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Production and Optimization of Biosurfactants from *Citrullus lanatus* Seeds and Activity Determination with *Pseudomonas aeruginosa* Isolate

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Authors' contributions

This work was carried out in collaboration among all authors. Author TOU conceived the research and performed the analysis, authors VEOO and OUN assisted in sourcing for the research materials and in designing the experiment, author GSH performed the statistical analysis All authors read and approved the final manuscript.

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ABSTRACT

Aims: To produce and optimize biosurfactants from *Citrullus lanatus* seeds.
Study Design: Randomized design.
Place and Duration of Study: Department of Biochemistry Lab. and Department of Microbiology lab. University of Nigeria, Nsukka, between April and August, 2017.
Methodology: Biosurfactants were produced in two fermentation media consisting of basal mineral medium+ watermelon (*C. lanatus*) seed (BMM+WMS), and Nutrient broth (NB). Optimization of production process was carried out with respect to time/duration of fermentation and pH of production which indicated 7 days fermentation period at pH 8.0. The *Pseudomonas aeruginosa* used was isolated from soil. The biosurfactants stability under some environmental conditions were studied using; Thermostability test at 30°C, 60°C, and 100°C; Halostability test with %w/v concentration of NaCl in; 2%NaCl, 5%NaCl, 7%NaCl, and 10%NaCl; pH stability test was conducted with pH 2.0, pH 4.0, pH 6.0, pH 8.0, pH 10.0, and pH 12.0.

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Results: Proximate analysis(%) showed; protein (0.317 ± 0.02), lipid (17.66 ± 0.17), carbohydrate (62.77 ± 0.21), ash (2.33 ± 0.24), fibre (4.84 ± 0.79), and moisture (12.06 ± 0.17).The Emulsification index (E24) for the crude biosurfactant solutions (supernatants) that resulted from the two production media, and sodium dodecyl sulfate 1% SDS in distilled water (control) using palm oil (PO), olive oil (OO), engine oil (EO), kerosene (KR), and petrol (PT) for BMM+WMS biosurfactant gave PO (79.66±1.52%), OO (64.66±7.23%), EO (15.33±4.93%), KR (12.66±6.50%), and PT (0.00±0.00%). Also, the NB biosurfactant resulted in; PO (71.00±2.00), OO (54.33±3.78), EO (42.66±6.42), KR (22.66±7.57), and PT (0.00 ± 0.00) E24 values. There were significant decreases ($p<0.05$) in biosurfactants activities in all vegetable oils and hydrocarbons when compared to the SDS surfactant. However, there was no significant decrease ($p>0.05$) in WMS biosurfactant activity in palm oil compared to SDS surfactant activity in palm oil.
Conclusion: The biosurfactants were shown to have high thermostablity as their E24 values increased with rise in temperature.

Keywords: Bioemulsifiers; optimum; thermostability; halostability; microorganism.

1. INTRODUCTION

Surfactants are amphipathic molecules that accumulate at interfaces of two immiscible liquids like water and oil. Biosurfactants are biologically derived surfactants produced by yeasts or bacteria**.** Surfactants of biological origins are mainly classified into two classes: low-molecular weight surface active agents called biosurfactants (lipopeptide, glycolipids) and bioemulsifiers (high molecular weight surface active agents; Liposan, Alasan, Emulsan). Biosurfactants are further divided into six classes: hydroxylated and cross linked fatty acids (mycolic acids), glycolipids, lipopolysaccharides, lipoproteins-lipopeptides, phospholipids and polymeric or particulate compounds [1]. Biosurfactants lower interfacial tensions, and form aggregate structures such as micelles. They can be used as emulsifiers, de-emulsifiers, wetting agents, foaming agents, functional food ingredients and detergents. These inherent properties of biosurfactants and bioemulsifiers are widely utilized in industrial processes like; agricultural, food, cosmetic and pharmaceutical factories [2,3]. Comparatively, biosurfactants have advantages over chemically synthesised surfactants because they are biodegradable, have low toxicity, can be used at extreme temperatures or pH values and are more environmental friendly [4]. However, economical considerations of their production show that synthetic-surfactants are yet preferred over bio-surfactant [5]. In addition to the industrial uses, biosurfactants environmentally has many applications which include; bioremediation, microbial enhanced oil recovery (MEOR), dispersion of oil spills, and transfer of crude oil [6]. The various assay methods for the determination of biosurfactants activity include; surface tension measurement, emulsification index (E24), heamolysis test, stability test, and antimicrobial test [5]. To achieve large scale of biosurfactant production, cheap and renewable substrates are made available to the producing microorganinsm in optimum conditions [7]. Watermelon (*Citrullus lanatus*) seed is one of the under-utilized seeds grown in the temperate part of the world. The mesocarp of watermelon is used for human consumption, while rind and seeds are major solid wastes [8,9]. This is a renewable and inexpensive carbon source for microbial production of surfactants. A number of microorganisms produce biosurfactants of diverse structure and classes. However, *Pseudomonas aeruginosa* has been shown to be capable of using different substrates for carbon source like; glucose, glycerol, fructose, mannitol, n-paraffins and vegetable oils, in the secretion of rhamnolipid-type biosurfactants [10,4]. The present study involves the production and determination of biosurfactant activities from *Citrullus lanantus* seeds with *Pseudomonas aeruginosa* isolate.

