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Optimization of Biosurfactant Production Using Bacteria Isolated from Hydrocarbon Contaminated Soil

***[1]Asiya Gidado Yabo, [2]Aliyu Sarkin Baki, [2]Abdullahi Bako Rabah, [2]Aliyu Mamuda, [2]Umar Musa Abdullahi, [1]Aisha Habibu, [1]Hannatu Bello, [2]Aisha Aminu Mode and [3]Bahira Bello Yabo**

[1]Department of Science Laboratory, Technology, Umaru Ali Shinkafi Polytechnic, Sokoto, Nigeria.
[2]Department of Microbiology, Faculty of Chemical and Life Sciences, Usmanu Danfodio University, Sokoto, Sokoto State, Nigeria.
[3]Department of Biology, State College of Basic and Remedial Studies, Sokoto, Nigeria.
*Corresponding Author's email: aseeyabo@gmail.com

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ABSTRACT
Biosurfactants are surface-active biomolecules produced by microorganisms that have different applications in many environmental problems. This research was aimed to isolate biosurfactant producing bacteria and optimize the conditions like pH, carbon source and incubation time for maximum biosurfactant production. Samples were collected from three points of hydrocarbon contaminated soil in Sokoto Metropolis. Primary screening test including hemolytic activity, drop collapse technique and oil displacement method were performed and species with the best results were picked for complementary screening test like emulsification activity and foaming activity. The viable aerobic heterotrophic bacterial count of all the samples ranges from $13.8x10^7$cfu/g to $2.5x10^7$ cfu/g. A total of sixteen isolates were identified. During primary and complementary screening tests, eight species showed hemolytic activity, five species (5) had drop collapsing activity and eight species showed oil displacement activity. Optimization studies revealed that production of biosurfactant was optimal at pH 6.5, 3% carbon source and 96 hours incubation time respectively. Finally, two strains *Psuedomonas aeruginosa* and *Bacillus subtilis* with a high amount of biosurfactant production were identified molecularly with their best hit match similarity was source from the GenBank of NCBI. Therefore, there is need to use biotechnology approaches with a view to improving the performance of bacterial strain.

INTRODUCTION

Microorganisms produce biosurfactants which are surface active agents having both hydrophobic and hydrophilic moieties. They are classified as glycolipids, lipopeptides, lipoprotein, lipopolysaccharides and phospholipids based on the chemical components found in these molecules (Ekprasert *et al*., 2020). Bacteria, yeast and molds produced biosurfactants, which are a varied and heterogeneous category of microbial compounds with wide ranges of applications.

A number of biosurfactants were studied by many researchers but bacterial biosurfactants (rhamnolipid, surfactin and licheysin produced from *Psuedomonas* and *Bacillus* species) were the most- thoroughly investigated (Brumano *et al*., 2016). These microbial compounds are very advantageous because of their structural variety,

great biodegradability, low environmental effect and toxicity, high specificity, stability and activity under harsh conditions and have been shown to remove petroleum hydrocarbon-contaminated waste stream like soil and refinery effluent in addition to many industrial applications (Luna *et al*.,2015). It also have applications in many unconventional such as cosmetics, pharmaceuticals, petroleum recovery polymerization, foods and environmental remediation, as opposed to synthetic surfaceactive agents which are used as laundry detergent and individual care product (Banat *et al*., 2010). According to Mulligan *et al.* (2005), biosurfactants are classified into six groups based on chemical structure: glycolipids, phospholipids, lipopeptides and lipoproteins, fatty acids, neutral acids, and polymeric and particulate surfactants. As a result of their functional and structural diversity, biosurfactants are an attractive group of molecules with the potential to be used in numerous biotechnological and industrial applications (Packwa & Zhang, 2011).

Biosurfactants are used for several important roles namely, emulsification, dispersal, solubilization, mobilization, wetting, surface tension reduction, formation of micelles and foam formation due to their ability to partition into different interfaces: liquid/solid, liquid/gas, liquid/liquid (Satpute *et al.,* 2017). *Candida bombicola, Candida lipolytica, Candida ishiwadae, Candida batistae, Aspergillus ustus*are the well-known fungi for the production of biosurfactants. The type of biosurfactant produced mainly by these strains are sophorolipids (Bhardwaj *et al.,* 2013). Biosurfactants are having a wide range of applications in the areas of bioremediation, food processing and pharmaceutical industries (Kaur *et al.*, 2017). Biosurfactants are preferred over synthetic surfactants because of their low toxicity, biodegradable nature. Biosurfactants are amphiphilic, consisting of two parts, a polar (hydrophilic) moiety and a non-polar (hydrophobic) group. The hydrophilic group consists of mono, oligo, or polysaccharides, peptides or proteins while the hydrophobic moiety usually contains saturated, unsaturated and hydroxylated fatty acids or fatty alcohols (Packwa & Zhang, 2011).

Hydrocarbons, derived from petroleum products, are major environmental pollutants due to industrial activities, transportation, and accidental spills. These compounds are persistent in the environment, leading to soil degradation, loss of fertility, and toxicity to organisms. Hydrocarbons can be categorized into alkanes, aromatics, asphaltenes, and resins, with varying levels of toxicity and biodegradability. The impact of these contaminants includes disruption of soil structure, alteration of microbial communities, and long-term ecological damage. (Elgazali *et al*., 2023).

Studies have shown that hydrocarbon pollution is particularly severe in oil-producing regions, with long-term effects on agricultural productivity and groundwater quality. Conventional remediation methods, such as incineration and chemical treatment, are often expensive and can cause secondary pollution, emphasizing the need for sustainable approaches (Liu *et al*., 2018).

The aim of this research is to determine the physicochemical characteristics, isolate and identified bacteria from hydrocarbon contaminated soil. Screen, optimize and molecularly identify the biosurfactant producing isolates

MATERIALS AND METHODS

Sample Collection and Sample Preparation

The sample was collected according to the methods described by (Ijah and Antai2003). Two hundred grams (200g) of a triplicate from three points (coded A, B and C) of hydrocarbon contaminated soil were collected from mechanical workshop at Abdullahi Fodio Road Sokoto. All the samples were packed in a zip lock bag, labelled and transported aseptically to the Microbiology Research Laboratory, Usman Danfodiyo University Sokoto for analysis. One gram (1g) of each sample were weighed out and homogenised into the test tube containing sterile distilled water. Sterile syringe was used to pour nine millilitres (9ml) of distilled water into seven test tubes each sample and autoclaved at 121^0C for 15minutes. One ml (1ml) of each sample was transferred into a sterilized test tubes obtaining dilution factors (10^1 to 10^7) for all samples (Aminu *et al*., 2023).

Physicochemical Properties of the soil samples

Determination of pH

The soil pH was determined according to the method of International Institute of Tropical Agriculture. Twenty (20) grams of airdried soil was sieved and placed in 50 ml capacity beaker. Twenty (20) millilitres of distilled water was added and allowed to stand for 30 minutes, while stirring occasionally with a glass rod. The pH meter was calibrated with buffer of pH 7.0 before use. The electrode of the pH meter inserted into the partly settled suspension and the reading on the pH meter were noted and recorded accordingly in triplicate and the mean was taken (Premei *et al*., 2020).

Determination of Moisture Content

An empty crucible was weighed (W_0) and 2g of soil was added and weighed again (W_1). Samples was dried in hot air oven at 105 ^{0}C until constant weight is achieved (W_2). Both the container and the dried samples were weighed again (Premei *et al*.,2020). The moisture content were calculated as follows

$$\%\ moisture\ = \frac{W_1 - W_2}{W_1 - W_2} \times 100 \quad \text{(Premei } et\ al\text{., 2020)} \qquad (1)$$

Determination of Organic Carbon Content

One gram of soil sample was weighed in duplicates and transferred to 250ml Erlenmeyer flask. Ten millilitres of

potassium dichromate solution and 20ml of concentrated H_2SO_4 were added and the contents of each flask were shaken gently until properly mixed. One hundred millilitres of distilled water were added and allowed to stand for 30 minutes. This was followed by adding four drops of methyl red indicator and titrating against 0.5N ferrous sulphate solution until the colour changed from blue to red (Abdallah, and Ibrahim, 2023).

