Biosurfactant Producing Potential of Bacteria Isolated from Sediment of Crude Oil Contaminated Iko River Estuary, Nigeria

Akpan Stephen Bernard [1], **Ome Kalu Achi** [1], **Nwachukwu Ejikeme** [1]

[1] *Michael Okpara University of Agriculture*
P. M. B. 7267 Umuahia Umudike, Abia State, Nigeria

DOI: 10.22178/pos.126-5

LCC Subject Category: T1-995

Received 25.12.2025
Accepted 28.01.2026

Corresponding Author:
Akpan Stephen Bernard

Abstract. There are several physical, chemical, and biological methods to remove petroleum hydrocarbons from contaminated environments. Biological processes are considered the most exciting, cost-effective, and environmentally friendly approaches for removing petroleum hydrocarbons compared to chemical or physical processes. Biosurfactants enhance the bioavailability of hydrophobic organic compounds, making them effective agents for environmental cleanup. Hence, this study aimed to isolate biosurfactant-producing bacteria from the estuarine sediment of the crude oil-contaminated Iko River estuary capable of degrading petroleum hydrocarbons using appropriate methods. The results revealed that *Pseudomonas aeruginosa, Bacillus subtilis, Micrococcus spp.* and *Bacillus spp.* exhibited haemolytic activity. All organisms except Bacillus cereus were positive for the emulsification, drop collapse, and oil spread tests. However, *Pseudomonas aeruginosa* demonstrated the highest emulsification capacity, followed by *Bacillus subtilis, Micrococcus spp., Bacillus spp.*, and *Bacillus cereus*, respectively. Degradation of crude oil and its components was faster when enhanced with a mixed culture of *Bacillus subtilis* and *Pseudomonas aeruginosa* than with individual bacterial isolates. Bacillus subtilis reduced the crude oil component from 257.27 mg/l to 120.42 mg/l. *Pseudomonas aeruginosa* reduced crude oil component 257.27 mg/l to 48.41 mg/l, whereas the mixed culture of *Bacillus subtilis* and *Pseudomonas aeruginosa* reduced crude oil components from 257.27 mg/l to 7.82 mg/l. Biosurfactant-producing bacteria with hydrocarbon-utilising capacity have the potential to enhance the biodegradation of total petroleum hydrocarbons. The key to increasing hydrocarbon degradation rates is bioaugmentation with a biosurfactant-producing bacterial consortium that can act synergistically to degrade hydrocarbons more effectively.

Keywords: Hydrocarbon; biodegradation; petroleum; bioaugmentation; bacterial consortium

INTRODUCTION

Hydrocarbons and thousands of other organic chemicals, including organometallic components, make up the complex mixture known as petroleum [1]. A complex mixture of hydrocarbons with varying molecular weights and other organic compounds found in geologic formations beneath the earth's surface combine to form this naturally occurring, poisonous, and combustible liquid. The soil's overall composition influences the breakdown of petroleum hydrocarbons by microorganisms. It is observed that the composition of petroleum hydrocarbons is highly complex, comprising hundreds of distinct compounds with diverse chemical, physical, and toxicological properties [2].

Thus, one practical approach to enhancing the process's effectiveness is the use of biosurfactant-producing microbes. Biosurfactants, also known as green surfactants, are surface-active compounds produced by microbes. These compounds are biodegradable and non-toxic, thus they do not accumulate in the environment [3]. Biosurfactants enhance the bioavailability of hydrophobic organic compounds, making them effective agents for environmental cleanup. The most engaging characteristic of biosurfactants is their balanced structure across different temperatures, pH, and salinity [4]. Biosurfactants can also be produced from waste materials, thereby reducing contaminant levels and lowering production costs, making biosurfactants a promising area for further research [4]. A range of microorganisms produce biosurfactants, which are a diverse group of chemical compounds with unique physicochemical and biochemical properties. Based on their microbial origin and chemical composition, biosurfactants are classified as lipopeptides, glycolipids, polymeric types, fatty acids, and particulate biosurfactants. The most reported biosurfactants are lipopeptides (rhamnolipids, surfactin, iturin, sophorolipids) and glycolipids. Bacillus strains are essential sources for the production of lipopeptides [5].

There have been many studies on the bioremediation of petroleum hydrocarbons by potential biosurfactant-producing microbes. However, most studies on biosurfactant-mediated biodegradation have focused on single cultures rather than mixed cultures [6]. Furthermore, ex situ biosurfactant production via co-culture with biosurfactant-producing hydrocarbon-degrading bacteria is less studied than research on biosurfactant addition alone. Hence, this study aimed to isolate biosurfactant-producing bacteria from the estuarine sediment of the crude oil-contaminated Iko River estuary capable of degrading petroleum hydrocarbons.

MATERIALS AND METHODS

Study Area. The study area was the mangrove ecosystem of the Iko River Estuary. Iko is located within the petroleum belt of the Niger Delta, Nigeria (70 30' N and 70 45'N, and longitude 70 30' E and 70 40'E). The Iko River estuary has semi-diurnal tides and a shallow depth of 1 to 7 m at flood and ebb tides. The estuary was more than 20 km long with an average width of about 5m. Iko River takes its course from Qua Iboe River catchments and drains directly into the Atlantic Ocean at the Bight of Bonny. It has many tributaries, some of which drain into the Imo River estuary, which opens into the Atlantic Ocean. Soft dark mudflats, usually exposed during low tide, mangrove swamps, shoals and sandbars, characterise the shoreline of the Iko River. The area was characterised by a humid tropical climate with rainfall reaching about 3,000mm per annum.

Sample Collection. The study selected three sites in the Iko River estuary to represent a wide range of crude oil contamination. The sampling procedure aseptically collected intertidal sediment samples using an Eckman sediment grab, placed them in plastic containers sanitised with 95% ethanol, and transported them to the Microbiology Laboratory within 24 hours for further analysis. The protocol stored the samples at ambient temperature.

