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Characterization of biosurfactant produced from submerged fermentation of fruits bagasse of yellow cashew (*Anacardium occidentale*) using *Pseudomonas aeruginosa*

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Biosurfactants are amphiphilic compounds produced by bacteria and fungi to reduce surface and interfacial tension. This work was designed to produce biosurfactants from the fermentation of submerge cashew bagasse (*Anacardium occidentale*) using a microorganism *Pseudomonas aeruginosa.* The proximate components of the biosurfactant was determined. Results show that yellow cashew fruit bagasse contains lipid (11.34 ± 0.16%), protein (26.67 ± 0.66%), carbohydrate (49.37 ± 0.60%), moisture (5.78 ± 0.17%), ash (2.70 ± 0.04%) and fibre (2.86 ± 0.09%). Biosurfactants activity was characterized based on emulsification index and oil displacement capacity. The yield of biosurfactants was, 0.71 g; when only cashew was used; 0.93 g for cashew and glucose and nutrient broth 0.12 g respectively. The preliminary biochemical characterization revealed that the biosurfactants contained carbohydrates and lipids designated as glycolipids. The biosurfactants showed antimicrobial activity against a range of Gram positive and Gram negative bacteria strains with diameters of zone and growth inhibition: *Escherichia coli,* 38.70 ± 1.30 mm; *Staphylococcus aureus,* 38.00 ± 2.94 mm; *Klebsiella,* 31.00 ± 2.20 mm and *Bacillus cereus,* 28.70 ± 1.70 mm. These results suggest that cashew fruits bagasse serve as cheap carbon source for the production of glycolipid biosurfactants with useful industrial applications.

Key words: Biosurfactants, fruit bagasse, yellow cashew, characterization and *Pseudomonas aeruginosa.*

INTRODUCTION

In recent years, industries have generated large amount of tropical agricultural residues. Their disposal causes several environmental problems such as accidental oil spillage or deliberate oil spillage occasion by vandals in the form of bunker. There has been an increased trend towards efficient utilization of agro industrial residues like oil cakes, wheat bran, soya bean waste, sesame waste, coconut waste, and cashew bagasse (Saharan et al.,

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2011). These residual by-products serve as ideal substrates for fermentation processes to produce different important compounds (Rosenberg, 1986). Most agricultural products are utilized as source of raw materials. They produce large quantities of biosufactants, contain large amount of applicable proteins and carbohydrates such as glycoprotien with some amount of oil residues. The facilities used for the storage and preservation of this plant produce are either not available or inadequate.

Some surfactants are synthetic molecules that concentrate at interfaces and decrease surface and interfacial tension (Rosenberg and Ron, 1999). These compounds find applications in wide variety of industrial production as emulsions, foams, detergents, wetting agents, dispersers or solubilization agent of phosphorus (Desai and Banat 1997; Banat et al., 2000). However, biosurfactant are derived from microorganisms and act as surface-active compounds; there are attracting attention as they offer several advantages over chemical surfactants, such as low toxicity, inherent good biodegradability and ecological acceptability (Banat, 2000; Samadhan et al., 2014). Biosurfactants are active compounds produced from microbial cells surfaces or excreted extracellularly by varieties of microorganisms (Jamal et al., 2012). They possess hydrophilic and hydrophobic moieties that reduce surface and interfacial tensions in colloidal. Also, surfactant molecules that adsorb at liquid-liquid interfaces, decrease the enthalpy and contribute to the overall free energy by reducing the tendency of the emulsion to destabilize by accumulating at the interface between two immiscible fluids (Saharan et al., 2011; Okpashi et al., 2013). Biosurfactants are classified based on their chemical structures and the organisms that produce them (Gomaa, 2012). They are helpful in the uptake and utilization of hydrocarbons to facilitate the biodegradation of toxic hydrocarbons. Other advantages include eco-friendly nature, high foaming capacity, and efficiency at variable temperatures, pH and salt concentrations (Salihu et al., 2009; Chandran and Das, 2010).

