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Screening of Bacterial Isolates from Spent Lubricating Oil Polluted Soil for Potential to Produce Biosurfactant

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ABSTRACT
Biosurfactants are surface – transitive biomolecules generated by microbes that have different capability in utilizing crude oil polluted soil, biosurfactants are better than chemical surfactants because of their cost effectiveness, complete degradability and environmentally friendly. The research focused in isolating, identifying and screening bacteria for potential of producing biosurfactants from spent lubricating oil polluted soil from automobile Mechanic Workshop Artillery Barrack, Kakuri, Kaduna, Kaduna state Nigeria. Soil samples were collected and processed. Bacteriological analysis of the soil sample was carried using standard methods. In all, fifteen (15) bacterial strains were isolated, identified and characterized following standard microbiological assay. The isolates include Pseudomonas aeruginosa (33%), Bacillus subtilis (20%), Bacillus cereus (27%), Staphylococcus aureus (13%) and Escherichia coli (7%) respectively with Pseudomonas having the highest occurrence while Escherichia coli had the least. The fifteen bacterial isolated were screened for potential of producing Biosurfactants using drop collapse, oil displacement as well as Emulsification activity (E-24) test. Nine isolates out of fifteen identified bacteria were positive for drop collapse test, displacement as well as emulsification activity (E-24) test. Isolate J12 had the highest emulsification activity of (43±0.2) followed by J6 (22±0.0) others are J9 (17±0.1). J15 (16±0.0), J2 (15±0.0), J10 (12±0.0), J13 (11±0.0), J1 (10±0.0) while no emulsification activity on J3, J4, J5, J7, J8, J11 and J14 . For oil displacement method, J12 had the highest with (4.1±0.0) followed by J6 and J15 having (3.0±0.0) each. J9 and J2 had 2.5±0.0 and 2.0±0.0 respectively.
KEYWORDS: *Biosurfactants, Analysis, lubricating oil, Screening and Bacteria*

INTRODUCTION

One of the most prevalent forms of energy in the world, crude oil is made up of an intricate and dynamic combination of hydrocarbons, which mostly asphaltenes, naphthenes, aromatics, saturates, alkanes, and resins (Sakthipriya *et al.*, 2015; Pereira *et al*., 2019; Jayasena and Perera, 2021). Environmental pollution caused by petroleum hydrocarbons has extensive and detrimental effects on public health, mental health, and the environment. Soil and water environments are frequently contaminated with oil hydrocarbons (OHC), polycyclic aromatic hydrocarbons (PAH), and other hydrophobic substrates, which frequently lead to severe environmental consequences (Ventriglio *et al*., 2021; Ajibade *et al.*, 2021; Sumudumali, and Jayawardana, 2021). In addition to its inherent poisoning and cancer-causing potential, crude oil has the potential to bioaccumulate, biomagnify, and resist biodegradation, which can cause it to remain in soil environments for an extended period of time following an oil spill (Al-Hawash et al., 2018; Naeem, and Qazi, 2020). Every year, about 2 million tonnes of crude oil enter marine environments as a result of sea-based activities (Zahed et al., 2011). Natural gases, aromatic compounds, and heterocyclic hydrocarbons combine to form petroleum, a multicomplex mixture. Spent engine oil is frequently disposed of in Nigeria, particularly by auto mechanics, into gutters, water drains, and open fields. This oil, also known as waste engine oil or wasted lubricant, is typically collected after car and generator engines have been serviced and drained; the majority of the oil is then thrown into the ground. The used oil contains a comparatively high concentration of hydrocarbons, including extremely hazardous PAH (Wang *et al*., 2014). heavy metals like iron, nickel, and lead These heavy metals may be linked to organic matter in the soil and retained as oxides, hydroxides, carbonates, and exchangeable cations. The presence of this used lubricating oil can alter the contaminated soil's chemical, physical, and microbiological characteristics. Environments contaminated by petroleum hydrocarbons must be cleaned up immediately. Furthermore, because different pollutants are constantly entering environments as a result of human activity, some remediation strategies—such as physical or chemical ones—are insufficient to completely decontaminate them. On top of this, these methods are typically expensive and time-consuming. Because of this, bioremediation has been widely advocated as an affordable, environmentally benign, and non-toxic biotechnology technique (Zaki *et al*., 2015; Dangi *et al*., 2019). Decontamination and reduction of pollutants from the anticipated contaminated environment through microbial activity is known as bioremediation, an important biotechnology technique. By making contaminants more bioavailable to oleophilic microorganisms living in contaminated aquatic and soil environments, biosurfactant—

