

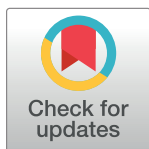
## RESEARCH ARTICLE

# Isolation and chemical characterization of the biosurfactant produced by *Gordonia* sp. IITR100

Arif Nissar Zargar<sup>1</sup>, Sarthak Mishra<sup>1</sup>, Manoj Kumar<sup>2</sup>, Preeti Srivastava<sup>1\*</sup>

**1** Department of Biochemical Engineering and Biotechnology, Indian Institute of Technology Delhi, New Delhi, India, **2** Indian Oil Corporation, R&D Centre, Faridabad, India

\* [preeti@dbeb.iitd.ac.in](mailto:preeti@dbeb.iitd.ac.in), [preetisrivastava@hotmail.com](mailto:preetisrivastava@hotmail.com)



## Abstract

Biosurfactants are amphipathic molecules produced from microorganisms. There are relatively few species known where the detailed chemical characterization of biosurfactant has been reported. Here, we report isolation and chemical characterization of the biosurfactant produced by a biodesulfurizing bacterium *Gordonia* sp. IITR100. Biosurfactant production was determined by performing oil spreading, drop-collapse, Emulsion index ( $E_{24}$ ), and Bacterial adhesion to hydrocarbons (BATH) assay. The biosurfactant was identified as a glycolipid by LCMS and GCMS analysis. The chemical structure was further confirmed by performing FTIR and NMR of the extracted biosurfactant. The emulsion formed by the biosurfactant was found to be stable between temperatures of 4°C to 30°C, pH of 6 to 10 and salt concentrations up to 2%. It was successful in reducing the surface tension of the aqueous media from 61.06 mN/m to 36.82 mN/m. The biosurfactant produced can be used in petroleum, detergents, soaps, the food and beverage industry and the healthcare industry.

## OPEN ACCESS

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## Introduction

Biosurfactants are a biologically-produced group of structurally diverse amphipathic molecules capable of assembling at the interface between two phases with degrees of polarity differing from each other [1]. These interfaces may be liquid/liquid (oil-water), liquid/gas (water-air) or solid/liquid (metal-water) [2]. The amphipathic nature of biosurfactants makes them proficient in forming microemulsions by reducing the interfacial tension between two phases and therefore enhances solubility of one phase in another [1,3–5]. A typical biosurfactant contains a hydrophilic part composed of anions/cations, hydrophilic amino acids or a sugar unit, while the hydrophobic part is composed of hydroxylated, saturated or unsaturated fatty acids (lipids) or hydrophobic peptides [3]. Most commonly reported biosurfactants are glycolipids in which long-chain fatty acids are coupled with carbohydrates. Other biosurfactants which include lipopeptides, heteropolysaccharides and lipopolysaccharides are relatively more complex in structure [1]. Biosurfactants have fostered interest in the past three decades due to significant advantages that they offer over their chemical counterparts. These include: low toxicity, high biodegradability, higher foaming, environmental compatibility, ability to form micelles at low Critical micellar concentration, low cost of production, and higher specific activity and

selectivity across a wide range of conditions including extremes of pH, temperature and salt concentration [1,3,6–9]. Biosurfactants also have antibacterial, antiviral and antifungal properties [1,10–12]. These properties make them versatile in terms of their applications and therefore are used in several industries such as agriculture [10], food [6,13,14], petroleum [15], cosmetic [16], healthcare [17–19], soaps and detergent [19], nanotechnology [8,20,21], paper and pulp [1,22], coal [17,23] and ceramics [1,24]. Major applications include bioremediation (oil spills, groundwater contaminated with hydrocarbons, sites with heavy metals and other pollutants) [17,25–29], cleaning oil sludge in tanks, microbial enhanced oil recovery (MEOR) [2,13,30] and replacement of chlorinated cleaning solvents [31].

In 2017, the global biosurfactant market was evaluated at over USD 1.85 billion with consumption of over 540 kilo tons predicted by 2024 [32]. Despite large demand alongside numerous advantages, their utilization is limited by the relatively higher cost of production and 10 to 40-fold lower CMC compared to chemical counterparts. Various strategies for biosurfactant production such as solid-state fermentation, biosurfactant co-production, specific yield augmentation by media modulations and immobilization for growth enhancement have been suggested for decreasing production costs [19]. Yet choice of the microorganism is a key factor in reducing the cost. A variety of biosurfactant-producing microorganisms have been listed by Banat [3] but the number of species remains low and those characterised need improvement in terms of production efficiency and versatility of growth substrate. There is therefore a requirement for biosurfactant production in new microbial species.

