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BIOSURFACTANTS PRODUCING BACTERIA FROM OIL-POLLUTED SOIL IN ABEOKUTA, OGUN STATE

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ABSTRACT

Biosurfactants are amphiphilic compounds produced by a variety of microorganisms as extracellular compounds. Biosurfactants are preferable over the chemical surfactant because of their effectiveness, degradability and environmentally friendly nature. This study focuses on the isolation, identification and screening of biosurfactants-producing bacteria. Soil samples were collected from different automobile shops in Abeokuta, Ogun State. Bacteria were isolated and screened for their ability to produce biosurfactant using different methods in a stepwise process such as haemolytic test, drop collapse test, bacterial adhesion to hydrocarbon test and the confirmatory test which was the emulsification test that was assayed every 4 hours. Forty-one bacteria isolates showed beta-haemolysis, 20 exhibited alpha haemolysis and 47 exhibited gamma haemolysis. All positive isolates from the beta haemolytic test were screened for drop collapse test and result showed that 14 isolates were positive to the drop-collapse test. The isolates adhered to hydrocarbon at different rates. *Proteus mirabilis* had the lowest hydrophobicity value of 41% and the highest was *Pseudomonas aeruginosa* with a hydrophobicity value of 72%. The result showed that *Pseudomonas aeruginosa* had the maximum emulsification capacity of 65% at 24 hours while the least emulsification capability for biosurfactant production was exhibited by *Proteus mirabilis* (43%). This result showed that *P. aeruginosa* is a better organism for biosurfactant production and are good choice of organisms in many industries because of their emulsion capability.

Keywords: Biosurfactant, Bacteria, Hydrocarbon, Soil, Screening

INTRODUCTION

Biosurfactants are amphiphilic compounds produced by microorganisms and they are produced extracellularly. They contain hydrophobic and hydrophilic moieties that reduce the interfacial or surface tension (Karanth *et al*., 2008; Banat *et al*, 2010). The microbial surfactants called bioemulsifier are microbial compounds with a distinct surface activity that exhibit a broad diversity of chemical structures such as: glycolipids, lipopeptides and lipoproteins, lipopolysaccharides, phospholipids, fatty acids and polymeric lipids. A host of interesting features of bioemulsifier have led to a wide range of potential applications in the medical field as antibacterial, antifungal, antiviral agents in environmental protection, including enhanced oil recovery (EOR), oil spills control, biodegradation and detoxification of oil-contaminated industrial effluents and soils (Yalcin and Ergene, 2009).

The use of biosurfactants represents an alternative and eco-friendly option for bioremediation technology in environments contaminated with hydrocarbons. Biosurfactants, which increase the surface area of hydrophobic water-insoluble substrates can be added to the bioremediation processes to stimulate the growth of oil-degrading bacteria and improve their ability to degrade hydrocarbons (Bodour *et al*., 2003). Unlike synthetic surfactants, microbial-biosurfactant are easily degraded, effective and particularly suited for environmental applications such as bioremediation and dispersion of oil spills (Mohan *et al*., 2006). The aim of this study was to isolate, characterize and screen bacterial species from different hydrocarbon polluted sites for biosurfactant production.

MATERIALS AND METHODS

Collection of Soil Samples

Hydrocarbon contaminated soil samples were collected from three different automobile workshop locations in Abeokuta: at Obantoko, Camp and Ilupeju area. Soil samples were taken at a depth of 0-10 cm and 10-20 cm. Control soil sample was collected in an uncontaminated location site at depth of 0-10cm and 10-20 cm. Collection of soil samples was done aseptically

using an auger that was surface sterilized with 75% alcohol prior to use. Samples were transported to the laboratory in ice-packs within one hour.

Isolation and Enumeration of Bacteria

Total Heterotrophic Bacterial Count (THBC)

The total heterotrophic bacterial count (THBC) was determined using the method of Rahman *et al.* (2003). One gram of each of the samples was serially diluted five-fold in sterile distilled water and 1 ml of the diluents was aseptically dispensed into sterile Petri-dishes. Using the pour plate method, plate count agar (Lab M, UK) was poured aseptically in the sterile plates. The plates were incubated at 28 °C for 24 h after which the colonies were counted. This was carried out in replicates. The various colonies were then sub-cultured to obtain pure colonies.

