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Original Research Article

Production of Biosurfactants *Aspergillus niger* and *Rhodotorula sp* Isolated from Sugar Cane Bagasse Dumpsite: A Comparative Study

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ABSTRACT

Biosurfactants are amphiphilic and surface-active compounds produced by microorganisms. Their relevance can be seen in petroleum, agriculture and pharmaceutics. Environmental pollution from agricultural wastes and by products has reached its peak and alternate applications of these wastes for other possible benefits are the current trends. This study compared the physicochemical properties and activity of biosurfactants produced from fungi isolated from sugar cane bagasse dumpsite. Two species of fungi were isolated and identified from sugar cane bagasse and identified as *Aspergillus niger* and *Rhodotorula* sp. by physiological and morphological tests. They were grown on mineral salt medium (MSM) for 14 days using sugar cane bagasse as carbon source. The microbial broths were assayed for biosurfactant production using drop collapse method, oil displacement method, and emulsification method. The emulsification activity of biosurfactants produced by *Rhodotorula* sp. recorded 51.133±4.964% which was significantly different when compared to that of *Aspergillus niger* (48.067±3.126%). The *Aspergillus niger* performed poorly in the drop collapse test assay (DCTA). There was a significant difference in the clear zone formation (CZF) produced by Rhodotorula sp (0.167±0.029 cm) when compared to that of the *Aspergillus niger* (0.133±0.289 cm). Biosurfactants produced were isolated using acid precipitate method and characterized primarily to yield high levels of carbohydrate and lipids. The biosurfactant produced by *Rhodotorula* sp. had a higher wetting and emulsification activity. This study suggests that the crude biosurfactants isolated from *Rhodotorula sp* shows promising possibilities for a wider application in pharmaceutical industries where emulsion is important towards efficiency in production.

Keywords: Biosurfactant, Micro-organism, Sugar cane Bagasse, Wetting property.

Introduction

The growing environmental threats posed by agricultural-based industrial wastes have necessitated a search for alternative application of these wastes. Such wastes including sugar cane bagasse, molasses and grain chaffs could serve as used substrates for fermentation processes in order to produce different commercial products like biogas, bioethanol, biofuel and biosurfactants.[1] Biosurfactants are surface-active compounds produced by microorganisms. They possess the characteristic ability to limit the surface and interfacial tension in a solution or solid using the same mechanisms as chemical surfactants.[2] Micro-organisms reported to produce biosurfactants include bacteria, fungi and yeasts.[1] Biosurfactants are grouped according to their mechanism of dissociation in water; based on their chemical composition (some of which include: glycolipids, lipopeptides and protein, surfactin, fatty acids, polymeric and particulate); their molecular weight (high and low molecular weight), their physico-chemical properties; their mechanism of action and their microbial origin.[3] Biosurfactants have diverse applications in industrial processes involving emulsification, foaming, detergents, wetting, dispersing or solubilisation.[4] These compounds are environmentally friendly, degradable by organisms and non-hazardous. The foaming properties and its selectivity is higher than the synthesized ones. They have been found to be stable at extreme pH, temperatures and salinity as well as being able to be produced from industrial wastes and by-products [5]. The complete structural elucidation of biosurfactants can be done by various chromatographic and spectroscopic techniques.

By-products of sugar cane have been used as substrates for biosurfactant production.[6] Lima and Souzar,[7] produced biosurfactants from sugarcane vinasse using *Bacillus sutilis* PC. Sugar cane (*Saccharum officinarum*) is a tall perennial grass of the genus *saccharum* tribe *andropogonae* mostly grown in template regions and used for sugar production. The main products of sugarcane are sucrose and ethanol produced from sugarcane industry in large quantity in Brazil. The worlds demand for sugar is what drives the sugarcane agriculture.[7] The sugarcane contains fibre (11-16%), soluble sugars (12-16%), non-sugars (2-3%) and water (63-73%). These figures can vary depending on crop management.

