Screening and Identification of Biosurfactant-Producing Bactria from Crude Oil Contaminated Soil and Water in the Niger Delta Area, Nigeria

Emeanuru P. C.* Ogbulie J. N. Adieze I. E. Nwachukwu I. N. and Braide, W.
Department of Microbiology, Federal University of Technology, Owerri, Imo State, Nigeria
* Corresponding author: peacesylversonco@gmail.com

Abstract: Crude oil pollution is *sine qua non* in oil exploration and exploitation activities. Biosurfactant increases bioavailability and help to enhance contact between the pollutants and the microorganisms and therefore facilitates uptake and degradation as well as amelioration (remediation) of hydrocarbon polluted environment. Biosurfactant is preferred to chemical surfactant in crude oil remediation. The study focus on screening and identification of biosurfactant-producing bacteria from crude oil contaminated Soil and water in the Niger Delta area, Nigeria. Water and soil/sediments from three communities, namely, Eleme, Omoku and Ogbia in the Niger Delta Area Nigeria was screened for biosurfactant-producing bacteria. Haemolytic activity, emulsification capacity and oil spread were used as index in the screening of biosurfactant-producing bacteria. Crude oil utilizing bacteria isolated from the water and soil/sediments in the three oil producing communities were identified by vapour phase method. Identification was done by 16s rRNA sequence from the isolates that produced highly similar sequences from the NCBI non-redundant nucleotide (nr/nt) database. The sequenced isolates were, *Alcaligenes faecalis, Bacillus altitudinsis, Bacillus siamensis, Bacillus velezensis, Cytobacillus horneckiae, Enterobacter roggenkampii, Morganella morganii, Providencia stuartii, Priestia flexa, and Pseudomonas aeruginosa*. Six isolates representing 60% of the total isolates have potentials for biosurfactant production. *Providencia stuartii, Alcaligenes faecalis, Bacillus altitudinis, Bacillus siamensis* and *Bacillus velezensis* are novel isolates for biosurfactant production in the Niger Delta region of Nigeria. The *Bacillus* species and *Pseudomonas aeruginosa* showed higher activities in crude oil degradation and biosurfactant production. *Pseudomonas aeruginosa* produce glycolipid type of biosurfactant used in bioremediation of oil sludge contaminated soils. The Niger Delta remains a potential reservoir for biosurfactant-producing bacteria with relevant biomarker genes that may prove significant in environmentally friendly clean up in the Niger Delta Area.
Key word: Biosurfactant production, bacteria, water, soil/sediments

INTRODUCTION

The Niger Delta, a region known for its rich biodiversity among the rainforest zones of Nigeria has unfortunately faced severe environmental degradation due to crude oil spills and exploration activities. In recent years, the search for eco-friendly solutions to remediate these polluted sites has led scientists to explore the potential of biosurfactant-producing bacteria involved in bioremediation (Adebusoye *et al*: 2008, Bharali *et al*., 2022 and Aruotu *et al.,* 2023). Adebusoye *et al*. (2008); Bharali *et al*. (2022) and Goveas *et al*. (2022) independently reported potential hydrocarbon-degrading bacteria such as *Pseudomonas aeruginosa*, *Bacillus subtilis* and *Staphylococcus* species. Biosurfactants are surface-active compounds produced by microorganisms that are capable of enhancing the availability and biodegradation of hydrophobic pollutants like crude oil by reducing interfacial tension which in turn increases the surface area of the immiscible phases (Fakruddin, 2012; Aulwar and Awasthi, 2016). The biosurfactant-producing bacterial isolates identified exhibited diverse substrate specificities for different hydrocarbons, enabling them to interact with a wide range of hydrophobic compounds; with some being able to degrade polycyclic aromatic hydrocarbons (PAHs) (Fatigue *et al*., 2023). Eras-Muñoz (2022) reported that biosurfactants producing bacterial isolates in the Niger Delta effectively emulsified crude oil components, enhancing their bioavailability to indigenous microorganisms which then improved pollutant biodegradation. The findings of these studies highlight the potential of indigenous microbial strains as a bioresource for the remediation of chronically polluted soils in the Niger Delta.This study reports on the screening and identification of biosufactant-producing bacteria isolated from crude oil contaminated sites.

MATERIALS AND METHODS

Sample collection: Composite soils contaminated with crude oil were randomly collected from four communities frequently exposed to oil spills and exploration activities in the Niger Delta regions in Nigeria. Composite samples were collected into sterile polyethylene bags and immediately taken to the laboratory within 1:30 minutes for isolation and characterization biosurfactant-producing bacteria. The microorganisms were isolated from composite crude oil contaminated soil samples according to the method of Ogbonna *et al*. (2020). Samples size was determined according to the method described by Kothari (2004).

