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ORIGINAL RESEARCH ARTICLE

Molecular identification of biosurfactant-producing bacteria obtained from oil-polluted soil samples in Abakaliki, Ebonyi State, Nigeria

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ABSTRACT

Several human activities have led to environmental pollution with crude oil and other related hydrocarbons. There are various technologies of remediating oil-polluted environments, but the use of biological agents such as biosurfactants are widely practiced, and are generally considered as safe and eco-friendly. This study aimed at isolating and identifying biosurfactant -producing bacteria obtained from oil polluted soil samples in Abakaliki, Ebonyi State, Nigeria using molecular techniques. Six waste oil-contaminated soil samples were collected from mechanic and generator repair workshops, and were analyzed using standard microbiological methods. Nine bacterial isolates were obtained from the waste oil-contaminated soil samples, and were suspected to be *Pseudomonas aeruginosa*, *Bacillus subtilis* and *Staphylococcus aureus*. Molecular identification of the biosurfactant-producing isolates was also carried out using PCR and the genes responsible for biosurfactant production targeted. Oil spreading technique was used to detect the oil displacement activity (ODA) of the isolates. The emulsification index (E_{24}) was calculated and the ability of the extracted biosurfactant to emulsify the hydrocarbons was determined. *P. aeruginosa* had the highest ODA of 28.1 cm while *S. aureus* had the least ODA of 5 cm. *P. aeruginosa* showed E_{24} of 70.1%, *B. subtilis* 57.1% while *S. aureus* showed 32.5%. Hence, *Pseudomonas* and *Bacillus* species have good potentials for biosurfactant production, and can be harnessed and utilized in the cleanup of oil polluted soil.

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INTRODUCTION

There is a world-wide concern about the liberation of hydrocarbon in the environment from both industrial activity and accidental spills of oils. Nowadays, use of petroleum and petroleum base polymers has increased to an alarming rate. These polymers facilitate us in many different ways, but their accumulation, non-biodegradability and contamination has been a concern-voiced by many (Rafeya & Nazia, 2020). Presence of these hydrocarbons in the environment is of global public health and ecological concern, due to their persistence and toxicity (Brinda *et al*., 2023). Different physical and chemical technologies such as excavation, thermal evaporation, soil-flushing extraction and soil-vapour extraction, etc are employed to clean oil-contaminated sites (Al-Marri *et al*., 2023). Despite the deleterious effect and hydrophobic nature of oil, the presence of live organisms has been observed in oil polluted areas. These organisms are capable of producing surfactants - surface active substances which reduce the surface tension, and increase spreading and wetting properties of oil droplets in soil and water

(Talreja *et al*., 2022). Surfactants include synthetic surfactants and biosurfactants. Majority of the synthetic surfactants are produced from petrochemicals, which unfortunately increase the level of environmental pollution, as they are hardly degraded by microorganisms, and are toxic in nature (Obayori *et al*., 2021). Biological means are employed to remediate oil polluted, hence production of biosurfactants. Several studies have shown that different microorganisms such as *Pseudomonas*, *Bacillus*, *Streptomyces*, *Azotobacter*, *Citrobacter*, *Stenotrophomonas*, *Lactobacillus*, etc present in oil contaminated sites have potentials to degrade different crude oil products, utilize them as energy sources, and sometimes encase them in their body biomass leading to production of active surfactants (Araújo *et al*., 2019; Farias *et al*., 2021; Talreja *et al*., 2022; Al-Marris *et al*., 2023). Biosurfactants are surface-active metabolites produced by microorganisms. They tend to solubilize hydrocarbons in the surrounding environment, by reducing surface tension and increasing carbon uptake (Nor *et al*., 2021). They are excreted by many microorganisms with emulsifying activities, and are bipolar in nature. They are composed of both hydrophilic and hydrophobic parts, which allow them to interact at the oil-water interface and optimize the microbial uptake of petroleum hydrocarbons by increasing the contact area with the contaminants (Ayoib *et al*., 2024). Biosurfactants have the capacity to create self-assembled molecular clusters known as micelles which houses both the hydrophilic head and the hydrophobic tail. They are complex molecules shrouding a wide range of different compounds such as peptides, fatty acids, phospholipids, glycolipids, antibiotics, and lipopeptides (Varjani & Gnansounou, 2017). Despite their molecular structure, biosurfactants display excellent surface activities since they originate from living organisms such as bacteria, fungi and yeasts (Sánchez, 2022). The biochemistry of surfactants permits the reduction of the interfacial tensions between liquids, solids, and gases.

