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Pseudomonas monteilii and *Citrobacter murliniae*, Biosurfactant-Producing Bacteria Isolated from Nigerian Soil

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Authors' contributions

This work was carried out in collaboration between all authors. Author CGA designed the study, performed the statistical analysis, wrote the protocol, and wrote the first draft of the manuscript and managed literature searches. Authors AIE, CCE, UCO, EJA and VNA managed the analyses of the study and literature searches. All authors read and approved the final manuscript.

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Short Communication

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ABSTRACT

Aims: To isolate bacterial species with good potentials for biosurfactant production and to determine the tenso-active characteristics of the active producers.
Study Design: Study on biosurfactant-production potential of bacteria in shake flask.
Place and Duration of Study: Department of Applied Microbiology and Brewing, Nnamdi Azikiwe University, Awka, Nigeria, between October 2013 and June 2014.
Methodology: Soils, polluted with spent oil, from different regions of Anambra state, Nigeria, were examined for biosurfactant-producing bacteria. The bacterial isolates were screened for biosurfactant production in mineral salt medium (MSM) and nutrient broth supplemented with olive oil(NB). Biosurfactant production assay fermentation broth, include emulsification index measurement, oil displacement test, drop collapse test and blue agar plate test. The active producers were identified based on 16S rDNA sequencing.
Results: Out of the twenty-nine bacterial species screened, two of the isolates were recovered as

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active biosurfactant producers. They were identified as *Pseudomonas monteilii* AF064458 and *Citrobacter murliniae* AF025369. The biosurfactant production assay carried out on mineral salt medium and nutrient broth supplemented with olive oil revealed that *P. monteilii* AF064458 had emulsification index (E24) of 76.67% and 64.85%, oil displacement diameter of 2.1 cm and 1.2 cm respectively. The drop collapse test was positive in both medium and the organism showed a positive blue-agar plate test of 0.8 cm in diameter. With *C. murliniae* AF025369, an emulsification index (E24) of 66.67% and 63.33%, and an oil displacement diameter of 1.8 cm and 1.6 cm were obtained in MSM and NB respectively. A positive drop collapse test in both medium, and a negative blue-agar plate test were observed. Biosurfactants produced Biosurfactants produced by *P. monteilii* AF064458 and *C. murliniae* AF025369 reduced surface tension of water from 72 mN/m to 34 mN/m and 42 mN/m, with critical micelle concentrations (CMC) of 50 mg/L and 60 mg/L respectively.

Conclusion: The findings indicate that *Pseudomonas monteilii* AF064458 and *Citrobacter murliniae* AF025369 are biosurfactant-producing bacteria.

Keywords: Biosurfactants; surface tension; emulsification index; critical micelle concentrations; Citrobacter murliniae; Pseudomonas monteilii; soil.

1. INTRODUCTION

Surfactants (surface active agents) are amphipathic molecules with both hydrophilic and hydrophobic moieties that partition preferentially at the interface between fluid phases with different degrees of polarity and hydrogen bonding such as oil/water or air/water interfaces. Biosurfactants can be obtained either by chemical syntheses from renewable resources, by microbial fermentation processes, or by enzymatic syntheses. Surfactants from renewable resources are reviewed in [1].

Biosurfactants can be classified into (i) glycolipids (e.g. rhamnolipids, sophorolipids, trehalose lipids), (ii) lipopeptides (e.g. surfactin, liposan), (iii) phospholipids, (iv) neutral lipids (e.g. corynomycolic acid), (v) polymeric surfactants (e.g. emulsan, liposan) and (vi) particulate biosurfactants (vesicles, whole cells).

Three main strategies could be used to make the production of biosurfactants more cost-competitive: (i) screening for overproducing non-pathogenic wild type, mutant or recombinant strains, (ii) the use of cheaper and/or waste substrates to lower the initial raw material costs involved in the process and (iii) development of more efficient bioprocesses including optimization of culture conditions, as well as cost-effective separation processes for maximum biosurfactant recovery [2].

