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ASSESSMENT OF BIOSURFACTANT PRODUCTION POTENTIAL OF HYDROCARBON UTILIZING BACTERIAL ISOLATES IN ALEX EKWUEME FEDERAL UNIVERSITY ENVIRONMENT

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ABSTRACT

The biosurfactant production potentials of hydrocarbon utilizing bacterial species isolated from petroleum hydrocarbon polluted environments within the University community were analyzed. Seven isolates were obtained from the oil polluted sites from four soil samples. These were assayed for biosurfactant production. The isolates were *Micrococcus* sp., (A), *Pseudomonas* sp. (B), *Bacillus* sp. (C), *Pseudomonas* sp. (D), *Klebsiella* sp. (E), *Pseudomonas* sp. (F) and *Bacillus* sp. (G). All the isolates were screened for haemolysis, isolates A, C and F were alpha haemolytic while B, D, E and G were beta haemolytic. The least hydrocarbon utilizers counts was that of Administrative zone area ($1.2x10^5$), the least percentage heterotrophs were those from the Research Lab area (3.2%) while the Auditorium temporary site area had the least total heterotrophic counts of $1.10x10^7$cfu/ml. The biosurfactant production using drop collapse assay and oil displacement test showed that B and D were positive for drop collapse while isolates B, C, D, E were positive for oil displacement test. Emulsification tests of isolates B, C, D, E on diesel and kerosene were 21,84,13,17 and 5,6,6,7 respectively. From the test result obtained, the isolates produced biosurfactants to varying degree, with the highest production from *Pseudomonas* sp. (B), *Pseudomonas* sp. (D) and *Klebsiella* sp. (E). These isolates can be harnessed in the cleanup of petroleum hydrocarbon polluted media.

INTRODUCTION

The development of petroleum industries has been contributing to an increase in the amount of poly aromatic hydrocarbons (PAHs) released into the environment. PAHs are toxic, carcinogenic, or teratogenic. A variety of microorganisms can degrade certain poly aromatic hydrocarbons completely to CO_2 and metabolic intermediates, enroute gaining energy and carbon for cell growth. Biosurfactants are amphiphilic compounds produced on living surfaces, mostly on

microbial cell surfaces, or excreted extracellularly and contain hydrophobic and hydrophilic moieties that confer the ability to accumulate between fluid phases, thus reducing surface and interfacial tension at the surface and interface respectively. Biosurfactants possess the characteristic property of reducing the surface and interfacial tension using the same mechanisms as chemical surfactants. Unlike chemical surfactants, which are mostly derived from petroleum feedstock, these molecules can be produced by microbial fermentation processes using cheaper agro-based substrates and waste materials. Biosurfactants are surfactants produced by microorganisms, either directly in microbial cell surfaces or by extracellular secretion. The advantages of biosurfactants include their biodegradability, their reduced toxicity and the possibility to be produced by renewable raw material (Mulligan and Wang, 2006). The main biosurfactant market is the petroleum industry, where they have been incorporated into oil formulations and utilized in petroleum production (enhanced oil recovery) and bioremediation (Pacheco *et al., 2010*). Biosurfactants have received considerable attention in the field of environmental remediation processes because of their efficacy as dispersion and remediation agents and their environmental friendly characteristics such as low toxicity and high biodegradability (Kiran *et al*., 2010). Due to their unique properties and vast array of applications, identification of new biosurfactant producing microbes is in great demand. Biosurfactants can be produced by microbial fermentation processes using cheaper agrobased substrates and waste materials. Therefore, this study is aimed at isolating, characterizing and screening of hydrocarbon utilizing bacterial species from petroleum hydrocarbon polluted sites for biosurfactant production.

MATERIALS AND METHODS

Sampling area: The soil samples for the study were collected from hydrocarbon polluted soils from the surroundings of the University generator houses and other environment of Federal University Ndufu Alike Ikwo,Ebonyi State. Soil samples from these sites were all polluted to various levels by different hydrocarbon oils such as diesel and engine oil.

Sample collection: Soil samples were collected from the soil surfaces spilled with oil with the aid of a sterile spatula into sterile sample containers. The samples were properly labeled and taken to the Laboratory in an ice packed container within one hour for analysis.

