ORIGINAL RESEARCH ARTICLE

Biosurfactant quality generated from fungi using agro-wastes (maize husk, rice husk, and augarcane bagasse)

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ABSTRACT
Biosurfactants are amphiphilic compound that contain hydrophilic and hydrophobic moieties produced extracellularly by microorganism on cell surface or excreted extracellularly thereby reducing surface and interfacial tension between molecules at the surface and interface, respectively. This study was aimed on the isolation and identification of biosurfactant producing fungi; the production of biosurfactants from the identified fungi in a submerged fermentation using sugarcane bagasse, rice husk and maize husk as carbon sources and the evaluation of quality of biosurfactants generated. The biosurfactant production was assayed for 21 days with constant agitation for at most four times daily in an incubator (30^0C). The emulsification ability of the biosurfactant produced was analyzed on two hydrocarbons: diesel and kerosene**.** In diesel, the emulsification index (EI) showed highest in biosurfactant produced by *Aspergillus niger* in Rice Husk (BAR) after 72 hours (E72) (80.30±0.30%) and lowest in biosurfactant produced by *Fusarum oxysporum* in sugarcane bagasse after 24 hours (E24) (BFS) (33.43±0.10%). In kerosene, the highest emulsification index was observed in biosurfactant produced by *Fusarum oxysporum* in Maize Husk (BFM) E72 (94.25±0.25%) and lowest in biosurfactant produced by *Fusarum oxysporum* in sugarcane bagasse (BFS) E48 (52.53±0.10%). Therefore, comparing the biosurfactants produced by *Fusarum oxysporum* and *Aspergillus niger*, *Fusarum oxysporum* exhibited more emulsification ability than *Aspergillus niger*. The result of EI showed that Maize Husk and Rice Husk are good substrates for biosurfactant production while *Fusarum oxysporum* is the better biosurfactant producing fungi. In comparing the hydrocarbons, more emulsion was noticed in diesel than kerosene. The result of oil spreading assay with spent engine oil showed that biosurfactants produced by *Fusarum oxysporum* grown on Rice Husk had the highest area of displacement (13.88±0.01cm^2) while biosurfactant produced by *Aspergillus niger* grown on Sugarcane Bagasse showed the least area of displacement (4.91±0.00cm^2). The carbon sources used in this work are good for the production of biosurfactant. Upscaling of the process will yield biosurfactants applicable in industries.

Keywords: *Agrowastes, Biosurfactants, Fungi.*

Introduction
The basic benefit of biosurfactants is to generate organic surfactants which will be useful in cleaning of oily and greasy wastes in industries, homes, offices and any other environment where human activities occur. Also, it is a way of converting wastes into useful substances as a result

help to reduce the accumulation of wastes in our environments. Biosurfactants have potential of being desirable products in industries; being organic in origin, it is expected that they have the capability of biodegrading after serving their dirt removal functions. Microbial surfactants or bio-surfactants are the surface-active molecules derived from a large number of microorganisms. They are amphiphilic compounds produced mostly on microbial cell surfaces or excreted extracellularly and contain hydrophobic and hydrophilic moieties that reduce surface and interfacial tensions between two immiscible fluids like oil and water (Anyanwu *et al*., 2011; Govindammal, 2013). Considerable attention has been given in the past to the production of surface-active molecules of biological origin because of their potential utilization in food processing, pharmacology, cosmetic, biomedical and petroleum industries (Emine and Aysun, 2009). Plant biomass are used as sources of energy for microbial fermentation with the aim of producing bio-surfactants. Recent advances have focused on the use of agricultural products, by products and wastes in bio-surfactant synthesis.

Materials and Methods

Carbon sources collection

Fresh and decomposing rice husk from rice mill, bagasse of sugarcane from old market, maize husk from Old BB areas of Wukari, Taraba State, Nigeria in April 2018, were obtained randomly without cost from processors. These fresh agro waste samples were washed with distilled water and dried; while the decomposing samples were carefully preserved in polyethylene bags kept in a refrigerator prior to isolation of microorganism.

Microorganism

Fungi for the research were isolated in the culture selection unit of the Department of Microbiology, Federal University Wukari from decomposing agrowastes (rice husk, sugarcane bagasse and maize husk) with Potato Dextrose Agar as growth medium according to manufacturer's recipe. Sub-culturing was carried out severally until individual pure strains were identified.