Biosurfactants are produced extracellularly or as part of the cell membrane by bacteria, yeasts and fungi [3], and are known to offer several benefits over their chemically synthesized counterparts. They are biodegradable, biocompatable and digestible, availability of raw materials and acceptable production economics, environmentally friendly and specific in their actions [4].

Biosurfactants have several applications in agriculture, medicine, petroleum and industry. In agriculture, they are used for hydrophilization of heavy soils to obtain good wettability and to achieve even distribution of fertilizer in the soil. They also prevent the caking of certain fertilizer during storage and promote spreading and penetration of the toxicants in pesticides [5]. Biosurfactants such as Fengycins have been reported to possess antifungal activity, and therefore, may be employed in biocontrol of plant diseases [6].

Some microorganisms, such as *Bacillus subtilis, Pseudomonas aeruginosa, Torulopsis bombicola* have also been reported to utilize crude oil and hydrocarbons by producing biosurfactant and thus can be used for oil spill clean-ups [7].

2. MATERIALS AND METHODS

2.1 Sample Collection

Soil samples were collected from spent oil-contaminated sites in Anambra state, Nigeria by random sampling technique.

2.2 Isolation of Bacterial Organisms

m-Cetrimide medium (Pancreatic digest of Gelatine, 40.0 g; Magnesium Chloride, 2.8 g;

Potassium Sulphate, 20.0 g; Cetrimide, 0.6 g; Glycerol, 10 g; Agar, 15 g; Water, 1L; pH, 7.2±0.2) [8] was used for the isolation of bacterial species. One gramme of soil sample was suspended in 10ml of sterile distilled water and the suspension diluted two-fold. 0.1 ml of 2^{-2} dilution was inoculated onto the m-cetrimide medium and the plates incubated at 30°C for 48 h. Pure cultures obtained were stored on Nutrient agar slants at 4°C.

2.3 Seed Inoculum

One loopful of a 24 h old culture of the isolate was inoculated into 10 ml of sterile Nutrient broth in a test tube and then incubated on a reciprocating shaker at 150 rpm for 24 h and 30°C.

2.4 Fermentation

A fermentation process was carried out following a modified method described by Guo-liang et al. [9]. A mineral salt medium (MSM) consisting of (g/L): KCl, 1.1; NaCl, 1.1; $FeSO_4.7H_2O$, 0.00028; KH_2PO_4, 3.4; K_2HPO_4, 4.4; $MgSO_4.7H_2O$, 0.5; Yeast extract, 0.5; 2 ml of Trace element solution ($ZnSO_4.7H_2O$, 0.29; $CaCl_2.4H_2O$, 0.24; $CuSO_4.5H_2O$, 0.25; $MnSO_4.7H_2O$, 0.17); $NaNO_3$, 1.5 and Glycerol, 2% w/v; H_2O, 1L. The pH of the medium was adjusted to 7.2. with 1N NaOH. A 50 ml of the medium in 100 ml Erlenmeyer flask was sterilized in autoclave at 121°C for 15 min, cooled and inoculated with 1 ml (ca 2.15 × 10^6 cell/ml) of the seed inoculum. The flask was incubated for 72 h on orbital shaker at 150 rpm and 30°C. Duplicate flasks were used and uninoculated flasks served as control.

2.5 Screening of Potential Biosurfactant Producing Organisms

Mineral salt medium and Nutrient broth(NB) supplemented with 2% olive oil [10] were used as the fermentation media for the screening of biosurfactant-producing bacteria. A 50 ml of the fermentation medium in 100 ml Erlenmeyer flask was inoculated with 1 ml of the seed inoculum. The flask was incubated for 72 h on orbital shaker at 150 rpm and 30°C. The fermentation broth was used for biomass estimation, drop collapse test, oil displacement test and emulsification index measurement. The tests were carried out in triplicates and the mean result was recorded.

2.5.1 Biomass estimation

Growth was determined from the fermentation broth using spectrophotometer (PerkinElmer Lambda 35 UV-VIS) at a wavelength of 600 nm.

