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Research Article

Biosurfactant Production Potentials of Autochthonous Bacterial Species Associated with Waxy Crude Oil

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Abstract

Microbial degradation of paraffinic compounds has gained considerable interest due to their easy handling, eco-friendliness, high operational safety for sustainable development, low toxicity, non-carcinogenic nature, non-combustibility, and widespread presence of microorganisms. Biosurfactants increase bioavailability and help enhance contact between pollutants and microorganisms, thereby facilitating uptake and degradation, as well as the amelioration (remediation) of hydrocarbon-polluted environments. It also acts as a waxy depressant, enhancing the flow of crude during production. This study investigated the biosurfactant-producing potential of autochthonous bacterial species associated with waxy crude oil. The culturable autochthonous heterotrophic bacterial density in the waxy samples ranged from $1.3 \pm 0.3 \times 10^4$ to $2.6 \pm 0.3 \times 10^4$ CFU/ml. Four distinct bacterial species were isolated, characterized, and identified as *Bacillus cereus*, *B. subtilis*, *Pseudomonas aeruginosa*, and *Micrococcus* sp. Analysis of the biosurfactant potentials of the isolates cell-free supernatants revealed varying biosurfactant potentials, with *Pseudomonas aeruginosa* culture extract having the best oil spread result and biosurfactant potential (% EC_{24} = 52.05), followed by *B. subtilis* (% EC_{24} = 50.0), then *Micrococcus* sp. (% EC_{24} = 48.7) and *B. cereus* (% EC_{24} = 47.5). All the isolates' cell-free extracts exhibited β-hemolysis. The potential of these autochthonous bacterial communities would be explored in the future as depressants for waxy crude oil treatment and for in the oil and gas sector, microbial enhanced oil recovery.

Keywords

Biosurfactant Production, Bacteria, Waxy Crude, Wax Treatment

1. Introduction

Bacteria are capable of degrading the crude oil substrate through metabolic or enzymatic processes. They can cleave the long-chain paraffins found in crude oil into shorter-chain paraffins using enzymes, genes, and by-products they secrete, thereby reducing the apparent molecular weight of the crude oil. Effective and rapid degradation of non-volatile high molecular weight hydrocarbons, such as waxes, is a critical factor in addressing wax precipitation and enhanced oil recovery (EOR) during crude oil production and transportation.

The main mechanisms of microbial wax degradation include reduction of interfacial tension (IFT), wettability alteration, oil-water emulsification, and oil viscosity reduction.

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The microbial metabolites, especially biosurfactants, are believed to be the key contributors [1]. Biosurfactants are amphiphilic surface-active compounds that can interact at the interface between aqueous and non-aqueous systems [2] due to the presence of both hydrophilic and hydrophobic ends. These features also allow them to reduce the surface and interfacial tension between liquids and solids [3].

Low toxicity to the environment, eco-friendliness, biodegradability, and acceptability have made microbial biosurfactants to gain renewed commercial interest, in recent times, over their synthetic counterparts. They are being used in the fields of agriculture, the food industry, and therapeutics [4, 5].

Reports have shown that a variety of biosurfactant-producing bacteria can be isolated from aqueous environments and polluted/unpolluted soils with crude oil or plant rhizosphere [3, 4, 6, 7], but information on the potential of bacteria associated with waxy crude is limited. Waxy crude oil is paraffin wax that has precipitated. This waxy crude oil is a significant challenge for the oil and gas industry across the production, transportation, and refining stages. They have a negative effect on the transportation of crude oil, such as restriction of crude oil flow, thus creating pressure abnormalities and leading to a reduction or interruption in production; higher pumping costs due to additional pressure drop caused by wax deposition; and increased capital, operational, and maintenance costs.

The carbon chain length or the proportion of paraffin compounds (ranging from C_{18} to C_{40}) present in a crude oil sample can serve as indicators of its paraffinic or waxy nature [8, 9]. Studies have also shown that microbial products and their enzymes are used as wax suppressants in the oil and gas industry [10-13]. There is a need to investigate the cost-effective sources of biosurfactant-producing bacteria that can be exploited for wider use. This study was thus designed to investigate the biosurfactant-producing potentials of waxy crude oil autochthonous culturable bacterial communities. The primary work involved isolation and characterization of bacterial strains from waxy crude-oil samples and screening their potential to produce biosurfactants.

2. Methodology

2.1. Source of Waxy Crude Oil

Waxy crude oil samples were collected from an oil field in the Niger Delta, and the investigation of the biosurfactant-producing potentials of the waxy crude oil autochthonous culturable bacterial communities was carried out at the Microbiology laboratory, University of Uyo, Nigeria.

