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Characterization of new glycosophorolipid-surfactant produced by *Aspergillus Niger* and *Aspergillus flavus*

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Abstract
Five fungal species were isolated from an abandoned heavy metals contaminated soil, identified as Aspergillus niger, Aspergillus flavus, Aspergillus fumigatus, Mucor mucedo and Trycophyton mentagrophyte and were screened for biosurfactants production using oil spreading technique and emulsification stability test. Aspergillus niger and Aspergillus flavus produced biosurfactants on the broth of Mineral Salt Medium containing 2% of crude oil. These biosurfactants were partially purified and characterized by Thin Layer Chromatography and Gas Chromatography-Mass Spectrometry analysis. The analyses signify Glycolipid biosurfactants specifically designated as glycosophorolipid Sf1 and glycosophorolipid Sf2 respectively, containing fatty acids with various functional groups.

Keywords: Glycosophorolipid, Fungi, TLC, GC-MS, R_f-value

1. Introduction
Biosurfactants are amphiphilic compound produced on living surfaces, mostly microbial cell surfaces or excreted extra-cellularly and contain hydrophobic and hydrophilic moieties that reduce surface tension and interfacial tension between individual molecules at the surface and interphase respectively (12). Biosurfactants are produced by a wide variety of bacteria, yeast and filamentous fungi under specific growth conditions (18). The production of biosurfactant such as glycolipid (Rhamnolipid), trehalolipid, and liopeptide (Surfactin) have been reported by *Pseudomonas aeruginosa, Arthobacter* species and *Bacillus subtilis* respectively to mention few (19). Though bacterial surfactants were studied along the years, fewer fungi such as *Candida lipolytica, Candida batistae, Candida ishiwadae, Aspergillus ustus, Ustilago maydis* and *Trichosporon ashii* with better biosurfacatnts (Sophorolipids) functionality have been reported (22, 16, 26,15, 1, 3). Study of biosurfactants was a focus in recent years because of several advantages it offers such as control of the bioavailability of toxicants in soils, soil heavy metals remediation without leaving behind secondary contaminant, usefulness in enhanced oil recovery, detergent formulation, food, cosmetic, pharmaceutics, medicine and self degradation amongst other (21). This study assesses *Aspergillus niger, Aspergillus flavus, Aspergillus fumigatus, Mucor mucedo* and *Trycophyton mentagrophyte* biosurfactants production potentials and identified the biosurfactants.

2. Materials and Methods
2.1 Fungal Isolation and Identification
Fungal were isolated from heavy metal contaminated soil collected from abandoned mine site at Itagumodi, Atakumosa West Local Government Area, Ilesha Osun State, Nigeria using Potato Dextrose Agar. A small portion of mycelia growth was carefully picked and placed in a drop of lactophenol cotton blue on a slide and covered with cover slip. Microscopic examination was carried out to determine the colour of aerial and substrate hyphae, shape and kind of asexual spores, presence of foot cell, sporangiophore, conidiophores, and characteristics of spore head and the fungi isolates were identified also by comparing their characteristics with those of known taxa using the schemes of (4)

2.20 Screening of Biosurfactant production
2.21 Emulsification Test
The emulsification capacity was evaluated by an emulsification index (E_{24}). 2ml of kerosene was added to equal volume of cell free supernatant and homogenized in a vortex at high speed for 2minutes. The emulsification stability was measured after 24hours and emulsification

index was calculated as; E_{24} = Total height of the emulsify layer / Total height of the liquid layer x 100
The calculation was done for all cultures individually and their emulsification was compared with each other (2).

2.22 Oil spreading Test
Fifty micro-litres (50µl) of crude oil and 50µl of the supernatant of the culture isolated were carefully added to the centre of petridishes containing thirty millilitres (30 ml) of water using micropipette. The diameter of the clear zones and the area covered by the oil was measured. This test was done for all isolates separately in triplicate (2).

2.3 Biosurfactant Production and Isolation
Fungal were grown in mineral medium containing Soluble Starch (0.5 g/l), Yeast Extract (0.5g/l), $MgS0_4$ (0.5g/l), Na_2HPO4 (3g/l), KH_2PO4 (1.0 g/l) and 2% crude oil at pH and temperature of 7.0 and 28°C respectively for 7days. The biosurfactants were extracted from cell free - broth by centrifugation at 15,000 rpm for 25minutes. The supernatant was subjected to acid precipitation by adding 1 MH_2SO4 to achieve a final pH of 2.0 and allowing it to precipitate at 4°C. The precipitate was pelleted at 10,000rpm rpm for 20min, re-dissolved in distilled water, adjusted to pH 7.0, freeze-dried, and weighed. The dried surfactant was extracted with chloroform and methanol with the aid of a rotary evaporator under vacuum (20).