Fungi such as *Aspergillus*, *Penicillium*, *Saccharomyces*, *Torula*, *Pichia*, *Rhodotorola* and bacteria including *Streptomyces*, *Pseudomonas*, and *Bacillus* are commonly reported as responsible for the deterioration of sugar.[8] *Bacillus* and *Pseudomonas* are known for their ability to breakdown compounds with carbon chain. These compounds could be sugars, cellulose, lignin, and hemi-cellulose as such contained in the sugar cane bagasses. In the process, these organisms can produce expolymer such as biosurfactant.[9] Various

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microorganisms were reported to produce biosurfactants. These microbes range from different bacteria, fungi and yeasts.[9]
Environmental pollution from agricultural wastes has reached its peak and alternate applications of these wastes for other possible benefits are being looked into by researchers. This study was therefore aimed at Comparing the biosurfactants produced by two different yeasts (*Rhodotorola* sp. and *Aspergillus niger.)* isolated from the sugar cane bagasse (chaff) dump site with a view of determining the one with better activity yield and potentials with respect to the physiochemical properties.

Materials and Methods

Collection of sample
Sugar cane bagasse were collected on march 2018 from a dumpsite at Ogurugu road, Odenigbo, Nsukka, Enugu state. They were air dried and pulverised.

Isolation and identification of microorganisms:
Preparation of Liquid Broth for Isolating of Micro Organisms
Sample from sugarcane bagasse was homogenized in a sterile medium containing 1 g sugarcane bagasse, 0.04 g of NH_4CL, 0.01 g of KH_2PO_4, 0.001 g of NaCl, 0.005 g of $MgS0_4.7H_2O$, 0.01 g of $ZnSO_4.7H_2O$, 0.01 g of $FeSO_4.7H_2O$, 0.01 g of $MnSO_4.7H_2O$, and 0.015 g of yeast extract. The mixture was incubated at 30°C for 48 hrs according to the method of El-Banna *et al*.[10] and a 10-fold serial dilution was carried out.

Inoculation of plates and sub-culturing
A loop from the liquid medium was streaked from each side of the plate onto the solid medium under the flame of Bunsen burner. The plates were later incubated at 35°C untill visible colonies were observed. All colonies with varying morphological properties were purified by a continuous streaking and sub-culturing on different plates. This process was continued till pure cultures were obtained.

Identification of microorganisms
The isolates (2 distinct colonies) were isolated using nutrient broth and identified by carrying out morphological and physiological tests on them using the microscope.

Media Used for biosurfactant production
Mineral Salt Medium (MSM) for Aspergillus niger:
sugarcane bagasse (1%) was homogenized in a sterile medium containing 100 mL of distilled water as carbon source (dried form), mineral salt medium (MSM) containing the following in g/L (0.8 KCl, 0.8 NaCl, 0.1 $CaCl_2$, 0.2 $MgSO_4$, 2.0 Na_2HPO_4, 2.0 NH_4cl 0.1 $FeSO_4$ and pH 6.2) and 0.015 % of yeast extract and was made up to 500 ml with distilled water. The medium was autoclaved as describe above. In preparing the media for *Rhodotorula* species, 1 g of sugarcane bagasse (dried form) was introduced into 100 mL of distilled water and 0.04 g of NH_4Cl, 0.01 g of KH_2PO_4, 0. 001 g of NaCl, 0.005 g of $MgSO_4.7H_2O$ and 0.015 g of yeast extract and was made up to 500 mL and autoclaved.[11]

Determination of microbial growth with substrate
The microbial growth was determined by taking spectrophotometric readings of the microbial broth every 24 hrs for 7 days at a wavelength of 600 nm. The optical densities were plotted against time to determine the growth curve.[12]

Screening for biosurfactant production:
This screening was done by carrying out the drop collapse test, emulsification activity, oil displacement test on the microbial broth.