2.2. Estimation of Microbial Loads in the Waxy Crude Oil Samples

Series of dilutions of the sample to achieve a 10^{-3} dilution factors were performed, according to the method of Cheesbrough [14]; 1 ml from each enrichment sample was measured and mixed in 90 ml of sterile water (aliquot) sample. Further dilution was carried out by transferring 1ml from the aliquot into a sterile 9 ml of dilution blank in a test tube. Standard microbiological techniques described by Horrgan and MacCance [15] and, Prescott [16] were employed for the microbiological analysis of these samples. Total Hydrocarbon Bacteria Count (THBC) was determined using the Pour plate's method. This procedure used mineral salt medium (see, Figure A1) as an analytical medium. Isolates obtained from this medium were inoculated on Nutrient agar. Plates were incubated for 24 - 48 Hours at 28 ℃, using a Gallenkamp incubator (see, Figure A2). Microbial colonies that emerged on the incubated plates were counted with the aid of a Quebec Colony counter and recorded as colony forming unit (CFU/ml) of samples.

2.3. Characterization and Identification of Positive Isolates

Standard characterization tests (such as Gram staining, catalase, coagulase, motility, starch hydrolysis, methyl-red, Vogues Proskauer, indole, citrate utilization, urease, spore staining, hydrogen sulfide production, and sugar fermentation) were performed. The details of the test procedures are presented in appendix A. The pure culture was identified based on its cultural, morphological, and physiological features with those in Bergey's Manual of Determinative Bacteriology [17, 18].

2.4. Enrichment of Biosurfactant-producing Autochthonous Culturable Bacterial Communities

For efficient isolation of the culturable autochthonous bacterial community, 3.27 g/L of sterilized Bushnell and Haas medium (BHM) (BDH Chemical Ltd.) supplemented with 2% of waxy crude (v/v) as the sole carbon source (pH 7.0) was inoculated with 1 mL volume of bacterial cultures (grown at 22 ±2 ℃ for 18-24 Hours with agitation in LB broth) with an OD_{600} between 0.6 and 1.0 and transferred to 100 mL of the carbon-amended BHM media. Inoculated media was incubated with continuous agitation (2 x g) at 30 ℃ for 7 days, and then the cell-free supernatant was collected by centrifugation (6500 g at 4 ℃ for 20 min). Removal of any residual oil and/or live bacterial cells present in the cell-free supernatant was accomplished by filtration using a 0.22 m Millipore filter and kept at 4 ℃ until further use.

2.5. Assay for Biosurfactant Production

Assays for biosurfactant production were carried out by subjecting the cell-free supernatants from each of the isolates to drop collapse assay, oil spread assay, hemolytic activity,

and emulsification assay [3, 19]. Isolates were considered to have significant biosurfactant production if the clearing zone they produced was at least 1.0 cm in diameter in the oil spreading assay and 3.0 mm in the drop collapse assay [19]. In all assays, Triton X-100 (10 mg/mL) was used as the positive control, while sterile deionized water and uninoculated hydrocarbon-amended BHM medium served as negative controls. All tests were carried out in duplicate. The best indicative biosurfactant producers were further screened using the emulsification assay and hemolytic assay.

2.5.1. Drop Collapse Assays

The drop-collapse test was performed according to Plaza *et al. [20]*. In this method, 5 µL of supernatant from each bacterial isolate was pipetted onto a microplate lid (12.7 ×8.6 cm) previously coated with crude oil and allowed to dry for 24 hours. The results were considered positive for biosurfactant production when the culture supernatant was flat within 2 minutes. Those that gave rounded drops were scored negative, an indication of the absence of biosurfactant production.

2.5.2. Oil Spread Technique

For this test, a thin oil film was formed by adding 10 µl of crude oil to the surface of 40 ml of distilled water in a petri dish. This was followed by the addition of 10 µl of the test bacterial culture supernatant gently on the center of the oil layer and observed for the formation of a clearing zone. After 30 seconds from the addition of the culture supernatant, the diameter of the clear zone was measured and correlated to surfactant activity. The measurement is expressed in BS units, known as biosurfactant units.

2.5.3. Hemolytic Activity

Approximately 150 L of filtered cell-free supernatant of each bacterial isolate was loaded into each well (6.5 mm in diameter) made by a cork borer in the blood agar plates and incubated at 30 ℃ for 24-48 hours. Biosurfactant biosynthesis was confirmed by hemolysis activity as indicated by the presence of clearing zones around the wells. The diameter of the lysis zone is scored as 'γ-hemolysis' (no lysis), 'α-hemolysis' (partial hemolysis, i.e., the clear zone becomes dark and greenish), or 'β-hemolysis' (complete hemolysis).