Interest towards these biomolecules has increased considerably, recently, they serve as potential fluids for many applications in industries such as petroleum, pharmaceutical, cosmetic, biomedical and food processing (Haba et al., 2003; Emine and Aysun, 2009). Large scale production of biosurfactants is achievable by finding cheap and renewable substrates; efficient microorganisms, optimization of the growth medium composition and conditions (Plaza et al., 2011; Jamal et al., 2012). Cashew (*Anacardium occidentale*) is widely grown and consumed by human. Often, due to lack of cashew marked in Nigeria, cashew is wasted and the bagasse that are rich in sugars, proteins and mineral salts are disposed as waste (Adebowale et al., 2011). This waste product is an interesting and inexpensive renewable carbon source for microbial fermentation to reducing bulk wastage of fruits. Among the microorganisms used in biosurfactant production, *Pseudomonas* species are the best known to grow in various substrates to produce rhamnolipids (Chandran and Das, 2010). This research work aimed to utilize cashew (*A. occidentale*) bagasse as substrates for *P. aeruginosa* to produce biosurfactants thereby converting low cost waste materials of environmental into products of superior value.

MATERIALS AND METHODS

Plant materials

Yellow cashew (*A. occidentale*) fruits bagasse was used as plant materials for this study. The cashew apples were collected from Ubogidi cashew plantation in Nsukka Local Government Area, Enugu state, Nigeria.

Preparation of plant material

The yellow cashew fruits bagasse was washed with normal saline and manually crushed to remove the juice using a pestle and mortar. The bagasse was air-dried for 4 days and pounded into powder. The powdered bagasse was packaged in an air-tight polythene bag and stored at room temperature.

Proximate composition

The proximate composition of the yellow cashew bagasse was determined using the method described by AOAC (1990).

Microorganism

P. aeruginosa was used in this study. The microorganism was obtained from the Culture and isolation Unit of the Department of Microbiology, University of Nigeria, Nsukka.

Preparation of P. aeruginosa growth culture

This process was achieved by adopting the method described by Atlas et al. (2010). Under a sterile working environment, A loop-full of *P. aeruginosa* colony from the culture medium in the Petri dish was inoculated into 100.0 mL nutrient broth contained in a 250 mL conical flask. The inoculum was shaken and left undisturbed for 4 h after which 1 ml of the inoculum was collected in a flamed environment and the absorbance was read at 600 nm using spectrophotometer. The readings were taken three times at 2 h interval. Growth was indicated by increase in absorbance. This primary inoculum was grown until the optical density reached to 1.459 kg/cm^3 and was used to inoculate the various fermentation media at 2% (v/v); that is 2 ml of inoculum for every 100 mL of media.

Screening of culture broth and P. aeruginosa

The growth of the organism was monitored by taking plate counts on nutrient agar. The plates were inoculated with 0.1 mL via serial dilutions using pour plate method described by Willey et al. (2013).

The plates were incubated for 24 h before the colonies were counted.

Extraction of biosurfactants

Biosurfactants produced were extracted using the method described by Suresh et al. 2012). The various culture media were centrifuged at 4000 rpm for 20 min to remove bacterial cells. The supernatant was precipitated by acidification to pH 2.0 with hydrochloric acid (HCl) and stored at 4°C overnight. The precipitate was collected for centrifugation at 4000 rpm for 20 min. Equal volume of chloroform: methanol (2:1) mixture was added. The resulting mixture was vortex for mixing. The contents were centrifuged at 4000 rpm for 20 min upon which the solvent was evaporated by air drying at 40°C.

Characterization of isolated biosurfactant

The isolated biosurfactants from the various culture broth supernatants were analyzed for carbohydrate, protein and lipid content. Chemical composition of the biosurfactant was analyzed following standard methods. Carbohydrate content of the biosurfactant was determined by the anthrone reagent method as described by Spiro (1966). D-glucose was used as standard. Lipid content was estimated adopting the procedure of Folch et al. (1956). Protein content was determined using bovine serum albumin as a standard following the method of Lowry et al. (1951).

