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The emulsifying effect of biosurfactants produced by food spoilage organisms in Nigeria

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Abstract

Food spoilage organisms were isolated using standard procedures on Nutrient Agar, Cetrimide Agar and Pseudomonas Agar Base (supplemented with CFC). The samples were categorized as animal products (raw fish, egg, raw chicken, corned beef, pasteurized milk) and plant products (vegetable salad, water leaf (*Talinium triangulare*), boiled rice, tomatoes and pumpkin leaf (*Teifairia occidentalis*).They were characterised as *Pseudomonas putida, Pseudomonas aeruginosa, Pseudomonas stutzeri, Burkholderia pseudomallei, Serratia rubidaea, Corynebacterium pilosum, Bacillus subtilis, Bacillus mycoides, Bacillus laterosporus, Bacillus laterosporus, Serratia marcescens, Bacillus cereus, Bacillus macerans, Alcaligenes faecalis and Alcaligenes eutrophus.* Preliminary screening for biosurfactant production was done using red blood haemolysis test and confirmed by slide test, drop collapse and oil spreading assay. The biosurfactant produced was purified using acetone and the composition determined initially using Molisch's test, thin layer chromatography and gas chromatography mass spectrometry. The components were found to be ethanol, amino acids, butoxyacetic acid, hexadecanoic acid, oleic acid, lauryl peroxide, octadecanoic acid and phthalic acid. The producing organisms grew readily on several hydrocarbons such as crude oil, diesel oil and aviation fuel when used as sole carbon sources. The purified biosurfactants produced were able to cause emulsification of kerosene (19.71-27.14%) as well as vegetable oil (16.91-28.12%) based on the emulsification index. This result suggests that the isolates can be an asset and further work can exploit their optimal potential in industries.

Keywords: Biosurfactants; Hydrocarbon; Emulsification; Heteropolymers

1 Introduction

Biosurfactants or microbial surfactants are surface metabolites produced by bacteria, yeast and fungi having very different chemical structures and properties (Ron & Rosenberg, 2001). These biosurfactants are amphiphilic molecules of microbial origin whose hydrophobic and hydrophilic domains depend on the carbon substrate and the organism strain. These hydrophobic and hydrophilic properties have found application in an extremely wide variety of industrial processes involving emulsification, foaming, detergency, wetting, dispersing or solubilization (Gautam & Tyagi, 2006). Nowadays, biosurfactants are used in industries as a cosmetic and special chemical substances, food, pharmaceutics, agriculture, cleansers, enhanced oil recov-

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ery and bioremediation of oil-contaminated sites (Kitamoto, Isoda, & Nakahara, 2002). They are potential alternatives of chemically synthesized surfactant in a variety of applications because of their advantages such as lower toxicity, higher biodegradability, better environmental compatibility, lower critical micelle concentration, ease of production, ability to be synthesized from renewable resources, higher foaming, higher selectivity, specific activity at extreme temperature, pH and salinity (Ilori, Amobi, & Odocha, 2005). Many microorganisms have the ability to produce a wide range of biosurfactants, as such initial classification was made into two; based on molecular weights, properties and cellular localizations. The low molecular weight biosurfactants e.g. glycolipids, lipopeptides, flavolipids, corynomycolic acids and phospholipids, lower the surface and interfacial tensions at the air/water interfaces while the high molecular weights are called bioemulsans (such as emulsan, alasan, liposan, polysaccharides and protein complexes) and are more effective in stabilizing oil-in-water emulsions (Franzetti et al., 2009). Scientists have commonly turned to contaminated sites as sources for the isolation of biosurfactant-producing and hydrocarbon-degrading microorganisms which have produced varied results such as *Peudomonas fluorescens* from petroleum-contaminated soil (Barathi & Vasudevan, 2001), naphthalene degrading *Bacillus pumilis* strain isolated from oil sludge (Calvo, Toledo, & Gonzalez-Lopez, 2004), and *Candida glabrata* UCP 1002 obtained from petroleum hydrocarbons contaminated soil samples (Sarubbo, de Luna, & de Campos-Takaki, 2006). An alternative and largely untapped source of biosurfactant-producing bacteria is food. The objective of this work is to isolate biosurfactant-producing bacteria from food waste as an alternative to environmental samples (water and soil) and to also screen for emulsification ability.