Community Deoxyribonucleic Acid (DNA) Extraction. A Zymo-Spin™ IV-HRC Spin Filter was first prepared by snapping off the base, inserting it into a collection tube, and spinning in a microcentrifuge at 8,000 xg for 3 minutes. The extraction protocol isolated genomic DNA by transferring 0.25 g of each sample into separate ZR BashingBead™ Lysis Tubes. The procedure added 750 µL of lysis solution to each tube and vortexed the mixture for 5–10 minutes. The method transferred each mixture to a bead beater fitted with a 2 ml tube holder assembly and centrifuged it at 10,000 × g for 1 minute. The protocol then transferred the supernatant to a ZymoSpin™ IV Spin Filter in a collection tube and centrifuged it at 7,000 × g for 1 minute.

The procedure added 1,200 µL of soil DNA binding buffer to the filtrate in the collection tube. It transferred 800 µL of the resulting mixture to a ZymoSpin™ IIC Column in a collection tube, then centrifuged at 10,000 × g for 1 minute. After discarding the flow-through, the method added 200 µL of DNA pre-wash buffer to the ZymoSpin™ IIC, placed it in a new collection tube, and centrifuged it at 10,000 × g for 1 minute. The protocol then transferred the column to a new collection tube, added 500 µL of sample DNA wash buffer, and centrifuged it at 10,000 × g for 1 minute.

The method transferred the ZymoSpin™ IIC to a clean 1.5 ml microcentrifuge tube, added 100 µL of DNA elution buffer to the column matrix, and centrifuged it at 10,000 × g for 30 seconds. The procedure transferred the eluted DNA to a prepared ZymoSpin™ IV-HRC Spin Filter in a clean 1.5 ml Eppendorf tube, then centrifuged at 8,000 × g for 1 minute. The resulting filtrate constituted the extracted genomic DNA.

Polymerase Chain Reaction and Next Generation Sequencing. The protocol subjected the extracted DNA samples to polymerase chain reaction (PCR) using a 96-well thermal cycler (2E™ UK). The amplification procedure used labelled Eppendorf tubes bearing sample codes and primer names on the top and sides. The assay amplified the target sequences using the universal primer pair 341F and 785R, targeting the V3 and V4 regions of the 16S rRNA gene.

The workflow gel-purified the resulting amplicons, end-repaired them, and ligated Illumina-specific adapter sequences to each amplicon using the NEBNext Ultra II DNA Library Prep Kit. After quantification, the protocol individually indexed the samples with NEBNext Multiplex Oligos for Illumina (Dual Index Primers Set 1) and performed an additional AMPure XP bead-based purification step. The sequencing procedure analysed the amplicons on the Illumina MiSeq platform using a MiSeq v3 (600-cycle) kit according to the manufacturer's instructions, generating approximately 20 Mb of paired-end reads (2 × 300 bp) per sample.

The bioinformatics analysis processed the sequencing data using the National Centre for Biotechnology Information (NCBI) BLAST tool (version 2.2.4) and the CLC Genomics Workbench (version 7.5.1).

Enumeration and Isolation of Oil Degrading Bacteria (ODB). The density of oil-degrading bacteria (ODB) was estimated using the vapour-phase transfer technique [7]. 1 g of the sample was diluted in a series of dilutions. The desired diluents were plated on MSM fortified with sterilised Bonny Light crude oil and were incubated at room temperature for 14 to 21 days, depending on the growth rate on the plates. Colonies of bacteria on plates treated with crude oil were enumerated.

The enrichment culture technique was employed. Precisely 1 g of sediment sample was inoculated into three sets of conical flask containing 50 ml of sterile Mineral Salt Medium [K_2HPO_4 – 6g, NaCl – 12g, KH_2PO_4 – 6g, $(NH_4)_2SO_4$ – 6g, $MgSO_4.7H_2O$ – 2.6g, $CaCl_2.2H_2O$ – 0.16g, per liter (pH 7.0 + 0.2)] (MSM) enriched with 1% crude oil as carbon source. The medium was incubated at 28°C in a shaker incubator (100 rpm) for 7 days. After 7 days of incubation, the samples were serially diluted with sterile water and plated on Nutrient Agar (NA) to obtain viable bacterial cells. The discrete colonies obtained were subcultured using the streak method as described by [8] to obtain pure cultures.

Maintenance of Pure Cultures of Oil-degrading Bacterial Isolates. Distinct colonies of the oil-degrading bacteria isolated from estuarine sediment were subcultured into McCartney bottles containing freshly prepared Nutrient Agar slants and incubated at 30 ± 2°C for 24 hours before storage at 4°C for characterisation.

Characterisation of Bacterial Isolate. The best crude oil-utilising bacterial isolates were characterised based on their cultural and morphological attributes and responses to standard biochemical tests, as described by [8]. Twenty-four-hour-old monocultures of bacteria obtained were subjected to Gram's staining, endospore staining, and several biochemical tests, including the Catalase test, Citrate Utilisation test, Oxidase test, Motility test, Endospore test, Methyl red and Vogues Proskauer test, and Indole test, as well as sugar fermentation tests.

Screening for Biosurfactant-Producing Potentials of the Bacterial Isolates. The study evaluated pure cultures of the bacterial isolates for biosurfactant-producing potential using haemolysis, emulsification capacity (%EC_{24}), drop-collapse, and oil-spread tests. The following section describes the test procedures in detail:

Haemolytic Activity. Microbiologists commonly use this test to classify certain microorganisms. The assay distinguishes three types of haemolysis: α, β, and γ. In α-haemolysis, the agar beneath the colony darkens and appears greenish; in β-haemolysis, the agar becomes light yellow and transparent; and in γ-haemolysis, the agar shows no visible change. Test bacterial isolates were streaked on freshly prepared blood agar plates and incubated at 37 oc for 48 hours. The plates were visually inspected for a clear zone around the colonies, an indication of biosurfactant production. The assay used the diameter of the clear zone as an indicator of biosurfactant production and classified the strain with the largest clear zone as a high biosurfactant producer.

Emulsification Capacity (% EC_{24}). The assay determined emulsification capacity using the Cooper and Goldenberg method. The procedure mixed equal volumes (1:1) of a 72-hour test culture supernatant and kerosene in a glass test tube (125 mm×15 mm), vortexed the mixture for 2 minutes, and allowed it to stand for 24 hours. The method calculated %$EC_{(24)}$ by dividing the height of the emulsified layer (mm) by the total height of the liquid column (mm) and multiplying the result by 100.

