COMPARATIVE STUDY ON IDENTIFICATION OF BIOSURFACTANT PRODUCING BACTERIA USING THREE SCREENING TESTS

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Abstract. This study ascertained the comparative effectiveness of three screening assays, β hemolysis, emulsification index (E24) and oil displacement as a preliminary screening test to identify biosurfactant producing bacterial isolates. Evidently, 52 bacterial species were isolated from environmental samples and 29 of them (56%) screened negative using β hemolysis whereas 23 (44%) were positive. The emulsification index and oil displacement were carried out on 4 hydrocarbons (kerosene, crude oil, petrol, diesel) and power vegetable oil. Most of the 52 bacterial isolates emulsified and displaced the test hydrocarbons and power vegetable oil to varying levels. The E24 showed that out of the 29 hemolytic negative bacteria, 18 (62.1%) emulsified kerosene; 8 (27.6%) emulsified crude oil, 13 (44.8%) emulsified vegetable oil; 22 (75.9%) emulsified petrol and 10 (34.5%) emulsified diesel. Furthermore 69% had emulsification index above 45% indicating the test isolates as potentially suitable for microbially enhanced oil recovery [MEOR]. The oil displacement results equally showed that 15 (51.7%) displaced kerosene, 22 (75.9%) displaced crude oil; 16 (55.2%) displaced vegetable oil and 15 (51.7%) displaced both petrol and diesel respectively. This study showed that comparatively, petrol and crude oil were emulsified and displaced respectively by the bacteria isolates more than other oil sources; hence it is evident that the type of hydrocarbon affects the E24. Consequently, E24 and oil displacement are recommended to be the preliminary screening tests for identification of biosurfactant producing bacteria as the lyses of red blood cells in β hemolysis by the bacteria might be affected by some inherent factors.

Keywords: Bio-surfactant; β hemolysis; emulsification index; hydrocarbons; oil displacement.

INTRODUCTION

Bio-surfactants are produced by diverse groups of micro organisms isolated from environmental samples. They are amphiphilic compounds that contain both hydrophobic and hydrophilic moieties and have been most recently the subject of interest as alternatives to chemical surfactants, which are used in environmental clean ups with negative effects like persistence, accumulation in the food chain and biorecalcitrance. Bio-surfactants have been found by many authors [1-4, 6, 10, 19, 20, 24, 26, 27] to have a wide range of applications in the oil, pharmaceuticals, cosmetics and food industries. This is due to the fact that they are non-toxic, biodegradable, bio-compatible, non-persistence, active at extreme temperatures etc.

Micro organisms being ubiquitous in nature occur in different environmental samples and to identify the bio-surfactant producers there is need to use the best screening method to obtain high yield bio-surfactant producing bacteria for specific applications. The choice of a particular method for identification can result in eliminating potential bio-surfactant producers that may have shown a negative result for one test and positive for another test [20]. Screening of bio-surfactant-producing bacteria from samples contaminated by hydrocarbons constitutes a powerful tool for the selection of strains with high emulsifying capacity.

There are different screening methods for identifying bio-surfactant producing bacteria. These include: β haemolysis test, Centriamide test (CTAB)/methylene blue test, oil displacement test, drop collapse, tilted glass slide, and emulsification index test. It is however, difficult to detect the biosurfactant producers using a single method. In view of this, it appears that combinations of screening methods are needed to understand the ability of a single microbe in producing biosurfactant [7, 20]. This study was undertaken to compare three screening methods, β haemolysis, emulsification index and oil displacement for biosurfactant production, with the view to elucidate the appropriate method to be recommended or applied as a preliminary screening test for identifying bio-emulsifying organisms for use in biodegradation of hydrophobic compounds.

MATERIALS AND METHODS

Sample collection

The samples used in this study were collected from both hydrocarbon contaminated and non hydrocarbon contaminated environments. The samples were soil from automobile workshop (AMW), crude oil polluted Ogoni (CSS), kerosene stand (KSS), petrol stand (PFS), diesel stand (DCS), palm oil mill (POM), abattoir (AS), metal dump sites (WMS), cassava mill (CMS), waste battery dump site (WBS), whereas the water samples were from crude oil polluted Ogoni river sample (CWS), swimming pools (PWS) and fish ponds (FPS). On each sampling day, samples were collected within 2 h using surface sterilized containers and immediately transported to the Environmental Biology Laboratory of Federal Polytechnic Nekede, Owerri for further analysis. Surface sterilization was carried out using modified method of Yee *et al.*, [28] as described by Ogbulie *et al*., [14, 15]. This was done by washing the containers and steeping in hypochlorite solution for 45 min, followed by rinsing with absolute ethanol for

30 min. Thereafter the containers were rinsed thrice with sterile water for 10 min per rinse.

