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SCREENING OF BIOSURFACTANT-PRODUCING *PSEUDOMONAS* AND *BACILLUS* SPECIES ISOLATED FROM ENGINE OIL CONTAMINATED SOIL FOR BIODECOLORIZATION OF TEXTILE EFFLUENTS

***Dalhat, S. M., Sanusi, S. B. and Shehu, I.**

Department of Microbiology, Faculty of Pure and Applied Sciences, Kaduna State University-Nigeria

*Corresponding authors' email: dalhatsalma91@gmail.com

ABSTRACT

Textile industrial activities have been characterized in its usage of large volumes of water and variety of chemicals. Surface-active chemical compounds produced by vast varieties of microbial species are known as biosurfactant. This study aimed at screening of biosurfactant-producing Pseudomonas and Bacillus species isolated from engine oil contaminated soil for biodecolorization of textile effluents. Engine-oil polluted soil sample was collected from a mechanic work shop in Abakpa Kaduna state. Bacteria from oil polluted soil was isolated by standard spread plate technique. Physical properties of the effluent sample were determined. Screening of isolates for biosurfactant production potential was achieved using standard method. The PCR amplification of the isolates was carried out using 16S rDNA primers. The results obtained revealed that Bacillus had the highest frequency of occurrence (26.6%), followed by Micrococcus, Pseudomonas and Staphylococcus (20% each) while Acinetobacter had (13.3%). isolate S9 had the highest emulsification index (52.5%). On the other hand, isolate S11 had the lowest emulsification index (44.8%). Pseudomonas aeruginosa S5 had 99% homologue with Pseudomonas aeruginosa MSSRFV220 while Bacillus subtilis S5 had 75.59% homologue with Bacillus subtilis BC5. Textile effluent with 1.0 mg of biosurfactant from Pseudomonas aeruginosa had the highest pH value (8.0) while textile effluent with 1.0 mg of biosurfactant from Bacillus subtilis recorded the least pH value (7.6). Textile effluent amended with biosurfactant from Bacillus subtilis showed a higher temperature value (37oC) while the least temperature value was (33oC). It is concluded therefore, that Bacillus and Pseudomonas species are good biosurfactant producers.

Keywords: Biosurfactant, Bacteria, Biodecolorization, Textile effluent

INTRODUCTION

The textile industry generates large amount of this effluent during textile production processes such as grinning, spinning, and weaving for the production of bed spread, pillow case, polyester/cotton fabrics, and so on, which requires high volume of water that eventually results in high wastewater generation, this effluent poses serious environmental concern particularly in developing countries like Nigeria (Ghoreishi and Haghighi, 2003; Shireesha *et al.,* 2017). Textile industrial activities have been characterized in its usage of large volumes of water and variety of chemicals (Qin *et al.,* 2007; Khan and Malik, 2018). Textile dye pollution to land and water have been reported to be one of the most important problems facing the environment (Olaganathan and Patterson, 2012; Prabha *et al.,* 2017). Also, light penetration of water body is reduced due to the influenced of color, which alter the some of the physicochemical properties of the water such as COD, BOD and pH, resulting to difficulty in survival for aquatic animals, and as such, textile effluents have been characterized to be of environmental concern (Mcmullan *et al.,* 2001; Khan and Malik, 2014). Unused dyestuff (10-50%) enters the wastewater directly due to inefficiency in dyeing processes and also all dyes do not bind to the fabric (Pandey *et al.,* 2007; and Kumar *et al.,* 2019). Sarka *et al.* (2017) in their study reported that compounds emitted from synthetic dyes and their degradation intermediates are detrimental as they are potential carcinoma and mutagens in humans and other animals.

