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Full Length Article

Morphological and Biochemical Characterization of Biosurfactants Producing Bacteria from Diesel Contaminated Soil

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Abstract

Two soil bacteria designated as S1 and S2 were isolated from diesel-contaminated soil in Covenant University, Nigeria. Characterization (morphologically and biochemically) and comparison of the isolates with standard reference suggests they were *Bacillus* (S1) and *Pseudomonas* (S2) species. These bacterial species were screened for biosurfactant production potentials using: drop collapse test, oil displacement test, foam activity, blood hemolysis test, emulsification activity and interfacial tension. The results showed that the isolates S1 and S2 grew and reduced surface tension of diesel. Optimum growth of the bacteria and biosurfactant production were observed at pH 7. The emulsification index E_{24} obtained using the *Pseudomonas* and *Bacillus* species on oil substrates (crude oil, petrol and kerosene) were 51.00 – 34.48 and 60.7 – 30.0%, respectively. The isolated bacterial species exhibited biosurfactant production and such bacterial species could have application for clean-up of oil spilled sites in the Niger delta regions of Nigeria.

Keywords: Biosurfactant; Diesel-contaminated soil; Emulsification activity; Foam activity; Blood hemolysis; Bacteria

Introduction

The Niger delta is the oil producing zone in Nigeria. It is a wetland, where majority of the population rely on subsistence farming and fishing as a means of livelihood. As a result of exploration and exploitation activities of the crude oil in this zone, several pristine arable lands have been inundated with hydrocarbon pollution rendering the land unsuitable for agricultural purposes. Currently, this region has been chequered with restiveness due to the direct impact of the hydrocarbon pollution to the economic, social and health welfare of the population in this region. According to United Nations Development Programme (2006), estimated amount of oil spillage in the zone is about 400,000 tons, and less than 70% of the spilled oil has been recovered.

Globally, there are examples of hydrocarbon spills with severe global consequences these include but not limited to the: Exxon Valdez spill in 1989 and British Petroleum Deepwater Horizon spill in 2010 (Atlas and Hazen, 2011), and the accident in Dalian, China 2010 (Xu *et al*., 2012). Readman *et al*. (1992) and Montagnolli *et al*. (2015) stated that oil production could reach about 3 billion tons per year and about 2 million tons of hydrocarbons are lost per year due to accidents. Evidently, with the severe social and economic consequences of oil spills, there is need to deploy alternative cleanup methods that will not impact negatively on the environment. Currently, surfactants have been used in cleanup of the hydrocarbon spilled sites. Conversely, there are negative impacts of the surfactant to the environment. These include increased toxicity to the biota, recalcitrance, and sorption to the sediments.

Alternatively, biosurfactants have advantages over synthetic surfactant. Biosurfactants possess advantages such as high biodegradability, low toxicity and irritancy, compatibility with human skin and ability to remain active under extreme conditions (Desai and Banat, 1997; Cameotra and Makkar, 2004; Mulligan, 2009; Pacwa-Płociniczak *et al*., 2011). Biosurfactants are surface-active amphiphilic molecules produced by many microorganisms on the surface of their microbial cells or excreted extracellularly (Banat *et al*., 2010). Some of the major examples of biosurfactants are lipoamino acids, glycolipids and lipopeptides, polymers (lipoproteins, lipopolysaccahrides), phospholipids, monoglycerides, diglycerides and fatty acids (Neu, 1996; Toren *et al*., 2004; Dastgheib *et al*., 2008). Biosurfactants apart from their natural role in enhancing bioavailability of hydrophobic substrates have industrial applications in enhanced oil recovery, bioremediation of oil polluted environmental compartments, metal sequestering, excellent detergency,

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replacement of chlorinated cleaning solvents, detergent industries, pesticide and herbicide formulation, and in food and cosmetic industries (Shepherd *et al.*, 1995; Rosenberg and Ron, 1998).

