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Biosurfactant Produced by *Bacillus subtilis* and *Pseudomonas aeruginosa* was Efficient to Degrade Hydrocarbons

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ABSTRACT

Biosurfactants are superficial amphipathic molecules that are produced from different organisms. This study investigates how efficient biosurfactants produced by microorganisms can degrade hydrocarbons. Bacteria isolates from three different automobile mechanic soil sites were screened for their ability to produce biosurfactants. Two isolates, *Pseudomonas aeruginosa* and *Bacillus subtilis* were further vetted to ascertain their bio-surfactant capability using hemolysis, emulsification and oil spreading test. *Pseudomonas aeruginosa* exhibited a larger zone of hemolysis (39±0.15 mm) on Petri dishes containing plasma agar than *B. subtilis* having a length of 37±0.21 mm. The oil spreading ability also showed that *P. aeruginosa* exhibited a diameter of oil displacement of 56±0.15 mm while *B. subtilis* exhibited a diameter of 22±0.2 mm. The emulsification capability of the natural surfactant was subjected to scrutiny on certain hydrocarbon products such as kerosene, diesel, engine oil and crude oil. *Pseudomonas aeruginosa* showed emulsification indices of 87±1.53%, 92±1.53%, 84±1.53% and 98±1.53% for kerosene, diesel, engine oil and crude oil respectively while *B. subtilis* showed emulsification indices of 81±1.53%, 86±1.53%, 80±2% and 93±1.53% for kerosene, diesel oil, engine oil and crude oil respectively. *Pseudomonas aeruginosa* exhibited higher surfactant ability than *B. subtilis* and the natural surfactant it yielded was extracted and used to compare with that of synthetic surfactant concerning their level of degradability on fuel. The region of clearance was observed with rhamnolipid showing a distance of 36±1.5 mm and sodium dodecyl sulfate a diameter or distance of 34±1.5 mm. Therefore, rhamnolipid, the natural surfactant synthesized by *P. aeruginosa* was efficient to remediate hydrocarbon-polluted soil environment.

Key words: Biosurfactant, *B. subtilis, P. aeruginosa*, hemolysis, inhibition, rhamnolipid

INTRODUCTION

The word surfactant is coined out of the phrase "superficial agile mediator". Surfactants can be defined as compounds that are capable of reducing the external as well as the thin boundary tightness or stiffness between two fluids or linking a fluid as well as a non-fluid[1]. When the external tightness is decreased, it allows the spreading of water and the degradation of oil droplets and other pollutants[2]. Surfactants are of two types which are the synthetic or chemical surfactants and bio-surfactants. Synthetic surfactants are synthetically produced compounds from petroleum. An example is

