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Extraction of Biosurfactants Produced from Bacteria Isolated from Waste-Oil Contaminated Soil in Abakaliki Metropolis, Ebonyi State

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Abstract: Biosurfactants are surface active compounds that reduce the surface and interfacial tension between two liquids or between a liquid and a solid. They are produced from a variety of microorganisms such as bacteria. This study focused on the extraction of the biosurfactants produced by bacteria isolated from waste oil contaminated soil. Four different waste oil-contaminated soil samples (Designated Samples A-D) were collected from mechanic sites and filling stations within Abakaliki metropolis, Ebonyi State, Nigeria and these were analyzed in the Microbiology Laboratory Unit of Ebonyi State University, Abakaliki using standard microbiological procedures. Twelve bacterial isolates were isolated from the waste oil-contaminated soil and were then tested for biosurfactant production using different techniques which included oil spreading test, emulsification test and blood haemolysis test. Out of the twelve bacterial isolates, six were found to produce biosurfactants. They were identified as Pseudomonas spp (4 strains), Bacillus sp and Staphylococcus spp. The Pseudomonas species were isolated from samples A, B, C and D, the Bacillus specie was from sample B and the Staphylococcus spp were from A and D. The result of their emulsification test showed that Pseudomonas b had the highest emulsification index (E), E24 in fuel (60.00%), followed by Pseudomonas a (50.00%). In kerosene, Pseudomonas a and Pseudomonas d had the highest E24 (47.50%), while in vegetable oil, Bacillus sp, Pseudomonas a and Pseudomonas b had the highest E24 (50.00%). Also, in paraffin oil, Pseudomonas a showed the highest E24 (71.43%) followed by Staphylococcus sp and Pseudomonas b (50.00%). The result of the oil spreading test showed that Pseudomonas d had the highest ODA (2.27cm2). This was followed by Pseudomonas and Pseudomonas c (1.77cm2) whereas Pseudomonas b had the least ODA (0.07cm2). Out of the six isolates, only three showed haemolytic activity (Pseudomonas b, d and Staphylococcus). The results showed that Pseudomonas represents a good candidate for the production of the biosurfactant when grown in both nutrient broth (NB) and mineral salt medium (MSM) supplemented with 2% glucose. The biosurfactants produced were extracted by centrifugation. Biosurfactant from Pseudomonas b had the highest weight (18.9g), followed by Pseudomonas a (18.8g) while that of Staphylococcus had the least weight (15.2g). The study was carried out on a small scale and if biosurfactant can be produced in large quantity, it can play an important role in the petroleum industry as well as in the bioremediation of oil-contaminated sites.

Keywords: Biosurfactants; Pseudomonas spp; Bacillus spp; Staphylococcus spp; Abakaliki, Nigeria.