Drop Collapse Test. Authors [9] developed this test to evaluate surfactants' ability to destabilise liquid droplets. The assay relies on the interaction between liquid droplets and an oil-coated solid surface. In the procedure, the method places drops of cell suspension or culture supernatant onto an oil-coated solid surface. When the liquid lacks surfactants, polar water molecules repel from the hydrophobic surface, and the drops remain stable. When the liquid contains surfactants, the drops spread or collapse because reduced interfacial tension weakens the interaction between the liquid droplet and the hydrophobic surface. Drop stability depends on surfactant concentration and correlates with surface and interfacial tensions.

The study performed the drop-collapse test by pipetting supernatant from each bacterial isolate onto a microplate lid (12.7 × 8.6 cm^3) previously coated with Tapis crude oil. If the supernatant spread flat within 1 minute, the assay recorded a positive result; beaded drops indicated a negative outcome.

Oil Spread Technique. This test assesses the biosurfactant's ability to alter the contact angle at the oil–water interface by displacing the oil as its surface pressure increases. For the assay, the procedure gently places 10 μL of culture supernatant at the centre of the oil layer. When the supernatant contains biosurfactant, the oil displaces and forms a clearing zone. The assay measures the diameter of the clear zone after 30 seconds, and this diameter correlates with surfactant activity, also referred to as oil-displacement activity. The method expresses the measurement in biosurfactant units (BS units).

Crude Oil Index of Hydrocarbon Utilisation Potential of Biosurfactant Producing Hydrocarbon Degrading Bacteria in vitro

Indirect assessment of degradation. The study determined the biodegradability of Bonny Light crude oil using the four most efficient hydrocarbon-utilising bacterial and yeast isolates by applying methods previously reported by [10]. The procedure prepared 99 ml of mineral salt broth (MSB) in thirty-five (35) 250 ml conical flasks and adopted a destructive sampling approach. The protocol sterilised the flasks by autoclaving and, after cooling, dispensed 0.1 ml of filtered Bonny Light crude oil into each flask. The experiment inoculated 0.1 ml of a 24-hour nutrient broth culture of the four bacterial isolates into 28 conical flasks, while five flasks remained uninoculated and served as controls. The flasks were incubated on a shaker (SGM-300 Gallemkamp, England) operating at 80 oscillations/min at room temperature (28±2°C) for 28 days. At 4-day intervals, the procedure collected 20 ml of sample from each set of inoculated flasks representing the four bacterial isolates for the determination of:

a) optical density, i.e. turbidity at 540nm using HACH RD/2010 spectrophotometer,

b) pH changes monitored with an electronic pH Meter (HACH Sension 3 pH Meter),

c) total viable count (TVC) determined by serially diluting the culture, plating on nutrient agar and incubating at room temperature (28+2°C) for 24 hours, after which counts were taken and expressed as colony-forming units per millilitre (cfu/ml),

d) free CO_2 determined by titrating 1 ml of the fermented broth against 0.05N NaCl solution using phenolphthalein as indicator and appearance of a stable pink colour as end point. The amount of CO_2 was calculated using the formula:

$$\text{Free } CO_2 \text{ (mg/l)} = \frac{Titre\ value\ x\ normality\ of\ NaOH\ x\ 1000\ x\ 44}{Volume\ of\ sample}$$

Percentage utilisation as weight loss of the incorporated crude oil was calculated as weight of crude oil (Control) minus weight of crude oil (degraded) divided by weight of crude oil (control) multiplied by 100.

Direct Assessment: Petroleum Hydrocarbons Degradability of the Test Isolate. The study employed extraction and column chromatographic analyses to examine the crude oil before (control) and after degradation by the microbial isolates:

Extraction. At the end of the 28-day incubation period, we removed the five Erlenmeyer flasks and extracted the residual crude oil with dichloromethane. We then separated the extract by column chromatography and quantified it in milligrams per litre. To extract the crude oil, we measured 100 ml of the broth sample into a separatory funnel after rinsing the sample container with dichloromethane. We added 10 ml of dichloromethane to the 100 ml broth sample and shook the mixture vigorously to extract all available organic material. We collected the organic extract in a receiving vial after passing it through a column packed with glass wool, silica gel, and anhydrous sodium sulphate; the silica gel facilitated extract cleanup by preventing the passage of debris. At the same time, anhydrous sodium sulphate acts as a dehydrating agent, removing all forms of moisture/water from the sample, as the two liquids are immiscible.

Gas Chromatographic Analysis. The analysis injected 1 μL of the concentrated organic extract into the gas chromatograph through a rubber septum using a hypodermic syringe. Separation occurs when vapour constituents partition between the gas and liquid phases. The flame ionisation detector (FID) automatically detected the aliphatic compound fractions (C8–C40) as they emerged from the column, with detector response depending on vapour composition. The study expressed the results in mg/L as the equivalent unit.

RESULTS AND DISCUSSION

Biosurfactant Producing Potential of Bacteria Isolated from Estuarine Sediment. The results of the screening test for the capability of bacteria isolated from estuarine sediment to produce biosurfactants are presented in Table 1. The results revealed that *Pseudomonas aeruginosa, Bacillus subtilis, Micrococcus spp.,* and *Bacillus spp*. exhibited hemolytic activity. All organisms except *Bacillus cereus* were positive in the emulsification, drop collapse, and oil spread tests. However, *Pseudomonas aeruginosa* demonstrated the highest emulsification capacity, followed by *Bacillus subtilis, Micrococcus spp., Bacillus spp*., and *Bacillus cereus*, respectively.

Table 1 – Biosurfactant-producing potential of hydrocarbon-degrading bacteria

Isolates	Haemolytic activity, mm	Emulsification test, %	Drop collapse test	oil spread test
Bacillus cereus	No activity	-(28)	-	-
Pseudomonas aeruginosa	Activity (0.3)	+(64)	+	+
Bacillus subtilis	Activity (0.1)	+(52)	+	+
Micrococcus spp.	Activity (0.1)	-(46)	+	+
Bacillus spp.	Activity (0.1)	-(40)	+	+

Notes: "+" – Positive; "-" – Negative.