The percentage carbon were calculated according to the formula:

% Organic Carbon = Me $K_2Cr_2O_7$ – Me $FeSO_4$ × 100 Weight (Wet soil – dry soil)

%OC=(M of OC/ TSM) x 100 (2)

Where M= Mass, TSM = Total Sample Mass

Determination of Nitrogen Content of the Soil

Total nitrogen were determined by Macro-Kjeldahl digestion method of Juo (1979). Five grams of soil samples were weighed into a 500ml Macro-Kjeldahl flask and 20ml of distilled water were added. The contentwere swirled for five minutes and allowed to stand for 30 minutes. One tablet of mercury catalyst and 10g of K_2SO_4 was added and 30ml of conc. H_2SO_4 was added through an automated pipette. The content of the flasks were heated gently in the digestion stand. After cooling, 100ml of distilled water were added and transferred into another clean macro-Kjeldahl flask (750ml) and the sand residue washed four times with 50mlof distilled water. All the washings were transferred into the same flask. Fifty millilitres (50ml) H_3BO_3 indicator solution was added into 500ml Erlenmeyer flask, which was placed under the condenser of distillation apparatus and 150ml of 10N NaOH were introduce. This was followed by distillation. For the condenser to remain cool (30^0C) and prevent frothing, sufficient cold water was allowed to flow through the condenser. Ammonium were determined in the distillate by treating against 0.01N standard H_2SO_4 using a 25ml burette graduated at 0.1ml interval. The colour changed at the end point from green to pink (Juo, 1979). Percentage nitrogen were calculated using the formula:

$$\%Nitrogen = \frac{\text{N}\times 0.014\times \text{Vd}\times 10}{\text{A}\times \text{Weight of sample}} \times 100 \quad (3)$$

Where: N = Normality of acid, Vd = Volume of the digest, A = Aliquot of digest (Juo, 1979).

Determination of Cation Exchange Capacity

Twenty millilitres of 0.1 M $BaCl_2$ saturating solution were added to 2g of air dried soil in a pre-weighed centrifuge tube (plastic) and continuously shaken for 2 hours in a thermolyneshaker at 300rpm and at 35^0C. After shaking, the solution was centrifuged (Marathon 3200R, Fisher Scientific, USA) at 3000 rpm for 10 minutes and decanted. This was followed by equilibrating the soil with three successive 20 ml increments of 0.002M $BaCl_2$. Each time the solution was sonified using a Vortex mixer (Model S8223, Genie, USA) for 30 seconds followed by shaking on a thermolyne shaker at 300rpm for 1 hour. The solution were centrifuged at 3000 rpm for 10 minutes and the supernatant discarded (Abdallah, and Ibrahim, 2023). The centrifuge tube plus soil and entrained 0.002M $BaCl_2$ of solution were weighed following the last decantation of the supernatant. Then, 10 ml of 0.005M $MgSO_4$ reactant solution was added to the soil and was gently shaken at 200 rpm for 1 hour in thermolyne shaker. The exchange capacity of the reactant suspension was measured and adjusted to the exchange capacity of 0.0015M $MgSO_4$ ionic strength reference solution by measuring the conductivity. After shaking the samples gently at 200rpm overnight, the conductivity of the reactant suspension was adjusted to that of the 0.0015 M $MgSO_4$ ionic strength reference solution using distilled water. (Abdallah and Ibrahim, 2023). The centrifuge tubes and contents was weighed to determine the volume of $MgSO_4$ or water that needed to be added for adjusting the conductivity. This was followed by centrifugation at 3000rpm for 10 minutes and decanting the supernatant that was retained for analysis. The solution was analyzed for magnesium using Perkins Elmer Analyst 300 Atomic Absorption Spectrophotometer (Perkin Elmer, USA) and the pH was also measured using pH meter (SympHony SB20, Mettler Toledo, USA). The CEC were calculated from the following equation:

$$CEC\ in\ meq/100\ g = \frac{100(0.01-\text{C}_1\text{V}_2)}{(\text{oven dry weight soil sample in gram})} \quad (4)$$

Where V_2 is the volume of final supernatant solution and C_1 is the concentration of Mg in the supernatant, meq= (milliequivalents/milliliter) (Abdallah and Ibrahim, 2023).

Determination of Soil Particle Size

The hydrometer method for the mechanical analysis of particle size distribution presented by Ibrahim *et al*., (2014) were used. Air dried soil was sieved using 425µm pore size sieve. Fifty one grams (51g) was transferred into 1 liter and shaken to mix. Fifty milliliters (50ml) of 5% sodium hexametaphosphate were added followed by 100ml of distilled water. The soil suspension was stirred thoroughly for 15 minutes and transferred into a cylinder containing hydrometer. Distilled water was added to the lower blue line of the cylinder. The volume changed to 1130ml and the hydrometer was removed. The top of the cylinder were covered with hand and inverted several times until all soil were in suspension. The cylinder were placed on a flat surface and time was noted. Hydrometer was placed in the suspension and. The suspension was allowed to stand for Three (3) hours (Ibrahim *et al*.,2014).

Microbiological Analysis of the Sample

Enumeration of Bacterial Loads in the Soil Sample

A stock solution for serial dilution was made by dispensing 1 gram of soil into 100ml of distilled water, shaking thoroughly, and transferring one (1) ml into a test tube containing 9ml of sterile distilled water, subsequently making a serial dilution up to 10^{-7} using spread plate

method technique, 0.1ml of suspension from dilution factors 10^{-5},10^{-6} and $10^{-7}$was plated aseptically on prepared Nutrient Agar (NA) incubated at 30^0C for 24 hours. The plates were determined by multiplying the number of counts with the dilution used and expressed as colony - forming units per gram (cfu/g) of soil (Premei *et al*., 2020).

Identification and Characterization of Bacterial isolates

Gram's Staining

Gram's staining was carried out as described by Harley and Prescott (2002). A Smear of the bacterial isolates were made on a clean grease-free glass slide using a drop of water. The smear was allowed to air dry and then passed over a flame in order to be fixed. After fixing, the smear was covered with a primary dye (i.e., crystal violet) for one minute until it behead with water. The slides was covered with Lugol"s iodine and washed after one minute. Ethanol was used to rapidly decolorize the smear and washed with water. Safranin was added and left for 30seconds and later washed with water. Back of the slides were cleaned with a cotton wool and allowed to air dry. The slides were examined using oil immersion objective lens (x100) of the microscope. Bluish or purplish colour indicates Gram positive while red or pinkish colour indicates Gram negative bacteria.

Spore Staining Test

A smear was made on a slide and heat fixed. Malachite green (5%) solution was applied and heated until steam rises and allowed to cool, and washed gently with cold water. The smear was counterstained with 0.5% safranin for 30 seconds and washed with water. The slide was blot dried and were examined under oil immersion objective lens for the presence of spores. Same procedure were applied for the remaining slides. Spores stained green while vegetative cells stained red (Cheesbrough, 2006).

Catalase Test

A drop of 3% (v/v) H_2O_2 was placed on a glass slide onto which a bacterial colonies were added. Presence of catalase were observed by the formation of oxygen bubbles (Harley and Prescott, 2002).

Oxidase Test

An oxidase reagent (1% Tetra-methyl-paraphenylene-diamine-dihydrochloride) was placed on Whatman paper filter and a bacterial colonies was smeared on the paper. Presence of the enzyme oxidase was observed by the appearance of a purple colour (Cheesbrough, 2006).

Starch Hydrolysis Test

Cultures of bacteria was grown on starch agar. The colonies were flooded with iodine. Clear area observed around the isolate indicated positive result (Harley and Prescott, 2002).

Urease Production Test

Slants of urea medium in universal bottles were inoculated with a loopful of the isolates by streaking. These were incubated at 37^0C for 4 days and examined daily. Change in colouration from pink to red indicated urease positive (Cheesbrough, 2006).

Methyl Red Reaction Test

To a prepared glucose phosphate medium in a test tube, a loopful of the isolates were inoculated and incubated at 37^0C for 24 hours. To the four-day old culture, drops of methyl red solution was added. They were shaken and examined. Appearance of red colour at the surface of reagent layer showed positive methyl red reaction (Cheesbrough, 2006).