In 2011, global biosurfactant market was estimated to be 1735.5 million USD and it is projected to reach 2.2105 billion USD in 2018. An impediment to the widespread economic exploitation of biosurfactants is cost of production. At present biosurfactant are more expensive than synthetic surfactant. Hence, it is imperative to explore ways to make biosurfactant to economically competitive to the synthetic counterparts. Among other factors are the choice of microbial species, which is a key determinant towards making biosurfactant to be economically competitive with synthetic surfactant. Examples of known microorganisms' genera that are capable of producing biosurfactant include: *Corynebacterium, Rhodococcus Pseudomonas* (Lotfabad *et al*., 2009; Gudiña *et al*., 2012; Xia *et al*., 2013), *Serratia* and *Bacillu*s (Yakimov *et al*., 1995; She *et al*., 2011), S*accharomyces* and *Candida* (Sarubbo *et al*., 2006; Ilori *et al*., 2008). So far the lists of biosurfactant producing microbial species are relatively few, hence there is a motivation to explore for efficient and substrate versatile biosurfactant producing microbial species. In addition, few researches on biosurfactant production have been mainly from the developed countries such as China (She *et al*., 2011; Wang *et al*., 2011; Xia *et al*., 2013), Brazil (Gudiña *et al.*, 2012), India (Pruthi and Cameotra, 2003), Iran (Lotfabad *et al*., 2009), and Germany (Yakimov *et al*., 1995) and very scanty report are from developing countries such as Nigeria.

The screening and isolation of new biosurfactant producers primarily have focused on the contaminated terrestrial environment (Das *et al*., 2010). In furtherance of this study for new biosurfactant producers, we explored the diesel contaminated site for a rich and diverse microbial community that can provide native isolates that may harbor interesting, extraordinary metabolic surface-active amphiphilic molecules that could be exotic in remediation of hydrocarbon (oil spilled) contaminated environment. Therefore, the current study attempted to bridge the knowledge gap by screening for biosurfactant producers using the drop collapse test, reduction of Interfacial tension and emulsification capacity of the biosurfactant.

Materials and Methods

Soil Sample

Diesel contaminated soil samples were collected at random spots around generator house at the students' hall of residence in Covenant University. The soil samples were collected at 0–12 cm depth with hand trowel and transferred into a sterile Ziploc bag. The soil samples are dark in color. The soil samples were sieved through a 0.2 mm wire mesh or and stored at 4^{o}C.

Isolation of Biosurfactant Producing Bacteria

The collected soil sampled were enriched on minimal salt (MS) medium that consisted of (g/L) 0.5 $(NH_4)_2SO_4$, 0.1 $MgSO_4 \cdot 7H_2O$, 0.076 $Ca(NO_3)_2.4H_2O$ and 40 mM phosphate buffer (pH 7.25). Solid MS medium was made by the addition of 1.8% Bacto-agar (Difco Laboratories, Detriot, MI USA). All chemicals used were of analytical grade unless otherwise stated; and were supplied from Merck Germany. Nutrient agar and nutrient broth were obtained from Micro Master India. Urease base agar, starch agar, methyl red and voges proskauer medium and peptone water were supplied by the Microbiology Laboratory, Covenant University. Biosurfactant producing bacteria were initially isolated by the conventional enrichment culture methods. For this, 5.0 g of the soil samples were mixed with 100 mL of MS medium contained in a conical flask (200 mL). The medium was amended with diesel (0.2% v/v) as the primary carbon source. The flasks were incubated on a shaker (Model H2Q-X 300) at 160 rpm at 35^{o}C for 7 days. Subsequent transfers from these enrichments were made weekly by using the same methods and conditions. After about four weeks, the enrichment cultures were transferred to a fresh medium using a 17% inoculum and continued cultivation under the same conditions.