Preparation of enrichment culture media for isolation of biosurfactant-producing bacteria: The content of the mineral source medium include monoptassium phosphate (1.00 g), K_2HPO_4 (1.00 g), $MgSO_4.7H_2O$ (0.20 g), $FeCL_3$ (0.05 g), $NaNO_3$ (1.5 g), $(NH_4)_2SO_4$ (1.5 g) and $CaCl_2$ (0.02 g), agar No. 1 (20.0 g) **(**Okerentugba *et al*., 2016**)**. Ten grams (10 g) of the contaminated soil sample was serially diluted in 90 ml of sterile physiological saline. After shaking thoroughly, a 10 fold (v/v) serial dilutions were made by transferring 1 ml of the stock solution into freshly prepared normal saline diluents to a range of 10^1, 10^2, 10^3 and 10^4 dilutions and 0.1 ml was inoculated into the mineral salt medium (Bushnell- Haas agar, BHA; Nutrient agar (NA), Mueller-Hinton agar (MHA), Mannitol Salt agar (MSA) and Cetrimide agar (CA). Inocula were uniformly spread with a sterile hockey stick-like glass spreader and plates were incubated at ambient temperature (28 ± 02^0C) for 24-48 h for other media and 3-7 days for BHA (Prescott *et al*., 2005).

Enumeration of total heterotrophic microorganisms: The total counts of heterotrophic bacteria in the soil sample was determined by the spread plate techniques described by Rakish (2020).

Isolation, characterization and identification of bacteria: The morphological characteristics of isolates were observed and recorded colonially, microscopically and biochemically (Cheesbrough, 2000). The identities of the microorganisms were confirmed with reference to standard identification manuals (Beishir, 1987, Holt *et al*., 1994; Buchanan and Gibbon, 2000; Harrigan and McCance, 2000).

Molecular characterization of bacterial isolates: Polymerase chain reaction (PCR) amplification of the 16S rRNA gene was followed by sequencing, allowing for bacterial species identification and phylogenetic analysis. High-quality genomic DNA was extracted using commercial kits or phenol-chloroform extraction methods, providing genetic material for further molecular studies. Specific genes related to biosurfactant production, such as rhlA, rhlB, and rhlC, were amplified using PCR, enabling the detection of biosurfactant-associated genetic markers (Das *et al*., 2020).

Inoculum of bacteria for screening: Five bacterial isolates were screened for petroleum degradation in a mineral salt medium. The isolate with highest degradation capabilities was selected for optimum performance (Harinatha, 2021).

Inoculum biomass build up: The bacteria was cultured and grown to a sizable population for 3 weeks and the biomass determined at 600 nm in digital spectrophotometer (model: SP-VG722, wavelength range:190 – 110nm) with optical density (OD) of 0.526 (Harinatha, 2021).

Screening of biosurfactant-producing organism: The following methods were used for the screening of biosurfactant:

Haemolytic activity: Bacterial isolates were screened on blood agar plates containing 5% (v/v) sheep blood and incubated at 37^{o}C for 48 h. Haemolytic activity was detected as the presence of a clear zone around bacterial colonies recorded (Mulligan *et al*., 1984; Carrilo *et al*., 1996; Vijayajkumar and Saravanan, 2015). This was done in triplicates.

Emulsification capacity: A mixture of 2 ml hydrocarbon and 1 ml cell free extract was

obtained after the centrifugation of sample culture. Two millilitre (2 ml) hydrocarbon and 1 ml cell free extract were homogenized by vortexing for 2 minutes. The emulsion activity was investigated after 24 h. The emulsification index (B24) was calculated by the total height of the emulsion divided by the total height of the aqueous layer and their multiplying by 100 (Cooper and Goldenberg, 1987; Pattanathu *et al*., 2008; Makkar and Cameotra, 1997; Van Dyke *et al*., 1993).

$$\text{EC24} = \frac{\text{height of the emulsion}}{\text{Height of the aqueous layer}} \times 100$$

Oil spreading method: Ten microliters (10 ul) of crude oil was added to the surface of 40 ul of distilled water in a Petri dish to form a thin oil film layer. Then, 10 ul of culture supernatant was gently place on the center of the oil layer (Noha *et al*., 2004). The diameter of clear zone on the surface was measured and compared to 10 ml distilled water as negative control. This was also done in triplicates.

Identification of bacterial isolates: The bacteria for petroleum degradation capabilities were identified based colonial microscopic, biochemical and molecular characterization (Cowan and Steel, 1993; Holt *et al*., 1994).