Voges-Proskauer test

To the culture in above, 0.6ml 5% α- napthol solution were added and shaken. The test tubes were sloped and examined after 15minutes. A red colouration indicated a positive VP reaction (Ochei and Kolhatkar, 2000).

Indole Production Test

A loopful of the isolates were inoculated in a sterile nutrient broth. Incubation was done at 37 ^{0}C for 48 hours. After incubation, 0.5ml Kovac's reagent were added and shaken. These were examined after one minute. A red colouration in the reagent layer indicated indole production (Cheesbrough, 2006).

Citrate Utilization Test

To a sterile Simon's citrate medium, a loopful of 24hour old isolates were inoculated aseptically and incubated at 37 ^{0}C for 24hours. The media were examined daily for turbidity for a period of 3 days. Turbidity indicated citrate utilization (Cheesbrough, 2006).

Motility Test

This was done as described by Ochei and Kolhatkar (2000). A little portion of each isolate was stabbed onto motility medium and incubated at 37^0C for 24 hours. Motility was observed by spreading of organisms outwards from the stabbed area.

Screening of the Isolates for the Production of Biosurfactant

The isolates were screened for biosurfactant production using the following screening tests.

Blood Hemolysis Test

Freshly prepared blood agar were inoculated with pure culture of bacterial isolates and incubated at 30°C for 24

hours. According to the clear zone observed, results were determined where, α-hemolysis is determined when the colony were surrounded by greenish zone, β-hemolysis when the colony were surrounded by a clear white zone and γhemolysis when there was no change in the medium surrounding the colony (El-Shahawy, 2014).

Drop Collapse Test

Drop collapse test were carried out according to the method described by Youssef *et al*. (2004). Ten microliter of cell free supernatant were placed on a grease free surface and drops of the cell free supernatant was spread or collapsed. The experiment was set up in triplicates. The presence of biosurfactant made the cell supernatant to collapse and were recorded as a positive. Those cultures that gave rounded drops were scored as negative indicative of a lack of biosurfactant production.

Oil Displacement Assay

The oil displacement test was done by adding 50ml of distilled water in petri dish followed by addition of 10ml of oil to the surface of the water. Then 10ml of the cell culture supernatant was dropped on the oil surface. The diameter of the clear zone on oil surface were visualized under visible light and measure after 30second (Rodrigues *et al*., 2006).

Determination of Emulsification Index (E24%)

Emulsification index of cell free broth were determined by adding 2 ml of oil to 2ml cell free broth, mixing with a vortex for 2minute, and leaving it undisturbed for 24hours. The E24 index is given as percentage of height of emulsified layer (mm) divided by total height of the liquid column (mm). The total height of liquid, as given by the expression: E24 =h emulsion / h total x 100.

Where: E24 is emulsion index after 24 hours, h emulsion is the height of emulsion layer, h total is the total height of the liquid (Ibrahim *et al*., 2013).

Biosurfactant Production

The mineral salt medium were supplemented with5 ml of stock solution of trace element containing g/l: 0.56 H_3BO_3, 0.42 $CoCl_2$. $6H_2O$; 1.0 $CuSO_4.5H_2O$, 1.78 $MnSO_4.4H_2O$, 0.39 Na $_2MO_4.2H_2O$ and 2.32 $ZnSO_4.7H_2O$, 1.0 EDTA, 0.004 $NiCl_2.6H_2O$ and 0.66 KI. The stock solution were sterilized by filtration and added to the medium. The pH of the medium was adjusted to 7.2 and sterilized at 121 ^{0}C for 15 min. Isolates were inoculated into flasks (250 ml capacity) containing 100 ml of the medium and incubated at 30^{0}C for 7 days (Abouseoud *et al*., 2008).

Optimization of Biosurfactants Growth Production Parameters

Different parameters that include carbon source that include carbon source concentration, pH and incubation time were optimized using one factor at a time (OFAT).

Determination of Optimum Carbon Source Concentration for Biosurfactant Production

Different concentrations of diesel 2%, 3% and 4% were added to the mineral salt broth in different conical flask thereby serving as the production media. The optimal concentration of the carbon source for biosurfactant production was determined by measuring the quantity of biosurfactant produced as described by (Imran *et al*., 2012).

Determination of Optimum pH for Biosurfactant Production

The mineral salt media were prepared by using different pH and keeping others at constants The pH of the medium was adjusted by adding NaOH or HCl (Imran *et al.,* 2012).

Determination of Optimum Incubation Time for Biosurfactant Production

Optimum incubation time for biosurfactant production was studied by settling the cultivation at different period of time that include 24, 48, 72, 96, 120, 144 and 168 hours. The best range incubation period where high production of biosurfactant occurred was identified (Khokar *et al*., 2012). Molecular Characterization of the most Potent Bacterial Isolates in Biosurfactant Production

Genomic DNA Extraction

The single pure colonies of the bacterial isolates were grown in Luria-Bertani (LB) broth overnight at 28^{0}C. Two (2) ml of the culture were centrifuged at 5000 rpm for 5 min and pellet was suspended in 200 ml of TE buffer in order to prevent it from degradation, at pH 8 containing RNase (50 ng /ml), then 400 ml of lysis buffer will be added followed by mixing well and incubation for 15 minute 37^{0}C with intermittent shaking for every 5 min. Immediately chloroform and isomyalcohol in the ratio (24:1) was taken and mixed by inversion. The Tubes were centrifuged at 10000 rpm for 5min, supernatant were transferred carefully to another micro-centrifuge tube. To the supernatant, 0.1 vol 3 M sodium acetate (pH = 5.2) and 0.6 vol isopropanol was added, mixed well by inversion and kept in the ice for 10 min followed by centrifugation at 1000 rpm for 10 min. The pellet was washed with 70% ethanol with gentle shaking and centrifuged at 10000 rpm for 3 minute. Supernatant was removed and pellet was air dried. Extracted DNA was visualized using 0.8% agarose gel electrophoresis and images were documented (Balakrishnan *et al*., 2022).

16s rRNA gene PCR Amplification

Using 16S ribosomal RNA gene specific universal primers 27F 50-AGA GTT TGA TCC TGG CTC AG-30 and 1492R 50-GGT TAC CTT GTT ACG ACT T-30 (Sigma), the 16S rRNA gene were amplified with 50 ll reaction mixture containing 1X reaction buffer (10 mMTris [pH 8.3], 50 mMKCl and 1.5 mM $MgCl_2$), 200 lldNTPs, 0.05 U Taq DNA polymerase enzyme (Sigma, USA), 0.5 lM of each primer and 1 ng template DNA. The thermal cycling conditions was: 5 minutes at 94 ^{0}C for initial denaturation; 31 cycles of 30 s at 95 0C,1 minute at 54 ^{0}C, 2 minutes at 74^{0}C, and a final extension for 5 minutes at 72 ^{0} C. The amplification reaction were performed with a thermal cycler (MyCycler, Bio-Rad, USA) and the PCR amplicons (approximately 1500 bp) were resolved by electrophoresis in 1% (w/v) agarose gel to confirm the expected size of the product (Balakrishnan *et al*., 2022).

PCR Product Purification

Isolates producing biosurfactant were purified by two procedures- Ammonium sulphate precipitation method and $ZnCl_2$ precipitation method. By Ammonium sulphate precipitation method: it consists of four steps, ammonium sulphate fractionation, chilled acetone, hexane treatment, and silica gel column chromatography. By $ZnCl_2$ precipitation method: 10 ml of the culture supernatants was concentrated by $ZnCl_2$ to final concentration of 75 mm. The precipitated material were dissolved in 10 ml Sodium phosphate buffer (pH 6.5), extracted twice with equal volumes of diethyl ether. Pooled organic phase was evaporated to dryness and pallets was dissolved in 100 µl of methanol. Further purification was achieved by preparative TLC (Jiraporn and Niran, 2003).

DNA Sequencing of 16S rRNA Gene Fragment

By using ABI DNA 3730 XL for sequencing sequencer (Applied Bio system), the 16S rRNA purified PCR product were submitted. Sequencing of the bacterial isolate's 16S rRNA gene were carried out in both directions. The bacterial species were determined with the obtained sequence which was searched for BLAST. The sequences were submitted to the NCBI Gen Bank after sequence matching percentages and accession numbers were obtained (Balakrishnan *et al*., 2022).

