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Research Article

Sport and Performance

Isolation, Screening, Characterization And Application Of Biosurfactant By *Achromobacter Xylos* Strain GSR21 Producing Bacteria From Hydrocarbons Contaminated Soil

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Abstract: Biosurfactants plays a key role in tertiary recovery (EOR), and production, excessive minerals discharge from water during petroleum refinery process, environmental utilization and eco-friendly. Biosurfactant producing bacteria found to be sufficient in hydrocarbon-polluted soil samples; it is expected to more amounts of agrichemicals contemporary in the clay. These bacteria establish itself soil and region specific. So, in this study we made an attempt to identify and characterize biosurfactant produced by *achromobacter xylos* strain GSR21 from hydrocarbon polluted soil in Andhrapradesh, India. A battery of biosurfactant screening methods engaged were haemolytic activity, oil spreading technique, lipase activity, emulsification index (E_{24}), emulsification assay, tilting glass slide, blood haemolysis test, drop-collapsed assay, and foaming activity. The organism isolate was studied based on molecular, phenotypic, and biochemical methods. Thin-layer chromatography I(TLC), Fourier transforms infrared spectroscopy (FTIR) and gas chromatography-mass spectrometry (GC–MS) investigates were used to identify and characterize the biosurfactant produced. The isolated biosurfactant was applied on chosen hydrocarbons to measure its emulsifying capacity.The phylogeny study of the 16S rRNA classified the isolate as A *chromobacter xylos* strain GSR21. The sequence secured from the isolate has been accumulated in GenBank covered by the accession number JQ746488. The result obtained from the study acknowledge high biosurfactant action with a maximum emulsification index (E_{24}) of 62 % compared to emulsification index (E_{24}) of 72% by sodium lauryl sulfate (SLS). Moreover, the biosurfactant appear emulsifying activity against the following hydrocarbons: diesel, methylbenzene, kerosene, dimethylbenzene, and petrol. The optimum cultural conditions (incubation time, carbon, pH, hydrocarbon, inoculum concentration, nitrogen, and temperature) for growth and biosurfactant produced by A *chromobacter xylos* GSR21 were analysed. The biosurfactant was characterized as a glycolipid using thin layer chromatography (TLC), while the gas chromatography-mass spectrometry (GC–MS) technique analyzed the glycolipid as dodecanoic acid-undecyl ester.Present study has exhibited the magnitude of *Achromobacter xylos* strain GSR21 isolated from hydrocarbon-polluted soil to produce biosurfactant and the effectiveness of the produced biosurfactant in emulsifying different hydrocarbons. Moreover, the biosurfactant produced was established to be held by the class, glycolipid based on the thin layer chromatography (TLC) and gas chromatography-mass spectrometry (GC–MS) analyses.

Keywords: *Achromobacter xylos* strain GSR21, Biosurfactant, Dodecanoic acid-undecyl ester, Hydrocarbon-polluted soil, and glycolipid.

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1. INTRODUCTION

Microbes those produce biosurfactant to be abundant in the environment; they occupy both water (new water, groundwater, and ocean) and land (soil, residue and slime). Moreover, they can be established in utmost circumstances (e.g., oil reservoirs) and develop at a comprehensive of salinity, temperature, and pH values[1]. I Moreover, they can be secluded from uninterrupted situations, where they have physical characteristics not necessitate the dissolving of aqua phobic poisons, for example, antimicrobial action, biofilm development or cycles of flexibility, and encampment of surfaces[2]. Although, hydrocarbon-degrading microbial neighbourhood remain the most reasonable condition for far reaching ability for biosurfactant production, Hydrocarbon-degrading bacterial community are mostly influenced by a few main categories, namely: *Actinobacteria, Klebsiella, Sphingomonas, Bacillus* and *Pseudomonas* in soils and powders, and *Acinetobacter, Alcanivorax, Halomonas*, and *Pseudoalteromonas* in marine biological systems[3]. It is not extraordinary so that a ton of biosurfactant or bioemulsifier makers have a place with these same categories. An assessment of the commonness of biosurfactant-creating strains inside a microbial culture cannot be simply resolved, as it builds upon the experimental methods used. It has been reported that 3–4 percent of screened communities in contemporary soils are biosurfactant-producing microorganisms. This number increases to 25 percent in polluted soils.[4-10]. In different circumstances, enhancement culture methods specific for hydrocarbon-degrading bacteria can show to a enough greater discovery of biosurfactant makers with assessment up to 85 percent[11-19] I. Biosurfactants produced by microbes are gathered into two various categories based on their different chemical constitution and relative molecular mass, viz., low relative molecular mass surface-active representatives called biosurfactants and high relative molecular mass biosurfactants referred to as bioemulsifiers. Examples of low relative molecular mass biosurfactants are the polymeric biosurfactant, lipopeptides and lipoprotein, crude biosurfactants, cholesterol, glycolipids, phospholipids, neutral lipids and while the high relative molecular mass biosurfactants are confidently of lipopolysaccharides, complex mixtures of these biopolymers, proteins, polysaccharides, lipoproteins. The outstanding applications of bioemulsifiers are the bioemulsio ns composed by various species of *Acinetobacter*[20-25].The various types of biosurfactant determined application in various industrial processes.The consideration accustomed to the creation of biosurfactants in modern days is primarily due to their potential applications in petroleum refineries, bioprocess industries, food preparing, pharmacology, beauty care products, oil investigation and abuse businesses, environmental circumstances, and agriculture[26]. One application of biosurfactant that is of intrigue to biotechnologist is in natural circumstances and natural removal. Biosurfactants I have been progressively authorized in the organic removal of unrefined petroleum dirtied destinations[27]. Glycolipids from *Pseudomonas aeruginosa* GN25 was used in the Haldia Petrochemicals oil slick in Malyavanthunipadu with 2% being enough to remove four times the oil on water at temperatures of 50°C and 90°C.In 1990, A strain of bacteria I accidentally Imported into Florida from the Middle East then spread to California where it is a very serious pest feeding on nearly all root crops and i poinsettias (oil eating bug) was created in the oil slick tidy up of the territory of Texas in the USA. This superbug was built initially by Dr.Anand Mohan Chakrabarty (Indian-born American) in 1979[27]. The bug which had the option to grow quickly and produce surface-dynamic substances that corrupt hydrocarbon was a half and half of *Pseudomonas putida*[28]. Different analyses with research center size of sand-stuffed segments and field preliminaries have effectively exhibited the viability of biosurfactants in tertiary **recovery** (MEOR)[29]. The application of biosurfactants in MEOR can be applied in two distinct ways as either an ex situ biosurfactant immunization or in situ biosurfactant production to reach an increase in oil recovery from underground pond[30-39]. ITwo of them are essential as the biosurfactants and their producing microbes are able to allow the rasping environmental circumstances, such as pressure , temperature, and high salinity. Even though there is unexpectedly lack of facts concerning the application of glycolipids biosurfactants, few researchers have described their application for oil recovery and environmental control and [40] exihibit that the character of biosurfactant, ethanol concentration, and quantity of the oil-to-water phase are the most important factors for processing and preserving dodecanoic acid-undecyl ester -based emulsions. Glycolipids are known to form major components of microbial membranes. Wiącek (2012) examined for the first time that analyze the accouterments of both polyelectrolyte ions and ethanol molecules on 1,2-dioleoyl-L-alpha-phosphatidylcholine hydrolysis by phosphorus. When certain hydrocarbon-degrading microbe cells or fungus are developed on paraffin substrates, the level of glycolipids increases greatly. For instance, using cetane-grown moraxellaceae family sp. HO1-N, glycolipid (mainly dodecanoic acid-undecyl ester) high saccules were produced[41]. Glycolipids have been quantitatively produced from *T. thiooxidans* that are responsible for wetting elemental sulphur necessary for development[42-45] . Dodecanoicacid-undecylester produced by *Torulopsis bombicola* grown on ester resulted in the lowering of surface tension between water and cetane to less than 2 mN m^{-1} and CMC of 20 mg L^{-1} I. In the present research paper, the isolation, screening, characterization, and application in hydrocarbon emulsification and removal of heavy metals from industrial effluents of biosurfactant by A *chromobacter xylos* strain GSR21 isolated from hydrocarbon-contaminated soil in Andhra pradesh, India.