RESULTS

Colonial and microscopic morphologies of bacteria isolated from samples contaminated with crude oil is shown in Table 1. Table 2 shows the biochemical and carbohydrate fermentation abilities of the isolates. Both characterization was done according to standard methods with reference to identification manuals.

Phylogenetic tree showing the evolutionary distance between the bacterial isolates is shown in Figure 1. The obtained 16s rRNA sequence from the isolate produced are highly similar sequences from the NCBI non-redundant nucleotide (nr/nt) database. The 16s rRNA of the isolate MI showed a percentage similarity to other species at 100%. The evolutionary distances computed using the jukes-cantor method were in agreement with the phylogenic placement of the 16s rRNA of the isolates. The sequenced isolates were, *Alcaligenes faecalis Bacillus altitudinsis, Bacillus velezensis, Bacillus siamensis, Cytobacillus horneckiae, Cytobacillus horneckiae, Enterobacter roggenkampii, Morganella morganii, Pseudomonas aeruginosa, Priestia flexa* and *Providencia stuartii* (Figure 1).

Table 3 shows the counts and percentage occurrence of bacteria isolated from the water and soil samples. *Bacillus* species and *Pseudomonas aeruginosa* were predominant in both samples, though more in the soil/sediment than water. The counts and percentage occurrence of the bacterial isolates from the three locations is shown in Table 4. Samples from Eleme and Ogbia recorded more isolates than samples from Omoku. The occurrence of the isolates are similar to that recorded in Table 4.

Table 5 shows the screening of biosurfactant-producing potentials of the bacterial isolates in relationship with emulsification capacities, oil spread and haemolytic activities. *Pseudomonas aeruginosa* and the *Bacillus* species rank highest in all the parameters measured. *Alcaligenes faecalis* and *Providencia stuartii* also showed high level of significance in the criteria for determining biosurfactant production. The percentage occurrence of biosurfactant-producing bacteria was significant in the soil/sediment samples than the water (Table 6). *Pseudomonas aeruginosa* and *Bacillus* species are dominant.

Table 1: Colonial morphology and microscopic characterization of bacterial isolates

Colonial morphology	Gram Morphology	Motility	Capsule Formation	Spore Production	Probable identity
Bluish green colonies on nutrient and cetrimide agar	-R	+	-	-	*Pseudomonas* sp
Very large grey white colonies, dry and sometimes look wet and blistery	+R	+	-	+	*Cystobacillus* sp
Moist and shiny smooth colonies	-R	+	-	-	*Enterobacter* sp
Colonies appear off-white and opaque in colour	-R	+			*Monganella* sp
Large grey white colonies with blistery appearance	+R	+	-	+	*Bacillus* sp
Creamy white rough colonies with slightly irregular margins on nutrient agar	+R	+	-	+	*Bacillus* sp
Surface flat, dry and wrinkle colonies	+R	+	-	+	*Bacillus* sp
Large grey opaque shiny smooth colonies	-R	+	-	-	*Providencia* sp
Whitish with a feathery thin flared irregular edged colonies	-R	+	-	-	*Alcaligenes* sp
Creamy white circular moist and shiny	+R*	+	-	+	*Priesta* sp

Key: *Staining variable

Table 2: Biochemical and carbohydrate fermentation of bacterial isolates

Cat	Oxi	Coag	In	MR	VP	Cit	Ure	S	L	M	Mn	G	Bacterial Isolates
-	+	-	-	+	-	+	+	-	-	-	+	+	*Pseudomonas aeruginosa*
+	-	-	-	-	+	-	-	-	+	-	-	+	*Cystobacillus horneckiae*
+	-	-	+	+	-	-	+	-	-	+	+	+	*Monganella morganii*
+	-	-	-	+	-	+	-	-	+	-	-	+	*Bacillus altitudinis*
+	-	-	-	+	-	+	-	-	-	+	-	+	*Bacillus velezensis*
+	-	-	-	-	+	+	-	-	-	-	-	+	*Bacillus siamensis*
-	-	-	+	+	-	+	+	+	-	-	+	+	*Providencia stuartii*
+	+	-	-	+	-	+	-	+	-	-	-	-	*Alkaligenes faecalis*
+	-	-	-	-	+	+	+	-	+	-	-	+	*Enterobacter roggenkampii*
+	+	-	-	-	+	+	-	+	-	+	-	+	*Priestia flexa*

Key: + = positive; - = negative; Cat, Catalase; Oxi, Oxidase; Coag, Coaglase; In, Indole; VP, Voges Proskaeur; MR, Methyl Red; Cit, Citrate; Ure, Urease; S, Sucrose; L, Lactose; M, Maltose; Mn, Mannitol; G, Glucose.

