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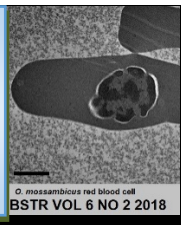

Biosurfactant Production by Bacteria Isolated from Hydrocarbon-impacted soil

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ABSTRACT

Advancement in biotechnology and its industrial application has led to increased demand for biological compounds of microbial origin. Such compounds are biosurfactant and their application in different sectors is increasing despite some challenges. In the present study, the ability of bacterial species growing in hydrocarbon impacted soil to produce biosurfactants was investigated with a view to isolate competent biosurfactant producers with desirable qualities for a large scale biosurfactant production. Soil samples contaminated with different kinds of hydrocarbon were collected from Mechanic workshop (MW) and Mai-kose (MK) area in Maiduguri Metropolis, Nigeria. Soil samples were cultured on nutrient agar and mineral salt agar by pour plate technique for enumeration of viable bacteria and isolation of oil utilising bacteria respectively. Samples from MW were observed to harbour fewer bacteria than MK area with 7.3×10^7 CFU/g and 4.0×10^8 CFU/g respectively. Fifteen bacterial species were isolated and identified to belong to the genus *Pseudomonas* using cultural, morphological and biochemical characteristics. The species were *P. aeruginosa, P. citronellolis* and *P. mendocina* with 66.7%, 26.6% and 6.6% rate of occurrence. A preliminary investigation for the isolates' ability to produce biosurfactants using haemolysis and hydrocarbon degradation tests revealed that all the isolates were haemolytic on blood agar and can utilise vegetable oil at different magnitudes. Biosurfactant assay using broth culture supernatants showed that all the isolates were able to produce surfactants that collapsed oil drop within the interval of 10-20 min and *Pseudomonas citronellolis* WB4 was most efficient in this respect (t = 10 min). With regards to oil spreading assay, *P. aeruginosa* KB1 produced largest diameter (d) of 4.21 cm after 15 min compared to other species ($d_{av.}$ = 3.44cm average $t_{av.}$ = 13 min). After 24 hrs of emulsion formation, it was observed that *P. aeruginosa* WB5 (E_{24} = 70.45%) and *P. citronellolis* WB4 (E_{24} = 70.35%) have the highest emulsification indexes (range 70.45% - 43.75%). Therefore, all isolates in this study were biosurfactant producers with *P. citronellolis* WB4 being the most suitable candidate for large-scale biosurfactant production. Hence, further studies are recommended to harness its full potentials.

INTRODUCTION

Surfactants are amphipathic molecules that act preferentially at the interface between fluid phases including oil-water or air-water interfaces [1]. They are surface active agents with a wide range of properties including lowering surface and interfacial tensions of liquid at interfaces. Surfactants, a short form for "surface-active-agents", are basically chemical compounds that reduce the surface tension of a liquid, interfacial tension between two liquids, or that between a liquid and a solid [2]. Biosurfactants, on the other hand, are microbially produced surface-active agents with properties similar to those of chemical surfactants. They aggregate at interfaces between fluids with different polarities such as water and hydrocarbons and reduce interfacial surface tension [3]. Biosurfactants are found as extracellular compounds or localized on the cell surface of microorganisms, where the microbial cell itself is a biosurfactant [4]. They primarily comprised glycolipids (sophorose lipid, rhamnose lipid), hydroxylated and cross-linked fatty acids, polysaccharides lipid complexes, lipoprotein-lipopeptides and phospholipids [5]. Biosurfactants are believed to be used by microorganisms for transportation across the membrane, protection against other organisms and in host-microbe interaction. In addition, they have the properties of wetting,

penetrating actions, spreading, the hydrophobicity of surfaces, metal sequestration, microbial growth enhancement, and anti-microbial activity [5]. They are specific in activity, and the specificity depends on the structure and functional group of a particular molecular. Currently, biosurfactants have a wide range of applications in different industries. They are used as emulsifying agents for drugs transport to infection sites, supplementary agents to pulmonary surfactant and adjuvants for vaccines [6].