Total Hydrocarbon Degrading Bacterial Count (THDBC)

The hydrocarbon utilizing bacterial count was carried out on mineral salt medium (MSM) agar on which dual purpose kerosene (DPK) was used as the sole carbon source. Prior to use, the DPK was filtered using a Whatman filter paper No.1. Two percent agar was added to solidify the medium. The MSM composition as described by Balogun and Fagade (2010) was made up of basal salt medium (BSM) and trace element solution. The BSM contained (g/L): K_2HPO_4, 1.8; KH_2PO_4, 1.2; NH_4Cl, 4.0; $MgSO_4.7H_2O$, 0.2; NaCl, 0.1; yeast extract, 0.1 and $FeCl_2.4H_2O$, 0.05. Trace elements solution contained (g/L): H_3BO_3, 0.1; $ZnSO_4.7H_2O$, 0.1; $CuSO_4.5H_2O$, 0.05 and $MnSO_4.H_2O$, 0.04 with the pH of 6.5. The basal salt medium and the trace elements solution were sterilized separately.

Ten millilitres of the trace elements solution was added aseptically to the sterilized basal salt medium to make it up to a litre. One millilitre of the serially diluted samples was aseptically dispensed into sterile Petri-dishes and using the pour plate method, the MSM was aseptically poured on the plates. The plates were then incubated at 28 °C for 5 days after which the colonies were counted. This was carried out in replicates and the various colonies were sub-cultured to obtain pure colonies (Balogun and Fagade, 2010)

Surface Active Bacterial Count (SABC)

Screening for surface-active bacteria was done on blood agar. The blood agar was made up of nutrient agar containing 5% (v/v) defibrinated rabbit blood. One milliliters of the serially diluted sample was plated on the blood agar using the pour plate method. The plates were incubated at 28 °C for 48 h after which the colonies that showed clear zone of beta-heamolysis were counted as surface active agent producer as reported by Tabatabaee *et al.* (2005). This was carried out in replicates and the colonies were then sub-cultured to obtain pure colonies.

Screening for Biosurfactants Production

The pure culture bacterial isolates were screened for biosurfactants production using the following methods:

Blood Haemolysis Test

This is a major screening assay for biosurfactant production potential of isolates. The fresh single colonies from the isolated cultures were taken and streaked on blood agar plates. These plates were incubated for 48 h at 37 °C. The plates were then observed and the presence of clear zone around the colonies indicated the presence of biosurfactant producing organisms (Tambekar and Gadakh., 2013).

Drop-Collapse Test

The bacterial isolates were inoculated in mineral salts medium with 0.1% crude oil and incubated for 48 h. Two microliter of crude oil was applied to the well regions delimited on the covers of 96-well microplates and these were left to equilibrate for 24 h. The 48 h culture was centrifuged at 12,000 rpm for 15 mins at 25 °C to separate the cells from the supernatant. Five microlitres of the supernatant was transferred to the oil-coated well regions and drop size was observed after 1 min with the help of a magnifying glass. The result was considered to be positive when the diameter of the drop was increased by 1 mm from that which

was produced by distilled water which was taken as the negative control (Youssef *et al.*, 2004).

Bacterial Adhesion to Hydrocarbons (BATH)
Bacterial cells were suspended in phosphate buffer salt solution g/L (K_2HPO_4: 16.9 and KH_2PO_4 : 7.3) pH 7 to give an optical density of 0.5 at 600 nm. One hundred microliter of kerosene was added to 2 ml of cell suspension and was vortexed for 2 min in test tubes. Aqueous phase was allowed to separate for 1 h. The optical density of the aqueous phase, A_0, was measured after 10 min. Hydrophobicity was measured as the percentage of cell adherence to hydrocarbon. The degree of hydrophobicity was calculated as:

$H = 1 - {}^{A}/_{A_0} \times 100\%$ (Goulart *et al.*, 2014)

where A is the absorbance of the aqueous phase after hydrocarbon was added and A_0 is the absorbance of the aqueous phase before hydrocarbon was added.

Determination of Emulsification index (E24%)
The surface active agents producing bacteria were grown on MSM, supplemented with 1% DPK for 7 days in an orbital incubator at 180 revolutions per minute (rpm) at 28 °C. Cell free supernatant obtained by centrifuging the broth culture at 15,000 rpm for 15 min was used for the experiment (Balogun and Fagade, 2010). The emulsification index for surface active agent-producing bacteria was carried out using the method of Bodour *et al.* (2004). Two millilitres of the supernatant of each organism was put in reaction tube and 2 ml of DPK added as hydrocarbon substrate. The mixture was vortexed at high speed for 2 min and observed for percentage emulsification at every 4 h for 24 h. Emulsification index (E24%) was recorded as a percentage of the height of the emulsified DPK to the total height of the mixture after 24 h (Tabatabaee *et al.*, 2005).

$$E_{24} = \frac{\text{height of emulsion}}{\text{total height}} \times 100\%$$

Identification of Isolates
Identification of the isolates was carried out using standard microbiological method. Shape, pigmentation, elevation, size, appearance and motility were observed for morphological characteristics. The following biochemical tests were carried out: Gram stain, catalase test, oxidase test, motility test, indole, coagulase test, nitrate test and urease test.