2.5.2 Drop collapse method of screening

The method described by Tugrul and Cansunar [11] was used for the test. A polystyrene microwell plates was used for the drop collapse test. Each well with a diameter of 8 mm and 0.03 mm depth was coated with 7 µl mineral oil and left for 24 h at room temperature. A 20 µl supernatant from the fermentation broth culture was then added to each well at an angle of 45°C using a sterile syringe. As a control, sterile distilled water replaced the supernatant. After one minute interval, the wells were observed for drop collapse.

2.5.3 The oil displacement technique

The method described by Morikawa et al. [12] was used. 40 ml of distilled water was placed in a large petri dish followed by the addition of 15 µl of crude oil to the surface of the water. 10 µl of the supernatant from the fermentation broth was slightly placed on the surface of the oil film. The diameter of the clear zone on the oil surface was measured.

2.5.4 Emulsification index

The emulsifying activity of biosurfactant produced by the isolates was determined according to the method described by Cooper and Goldenberg [13]. A mixture of 2 ml supernatant and 2 ml kerosene was vortically stirred for 2 min and the height of emulsion layer was measured after 24 h. Water was used as a negative control. Emusification index was calculated by measurement of the height of the emulsion layer (a), divided by the total height (b), and then multiplied by 100 (EI = a /b × 100).

2.6 Identification of Selected Isolates

The active isolates selected were subjected to molecular assessment. The isolates were identified by 16S rDNA sequence analysis using the FASTA algorithm with the Prokaryote database from European Bioinformatic Institute (EBI).

2.7 Blue Agar Plate Test for Glycolipid-type of Biosurfactant by the Active Biosurfactant Producers

The method described by Satpute et al. [14] was used. Mineral salt agar (MSA) medium supplemented with glucose, 2%; cetyltrimethylammonium bromide (CTAB), 0.2 g; methlyene blue, 0.005 g; water, 1L; pH, 7.2±0.2 was used for the assay. A drop of cell free supernatant of the isolate grown in the mineral salt medium for biosurfactant production was placed on the agar plate and incubated at 37°C. After 48-72 h, the plate was observed for the formation of light blue halo around the culture.

2.8 Surface Tension Measurement

The surface tension of the cell free culture broth of the active biosurfactant producers was determined by capillary rise method [15]. The cell free culture broth was added to 1 L of sterile distilled water in increasing concentration (1-8 mg). The height of the water in the capillary tube (0.01 cm diameter) placed inside the solution was read, and surface tension measured using the equation: surface tension (γ) = [(ρga) /2]h. The CMC value was determined by plotting the surface tension as a function of the biosurfactant concentration.

3. RESULTS AND DISCUSSION

Cetrimide agar is a selective medium for bacteria of the genus *Pseudomonas.* The isolation of *Pseudomonas monteilii* from spent-oil contaminated soil sample with cetrimide agar in this study is in line with the work of Onwosi and Odibo [16], who isolated *Pseudomonas nitroreducens* from petroleum contaminated soil sample using the same medium. Rashedi et al. [17], also isolated *Pseudomonas aeruginosa* from oil well using cetrimide agar. Although cetrimide agar is a selective medium for isolation of *Pseudomonas,* the isolation of *Citrobacter murliniae* with cetrimide agar in this study is supported by de Ther – allonne [18], who observed that strains of *Klebsiella*, *Enterobacter*, *Citrobacter*, *Proteus*, *Providencia*, *Alcaligenes* and *Aeromonas* may also grow on cetrimide agar, causing a slight yellowing of the medium. Twenty-nine bacterial species isolated were screened for biosurfactant production in a mineral salt medium (MSM) and a nutrient broth (NB) supplemented with olive oil. Table 1 shows screening test results of the bacterial isolates grown on mineral salt medium, while Table 2 shows the screening test results of the bacterial isolates grown on nutrient broth. The drop collapse tests showed that 93.1% of the isolates had positive results and 6.9% negative results on mineral salt medium, and 96.6% positive results and 3.4% negative results on nutrient broth (Table 1) isolates grown on mineral salt medium showed 75.9% positive emulsification activity (E24) and 24.1% had zero emulsification activity (Table 1) on nutrient broth, 93.1% of the isolates gave positive emulsification activity, while 6.9% did not show any. The bacterial isolates produced oil displacement diameter ranging from 0.2-2.1 cm on MSM (Table 1) and 0.3 - 1.6 cm on NB (Table 2).