2. MATERIALS AND METHODS

2.1 Materials

2.1.1 Microorganism

The organism, *Pseudomonas aeruginosa* was used in this study. The microorganism was isolated from soil samples collected within University of Nigeria, Nsukka campus.

2.1.2 *Citrullus lanatus* seeds

Fresh fruits of watermelon (*Citrullus lanatus*) were sourced from fruit dealers around Nsukka in

Enugu state Nigeria. The fruits were washed and manually processed to extract the seeds from the fleshy mesocarp. The fresh seeds weighing 387g was sun dried, reweighed (342 g).

2.2 Methods

2.2.1 Proximate analysis

The proximate composition of the *Citrullus lanatus* seed was determined using the method described by AOAC [11].

2.2.2 Isolation of *Pseudomonas aeruginosa*

Microbial isolation and identification was by standard microbiological techniques as described by Atlas, [12]. One gram samples were homogenized in 9mL normal saline and serially diluted (ten-fold dilutions). Aliquot 0.1 mL of appropriate dilutions was spread inoculated in duplicate onto Nutrient agar (Cetrimide medium). Inoculated Nutrient Agars were incubated at 37± 2°C for 48 hrs for the growth of heterotrophic bacteria. A distinct colony was thereafter enumerated at the end of incubation period with plates which were between 30-300 colonies. Purification and preservation of pure isolate was done by picking representative of each different microbial isolates colony from the primary plates and sub cultured onto sterile plates by streaking method. The isolates were sub cultured by repeated streaking to obtain pure cultures. All the pure cultures were kept in duplicate on nutrient agar slants in bijou bottle as working and stock cultures. The slants were stored in the refrigerator at 4°C.

2.2.3 Characterization and Identification of microbial isolate

The screened microorganism was subjected to characterization tests as described by Chessbrough, [13]. Colonial morphology, Gram staining, biochemical and carbohydrate (sugar) fermentation tests and growth on Cetrimide selective Agar were used to characterize the isolate. The identification was based on comparison of observed characteristics with those described in the Bergey's Manual of Determinative Bacteriology.

2.2.4 Media preparation

Two different culture media were prepared according to the method described by Atlas [12] The two different culture media were prepared in triplicates. Each culture medium was inoculated with 5mL of *Pseudomonas aeruginosa*. Proper working environment was maintained by aseptic means. This was achieved by working in laminar flow machine. Also, sodium dodecyl sulphate (SDS) standard solution was prepared and used as standard surfactant solution.

2.2.5 Preparation of basal mineral medium (BMM)

BMM was prepared as described by Atlas [12]. The trace element solution was prepared first by adding components (0.232 g H_3BO_3, 0.174 g $ZnSO_4.7H_2O$, 0.116 g $FeSO_4$ $(NH_4)_2SO_4.6H_2O$, 0.096 g $CoSO_4.7H_2O$, 0.022 g $(NH_4)_6Mo_7O_{24}.4H_2O$, 8.0 mg $CuSO_4.5H_2O$, 8.0 mg $MnSO_4.4H_2O$) to distilled water and bringing its volume to 1.0 L. Finally, the basal mineral medium was prepared by adding components (12.5 g K_2HPO_4, 3.8 g KH_2PO_4, 1.0 g $(NH_4)_2SO_4$, 0.1 g $MgSO_4.7H_2O$ plus 5.0 mL of the trace elements solution) to distilled water and bringing the volume to the 900.0mL mark. The solution was mixed thoroughly, gently heated and brought to boiling. It was then autoclaved for fifteen minutes at 15 psi pressure 121°C and cooled to 45-50°C.

2.2.6 Preparation of sodium dodecyl sulphate SDS (standard surfactant solution)

1 g of SDS was weighed and dissolved in about 30 mL of distilled water and then made up to 100 mL using distilled water. This gave 1% SDS solution.

2.2.7 Preparation of media composed of basal mineral medium and *Citrullus lanatus* seed (carbon source)

14 g of powdered carbon source (*Citrullus lanatus* seeds*)* were added into three empty 250 mL conical flasks,100 mL of freshly prepared basal mineral medium (BMM) contained in three conical flasks. The flasks and their contents (carbon source, and BMM) were sterilized by autoclaving for fifteen minutes at 15 psi pressure and 121°C. The BMM flasks were transferred to the flasks containing the carbon source and labelled (BMM/WMS1, BMM/WMS2, and BMM/WMS3).