Microbial isolation

The biosurfactant producing bacteria were isolated from the soil samples and water samples using the method of Kumari *et al*., [10] with slight modification. The test soil samples (2 g each) and 5 ml of water samples were transferred separately into 100 ml of sterilized minimal salt medium (MSM) at pH 7 ± 0.2 in 750 ml Erlenmeyer flask and incubated at 37^{o}C for 2 days. The MSM composition was 2 g mono potassium phosphate (KH_2PO_4), 0.5 g sodium citrate, 1 g ammonium sulphate $(NH_4)_2SO_4$, 0.1 g magnesium sulphate ($MgSO_4$) in 1L of distilled water. After 2 days of incubation, 1 ml of active inoculum was transferred to a flask containing fresh 100 ml sterilized MSM supplemented with crude oil (0.5 ml). The Ogoni soil and river samples were supplemented with both crude oil and diesel as the carbon source (CSS_D and CSS_C; CWS_D and CWS_C). These were incubated at 37^{o}C for one week after which aliquots of the active inocula were spread over solidified nutrient agar. Subsequent incubation was at 37^{o}C for 24 h and discrete colonies were selected, subcultured by streaking to obtain pure culture, and preserved in a slant at 4^{o}C prior to screening for bio-surfactant production.

Screening of isolates for biosurfactant production

Bio-surfactant production activity of bacterial isolates was determined using different screening assays such as β-hemolysis, oil spreading test and emulsification index test (E_{24}).

β-hemolysis test

This was carried out by streaking each solate on blood agar medium and incubating at 37^{o}C for 72 h. Thereafter the plates were observed for β- hemolytic activity evidenced by defined clear zone around the colony, indicative of bio-surfactant production [1, 5, 12, 16-18, 25].

Oil spreading test

It is a method to determine the diameter of the clear zone which occurs after adding biosurfactant containing solution on an oil-water interface. The diameter evaluation is proportional to the surface tension reduction efficiency of a given biosurfactant. This was carried out by adding 50 ml of distilled water into large Petri plates (9 cm diameter) followed by addition of 1 ml of oil to the centre of the plates containing distilled water (i.e. onto the water surface). Then 0.1 ml of the supernatant of the isolate cultures (cell-free extracts) was added to the centre of the oil. The diameter and the clear halo visualized under visible light were measured after 30 sec to determine the displacement values [1, 5, 7, 16-21].

Emulsification index test (E_{24})

To determine the emulsification capacity, a mixture of ml of oil (diesel, engine oil, kerosine, crude oil) was added to the same amount of cell free supernatant in test tubes and vortexed at high speed for 2 min and left to stand for 24 h. The emulsification index (E_{24}) was calculated as the height of emulsified layer (mm) divided by total height of the liquid/aqueous column (mm), multiplied by 100 [5, 7, 13, 17-21, 24, 25].

RESULTS

β-hemolysis screening for biosurfactant production

The β hemolysis test is a commonly preferred method to screen bio-surfactant producing bacteria but this study found that varying positive results were recorded for other screening test methods for all the hemolytic negative isolates. The β hemolysis test showed that out of the 52 solates, 23 isolates (44%) were positive and 29 isolates (56%) were negative for bio-surfactant production (Tables 1, 2). These hemolytic positive bacteria were isolated from abattoir soil (AS 1, 2, 3), automobile workshop (AMW 2, 3, 5), crude oil polluted Ogoni soil ($CSS\ 1_D$), cassava mill soil (CMS 1 - 6), kerosene soil (KSS 1, 2), palm oil mill soil (POM 1& 4), waste metal dumpsite soil (WMS 1- 3) and waste battery dumpsite soil (WBS 1, 3, 6).

Emulsification index (E24) and Oil displacement screening tests for biosurfactant production

The E24 showed that 38.46%, 73.1%, 51.9%, 19.2% and 67.3% of the 52 isolates could not emulsify kerosine, crude oil, vegetable oil, petrol and diesel, respectively [Fig 1-5]. This indicates that the type of hydrocarbon also affects the E24; hence petrol was emulsified more by the isolates for recording the lowest failed emulsifying activity. Eleven (11) of the 23 bacteria isolates that screened positive for β haemolysis exhibited emulsification index values (E24) below 45% on the different hydrocarbons whereas, the other 12 exhibited high E24 values, including AS2 (46.88% on Petrol [Fig 4]), AMW4 (51.4% on Kerosene [Fig 1]), AMW5 (53.31% on Kerosene [Fig 1]; 47.2%, on Diesel [Fig 5]), $CSS1_D$ (45.95% on Kerosene [Fig 1]; 57.14% on Diesel [Fig 5]), CMS1 (57.41% on crude oil [Fig 2]; 68.52% on Vegetable oil [Fig 3]), CMS2 (58.33% on crude oil [Fig 2] ; 64.15% on Vegetable oil [Fig 3]), CMS4 (48% on Vegetable oil [Fig 3]), WMS1 (45% on Petrol [Fig 4]) WMS2 (51.61% on Petrol [Fig 4]), WMS5 (46.67% on Petrol [Fig 4]), WBS1 (78.13% on Kerosene [Fig 1]), WBS3 (45% on Petrol [Fig 4]).