The screening procedures used in this study were consistent with previous works [14,16,]. In drop collapse test, drops of cell suspension containing biosurfactants collapsed, whereas non-surfactant containing drops remain stable [11]. Distilled water which served as control, did not collapse on the oily surface of the well but appeared as a bead. This is because, the oily surface is hydrophobic and, therefore, the force causes aggregation [19]. The increased positive drop collapse result agrees with the work of Thavasi et al. [20], who screened bacterial strains for biosurfactant production and noted that 78.1% were positive for drop-collapse activity. They, therefore, recommended drop-collapse and oil spreading assays as reliable methods for screening large numbers of samples. However, contrary to our findings, Sabina et al. [21], screened bacterial isolates and had only 3.4% positive result in drop collapse test, even though better result was obtained in oil spreading test.

The oil displacement test is an indirect measurement of the surface activity of a surfactant sample tested against oil; a larger diameter represents a higher surface activity of the testing solution [22]. The bacterial isolates produced oil displacement diameters ranging from 0.2-2.1 cm (Tables 1 and 2) which is contrary to the works of Hesham et al. [23] and Jaysree et al. [24]. While Hesham et al., obtained rate of oil displacement ranging from 2.8 cm to 4.1 cm in the screening of *Candida* species for biosurfactant production, Jaysree et al., recorded displacement diameters ranging from 3.0 cm to 4.2 cm in their work on biosurfactant production by halophilic bacteria. The variations in displacement diameters are likely to be strain dependent.

Emulsification activity is one of the criteria used for the selection of potential biosurfactant producers [14]. Cell-free culture broth containing biosurfactant usually emulsifies the hydrocarbon present in the test solution [25]. The increased positive emulsification test result in this study (Table 1 and 2), is contrary to the result obtained by Ellaiah et al. [26], who screened 68 bacterial isolates for biosurfactant production on mineral salt medium and found only 6% of the isolates had emulsification activity up to 61%. However, Bodour and Maier [27], suggested that a maximum of two or three screening methods should be used for the selection of biosurfactant producers.

Several researchers have reported that *Pseudomonas* spp. are capable of biosurfactant production [28-31]. The isolation of *P. monteilii* as a biosurfactant producer in this study suppports the views of these researchers. The isolation of *Citrobacter murliniae* as biosurfactant producer is also supported by the works of Thavasi et al. [20] and Mandal et al. [32]. While Thavasi et al. isolated *Citrobacter intermedius* alongside *Klebsiella ozaenae* as biosurfactant producers, Mandal et al. isolated and characterized *Citrobacter* and *Enterobacter* as lipopeptide biosurfactant producers.

Pseudomonas monteilii was positive for blue agar plate test by forming a dark blue halo of 0.8 cm diameter (Fig. 1), while *Citrobacter murliniae* appeared negative. This indicates that the biosurfactant produced by *P. monteilii* is likely to be a glycolipid-type of biosurfactant.