2.2.8 Preparation of media composed of nutrient broth (NB)

A quantity, 14 g of powdered nutrient broth was dissolved in 100 mL of distilled water. The

mixture was swirled properly and sterilized by autoclaving for fifteen minutes at 15 psi pressure-121ºC. The above preparation was done in triplicate in 250 mL conical flasks and labelled accordingly (NB1, NB2 and NB3).

2.2.9 Preparation of *Pseudomonas aeruginosa* culture

This process was achieved by adopting the method described by Atlas [12]. Under a sterile working environment, a loop of *Pseudomonas aeruginosa* colony from the culture medium in the Petri dish was inoculated into a 100.0 mL sterilized distilled water contained in a 250 mL conical flask. The inoculum was shaken and left undisturbed for four hours.

2.2.10 Innoculation of *Pseudomonas aeruginosa* into fermentation media

Each of the previously prepared media (BMM + WMS and NB) was inoculated with inoculums from the seed culture as described by Atlas [12]. In an aseptic environment 5 mL of inoculum was inoculated into each of the six 250 mL conical flasks containing the different media (BMM + WMS and NB) above; which means 5 mL of inoculum for every 100 mL of media. The culture broths (media plus inoculum) were left for seven days at room temperature with continuous shaking in a shaker machine.

2.2.11 Fermentations of the production media

Seven days fermentation period were used for biosurfactant production, using 100 mL of the broth in 250 mL flasks under agitation in a Shaker machine at 120 rpm. Also the initial seeding material standardized in a culture medium at room temperature. This method of fermentation is in line with that of Rashediet al. [14] and Saharan et al. [15].

2.2.12 Screening the culture broth for growth of *Pseudomonas aeruginosa*

The growth of the organism was monitored by taking plate counts on nutrient agar. The plates were inoculated with 0.1 mL of serial dilutions using the pour plate method described by Willey et al. [16]. The plates were incubated for twenty-four hours before the colonies were counted.

2.2.13 Optimization of biosurfactant production

Optimization of the production conditions for biosurfactant was carried out before mass production to enhance the biosurfactant yield in the course of the fermentation process. The two optimized parameters are; pH of the media, and duration of fermentation. The best pH for the operation was determined using; 4.5, 5.0, 5.5, 6.0, 6.5, 7.0, 7.5, 8.0, 8.5, and 9.0 pH range values. Also the best duration of fermentation period was carried out within; 1-10 days.

2.2.14 Harvest of the fermentation media

The fermentation media (flasks) were harvested by centrifugation at 10,000rpm for 20 minutes and the supernatant used as crude biosurfactant solution.

2.2.15 Screening of the supernatant for biosurfactant activity

Biosurfactant activity in each of the production media were carried out using; emulsification index (E24), haemolytic assay, surface tension reduction assay, antimicrobial test, also the stability of the biosurfactant were analyzed using; thermo stability, halostability, and pH stability

2.2.16 Emulsification index (E_{24})

The emulsification test was carried out as described by Cooper and Goldenberg, [17]. Sterile biosurfactant solution (1.0 mL) was added into each test-tube (in a set of three) containing 1.0 mL of the substrates (Olive oil, Palm oil, Engine oil, kerosene, and petrol). The content of the tubes were vigorously shook for uniformity and left undisturbed for twenty-four hours. The volumes of the total content of the test tubes were measured as total height (TH), and the intermediate layer measured too as emulsified height (EH). Their emulsification index after twenty-four hours (E_{24}) was determined and expressed as percentage of height of emulsified layer in centimetre divided by total height of the liquid column in centimetres. E24 provides the knowledge of how stable the resulting emulsion is. Emulsification index of value greater than 50% was indicative of a positive result.

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

2.2.17 Stability tests

Stability studies were carried out by the procedure described by Ali and Shereen, [18]. The cell-free broth was obtained by centrifuging the cultures at 10,000 revolutions per minute

(rpm) for twenty minutes. The pH of the biosurfactant (15.0 mL) was adjusted to 2.0, 4.0, 6.0, 8.0, 10, and 12.0 using sodium hydroxide (1M NaOH) and/or hydrochloric acid (3N HCl) after which the emulsification index (E_{24}) was determined. Heat stability of the biosurfactant was determined by heating 1mL in test tubes to 30°C, 60°C, and 100°C in a water bath, cooled to room temperature and emulsification index (E_{24}) was determined. Stability was also analyzed with sodium chloride salt concentrations ranging from 0 to 10% w/v.

