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Potential of biofilm producing bacteria in remediation of crude oil polluted soil

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ABSTRACT

Bioremediation is an environmentally friendly approach to remediate polluted environments. This study aimed to evaluate the potential of biofilm-producing bacteria for the remediation of crude oil-polluted soil. The crude oil-polluted soil sample was collected from Biara, in Gokana Local Government Area of Rivers State, Nigeria. The soil was analyzed using standard procedures. The hydrocarbon-utilizing bacterial isolates were screened for biofilm production (BP), and those positive for BP were identified using biochemical methods. Three strong biofilm-producing bacteria (*Klebsiella, Pseudomonas* and *Bacillus* spp.) were selected and used for laboratory-scale bioremediation experiments. The experiments were carried out in eight microcosms, where the isolates were used singly and in combination, representing the various treatment options and the control (sterile soil). The setups were maintained for 56 days; samples were taken out every 7 days to analyze for the total hydrocarbon content (THC) using a spectrophotometer, nitrate, phosphate, total heterotrophic bacteria (THB) and hydrocarbon-utilizing bacteria (HUB). The results obtained revealed that THC, nitrate, pH and phosphate of the crude oil polluted soil were 18,403±3, 627, 92.6±0.3, 6.11 and 4.36±0.12 mg/kg, respectively on day 1. The THB and HUB populations in the soil were 1.68±0.07 × 10^7 and 1.54±0.06× 10^3 CFU/g. THB and HUB in the microcosms on days 1 to 56 ranged from 1.02±0.01 to 9.65±0.21 × 10^8 and 7.00±1.41 × 10^3 to 5.20±0.14 × 10^8 CFU/g, respectively. A general decrease in THC, nitrate and phosphate from days 1 to 56 in all the microcosms, was observed. The extent of hydrocarbon degradation (EHD) in the microcosms at day 56 was observed to be highest for the single isolates than the combinations, with *Bacillus* sp having the highest EHD of 97.40%, *Klebsiella* sp at 96.0% and *Pseudomonas* sp at 95.60%. It was observed that for the combinations, *Klebsiella* plus *Pseudomonas* gave 95.0%, *Pseudomonas* plus *Bacillus* gave 93.9%, and *Klebsiella* plus *Bacillus* gave 89.34%. The concentration of crude oil degraded is statistically significant when compared to the control, but when the EHD of the single isolates were compared, there was no significant difference between the results. When the three isolates were combined, 85.69% EHD was observed, while the control gave the least EHD of 12.0%. This study has shown that BPB has the potential to biodegrade crude oil pollutants in soil and may be deployed for the bioremediation of crude oil-polluted soil.

Keywords: Bioremediation, potentials, biofilm-producing bacteria, polluted soil, consortium.

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INTRODUCTION

Crude oil is a major source of income in Nigeria, accounting for over 70% of the Government's revenue and 83% of total export earnings (Nuhu et al., 2021). However, over the years there has been an increased release of crude oil and its products into the environment, resulting in the contamination of the environment: soil, water, and the atmosphere (Linden and Palsson, 2013). Oil spills are frequent events in Nigeria now and are a major potential environmental hazard in the Niger Delta region (Albert et al., 2018). Most of the Nigeria's oil and

gas production takes place in the Niger Delta. Although this generates billions of dollars for the Nigerian economy, it also causes major environmental problems for the inhabitants and the ecosystem in that area.

Bioremediation is one of the most effective ways of restoring soils or other environments contaminated with petroleum hydrocarbon due to oil spillage. Many studies have reported its effectiveness in removing numerous pollutants from various contaminated sites (Akani et al., 2020; Douglas et al., 2020). Different techniques are employed depending on the degree of saturation and aeration of an area (Vidali, 2001). Microorganisms with the capacity to degrade hydrocarbon are central to the bioremediation of oil-contaminated environments. Bacteria generally implicated in bioremediation include species from the genera *Bacillus, Pseudomonas, Vibrio, Micrococcus* and *Alcaligenes* (Ijah and Antai, 2003; Douglas and Green, 2015). Other specific microorganisms reported so far include *Aspergillus niger*, *Pseudomonas aeruginosa*, *Serratia marcescens*, and *Alcaligenes faecalis* (Uba, 2019). A major limitation of bioremediation is the bioavailability of the contaminant to the degrading microbes (Dasgupta et al., 2013).

Biofilms are structured microbial communities formed on the surface of solid materials or interfaces (Watnick and Kolter, 2000). It has been proved that environmental microorganisms exist predominantly as biofilms and gain high tolerance to physical, chemical, and biological stresses (Gorbushina and Broughton, 2009). Biofilm-associated cells exhibit specific gene expression, many times controlled by quorum sensing systems or dormancy, to allow their increases in resistance (Dickschat, 2010). Thus, forming biofilms is considered a natural strategy of microorganisms to construct and maintain a favourable niche in stressful environments (Shemesh et al., 2010). Single microbial species (Bacterial, Fungal or Yeast) as well as microbial consortiums can form biofilm. Many studies have reported the efficiency of microbial aggregates in the biofilm communities in bioremediation. Biofilm matrix protects the microorganisms during stress, allowing hydrocarbon-degrading bacteria a better chance of adapting to contaminants (Klein et al., 2010; Dasgupta et al., 2013). Biofilm-producing bacteria have been reported as better degraders than their planktonic counterparts. Shimada et al. (2012) reported efficient degradation of naphthalene by biofilm-associated *Pseudomonas stutzeri* T102 over planktonic form.

Similarly, Gertler et al. (2009) reported faster and more intense degradation of straight and branched hydrocarbons by the biofilm microbial community of *Alcanivorax borkumensis*. Some advantages of biofilm communities are their diversity and relative stability over time, these features are particularly important in biodegradation of hydrocarbons (Gertler et al., 2009). This study was therefore carried out to evaluate the potential of biofilm-producing bacteria in the bioremediation of crude oil-contaminated soil.

MATERIALS AND METHODS

Sample collection

Soil sample was collected from a crude oil polluted site in Biara, Gokana Local Government Area of Rivers State, Nigeria. The samples were collected from a depth of 0 - 15 cm using soil auger at different points of the site and bulked together for homogeneity to form a composite soil. The soil samples were placed in an ice-packed cooler and transported immediately to the Microbiology Laboratory of Rivers State University, Port Harcourt, for analysis.

Physicochemical and microbiological analyses of the soil sample

The soil sample was analyzed for total hydrocarbon content, nitrate, phosphate (APHA, 2012), total heterotrophic bacteria, and hydrocarbon-utilizing bacteria (Douglas and Green, 2015). All samples were analysed in duplicate for all parameters tested.