an environmentally friendly and efficient compound with the characteristics of surfactants produced by certain microorganisms—can improve the process of bioremediation and accelerate the breakdown of petroleum hydrocarbons. Thus, a biosurfactant-optimized bioremediation procedure is essential (Ławniczak, 2013; Rylott, and Bruce, 2020).

The production of emulsifiers and biosurfactants is one fairly effective strategy for the bioremediation of such pollutants by microbial consortia (Jayasena and Perera, 2021). Biosurfactants are surface active metabolites that contain hydrophobic and hydrophilic moieties that reduce surface and liquid-liquid or solid-liquid interfacial tensions (Su-mudumali and Jayawardana 2021). They also improve the solublization of hydrocarbons into water, which eventually leads to better degradation of these pollutants. Additionally, surface active compounds have applications in enhanced oil recovery, food processing, pharmaceuticals, etc. that can be profitably (Su-.mudumali and Jayawardana 2021). Microorganisms that produce biosurfactants, specifically bacteria and yeasts, have been documented. These include species of Bacillus, Lactobacillus, Streptococcus, Nocardioides, Aeromonas, Serratia, Rhodococcus, and Candida, as well as Pseudomonas (Chen et al., 2020). According to Saktikriya et al. (2015), these microbes are prevalent in soil and water contaminated by hydrophobic organic compounds, such as waste from refineries. There are currently very few commercially available biosurfactants, such as rhamnolipids, sophorolipids, and surfactin. Many synthetic, chemical surfactants primarily derived from petroleum are used to meet the massive market demand for surfactants; these surfactants are typically hazardous to the environment and nondegradable (Ventriglio et al., 2021). Furthermore, because of their selective action, capacity to degrade biologically, and stability at high temperature, pH, and salinity, biosurfactants are more effective and adaptable than many synthetic surfactants. The advancement of this field of study is crucial, particularly in light of the current environmental protection concerns. This study aims to screen microorganisms that produce biosurfactants for the purpose of bioremediating soil contaminated by discarded lubricating oil.

MATERIALS AND METHODS

Study site / Sample Collection

Lubricating oil polluted soil sample were collected randomly from four different automobile Mechanic Workshop Artillery Barrack, Kakuri, Kaduna, Kaduna state Nigeria. The soil samples were collected by digging ground 5cm deep which was collected using sterile spatula and was placed on sterile polythene bag and immediately transported to the laboratory for analysis.

Bacteriological Analysis of Spent Lubricating Oil Polluted Soil

Enumeration, Identification and Characterization of Bacterial isolates

One gramme (1g) of each polluted soil was inoculated in 100ml of mineral salt medium (MSM), which is composed of (k2HPO4 0.3g, MgSO47H2O 0.05g, Soluble starch 0.5g, Dextrose 0.5g, Sodium pyruvate 0.3g, Peptone 0.25g, Casamino acid 0.5g, distilled water 1L, pH 7.2) supplemented with 1% crude oil and incubated at 30OC for 72 hours. The process of enrichment and bacterial isolation was done according to Erum et al. (2012). Bacterial isolation was done by spreading 0.1 ml of each culture to the MSM agar plate containing 1% of crude oil. After 48 hours of incubation morphologically distinct colonies were selected and characterized based on cellular and biochemical characteristics including Gram's reaction, cell shape. Motility, aerobic growth, pigmentation, catalase, oxidase, urease, indole, coagulase, methyl red and Voges ProsKauer, citrate utilization, starch hydrolysis and spore formation. The procedures outlined by Cowan and Steel (2004) were followed in conducting these experiments. For additional screening, the pure cultures were kept in a refrigerator (4OC) on nutritional agar slants.

Screening of bacterial isolates for biosurfactants producing potential

The ability of each bacterial isolate to produce biosurfactants was assessed by inoculating 10 ml of nutrient broth medium, centrifuging the mixture at 3000 rpm for 30 minutes, and then evaluating the supernatant using three different techniques: the drop collapse test, the oil displacement method, and the emulsification activity (E-24) test.