Surface-active chemical compounds produced by vast varieties of microbial species are known as biosurfactant. It is reported that some these biosurfactants are of significant importance, with wide applications in different sectors such as antibiotics, antifungal or antiviral compounds as well as in the degradation/remediation of environmental pollutants (Shekhar *et al.,* 2015). Biosurfactants are synthesized by diverse bacteria and fungi species. These synthesized biosurfactants are grouped into six major classes based on the producing microorganism. They include; phospholipids, glycolipids, polysaccharide–lipid complexes, lipoproteins–lipopetides, hydroxylated and cross-linked fatty acids, and the complete cell surface (Urum and Pekdemir, 2004). Lipopeptides and glycolipids are known to be the two major classes of biosurfactants and are synthesized majorly by *Bacilli* and other species, as well as *Pseudomonas* species respectively (Shekhar *et al.,* 2015). Biosurfactants have been given considerable attention scientifically due to so many advantages it confers such as higher biodegradability, lower toxicity, high-throughput at extreme temperatures, pH and salinity and more importantly, possibility of their production through fermentation using low cost agro-based as substrate (Shekhar *et al.,* 2015).

In most developing countries like Nigeria, most industries dispose their effluents without treatment. These industrial effluents have hazardous effects on water quality, habitat quality, and complex effects on flowing water and may also lead to a lot of adverse effects to humans (can be carcinogenic and mutagenic) and the environment at large (Bound and Voulvoulis, 2005). Through the use of microbes for decolorization and degradation of effluent, it leads to the detoxification of the effluent effectively leaving no residue. Therefore, this study was carried out with the aim of screening biosurfactant-producing *Pseudomonas* and *Bacillus* species isolated from engine oil contaminated soil for biodecolorization 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.

Bacteriological Screening of Isolates

Bacteriological screening of the soil polluted 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^{o}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).

Physical Characterization of Textile Effluent

The pH and electrical conductivity was determined using HANNA instruments model HI 9813-6 on 1:2.5 (v/v) (Obiakalaije *et al.,* 2015). 200 mL of effluent was placed in a glass beaker and. The effluent was allowed to stand for 2 min. while being stirred gently and occasionally using a glass rod. The protective cap was removed from the electrode probe and rinsed with a small amount of sample solution and then discarded. The electrode tip was then submerged approximately 3 cm (1¼) into the sample to be tested and stirred gently (making sure the electrode junction was completely submerged). Stability time for the electrode was allowed in the sample before the reading was taken, by pressing the pH key and then the temperature key.

Screening of Isolates for Biosurfactant Production Potential

The isolates were screened for ability to produce biosurfactant using the following methods: Drop collapse test, oil displacement method and emulsification activities.

Drop collapse test

Two micro-liter of crude oil was applied to each cavity of a glass cavity slide. The slide was equilibrated for 1 hour at room temperature and then 5 µL of the cultural supernatant was added to the surface of oil (test). In the control, uninoculated medium was added instead of culture supernatant. The shape of the drop on the oil surface was observed after 1 minute. Biosurfactant producing cultures giving flat less convex drops were indicated positive (+) result; while the cultures that produced round convex drops indicated negative (-) result for biosurfactant production (Patowary *et al*. 2016).

Oil displacement method

Oil displacement method according to Hassanshahian (2014) was used to determine the diameter of the clear zone, which occurred after adding surfactant-containing solution on an oil-water interphase. Distilled water weighing Fifty (50) mL of was poured into a petri dish and 100 µL of crude oil was aseptically added to the water surface. Thereafter, 10 µL of the cell-free culture supernatant which was obtained after the centrifugation was equally added and allowed to mix. Observations and reading of result were taken on the diameter of the oil displaced by the cell-free supernatant and the clear zone formed as visualized under visible light after 30seconds ((Nayak *et al.,* 2020).