Determination of total hydrocarbon content

The total hydrocarbon content (THC) in the soil sample was determined using the spectrophotometric method as described in Peekate et al. (2022). In this method, 10 ml hexane was added to 5g of the soil sample. The mixture was agitated and filtered using a Whatman No. 1 filter paper. The filtrate was subjected to absorbance measurement at 420nm using a 721 VIS Spectrophotometer (Huanghua Faithful Instrument Co. Ltd, China). The absorbance reading was used to determine THC with the aid of a previously obtained calibration graph.

pH of the soil

The pH of the soil sample was determined using APHA (2012). The meter was switched on and allowed for some time. It was then calibrated with buffer solutions of a high pH range between 8 and 9 as well as a lower pH range between 1 and 6 by dipping the electrode into the buffer solutions. Ten grams (10g) of soil was weighed into a 100 ml beaker, 50 ml of distilled water was then added to allow immersion of the electrode, and mixing was carried out by stirring frequently for a few minutes. The beaker was allowed to stand for 15 minutes. The electrode was immersed into the sample. The pH values for each sample were recorded accordingly.

Determination of nitrate concentration

The nitrate concentration in the soil sample was determined using a modification of the APHA method

4500-NH_3 F (APHA, 2012), the indophenol blue method. Three grams (3g) of soil sample and 1 g piece of Aluminium foil were placed in a 250 ml Buchner flask, and 90 ml distilled water was added. The side arm of the Buchner flask was connected to an extracting solution (98 ml of 1.0 M HCl plus 1 ml of Phenol-nitroprusside solution plus 1 ml of alkaline hypochlorite solution) in a 250 ml beaker with the aid of a rubber hose. About 10 ml of 0.5 M (aq.) was added to the content of the Buchner flask, and the flask was immediately sealed. The setup was then allowed to stand for about 1 hour at which time effervescent in the Buchner flask was seen, and blue colour development in the extracting solution must have completed. The resulting blue colour solution was then subjected to absorbance measurement at 630 nm using a 721 VIS Spectrophotometer (Huanghua Faithful Instrument Co. Ltd, China). The absorbance reading was used to extrapolate the concentration of ammonium (NH_4^+) with the aid of a previously obtained calibration graph, and then the nitrate concentration was derived by multiplying the determined NH_4^+ concentration (mg/kg) by 3.44, the stoichiometric conversion factor.

Determination of phosphate concentration

The phosphate concentration in the soil sample was determined using a slight modification of the ascorbic acid method (APHA, 2012). In this method, 30 ml of 0.5 M HCl was added to a 3 g dried soil sample and allowed to stand for 30 minutes. Afterwards, the mixture was agitated and filtered through a Whatman No. 1 filter paper. The filtrate was transferred into a 100 ml capacity volumetric flask, and 13 ml of the analyzing reagents (373.2 ml distilled water plus 0.06g Potassium antimony tartrate plus 2.4g Ammonium molybdate plus 2.12g Ascorbic acid plus 26.8 ml conc. sulphuric acid) was added. The content of the flask was made up to the 100 ml mark using distilled water. The flask was agitated a little and allowed for about 30 minutes for blue colour development. The resulting blue colour solution was then subjected to absorbance measurement at 880nm using a 721 VIS Spectrophotometer (Huanghua Faithful Instrument Co. Ltd, China). The absorbance reading was used to extrapolate the concentration of phosphate with the aid of a standard phosphate-absorbance graph previously obtained.

Determination of total heterotrophic bacterial population

Total heterotrophic bacteria for the sample were enumerated using the spread plate technique described by Douglas and Green (2015) and Douglas et al. (2020). A gram of the soil was added to 9ml of sterile normal saline and serially diluted to 10^{-7}. Aliquot (0.1ml) of 10^{-4} to 10^{-7} dilutions were aseptically transferred onto properly dried nutrient agar plates in triplicate, spread evenly using a bent glass rod, and incubated at 37^{o}C for 24 hours. After incubation, the bacterial colonies that grew on the plates were counted, and the counts were used to calculate the Total Heterotrophic Bacterial population. Subsequently, selected colonies were sub-cultured onto fresh nutrient agar plates, and stock cultures of the isolates were prepared for preservation and identification. The slants were refrigerated at 4^{o}C until required for use (Douglas et al., 2020).

Determination of hydrocarbon utilizing bacterial population

Hydrocarbon utilizing bacterial population was determined by inoculating 0.1ml aliquot of the 10^{-2} to 10^{-3} dilutions onto mineral salt agar (MSA) plates using the spread plate technique. The composition of the MSA is as follows: agar (15g), K_2HPO_4 (0.5 g/L), $MgSO_47H_20$ (0.3 g/L), NaCl (0.3 g/L), $MnSO_4H_2O$ (0.2 g/L), $FeSO_4.6H_2O$ (0.2g/L), $NaNO_2$ (0.3g/L), and $ZnCl_2$ (0.3g/L). An antifungal agent (Amphotericin B, 250mg/L) was added after sterilization and cooling to 50°C of the MSA medium. Aliquots of 0.1ml from 10^{-2} to 10^{-3} dilutions were inoculated in triplicate and spread evenly using a bent glass rod. The vapour phase transfer method was adopted in supplying hydrocarbons in the inoculated plate. The plates were incubated at 32°C for 5 to 7 days. Ensuing colonies were counted and used to calculate the hydrocarbon utilizing bacterial population. Discrete colonies on the HUB plates were picked randomly using a sterile wire loop and streaked on a fresh nutrient plate (Douglas et al., 2020).

Identification of the bacteria

The pure culture of bacterial isolates was described based on colour, shape, elevation, size, texture, and opacity. The isolates were subjected to gram staining and microscopic examination, motility, oxidase, catalase, coagulase, indole, methyl red, Voges-Proskauer, citrate utilization, starch hydrolysis, and sugar fermentation tests using glucose, maltose, lactose, and sucrose. All the tests were carried out as described in Peekate (2022) and Cheesbrough (2006). Identities of the isolates were confirmed by comparing the characteristics of the isolates with standard taxa as outlined in Bergey's Manual of Determinative Bacteriology (Holt et al., 1994).

