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Full Length Research Article

BIODECOLOURIZATION OF TEXTILE EFFLUENT USING BIOSURFACTANT PRODUCED BY *PSEUDOMONAS AERUGINOSA* AND *BACILLUS SUBTILIS* ISOLATED FROM ENGINE OIL CONTAMINATED SOIL

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ABSTRACT
Biosurfactants can be characterized according to their chemical composition, molecular weight, physicochemical properties, mode of action and microbial origin. Microbial decolorization and degradation is an environment friendly and cost-competitive alternative to chemical decomposition processes. This study therefore was carried to with the aim to produce and characterized biosurfactants produced by *Pseudomonas* and *Bacillus* species for the decolorization of textile effluents. Bacteria from oil polluted soil was isolated by standard spread plate technique using nutrient agar. Extraction of biosurfactant was done using the acid precipitation method and the weight of the biosurfactant was determined. GCMS and FTIR analysis of the biosurfactant were carried out according to standard method, and the extent of decolourization caused by the biosurfactant was estimated and expressed as percentage (%) decolourization. The result indicated that the dry weight of biosurfactant produced by *Pseudomonas aeruginosa* was estimated to be (1.2±0.03), while *Bacillus subtilis* was 1.38±0.02. The GC/MS analysis of the biosurfactant produced by *Pseudomonas aeruginosa* was a rhamnolipid and detected 12 compounds present in the biosurfactant while the biosurfactant produced by *Bacillus subtilis* was glycolipid and the constituents present in the extract where 17 in numbers. Biosurfactants produced by *Bacillus subtilis* decolorized textile effluent by 67.7%; on the other hand, biosurfactant produced by *Pseudomonas aeruginosa* decolorized textile effluent by 13.7%. *Bacillus subtilis* and *Pseudomonas aeruginosa* proved to be good biosurfactant producers and hence good in decolorization of textile dyes and effluents.

Keywords: Production, Characterization, Biosurfactant, Bacterial species, and Decolourization, Textile effluents.

INTRODUCTION

Biosurfactants are amphiphilic compounds produced on living surfaces, mostly microbial cell surfaces, or excreted extracellularly and contain hydrophobic and hydrophilic moieties that reduce surface tension and interfacial tensions between individual molecules at the surface and interface, respectively (Kaur *et al.,* 2023a). A biosurfactant may possibly have one of the following structures: mycolic acid, glycolipids, polysaccharide–lipid complex, lipoprotein or lipopeptide, phospholipid, or the microbial cell surface itself. Biosurfactants can be characterized according to their chemical composition, molecular weight, physicochemical properties, mode of action and microbial origin (Kaur *et al.,* 2023a). The composition and emulsifying activity of the biosurfactant not only depends on the producer strain but also on the culture conditions, therefore, the nature of the carbon source, the nitrogen source in addition to the C:N ratio, nutritional limitations, chemical and physical parameters such as temperature, aeration, divalent cations and pH not only the amount of biosurfactant produced but also the type of polymer produced (Ansari *et al.,* 2023).

The dyes constitute only a small portion of the total volume of waste discharge in textile processing. These compounds are not readily removed by typical microbial-based waste-treatment processes (Lan *et al.,* 2022). Fundamental work has revealed the existence of a wide variety of microorganisms capable of decolorizing a wide variety of dyes (Sharma *et al.,* 2016). Many microorganisms belonging to different taxonomic groups of bacteria, fungi, actinomycetes and algae have been reported for their ability to decolorize dyes. Bacterial degradation of these dyes requires their intracellular uptake while the fungi degrade these by extracellular enzymes (Singh *et al.,* 2015).

Due to usage of dyes and chemicals, effluents are dark in colour, which increase the turbidity of water body. This in turn reduces sunlight penetration which hampers the photosynthesis process, causing alteration in the habitat and toxicity of fish and mammals (Sharmar *et al.,* 2016). Using whole organisms has been done a lot but the use of products produced by the organisms is not widespread. Microbial decolorization and degradation is an environment friendly and cost-competitive alternative to chemical decomposition processes (Verma and Madamwar, 2003; Parmer and Shuklar, 2018). This study therefore was carried to with the aim to produce and characterized biosurfactants produced by *Pseudomonas* and *Bacillus* species for the decolorization of textile effluents.