Enumeration of total heterotrophic bacteria and hydrocarbon utilizing bacteria: The standard plate count technique was used for the total aerobic heterotrophic bacterial populations in the soil. Ten fold serial dilutions of soil samples were prepared and 0.1 ml of dilutions 10^{-7} to 10^{-8} were plated on nutrient agar plates, using the pour plate method. The plates were incubated at 28±2°C for 24 h and recorded as CFU/g of soil. For hydrocarbon utilizing bacteria (HUB) count, aliquot (0.1 ml) of 10^{-3} to 10^{-5} dilutions of the soil sample suspensions were plated on the mineral salts medium using a sterile glass rod. Whatman No. 1 sterile filter paper saturated with diesel were aseptically placed onto the covers of inoculated inverted plates and incubated for 5 to 7 days at 28±2°C. Colonies were recorded as CFU/g of soil.

Isolation and Identification of Hydrocarbon Utilizing Bacteria: Colonies of hydrocarbon utilizing species were selected based on their colony characteristics and colour in different plates was selected. Sugar fermentation tests and other biochemical tests were used for identification according to Cheesbrough, 2006.

Screening of isolates for petroleum hydrocarbon utilization: Bacterial isolates were screened for utilization of diesel, kerosene and spent engine oil. A 24 h culture suspension of each isolate was standardized and inoculated into the sterilized mineral salts broth (Okpokwasili and Amanchukwu, 1988) supplemented with 1.0% carbon source (diesel, kerosene and engine oil) and incubated at 28±2°C for 7 days with a mechanical shaking at 150 rpm. Absorbance of the culture was measured with spectrophotometer and the optical density (OD) was recorded for the various treatments. Isolates producing intense turbidity with visible disappearance of oil were selected and stored for further use.

Biosurfactant production screening assay: Culture broths of 7 bacterial isolates that showed intense turbidity and visible disappearance of hydrocarbons were tested for the presence of biosurfactant using the drop collapse method, oil spreading technique, and emulsification activity. Haemolytic activity of the isolates was also assayed.

Drop collapse test:

Aliquots of the culture isolates obtained from shake flasks grown for 5 days on mineral salts medium supplement with 1% diesel oil were centrifuged at 10,000 x g for 20 min. Supernatants obtained were used in drop-collapse test as described by Jain *et al.,* (1991). Drops of the supernatant from each of the isolates were placed on a clean glass slide previously coated with diesel. A rapid collapse of the drops indicates presence of biosurfactant while, non-biosurfactant containing drops form balls and does not collapse.

Oil spreading technique:

Thirty (30 ml) of distilled water was added to a 100 mm petri dish and 10 µl of oil was added to the surface of water to form a film. 10 µl of supernatant from the culture broth of the isolates were dropped on top of the surface of the film. Dispersion or non dispersion of oil was observed and recorded as positive or negative. Drop of distilled water on the surface of the film was used as a negative control (Morikawa *et al*., 2000)

Haemolytic activity:

Isolates were screened on nutrient agar plates containing 5% (v/v) human blood and incubated at 28±2°C for 24 h. β-haemolytic activity was detected by clear zones around colonies (Carrillo *et al*., 1996).

Emulsification activity assay: The supernatant of isolates positive for the drop collapse assay were obtained and assayed for emulsification activity. The clear supernatant served as source of crude biosurfactant. Emulsification activities of the biosurfactants were determined by the addition of oil (diesel oil, kerosene or spent engine oil) to 5ml of biosurfactant in a graduated screw-cap test tubes. The tubes were vortexed for 2 min and left to stand for 24 h. The emulsification index (E_{24}) was calculated thus: (E_{24}) % = (Height of the emulsion layer/Total height of mixture) × 100

RESULTS

Results of the total heterotrophic bacterial count, hydrocarbon utilizing bacterial count and percentage heterotrophes that are hydrocarbon utilizing from the various polluted soils in the campus as shown in Table 1. The highest total heterotrophic count and hydrocarbon utilizing bacterial count, (6.20×10^7 cfu/ml and 1.0×10^6cfu/ml) were observed from soil samples at Research Lab area and beside Medical centre area, FUNAI. The least total heterotrophic counts were observed in the soil sample from Auditorium temp site area , while the least hydrocarbon utilizing bacterial counts were observed in the soil samples from Administrative zone area. The highest percentage heterotrophs that are hydrocarbon utilizing (7. 0%) was observed from the soil sample from Administrative zone area of FUNAI, while the least was observed in soil sample from Research Lab area with 3.2%. The probable identities of isolated hydrocarbon utilizers screened for biosurfactant production were shown in table 2. The result showed that a total of twelve microbial species were isolated. Three species of *Pseudomonas,* two species of *Bacillus,*one specie of *Micrococcus* and *one* specie of *Klebsiella*. From the utilization of the various carbon sources (diesel) by the isolates in mineral salts broth, diesel was the best hydrocarbon substrates for the isolates (table 3). The isolates *Pseudomonas* sp., B and *Pseudomonas* sp., D produced high turbidity (+++) with visible disappearance of hydrocarbons in broth table 3. Emulsification activities of isolates on diesel and kerosene were shown in table 6.The emulsification activities of the isolates was best on diesel followed by kerosene. *Bacillus* sp. C showed the best emulsification on the diesel while the *Klebsiella* sp. E showed the best emulsification on the kerosene sample. The least emulsification index of diesel and kerosene were observed in *Pseudomonas* sp. B and *Pseudomonas* sp. D.