Isolation, Purification and Identification of the Bacterial Species

Pure cultures from the diesel–enriched media were isolated by plating 1.0 mL of the enriched cultures onto minimal salt (MS) agar, sprayed with diesel on the surface. This was incubated in dark at 28–32^{o}C for five days. Colonies were periodically transferred to MS agar to obtain the pure culture. Using the morphological examinations, bacterial species were isolated and sub-cultured onto separate agar plates. The pure cultures were incubated at 37°C for 18–24 h. Six pure isolates were obtained, however we screened down to two most rapid growing organisms. For the purpose of initial identification we named the pure isolates as S1 and S2. These organisms were further classified using the standard, cultural and morphological techniques and comparison with standard reference organisms (Cowan, 1985; Olutiola *et al*., 1991). The following tests were carried out:

Gram stain, morphology, catalase, oxidase, colony motility, methyl red, voges proskauer, indole, nitrate reduction, starch hydrolysis, citrate and sugar utilization.

Screening/Extraction of the Yielded Biosurfactant

Preliminary screening of the biosurfactant –producing bacterial species were carried out by adding about 17% of the different organisms' inoculums on different freshly prepared 200 mL conical flask containing 100 mL sterile MS medium supplemented with diesel (0.4% v/v) and stoppered with cotton wool. The flasks were incubated on a

shaker (Model H2Q-X 300) at 120 rpm/min at 30ºC for 48 h. The biosurfactant yield was assayed from the crude cell-broth by acid precipitation (Queiroga *et al*., 2003; Nitschke *et al*., 2004; Dehghan-Noudeh *et al*., 2005). In this, the growth mediums containing the different inoculums were centrifuged in 50 mL test tubes, respectively. The cell free supernatant were collected and acidified to pH 2 with HCl (6 M) at 4ºC and left overnight for complete precipitation of biosurfactant. The acidified precipitate was then separated and distilled water added (Pornsunthorntawee *et al*., 2008). The biosurfactant was extracted by adding chloroform and ethanol in the ratio (2:1, v/v) mixture at room temperature. After 24 h, the bottom layer was collected and the solvent removed. The biosurfactant was concentrated using a rotary evaporator. Four parameters were used to assess the efficacy of the produced biosurfactants.

Oil Spreading Technique

Oil spreading technique depends on the destabilization of liquid droplets by the biosurfactants (Morikawa *et al*., 1993). In this, drops of cell suspension or of culture supernatant are placed on an oil coated surface. If the liquid cell free broth does not contain biosurfactants, the polar water molecules are repelled from the hydrophobic surface and the drops remain stable. If the liquid cell free broths contain biosurfactants, the drops spread or even collapse because the force or interfacial tension between the liquid drop and the hydrophobic surface is reduced. The stability of drops is however dependent on biosurfactant concentration and compares with surface and interfacial tension.

In this assay, 30 mL of distilled water was added to a petri dish with 100 µL of crude oil. 10 µL of cell free broth was then dropped on the surface of the oil (Rodrigues *et al*., 2006). These were done, respectively. If the drop contained biosurfactant, it was shown to collapse whereas non-biosurfactant containing drops remained stable. The diameters of the clear zone were measured.

Emulsification (E24) Test

Emulsifying action is one of the core activities of surfactants. This activity occurs when a liquid phase is dispersed as microscopic droplet in another liquid even when the two phases were previously immiscible. We analyzed this activity for our obtained isolates (S1 and S2) using the original method adapted from Cooper and Goldenberg, 1987; Ilori *et al*., 2005; Montagnolli *et al*., 2015).

The emulsification index were determined by adding to different test tubes 5 mL of the hydrocarbons (crude oil, kerosene and petroleum) and 5 mL of the cultures in the test tubes and homogenized by vortexing at high speed for 2 min and left to stand for 24 h. The E24 was determined as the percentage of height of the emulsified layer cm divided by the total height of the liquid column (cm). The emulsification activity was assessed by E24 using Eq. (1):

$$E24 = HEL/HS \times 100\% \quad (1)$$

Where, HEL is the height of the emulsified layer cm and HS is the height of the total solution.