1.1 Sample collection

The hydrocarbon polluted soil samples (0-15cm) used for bacterial isolation were obtained from the Tadepalli community of amaravathi local Government area (Guntur), located at a latitude of 16.5532° N"N and a longitude of 76.9301° E in the Andhrapradesh delta region of Amaravathi. For each dirt source, soil tests were haphazardly gathered from various focuses at profundities somewhere in the range of 0 and 25 cm utilizing a hand-held soil drill and afterward built to get a composite example. The examples were shipped aseptically in sterile polythene packs to the research centre for the examination. The examples were put away at encompassing temperature for additional utilization[46-50].

1.2 Isolation of bacteria

Sequential dilution changed into led with the aid of the method depicted IIby[46](Nandhini and Josephine 2013). Nine milliliters (9 mL) of ordinary saline (0.85 % NaCl in refined water) was initially administered into each spotless test tube, sanitized in an autoclave at 121 °C (15 psi) for 15 min and

permitted to cool. To plan stock arrangement, 10 g of the dry soil test was disintegrated in 90 mL of clean typical saline; from this stock arrangement 10^{-1} , 10^{-2} , 10^{-3} , 10^{-4} , 10^{-5} , and 10^{-6} dilutions were made. Hundred microliters (100 µl/0.1 mL) of 10^{-3}, 10^{-5} , and 10^{-6} dilutions were spread-plated on the MSM described by Techaoei et al. (2011), containing the following ingredients (in 1 L distilled H_2O): glycerol-5g; asparagines-1g; K_2 HPO_4-1 g; $MgSO_4.7H_2O$-5g; KCl-1.0 g; agar powder-15 g; and 1 mL of trace solution containing (in 1 L of distilled water) $MgSO_4.7H_2O$-0.5 g, $CuSO_4.5H_2O$-0.16 g, and $FeSO_4$. $7H_2O$-0.015g and incubated at 30 °C for 72 h. Morphologically distinct colonies were recognized and refined. The all out suitable cell check (TVC) was resolved. The bacterial confines were put away in MSM inclines and held under refrigerated condition (4°C) for additional studies.

1.3 Screening of biosurfactant-producing bacteria

The following screening methods used for obtaining biosurfactant-producing strains. Oil-spreading technique, tilted glass slide test, lipase activity, emulsification index (E_{24}), emulsification assay, and Haemolytic activity were employed. The selection of the biosurfactant producer was based on the ability of a given strain to give positive results in all the screening tests performed.

1.4 Haemolytic Activity

This is a qualitative-screening test for the detection of biosurfactant producers[47] (Satpute et al. 2010). Nutrient agar[48] (NA) supplemented with 5 % (v/v) fresh blood was used according to Banat 1993 and Carrillo et al. 11996; Reddy G.S. et.al.,2017). The plates were incubated at 37°C for 24 h. After incubation, the plates were then observed for the presence of clear zone around the colonies.

1.5 Oil Spreading Technique

Bacterial isolates were inoculated, separately, into sets of 10 ml of broth media and incubated at 30 °C for 72 h. Supernatants were collected by centrifuging culture media at 3000 rpm for 30 min. They will be used for the various biosurfactant screening tests. The oil spreading test was carried out by adding 1 ml of vegetable oil on the surface of 30 ml of distilled water (contained into a petri dish bottom). On the center of the oil layer, 10 µl of the culture supernatant were gently added, and the observations were recorded after 1 min. The presence of biosurfactant will cause oil displacement, and a clear zone will appear[49] . The displacement diameter was measured in (mm), known as oil-displacement activity. Replicates for each isolate were carried out, and a water drop was used as a negative control.

1.6 Lipase Activity Using Tributyrin Clearing Zone (TCZ)

The predominant bacteria in the nutrient agar plate were isolated and screened for lipolytic activity. Lipolysis is observed directly by changes in the appearance of the substrate such as tributyrin which are emulsified mechanically in various growth media and poured into a petri dish. The bacterial isolates were screened for lipolytic activity on agar plates containing tributyrin (1%, w/v), agar (2%, w/v), peptone(0.5g), beef extract(0.3g).Lipase production is indicated by the formation of clear halo zone around the colonies grown on tributyrin-containing agar plates[50]

1.7 Emulsification index (E_{24})

Emulsifying capacity was evaluated as an emulsification index (E_{24}). E_{24} of culture sample was determined by mixing 2 mL of kerosene and 2 mL of cell-free broth for 2 min and allowing the mixture to stand for 24 h. E_{24} was calculated by dividing the height of the emulsion layer by the mixture total height and then multiplying by 100[51] (Techaoei, S., et al 2011).

$$\%\text{Emulsification index}(E_{24}) = \left(\frac{\text{Total height of the emulsified layer}}{\text{Total height of the liquid layer}}\right) * 100$$

1.8 Emulsification Assay

A volume of 1 mL of the cell-free supernatant was added to 5 mL of 50 mM Tris buffer (pH 8.0) in a 30 mL screw-capped test tube. Crude oil was tested for emulsification activity. Crude oil (5 mg) was added to both layers and vortexed for 1 min and then the emulsion mixture was allowed to settle for 20 min. The optical density of the emulsified mixture was measured at 610 nm[52] . A negative control consisted of only buffer solution and crude oil with Triton X-100 was used as the positive control.

1.9 Tilting Glass slide Test

This technique is effectively a modification of the drop collapse method[52] I. Isolates were grown for 24 h on nutrient agar plates. A sample colony was mixed with a droplet of 0.85 % NaCl at one end of the glass slide. The slide was tilted and droplet observed. Biosurfactant producers were detected by the observation of droplet collapsing down[53] I

1.10 Foam height analysis

Foaming ability was determined according[58] to (Techaoei, S., et al 2011; Reddy G.S. et.al.,2018). Isolated strains was grown in 250 mL Erlenmeyer flask, containing 50 mL of nutrient broth medium. The flask was incubated at 33 °C on a shaker incubator (200 rpm) for 96 h. Foam activity was detected as the duration of foam stability, foam height and foam shape in the graduated cylinder.

1.11 Drop Collapse Assay

The wells of a polystyrene 96 well micro-plate lid (Corning Incorporated, United States) were coated with 2 µL of crude oil and left to dry for 24 h at 22°C. Filtered cell-free supernatant (5 µL) was transferred to the center of the oil coated well. The results were recorded after 1–2 min and considered positive for biosurfactant production when the oil drop was flat. Those that gave rounded drops were scored negative,that was an indication of the absence of biosurfactant production[59].

1.12 Blood hemolysis l

The hemolytic activity was the first screening test to identify biosurfactant producing bacteria[60].Blood agar plates were prepared by adding 5 ml sheep blood on 1 L nutrient agar medium. Freshly prepared colonies were streaked on blood agar plates and incubated for 72 h. at 30°C. The presence of greenish color or clear zone around the colonies (á- or â-hemolysis) indicating that the bacterial isolate under investigation may has biosurfactant production ability[61].

1.13 Penetration Assay

[62]R.Sumathi and N.Yogananth. (2016) developed another assay suitable for high throughput screening, the penetration assay. This assay relies on the contacting of two unsoluble phases which leads to a color change.For this assay, the cavities of a 96 well microplate are filled with 150 µl of a hydrophobic paste consisting of oil and silica gel. The paste is covered with 10 µl of oil. Then, the supernatant of the culture is colored by adding 10 µl of a red staining solution to 90 µl of the supernatant. The colored supernatant is placed on the surface of the paste. If biosurfactant is present, the hydrophilic liquid will break through the oil film barrier into the paste. The silica is entering the hydrophilic phase and the upper phase will change from clear red to cloudy white within 15 minutes. The described effect relies on the phenomenon that silica gel is entering the hydrophilic phase from the hydrophobic paste much more quickly if biosurfactants are present. Biosurfactant free supernatant will turn cloudy but stay red.