Table 1: Total heterotrophic and hydrocarbon utilizer's counts

Samples Location	Total heterotrophic counts	Hydrocarbon utilizer's counts	Percentage heterotrophs that are hydrocarbon utilizers (%)
(1) Auditorium temp site area	1.10×10^7	Growth absent	0.0
(2) Research Lab area	6.20×10^7	1.8×10^5	3.2
(3) Beside medical centre area	1.82×10^7	1.0×10^6	6.0
(4) Administrative zone area	2.00×10^7	1.2×10^5	7.0

Table 2: Gram reactions, biochemical reactions and probable identities of the screened of bacterial isolates

Sample Code	Gram Stain	Microscope	Indole test	Citrate	Glu	LAC	Slant	But	GAS	Acid	Motility	MR	VP	Oxide	Catalase	Probable Organisms
A	+	Cocci	-	+	+	-	B	A	-	-	-	-	+	+	+	*Micrococcus* spp.
B	-	Rod	-	+	-	-	B	B	-	-	+	+	-	-	+	*Pseudomonas* spp
C	+	Rod	-	+	-	-	B	A	-	-	+	-	+	-	+	*Bacillus* spp
D	-	Long Rod	-	+	-	-	B	B	-	-	+	+	-	+	+	*Pseudomonas* spp.
E	-	Rod	-	+	+	-	A	A	-	-	-	+	-	+	+	*Klebsiella* spp.
F	-	Rod	-	+	-+	-	B	B	+	-	-	-	+	+	+	*Pseudomonas* spp.
G	+	Straight Rod	-	+	+	+	B	A	-	-	+	-	+	+	+	*Bacillus* spp.

Key: Gm –, Gram negative; Gm +, Gram positive; +, Present; - , Absent.

Table 3: Diesel utilization by the different isolated HC utilizer

Isolates	Diesel
Micrococcus spp. A	+
Pseudomonas spp. B	+++
Bacillus spp. C	++
Pseudomonas spp. D	+++
Klebsiella spp. E	+++
Pseudomonas spp. F	++
Bacillus spp. G	++

Key: - No Turbidity, + Low turbidity, ++ Moderate Turbidity, and +++ High Turbidity.

Table 4 : The growth (OD at 420 nm) of diesel hydrocarbon with time

Isolates	Day 1	Day 2	Day 3	Day 4
Micrococcus spp. A	0.094	0.293	0.387	0.363
Pseudomonas spp. B	0.243	0.514	0.545	0.475
Bacillus spp. C	0.238	0.481	0.509	0.464
Pseudomonas spp. D	0.206	0.518	0.527	0.450
Klebsiella spp. E	0.252	0.492	0.504	0.466
Pseudomonas spp. F	0.218	0.498	0.480	0.449
Bacillus spp. G	0.248	0.478	0.487	0.469

Key: OD - Optical density

Table 4: Haemolytic activity of the different Isolates

Isolates	Haemolytic activity
Micrococcus spp, (A)	Alpha (α) Haemolysis
Pseudomonas spp. (B)	Beta (β) Haemolysis
Bacillus spp. (C)	Alpha (α) Haemolysis
Pseudomonas spp. (D)	Beta (β) Haemolysis
Klebsiella spp. (E)	Beta (β) Haemolysis
Pseudomonas spp. (F)	Alpha (α) Haemolysis
Bacillus spp. (G)	Beta (β) Haemolysis

Four out of seven isolates were beta (β) haemolytic while the rest were alpha haemolytic.

