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BIOSURFACTANT PRODUCTION POTENTIALS OF MICROORGANISMS ISOLATED FROM ATMOSPHERE OF FIVE PETROLEUM STATIONS AT TANKE, ILORIN, KWARA STATE, NIGERIA

*Adetitun D.O., Oni P.K. and Tomilayo R.B.

Department of Microbiology, Faculty of Life Sciences, University of Ilorin, Nigeria

*Corresponding Author Email Address: adetitun.do@gmail.com , adetitun.do@unilorin.edu.ng

ABSTRACT
Biosurfactants aid in bioremediation by improving the bioavailability of hydrocarbon contaminants. The objective of this work was to isolate, enumerate and identify bacteria and fungi for their biosurfactant production potential in the atmosphere of five petroleum stations at Tanke, Ilorin, Kwara State, Nigeria using appropriate, standard microbiological methods (haemolysis test, emulsification index test, drop collapse test and oil displacement test). Fourteen bacteria and ten fungi were isolated in this study. The bacterial isolates belong to the genus *Bacillus*, *Corynebacterium*, *Micrococcus*, *Staphylococcus*, *Listeria, Clostridium*. The fungal isolates are of the genus *Neurospora*, *Curvularia*, *Aspergillus*, *Fusarium*, *Rhizopus*, *Cladosporium*, *Colletotrichum*, *Sclerotinia*. The mean number of bacteria counted during the sampling ranged from $3.48\pm1.15*10^2$ CFU to $4.82\pm1.69*10^2$ CFU, with petrol station 4 having the highest bacteria count ($2.41*10^2$ CFU) and petrol station 3 having the lowest bacteria count ($1.74*10^2$ CFU). The mean fungal count ranged from 7 ± 3.7 CFU to $8.6\pm4.7*10^2$ CFU, with petrol station 4 having the highest fungal count (4.3 *10 CFU) and petrol station 3 having the lowest fungal count (3.4*10 CFU). *Bacillus* species showed promise of biosurfactant production after screening. *Aspergillus fumigatus* was the most prevalent fungus isolated (24%) while *Staphylococcus epidermidis* was the most prevalent bacterium isolated (11%). Some of these organisms are known opportunistic pathogens therefore, improved ventilation and sanitation of the petrol stations should be carried out to reduce the microbial load in the air. Also, bacteria that produce biosurfactant can be isolated from air of gas stations.

Keywords: Biosurfactant, Bacteria, Fungi Air microflora, *Aspergillus niger*

INTRODUCTION

A basic essential for living is clean air. Since the dominant microorganisms in an environment represent the various human activities (Baberan *et al*., 2015), as well as their roles in various ecosystems (Adams *et al*., 2016), there has been a noticeable increase in awareness of the air quality of both indoor and outdoor systems. However, in densely populated places, airborne microbial pollutants, particularly emerging pathogens, can negatively impact human health and well-being by causing infections, inflammation, and toxic consequences (Shiaka &Yakubu 2013; Wei *et al*., 2017). Air does not have an indigenous micro flora, though a number of microorganisms are present in the air. Air is not a natural environment for microorganisms as it doesn't contain enough moisture and nutrients to support their growth and reproduction (Fuhrman, 2009). One of the most common sources of air microflora is the soil. Soil microorganisms when distributed by the wind are blown into the air and remain suspended for a period of time (Baberan *et al.,* 2015). Man-made actions like digging or ploughing the soil may release soil born microbes into the air. Similarly, microorganisms found in water may also be released into the air in the form of water droplets or aerosols, splashing of water by wind action and tidal action may also produce droplets or aerosols (Shiaka & Yakubu 2013).