Sterilization Techniques

All glass wares used in this research were sterilized in a dry, ventilated oven at 160 °C for 2 hours after being thoroughly washed with detergents and clean water. All media were sterilized with the aid of autoclave for 20 min at a temperature of 121 °C and 15 psi. Needles were sterilized in 70% ethanol followed by heating to hot red in flame (Bunsen burner) before inoculation was carried out.

Preparation of culture medium

Potato Dextrose Agar (PDA) LAB M quality was used in fungal media preparation. Preparation of media was according to producer's protocol of inclusion of 39g PDA in a liter of water, which served in both sustenance and culture of isolates. For 20 minutes and at 121 °C and 15 psi, complete uniform mixing and solution of the medium was achieved following cooling to 45 °C and inclusion of a chloramphenicol capsule into prepared PDA equivalent of 500 ml to prevent bacterial growth (Green et al., 1994). Dispensing of 15 ml of the bactericidal PDA was into 86 mm diameter petri dish pre-sterilized. After solidification of medium, incubation was carried out at 28 °C (ambient temperature) for 24 hours (Cheesebrough, 1984).

Fungal Isolation

Onyike and Maduewesi (1985) had previously reported a method of isolation employed. Measured quantity of rice husk, sugar cane bagasse and maize husk already putrefied were kept

at patterned equal distance from one another on the solidified agar and incubated for 7 days at 27 ± 2 °C. These agro-waste samples in agar were observed for fungal growth and subsequently individually isolated through sub-culturing until pure cultures from each sample was obtained (Okigbo and Ikediugwu 2000).

Fungal isolates Identification

Microscopic and macroscopic method was used to observe the physical characteristics of the isolates. Pattern of growth, colour of colony, type of hyphae (loose, arial or compact) as well as texture (cottony, coarse or velvety) were also employed in fungal identification. Isolates were also stained, and slides prepared and viewed in low powered light microscopes to match the features outline by the guides and manual of identification by Alexopoulos (1961); Nelson *et al*. (1983); Rippon (1988); Samson *et al*. (1991); and Snowdon (1991).

Further Identification: Biochemical Test

Citrate Test: Blue to greenish colour was indicative of positive while non colour variation was a negative indicator of isolates in a citrate Simond agar at 30^0 C after 24 hours.

Catalase test: Presence of organisms in hydrogen peroxide with evolution of hydrogen bubble is a positive catalase test while absence of hydrogen bubble is a negative indicator.

Sugar fermentation test: Production of gas by fungi causing pH change and colour variation in the presence of phenol red shows positive test for sugar fermentation.

Bioemulsifier/Biosurfactant Production

Agro Wastes (Rice husk, sugarcane bagasse and maize husk) properly washed with solid wastes sorted out were for 24 hours dried at 80ºC, grounded (size < 3mm) and stored (-20° C). Spent Vegetable Oil (SVO) obtained opposite Federal University Wukari gate used for 6 hours per day for 3 consecutive days at 70-210^o C from pastry makers was employed.

Ingredients include: 10g waste (rice husk, sugarcane bagasse, and maize husk) in 250ml of distilled water with inclusion of 4% spent vegetable oil and 2.5g of yeast extract adjusted to pH of 5. Culture media (25mL) outlined above in 125 ml Erlenmeyer flasks was autoclaved at 121°C for 15 minutes, cooled and inoculated in triplicate with well identified fungi. However, negative control involved media with no inoculation. Incubation period of 21 days at 30 ± 2°C with 100 rpm using orbital shaker was applied and in absence of electricity, physical agitation of media was carried out for 90 minutes daily. At completion of incubation, filtration was carried out with a filter paper membrane having 0.45 mm porosity coupled with the aid of sterile 20 mL syringe (Figure 1).

Figure 1. Synthesized Bio-surfactants

Estimation of Biosurfactant Quality

Emulsification index (E24)

In this research Emulsification Index (EI) (%) after 24 hours, 48 hours, and 72 hours technique adopted, was a modification of that described by Cooper and Goldenberg (1987) and adapted by Kiran et al. (2010). Filtered 2 mL cell-free broth (of the tests and negative control produced biosurfactants) were each blended in 2mL of diesel and kerosene (hydrocarbon compounds) in a 150 x 16 mm glass pipe and agitated vigorously for 2 minutes. Emulsion formation after 24 hours of blending and agitation was compared to the total volume of added hydrocarbon.

$$\boldsymbol{EI}\ (\%) = \left(\frac{\boldsymbol{Emulsion\ layer\ height}}{\boldsymbol{Total\ height}}\right) \times \mathbf{100}$$

Where, E_{24} (%) = Emulsification Index after 24 hours etc.

