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Original article

SCREENING OF BACTERIAL ISOLATES FROM CRUDE OIL CONTAMINATED SOIL FOR POTENTIAL TO PRODUCE BIOSURFACTANTS

***Garba, B. S[1]., Ijah, U. J. J[2]. and Musa F. M[1].**

[1]Department of Microbiology, Faculty of Science, Kaduna State University, Kaduna, Nigeria

[2]Department of Microbiology, Federal University of Technology, Minna, Nigeria

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ABSTRACT

Biosurfactants are heterogeneous group of surface active molecules produced by microorganisms, which adhere to cell surface or excreted extracellularly in the growth medium. This study was conducted to screen bacterial isolates from crude oil contaminated soil for potential to produce biosurfactants. Sixteen bacterial species were isolated and screened for biosurfactant production using the drop collapse test, oil displacement method and emulsification activity. Based on the screening test, nine bacterial isolates were identified as biosurfactant producing bacteria. The isolates were species of *Klebsella*, *Micrococcus, Bacillus, Staphylococcus, Pseudomonas*, *Corynebacterium*, *Acinetobacter and Comamonas*. Two efficient biosurfactant producing bacteria were confirmed by 16S rDNA sequencing as *Acinetobacter baumanni* strain and *Comamonas testosteroni*. Biosurfactants produced by the two bacteria were extracted by acid precipitation method. *Acinetobacter baumanni* produced 1.2g/L of biosurfactant after 7 days while *Comamonas testosteroni* produced 0.6g/L of biosurfactant over the same period. The biosurfactants were characterized using FTIR, GC-MS and Physicochemical content. The biosurfactant produced by *Acinetobacter baumanni* indicated the biosurfactant was a lipopeptide having characteristics lipid and peptide peak values, containing 35.88% protein, 18.32% lipid and 24.44% Carbohydrate, while the biosurfactant produced by *Comamonas testosteroni* was a glycolipid (rhamnolipid) containing 35.28%, 18.65% lipid and 26.28% Carbohydrate. This study shows that the biosurfactants produced (lipopeptide and rhamnolipid) may be useful in the management of oil spills in the environment.

Keywords: Biosurfactant, crude oil, soil; screening, bacteria.

*** Corresponding Author**: Bgs3497@gmail.com: TEL:234 -7034972586

INTRODUCTION

Environmental pollution due to hydrocarbons, chemicals, solvents and heavy metals are very serious issues that the current world is facing. These pollutants are really harmful to living organisms including human beings and also indirectly contribute to the economic losses in developing countries [1]. Few of these toxic compounds and xenobiotics including crude oil can be naturally degraded to an extent by indigenous microorganisms through biodegradation processes [2]. The most widely distributed environmental pollution can be attributed to oil contamination, caused by tanker accidents, storage tank rupture, pipeline leaks and transport accidents [3,4]. This contamination causes significant environmental impacts and presents substantial hazards to human health [5, 6, 7].

Biosurfactants are diverse groups of surface-active chemical compounds that are produced by a wide variety of microorganisms [8]. Biosurfactants can be synthesized by different microorganisms and are grouped into six major classes based on the producing microorganisms. These classes are glycolipids, phospholipids, polysaccharide–lipid complexes, lipoproteins-lipopetides, hydroxylated and cross-linked fatty acids, and the complete cell surface [8]. Biosurfactants are widely used for various purposes such as food processing, oil recovery process, crude oil drilling, cleaning purpose, and bioremediation of oil contaminated sites [9, 10, 11]. Compared to chemical surfactants, biosurfactants have potential advantages, that is, they are eco-friendly, easily degradable, active in any extreme conditions like high salinity/temperature regions and can be produced using cheap organic sources, which facilitate commercialization [12]. Many studies have reported the application of biosurfactant producing microbes in the petroleum contaminated environments to remove hydrocarbon and remediate the environment [13, 14, 15]. Some biosurfactant producing microorganisms are species of *Bacillus, Pseudomonas*, *Saccharomyces*, *Candida*, *Acinetobacter* and *Nocardia* [16, 1, 17, 11]. These microorganisms are widely distributed in the environment and they are easy to obtain. However, the need to search for more prolific biosurfactant producing bacteria arises due to the fact that demand by industries has increased while production is still at low level. The aim of this study was to screen bacterial isolates from crude oil contaminated soil for potential to produce biosurfactants.

MATERIALS AND METHODS

Collection of Samples

Crude oil contaminated soil samples were collected in sterile sample bottles from an oil contaminated site in parts of Kaduna Refinery and Petrochemical Company (KRPC), Kaduna, Nigeria. The samples were transported to Microbiology laboratory, Kaduna State University (KASU), Kaduna for the study.

Escravos light crude oil (ELC) was collected from KRPC in sterile sample bottles and transported to the Microbiology laboratory, KASU, Kaduna, Nigeria

Isolation of Bacteria

Bacteria from crude oil polluted soil were isolated by spread plate technique using Nutrient agar. Ten gram of the crude oil polluted soil was measured into a sterile conical flask containing 90ml of sterile water, and shaken vigorously. The suspension was serially diluted and 1ml of the sample was placed on Nutrient agar and was incubated at room temperature

(28±2^{0}C) for 48 hours. Colonies which developed on the agar plate were sub-cultured repeatedly on Nutrient agar to obtain pure isolates, the pure isolates were maintained on agar slants for further characterization and identification.

Screening of Bacterial Isolates for Biosurfactant Production

The bacterial isolates were screened for ability to produce biosurfactants using three methods: Drop collapse test, oil displacement method, and emulsification test:

Drop collapse test

Two micro-liter of crude oil was applied to each cavity of a glass cavity slide. The slide was equilibrated for 1 hour at room temperature and then 5 µl of the Bacterial culture supernatant was added to the surface of oil (test). In the control, uninoculated medium was added instead of Bacterial culture supernatant. The shape of the drop on the oil surface was inspected after 1minute. Biosurfactant producing cultures giving flat/less convex drops were scored as positive (+). Those cultures which produced round convex drops were scored as negative (-), indicative of lack of biosurfactant production [18].

Oil displacement method

Oil displacement method according to Hassanshahian [19] was used to determine the diameter of the clear zone, which occurs after adding surfactant-containing solution on an oil-water interphase. In this test, 50ml of distilled water was added to a Petri dish (90mm in diameter), 100 µl of crude oil was added to the water surface, followed by the addition of 10 µl of the cell free culture supernatant obtained after the centrifugation of eighteen hours old broth culture at 6000rpm for 30minutes. The diameter of the oil as displaced by the cell free supernatant and the clear zone formed was visualized under visible light and this was measured after 30 seconds [20].