Here, we report the identification and characterization of the biosurfactant produced by *Gordonia* sp. IITR100. The strain was previously isolated in our lab from petroleum-contaminated soil by enrichment culture technique using 4,6-dimethyl dibenzothiophene as the sole sulphur source [33]. The bacterium has previously been characterized for its heavy oil biodesulfurization and viscosity reduction potential [34]. It has been reported to desulfurize 76% sulfur from crude oil and also reduces the viscosity of heavy crude oil by 31%. Both the processes are restrained by the limited availability of the polyaromatic hydrocarbons to the degrading microbes. Identification and Characterization of the biosurfactant will help in designing strategies for minimizing the mass transfer limitations and designing the process such that the microbes will have a maximum access to the hydrocarbon fraction. This will help in enhancing and improving heavy oil biodesulfurization and viscosity reduction. Most species of the genus *Gordonia* have the capability to degrade environmental pollutants, xenobiotics and biodegradable natural polymers [35]. Multiple experiments including drop-collapse, microplate assay, stable emulsion index ( $E_{24}$ ) assay, surface tension reduction, oil spreading, and BATH assay were performed to determine biosurfactant production.  $E_{24}$  tests were performed for different values of temperature, pH and salt concentration to determine the ideal conditions for emulsion stability. For identification of the nature of biosurfactant, chromatographic studies were performed and the biosurfactant was chemically characterized using LCMS, GCMS, NMR and FTIR.

## Materials and methods

*Gordonia* sp. IITR100 (MCC 2877), a biodesulfurizing bacterium previously isolated in our lab from a petroleum-contaminated soil sample using an enrichment culture, was used in the present study [33].

## Screening for biosurfactant production

*Gordonia* sp. IITR100 was screened for its ability to produce biosurfactant by five different methods. The isolated strain was inoculated in 200 ml of Luria broth by transferring 1% of the

overnight grown seed culture (containing  $2.3 \times 10^6$  CFU/ml) into the flask. The flask was incubated in a rotatory shaker for 7 days at 30°C and 180 rpm. The flasks were harvested by centrifuging the culture at 8400 g and 4°C for 30 min. The supernatant (cell-free culture) was collected and used for performing biosurfactant screening assays, emulsion stability assays and surface tension reduction measurement. Assays performed to determine biosurfactant production include: drop collapse assay, oil spreading assay, microplate assay, emulsion index assay and surface tension measurements. The experiment was repeated twice in triplicates.

### Drop-collapse assay

Drop-collapse assay was performed in polystyrene 48-well microplates and on glass slides, as illustrated by Mohanram et al [27]. Wells and glass slides were coated by addition of 20 µl of burnt engine oil followed by 10 µl of culture supernatant (cell-free extract) or controls added to the centre of the oil. The drop added was left undisturbed for 2–3 min and its collapse was monitored. If the drop collapsed, the supernatant was scored positive for the presence of biosurfactant. Approximately 10% SDS was taken as positive control and water was taken as negative control. Tests were repeated 5 times.

### Oil spreading assay

The oil spreading assay was carried out as illustrated by Youssef et al [36]. Distilled water (25 ml) was poured in a petri dish. One hundred microlitre of used engine oil was poured carefully on the surface of the water. Ten microlitre of culture supernatant (cell-free extract) was added to the centre of the surface of petrol and the formation of a clearing zone as a result of displacement of petrol was monitored. Tests were conducted in triplicate.

### Microplate assay

The microplate assay was performed as illustrated by Vaux and Cottingham 2007 [37]. One hundred microlitres of supernatant (cell-free extract) were added to a well of a 48-well microplate and the plate was viewed against the backing sheet containing grids. Optical distortion of background grids was monitored and the assay was scored positive if distortion was observed. This optical grid distortion provides qualitative assessment for detecting the presence of biosurfactant(s).

### Emulsion index ( $E_{24}$ )

The stable emulsion index ( $E_{24}$ ) was determined as illustrated by Mohanram et al [27]. Equal volume (2.5 ml) of cell-free supernatant and petrol was added to a test tube. The resulting mixture was vortexed vigorously for 3 min and left undisturbed overnight. After 24 hours, the stable emulsion index ( $E_{24}$ ) was determined as the percentage of height of the emulsified layer (cm) divided by height of the entire liquid column (cm). The test was repeated with diesel, mineral oil and hexadecane in place of petrol. Approximately 10% SDS was taken as positive control and water was taken negative control. Tests were performed in triplicate.