Emulsification activity (E_{24})

The emulsification activities of the biosurfactant solution were determined by measuring the emulsion index (E_{24}) at 25^{o}C as described by Wang *et al*. (2014). Four milliliters (4 mL) of crude oil was poured separately into a test tube containing 4 mL of biosurfactant solution obtained after the centrifugation of eighteen hours (18hours) broth culture at 6000 rpm for 30 min using IECFL 40R centrifuge, USA. The mixture was homogenized by vortexing at high speed for two minutes using Stuart auto vortex mixer (AE-11D, Great Britain). The homogenized mixture was allowed to stand for 24 hours undisturbed. After 24 hours, the height of the stable emulsion layer and total height of the mixture was measured by using a meter rule, the values obtained was used to calculate the emulsification index (E_{24}) thus:

$$E24 = \frac{\text{Height of emulsion layer}}{\text{Total height of solution}} \times 100$$

Molecular Confirmation of Biosurfactant Producing Isolates

The PCR amplification of the isolates was carried out using 16S rDNA primers (27F.1 forward 5'AGRGTTTGATCMTGGCTCAG 3 and 1492R reverse 5'GGTTACCTTGTTACGACTT 3'). The reaction was a 20µl reaction. For reaction set-up, templates, specific primers and water were added to the premix. For the reaction set up, 1µl of each of the reverse and forward primers were mixed with 2µl of the templates and 16µl of deionized water in the hot start PCR premix tube to make 20µl for the PCR run. For the negative control, 18µl of deionized water was added to the premix and 2µl of the primer was placed in the PCR machine. The PCR condition: Pre- Denaturation: 5min at 94^{0}C, denaturation: 30sec at 94^{0}C. Annealing: 30sec at 52^{0}C, extension: 1min at 72^{0}C 35 cycles, final extension: 5min at 72^{0}C (Pourfadakari *et al.,* 2021). Agarose-gel electrophoresis was carried out. Sequencing analysis was done at Inqaba Biotechnology Pty South Africa and the sequences in the forward and reverse files were analyzed using the Sequence Scanner Software v1.0 (Applied Biosystems Themo Fisher Scientific). The similarity search was conducted *in-silico* using the Nucleotide Basic Local Alignment Search Tool at the Nation Centre for Biotechnology Institute (NCBI) server.

RESULTS AND DISCUSSION

A total of fifteen (15) bacteria were isolated from the soil sample collected from mechanic workshop. The microscopic and biochemical characteristics of the bacterial isolates are shown in Table 1 below. Bacterial isolates were observed to belong to five genera. The genus, *Bacillus* had the highest frequency of occurrence (26.6%), followed by *Micrococcus*, *Pseudomonas and Staphylococcus* (20% each) while *Acinetobacter* had 13.3%.

Screening of Bacterial for Biosurfactant Production

Drop collapse test

The fifteen (15) bacterial isolates were all tested and six (6) tested positive to this test. The drop of crude oil collapsed almost immediately in these six isolates thus indicating the ability of the bacteria to produce biosurfactant. The remaining

nine (9) isolates gave a negative result. Table 4.2 shows the drop collapse results for all the bacterial isolates ion this study.

Oil displacement method

The fifteen (15) bacterial isolates were observed to see if they would displace the oil layer. Nine (9) of the bacterial isolates displaced the oil layer and spread on the water indicating positive results. The remaining six (6) bacterial isolates were not able to displace the oil layer significantly. Table 4.3 shows the zone of displacement of all the isolates tested in this study. Isolate S9 had the highest zone of displacement (1.96cm).

Emulsification activity (E_{24})

The ability of bacterial isolates to emulsify oil was tested and the results are shown in Table 4.4. It was observed from the experiment that isolate S9 had the highest emulsification index (52.5%). On the other hand, isolate S11 had the lowest emulsification index (44.8%).