Phylogenetic analysis

The 16 rRNA gene sequence obtained in this study was aligned with the sequences published in National Center for Biotechnology Information (NCBI). Aligned sequences were edited and phylogenetic tree was constructed using MEGA (version 6) software. The phylogenetic tree for relationship among strains were constructed by the Maximum Likelihood Method with a bootstrap of 500 using Kimura-2 parameter (Balakrishnan *et al*., 2022).

Data Analysis

All experiments in this study were carried out and studied in triplicate and almost all results in this study are expressed in a form of mean ± standard deviation. Some measures that are commonly used to describe the data set in descriptive statististics are measures of central tendency which include mean, median and mode, then measures of variability such as standard deviation (or variance). Bioinformatics tools such as sequence BLAST in NCBI, and Mega X were also used in analysing the data obtained.

RESULTS AND DISCUSSION

Physicochemical Characteristics of Hydrocarbon Contaminated Soil

Present the physicochemical properties of soil from three hydrocarbon contaminated soil coded A, coded B and coded C. The mean of pH of all the samples was slightly acidic ranging from (6.95 +0.02 to 6.99 +0.02). Sample C were found to have the highest electrical conductivity with 2.4±0.20 cmol/kg while sample B had the least (2.0±0.20cmol/kg). Sample C were recorded with highest percentage carbon (1.5±0.15%) while sample A had the least (1.2±0.15%). Sample B were also recorded with highest percentage of Nitrogen (0.16±0.05%) whereas sample A and sample C (0.07±0.05%).

Aerobic Heterotrophic Mean Bacterial Count from the Hydrocarbon Contaminated Soil

Table 2 shows the Bacteriological Counts of Soil Samples from the three Sampling Sites, A, B and C Showing both mean bacterial colony count for each site, coded B has a mean bacterial count of 3.5× 10^7 cfu/g, coded C has the lowest bacterial count of 2.5X 10^7cfu/g. However, coded A shows the highest bacterial count of 13.8X 10^7cfu/g.

Morphology and Biochemical Characterization of the Isolates

Table 3 presents the results of biochemical identification data of various bacterial isolates, showing several key characteristic essential for species diffentiation. The shape of the bacteria (either rod or cocci) is indicated in the first column, the second column showing the gram reaction of the isolates and third column showing whether the organism produce spores. The table also includes a series of biochemical test: catalase, oxidase, methyl red (M- R), voges-proskauer, indole production, urease, fermentation of glucose, lactose and sucrose as well as the hydrogen sulfide production and gas production. From the data, it can be seen organism like *Bacillus subtilis, Bacillus pumilis* and *Staphylococcus epidermidis* showsthe distinct biochemical reactions such as positive spore formation and certain fermentation patterns. In contrast *Pseudomonas aeruginosa* display negative results for spore formation and distinct biochemical characteristics including catalase positive and specific fermentation profile.

Table 1: The Physicochemical Properties of the Soil Collected from Mechanic Workshops Abdullahi Fodio Road Sokoto

Samples	pH	EC µS/CM	Ca cmol/kg	Mg cmol/kg	Na cmol/kg	K cmol/kg	CEC cmol/kg	%N	%O.C	%Sand	%Silt	%Clay	PO_4 mg/kg
A	6.95	141.8	0.75	1.05	0.48	0.58	2.2	0.07	1.2	91.7	5.9	2.4	0.14
B	6.96	187.1	0.65	0.55	0.57	0.65	2.0	0.07	1.4	93.7	3.9	2.4	0.15
C	6.99	67.9	0.55	1.3	0.65	0.88	2.4	0.16	1.5	95.6	4.0	0.4	0.15
Mean ±	6.97 ±	132.27	0.65 ±	0.97 ±	0.57 ±	0.70 ±	2.20 ±	0.10 ±	1.37 ±	93.67±	4.60 ±	1.73 ±	0.15 ±
SD	0.02	± 60.17	0.10	0.38	0.09	0.16	0.20	0.05	0.15	1.95	1.13	1.15	0.01

Keys: Oc=Organic Carbon , N=Nitrogen, $P0_4$ =Phosphate , Na=Sodium, CEC = Cation Exchange Capacity, Ec= Electrical conductivity and %= Percentage

Table 2: Aerobic heterotrophic mean bacterial count from the hydrocarbon contaminated soil

Sample	Mean of bacterial colony count (cfu/g)
A	$13.8X\ 10^7$
B	$3.5X\ 10^7$
C	$2.5X\ 10^7$

Table 3: Biochemical Characterization of Bacterial IsolatedFrom Hydrocarbon Contaminated Soil

S/N	Isolate ID	Shape	Gram rxn	Spore	Catalase Spore	Citrate	Indole	MR	VP	Urease	Oxidase	Glucose	Lactose	Sucrose	H_2S	Gas	Organism Identified
1	A1	R	+	+	+	-	+	+	+	+	+	+	-	+	-	-	*Bacillus pumilus*
2	A2	R	+	+	+	-	+	+	+	+	-	+	-	+	-	-	*Bacillus pumilus*
3	A3	R	+	+	+	-	+	+	+	+	+	+	-	+	-	-	*Bacillus pumilus*
4	A4	R	+	-	+	+	-	-	+	+	-	+	-	+	-	-	*Bacillus pumilus*
5	A5	R	-	-	+	+	-	-	-	+	+	+	-	-	-	-	*Psuedomonas aeruginosa*
6	A6	C	+	+	+	+	-	+	+	+	-	+	-	+	-	-	*Staphylococcus epidermis*
7	B1	R	+	+	+	+	-	-	-	-	-	+	-	+	-	-	*Bacillus subtilis*
8	B2	R	-	-	+	+	-	-	-	+	+	+	-	-	-	-	*Psuedomonas aeruginosa*
9	B3	R	-	-	+	+	-	-	-	+	+	+	-	-	-	-	*Psuedomonas aeruginosa*
10	B4	R	-	-	+	+	-	-	-	+	+	+	-	-	-	-	*Psuedomonas aeruginosa*
11	B5	R	-	-	+	+	-	-	-	+	+	+	-	-	-	-	*Psuedomonas aeruginosa*
12	C1	R	-	-	+	+	-	-	-	+	+	+	-	-	-	-	*Psuedomonas aeruginosa*
13	C2	R	+	+	+	+	-	-	-	-	-	+	-	+	-	-	*Bacillus subtilis*
14	C3	R	+	+	+	+	-	-	-	-	-	+	-	+	-	-	*Bacillus subtilis*
15	C4	R	+	+	+	+	-	-	-	-	-	+	-	+	-	-	*Bacillus subtilis*
16	C5	R	+	+	+	+	-	-	-	-	-	+	-	+	-	-	*Bacillus subtilis*

Frequency of Occurrence of the Identified Isolate

Table 4 presents the frequency of occurrence of the identified isolates. A total of sixteen (16) bacterial isolates with different frequency of occurrence were identified from the samples. The four (4) bacterial genera are *Pseudomonas aeruginosa, Bacillus subtilis, Bacilllus pumilus* and S*taphylococcus epidermidis*.

Screening of Biosurfactant Production by Bacterial Isolates

All the sixteen (16) bacterial isolates were screened for biosurfactant production, eight (8) isolates were positive for blood haemolysis, oil displacement assay, five (5) isolates were positive on from drop collapse assay and three were negative as shows in Table 4.5.