Crude Oil Index of Hydrocarbon Utilisation Potential of Biosurfactant Producing Hydrocarbon Degrading Bacteria in vitro

Indirect assessment of degradation. Analysis of the optical density of the test medium during the degradation process, under monoculture and mixed culture conditions, revealed that optical density increased over time (Figure 1). The analysis showed that attendance levels varied with treatment. Measurements for *Bacillus subtilis* ranged from 0.240 to 0.511 nm, whereas measurements for *Pseudomonas aeruginosa* cultures ranged from 0.510 to 0.695 nm. Cultures containing bacterial consortia exhibited slightly higher attenuance levels (0.712–0.963 nm). The post hoc test revealed statistically significant differences ($p < 0.05$) in optical density between the control medium and the medium containing bacterial culture, as well as between the press containing *Bacillus subtilis* and the press containing *Pseudomonas aeruginosa*.

The pH of the test substrates decreased over time, indicating a higher catabolic activity (Figure 2). The increase in acidity was slightly higher in Bacterial consortia (*Bacillus subtilis* and *Pseudomonas aeruginosa* augmented medium. However, the pH changes in the control medium and in media with single bacterial cultures or the mixed culture were not statistically significant ($p > 0.05$). The results of the indirect assessment using total viable cells are presented in Figure 3. Total viable counts revealed that microbial biomass increased over time during degradation. The feasible counts in both monoculture and mixed-culture media increased steadily until day 21, then began to decrease. The rate of increase was apparently higher in the mixed culture (*Bacillus subtilis* and *Pseudomonas aeruginosa*) degradation process than in the monoculture degradation process. However, no growth was observed in the control medium during the degradation study. The difference in viable counts between the bacterial consortia medium and the monoculture medium was significant ($p < 0.05$).

Analysis of CO_2 evolution in the test medium during the degradation process revealed an increase in CO_2 production over time (Figure 4). CO_2 evolution levels varied across treatments. *Bacillus subtilis* produced CO_2 in the range of 15-40 ml, while the range for *Pseudomonas aeruginosa* was 11-81 ml. The bacterial consortia have even higher CO_2 production, ranging from 36 to 97 ml. The control medium showed no CO_2 evolution, indicating that CO_2 production was specific to the bacterial treatments. CO_2 evolution in the control medium was significantly different from that in the medium containing bacterial culture ($p < 0.05$); furthermore, CO_2 evolution by Bacillus subtilis was statistically different from that of bacterial consortia ($p < 0.05$).

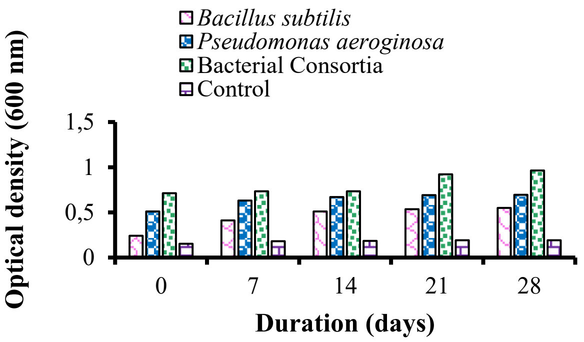

Figure 1 – Changes in optical density of test media during hydrocarbon biodegradation by bacterial isolates from the estuarine sediment ecosystem

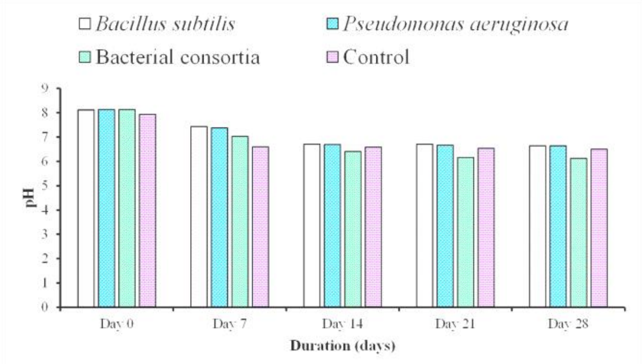

Figure 2 – Changes in pH of test media during hydrocarbon biodegradation by bacterial isolates from the estuarine sediment ecosystem

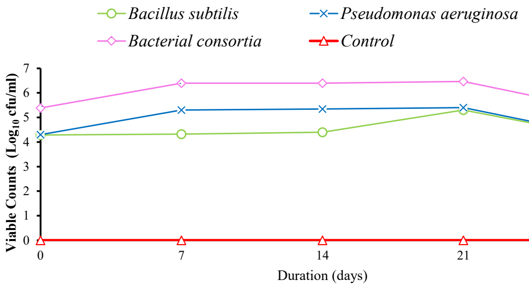

Figure 3 – Changes in biomass production during hydrocarbon biodegradation by bacterial monoculture and mix-culture

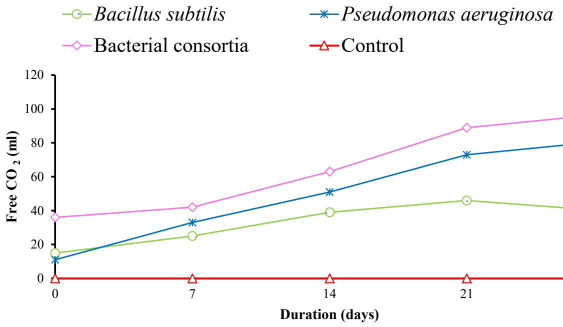

Figure 4 – Changes in Free CO2 of test media during hydrocarbon biodegradation by bacterial isolates from the estuarine sediment ecosystem

Direct Assessment: Petroleum Hydrocarbons Degradability of the Test Isolate

The biodegradability of the significant components of Bonny light crude oil, as determined by column chromatographic analysis after 28 days of incubation, is presented in Table 2. Degradation of crude oil and its components was faster when enhanced with a mixed culture of *Bacillus subtilis* and *Pseudomonas aeruginosa* than with individual bacterial isolates. *Bacillus subtilis* reduced the crude oil component from 257.27 mg/l to 120.42 mg/l. *Pseudomonas aeruginosa* reduced crude oil component 257.27 mg/l to 48.41 mg/l, whereas the mixed culture of *Bacillus subtilis* and *Pseudomonas aeruginosa* reduced crude oil components from 257.27 mg/l to 7.82 mg/l. The results have shown a remarkable reduction in the petroleum content of the test substrates. The best degradation was obtained with a bacterial consortium (*Bacillus subtilis* and *Pseudomonas aeruginosa*) in an augmented medium, with 96.96 % degradation, followed by *Pseudomonas aeruginosa* ADR61 (81.18 %) and Bacillus subtilis (58.19 %).