Emulsification activity (E24)

The emulsification activity of the biosurfactant solution was determined by measuring the emulsion index (E_{24}) at 25^{o}C as described by Wang *et al.* [21]. Two milliliters (2ml) of crude oil was poured separately into a test tube containing 2mL of cell free bacterial supernatant obtained after the centrifugation of eighteen hours broth culture at 6000rpm for 30minutes, the mixture was homogenized by vortexing at high speed for two minutes using Stuart auto votex mixer. The homogenized mixture was then allowed to stand for 24 hours undisturbed. After 24 hours, the height of the stable emulsion layer and total height of the mixture was measured, the values obtained were used to calculate the emulsification index (E_{24}) thus:

$$E_{24\,(\%)} = \frac{\text{Height of emulsion layer}}{\text{Total height of solution}} \times 100$$

Characterization and Identification of Biosurfactant Producing Bacterial Isolates

Bacterial isolates were identified on the basis of microscopic examination, cultural characteristics, morphological characteristics and gram staining reaction. Relevant biochemical tests such as [production of catalase Oxidase, Spore, Sugar fermentation, Indole, Methyl red, Voges proskaur and Gelatin hydrolysis, were also carried out. Confirmatory identities of the bacteria were made using Bergey's Manual of Systemic Bacteriology [22].

Molecular identification of bacterial isolates

DNA extraction

The Accuprep DNA Extraction kit was used for DNA Extraction. Proteinase K was dissolved completely in 1.250µl of nuclease – free water. RNase A was also dissolved in 600µl of nuclease free water and absolute ethanol was corrected to WA1 buffer and kept to be used. Cultural cell was centrifuged for 5minutes, supernatant was carefully discarded without disturbing the pellet. Two hundred microliters (200µl) of phosphate buffer saline (PBS) was suspended into pellet. Twenty microlitres (20µl) of prepared proteinase K and 10µl of RNase was added to pellet and mixed thoroughly and incubated for 2minutes at room temperature. Two hundred microliters (200µl) of GB buffer was added and mixed immediately using vortex mixer and was incubated at 60°C for 10 minutes. Exactly 400µl of absolute ethanol was added in mixed well by pipetting. Lysate was transferred into the upper reservoir of the binding column tube, closed and centrifuged at 8,000 rpm for 1 minute. Solution in collection tube was discarded and reused. Five hundred microliters (500µl) of washing buffer 1 (W1) was added, the tube was closed, and centrifuged at 8,000 rpm for 1minute. The tube was opened and the solution was poured from the 2 mL tube into a disposal bottle. Five hundred µl of washing buffer 2 (W2) was added, closed and centrifuged at 8,000rpm for 1 minute. It was centrifuged at 12,000 rpm for 1 minute to completely remove ethanol. The binding column tube was transferred to a new 1.5 ml tube for elution, 50µl of elution buffer was added onto binding column tube and allowed for 1 minute.at 15^{o}C centrifuged at 8,000 rpm for 1 min to elute [23].

Polymerase chain reaction (PCR) amplification

The PCR amplifiability was checked using 16S ribosomal DNA primers (27F.1 Forward 5'AGRGTTTGATCMTGGCTCAG 3 and 1492R reverse 5'GGTTACCTTGTTACGACTT 3'). The reaction was a 20μl reaction. For reaction set-up, templates, specific primers and water were added to the premix. For the reaction set up, 1μl of each of the reverse and forward primers were mixed with 2μl of the templates and 16μl of deionized water in the hot start PCR premix tube to make 20μl for the PCR run. For the negative control, 18μl of deionized water was added to the premix and 2μl of the primer and was placed in the PCR machine. The PCR Conditions: Pre- Denaturation: 5min at 94^{o}C, Denaturation: 30sec at 94^{o}C, Annealing: 30sec at 52^{o}C, Extension: 1min at 72^{o}C 35 cycles, Final extension: 5min at 72^{o}C [23].

Agarose gel electrophoresis

One point five grams (1.5g) agarose gel was dissolved into 100ml Tris Acetate EDTA (TAE) buffer solution and was heated in a microwave until agarose was completely dissolved. It was allowed to cool in a water bath set at 55^{o}C. Gel casting tray was prepared by sealing ends of gel chamber with appropriate casting system. The combs were then placed in gel tray. 5ul of ethidium bromide was added to cooled gel and was poured into gel tray. It was allowed to cool for 30 minutes at room temperature. The comb(s) placed in electrophoresis chamber were removed and the gel was covered with buffer (TAE). DNA and standard (Ladder) were loaded onto gel and electrophoresed at a given Voltage for 1hour. The DNA bands were visualized using UV light box.

Sequencing

The PCR product was purified and analyzed by Sanger (dideoxy) sequencing Technique to determine the nucleotide sequence of the specific microorganism isolated using automated PCR cycle-Sanger Sequencer™ 3730/3730XL DNA Analyzers from Applied Biosystems. Sequencing

analysis was done at Inqaba Biotechnology Pty South Africa and the sequences in the forward and reverse files were analyzed using the Sequence Scanner Software v1.0 (Applied Biosystems Thermo Fischer Scientific).

Basic local alignment search tool (BLAST)

The multiple alignment conducted on the European Molecular Biology Laboratory-European Bioinformatics Institute (EMBL-EBI) server on the Clustal Omega. The similarity search was conducted *in-silico* using the Nucleotide Basic Local Alignment Search Tool at the National Centre for Biotechnology Institute (NCBI) server. The phylogenetic and molecular evolutionary analyses were conducted using MEGA version 7 using the neighbor-joining method.

Production of Biosurfactant by the Isolates

The potential biosurfactant producing bacterial isolates were inoculated into sterile Muller Hinton broth and were incubated at 37^{o}C for 24 hours, then 1ml of the 24 hours old culture was transferred into 100ml mineral salts medium [24] in Erlenmeyer flask and was incubated at 25^{o}C for 7 days with shaking at 300 oscillations per minute using incubator shaker.

Biosurfantant extraction

Extraction of biosurfactant was done using the acid precipitation method [25], the bacterial isolates were removed after 7days of incubation by centrifugation at 6000rpm for 30 minutes. The cell free culture supernatant was acidified with IM of freshly prepared hydrochloric acid (HCl) to obtain a pH of 2.0. To every 100ml of the acidified cell free supernatant, 100ml of mixture of chloroform: methanol in the ratio of 2: 1 (v/v) was added. The mixture was allowed to react for 30 seconds, after which it was shaken vigorously until two phase separation was obtained. The upper layer containing majorly the reagents was decanted and the lower containing the biosurfactant was concentrated using a rotary evaporator, where most of the solvent was evaporated and the left over sediment was poured into a test tube and centrifuged at 600rpm for 20 minutes. The lower phase (whitish colour sediment) containing the extracts was then concentrated in an oven set at 40^{o}C to a dried crude biosurfactant [26].