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1. Introduction

The impact of environmental pollution to man, animals, and plants cannot be over emphasized. The release of contaminants into the environment, such as petroleum and petroleum-derived products, is one of the main causes of global contamination [1]. Such pollution has led to decrease in agricultural produce, loss of aquatic lives, increase in the incidence of diseases, and loss of aesthetic value of the natural environment. It is also a risk for human and animal health since many of these contaminants have demonstrated to be toxic and carcinogenic [2]. Most environmental pollutants are known to be persistent for quite a long period of time. Hydrocarbon molecules that are released into the environment are difficult to remove, since they adsorb to surfaces and are trapped by capillarity in a water-immiscible phase. Petroleum-derived hydrocarbons are among the most persistent soil contaminants, and some hydrocarbon degrading microorganisms can produce biosurfactants to increase bioavailability and degradation. Bioremediation has proven to be an alternative to reduce the effects caused by hydrocarbon contamination of soil and water, using the metabolic capacities of microorganisms that can use hydrocarbons as source of carbon and energy. The efficiency of removal is directly related to the compounds' chemical structure, to its bioavailability (concentration, toxicity, mobility and access) and to the physicochemical conditions present in the environment [3]. Biosurfactants are biological surface-active agents (surfactants) capable of reducing interfacial tension between liquids, solids and gases, thereby allowing them to mix and disperse readily in water or other liquids. The microbial surfactants (MS) are complex molecules covering a wide range of chemical types including peptides, fatty acids and phospholipids [4]. Microbial surfactants are mainly produced by aerobic microorganisms, using carbohydrates, hydrocarbons, animal and vegetable oils or a mixture of them as carbon source Bognolo [5] and Fiechter [6]. Bacteria are the main group of biosurfactant-producing microorganisms, although they are also produced by some yeast and filamentous fungi [7]. Hydrophobic pollutants present in petroleum hydrocarbons, soil and water environment require solubilization before being degraded or metabolized by microorganisms. Surfactants can increase the surface area of hydrophobic materials such as pesticides and crude oil in soil and water environment thereby increasing their water solubility. Hence, the presence of surfactants may increase microbial degradation of pollutants [8]. There exist many negative impacts of petroleum and its products on the atmosphere, hydrosphere, and the soil cover of the earth and this is caused by several factors such as an active and growing use of hydrocarbon resources in several sectors of human activity. Biosurfactants can be produced intracellularly and/or can be excreted to the culture media [9]. When the biosurfactants are intracellular, their structure includes membrane lipids, and promotes the transport of insoluble substrates through the membrane. But when they are extracellular, the biosurfactants help in the substrate solubilization and are usually a complex structure of lipids, proteins and carbohydrates [2]. The main difference in the chemical nature of the different biosurfactant molecules is in the hydrophilic head; allowing for a wide range of variation in their physical and biological properties [10]. Several properties and physiological functions in the producer organisms have been described for different groups of biosurfactants, which include solubility of hydrophobic compounds, heavy metal binding, bacterial pathogenesis, cell adhesion and aggregation, quorum sensing and biofilm formation [11, 12]. Almost all classes of microorganisms produce biosurfactants [13]. A number of high molecular weight biosurfactants and bioemulsifiers are produced by both bacteria and fungi. The genera of soil microorganisms that are known to degrade hydrocarbons include *Pseudomonas*, *Flavobacterium*, *Achromobacter*, *Arthrobacter*, *Bacillus*, *Micrococcus* and *Acinetobacter [14]*. The microbial surfactants are not yet able to compete economically with the chemically synthesized biosurfactants in the market, basically due to their high cost of production [15]. Xu , *et al.* [16] also reported that the use of microbial surfactant in industry is limited due to lack of public acceptance of biosurfactant producing microorganisms, and the high purity necessary for food, cosmetics and pharmaceutical applications. For these reasons they are mainly used for the environmental treatments. Thus this study presumptively evaluated the extraction of Biosurfactants from bacteria isolated from waste-oil contaminated soil in Abakaliki Metropolis, Ebonyi State, Nigeria.

2. Materials and Methods

2.1. Isolation and Identification of Biosurfactant-Producing Bacteria from the Waste Oil-Contaminated Soil

Four soil samples were collected from mechanic sites and petrol stations in Abakaliki metropolis in Ebonyi State, Nigeria; and 10 grams of each soil sample was placed into 250 ml flask containing 100 ml of distilled water and incubated at 23^{o}C on a shaker at 200 rpm for about 72 hours. On the third (3rd) day, samples from each slurry was serially diluted, plated on nutrient agar medium (i.e. an aliquot of 10^{-4}, 10^{-5} and 10^{-6}) and incubated for 48 hours in duplicates. After incubation, plates were enumerated and morphologically different bacterial colonies were subcultured on nutrient agar for 24 hours to obtain the pure culture. The cultures were stored in agar slants and used for biosurfactant screening [17].

2.2. Production of Biosurfactants by the Selected Bacterial Isolates

Twelve purified isolates were selected and tested for biosurfactant production. The Isolated colonies were inoculated into 5 ml mineral salt medium (MSM) containing 2% glucose as the sole carbon and energy source. The MSM was a mixture of two solutions. Solution A contained (per liter) 2.5 g of $NaNO_3$, 0.4 g of $MgSO_4.7H_2O$, 1.0 g of NaCl, 1.0 g of KCl, 0.05 g of $CaCl_2.2H_2O$, and 10 ml of concentrated phosphoric acid (85%). The solution was adjusted to pH 7.2 with KOH pellets. Solution B contained (per liter) 0.5 g of $FeSO_4.7H_2O$, 1.5 g of $ZnSO_4.7H_2O$,

1.5 g of $MnSO_4.H_2O$, 0.3 g of K_3BO_3, 0.15 g of $CuSO_4.5H_2O$, and 0.1 g of $Na_2MoO_4.2H_2O$. One (1) ml of solution B was added to 1000 ml of solution A to form the MSM. The broth cultures were incubated in a shaker (at 200 rpm) for 7 days at room temperature. Suspensions were then tested for the presence of surfactant [17, 18]. After incubation, the culture media were centrifuged at 4000 rpm for 30 min to obtain a cell free supernatant. The culture free supernatant was tested for the presence of biosurfactant [19].