Table 2 – Level of Petroleum Hydrocarbon Degradation by Bacterial Species, %

Parameter	Amounts (mg/l)			
	Bacillus subtilis	*Pseudomonas aeruginosa*	*Bacterial consortia*	Control
C8	-	-	-	0.00638235
C9	0.126086	0.0171718	0.00729280	0.351909
C10	0.794891	0.581209	0.174327	1.60144
C11	0.414668	1.09134	0.195431	1.75683
C12	1.51339	1.39523	0.291757	2.87754
C13	2.20827	2.07605	0.183832	4.31528
C14	4.07451	0.938701	0.401725	7.08713
C15	2.15146	1.31650	0.259764	20.61457
C16	7.59096	6.06258	0.515578	14.88639
C17	2.66251	1.32855	0.418369	13.31253
Pristane	10.53049	2.92437	0.221866	23.42837
C18	5.50961	3.83805	0.710863	11.10784
Phytane	23.46868	1.29344	0.353275	54.98965
C19	2.76538	1.73043	0.329061	10.24372
C20	4.79272	1.12977	0.427050	8.16063
C21	3.02830	1.82163	0.340121	5.65116
C22	1.81357	1.25967	0.331808	3.86701
C23	6.91442	2.57970	0.456157	13.91204
C24	5.30397	0.830658	0.303840	12.89445
C25	3.46856	1.99760	0.141590	5.36759
C26	1.98596	1.09786	0.255068	4.32024
C27	3.14694	3.87969	0.203085	5.26862
C28	3.07136	1.35765	0.0906554	8.66557
C29	3.65848	1.37505	0.0866033	6.60356
C30	3.37006	0.855817	0.0554818	2.00209
C31	3.95153	0.993637	0.169606	1.87573
C32	5.16019	1.83126	0.112001	6.32784
C33	1.47153	1.77151	0.0980740	2.83015
C34	5.22323	0.0225201	0.329029	2.34273
C35	0.188127	0.937646	0.261917	0.247445
C36	0.0603991	0.0788695	0.0910686	0.314919
C37	-	-	-	0.0422648
C38	-	-	-	
C39	-	-	-	
Total	120.42025 (58.19)	48.41417 (81.18)	7.81630 (96.96)	257.27362

In this study, four methods were employed to screen the biosurfactant-producing potential of the hydrocarbon-degrading isolates. The methods used were two qualitative methods (the drop collapse and oil dispersion tests) and two quantitative methods (the emulsification and haemolytic activity tests). The drop collapse test revealed that 4 (*Pseudomonas aeruginosa, Bacillus subtilis, Micrococcus spp.* and *Bacillus spp.*) out of the 5 hydrocarbon-degrading isolates (80%) tested positive, with the only negative result coming from Bacillus cereus. This test measures the destabilisation of liquid droplets by the surfactant activity of the biosurfactants produced by the isolates. Authors [11] explain that the presence of biosurfactants causes the drops to spread or collapse due to reduced interfacial tension. This study corroborates the findings of authors [12], who reported strongly positive results for the drop collapse test, suggesting that the hydrocarbon-degrading isolates in this study have strong biosurfactant production potential; this is an essential finding as biosurfactants play a crucial role in improving the efficiency of hydrocarbon degradation by increasing the surface area of the oil available for microbial attack.

Screening for haemolytic activity revealed that 4 out of 5 hydrocarbon-degrading isolates (80.0%) exhibited haemolytic activity, while the remaining isolate (20.0%) did not. Some researchers have considered haemolytic activity as an indicator of biosurfactant production [13, 14], although this is not always a reliable indicator, as other metabolic products can also cause haemolysis. Despite this, there appears to be a strong relationship between surfactant production and haemolysis as evidenced in this study. In the oil spreading test, four hydrocarbon-degrading isolates, namely *Pseudomonas aeruginosa, Bacillus subtilis*, *Micrococcus spp*. and *Bacillus spp*., displayed positive results; this was attributed to the biosurfactants produced by these isolates, which reduced the surface tension between the supernatant drop and the hydrophobic oil surface, permitting the drop to spread more readily. This observation aligns with the research of authors [15], who reported that reduced surface tension is responsible for the oil-spreading effect observed in some bacteria. This finding further supports the potential of these isolates for hydrocarbon degradation and bioremediation, as their biosurfactant production enables the efficient degradation of hydrophobic hydrocarbons.

Researchers use the emulsification test as a straightforward quantitative method to identify biosurfactant-producing microorganisms, because emulsifying activity (E24) allows them to estimate bioemulsifier productivity. An emulsification index (E_{24}) higher than 50% is often used as a criterion for selecting biosurfactant-producing isolates. In various screening processes for hydrophobic compounds, kerosene is commonly used due to its relatively high viscosity and tendency to form emulsions [16, 17]. The type of biosurfactant produced by each bacterial genus and strain significantly influences emulsification capacity. *Pseudomonas aeruginosa* and *Bacillus subtilis* achieved high kerosene emulsification (64% and 52%, respectively), indicating that emulsification tests can effectively identify highly productive biosurfactant-producing cultures. Cultures that showed emulsification activity in the range of 24-48% were also positive for biosurfactant production in one or two other methods, indicating that emulsification tests can be a valuable tool for biosurfactant screening. The measurement of emulsification units can also help select the optimal carbon and energy sources for biosurfactant production. It is important to note that multiple screening methods are recommended for effective biosurfactant screening [18, 19], as a single method may not be effective for all biosurfactant types.

The researchers further analysed the best biosurfactant-producing oil-degrading bacteria (*Pseudomonas aeruginosa* and *B. subtilis*) for crude oil degradation in vitro. The extent of hydrocarbon degradation was measured both indirectly, by examining the density of viable cells produced, carbon dioxide production, changes in optical density and pH of the culture medium; and directly, by quantifying the rate of degradation of petroleum hydrocarbon contents in Bonny Light Crude oil over time. The results of the biodegradation study showed that monocultures of *Pseudomonas aeruginosa* and *Bacillus subtilis*, and a consortium culture of both species, exhibited different rates of hydrocarbon utilisation.