Moreover, their potential applications in bioremediation, oil recovery, food emulsification and cosmetics have also been reported [7]. The petrochemical industry is believed to be the major beneficiary of biosurfactants as it is found applicable in petroleum production, incorporation into oil formulations, oil spill bioremediation, removal of oil sludge from storage tanks and enhanced oil recovery [1]. The wide range of application of biosurfactants has recently made studies in this area to expand rapidly. Biosurfactants have many advantages over chemical surfactants that include biodegradable, diversity, low toxicity, biocompatible, digestible and effective even in extreme conditions such as high temperature, pH and salinity [1,8]. The most significant advantage of biosurfactants over chemical surfactants is their ecological acceptance since they are biodegradable and nontoxic to natural environments [9].

A number of microorganisms have been known to produce biosurfactants using different substrates. Majority of known biosurfactants are synthesised by microorganisms grown on water immiscible hydrocarbons, but some are produced on water-soluble substrates as glucose, glycerol and ethanol [10]. Agricultural raw materials and wastes such as cassava, sweet potato, sweet sorghum, sugar beets, sugarcane bagasse, wheat and rice bran are important substrates for biosurfactant production. Some bacterial species belonging to genera such as *Serratia, Arthrobacter, Nocardia, Lactobacillus, Rhodococcus, Thiobacillus* and fungal species like *Candida, Torulopsis, Debaryomyces* and *Trichosporonasahii* have been reported as biosurfactant producers [11]. Members of the genera *Bacillus* and *Pseudomonas* are often predominant.

In spite of many laboratory-based successes in biosurfactant production and its immense commercial application, its production in commercial scale remains a challenging issue as the composition of the final product is affected by the nutrient, micronutrient and environmental factors, in addition to competent microbial cells capable of producing large-scale surfactants. It is against this that the search for efficient microbial species is continuous. Since it is expected that in the future, super active microbial strains will be developed using genetic engineering for producing biosurfactants at industrial level using renewable raw materials, there is also a need for the continuous search of competent microorganisms with desirable qualities for large-scale and effective biosurfactant production. This study aims to examine the biosurfactant production by bacteria isolated from hydrocarbon impacted soil.

MATERIALS AND METHODS

The study area, sampling sites and sample collection

This study was conducted in Maiduguri, Borno State. The city is located at the Northeastern part of Nigeria that lies within latitude 11.15 ºN and longitude 30.05 ºE in the Sudano-Sahelian savanna zone. It is known for its dryness with a light annual rainfall of above 300 to 500 mm and the average daily temperature ranging from 22 to 35ºC with a mean of daily maximum temperature exceeding 40 ºC between March and June. It mainly has sandy loam soils [12]. The soil sampling sites are a mechanical workshop and Mai-Kosai (a local fried bean paste staple) areas where different hydrocarbons are used. Soil samples (5 g) were collected from each Mechanic Workshop (MW) and Mai-Kose (MK) areas at the depths of 3 - 5 cm using sterilised Auger. The samples were packaged in sterile polythene bags and transported to Microbiology Laboratory, Faculty of Science, the University of Maiduguri at a controlled temperature.

Enumeration, isolation and identification of bacteria

Soil samples were serially diluted and inoculated using pour plate techniques. Aliquots (1ml) of six-fold diluted soil samples were inoculated in to petri dish onto which nutrient agar (NA; Oxoid) and mineral salt agar (MSA; composed of (per litre at pH 7.4): 1.2 g KH_2PO_4, 1.8 g K_2HPO_4 4.0 g NH_4Cl, 0.2 g $MgSO_4.7H_2O$, 0.1 g NaCl, 0.01 g $FeSO_4.7H_2O$, 20 g agar and 0.01% v/v vegetable oil) were added for enumeration and isolation of total viable and hydrocarbon utilising bacteria, respectively. Colonies growing on NA were counted after 24 hrs incubation and expressed as CFU/g of soil. Cultures on MSA were incubated for 72 hrs at 35ºC. Colonies emerged were sub cultured on NA to obtain pure cultures and subsequently transferred into NA slants. The slants were kept in a refrigerator at 9ºC as a stock culture. A pure culture of isolates was characterised and identified based on their cultural, morphological and biochemical characteristics in accordance with determinative schemes of [13, 14].