2.3. Screening for Biosurfactant Production

The isolated colonies were tested for their biosurfactant producing ability in three ways:

i) Oil Spreading Test

The produced biosurfactants were separated from culture media by centrifugation to get culture supernatant and characterized by measuring diameters of clear zones produced when a drop of a biosurfactant-containing solution is placed on an oil-water surface. Fifty (50) ml of distilled water was added to a large Petri dish (15 cm diameter) followed by addition of 2 drops of condemned engine oil to the water surface. And 1 drop of culture supernatant was gently placed on the center of the oil layer. The presence of biosurfactant was detected by the displacement of the oil and hence the appearance of clear zone after 30 sec. The diameter of the clear zone on the oil surface correlates to surfactant activity, also called oil displacement activity. The area of this circle was measured and calculated for oil displacement area (ODA) using the equation below and as previously reported [18]:

ODA = 22/7 $(radius)^2$ cm^2.

ii) Emulsification Assay (Emulsification index E_{24})

In a screw caped tube, 4 ml of the cell free supernatant was added to 4 ml of each of the following oils: kerosene, fuel, paraffin and vegetable oil. The tubes were vortexed at high speed for 2 minutes. The mixture was allowed to settle for 24 hours and the emulsification index (E_{24}) was measured as the ratio of the height of the emulsion layer and the total height of the mixture and then multiplied by 100 [20, 21].

$$E_{24} = \frac{\text{The height of the emulsion layer}}{\text{The total height of the mixture}} \times 100$$

E_{24} correlates to the biosurfactant concentration.

iii) Blood Haemolysis Test

Isolated colonies were screened for haemolytic activity on blood agar plates containing 5 % (v/v) human blood. The isolated colonies were streaked on the blood agar medium and incubated for 48 hours at 37^o C. The plates were visually inspected for clear zones around the colonies, indicative of biosurfactant production [22, 23].

iv) Extraction of the Biosurfactant

This was carried out using the cell free supernatant technique. The pH of the supernatant was first adjusted to 2.0 using concentrated HCL; and 5 ml each of chloroform and methanol in the ratio of 2:1 respectively were added to the supernatant. The mixture was shaken for proper mixing and was left overnight for evaporation. White coloured sediments were obtained as the biosurfactants which were then dried and weighed.

v) Dry Weight of the Biosurfactants

A dry, clean conical flask was weighed. The sediment was poured into the flask and placed in hot air oven at 200^{o}C for 15 minutes to dry. After drying, the conical flasks were weighed again and the dry weights of the biosurfactants were calculated using the formula:

Dry weight of biosurfactant = Weight of the conical flask after drying - Weight of the empty flask [24, 25].

3. Results and Discussion

This study was designed to isolate biosurfactant producing bacteria from waste oil contaminated soil and extract the surfactants produced. Four soil samples (designated as samples A-D) were collected within Abakaliki metropolis and were taken to Microbiology laboratory unit of Ebonyi State University, Abakaliki for processing. Standard microbiological and biochemical tests were used to isolate, characterize and identify the bacterial isolates. From the four samples analyzed, a total of 12 isolates were obtained. On characterization, it was discovered that 6 of the isolates produced biosurfactants after screening on MSM-glucose broth and as was indicated from the development of clear zones (Figure 1). Out of the 6 organisms isolated, four were gram negative rods and were identified as *Pseudomonas* species, one was gram positive rod and was identified as belonging to the genus *Bacillus,* and the other one was gram positive coccus identified as *Staphylococcus* specie. These organisms demonstrated biosurfactant production to an extent. This study is in agreement with the study carried out by Al-Saleh, *et al.* [26] which revealed that *Staphylococcus*, *Bacillus* and *Pseudomonas* species can produce biosurfactants.

Although rod shaped bacteria are more frequently reported to produce biosurfactants, *Staphylococcus,* a coccus and Gram positive bacteria, was also isolated from the soil sample. Different techniques such as oil spreading test, emulsification test and blood haemolysis test were used to identify the organisms as biosurfactant producers. The oil spreading test is a good indicator for biosurfactant production because it is very sensitive and requires small sample to undertake as previously reported [21]. It is rapid, easy to be carried out and does not require any specialized

equipment [27], and it depends on the decrease in water-oil-interfacial tension caused by the biosurfactant regardless of its structure [28].