Antibacterial activity assay

The antimicrobial activity of the biosurfactant was studied against different Gram positive and Gram negative bacteria. The antibacterial activity was evaluated by agar disc diffusion method. Sterile discs of 0.6 cm diameter was soaked in biosurfactant and methanol were assayed on the surface of a Mueller-Hinton agar inoculated with the test microorganisms. After incubation period of 24 h at 25-37°C, the diameters of zones of inhibition were measured. The same solvents used to obtain the extract were used as negative controls. Standard antibiotics loaded on discs were used as positive controls.

Oil spreading test

The oil displacement test is a method used to measure the diameter of the clear zone, which occurs after dropping a surfactant-containing solution on an oil-water interphase. The binomial diameter allows an evaluation of the surface tension reduction efficiency of a given biosurfactant. The oil displacement test was done by adding 50 mL of distilled water to a Petri dish with a diameter of 15 cm. After that, 20 µl of oil was dropped onto the surface of the water, followed by the addition of 10 µl of cell culture supernatant. The diameter and the clear halo visualized under visible light were measured after 30 s (Rodrigues et al., 2006).

Emulsification test

The emulsification test was carried out as described by Balogun and Fagade (2010). Sterile biosurfactant solution (1.0 mL) was added into each test-tube (in a set of three) containing 2.0 mL of the substrates (crude oil, olive oil and kerosene). The content of the tubes were vigorously shaken for uniformity for 2 min and left undisturbed for 24 h. The volume of oil that separated after 24 h was measured, that is, the ability of a molecule to form emulsion.

Their emulsification index after 24 h (E_{24}) was determined and expressed as percentage of height of emulsified layer in centimeter divided by total height of the liquid column in centimeters. Emulsification index of value greater than 50% was indicative of a positive result:

$$E24 = \frac{\text{Height of emulsified layer}}{\text{Total height}} \times 100\%$$

Haemolysis test

The hemolysis test was carried out as an indicator of biosurfactant activity using blood agar plates as described by Carrillo et al. (1996). Human blood was collected by swabbing and tying a tourniquet on the upper arm. Then a sterile needle was used to collect blood from the visible vein. The microorganisms were screened by plating on blood agar plates containing 5% (v/v) human blood and incubated at room temperature for twenty-four hours. A clear zone (zone of hemolysis) around the colonies after this period was indicative of biosurfactants.

Stability tests

Stability studies were carried out by the procedure described by Chandran and Das (2010). The cell-free broth was obtained by centrifuging the cultures at 4000 revolutions per minute (rpm) for 20 minThe pH of the biosurfactant (4.0 mL) was adjusted to 2.0, 4.0, 6.0, 8.0 and 10.0 using sodium hydroxide (NaOH) or hydrochloric acid (HCl) after which the emulsification index (E_{24}) was determined. To test the heat stability of the biosurfactant, the broth was maintained at different temperatures (10-100°C) in a water bath for fifteen minutes, cooled at room temperature and emulsification index (E_{24}) was determined. Stability was also analyzed with sodium chloride salt concentrations ranging from 0 to 20% w/v.

Carbohydrate content

The carbohydrate content of the isolated biosurfactant was analyzed by the anthrone method described by Umeji et al. (2010). A quantity 2.0 mL of the biosurfactant was added into appropriately labeled sterile test tubes, after which distilled water 3 mL and 10.0 mL of 0.2% solution of anthrone reagent (containing 0.2% anthrone in 95% H_2SO_4) was added to each of the test tubes. The absorbance of each of the preparation was read at 520 nm against a blank composed of distilled water and anthrone reagent. 2 g/l of glucose was used as the standard:

$$\text{Total carbohydrate } \left(\frac{g}{L}\right) = \frac{\text{Abs sample}}{\text{Abs standard}} \times \text{concentration of standard}$$

Protein content

A known weight, 0.5 g of oven-dried sample was placed into 30 ml Kjeldahl flask and 15.0 ml of concentrated H_2SO_4 was added with 1 g of the catalyst mixture. The mixture was heated cautiously in digestion rack under a fume cupboard until a greenish solution appeared. After the digest was cleared, it was heated further for 30 minand allowed to cool. About 10.0 ml of distilled water was added to avoid caking. The mixture was transferred to the Kjeldahl distillation apparatus. A receiver flask containing 5.0 ml of boric acid was placed under the condenser of the distillation apparatus. Ten