The optical density of both monocultures and the consortium medium increased over time, suggesting that the test organisms increased their biomass while utilising the hydrocarbon pollutants as their sole carbon source. This increase in optical density is consistent with the findings of authors [20], who also observed a gradual increase in optical density due to hydrocarbon biodegradation. Biodegradation of hydrocarbons by microorganisms requires favourable environmental conditions, such as sufficient water, oxygen, nutrients, and an appropriate pH range, which support microbial growth and hydrocarbon degradation [21, 22]. The pH values obtained during the degradation studies ranged from 6.12 to 8.13, which is considered ideal for microbial growth and hydrocarbon degradation. Upon terminal oxidation, the alcohol resulting from hydrocarbon degradation is further oxidised to an aldehyde and a fatty acid by enzymes known as pyridine nucleotide-linked dehydrogenases. This metabolic process results in the production of organic acids and other by-products during microbial hydrocarbon degradation [23, 24]. The production of organic acids by the hydrocarbon-degrading microorganisms, resulting from their utilisation of crude oil as a carbon and energy source, explains the decrease in pH observed in the culture media. These acidic metabolic products could account for the observed decrease in pH. The reduction in pH of the culture medium in the experimental flasks over the 28-day incubation period also confirms chemical changes of the hydrocarbon substrates, which must have been facilitated by microbial enzymes [25].

The amount of free carbon dioxide produced from the bacterial substrates increased significantly over the 28-day biodegradation study. In contrast, the control medium recorded no free CO_2 evolution, suggesting that CO_2 production was specific to the presence of the bacterial treatments. This significant increase in carbon dioxide production indicates that the microbial isolates were effectively utilising the petroleum hydrocarbon fractions as a source of carbon and energy. The ultimate products of petroleum hydrocarbon degradation are carbon dioxide and water, which also serve as measures of microbial respiration and activity in ecosystems. The results of this study are consistent with previous work by authors [20], who reported a progressive increase in carbon dioxide production during the first 4 weeks of biodegradation. Microbial biodegradation of petroleum products produces microbial cells and carbon dioxide as end products. The measurement of total carbon dioxide production is an effective method to assess the biodegradability potential of hydrocarbons in contaminated environments [22]. This approach provides a rapid, unambiguous assessment of hydrocarbon biodegradation over time, suitable for evaluating the effectiveness of various biological treatment options, such as nutrient addition and microbial inoculation. Carbon dioxide evolution can serve as an indicator of active hydrocarbon degradation during full-scale bioremediation. In this study, the results showed that the culture medium containing a microbial consortium of *Pseudomonas aeruginosa* and *B. subtilis* from the estuary ecosystem had the highest free carbon dioxide production (97 mg L^{-1}) compared to monoculture media.

The bacterial isolates showed varying growth patterns during the biodegradation studies. The microbial consortium of *Pseudomonas aeruginosa* and *B. subtilis* from the estuary ecosystem demonstrated the highest growth rate (10^6) during the biodegradation period, as determined by total viable count. The viable counts in the bacterial consortia medium were significantly higher ($p < 0.05$) than in the single-culture medium, suggesting that the mixed culture of *Bacillus subtilis* and *Pseudomonas aeruginosa* promoted microbial growth and enhanced degradation. The higher abundance of *Pseudomonas aeruginosa*, a Gram-negative bacterium, compared to *B. subtilis*, a Gram-positive bacterium, is consistent with the findings of [26], who also observed that Gram-negative bacteria tend to be better degraders of crude oil than Gram-positive bacteria. Gram-negative bacteria possess a combination of factors that may enhance their ability to utilise hydrocarbons for growth and metabolism effectively. These factors include chromosomal or plasmid-encoded genes involved in hydrocarbon degradation, as well as porins in their cell walls, which allow the uptake of essential molecules and the extrusion of harmful compounds. After 21 days of exposure, both monoculture and consortium cultures exhibited a sharp decline in bacterial population; this may be attributed to nutrient depletion (hydrocarbons), which are essential for the growth of hydrocarbon-degrading microorganisms. Authors [7] suggested that the population of hydrocarbon degraders within a microbial community can serve as an indicator of environmental exposure to hydrocarbons.

Hydrocarbon biodegradation typically begins with the degradation of alkanes and light aromatic fractions. In contrast, the degradation of higher-molecular-weight aromatics, resins, and asphaltenes is often considered more challenging due to their complex structures and high hydrophobicity [27]. However, it is essential to note that most components of crude oil are ultimately biodegradable, despite the increased stability and hydrophobicity that often accompany larger polycyclic aromatic hydrocarbons (PAHs).

A crude oil degradation study using both single bacterial isolates and a bacterial consortium, with Gas chromatography coupled with Flame ionisation Detector (FID), revealed that crude oil degradation was enhanced and accelerated with the use of the bacterial consortium (*Pseudomonas aeruginosa* and *B. subtilis*). Compared to single bacterial isolates, the bacterial consortium achieved a 96.96% crude oil degradation rate, resulting in a significant reduction in crude oil concentration from 257.274 mg/l to 7.83 mg/l. This result is in line with the findings of authors [28], who reported that a microbial consortium achieved a higher crude oil degradation efficiency (85.26%) within 15 days. In contrast, single strains exhibited lower degradation efficiencies ranging from 54.9% to 68.0%; this suggests that microbial consortia may be more efficient at degrading crude oil than single strains, perhaps due to the synergistic effects of different microbial species working together to utilise crude oil as a carbon and energy source efficiently. Both *Pseudomonas aeruginosa* and *B. subtilis* were shown to produce biosurfactants that accelerate the degradation of petroleum hydrocarbons through several processes, including emulsification, oil spreading, and cell-surface hydrophobicity measurements. The secretion of extracellular biosurfactants by bacterial cells increases the solubility and accessibility of hydrophobic hydrocarbons, while altering the cell surface hydrophobicity enhances the interaction between the cells and hydrocarbons. Both of these strategies play a crucial role in the degradation of hydrophobic hydrocarbons, and can occur simultaneously or separately in different bacterial strains [29, 30].