It was evident that 44.2% of the bacterial isolates from the test soil samples failed to displace kerosene in the oil displacement assay. Likewise, 19.2%, 48.1% and 51.9% could not displace crude oil, vegetable oil and petrol/diesel, respectively [Fig 6-10]. Crude oil was displaced more by the test isolates indicating that the oil displacement method using crude oil will select more potential biosurfactant producing isolates. Furthermore, 62.1%, 27.6%, 44.8%, 75.9% and 34.5% of the isolates that showed negative β-hemolytic ability

Table 1. Positive bio-surfactant producers based on β hemolytic test

S/N	Isolates	β-Haemolysis	Emulsification index (E24%) on Hydrocarbon					Oil displacement test on hydrocarbon (cm)				
			Kerosene	Crude oil	Vegetable oil	Petrol	Diesel	Kerosene	Crude oil	Vegetable oil	Petrol	Diesel
1.	KSS 1	**+ve**	0	0	28.60	33.30	0	2.00	4.50	0	0	2.00
2.	KSS 2	**+ve**	28.10	0	0	0	0	0	0	0	0	0
3.	AMW 2	**+ve**	31.40	0	0	0	0	0	1.00	1.00	1.00	4.50
4.	AMW 3	**+ve**	32.30	0	0	0	0	0	4.00	0	0	0
5.	AMW 5	**+ve**	53.31	0	28.60	25.60	47.20	1.00	6.00	0	0	0
6.	AS 1	**+ve**	42.85	0	0	32.26	0	0	1.50	0.50	0	0
7.	AS 2	**+ve**	0	0	5.13	46.88	40.00	2.50	4.50	1.00	0	0
8.	AS 3	**+ve**	37.10	0	0	16.10	0	1.00	2.00	1.00	2.00	4.00
9.	$CSS1_D$	**+ve**	45.95	6.06	2.56	57.14	0	0	0.20	3.50	0	0
10.	WMS 1	**+ve**	14.29	0	0	45.00	36.66	3.50	2.50	0.50	0	2.30
11.	WMS 2	**+ve**	50.00	0	0	51.61	32.26	1.40	1.50	0.10	1.30	2.50
12.	WMS 3	**+ve**	50.00	0	5.00	53.13	0	5.00	4.00	0.50	1.50	3.00
13.	WBS 1	**+ve**	78.13	0	0	22.60	0	2.50	3.00	0	2.00	2.00
14.	WBS 3	**+ve**	15.63	0	0	45.00	0	1.00	0.20	0	1.50	0
15.	WBS 6	**+ve**	42.42	0	0	38.70	48.39	1.00	1.00	0.50	0	0
16.	POM 1	**+ve**	31.25	0	0	33.33	0	1.00	0.40	0	0.50	0.40
17.	POM 4	**+ve**	0	0	0	20.00	37.50	1.00	1.00	1.50	0	0
18.	CMS 1	**+ve**	0	57.41	68.52	28.00	0	0	0.20	0	0	0
19.	CMS 2	**+ve**	0	58.33	64.15	33.33	0	1.00	4.50	1.50	1.00	0
20.	CMS 3	**+ve**	0	0	21.57	44.44	0	0.30	0	0.70	0.30	2.00
21.	CMS 4	**+ve**	0	17.14	48.00	40.63	0	0	0	0.60	1.00	0
22.	CMS 5	**+ve**	0	29.27	0	44.40	0	0.5	1.5	0	0	0
23.	CMS 6	**+ve**	0	32.65	28.89	35.71	0	0.50	3.00	0	0	0.70

Legend: KSS - soil from automobile workshop (AMW), CSS - crude oil polluted Ogoni soil sample, CWS - crude oil polluted Ogoni river sample, KSS - soil from kerosene stand, POM - soil from palm oil mill, AS - soil from abattoir, WMS - soil from metal dump sites, CMS - soil from cassava mill, WBS - soil from waste battery dump site.