2.4 Characterization of Biosurfactant
Capillary tube was used to spot crude biosurfactants on silica plate. The biosurfactants was separated on the plate using chloroform: methanol (10:5). Throne reagent was sprayed to detect glycolipid biosurfactants and the retention factor (R_f) was calculated as per the standard database of biosurfactants (11). The purified supernatant of the extracted biosurfactants was analysed for component identification of the fatty acid present using GC-MS (ALS Vial) maintained at 120°C for 6 minutes and later increased to 270°C at the flow rate of 15°C/minute for 12minutes.

Results
Morphological Characteristics and identification of rhizospheric fungi as described in (Table 1) showed the presence of *Aspergillus flavus, Aspergillus niger, Mucor mucedo, Aspergillus fumigatus* and *Trichophyton mentagrophyte.* Biosurfactant producing ability was found only in *Aspergillus niger*- Sf1 and *Aspergillus flavus*-Sf2 indicated by formation of 14 - 17mm oil displacement zone and 61.30 – 57.30 % emulsification activity in oil spreading and emulsification test (Table 2 and 3) respectively. *Aspergillus fumigatus, Mucor mucedo* and *Trycophyton mentagrophyte* showed very low or negative result Table 2. *Aspergillus niger* had higher biosurfactant yield of 0.28g and *Aspergillus flavus* had the least of 0.20g per 50ml of broth/ growth medium. TLC analysis showed brown spot of Sf1 and Sf2 biosurfactants when sprayed with anthrone reagent with retention factor (R_f) value ranged from 0.16 - 0.46 (Table 4). The GC-MS fatty acid analysis of *Aspergillus niger*-Sf1 and *Aspergillus* flavusSf2 showed six major peaks each corresponds to long chain poly aliphatic and unsaturated compounds consistent with fatty acid methyl esters linked with decanoic acid (Table 5 and 6). *Aspergillus niger*-Sf1 and *Aspergillus flavus* Sf2 chromatograms and Mass spectra structural results are shown in Figure 1, 2, 3 and 4 respectively.

Table: 1 Morphological Characteristics and identification of rhizospheric fungi

Isolate code	Colour of Substrate Hyphae	Colour of Aerial Hyphae	Shape of Sexual Spore	Nature of Hyphae	Presence of Special Structure	Appearance of Sporangiophore or Conidiophores	Characteristic of Spore Head	Organism
CSF1	Yellow	White	Oval Green Conidia	Septate Multinucleate	Foot Cell present	Long Erect Non-Septate Conidiophore	Small and Uninucleate	*Aspergillus flavus*
CSF2	Brown	Whitish Black	Oval Green Conidia	Septate	Foot Cell present	Long Erect Non-Septate Conidiophore	Small and Multinucleate	*Aspergillus niger*
CSF3	Dark-Grey	White	Round and Black	Non Septate	Round and Black	Sporangiophore Unbranched	Round and Black	*Mucor mucedo*
CSF4	Brown	Green	Oval	Septate Multinucleate	Foot Cell present	Long Erect Non-Septate Conidiophore	Multinucleate Globose	*A. fumigatus*
CSF5	Yellowish Brown	White to Cream	Hyaline, Smooth	Spiral Hypae	Hyaline, Smooth	Spherical Chlamydospores	Nucleate	*Trycophyton mentagrophyte*

Table: 2 Result of Oil Spreading Test

Organisms	Zone of Clearance (mm)	Time(s)	Interpretation
Aspergillus niger	17	2	+ve
Aspergillus flavus	14	5	+ve
Aspergillus fumigatus	3	30	-ve
Mucor mucedo	-	30	-ve
Trycophyton Mentagrophyte	-	30	-ve

Key: -ve = Negative result +ve = Positive result

Table: 3 Emulsification activities of the experimental organisms

Organisms	Emulsified layer (Mm)	E_{24}% ±Se
Control A	0.00±0.00	0.00±0.00
Aspergillus niger	22.00±1.00	61.30±0.20
Aspergillus flavus	14.00±1.00	57.30±0.60
Aspergillus fumigatus	04.00±0.00	10.30±0.50
Mucor mucedo	03.00±0.00	07.60±0.60
Trycophyton Mentagrophyte	0.00±0.00	0.00±0.00

Values are means of replicates ± standard error.