Screening of isolates for biosurfactant production

All the pure isolates were subjected to screening for their ability to produce biosurfactants using oil utilisation test and blood haemolysis test as described by [4, 15].

i. Oil utilisation test

The bacterial isolates from an overnight culture were transferred to 100 mL sterile mineral salt broth (MSB) with 0.1% (v/v) of vegetable oil as carbon and energy source. The cultures were grown at 30°C for seven days with shaking. The broth cultures were observed for turbidity.

ii. Blood haemolysis test

Isolates were grown on blood agar plates containing 5% (v/v) sheep blood and incubated at room temperature for 24 hrs. Haemolytic activity was detected as the occurrence of define clear zone around a colony.

Biosurfactant activity assay

Three methods were used to assay the biosurfactants produced by the isolates, which are the emulsification index, oil spreading, and drop collapse methods as described by [15]. Isolates were grown in MSB containing 0.5% (v/v) vegetable oil as substrate. The culture was incubated for seven days at 30°C with regular shaking. After the incubation period, the broth of each isolate was centrifuged at 6000 rpm for 10 min, and the supernatants were filtered to obtain cell-free supernatants. The supernatants were used for the assay.

i. Drop collapse test

A drop of crude oil was placed on a grease free slide, and one drop of the cell-free supernatant was placed at the centre of the oil drop. The shape of drop on the oil surface was observed for flattening. The collapse of the drop was due to the reduction of interfacial tension between the liquid drops, and the hydrophobic surface of the oil. Additionally, the time required by the oil drop to collapse was recorded.

ii Oil spreading method

Oil spreading technique was carried out according to the method described by [16]. 40 mL of distilled water was added to Petri dishes followed by the addition of 100 µL of vegetable oil on the surface of the water. Then, one drop of the supernatant was put on the vegetable oil surface. The diameter of the clear zone on oil surface was measured using a meter rule. The time taken to achieve the spread was also noted.

iii Emulsification capacity test

Emulsification activity of the biosurfactants was carried out. Four ml (4m) of vegetable oil was added to an equal amount of cell-free supernatant and Vortexed at 500 r.p.m for 10 min. After 24 hours, the height of the stable emulsion layer was measured using a meter rule. The emulsification index (E_{24}) was calculated as the ratio of the height of the emulsion layer and the total height of the liquid. As given by the expression:

$$E_{24} = \frac{h\ emulsion}{h\ total} \times 100$$

where: E_{24} is emulsion index after 24 hours, *h* emulsion is the height of the emulsion layer, *h* total is the total height of the liquid.

RESULTS AND DISCUSSION

The study of biosurfactant production by bacteria isolated from hydrocarbon contaminated soil was employed in samples collected from Maiduguri metropolis. Bacterial counts revealed that sample from the MK area had more viable bacteria with 4.0×10^8 CFU/g compared to MW which had 7.3×10^7CFU/g. The difference in bacterial count could be a function of environmental adaptation and the oil substrate whereby MK area is mainly contaminated with oil containing fatty acids that are readily assimilated compared to petroleum hydrocarbons, which can be toxic (e.g. PAHs) to some bacterial species. For an effective biodegradation process, most aerobic microbes convert hydrocarbon molecules to fatty acid and eventually oxidise the product through beta oxidation [17]. In general, population levels of hydrocarbon utilisers and their proportion within the microbial community appear to be sensitive to environmental exposure to hydrocarbons, their composition and inherent biodegradability.