2 Materials and Methods

2.1 Sample collection

Food samples were obtained from kitchen wastes and transported to the laboratory in sterile plastic containers. The crude oil, aviation fuel, diesel oil, kerosene and cooking oil (Gino vegetable oil) were obtained from local sources.

2.2 Isolation of organisms

The microbial population in the food samples was assayed by standard plate count method using the spread plate technique. For each sample, 20 g was aseptically removed and homogenised for 2 min in 180 ml Peptone water (Lab m laboratories, UK). A tenfold serial dilution (10^{-1} to 10^{-8}) was prepared and an aliquot of 0.1 ml plated in triplicate on Nutrient Agar (Biotec laboratories, UK), Pseudomonas Agar base (supplemented with CFC) (Biomark laboratories, India) and Cetrimide Agar (Lab m laboratories, UK).

2.3 Preliminary test for biosurfactant production

Haemolysis

This was done using the method developed by Mulligan, Cooper, and Neufeld (1984). Colonies from 18 h old cultures of the isolates were inoculated on Sheep Blood Agar plates and incubated for 48-72 h at 37°C. Positive strains cause lysis of the blood cells and exhibit a colourless, transparent ring around the colonies.

Oil spreading assay

The oil spreading assay was developed by Morikawa, Hirata, and Imanaka (2000). This was done by placing 10 μl of crude oil on the surface of 40 ml of distilled water in a Petri dish to form a thin oil layer. Culture supernatant (10 μl of 10^{-6} cfu/ml) was gently placed on the centre of the oil layer. If biosurfactant is present in the supernatant, the oil is displaced and a clearing zone is formed. The diameter of this clearing zone on the oil surface is the surfactant activity which is the oil displacement activity.

Drop collapse assay

Cell free supernatants obtained from the isolates when grown in Nutrient Broth (Lab m laboratories, UK) for 24 h were tested for their ability to

collapse on the surface of an oil drop, as described by Jain, Lee, and Trevors (1992). This was done by adding 40 μl of cooking oil (vegetable oil) to the surface of a clean glass slide and allowing to equilibrate to room temperature (25°C) for 1 h. A loopful of an overnight culture of each isolate was suspended in 150 μl sterile distilled water and 50 μl of the cell suspension was then added to the surface of an oil drop. The shape of the drop on the surface of oil was inspected visually after 1 – 2 min. Biosurfactant-producing isolates produced collapsed drops while those that did not remained stable. Sterile distilled water was used as the control.

Slide test

A wire loop was aseptically used to pick an inoculum from a 24 h old culture on Nutrient Agar (Lab m laboratories, UK). A droplet of Normal Saline (0.85% Sodium Chloride) was placed on it to make a wet preparation of the bacterial isolate on a grease free slide. The slide was slanted at 45°C and then observed visually for the flow of the wet preparation over the surface of the glass slide. Flow of the wet preparation of bacteria over the glass slide was recorded as a positive result (Olutola, Famurewa, & Sonntag, 2000)

2.4 Data Analysis

The biosurfactant-producing isolates were characterized and identified on the basis of their cultural and cellular morphologies, Gram reactions and several biochemical reactions using the taxonomic Schemes of Bergey's Manual of Determinative Bacteriology (Holt, Krieg, Sneath, Stalely, & Williams, 1994) and API 20 NE (Analytical Profile Index) kit (BioMérieux, USA).