Table: 4 TLC (Rf) Values of Biosurfactants

Biosurfactant code	Number of spots	Rf value	
		Lower	Upper
SB1	2	0.16	0.42
SB2	2	0.20	0.46

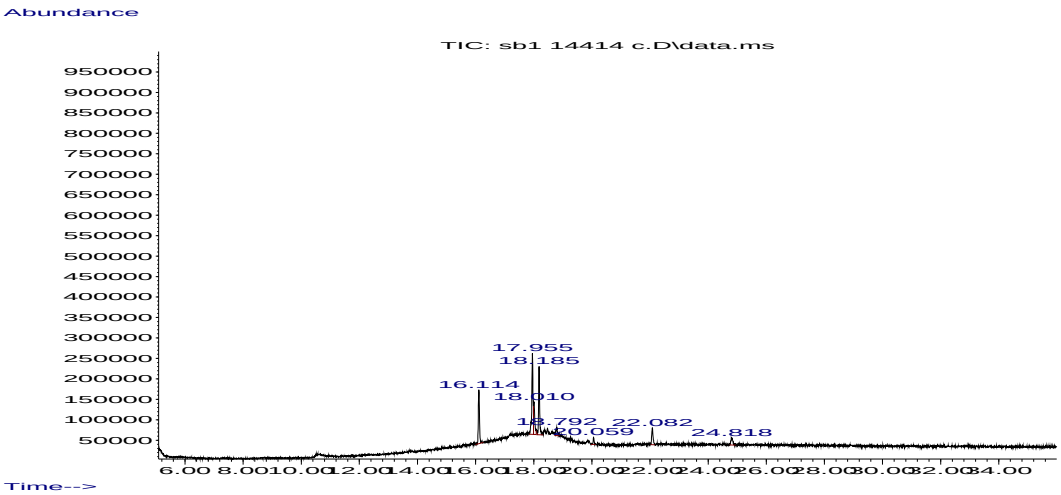
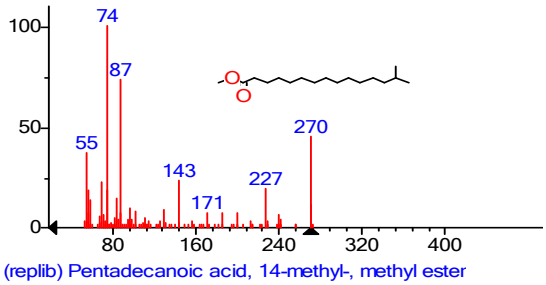


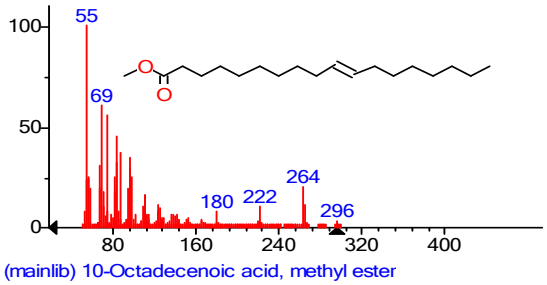
Figure 1 Chromatogram of *Aspergillus niger* biosurfactant

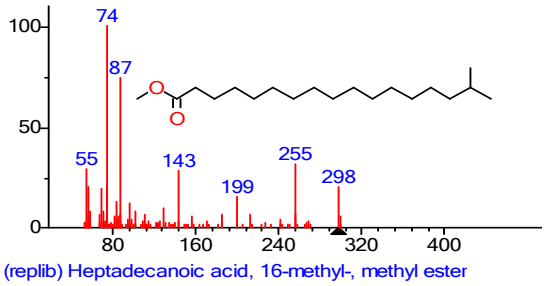
Table 5: Library Search Results of *A. Niger* Biosurfactants by GC-MS Analysis