Collapse qualitative Oil drop test

Produced surfactants in triplicate (filtered cell-free extract 3.5mL), placed in petri dishes (60 x 12 mm) had oil drops added to them and observed for 1, 5, 30 min and 72 hours duration. For negative and positive controls respectively, 3.5mL fungus-free extract and 3.5mL 1M dodecyl sulfate sodium (DSS) surfactant solution was used.

Biodegradability test using the redox 2, 6-dichlorophenol indophenol (DCPIP) indicator

The redox 2, 6-dichlorophenol indophenol (DCPIP) method was used in biodegradability test (Hanson *et al*., 1997). Polystyrene plates with 96 wells were used for the biodegradability test. Measured 200µL DCPIP solution from 0.010g/mL DCPIP concentration was added to 10µL of spent vegetable oil (SVO) and hyphae of fungi from different bio-surfactants produced were mixed. Measured 3 mm from the mixture of DCPIP and SVO illustrated above was dispensed into the wells. Duration of 24 to 48 hours at 27±2°C was used to determine medium discolouring-time. Positive and negative control involved DCPIP with oil and without strain respectively.

Area Test of Oil displacement

Measurable diameters results when surfactants encounter oil layers thus creating non-oil water clear surfaces. This is the principle of oil displacement which is also a noteworthy determination of bio surfactant efficacy. Briefly, 10µl of spent engine oil (SVO) was layered on the surface of 30 ml distilled water in a 15 cm petri dish. The known produced bio surfactants (5µl) each was carefully dropped on the center of various layered oil. Clear diameter (figure 2) seemingly forming oil spread halo was observed after 30 second and area of dispersion calculated (Pornsunthorntawee *et al.,* 2008).

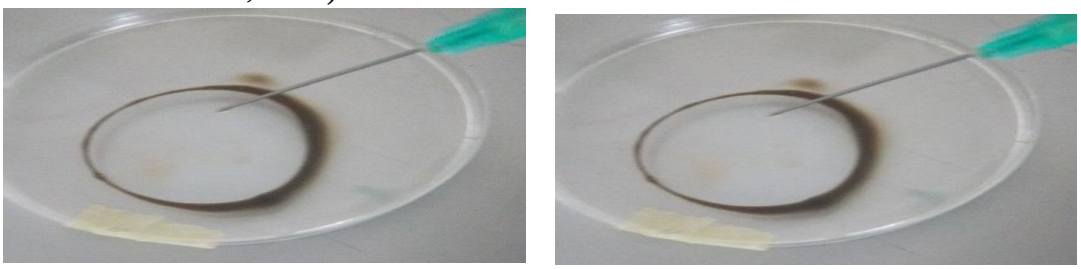

Figure 2: Oil spreading by Bio-surfactants

Test for Iodine
Few drops (4 drops) of iodine solution were added to small quantity of biosurfactant and gently agitated. Colour change was observed for positive result or otherwise (Mahesh *et al*., 2006).

Test for Saponification
Little quantity of biosurfactant was gently mixed with 2 mL NaOH (2%) solution and vigorously agitated to observe for soap frosty formation (Mahesh *et al.,* 2006).

Statistical Analysis
Analyzed of data was carried out using SPSS version 20 descriptive statistics and the results presented as Mean ± Standard Deviation.

RESULTS
Microbial Identification
The morphological identification showed that two of the fungi isolates were fungi A identified as *Fusarium oxysporum (*figure 3) and fungi B (figure 4) as *Aspergillus niger*.