Table 1: Microscopic and Biochemical Characteristics of Bacterial Isolates from Soil Samples

Isolate Code	Biochemical characteristics														Probable org
	Gram	Rxn	Mot	Cat	Cit	Ind	MR	VP	H2S	Oxi	G	L	S	Mann	
S1	Cocci	+ve	_	_	+	_	+	_	_	+	A	A	A	A	*Micrococcus* sp
S2	Rods	+ve	+	+	+	_	_	+	_	_	A	A	A	_	*Bacillus* sp.
S3	Cocci	+ve	_	+	+	_	+	+	_	_	A/G	A/G	A/G	A/G	*Staphylococcus* sp.
S4	Rods	–ve	_	+	+	_	+	_	_	+	A	_	_	A	*Acinetobacter* sp.
S5	Rods	–ve	+	+	+	_	+	_	_	+	A/G	A	_	A	*Pseudomonas* sp.
S6	Cocci	+ve	_	+	+	_	+	+	_	_	A/G	A/G	A/G	A/G	*Staphylococcus* sp.
S7	Cocci	+ve	_	_	+	_	+	_	_	+	A	A	A	A	*Micrococcus* sp.
S8	Rods	–ve	+	+	+	_	+	_	_	+	A/G	A	_	A	*Pseudomonas* sp.
S9	Rods	+ve	+	+	+	_	_	+	_	_	A	A	A	_	*Bacillus* sp.
S10	Rods	–ve	+	+	+	_	+	_	_	+	A/G	A	_	A	*Pseudomonas* sp
S11	Cocci	+ve	_	_	+	_	+	_	_	+	A	A	A	A	*Micrococcus* sp.
S12	Rods	+ve	+	+	+	_	_	+	_	_	A	A	A	_	*Bacillus* sp.
S13	Cocci	+ve	_	+	+	_	+	+	_	_	A/G	A/G	A/G	A/G	*Staphylococcus* sp.
S14	Rods	+ve	+	+	+	_	_	+	_	_	A	A	A	A	*Bacillus* sp.
S15	Rods	–ve	_	+	+	_	+	_	_	+	A	_	_	A	*Acinetobacter sp.*

Key: +: positive; _: negative; G: glucose; L: lactose; S: sucrose; Mann: mannitol; A/G: acid/gas

Table 2: Drop Collapse Result for the Bacterial Isolates

Isolate code	Observation
S1	Negative
S2	Positive
S3	Negative
S4	Negative
S5	Positive
S6	Negative
S7	Negative
S8	Positive
S9	Positive
S10	Positive
S11	Negative
S12	Negative
S13	Negative
S14	Positive
S15	Negative

Table 3: Oil Displacement by Bacterial Isolates

Isolate code	Extent of spread (cm)
S1	0.78±0.42c
S2	1.3±0.14ab
S3	1.5±0.21a
S4	1.1±0.12b
S5	1.94±0.14a
S6	0.55±0.14c
S7	0.1±0.42c
S8	0.88±0.61c
S9	1.96±0.23ab

S10	1.65±0.12b
S11	0.23±0.28c
S12	0.55±0.23c
S13	0.72±0.25c
S14	1.35±0.41b
S15	0.15±0.34c

Table 4: Emulsification Activity (E24) for Bacterial Isolates

Isolate code	Emulsification layer	Total lipid layer (cm)	E_{24} (%)
S1	2.9	5.9	49.1
S2	2.6	5.2	50
S3	2.9	6	48.3
S4	3	5.8	51.7
S5	2.8	5.8	48.2
S6	2.7	5.9	45.7
S7	2.9	6	48.3
S8	2.9	5.6	51.7
S9	3.1	5.9	52.5
S10	2.6	5.2	50
S11	2.6	5.8	44.8
S12	2.7	5.9	45.7
S13	2.8	5.8	48.2
S14	2.9	6	48.3
S15	2.7	5.9	45.7

Molecular Characterization of Bacterial Isolates obtained from Soil Sample

The gel electrophoresis of the two most effective biosurfactant_producing bacteria (*Pseudomonas aeruginosa* S5 and *Bacillus subtilis* S9) is presented in Figures 1 and 2 respectively. The BLASTn for the bacteria submitted to GenBank revealed that *Pseudomonas aeruginosa* S5 had 99% homologue with *Pseudomonas aeruginosa* MSSRFV220 and an accession number of HQ455033.1 while *Bacillus subtilis* S5 had 75.59% homologue with *Bacillus subtilis* BC5 and an accession number of JN660081.1