Table 2. Negative bio-surfactant producers based on β hemolytic test

S/N	Isolates	B-Hemolysiss	Emulsification index (E24%) on Hydrocarbon					Oil displacement test on hydrocarbon (cm)				
			Kerosene	Crude oil	Vegetable oil	Petrol	Diesel	Kerosene	Crude oil	Vegetable oil	Petrol	Diesel
1.	KSS 3	**-ve**	8.10	0	0	14.30	0	1.00	6.00	0	0	0
2.	KSS 4	**-ve**	0	0	0	0	15.60	0	2.50	1.00	0	0
3.	KSS 5	**-ve**	50.00	0	0	0	0	2.00	0	1.00	0	6.00
4.	KSS 6	**-ve**	45.50	0	11.60	0	0	1.00	5.00	0	1.00	0
5.	AMW 1	**-ve**	31.40	0	0	0	0	0	5.00	2.00	1.00	1.00
6.	AMW 4	**-ve**	51.40	0	10.60	15.60	44.40	0	4.50	0	1.00	2.00
7.	AMW 6	**-ve**	53.10	0	0	0	0	0	4.50	1.00	1.00	0
8.	$CSS\ 1_C$	**-ve**	42.86	0	2.70	56.00	0	0	0	0	4.50	1.50
9.	$CSS\ 2_C$	**-ve**	60.00	9.38	2.63	68.00	36.36	1.00	5.00	0	1.00	0
10.	$CSS\ 2_D$	**-ve**	50.00	5.88	0	51.43	0	1.00	5.00	1.00	0	5.00
11.	$CSS\ 3_C$	**-ve**	3.23	0	0	67.86	0	0	4.00	2.50	5.00	0
12.	$CSS\ 3_D$	**-ve**	11.43	0	0	53.57	0	0	1.50	0	0	0
13.	WMS 4	**-ve**	0	0	13.51	41.94	2.70	2.00	0	0	1.50	1.50
14.	WMS 5	**-ve**	40. 00	0	2.56	46.67	25.00	0	0.30	0	0	0
15.	WMS 6	**-ve**	0	0	5.00	37.50	45.16	2.00	0	0	0.50	1.50
16.	WBS 2	**-ve**	48.39	0	12.50	0	0	0	0.50	0.60	0	3.00
17.	WBS 4	**-ve**	31.25	0	0	0	0	0	1.50	0.60	0	0
18.	WBS 5	**-ve**	3.33	0	0	3.44	31.25	0	0.50	2.00	0	1.00
19.	POM 2	**-ve**	46.90	0	0	37.50	0	0	0	0	1.00	0
20.	POM 3	**-ve**	17.24	0	0	35.71	42.85	0	0	0	0	0
21.	POM 5	**-ve**	0	0	0	34.37	0	1.00	1.50	0	0.40	2.00
22.	POM 6	**-ve**	15.63	0	0	45.00	15.62	0	6.00	2.00	0	1.00
23.	$CWS\ 1_C$	**-ve**	0	0	66.67	17.39	40.0	3.00	0.40	0	0	1.00
24.	$CWS\ 1_D$	**-ve**	0	50.00	54.55	37.50	0	0.50	6.00	0.20	2.00	0
25.	$CWS\ 2_C$	**-ve**	0	13.89	39.22	7.80	45.00	1.00	0.60	0.30	0	1.70
26.	$CWS\ 2_D$	**-ve**	0	50.00	57.69	42.86	0	0	1.20	0	1.00	0.50
27.	$CWS\ 3_C$	**-ve**	0	75.00	30.00	26.00	0	0	2.00	1.00	0.60	0.30
28.	$CWS\ 3_D$	**-ve**	0	7.50	0	47.62	0	0.70	0	3.00	0.50	0
29.	$CWS\ 4_D$	**-ve**	0	2.50	38.46	42.86	0	2.00	0.10	0	0	0

Legend: KSS - soil from automobile workshop (AMW), CSS - crude oil polluted Ogoni soil sample, CWS - crude oil polluted Ogoni river sample, KSS - soil from kerosene stand, POM - soil from palm oil mill, AS - soil from abattoir, WMS - soil from metal dump sites, CMS - soil from cassava mill, WBS - soil from waste battery dump site.

emulsified kerosene, crude oil, vegetable oil, petrol and diesel respectively [Fig 11-15]. On the other hand, the oil displacement assay on the β-hemolytic negative test isolates depict the displacement of 51.7%, 75.9%, 55.2% and 51.7% of kerosene, crude oil, vegetable oil and petrol/diesel respectively [Fig 16-20]. However, Figures 11-15 illustrates that 20 of the 29 negative β-hemolytic bacterial isolates had E_{24} values above 45% whereas the other 9 had values below 45%.

Varying emulsification index (E_{24}%) and oil displacement tests (O.D cm) values were observed amongst these 20 bacteria isolates per test soil samples. The recorded performance values of isolates from the different test soil samples (Table 2) depict that isolates from KSS5 emulsified 50% of Kerosene and displaced 2 cm, 1 cm, 6 cm of Kerosene, Vegetable oil, diesel respectively; KSS6 also emulsified 45.5% of Kerosene and displaced 1 cm, 5 cm, 1 cm O.D of Kerosene, Crude oil, Petrol respectively; AMW6 emulsified 53.1% of Kerosene and displaced 4.5 cm, 1 cm, 1 cm of Crude oil, Vegetable oil, Petrol respectively ; $CSS1_C$ emulsified 56% of Petrol and displaced 4.5 cm, 1.5 cm O.D of Petrol, Diesel respectively; $CSS2_C$ emulsified 60% & 68% of Kerosene & Petrol, and displaced 1 cm, 5 cm, 1 cm of Kerosene, crude oil, Petrol respectively; $CSS2_D$ emulsified 50% & 51.43% of Kerosene and Petrol, and displaced 1 cm, 5 cm, 1 cm, 5 cm of Kerosene, crude oil, Vegetable oil, Diesel respectively; $CSS3_C$ emulsified 67.86% of Petrol and displaced 4 cm, 2.5 cm, 5 cm of Crude oil, Vegetable oil, Petrol respectively, $CSS3_D$ emulsified 53.57% of Petrol and displaced 1.5 cm of only Crude oil; WMS3 emulsified 50% & 53.13% of Kerosene and Petrol, and displaced 5 cm, 4 cm, 1.5 cm, 3 cm of Kerosene, Crude oil, Petrol, Diesel respectively; WMS6 emulsified 45.16% of Diesel and displaced 2 cm, 1.5 cm of Kerosene, Diesel respectively, WBS2 emulsified 48.3% of Kerosene and displaced 3 cm of only Diesel; WBS6 emulsified 48.39% of Diesel and displaced 1 cm O.D of Kerosene and Crude oil each; POM2 emulsified 46.9% of Kerosene and displaced 1 cm of only Petrol; POM6 emulsified 45% of Petrol and displaced 6 cm, 2 cm, 1 cm of Crude oil, Vegetable oil, Diesel respectively; $CWS1_C$ emulsified 66.67% of Vegetable oil and displaced 3 cm, 1 cm of Kerosene, Diesel respectively, $CWS1_D$ emulsified 50% & 54.55% of Kerosene and Vegetable oil, and displaced 6 cm, 2 cm of Crude oil, Petrol respectively, $CWS2_C$ emulsified 45% of Diesel and displaced 1 cm, 1.7 cm of Kerosene, Diesel respectively; $CWS2_D$ emulsified 50% & 57.69% of Crude and Vegetable oil, and displaced 1.2 cm, 1cm of Crude oil and Petrol; $CWS3_C$ emulsified 75% of Kerosene and displaced 2 cm, 1cm of Crude oil and Vegetable oil respectively, while $CWS3_D$ emulsified 47.62% of Petrol and displaced 3 cm of Vegetable oil.