2.2.18 Haemolysis test

Blood Agar plates containing 5% (v/v) human blood was used for this Assay according to the method described by Saravanan and Vijayakumar, [19]. A sterile needle was used to collect human blood. Two plates were prepared by dissolving 5 g of the agar in 118 mL of distilled water from the stock formula, autoclaved at 151°C for 15 minutes and after 3 hrs two drops of the two supernatants (WMS + BMM, and NB) were made in each plate and labelled accordingly and incubated at room temperature. A clear zone (zone of haemolysis) around the colonies after this period was indicative of haemolytic biosurfactant activity.

3. RESULTS AND DISCUSSION

3.1 Results

3.1.1 Proximate composition of watermelon seed (*Citrullus lanatus* seed)

From the results shown in Table 1, *Citrullus lanatus* seed contains appreciable quantities of carbohydrates, lipids and proteins with carbohydrates being the highest, followed by lipids, and protein. The moisture, fibre, and ash contents were quantitatively shown in the following ascending order of magnitude; ash, fibre, and moisture.

Table 1. Proximate composition of *Citrullus lanatus* seeds

PARAMETER	PROXIMATE COMPOSITION (%)
Moisture	12.06 ± 0.17
Fibre	4.84 ± 0.79
Ash	2.33 ± 0.24
Protein	0.317 ± 0.02
Lipid	17.66 ± 0.17
Carbohydrate	62.77 ± 0.21

Values are presented as Mean ± SD, where n = 3

3.2 Characterization and Identification of Microbial Isolate

Pseudomonas aeruginosa, the organism used in this study was isolated from soil samples collected within the university environment. It was authenticated by media and culture selection unit of the Department of Microbiology University of Nigeria, Nsukka. Table 2 shows the microbial and biochemical tests carried out on the microorganism.

Table 2. Result of Identification tests on the microbial isolate

Test	Result
Gram Reaction	Gram negative short rod.
Citrate	+
Coagulase	-
Catalase	+
Oxidase	+
Growth Cetri. Agar	+
Motility	+
Glucose	W
Starch	W
Mannitol	W/g
Galactose	W
Lactose	-
	-

Keys; + = positive, - = negative, w = weak, s = strong, and g = gas evolution

3.3 Viability Result of *Pseudomonas aeruginosa*

The growth curve of the microorganism in Fig. 1 shows a rapid increase in the colony forming units (cfu), and attained stationary phase from day four which lasted till day eight. However a decline in the cfu was observed in the two media after the 8th day of the fermentation. This shows reduction in the microbial cells available for fermentation

3.4 Emulsification Index (E24) Test Result

Fifty percent (50%) emulsification index (E24) and above shows a high biosurfactant activity. Fig. 2 contains the result of the emulsification index analysis. The two culture supernatants generally showed high biosurfactant activity with vegetable oils; Olive oil, and Palm oil. The E24 in Engine oil was moderate. Low emulsification index was observed in Kerosene and Petrol hydrocarbons. Sodium dodecyl sulphate SDS (a synthetic surfactant) toped emulsification index

values across the two media supernatants. There were significant decrease ($p < 0.05$) in the E24 values across the results of the biosurfactants as compared to the SDS synthetic surfactant, however WMS derived biosurfactant had non-significant ($p > 0.05$) decrease in palm oil when compared to the activity of SDS in palm oil.

3.4.1 Thermostability result

The temperature of both WMS + BMM, NB supernatants, and SDS surfactant were uniformly varied by heating 1 mL of each in test tubes to 30°C, 60°C and 100°C and E_{24} of each determined after cooling to room temperature. The E24 values for the biologically derived surfactants increased steadily with unit increase in temperature, implying high temperature stability of the biosurfactants unlike the SDS. There were significant ($p < 0.05$) decrease in E24 values biosurfactants in the three temperature ranges when compared to the SDS standard.

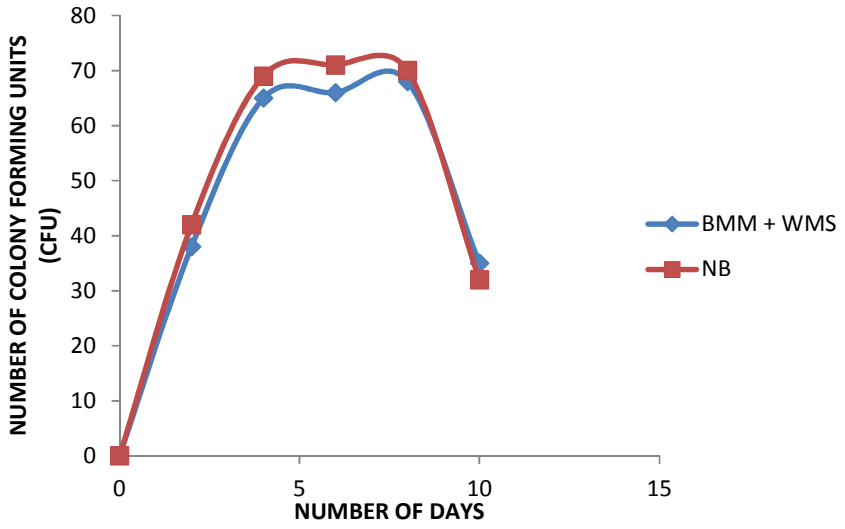

Fig. 1. Graph showing the growth of *Pseudomonas aeruginosa* in the two fermentation media