Table 4: Frequency of Occurrence of the Identified Isolated from Hydrocarbon Contaminated Soil

S/N	Identified isolates	Frequency of occurrence	% Frequency of occurrence
1	*Pseudomonas aeruginosa*	6	13.1
2	*Bacillus subtilis*	5	23.9
3	*Bacillus pumilus*	3	30.4
4	*Staphylococcus epidermis*	1	32.6
		16	100

Table 5: Screening of Biosurfactant Production using Bacterial Isolated from Hydrocarbon contaminated soil

S/N	Samples Code	Haemolysis	Drop Collapse	Oil displacement
1	A1	+	-	+
	A2	+	-	+
	A3	+	-	+
2	B1	+	+	+
	B2	+	+	+
	B3	+	+	+
3	C1	+	+	+
	C3	+	+	+

Keys: + = Positive; -= Negative

The BLAST of Similarity between the Sequence Queried and the Biological Sequences within the NCBI Database

Table 6 presents the results of a BLAST (Basic Local Alignment Search Tool) search, indicating the degree of similarity between the queried sequence from the sample and sequences in the NCBI (National Center for Biotechnology Information) database. In this case, the sample identified as "C3" has been compared to the database, and it shows a similarity of 99.77% with the organism *Bacillus subtilis*. And “B5” showed a similarity of 99.32% with the organism *Pseudomonas aeruginosa*. This percentage reflects the proportion of sequence identity between the queried sequence and the reference sequence in the database, signifying a high degree of genetic homology. A higher percentage of similarity, in this case, suggests that *Bacillus subtilis* and *Pseudomonas aeruginosa* are the most likely organism in the database that aligns with the queried sequence, confirming its potential biological identity.

Table 6: The BLAST Results Showing Similarity Between the Sequence Queried and the Biological Sequences Within the NCBI Database

Sample ID	Predicted Organism	Percentage Similarity (%)	Accession Number
C3	*Bacillus subtilis*	99.77	NR 12116.2
B5	*Pseudomona saeruginosa*	99.32	ON 91428.1

Key: % = Percentage

Figure 1 shows the percentage emulsification activity by the isolates were by code C1 and C3 had the highest emulsion activity (60%) while code A1 with least emulsion activity (42%).

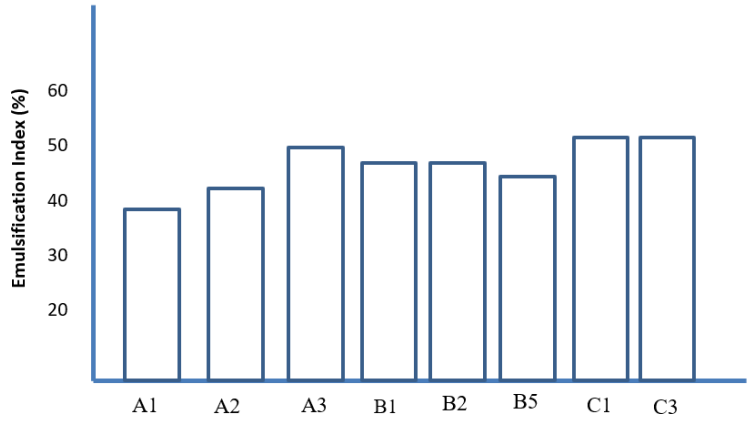

Figure 1: Emulsification Index (E1) for the respective isolates

Figure 2 shows the effect of carbon source on the biosufactant that ranges from 2%,3% and 4%. Biosuirfactant increased of activity1.4mg/ml at 3% diesal carbon source and biosurfactant decreased at activity 0.6mg/ml.

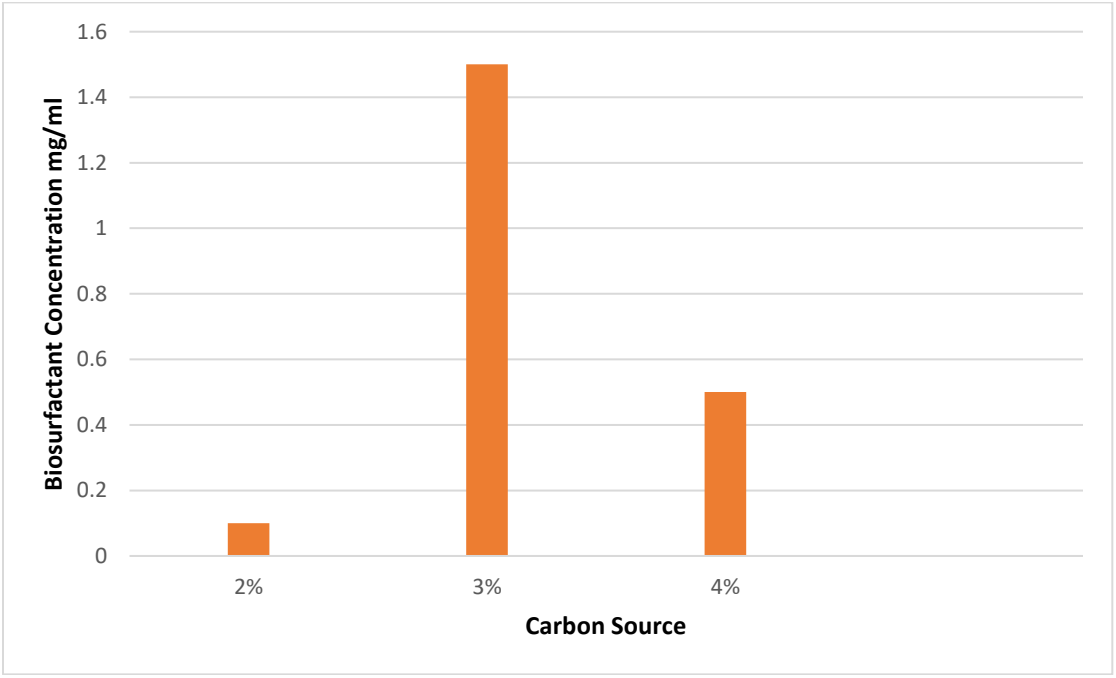

Figure 2: Effect of Carbon Source on the Production of Biosurfactant

Figure 3 show the effect of pH range of 4-7 on biosurfactant production. The result shows that the pH significantly affects the biosurfactant production. The maximum pH to produce biosurfactant was observed at pH.6.5.

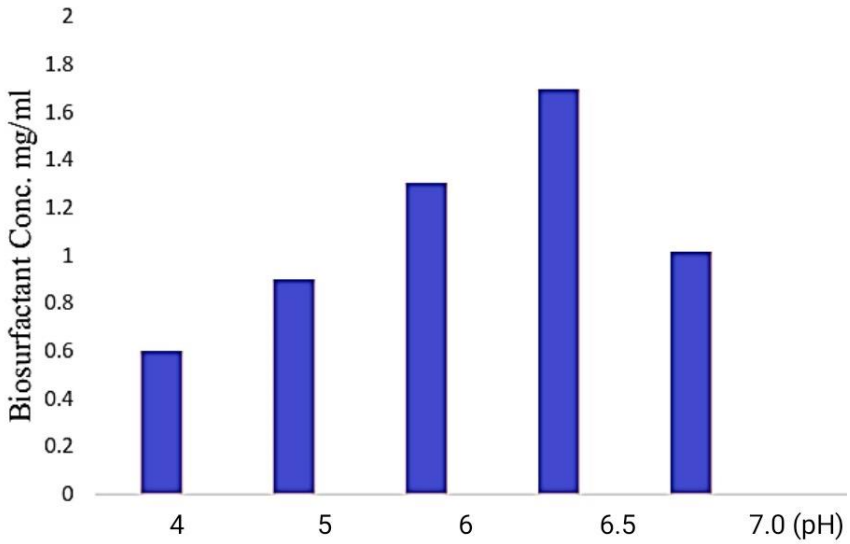

Figure 3: Effect of pH on the Production of Biosurfactant

Figure 4 show the effect of incubation period of 24 t0 168 hours on biosurfactant production. From the result, the optimal incubation time was observed at 96 hours.