The other 9 isolates with E24 values less than 45% for all the five test hydrocarbons were those isolated from KSS3, KSS4, AMW1, WMS4, WBS4, WBS5, POM3, POM5 and $CWS4_D$. Isolates from KSS3 had the highest displacement value of 6 cm on Crude oil followed by AMW1 (5 cm) that also displaced 2 cm of Vegetable oil, 1 cm of Petrol and 1 cm of Diesel. Isolate from KSS4 had displacement values of 2.5 cm and I cm on Crude oil and Vegetable oil respectively. Similar displacement value of 1.5 cm each was respectively recorded for isolates from WMS4 (on Petrol and diesel) and WBS4 (crude oil). WBS5 on the other hand, had 2 cm and 1 cm displacement values on vegetable oil and diesel whereas POM5 respectively recorded O.D value of 1 cm, 1.5 cm and 2 cm for Kerosene, Crude oil and Diesel. While $CWS4_D$ had O.D value of 2 cm for only Kerosene, POM3 failed to displace any of the hydrocarbons (Table 2).

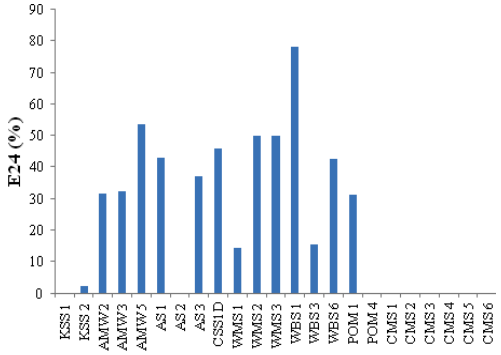

Figure 1. Emulsification index of β-hemolytic positive isolates on Kerosine

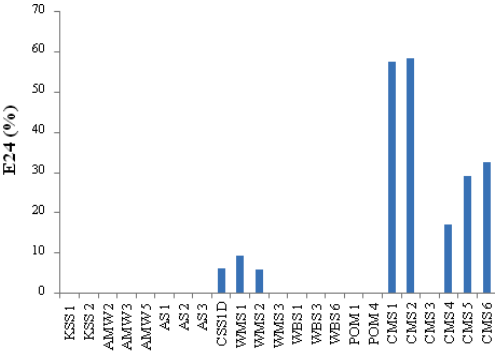

Figure 2. Emulsification index of β-hemolytic positive isolates on crude oil

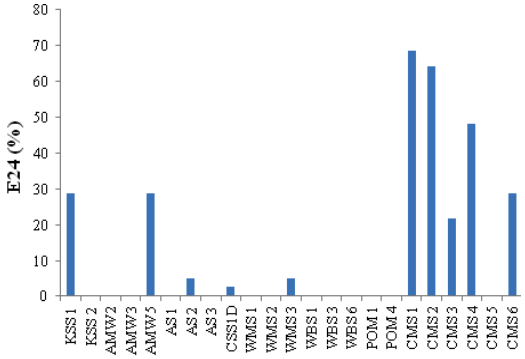

Figure 3. Emulsification index of β-hemolytic positive isolates on vegetable oil