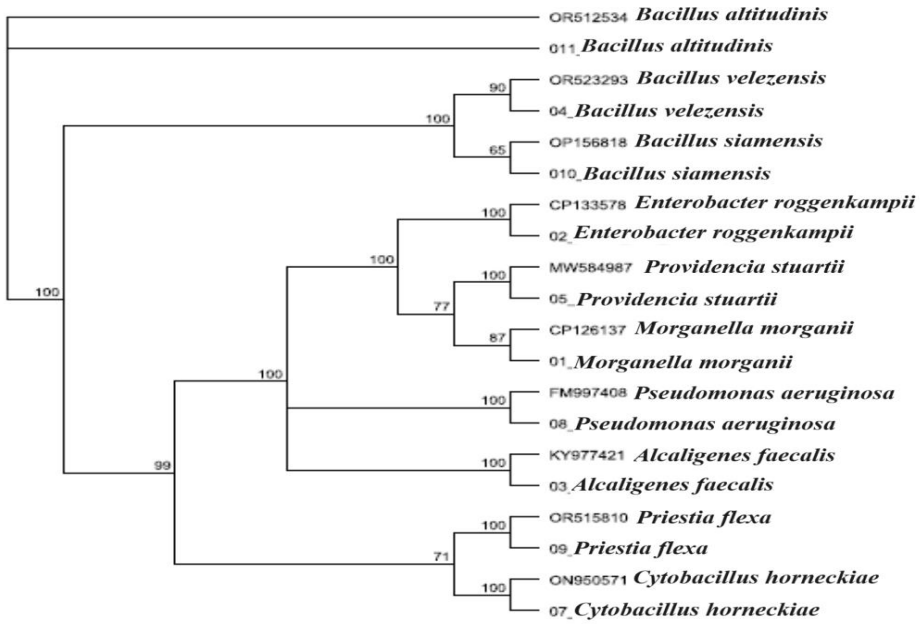

Figure 1: Phylogenetic tree showing the evolutionary distance between the bacterial isolates

Table 3: Percentage occurrence of bacteria isolated from samples

Bacterial Isolates	Water	Soil
Pseudomonas aeruginosa	32(19.2)	47(17.4)
Cystobacillus horneckiae	7(4.2)	11(4.1)
Monganella morganii	6(3.6)	11(4.1)
Bacillus altitudinis	19(11.4)	33(12.2)
Bacillus velezensis	31(18.6)	48(17.8)
Bacillus siamensis	21(12.6)	35(13.0)
Providencia stuartii	17(10.2)	35(13.0)
Alkaligenes faecalis	19(11.4)	28(10.4)
Enterobacter roggenkampii	6(3.6)	10(3.7)
Priestia flexa	10(6.0)	15(5.6)

Key: Figures in parenthesis represent percentage

Table 4: Percentage occurrence of bacteria isolated from sampling locations

Bacterial Isolates	Omoku	Eleme	Ogbia
Pseudomonas aeruginosa	31(22.0)	45(19.8)	44(18.9)
Cystobacillus horneckiae	4(2.8)	7(3.1)	2(0.9)
Monganella morganii	6(4.3)	3(1.3)	6(2.6)
Bacillus altitudinis	16(11.4)	22(9.7)	33(14.2)
Bacillus velezensis	21(14.9)	36(15.8)	48(20.6)
Bacillus siamensis	18(12.8)	39(17.2)	31(13.3)
Providencia stuartii	12(8.5)	20(8.8)	27(11.6)
Alkaligenes faecalis	18(12.8)	38(16.7)	33(14.2)
Enterobacter roggenkampii	8(5.7)	11(4.8)	3(1.3)
Priestia flexa	6(4.3)	8(3.5)	8(3.4)

Key: Figures in parenthesis represent percentage

Table 5: Emulsification capacity, oil spread and haemolytic activity test of biosurfactant-producing organisms

Biosurfactant Producing Organisms	Oil Spread	Haemolytic Activity	Emulsification Capacity E24%
Pseudomonas aeruginosa	+++	++	3.6
Bacillus altitudinis	+++	++	3.9
Bacillus velezensis	+++	++	3.6
Bacillus siamensis	++	++	3.3
Alcaligenes faecalis	++	+	3.0
Providencia stuartii	++	+	3.0

Key: + alpha haemolysis (partial); ++/+++, beta haemolysis (complete)

Table 6: Percentage occurrence of biosurfactant-producing bacteria from samples

Bacterial Isolates	Water	Soil/Sediment
Providencia stuartii	14(7.7)	31(10.5)
Alcalegenes faecalis	21(11.6)	39(13.3)
Bacillus altitudinis	18(9.9)	51(17.3)
Bacillus velezensis	37(20.4)	44(15.0)
Bacillus siamensis	42(31.1)	59(20.1)
Pseudomonas aeruginosa	51(28.1)	72(24.)