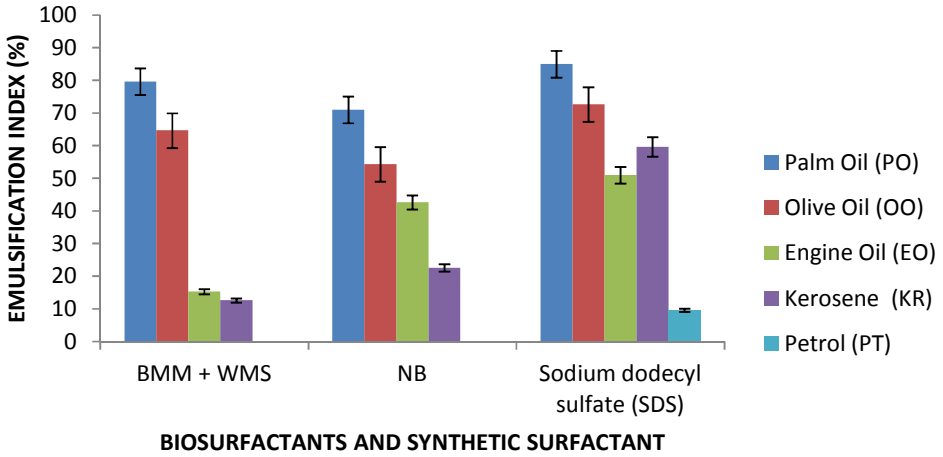

Fig. 2. Emulsification Index (E24) result in percentage (%) for the crude biosurfactants from *Citrullus lanatus* seeds supernatant, Nutrient broth supernatant, and SDS with different vegetable oils and hydrocarbons

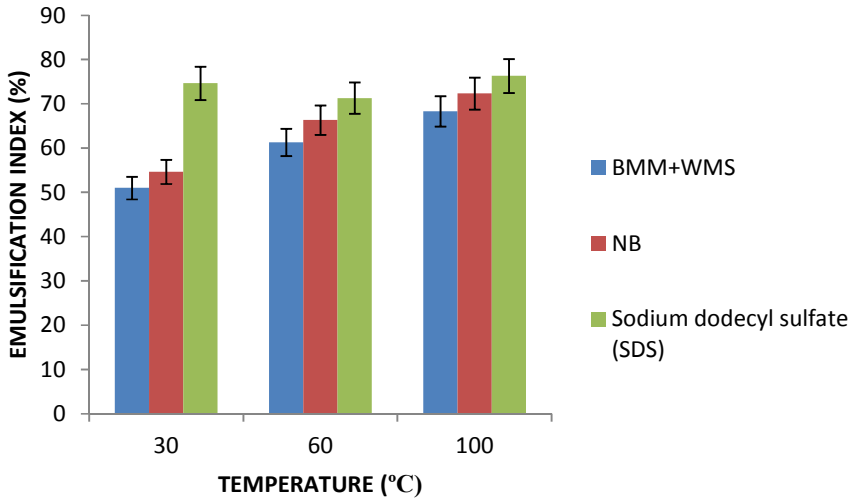

Fig. 3. Thermostability E24 test result in percentage (%)

3.4.2 Salinity stability test

NaCl solutions were made in 2%, 5%, 7% and 10% w/v using the surfactants and the E24 was determined accordingly as shown above; Increasing Salinity content of the crude biosurfactant solution resulted in a sensitive decrease in E24 values indicating rapid loss of activity. There were significant ($p < 0.05$) decrease in E24 values of the biosurfactants in all the salt concentration ranges when compared to the SDS synthetic surfactant.

3.4.3 PH stability test

The pH range of 2.0, 4.0, 6.0, 8.0, 10.0, and 12.0 in both supernatants were adjusted using 1 N HCl, and 0.4 M NaOH. The highest biosurfactant activity was recorded in pH 8 followed by pH 10.0, pH 6.0, pH 4.0, and pH 2.0. This shows that optimum pH for the biosurfactant is tilted towards the alkaline medium. Again across the E24 values for pH stability test biosurfactants had significantly ($p < 0.05$) lower activities when compared to the SDS surfactant.