MATERIALS AND METHODS

Collection of Samples

Engine-oil polluted soil sample was collected at a depth of 0-20 cm, from a mechanic work shop in Abakpa Kaduna state. Following this, the soil sample was transported in a pre-cleaned polyethylene bag to the laboratory at the Department of Microbiology, Kaduna State University, Kaduna, Nigeria and stored at 4^{o}C until analyses were carried out (Agbo *et al.,* 2021). The effluent was collected in sterile plastic bottles from African Textile Manufacturers Kano, Kano state, Nigeria and transported to the Microbiology Laboratory of Kaduna State University, Kaduna for further analysis.

Isolation and Identification of Bacteria Isolates

Bacteria from oil polluted soil was isolated by standard spread plate

technique using nutrient agar. Ten (10-fold dilution) serial dilutions were carried out. Then 0.5 mL of each of the 10^{-3} and 10^{-4} dilution factor was inoculated on solidified nutrient agar using spread plate technique. The plates were incubated at 37°C for 24 hours. Colonies were counted and results were expressed as colony forming unit per gram (cfu/g), the isolates were thereafter sub-cultured repeatedly to obtain pure culture. Pure isolates were maintained on agar slants for further study. Bacterial isolates were identified on the basis of microscopic examination, cultural characteristics, morphological characteristics and gram staining reaction. Relevant biochemical tests were also carried out as described below. Confirmatory identities of the microorganisms were made using Bergey's Manual of Systematic Bacteriology (Hassan *et al.,* 2017).

Production of Biosurfactants

The potential biosurfactant producing bacterial isolates were inoculated into a sterile Muller Hinton broth and incubated at 37°C for 24 hours, then 1.0 mL of the 24 h old culture was transferred into 100 mL mineral salts medium (Umar *et al.,* 2021) in Erlenmeyer flask and was incubated at 25°C for 7 days with shaking at 300 oscillations per minute using flask shaker.

Biosurfactant Extraction

Acid precipitation method

Extraction of biosurfactant was done using the acid precipitation method (Adamu *et al.,* 2015), the bacterial isolates were removed after 7days of incubation by centrifugation at 6000 rpm for 30 min. The cell free culture supernatant was acidified with 1 M of freshly prepared hydrochloric acid (HCL) to obtain a pH of 2.0. The acidified cell free supernatant was then used for the extraction of the biosurfactant. To every 100 mL of the acidified cell free supernatant, 100 mL of mixture of chloroform: methanol in the ratio of 2: 1 (v/v) was added. The mixture was allowed to react for 30 seconds, after which it was shaken vigorously until two phase separation was obtained. The upper layer containing majorly the reagents was decanted and the lower containing the biosurfactant was concentrated using a rotary evaporator, where most of the solvent was evaporated and the left over sediment was poured into a test tube and centrifuged at 6000 rpm for 20 minutes. The lower phase (whitish colour sediment) containing the extracts was then concentrated in an oven set at 40°C to a dried crude biosurfactant (Singh *et al.,* 2015).

Determination of dry weight of biosurfactants

Using the method of Chandran and Das (2010), the weight of the biosurfactant was determined. The cell free culture was centrifuged at 600rpm for 30 minutes and extracted with chloroform and methanol (2:1, v/v). The initial weight of a sterile Petri plate was taken, and then the extracted biosurfactant was poured into the plates. This was placed in the hot air oven at 100°C for 30 min. After drying, the plates and contents were reweighed. The weight of biosurfactant produced was determined using the formula (Singh *et al.,* 2015): DWB = WPBAD – WEP

Where:

DWB= dry weight of biosurfactant

WPBAD= Weight of the petri dish containing biosurfactant after drying

WEP= weight of the empty petri dish.