In all the biosurfactant screening assays, SDS (10%) and cell-free extracts from other biosurfactant-producing microbes (*Rhodococcus* sp. IITD102 and *Paenibacillus* sp. IITD108) were taken as positive control and water was taken as negative control.

### Surface tension measurements

To confirm the production of surfactant, surface tension of the supernatant (cell-free broth) was measured and compared to that of the control (autoclaved uninoculated medium). A

decrease in surface tension of cell free supernatant due to the growth of microorganisms would confirm the production of biosurfactant. A digital K12 –Kruss tensiometer, Germany, was used for determining the surface tension of the cell-free broth and the control medium. Surface tension was measured using Du Noüy ring method (ASTM D1331). Here also, cell-free extracts from other biosurfactant-producing microbes (*Rhodococcus* sp. IITD102 and *Pae-nibacillus* sp. IITD108) were taken as positive control and uninoculated culture medium was taken as negative control.

### Confocal microscopy of the emulsion

A confocal microscope (Leica TCS SP8 Confocal Laser Scanning Microscope) was used to examine the microstructure of the W/O emulsion droplets. Before analysis, the emulsions were gently stirred to ensure a homogenous sample. Approximately 20 µl of the samples were placed on the glass slide, and a cover slip (0.17 mm thickness) was placed on top to ensure that no air gaps (or bubbles) were caught between the sample and the coverslip. The samples were scanned using a 20X objective lens at room temperature (25°C). Image acquisition was performed using LAS X application software.

### Biosurfactant extraction

The cell-free broth after harvesting the culture was collected separately and used for extraction of surfactant. For biosurfactant extraction, the pH of the cell-free broth was adjusted to 2 using 11.5 N HCl solution and was stored overnight at 4°C. Surfactant was extracted by using a solution of chloroform and methanol in the ratio 2:1 [38,39]. Chloroform-methanol mixture was added to cell-free broth in equal volume and mixed vigorously for 10 min. The mixture was then left undisturbed for 20 min and allowed to settle, after which the upper phase (aqueous phase/methanol phase) was pipetted into a fresh beaker. The lower phase containing the surfactant was left to evaporate at room temperature inside a fume hood. Extraction was repeated three times. Honey coloured crude biosurfactant was recovered from the bottom of the beakers using 5 ml of the 2:1 chloroform and methanol mixture. This solution was concentrated to 3 ml and used for chromatographic analysis and identification of biosurfactant.

### Emulsion stability studies

Emulsion stability assays were performed as described by Campos et al. to determine the ideal conditions for stability and functioning of biosurfactant produced by *Gordonia* sp. IITR100 [40]. Emulsion stability at different temperatures, pH and salt concentrations was determined by performing  $E_{24}$  assay (with petrol) under each condition.

The effect of temperature on the stability of emulsion was determined by performing  $E_{24}$  assay of cell-free culture with petrol and incubating the emulsion formed at 4°C, 20°C, 30°C, 37°C, 50°C and 60°C. The effect of pH and salinity on the stability of emulsion was determined by adjusting the pH (using 1 N HCl or 1 N NaOH) or salt concentration (by addition of NaCl) of the cell-free broth to the desired value and calculating  $E_{24}$  indices after 24 hours. The pH was varied from 2–10 (2, 4, 6, 7, 8 and 10) and salt concentration from 0–8% (0%, 0.5%, 1%, 2%, 4%, 6% and 8%).

### BATH assay

The Bacterial adherence to Hydrocarbons (BATH) assay was performed as illustrated by Mohanram et al. 2016 with slight modifications. Flask containing 100 ml of Luria Broth was inoculated by transferring 1% of overnight grown seed culture (containing  $3.2 \times 10^6$  CFU/ml)

of *Gordonia* sp. IITR100. The flasks were incubated at 30°C with constant shaking at 180 RPM for 3 days to reach its logarithmic phase. The cells were then harvested at 8400 g at 4°C for 30 min. The supernatant was collected separately while the cells were resuspended in 25 ml of phosphate urea magnesium sulfate (PUM) buffer. The composition of PUM buffer was 16.948 g/l of K<sub>2</sub>HPO<sub>4</sub>, 1.8 g/l of urea, 7.26 g/l of KH<sub>2</sub>PO<sub>4</sub> and 0.2 g/l of MgSO<sub>4</sub>·7H<sub>2</sub>O. To 6 ml bacterial suspension, 3 ml of petrol was added in a test tube. The test tube was pre-incubated at 30°C for 10 min, after which it was vortexed for 5 min and left to settle for 45 min. The 2 phases were allowed to separate. Using a spectrophotometer, the optical density (OD) of the aqueous phase before (OD<sub>1</sub>) and after petrol treatment (OD<sub>2</sub>) was determined at 400 nm. The values were expressed in terms of percentage of bacterial cells adhering to petrol as compared to the control (suspension without addition of petrol). The test was repeated with petrol in place of hexadecane.