Screening the bacterial isolates for biofilm production

The hydrocarbon-utilizing bacterial isolates were screened for biofilm production using the Congo red and

tube methods (Bose et al., 2009; Douglas et al., 2022). In the Congo red method, bacterial isolates were inoculated on a complex medium containing Congo red. The composition of the medium is as follows: Brain heart infusion broth (37 g/L), sucrose (5 g/L), agar (10 g/L), and Congo red dye (0.8 g/L). Inoculated plates were incubated at 37°C for 24 to 48 hours. Black colonies with dry crystalline consistency indicated biofilm production (Williams et al., 2021).

In the tube method, 10 ml Trypticase soy broth containing glucose (1%) was inoculated with a loopful of bacteria from overnight culture on nutrient agar. The inoculated broths were incubated at 37°C for 24 hours. After incubation, the cultures were decanted, and the tubes were washed with phosphate buffer saline (pH 7.3). The tubes were dried and stained with 0.1% crystal violet. The excess stain was washed off with deionized water, and the tubes dried in an inverted position. Visible stained film seen lining the wall and bottom of the tube was indicative of biofilm production (Christensen et al., 1995).

Bioremediation experimental setup

From the results of the screening for biofilm production, three strong biofilm-producing bacteria were selected and evaluated for their bioremediation potential, singly and in combination, using a laboratory scale. Each microcosm contained 1kg of the polluted soil in well-labeled clay pots. Standard inoculums of the biofilm-producing bacteria were prepared using nutrient broth. The bioremediation experimental setups were prepared as shown in Table 1. NPK (15:15:15) fertilizer was added to stimulate the growth of the organisms for the remediation process. The control was sterile polluted soil, with no organism added. The setups were maintained for 56 days, soil moisture content was maintained at 10 - 30% using a sterile Mineral Salt Medium (MSM). The microcosms were kept in a greenhouse at ambient temperature; the contents were tilled every three days for proper mixing and aeration (Douglas et al., 2020). The microcosms were monitored on days 1, 7, 28, and 56 for Total Hydrocarbon Concentration (THC), Total Heterotrophic Bacteria (THB), Hydrocarbon Utilizing Bacteria (HUB) (Douglas *et al*., 2020), nitrate, and phosphate concentrations (APHA, 2012).

Statistical analysis

All data obtained from the experiment were analysed and presented in Tables and Charts. Statistical analysis was carried out using SPSS tools.

Table 1. Bioremediation experimental setup.

N	Setup code	COS (Kg)	BPB1 (ml)	BPB2 (ml)	BPB3 (ml)
1	CB1	1	50	-	-
2	CB2	1	-	50	-
3	CB3	1	-	-	50
4	CB12	1	25	25	-
5	CB13	1	25	-	25
6	CB23	1	-	25	25
7	CB123	1	16.7	16.7	16.7
8	CT (control)	1	-	-	-

Key: COS: Crude oil polluted soil, BPB: Broth culture of biofilm producing bacteria.

RESULTS

Physicochemical and microbiological parameters of the crude oil polluted soil

Phosphate, nitrate, pH and total hydrocarbon concentration (THC) in the crude oil-polluted soil were 4.36 ± 0.12 mg/kg, 92.6 ± 0.3, 6.11 and 18,403 ± 3,627 mg/kg, respectively. The populations of total heterotrophic bacteria (THB) and hydrocarbon-utilizing bacterial (HUB) counts were $1.68\pm0.07\times10^{7}$ CFU/g and $1.54\pm0.06\times10^{3}$ CFU/g, respectively. The population of HUB was significantly ($P < 0.05$) lower than the population of THB (Table 2).

Table 2. Microbiological and physicochemical parameters in the crude oil polluted soil (Baseline).

Parameters	MN ± SD
THB (CFU/g)	$1.68\pm0.07 \times 10^{7}$
HUB (CFU/g)	$1.54\pm0.06\times 10^{3}$
pH	6.11
THC (mg/kg)	18,403 ± 3,627
Phosphate (mg/kg)	4.36 ± 0.12
Nitrate (mg/kg)	92.6 ± 0.3

Biofilms production by isolated hydrocarbon utilizing bacteria

Responses of the hydrocarbon utilizing bacterial isolates,

isolates A to J, to the two methods used in screening for biofilm production are presented in Table 3. The responses in the Table revealed that isolates C, D, F, G, I, and J are all biofilm producers, but isolates G. I. and J were strongly positive for biofilm production.

Identity of isolated biofilm-producing bacteria

The biofilm-producing bacteria isolated from the crude oil-polluted soil were coded A to J. Their morphological and biochemical characteristics are presented in Tables 4a and 4b respectively**.** They were identified as follows: A – *Staphylococcus* sp., C – *Serratia* sp., D – *Alcaligenes* sp., F – *Escherichia coli*, G – *Klebsiella* sp., I – *Pseudomonas* sp., and J - *Bacillus* sp.

Changes in bacterial populations during the bioremediation

The total heterotrophic bacterial population (THBP) in the microcosms on days 1 – 56 ranged from 1.02±0.01 to 9.65±0.21 × 10^8 CFU/g. THBP was highest on days 7 and 56 in setup CB3, on day 14 in setup CB12, and on day 28 in setup CB13 as revealed in Figure 1. On the other hand, THBP was lowest on day 1 in microcosm CB123, on days 14 and 28 in microcosm CB3, and on day 56 in microcosm CT. Analysis of Variance (ANOVA) of THBP of the microcosms for the different days revealed that THBP in the setups was significantly different ($P < 0.05$) on all the days.

Hydrocarbon-utilizing bacterial populations (HUBP) in the microcosms ranged from 7.00±1.41 × 10^3 to 5.20±0.14 × 10^8 CFU/g. HUBP was highest on days 1, 7 and 14 in setup CB123, on day 28 in setup CB12, and on day 56 in setup CB3, as revealed in Figure 2. On the other hand, HUBP was lowest on days 1, 7 and 14 in setup CB1, on day 28 in setup CB123, and on day 56 in setup CT. ANOVA of HUBP of the setups for the different days revealed that HUBP in the setups were significantly different ($P < 0.05$) on all the days. The proportion of total heterotrophic bacteria that were hydrocarbon-utilizing bacteria in the microcosms was higher on days 7 (0.053 %) and 14 (0.051 %) in microcosm CB123 as seen in Figure 3; on day 28 it was highest (0.032 %) in setup CB3 and on day 56 in microcosm CB2 (0.103 %).

Table 3. Biofilms production by hydrocarbon utilizing bacterial isolates.