sodium dodecyl sulfate which is an organic sodium and it is mainly applied as detergents in industries for cleaning whereas Bio-surfactants or Natural surfactants are inherently or naturally occurring surface-active molecules resulting from a great number of microorganisms[3]. They have exceptional properties such as better ability to biodegrade, less toxic level, more environmentally friendly and great elasticity[4]. However, biosurfactant synthesis on a large industry level or a large scale is still a task and it is more expensive than the artificial surfactant reason being that the source of nourishment required for its growth is expensive[5]. However, this challenge has been overcome by the usage of remains from industries. The prevailing deleterious impacts of hydrocarbons in the soil environment are triggered by numerous factors an instance is the vigorous application of petroleum products in every area of life. The hydrophobic pollutants present in these hydrocarbons end up polluting the soil milieu[6] whose existence is detrimental. To be able to remove pollutants caused by hydrocarbon, established procedures were incorporated which includes chemical, physical and mechanical treatments. Nonetheless, bioremediation which involves usage of oil-degrading organisms happens to be the sole natural way that permits the entire transformation of these contaminants to innocuous amalgams[7]. These degraders utilize petroleum products or hydrocarbons as their sole carbon source as well as their source of energy, transforming them to the total microbial mass, carbonic acid gas as well as several harmless metabolites. A solid restraining issue surrounding the breakdown of these products of petroleum was based on their inability to be absorbed by water, a capable way to overcome this problem is the application of natural surfactants. The capability of decreasing the external as well as the thin boundary tightness or stiffness flanked by dual fluids that cannot mix such as hydrocarbons as well as the liquid is done with these bio-surfactants. In other words, they are exterior agile mediators that gather at the outer edge flanked by two fluids that cannot mix otherwise flanked by a solid and a liquid[8]. The massive commercial and health care capabilities of natural surfactants as well as recent demand in the market due to its inexpensive or cheap cost, low toxicity, high foaming capacity, higher biodegradability, mild process conditions and pleasantness in the milieu has increased its replacement with artificial surfactants and has also fascinated around the sphere researchers to study more on the sequestration as well as the assessment of organisms that are capable of synthesizing natural surfactants. These features which are seen in biosurfactants necessary for different manufacturing practices which are improved recovery of hydrocarbons, sanitization of the milieu using biological agents, handling and managing of diet, pharmaceutical formulations[9], cosmetics, biomedicals, etc. In as much as the synthesis of natural surfactant relies on the kind as well as the capacity of organisms, factors such as nitrogen, degree of cold or heat, carbon, air as well as other macromolecules similarly disturb the production of biosurfactant via the microorganisms. Contaminants that cannot be absorbed by water existing in the products of petroleum, soil and aquatic surroundings need to be soluble to enable them to be broken down by the cells of bacteria. Biosurfactants can increase water solubility by raising the external part of petroleum products that do not have an affinity for water in the soil and aquatic environment. The current study aimed to isolate and compare the biosurfactant producing capacity of the most frequently occurring organisms from different auto-mechanic soil environments in Port Harcourt, Nigeria.

MATERIALS AND METHODS

This study was carried out in Nigeria in 2019 in Port Harcourt Local Government Area of Rivers State, Nigeria.

Sampling sites: Samples of soil were taken from three auto-mechanic workshops in Port Harcourt Local Government Area of Rivers State; Ikoku at Amigbo off Ikwerre road, Mile 3 at Nkpolu Oroworukwo Community Secondary school off Ada George Junction and Elekahia at Elekahia police station off stadium road. The samples of soil were taken at 3 distinct spots from the various auto-mechanic areas at the top soil and subsoil (at a depth of 12 cm), pooled together and placed in a sterile air-tight polythene bag[10]. An unpolluted sample of soil was also collected as control from an unpolluted area in Mile 3. Soil analysis was done to ascertain the soil condition using atomic absorption spectrophotometer[11].

Method: One gram of soil sample from each of the respective auto-mechanic workshops were weighed and diluted serially into test-tubes containing 9 mL of 0.85% purified brine water. The samples of soil were diluted serially to 10^{-6} dilution[12]. The spread plate technique using nutrient agar was applied for the sequestration. All the dilutions were done in threefold and distributed on the Petri dishes containing the nutrient agar. Then, the Petri dishes were kept at a temperature of 37°C for about 24-48 h. After incubation, the plates were counted and distinct wholesome bacteria sequesters were described and classified based on their form and structure[13].

Enumeration and isolation of bacteria: A 0.1 mL of the sample (an aliquot) was transferred into sterile nutrient agar plates in triplicates. A sterile glass spreader was then used in spreading the mixture uniformly (spread plate method) and incubated in an inverted position for 1 day at a temperature of 37°C. The Petri dishes were observed for grown colonies and counted as well as recorded after incubation[14]. For the culturing of biosurfactant-producing organisms from polluted soil samples from the various automobile mechanic soil environments, a ten-fold serial dilution of six dilutions in test-tubes was conducted to decrease the microbial load in the soil samples to avoid overcrowded organisms after incubation which might be too numerous to count[15]. The diluents from the sixth test-tube was then spread on Petri dishes containing nutrient medium to enhance or support the growth of organisms, this is called primary isolation. The total bacteria count from the various sampling sites after the primary isolation using the spread-plate method of isolation.