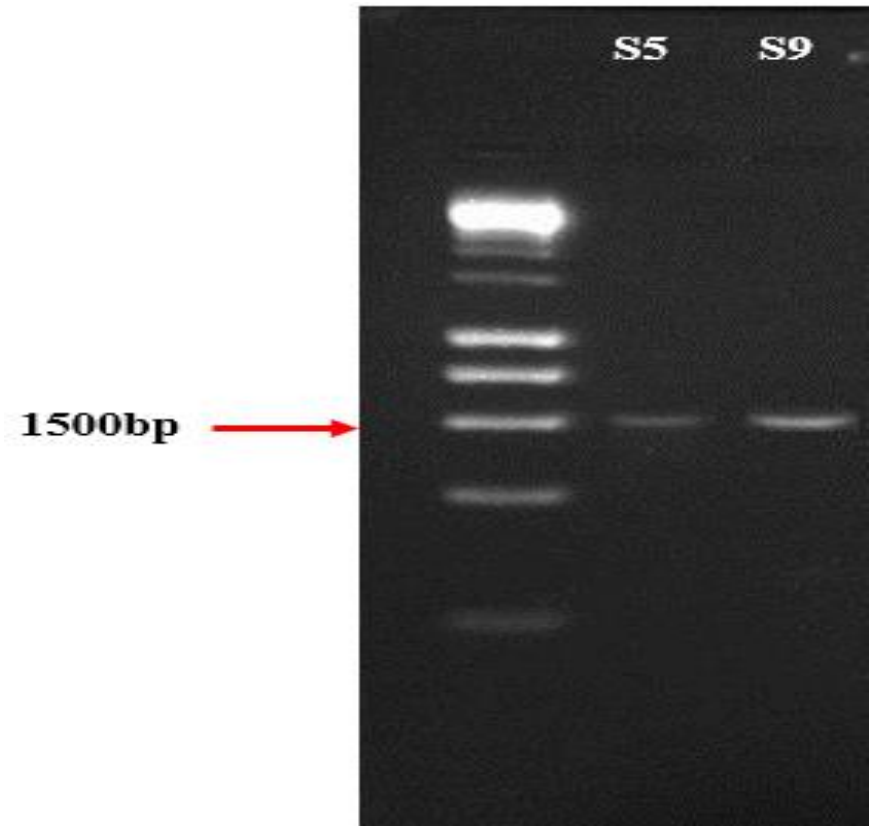

Figure 1: Gel Electrophoresis for Amplified 16S rDNA gene of the Isolates S5 (*Pseudomonas aeruginosa*), and S9 (*Bacillus subtilis*)

Physical Properties of Textile Effluent During Decolorization

Figure 2 shows the changes in the pH value of the textile effluent amended with 1mg of *Pseudomonas aeruginosa* surfactant and 1mg of surfactant from *Bacillus subtilis* during the study. The pH value ranged from 7.6 to 8.0 throughout the study in all samples. At the end of the study, it was observed that textile effluent with 1.0 mg of biosurfactant from *Pseudomonas aeruginosa* had the highest pH value (8.0) while textile effluent with 1.0 mg of biosurfactant from *Bacillus subtilis* recorded the least pH value (7.6) at the end of the study.