1. Drop collapse test

Each cavity of a glass cavity slide was filled with two microliters of crude oil; the slide was allowed to acclimatize to room temperature for an hour, after which 5 µl of the bacterial culture supernatant was added to the oil's surface (test); in the control, inoculated medium was substituted for the bacterial culture supernatant; the shape of the drop on the surface was noted after a minute; cultures that produced biosurfactants and produced flat or less convex drops were scored as positive (+).

2. Oil displacement method

According to Hassanshahian (2014), the diameter of the clear zone, which develops after applying a solution containing surfactants on an oil-water interphase, was measured using the oil displacement method. In this experiment, a Petri dish measuring 90 mm in diameter was filled with 25 ml of distilled water, 100 µl of crude oil was added to the water's surface, and then 10 µl of the cell-free culture supernatant—obtained by centrifuging a broth culture that had been incubating for eighteen hours at 6000 rpm for thirty minutes—was added. After 30 seconds, the diameter of the oil as displaced by the cell-free supernatant and clear zone created was measured under visible light.

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3. Emulsification activity (E24)

The emulsion index (E24) at 25 °C was used to measure the biosurfactants solution's emulsification activity, in accordance with Wang et al. (2014). After centrifuging an eighteen-hour broth culture at 6000 rpm for thirty minutes, two millilitres (2 mL) of crude oil was separately added to a test tube holding two millilitres (2 mL) of cell-free bacterial supernatant. The mixture was then homogenized by overtaxing it at a high speed for two minutes using a Stuart auto votex mixer. After the homogenized mixture was left to stand for 24 hours, measurements were taken of the mixture's overall height and the height of the stable emulsion layer. The results were used to compute the emulsion index (E24), which is as follows:

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

RESULTS AND DISCUSSIONS

A total of fifteen bacterial strains belonging to four genera were isolated and identified in this research as follows *Pseudomonas aeruginosa* (33%), *Bacillus cereus* (27%), *Bacillus subtilis* (20) *staphylococcus aureus* 13 % and *E. coli* 7 % respectively. The results showed that soil samples used harboured various bacteria as showed in table 1. Sixty percent (60) of the total bacteria isolated were gram positive while fourty percent (40) were gram negative. Most of the bacteria isolated in this study were linked with the ability to produce emulsifiers which degrade oil polluted soil and water (Femi- Ola *et al*., 2015). Several different genera of similar organisms were isolated by other researchers from spent lubricating soil samples (Al-Mailem *et al*., 2017; Ebakota *et al*., 2017)**.** The results also showed that *Pseudomonas* and *Bacillus* are more dominant than other bacterial isolates this could probably be that both strains form spores and can withstand harsh environmental conditions this is similar to the findings of (Al-Mailem *et al*., 2017). The frequency of occurrence of gram positive bacteria was higher than gram negative this could be as a result of their distinctive structure and strong cell envelope that can allowed them to proliferate more easily in harsh environmental conditions than gram negative bacteria. This is similar to the findings of (Ebakota *et al*., 2017). The capacity to isolate significant number of certain bacteria from oil contaminated environment is generally interpreted as proof that these microbes are the active degraders of the contaminants in that ecosystem (Okerentugba and Ezeronye, 2003).

Table 1:
Morphological and Biochemical Identification of Bacterial Isolates in spent lubricating polluted soil

S/N	G/R	Cell shape	Aero grt	Pigments	Motility	Indole	Catalase	Coagulase	Spore	Oxidasase	MR	VP	Citrate	Urease	Nitrate reduction	Glucose	Lactose	Maltose	Sucrose	Mannitol	Fructose	Galactose	Mannnese	Starch hydralysis hydrolysis	DNASE	Probable Organisms
1	+	R	+	-	+	-	+	-	+	-	-	+	+	-	+	+	-	+	+	-	+	-	-	+	-	*B. cereus*
2	+	C	+	+	-	-	+	+	-	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	*S. aureus*
3	+	R	+	-	+	-	+	-	+	+	+	-	+	-	+	+	-	+	-	+	+	+	-	-	+	*B.subtilis*
4	-	R	+	+	+	+	+	-	-		+	-	-		-	+	+	+	-	+					-	*E.coli*
5	-	R	+	+	+	-	+	-	-	+	-	-	+	-	+	+	-	-	-	+	-	+	-	-	-	*P. aeruginosa*