Fifteen morphologically distinct bacterial colonies were isolated and identified. The isolates were observed to be gram-negative rods, non-spore forming; negative for indole, methyl red, Voges-Proskauer, ureases and unable to ferment sugars in triple sugar iron medium but showed positive results for catalase, oxidase, citrate and nitrate reduction. Based on cultural, morphological and biochemical characteristics, all the isolates were identified to be the members of the genus *Pseudomonas* (Table 1). *Pseudomonas* spp. are common soil inhabitants, and their occurrence in contaminated soil have been severally and recurrently reported. This might be attributed to their ability to tolerate high concentrations of hydrocarbons with a high capability for their degradation [18]. *Pseudomonas aeruginosa* was the most prevalent isolate in the soil samples with occurrence percentage of 66.7% followed by *P. citronellolis* (26.6%) and *P. mendocina* (6.6%). Results for the utilisation of hydrocarbon showed that *P. aeruginosa* KA3, *P. aeruginosa* KA4, *P. aeruginosa* WA2 and *P. citronellolis* WB4 (making about 50% of the isolates) used the vegetable oil luxuriantly. Only two species, which are *P. aeruginosa* KA1 and *P. aeruginosa* KB1, used the vegetable oil poorly as shown in Table 2. The ability of these organisms to effectively utilise the vegetable oil was due to the capacity of the organisms to use hydrocarbons as a major or sole energy and carbon source. Studies by [19, 20] have demonstrated the biodegrading ability of these organisms even with complex hydrocarbons like diesel and anthracene.

Table 1. Characteristics of identified isolates.

Isolate	Occurrence (%)	Cultural properties		
		Size	Pigment	Edges
P. aeruginosa	10 (66.7)	Large	Greenish	Rough
P. citronellosis	4 (26.6)	Large	Milky	Smooth/round
P. mendocina	1 (6.6)	Large	Yellow-green	Rough

Table 2. Rate of hydrocarbon utilization by the isolates.

Isolate	Turbidity
P. aeruginosa KA1	+
P. aeruginosa KA2	++
P. aeruginosa KA3	+++
P. aeruginosa KA4	+++
P. aeruginosa KB1	+
P. aeruginosa KB2	++
P. aeruginosa KB3	++
P. aeruginosa WA1	++
P. aeruginosa WA2	+++
P. citronellosis WA3	+++
P. mendocina WB1	++
P. citronellosis WB2	+++
P. aeruginosa WB3	+++
P. citronellosis WB4	+++
P. aeruginosa WB5	++

Key: + poorly, ++ moderately, +++ luxuriantly

All the 15 bacterial isolates were screened for biosurfactant production with the result shown in Table 3. All the isolates were shown to be haemolytic on blood agar. Haemolytic activity has been regarded by some authors as an indication of biosurfactants production [4]. Although not 100% reliable due to the presence of other metabolic products that may cause haemolysis like virulence factors, there is a strong correlation between surfactant production and haemolysis as demonstrated by this study.

It was observed that the supernatant of all isolates was positive for drop collapse and oil spreading tests. All the surfactants produced by the *Pseudomonas* species collapsed the oil drop between 10-20 min (Table 3). *P. citronellolis* WB4 was able to rapidly collapse the oil drop within 10 min, in contrast, *P. aeruginosa* KA4 was observed to cause the collapse of oil drop after 20 min. A study by [19] observed that medium containing a strain of *P. citronellolis* showed the highest reduction in surface tension compared to that of *P. aeruginosa* while growing in medium containing anthracene thus supporting the findings of this present study. In oil spreading assay, *P. aeruginosa* KB1 produced the largest zone of 4.21 cm and followed by *P. aeruginosa* KB1 with 3.85 cm during 15 min period. In contrast, *P. aeruginosa* KB3 and *P. aeruginosa* KA3 produced 2.84 cm and 2.43 cm after 10 and 15 min, respectively. For emulsification and oil spreading assays, however, *P. aeruginosa* WB5 (E_{24} = 70.45%) and *P. citronellolis* WB4 (E_{24} = 70.35%) were observed to produce better emulsification than other species after 24 hrs, respectively.