Despite numerous studies indicating that bacterial consortia degrade crude oil more effectively than single strains, few studies have examined the biodegradation efficiencies of individual crude oil components, such as petroleum hydrocarbons with varying carbon chain lengths (C8–C36). In this study, the biodegradation of individual hydrocarbons was analysed by single strains and a bacterial consortium. The bacterial consortium, composed of Pseudomonas aeruginosa and B. subtilis, exhibited complementary degradation advantages, particularly for carbon chains ≤ C33, and outperformed the single bacterial species. The metabolic complementation of these two strains could serve as an actual strategy for the effective degradation of crude oil. It is well-established that the susceptibility of different petroleum hydrocarbons to bacterial degradation varies significantly [31]. Low-molecular-weight alkanes (C8-C17) with simple structures and low hydrophobicity are more readily absorbed by bacterial cells, resulting in high biodegradation efficiency. In contrast, the biodegradation of high-molecular-weight alkanes is more challenging due to their complex structures and low solubility, necessitating the use of biosurfactants to enhance absorption and assimilation [32]. Therefore, enhanced degradation efficacy of the bacterial consortium is attributable to two key factors:

a) the metabolic complementarity of the two bacterial strains, which facilitates efficient crude oil degradation, and

b) the presence of biosurfactant-producing bacteria within the consortium, which improves the dispersion, emulsification, and bioavailability of crude oil, facilitating its degradation [29, 33].

In monoculture conditions, *Pseudomonas aeruginosa* showed superior crude oil degradation, achieving 81.18%, while *Bacillus subtilis* exhibited a lower degradation rate of 58.19%. This finding is in line with previous research highlighting that *Pseudomonas spp*. has superior hydrocarbonoclastic capability compared to other bacterial species such as *Bacillus subtilis* [34].

CONCLUSIONS

The results demonstrate that the Iko River Estuary hosts diverse biosurfactant-producing microbial communities. The study showed that biosurfactant-producing bacteria with hydrocarbon-utilising capacity have the potential to enhance the biodegradation of total petroleum hydrocarbons. The key to increasing hydrocarbon degradation rates is bioaugmentation with a biosurfactant-producing bacterial consortium that can act synergistically to degrade hydrocarbons more effectively.

REFERENCES

1. Vignier, J., Volety, A., Soudant, P., Chu, F., Loh, A. N., Boulais, M., Robert, R., Morris, J., Lay, C., & Krasnec, M. (2019). Evaluation of the Toxicity of the Deepwater Horizon Oil and Associated Dispersant on Early Life Stages of the Eastern Oyster, Crassostrea virginica. In *Separation Science and Technology* (pp. 169–198). doi: 10.1016/b978-0-12-815730-5.00008-9

2. Mao, D., Lookman, R., Van De Weghe, H., Weltens, R., Vanermen, G., De Brucker, N., & Diels, L. (2009). Estimation of ecotoxicity of petroleum hydrocarbon mixtures in soil based on HPLC–GCXGC analysis. *Chemosphere, 77*(11), 1508–1513. doi: 10.1016/j.chemosphere.2009.10.004

3. Bustamante, M., Durán, N., & Diez, M. C. (2012). Biosurfactants are useful tools for the bioremediation of contaminated soil: a review. *Journal of Soil Science and Plant Nutrition, ahead.* doi: 10.4067/s0718-95162012005000024

4. Primeia, S., Inoue, C., & Chien, M. (2020). Potential of Biosurfactants' Production on Degrading Heavy Oil by Bacterial Consortia Obtained from Tsunami-Induced Oil-Spilt Beach Areas in Miyagi, Japan. *Journal of Marine Science and Engineering, 8*(8), 577. doi: 10.3390/jmse8080577

5. Sarwar, A., Brader, G., Corretto, E., Aleti, G., Abaidullah, M., Sessitsch, A., & Hafeez, F. Y. (2018). Qualitative analysis of biosurfactants from Bacillus species exhibiting antifungal activity. *PLoS ONE, 13*(6), e0198107. doi: 10.1371/journal.pone.0198107

6. Floris, R., Rizzo, C., & Lo Giudice, A. (2019). Biosurfactants from Marine Microorganisms. In *IntechOpen eBooks*. doi: 10.5772/intechopen.80493

7. Chikere, C. B., Okpokwasili, G. C., & Ichiakor, O. (2009). Characterisation of hydrocarbon-utilising bacteria in tropical marine sediments. *African Journal of Biotechnology, 8*(11), 2541-2544.

8. Cheesbrough, M. (2010). *District Laboratory Practice in Tropical Countries. Part 2.* (2nd Ed.). Cambridge University Press

9. Jain, D., Collins-Thompson, D., Lee, H., & Trevors, J. (1991). A drop-collapsing test for screening surfactant-producing microorganisms. *Journal of Microbiological Methods, 13*(4), 271–279. doi: 10.1016/0167-7012(91)90064-w

10. Itah, A. Y., & Essien, J. P. (2005). Growth Profile and Hydrocarbonoclastic Potential of Microorganisms Isolated from Tarballs in the Bight of Bonny, Nigeria. *World Journal of Microbiology and Biotechnology, 21*(6–7), 1317–1322. doi: 10.1007/s11274-004-6694-z

11. Batista, S., Mounteer, A., Amorim, & Tótola. (2005). Isolation and characterisation of biosurfactant/bioemulsifier-producing bacteria from petroleum-contaminated sites. *Bioresource Technology, 97*(6), 868–875. doi: 10.1016/j.biortech.2005.04.020

12. Saminathan, P., & Rajendran, P. (2016). Molecular Identification and Characterisation of the Biosurfactant Produced by Pseudomonas aeruginosa-PSPA15 from the Oil Contaminated Soil. *International Journal of Current Microbiology and Applied Sciences, 5*(8), 708–720. doi: 10.20546/ijcmas.2016.508.080

13. Atlas, R. M. (1981). Microbial degradation of petroleum hydrocarbons: an environmental perspective. *Microbiological Reviews, 45*(1), 180–209. doi: 10.1128/mr.45.1.180-209.1981

14. Ismail, H., Isa, T., Ngoshe, I., & Gajere, H. (2018). Biosurfactant Production by Bacteria Isolated from Hydrocarbon-impacted Soil. *Bioremediation Science and Technology Research, 6*(2), 1–4. doi: 10.54987/bstr.v6i2.430