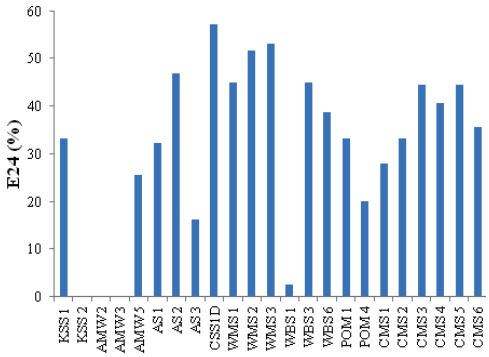

Figure 4. Emulsification index of β-hemolytic positive isolates on Petrol

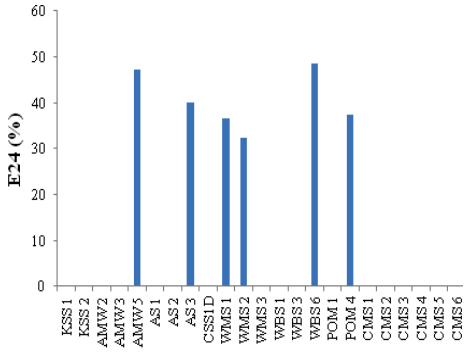

Figure 5. Emulsification index of β-hemolytic positive isolates on diesel

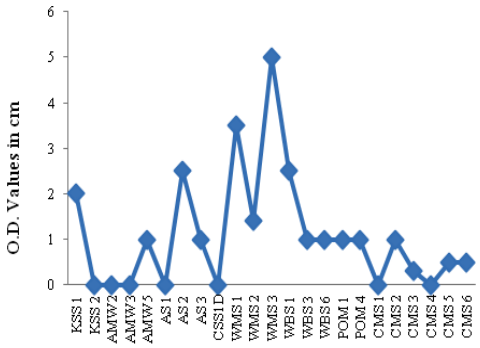

Figure 6. Oil displacement of kerosene by β-hemolytic positive isolates

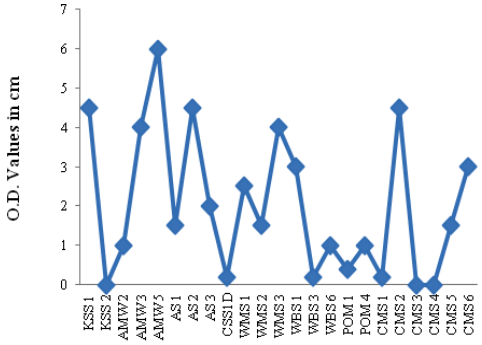

Figure 7. Oil displacement of crude oil by β-hemolytic positive isolates

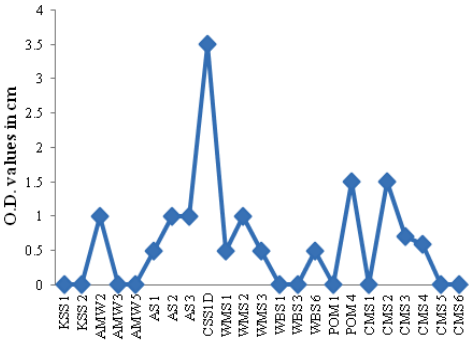

Figure 8. Oil displacement of vegetable oil by β-hemolytic positive isolates

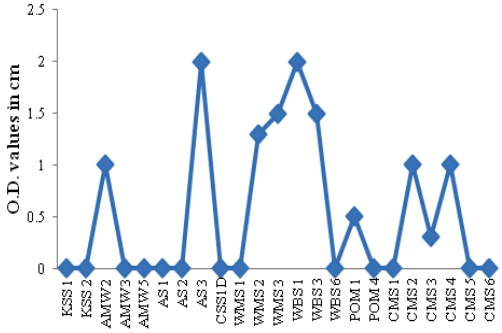

Figure 9. Oil displacement of petrol by β-hemolytic positive isolates

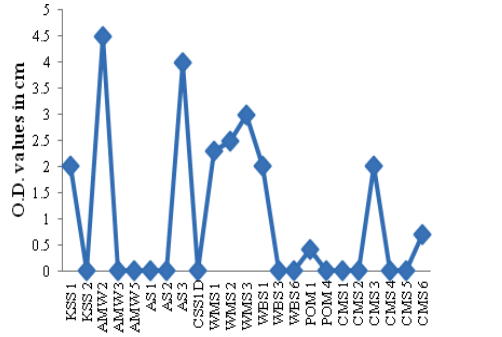

Figure 10. Oil displacement of diesel by β-hemolytic positive isolates

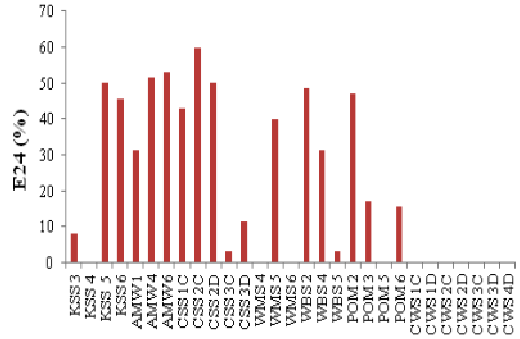

Figure 11. Emulsification index E24 of β-hemolytic negative isolates on kerosene

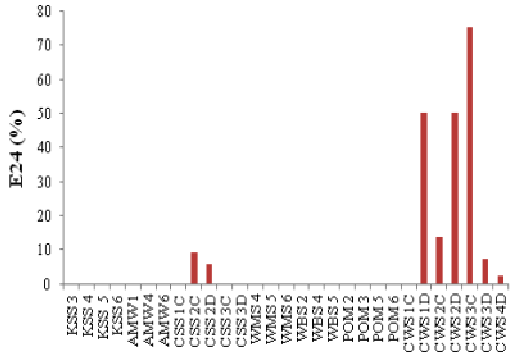

Figure 12. Emulsification index E24 of β-hemolytic negative isolates on crude oil

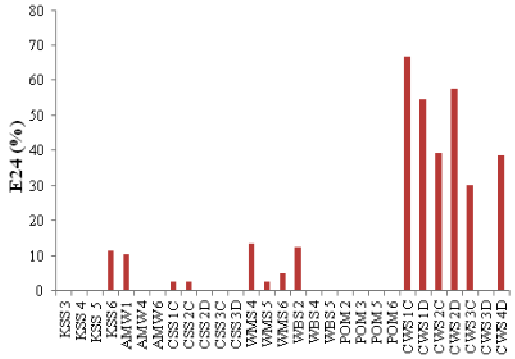

Figure 13. Emulsification index E24 of β-hemolytic negative isolates on vegetable oil