2.5 Biosurfactant production and hydrocarbon utilization assay

The isolates were cultured on Nutrient Agar, incubated at 37°C for 24 h. After 24 h, a loopful of each isolate was inoculated into 9 ml of sterile distilled water, shaken thoroughly for even distribution and 1 ml aliquot transferred into separate 100 ml minimal salt media, supplemented with 1% hydrocarbon as carbon source (Barathi & Vasudevan, 2001). Crude oil, diesel oil and aviation fuel were used. It was incubated at 30°C on a shaker incubator (Sheldon Manufacturing, Inc., USA) at 120 rpm for 21 days. Hydrocarbon utilization assay was carried out by monitoring the growth pattern of isolates every 72 h by cell count and visual observation. To obtain a viable cell count, a tenfold serial dilution (10^{-1} to 10^{-8}) was plated in duplicate using the pour-plate technique on Nutrient Agar and incubated aerobically at 37°C for 24h with an un-inoculated flask as control. Increase in cell number and physical disappearance of the hydrocarbon indicates utilization (Adebusoye, Amund, Ilori, Domeih, & Okpuzor, 2008).

2.6 Biosurfactant extraction and purification

Pure culture of each of the biosurfactant-producing isolates was inoculated into 100 ml minimal salt media, supplemented with hydrocarbon as carbon source and incubated for 21 days. Culture broth was centrifuged at 5,000 rpm for 20 min at 4°C to obtain cell free supernatant. The biosurfactant was extracted by adding equal volume of acetone to the supernatant and incubated at 4°C for 24 h. The mixture was centrifuged at 5,000 rpm for 20 min at 4°C and the pooled extracts evaporated to dryness over a water bath at 45-50°C (Patil & Chopade, 2001).

2.7 Biochemical characterization of biosurfactants

Test for carbohydrate

This test was carried out using Molisch's test (Pinzon & Ju, 2009). Two drops of Molisch's reagent (α-naphtol dissolved in ethanol (Sigma-Aldrich, Dorset, UK)) was added to 2 ml of the cell free supernatant obtained by centrifugation and then mixed together. After mixing, 2 ml of concentrated sulphuric acid was added slowly down the side of the test tube placed in a sloppy condition without mixing to form a bottom layer. The positive reaction was indicated by the ap-

pearance of a purple ring at the interface between the acid and the test layers.

Test for peptides and lipids

Thin layer chromatography (TLC) of the extracted biosurfactants from the I8 isolates was carried out by spotting 10 μl volume of extracted biosurfactant dissolved in methanol (Sigma-Aldrich, Dorset, UK) onto silica gel aluminium TLC plates (FMC Biopolymer, Philadelphia, PA, USA)and developed in chloroform:methanol:water (65:15:2, vol/vol/vol) (Sim, Ward, & Li, 1997). For detection of peptides the plates were air dried, sprayed with ninhydrin (0.2% in 95% ethanol), air dried once more and heated at 110°C for 10 min. Plates were also viewed under UV light before staining with ninhydrin while for detection of lipids, the plates were stained with iodine. Migration distances of sample spots, before and after staining, relative to the mobile phase (Rf values) were calculated.

2.8 Component identification of biosurfactants

The biosurfactants were analyzed by gas chromatography mass spectrometry (GC-MS) for components identification (Yakimov, Timmis, Wray, & Fredrickson, 1995). The partially purified biosurfactant was converted into a volatile compound using exane (Sigma-Aldrich, Dorset, UK).The exane was removed from the sample by decanting it into a beaker using a separating funnel, cotton wool was put in a Pasteur pipette then anhydrous sodium sulphate (Sigma-Aldrich, Dorset, UK) was poured into it before pouring the sample to remove the remaining moisture from the sample. The filtered sample was then allowed to stand for 2 min to dry before pouring into a GC vial. GC-MS analysis was performed using Agilent 6890N with Agilent Technologies (United States) 123-5032 DB-5 (30 m × 0.25 mm × 0.25 μm) capillary column and SGE 10 μL syringe. Distillate samples (1 μL) were injected and oven temperatures was programmed from 60°C to 200°C at the rate of 6°C/min and then isothermally held for 11 min until the analysis was completed then the peaks were compared with standards.