$$\%BATH = (1 - (OD_2/OD_1)) \times 100.$$

### Determination of critical micelle concentration (CMC)

CMC of a biosurfactant is the concentration at which micelles begin to form and surface tension reaches its lowest value. To evaluate the CMC of the biosurfactant, dried extracted biosurfactant was dissolved in milliQ water at various concentrations ranging from 10 mg/l to 500 mg/l, and the surface tension of the resulting mixtures was measured using a digital K12 – Kruss tensiometer and the Du Noüy ring method. The biosurfactant's CMC value was determined as the inflection point of the surface tension versus biosurfactant concentration curve.

### Kinetics of biosurfactant production

Kinetic studies on biosurfactant production were performed in a stirred tank bench top reactor (Applikon) with a tank diameter of 0.16 m and working volume of 3.5 l. The vessel was equipped with two Rushton turbine impellers of diameter 0.06 m. The composition of the culture medium used for the growth of the microbial strain and for studying the kinetics of the biosurfactant production was: 2.00 g/l Na<sub>2</sub>HPO<sub>4</sub>, 1.00 g/l KH<sub>2</sub>PO<sub>4</sub>, 4.25 g/l ammonium oxalate, 0.40 g/l MgCl<sub>2</sub>, 0.025 g/l ammonium sulphate and 17 g/l sucrose. The medium was supplemented with trace elements (1 ml/l) of composition: 0.05 g/l KI, 0.05 g/l LiCl, 0.80 g/l MnCl<sub>2</sub>·4H<sub>2</sub>O, 0.50 g/l H<sub>3</sub>BO<sub>3</sub>, 0.10 g/l ZnCl<sub>2</sub>, 0.10 g/l CoCl<sub>2</sub>·6H<sub>2</sub>O, 0.10 g/l NiCl<sub>2</sub>·6H<sub>2</sub>O, 0.05 g/l BaCl<sub>2</sub>, 0.05 g/l (NH<sub>4</sub>)<sub>6</sub>Mo<sub>7</sub>O<sub>24</sub>·2H<sub>2</sub>O, 0.50 g/l SnCl<sub>2</sub>·2H<sub>2</sub>O and 0.10 g/l Al(OH)<sub>3</sub>. Culture medium was prepared and added to the vessel. The component parts were mounted on the vessel and the entire reactor was sterilized by autoclaving at 121°C for 15 mins. After cooling down, the reactor was inoculated with 1% seed culture (containing 1.5 × 10<sup>7</sup> CFU/ml) and operated at 30°C and 350 rpm till the stationary phase was reached. The pH of the broth was maintained at 7 by addition of 2 M NaOH or 2 M HCl in response to the change in pH value. During the course of the reactor run, regular liquid sampling was performed during which 20 ml of the sample was withdrawn from the reactor. Biomass growth was determined by measuring the light absorbance of the liquid samples at a wavelength of 600 nm. The collected sample was centrifuged at 8400 g for 15 min at 4°C and the cell free supernatant was used for extraction of the biosurfactant, determination of emulsion index and determination of the sugar uptake. HPLC equipped with a refractive index detector (Agilent, USA) was performed for detection of sucrose uptake as described by Zargar et al. [41].

### Characterization of biosurfactant by thin layer chromatography

To determine the nature of the biosurfactant, approximately 4 µl of extracted biosurfactant was loaded onto a TLC plate. TLC was performed using methanol as mobile phase in a

saturated chamber. After completion of the run, TLC plates were analysed using UV and developed using iodine, ninhydrin, anthrone and anisaldehyde. The plates were taken out and  $R_f$  values were calculated.

Iodine vapor staining was performed to detect the presence of lipids [42]. A glass chamber was saturated with iodine ( $I_2$ ) vapours using iodine crystals. The TLC plate was placed inside the saturated chamber and covered with a glass plate. The appearance of yellow-brown spots due to reversible reaction of iodine vapours with lipids was monitored.