Different environmental conditions such as temperature, UV light, dryness and humidity, play a role in controlling the load of airborne particles. Microbes manage to reach new hosts through the air for its survival (Sheik *et al*., 2015). Poor ventilation, crowded conditions and increase in number of air conditions inside building nowadays can facilitate the spreading and the survival rates of airborne particles and also can increase the chance of people at risk of airborne infections (Jacob *et al*., 2016). Among dust particles present in the environment, fungus which reproduce by forming spores, some bacteria especially/ Gram positive bacteria and some viruses can survive for a long time in the air (Sheik *et al*., 2015; Jacob *et al*., 2016).
Surface-active substances known as biosurfactants are created by microorganisms. They increase the solubility, bioavailability, and eventual biodegradation of the hydrophobic or insoluble organic compounds by accumulating at the interface of immiscible fluids, which lowers surface and interfacial tension (Van-Hamme *et al*., 2006). According to Thavasi *et al*. (2011), they are made up of a heterogeneous set of chemical molecules such as glycolipids, lipopolysaccharides, oligosaccharides, and lipopeptides. Chemical characteristics and molecular sizes differ across biosurfactants. Their ability to interact across fluid phases allows them to reduce surface and interfacial tension at the surface and interface, respectively (Satpute *et al*., 2009). They also contain hydrophobic and hydrophilic moieties (Satpute *et al.,* 2009).

Due to their variety, environmental friendliness, potential for production through fermentation, and potential applications in fields like environmental protection, crude oil recovery, healthcare, and food processing industries, microbial surfactants have seen a steady rise in interest in recent years (Makkar *et al*., 2011). According to several studies (Desai & Banat, 1997; Tabatabaee *et al*., 2005; Makkar *et al*., 2011), the majority of biosurfactant synthesis involved the utilization of microorganisms cultured on water immiscible hydrocarbons and some water-soluble

substrates. Bacteria especially *Bacillus* species, are among the best known biosurfactant producers. Previous studies have shown the ability of *Bacillus subtilis* and *Bacillus licheniformis* to produce large quantities of biosurfactants using different substrates (Joshi *et al*., 2008; Jaysree *et al*., 2011).

The objective of this work is to isolate, enumerate and identify bacteria and fungi for their biosurfactant production potential in the atmosphere of five petroleum stations at Tanke, Ilorin.

MATERIALS AND METHODS

Sampling Site, Sample collection and Isolation of Microorganisms

Air samples were taken from five petroleum stations at Tanke, Ilorin for a period of 5 days. The sedimentation technique was used for sample collection. The samples were collected in duplicates for each agar medium. The plates were placed 1m from the ground and from the wall and then exposed to air for 10 minutes (Cosentiano *et al*., 2008). The Nutrient Agar plates were incubated at 37°C for 24 hours while the potato dextrose agar plates were incubated at 25±2 ^{o}C for 48-72 hours. The number of colonies were counted, recorded and characterization was carried out. The CFU range was calculated using the Omelyansky's formula: $N = 5a \times 10^{4}$ (bt)-1 (Adetitun and Abioye, 2017).
where N = CFU/m^3, a = number of colonies per Petri dish, b = dish square centimeter, t = exposure time in minutes.

Characterization and Identification of Bacteria Isolates

The characterization of each isolate was based on colonial morphology, cellular morphology and biochemical tests. Colonial morphology such as shape, colour, edge, elevation, surface texture and optical characteristics was observed and recorded according to Fawole and Oso (2007). Cellular morphology of the isolates was determined through. Gram staining, spore staining capsule staining. catalase test, indole test, motility test, coagulase test, starch hydrolysis, oxidase test, triple sugar iron (TSI) test, oxygen relationship test, citrate test. Voges Proskaeur test, sucrose test and inositol test. Isolates were identified according to Bergey's manual of systemic bacteriology (Buchanan *et al*., 2012).

Characterization and identification of fungal Isolates

The isolates morphological and microscopic characteristics were used for their characterization. The growth characteristics of the fungi on the Petri dish plates where recorded. The colour on the upper side and underneath were also recorded. Lactophenol in cotton blue was used to mix picked mycelium on a clean glass slide and teased with inoculating needle. This was observed under X40 objective lens of the binocular microscope. The appearance of the mycelium under the microscope was recorded (Fawole and Oso, 2007). Identification of the moulds was done by comparing their characteristics with those found in standard mycology texts (Samson *et al*., 1996).

