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Biodegradation potential of indigenous bacteria isolated from a crude oil polluted soil

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ORIGINAL RESEARCH ARTICLE

ABSTRACT

Microorganisms play a crucial role during bioremediation of crude oil polluted environment. This is due to their ability to utilize pollutant as carbon source thus reducing the concentration of the offending pollutant. However, not all indigenous microorganisms (bacteria) in polluted environment have same pollutant degradation potential. This study was carried out to determine the biodegradation potential of indigenous hydrocarbon-utilizing bacteria (HUB) isolated from a crude oil polluted site in Bodo West (Gokana, Rivers State, Nigeria). Crude oil polluted soil sample was collected and analyzed for total petroleum and aromatic hydrocarbons. Culturable HUB were isolated using vapour-phase transfer method, and the isolates were further screened for crude oil degradation potential using Okono medium crude oil. The 16S rRNA segment of each HUB isolate was amplified using a universal primer set (27F and 1492R). The total petroleum hydrocarbon (TPH) content of the polluted soil was 3376 mg/kg. Thirty HUB were isolated, out of which 43.33% were observed to have strong crude oil biodegradation potential, whilst 36.67% and 20% were scored as intermediate and weak, respectively, with regard to degradation potential. Out of the 30 HUB isolates, 15 were successfully sequenced and all were Gram negative. The sequenced isolates covered 7 bacterial genera namely: *Alcaligenes*, *Escherichia*, *Proteus*, *Providencia*, *Pseudomonas*, *Vibrio* and *Shewanella*. Members of *Escherichia*, *Proteus*, and *Providencia* were among those that showed strong crude oil degradation potential with optical density > 1.50, following 21 days of incubation at 37 ºC. This study showed that in a crude oil polluted site such as Bodo West, indigenous HUB have different biodegradation potentials. This varying degree of crude oil degradation potential by different HUB isolates could be explored for future bioremediation studies in the polluted site.

KEYWORDS

crude oil; degradation; indigenous hydrocarbon-utilizing bacteria; pollution

1. INTRODUCTION

Petroleum hydrocarbon-utilizing microorganisms are widely distributed in nature. Bacteria, fungi, cyanobacteria and microalgae are examples of microbial group, which utilize hydrocarbons as sources of carbon and energy for growth and other metabolic activities. Microbial metabolic process is crucial for the breakdown or transformation of pollutants from a more harmful state to an innocuous state (Bamforth and Singleton 2005; Kappell et al., 2012). Petroleum hydrocarbons enter any environment (water, soil, and air) by either naturally or anthropological means (Gao et al., 2015; Nkem et al., 2016). Bioremediation as an attractive technology to decontaminate polluted environment (Tyagi et al., 2011) has gained popularity in the global conservation and sustainability strategies owing to its eco-friendly and cost effective features (van Elsas et al., 2007; Chikere et al., 2009). It has been reported that pollution or contamination of an environment with hydrocarbon alters overall microbial community structure leading to dominance of some taxa in polluted environment (Wolicka et al.,

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2009; Kumar and Khanna, 2010). Nature and state of pollutant, and natural selection pressure prevailing in polluted environment are some of the factors that determine which microbial groups are to be found in polluted environment at a given time (Head et al., 2006). Microbial communities exposed to hydrocarbon adapt by exhibiting some genetic changes beneficial to their population; this in turns results in increased proportions of hydrocarbon-utilizing communities and their catabolic genes in polluted environment (Quatrini et al., 2008). Nevertheless, the majority of petroleum hydrocarbons are very hydrophobic in nature therefore limiting the capabilities of microbes, which generally exist in aqueous phase, to access and utilize the hydrocarbons as carbon and energy sources. Indigenous hydrocarbon-utilizing bacteria can overcome such limitation by producing biosurfactants, which cause desorption of pollutants thus increasing contact (mass transfer) with degrading microbial population (van Hamme et al., 2003, Wang et al., 2014).