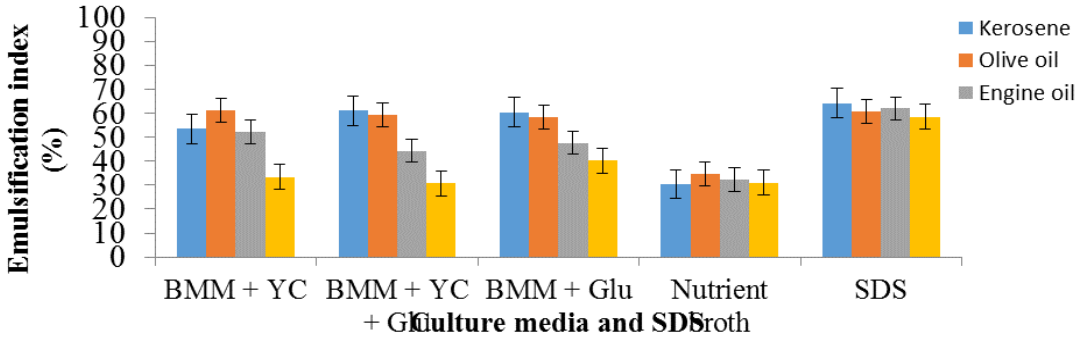

Figure 1. Emulsification index of the various fermentation broth culture supernatants with different vegetable and hydrocarbon oils. BMM = Basal mineral medium; YC = Yellow cashew; SDS = Sodium dodecyl sulphate; GLU = Glucose

millilitres (10.0 ml) of 40% NaOH solution was added to the digested sample. Distillation was stopped when the distillate reached the 35 ml mark on the receiver flask. The distillate was titrated to first pink colour with 0.1 M HCl. The crude protein content was calculated as:

$$\% \text{ Crude protein} = \frac{(\text{titre value} \times 14.01 \times 0.1 \times 100 \times 6.25 \times \text{dilution factor})}{1000 \times \text{sample weight}}$$

Lipid content

Soxhlet flask was dried in an oven at 100°C, allowed to cool and weighed (W_1). Five grammes (5 g) (W_2) of the sample was transferred to a thimble and its contents into the Soxhlet extractor. Hexane was used for the extraction. After three hours, the thimble was removed and the solvent distilled off from the flask. The flask was disconnected and placed in an oven set at 60°C for two hours, cooled and weighed (W_3). Lipid content was calculated as:

$$\% \text{ Lipid} = \frac{(W_3 - W_1)}{W_2} \times 100$$

Statistical analysis

All investigations were carried out in triplicate and data obtained were presented as mean ± standard deviation using descriptive statistics. Analysis was conducted using SSPSS version v16, for the determination of mean values.

RESULTS

Emulsification index (EI)

Emulsification index indicated 50% biosurfactant activity ss shown in Figure 1. The results reveal that all the culture supernatants formed emulsion when tested with different vegetables and hydrocarbon oils. Poor emulsion formation was observed with nutrient broth. The highest emulsification index was observed in sodium dodecyl sulphate (SDS), a synthetic surfactant.

Pseudomonas aeruginosa growth culture

Freshly prepared and sterilized nutrient broth solution containing 1.3 g of nutrient broth salt in 100 mL of distilled water was used as the blank in the spectrophotometric readings. Observed increase in absorbance over time indicated growth and viability for subsequent inoculation into various fermentation media (Figure 2).

Hemolysis test

The culture broth supernatants showed positive hemolytic activity, as clear zones of hemolysis were observed on blood agar. More hemolytic activity (wider zones of hemolysis) was produced by the biosurfactants from the Basal mineral medium (B.M.M) + Yellow cashew medium. This is presented in Table 1.

Oil spreading test

As shown in Table 5, the oil spreading activity is represented by the diameter of the clear zone formed. The supernatants of the culture media showed different diameters of clear zones. The highest activity was observed in sodium dodecyl sulphate (SDS) a synthetic surfactant while the lowest activity was observed in the nutrient broth supernatant.