Chemical Characterization of Biosurfactants

Gas chromatography and mass spectroscopy (GC-MS)

GCMS analysis of the biosurfactant was carried out according to the method used by Nigerian institute of science and technology Ibadan, NISLT (2007). The gas chromatographic model: 7890A (GC) analysis was done on an Agilent technology interfaced with mass selective detector model: 5975C (MSD). The electron ionization was at a 70v with an ion source temperature at 250°C. Highly pure helium gas (99.9 purity) was used as carrier gas, while HP-5ms (30mm X 0.25mm X0.320mm) was used as the stationary phase. The oven temperature was at 80°C for 4 minutes and then it was ramped to 270°C at the rate of 3.5°C/min holding for 6 minutes. One (1.0) m/l was also be injected. The extraction of the active ingredient was carried out by dissolving 10g of the milled powdery biosurfactant in 20 mL 99.999% pure n-hexane in a well corked reagent bottle. This was thoroughly mixed using an ultra sonicator for a period of five hours. The mixture was allowed to stand for 72 h and filtered into a beaker; the mixture was rewashed with 20 mL n-hexane for two more consecutive times. The combined aliquots was evaporated on a steam bath to 5 mL and filtered through a pasture pipette stocked with glass wool (membrane) with packed anhydrous sodium sulfate to remove the left over moisture. The filtrate was concentrated to 1.0 mL in the vial bottle and was taken to analyze on Gas chromatography for the chemical composition.

Fourier transform infra-red (FTIR) analysis

FT-IR spectroscopy can be used to elucidate the chemical structures of some components in an unknown mixture by identifying the types of chemical bonds or the functional groups present in their chemical structures (Pornsunthorntawee *et al.,* 2008; Ahmed *et al.,* 2016). In order to determine the functional groups in the biosurfactant, FTIR analysis was carried out according to the method used by National Research Institute for Chemical Technology, Zaria NARICT (2017), One milligram of the extracted biosurfactant was grounded with 100mg of potassium bromide (KBr) and pressed with a silver coated hand presser at 7,500 kg of pressure for 30 seconds to obtain translucent pellets. The pellet obtained was inserted into Fourier Transform Infrared Spectrophotometer (FTIR-8400S, Shinmadzu, Japan) where the infrared spectra were recorded within the range of 4500-500cm^{-1} wave number. All measurements consisted of 500 scans, and KBr pellet were used as background reference.

Decolorization of Textile Effluent using Biosurfactant

For the decolourization process, 100 mL of the sterile textile wastewater was dispensed into nine already sterilized Erlenmeyer flasks each and then inoculated with concentration of 1 mg of biosurfactant powder. This concentration was inoculated in 2 flasks. The flasks was cocked with sterile cotton wool and incubated at room temperature for 15 days. Samples were drawn aseptically at 5-day intervals over 15 days for analysis. Decolourization of the effluent was determined by checking the absorbance of the effluent at the predetermined wavelength 450nm using a UV-visible spectrophotometer by centrifuging 4 mL of the mixture at 5000 rpm for 15 minutes and quantitatively analyzing the absorbance of the supernatant . Distilled water was used as blank and decolourization activity was calculated (Sharma *et al.,* 2016). The extent of decolourization was expressed as percentage (%) decolourization and estimated as;

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$\%\ decolourisation \frac{A0-At}{A0}\ X\ 100\%$

Where A0 is the initial absorbance of the textile effluent in ml/l and At is the final absorbance of the textile effluent in ml/l

RESULTS AND DISCUSSION

Production of biosurfactants

Production was done in 100 mL MSM broth containing 1% (v/v) crude oil. After incubation for 7days, the broth was centrifuged to remove cells and the cell free culture supernatants were used for extraction. The recovery of biosurfactants produced by *Pseudomonas aeruginosa* and *Bacillus subtilis* was carried out. Biosurfactants was precipitated and extracted with a solvent system $CHCl_3$: C_2H_5OH in a ratio 2:1. The organic phase was evaporated in rotary evaporator to give a white powder. The dry weight of biosurfactant was calculated by using the following formula:

Dry weight of biosurfactant = Weight of plate after drying – weight of empty plate (Table 1).