Biosurfactant production assay

In order to determine the capability of the isolated bacteria to produce biosurfactant, the following tests were carried out to test if the bacteria isolated could produce biosurfactant: haemolytic assay, drop collapse assay, oil displacement assay and emulsification test.

Haemolytic Assay

The Isolated bacteria were inoculated on human blood agar plates and incubated for 48 hours at 25 °C. Biosurfactant activity of the bacterial strains is indicated by the lysis of the blood cells and exhibit a colorless, transparent ring around the colonies (Ghasemi *et al*., 2019).

Drop Collapse Assay

The drop collapse technique depends on the principle that a drop of a liquid containing a biosurfactant will collapse and spread completely over the surface of oil. This assay relies on the destabilization of liquid droplets by surfactants. Therefore, drops of a cell suspension or of culture supernatant are placed on an oil coated, solid surface. If the liquid does not contain surfactants, the polar water molecules are repelled from the hydrophobic surface and the drops remain stable (Jain *et al*., 1991; Walter *et al*., 2020).

Oil displacement Assay

A Petri dish was filled with distilled water (35 mL), and a burned lubricating oil (100 µL) was added to the water surface. Subsequently, 10 µL of a bacterial cell culture supernatant was added to the center of the oils' surface. The positive control and negative control were obtained with a 1% sodium dodecyl sulfate (SDS) solution and distilled water, respectively. The result was considered positive when the drop was totally or partially scattered and negative when it remained unchanged. The diameter of the spread is measured and recorded (Thavasi *et al*., 2006).

Emulsification Test

Exactly 4 ml of a known hydrocarbon source was homogenised with 4 ml of cell free supernatant by vortexing at 1000-1500 rpm for 2-5 minutes to form an emulsion layer. The mixture was allowed to stand for 24 hours before calculating the emulsification index. The emulsion index was calculated as the ratio of the height of the emulsion layer and the total height of liquid (Charan and Patel, 2012).

Statistical Analysis

The data were analyzed with SPSS. All results are presented as the mean value (±SD). A statistical significance level of $p<0.05$ was applied during the statistical analyses.

RESULTS

Microorganisms Isolated

From the research work carried out, fourteen bacteria and ten fungal isolates from the sampling sites was successfully isolated. The bacteria isolates have been tentatively identified as: *Micrococcus leutus, Bacillus subtilis, Corynebacterium pseudotuberculosis, Staphylococcus epidermidis, Bacillus coagulans, Bacillus cereus, Clostridium* sp, *Staphylococcus saprophyticus, Staphylococcus aureus, Bacillus megaterium, Clostridium perfringens, Listeria monocytogenes, Corynebacterium diphtheria, Bacillus circulans*. The fungal isolates were identified as *Aspergillus niger, Fusarium oxysporum, Rhizopus stolonifer, Cladosporium herbarum, Colletotrichum gloeosporioides, Sclerotinia sclerotiorum, Curvalaria lunatus, Aspergillus fumigatus, Aspergillus flavus,* and *Neurospora crassa*.

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Bacteria isolates

The isolated bacteria are of 6 genera in which the *Bacillus* genera had the highest occurring specie (5 species). The mean number of bacteria count throughout the sampling sites ranging from 3.48±1.15*10 CFU to 4.82±1.69*10 CFU with petrol station 4 having the highest bacteria count and petrol station 3 having the lowest bacteria count and the mean bacteria count ranging from 2.28±3.6*10 CFU on day 3 of sampling to 6.32±1.63*10 CFU on day 4 of sampling are shown in **Table 1**. The bacteria contamination load ranged from $2.48*10^4$ cfu/m^3 on day 4 to $8.*10^3$ cfu/m^3 on day 3 and $1.89*10^4$ cfu/m^3 at petroleum station 4 to $1.36*10^4$ cfu/m^3 at petroleum station 3.