Emulsification index of the various culture supernatants at different temperatures

As shown in Figure 3, the biosurfactant was able to maintain its activity measured with emulsification index at variable temperatures. The emulsification indices of the biosurfactant was above 50% and increased due to

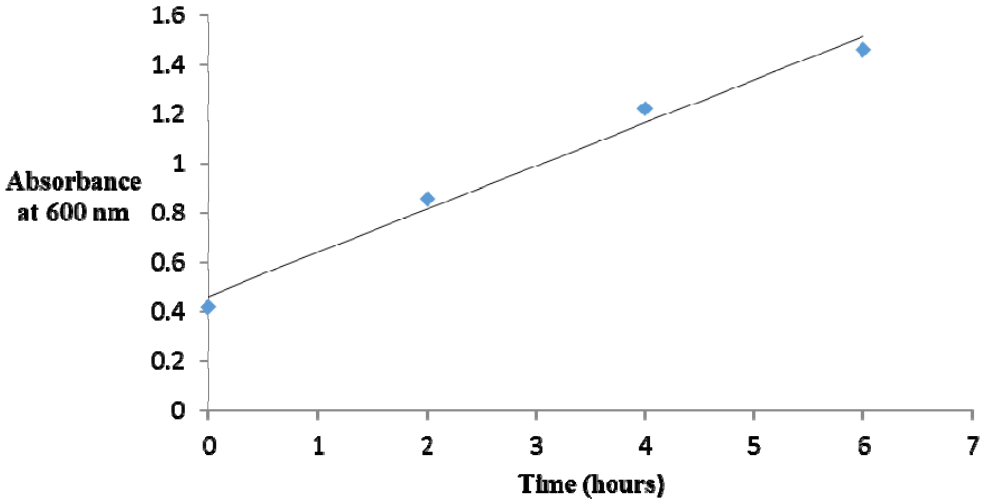

Figure 2. *Pseudomonas aeruginosa* growth culture.

Table 1. Red blood cell lysis of culture broth supernatants.

Culture broth	Hemolysis activity
B.M.M + yellow cashew	++
B.M.M + yellow cashew + glucose	+++
B.M.M + glucose	++
Nutrient broth	+

+, Slight activity; ++, Moderate activity; +++, High activity.

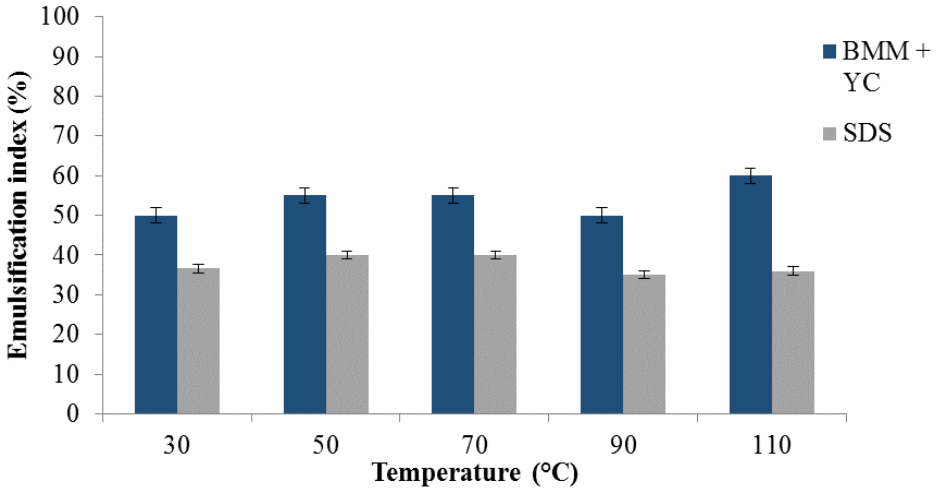

Figure 3. Emulsification index of the various culture supernatants at different temperatures. BMM = Basal mineral medium; YC = Yellow cashew; SDS = Sodium dodecyl sulphate.

temperature increased. It stability was reduced at 70°C. The highest emulsification index was observed at 110°C. Sodium dodecyl sulphate, a synthetic surfactant showed emulsification indices which was low at different temperature. There was a periodic rise in emulsification index from 30-70°C, after which it dropped at 90 and