Key: Figures in parenthesis represent percentage

DISCUSSION

Biosurfactant plays significant role in the industry, agriculture and bioremediation. The current dominate players in the market are chemically synthesized surfactants such as Tween 20/80, Triton X-100, and Brij35 (Lamichhane *et al*., 2017; Poonguzhali *et al*., 2022). However, such chemically synthesized surfactants generally have concerns of toxicity and low biodegradability (Poonguzhali *et al*., 2022). Moreover, as these products are derived from fossil fuels, their production is not sustainable in a long run and the production

costs are subjected to the price variance of fossil fuels (Otzen, 2017). In view of these limitations, in the past decades, extensive research efforts have been placed in the development of environmental friendly, renewable and non/less-toxic alternatives such as biosurfactants, which are surfactant molecules produced by microorganisms during their growth (Vijayakumar and Saravanan, 2015).

Some studies demonstrated that the type of biosurfactant produced by some bacterial strains is modified when growing on hydrocarbons and oils (Rashedi *et al*., 2005; Makkar and Cameotra, 2016). Some organic pollutants in form of crude petroleum, are commonly assumed to be an unfriendly habitat/medium for microbes to thrive upon. However, in spite of its high toxicity and hydrophobicity, mounting evidence has revealed the presence of live microbes (particularly bacteria) in crude oil. A wide distribution of microbial diversity, including *Pseudomonas, Bacillus, Streptomyces and Stenotrophomonas* species, were associated with oil wells (Rashedi, 2005). These organisms have the potential to degrade numerous hydrocarbons for use as carbon sources and encapsulate heavy metals and/or hydrocarbons through the production of active surfactants (Varjani and Gnansounou, 2017). Isolation and characterisation of biosurfactant-producing bacteria had been reported by Ayebabogha *et al*. (2021). Water and soil/sediment from the three communities in the Niger Delta region of Nigeria showed evidence of indigenous biosurfactant-producing bacteria.

Owing to their unique properties and vast array of applications, identification of new biosurfactant-producing microbes is of great importance. There are nine different screening methods that have been reported as criteria to screen biosurfactant producing microbes. These include haemolytic assay (Volchenko *et al*., 2007), bacterial adhesion to hydrocarbons (BATH) assay (Benat *et al*., 2010), drop collapse assay (Bodour and Miller, 1998), oil spreading assay (Yonebayashi *et al*., 2000), emulsification assay (Makkar and Cameotra, 1997; Yonebayashi *et al*., 2000; Venessa *et al*., 2013), surface tension measurement, tilted glass slide test, blue agar plate and hydrocarbon overlay agar assay (Soltanighias *et al*., 2019). All these tests have variable sensitivity and specificity. This study reported on the haemolytic assay, oil spread and emulsification assay in the screening of biosurfactant producing bacteria from contaminated soil/sediment and water. *Bacillus altitudinsis, Bacillus velezensis, Bacillus siamensis*, *Providencia stuartii, Pseudomonas aeruginosa, Alcaligenes faecalis* showed great affinity in biosurfactant production reported in this study.

CONCLUSION

This study demonstrated the ability of new species of bacteria such as *Alcaligenes faecalis, Bacillus altitudinsis, Bacillus velezensis, Bacillus siamensis, Pseudomonas aeruginosa* and *Providencia stuartii* that showed great affinity in biosurfactant production with great potentials in biosurfactant production. The soil/sediment and water from crude oil contaminated soils are reservoiur for biosurfactant-producing organisms. Biosurfactant-producing bacteria in the Niger Delta area of Nigeria hold immense potential for sustainable solutions in bioremediation, agriculture and industrial applications. Addressing the challenges through innovative research methodologies and interdisciplinary collaborations is essential. Comprehensive studies bridging knowledge gaps will not only deepen our understanding of biosurfactant biology but also pave the way for novel applications, driving the field toward a more environmentally friendly and economically viable future. A holistic approach, integrating molecular studies, environmental monitoring and responsible antibiotic usage is essential to mitigate antibiotic resistance in biosurfactant-producing bacteria. Strict regulations and continuous research efforts are vital to harness the benefits of biosurfactants while minimizing the risks

associated with antibiotic resistance in environmental applications.

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