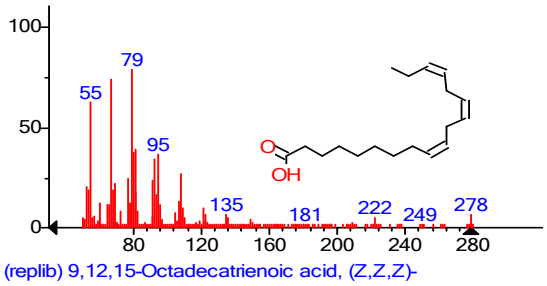
S/n	RT	Name of Compound	Mf	Mw	Quality (%)
1	16.113	Pentadecanoic acid, 14-methyl-, methyl ester	$C_{17}H_{34}O_2$	270.45	98
2	17.956	10 - Octadecenoic acid, methyl ester	$C_{19}H_{36}O_2$	296.48	99
3	18.185	Heptadecanoic acid, 16-methyl-,methyl ester	$C_{19}H_{38}O_2$	298.50	98
4	18.791	9,12,15 -Octadecatrienoic acid, (Z,Z,Z)-	$C_{18}H_{30}O_2$	294.50	90
5	22.081	Docosanoic acid, methyl ester	$C_{23}H_{46}O_2$	354.61	99
6	18.185	13,16-Octadecadiynoic acid,methyl ester	$C_{16}H_{30}O_2$	254.40	90

Key; S/n = Serial number, RT = Retention time, Mf = Molecular Formular
Mw = Molecular weight.









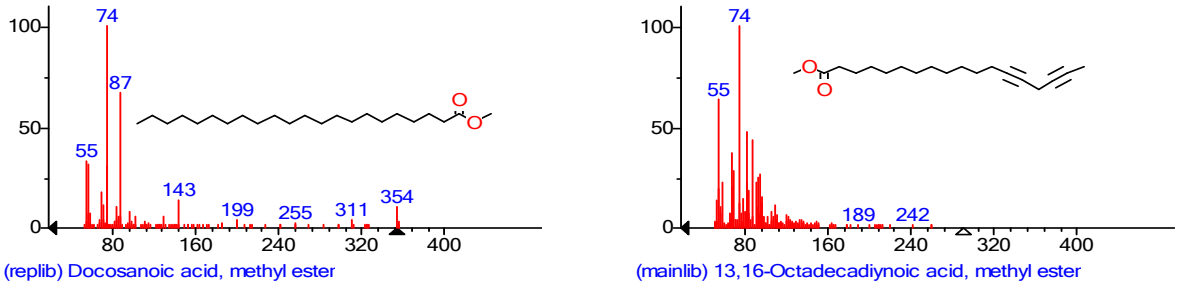


Fig: 2 (a, b, c, d e and f): GC-MS of biosurfactant sample of *A. niger*.

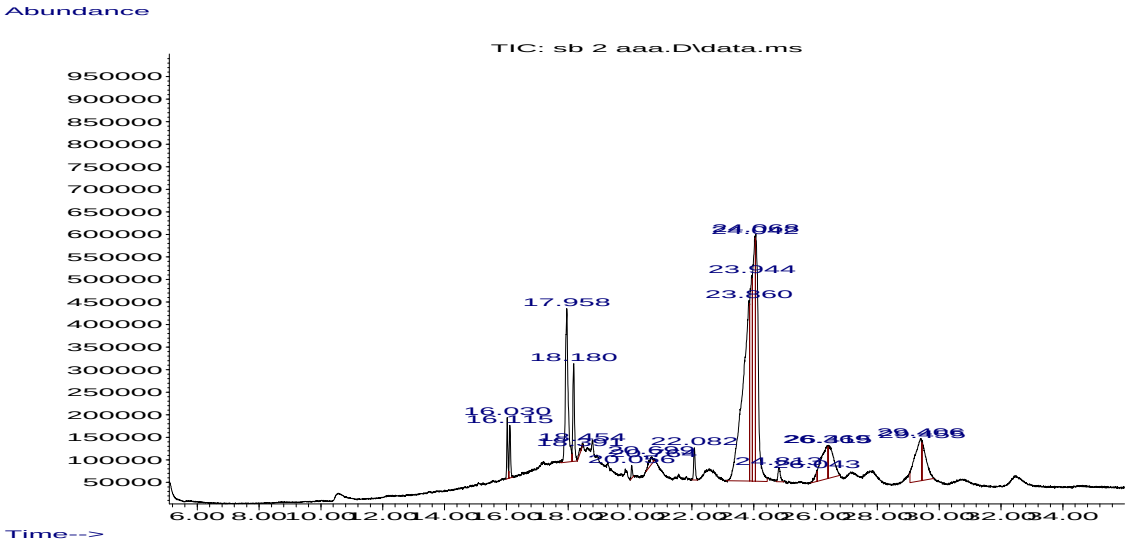
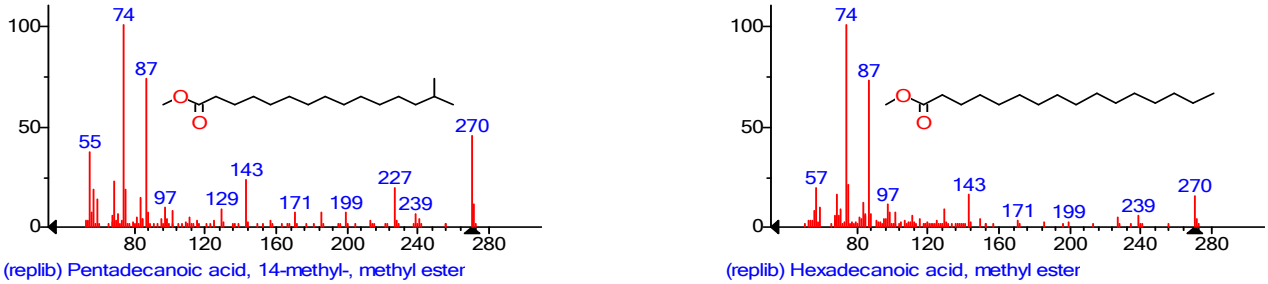