Hemolysis Test

Hemolytic activity is known to be related with organisms capable of producing biosurfactants. Thus assaying for the hemolytic activity is one way of determining organisms with capacity to produce biosurfactants. Assessment of hemolytic activity was done for quick identification of our isolates that were capable of producing biosurfactant. This was done by streaking our isolates on blood agar at 37^0C for 48 h (Mulligan *et al*., 1984; Carrillo *et al*., 1996; Ghojavand *et al*., 2008; Pornsunthorntawee *et al*., 2008). The development of clearing zones of inhibition around the colonies showed that our isolates were positive for hemolytic activity and possesses ability of producing biosurfactant.

Foam Activity Characterization

Foam activity is part of the determinative characteristics for biosurfactant. This was done by growing the different isolates in a 200 mL conical flask containing 50 mL nutrient broth and incubated at 37ºC for 96 h. The foam activity was produced by shaking the 96 h different cultures supernatant using the shaker incubator at 200 rpm for few minutes. This method was carried out for each of the isolates.

Influence of pH on Biosurfactant Activity and Growth Optimization

We determined the effect of pH on biosurfactant activity. In this, the pH of the biosurfactant was amended between 6.0–8.0 and the interfacial tension determined. The Interfacial tension is to some extent similar to surface tension in that cohesive forces are also involved. However, the main forces involved in interfacial tension are adhesive forces between the cell free culture broth containing the biosurfactant and the diesel. The interaction occurs at the surfaces of the substances. A tensiometer (Kruss GmbH Hamburg Germany) was used by submerging the platinum ring in the cell free culture broth and recording the force required to pull out. In addition, we determined the optimum pH favorable for the growth of the isolates. The pH was varied between 6, 7 and 8. The growth profiles of the isolates in diesel were monitored by measuring the optical density fluxes using Genesys 10 UVS Spectrophotometer at 600 nm.

Results

Several bacterial species were isolated and two best (S1 and S2) bacterial species that showed the significant collapsing drop were selected. The isolates S1 had a flat

appearance, non-motile, gram positive during the grams reaction. The organism was catalase and citrate positive. The isolate could not produce Hydrogen sulphide and exhibited negative results to Methyl red and Voges proskauer tests. The isolate utilized glucose, sucrose and lactose but not galactose. The isolate was putatively named *Bacillus*. The isolate S2 showed a raised shape with creamy to brown texture. It showed green colour when left for 48–72 h. The organism was motile, gram negative and produced hydrogen sulphide. The S2 was negative to Methyl red and Voges proskauer tests. The organism showed a positive result to catalase, citrate, galactose and glucose tests. The S2 could not utilize sucrose nor lactose. The data of Table 1 showed that the isolate S2 were putatively the members of the genus *Pseudomonas.* The initial primary screening of the physiological responses of the isolates to the hemolysis, oil displacement, biosurfactant and foam tests is as shown in Table 2. The isolates *Bacillus* and *Pseudomonas* were positive for the initial assay. In Table 3, we used crude oil, petrol and Kerosene as hydrocarbon substrates for emulsification by the biosurfactant. Kerosene was the best substrate while Petrol showed a poor result. However it was evident that our isolates *Bacillus* and *Pseudomonas* had ability to emulsify the hydrocarbons. The activities of the biosurfactants were enhanced at pH of 6 and 7. The pH ranges of 6, 7 and 8 were used for the optimization of pH for the growth of our isolates. Both organisms exhibited higher growth rate at pH of 7 but *Pseudomonas* spp. showed more growth than *Bacillus* species (Fig. 1).

Discussion

In Nigeria, the incessant oil spills in the Niger delta region has called for concerted efforts for massive cleanup of such contaminated sites. The use of chemical surfactants may not be the best option because it has been found to persist and even exacerbate the contamination of the environment (Ilori *et al*., 2005). Biosurfactant an amphiphilic microbial product is the best alternative due to the great advantages it offers. Thus, it is of great significance to screen and isolate various versatile biosurfactant producers from a hydrocarbon contaminated source.