Figure-1. Photographs showing oil spreading activity of the biosurfactant produced (arrow).

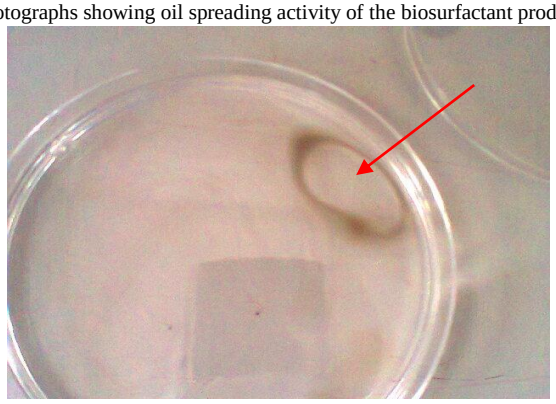

Table-1. Oil displacement area, ODA (cm^2) of some selected bacteria isolates

Isolated Bacteria	Oil Displacement Area (cm^2)
Pseudomonas a	1.77
Pseudomonas b	0.07
Pseudomonas c	1.77
Pseudomonas d	2.27
Bacillus sp	0.79
Staphylococcus spp	0.20

Table 1 and Figure 2 show the results of the oil displacement area of some selected bacteria. The result of the oil displacement area showed that *Pseudomonas* d had the highest oil displacement activity (Table 1). *Pseudomonas* d had oil displacement area of 2.27 cm^2. This implies that surfactants produced by this organism can be used in the remediation of oil contaminated soil. It was followed by *Pseudomonas* a and *Pseudomonas* c which had ODA of 1.77 cm^2. *Pseudomonas* b had the least ODA (0.07cm^2). Table 2 shows the emulsification index (E_{24}) of the selected bacterial isolates in fuel, kerosene, vegetable oil and paraffin oil.

Figure-2. Graph of oil displacement area (cm^2) of the selected bacterial isolates.

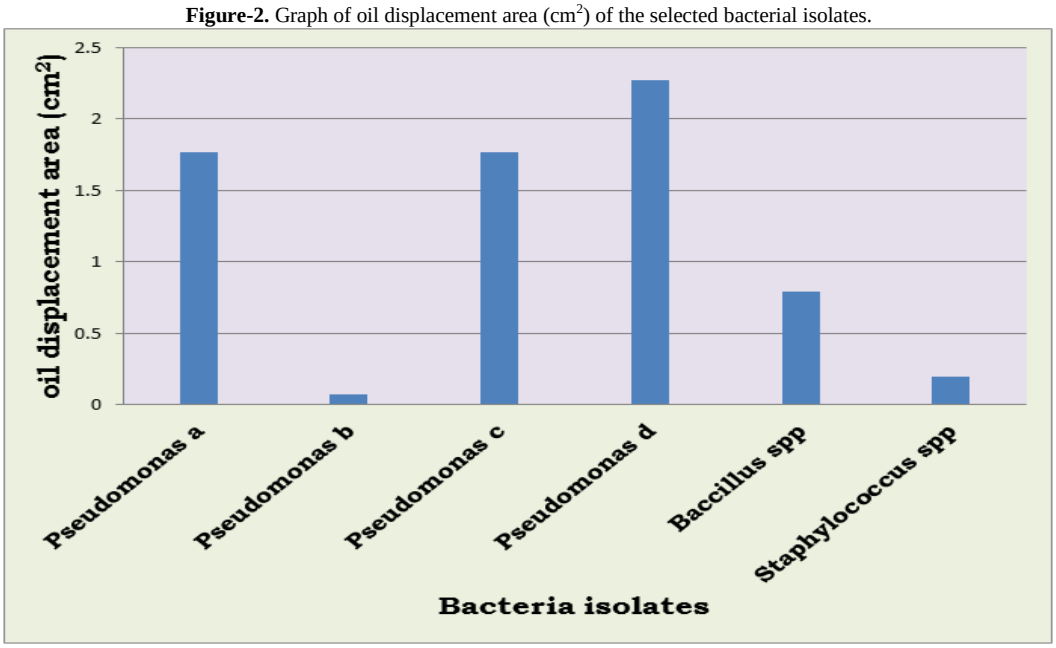

Table-2. Emulsification index (E_{24}) of the selected bacterial isolates in fuel, kerosene, vegetable oil and paraffin oil expressed in percentage.