The result of the SPSS showed a statistically significant difference (F= 8.24, p= 0.0004) between the mean concentration of airborne bacteria on day 4 (63.2±16.3) and day 3 (22.8±3.6) with a=0.05. This can be due to the windy weather on day 4 during sample collection which will result in increased transportation of microorganisms in form of droplet nuclei in the dust particles. There was no significant difference in the mean concentration of airborne bacteria for petrol station 4 and petrol station 3 (F=0.59, p=0.67, a=0.05).

Figure 1 shows the percentage occurrence of the bacteria isolates. *Staphylococcus epidermidis* had the highest percentage occurrence (11%). *Corynebacterium diphtheria* showed the least percentage occurrence (4%).

Biosurfactant production

Table 2 depicts the biosurfactant analysis results. *Staphylococcus aureus*, *Bacillus circulans* and *Staphylococcus saprophyticus* showed positive oil displacement for all the oils tested. *Bacillus circulans* showed positive for drop collapse assay using crude oil. Only *Bacillus subtilis* showed positive for drop collapse assay using kerosene. *Bacillus coagulans* was positive for emulsification test with all the oils tested and had the highest value of 0.7cm for engine oil. Kerosene and crude oil gave the most positive results for emulsification. Five isolates showed Beta hemolysis (*Listeria monocytogenes, Corynebacterium pseudotuberculosis, Clostridium* sp, *Staphylococcus aureus, Bacillus coagulans).* Four isolates showed alpha hemolysis (*Bacillus cereus, Staphylococcus epidermidis, Bacillus circulans, Staphylococcus saprophyticus).* The remaining isolate did not undergo hemolysis (gamma hemolysis).

Fungal isolates

In total 10 fungal species belonging to 7 genera were isolated from the petroleum stations. Three of them were identified as *Aspergillus* sp which showed that *Aspergillus* was the most frequent genus with Aspergillus isolates observed in all the sampling site throughout the period of sampling. The fungal Isolates were *Neurospora crassa, Curvalaria lunatus, Aspergillus fumigatus, Aspergillus flavus, Colletotrichum gloeosporioides, Sclerotinia sclerotiorum, Fusarium oxysporum, Aspergillus niger, Rhizopus stolonifer, Cladosporium herbarum.*

The mean of fungal count throughout the sampling sites ranging from 7±3.7 CFU to 8.6±4.7 CFU with petrol station 4 having the highest fungal count and petrol station 3 having the lowest fungal count and the mean fungal count ranging from 4.2±1.2 on day 2 of sampling to 14.4±2.0 on day 4 of sampling are shown in **Table 3**. The fungal contamination load ranged from $2.67*10^3$ cfu/m^3 at petroleum station 3 to $3.38*10^3$ cfu/m^3 at petroleum station 4 and also from $1.65*10^3$ cfu/m^3 on day 2 to $5.66*10^3$ cfu/m^3 on day 4 using the Omelyansky formula for counting microbes isolated from air (Adetitun and Abioye, 2017).

The result of the SPSS showed no statistical significant difference (F= 28.72, p= 4.89, a=0.05) between the mean concentration of airborne fungi on day 4 and day 2. There was also no significant difference in the mean concentration of airborne fungi for petrol station 4 and petrol station 3 (F=0.12, p=0.97, a=0.05). **Figure 2** shows the percentage occurrence of the fungal Isolates where *Aspergillus fumigatus* showed the highest percentage occurrence of 24% and *Neurospora crassa* showed the least percentage occurrence of 4%.