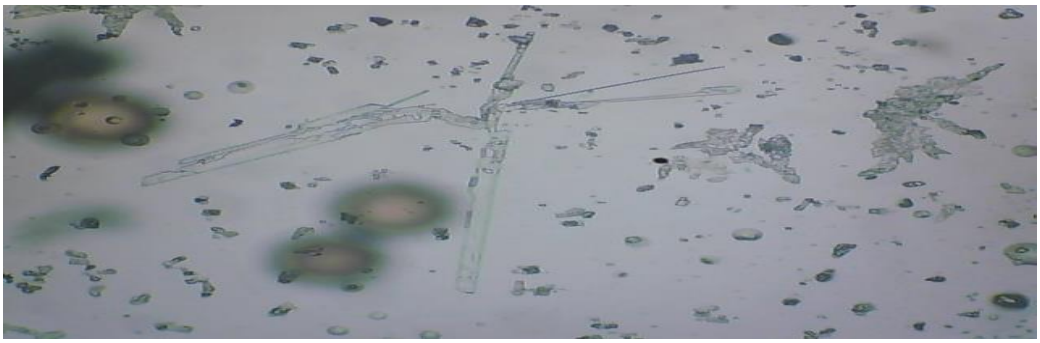

Figure 3: Fungi A (*Fusarium oxysporum*)

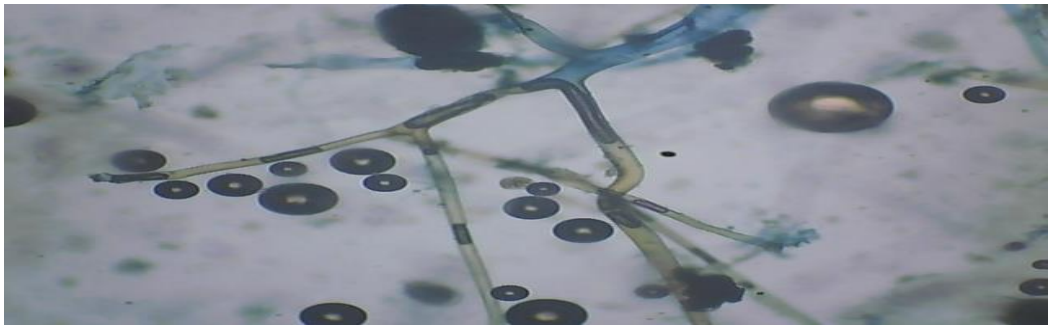

Figure 4: Fungi B (*Aspergillus niger*)

Table I: The Biosurfactant Fungi Producing Isolates Colony Morphology

Features	Fungi A	Fungi B
Shape	**Irregular**	**Irregular**
Colony Type	**Compact**	**Compact**
Texture	**Velvety**	**Cottony**
Size	**1 mm**	**1 mm**
Colour	**Gray**	**Black**
Diameter	**75mm**	**75mm**
Hyphae	**Aerial hyphae**	**Aerial hyphae**

The identification of fungi: Biochemical test

Table II: Biochemical test for identification of fungi

Test	Fungi A	Fungi B
Peptone/Covax Test	+	+
Citrate	+	+
Catalase	+	+
Triple Sugar Identification (TSI):		
Glucose	-	+
Lactose	-	-
Sucrose	-	-
Hydrogen Sulphide	-	-

Fungi A = *Fusarium oxysporum*, Fungi B = *Aspergillus niger*
Key - = Negative Test, + = Positive Test.

The result for fungi A and B shows positive in peptone, citrate and catalase test. Glucose, lactose and sucrose test shows negative for fungi A, while glucose test was positive for fungi B and lactose and sucrose were negative.

Biosurfactant Test
Emulsification Index (%)
The percentage emulsification index was analyzed using SPSS version 20 and the result is presented as Mean ± Standard Deviation in figure 5 below.