RESULTS
Table 1 shows the description of different soil samples collected with their different code. Soil samples were collected at different depths to determine or identify the biosurfactant potential isolate depth in the soil.

Table 1: Description and Codes of Soil Samples collected from Automobile workshops in Abeokuta.

S/N	Location	Depths (in cm)	Sample codes
1	Ilupeju (A)	0-10 cm	A1
		10-20 cm	A2
	Ilupeju (B)	0-10 cm	B1
		10-20 cm	B2
2	Camp (C)	0-10 cm	C1
		10-20 cm	C2
	Camp (D)	0-10 cm	D1
		10-20 cm	D2
3	Obantoko (E)	0-10 cm	E1
		10-20 cm	E2
	Obantoko (F)	0-10 cm	F1
		10-20 cm	F2
4	Control (G)	0-10 cm	G1
		10-20 cm	G2

Soil samples were collected at different depths to identify the biosurfactant producing isolate that could be found in the soil

Bacterial Counts

From the soil samples obtained, the highest total heterotrophic bacterial count (THBC) was $9.72x10^6$ CFU/g obtained at location F2 and the lowest heterotrophic bacterial count was $1.16x10^6$ CFU/g obtained at location D1. The highest total hydrocarbon degrading bacterial count (THDC) was $4.65x10^6$ CFU/g obtained at location F2 and the lowest was $1.25x10^6$ CFU/g from location A2 while the highest surface active bacterial count (SABC) was $8.0x10^5$ CFU/g at A1 and the lowest surface active bacterial count was $2.0 x10^5$ CFU/g obtained at location B1 and E2 (Table 2).

Table 2: Total Bacterial Count of the Soil Samples collected from Automobile workshops.

S/N	Sample code	Depth (cm)	THBC (10^5)	THDC (10^5)	SABC (10^5)
1	A1	0-10	31.0± 4.1	15.8± 1.2	8.0± 2.5
	A2	10-20	24.0± 1.6	12.5± 3.8	4.0± 1.1
2	B1	0-10	63.7± 8.5	27.4± 1.7	2.0± 0.6
	B2	10-20	81.2± 2.1	16.3± 2..1	5.0± 1.0
3	C1	0-10	56.0± 1.7	13.0± 0.6	2.1± 0.8
	C2	10-20	32.28± 4.1	21.5± 2.5	4.0± 0.3
4	D1	0-10	11.6± 3.6	13.0± 1.3	6.0± 0.1
	D2	10-20	22.7± .2.4	15.6± 1.18	6.0± 0.2
5	E1	0-10	23.4± 5.3	15.8± 3.4	3.0± 0.2
	E2	10-20	38.6± 1.9	24.4± 4.1	2.0± 0.1
6	F1	0-10	21.36± 1.3	17.2± 2.5	4.0± 1.2
	F2	10-20	92.7± 1.7	46.5± 4.8	5.0± 0.4
7	G1	0-10	71.8± 2.9	21.0± 2.5	6.6± 1.5
	G2	10-20	62.8± 3.5	40.0± 5.3	5.0± 0.8

Key: THBC = Total heterotrophic bacterial count, THDC = Total hydrocarbon degrader count, SABC = Surface active bacterial count

Screening for Biosurfactant Production

One hundred and eight bacterial isolates were obtained from the different counts (THBC, THDC and SABC). The isolates (THBC, THDC and SABC) were subcultured on blood agar medium for haemolytic screening. Result revealed that the isolates could be separated into: 41 (beta-haemolytic ability), 20 (alpha haemolytic ability) and 47 (gamma haemolytic ability). This was shown through their respective colour on the medium. Alpha haemolysis was shown by a greenish colouration around bacterial colony, beta haemolysis was shown by clear zone around bacterial colony and gamma haemolysis showed no clear zone of haemolysis. Those organisms which showed beta-haemolysis were identified by various biochemical tests. Figure 1 showed the occurrence of the different isolates identified using biochemical test. All positive 41 beta haemolytic isolates were screened for drop collapse test and result showed that the 14 isolates were positive to the drop-collapse test.