In polluted environments, bioremediation activities can be enhanced either by exogenous addition of microbes (bioaugmentation) or by substrates addition (biostimulation) (Azubuike et al., 2016). Both approaches have recorded successes towards hydrocarbon removal from polluted sites (Chikere et al., 2015; Nwogu et al., 2015). Other environmental factors such as: temperature, pH, moisture content, oxygen concentration, and conductivity are also important factors considered towards enhancing bioremediation process (Lu et al., 2014; Smith et al., 2015; Sarkar et al., 2016). It has been demonstrated that microbial consortia exhibit better pollutant removal efficiency compared to single bacterial species (Hassanshahian et al., 2014). Little is known on the indigenous hydrocarbon-utilizing bacteria that play a crucial role during intrinsic bioremediation in Bodo West, Gokana Local Government Area of Rivers State, Nigeria. More information (especially on degradative potentials of resident microbes in the polluted site) is needed in order to understand the microbial ecological processes taking place in such polluted site. Such information will be pivotal in future designing and/ or selecting of any bioremediation approach aimed at enhancing pollutant removal from the polluted site. Therefore, this study investigated crude oil biodegradation potential of indigenous culturable hydrocarbon-utilizing bacteria isolated from Bodo West, Gokana, Rivers State, Nigeria.

2. MATERIALS AND METHODS

2.1. Site description and sample collection

Crude oil polluted soil samples were collected at depth of 15-30 cm from different sampling points in Bodo West community, Gokana Local Government Area of Rivers State, Nigeria. The community is one of the largest indigenous communities in the Niger Delta, which hosts the Shell Petroleum Development Company (SPDC) of Nigeria's Trans-Niger pipelines (24 and 28 inches). The crude oil spill that goes unchecked from oil spills have clearly devastated about 20 km^2 network of creeks and inlets within the community. Following the sample collection, the samples were aseptically transported to laboratory as described by Sojinu et al. (2010) and were mixed to obtain composite samples, which were used for further analyses.

2.2. Determination of the physicochemical conditions and hydrocarbon content of the polluted soil sample

Composite polluted soil sample was analyzed for physicochemical parameters such as: temperature, pH, electrical conductivity, total organic carbon (% TOC), moisture content, total nitrogen and phosphorus contents as described (APHA 2008). Residual total petroleum hydrocarbon (TPH) and polycyclic aromatic hydrocarbons (PAHs) were extracted from soil sample and quantified using gas chromatograph-flame ionization detector (GC-FID) according to the methods of ASTM (2010) and USEPA (2001).

2.3. Enumeration of total culturable heterotrophic and hydrocarbon utilizing bacteria

One gram of soil samples was suspended in 9 mL of 0.85% NaCl (physiological saline), which served as a solvent. The sample suspension was serially diluted to 10-fold using the same 0.85% NaCl as diluent. For the enumeration of total culturable heterotrophic bacteria (TCHB), 0.1 mL of each dilution was transferred to plate count agar, following the serial dilution. This procedure was carried out in duplicate for each of the dilution. The inoculated agar plates were incubated for 24 h at 37 ^oC. On the other hand, culturable hydrocarbon-utilizing bacteria (CHUB) were enumerated in a similar manner. However, mineral salt agar medium (Bushnell-Hass) in lieu of plate count agar was used for the procedure.

In addition, Okono medium crude oil served as the sole carbon source and was supplied in a vapour-phase transfer method as described (Hamamura et al., 2006; Nwogu et al., 2015). After incubation of different sets of agar plates, CHUB were tentatively identified based on their morphological and biochemical features with reference to Holt et al. (1994).

2.4. Crude oil biodegradation screening of CHUB

The crude oil degrading activities of each culturable hydrocarbon-utilizing bacteria (CHUB) isolate was determined as described (Peressutti et al., 2003; Chikere and Ekwuabu, 2014). In brief, 2 mL of crude oil was transferred into 100 mL flask containing 18 mL of 6 h culture in Bushnell-Haas broth (BHB). The culture was further incubated at 37 ºC for a period of 21 days. Following the incubation, crude oil degradation potential of each CHUB isolates was scored based on optical density at 600 nm.