1.14 Optimization of cultural conditions for l biosurfactant production

The effect of various cultural conditions (incubation time, pH, temperature, nitrogen source, inoculum concentration, and carbon source) on the growth of the selected bacterial isolates, and the ability of the strain to produce biosurfactant was determined. The inoculum for the optimization used was first standardized using MacFarlane's standard. The optimum incubation time for growth and biosurfactant production by the selected strain was studied by varying the incubation time (24, 48, 72, 96, 120, 144, and 168 h) of the culture medium. The culture medium was inoculated with a 24 h culture broth containing a total viable cell count (TVC) of 9.8×10^6 cfu/mL of the selected isolate and incubated at 35 °C for 48 h in a rotary shaker incubator. Biosurfactant production was measured using E_{24}, while growth was determined using a spectrophotometer. The bacterial isolate was incubated at different temperatures (25, 30, 35, 40, and 45 °C) for 48 h, after which the biosurfactant production and growth of the strain were determined. The optimum pH for growth and biosurfactant production by the bacterial isolate was studied by varying the pH (5, 6, 7, 8, 9, 10, and 11) of the culture medium. After 48 h of incubation, biosurfactant production and growth were determined. The bacterial isolate was incubated with different carbon sources (dextrose, fructose, glucose, glycerol, starch, and sucrose) for 48 h, after which biosurfactant production and growth were determined. The bacterial isolate was incubated with different nitrogen sources (asparagine, NH_4NO_3, peptone, urea, and yeast extract) for 48 h, after which biosurfactant production and growth were determined.

1.15 Production of biosurfactant

The optimized parameters were used in setting up the biosurfactant production media. The production was carried out in a 500 mL Erlenmeyer flask containing 200 mL of the production.

1.16 Bacterial identification

Biochemical and phenotypic characterization was carried out on the positive biosurfactant-producing isolate using Bergey's Manual of Determinative Bacteriology as a guide[63] The identity of the selected isolate was confirmed based on 16S rRNA gene sequence analysis. Genomic DNA was isolated from the bacterial sample using Bacterial 16S rDNA PCR Kit Fast (800)-TAKARA. The universal primers of 16S rDNA fragments, 10F and 800R, were used to amplify the 16S rDNA. The sequences of primers were as follows: (10F) CAGTTGCATTTGGCAGACC and (800R) 5'TACCAGGGTATCTAATCC3' (Bioserve Biotechnologie,Hyderabad). A phylogenetic tree was constructed using partial 16S rRNA gene sequences of the isolate and the other sequences, closely related with the reference strain, obtained from NCBI database. Clustal Omega was used for multiple sequence alignment of sequences. Neighbor joining tree was constructed with complete deletion using bootstrapping at 10,000 bootstraps trials with Kimura-2 parameter using MEGA 6.0 software (Das and Tiwary 2013). The GSR 21 was finally identified as *Achromobacter xylosoxidans*. The sequence of the 16S rRNA gene of the strain GSR 21 is available in NCBI under the GenBank accession number MK079349.1

1.17 Preliminary classification of the biosurfactant

The following analyses: CTAB/methylene-blue agar test[64] (Siegmund and Wagner 1991; Kayganich and Murphy 1991); Biuret test[65] (Feigner et al. 1995); and phosphate test[66] (Okpokwasili and Ibiene 2006) were carried out to determine the class of the biosurfactant produced.

1.18 Thin-layer chromatography

The detection of glycolipid was done using the glycolipid–protein interaction specificity spray method described by Pablo H. H. Lopez and Ronald L. Schnaar (2008)[67]. Brownish copper (0.09 g) was placed in a solution of 0.45 g ammonium heptamolybdate in 2 mL of distilled water. The mixture was chilled and 2 mL of concentrated lactic acid added; the deep blue solution was then shaken. The reaction mixture was kept for 3 h at room temperature with occasional shaking. Fifty millilitres (50 mL) of distilled water were thereafter added and the content shaken; a colour change from deep blue to light brown was observed and noted. The brownish copper metal was then removed, and 4.4 mL of concentrated lactic acid was added; the resulting solution remained light brown. The solutions to be tested were applied on pre-coated thin-layer-plate silica gel (G-368 of 0.56 mm thickness) and sprayed with the reagent. The plate was then kept in an oven at 70–75°C for 10 min; it was removed and again sprayed with the reagent and kept for an additional 7–8 min in the oven. Glycolipids stained blue against a light blue background; all other compounds did not give any colour. Overheating produced a pink coloration of the cholesterol, which ultimately turned greenish grey against a light blue background. The plate was developed with chloroform–methanol–glacial acetic acid 65:15:5 (v/v/v), air dried, and then sprayed with the reagent. This procedure can detect as little as 1µg of glycolipids (Pablo H. H. Lopez and Ronald L. Schnaar 2008)[67].

1.19 GC–MS analysis

The partially purified glycolipid-biosurfactant fractions (15 mg) for the GC/MS analysis were saponified with 1 M NH_4OH, mixed with X9-BB and X7-FGH (internal standards), esterified with 4,4′-dibromooctafluorobiphenyl in *N*-Trimethylsilyl-*N*-methyl trifluoroacetamide, and extracted into isooctane. Thereafter, 2 µl of the extracted solution was injected into Agilent GC-MS 5975 (Agilent Technologies, UK), which was set to scan from m/z 60 to m/z 870 at a scan rate of 1.5 scans per second. The capillary column used was an Agilent technologies G3172A ms Ultra Inert (30 m × 0.531mm inner diameter; 0.35 µm film thickness) GC column. The oven temperature was programmed from 140 °C to 250 °C at 4°C min^{-1}. For the present study, , the temperature of the injector port was 230°C, while the transfer line temperature was 280°C. Helium was used as the carrier gas, with a constant flow rate of 2.0 mL/min.

1.20 Application of the biosurfactant on hydrocarbon emulsification

The biosurfactant produced was applied on various hydrocarbons (xylene, petrol, diesel, kerosene, and toluene) and the ability to emulsify these hydrocarbons determined using emulsification index.

1.21 Preparation of metal solutions

The protocol of samples were prepared by dissolving specific weight of metal salts (cadmium ($Cd(NO_3)_2.4H_2O$), lead ($Pb(NO_3)_2$), nickel ($NiCl_2.6H_2O$), barium (BaCl) zinc (ZnCl) and copper (Cl) in 1000 ml of deionized water to produce metal solution (500mg/L). The pH of each solution was measured using a pH meter .

1.22 Experimental design

Aliquots of 10 ml samples (500 mg/L) of the metal solution were transferred into four test tubes. From each test tube, 1 ml sample was replaced by either 1 ml of deionized water as control or with 1 ml of 20ppm, 40ppm or 80ppm concentrations of g lycolipids solution. Samples were then incubated at room temperature for one hour, prior to filtration through whatman filter paper using a buchner funnel. The concentration of the heavy metal in the filtrate was measured using an inductively coupled plasma optical emission spectrophotometer (ICP –OES vista –PRO, Libyan Petroleum Institute, Tripoli- Libya). All experiments were made in triplicate, and the average value was calculated and presented as a percentage of metal removed.The percentage of metal removed (η) was calculated based on the initial metal content (control) in the aqueous solution using the following equation:

$$\eta = \left(\frac{\text{Initial concentration of the heavy metal} - \text{Final concentration of heavy metals}}{\text{Initial concentration of the heavy metal}}\right)$$

2. STATISTICAL ANALYSIS

All of the experiments were accomplished three times and studied in triplicate. Experimental results represent ed the mean ± standard deviation. One-way analysis of variance (ANOVA) with the least significant difference (LSD) test was conducted to analyze the significant differences in hydrocarbon degradation efficacy of the bacterial strain at different time periods. SPSS ver.18 software (Chicago, IL) was used to carry out the statistical analysis.

Physiochemical analysis of the soil sample

The physical and chemical properties of the soil sample are represented in Table 1. The hydrocarbon-polluted soil had a pH of 6.2 ± 0.1. The temperature of the soil was 27.5 ± 0.4°C. The soil types ranged from humus soil to humus soil mixed with crude oil, and the TPH (mg/kg) value of the soil was 9528.

Table 2: Physiochemical properties of soil samples

Parameter	Hydrocarbon polluted soil (HPS)
pH	6.2 ± 0.1
Temperature	27.5 ± 0.4 °C
Type of Soil	humus soil mixed with crude oil
TPH (mg/kg)	9528

Values are mean±SD; (n=6) P<0.01 when compared with control

Screening and selection of the biosurfactant producers

Out of the 45 bacterial isolates screened, four isolates were selected as biosurfactant producers based on their ability to give positive results to all the screening methods employed. From the four biosurfactant-producing bacteria, the isolated GSR-21 was chosen for optimum and results are represented in Table 3 and 4.