2.9 Emulsification capacity assay

The assay for emulsification capacity of biosurfactants was developed by Cooper and Goldenberg (1987). Kerosene and cooking oil (vegetable oil) were used. The isolates were grown in Nutrient Broth at 37°C for 24 h; the supernatant of the biosurfactant-producing isolates was obtained by subjecting each isolate contained in the Nutrient Broth to centrifugation (5 min, 5000 rpm). Three ml of kerosene and cooking oil was added to 2 ml of cell free supernatant for each isolate differently and then vortexed for 2 min using a vortex mixer (Eppendorf, North America). It was then allowed to stand for 24 h and the emulsification index was measured and recorded using the formula below. Sterile Nutrient Broth served as a control. The emulsion index E_{24} is calculated as the ratio of the height of the emulsion layer and the total height of liquid:

$$E_{24} = \frac{\text{Height of emulsion formed}}{\text{Total height of solution}} \times 100 \quad (1)$$

3 Results and Discussion

3.1 Results

Isolation

A total of 54 bacterial isolates were isolated from the food wastes. Twenty organisms were isolated from Nutrient Agar (NA), 23 from *Pseudomonas* Agar Base (PAB) and 11 from Cetrimide Agar (CE). The isolates were designated R_1, Fish PAB, TO_2 CE, TO_1.....$EPAB_2$1.

Biosurfactant producing isolates

Eighteen isolates out of the 54 isolates obtained were found to produce biosurfactants (Table 1).

Identification of isolates

The phenotypic characterization of the isolates revealed the presence of *Pseudomonas*

Table 1: Biosurfactant producing ability of isolates

S/NO	Isolate code	Organisms	Origin	Hemolysis	Slide test	Drop Collapse	Oil Spreading Assay
1	R_1	*Pseudomonas stutzeri*	rice	+[a]	-[b]	+	+
2	Fish PAB	*Burkholderia pseudomallei*	fish	+	+	+	+
3	$EPAB_2^2$	*Serratia rubidaea*	egg	+	+	-	+
4	ENA_12	*Alcaligenes eutrophus*	egg	+	+	+	+
5	$EPAB_21$	*Burkholderia pseudomallei*	egg	+	+	+	+
6	W_1 CE	*Pseudomonas putida*	water leaf	+	-	+	+
7	W_2 CE	*Pseudomonas aeruginosa*	water leaf	+	-	+	+
8	W_3 CE	*Bacillus mycoides*	water leaf	+	+	+	+
9	W_4 PAB	*Pseudomonas stutzeri*	water leaf	+	+	+	+
10	CB_2 LP	*Bacillus laterosporus*	corned beef	+	+	+	+
11	TO_1	*Alcaligenes faecalis*	tomatoes	+	+	+	+
12	TO_2	*Bacillus macerans*	tomatoes	+	+	+	+
13	PL	*Bacillus cereus*	pumpkin leaf	+	-	+	+
14	$SPAB_2$	*Corynebacterium pilosum*	salad	+	+	+	+
15	MNA_1	*Serratia marcescens*	milk	+	-	+	+
16	$MNA1_3$	*Bacillus circulans*	milk	+	+	+	+
17	M_3PAB	*Bacillus subtilis*	milk	+	+	+	+
18	CHPAB	*Pseudomonas stutzeri*	chicken	+	+	+	+

Key: [a] Positive; [b] Negative

putida, Pseudomonas aeruginosa, Pseudomonas stutzeri, Burkholderia pseudomallei, Serratia rubidaea, Corynebacterium pilosum, Bacillus subtilis, Bacillus mycoides, Bacillus laterosporus, Serratia marcescens, Bacillus cereus, Bacillus macerans, Alcaligenes faecalis and Alcaligenes eutrophus. This indicates that these spoilt food samples contained 33.3% of *Bacillus* spp., 27.8% of *Pseudomonas* spp., 11.1% of *Alcaligenes* spp., 11.1% of *Burkholderia* spp., 11.1% of *Serratia* spp. and 5.6% of *Corynebacterium* spp.