Interests in the production of biosurfactant have gained considerable value owing to its lower toxicity, ease of preparation, flexibility, eco-friendly and wide application in the petroleum industry and other medical applications (Nitschke and Costa, 2007; Makkar *et al*., 2011). In this study, two new biosurfactant producing bacterium were isolated from a hydrocarbon (diesel) contaminated site. Because our organisms were isolated from a hydrocarbon contaminated site, they may have been stressed to induce production of biosurfactants as a means of metabolizing the hydrocarbon as carbon or energy sources. The isolates (*Bacillus* and *Pseudomonas*) showed significant effect to the characteristics of biosurfactants such as the oil displacement test, foam activity and hemolysis test Table 2. In our study these organisms were isolated following enrichment from diesel contaminated soil in line with (Ghayyomi *et al*., 2012). The isolates S1 and S2 was selected for further studies based on the primary abilities to exhibit biosurfactant activities such as hemolytic activity, oil displacement, drop collapse and Emulsion activity. Youssef *et al*. (2004) and Jain *et al*. (1991) stated that drop collapse test and oil displace test are indicative of biosurfactants surface wetting activity. Satpute *et al*. (2008) suggested that more than one screening method should be included in the primary screening as to identify potential biosurfactant producers. Data presented in Table 2, show the screening methods for biosurfactant producing from the isolated bacteria. Our isolates *Bacillus* and *Pseudomonas* species

Table 1: Cultural, morphological and biochemical features of isolates S1 and S2

Morphological Features	S1	S2
Cultural characteristics Texture Surface pigment	Flat creamy colonies with irregular edges creamy to brown color	Raised creamy to brown green color
Motility	-	+
Gram stain	+	-
H2S production	-	+
Indole	-	-
Methyl red	-	-
Vogues proskauer	-	-
Starch hydrolysis	+	-
Catalase	+	+
Citrate	+	+
Glucose	+	+
Sucrose	+	-
Lactose	+	-
Galactose	-	+
Probable organism	*Bacillus* spp	*Pseudomonas* spp

Table 2: Primary screening for biosurfactant

Tests	*Bacillus* species	*Pseudomonas* species
Hemolysis test	+	+
Biosurfactant production	+	+
Foam test	+	+
Oil displacement test	+	+

+ = positive reaction, - = negative reaction

Table 3: Evaluation of hydrocarbon substrates for emulsification by the biosurfactants (E24)

Substrate	*Bacillus* spp (%) (emulsion index E24)	*Pseudomonas* spp (%) (emulsion index E24)
Crude oil	30	34.48
Petrol	32.1	17.2
Kerosene	60.7	51.7

Table 4: Shows the interfacial tension of the cell free broth of the bacterial species at different pH

	Bacillus subtilis (mN/m)	*Pseudomonas aeruginosa* (mN/m)
pH 6.0	0.8615 ± 0.05	0.8661 ± 0.02
pH 7.0	0.9143 ± 0.02	1.0451 ± 0.03
pH 8.0	0.6245 ± 0.02	0.6432 ± 0.02