The least among the organisms was *P. aeruginosa* WA2 with 43.75% emulsion. The high E_{24} indexes observed in this study are indicative of nature of surfactants produced as high molecular surfactants are believed to cause better emulsion than low molecular compounds [19]. High E_{24} index of *P. citronellolis* WB4 was quite interesting to note considering that previous studies [20, 21] have reported E_{24} rates of similar species in relatively close range with the findings of this study even with different substrates. Despite the organisms being in the same genus, there were little differences in their emulsification indexes

probably due to genetic variations. Some studies that used organisms of the same genera have also shown similar differences [15, 22, 23]. *P. mendocina* WB1 was the only species of its kind in this study, to collapse oil drop and spread oil moderately during 11 and 15 min period, respectively. The E_{24} value was 52.41% and the diameter (3.60 cm) produced while spreading the oil was above average. Previous studies by [24, 25] have reported the biodegradation and biosurfactant production potential of *P. mendocina.* Many hydrocarbon utilising organisms possess emulsifying activities due to the release of surface-active compounds. It is widely believed that for effective biodegradation of hydrocarbons, production of biosurfactants by microbes is essential because low solubility and high hydrophobicity of many hydrocarbon compounds can make them unavailable to microorganisms. Biosurfactant is known to reduce surface tension, interfacial tension between the bacterial cell wall and hydrocarbon molecules, modify bacterial membrane and enhance the dispersion of hydrocarbons [22]. Therefore, biosurfactant production helps the hydrocarbon-degrading bacteria to gain better access to their hydrophobic substrates.

Table 3. Properties of the biosurfactant produced.

Isolate	Hemolysis	E_{24} (%)	Drop collapse		Oil Spreading		
			Result	Time (min)	Result	Diameter (cm)	Time (min)
P. aeruginosa KA1	+	60.71	+	15	+	3.70	15
P. aeruginosa KA2	+	52.63	++	17	++	3.82	15
P. aeruginosa KA3	+	57.89	+++	17	+++	2.43	15
P. aeruginosa KA4	+	52.63	+++	20	+++	3.60	10
P. aeruginosa KB1	+	54.54	+	10	+++	4.21	15
P. aeruginosa KB2	+	51.78	++	12	++	3.43	10
P. aeruginosa KB3	+	52.63	++	15	++	2.84	10
P. aeruginosa WA1	+	55.44	++	15	++	3.43	13
P. aeruginosa WA2	+	43.75	+++	15	+++	3.62	11
P. citronellolis WA3	+	54.32	+++	17	+++	2.91	11
P. mendocina WB1	+	54.21	++	11	++	3.60	15
P. citronellolis WB2	+	52.63	+++	13	+++	3.85	15
P. aeruginosa WB3	+	52.63	+++	15	+++	3.40	15
P. citronellolis WB4	+	70.35	+++	10	+++	3.41	15
P. aeruginosa WB5	+	70.45	++	15	++	3.46	10

CONCLUSION

This study has revealed the ability of hydrocarbon contaminated soil in harbouring a large number of bacterial cells and utilising the contaminants as carbon and energy source. Fifteen bacterial isolates belonging to the genus *Pseudomonas* have demonstrated their efficiency to utilise vegetable oil and produce biosurfactants. All the isolates were biosurfactant producers and the compounds produced were shown to exhibit all the desirable qualities of biosurfactants. Surfactants produced by *P. aeruginosa* WB5 and *P. citronellolis* were the most effective in emulsion formation while those produced by the latter could be more desirable considering their oil spreading activity. Therefore, characterisation and optimisation of *P. citronellolis* surfactants are essential for potential application in large-scale biosurfactant production.

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