegradation was directly proportional to TPH reduction. This report is also in agreement with the findings of Yan *et al.,* (2012) who asserted that the biodegradation process follows kinetic models, and indeed it was found to follow 2nd order kinetics. Total petroleum hydrocarbons reduction from the surface of Lake Albert water gets bulky as bioremediation rate gets greater and when petroleum hydrocarbons dilution grows lesser, the bioremediation rate grows lesser too (Zhang, 2008).

Studies involving the measurement of the rate of degradation can provide important information about the biodegradability of industrial waste compounds, including petrochemicals. In this study, assessment of metabolic compounds indicates the degradation of petroleum products.

The metabolic compounds from the degradation of the petrochemicals were largely degraded. During the initial period of biodegradation, catabolic activity was more rapid. It might be due to the high potential of *Pseudomonas* strains and their consortium in the test soil having efficiency in utilizing the metabolic compounds as sources of carbon and energy (Antai, 1990). The higher concentrations of the metabolic compounds, during initial period of biodegradation exhibited an increase in biomass confirming the isolates being capable of exhibiting growth by breaking down petroleum hydrocarbon.

The important role of *Pseudomonas* species in achieving significant percentage reduction of phenolic compound, and asphaltene and polar metabolic compounds was observed. This observation was similar with researches reported by Kostal *et al.,* (1998); Balachandran *et al.,* (2012), who observed that *Pseudomonas* C12B, an SDS degrading strain, harbours a plasmid coding for degradation of medium chain length n-alkanes. Mander and Williams, (2003) also reported massive reduction of phenolic compound during the oxidative degradation of benzenoid hydrocarbons. Highest rate of degradation of the phenolic compound was recorded on day 7 which actually correlates the report of Yu *et al*. (2007); Mishra *et al.* 2014). The report showed that among the assessed organisms, *Pseudomonas* species emerged as the best degrader with about 73.7 % rate of degradation. This was demonstrated by its rapid discolouration of the redox indicator.

The result revealed that the percentage reduction of aromatic compound and saturated hydrocarbon increased with increased degradation periods. Consortium of *Pseudomonas* strains was observed to offer a better metabolic activity on aromatic compounds and saturated hydrocarbon. This observation agrees with the works of other authors (Hadibarata and Kristanti, 2012; Zhang *et al.,* 2011; Tao *et al.,* 2007). However, during the biodegradation, percentage reduction ranged between 32% and 97% for the period of 28 days. This was contrary to the results of Cai *et al*., (2013) who reported that the percentage reduction ranged from 43.03–99.9 % for 90 days. It has been reported also that *P. putida* had offered a better catabolic activity on saturated hydrocarbon (Hadibarata and Kristanti, 2012).

The dominant mechanism that breaks down these petroleum products is biodegradation, which is carried out by natural microbial population (Margesin and Schinner, 2001). Biodegradation of the complex hydrocarbons require the cooperation of more than one indigenous species (consortium), each strain contributing in hydrocarbon transformation process by metabolizing limited range of hydrocarbons (Kim and Crowely, 2007). In the present study *Pseudomonas* strains were able to degrade petrochemicals which were used as carbon and energy source

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CONCLUSION

This study concluded that indigenous consortium of *Pseudomonas* strains showed more efficient biodegradative potentials. This is evidenced by the high rate of degradation of total petroleum hydrocarbon (TPH) and the metabolic compounds. The assessment of metabolic compounds indicated the degradation of petroleum products. Hence, these strains and the assessment technique can be employed in biodegradation of petrochemical contaminated environment and also in the monitoring of biodegradation studies.

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