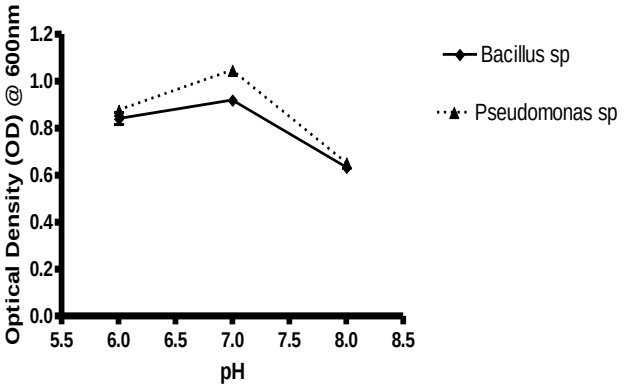

Fig. 1: Optimization of pH for Biosurfactant production

a. *Pseudomonas* spp b. *Bacillus* spp

Plate 1: Foam test of our isolates

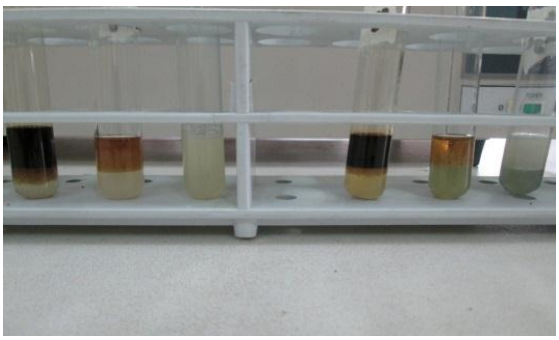

Plate 2: Emulsification test

both lysed blood agar after 24 h of incubation at 37°C. This agrees with the findings of Ghojavand *et al*. (2008) and Mulligan *et al.* (1984, 1989). Our *Bacillus* species showed greater emulsifying ability than *Pseudomonas* species in kerosene. This difference could be due to the difference in their cell membrane architecture; which is different between gram positive and gram negative organisms. In this study the result obtained for the kerosene shares some level of similarity as the diesel. In the study of Nyer and Skladany (1989), they compared kerosene and diesel fuel no. 1 in terms of their structure and found both to be possessing about 11 to 12 carbon hydrocarbons with both normal and branched hydrocarbon present. In addition they found normal alkanes, cycloalkanes, aromatic and mixed aromatic cycloalkanes to be present. Also, kerosene and diesel fuel contained low to non-detectable levels of benzene and polyaromatic hydrocarbon while petrol (gasoline) contains 6-10 carbons in length with cycloalkenes and alkylbenzenes present.

Biosurfactant could be substrate specific, solubilizing and emulsifying different substrates at different rates (Ilori and Amund, 2001). The different emulsifying values obtained for crude oil, petrol and kerosene corroborated with the findings of Ilori and Amund (2001). Thus, it is the ability of biosurfactant producer to reduce interfacial surface tension, which has important tertiary oil recovery and bioremediation consequences (Bento *et al.*, 2005).

Both *Pseudomonas* and *Bacillus* species produced biosurfactant with high foam formation activity when transferred to nutrient broth at 37°C for 96 h in a shaker incubator at 200 rpm. Desai and Banat (1997), reported that an effective biosurfactant is one that reduces the surface tension of water. Thus these organisms exhibited such when grown at different pH. Desai and Banat (1997) mentioned pH as environmental factor that can affect the physiological responses of organisms. In the present study, biosurfactant emulsification activity at pH 7 showed more activity in reduction of interfacial tension measured See Table 3, Fig. 1. It was also noticed that at pH 7 our organisms exhibited more growth resulting to increase in turbidity measured by OD at 600 nm. This shows that pH 7 may likely be the prevailing pH in the diesel contaminated site where our organisms were isolated. Gobbert *et al*. (1984) reported that pH of the medium plays an important role in biosurfactant production. In furtherance of this, Jagtap *et al.* (2010) showed that maximum production of bioemulsifier was at pH 7 though less activity was at pHs 6, 8 and 9. The pH 7 was the optimum for production of biosurfactant by our organisms.

In conclusion, this study has revealed that our bacterial species isolated from diesel spilled sites could use diesel as carbon source, and emulsify other hydrocarbons such as crude oil, kerosene and petrol at optimum pH. The results presented in this study have extended our knowledge about the diverse bacteria species that could be explored for biosurfactant production in the Niger delta zones of Nigeria and other tropical ecosystem. Given the significance of this study to environmental management and economics, the result of this study could be an avenue for revenue for Nigeria in the ever-increasing biosurfactant industry.

Competing Interests

Authors have declared that no competing interests exist.

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