Table 1: Determination of dry weight of biosurfactant

Isolates code	weight of empty plate (Mg)	weight of plate after drying (Mg)	weight of biosurfactant Mg/1000 mL)
P_1	101.6±0.0[a]	102.8±0.01[a]	1.2±0.03[a]
B_2	85.2±0.0[a]	86.56±0.03[b]	1.38±0.02[b]

Values are mean±SD

KEY: P_1: *Pseudomonas aeruginosa*; B_2: *Bacillus subtilis*

Gas Chromatography and mass and mass spectrophometric analysis

The GC/MS analysis of the biosurfactant produced by *Pseudomonas aeruginosa* was a rhamnolipid and detected 12 compounds present in the biosurfactant. The major compounds revealed by the GC/MS library were Pentobarbital, Dodecane, Heneicosane and Tricosane The GC-MS analysis showed that the biosurfactant produced by *Bacillus subtilis* was glycolipid. The constituents present in the extract where 17 in numbers. Heptadecanoic acid, 16-methyl-, methyl ester, D-Limonene, Hexadecanoic acid, methyl ester and 10-Octadecenoic acid, methyl ester had the highest percentage (Table 2).

Table 2: Major compounds detected in Rhamnolipid produced by *Pseudomonas aeruginosa*

Retention tine (s)	Area Peak (%)	Main molecule
23.834	7.98	Dodecane
23.346	5.88	Pentobarbital
19.749	6.64	Naphthalene, 1-methyl-
18.823	5.62	Tricosane
20.537	4.68	Dodecane
17.034	6.15	Dodecane
13.657	1.98	Heneicosane
13.269	4.19	Dodecane
8.609	3.02	Benzene, 1,2,3-trimethyl-

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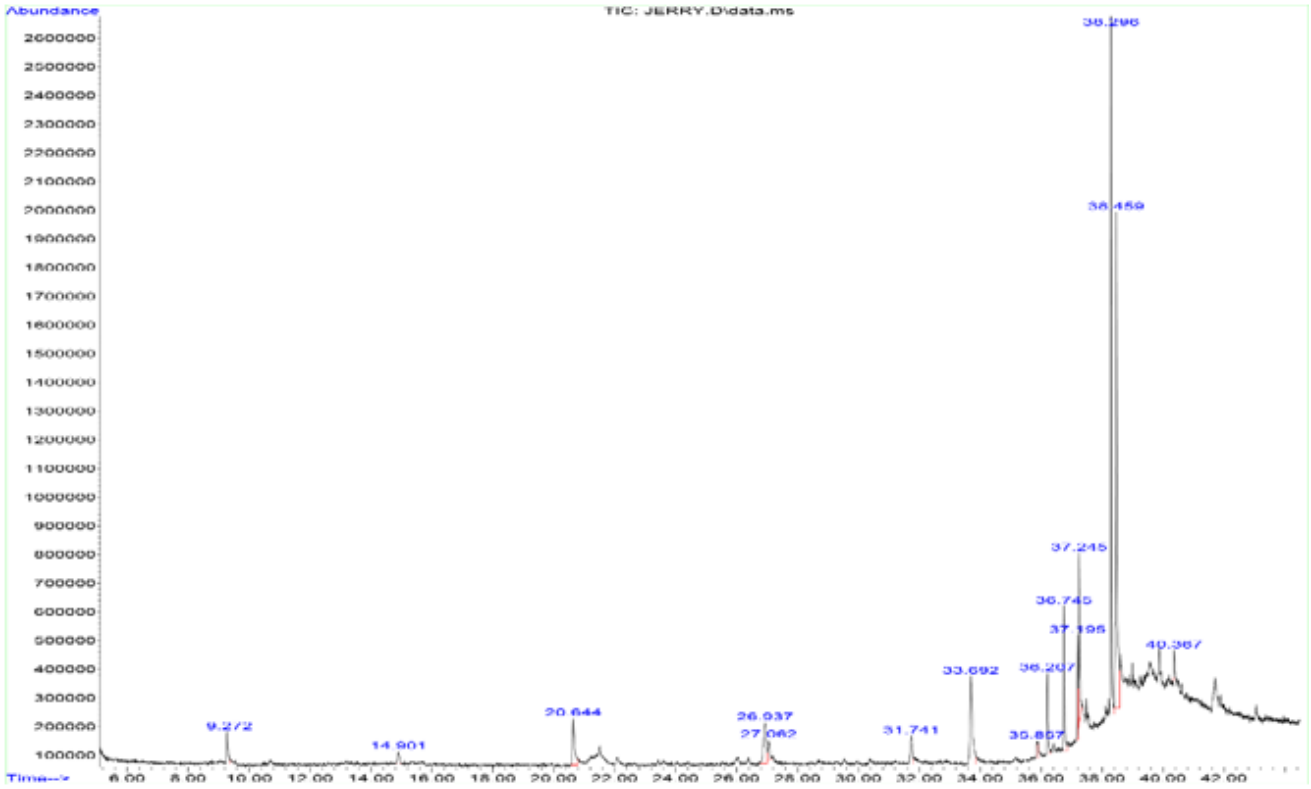