Table 3: Comparison of screening characteristics of isolate GSR-21 with other biosurfactant-producing bacterial isolates

Isolate codes	Source of sample	Lipase Test (mm)	Oil spreading test (mm)	Emulsification assay (@400nm)	Emulsification index (E_{24} %)
GSR-2	TPH	15±2	31.3±0.89	0.6135±0.0034	13.1±2.0
GSR-15	TPH	10±2	21.6±0.88	0.6185±0.0024	43.5±1.0
GSR-21	TPH	13±1	88.6±0.89	0.5030±0.003	48.7±1.0
GSR-39	TPH	18±2	179.9±4.15	0.4330±0.002	28.1±2.0

Table 4: Comparison of screening characteristics of isolate GSR-21 with other biosurfactant-producing bacterial isolates

Isolate codes	Source of sample	Foaming activity	Blood hemolysis	Drop collapsing test	Tilting glass	Haemolytic
GSR-2	TPH	+	γ hemolysis	+	+	5±2.0
GSR-15	TPH	+	γ hemolysis	+	+	14±2.0
GSR-21	TPH	+	γ hemolysis	+	+	7±2.0
GSR-39	TPH	+	γ hemolysis	+	+	3±0.6

Optimization of cultural conditions for enhanced biosurfactant production

From the results obtained, the optimum incubation time for both growth and biosurfactant production was 48 and 120 h with the OD (optical density) reading of 1.7300 ± 0.013 and E_{24} value of 22.00 ± 1.61 %, respectively. The result of the effect of incubation time on growth and biosurfactant production is presented in Fig. 1a.

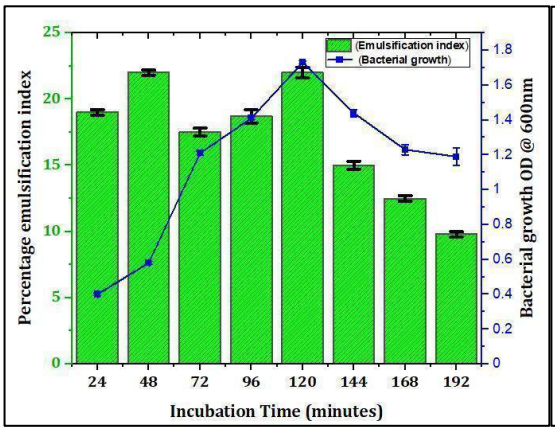

Fig 1a: Effect of incubation time

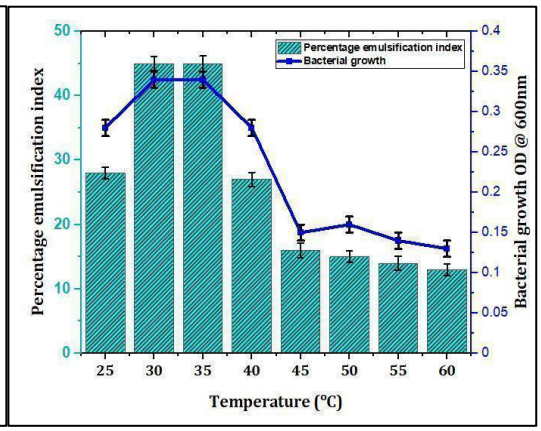

Fig 1b: Effect of Temperature

The effect of different incubation temperatures on growth and biosurfactant production showed the optimum incubation temperatures as 30 and 35°C for growth and biosurfactant production by the bacterium, respectively (Fig. 1b). The impact of various pH esteems on development and biosurfactant generation demonstrated the ideal pH as 7 and 8 for development and biosurfactant creation, respectively. The optimum pH Optimcal density was 0.6100 ± 0.005, while the optimum pH for biosurfactant production had E_{24} of 29.0 ± 2.41 %. Figure 1c shows the results of the pH optimization for growth and biosurfactant production.

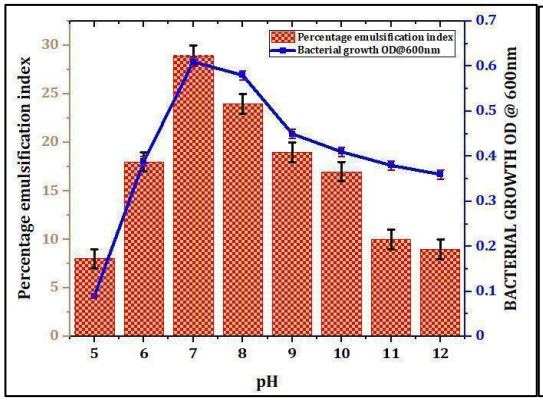

Fig 1c: Effect of pH

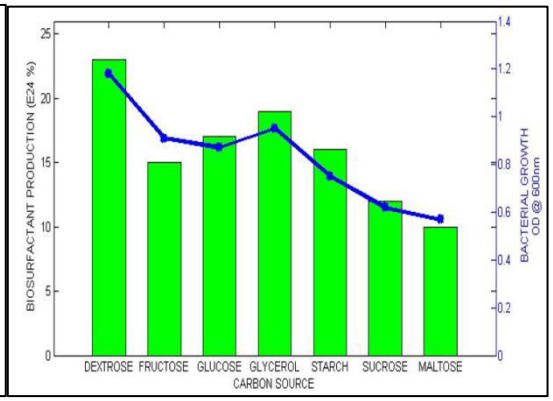

Fig 1d: Effect of carbon source

Figure 1d shows the effect of different carbon sources on the growth of the bacterial isolate and ability to produce biosurfactant. The result obtained shows that glycerol had the highest effect on bacterial biomass. Meanwhile, dextrose had the best effect on the production of biosurfactants by the bacterial strain with E_{24} of 23.00 ± 1.41 %. Figure 1e shows that NH_4NO_3, as a nitrogen source, had the best effect on the production of biosurfactant by the bacterial strain, while asparagine had the highest effect on bacterial growth with the OD reading of 1.4100 ± 0.021.

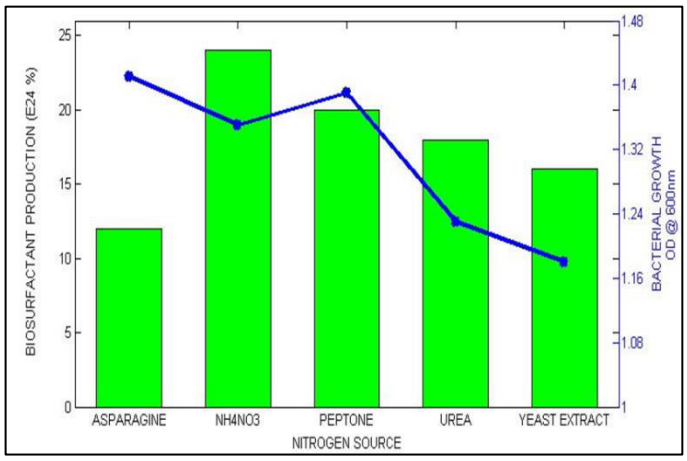

Fig 1e: Effect of nitrogen source on bacterial growth and biosurfactant production

Identification of the isolate

Phenotypic and biochemical characteristics placed on the isolate (GSR-21) in the family *Anchromobacter* having a place with the phylum, Proteobacteria, class, Beta proteobacteria; arrange, Burkholderiales , and family, Alcaligenaceae (Table 5). The phylogenetic investigation based on the 16S rRNA gene of the sequence generated from the isolate classified the isolate as *Achromobacter xylos* strain GSR21 (Figs. 2, 3). The sequence has been deposited under the accession number, JQ746488.1.