Drop collapse test:
Qualitative drop collapse test was performed according to the method described by Chandran and Das.[13] A quantity, 2 µl of kerosene was added to a 96 well microtitre plates. The plates equilibrated for 1h at room temperature. The culture supernatant (5 µL) was added to the surface of the kerosene and the shape observed after 1min.

Oil displacement test
The oil displacement test was carried out according to the method described by Morikawa *et al*.[14] A quantity, 30 mL of distilled water was added to a petri dish with a diameter of 15 cm. Thereafter 20 µL diesel was dropped into the surface of the water, followed by the addition of 10 µL of cell culture supernatant. The diameter of the clear halo visualized under visible light was measured after 30s.

Determination of emulsification index
This was determined as described by Rodrigues *et al*.[15] A quantity, 1ml of kerosene was added to 1ml of cell free culture broth in a centrifuge tube, vortexed for 2min and left overnight. The emulsification index was determined by the equation below.

$$emulsification\ index = \frac{Height\ of\ emulsified\ layer}{Total\ height\ of\ liquid} \times \frac{100}{1} \quad \text{-------- 1}$$

Biosurfactant extraction
Biosurfactants produced were isolated using acid precipitate method and characterized primarily. The broth was centrifuged at 13,000 rpm for 15 min. The pH of the supernatant (often referred to as unrefined biosurfactant) was reduced to pH 2.0 using 2 N HCl and left to stand for 24 hr at 4°C for complete biosurfactant acid precipitation. A batch of the biosurfactant was also extracted with an equal volume of either acetone, dichloromethane, ethyl acetate or chloroform/methanol (2:1).[21]

Determination of some proximate composition of the biosurfactant
Estimation of the lipid content
The lipid content was estimated as described by Frings and Dunn.[16] A known volume, 0.1 mL of the biosurfactant was taken in a tube and mixed with 1.9 mL of 95% conc. sulphuric acid and placed in a boiling water bath for 15 mins. The mixture was allowed to cool, 0.2 mL of the sample was drawn and mixed with 5.0 ml of vanillin reagent. The tube was let to stand for 30 minutes and the absorbance was measured at 540 nm. A standard curve was plotted using 100-500 µg/mL concentrations of olive oil.
Calculation:

$$total\ lipids\ in \frac{mg}{dl} = Absorbance\ of\ sample\ conc \times Absorbance\ of\ sample\ conc \quad \text{---- 2}$$

Estimation of the carbohydrate content:
The carbohydrate content was evluated following the Anthrone method as described by Ilori *et al*.[17] and Umeji *et al*.[18] Two millilitres (2.0mL) of the biosurfactant solution (0.1g biosurfactant in 1000ml distilled water) was added into test tubes, 3.0ml distilled water and 10.0mL of 0.2% solution of anthrone reagent (containing 0.2% anthrone in 95% H_2SO_4) was added to the same test tube. Read at 630 nm against a blank constituting water and anthrone reagent. Glucose was used as standard and standard curve prepared.

Estimation of the protein content:
Protein content estimation was determined using the biuret's method. A standard kit that makes use of biuret's method. A kit which has a standard concentration of 60 g/L. 20 µl of biuret's standard was put in a test tube, 20 µl of the biosurfactant was also put in a separate test tube and 20 µl of distilled water was put in another test tube. 1µl of biuret's reagent was put into three test tubes and incubated at room temperature for 10 mins. Then absorbance at 546nm was taken

$$protein\ conc\ in\ g/L = \frac{change\ in\ sample\ absorbance}{change\ in\ standard\ absorbance} \times \frac{standard\ conc}{1} \quad \text{---- 3}$$

Statistical analysis
Data were collected in triplicates and expressed as mean ± standard deviation. One-way analysis of variance was used in separating the mean and comparing the differences. Statistical Product and Service Solution (SPSS) version 16.0 was used as the statistical package. The level of significance was observed at $p < 0.05$

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Results and Discussion

This present study was explored to determine biosurfactant production using microorganisms isolated from sugar cane bagasse. The sugar-cane bagasse was used as substrate.