Isolate code	Identity	Congo red method	Tube method
A	*Staphylococcus* sp.	+	-
B	ND	+	-
C	*Serratia* sp	+	+
D	*Alcaligenes* sp	+	+
E	ND	-	-
F	*Escherichia coli*	+	+
G	*Klebsiella* sp.	+	++
H	ND	-	-
I	*Pseudomonas* sp.	+	++
J	*Bacillus* sp	+	++

Key**:** + = Moderately positive, ++ = Strongly positive - = Negative ND: Not identified.

Table 4a. Cultural characteristics of the biofilm producing bacteria (BPB).

Isolate code	Colour	Elevation	Opacity	Size	Texture
C	Red	Round	Opaque	Small	Smooth
D	Cream	Round	Translucent	Small	Smooth
F	Cream	Round	Translucent	Small	Smooth
G	Cream	Flat	Opaque	Large	Mucoid
I	Greenish	Round	Opaque	Small	Smooth
J	White	Round	Opaque	Small	Mucoid
K	Yellow	Round	Translucent	Small	Smooth

Total hydrocarbon concentration in the microcosms

There was a general decrease in total hydrocarbon concentration (THC) from day 1 (18,403mg/kg) to 56 in all the setups as shown in Figure 4. THC was highest on days 7 (11,052 ± 1,215 mg/kg) and 28 (10,750 ± 478

Table 4b. Morphological and biochemical characteristics of the BPB.

IC	GS	MP	CT	IN	CG	MT	OX	MR	VP	SH	CU	GF	MF	LF	SF	SO
C	-	R	+	-	-	+	-	-	+	-	+	AG	-	AG	AG	*Serratia* sp
D	-	R	+	+	-	+	+	+	-	+	+	AG	AG	AG	AG	*Alcaligenes* sp
F	-	R	+	+	-	+	-	+	-	+	+	AG	AG	AG	AG	*Escherichia coli*
G	-	R	+	+	-	+	-	-	+	+	+	AG	AG	AG	A	*Klebsiella* sp
I	-	R	+	-	-	+	+	+	-	-	-	-	-	-	-	*Pseudomonas* sp.
J	+	R	+	-	-	+	-	-	+	+		AG	A	-	A	*Bacillus* sp
K	+	C	+	-	+	+	+	+	+	+	+	AG	AG	AG	AG	*Staphylococcus* sp.

IC: Isolate code, GS: Gram stain reaction, MP: Morphology, CT: catalase, IN: Indole, CG: Coagulase, MT: Motility, OX: Oxidase, MR: Methyl red, VP: Voges-Proskauer, SH: Starch hydrolysis, CU: Citrate utilization, GF: Glucose fermentation, MF: Maltose fermentation, LF: Lactose fermentation, SF: Sucrose fermentation, SO: Suspected organism, R: Rods, C: Cocci, AG: Acid and gas, A: Acid only.

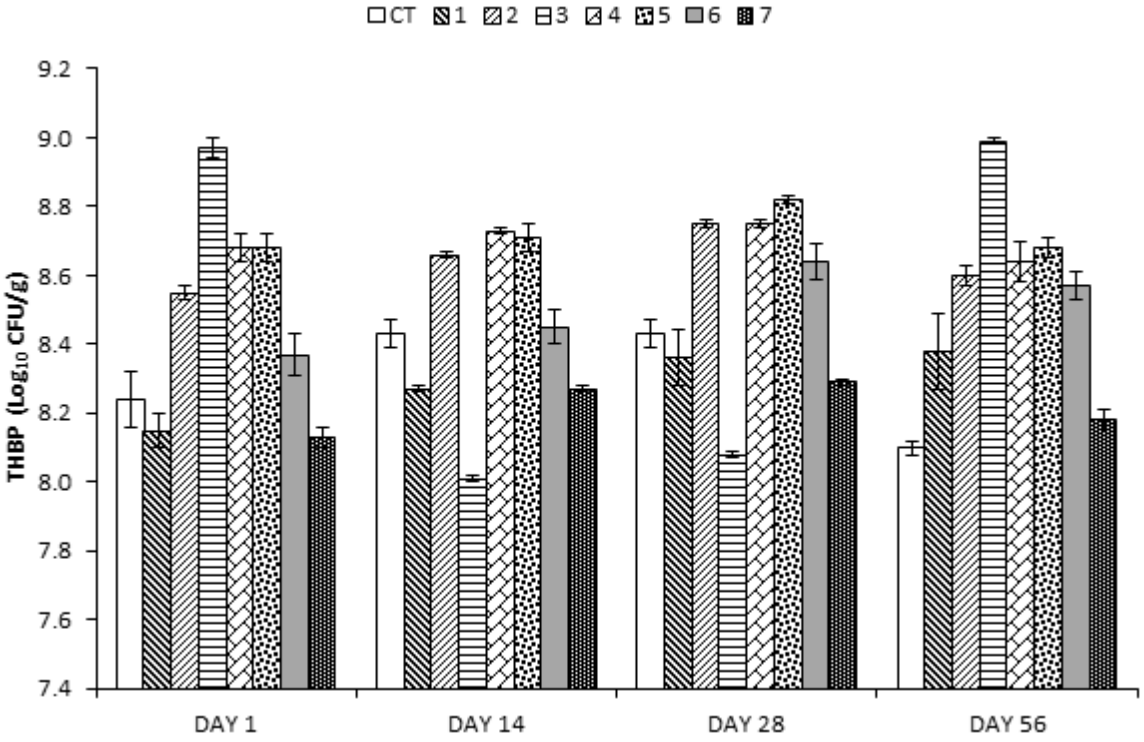

Figure 1. Total heterotrophic bacterial population (THBP) in the microcosms.
CT: Control setup; 1, 2, 3, 4, 5, 6, and 7 represents setups CB1, CB2, CB3, CB12, CB13, CB23, and CB123 respectively.

mg/kg) in setup CB23 and on day 56 (6,172 ± 272 mg/kg) in the control setup (CT). On the other hand, THC was least on day 7 (4,495±469 mg/kg) in setup CB12, on day 28 (2,798 ± 1,565 mg/kg) in setup CB123, and on day 56 (477± 424 mg/kg) in setup CB3. ANOVA of THCs in the setups for the different days revealed that THCs in the setups were not significantly different ($P > 0.05$) on days 1 and 56, but were significantly different ($P < 0.05$) on day 28. The extent of hydrocarbon degradation (EHD) in the setups on day 56, with THC values on day 1 as the initial reference point, was highest (94.52 %) in setup CB3 and lowest (42.08 %) in setup CB123 (Table 5).