2.5.4. Emulsification Assay (% EC $_{24}$)

The same volume of a test culture supernatant and kerosene in a ratio of 1:1 was mixed in a glass test tube (125 mm × 15 mm). Then, the combination was vortexed for 2 min and left to stand for 24 Hours. The $\%EC_{24}$ was calculated by dividing the height of the emulsified layer (mm) by the total height of the liquid in the glass test tube (mm) and then multiplying by 100. This was used as the percentage yielded.

3. Results and Discussion

Microorganisms are distributed in the biosphere based on natural selection [21]. One of the indices of microbial adaptation in any ecosystem is its biomass. The culturable autochthonous heterotrophic bacterial density in the waxy samples ranged between 1.3 ± 0.3 x 10^4 and 2.6 ± 0.3 x 10^4 CFU/ml (Table 1). The bacterial density in the waxy crude was lower than the density reported by Henry *et al. [22]* for crude oil-contaminated soil (4.6 x 10^7 CFU/g). Four distinct bacterial colonies were obtained, purified, and screened for biosurfactant production. The colonial and morphological characteristics of the isolates are presented in Table 2.

Table 1. *Density of culturable autochthonous heterotrophic bacteria in the waxy crude.*

Samples	THBC (x 10^4 CFU/ml)
Waxy crude 1	2.6 ±0.3
Waxy Crude 2	1.3 ±0.3

Table 2. *Colonial and Morphological Characteristics of the isolates.*

Isolate Code	Colonial	Gram Morphology	Motility	Endospore
WAX-1	Large, flat, and irregularly shaped colonies with a frosted glass-like surface	+ve Rod	+	+
WAX-2	Creamy white rough colonies with slightly irregular margins	+ve Rod	+	+
WAX-3	Bright mustard-yellow colonies	+ve Cocci	-	-
WAX-4	Greenish pigmented colonies on nutrient agar	-ve Rod	+	-

Two of the isolates (WAX-1, WAX-2) were Gram-positive rods, one (WAX-3) was Gram-positive cocci, while the last

(WAX-4) was a Gram-negative rod. Biochemical characterization of the isolates revealed the identities (Table 3).

Biosurfactant plays a significant role in industry, agriculture, and bioremediation. The current dominant players in the market are chemically synthesized surfactants such as Tween 20/80, Triton X-100, and Brij-35 [23, 24]. However, such chemically synthesized surfactants generally have concerns of toxicity and low biodegradability [24]. Moreover, as these products are derived from fossil fuels, their production is not sustainable in the long run, and the production costs are subject to the price variance of fossil fuels. In view of these limitations, extensive research efforts have been placed in the development of environmentally friendly, renewable, and non/less-toxic alternatives such as biosurfactants, which are surfactant molecules produced by microorganisms during their growth [25].

Table 3. *Biochemical Characterization and Identification of Bacteria Isolated from Waxy crude and synthetic crude samples.*

Isolate Code	Gram Reactions	Catalase	Motility	Starch hydrolysis	Citrate	Urease	Spore formation	H_2S	Oxidase
WAX-1	+ve	+	+	+	-	-	+	-	-
WAX-2	+ve	+	+	+	+	-	+	-	-
WAX-3	+ve	+	-	+	+	+	-	-	+
WAX-4	-ve	+	+	-	+	-	-	+	-

Table 3. *Continued.*

Isolate Code	Glucose	Maltose	Xylose	Lactose	Fructose	Sucrose	Mannitol	Galactose	Probable organisms
WAX-1	AG	A	-	-	A	A	-	A	*Bacillus cereus*
WAX-2	AG	A	A	-	A	-	-	A	*Bacillus subtilis*
WAX-3	-	A	A	-	A	-	A	A	*Micrococcus sp*
WAX-4	A	-	-	AG	AG	AG	AG	AG	*Pseudomonas aeruginosa*

The cell-free supernatants of the test isolates exhibited varying biosurfactant potentials (Table 4). *P. aeruginosa* and *B. subtilis* broth supernatants exhibited moderate drop collapse potentials, with collapse zones ranging from 6 to 10 mm, while the cell-free supernatant from *B. cereus* and *Micrococcus* sp. exhibited a weak drop collapse potential. Findings of the oil spread assay of the culture supernatants revealed that *P. aeruginosa* culture supernatant exhibited strong (> 15 mm) oil spread potential. *B. subtilis* and *Micrococcus* sp. culture supernatant exhibited moderate (60 to 10 mm) oil spread potential, while *B. cereus* culture supernatant had a weak oil displacement potential.

Analysis of the quantity of biosurfactants elaborated by each of the isolates was determined based on the emulsification index of the cell-free culture supernatants. *Pseudomonas aeruginosa* culture supernatant exhibited the highest biosurfactant elaborating potential (%EC_{24} = 52.05), followed by *B. subtilis* (%EC_{24} = 50.0), then *Micrococcus* sp. (%EC_{24} = 48.7) and *B. cereus* (%EC_{24} = 47.5), (see Figure 1). All the isolates cell-free extracts exhibited β-hemolysis.