Fig. 1: GC-MS of Biosurfactant produced by *Pseudomonas aeruginosa*

Table 3: Major compounds detected in Glycolipid produced by *Bacillus subtilis*

Retention tine (s)	Area Peak (%)	Main molecule
21.234	6.72	Dodecane
13.432	3.32	Heptadecanoic acid
18.749	7.48	D-Limonene
18.823	7.24	Naphthalene, 1,2,3,4-tetrahydro-5- methyl-
22.456	5.67	methyl ester
19.044	5.21	methyl ester
18.467	1.99	10-Octadecenoic acid
16.345	4.23	D-Limonene
9.457	2.13	Benzene, 1,2,3-trimethyl-

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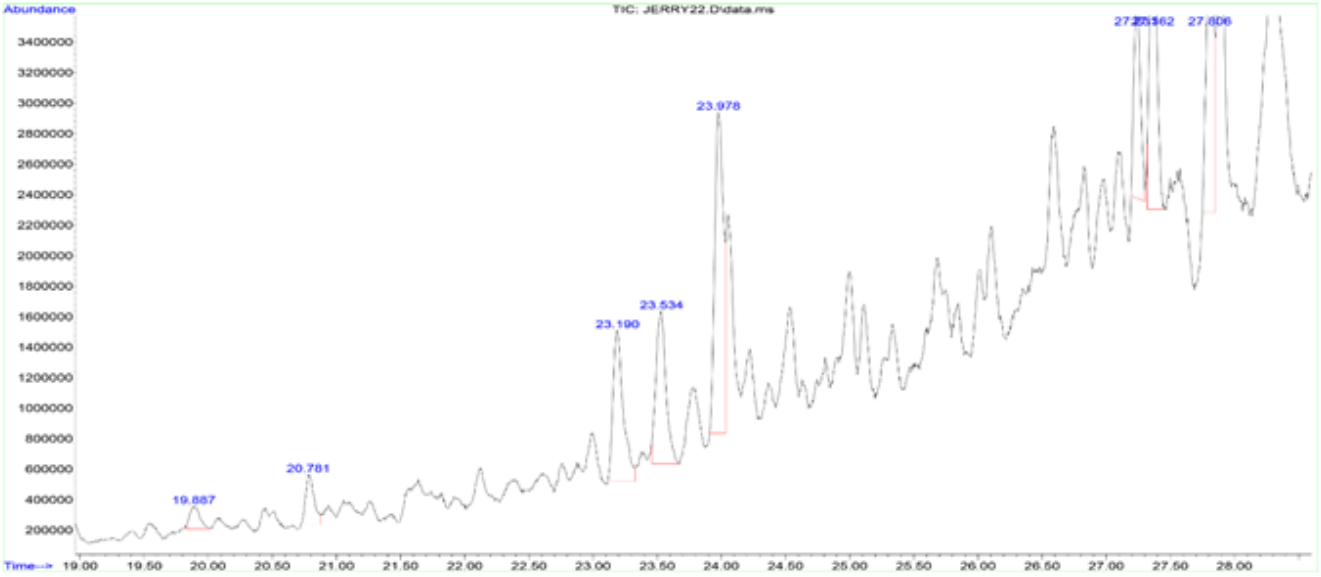