Concentrations of physicochemical parameters monitored in the setups

Concentrations of phosphate and nitrate in the setups on days 1 and 56 are presented in Figures 5 and 6. There was a reduction in phosphate concentration in all the setups as shown in Figure 6; Setup CB3 had the highest phosphate concentration (11.3 mg/kg) on day 1, while setup CB2 and CB123 had the least (1.3 mg/kg) on day 56. There was a huge reduction in nitrate in all the setups (Figure 6). Setup CB2 had the highest nitrate concentration of 669.1 mg/kg on day 7; by day 56 nitrate

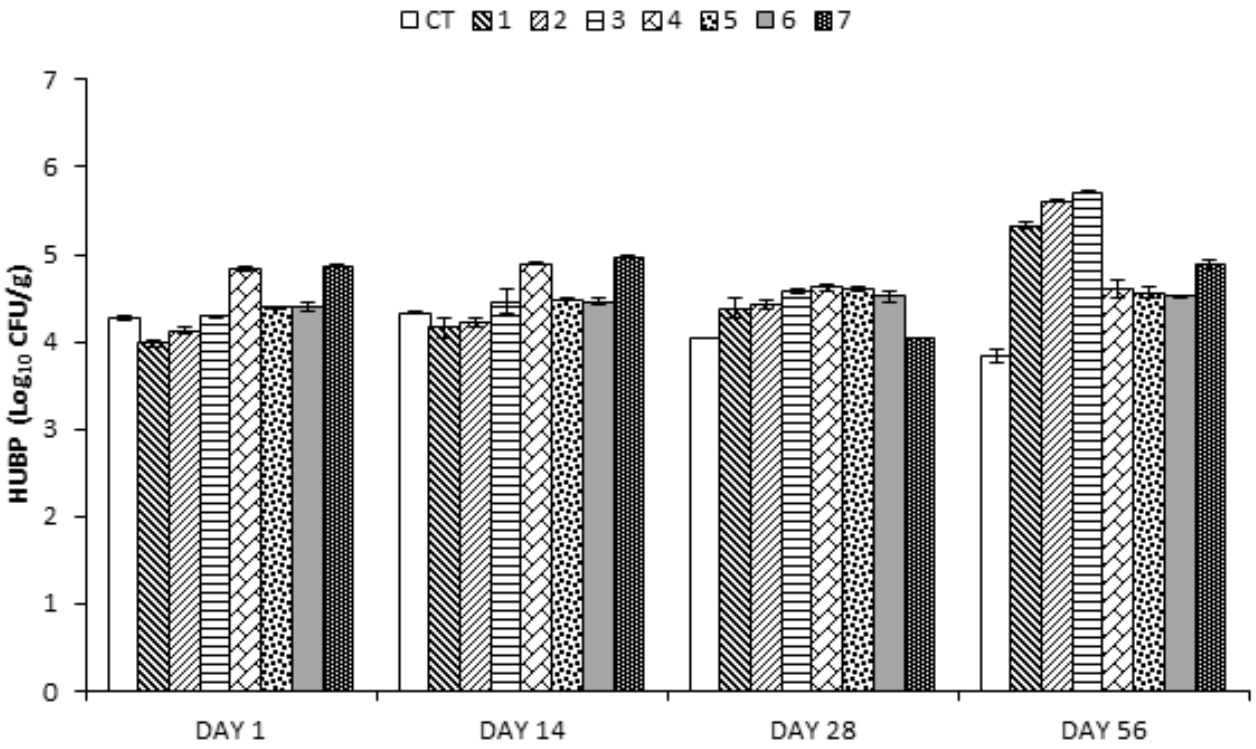

Figure 2. Hydrocarbon utilizing bacterial population (HUBP) in the setups.

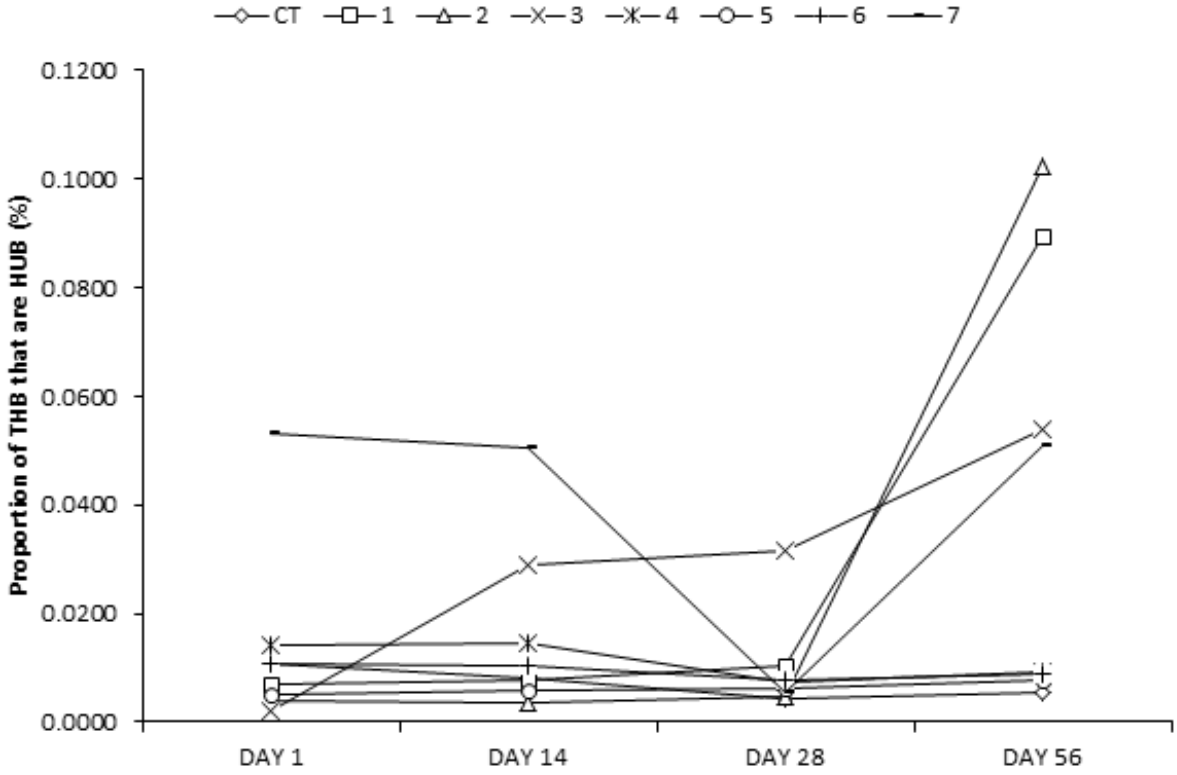

Figure 3. Proportion of total heterotrophic bacteria (THB) that are hydrocarbon utilizing bacteria (HUB) in the setups. CT: Control setup; 1, 2, 3, 4, 5, 6, and 7 represents setups CB1, CB2, CB3, CB12, CB13, CB23, and CB123 respectively.