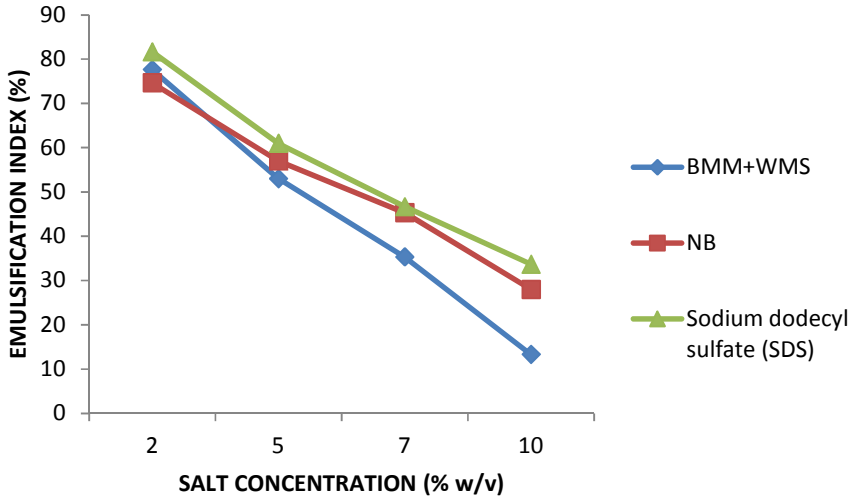

Fig. 4. E24 Result in percentage (%) for salinity stability test of the biosurfactants

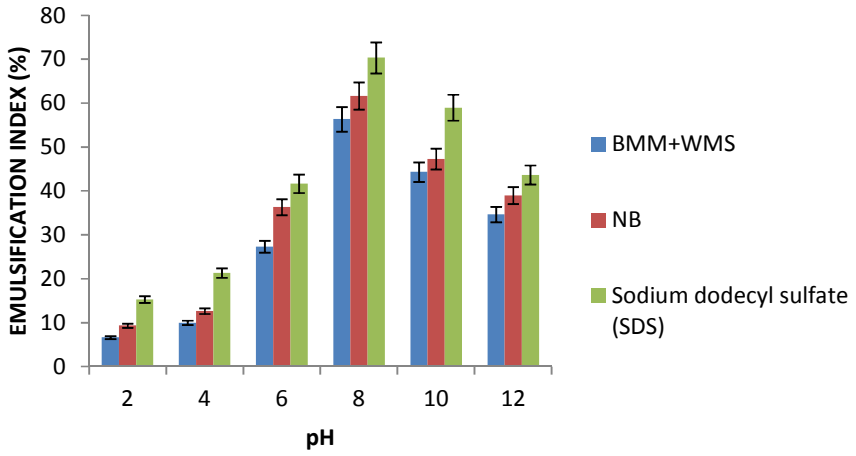

Fig. 5. E24 Result in percentage (%) for pH stability test

3.4.4 Haemolysis test

Result of the haemolytic test as viewed directly did not show a clear zone (zone of haemolysis) around the colonies after this period was indicative of non-haemolytic biosurfactant activity by the biosurfactants produced from *Citrullus lanatus* seeds.

3.5 Discussion

This research was conceived to harness local resources in the environment and convert them to useful biosurfactant that will be environmentally friendly and will pose no health hazard in contrast to the existing ones. Hence all the materials were carefully selected from the local environment. Activity determination in each of the production media were carried out using; emulsification index (E24), haemolytic assay, surface tension reduction assay, antimicrobial test, also the stability of the biosurfactant were analyzed using; thermo stability, halostability, and pH stability.

Cetrimide agar is a selective medium for bacteria of the genus *Pseudomonas* [20]. Pure strain of *Pseudomonas aeruginosa* was isolated using standard method described by Atlas [12] and inoculated into the two production media upon harvest of the crude biosurfactant on the 7th day of fermentation and the screening result for the activity of the biosurfactant which was positive indicated the presence of the surfactant of biological origin in the two media. This goes to confirm the actual isolation of *P. aeruginosa* with biosurfactant producing ability from the native soil as against many works in literature that promoted isolation of microbes from crude oil contaminated soil for biosurfactant production. This could be attributed to the high versatility of *P. aeruginosa* in terms of their carbon source utilizing ability [21,22]. However, prior to use the isolate was reactivated by sub-culturing from the bijou bottle slant stock to obtain a 24 hrs more active cells for fermentation. The 24 hrs grown microbial cells have been shown to be most suited for studies [23]. This could be that their cellular constituents like; enzymes, cell structures, and nutrient utilizing capacity are stable enough to withstand varying environmental conditions which could arise from nutrient, temperature, pH, or ionic stress from their new medium.