***Key**: Positive (+), Negative (-), Cell shape (Rod) or (Cocci), Gram's reaction (G/R), Methyl red (MR), Voges Proskauer (VG), Aero/grt, (Aerobic growth), B (Bacillus), S (Staphylococcus), E (Escherichia) and P (Pseudomonas)*

Table 2:
Percentage Occurrence of Bacteria Isolate from Soil Sample

Bacterial Isolate	Frequency	Percentage (%)
Staphylococcus aureus	2	13
Bacillus cereus	4	27
Bacillus subtilis	3	20
Escherichia coli	1	7
Pseudomonas aeruginosa	5	33

All the bacterial isolates identified in this research were screened for biosurfactant production potential using three different methods (drop collapse, oil displacement and emulsification test E24) (Table 3). The results showed that 9 strains JI, J2, J5, J6, J9, J10, J12, J13 and J15 were positive to drop collapse test with *Pseudomonas aeruginosa* having (33%), *Bacillus cereus* (13%) and *Bacillus subtilis* and *Staphylococcus aureus* having 6.6 % each while 6 strains were negative (Table 3). The bacterial strains that showed the highest potential belonged to the genera *Pseudomonas* and *Bacillus* respectively (Table 3) these bacteria have been known to have ability to survive heavily hydrocarbon contaminated environments. These is similar to the findings of Adebajo, *et al*., (2017). The level of emulsification activity E-24 and oil displacement was highest by bacterial strains J12 with 43 and 4.1cm followed by strain J9 which had 17 and 2.5cm respectively (Table 3).

Table 3:
Screening of bacterial isolates for potential of biosurfactant production

Strains	Biosurfactants production potential using various methods			Organisms
	Oil displacement (cm)	E 24	drop collapse	
JI	0.5±0.1	10±0.0	+	*Bacillus cereus*
J2	2.0±0.0	15±0.0	+	*Pseudomonas aeruginosa*
J3	-	-	-	*Bacillus cereus*
J4	-	-	-	*Escherichia* coli
J5	1.5±0.2	0.0±0.1	+	*Pseudomonas aeruginosa*
J6	3.0±0.0	22±0.0	+	*Pseudomonas aeruginosa*
J7	-	-	-	*Bacillus subtilis*
J8	-	-	-	*Escherichia* coli
J9	2.5±0.0	17±0.1	+	*Pseudomonas* spp
J10	1.8±0.1	12±0.0	+	*Bacillus cereus*
J11	-	-	-	*Bacillus subtilis*
J12	4.1±0.0	43±0.2	+	*Bacillus subtilis*
J13	0.6±0.2	11±0.0	+	*Staphylococcus* spp
J14	-	-	-	*Pseudomonas* spp
J15	3.0±0.1	16±0.0	+	*Pseudomonas* spp

The results presented in Table 3 the dispersion action shown changes in the dispersion possibilities of the various isolates studied; these variations suggest that the isolates have varying degrees of the biosurfactants activity, which is species-specific and had been previously demonstrated by (Al-Bahry et al., 2013). The variation can also be attributed to different enzymatic activities exhibited by different isolates. The results obtained from emulsification activity in this present study is similar to the findings of (John *et al*., 2020). For oil displacement zone formation, various bacterial isolates showed varying oil displacement zone formation with the highest produced by *Bacillus spp.* (4.1cm) similar results was showed by work carried out by Adna *et al*., 2015) which gave oil displacement zone value of 25mm. these was similar with those obtained by Ibrahim *et al*. (2013); Elemba *et al*. (2015) respectively

CONCLUSION

In conclusion, the present study showed that spent lubricating polluted soil harbored the following bacteria *Pseudomonas aeruginosa, Bacillus, Staphylococcus, Escherichia coli.* The results also showed that spore forming bacteria like *Pseudomonas spp.* and *Bacillus spp.* are more predominantly present in oil polluted soil compared to others. The screening results showed that *Pseudomonas aeruginosa* and *Bacillus cereus* have the best Biosurfactants producing potential.

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