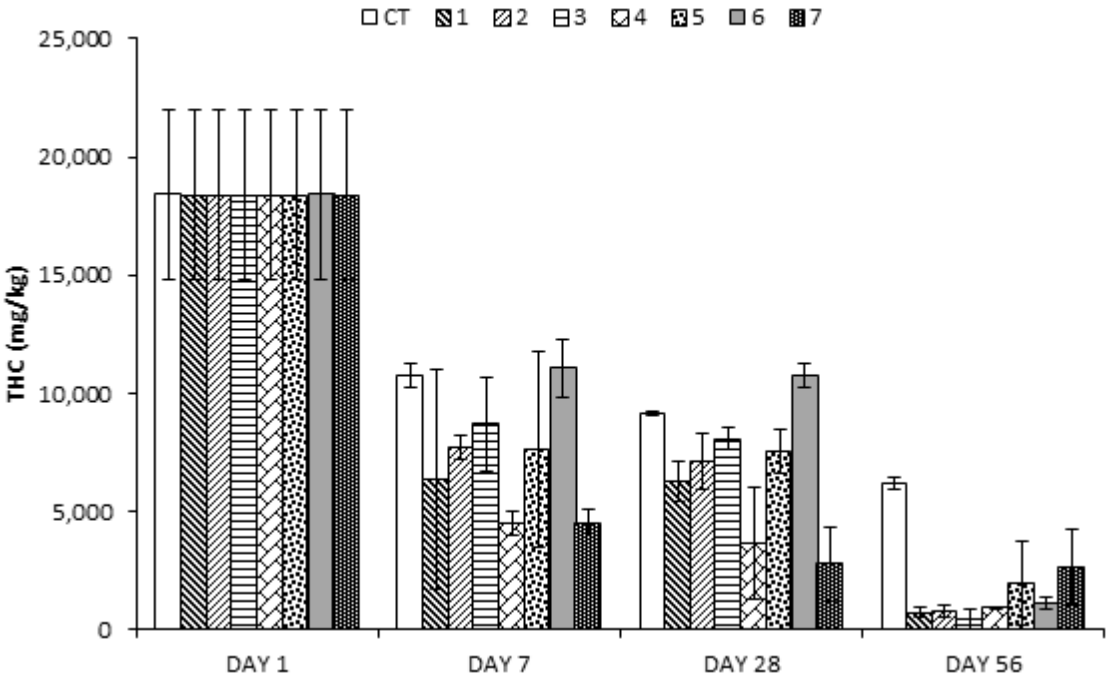

Figure 4. Total hydrocarbon concentration (THC) in the setup.
CT: Control setup; 1, 2, 3, 4, 5, 6, and 7 represents setups CB1, CB2, CB3, CB12, CB13, CB23, and CB123 respectively.

Table 5. Extent of hydrocarbon degradation (EHD) in the microcosms.

N	SC	IBAS	THC on day 1 (mg/kg)	THC on day 56 (mg/kg)	EHD (%)
1	CB1	*Klebsiella* (K)	18,403±3,627	735 ± 186	96.00
2	CB2	*Pseudomonas* (P)	18,403±3,627	811 ± 244	95.60
3	CB3	*Bacillus* (B)	18,403±3,627	477 ± 424	97.40
4	CB12	K+P	18,403±3,627	919 ± 505	95.00
5	CB13	K+B	18,403±3,627	1,962 ± 575	89.34
6	CB23	P+B	18,403±3,627	1,124 ± 221	93.90
7	CB123	K+P+B	18,403±3,627	2,634 ± 751	85.69
8	CT	Sterile soil	18,403±3,627	16,172 ± 272	12.00

SC: Setup code, IBAS: Identity of Biofilm producing bacteria added in setup, THC: Total hydrocarbon concentration.

$$\text{EHD (\%)} = \frac{\text{THC on day 1} - \text{THC on day 56}}{\text{THC on day 1}} \times 100$$

concentration in all the setups had dropped below 4.0 mg/kg.

DISCUSSION

The physicochemical and microbiological (baseline) properties of crude oil-contaminated soil investigated include total hydrocarbon content, nitrate, pH, phosphate, total heterotrophic bacteria, and hydrocarbon-utilizing bacteria. The phosphate concentration in the polluted soil was 4.36 ± 0.12 mg/kg, pH 6.11, and the nitrate concentration was 92.6 ± 0.3 mg/kg. These values in the soil were adequate to allow for the adaptability and growth of bacteria despite the high concentrations of the hydrocarbon pollutant (Table 2). The results correlate positively with the findings of Ogbonna and Amajuoyi (2009), who analysed the physicochemical properties of a crude oil polluted site and reported that the oil pollution made the values of the parameters above the specified limits as recommended by the Department of Petroleum Resources (DPR). The initial total hydrocarbon content of 18,403 ± 3,627mg/kg was recorded in this study, which exceeds the DPR intervention limits of 5000 mg/kg