Hydrocarbon utilization

Isolates identified as *Pseudomonas putida, Pseudomonas aeruginosa, Pseudomonas stutzeri, Burkholderia pseudomallei* and *Alcaligenes faecalis* were able to utilize the three hydrocarbons (crude oil, diesel oil and aviation fuel) for growth while *Bacillus cereus* and *Bacillus subtilis* were able to utilize crude oil and diesel oil (Table 2). The growth pattern of the organisms that were able to utilize crude oil was determined by plating out an aliquot every 72 h (Figure 1).

Biochemical characterization of biosurfactants

Preliminary biochemical characterization of the purified biosurfactants confirmed the presence of carbohydrate while TLC analysis confirmed the presence of lipid and protein moieties with Rf values ranging from 0.79-1.00 (Table 3).

Component identification of biosurfactants

GC-MS detected the presence of ethanol, amino acids, butoxyacetic acid, hexadecanoic acid, oleic acid, lauryl peroxide, octadecanoic acid and phthalic acid (Figure 2).

Emulsification assay

The highest emulsification index for kerosene was 27.14% while the lowest was 19.71% and that of vegetable oil was 28.12% while the lowest was 16.91% (Table 4).

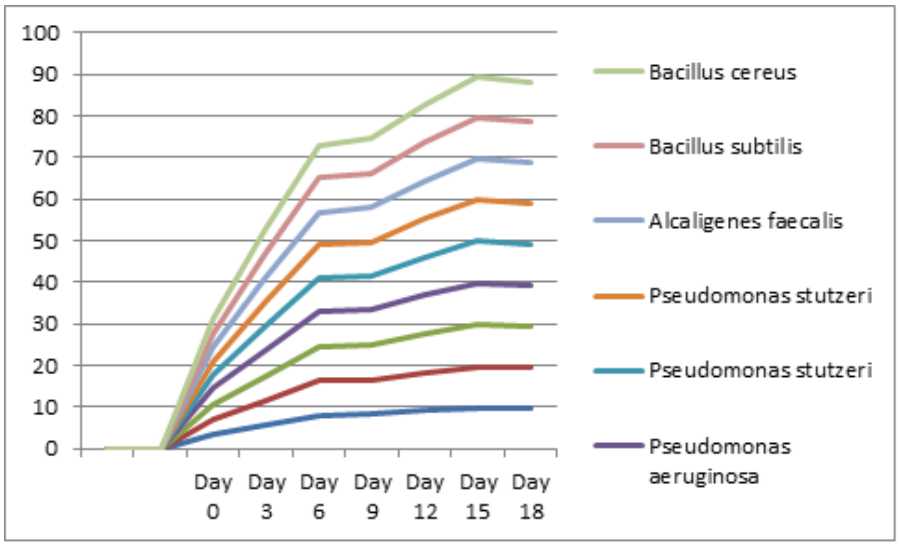

Figure 1: Growth pattern of biosurfactanct producing organisms on crude oil within 21 day incubation period

Table 2: Rate of hydrocarbon utilization

Organisms identified	**Crude oil**	**Diesel oil**	**Aviation fuel**
Pseudomonas putida	+++[a]	+++	+++
Pseudomonas aeruginosa	+++	+++	+++
Pseudomonas stutzeri	+++	+++	+++
Pseudomonas stutzeri	+++	+++	+
Burkholderiapseudomallei	+++	+[c]	+
Burkholderiapseudomallei	+++	+++	+++
Bacillus subtilis	+++	+++	-[d]
Alcaligenesfaecalis	+++	++	++[b]
Bacillus cereus	+++	+++	-
Control	-	-	-

Key: [a] 100% utilization of hydrocarbon; [b] 80% utilization of hydrocarbon;
[c] 40% utilization of hydrocarbon; [d] 0% utilization of hydrocarbon