Soil pollution occurs when there is accumulation of different chemical compounds arising from either natural or industrial processes, and this is a major environmental problem. Chemical remediation which involves the use of chelating agents and different chemical surfactants is an alternative but they have biocidal effects on the native organisms present in the affected environments, and also non-biodegradable and can produce secondary pollutants (Huang *et al*., 2020; Zang *et al*., 2021; Farias *et al*., 2021). Increasing environmental concerns have led to the search of natural surfactants as an alternative to the traditional products (Suaibu *et al*., 2021). Natural surfactants synthesized by microorganisms offer several advantages over chemical surfactants such as low toxicity, biodegradability, ecological acceptability, and eco-friendly, stability at extreme temperature, salinity conditions and possibility of production from renewable sources (Scrivastava *et al*., 2022; Karnwal *et al*., 2023). In addition to these characteristics, biosurfactants have a wide variety of potential applications, which make them more interesting (Mohy-Eldin & Hossam, 2023). The main applications of these biological compounds are in advanced oil recovery and in the bioremediation fields, however, they can still be used as emulsifiers, functional ingredients, pharmaceutical and therapeutic agents, and as additives in health and beauty products (Karnwal *et al*., 2023). It is important to employ biotechnological tools to enhance the identification of biosurfactant producers because of their excellent properties. Hence, this present study is aimed at isolating and characterizing biosurfactant-producing bacteria from oil-polluted soil samples in Abakaliki, Ebonyi State, Nigeria, using molecular techniques.

MATERIALS AND METHODS

Sample collection

Six soil samples were collected from different waste oil contaminated sites using an auger at a depth of 15-20 cm deep in sterile polythene bags and transported to the Department of Applied Microbiology Laboratory, Ebonyi State University, Abakaliki for analyses.

Isolation of bacteria

One grams of each soil sample was dissolved in 9 ml of distilled water and incubated in a shaker for 2-3 days at room temperature. The samples were allowed to settle and serial dilutions were performed on the samples. After preliminary trials, dilutions of 10^{-2} – 10^{-3} had growth that were too numerous to count on the culture media, hence, dilutions of 10^{-5} were plated onto Nutrient agar, Cetrimide agar and Bushnell Haas agar plates respectively by pour plate method and incubated at 37°C for 24 - 48 hours. Distinct colonies were selected randomly and purified by sub-culturing onto nutrient agar by streak plate method for purification of isolates.

Media preparation

All media used were prepared based on the manufacturers' specifications. Mineral Salt medium (MSM) was also formulated by mixing solutions A and B in 1 liter of distilled water (Ndibe *et al*., 2019).

Identification of biosurfactant producers

The biosurfactant producing isolates were identified based on their morphological, cultural and biochemical characteristics. Gram staining and biochemical tests were carried out based on the standard procedures and organisms were identified using Bergey's Manual of Determinative Bacteriology (Holt *et al*., 2002).

Screening of isolates for biosurfactant production

The isolated organisms were grown in MSM and incubated in a shaker operated at 200 rpm for seven (7) days. At the end of the incubation periods, the cultures were centrifuged at 4000 rpm for 30 minutes for the removal of cells. The cell free broth cultures (supernatant) were then tested using different oil such as fuel, kerosene, vegetable oil and paraffin oil for the activities of biosurfactants using the following test:

Oil spread test

Thirty (30) mL of distilled water was poured into four large petri

dishes (15 cm diameter), 2 drops of each of the oil was added to the water surface. 1 drop of cell free extract was placed gently in the middle of the oil layer. The appearance of zones of clearance observed by the displacement of oil after 30 seconds indicates the presence of biosurfactants, and the diameter of the clear zone on the oil surface signifies biosurfactant activity, also called oil displacement activity. The area of this circle was measured and calculated for oil displacement area (ODA) using the formula: ODA = 22/7(radius)^2 cm^2(Liu *et al*., 2018; Ibrahim *et al*., 2020).

Emulsification activity

Four (4) mL of the supernatant culture medium was introduced into a screw capped tube containing 4 mL of each of the following oil: fuel, kerosene, vegetable oil and paraffin oil. The tubes were shaken for 2 minutes (Ewida and Mohamed, 2019). The mixture was then allowed to settle for 24 hours and the emulsification index was measured using the formula:

E_{24} = Height of the emulsion layer/Total height of mixturex100

Molecular identification of biosurfactant producers

The molecular identification of all isolates obtained from this research was carried out at the Biotechnology Research and Development Centre (BRDC), Ebonyi State University, Abakaliki. PCR was used to screen selected isolates for the presence of genes involved in biosurfactants production using the following methods:

DNA Extraction

DNA extraction from the isolates was performed using Cetyltrimethylammonium Bromide (CTAB) method, as modified by Allen *et al*., 2006. Genes of the nine isolates obtained from waste oil contaminated soil samples which showed biosurfactant activity were screened using real time PCR.