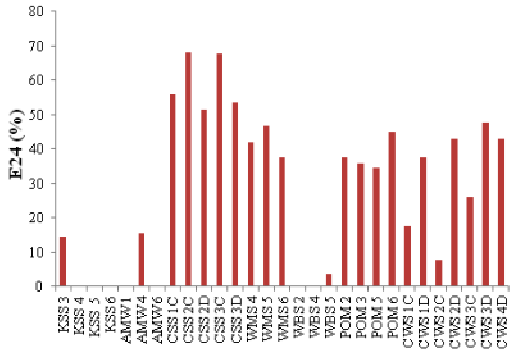

Figure 14: Emulsification index E24 of β-hemolytic negative isolates on petrol

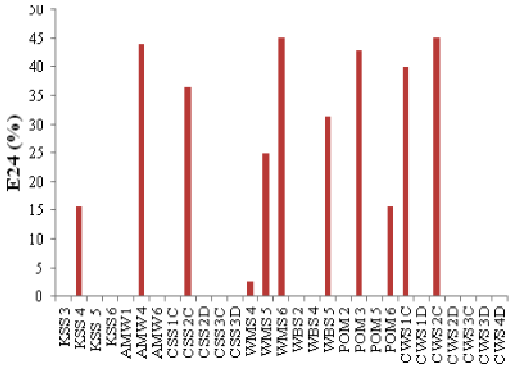

Figure 15. Emulsification index E24 of β-hemolytic negative isolates

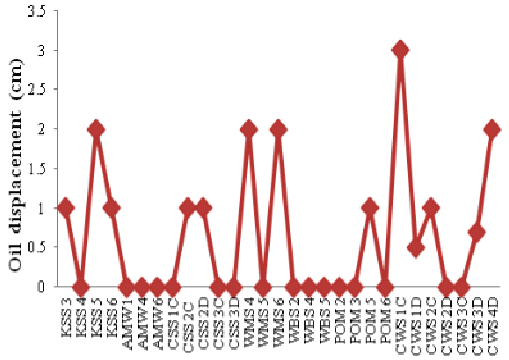

Figure 16. Oil displacement of kerosene by β-hemolytic negative isolates on diesel

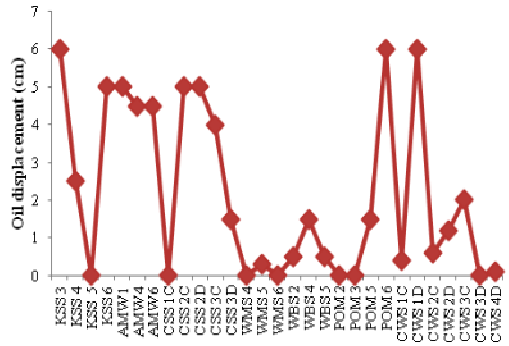

Figure 17: Oil displacement of crude oil by β-hemolytic negative isolates

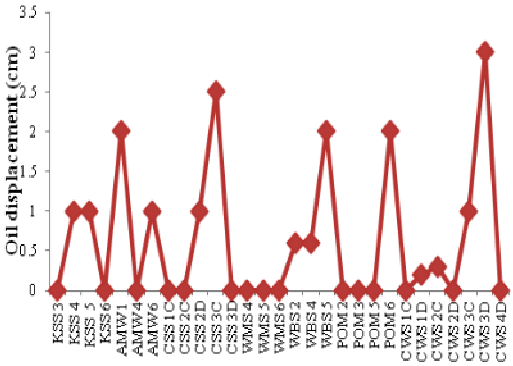

Figure 18: Oil displacement of vegetable oil by β-hemolytic negative isolates

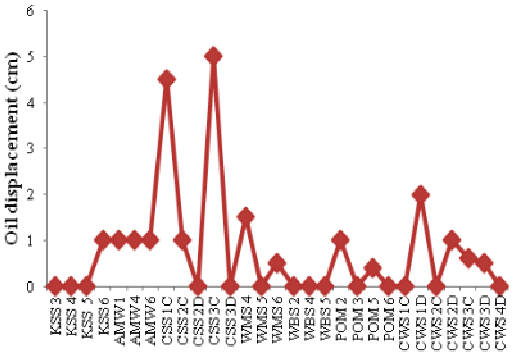

Figure 19: Oil displacement of petrol by β-hemolytic negative isolates

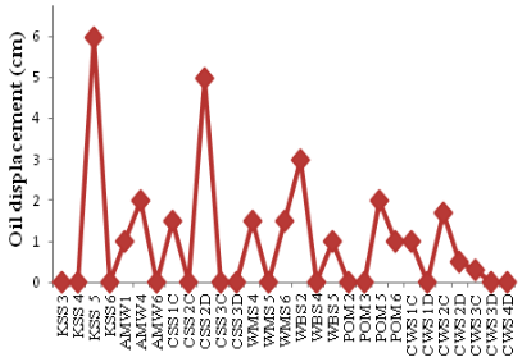

Figure 20. Oil displaement of diesel by β-hemolytic negative isolates

DISCUSSION

Comparative effectiveness of three screening assays were carried out which include, β hemolysis, emulsification index (E24) and oil displacement as a preliminary screening test to identify biosurfactant producing bacterial isolates. Evidently, 52 bacterial species were isolated from environmental samples and 29 of them (56%) screened negative using β hemolysis whereas 23 (44%) were positive. Most of the 52 bacterial isolates emulsified and displaced the test four hydrocarbons and vegetable oil to varying levels