Microorganisms isolated were identified as *Aspergillus niger* and *Rhodotorula* sp. The *Aspergillus niger* isolate cells had large, globule, dark brown conidial heads. The colony cells had a basal white to yellow dense layer of dark brown to black conidial heads while The isolate cells of *Rhodotorula sp.* where oval in cell shape when viewed under the microscope with magnification of 40x, smooth in colony texture, and light pink in colour. The growth patterns of *Aspergillus niger* on substrate showed minimum growth on day 2 and maximum growth on day 10. There was an increase in growth on the day3, day 7 and day 10 and a continuous decrease in growth from the day 10 as shown in Figure 1 below. While the growth patterns of *Rhodotorula sp* showed minimum growth on day 2 and maximum growth on day 9. There was a steep increase in growth on the day 3 and a continuous decrease in growth from the day 9 as also seen in Figure 1. The minimum growth could be referred to as its lag phase where the organisms are still acclimatizing to their new environment while their maximum growth or point were conditions were availability of resources and nutrients are easily taken up by the microbes. The increase in growth of the *Aspergillus niger* on the different days could be as a result of acclimatisation of the organism to the environment it found itself. The reduction in growth may be due to the depletion in nutrient due to consumption by the organism *Asergillus niger*. This cis in tandem with the steep increase in growth noticed for *Rhodotorula sp* and the decrease in growth observed. The drop collapse test run for the preliminary screenings of biosurfactants are qualitative tests which are indicative of the surface and wetting activities.[19] This relies on the destabilization of liquid droplets by biosurfactants. The stability of the drop depends on the concentration of the biosurfactants. In this work, wetting activities of the unrefined biosurfactants were probed in comparison with each other. Drop collapse test of that of *Rhodotorula* sp. had a moderately positive result than that produced by *Aspergillus niger*. This shows that *Rhodotorula* sp. has a higher concentration of biosurfactant produced than that of *Aspergillus niger*. This occurs when the interfacial tension between two immiscible liquids get reduced as can be seen in Table 1.

Emulsification index is reliable in checking for biosurfactant production. The stability of the emulsification is an indication of strength of the surfactant. The emulsification index of the *Aspergillus niger* was 47.200 ± 2.800%, 43.633 ± 1.521% and 48.067 ± 3.126% on the 3rd, 7th and 14th day respectively while the *Rhodotorula sp* had 49.433 ± 1.499%, 50.467 ± 1.805% and 51.133 ± 4.964% as its emulsification activity on day 3, 7 and 14 respectively. A study by Chandran and Das,[13] *Rhodotorula muciliginosa* and *Candida rugosa* were able to form stabilized emulsions (86 ± 0.7%) with diesel. Chandran and Das[13] also reported that biosurfactant obtained from yeast species can be applied in environmental conditions with high and low pH. The biosurfactant produced by *Aspergillus niger* in this study has a low emulsification activity with kerosene, this was also reported by Kannahi and Sherley.[20] Conpairing the emulsification activity of the biosurfactant produced by *Aspergillus niger* with that of *Rhodotorula sp*, *Rhodotorula sp* had higher emulsification activity.

The clear zone formation in oil displacement is a measure of the concentration of the biosurfactants produced by these two microbes as can be seen in Table 2. The diameter of the clear zones formed by the *Aspergillus niger* on the day 3 had the same measurement with that of *Rhodotorula sp* on day 7 of the fermentation days which could be as a result of equal concentration of the biosurfactant produced on that day. On the day 14 the *Rhodotorula sp* had a higher measurement than that of the *Aspergillus niger* which could also be as a result of an increased concentration of the biosurfactant produced by *Rhodotorula sp*. The drop collapse test, oil displacement and the emulsification activities that were carried out seemed to be growth associated. This is also in line with the biosurfactants produced by *Rhodotorula* species and some bacteria (*Pseudomonas sp*. and *Bacillus sp*.) species that were grown on diesel.[17] Biochemical characterization of the isolated biosurfactants, gave the usual profile of the produced biosurfactants which had carbohydrates and lipids as major constituents (glycolipids) but in different concentrations as seen in Table 4. The biosurfactant produced by Rhodotorula specie in this study has carbohydrate and lipid constituent concentration in percentage as 31.11% and 65.46%. Das and Chandran[13] also reported biosurfactant with 30.78 % and 60.23% carbohydrate and lipids respectively produced by *Rhodotorula sp*. The biosurfactant produced in this work by the *Rhodotorula sp* had higher physiochemical properties with reference to surface activity when juxtaposed to that of *Aspergillus niger* in the assays done in this work.