Characterization and identification of bacterial isolates: The preparation of bacterial standard inoculums was done according to the method by Hamzah *et al.*[16]. Characterization of sequesters was done in respect of their cultural morphology (morphological), microscopic examination (physical) and biochemical tests. Morphological studies were conducted on different media plates used for the sequestration of the bacteria; pure clusters were sequestered based on colony size, shape, pigmentation, elevation and texture of the individual organisms after 48 h of growth at 30°C. Pure isolates from the respective media were described and classified based on their morphological, physical and biochemical features.

Supernatant screening for the activity of Biosurfactant: Several tests such as hemolysis test, oil spreading technique and emulsification index test were conducted on the supernatants to observe the activity of biosurfactant.

Hemolysis test: The hemolysis test was conducted using a blood agar plate[17]. This test aimed to ascertain if the colonies of bacteria can grow on plasma agar. Growth affirmed hemolysis. Bacterial colonies were plated by streaking on Petri dishes containing blood agar and incubation was done at a temperature of 30°C for 1 day. The colonies were noted and a zone of hemolysis after 24 h was suggestive of biosurfactants.

Oil spreading technique test: The oil spreading test was used to ascertain the distance of the zone that is cleared after a dip of the biosurfactant-containing mixture was dropped in water containing oil. The aim was to establish if bio-surfactant can change the angle of contact at the exterior of the oil and water mixture by reducing the thin layer tightness between them. Fifty millilitres of purified water were put on plates and 20 µL of the lubricant was added at the exterior of the water and then 10 µL of supernatants of culture broth dropped at the center of the lubricant layer. The distance of the displaced area was measured after 30 sec[18].

Emulsification index test: This test aimed to ascertain the formation of steady suspension as illustrated by Jain *et al.*[19]. Two millilitres (2 mL) of hydrocarbons were annexed to the supernatant and subjected to spinning for two minutes, it stood for 1 day and the quantity of the hydrocarbons that were detached after 24 h of standing was measured. The emulsification index (E24) is given as Eq. 1.

$$E24 = \frac{\text{Measured height of emulsified layer}}{\text{Overall height}} \times 100\% \quad (1)$$

Synthesis of Biosurfactant: The synthesis of biosurfactant was done with diverse sources of carbon such as glucose and several hydrocarbons. These carbon sources are utilized by bacteria that are capable of synthesizing bio-surfactant[20,21].

Extraction of Biosurfactants: Centrifugation of the supernatant was done to extract the cells and the debris[22]. The supernatant was then precipitated by acidification with Hydrochloric acid and methanol. It was shaken vigorously and kept for 1 day for dehydration and extraction according to the method of Anandaraj and Thivakaran[23].

Statistical analysis: Mean±standard deviations of triplicate determinations were used to analyze data. Data obtained was compared by one-way analysis of variance (ANOVA) using the IBM Statistical package of Biological and Social Sciences (SPSS). At $p<0.05$, differences between groups were considered statistically significant.

RESULTS AND DISCUSSION

Biosurfactants were synthesized by *B. subtilis* and *P. aeruginosa* with the latter exhibiting more biosurfactant activity than the former and its ability was likened with a chemical surfactant based on their degradability level on hydrocarbon[24]. The growth of the bacteria isolates in the nutrient medium in the various sampling sites was observed by the determination of the number of colony-forming units per gram as shown in Fig. 1A-E.