Table 1. Screening tests of bacterial isolates for biosurfactant production on Mineral salt medium

Isolate No.	OD(600nm)	Emulsification index (%)	Oil displacement(cm)	Drop collapse
1	0.854	20.00	0.3	+++
2	0.725	26.67	0.3	++
3	1.902[b]	66.67[b]	1.8[b]	+++[b]
4	0.356	20.00	0.5	++
5	0.307	43.33	P	+
6	0.395	6.67	P	+
7	2.015	63.33	1.0	++
8	1.421	70.00	0.5	++
9	0.985	40.00	0.6	+
10	0.348	33.33	0.6	++
11	1.623	73.33	0.6	++
12	2.020	16.67	0.3	+
13	0.210	63.33	1.0	+
14	1.754[a]	76.67[a]	2.1[a]	+++[a]
15	0.368	63.33	0.7	+
16	0.248	0.00	0.3	+
17	0.333	17.14	0.6	++
18	1.495	20.00	0.5	+
19	1.980	2.86	0.6	++
20	0.042	0.00	1.0	+
21	0.104	0.00	1.2	+
22	0.154	6.67	0.2	-
23	0.087	0.00	P	-
24	0.075	0.00	0.6	+
25	0.058	6.67	1.5	+
26	0.077	0.00	1.5	+++
27	1.060	0.00	0.5	+
28	0.582	33.33	2.0	++++
29	1.305	63.33	0.5	++

Key : + = Positive, - = Negative, P = Poor, OD = Optical density, [a] represents results for Pseudomonas monteilii , [b] represents results for Citrobacter murliniae

Table 2. Screening tests of bacterial isolates for biosurfactant production on nutrient broth

Isolate No.	OD(600nm)	Emulsification index (%)	Oil displacement (cm)	Drop collapse
1	1.475	16.67	0.4	+
2	3.780	50.00	0.3	+
3	3.800[b]	63.33[b]	1.6[b]	++[b]
4	2.800	0.00	0.4	++
5	1.843	50.00	P	++
6	1.808	60.67	P	++
7	2.550	50.00	1.1	++
8	2.835	60.67	0.4	++
9	2.255	53.33	1.0	++
10	1.944	10.00	0.3	+
11	1.080	63.33	0.5	++
12	2.020	50.00	0.5	+
13	2.081	16.67	0.4	+
14	2.395[a]	64.85[a]	1.2[a]	++[a]
15	2.370	0.00	0.5	+
16	1.573	31.43	0.5	+
17	1.430	54.29	0.4	++
18	1.229	22.86	0.3	+
19	2.185	17.14	0.6	+
20	0.520	8.57	0.4	+
21	1.480	54.29	1.0	++
22	1.732	43.33	0.5	-
23	1.774	60.00	1.0	+
24	1.882	56.67	1.0	+
25	0.960	13.33	0.6	+
26	2.207	56.67	0.6	++
27	1.945	57.14	1.0	+++
28	2.028	57.14	1.0	+++
29	2.023	33.33	0.6	

Key : + = Positive, - = Negative, P = Poor, OD = Optical density, [a] represents results for Pseudomonas monteilii , [b] represents results for Citrobacter murliniae

Fig. 1. Positive blue agar plate (blue halo) by *P. monteilii*

Surface tension measurement of the cell-free culture broth obtained in this study showed that *C. murliniae* was able to reduce surface tension of water from 72 mN/m to 42 mN/m (Fig. 2), while *P. monteilii* reduced surface tension of water to 34 mN/m (Fig. 3). Critical micelle concentration (CMC) was reached at 60 mg/L and 50 mg/L respectively. Khopade et al. [33], observed that the addition of biosurfactant produced by *Streptomyces* strain reduced surface tension of water to 30 mN/m having CMC of 110 mg/L. Biosurfactant secreted by *Burkholderia glumae* reduced surface tension of hexadecane from 40 mN/m to 1.8 mN/m, displaying CMC of 25 mg/L [34].