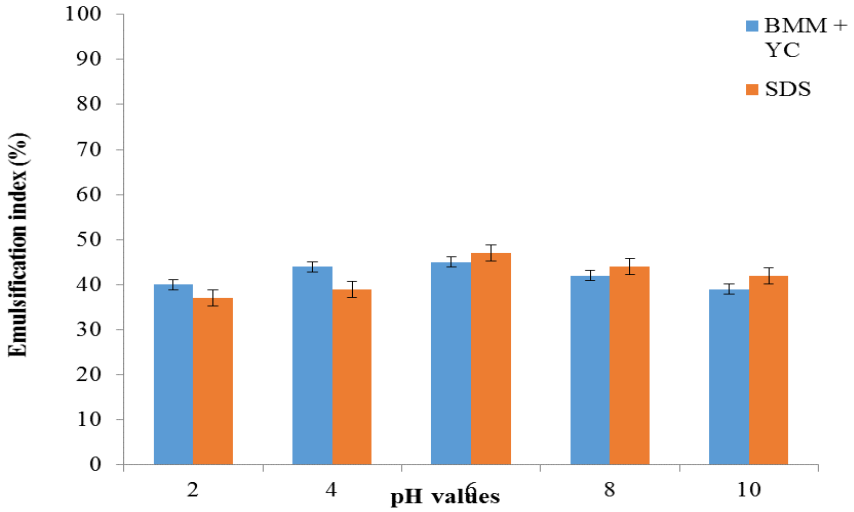

Figure 4. Effect of pH on the emulsification index of the B.M.M + Yellow cashew broth supernatant. BMM = Basal mineral medium; YC = Yellow cashew; SDS = Sodium dodecyl sulphate.

110°C.

Temperature stability test

The stability of the emulsion, biosufactant and sodium dodecyl sulphate was determined at variable temperature. At 70°C stability was observed. The highest observed temperature was at 110°C. There was a periodic rise in emulsification index from 30-70°C, after which it dropped at 90°C and 110°C.

pH stability test

As seen in Figure 4, pH had a negative effect on the stability of emulsion where B.M.M + Yellow cashew supernatants and sodium dodecyl sulphate (SDS) values were below 50%. A rise in emulsification index was observed from pH 2-6. At pH 8-10 the emulsification index decreased slowly. The emulsifying ability is favorable at pH 6.

The growth of inhibition and diameter zone

The growth inhibition and diameter zone is shown in Table 5

Salt concentration stability test on B.M.M + yellow cashew broth supernatant

As depicted in Figure 5, the biosurfactant and sodium dodecyl sulphate formed emulsion at different salt concentrations. There was slight decrease in emulsification index in biosurfactant and sodium dodecyl sulphate as the salt concentration increased from 0-20%.

DISCUSSION

The preliminary biochemical characterization of biosufactants and its associate products, extracted from various cultures contained carbohydrate and lipid, while protein was below the threshold of detection. Hemolysis test of the culture broth supernatants showed positive hemolytic activity, as clear zones of hemolysis were observed on blood agar. More hemolytic activity (wider zones of hemolysis) was produced by the biosurfactants from the B.M.M + Yellow cashew medium as presented in Table 1.

Still on the preliminary biochemical characterization, the extracted biosurfactant could be described as a glycolipid. This finding is in line with previous reports (Haba et al., 2003; Priya and Usharani, 2009). Saharan et al. (2011) reported that *P. aeruginosa* produced glycolipid biosurfactants, especially the rhamnolipids. In Figure 4, pH had a negative effect on the stability of emulsion where B.M.M + Yellow cashew supernatants and sodium dodecyl sulphate (SDS) values were below 50%. A rise in emulsification index was observed from pH 2-6. At pH 8-10 the emulsification index decreased slowly. The emulsifying ability is favorable at pH 6.