There were more bacteria growth or counts of 265±6.52 cfu g^{-1} in Ikoku automobile mechanic workshop (Table 1) than Elekahia and Mile 3 auto-mechanic workshops with the control (an unpolluted soil environment) having the least number of bacteria counts (135±1.0 cfu g^{-1}). Biosurfactant producing organisms have been isolated from hydrocarbon-contaminated sites[25,26]. The primary isolates from the various sampling sites were then sub-cultured using the streak-plate method of isolation to obtain pure isolates, known as the secondary isolates as seen in Fig. 1E. The pure isolates were further identified and characterized based on their morphological, biochemical and physical description (Table 2). Nine organisms were identified namely *B. licheniformis, B. cereus, B. megaterium, E. coli, Micrococcus* sp., *B. subtilis, P. aeruginosa, S. aureus* and *Proteus* sp. The identified and characterized organisms are shown in Fig. 1. From Table 3, two bacteria isolates, *B. subtilis* and *P. aeruginosa* were selected as a result of their frequent occurrence for the synthesis of bio-surfactant. The supernatant produced from *P. aeruginosa* and *B. subtilis* are 1.38 and 1.2 g L^{-1}, respectively, were extracted and was creamy in color. Screening tests were conducted on the extracted supernatant to check for the activity of bio-surfactant using hemolysis test, oil spreading test and emulsification index test. It came into the realization that the supernatant tested positive for biosurfactant activity. The result in Table 4 showed both the bisques of *P. aeruginosa* and *B. subtilis* having positive hemolytic activity. *Pseudomonas aeruginosa* exhibited a larger zone of hemolysis at a distance of 39±0.15 mm and *B. subtilis* had a

Table 1: Total bacteria counts from sampled sites after the primary isolation

SITE	Bacterial count	CFU g^{-1} ($\times 10^9$)
Ikoku	265±6.51[a]	2.65
Mile 3	219±3.51[b]	2.19
Elekahia	223±2.52[c]	2.23
Control	135±1.0[d]	1.35

Values are expressed as mean±SD; n = 3, [a,b,c] and [d] are considered statistically significant at p<0.05 when one site is compared to the other

Table 2: The Biochemical, physical and morphological characterization of isolates

S/No.	Organism	GR	SHP	CAT	OX	CTR	MR	VP	IND	MOT	STH	SUC	GLU	FRU	LAC
1	*B. licheniformis*	+ve	R	+ve	+ve	-ve	+ve	+ve	-ve	+ve	+ve	A	A	A	A
2	*Bacillus cereus*	+ve	R	+ve	-ve	+ve	-ve	+ve	-ve	+ve	+ve	-	A	A	-
3	*Bacillus megaterium*	+ve	R	+ve	+ve	-ve	+ve	+ve	-ve	+ve	+ve	A	A	A	-
4	*Escherichia coli*	-ve	R	+ve	+ve	+ve	+ve	-ve	+ve	+ve	-ve	-	A	A	AG
5	*Micrococcus* sp.	+ve	C	+ve	+ve	-ve	+ve	+ve	-ve	+ve	-ve	A	A	A	A
6	*Bacillus subtilis*	+ve	R	+ve	-ve	+ve	+ve	-ve	-ve	+ve	+ve	A	A	A	-
7	*P. aeruginosa*	-ve	R	+ve	-ve	+ve	-ve	+ve	-ve	+ve	-ve	-	-	-	-
8	*S. aureus*	+ve	C	+ve	+ve	+ve	+ve	+ve	-ve	+ve	-ve	A	A	A	-
9	*Proteus* sp.	-ve	R	+ve	+ve	+ve	+ve	-ve	+ve	+ve	-ve	-	A	-	A