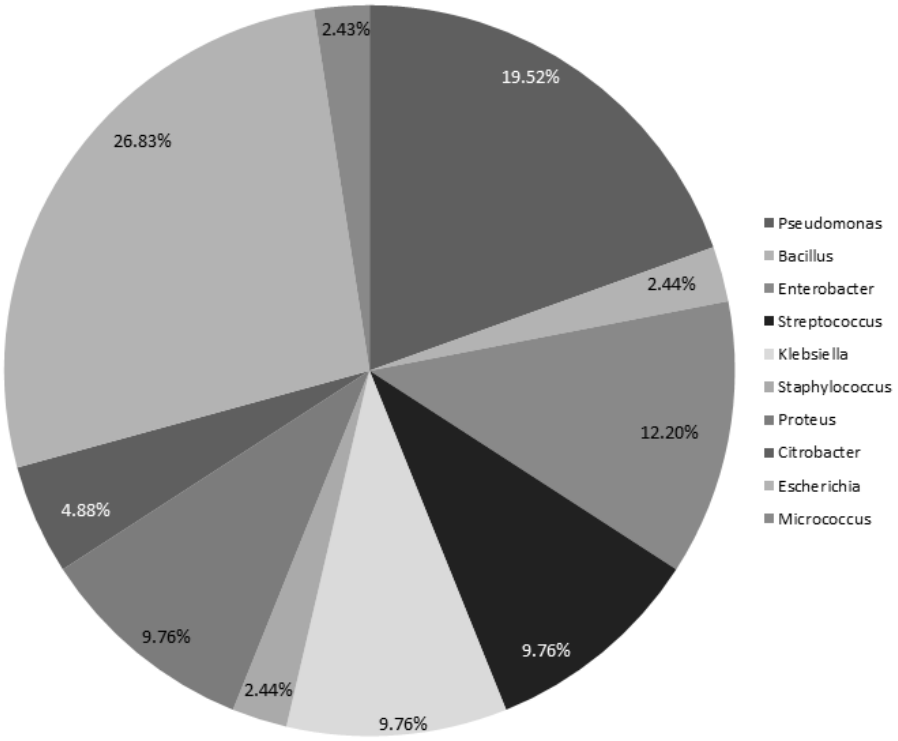

Figure 1: Occurrence of the Various Genera Identified in the Soil Sample

Fourteen positive drop collapse test isolates were subjected to BATH test and emulsification activity assay. Table 3 shows the result of the ability of the isolates to adhere to hydrocarbon, i. e (BATH). *Proteus mirabilis* had the lowest hydrophobicity value of 41% and the highest obtained was *Pseudomonas aeruginosa* with a hydrophobicity value of 72 %.

The emulsification activity was monitored at intervals following mixing at high speed with the vortex. A time interval of 4 h in 24 h was taken into consideration. Figures 2 to 8 show the emulsification ability of the different isolates. Isolates ability to emulsify kerosene showed that *Pseudomonas aeruginosa* had the highest emulsification index (E24%) of 65% while *Proteus mirabilis* had the lowest emulsification index of 43% after 24 hours.

Table 3: Percentage of Bacterial Adhesion to Hydrocarbon

Isolate code	Isolates	Degree of Hydrophobicity (H%)
A	*Proteus* spp.	44%
B	*Citrobacter aerogenes*	56%
C	*Escherichia coli*	48%
D	*Streptococcus* spp.	52%
E	*Bacillus subtilis*	64%
F	*Pseudomonas aeruginosa*	72%
G	*Proteus mirabilis*	41%
H	*Klebsiella oxytoca*	50%
I	*Enterobacter cloaca*	44%
J	*Enterobacter* spp.	56%
K	*Pseudomonas fluorescens*	52%
L	*Klebsiella* spp.	46%
M	*Staphylococcus saprophyticus*	50%
N	*Micrococci* spp.	54%