Table 3: Thin layer chromatography biosurfactants

S/NO	**Organisms isolated**	**Origin**	**R*f* Values**
1	*Pseudomonas putida* (W_1 CE)	water leaf	0.85, 0.98
2	*Pseudomonas aeruginosa* (W_2 CE)	water leaf	0.88, 0.98
3	*Pseudomonas stutzeri* (R_1)	rice	0.79, 0.96
4	*Pseudomonas stutzeri* (W_4PAB)	water leaf	0.86,0.98
5	*Burkholderia pseudomallei* ($EPAB_1^2$)	egg	0.87, 0.98
6	*Burkholderia pseudomallei* (FISH PAB)	fish	1.00
7	*Bacillus subtilis* (M_3PAB)	milk	0.86,0.98
8	*Alcaligenes faecalis* (TO^1)	tomatoes	0.82, 0.96
9	*Bacillus cereus* (PL)	pumpkin leaf	0.86, 0.98

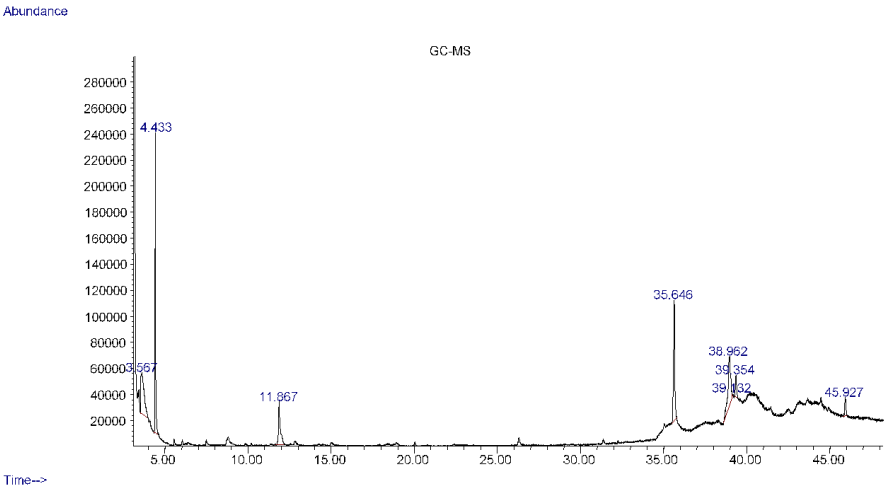

Figure 2: GC-MS of biosurfactant

Table 4: Thin layer chromatography biosurfactants

Organisms identified	**Emulsification index (%) on kerosene**	**Emulsification index (%) on vegetable oil**
Pseudomonas putida	24.41	28.12
Pseudomonas aeruginosa	25.37	26.41
Pseudomonas stutzeri	23.53	23.28
Pseudomonas stutzeri	22.79	25.76
Burkholderia pseudomallei	27.14	26.96
Alcaligenes faecalis	21.43	17.91
Burkholderia pseudomallei	24.31	26.36
Bacillus cereus	19.71	16.91
Bacillus subtilis	26.38	27.94

3.2 Discussion

Eighteen isolates belonging to six genera were found to be biosurfactant producers. All 18 bacterial isolates tested positive for biosurfactant producing ability as shown by haemolysis, slide test, drop collapse test and oil spreading assay. Mulligan (2005) recommended blood agar lysis as a preliminary screening method for biosurfactant production. However, in some cases haemolytic assay excluded many good biosurfactant producers; hence in this present study, the presence was confirmed as positive results using slide test, drop collapse test and oil spreading assay. It has been reported by Nitschke and Pastore (2006) that microbes produce biosurfactants, especially during growth on water-immiscible substrates. Crude oil, diesel oil and aviation fuel were used as sole carbon and energy sources in this study to assess the emulsification ability of these organisms and concomitant biosurfactant production. All nine isolates tested positive for the utilization of at least two out of the three hydrocarbons used which was confirmed by the increase in the number of viable cells as well as turbidity and reduction of hydrocarbon through visual inspection (Table 2 and Figure 1). The ability of these organisms to utilize these water-immiscible substrates was due to their ability to emulsify these substrates thereby