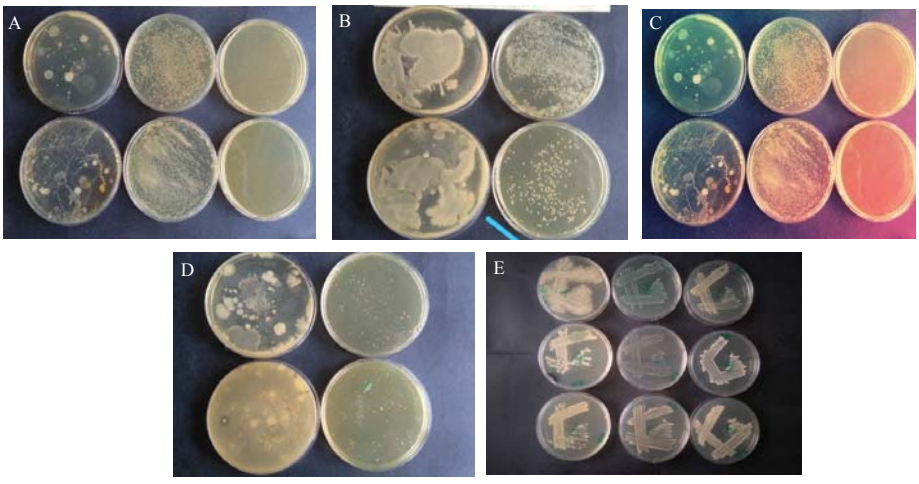

Fig. 1: Primary and secondary cultures of organisms isolated from the soil. (A): Primary cultures of organisms isolated from the soil at Ikoku, (B): Primary cultures of organisms isolated from the soil at mile 3, (C): Primary cultures of organisms isolated from the soil at Elekahia, (D): Primary cultures of organisms isolated from an uncontaminated soil acting as the control and (E): Secondary cultures of organisms from distinct colonies obtained from the primary culture

Table 3: Distribution of microorganisms from the sample location

S/N	Control	Mile 3	Elekahia	Ikoku
1	*Bacillus licheniformis*	*Bacillus cereus*	*P. aeruginosa*	*Staphylococcus* sp.
2	*Bacillus cereus*	*Bacillus subtilis*	*Proteus* sp.	*P. aeruginosa*
3	*Escherichia coli*	*Proteus* sp.	*Bacillus licheniformis*	*Bacillus megaterium*
4	*Micrococcus* sp.	*Staphylococcus aureus*		*Bacillus subtilis*
5	*Bacillus megaterium*	*Micrococcus* sp.		
6	*Bacillus subtilis*			
7	*Pseudomonas aeruginosa*			
8	*Bacillus niacin*			
9	*Staphylococcus aureus*			

Table 4: Hemolysis test result of the culture broth supernatants

Bacterial strain	Diameter of clear zone (mm)	Interpretation
Pseudomonasa aeruginosa	39±0.15[a]	Positive
Bacillus subtilis	37±0.21[b]	Positive

Values are expressed as mean±SD; n = 3. [a,b] are considered statistically significant at p<0.05

Table 5: Oil spreading test result of the culture broth supernatants

Bacterial strain	Diameter of clear zone (mm)	Interpretation
Pseudomonasa aeruginosa	56±0.15[a]	Positive
Bacillus subtilis	22±0.20[b]	Positive

Values are expressed as mean±SD; n = 3. [a,b] are considered statistically significant at p<0.05

Table 6: Emulsification index test result of the culture broth supernatants

	Emulsification indices (%)			
Bacterial strain	Kerosene	Diesel	Engine oil	Crude oil
P. aeruginosa	87±1.53[a]	92±1.53[a]	84±1.53[a]	98±1.53[a]
Bacillus subtilis	81±1.53[a]	86±1.53[a]	80±2.00[b]	93±1.53[a]

Values are expressed as mean±SD; n = 3. [a,b] are considered statistically significant at p<0.05

diameter of 37±0.21 mm. The hemolytic test has been recommended as a preliminary test for biosurfactant activity and it serves as a simple and easy method[27]. The positive hemolytic test is in line with the report of Das and Mukherjee[28] in their study on biosurfactant production by *Bacillus* sp. Isolated from Petroleum Contaminated Soils of Sirri Island. A positive oil spreading or displacement test result of the supernatant in the culture broth of *P. aeruginosa* and *B. subtilis* was reported in Table 5. The oil displacement test is a sign of the surface and wetting activities of a biosurfactant. *P. aeruginosa* had a larger diameter of 56±0.15 mm while *B. subtilis* had a lesser diameter of 22±0.20 mm. Additionally, a larger diameter represents a higher surface activity[29]. Hence, *P. aeruginosa* exhibited a higher spreading capacity compared to *B. subtilis.* The ability of the supernatants to form stable emulsions was analyzed for biosurfactant activity on some hydrocarbons such as kerosene, diesel, engine oil and crude oil. From the result in Table 6, *P. aeruginosa* showed emulsification indices of 87±1.53%, 92±1.53%, 84±1.53%