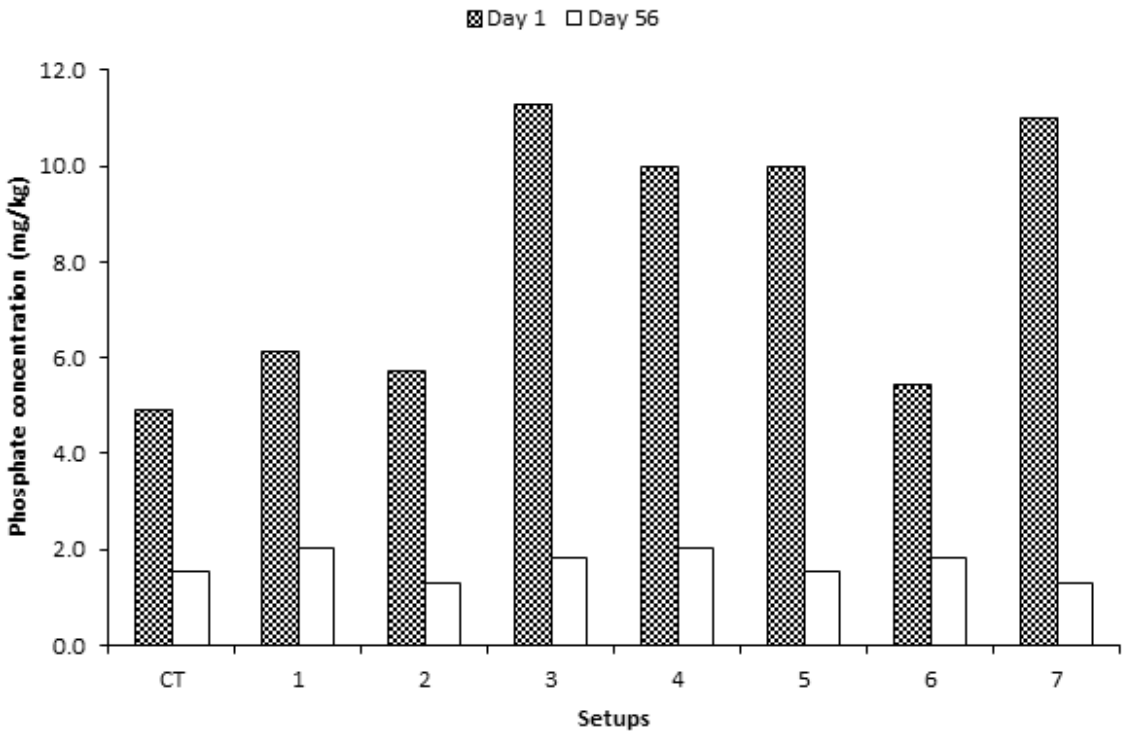

Figure 5. Phosphate concentrations in the setups.
CT: Control setup; 1, 2, 3, 4, 5, 6, and 7 represents setups CB1, CB2, CB3, CB12, CB13, CB23, and CB123 respectively.

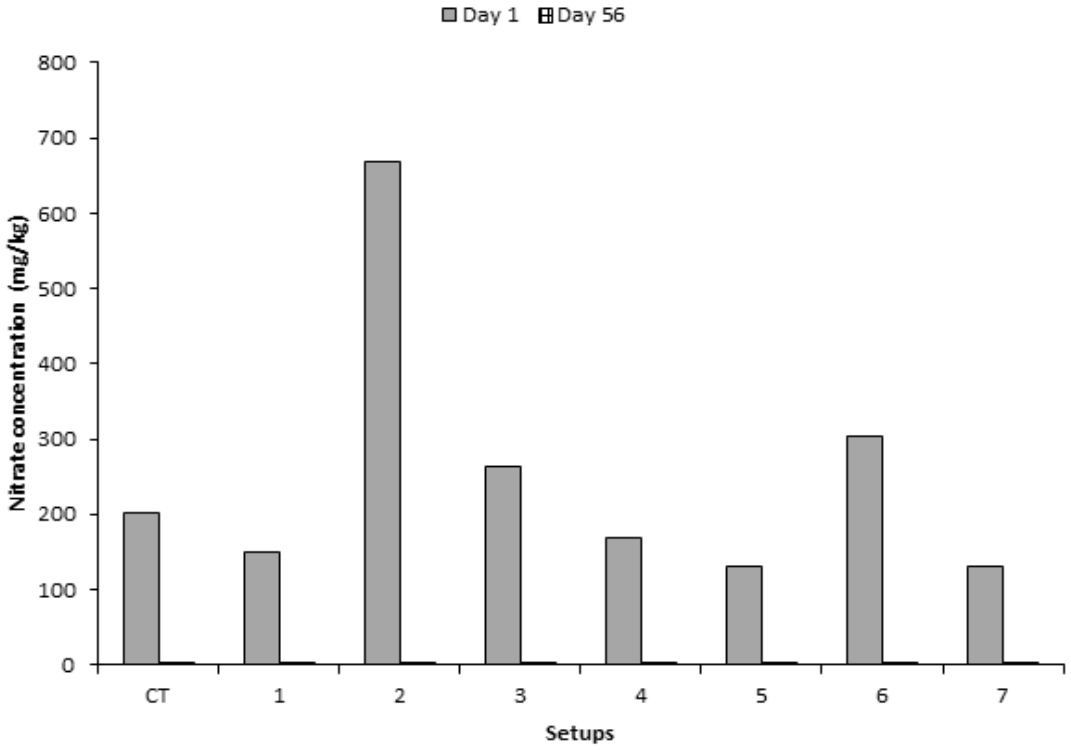

Figure 6. Nitrate concentration in the microcosms.
CT: Control setup; 1, 2, 3, 4, 5, 6, and 7 represents setups CB1, CB2, CB3, CB12, CB13, CB23, and CB123 respectively.

(EGASPIN, 2018).

The bacterial counts of the contaminated soil sample showed moderate growth of THB and HUB, which indicates the presence of active microbial populations in the soil, which may be due to the presence of organic carbon and other environmental conditions that encourage the growth of the microorganisms. The THB and HUB (CFU/g) count in this study was lower when compared with the findings of (Douglas et al., 2022), who recorded higher counts of total and hydrocarbon utilizing bacteria of crude oil-contaminated soil. The lower counts recorded in this study may be attributed to the toxicity effect of the crude oil on the microorganisms while leaving only the crude oil degraders that survived the pollution (Douglas et al., 2022).

The following hydrocarbon-utilizing bacterial species were identified: *Bacillus, Pseudomonas, Staphylococcus, Serratia, Alcaligenes, Klebsiella,* and *Escherichia coli*. Most of these isolates have been identified by previous researchers as hydrocarbon utilizers (Akani et al., 2020; Douglas et al., 2022). The presence of these organisms in an environment with such pollutants is an indication of their ability to withstand the toxic effect of the pollutant and their capacity to use up the pollutant, which in turn brings about degradation.