Table 1. Physicochemical properties of the polluted soil sample

Parameter	Concentration
pH	6.95
Total nitrogen	0.28 mg/kg
Total phosphorus	0.31 mg/kg
Temperature	27 ºC
Conductivity	3320 µS/cm
Moisture content	21.8%
Total organic carbon (TOC)	0.8%
Total hydrocarbon content (THC)	2,835 mg/kg
Total petroleum hydrocarbon (TPH)	3,376 mg/kg
Polycyclic aromatic hydrocarbon (PAH)	15.63 mg/kg

2.5. Genomic DNA extraction and microbial community analysis

Total community DNA was extracted from each isolates using a ZR Soil Microbe DNA MiniprepTM (Zymo Research Corp. USA). The extracted DNA was quantified with a NanoDropND-1000 spectrophotometer (NanoDrop Technologies, USA) and was stored in MilliQ water at –20 °C. Subsequently, each genomic DNA extract was further analyzed in an electric field using 1% agarose gel. Next, the purified DNA extracts were amplified using a 16S rRNA universal primer set (27F 3'GAGTTTGATCCTGGCTCAG5' and 1492R 5'CTCAAACTAGGCCAGTC3'). The PCR reaction was carried in a thermal cycler with the initial denaturation temperature at 95 ºC for 5 min, denaturation at 95 ºC for 30 sec, annealing at 52 ºC for 30 sec, extension at 72 ºC for 45 sec, and final extension at 72 ºC for 3 min. The amplicons were further purified and sequenced as described (Zhou et al., 2015). The gene sequences of each isolate obtained in this study were compared with known 16S rRNA gene sequences in the GenBank database using the BLAST algorithm.

3. RESULTS AND DISCUSSION

3.1. Determination of physicochemical properties and hydrocarbon content of the polluted soil sample

Crude oil polluted soil sample collected from the site was analyzed for the total petroleum hydrocarbon (TPH) and polycyclic aromatic hydrocarbon (PAH) contents in order to quantify the extent of pollution. Other physicochemical parameters of the sample determined were: total nitrogen, total phosphorus, temperature, pH, total hydrocarbon content, electrical conductivity, moisture content and total organic carbon. The result showed that the TPH and PAH values were 3376 mg/kg and 15.63 mg/kg, respectively (Table 1). The result obtained for total nitrogen and total phosphorus were 0.2 mg/kg and 0.31 mg/kg, respectively. Soil environments are variable in terms of physiochemical properties, which are important for microbial growth and bioavailability of contaminants (Ramirez et al., 2012; Sarkar et al., 2016). The total petroleum hydrocarbon (TPH) directly reflects the real contamination posed by external contaminants as it excludes other polar hydrocarbons, which can accumulate during bioremediation processes (Head et al., 2006). The pH of the soil sample was 6.95, which could be considered optimal for microbial activity. Studies have demonstrated that optimal pH range for biodegradation range from 6.5 to 8.5 (Sarma and Sarma, 2010; Aparna et al., 2010). Similarly, Mikkonen et al. (2011) proposed that neutral range of pH is favourable for crude oil degradation by bacteria. The temperature of the polluted soil sample was 27 ºC. It has been reported that temperature affects the solubility of hydrocarbons as well as their physiology, and diversity of micro flora (Jain et al., 2011). The total nitrogen level in the polluted soil sample was low (0.28 mg/kg) and was attributable to high level of contamination (Bissette et al., 2013). Nutrient especially nitrogen is