Fig. 2: GC-MS of Biosurfactant produced by *Bacillus subtilis*

Fourier transform infra-red (FTIR) analysis

The presence of 3486.65 and 3360.07cm^{-1}peack in the sample *Bacillus* Biosurfactant (BB) section clearly indicate in sample BB the presence of OH and NH group in glycoprotein. But in sample *Pseudomonas* Biosurfactant (PB) 3025.28 and 291971 cm^{-1}is assigned to the symmetric stretch (-CH) of CH_2 and $CH_3$of aliphatic chains. Also 3060.30 and 3025.49 and 2919.87cm^{-1} in sample BB indicates the presence of CH of –CH_2 and CH_3 of aliphatic chains. The OH and NH group in glycoprotein is not present in sample PB, which is a structure of Biosurfactant.

Important peak observed at 2919.87 indicate CH_2-CH_3 stretching ad this occurs on PB at 2919.71 and 2851.13 cm^{-1} which are CH_2-CH_3 stretch too (Fig 3).

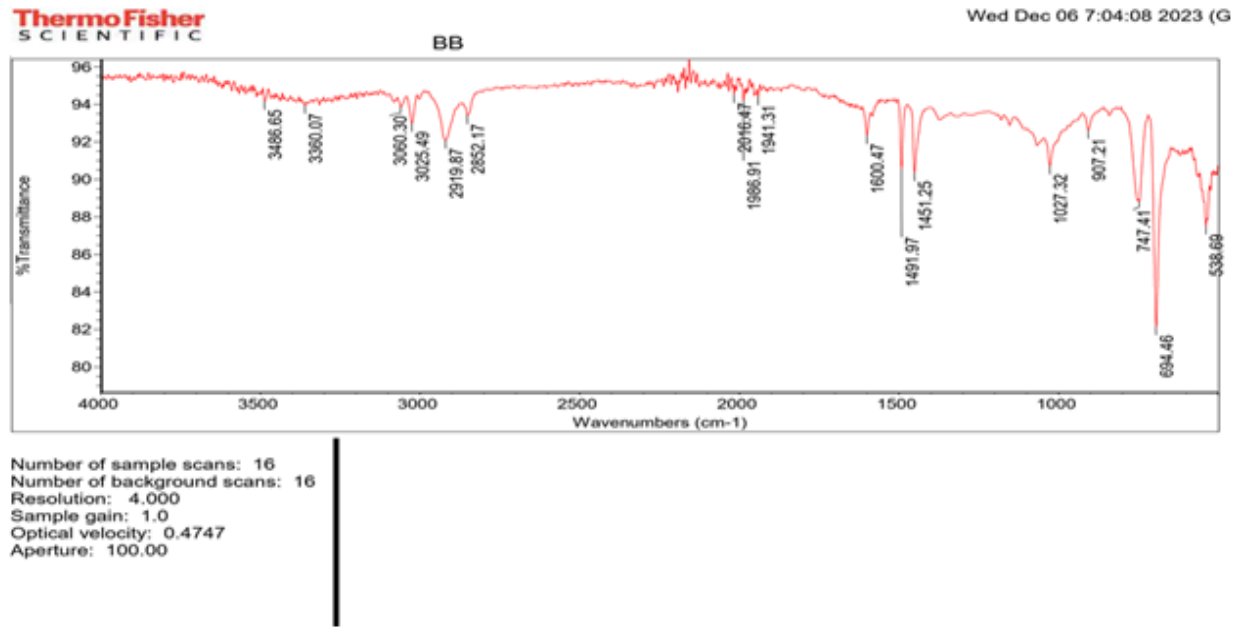

Fig. 3: FTIR of Biosurfactant Produced by *Bacillus subtilis*

At 1986.91, 2016.47 and 1941.31cm^{-1} there is in sample B the presence of C= O stretching of carbonyl group and NH bending which is linked to peptide. In PB at 1600 the presence of carbonyl group of –C =O bond in – COOH is presence with – C=O bond.