Table 5. Biochemical characteristics of the biosurfactant producing isolate

Characterization Tests	**GSR-21**
Colony shape	Circular
Colour	White
Gram staining	-(rods)
Citrate	+
Shape	Short rod
Arrangement	Single
Motility	+
Oxidase	+
Catalase	+
Indole	-
Urease	-
MR	-
VP	-
Starch hydrolysis	-
Gelatin hydrolysis	+
Sugar fermentation	
Maltose	-
Dextrose	+
Lactose	-
Mannitol	-
Xylose	-
Arabinose	-
Sucrose	-
Raffinose	-
Celloboise	-
Sorbitol	-
Fructose	-
Probable genus	***Achromobacter xylos***

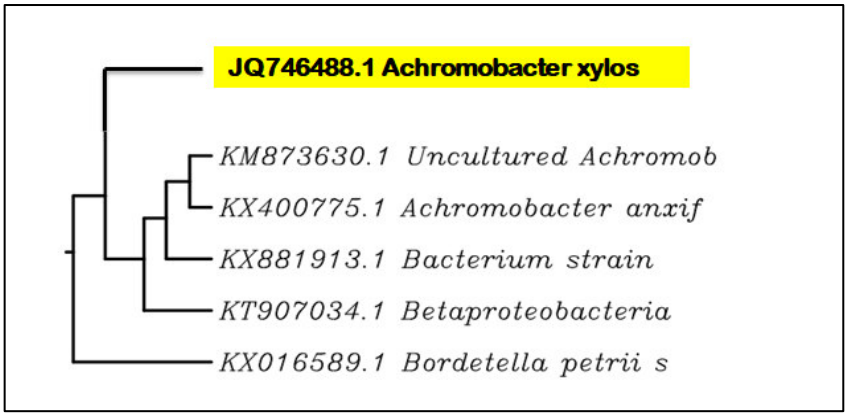

Fig. 2 Neighbour-joining phylogenetic tree of isolated GSR21 made by MEGA 6.0 (Tamura et al.2013). Bootstrap values of >50% (based on 100 replicates) are given in the nodes of the tree. Nucleotide substitution model used jukes and cantor.

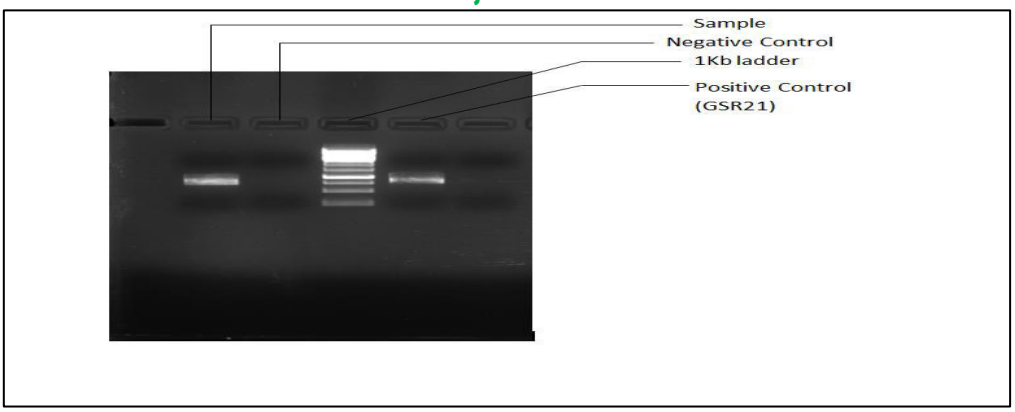

Fig. 3 PCR amplification images of the 16S rRNA gene bands of the biosurfactant-producing bacterium

Characterization of biosurfactant produced

The preliminary analyses of the biosurfactant placed it in the class glycolipids (Tables 6, 7). Furthermore, the result of the thin-layer chromatography showed that the biosurfactant produced, belonged to the class glycolipids (Figs. 4, 5), whereas the GC–MS analysis identified the glycolipids, dodecanoic acid undecyl ester ($C_{23}H_{46}O_2$) with molecular weight (MW) 354 g/mol as the most abundant component (Fig. 5). The components of the cell-free broth are presented in Table 8, and they include: esters, dodecanoic acid undecyl ester, with fatty acids such as palmitic acid and oleic acid.

Table 6: Physicochemical characterization of the biosurfactant produced by *Achromobacter xylos* strain GSR21

Material	Surface tension (mN/m)	Emulsification assay (@400 nm)	Emulsification index (E24) %	Tilting glass slide test	Oil-spreading test (mm^2)
DH_2O	71 ± 0.9	-	-	-	-
Partially purified bio surfactant	35 ± 0.5	0.5424±0.002	62±2.0	+	261±6.2
SDS	35 ± 0.8	0.6114±0.003	72±1.8	+	285.5±3.15

Table 7: Preliminary result showing the class of the biosurfactant produced

Biosurfactant Test	Biosurfactant aimed at detecting	Result
Biuret test	Rhamnolipid	-
CTAB/methylene- blue agar test	Glycolipid biosurfactant	+
Phosphate test	Phospholipids	-

Table 8: Composition of the cell free broth			
Sample	**Amino acids**	**Fatty acids**	**Others**
Cell free broth	Arginine	Oleic acid	Dodecanoic acid
	Leucine	Palmitic acid	undecyl ester
	Glycine		

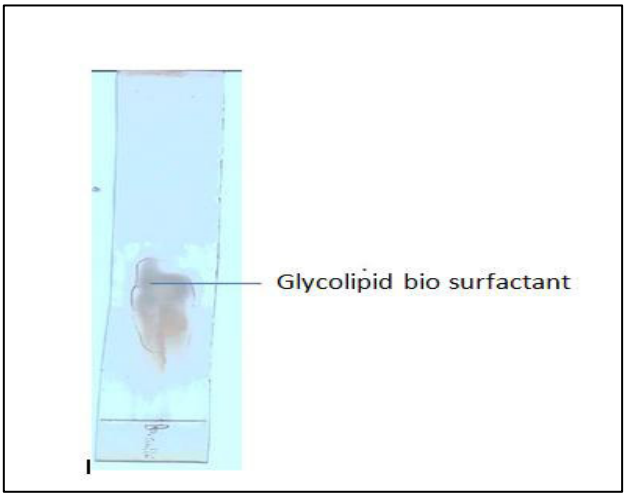

Fig. 4 Glycolipid produced by the *A chromobacter xylos* GSR21 on thin layer plate (*Light brown* colouration visible on the plate is an indication of the presence of glycolipids)

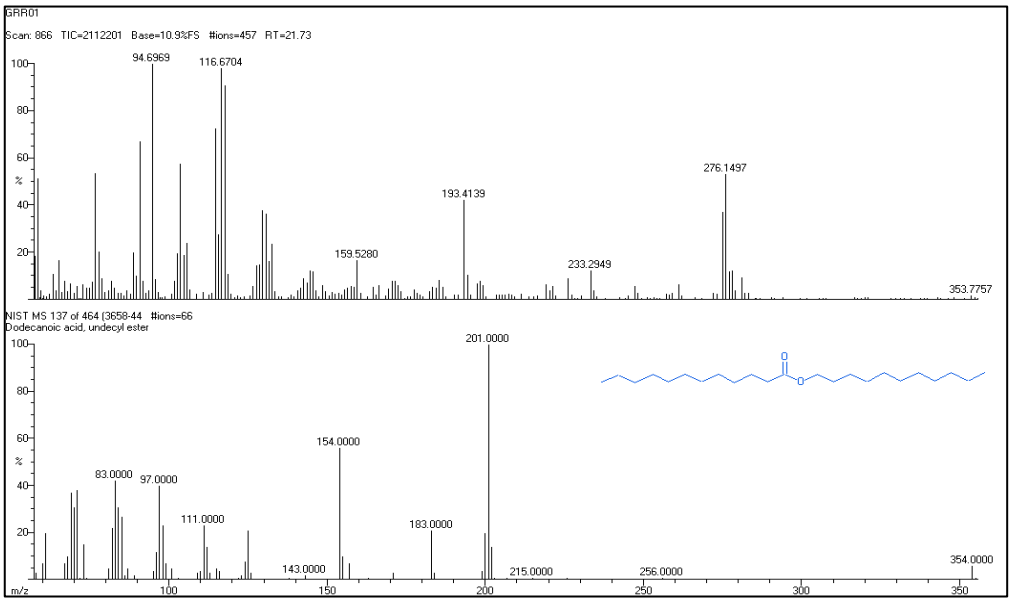

Fig. 5 Mass spectrum of partially purified glycolipid-biosurfactant produced by Achromobacter xylos GSR21 using silica column chromatography (Dodecanoic acid-undecyl ester; MW: 354)

Fourier transforms infrared spectroscopy

Infrared spectroscopy is the investigation of infrared light with the issue [12]. The essential estimation acquired in infrared FTIR spectroscopy is in the infrared range, which is a plot of estimated infrared force versus wavelength (or wavenumber in cm^{-1}). FTIR spectroscopy is delicate to the nearness of substance practical gatherings in the readied test. A basic gathering is an auxiliary part inside an atom.