15. Abubakar, U., Ibrahim, U. B., Fardami, A. Y., Kawo, A. H., & Dankaka, S. M. (2020). Biosurfactant production potential of Bacillus obtained from dye effluent. *African Journal of Microbiology Research, 14*(2), 71–76. doi: 10.5897/ajmr2019.9174

16. Iqbal, S., Khalid, Z., & Malik, K. (1995). Enhanced biodegradation and emulsification of crude oil and hyperproduction of biosurfactants by a gamma ray-induced mutant of Pseudomonas aeruginosa. *Letters in Applied Microbiology, 21*(3), 176–179. doi: 10.1111/j.1472-765x.1995.tb01035.x

17. Chen, C., Baker, S. C., & Darton, R. C. (2007). The application of a high-throughput analysis method for the screening of potential biosurfactants from natural sources. *Journal of Microbiological Methods, 70*(3), 503–510. doi: 10.1016/j.mimet.2007.06.006

18. Satpute, S. K., Bhawsar, B. D., Dhakephalkar, P. K., & Chopade, B. A. (2008). Assessment of different screening methods for selecting biosurfactant-producing marine bacteria. *Indian Journal of Marine Science, 37*(3), 243-250.

19. Adeyemi, J. O., Onajobi, I. B., Adebajo, L. O., Orji, F. A., O., Ilusanya A. F., & Samson, O. J. (2019). Biosurfactant Production Potentials of Some Bacteria Recovered from Agro-Industrial Wastes. *Fuw Trends in Science & Technology Journal, 4*(3), 870 – 873

20. Nkanang, A. J., Antai, S. P., Asitok, A. D., & Ekpenyong, M. (2018). Hydrocarbonoclastic potentials of Enterobacteriaceae isolated from the crude oil-polluted Iko River estuary and freshwater ecosystem of the Niger Delta Region of Nigeria. *GSC Biological and Pharmaceutical Sciences, 2*(1), 038–046. doi: 10.30574/gscbps.2018.2.1.0058

21. Kebede, G., Tafese, T., Abda, E. M., Kamaraj, M., & Assefa, F. (2021). Factors influencing the bacterial bioremediation of hydrocarbon contaminants in the soil: mechanisms and impacts. *Journal of Chemistry,* 1–17. doi: 10.1155/2021/9823362

22. Darsa, K., Thatheyus, A. J., & Ramya, D. (2014). Biodegradation of Petroleum Compounds Using the Bacterium Bacillus subtilis. *Science International, 2*(1), 20–25. doi: 10.17311/sciintl.2014.20.25

23. Idorenyin, U., Peter, U., Paul, B., & Peter, E. (2017). Hydrocarbons Degrading Potential of Stimulated Cultures of Bacteria Isolated from Humic Fresh Water Sediment of Eniong River in the Niger Delta of Nigeria. *Microbiology Research Journal International, 21*(3), 1–13. doi: 10.9734/mrji/2017/35647

24. Hassan, F. I., & AI-Jawhari, H. (2014). Ability of Some Soil Fungi in Biodegradation of Petroleum Hydrocarbons. *Journal of Applied & Environmental Microbiology, 2*(2), 46-52.

25. Ajao A. T., Yakubu S. E., Umoh V. J., & Ameh J. B. (2014). Enzymatic Studies and Mineralisation Potential of Burkholderia cepacia and Corynebacterium kutscheri Isolated from Refinery Sludge. *Journal of Microbiology Research, 4*(2), 29-42

26. Romanus, A. A., Ifeoma, O. G., Ikechukwu, E. F., Femi, & Justus, I. (2018). Screening for Plasmid-Mediated Multidrug-Resistant Bacteria in Ikpoba River Water Samples. *International Journal of Microbiology and Biotechnology, 3*(2).

27. Pandolfo, E., Caracciolo, A. B., & Rolando, L. (2023). Recent advances in bacterial degradation of hydrocarbons. *Water, 15*(2), 375. doi: 10.3390/w15020375

28. Xia, M., Fu, D., Chakraborty, R., Singh, R. P., & Terry, N. (2019). Enhanced crude oil depletion by a constructed bacterial consortium comprising a bioemulsifier producer and petroleum hydrocarbon degraders. *Bioresource Technology, 282*, 456–463. doi: 10.1016/j.biortech.2019.01.131

29. Chen, W., Kong, Y., Li, J., Sun, Y., Min, J., & Hu, X. (2020). Enhanced biodegradation of crude oil by a constructed bacterial consortium comprising salt-tolerant petroleum degraders and biosurfactant producers. *International Biodeterioration & Biodegradation, 154*, 105047. doi: 10.1016/j.ibiod.2020.105047

30. Wang, X., Nie, Y., Tang, Y., Wu, G., & Wu, X. (2012). n -Alkane Chain Length Alters Dietzia sp. Strain DQ12-45-1b Biosurfactant Production and Cell Surface Activity*. Applied and Environmental Microbiology, 79*(1), 400–402. doi: 10.1128/aem.02497-12

31. McGenity, T. J. (2013). Hydrocarbon biodegradation in intertidal wetland sediments. *Current Opinion in Biotechnology, 27*, 46–54. doi: 10.1016/j.copbio.2013.10.010

32. Hennessee, C. T., & Li, Q. X. (2016). Effects of polycyclic aromatic hydrocarbon mixtures on degradation, gene expression, and metabolite production in four Mycobacterium species. *Applied and Environmental Microbiology, 82*(11), 3357–3369. doi: 10.1128/aem.00100-16

33. Abalos, A., Viñas, M., Sabaté, J., Manresa, M., & Solanas, A. (2004). Enhanced biodegradation of Casablanca crude oil by a microbial consortium in the presence of a rhamnolipid produced by Pseudomonas aeruginosa AT10. *Biodegradation, 15*(4), 249–260. doi: 10.1023/b:biod.0000042915.28757.fb

34. Wu, B., Xiu, J., Yu, L., Huang, L., Yi, L., & Ma, Y. (2023). Degradation of crude oil in a co-culture system of Bacillus subtilis and Pseudomonas aeruginosa. *Frontiers in Microbiology, 14*, 1132831. doi: 10.3389/fmicb.2023.1132831