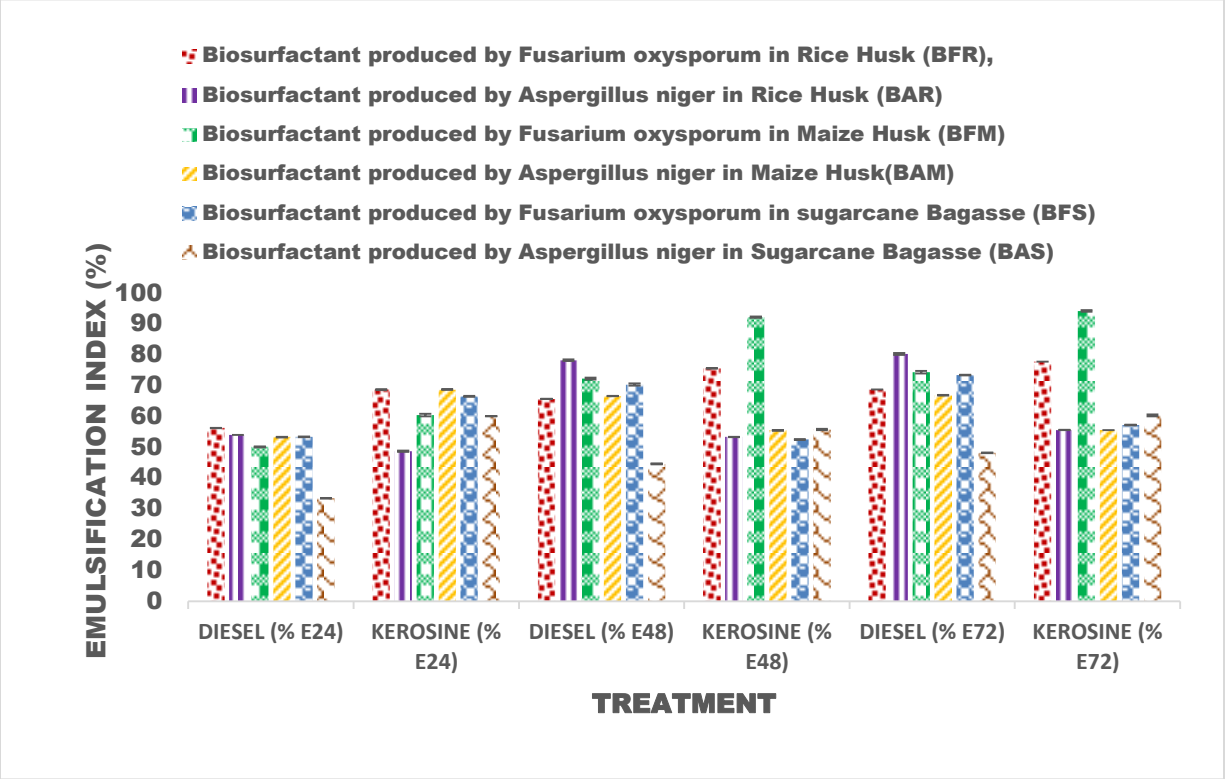

Figure 5: Emulsification Index (%) test using diesel and kerosene

BFR= Biosurfactant produced by *Fusarium oxysporum* in Rice Husk, BAR = Biosurfactant produced by *Aspergillus niger* in Rice Husk, BFM = Biosurfactant produced by *Fusarium oxysporum* in Maize, BAM= Biosurfactant produced by *Aspergillus niger* in Maize Husk, BFS= Biosurfactant produced by *Fusarium oxysporum* in sugarcane Bagasse, BAS= Biosurfactant produced by *Aspergillus niger* in Sugarcane Bagasse.

Result is represented with mean ± standard deviation of group obtained (n=2).

Oil Drop Collapse Qualitative Test

The result of oil drop collapse assay can be represented in table 4.4 below.

Table III: Oil drop collapse qualitative test result.

SUBSTRATE	TIME				
	0min	1min	5min	30min	72hours
BFR	-	-	-	-	-
BAR	-	-	-	-	+
BFM	-	-	-	-	+
BAM	-	-	-	-	+
BFS	-	-	-	-	+
BAS	-	-	-	-	+

Key: + = positive collapse, - = no collapse

BFR= Biosurfactant produced by *Fusarium oxysporum* in Rice Husk, BAR = Biosurfactant produced by *Aspergillus niger* in Rice Husk, BFM = Biosurfactant produced by *Fusarium oxysporum* in Maize, BAM= Biosurfactant produced by *Aspergillus niger* in Maize Husk, BFS= Biosurfactant produced by *Fusarium oxysporum* in sugarcane Bagasse, BAS= Biosurfactant produced by *Aspergillus niger* in Sugarcane Bagasse.

Oil Displacement/Spreading Test Result

Oil spreading or displacement ability was observed in all culture supernatants as an indication of biosurfactant activity. The oil spreading test is indicative of the surface and wetting activities of a surfactant sample, thus a larger diameter represents a higher surface activity. The engine oil area in square centimetre displaced by the biosurfactant was analyzed using SPSS version 20 and presented as Mean ± Standard Deviation below.

Table IV: Result for Oil Displacement Area (Cm^2) Test

BFR	BFM	BFS	BAR	BAM	BAS
13.88 ± 0.01^{f}	8.05 ± 0.00^{c}	5.74 ± 0.01^{b}	10.76 ± 0.01^{e}	9.63 ± 0.27^{d}	4.91 ± 0.00^{a}

Result represent mean ± standard deviation of group result obtained (n=2). Mean in the same row, having different letters of the alphabet are significantly different ($p<0.05$).