Determination of dry weight of biosurfactants

The initial weight of a sterile glass Petri dish was taken, and the extracted biosurfactant was introduced into the Petri dish. It was then placed in the hot air oven set at 100^{0}C for 30 minutes. After drying, the plates and contents were reweighed. The weight of biosurfactant produced was determined using the formula: [27]. DWB = WPBAD – WEP

Where:

DWB= dry weight of biosurfactant

WPBAD= Weight of the Petri dish containing biosurfactant after drying

WEP= weight of the empty Petri dish.

Fourier transform infra-red (FTIR) analysis

The biosurfactants functional groups were further analyzed using FTIR [28]. Pellets for the infrared analysis were obtained by grinding a mixture of 1mg of the extracted biosurfactant with 100mg of potassium bromide (KBr). FTIR spectra were recorded in the region of 4500-500cm^{-1} wave number.

Gas chromatography and mass spectroscopy (GC-MS) analysis of the biosurfactants

The extraction of the active ingredient was carried out by dissolving 100mg of the milled powdery biosurfactant in 20ml pure n-hexane (99.999% purity) in a well corked reagent bottle. This was thoroughly mixed using an ultra sonicator for a period of five hours. The mixture was allowed to stand for 72hours and filtered into a beaker, the mixture was rewashed with 20ml n-hexane for two more consecutive time. The combined aliquots was evaporated on a steam bath to 5ml and filtered through a pasture pipette stocked with glass wool (membrane) with packed anhydrous sodium sulfate to remove moisture. The filtrate was concentrated to 1ml in the vial and was analyzed on Gas chromatography for chemical composition.

GCMS analysis of the biosurfactant was carried out according to the method used by Nigerian Institute of Science Laboratory Technology Ibadan [29]. The gas chromatographic (GC) analysis was performed on an Agilent Technologies interfered with mass selective detector. The electron ionization was at 70v with an ion source temperature at 250^0C. Highly pure helium gas (99.9% purity) was used as carrier gas, while HP-5ms (30mm x O.25mm x 0.32 μm) was used as the stationary phase. The oven temperature was at 80^0C held for 4 minutes and ramped to 270^0C at the rate of 3.5^0C/ minutes holding for 6 minutes.

Analysis of Biosurfactants for Physiochemical Properties

Determination of moisture content

Moisture content of the biosurfactant sample was determined based on mass loss after two hours at 105°C under N_2 purge. Approximately 0.1 g of air-dried sample was weighed into a ceramic crucible. The samples were placed inside a Lindberg muffle furnace, which was initially purged with N_2 gas for ≥20 min at a flow rate of 3 L min^{-1}, to ensure removal of all oxygen. After the 2 h heating, the furnace was turned off and samples were transferred immediately to a desiccator, left to cool for one hour and then weighed [30].

$$\% \text{ MC} = \frac{Wc \cdot Dc}{Dc} \times 100$$

Where:

Wc is the Air dried weight of sample

Dc is the Oven dried weight of sample

MC = Moisture content.

Determination of ash content (AC)

Ash content of the biosurfactant sample was determined by heating the sample to 730^0C in an air atmosphere using muffle furnace. To ensure complete combustion, crucible lids were removed and a low flow of house air (1.5 L min^{-1}) was constantly flushed through the furnace. The furnace was heated to 730^0C and held at that temperature overnight (8–10 h). After ashing, the furnace was switched off and allowed to cool for one hour before the samples were transferred to a desiccator to cool. The crucibles were weighed and ash mass was determined by subtracting the empty crucible weight. All reported proximate analysis data were done in triplicate measurements [30].

$$\text{AC (\%)} = \frac{\text{(Weight of Ash, g) x 100}}{\text{(Sample weight, g)}}$$

Determination of nitrogen content

Nitrogen of the sample was determined using Kjeldahl titrimetric method. Zero point one gram (0.1g) of sample was introduced in a 600 ml distillation flask containing 250 mL of water. Five millimeters (5 mL) of 40% NaOH solution was added gradually (for digestion), which converts the ammonium sulfate to ammonia. The flask was then connected to

a reflux condenser and heated. One hundred Millimeter (100 mL) of the distillate was introduced into a 15 mL of 0.1M HCl. This was then titrated with standard NaOH (0.1M) to determine the remaining amount of unused acid, using methyl red indicator. The acid used to neutralize ammonia is equivalent to the Nitrogen content in the sample. This procedure was also followed for blank sample [30].

The relevant calculation is:

$$N = \frac{[(A-C)-B] \times 0.0014 \times 100}{W}$$

A = mL of standard acid (0.1M HCl) taken to receive ammonia

C= mL of standard alkali (0.1M NaOH) used in titration

W = weight of the sample taken

B= mL of standard alkali used in the blank.

1 mL 0.1M HCl = 0.0014 g N

Determination of lipid content

The Folch method employs the use of chloroform–methanol (2:1 by volume) for extraction of lipids from endogenous cells. The homogenized cells were equilibrated with one-fourth volume of saline solution and mixed well. The resulting mixture was allowed to separate into two layers and lipids settled in the upper phase [30].

Determination of Protein content

The nitrogen content of the samples was determined by Micro - Kjeldahl method.The nitrogen value obtained was multiplied by 6.25 to convert it to protein [30].

Protein = % Nitrogen x 6.25

Determination of carbohydrate determination

The carbohydrate content was calculated using:

Carbohydrate (%) = 100 – (Protein (%) +Moisture (%) + Ash (%) + Nitrogen (%) + Lipid (%))

RESULTS

Bacterial Isolates and their Biosurfactact Producing Potentials

A total of sixteen (16) bacterial isolates were obtained from the crude oil contaminated soil. The isolates were coded 3A, 3B, 3C, 3D, 4A, 4B, 4C, 4D, 5A, 5B, 5C, 5D, 6A, 6B, 6C and 6D.

Of the sixteen isolates, nine isolates (3A, 3B, 4D, 5B, 5C, 6A, 6B, 6C, 6D) showed positive results (56.25%) in drop collapse test, thereby indicating the presence of biosurfactant in the culture media. The drop of crude oil collapsed immediately or within 1minute of addition of culture broth. The remaining bacterial cultures (43.75%) could not collapse the drop of crude oil even after 1min (Table 1).

Ten isolates (62.5%) (3A, 3B, 4D, 5A, 5B, 5C, 6A, 6B, 6C, 6D) significantly displaced the oil layer which spread in the water, showing a zone of displacement (Isolate 6A had the highest diameter of 2.10 $\pm$ 0.14cm, zone of spread followed by 5B (1.65$\pm$0.12cm) and 6D (1.60$\pm$0.14cm) while 3D had the least diameter zone of spread (0.10$\pm$0.14cm). The results are presented in Table 1.