Proximate analysis of *Citrullus lanatus* seed shows that it contains good amount of carbohydrates, lipids and protein, which is in line with the works of Seyed et al. [24], Fila et al. [25]. Hence it is nutritious for *P. aeruginosa* growth in the fermentation media. The moisture content of the seed is relatively high. This was as a result of the mild drying condition with the intention of maintaining intact seed components like the fatty acids which could undergo some degradation in the form of rancidity when subjected to excessive heat of the sun as reported by Taiwo et al. [26]. Partly the goal of this work was achieved with respect to utilization of the pulverized seed as carbon source by the microorganism certifying the cheap microbial substrates for biosurfactant production.

Fermentation, and inoculation (cultivation strategies) described for rhamnolipid and other biossurfactants include shake flask, batch, fed-

batch, continuous, and integrated microbial/ enzymatic processes [27,15]. The present study utilized batch fermentation which was carried out in six conical flasks fermentation media. Batch fermentation method seems to favour laboratory research in biosurfactant production due to its simplicity, unlike the bioreactors employed industrially which are automated and provide the needed environmental conditions like; Temperature, Oxygen transfer, and pH during the fermentation process, and allows up scaling studies [28,29]. Optimization of aeration, and also even surface area for the culture conditions were provided by planting the flasks in a shaker machine throughout the fermentation period. Shaking of the fermentation media has been proven by head researchers in biosurfactant production to have a positive effect on the yield of biosurfactant.

The fermentation process was brought to a halt at the 7^{th} day as the two triplicate media were pulled together accordingly forming two bulk media. This was done to ensure homogeneity before centrifugation. Determination of growth of *P. aeruginosa* microorganism by counting the number of colonies formed in the two culture media was used to monitor the viability and density of the fermenting microorganism with time. This showed lag, log, stationary, and decline phases of the microbe indicating their functionality ability. Nutritional diversity of *P. aeruginosa* was revealed from the rapid increase in the number of colonies formed in the nutrient broth medium, and basal mineral medium plus Watermelon seed. This support the work of Priya and Usharani, [23] who reported the ability of the microorganism to virtually utilize a good number of substrate as carbon source making it ubiquitous.

Optimization of the production conditions for biosurfactant was carried out before mass production to enhance the biosurfactant yield in the course of the fermentation process. pH of the media, and duration of fermentation were optimized. The best pH for the operation was determined to be pH 8.0 which gave the highest E24 value. This shows that the pH of production is tilted in the alkaline medium, suggesting that the enzyme catalytic sites for the secretion of biosurfactant is most favoured in the alkaline medium. On the other hand best time/day for the harvest of biosurfactant from the production media was found to be on the 7^{th} day going by the highest value of the E24 in the time cost study among the ten days of analysis. This is in line with the works of Santa et al. [30], and Anna and Parthasarathi, [31]. Explanation of this could be that *P. aeruginosa* producing microorganism which attained stationary phase from the fourth day of fermentation gets saturated of the secondary metabolite (biosurfactant) from the 7^{th} day and developed the ability of absorbing biosurfactant into their cell walls and hence serve as nutrient for the microbes thereby reducing the amount of biosurfactant in the media as the stationary phase continued even after the 7^{th} day. This suggests that there were switch of nutrient source from primary to secondary metabolites by the microorganism.

The biosurfactant activity in the two supernatants resulting from the two fermentation media were tested using emulsification index (E24) test on the crude biosurfactant, stability tests, and haemolysis test. The result of the emulsification index test, revealed biosurfactant activity in all the media supernatants and were able to form stable emulsions with olive oil, palm oil, and engine oil. However, emulsion formation in olive oil, and palm oil were sufficiently greater than that in engine oil, this could be as a result of the *P. aeruginosa* producing chiefly glycolipids (rhamnolipid) biosurfactants when the carbon source is purely carbohydrate [32,33,34]. Or because of the structures of the vegetable oils. The emulsification index in Kerosene was quit poor, worst still there were no emulsification of petrol hydrocarbon at all in the two crude biosurfactant solutions bringing their (kerosene, and petrol) E24 values to be far less than 50%. This still may been as a result of the carbon source or the fact that the fermenting microorganism was isolated from hydrocarbon non-contaminated soil hence lack the ability to secret biosurfactant with hydrocarbon emulsifying capacity. The E24 results show significant ($p < 0.05$) decrease in the E24 results of the biosurfactants as compared to the SDS synthetic surfactant, however WMS derived biosurfactant had non-significant ($p > 0.05$) decrease in palm oil when compared to the activity of SDS in palm oil. Emulsification indexes obtained in this work could equate some work in the literature. However, the low activity of the surfactants unlike some works of; Rashedi et al. [14], and Celia et al. [33]. This could be from the novel carbon source with *P. aeruginosa*, inadequate optimization of production process like; temperature, trace elements for microbial growth, types of vegetable oils or hydrocarbons may have contributed to this fact. Also the crude biosurfactants (supernatants) used instead of the

pure biosurfactant solution in this emulsification index analysis could affect the result as well. Release of some cellular components other than biosurfactants could also take place in the harvesting process with the centrifuge machine.