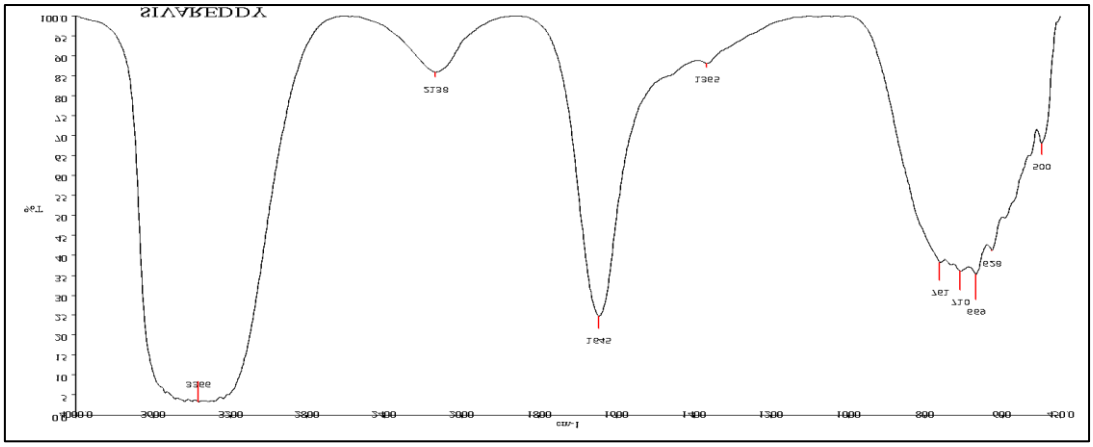

Fig.6: FTIR Spectrum of partially purified biosurfactants produced by *Achromobacter xylos* GSR21.

Application of the biosurfactant on hydrocarbon emulsification

When the biosurfactant produced was applied on different hydrocarbons, it showed varying degrees of emulsification. In addition, the biosurfactant-producing bacterium was able to grow on the different hydrocarbons. The highest emulsification was observed with kerosene, while the least emulsification was observed with kerosene (Fig. 7). Furthermore, the hydrocarbon that supported the growth of the isolate mostly was petrol, while diesel had the least support for the growth of the isolate (Fig. 8).

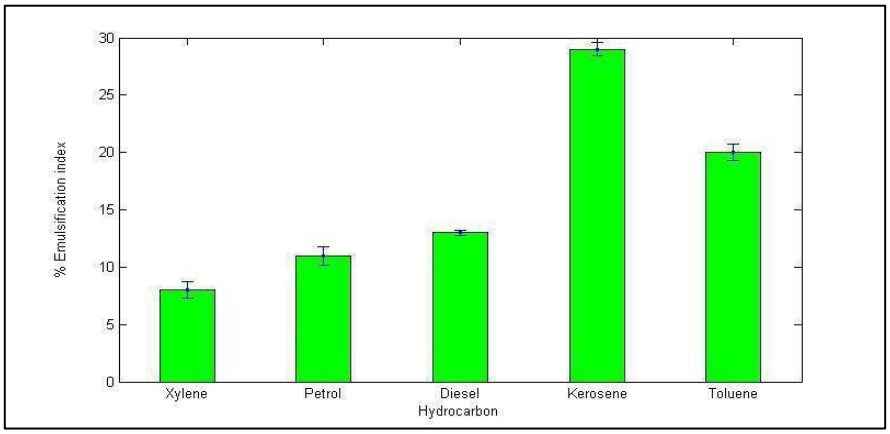

Fig.7: Emulsification of different hydrocarbons by the bio surfactant produced by *Achromobacter xylos* GSR21

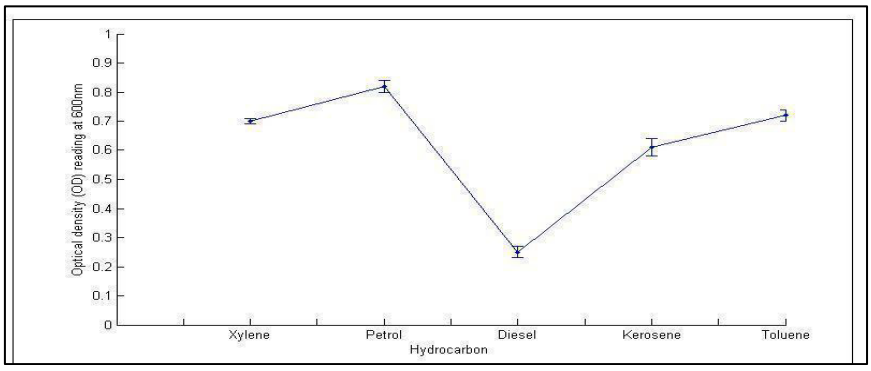

Fig.8: Effect of different hydrocarbons on the growth of *Achromobacter xylos* GSR21.

Compared to control, a significant reduction in the concentration of metal was observed after the addition of biosurfactant (Glycolipid). A significant decrease in Cadmium and Lead concentration was high with glycolipid at 90ppm concentration compared to 30 and 60 ppm concentrations.

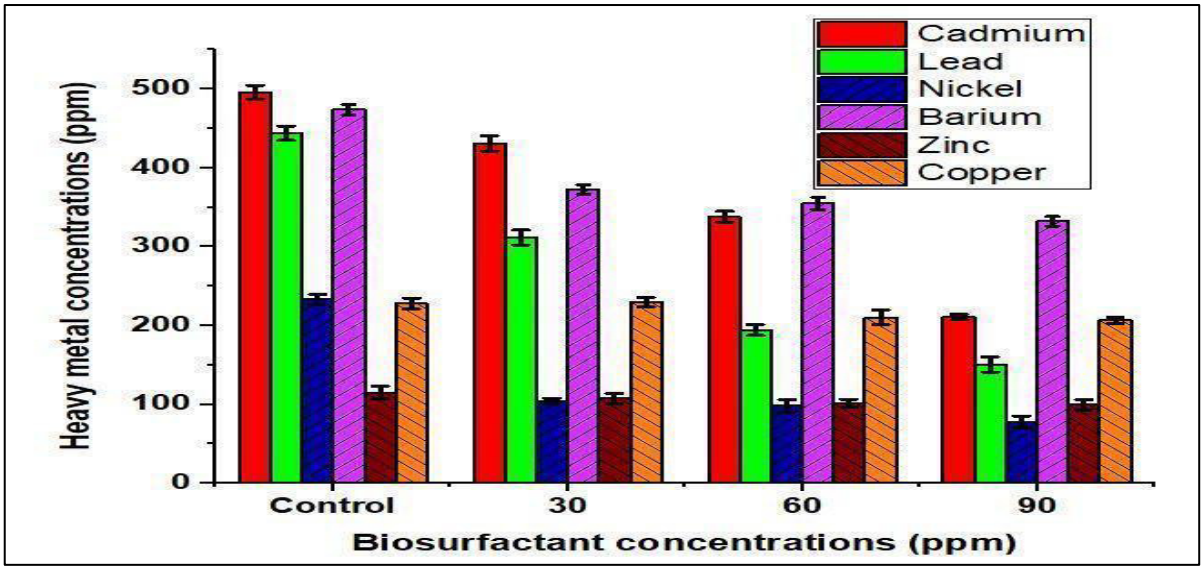

Fig.9. Effects of glycolipid concentration on the removal of heavy metals compared to control.

Glycolipid at 90 ppm was found to be a superior adsorbent in removing cadmium and lead from aqueous solution. Fig.10 shows the percentage efficiency of bio-surfactant in reducing metals contamination from water.

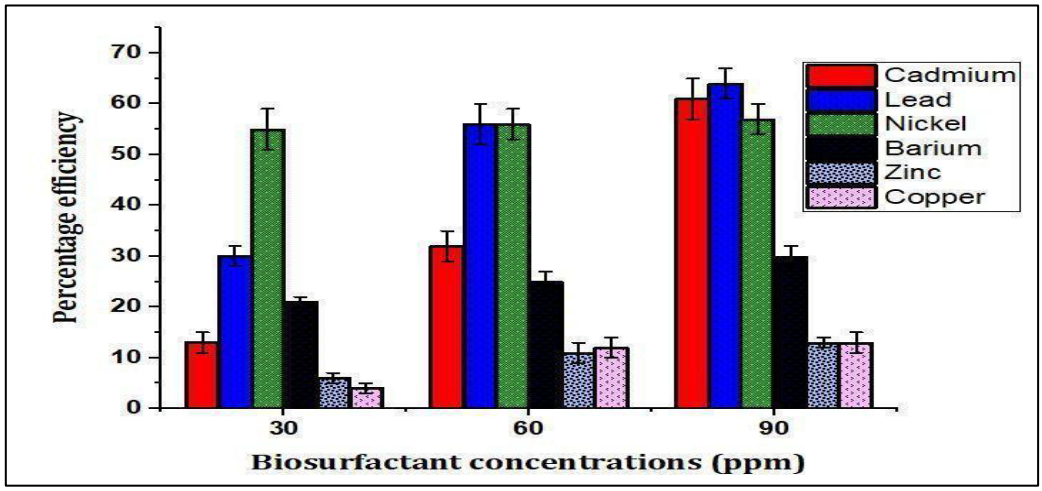

Fig.10. The desorption efficiency (%) of heavy metals by Glycolipid.