The Bacterial isolates were tested for their ability to emulsify crude oil and the results revealed that isolate 6D had the highest emulsification index of 57% while isolate 5A had the lowest emulsification index of 44%. Other isolates had emulsification index ranging from 46.43% to 55.17% Table 1

Table 1: Extent of drop oil collapsed caused by bacterial isolates

Coded bacterial Isolates	Reaction	Oil spread / displacement caused by bacterial isolates	Emulsification activity: Emulsification activity E_{24} (%)
3A	+	1.45±0.07	51.85
3B	+	1.30±0.14	55.17
3C	-	0.15±0.21	48.15
3D	-	0.10±0.14	46.43
4A	-	0.40±0.57	46.67
4B	-	0.30±0.42	46.43
4C	-	0.20±0.28	48.15
4D	+	0.55±0.64	48.15
5A	-	0.55±0.14	44.44
5B	+	1.65±0.12	46.43
5C	+	1.35 ±0.21	50.00
5D	-	-	-
6A	+	2.10±0.14	53.57
6B	+	1.40±0.14	50.00
6C	+	1.25±0.07	53.57
6D	+	1.60±0.14	57.14

+: Positive, -: Negative, No displacement of oil drop or no emulsificatuion, values are mean ± SD

Identification of Potential Biosurfactant Producing Bacterial Isolates

A total number of nine isolates (56.25%) out of sixteen bacterial isolates obtained from crude oil contaminated soil were positive for all three screening test used for screening of the isolates for biosurfactant production. The nine biosurfactant producing bacteria were characterized using morphological and biochemical properties. Six (66.66%) of the nine bacterial isolates were Gram positive bacteria and 3 (33.33%) isolates were Gram negative bacteria. The biosurfactants producing isolates were identified as *Bacillus* sp, *Klebsiella pneumoniae, Pseudomonas aeruginosa*, *Micrococcus luteus*, *Staphylococcus aureus*, *Corynebacterium* sp, *Comamonas testosteroni, and Acinetobacter baumanni.*

The gel electrophoresis of two more effective biosurfactant producing bacteria, *Acinetobacter baumanni* (6A and 6D) *Comamonas testosteroni* were viewed as presented in Figures 1 and 2. Blast for

isolate 6A submitted to Gen Bank revealed with accession number MH210899.1 showed 94.10% homologous with *Acinetobacter baumanni*, while Blast for 6D submitted to Gen Bank revealed with accession number MH255596.1 showed 90.35% homologous with *Comamonas testosteroni*. .

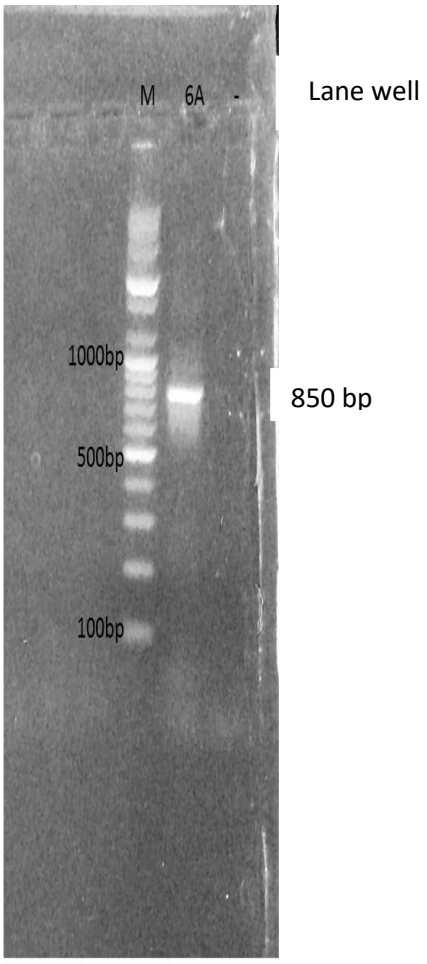

Figure 1: Gel Electrophoresis for Amplified DNA of Isolate 6A (*Acinetobacter baumanni*)

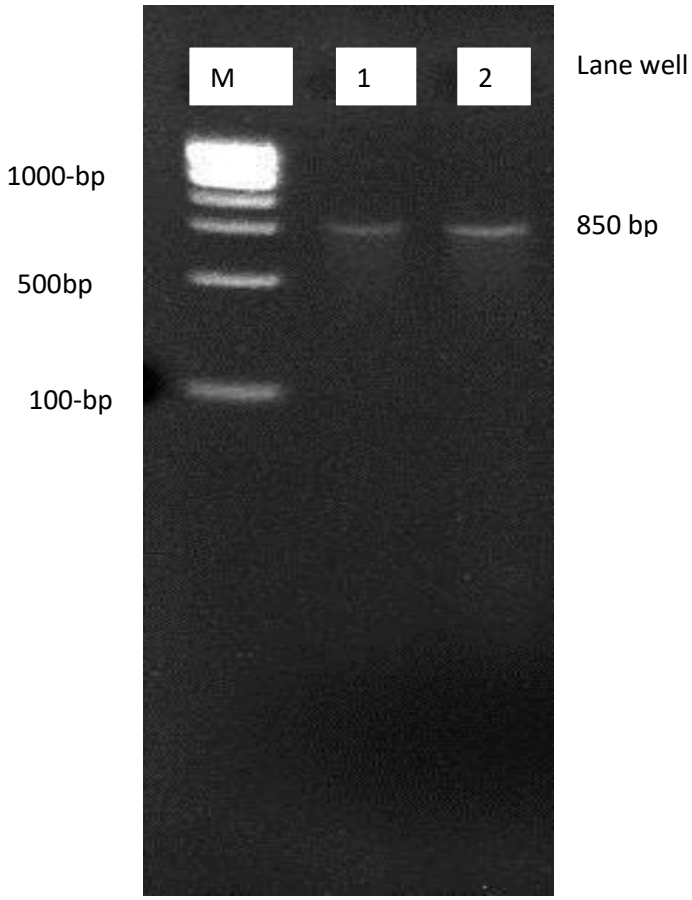

Figure 2: Gel Electrophoresis for Amplified DNA of Isolate 6D (*Comamonas testosteroni*)

Production of biosurfactants

Extraction of the biosurfactants

Two bacterial isolates with high biosurfactants producing potential were used for production of the biosurfactants. The bacterial isolates were *Acinetobacter baumanni* and *Comamonas testosteronni*.

The dry weight of the biosurfactants by the two bacterial isolates ranged between 0.6g/L and 1.2g/L. *Acinetobacter baumanni* produced 1.2g/L of biosurfactant after 7days while *Comamonas testosteroni* produced 0.6g/L of biosurfactant over the same period. Thus, *A. baumanni* was a more efficient producer of biosurfactant than *C. testosteroni*.