Table 2. Microbial identity and crude oil biodegradation score

Isolate code	Biochemical identification	Molecular identification	% Similarity	Accession number	Final OD	Score
HUBAS6	*Proteus* sp.	Bacterium COD56 (2015)	99	KT6693303	1.594	a
HUBA42	*Proteus* sp.	*Proteus mirabilis* HUB1	99	KT6693299	1.526	a
HUBA26	*Proteus* sp.	*Proteus mirabilis* HUB2	99	KT6693306	1.548	a
HUBAS9	*Vibrio* sp.	Endophytic bacterium SV754	99	KT6693307	1.554	a
HUBA8	*Pseudomonas* sp.	FS			1.309	b
HUBA18	*Pseudomonas* sp.	FS			1.310	b
HUBA6	*Pseudomonas* sp.	*Pseudomonas* sp. LDC -17116S	99	KT6693296	1.190	b
HUBA27	*Pseudomonas* sp.	*Alcaligenes* sp. COD24	99	KT6693300	1.190	b
HUBA13	*Providencia* sp.	FS			1.299	b
HUBA3	*Pseudomonas* sp.	*Pseudomonas* sp. H2	99	KT6693295	1.630	a
HUBA22	*Vibrio* sp.	*Vibrio fluvialis* strain MCCB130	99	KT6693298	1.475	b
HUBAS8	*Vibrio* sp.	*Vibrio* sp.W-137-8	99	KT6693304	1.598	a
HUBA13	*Providencia* sp.	*Providencia vermicola* strain PDMZnCd1502	99	KT6693297	1.554	a
HUBA14	*Escherichia coli*	*Escherichia coli* 3L	99	KT6693308	1.624	a
HUBA31	UI	*Providencia sp.* 11CDBZ10	99	KT6693305	1.622	a
HUBA16	*Escherichia coli*	FS			1.472	b
HUBA36	UI	FS			1.534	a
HUBA37	*Serratia* sp.	FS			1.914	a
HUBA15	*Serratia* sp.	FS			0.895	c
HUBA38	*Serratia* sp.	*Shewanella* sp. XC-2	99	KT6693302	1.231	b
HUBA28	*Vibrio* sp.	*Vibrio fluvialis* MBTD_CMFRI_VFO2	99	KT6693301	1.072	b
HUBA19	*Pseudomonas* sp.	*Pseudomonas aeruginosa* strain PAM8	99	KT6693294	0.875	c
HUBA29	*Pseudomonas* sp.	FS			1.604	a
HUBA30	*Serratia* sp.	FS			1.606	a
HUBA46	*Serratia* sp.	FS			1.385	b
HUBA24	UI	FS			1.594	a
HUBAS5	UI	FS			0.816	c
HUBA43	UI	FS			0.904	c
HUBAS11	*Serratia* sp.	FS			0.915	c
HUBAS4	*Pseudomonas* sp.	FS			0.690	c

UI: unidentified; FS: failed sequence; strong ([a]), intermediate ([b]) and weak ([c]) degradability potentials

crucial for a successful biodegradation of hydrocarbon pollutants.

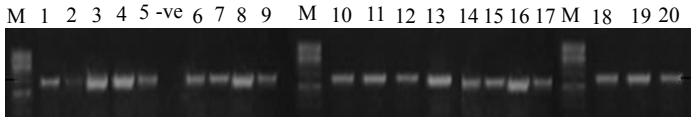
3.2. Enumeration of total culturable heterotrophic and hydrocarbon utilizing bacteria

The total culturable heterotrophic bacteria (TCHB) count was $0.5 \pm 2.0 \times 10^7$ CFU/g, while for hydrocarbon-utilizing bacteria (HUB) it was $0.2 \pm 0.6 \times 10^6$ CFU/g. This showed that TCHB were more in the polluted site compared to HUB. The result of the tentative identification based on morphological and biochemical properties showed that the HUB isolates were mainly of *Pseudomonas*, *Providencia*, *Proteus*, *Serratia*, *Escherichia coli* and *Vibrio* genera. These isolates were mainly Gram-negative bacteria, which were dominated by members of the class Gammaproteobacteria.

The dominant genera observed were *Pseudomonas*, *Serratia* and *Vibrio*. Based on the biochemical characteristics five isolates could not be identified as a result they were designated as unidentified (Table 2). In this study, it was observed that higher count of total culturable heterotrophic bacteria (TCHB) was obtained compared to that of culturable hydrocarbon utilizing bacteria (CHUB). This might be due to the fact that the site has been polluted for a longer time. This probably resulted in the contaminant (hydrocarbon) being non-bioavailable to the indigenous HUB, and in turn favoured the growth of heterotrophic bacteria.