Polymerase chain reaction (PCR)

PCR amplification was carried out in volume of 25 µL made up of 2.0 µL 100 ng DNA, 2.5 µL of 10x Buffer (Bioline), 1.5 µL of 50 mM $MgCl_2$(Bioline), 2.0 µL of 2.5 mM dNTPs (Bioline), and 0.2 µL 500U *Taq* DNA polymerase (Bioline), 1.0 µL of 10 µM each of the primers and 15.05 µL DEPC-treated water (Invitrogen Corporation). The PCR cycling profile used for the reaction is made up of an initial step at 96 °C for 5-7 min., 35 cycles of 94 °C for 35 s, 58 °C to 60 °C for 1 min., and 72 °C for 1min., and a 10-min final extension at 72 °C. For agarose gel electrophoresis, 10 µL of amplified PCR products of each test isolate together with appropriate DNA standards (1 Kb and 100 bp step DNA ladders) were separated on 1.5% agarose gel for 2 hours at 100 Volts. The gel was stained with Ethidium bromide and the resulting bands viewed under ultraviolet light.

RESULTS AND DISCUSSION

Several strains of bacterial isolates were detected, but three representative organisms were selected from each of the isolated bacterial species, and were designated as PS*a*, PS*b*, PS*c* (for *P. aeruginosa*); BS*a*, BS*b*, BS*c* (for *B. subtilis*) and SS*a*, SS*b*, SS*c* (for *S. aureus*). The isolates were subjected to PCR identification. Molecular identification of the nine isolates was carried out using OPrL (F/R) primers, PS was amplified at 480 bp (Figure 1). PS*a* was also amplified with amplicon size of 249 bp for OPrL (F/R) primer pair. The Bac 463 (F/R) primer pair, specific for *Bacillus* did not amplify any of the isolates (Figure 2).

The universal primers (16SrRNAR/16SRrDNA63F and 16SRrDNA 1389R/16SrRNA F), which were also used in this study, amplified the isolates at different target sites with different *amplicon* sizes. PS*a* was amplified at 400 bp, PS*b* had amplicon sizes of 400 and 500 bp respectively while PS*c* had amplicon sizes of 400, 500 and 1200 bp (Figure 2). BS*a* was amplified at 1200 bp, while BS*b*, BS*c*, and SS*a*, SS*b* were all amplified at 400 and 500 bp respectively (Figure 2). The isolates suspected to be *P. aeruginosa* was amplified. Research has shown that *Pseudomonas* contain L and I lipoproteins in their outer membrane, thus this could be a reliable factor for rapid identification, hence for the primer pair OPrI and OPrL (F/R), only PS*c* was amplified at 480 and 240 bp respectively (Figures 1 and 2). This agreed with the research of Haque *et al*. (2020), who noted that all *P. aeruginosa* strains contained OPrI and OPrL genes. Similarly, Al-Marris *et al*. (2023), noted that *P. aeruginosa* strains contained same genes and exhibit remarkable potential to produce biosurfactants using various carbon sources. For 463 (F/R) pair of primers, there was no amplification. *Bacillus* has different species; hence, it could be possible that the Bac 463 primer pair is specific for a different species and not *B. subtilis*, thus was not amplified at 463 bp. This was also revealed by Brinda *et al*. (2023), who used GC-MS analysis to amplify *Bacillus* sp. MN 243657 at Bac 463 primer pair. 16SrRNAR/16SRrDNA63F amplified successfully at 1000bp, while the primer pair of 16SRrDNA 1389R/16SrRNAF also amplified successfully with different amplicon sizes. PS*a* was amplified at 400 bp, PS*b* had an amplicon size of 400 and 500 bp respectively while PS*c* had amplicon sizes of 400, 500 and 1200 bp. BS*a* amplified at 1200bp, while BS*b*, BS*c*, SS*a* and SS*b* were all amplified at 400 and 500 bp respectively. Studies carried out by Liu *et al*. (2018), corroborated the present study as they carried out partial sequencing of 16S rDNA of their isolated strain and identified it as *P. aeruginosa* SNP0614.