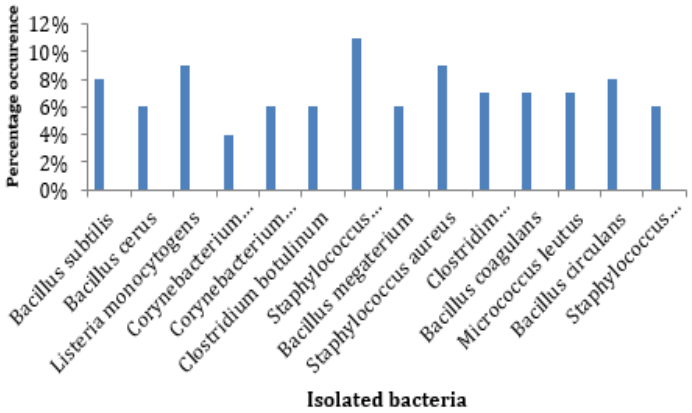

Figure 1: Percentage Occurrence of the Bacteria Isolates

Table 1: Average number of bacteria count over the period of sampling

Sampling period	Petrol station 1 (CFU)	Petrol station 2 (CFU)	Petrol station 3 (CFU)	Petrol station 4 (CFU)	Petrol station 5 (CFU)	Mean
Day 1	40	44	45	40	31	40±4.9
Day 2	35	27	40	49	37	37.6±3.6
Day 3	24	28	17	21	24	22.8±3.6
Day 4	70	87	49	69	41	63.2±16.3
Day 5	49	44	23	62	47	45±12.6
Mean	43.6±15.4	46±21.7	34.8±11.5	48.2±16.9	36±7.9	

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Table 2: Biosurfactant production by the isolated bacteria

	Displacement test (mm)			Drop assay test			Emulsification test (cm)			Hemolysis test
Isolate	KS	EG	CD	CD	EG	KS	CD	EG	KS	
Bacillus cereus	0	0	0	-	+	-	0	0	0.1	Alpha
Bacillus subtilis	0	0	4	-	+	+	0	0	0.2	Gamma
L. monocytogenes	0	0	0	-	-	-	0	0	0	Beta
C. diphtheria	0	0	0	-	-	-	0	0	0	Gamma
C. pseudotuberculosis	0	0	3	-	+	-	0.2	0	0.4	Beta
Clostridium sp	4	0	6	-	+	-	0.3	0	0.1	Alpha
S. epidermidis	0	0	0	-	-	-	0.1	0	0.5	Gamma
Bacillus megaterium	9	0	6	-	+	-	0.1	0	0.3	Beta
Staphylococcus aureus	5	8	8	-	+	-	0	0	0	Gamma
Clostridium perfringens	0	0	0	-	-	-	0	0	0	Beta
Bacillus coagulans	0	0	9	-	-	-	0.1	0.7	0.4	Gamma
Micrococcus leutus	0	0	0	-	-	-	0.2	0	0.1	Gamma
Bacillus circulans	10	8	20	+	+	-	0.1	0	0.2	Beta
S. saprophyticus	10	8	10	-	-	-	0.3	0	0.2	Alpha

Key: - = negative, **+**= positive, **KS**=Kerosene, **CD**=Crude oil, **EG**=Engine oil

Table 3: Average number of fungi count at the sampling sites during the sampling period

Sampling period	Petrol station 1	Petrol station 2	Petrol station 3	Petrol station 4	Petrol station 5	Mean
Day 1	5	6	2	5	5	4.6±1.4
Day 2	5	3	3	4	6	4.2±1.2
Day 3	8	7	9	10	8	8.4±1.0
Day 4	14	16	11	17	14	14.4±2.0
Day 5	5	9	10	7	9	8±1.8
Mean	7.4±3.5	8.2±4.4	7±3.7	8.6±4.7	8.4±3.1	