Literature review shows that *P. aeruginosa* produces protein emulsifier from hydrocarbon and acetyl alcohol substrates but not from glucose, glycerol and vegetables oils where it was observed to produce glycolipid (Plaza et al., 2011; Saharan et al., 2011). Also, Figure 1 illustrate

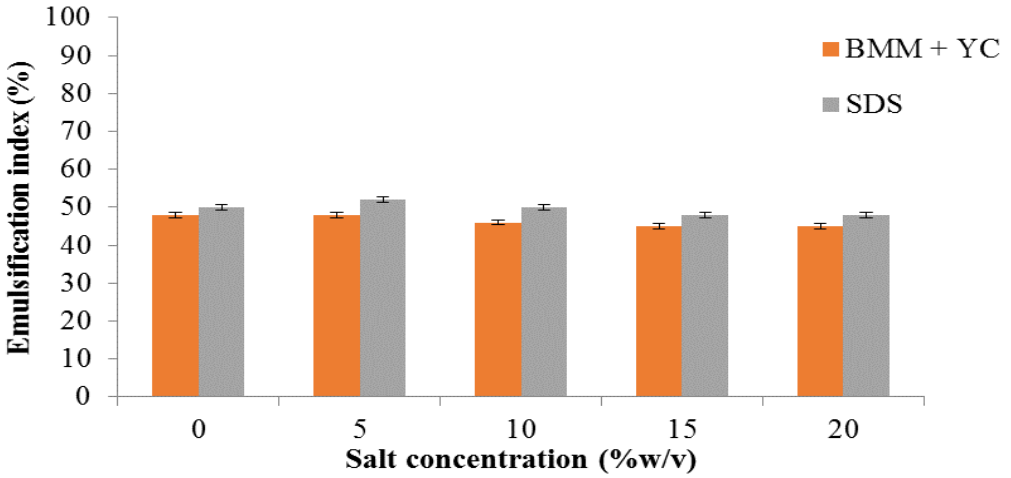

Figure 5. Effect of salt concentration on the emulsification index of the B.M.M + Yellow cashew broth supernatant. BMM = Basal mineral medium; YC = Yellow cashew; SDS = Sodium dodecyl sulphate.

emulsification index (EI). Emulsification index indicated 50% biosurfactant activity. The results reveal that all the culture supernatants formed emulsion when tested with different vegetables and hydrocarbon oils. Poor emulsion formation was observed with nutrient broth. The highest emulsification index was observed in SDS, a synthetic surfactant. From these, the uniformity of the chemical composition of the biosurfactants produced in the four culture media could be attributed to the microbial origin (*P. aeruginosa*) as well as the nature of the carbon source in the four different culture media as indicated in Table 5. The antimicrobial activity of several biosurfactants has been reported in the literature for many different applications. From the results, the biosurfactant showed antibacterial activity with the following diameters of zone of growth inhibition against four bacterial species namely *Bacillus cereus, Klebsiella*, *Staphylococcus aureus* and *Escherichia coli*.

The antibacterial activity screening was negative for the following bacterial species *P. aeruginosa* and *Salmonella* (Table 2). The point of attack of surface-active agents is the biological membrane. The antimicrobial effect of biosurfactants is explained by the structures of biosurfactants. Being amphipathic molecules, their insertion into cell membranes could cause significant structural changes in the cells. Another explanation of their antimicrobial effect is the adhering property of biosurfactants to cell surfaces causing deterioration in the integrity of cell membrane. Findings of the present study correspond with those by Haba et al. (2003); and Gomaa (2012) which stated that *P. aeruginosa* produced rhamnolipid biosurfactants which have antimicrobial activities. These results further demonstrate that the biosurfactant produced in this research could be a an effective antimicrobial agent. As shown in Figure 5, the biosurfactant and sodium dodecyl sulphate formed emulsion at different salt concentrations. There was slight decrease in emulsification index in biosurfactant and sodium dodecyl sulphate as the salt concentration increased from 0-20%. Antimicrobial activity was not observed on the same species of the producing organism. This is attributed to the fact that biosurfactants are produced as substances that inhibit the growth of other microorganisms in the natural environment as they compete for nutrients. In Figure 2, *P. aeruginosa* Growth Culture freshly prepared and sterilized nutrient broth solution containing 1.3 g of nutrient broth salt in 100 ml of distilled water was used as the blank in the spectrophotometric readings. Observed increase in absorbance over time indicated growth and viability for subsequent inoculation into various fermentation media.