Oil displacement activity is often used to test the ability of microorganisms to produce biosurfactants. Result of the oil displacement activity (ODA) of the isolates showed that all the isolates exhibited ODA. *P. aeruginosa*, *B. subtilis* and *S. aureus* isolated from this study revealed that they exhibited potentials for biosurfactant production. *B. subtilis a* and *b* showed the highest ODA of 28.1 cm in kerosene and 25.5 cm in fuel. *P. aeruginosa a* exhibited the highest ODA in fuel (19.6 cm) and the least (13.2 cm) in paraffin oil. Of the three bacteria isolated from this study, *S. aureus* showed the lowest ODA of 5.1 cm in paraffin oil. Both *P. aeruginosa* and *S. aureus* showed similar oil displacement patterns of fuel>kerosene>vegetable oil>paraffin oil (Table 1).

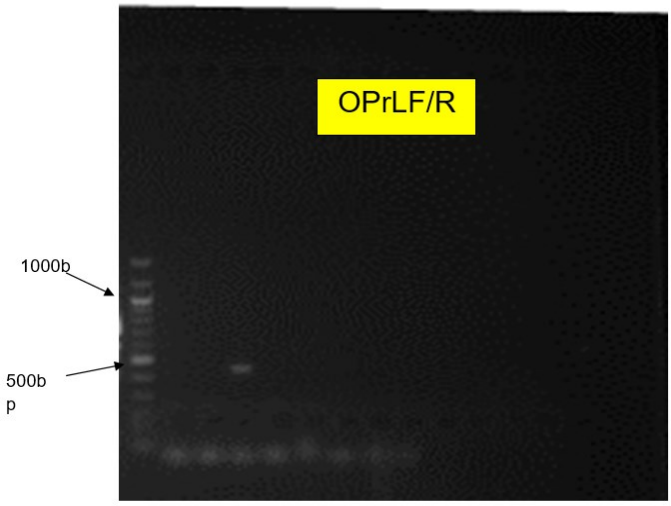

Figure 1. *Nine DNA samples, Pseudomonas aeruginosa c amplified Key: PSa, PSb, PSc (for P. aeruginosa a, b, and c); BSa, BSb, BSc (for B. subtilis a, b, and c) and SSa. SSa, SSc (for S. aureus a, b, and c).*

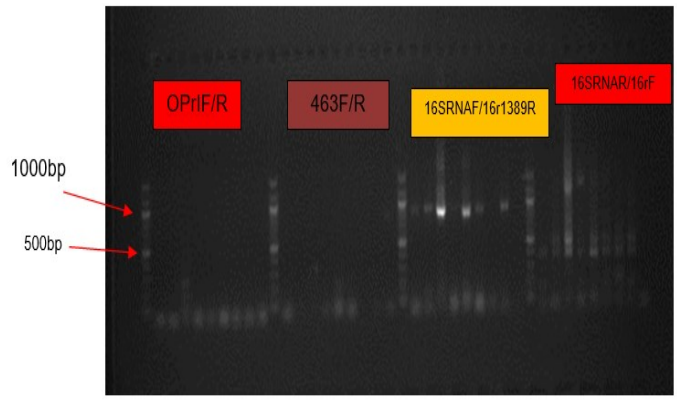

Figure 2. *Nine DNA samples each amplified with four primers Key: PSa, PSb, PSc (for P. aeruginosa a, b, and c); BSa, BSb, BSc (for B. subtilis a, b, and c) and SSa, SSb, SSc (for S. aureus a, b, and c).*

That is, the more viscous the oil, the less the ability of the bacteria to displace them. This agreed with the result of Diallo *et al.* (2021), who revealed that biosurfactant activity increases with decrease in viscosity. The ODA exhibited by all the isolates in this study is in agreement with the research of Nayarisseri *et al.* (2020), in which biosurfactant producers exhibited the ability to displace oil, hence, producing clearance zone. However, the reliability of the result obtained in this study contradicts the study of Sánchez (2022), who reported ODA as indicative of the surface and wetting activities of biosurfactants.

Emulsification index is an indirect method used to screen biosurfactants production by microorganisms (Derguine-Mecheri *et al.*, 2021). In this study, all isolates emulsified the waste contaminated oil used at different percentages. *P. aeruginosa a* and *c* exhibited highest emulsification index of 70.1 % and 68.2 % in kerosene, and this reveals that biosurfactants produced by *P. aeruginosa* isolates can be effectively used in degrading kerosene, and in fact, all the oil used in this study. *B. subtilis c* showed E_{24} of 57.1 % in kerosene, 55.6 % in vegetable oil and 50.5 % in paraffin oil. Hence, the viscosity of the contaminant has an effect on the rate of emulsification, that is, the more viscous the oil, the less the emulsification index. *S. aureus a* exhibited highest emulsification activity of 40.5 % in kerosene, while the least was observed with *S. aureus a* and *c* (32.5 %) respectively in paraffin oil (Table 2).