The results from stability tests on the crude biosurfactant which were analyzed by varying some parameters like; pH, temperature, and the salinity (NaCl), to a certain degree and the emulsification index values determined accordingly showed that; the biosurfactants are most stable at pH 8.0 as revealed from the E24 values showing that alkaline medium supported the biosurfactant activity preferentially more than the acidic medium as there was a gradual increase in E24 value from acidic to alkaline region. Though this finding contradict some reviewed works; Anna and Parthasarathi, [31], and Iroha et al. [35] who promoted best pH for biosurfactant production to be 7.0 and 6.0 respectively. Optimum pH for this work (pH 8.0) could have resulted from the nature of the carbon source, and/or other environmental conditions prevailing in the course of biosurfactant production. This indicates that the biosurfactant is most useful in an alkaline environment. pH 8.0 was also reported as the optimal pH of production according to Saharan et al. [15]. More over the highest recovery of rhamnolipid was obtained with pH 8.0 according to Pimienta *et al.* [32].

The temperature stability test showed an increase in E24 values with unit increase in temperature, indicating increase in biosurfactant activity. The increase in E24 value with temperature increase could be that upon heating of the crude biosurfactant, there were liberation of more biosurfactant molecules from the solution which remained active even after cooling to the room temperature. The variation increase in E24 value with temperature is in tandem with many reviewed works; Silva et al. [36], and Anna and Parthasarathi, [31]. Though molecular explanation to this phenomenon is yet to be satisfactorily unravelled. The increase in the activity of surfactants of biological origin suggest their applicability in some industrial settings that use high temperature in their mode of operations. Worthy of note is the haphazard increase and decrease in the activity of the SDS synthetic surfactant in the varying temperature unlike the biologically derived surfactants that had steady increase in their activity.

Result of the salinity stability of the biosurfactant solution showed an abrupt decrease in the activity of the surfactants starting from the 2% NaCl concentration. The super sensitivity of the surfactants on even the slightest increase in sodium chloride concentration is an express indication that the surfactant cannot withstand industrial application that deals with NaCl (Na^{+}&Cl^{-}) and the similar ions. Although this contradicts the work of Gesheva et al. [37], however, they used halo-tolerant *Rhodococcus fascians* for biosurfactant production. Secondly it shows that biotechnologically, it cannot be used in bioremediation of marine geographical locations. The destruction of the surfactant activity in NaCl solution could be that there was ionic interference in the native structure of the biosurfactant leading to destruction, and polarization of the constituting molecules and/or atoms thereby resulting in permanent loss of activity.

Worthy of note is the streamlined pattern of E24 results in all the stability tests carried out, it was observed that SDS synthetic surfactant recorded the highest E24 in all, followed by NB medium derived biosurfactant, and then BMM+WMS medium produced biosurfactant. This further shows that regardless of the two different media of their production, the surfactants are of the same characteristics; this could be as a result of same microorganism used in their production. However in most cases there were negligible differences between NB surfactant, and BMM surfactant activity. It was observed in all the stability tests conducted that the two biosurfactants activities were significantly ($p < 0.05$) lower than that of sodium dodecyl sulphate (SDS) synthetic surfactant. Explaining this could come from the level of purity among the surfactants.

Rating the crude biosurfactant activity and the purified SDS synthetic surfactant by virtue of their E24 results, for instance 85.00 ± 4.35%, and 79.66 ± 1.52% corresponding to SDS surfactant, and WMS derived biosurfactant E24 values with palm oil, shows that purification of the surfactant of biological origin would result in a surfactant that will far out weight the synthetic surfactant in every aspect.

Hemolytic test was negative in the two biosurfactants solutions. This shows that they may not cause anemia if by chance got exposed to human erythrocytes in the course of their usage.

4. CONCLUSION

In this research, biosurfactant was produced using locally sourced *Citrullus lanatus* seeds as the raw material in an attempt bit to find alternative to synthetic products which are often of less quality. It was optimized using *Pseudomonas aeruginosa* isolated locally from the soil within the University of Nigeria, Nsukka campus. The biosurfactant produced showed high activity marked by high emulsification index (E24) which value increased with rise in temperature but decreased with increasing salt (NaCl) content of the biosurfactant solutions, high thermostablity, negative haemolytic effect and optimum pH value of 8. When compared to biosurfactants from synthetic sources, that from *Citrullus lanatus* seeds was of better quality.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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