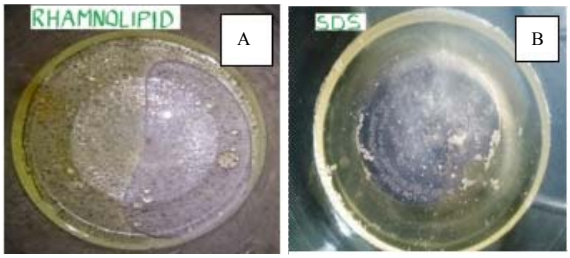

Fig. 2: The zone of inhibition exhibited between, (A) Rhamnolipid and (B) Sodium dodecyl sulfate by *P. aeruginosa*

and 98±1.53% for kerosene, diesel, engine oil and crude oil respectively while *B. subtilis* showed emulsification indices of 81±1.53%, 86±1.53%, 80±2% and 93±1.53% for kerosene, diesel oil, engine oil and crude oil respectively. The capability of these culture broths to form stable emulsions with different hydrocarbons is a key feature for biosurfactant activity. Based on this result, *P. aeruginosa* exhibited more surfactant ability than *B. subtilis*. These findings agree with that obtained by Chia-Wei *et al.*[25] and Ainon *et al.*[26] who reported that *Pseudomonas aeruginosa* is a common bacterium capable of degrading hydrocarbons and therefore encountering them in areas receiving petroleum waste discharges was not rare. The last stage of the research was to compare the rhamnolipid produced with sodium dodecyl sulfate, a chemical surfactant on their level of degradability. From Table 6, rhamnolipid displayed a larger diameter of clearance (36±1.5 mm) on fuel than sodium dodecyl sulfate having a diameter of 34±1.5 mm. Kaya *et al.*[30] reported that *P. aeruginosa* 78 and 99 produced rhamnolipid biosurfactants from petroleum-contaminated soil from Batman Province, Turkey. Similarly, Liu *et al.*[31] reported that *Pseudomonas* sp. is known to produce different types of rhamnolipids. SDS was not broken down as observed in Fig. 2B. There were deposits of whitish debris after the degradation and this is an attestation of its ability to accumulate in the environment which could be toxic to the environment. On the other hand, Rhamnolipid as

observed in Fig. 2A was readily broken down making them suitable for environmental remediation.

CONCLUSION

Microbial degradation is measured as a key factor in the clean-up strategy for petroleum hydrocarbon remediation due to its level of biodegradability and low toxicity. Most hydrocarbon polluted environment is loaded with microorganisms making them available, cost-effective and eco-friendly. Bacteria that synthesize bio-surfactant were sequestered as well as vetted and compared with their chemical counterparts. It is clear that the natural surfactant synthesized by *P. aeruginosa* called Rhamnolipids isolated from automobile mechanic polluted soil environment when compared with *B. subtilis*, is a more efficient hydrocarbon-degrading biosurfactant producer since it showed a larger zone of clearance or inhibition when compared to a chemical surfactant. The production of natural surfactants and their applications cannot be overemphasized. Based on this work, it can be inferred that the breakdown of hydrocarbons by natural surfactants is a vital factor in the remediation of hydrocarbon contaminated sites or milieu and the importance of features exhibited by them qualifies them as such. Polluted land is said to be safe not only when it is remedied, but also when it is devoid of harmful substances by the agent of remediation that could subsequently pose an environmental hazard.

COMPETING INTEREST

The author declares that there is no conflict of interest regarding the publication of this manuscript. Also, the ethical issues, including plagiarism, informed consent, misconduct, data fabrication and/or falsification, double publication and/or submission and redundancy have been completely observed by the authors.

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