Table 4. *Biosurfactant-producing potentials of the isolates.*

Isolate	Drop Collapse Assay	Oil Spread Technique	Hemolytic Activity
Bacillus cereus	+	+	β
Bacillus subtilis	++	++	β
Micrococcus sp	+	++	β
Pseudomonas aeruginosa	++	+++	β

Key: + = 1-5 mm (weak); ++ = 6-10 mm (moderate); +++ = 11-15 mm (strong); ++++ = >15 mm (strongest)

The findings of this study agree with several studies that have posited *Pseudomonas* species as promising [26, 27].

Surfactants have been shown in several studies to improve biodegradation by increasing the bioavailability of contaminants [28]. Cameotra and Singh [29], in their study on enhanced bioremediation of oil sludge using biosurfactants and a microbial consortium consisting of two isolates of *Pseudomonas aeruginosa* and one isolate of *Rhodococcus erythropolis* from soil contaminated with oily sludge, reported a 90% degradation of hydrocarbons in 6 weeks in liquid culture.

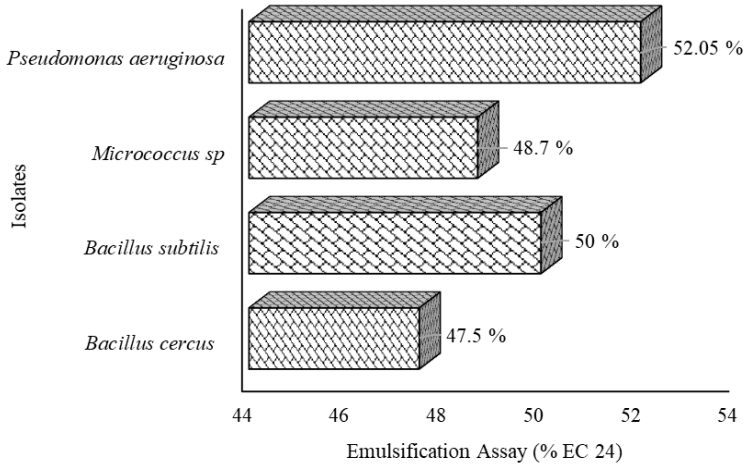

Figure 1. *Emulsification Index of the Cell-Free Supernatants.*

4. Conclusion

This study demonstrated the ability of culturable autochthonous bacteria species (*P. aeruginosa*, *B. subtilis*, *B. cereus*, *and Micrococcus* sp.) associated with waxy crude oil to elaborate biosurfactants. Biosurfactant-producing bacteria in the Niger Delta area of Nigeria hold immense potential for sustainable solutions. The culturable autochthonous heterotrophic bacterial density in the waxy samples ranged between $1.3 \pm 0.3 \times 10^4$ and $2.6 \pm 0.3 \times 10^4$ CFU/ml. Four distinct bacterial colonies were obtained, purified, and screened for biosurfactant production. The colonial and morphological characteristics of the isolates: two of the isolates (WAX-1, WAX-2) were Gram-positive rods, one (WAX-3) was Gram-positive cocci, while the last (WAX-4) was a Gram-negative rod. Biochemical characterization of the isolates revealed the identities. The cell-free supernatants of the test isolates exhibited varying biosurfactant potentials. Analysis of the quantity of biosurfactants elaborated by each of the isolates was determined based on the emulsification index of the cell-free culture supernatants.

The potentials of these autochthonous bacterial communities should be explored as waxy depressants in the oil and gas sector, microbial enhanced oil recovery, bioremediation, agriculture, and industrial applications.

Abbreviations

EOR	Enhanced Oil Recovery
THBC	Total Hydrocarbon Bacteria Count
BS Units	Biosurfactant Units
CFU	Colony Forming Unit
BHM	Bushnell and Haas Medium
IFT	Interfacial Tension
A	Acid
AG	Acid and Gas

Author Contributions

Festus Sunday Friday: Conceptualization, Resources, Investigation, Formal Analysis, Writing – original draft

Aniefiok Livinus: Supervision, Resources, Writing – review & editing

Akpabio Udoh Julius: Supervision, Writing – review & editing

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Data Availability

The datasets used and/or analysed during the current study available from the corresponding author on reasonable request.

Conflicts of Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Appendix

The major experiments carried out involve morphological and biochemical characterization of the bacterial Isolates, using standard characterization tests - gram staining, catalase, motility, starch hydrolysis, citrate utilization, urease, spore staining, hydrogen sulfide production, heamolysis and sugar fermentation, as described by Cowan [17] and Buchanan and Gibbons [18].

Figure A1. *Mineral salt.*

Figure A2. *Subculture plates on Nutrient Agar.*

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