Bacteria	EI (E_{24}) Fuel (%)	EI (E_{24}) Kerosene (%)	EI (E_{24}) Vegetable Oil (%)	EI (E_{24}) Paraffin oil (%)
Pseudomonas a	50.00	47.50	50.00	71.43
Pseudomonas b	60.00	43.90	50.00	50.00
Pseudomonas c	32.50	42.86	40.00	37.50
Pseudomonas d	32.50	47.50	42.86	33.33
Bacillus sp	28.57	42.87	50.00	32.50
Staphylococcus spp	31.58	30.00	30.00	50.00

Key: EI = E = Emulsification Index

The result showed that out of the six (6) bacterial isolates, *Pseudomonas* b showed the highest biosurfactant activity in fuel with emulsification index of 60.00 %. This shows that *Pseudomonas* b will be the most effective in degrading fuel when compared to other bacterial isolates in this study. This was followed by *Pseudomonas* a (50.00 %) and *Bacillus* sp. (28.7%) which showed the least emulsification index. *Pseudomonas* a and *Pseudomonas* d showed the highest biosurfactant activity in kerosene. They both had emulsification index of 47.50 %. This shows that *Pseudomonas* a and *Pseudomonas* d will be most effective in degrading kerosene when compared to other bacterial isolates in this study. *Pseudomonas* a, b, and *Bacillus* sp showed the highest biosurfactant activity in vegetable oil. They had emulsification index of 50.00 %. This shows that *Pseudomonas* a, d, and *Bacillus* sp are the most effective in degrading vegetable oil when compared to other bacteria isolates in this study. The use of vegetable oil and their wastes as source of biosurfactants is promising but required more efforts and research to full realization; and their study compares to those obtainable in this study [29, 30]. *Pseudomonas* a showed the highest biosurfactant activity in paraffin oil. It had emulsification index of 71.43 %. It was followed by *Pseudomonas* b (50.00%) and *Staphylococcus* spp (50.00%) and least by *Bacillus* sp (32.00 %). In all, *Pseudomonas* showed the highest emulsification index, just as also reported by Monterio, *et al.* [31]. It therefore implies that *Pseudomonas* is a good candidate in the bioremediation of oil contaminated sites. Blood haemolysis carried out on the isolates showed that *Pseudomonas* b, d and *Staphylococcus* were haemolytic.

Table-3. Blood Haemolysis test

Isolates	Haemolysis
Pseudomonas a	-
Pseudomonas b	+
Pseudomonas c	-
Pseudomonas d	+
Bacillus sp	-
Staphylococcus spp	+

Blood haemolysis is also useful in testing for the presence of biosurfactants as it has been previously reported that biosurfactants can cause the lysis of erythrocytes [32]. From our study, we discovered that not all the isolates were haemolytic. Jain, *et al.* [33] reported that the method has some limitations because it is not specific as lytic enzymes can also lead to clearing zones. Schulz, *et al.* [34] showed that some biosurfactants do not show haemolytic activity at all. Youssef, *et al.* [35] and Plaza, *et al.* [27] also reported the poor specificity of this method. Mulligan, *et al.* [32] recommend the blood agar method as a preliminary screening method which should be supported by other techniques based on surface activity measurement.

Table-4. Dry weight of the biosurfactant produced

Isolates	Weight of Biosurfactants (g)
*Pseudomonas*a	18.8
Pseudomonas b	18.9
Pseudomonas c	18.5
Pseudomonas d	18.6
Staphylococcus	15.2
Bacillus	18.3

The biosurfactant produced were quantitatively small but this could be as a result of the fact that the research was carried out in a laboratory scale, hence, if exploited on a large scale; higher volumes of biosurfactant will be produced. Production of lower volumes of biosurfactants may also be as a result of certain factors such as temperature effect, pH effect, salinity content, the presence of antibiotics and metal ions as previously reported [36]. Conclusively, biosurfactants have proven to be promising agents for bioremediation of hydrocarbon contaminated sites. Thus, microbial organisms should therefore be screened for potential source of these biosurfactants – owing to

their immense economic, environmental and industrial importance. Hydrocarbon contaminated soil can be a good enrichment site for the selection of hydrocarbon-degrading and/or biosurfactant producing bacterial strains. Using these organisms, biosurfactants can be produced in large quantity and exploited commercially for application in bioremediation, production of degradable detergents and even in oil recovery processes.

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