The desorption efficiencies achieved with glycolipid at 90ppm were 61% and 64% for cadmium and lead respectively. Glycolipid has also shown a significant effect in reducing the Nickel concentration. Glycolipid with a concentration of 30 ppm, had led to a desorption efficiency of 55% for nickel, however, no significant change after concentration of glycolipid was increased to 60ppm and 90ppm. The desorption efficiencies of glycolipid on barium, zinc and copper at concentration of 90 ppm were 29%, 13% and 13% respectively. In the case of zinc, no effect was shown probably due to the pH of metal solution (≤ 4) on glycolipid action that was less than the optimum pH of working glycolipid is 7.2. For barium, the highest effect was observed in glycolipid concentration of 90ppm while for copper, the highest effect was observed in glycolipid concentration of 60 ppm.

3. DISCUSSIONS

This study evaluated the isolation, characterization, and application of glycolipid by ***Achromobacter xylos*** **strain GSR21** isolated from hydrocarbon polluted soil in Andhrapradesh, India. Baseline physicochemical parameters of the soil sample from which the biosurfactant-producing bacterium was isolated revealed a hydrocarbon-contaminated soil. Many studies have reported the isolation and distribution of biosurfactant producing bacteria in hydrocarbon-polluted sites[68]. Although biosurfactant-producing bacteria are ubiquitous in nature, they are mostly found in hydrocarbon-contaminated environments.The screening methods employed were emulsification assay, emulsification index (E_{24}), lipase activity, haemolytic assay, oil spreading, and tilted glass slide. These methods have been previously reported for the identification of biosurfactant-producing bacteria: tilted glass slide[69], haemolytic assay[70], emulsification assay[70], lipase activity[70], oil spreading[70], and emulsification index[70]. The isolates screened in this study showed varying results for the different screening methods.The biosurfactant-producing bacterium was selected based on its ability to give positive results to all the screening methods. Haemolytic assay, tilting glass slide, and lipase are qualitative-screening techniques, while emulsification index and oil-spreading technique are both qualitative and quantitative techniques[70]. The use of these techniques is similar to the report of Satpute et al. (2008), who used the combination of oil spreading, drop collapse, tilted glass slide,

and emulsification index to select biosurfactant producers.[65-70]suggested that a single method is not suitable to identify all the types of biosurfactants, and recommended the combination of methods. In addition, Chandran and Das (2011) used different screening methods, such as emulsification capacity, oil-spreading assays, hydrocarbon overlaid agar, and modified drop collapse methods to detect biosurfactant production. Deepika and Kannabiran (2010) [66-70] reported the confirmation of biosurfactant production by the conventional screening methods, including haemolytic activity, drop collapsing, and lipase production activity. The effect of incubation time (24, 48, 72, 96, 120, 144, 168 and 192 h) on the ability of the test isolate to grow well and produce biosurfactant was investigated in this study. The optimum biosurfactant production (22.00 ± 1.61 %) was observed after 48 h (2 days) of incubation time. The value (22.00 ± 1.61 %) obtained for biosurfactant production after 48 h was similar to that obtained after 120 h (5 days) of incubation. However, the optimum growth (1.7300 ± 0.013) was observed after 120 h (5 days) of incubation. This result is similar to that obtained by Nwaguma et.al, (2016) who reported optimum growth and biosurfactant production after 96 h of incubation with *Klebsiella pneumoniae* strain IVN51. Optimization of the cultural temperature of ***Achromobacter xylos* strain GSR21** showed the highest biosurfactant production (45.0 ± 1.63 %)) and growth (0.3470 ± 0.003) at temperatures 30 and 35 °C, respectively, after 48 h of incubation. Similar results have been reported by several authors. Nwaguma et.al, (2016) reported maximum biosurfactant production at the temperature of 30 and 35°C for *Klebsiella pneumoniae* strain IVN51 isolated from oil contaminated soil samples. At temperatures less than or greater than 30 and 35°C, the isolate showed lower biosurfactant-producing ability. Different bacterial species produce biosurfactants at different temperature ranges. However, most of them produce at a temperature range of 30–37 °C (Chander et al. 2012). Youssef et al. (2004) [70]reported that a change in temperature can cause alteration in the composition of biosurfactants. The result of pH optimization for growth and biosurfactant production by ***Achromobacter xylos* strain GSR21** is consistent with that obtained by [66-70] Nwaguma et.al, (2016), Hamzah et al. (2013). Hamzah et al. (2013) reported maximum biosurfactant production by *Pseudomonas aeruginosa* UKMP14T. In addition, Gumaa et al. (2010) [69-70] obtained maximum biosurfactant production at pH 8 and maximum biomass at pH 9 with *Serratia marcescens* N3. Nwaguma et.al, (2016) reported maximum biosurfactant production by *Klebsiella pneumoniae* strain IVN51. The result showed that while maximum biosurfactant was achieved at neutral pH, the bacteria grow best at slightly alkaline pH. Studies [67-70] have reported the effect of pH on biosurfactant production by bacteria. Meanwhile, Mata-Sandoval et al. (2001), Al-Araji and Issa (2004), Rashedi et al. (2005), and Kannahi and Sherley (2012) reported maximum biosurfactant production at pH below 7. The effect of different carbon sources (dextrose, fructose, glucose, glycerol, starch, and sucrose) on biosurfactant production and the growth of ***Achromobacter xylos* strain GSR21** investigated in this study revealed that the maximum biosurfactant production was obtained when grown in a mineral salt medium amended with dextrose; maximum growth (1.1810 ± 0.003) was achieved with glycerol as the carbon source. Although the isolate was able to grow in the presence of other carbon sources, dextrose and glycerol gave the highest result for biosurfactant production and growth, respectively.Nitrogen plays an important role in the production of surface-active compounds by microorganisms (Mercade et al. 1996). The effect of different nitrogen sources (asparagine, NH_4NO_3, peptone, urea, and yeast extract) on the biosurfactant production and growth of ***Achromobacter xylos* strain GSR21** was studied. There are observations that different nitrogen sources can stimulate biosurfactant production by some microorganisms. The result showed maximum biosurfactant production when grown in a mineral salt medium amended with NH_4NO_3 and maximum growth (1.3500 ± 0.011) when grown in a mineral salt medium amended with asparagine. This finding is similar to that obtained by Nwaguma et.al, (2016) and shekhawat et al. (2014), who reported maximum biosurfactant production and growth of *Klebsiella pneumoniae* strain IVN51 and *Bacillus* sp. with NH_4NO_3 as a source of nitrogen. Other researchers have reported maximum biosurfactant production with other nitrogen sources. [70]Hamzah et al. (2013) reported maximum biosurfactant production by *Pseudomonas aeruginosa* UKMP14T with $(NH_4)_2SO_4$ as the nitrogen source. Similar results were obtained by Karkera et al. (2012) for *Pseudomonas aeruginosa* R2, and the optimum nitrogen source was found to be NH_4NO_3 (0.4 %). [70]Patil et al. (2014) reported KNO_3 as the optimum nitrogen source for biosurfactant production. The difference observed in the production of biosurfactants when ***Achromobacter xylos* strain GSR21** was grown in the presence of different nitrogen sources may be due to the preferential demand for a particular nitrogen source for growth and secondary metabolites production by the bacterium.Preliminary performance of the biosurfactant carried out, excluded the presence of glycolipids, rhamnolipids and lipopeptide, with a positive result for glycolipids using CTAB/methylene- blue agar test. CTAB/methylene- blue agar test test has been applied by [70]Nwaguma et.al, (2016) in determining the presence of glycolipid biosurfactants. They reported that the formation of blue colour, which may be followed by slow formation of a fine blue precipitate on reaction of 5 % ammonium molybdate and 6 M HNO_3 with the biosurfactant extract, indicated the presence of glycolipid biosurfactant. The thin-layer chromatographic analysis of the crude biosurfactant confirmed that the biosurfactant was of the glycolipid class. The isolation of phospholipids from ***Achromobacter xylos*** has been reported (Jamal et al. 2011); however, they did not identify the type of glycolipid responsible for the biosurfactant activity. This study went further to identify the type of glycolipid-biosurfactant. The GC–MS analysis carried out on the partially purified biosurfactant showed that the glycolipid-biosurfactant produced by the isolate was dodecanoic acid undecyl ester with molecular weight (MW) 354. Dodecanoic acid undecyl ester is the most abundant membrane glycolipid in many prokaryotic cells. The glycolipid-biosurfactant produced by the isolates showed emulsification properties against a wide range of hydrocarbons. Other researchers have reported glycolipid-biosurfactant production and the effect of certain conditions on the emulsifying capacity[67-70]. The GC–MS analysis of the cell-free broth revealed that it contained the following components: esters, dodecanoic corrosive undecyl ester, with unsaturated fats, for example, palmitic corrosive and oleic corrosive. These components have been associated with glycolipid biosurfactant[70] (Adamu et al. 2015).The application of the glycolipid-biosurfactant produced in this study against different hydrocarbons showed varying degrees of emulsification against the tested hydrocarbons. There is