For emulsification activity of isolates in fuel, the reaction broke the container and spilled part of its contents, hence, the results available are for kerosene, vegetable oil and paraffin oil. From the results obtained in this study, *P. aeruginosa*, *B. subtilis and S. aureus* were isolated from the waste oil contaminated soil samples. This agreed with result findings of Thirumurugan *et al.* (2023), who reported the isolation of *Bacillus* species from crude oil and anthracene; Djahnit *et al.* (2019), isolated *Pseudomonas* species from the Algerian centre coast, spilled with crude oil, and Pal *et al.* (2021), in his study of exploring the diversity and hydrocarbon bioremediation potential of microbial community in the waste sludge of Duliajan oil field, in Assam India, reported that *S. aureus* was isolated and exhibited degrading potentials. *S. aureus* can emulsify the three oils used in this study but not as efficiently as the *P. aeruginosa* and *B. subtilis*. These results agree with Muthukumar *et al.* (2022), who opined that emulsification is a property of biosurfactant-producing organisms. The excellent emulsification activities exhibited by the isolated bacteria in this study is also in line with Ayoib *et al.* (2024), who revealed that emulsification indicates the presence of biosurfactants.

Table 1. Oil displacement activity (ODA) of bacteria isolated from waste oil-contaminated soil grown in mineral salt medium (cm).

Organism	Kerosene	Vegetable oil	Fuel	Paraffin oil
PSa	16.0	13.8	19.6	13.2
PSb	12.6	10.8	18.1	9.1
PSc	8.0	12.8	12.6	9.1
BSa	28.1	12.6	25.5	11.3
BSb	16.0	19.6	20.4	12.6
BSc	12.3	12.0	14.5	10.2
SSa	8.0	5.6	7.1	5.1
SSb	7.7	5.8	7.1	5.3
SSc	7.0	6.1	6.5	5.8

Table 2. Percentage emulsification ability of test isolates from waste oil-contaminated soil grown in MSM.

Organism	Kerosene	Vegetable oil	Paraffin oil
PS_a	70.1	60.6	59.8
PS_b	65.00	63.8	55.1
PS_c	68.20	61.8	52.6
BS_a	53.33	50.6	50.2
BS_b	52.6	49.6	45.4
BS_c	57.1	55.6	50.5
SS_a	40.5	37.9	32.5
SS_b	35.7	38.8	37.1
SS_c	35.2	33.14	32.5

Conclusion

The 16s rRNA technique was employed in this study to identify bacterial isolates obtained from oil-polluted soil samples in Abakaliki, Ebonyi State, Nigeria which had potentials to produce biosurfactants. The identified bacterial strains were *P. aeruginosa*, *B. subtilis* and *S. aureus*. *P. aeruginosa* and *B. subtilis* showed the highest degrading ability as well as the highest biosurfactant production potential with E_{24} of 70.1% and 65.5% respectively. On the other hand, *S. aureus* had the least biosurfactant producing ability with E_{24} of 32.5%, hence, has no good potential for bioremediation. Biosurfactants produced from *P. aeruginosa* and *B. subtilis* should be produced on a large scale, and utilized in the clean-up or bio-removal of oil contaminated soil. The result of this research will help the Government of Ebonyi State, Nigeria, to design a pilot-scale study for the biodegradation of hydrocarbon-contaminated soil, which will help us to translate the laboratory-scale findings into the industrial applications.

DECLARATIONS

Authors' contribution: Conceptualization, investigation, methodology, formal analysis and Writing original draft: Nwachi, A.C.; Formal analysis, Data curation, investigation and methodology: Ogene, L.N. and Nwakaeze, E.A.; Methodology, Formal analysis, Writing-original draft, Writing-review and editing, Project administration and Supervision: Okoye, E.C.S.; Methodology, Validation, Project administration and Resources: Elom, E.E. All authors have read and agreed to the published version of the manuscript.

Conflicts of interest: The authors declare no conflict of interest.

Ethics approval: This study did not involve any animal or human participant and thus ethical approval was not applicable.

Consent for publication: All co-authors gave their consent to publish.

Data availability: The data that support the findings of this study are available on request from the corresponding author.

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