Chemical Characterization of biosurfactants

Fourier transform infra-red (FTIR) analysis

The spectra for biosurfactants produced from the FTIR analysis revealed peaks of different shapes (broad, asymmetric, strong, weak, stretching, and bending) each represents specific functional groups that are present on the molecular chain in the biosurfactants studied.

Figure 3 shows the spectra peaks of compound in biosurfactant produced by *Acinetobacter baumanni,* while Figure 4 shows the spectra peaks of compounds in biosurfactant produced by *Comamonas testosteroni.* Table 2 shows the interpreted results from the correlation table of infrared absorption bands and functional

groups showing the frequencies of peaks, vibration types and anticipated fuctional groups of the compounds. The main functional groups detected from biosurfactant produced by *Acinetobacter baumanni* are O-H stretch of carbonyl group, C-H stretching and bending from alky group, C≡C triple bond from alkyne group, C=O double stretch bond from aliphatic group, C-N from aromatic amino, C-O from ether and N-H with a medium stretch from aromatic secondary amine. Table 3 shows the main functional groups detected from biosurfactant produced by *Comamonas testosteroni*. The functional groups are O-H stretching from alcohol, N-H with a medium stretch from aromatic secondary amine, C=O stretch from aliphatic group, C-H bending from alkene group, C-C from aromatic group and CH_2 from aliphatic group.

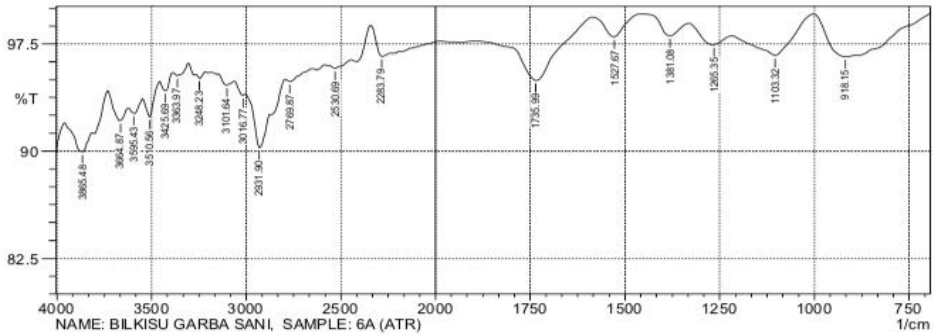

Fig. 3: Fourier transform infra-red (FTIR) of Biosurfactant produced by *Acinetobacter baumanni*

Table 2: FTIR analysis results of the biosurfactant produced by *Acinetobacter baumanni*

Peaks	Vibration Type	Functional groups
3865.40	Stretching	O-H
3510.88	Stretching	N-H
3248.28	Stretching	OH
3101.64	Stretching	NH_4^+
2931.90	Stretching & Bending	C-H
2283.97	Stretching	C≡C
1735.99	Stretching	C=C
1527.67	Stretching	N-O
1381.08	Asymmetric	C-H
1265.35	Stretching	C-N
1103.32	Stretching	C-O
918.15	Bending	C-H

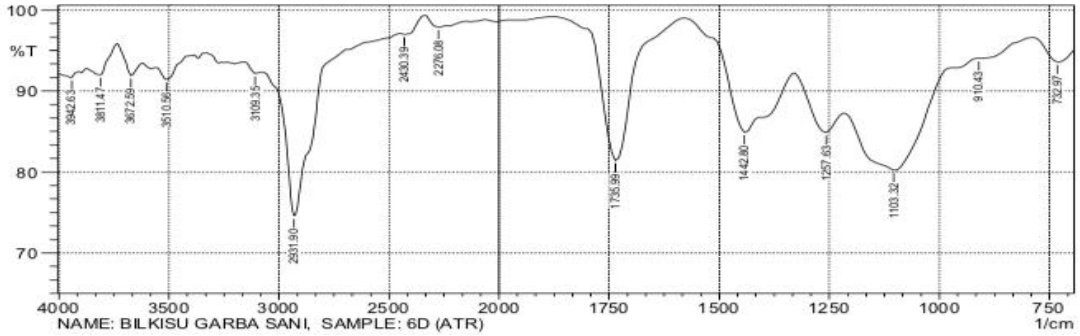

Fig. 4. Fourier transform infra-red (FTIR) of biosurfactant produced by *Comamonas testosteroni*

Table 3: FTIR analysis results of the biosurfactant produced by *Comamonas testosteroni*

Peaks	Vibration type	Functional Groups
3672.59	Stretching	O-H
3510.88	Stretching	N-H
3109.35	Stretching	NH_4^+
2939.9	Stretching & Bending	C-N
1735.99	Stretching	C=O
1442.8	Stretching	C-C
1257.63	Stretching	C-N
1103.32	Stretching	C-O
910.43	Bending	C-H
732.97		CH_2

Gas Chromatography and mass spectrometry of biosurfactants

The results of Gas Chromatography and mass spectrometry (GC-MS) analysis of biosurfactant by *Acinetobacter baumanni* are presented in Figure 5 and Table 4. Figure 5 shows the GC-MS report Thirty peaks were obtained in the biosurfactant and Table 6 shows the interpretation based on peak spectra where each peak has a chemical compound name, retention time and area percentage.Compounds such as Trichloromethane, oxalic acid, carbonoic acid and hexadecanoic acid were detected.

The result of gas chromatography and mass spectrometry (GC-MS) analysis of biosurfactant by *Comamonas testosteroni* are presented in Figure 6 and Table 5. Figure 6 shows the GC-MS report Thirty (30) peaks were obtained in the biosurfactant and Table 7 shows the interpretation based on peak spectra where each peak has a chemical compound name, retention time and area percentage. Most of the compounds were Trichloromethane followed by methyl ester and hexadecanoic acid.