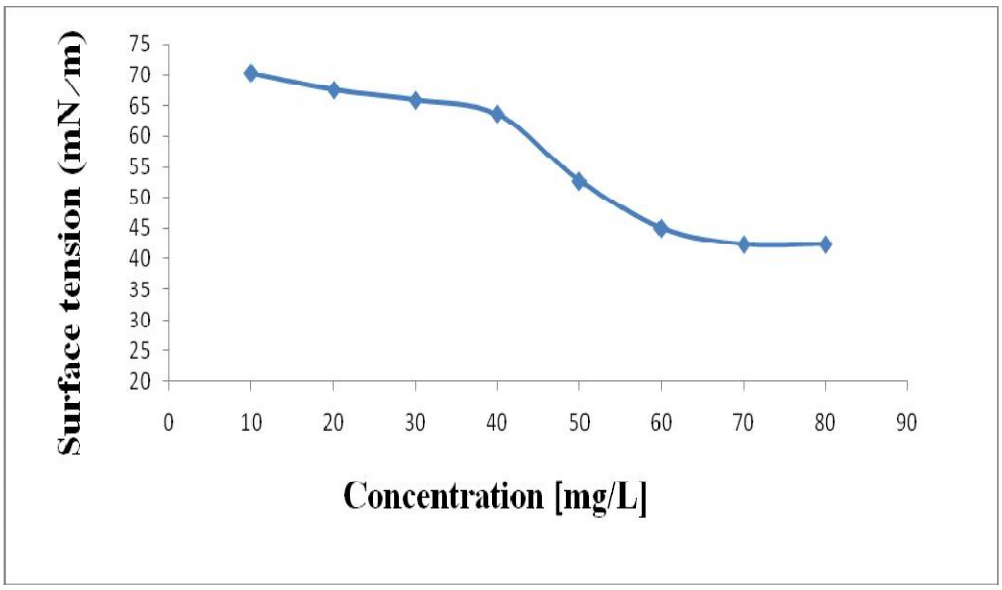

Fig. 2. Surface tension measurement of biosurfactant produced by *Citrobacter murliniae*

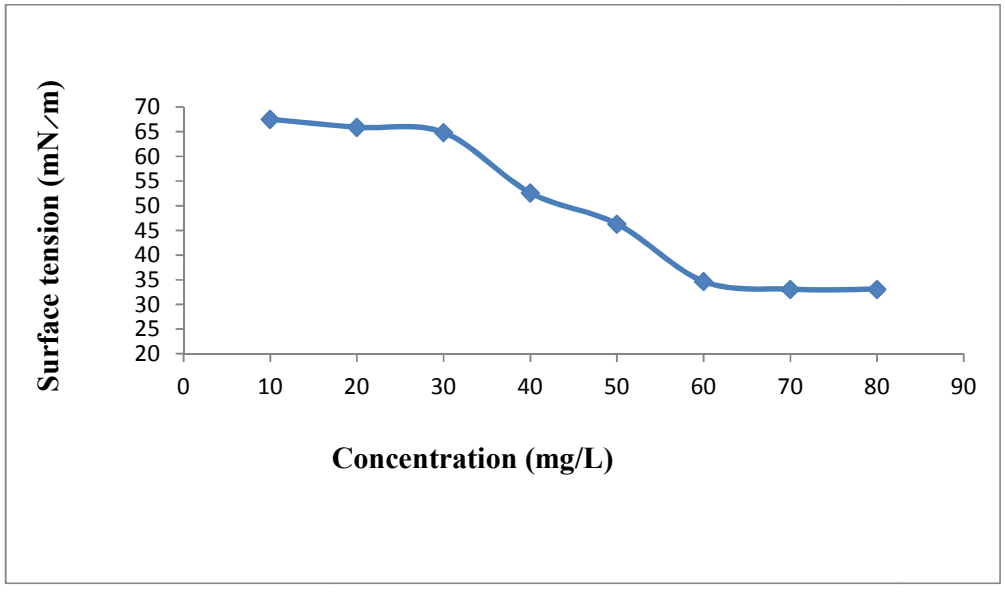

Fig. 3. Surface tension measurement of biosurfactant produced by *Pseudomonas monteilii*

4. CONCLUSION

The preliminary screening in this study revealed that apart from mineral salt medium, nutrient broth supplemented with appropriate carbon source can serve as a fermentation medium for biosurfactant production. *Citrobacter murliniae and Pseudomonas monteilii* are good producers of biosurfactants which have potentials for biostimulation in crude oil bioremediation.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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