Biosurfactant produced by *Fusarium oxysporum* in Rice Husk (BFR), Biosurfactant produced by *Aspergillus niger* in Rice Husk (BAR), Biosurfactant produced by *Fusarium oxysporum* in Maize Husk (BFM), Biosurfactant produced by *Aspergillus niger* in Maize Husk (BAM), Biosurfactant produced by *Fusarium oxysporum* in sugarcane Bagasse (BFS), Biosurfactant produced by *Aspergillus niger* in Sugarcane Bagasse (BAS).

Iodine, Saponification and Biodegradability Test Results

The following are the core biochemical test for biosurfactant quality used in this work; iodine and saponification test as well as biodegradability test using a redox 2, 6-dichlorophenolindophenol are presented in table 5 below.

Table V: Results of Iodine Test, Saponification Test and Biodegradability Test

Bio surfactants by Fungi in a given Substrate	Iodine Test	Saponification Test	Biodegradability Test (using 2,6-dcpip)
BFR	_	+	+
BFM	_	+	+
BFS	_	_	+
BAR	_	+	+
BAM	_	+	+++
BAS	_	_	+

Key: - = Negative Test + = Low Biodegradability/ Positive Test, +++ = High Biodegradability, Biosurfactant produced by *Fusarium oxysporum* in Rice Husk (BFR), Biosurfactant produced by *Aspergillus niger* in Rice Husk (BAR), Biosurfactant produced by *Fusarium oxysporum* in Maize Husk (BFM), Biosurfactant produced by *Aspergillus niger* in Maize Husk (BAM), Biosurfactant produced by *Fusarium oxysporum* in sugarcane Bagasse (BFS), Biosurfactant produced by *Aspergillus niger* in Sugarcane Bagasse (BAS).

Discussion

Fungi A and B (*Fusarium oxysporum* and *Aspergillus niger*) were isolated from decomposing rice husk, maize husk and sugarcane bagasse with stimulated degradation. These wastes were subjected to dampness using distilled water and allowed for a period of 7 days for signs of fungal appearance. Isolation of the fungi was done using several biochemical tests as seen in table II above. In this study, the biosurfactant production by the isolated fungi (Table I and II) was produced from fresh substrates (Rice Husk, Maize Husk and Sugarcane Bagasse). Bio-surfactants are known for their potency in producing emulsion when in contact with vegetable oil and hydrocarbons. The emulsification (E) ability at different duration of activity of hydrocarbon with the biosurfactant was analyzed on diesel and kerosene. From the result in figure 5, emulsification index (EI) after 24 hours (E24), 48 hours (E48) and 72 hours (E72) on diesel from biosurfactant produced by *Fusarium oxysporum* in Rice Husk (BFR) showed emulsification indices of 56.28 ±0.03%, 65.68±0.05% and 68.75 ±0.00%; for biosurfactant produced by *Aspergillus niger* in Rice Husk (BAR) showed emulsification indices of 54.03±0.03%, 78.25±0.25% and 80.30±0.30%; for biosurfactant produced by *Fusarium oxysporum* in Maize Husk (BFM) showed EI of 50.15±0.15%, 72.30±0.30% and 74.40±0.40%; for biosurfactant produced by *Aspergillus niger* in Maize Husk (BAM) had EI 53.38±0.05%, 66.62±0.05% and 66.82±0.15; for biosurfactant produced by *Fusarium oxysporum* in Sugarcane Bagasse (BFS) had EI of 53.39±0.06%, 70.35±0.35% and 73.45±0.10%; for biosurfactant produced by *Aspergillus niger* in Sugarcane Bagasse (BAS) had 33.43±0.10%, 44.59±0.15% and 48.25±0.10%, respectively. For E24, E48 and E72 using kerosene, the following emulsification indices were recorded; for BFR, it had 68.74±0.15%, 75.66±0.10% and 77.78±0.00; for BAR, it showed 48.69±0.20%, 53.38±0.05 and 55.61±0.05%; for BFM, it showed 60.45±0.45%, 92.25±0.25% and 94.25±0.25; for BAM, it showed 68.69±0.20%, 55.54±0.03% and 55.55±0.01%; for BFS, it showed 66.52±0.15%, 52.53±0.15% and 57.25±0.10%; for BAS, it