Fig. 5 GC-MS of biosurfactant produced by *Acinetobacter baumanni*

Table 4: Chemical constituent of chloroform:methanol extract of *A. baumanni* identified by gas chromatography and mass spectrophotometry (GC-MS)

Peak Number	Retention time(min)	Area (%)	Compounds
1	5.1419	0.5715	Trichloromethane
2	5.1636	0.7624	Trichloromethane
3	5.1888	0.5193	Trichloromethane
4	5.2059	0.7255	Trichloromethane
5	6.1597	1.4755	Trichloromethane
6	6.3722	0.4758	Trichloromethane
7	6.6697	1.3961	Trichloromethane
8	6.774	1.2355	Trichloromethane
9	7.0622	0.6989	Trichloromethane
10	7.2308	2.6531	Trichloromethane
11	8.7397	11.2401	D-Limonene
12	9.6364	1.0047	Methane, oxybis[dichloro-
13	19.4918	0.5656	1,6:3,4-Dianhydro-2-deoxy-.beta.-d-lyxo-hexopyranose
14	29.1417	0.5504	Octadecane, 2,2,4,15,17,17-hexamethyl-7,12-bis(3,5,5-trimethylhexyl)-
15	30.414	0.8991	Oxalic acid, cyclobutyl octadecyl ester
16	30.4647	0.8953	Isobutyl hexadecyl ether
17	30.6232	13.3298	Hexadecanoic acid, methyl ester
18	30.7352	1.3427	Hexadecanoic acid, methyl ester
19	31.0133	1.2798	Carbonic acid, decyl tridecyl ester
20	32.1263	24.1732	trans-13-Octadecenoic acid, methyl ester
21	32.2448	1.3023	9-Octadecenoic acid (Z)-, 2,3-dihydroxypropyl ester
22	32.3053	6.0488	Methyl stearate
23	32.3458	5.31	Methyl trans-9-(2-butylcyclopentyl)nonanoate
24	32.6298	2.1215	Naphthalene, 6-chloro-1-nitro-
25	32.6476	2.3093	Cyclohexane, 1,1'-(2-propyl-1,3-propanediyl)bis-

26	33.194	11.2841	Tributyl acetylcitrate
27	33.3236	1.4522	Silicic acid, diethyl bis(trimethylsilyl) ester
28	34.8634	2.0028	Cyclotrisiloxane, hexamethyl-
29	34.9093	0.436	1,4-Bis(trimethylsilyl)benzene
30	35.2737	1.9388	Adamantane, 1-isothiocyanato-3-methyl-

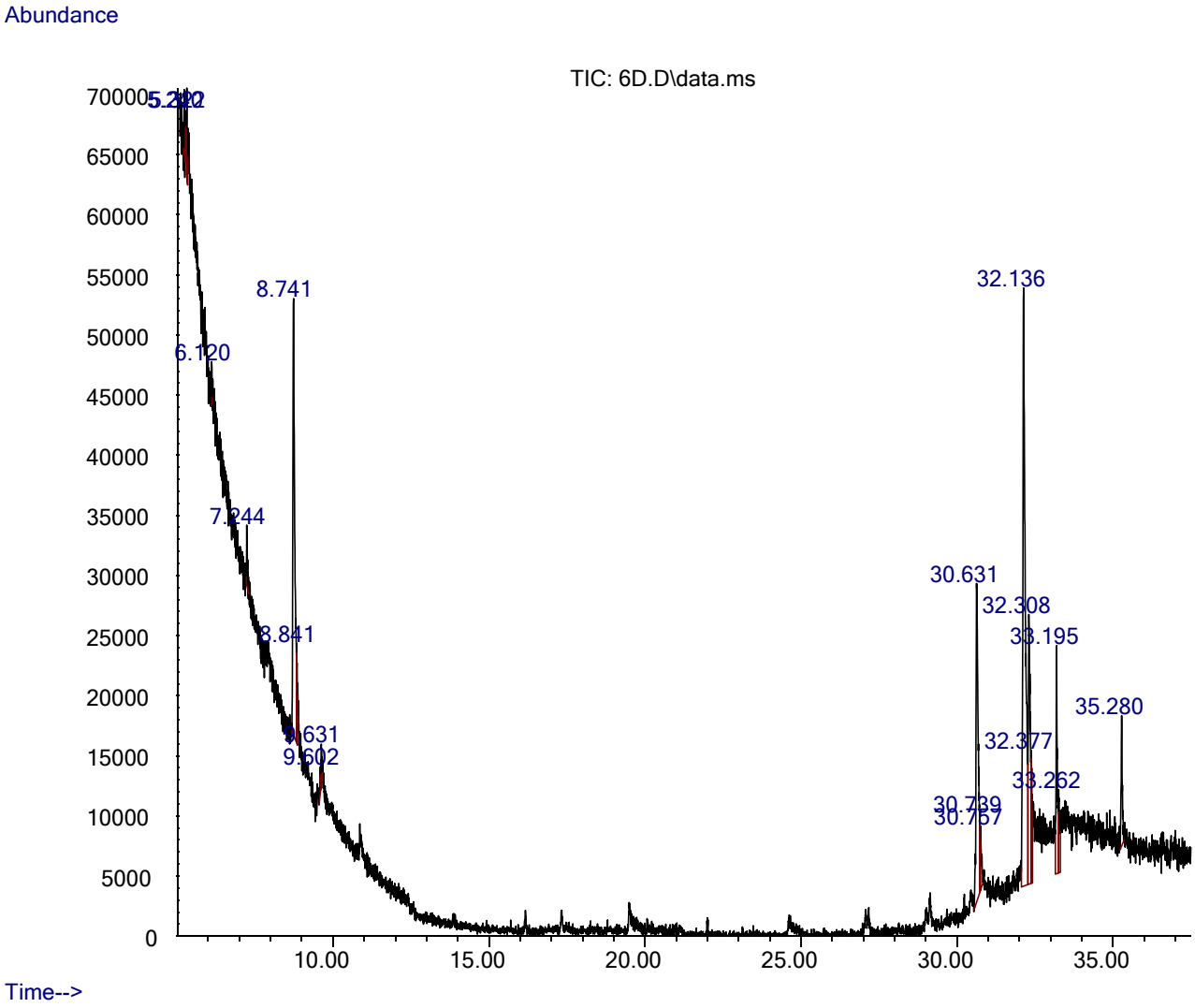

Fig. 6 GC-MS of Biosurfactant produced by *Comamonas testosteroni*

Table 5: Chemical constituent of chloroform: methanol extract of *C. testosteroni* identified by gas chromatography and mass spectrophotometry (GC-MS)

Peak number	Retention Time(min)	Area (%)	Compounds
1	5.2401	0.8246	Trichloromethane
2	5.3218	1.1219	Trichloromethane
3	6.1204	0.435	Trichloromethane

4	7.2438	0.8957	Trichloromethane
5	8.741	17.8217	D-Limonene
6	8.8411	1.4319	Trichloromethane
7	9.6022	0.4115	Trichloromethane
8	9.6314	0.829	Trichloromethane
9	30.6313	15.1821	Hexadecanoic acid, methyl ester
10	30.7386	0.6735	Tetradecanoic acid, 12-methyl-, methyl ester, (S)-
11	30.7569	1.4971	Hexadecanoic acid, methyl ester
12	32.1362	33.5964	10-Octadecenoic acid, methyl ester
13	32.3083	10.7831	Heptadecanoic acid, 16-methyl-, methyl ester
14	32.3769	2.8524	9-Octadecenoic acid (Z)-, methyl ester
15	33.1954	6.1024	Butyl citrate
16	33.2623	2.2138	Hexasiloxane, 1,1,3,3,5,5,7,7,9,9,11,11-dodecamethyl-
17	35.2798	3.3277	1,4-benzenedicarboxylic acid, mono(1-methylethyl) ester

Physicochemical Properties of Biosurfactants

The physicochemical analyses showed that biosurfactant produced by *Acinetobacter baumanni* had 35.88% protein, 18.32% lipid and 24.44% carbohydrate. Biosurfactant from *Comamonas testosteroni* contained 35.25% protein, 18.65% lipid and 26.28% carbohydrate (Table 6). Ash and nitrogen contents of the biosurfactants were 2.70% and 5.74% respectively for *A. baumanni* while *C. testosteroni* produced biosurfactant contained of 2.30% ash and 5.32% protein (Table 6). The biosurfacatants had moisture content ranging from 12.20% to 12.70%.