These isolates identified from the polluted soil were evaluated for biofilm production, and the following: *Staphylococcus*, *Serratia*, *Alcaligenes*, *Escherichia coli*, *Klebsiella*, *Pseudomonas*, and *Bacillus* species were confirmed biofilm producers. While *Bacillus, Pseudomonas* and *Klebsiella* spp. were confirmed as strong biofilm formers. The isolates identified in this study correspond to the findings of Douglas et al. (2022) and Williams et al. (2021), who also identified *Staphylococcus, Escherichia coli, Bacillus* and *Pseudomonas* spp. as biofilm-producing bacteria. According to Thompson et al. (2005), the formation of biofilms is a natural process used by microorganisms to create and preserve a favourable niche in stressful situations, including oil pollution. Biofilm bacterial isolates have adhering features like flagella that assist movement to receptor sites, which enable them to form biofilms (substratum). As recorded in previous studies, most microorganisms are found or occur as biofilms, and they have recently been shown to be highly resilient to chemical, physical, and biological stresses (Gorbushina and Broughton, 2009; Douglas et al., 2022). To enable their increase in resistance, biofilm-associated cells display particular gene expressions frequently regulated by quorum sensing systems or dormancy (Dickschat, 2010). Three of the isolates (*Pseudomonas*, *Bacillus*, and *Klebsiella* species) demonstrated stronger biofilm production and were used singly and in combination for the bioremediation experiment. The addition of nutrients in the form of NPK fertilizer also stimulated the growth of the organisms which in turn resulted in the decrease in the THC. Ekwuabu et al. (2016) reported that the application of nutrients in the form of NPK is an effective biostimulant that speeds up the bioremediation process and replaces the nutritional deficiencies in the soil.

The THC after 56 days of crude oil degradation for the single isolates and their mixed cultures showed a decrease in the value of THC compared with the control; this may be due to the utilization of the hydrocarbon components in the crude oil by the microorganisms. The results correlated positively with the research done by Douglas and Green (2015), who observed a reduction in the level of THC after the degradation of used engine oil using bacteria.

Total hydrocarbon concentration generally decreased in all microcosms from day 1 to day 56. With THC values on day 1 serving as the initial reference point, the microcosm with the highest hydrocarbon degradation (EHD) at day 56 were those treated with single biofilm producers, including *Pseudomonas*, *Bacillus*, and *Klebsiella* species. The extent of hydrocarbon degradation (EHD) in the microcosm at day 56 was observed to be highest for the single isolates than the combinations, with *Bacillus* sp having the highest EHD of 97.40 %, *Klebsiella* sp at 96.0% and *Pseudomonas* sp at 95.60%. It was observed that for the combinations, *Klebsiella* plus *Pseudomonas* gave 95.0%, *Pseudomonas* plus *Bacillus* gave 93.9%, and *Klebsiella* plus *Bacillus* gave 89.34%. The concentration of crude oil degraded is statistically significant compared to the control, but when the EHD of the single isolates were compared, there was no significant difference. When the three isolates were combined, an EHD of 85.69% was observed, while the control gave the least EHD of 12.0%. Research findings have shown that biofilm-forming microorganisms are adapted to bioremediation because they are immobilized in the EPS where the pollutants are also immobilized during degradation (Sutherland, 2001; Mitra and Mukhopadhyay, 2016). They acquire relatively limited oxygen and nutrients compared to the planktonic cells due to the diffusional mode of transport instead of conventional transport. Studies have shown that the three-dimensional structure of the EPS, which has reduced oxygen concentrations at the middle, brings into close contact the aerobic and anaerobic, heterotrophic organisms with nitrifiers, sulphate reducers and oxidizers that promote rapid biodegradation of hydrocarbon pollutants in natural and engineered environments (Field et al., 1995). Biofilm-producing *Pseudomonas* strain has been shown to work as a bio-filter for breaking down hydrocarbon and producing biosurfactants that aid biodegradation (Sar et al., 2023). EPS-containing surfactants may also aid solubilisation of hydrophobic or other refractory substrates which would otherwise be inaccessible to microorganisms (Iwabuchi et al., 2002). Mnif et al. (2015) have shown that microbial consortia containing *Bacillus subtilis* and *Acinetobacter radioresistens* with biosurfactant-producing strains showed to be better degrader than consortia containing

only degraders.

When compared to other single-species microcosm and the control, the microcosm involving the consortium of the three biofilm-producing bacterial species showed a lower percentage of THC reduction. This is explained by the fact that the reduction in total hydrocarbon content that takes place during bioremediation with bacteria that form biofilms is caused by a variety of processes. The ability of bacteria to stick to solid surfaces and form complex structures that resemble matrices known as biofilm is the first characteristic of these bacteria (Beolchini et al., 2010). These bacteria can obtain nutrients and survive in harsh environments because of the protective environment this biofilm provides. Bacteria produce a wide range of enzymes that are involved in the hydrocarbon breakdown process, such as oxygenases and hydroxylases, these enzymes help break down complex hydrocarbons into simpler substances that the bacteria can readily metabolize (Beolchini et al., 2010). The current finding agreed with the findings of Fatuyi et al. (1995), who showed that mixed cultures exhibited a lower percentage of petroleum hydrocarbon degradation than single isolates. However, in line with Sunita et al. (2013) research that revealed that mixed cultures used hydrocarbons more quickly than individual strains. The results are also in agreement with Fretsche and Hofrichter (2005), Heinaru et al. (2005) and Ghazali et al. (2004), who reported the need for a mixed consortium of microorganisms in degrading recalcitrant environmental contaminants since each organism would possess varying enzymatic capacities.

CONCLUSION AND RECOMMENDATIONS

This study has revealed the potential of biofilm-producing bacteria in the bioremediation of crude oil-contaminated soil and may be applied as a biological tool used in cleaning up crude oil pollution from the environment. The Results from this study revealed strong biofilm-producing bacteria, which include; *Pseudomonas, Bacillus,* and *Klebsiella* species, which were used singly and in combination for the bioremediation setup. The success of the bioremediation observed after 56 days suggests a significant reduction in hydrocarbon content, which may be due to the synergistic action of the bacteria and their biofilm-forming capabilities. The extent of hydrocarbon degradation (EHD) in the microcosms on day 56 was observed to be highest for the single isolates than the combinations, with *Bacillus* sp having the highest EHD of 97.40 %, *Klebsiella* sp at 96.0% and *Pseudomonas* sp at 95.60%. It was observed that for the combinations, *Klebsiella* plus *Pseudomonas* gave 95.0%, *Pseudomonas* plus *Bacillus* gave 93.9% and *Klebsiella* plus *Bacillus* gave 89.34%. The concentration of crude oil degraded is statistically significant compared to the control, but when the EHD of the single isolates were compared, there was no significant difference between them. When the three isolates were combined, an EHD of 85.69% was observed, while the control gave the least EHD of 12.0%. This study has shown that BPB has the potential to biodegrade crude oil pollutants in soil and may be deployed for the bioremediation of crude oil-polluted soil. Thus, careful monitoring, detailed analysis, and consideration of environmental factors are essential for the sustainable and effective application of such bioremediation strategies.

Conflict of interest

The authors declare no conflicts of interest regarding this paper.

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