At 694.80cm^{-1} in sample PB is the presence of –CH_2 group which also occurs in sample BB at ware number 694.64cm^{-1}. The information from the wavenumbers of the two sample indicates them to be glycolipid native of biosurfactant (Fig 4).

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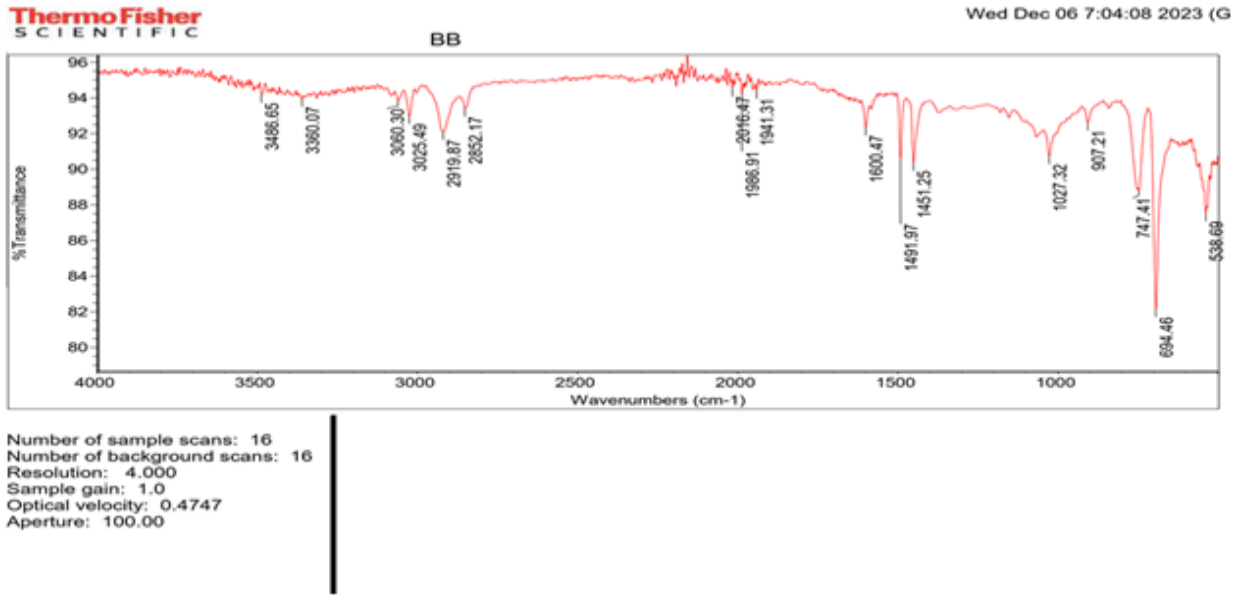

Fig. 4: FTIR of biosurfactant produced by *Pseudomonas aeruginosa*

Decolorization of Textile Effluent using Biosurfactant

In this study, it was observed that biosurfactant produced by *Pseudomonas aeruginosa* and *Bacillus subtilis* decolourized textile effluent effectively. The visual examination of the extent of decolorization of textile effluent is shown in Appendix XX. Table 4.6 shows the absorbance taken at different intervals (Day 0, Day 5, Day 10 and Day 15) of the textile effluent after being amended with biosurfactant produced by *Pseudomonas aeruginosa* and *Bacillus subtilis.* At day 0, the absorbance of all the samples was the same (0.248). The absorbance for samples amended with biosurfactant produced by *Pseudomonas aeruginosa* for day 5, 10, 15 were 0.273, 0.385 and 0.214 respectively. On the other hand, absorbance for samples amended with biosurfactant produced by *Bacillus aeruginosa* for day 5, 10, 15 were 0.273, 0.385 and 0.214 respectively as presented in (Table 4) below.