The findings of this study showed that petrol was emulsified more by the bacteria isolates as only 19.2% of the 52 bacterial isolates failed to emulsify it while the oil displacement test showed that crude oil stands a better choice of hydrocarbon/substrate for selecting potential biosurfactant producers since it also recorded only 19.2% of failed displacement by the test isolates. However, in relation to individual isolate per source, the rate of emulsification of kerosene was highest (78.13%) by isolates from WBS1, Crude oil (75%) by $CWS3_C$, vegetable oil (68.52%) by CMS1, petrol (68%) by $CSS2_C$ and diesel (48.39%) by WBS6. Of these 5 bacteria isolates that produced the highest results, only 2 (WBS1 and CMS1) screened positive for biosurfactant production using β-hemolysis. In the displacement assay, the rate of oil displacement of kerosene was highest (5 cm) by WMS3, crude oil (6 cm) by KSS3, AMW5, POM6 and $CWS1_D$, vegetable oil (3.5 cm) by $CSS1_D$, petrol (5 cm) by $CSS3_C$ and diesel (6 cm) by KSS5. However, only 2 (AMW5 and $CSS1_D$) of these 8 bacteria isolates screened positive for β hemolysis test.

Furthermore, the result of this study suggest that β hemolysis test should not be used as a sole preliminary screening method for biosurfactant production; E_{24} and oil displacement tests, or both should be used. This is in agreement with the findings of Satpute *et al*., [23] and Kiran *et al.*, [9] who worked on the assessment of different screening methods for selecting biosurfactant producing marine bacteria. Their results suggest that β hemolysis test is not totally reliable and that a single method is not suitable to identify all types of biosurfactant. They also recommended that the drop collapse, tilted glass slide test, oil displacement test and E24 assay are more suitable for primary screening of potential biosurfactant producing bacteria. Karthik *et al*., [8] equally reported that the efficacy of the blood agar lysis in predicting biosurfactant production was not wholly reliable and suggested that the drop collapse method is well suited for primary screening method of biosurfactant production and the oil spreading method is good to quantify the biosurfactant. In the study of Youssef *et al.*, [29], β hemolysis gave 16% false positives and it excluded many potential biosurfactant producers, thus they suggested screening the cultures first using the drop collapse method, followed by spreading techniques and emulsification index. The report of Maneerat and Phetrong, [13] showed only 13.5% β hemolytic strains out of 200 marine bacteria isolated and the others were screened biosurfactant producers using drop collapse method and oil spreading method. Iqbal *et al.*, [6] and Lin *et al*., [11] contributed that other microbial products, such as virulence factor, lyses blood agar and biosurfactants that are poorly diffusible might not lyse blood cells and therefore it is not clear if blood agar lysis should be used to screen for biosurfactant production, although such screening can be used as a rapid method if positive results are subsequently checked in the emulsification index assay.

In contrast, Carrillo *et al.,* [3]; Fiebig *et al*., [4] and Thenmozhi & Nagathya, [26] observed that biosurfactant producing capacity in liquid medium was found to be associated with β hemolysis activity and concluded that β hemolytic activity therefore appears to be a good screening criterion for surfactant producing strains. An example is seen in the comparative results of the three screening methods which showed that the qualitative screening method β hemolysis is not as reliable in identifying potential biosurfactant producers as the two quantitative screening methods, the E_{24} and oil displacement tests. This was inferred from the 29 potential biosurfactant producers that screened negative using β hemolysis. The 29 bacteria isolates emulsified and displaced the different hydrocarbons used in the E_{24} test and oil displacement test to varying levels. Generally, the results indicated that 69% of the isolates that were eliminated using β hemolysis had emulsification indices above 45% indicating the test isolates as potentially suitable for microbial enhanced oil recovery [MEOR]. This corroborates the findings of Astuti *et al*., [2] who carried out research on screening and characterization of biosurfactants produced by *Pseudoxanthomonas* specie G3 and its applicability for enhanced oil recovery.

Indeed, there is need to explore more than two screening methods for identifying potential biosurfactant producers. This is to guide to avoid excluding positive producers that will be screened negative with a single method. The use of up to three screening methods or more is appropriate and from the findings of the study it is recommended that E_{24} and oil displacement be amongst the preliminary screening

tests for identification of potential biosurfactant producing bacteria as the ability to lyse red blood cells in β hemolysis test by the bacteria might be affected by some intrinsic factors in the bacteria. This study also demonstrated that the type of hydrocarbon could be a determining factor in the E_{24} rate. In addition, biosurfactant producing organisms of great significance in environmental cleanup operations can be found in other soil sources beside those previously contaminated with hydrocarbons.

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