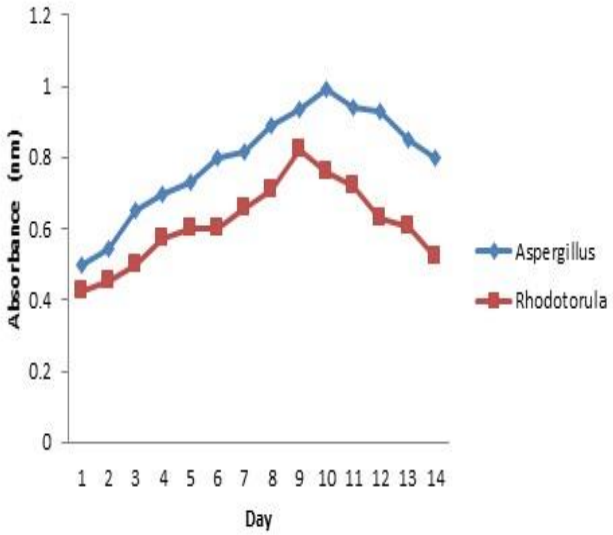

Figure 1: Growth curve of *Aspergillus niger* and *Rhodotorula sp* within fourteen days.

Table 1: Drop Collapse Test Result for *Aspergillus niger* and *Rhodotorula sp*

S/N	Characterization	Day 3	Day 7	Day 14
1	Distilled water	–	–	–
2	Supernatant of *Aspergillus niger*	+	+	+
3	Supernatant of *Rhodotorula sp*	+	++	++

– indicate negative drop collapse test
+ indicate low positive drop collapse test
+ + indicate moderate positive drop collapse test

Table 2: Oil Displacement Test Result for *Aspergillus niger* and *Rhodotorula sp*

S/N	Characterization	Day 3	Day 7	Day 14
1	Distilled water	Negative	Negative	Negative
2	Supernatant of *Aspergillus niger*	0.117 ± 0.029 cm	0.083 ± 0.076 cm	0.133 ± 0.289 cm
3	Supernatant of *Rhodotorula sp*	0.100 ± 0.00 cm	0.117 ± 0.029 cm	0.167 ± 0.029 cm

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Table 3: Emulsification Index of *Aspergillus niger* and *Rhodotorula Sp*

S/N	Day	*Aspergillus Niger*	*Rhodotorula sp*
1	3	47.200 ± 2.800%	49.433 ± 1.499%
2	7	43.633 ± 1.521%	50.467 ± 1.805%
3	14	48.067 ± 3.126%	51.133 ± 4.964%

Table 4: Percentage Concentration of the biosurfactants

Organism	Carbohydrate%	Lipid%	Protein%
Rhodotorula sp	31.11	65.46	3.43
Aspergillus niger	31.49	55.81	12.70

Conclusion

Aspergillus niger and Rhodotorula sp produced biosurfactants using sugar cane bagasse. The biosurfactant produced by *Rhodotorula sp* had a higher wetting activities and emulsification activity. As such this biosurfactant producing *Rhodotorula sp* should be exploited for bioemulsifiers and biosurfactants for proper applications in the industries

Conflict of interest

The authors declare no conflict of interest.

Authors' Declaration

The authors hereby declare that the work presented in this article is original and that any liability for claims relating to the content of this article will be borne by them.

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