Table 4: Changes in Optical Density (OD) of textile effluent treated with biosurfactant produced by the test organism

	Day 0 (nm)	Day 5 (nm)	Day 10 (nm)	Day 15 (nm)
Control	0.248	0.244	0.256	0.244
TEPS	0.248	0.273	0.385	0.214
TEBS	0.248	0.251	0.228	0.118

KEYS: TEPS: Textile effluent with *Pseudomonas* surfactant
TEBS: Textile effluent with *Bacillus* surfactant

Decolorization Percentage of Textile Effluents

The percentage decolorization of the textile effluents was calculated using the initial and final absorbance during the study. Table 4.7 showed the difference in percentage decolorization of textile effluents over the period of 15 days. Textile effluent amended with surfactant from *Bacillus subtilis* (67.7%) decolorized textile effluent far more than that amended with *Pseudomonas aeruginosa* (13.7%). Decolorization of textile effluent also occurred in the control option with a value of 1.6% (Table 5).

Table 5: Percentage Decolorization of Textile Effluents

Test option	Initial absorbance (nm)	Final absorbance (nm)	Percentage decolourization (%)
TEPS	0.248	0.214	13.7
TEBS	0.248	0.118	52
Control	0.248	0.244	1.6

DISCUSSION

The isolation of *Pseudomonas aeruginosa* and *Bacillus subtilis* in this study from crude oil contaminated soil confirms the reports of previous studies Murugalatha *et al*. (2010) that organism capable of utilizing any environmental waste could be isolated from such an environment. GCMS analysis showed that rhamnolipid produced by *Pseudomonas aeruginosa* had 12 compounds and the major compounds revealed by the GC/MS library were Pentobarbital, Dodecane, Heneicosane and Tricosane. This was similar with the research Kumar *et al*. (2015) who detected Dodecane and Pentobarbital in their research work. Glycolipid biosurfactant produced by *Bacillus subtilis* revealed Heptadecanoic acid, 16-methyl-, methyl ester, D-Limonene, Hexadecanoic acid, methyl ester and 10-Octadecenoic acid as compounds present in the surfactant. Kumar *et al.* (2015) had a contradictory observation where they recorded 23 compounds in the glycolipid surfactant. Changes in the number of compounds observed can be due to the difference in the place of collection of the bacteria.

The spectrophotometric data in this study showed tangible differences in the decolorization of the textile effluents. Surfactant produced by *Bacillus subtilis* decolorized the textile effluent (67.7%) far more than the surfactants produced by *Pseudomonas aeruginosa* (13.7%). The control option had the least (1.6%)

percentage decolorization of textile effluent recorded in this study. In a similar study, it has been reported that treatment of the textile effluent with mutagenized strains revealed biodecolourization ability of 60.30 ±0.04% (John *et al.,* 2013). This attests to the efficacy of chemical and physical mutagens as tools for genetic improvement of biodegradation abilities of micro-organisms. This result agrees with previous study who reported hyper biodecolourization of textile effluents using mutant strains of *Pseudomonas aeruginosa* and *Bacillus subtilis* (Okerentugba and Ezeronye, 2003). The result from this study validates the report of Parmar and Madamwar (2018) who suggested that *Bacillus* species are the best in dye decolorization. This could be as a result of their robust cellular machinery and ability to resist attack from the chemicals in dyes. *Pseudomonas aeruginosa* also decolorized textile effluent in this study and this corresponds to the report of Kumar *et al*. (2015) who also observed tangible decolorization of textile dyes by *Pseudomonas* sp. The decolorization observed in the control option could be due to physicochemical parameters such as fluctuation in pH or temperature. Light intensity also could be another reason for the decolorization observed in the control option which had no biosurfactant in it.

Conclusion

GCMS showed that rhamnolipid produced by *Pseudomonas aeruginosa* had 12 compounds with the major one as pentobarbitol. Glycolipid surfactant produced by *Bacillus subtilis* showed Heptadecanoic acid, 16-methyl ester and 3 other compounds present. Biosurfactants produced by *Bacillus subtilis* decolorized textile effluent by 67.7%; on the other hand, biosurfactant produced by *Pseudomonas aeruginosa* decolorized textile effluent by 13.7%. *Bacillus subtilis* and *Pseudomonas aeruginosa* proved to be good biosurfactant producers and hence good in decolorization of textile dyes and effluents.

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