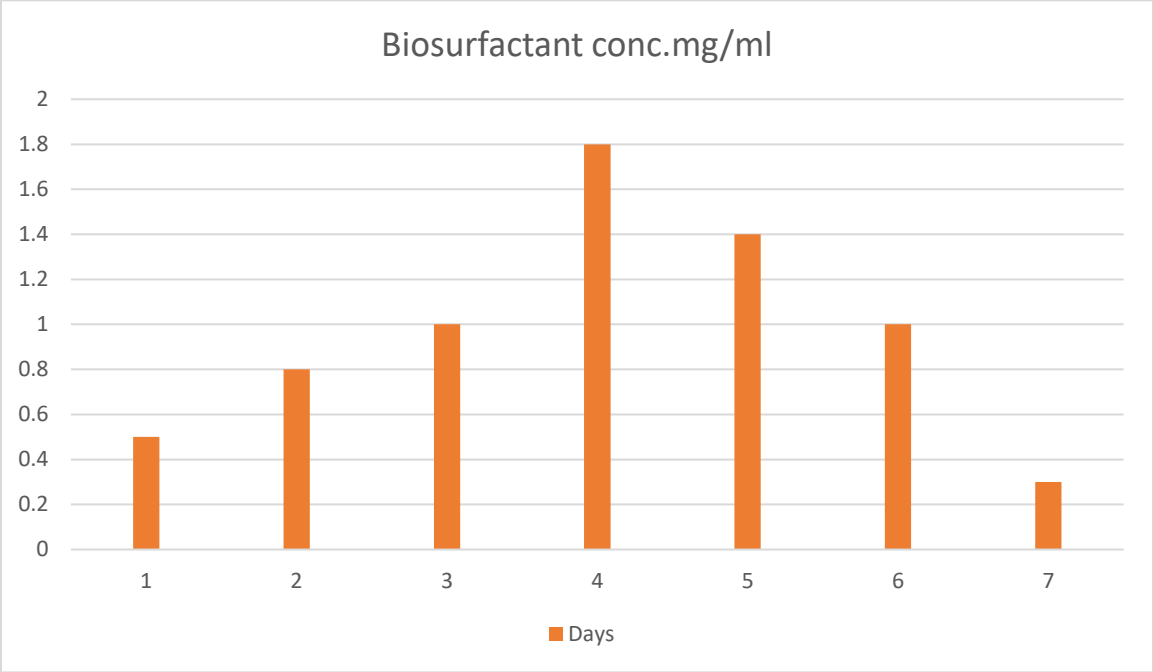

Figure 4: Effect of Incubation Time on the Production of Biosurfactant

Molecular Analysis of the Isolates

Agarose Gel Image of Bacillus subtilis and Psuedomonas aeruginosa

Figure 1 shows an agarose gel indicating of the 16s target region of B5-907R as *Psuedomonas aeruginosa* and C3-907R as *Bacillus subtilis* as heynesii. The anticipated band of 1.0kb of 16s gene was successfully amplified as shown gel. The band are strong to be purified for sequencing to confirm the identity of the isolates in the present research.

Phylogenetic Analysis of 16s rRNA of Bacteria

The evolutionary relationships between different identified species. The relationship are based on similarities and differences in the evolutionary genetic characteristics of the specie and their result from the GenBank of NCBI database. Their neighbor joining techniques was used to phylogenetic tree using the nearby members found in NCBI GenBank.

Hits Similarity of Isolates from GenBank of NCBI

Table 6 presents the BLAST results corresponding to the similarity between the queried and the biological sequences within the NCBI database. The results show to as *Bacillus subtilis* strain as 907R at 99.77% and B5-907R as *Psuedomonas aeruginosa* at 99.32%.

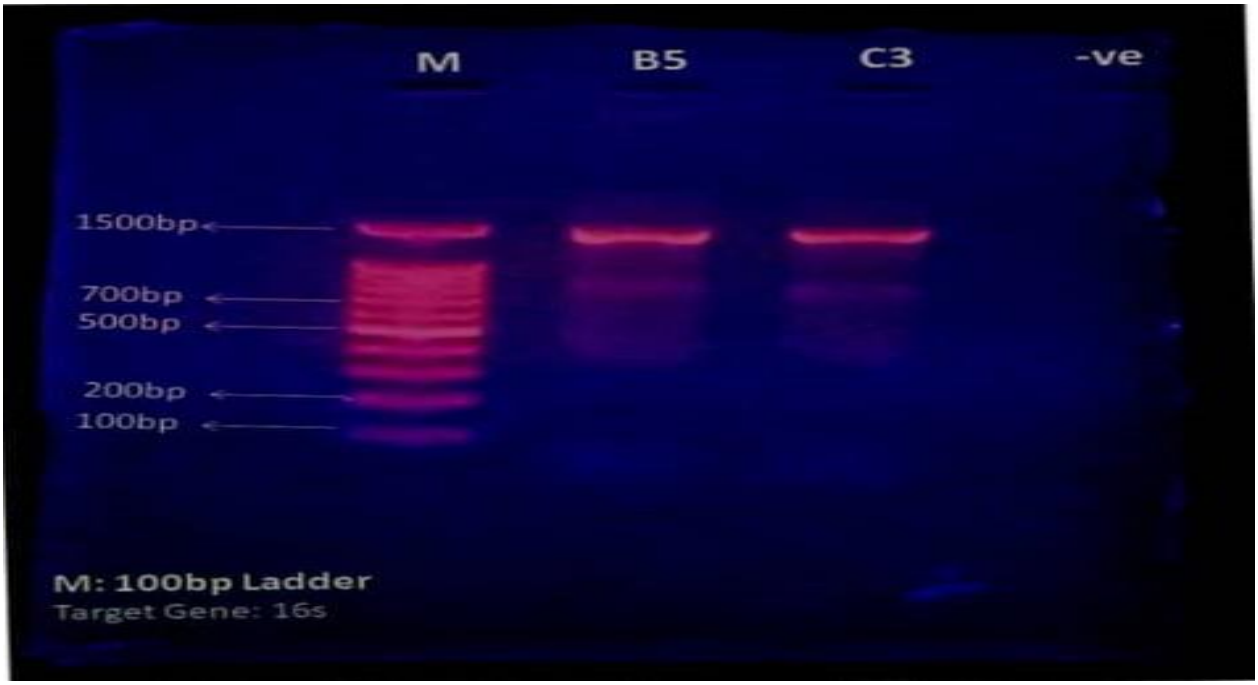

Figure 5: An agarose Gel Indicating the Amplification of the 16s rRNA Gene PCR Typically gives a Product of 1500 bp, and both Shows B5-907R and C3-907R Show strong bands around the size

Key: B5=*Psuedomonas aeruginosa;* C3=*Bacillus subtilis;* M=DNA Ladder

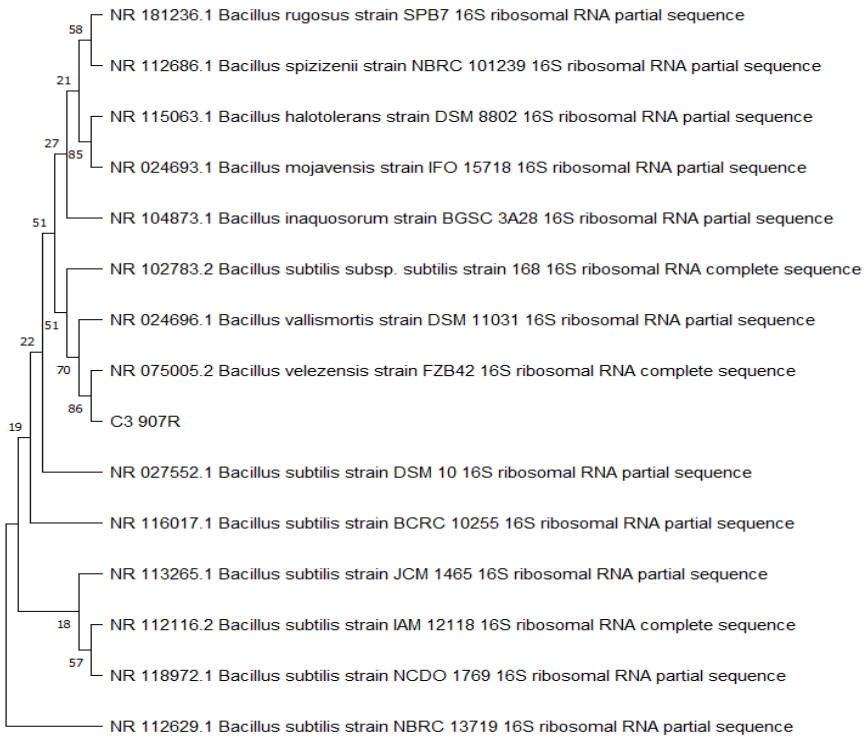

Figure 6: The Neighbour-joining phylogenetic dendrogram based on 16s rRNA gene sequences showing the relationship between the isolate *Bacillus subtilis* strain SPB7 and closest taxa from NCBI. Boostrap values are shown at branching point (greater than 50%)