Table 6: Physicoochemical properties of biosurfactants produced by *A. baumanni and C. testosteroni*

Parameter	*Acinetobacter baumanni*	*Comamonas testosteroni*
Moisture (%)	12.70	12.20
Ash (%)	2.70	2.30
Nitrogen (%)	5.74	5.32
Protein (%)	35.88	35.25
Lipid (%)	18.32	18.65
Carbohydrate (%)	24.66	26.28

DISCUSSION

Isolation and Screening of Bacterial Isolates for Biosurfactants Production

Environmental samples contaminated by hydrocarbon pollutants have been known to be more likely to harbour biosurfactant producing organisms [31]. Biosurfactants produced by a variety of microorganisms mainly bacteria, fungi and yeasts are diverse in chemical composition and their nature and the amount depend on the type of microorganism producing a particular biosurfactant.

Different screening methods were used to select potential biosurfactant producing bacterial isolates. The tests comprised oil displacement test, drop collapse test, and emulsification index, chosen for biosurfactant screening due to their advantages of low cost, high clarity and use of common equipment that can be easily obtained [32]. Among the screening methods, oil displacement method was considered better, since the oil displacement area (clearing zone) in this assay was directly proportional to the concentration of the biosurfactant in the solution [33]. These methods have been previously used by other researchers to identify biosurfactant producing bacteria; oil spreading test [34], drop collapse method [35] and emulsification index [36, 37].

The bacterial isolates in the present study showed positive results (56.25%) in drop collapse test. The increased positive drop collapse agrees with the work of Thavasi *et al.* [38] who screened bacterial strains for biosurfactant production and noted that 78.1% were positive for drop collapse activity. Similarly, Anaukwu *et al.* [39] who screened bacterial strains for biosurfactant production noted that 93.1% of the isolates had positive results for drop collapse test. The investigators therefore, recommended drop collapse test assays as a reliable method for screening large number of samples. However, contrary to the finding of this study, Sabina *et al.* [2010] screened bacterial isolates and had only 3.4% positive result in drop collapse test, even though better result was obtained in oil spreading test.

Oil displacement test was developed where clearing zone was formed as oil was being displaced by the presence of biosurfactant [33, 41]. In Oil displacement method, the quantity of biosurfactant secreted determines the extent of oil spreading activity of a given microorganism [42]. The oil displacement method is also a rapid and easy method to be carried out. In addition, this method can detect even low activity and quantity of biosurfactant present [32]. A study by Hamzah *et al*. [43] using oil displacement test was able to screen nine potential biosurfactant-producing isolates instead of only one isolate by using the drop-collapse test. There were other studies that also supported oil displacement test as a reliable and sensitive test [41, 44]. The bacterial isolates in this study produced oil displacement ranging from 0.1cm to 2.1cm, which agrees with the report of Anaukwu *et al.* [39] who recorded displacement diameters ranging from 0.2cm to 2.1cm in their work on biosurfactant production by bacteria isolated from Nigerian soil. However, the findings of the present study are contrary to the works of Hesham *et al*. [45] and Jaysree *et al*. [46]. While Hesham *et al.* [45] obtained rate of displacement ranging from 2.8cm to 4.1cm in the screening of *Candida* species for biosurfactant production, Jaysree *et al*. [46] recorded displacement diameters ranging from 3.0cm to 4.2cm in their work on biosurfactant production by halophilic bacteria, the variations in the displacement diameters are likely to be strain dependent.

Emulsification assay is an indirect method used to screen biosurfactant production. It was assumed that if the cell free culture broth used in this assay contains biosurfactant then it will emulsify the hydrocarbons present in the test solution However, emulsification activity by biosurfactant present does not always correlate with surface activity; therefore, this method just gives an indication of presence of biosurfactant [47]. According to Satpute *et al*. [34], biosurfactant production was determined by good emulsification activity exhibited by bacteria which is measured to be more than 30% in E24. The best biosurfactant producer was defined to be able to maintain at least 50% of its original emulsion after 24 hours of emulsification [48]. Purified glycolipid biosurfactant recorded an increase in E24 values from 77.5% to 82% when compared to the crude biosurfactant [49]. This finding showed that emulsification test is one of the strong screening methods in the determination of biosurfactant producer. Based on the results obtained in this study *Acinetobacter baumanni* had emulsification index of 57.14% while *Comamonas testosteroni* had the highest emulsification index of 57%. The emulsification test result in a study by Anaukwu *et al*. [39] who screened 29 bacterial isolates for biosurfactant indicated that 93.1% of the isolates gave positive emulsification activity while 6.9% were negative. Similarly, a study by Ellaiah *et al.* [37], who screened 68 bacterial isolates for biosurfacatant production found that only 6% of the isolates had emulsion activity up to 61%. However, Bodour and Maier [50] suggested that a maximum of two or three screening methods should be used for the selection of biosurfactant production.

The nine biosurfactant producing bacteria were identified using morphological and biochemical tests. Amongst the isolates, 6(66.67%) out of the 9 isolates were gram positive while 3 (33.33%) were Gram negative. A study by Bodour *et al.* [50] reported more Gram positive bacterial isolates from contaminated site than Gram negative. Contrary, Ramankutty and Nedunche – zhiyan [51] reported that Gram negative bacteria were abundant and were more tolerant to hydrocarbon contamination. However, both types of bacteria may have equal potential to co-exist within hydrocarbon contaminated sites

The nine biosurfactant producing bacterial isolates were *Bacillus* sp, *Klebsiella* sp*, Pseudomonas* sp, *Micrococcus* sp, *Staphylococcus* sp, *Staphylococcus* sp, *Corynebacterium* sp, *Comamonas testosteroni and Acinetobacter baumanni. Pseudomonas aeruginosa* has been widely reported [36, 53, 54, 55] for its ability to produce biosurfactant especially rhamnolipid. Production of biosurfactants by *Bacillus subtilis* has been reported by many researchers [56, 57, 58, 59]. Eddouaouda *et al*. [60] reported the production of biosurfactant by *Staphylococcus* sp. while Patil and Shendre [61] reported the production of biosurfactant by *Micrococcus* sp. Production of biosurfactant by *Comamonas* sp was reported by More *et al*. [62]. Dwivedi *et al*. [63] reported the production of biosurfactant by *Corynebacterium* sp. Mishra *et al*. [64], Jamal *et al.* [65] and Nwaguma *et al.* [66] reported the production of biosurfactant (phospholipid) by *Klebsiella pneumoniae.* The results obtained have revealed that the potential to produce biosurfactants is exhibited by bacteria of diverse genera.