reducing surface tension and interfacial tensions between individual molecules at the surface and interface, respectively. This is supported by the work of Calvo et al. (2004) where it was reported that biosurfactants produced by microbes during growth on water-immiscible substrates, permit their growth on such substrates by reducing the surface tension thereby making the hydrophobic substrate more readily available for uptake and metabolism. Kim, Lee, and Hwang (2000) also found that very often, the growth of microorganisms on hydrocarbons is accompanied by the emulsification of insoluble carbon sources in the culture medium and in most cases has been attributed to the production of emulsifying agents in the presence of these substrates. Several authors have reported diesel oil and crude oil as excellent substrates for microbial growth and also for biosurfactant production (Ilori et al., 2005; Adebusoye, Ilori, Amund, Teniola, & Olatope, 2007). Tuleva et al. (2009) reported the production of rhamnolipids by *P. putida* 21BN when grown on *n*-hexadecane, while Kumar et al. (2006) showed that *P. putida*IR1 also produced biosurfactants upon its growth on 2-, 3- and 4-ring PAHs, but not hexadecane and octadecane, as a sole carbon and energy source.

Partial biochemical characterization of the biosurfactants produced by these isolates indicated they were heteropolymers. The analysis of the purified biosurfactants by TLC showed respective single and double spots of lipopeptides (Table 3) while the Molisch's test showed the presence of carbohydrate. These surfactants can therefore be classified as peptidoglycolipids. Peptidoglycolipids have been isolated from environmental samples by several researchers. Adebusoye et al. (2008) reported peptidogycolipid biosurfactant production from C*orynebacterium* spp. DDV1, *Micrococcus roseus* DDV3, *Pseudomonas aeruginosa* DDV4 and *Saccharomyces cerevisae* DDV5 isolated from water samples collected from University of Lagos lagoon front. Ilori and Amund (2001) reported peptidoglycolipid production by *Pseudomonas aeruginosa* isolated from polluted soil; Sarubbo et al. (2006) also reported peptidoglycolipid production by *Candida glabrata* UCP 1002 obtained from petroleum hydrocarbons contaminated soil samples. However, this is the first report of this type of biosurfactant from food waste materials. This may present an additional source of biosurfactant for the petroleum industry. The presence of various organic acids (butoxyacetic acid, hexadecanoic acid and octadecanoic acid) in the purified biosurfactants which was detected by gas chromatography is a pointer to antimicrobial properties of these biosurfactants which can be employed in the medical and pharmaceutical industries.

Biosurfactant-producing isolates were evaluated for their ability to emulsify vegetable oil and kerosene. The emulsification index can vary with bacterial growth phase, bacterial interactions and hydrophobic compound tested (Krepsky, Da Silva, Fontana, & Crapez, 2007). The emulsification index values obtained in this work were low (16.91-28.12%) compared with those from environmental samples (50-80%); this might be due to substrate specificity and rates of occurrence on different hydrophobic substrates. The observed variation in activity of bacterial strains isolated from different sources could suggest that such activity is not really required for growth on spoilt foods. The ability of biosurfactants to form stable emulsions with vegetable oils and fats suggests potential application as cleaning and emulsifying agents in the food industry (Nitschke & Pastore, 2006). The ability of these isolated biosurfactants to form stable emulsions with different hydrocarbon and vegetable oils suggests considerable potential applications in the petroleum, food and pharmaceutical industries.

4 Conclusions

Microorganisms from food wastes such as spoilt milk, egg, fish, tomatoes, pumpkin leaf (*Telfairia occidentalis*), water leaf (*Talinium triangulare*), rice, and vegetable salad have the ability to produce biosurfactants with emulsification activity on kerosene and vegetable oil. The hydrocarbon catabolic property coupled with biosurfactant-producing capabilities and emulsification activity of these organisms is an asset that could be exploited for clean-up of oil-contaminated environment, enhanced oil recovery and also in food industry as food formulation ingredients and anti-adhesive agents. Further work should

be done to elucidate the functional components of these bioactive molecules and mode of action, to fully characterize their properties quantitatively and to study the best environmental conditions for these strains in order to optimize biosurfactant production and make it economically acceptable. Super-active microbial strains should be developed using genetic engineering (recombinant DNA techniques) for the manipulation of biosurfactant production at industrial level using renewable substrates as raw material.

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