showed 60.10±0.10%, 55.76±0.20% and 60.35±0.35 respectively. In diesel, the emulsification index showed highest in BAR whose E72 gave the value 80.30±0.30% and lowest in BAS E24 with the value 33.43±0.10%; while in kerosene, the highest emulsification index (94.25±0.25%) is seen in BFM at E72 and lowest (52.53±0.15%) in BFS at E48. The EI variation could be due to high emulsion formation between the biosurfactants and the hydrocarbon involved as well as the degree of large fat globule structure dissociation. The variation may also result from duration of emulsification and the pass rate of emulsification. The emulsification index in this work showed a positive correlation with the concentration of the biosurfactant in solution. This is similar to the finding of Rahman (2002).

As shown in table 3, when oil was dropped on the produced biosurfactants, none showed positive collapse instantly, 1 minute, 5 minutes and 30 minutes. But biosurfactant produced by *Aspergillus niger* in Rice Husk (BAR), biosurfactant produced by *Fusarium oxysporum* in Maize Husk (BFM), biosurfactant produced by *Fusarium oxysporum* in sugarcane bagasse (BFS) and biosurfactant produced by *Aspergillus niger* in sugarcane bagasse (BAS) was able to show oil drop-collapse at 72 hours, while biosufactant produced by *Fusarium oxysporum* in Rice Husk (BFR) did not show any positive result for the oil collapse test.

In table IV, oil spreading or displacement ability was observed in all the cultured supernatants as indication of biosurfactant activity. The oil dispersion test indicates the breakdown of oil and wetting activities of test biosurfactants, thus a greater halo formation or higher displaced area (cm^2) represents a more surface activity (Chandran and Das, 2010). Biosurfactant produced by *Fusarium oxysporum* in Rice Husk produced better oil displacement in area/cm^2 compared to the rest, showing the value of 13.88±0.01cm^2, this was followed by biosurfactant produced by *Aspergillus niger* in Rice Husk, showing the value of 10.76±0.01cm^2; an indication that Rice Husk is a model for the production of biosurfactants. Possibly, the Rice Husk contains more carbohydrate utilizable by the fungi to carry out their metabolic production resulting in the production of more quality biosurfactant. Biosurfactant produced by *Fusarium oxysporum* in Rice Husk (BFR) had the highest oil area displacement (13.88±0.01cm^2) while biosurfactant produced by *Aspergillus niger* in sugar cane bargasse (BAS) showed the least oil area displacement (4.91±0.00cm^2). This ability to disperse oil is due to the tension active properties of the biosurfactant molecules in the various culture supernatants. Biosurfactants are known to reduce surface and interfacial tension by accumulating at the interface between two immiscible fluids such as oil and water (Saharan *et al*., 2011). This property thus gives reason for the application of these biosurfactants in pollution control especially in oil spillages. The saponification and iodine test are biochemical assays (table V). The entire biosurfactant tested negative with iodine. These reveal that the types of biosurfactants produced by the fungi are not of the polysaccharide forms. The absence of blue or reddish-brown complex indicates the absence of polysaccharide and presence of di- or monosaccarides. However, in saponification test, the lipid layers present in the biosurfactants were saponified by NaOH. This therefore indicates the presence of lipid in the biosurfactant formed.

Conclusion

From the method applied in the screening of fungi, the emulsification index and the oil displacement assay, all indicate the production of biosurfactant from the isolated fungi. The carbon sources used in this work are good for biosurfactant production. In addition, findings from this work showed that rice husk followed by maize husk are the ideal carbon substrates suitable for fungal activities for the production of biosurfactants. Also, *Fusarium oxysporum* is a

good microorganism that can be used to produce high quality biosurfactant in all the substrates used. This study revealed that increased utilization of solid agro-waste for microbial growth and effective production of biosurfactant is feasible and has promising application with a view to enhancing the bioavailability and bioremediation of recalcitrant environmental condition. From the results and discussion presented, bioconversion of agro industrial wastes into useful products has the potential of being a source of new materials and can convert wastes into commercial products and still reduce pollution. There is no doubt that the biosurfactant produced, indicates that rice husk was able to produce better and quality biosurfactant than maize husk and sugarcane bagasse, given the fungi utilized in this particular research. The biosurfactant produced can be applied as cleaning, wetting and emulsifying agent in petroleum and other allied industries.

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