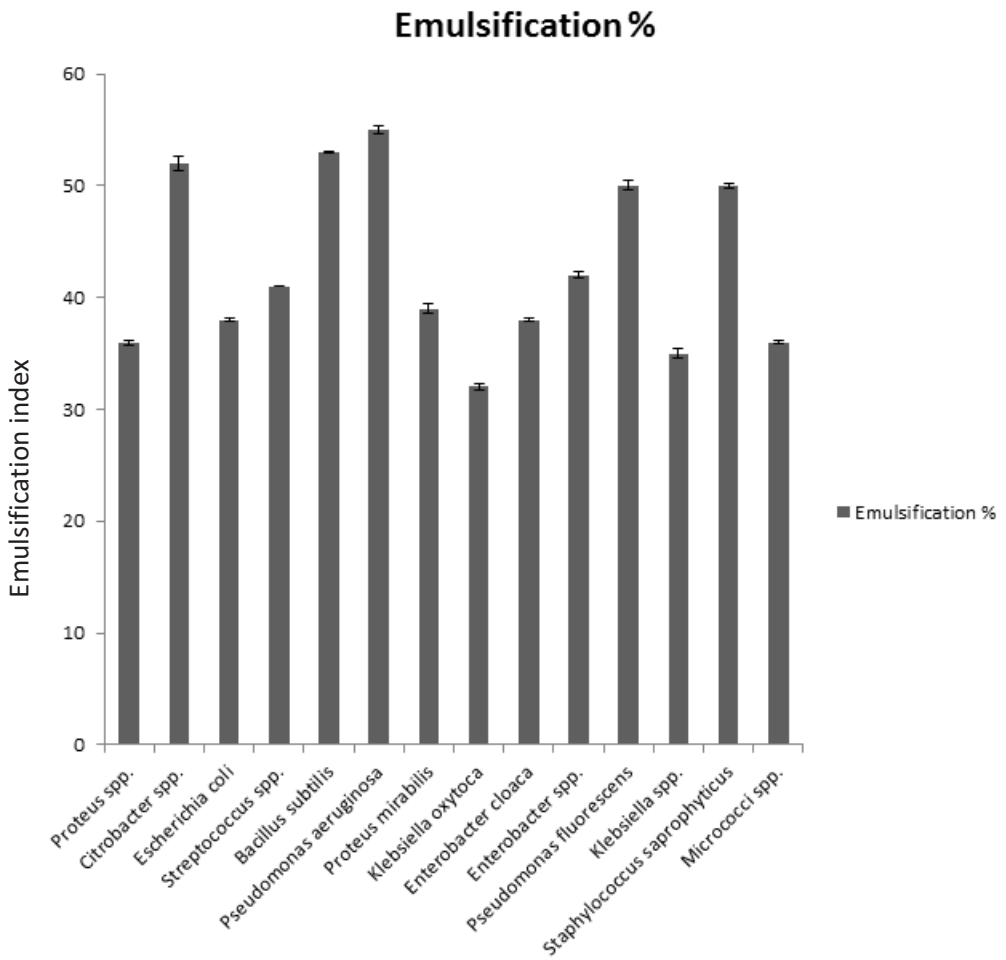

Figure 2: Percentage Emulsification Activity of Isolated Bacteria at 0 h

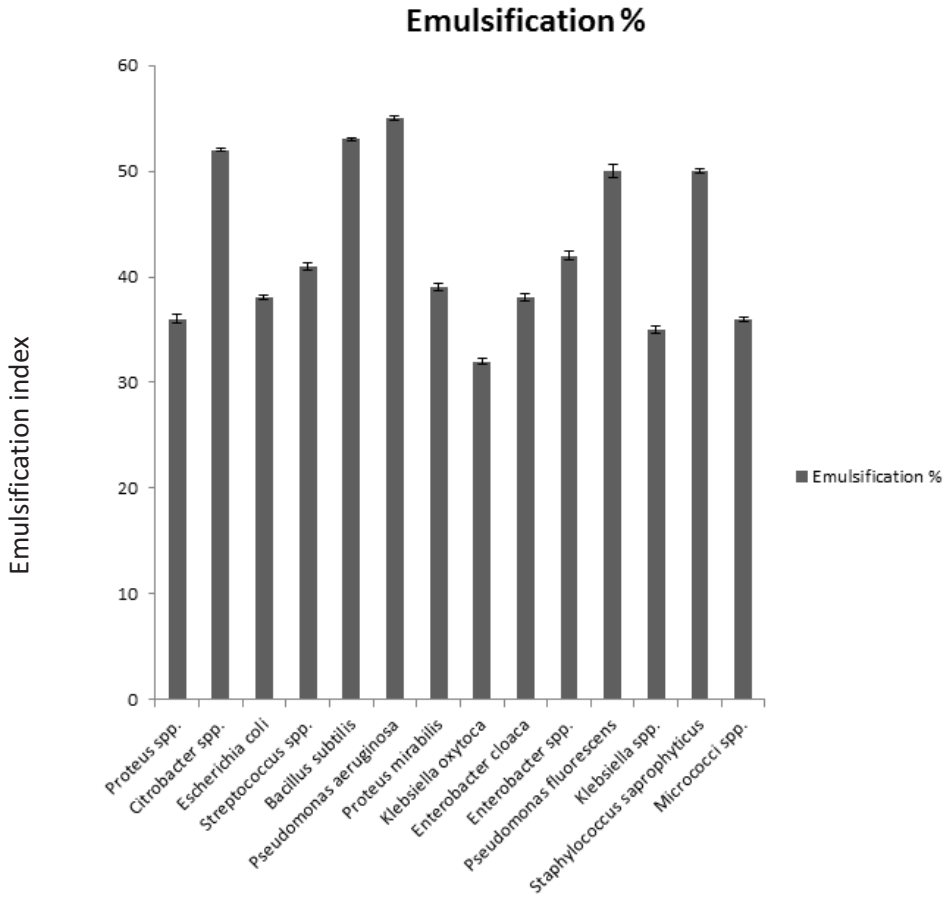

Figure 3: Percentage Emulsification Activity of Isolated Bacteria at 4 h

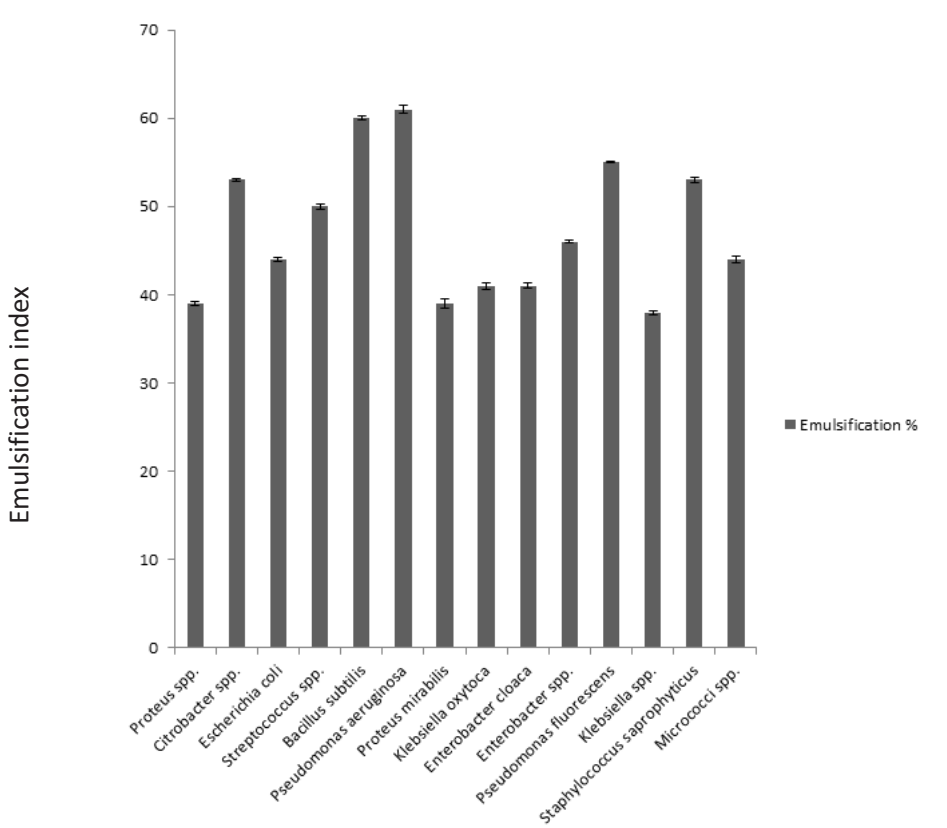

Figure 4: Percentage Emulsification Activity of Isolated Bacteria at 8 h

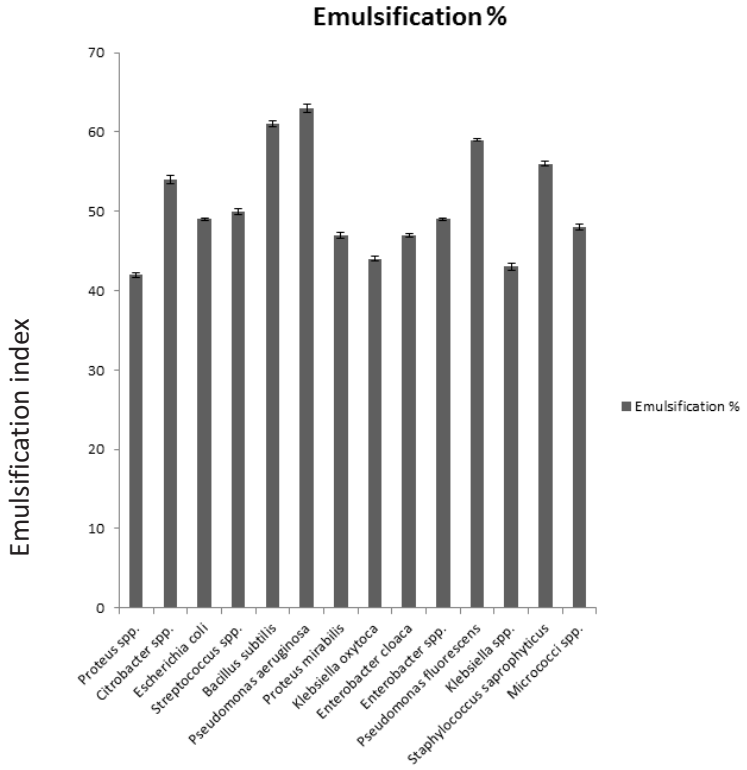

Figure 5: Percentage Emulsification Activity of Isolated Bacteria at 12 h

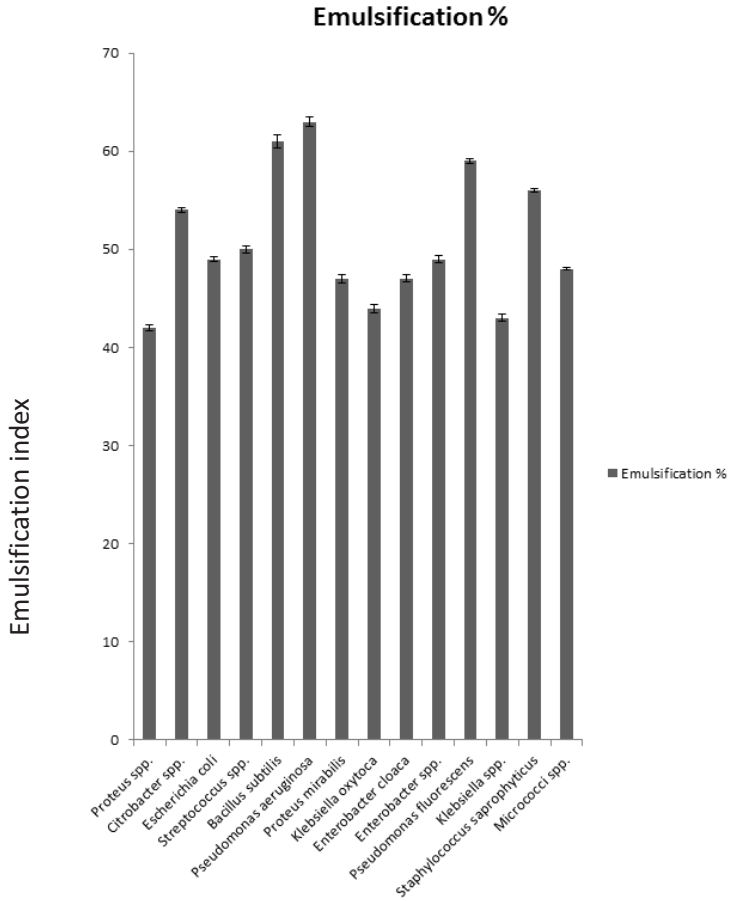

Figure 6: Percentage Emulsification Activity of Isolated Bacteria at 16 h