In conclusion, the biosurfactant family constitutes an interesting group of microbial secondary products with various useful applications. Selection of suitable alternative substrates in place of the hydrophobic ones and the design of feasible processes for cost-effective production which involves media and process optimization are the main research focus for their commercial production. Based on the results obtained, biosurfactants produced by *P. aeruginosa* using yellow cashew apple fruit bagasse as carbon source, on partial characterization, is probably glycolipid. The biosurfactant also showed fair emulsification index as illustrated in Figure 2 and oil displacement capacity in Table 3. Emulsification index of the various culture supernatants at different temperatures is shown in Figure 3; the biosurfactant was able to maintain its activity measured with emulsification index at variable temperatures. The emulsification indices of the bio surfactant were above 50% and increased due to temperature increased. It also

Table 2. Emulsification indices of culture broth supernatant and SDS.

Culture supernatant	E24 with kerosene	E24 with olive oil	E24 with petrol	E24 with engine oil
Medium I (yellow cashew)	53.50±1.19	61.20±1.37	33.40±0.45	52.10±0.86
Medium II (yellow cashew and glucose)	61.10±1.10	59.3±0.93	44.30±0.99	44.30±0.41
Medium III (glucose)	60.40±0.91	58.50±1.09	40.30±1.32	47.70±0.52
Medium IV (nutrient broth)	48.70±1.23	40.6±1.12	49.40±0.91	30.97±1.17
SDS	60.3±0.57	30.6±1.15	36.3±1.47	59.0±1.20

Values are Mean ± SD, where n=3. Emulsification index was calculated as height of emulsified layer (cm)/total liquid column (cm) × 100%.

Table 3. Oil spreading activity of the various broth supernatants.

Culture supernatant	Diameter (cm)
Yellow cashew	3.66±0.05
Yellow cashew + glucose	3.10±0.08
Nutrient broth	1.07 ± 0.05
Glucose	3.53 ± 0.05
SDS	8.27±0.21

Values are mean ± SD, where n = 3.

Table 4. Temperature stability test.

Temperature (°C)	Biosurfactant	Sodium dodecyl sulfate (SDS)
30	50.00 ±0.57	36.60 ±0.76
50	55.00 ±1.15	40.00 ± 0.87
70	55.00 ±0.50	40.00±0.28
90	50.00 ±0.29	35.00 ±1.04
110	60.00 ±0.76	36.00±0.77

Values are Mean ± SD, where n=3.

Table 5. Diameter of zone of growth inhibition.

Microorganism	Positive control (mm)	Biosurfactant	Negative control
Bacillus cereus	31.67±2.52	28.70±1.70	-
Escherichia coli	33.70±1.15	38.70±1.30	-
Salmonella	23.00±1.00	-	-
Staphylococcus aureus	39.00±1.00	38.00±2.94	-
Klebsiella	37.67±1.53	31.00±2.20	-
Pseudomonas aeruginosa	23.67±2.08	-	-

Values are Mean ± SD, where n=3. Positive control represents standard antimicrobial agents loaded on the discs. Biosurfactant solution was prepared by dissolving 0.2 g in methanol. Negative control is methanol.

showed antimicrobial activity against some Gram positive and Gram negative microorganisms as indicated in Table 4. Thus, it could be used as oil dispersant in pollution control and remediation and its application as antimicrobial and pharmaceutical agent. The stability of the emulsion, biosufactant and sodium dodecyl sulphate was determined at variable temperature. At 70°C stability was observed. The highest observed temperature was at

110°C. There was a periodic rise in emulsification index from 30-70°C, after which it dropped at 90°C and 110°C. Table 4 explains the temperature stability. The yield and chemical composition of the biosurfactant may have been influenced by the physicochemical nature of the carbon source (yellow cashew apple fruit bagasse) and the microorganism (*P. aeruginosa*) used in the production. Hence, yellow cashew apple is a fairly good substrate for biosurfactant (glycolipid) production by *P. aeruginosa*.

Conflict of interest

The authors did not declare any conflict of interest.

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