In the present study, the best two biosurfactants producing isolates were selected (6A and 6D) and their identities were confirmed using molecular techniques as *Acinetobacter baumanni* strain LacOIV and *Comamonas testostreoni* strain respectively. Biosurfactant produced

by *Acinetobacter baumanni* was 1.2g/L of biosurfactant. The yield of biosurfactant produced was higher than that reported by Bao *et al.* [67]. *Comamonas testosteroni* produced 0.6g/L of biosurfactant. The yield of biosurfactant produced was higher than that (0.31g/L) reported by More *et al*. [62] by the same organism. The differences in results could be due to differences in metabolic capability of the organisms and substrates used.

FTIR analysis of biosurfactant produced by *Acinetobacter baumanni* indicated that the protein product contained peptide-like moiety as well as aliphatic hydrocarbons. The results obtained are comparable with the reports of several authors [69, 70, 71, 72]. The FTIR analysis revealed that, the biosurfactant produced by *A. baumanni* had peptide due to the presence of C-N bonds. Also. Presence of C=O bonds indicated ester structure. The FTIR spectrum implies that the biosurfactant produced is a lipopeptide. Although, lipopeptide production is mostly associated with *Bacillus* species, for example *Bacillus aryabhattai* [73]. Several *Acinetobacter s*pecies have been identified as lipopeptide producers [62, 74, 75].

The FTIR spectra of *Comamonas testosteroni* revealed –OH stretching vibration for hydroxyl group, - C-H stretching vibration of hydrocarbon chain of alkyl group (CH_2 and CH_3). The ester carbonyl group (-C=O) elucidate the lipid moiety in glycolipid biosurfactant as rhamnolipid.

As far as we are aware this is the first study to describe the chemical characterization of biosurfactant by *Comamonas* sp. As *Comamonas* species are related to *Pseudomonas* species, the possibility of rhamnolipid production by *Comamonas* cannot be ruled out. The FTIR spectra of the biosurfactant was closely related to those reported for other glycolipid biosurfactant by *Pseudomonas* sp [76, 77, 55].

The GC-MS analysis showed that the compounds produced by *Acinetobacter baumanni LacOIV* was a lipopeptide derivatives. The hydrophobic moiety was envisaged to be an Octadecanoic acid methyl ester. The constituents present in the extract were D- limone (RT 11.24), Hexadecanoic acid, methyl ester (RT 30.46), 1.6:3, 4- Dan-hydro - 2- deoxy-beta (RT 19.49), 9 Octadecenoic acid (RT 32.24), trans 13- Octodecenoic acid, methyl ester (RT 32.12), diethyl bis (triemethylsilyl) ester (RT 32.32), tributyl acetylatrate (RT 33.19) and 1- isothiocynato- 3- methyl (RT 35.27). The result showed that the biosurfactant activity of the major chemical constituent of chloroform: Methanol extract of *Acinetobacter baumanni LacOIV* was identified due to the presence of Octadecenoic acid. This result is in accordance with Moussa and Abdel Azeez [78] and Anitha *et al*. [35]. Ibrahim *et al*. [16] reported that lipopeptide based biosurfactant contains fatty acids such as Octadecanoic acid and 9 - Octadecenoic as major components. Another fatty acid compound hexadecanoic acid was also detected in the biosurfactant [79].

GC-MS analysis of biosurfactant produced by *Comamonas testosteroni* was glycolipid (rhamnolipid) derivatives. The constituents present in the extract were 10 – Octadecenoic acid, methyl ester (RT 32.59), 9 – Octadecenoic acid (RT 32.37), Hexadecanoic acid, methyl ester (RT 30.75 and 30.63), Heptadecanoic acid (RT 32.30), Butyl citrate (RT 33.19), Hexasiloxane (RT 33.26) and 1, 4 – benzenedicarboxylic acid (35.27). The GC-MS showed major peak for Octadeonic acid, methyl ester, a faty acid with area percentage of 33.60%. Thisresults is in accordance with Sharma *et al*. [80].

The physicochemical analyses showed that biosurfactant produced by *Acinetobacter*

baumanni had 35.88% protein, 18.32% lipid and 24.44% carbohydrate, while biosurfactant from *Comamonas testosteroni* contained 35.25% protein, 18.65% lipid and 26.28% carbohydrate. These compounds are quite similar in quantity. However, the quantities of these components were lower with the exception of lipid than those reported by Jagtap *et al*. [81] that the biosurfactant produced by *Acinetobacter* sp was composed of 53% protein, 42% carbohydrate and 2% lipid. Similarly, Adetungi and Olaniran [75] reported that the biosurfactants produced by *Acinetobacter* sp Ab9ES was composed of carbohydrate (66.5%), and protein (31%) while that of *Acinetobacter* sp. Ab33-ES was composed of carbohydrate (67.8%) and protein (25%).

The biosurfactant in *Comamonas testosteroni* had a high protein content. The result is in contrast to the findings of Hatef and Khudier [82] who reported that the biosurfactants produced by *Pseudomonas putida* PS6 sp Ab9ES was composed of lipid (60.38%) carbohydrate (36.29%), and protein (3.32%). On the other hand, Thavas *et al*. [38] reported that the biosurfactant produced by *Pseudomonas* sp was composed of 50.2% protein, and 49.8% lipid.

CONCLUSION

Sixteen bacterial isolates were obtained from crude oil contaminated soil and screened for the production of biosurfactants. The biosurfactant producing bacterial isolates were characterized and identified as species of *Klebsella, Micrococcus, Bacillus*, *Staphylococcus*, *Pseudomonas*, *Corynebacterium, Acinetobacter* and *Comamonas.* However, the best two most effective isolates were characterized to molecular level. They were *Acinetobacter baumanni* and *Comamonas testosteroni, and* produced 1.2g/L and 0.06g/L respectively biosurfactants after 7 days. The biosurfactant produced by *Acinetobacter baumanni* was a lipopeptide, while the biosurfactant produced by *Comamonas testosteroni* indicated the biosurfactant was a glycolipid (rhamnolipid), both having high amount of protein, carbohydrate and Lipid.

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