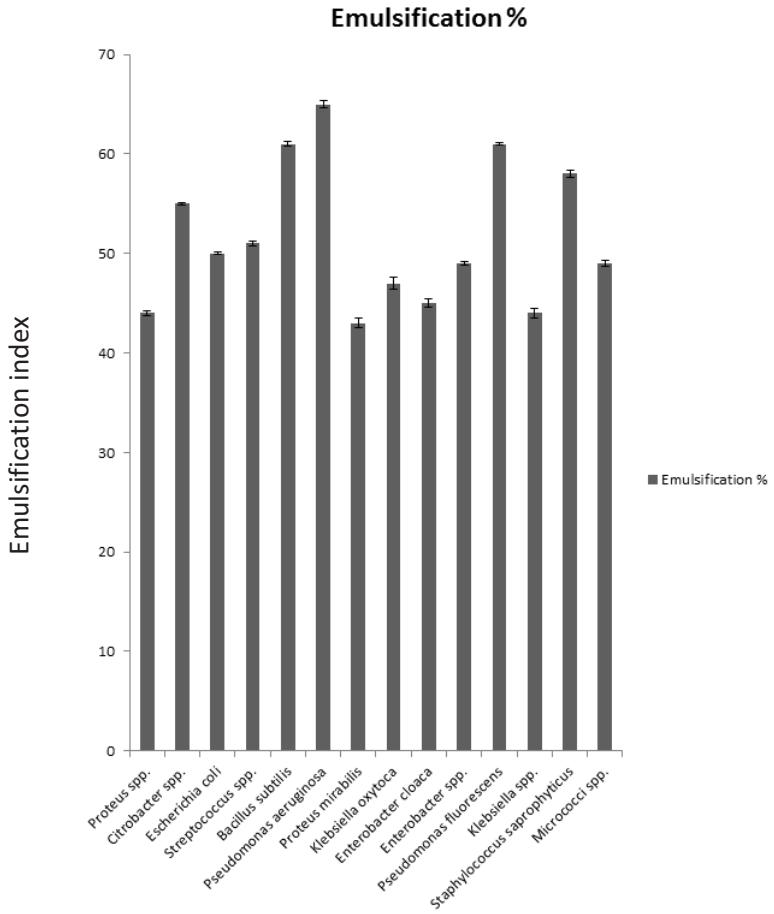

Figure 7: Percentage Emulsification Activity of Isolated Bacteria at 20 h

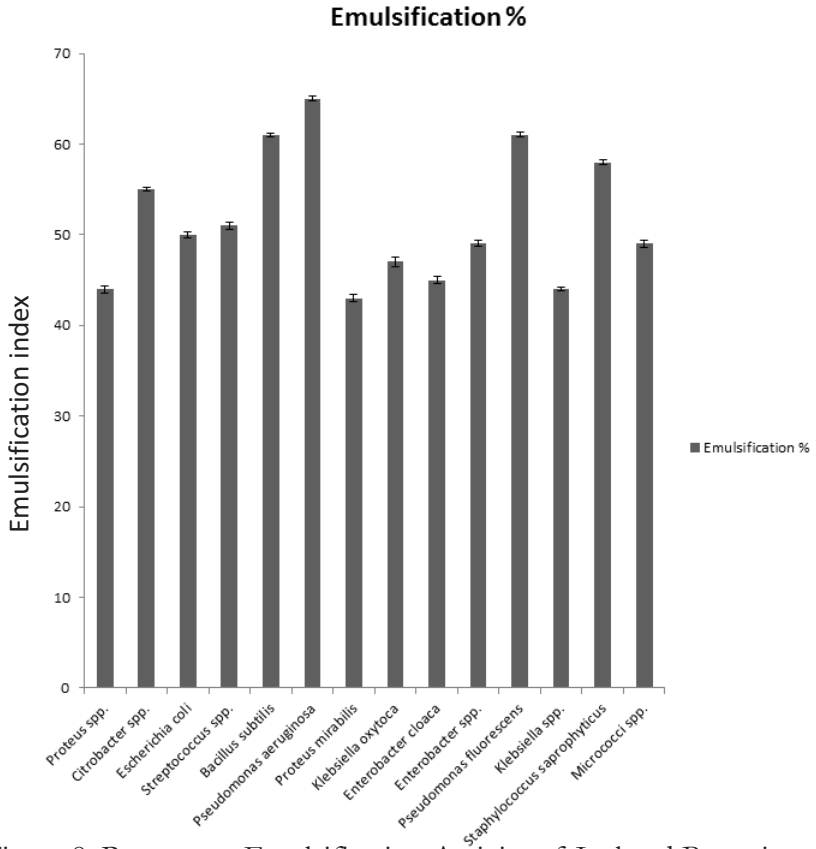

Figure 8: Percentage Emulsification Activity of Isolated Bacteria at 24 h

DISCUSSION

Occurrence of Gram negative biosurfactants producing organisms has been reported by some workers (Bodour *et al.*, 2003). Biosurfactant-producing Gram-positive rods have also been reported by many workers (Yakimov *et al.*, 1995). Tabatabaee *et al.*, 2005 and Rashedi *et al.*, 2005 reported the isolation of Gram positive and Gram negative emulsifying biosurfactant-producing bacteria as an indication of biosurfactant production.

The different bacterial isolates obtained from the oil contaminated soils showed that the soils supported the growth of a wide diversity of heterotrophic bacteria and this affirmed the ubiquity of microorganisms (Willey *et al.*, 2008).
Also, the biosurfactant-producing bacterial isolates screened from the oil-contaminated soils showed very good tension-active and emulsifying activities. The drop collapse test is indication of the surface and wetting activities.

Ability of the isolates to test positive for surface active test, drop collapse test, bacterial adhesion to hydrocarbon assay and emulsification assay points to the fact that the organisms are good and potential isolates for biosurfactant production.

CONCLUSION

From this study, different bacterial isolates were isolated from hydrocarbon-contaminated soil samples from three different automobile workshop locations in Abeokuta and from this isolates, *Pseudomonas aeruginosa* showed good potential for producing biosurfactants; however more research needs to be done to optimize this potential.

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