3.3. Crude oil biodegradation screening of CHUB

A total of 55 bacterial isolates were obtained from the soil sample as putative HUB using Bushnell-Haas (BH) broth with crude oil supplied as carbon source. The isolated HUB were further screened for their degradation potential and the results were scored as strong, intermediate, and weak depending on the final optical density observed during the study period (24 days). The results showed that 30 bacterial isolates were able to degrade crude oil evidenced by turbidity (biomass increase) and increased optical density (OD) determined with a spectrophotometer. Out of the 30 HUB isolates, 43.33% were scored as strong, 36.67% as intermediate, while 20% were scored as weak with regard to crude oil biodegradability potential. Generally, in all the screening, the OD increased with increasing incubation period (Table 2). The biodegradation screening showed that each HUB isolate has its different capacity or tolerance toward crude oil as evidenced in the optical density readings (Table 2). The genera *Proteus*, *Providencia*, *Escherichia* and some *Pseudomonas* and *Vibrio* spp. isolated in this study performed well during the biodegradation studies. This might be due to the production of biosurfactant or bioemulsifier by some of these isolates. Hydrocarbon-utilizing bacteria have been demonstrated to produce bioemusifiers and biosurfactants, which greatly enhance bioavailability and bioaccessibility by transporting hydrocarbon to bacterial cell by efficient uptake mechanism (Satpute et al., 2010, Cho et al., 2011). The method of hydrocarbon degradation screening used in this study has proven to be efficient and reliable (Sarma and Sarma, 2010; Thenmozhi et al., 2012). All HUB isolated in this study belonged to the Gram-negative bacterial group and proteobacteria phylum. This observation is in agreement with other studies (Oboh et al., 2006; Militon et al., 2010; Alonso-Gutierrez et al., 2009), which highlighted the importance of proteobacteria especially the gamma division in hydrocarbon-polluted soil. Studies have notably demonstrated *Pseudomonas* to be a key player in hydrocarbon degradation in polluted sites (Moorthy et al. 2010; Prakash and Irfan 2011; Li et al., 2012; Zhang et al., 2012; Ren et al., 2015).



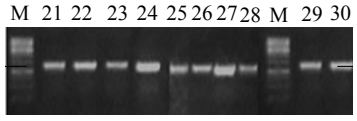


Figure 1. One percent agarose gel electrophoresis of genomic DNA (amplicons) extracted from culturable hydrocarbon-utilizing bacteria (CHUB) and amplified using a universal primer set. Lanes 1-30: amplified bacterial 16S gene of the isolated CHUB; Lanes M: 1.5 kb marker; -ve: negative control (sterile distilled water).

3.4. Genomic DNA extraction and microbial community analysis

The genomic DNA of the 30 HUB isolates that were scored positive for crude oil degradation were successfully amplified using universal primer set (27F and 1492R) (Figure. 1). The amplicon bands were purified and sequenced. Electropherograms obtained from the sequences were called using Chromas Lite

software version 2.01. Identification of bacterial 16S rRNA sequences was done using BLAST search facility of National Centre for Biotechnology Information (NCBI) database. The sequences alignment gave 99% similarity with those deposited in the GenBank and were considered close relatives, and used to assign identities to submitted sequences. Unfortunately, only 50% of the amplified sequences were successfully sequenced (Table 2).

4. CONCLUSIONS

This study showed that in a crude oil polluted soil such as the Bodo West in Rivers State, Nigeria, indigenous hydrocarbon-utilizing bacteria (HUB) exhibited different degradation potentials towards crude oil. Microbial genera belonging to *Proteus*, *Providencia*, and *Escherichia* spp exhibited strong degradation potential of the crude oil used in this study, whilst some spp of *Pseudomonas* and *Vibrio* were scored as intermediate with regard to crude oil degradation. Biodegradation screening of HUB isolates sheds light to their degradation abilities, and will prove useful especially during bioaugmentation geared towards enhancing bioremediation process.

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