dearth of information on the emulsification ability of different hydrocarbons by biosurfactants. This study has provided information on the emulsification capacity of the glycolipid-biosurfactant produced. The biosurfactant showed higher emulsification activity against straight chain hydrocarbons when compared with aromatic and cyclic-aromatic hydrocarbons. Moreso, lighter crude oil portions (petrol), supported the growth of the bacterium more than heavier portions (diesel). Emulsification capacity may be important in the bioremediation of crude oil contaminated environments. The use of biosurfactants for the bioremediation of hydrocarbon contaminated soil has been widely studied[65-71]. The isolate Achromobacter *xylos* GSR21 used in this study showed similarity with the following Genebank isolates: Achromobacter *xylos* JQ746488, 98 %; *Anchromobacter anxiety* KX400775, 97 %; *Bacterium strain* KX881913, 97 %; *Betaproteobacteria* KT903074, 97% *Bordetella Petris* KX016589,98%. The production of biosurfactants by Achromobacter *xylos* has been reported[66-70], although not widely. This work has, therefore, further validated the production of biosurfactants from Achromobacter *xylos* GSR21. The safety aspect of the isolate used in this study was taken into consideration. As a result of C–H stretching vibrations and N–H stretching vibrations, a broad absorbance peak (centred around 3366 cm^{-1}) with wave numbers ranging from 3800 cm^{-1} to 3000 cm^{-1} was observed (Figure 6). This is typical of carbon-containing compounds with amino groups. Sharp absorbance peaks are observed at 2138 cm^{-1}, 1645 cm^{-1}, and 1365 cm^{-1}, and are indicative of ester carbonyl chains (C = O in COOH). These peaks reflect the presence of alkyl chains in the compound. A strong band was also observed at 1645 cm^{-1}. This is due to a carbonyl group. The presence of C=O bonds causing C=O stretching vibrations leads to absorbance peaks in these regions. The FTIR spectrum implies the production of a glycolipid biosurfactant and results are represented in figure.6. Bioremediation of industrial wastes containing heavy metals has been demonstrated by several biotechnology companies employing bioaccumulation[70]. Biosorption, bioprecipitation, and uptake by purified biopolymers derived from microbial cells provide alternative and/or additive processes for conventional physical and chemical methods. Intact microbial cells live or dead and their products can be highly efficient bioaccumulators of both soluble and particulate forms of metals[70]. Various microbial species, mainly *Anchromobacter xylos*, have been shown to be relatively efficient in bioaccumulation of uranium, copper, lead and other metal ions from polluted effluents, both as immobilized cells and in the mobilized state for example, *Acinetobacter* RAG-1 was found to bind up to 240 µg uranium (UO22+)/mg emulsion[70]. Similarly, a *Pseudomonas* exopolysaccharide bound up to 96 µg uranium/mg exopolymer[66-70]. The cell surfaces of all microorganisms are negatively charged owing to the presence of various anionic structures[70]. This gives bacteria the ability to bind metal cations[70]. A study of *Cadmium-Arthrobacter exopolysaccharide* complexation showed that cadmium binding (3.3 µg/mg exopolymer) was significantly less than that of uranium[70]. Other study of several marine *Pseudomonas sp*. Exopolysaccharides showed complexation of copper, iron, lead, nickel, and zinc[70]. There is a little information about the effect of bio-surfactant-metal interactions on metal structure. Our earlier work has concluded that the isolated bacteria from the soil of local site were found to have the ability of producing the biosurfactant (Glycolipid) in the form of biological molecules[70]. This study presents experimental results that evaluate the capability of glycolipids and their ability on enhancing removal of heavy metals, in the water systems contaminated with heavy metals. The goal of the addition of a bio-surfactant may promote desorption of heavy metals from contaminated water through complexation of the free form of the metal residing in solution. This decreases the solution-phase activity of the metal resulting in direct contact between the bio-surfactant and the sorbed metal and, therefore, promotes desorption. Clearly, bio-surfactant structure size and charge will affect movement of bio-surfactant-metal complexes. In addition, structure size and charge will also affect the access of biosurfactants to filter pores[65-70]. Therefore the glycolipid solution pH was optimized to minimize the size of the metal–ligand complex. The size of glycolipid aggregates is pH dependent and they are predominantly small vesicles and micelles at pH > 6.0. The use of this technique allowed significant values of removal rates of cadmium, lead, and nickel. The removal efficiency of barium, zinc and copper were not completely encouraging, may be due to some errors related to the use of glycolipid concentration. Hence, the evolution of glycolipid effective concentration in barium, zinc and copper, the removal is a matter of investigation.

4. CONCLUSION

This study has shown that the bacteria *Anchromobacter xylos* starin GSR21 isolated from hydrocarbon-polluted soil was capable of producing biosurfactant and that the biosurfactant produced was a glycolipid based on the result obtained from GC–MS analyses. The biosurfactant produced was also found to demonstrate emulsification activity against the following hydrocarbons: xylene, kerosene, petrol, diesel, and toluene, a feature, which is attractive for application in the biodegradation of petroleum hydrocarbons. In addition, it was observed that temperature, pH, incubation time, carbon sources, and nitrogen sources all impacted on the ability of the isolate to produce biosurfactant. The result of the optimization process can be useful in enhancing the production of surface-active agents, making them attractive options for application at industrial level. The conclusions drawn from this study are that; Bio-surfactant addition to heavy metals contaminated water at concentrations above their CMC values generally enhance desorption of heavy metals. Using an inductively coupled plasma optical emission spectrometer established that 980 ppm of glycolipid can reduce up to 61% of cadmium, 64% of lead, and 57% of nickel respectively. Little effect (≤ 30% desorption efficiency) of glycolipid on barium, zinc and copper removal was observed. The effect of glycolipid on barium, zinc and copper suggest further research to be conducted.

5. AUTHORS CONTRIBUTION STATEMENT

Golamari Siva Reddy ,Nadeem Siddiqui conceived of the presented idea. Golamari Siva Reddy and , Pulapa Sahitya ,Kotari Ayyappa ,Tumu Teja , Kotharu Sivaramakrishna Akhil ,Mundunuri Venkata Satyanarayana Raju , Mallu Maheswara Reddy , N Konda Reddy, Varakala Nikhil Reddy,Divyansh Dhakate and Venkata Ramana Avula developed the theory and performed the computations.G Siva Reddy verified the analytical methods. G Siva Reddy encouraged Pulapa Sahitya ,Kotari Ayyappa ,Tumu Teja , Kotharu Sivaramakrishna Akhil ,Mundunuri Venkata Satyanarayana Raju to investigate design expert software for medium component analysis] and supervised the findings of this work. All authors discussed the results and contributed to the final manuscript. All the

authors read and approved the final version of the manuscript. discussed the results and contributed to the final manuscript. All the authors read and approved the final version of the manuscript.

6. CONFLICT OF INTEREST

Conflict of interest declared none.

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