Fig: 3 Chromatogram of *Aspergillus flavus* biosurfactant

Table: 6 Major Compounds Identified from *A. Flavus* Biosurfactants by GCMS Analysis.

S/n	RT	Name of Compound	Mf	Mw	Quality (%)
1	16.027	Pentadecanoic acid, 14-methyl-, methyl ester	$C_{17}H_{34}O_2$	270.45	98
2	16.113	Hexadecanoic acid methyl ester	$C_{17}H_{34}O_2$	270.25	99
3	17.957	10 - Octadecenoic acid, methyl ester	$C_{19}H_{36}O_2$	296.48	99
4	18.791	Methyl stearate	$C_{19}H_{38}O_2$	298.50	98
5	22.081	1,30-Triacontanediol	$C_{30}H_{62}O_2$	454.81	90
6	18.185	1-Naphthalenepropanol, a – ethyldecahydro-a,5,5,8a-	$C_{20}H_{36}O_2$	292.49	90

Key; S/n = Serial number, RT = Retention time, Mf = Molecular Formula
Mw = Molecular weight.



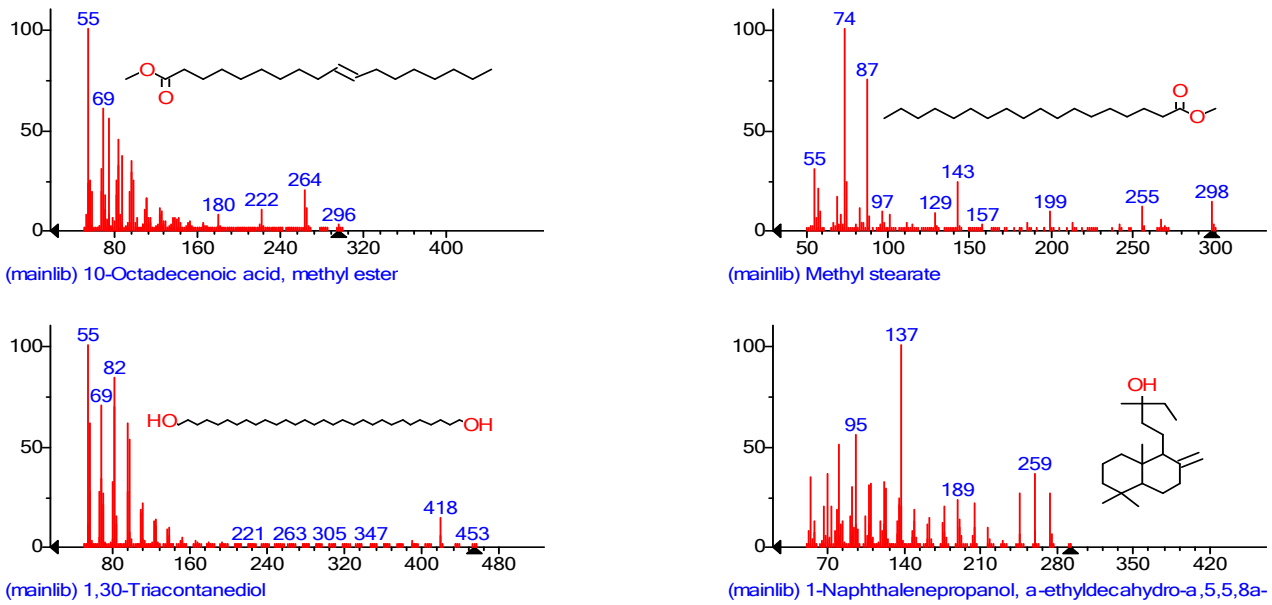


Fig: 4 (a, b, c, d, e and f as represented by 1-6 in Table - above): GC-MS of biosurfactant sample of *A. flavus*