Table 5: Response of the isolates to drop collapse and Oil displacement tests

ISOLATES	DROP COLLAPSE	OIL DISPLACEMENT
Pseudomonas spp. (B)	+	+
Bacillus spp. (C)	-	+
Pseudomonas spp. (D)	+	+
Klebsiella spp. (E)	-	+

Key: + slow displacement /drop collapse, ++ vigorous/rapid displacement/drop collapse

Drops of supernatant from *Pseudomonas* sp.,B and *Pseudomonas* sp.D collapsed oil rapidly while *Pseudomonas* sp. B, *Bacillus* sp. C, *Pseudomonas* sp. D and *Klebsiella* sp. E displaced oil rapidly while *Bacillus* sp. C and *Klebsiella* sp.,E had a negative reaction.

Table 6: Emulsification activity of isolates' crude surfactants on hydrocarbons sources

S/N	ISOLATES	DIESEL	KEROSENE
1	*Pseudomonas* spp. B	21	5
2	*Bacillus* spp. C	84	6
3	*Pseudomonas* spp. D	13	6
4	*Klebsiella* spp. E	17	7

DISCUSSION

The result obtained showed that the sample from the Research lab area in FUNAI had the highest total heterotrophic bacterial count of 6.2 $x10^7$ and then beside the medical centre generator house had the highest hydrocarbon utilizing bacteria count of 1.0 x 10^6. Meanwhile, the Administrative zone area where the generator house was kept in FUNAI had the highest percentage of heterotrophs (7.0%) that are hydrocarbon utilizers. This result indicates that the site maybe actively receiving hydrocarbons. This corroborates the work of Abu and Ogiji (1995) who stated that bacteria with the ability to degrade a wide range of crude oil components exist ubiquitously in the environment and do appear to respond quite rapidly to the presence of petroleum hydrocarbons in the environment. The least hydrocarbon utilizers counts was that of Administrative zone area ($1.2x10^5$), the least percentage heterotrophs were those from the Research Lab area (3.2%) while the Auditorium temporary site area has the least total heterotrophic counts of $1.10x10^7$cfu/ml. The low hydrocarbon utilization observed here maybe as a result of the fact that hydrocarbon contaminants in the site have been reduced over the years as a result of microbial activities and other environmental factors. This corroborates the work of Atlas and Bartha, 1998. The identification of the isolates showed that seven bacterial isolates belonging to four genera were obtained. These were two species of B*acillus*, three species of *Pseudomonas*, one specie of *Micrococcus* and *Klebsiella*. Bacteria are the most active agents in petroleum degradation, and they work as primary

degraders of spilled oil in the environment. The presence of these species corroborates the works of Broajimans *et al*., 2009; Rahman *et al.,* 2002. From the utilization of the various carbon sources (diesel, kerosene and spent engine oil) by the different bacterial isolates in mineral salt broth medium, diesel was the best hydrocarbon source of the isolates 3 isolates used diesel producing high turbidity as against kerosene (tables 3,4). Diesel is said to be an excellent substrate for microbial growth given that diesel consists of long chain aliphatic hydrocarbons, which are more readily utilized hydrocarbon degrading organisms. This is in agreement with the work of Bento and Gaylarde, 1996. The same was observed in the measured optical density of the isolates in growth broth with diesel as the sole carbon sources. The isolates *Pseudomonas* spp (B), *Pseudomonas* spp (D) and *Klebsiella* spp. (E) produced high turbidity with visible disappearance of oil in the broth supplemented with diesel as the carbon source.The haemolytic activity of the isolates showed that four out of seven of the isolates were beta haemolytic while the rest of the remaining four are alpha haemolytic. This corroborates the work of Youssef et al.,2004 and Carrillo *et al*.,1996. The four best isolates which produced Beta (β) haemolytic activity were used for the drop collapse test and 2 out 4 of the isolates scored positive in the drop collapse assay and in the oil displacement assay using diesel where four of the isolates tested positive. This showed that they were biosurfactant producers. Emulsification of diesel, and kerosene by crude biosurfactant from cultures of isolates were shown in table 6. *Bacillus* spp (C) showed the best emulsification activity for diesel and for kerosene, *Klebsiella* spp. showed the best emulsification. Kerosene had low emulsification from the isolates compared to diesel. This could be because it contained components such as Polyaromatic hydrocarbons which were toxic to the isolates used.

CONCLUSION

These biosurfactants can help to effect environmental bioremediation. *Pseudomonas* spp. (B) and *Pseudomonas* spp. (D) grew well in used engine oil as a carbon source. This means that biosurfactants from these isolates identified can be mass produced using cheap sources of substrates. The isolates can equally be used in bioaugmentation for the cleanup of media polluted by diesel.

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