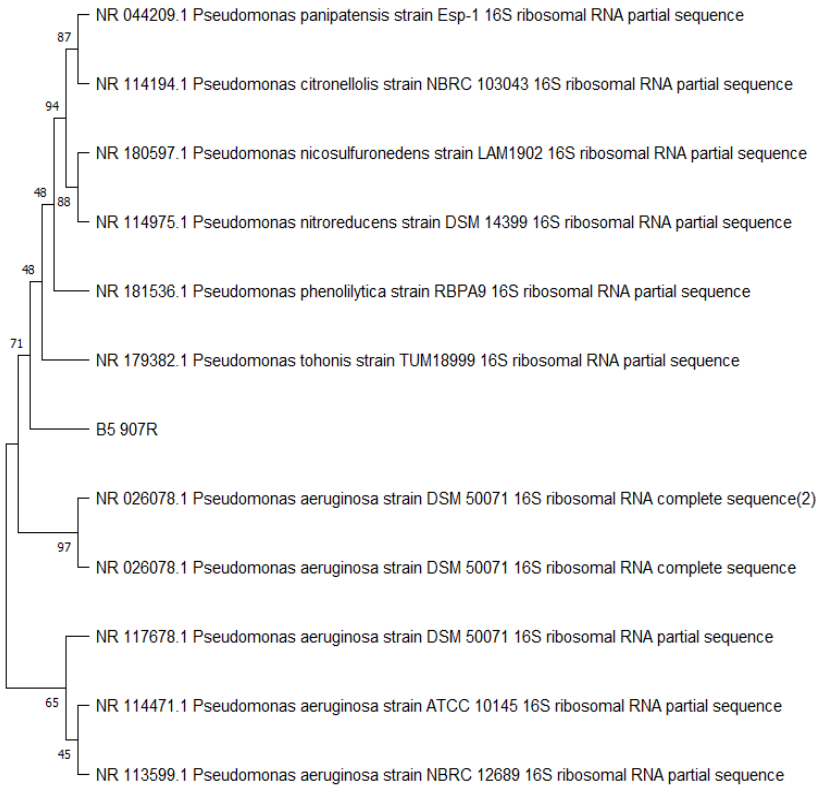

Figure 7: The Neighbour-joining phylogenetic dendrogram based on 16s rRNA gene sequences showing the relationship between the isolate*Psuedomonas aeruginosa* strain Esp-1 and closest taxa from NCBI. Boostrap values are shown at branching point (greater than 50%)

Discussion

Biosurfactants producing bacteria can be found in various environments, although the regions that are impacted by hydrophobic pollutants such as petroleum products are more yielding than uncontaminated onAes (Soltanighias *et al.,*2017). According to the previous report, 25% of microbial communities in hydrocarbon polluted soils belong to Biosurfactants producing microorganisms, which is relatively high as compared to unpolluted soil with the value of 2% (Saimmai *et al*., 2013). Our results on the colony forming units (CFU) of bacteria isolated confirmed the presence of Biosurfactant producing bacteria in the oil-contaminated sites. Previously, Biosurfactant producing bacteria were isolated from diverse environments like, plant leaf surfaces (Oso *et al*., 2019), hot springs (Mehetre *et al.*, 2019), seawater (Durval *et al*., 2018), solid waste management site contaminated with petroleum hydrocarbons (Xia *et al*. 2019) and chlorination tank (Felix *et al.,* 2019).

In this research, physicochemical properties of soil samples collected from hydrocarbon contaminated soil provide significant insights into the soil capacity to support bacterial communities capable of biosurfactant production. The results of pH recorded in this study where mainly neutral within range of 6.99, which is typically conducive to a wide range of microbial activities, including the breakdown of complex organic compounds (Hiltunen *et al*., 2017). Soil Ph influence microbial diversity, enzymatic activity and the availability of nutrients, all of which play a role in the biodegradation process (Monda *et al*., 2024). This finding is similar to the finding of Kumar *et al.*, (2019) who reported that soil with a pH range of 6.5 to 7.0 promotes microbial communities capable of degrading contaminants. The result of electrical conductivity of this research ranges from 67.9-187.1. This range is relatively similar to the finding of Ejairu and Okiotan, (2022) who had the EC that ranged from 122-185.

In this study, percentage result of sand ranged from 91.7-95.6 and the result of the percentage of silt and clay from this study ranges from 3.9-5.9 and 0.55-0.75 respectively. The reason may be the soil of the study area is more of sand and silt than in clay in that the soil of the study area is more of sand and silt than in clay in percentage. This study agreed with the findings of Aminu *et al*., 2023, whose percentage for clay ranges between 0.95 and 1.10 respectively.

Furthermore, nitrogen content in the soil ranging from 0.7 to 0.16% is another important factor influencing microbial activity, as nitrogen a key element in protein synthesis and overall microbial growth. Although the nitrogen content is relatively low, it is still within a range that could support the growth of nitrogen fixing bacteria, which may be present in

the soil and play a role in enhancing the biodegradation of contaminants (Xu *et al*., 2022).

Bacteriological Analysis of Soil Sample

Enumeration of Aerobic heterotrophic bacteria from hydrocarbon contaminated soil

Total heterotrophic bacterial count of the soil samples was determined and ranged from 2.5 x 10^7cfu/g to 13.8 x 10^7cfu/g (Table 2). This could be attributed to the fact that bacteria are abundant in the soil environment among which some are capable of producing biosurfactant. This result is not similar with that of Ogru, and Olannye, (2021) because his bacterial count ranged between 1.0-1.2 x 10^6cfu/g. It also disagreed with the results of Fardami *et al.*, (2022). As He had the bacterial count that ranged between 1.10-8.4x 10^6cfu/g. However, this might be due to differences in sample location and physicochemical characteristics of hydrocarbon contaminated soil. This result corresponds with the results of Aminu *et al*., 2023 as he had the bacterial count that ranged between 3.0 x 10^5cfu/g.

Isolation and identification of Aerobic Bacteria from Hydrocarbon Contaminated Soil

This study revealed that, sixteen (16) bacteria were isolated and identified from hydrocarbon contaminated soil and were identified such as *Pseudomonas aeruginosa*, *Bacillus subtilis*, *Bacillus pumilis* and *Staphylococcus epidermidis*. The occurrence of all these isolates in hydrocarbon contaminated soil could be attributed to the abundance of microorganisms found in soil isolation of *Pseudomonas aeruginosa* is the most predominant in this study and it is in agreement with the research conduct by Fardami *et al*., (2022) who reported *Pseudomonas aeruginosa* as good producer of biosurfactant.

Bacillus subtilis is the second most predominant throughout the study. This might be due to the ability of *Bacillus* species to survive in a wide temperature and pH. As such its capable of forming an endospore. This agreed with the findings of Fardami *et al*., 2020 who identified and reported bacillus species are good producers of biosurfactants. *Staphylococcus epidermis* is the least predominant isolate encountered in this study, it corroborated with the work of Zhang *et al*., 2021, who has analysis of bacterial abundance in soil.

Screening of the Bacterial Isolates for Biosurfactant Production

Blood haemolysis test was employed from which ten (10) out of the sixteen (16) isolates showed positive results. This may be due to the fact that many hydrocarbon degrading bacteria are capable of producing biosurfactant due to the presence of transparent clearing zones in blood agar plates. This technique has been used by various authors including Habib and Ibrahim (2023) Chigede *et al*., (2023) to screen biosurfactant production using haemolysis by bacteria.

Oil spreading technique, drop collapse and emulsification test wofere carried out in this research. Out of sixteen isolate. Eight (8) strains had beta-haemolytic activity (Table 5), Five (5) were able to collapse oil and eight (8) could spread oil. Finally eight (8) isolates were selected for complementary techniques after performing primary differential tests. These isolates show emulsification activity (E24) from 43% to 60% (Fig.1). According to results and data analysis, strain B5 and C3 showed the best biosurfactant production rate. Oghoje, (2023). Chigede *et al*., (2023) also have advocated the use of these techniques for screening microorganisms with potential to produce biosurfactants.