Discussion

In this study, Glycolipid named Sophorolipid was produced by *A. niger* and *A. flavus.* Although, no biosurfactant production had been reported for *A. niger* and *A. flavus* but Sophorolipid was reported in fewer fungi specifically *Candida* species (7). *Aspergillus flavus* and *A. niger* isolated from heavy metal contaminated soil showed higher tolerance potentials, the tolerance could be due to presence of biosurfactant which is capable of forming complexes with metals thereby promote bioavailability of metal ions in the soil (24).

The culture supernatants of *A. niger* and *A. flavus* displaced the oil and shown a clear zone as indicated for positive result for oil spread test. The displacement of oil by culture supernatant of an organism may be an indication of bio active substances synthesis (13). Emulsification activity of biosurfactant obtained from *A. niger* and *A. flavus* revealed that the samples were able to form stable emulsions of (E_{24} = 61% and E_{24} = 57%) respectively with kerosene for 24 hours. Emulsification capacity varied among isolates as *Aspergillus fumigatus, Mucor mucedo* and *Trycophyton mentagrophyte* shown none probably due to lack of ability to produce extracellular emulsifying agents or hydrocarbon degrading enzymes during growth of in the medium (25).

The lower and upper retention factors (R_f) values were found to be 0.19 and 0.42 for *A. niger* and 0.20 and 0.46 for *A. flavus*. Retention factor R_f values of 0.38 and 0.46, corresponding to D-mannose and meso erythritol, Sophorolpids biosurfactants by *Candida* species had been reported (7). (R_f) value of 0.40, 0.19 and 0.39 had been reported for glycolipid, Dirhamnolipid and mono-rhamnolipid respectively by *Bacillus subtilis* (17, 18). The lower and upper R_f values for *A. niger* and *A. flavus* in this study correspond to glycolipid and sophorolipid biosurfactants when compared with other research works and standard Sophorolipid produced by *Candida* species.

The C_{18} fatty acid (Octadecanoic acid group) of *A. niger* and *A. flavus* correspond to 17-hdroxyloctadecenic acid and 18-hydroxyloctadecenoic acid, standard sophorolipid biosurfactant sample produced by *Starmerella bombicola* reported by (7). 10-Octadecenoic acid methyl ester listed above, peak 2 and 3 fatty acid of *A. niger* and *A. flavus* had been revealed as sophorolipid fatty acid fragment synthesised by *Candida batistae* (16). The fragment 9, 12, 15 – Octadecatrienoic acid of *A. niger* is synonymous to α-linolenic and methyl-trans- oleate reported to be the major fatty acid of Sorphorolipid (9). Pentadecanoic acid, 14-methyl-, methyl ester (Isopalmitic acid) present both as first major peak fatty acids of *A. niger and A. flavus* within 16.113 and 16.027 retention time respectively was also investigated and reported to have low micelle Critical Micelle Concentration indicating it is probable use as a detergent with less effect on environment (6). The peak 1, 2, 3 and 4 reported for both *A. niger* and *A. flavus* are similar to the glycolipids fatty acids of *P. aeruginosa* suggesting the fatty acids are glycolipid.

Conclusion

Five fungal species identified as *Aspergillus niger, Aspergillus flavus, Aspergillus fumigatus, Mucor mucedo* and *Trycophyton mentagrophyte* which were isolated from an abandoned heavy metals contaminated mining site were found to be effective biosurfactants producing organisms (0.20-0.28g/50ml), with great oil displacement (14-17mm) and emulsification activity (61.30-57.30%). Through comparing R_f values with standards, Physical and biochemical analysis of the biosurfactants pointed to the presence of sophorolipid. Structure identification and quantification analysis carried out using GC-MS revealed glycolipid (sophorolipid). Although, research has not dwell much into the biosurfactant production potentials of fungi and the chemical structures of such biosurfactant but the fatty acid fragment detailed in this work is close to glycolipid, specifically sophorolipids and *A. niger* and *A. flavus* are likely to have produced glycol-sophorolipid Sf1 and Sf2 respectively, subject to further research.

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