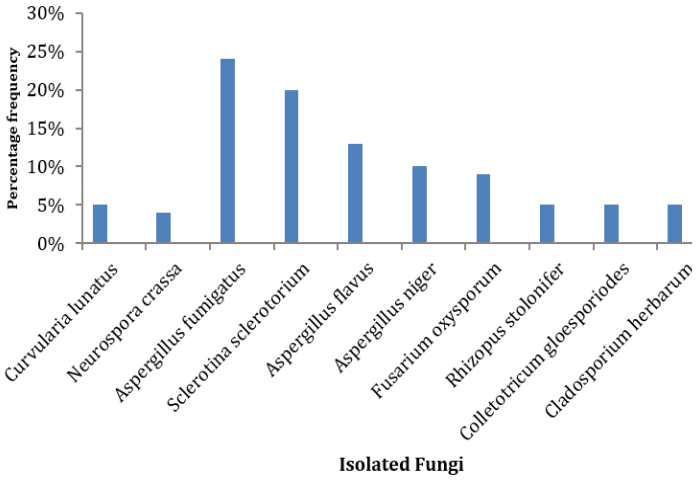

Figure 2: Percentage occurrence of the fungal isolates

DISCUSSION

Fourteen bacteria and ten fungi were identified from the sampling sites and the presence of these organisms is affected by some factors which includes level of activities in the sampling area and surrounding. Hence, the type of organisms that falls on exposed plate depends on the time of exposure, movement of individuals, the current content of the air passing at that time and the method used in sampling (Micheal *et al*., 1993). The discharge of these microorganisms could be through sneezing, coughing, talking, the soil, dust particles, contact with equipment and machineries, uncontrolled movement in and out of the petrol station, overcrowding, etcetera.

In this study the mean fungal load was $1.65*10^3$ cfu/m^3 to $5.66*10^3$ cfu/m^3 and the bacteria load ranged from $8.96*10^3$ cfu/m^3 to $2.48*10^4$ cfu/m^3, which is larger compared to the work done by Ekhaise *et al*. (2010) on airborne microflora in hospitals at University of Benin teaching hospital where the bacteria load was recorded as $3.0*10^0$ cfu/m^3 to $7.6*10^1$ cfu/m^3 and the fungal load ranged from $6.0*10^0$ cfu/m^3 to $4.5*10^1$ cfu/m^3. The variations in the fungal and bacteria load could be due to differences in environmental variables, compound sanitation, building conditions, and concentration of coarse airborne solid particles (dust) and the study samples have been collected at petroleum station which is an outdoor environment with high activity and overcrowding which may contribute to the difference in microbial load.

In this present study, various species of bacteria and fungi have been isolated which agrees partly with earlier studies of Humbat *et al*. (2018) that worked on health effect of bioaerosol and Stryjakowska-Sekulska *et al*. (2007) who worked on microbiological quality of university rooms. The isolated fungal and bacteria species variation could be triggered due to variability in temperature, humidity, building condition, study settings, and other physical parameters in the petroleum station environment. This research work also agrees with Makut *et al*. (2014) that worked on air microflora from outdoor air of Keffi metropolis where the *Aspergillus* genera was the highest isolated genera for fungi and *Bacillus* was the highest isolated genera for bacteria.

The fungi isolated most frequently in this study are the *Aspergillus* sp. with *Aspergillus fumigatus* having the highest frequency of isolation with percent occurrence of 24%, this is in concordance with the work of Ekhaise and Ogboghda (2011), where it was stated that members of the genus *Aspergillus* were the most isolated. Aydogdu *et al*. (2008) also reported *Aspergillus* as one of the predominant genus in outdoor air which also concur with Nayak, (2013). *Aspergillus* species are filamentous fungi that are commonly found in soil, decaying vegetation, and seeds and grains, where they thrive as saprophytes. In humans, *Aspergillus fumigatus* is the most common and life-threatening airborne opportunistic fungal pathogen, which is particularly important among immunocompromised hosts (Patterson and Strek, 2010).

Aspergillus niger is less likely to cause human disease than some other *Aspergillus* species, but if large amount of spores are inhaled, a serious lung diseases: Aspergillosis can occur (Heitman, 2011). It appears therefore, that the atmosphere at the study locations could be so dangerous contaminated with either spores or fragments of these medically important species. In agreement to this research *Aspergillus niger* and *Aspergillus flavus* among others have been isolated from air by Ekhaise and Ogboghodo (2011) who worked on microbiological air quality of hospital, also Han *et al*. (2023) also isolated *Aspergillus* species and *Fusarium* species from air and wall surface samples of duck-breeding farms in South

Korea.