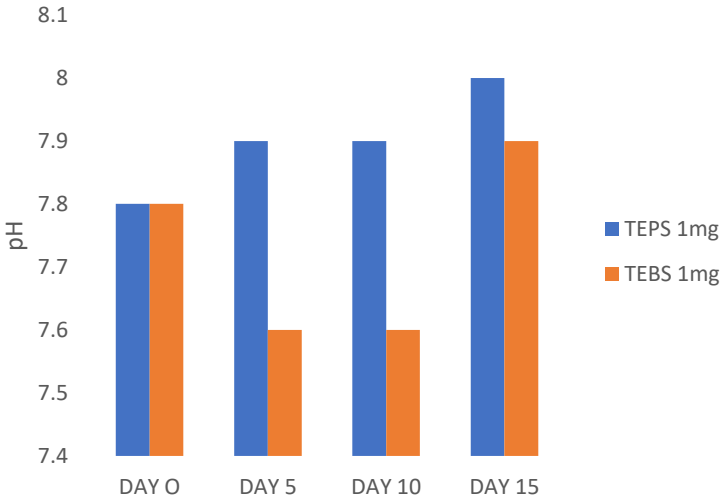

Figure 2: Changes in pH of Textile Effluent Amended with 1mg of Bacterial Surfactant
Key: TEPS: Textile effluent with *Pseudomonas s*urfactant; TEBS: Textile effluent wit *Bacillus* surfactant; Mg: milligram

Similarly, the temperature of all samples was observed throughout the study period. Figure 3 shows the changes that occurred in the temperature of the textile effluent after been amended with biosurfactant from the two test bacteria. Textile effluent amended with biosurfactant from *Bacillus subtilis* showed a higher temperature value (37) at the end of the study. Meanwhile, the least temperature value (33) recorded in this study was observed in all samples at the beginning of the study (day 0).

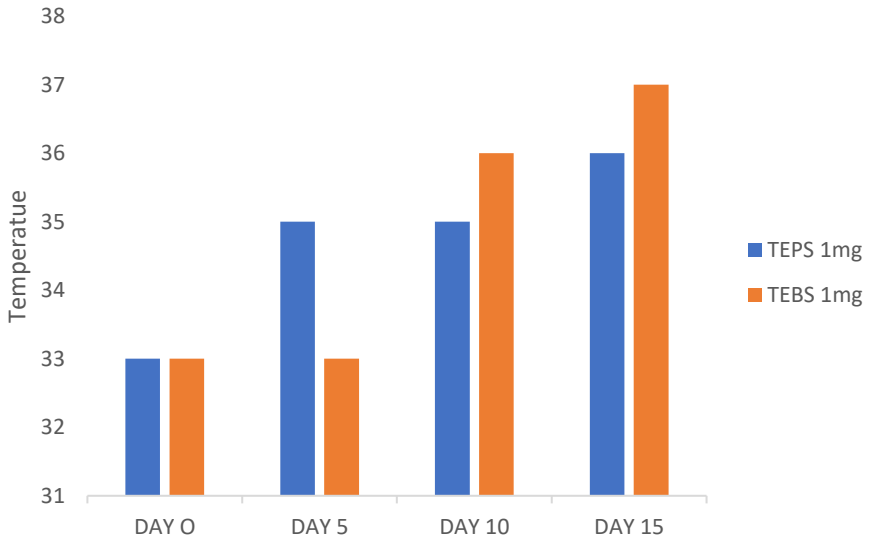

Figure 3: Changes in temperature of textile effluent amended with 1mg of bacterial surfactant
Key: TEPS: Textile effluent with *Pseudomonas* surfactant; TEBS: Textile effluent with *Bacillus* surfactant; Mg: milligram

Discussion

Soil polluted with different types of hydrocarbon was used to isolate bacteria capable of decolorizing effluents from textile industry. Decolorizing textile effluent is in the ability of the bacteria to produce biosurfactant. According to colony morphology, physiology and biochemical characterization, bacteria isolated in this study were presumptively identified to belong to five (5) genera. Three were Gram positive (*Micrococcus, Bacillus, Staphylococcus*) while the remaining two (*Acinetobacter, Pseudomonas*) were Gram negative. This observation corresponds to the report of Bodour *et al*. (2003) who isolated more Gram positive bacteria than Gram negative bacteria in hydrocarbon contaminated soil. On the contrary, Ramankutty and Nedunchezhiyan (2015) isolated more Gram

negative bacteria than Gram positive bacteria. The difference in the ratio between the types of bacteria in different studies could be due to the type of soil and hydrocarbon present in the soil. However, both types of bacteria can exist in soil contaminated with hydrocarbons.