Optimization of the Bacterial Isolates for Biosurfactant Production.

The result for the optimization of biosurfactant production revealed that the biosurfactant production was optimal at 3% diesel concentration, pH 6.5 and 96hours incubation period. Biosurfactant-producing microorganisms are frequently aerobic and as diesel was used in this study, higher diesel concentration would invariably produce lower biosurfactant. This is due to the fact that high diesel concentration creates relatively anoxic condition *because* of the low capacity of oxygen to dissolve in oil which restricts the microbial metabolism. To facilitate oxygen dissolution, the use of soluble substrate as carbon sources have been suggested (Hu *et al.,*2015).The lower or higher pH conditions led to decrease in biosurfactant production which might be due to the fact that the pH below or above the optimum. This results are in agreement with the work of Fardami *et al*., (2022) who made similar observations.

Molecular Characterization of the Most Potent Bacterial Isolated from Hydrocarbon Contaminated Soil by 16Rrna Analysis

The most potent bacterial isolates that were found to excel in the screening for biosurfactant production in this present research were *Pseudomonas aeruginosa* and *Bacillussubtilis*. The result of hit similarity on BLAST confirmed *Pseudomonas aeruginosa* strain B5 with 99.32% (See Appendix vii) with accession no. ON 491428.1and *Bacillus subtilis* strain C3with 99.77% (See Appendix vi) with accession No. NR 112116.2 respectively. Molecular identification of *Bacillus sp*. strain was identified by Aminu *et al*., (2023) as biosurfactant producing bacteria.

CONCLUSION

It can be concluded that the biosufactant produced by isolated bacteria from hydrocarbon contaminated soil have shown good capabilities for biosurfactant production

at different physicochemical conditions. The maximum production of biosurfactant occurred at optimum pH, carbon source and incubation time used in this study revealed that biosurfactant production was optimal at pH 6.5, 3% carbon concentration and 96hrs incubation period respectively. The most potent bacterium species was molecularly identified using 16s rRNA belonged to *Pseudomonas aeruginosa* and *Bacillus subtilis* have a great capacity to produce biosurfactants from the environment. Therefore, Biosurfactants production conditions can greatly improve the bacterial strains for large-scale biosurfactant production.

The research is recommended to manage and enhance bacterial activity from hydrocarbon contaminated sites by plot a large scale application in bioremediation projects.

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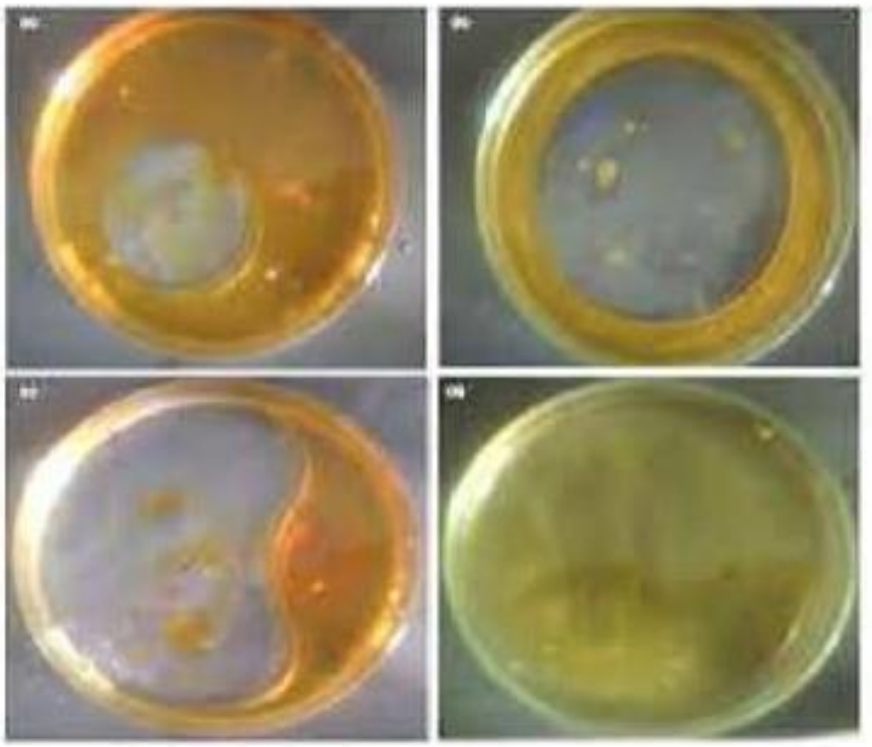
APPENDICES

APPENDIX 1: Drop Collapse of Biosurfactants Produced by Isolates

APPENDIX 2: Biosurfactant Production Setup by the Isolates

APPENDIX 3: Foam Activity of Biosurfactants Produced by Isolates

APPENDIX 4: Oil Displacement Assay by Isolates



APPENDIX 5: Sequence Prediction Report

>C3_907R

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APPENNDIX 6: Sequence Prediction Report

>B5_907R

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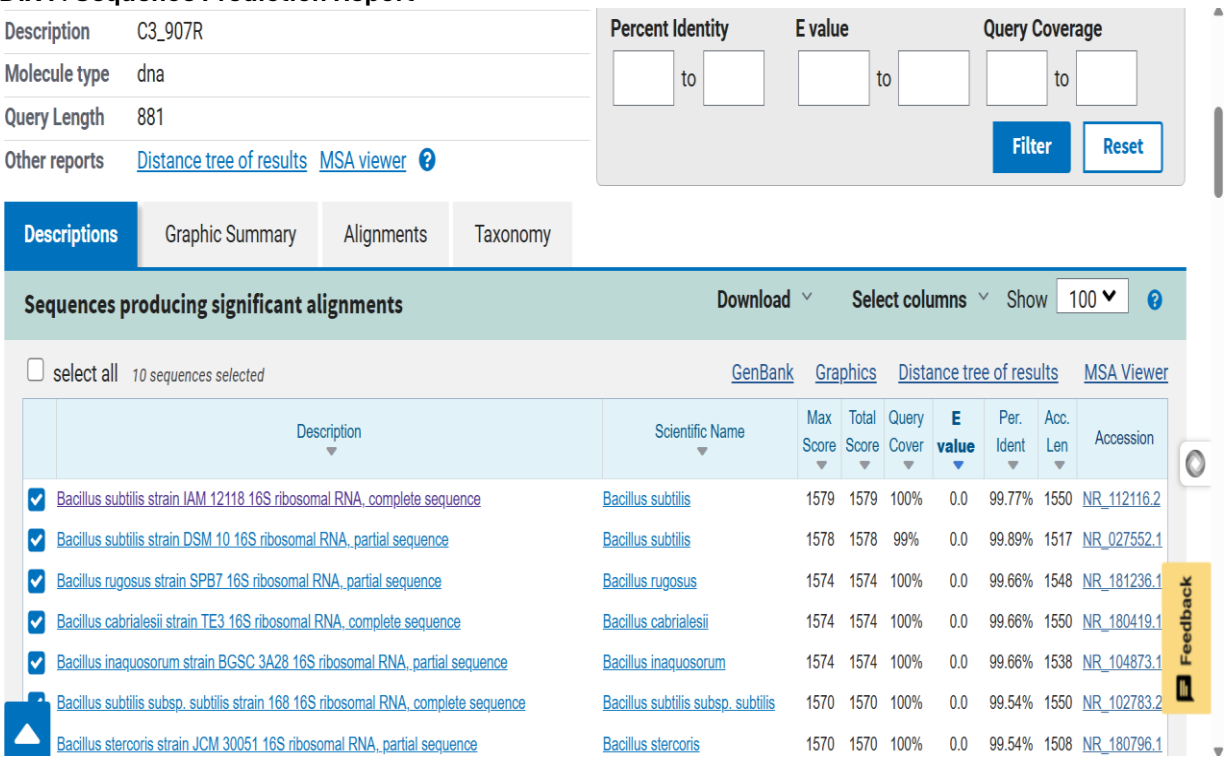
APPENDIX 7: Sequence Prediction Report



APPENDIX 8: Sequence Prediction Report