The isolation of *Staphylococcus epidermidis* which is the highest occurring bacteria in this study and *Staphylococcus aureu*s is a big concern because, despite being a common bacterium of the human microbiota, studies have shown these bacteria as emerging pathogens in nosocomial infections and reported as a pathogen on generalized infections (Gizaw *et al*., 2016). The prevalence of *Staphylococcus aureus* could be attributed to its many ways of transmission through agents such throat, skin, cuts, boils, nails and nasopharynx (Ekhaise *et al*., 2008) and other activities. *Staphylococcus* species are typical skin organisms and are known to be normal flora of the nose. *Staphylococcus epidermidis* commonly occurs as a harmless commensal on human and animal skin, however like many other coagulase negative Staphylococci, they may occasionally cause infection in immuno-compromised individuals (Shanson, 1989). This research is also in conformity with the result obtained by Badri *et al*. (2016), who isolated *Staphylococcus aureus* and *Staphylococcus epidermidis*.

The Isolation of the *Bacillus* species is supported by the fact that they are relatively abundant in air which could be attributed to their ability to form refractive endospores which enable them to resist adverse conditions (Michael and Idemudia, 2022). Some of them cause disease in humans and are of economic importance in food industry. *Bacillus subtilis* is widely distributed in the air and is a ubiquitous soil microorganism (Satpute *et al*., 2008). Its presence in the petrol stations can be due to movement of staff and customers in and out of the station, dust raising activities like driving of vehicles into the petroleum stations.

Among the isolates obtained from the different sampling site, the potential biosurfactant- producing isolates were confirmed as *Bacillus* species. The result supported the report of Okore *et al*. (2017) who isolated *Bacillus* species from different contaminated soils. Previous studies by El-Sheshtawy (2013) and Okore *et al*. (2013) had revealed the presence of different *Bacillus* species in oil and non-oil contaminated environments. The biosurfactant results gotten in this study is also in consonance with the findings of Shafi and Khanna (1995) and Michael and Idemudia (2022) who reported that *Bacillus* species possesses the potential of producing biosurfactants when grown in carbohydrate and nitrogen-base mineral salt medium as sole carbon source.

The *Bacillus* isolates showed varying oil displacement zone formation. An oil spreading test showed that *Bacillus circulans* produced the highest oil spreading or displacement ability of 20 mm using crude oil. A similar result was shown in the work of Adnan *et al*. (2015) which gave oil displacements zone value of 25 mm. The oil spreading observed indicated that the broth used has surface activities, hence, the larger the spreading diameter, the higher the surface activity (Chandran and Das, 2010). *Bacillus circulans* when subjected to biosurfactant production tests showed zone of γ (gamma)-haemolysis (Satpute *et al*., 2008). *Bacillus circulans* in this study revealed positive effect for oil spreading test with diameter of 20mm for crude oil, 8mm for engine oil, 10mm for Kerosene and positive for drop-collapse in crude oil and engine oil. The hydrophobic surface of oil reduction indicates the incidence of biosurfactant in cell supernatant and the significance of these tests is to check the biosurfactant production capacity of the bacterial isolates (Michael and Idemudia, 2022). *Bacillus circulans* showed capability to displace oil on water surface. This suggests biosurfactant production that destabilizes liquid droplets with drop of cell-free supernatant on solid microplate. The drop collapse ability is on biosurfactant concentration correlating surface tension (Morikawa *et al*., 2000).

In conclusion, the analyzed Petrol stations are potential reservoirs of infectious and opportunistic microorganisms and have a high microbial load. Certain measures must be taken to reduce the microbial load in the air to ensure air quality void of pathogenic microbes. Some of these measures include the use of de-humidifier to control moisture as well as humidity which may serve as catalyst for the growth of microorganisms, fumigation can be carried out to spray the air and kill or inhibit air microflora.

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