Species of *Bacillus*, *Micrococcus*, *Pseudomonas*, *Staphylococcus* and *Acinetobacter* were isolated in this study as biosurfactant producing bacteria. Other researchers isolated similar biosurfactant producing bacteria (Singh *et al.,* 2016). Out of fifteen bacteria isolated, only two showed better biosurfactant producing potential. These bacteria were identified using molecular techniques as *Bacillus subtilis* and *Pseudomonas aeruginosa*. According to Mahbub *et al*. (2015), *Bacillus* sp. is commonly found to degrade various textile dyes isolated from effluents of textile and printing press industries due to their biosurfactant producing capabilities (Wardlow *et al.,* 2017). The versatility of this bacterium cell would have given it the edge in producing biosurfactant. *Pseudomonas aeruginosa* on the other hand has also been reported of its ability to produce biosurfactant (especially rhamnolipid) (Kumar *et al.,* 2015) suggested that optimization of environmental condition and incubation period can improve the biosurfactant producing ability of these bacteria.

Different tests were carried out so as to ascertain the biosurfactant production potential of the isolated bacteria. Oil displacement test, drop collapse test, and emulsification index were chosen for biosurfactant screening. Plaza *et al*. (2006) revealed that these tests are of low cost, clear and the equipment used are readily available. Due to the simplicity of these tests, other researchers have used these tests (Satpute, 2010; Anitha *et al.,* 2015; Barbusiński *et al.,* 2021). About 49% of the isolated bacteria were positive for the drop collapse test in this study; 60% of the isolated bacteria were able to displace the oil layer. The emulsification of oil by isolated bacteria ranged from 44.8 – 52.5%. The variance observed in the screening of bacteria for biosurfactant production corresponds to the report of Sani *et al*. (2023) who also had varying results. The changes observed can be attributed to several factors such as the different source of isolating the organisms, different hydrocarbon used for the tests or the difference in materials used for the screening. The two samples S5 and S9 which had the best biosurfactant producing activities after drop collapse, oil displacement and emulsification index. After molecular identification was done using 16s rRNA gene sequencing, S5 had 75.59% homologue with *Bacillius subtilis* BCS, while S9 had 99% with *Pseudomonas aeruginosa* MSSRFV220. These two bacteria were used for the production of biosurfactants. After incubation for 7 days the biosurfactant was precipitated and extracted then evaporated. *Pseudomonas aeruginosa* had a dry weight 1.2 ± 0.03^{a}, while *Bacillus subtilis* had a dry weight of $1.38\pm0.02.^{b}$

The pH and temperature of the mixture influenced the decolorization of textile effluent in this study. It was observed that the pH of amended samples increased with time. Effluents amended with 1mg of *Pseudomonas aeruginosa* surfactant had the highest pH (8.0) while effluent amended with 1mg of *Bacillus subtilis* surfactant had the lowest pH (7.6). The difference in pH value between the two options could be due to different composition and mechanism of actor of the bio surfactants. In contrast, the temperature of the option amended with *Bacillus subtilis* (37ºC) surfactant was higher than that of *Pseudomonas aeruginosa* (33ºC).

CONCLUSION

Two of the fifteen isolated proved to be good biosurfactant producers after drop collapse, emulsification index and oil displacement test were done, isolate S9 had the highest displacement of 1.96cm. It also had the highest emulsification index of 52.5% while S5 had 1.94cm and 48.2% emulsification index. Both of them were positive for drop collapse test. They were both identified molecularly as *Bacillus subtilis and Pseudomonas aeruginosa b*y 16s rRNA gene sequencing.

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