Ninhydrin test was performed to detect the presence of peptides and amino acids. One percent (100 mg/ml) solution of glycine was used as positive control and 1% solution of glucose was used as negative control. Ninhydrin stain was prepared by adding 0.4 g of ninhydrin in 20 ml butanol and 0.6 ml acetic acid. This solution was sprayed on the TLC plate. The plate was then transferred to an incubator at 110°C and checked after every 5 minutes for development of any coloured spots.

Anthrone test was performed to detect the presence of sugars [43]. One percent (100mg/ml) solution of glucose was used as positive control and 1% solution of glycine was used as negative control. Anthrone stain was prepared by adding 0.2 g of anthrone in 5 ml  $H_2O$  and 95 ml  $H_2SO_4$ . This solution was sprayed on the TLC plate. The plate was then transferred to an incubator at 110°C and checked after every 5 minutes for development of green or greenish-blue spots.

Anisaldehyde test was performed to detect the presence of sugars that usually contain nucleophiles such as alcohols, ketones or aldehydes. One percent (100 mg/ml) solution of glucose was used as positive control and 1% solution of glycine was used as negative control. p-Anisaldehyde stain was prepared by adding 2 ml p-Anisaldehyde in 1 ml  $H_2SO_4$  and 48 ml acetic acid. This solution was sprayed on the TLC plate. The plate was then transferred to an incubator at 110°C and checked every 5 minutes for development of any coloured spots. Commercial rhamnolipid from AGAE Technologies, USA (Lot No. A79212328003) was used as a positive control during TLC characterization of the biosurfactant.

Purification of the crude biosurfactant was performed using a silica column as suggested by Zargar et al. [41].

## Chemical identification of biosurfactant

Biosurfactant was identified by performing liquid chromatography mass spectrometry (LCMS) using LCMS Agilent Technologies 1260 Infinity LC equipped with 6410 Triple Quad MS, USA and ZORBAX C18 column. Two microlitre of extracted biosurfactant was injected into the column. Chloroform/methanol mixture (3:1) at a flowrate 0.1 ml/min was used as the mobile phase. Detection time of 20 min was set in which m/z ranges from 100 to 1200 were scanned. LCMS was performed in positive electrospray (ES) mode and the m/z values obtained were analysed using METLIN.

Further characterization of the extracted biosurfactant was done by performing gas chromatography mass spectrometry (GCMS) analysis. For GCMS, 10  $\mu$ l sample was injected into the column. For identification of the biosurfactant, ions were analysed and identified using 5977B GC-MSD gas chromatography mass spectrometer (Agilent). The injection temperature was maintained at 260°C. The oven temperature was programmed to start at 40°C, which was held for 2 min, and then ramped to 280°C at a rate of 6°C/min and finally to 280°C at a rate of 10°C/min. MS source and MS Quad temperatures were maintained at 230°C and 150°C.

To determine the functional groups and the type of bonding present, Fourier-transform infrared spectroscopy (FTIR) of the extracted biosurfactant was performed using FTIR Thermo Fisher Scientific Nicolet iS50 in ATR mode between 400 and 4000  $cm^{-1}$ .

To further characterize and confirm the structure of the extracted biosurfactant,  $^1\text{H}$  and  $^{13}\text{C}$  nuclear magnetic resonance (NMR) was performed using Bruker Avance AV-III type spectrometer. For  $^1\text{H}$  NMR, 5 mg of extracted biosurfactant was dissolved in 1 ml of  $\text{CDCl}_3$  while in  $^{13}\text{C}$ , 30 mg of sample was dissolved in 0.5 ml of  $\text{CDCl}_3$ . The spectrum was recorded at 297.9 K at a frequency of 500 MHz.

### Bioinformatic analysis of the genome

Bioinformatic analysis of the genome was performed to check for the presence of genes involved in the synthesis of glycolipid biosurfactants. The microbial pathways for the biosynthesis of glycolipid biosurfactants (especially rhamnolipids) have been extensively studied and the formation of rhamnolipids has been found to occur in 3 steps [44,45]:

1. Synthesis of fatty acid moiety (Hydroxy acyl-ACP) from acyl-CoA.
2. Conversion of dTDP D-glucose to dTDP L-rhamnose.
3. Coupling of dTDP L-rhamnose with hydroxyalkanoyloxy alkanolic acid to form rhamnolipid.

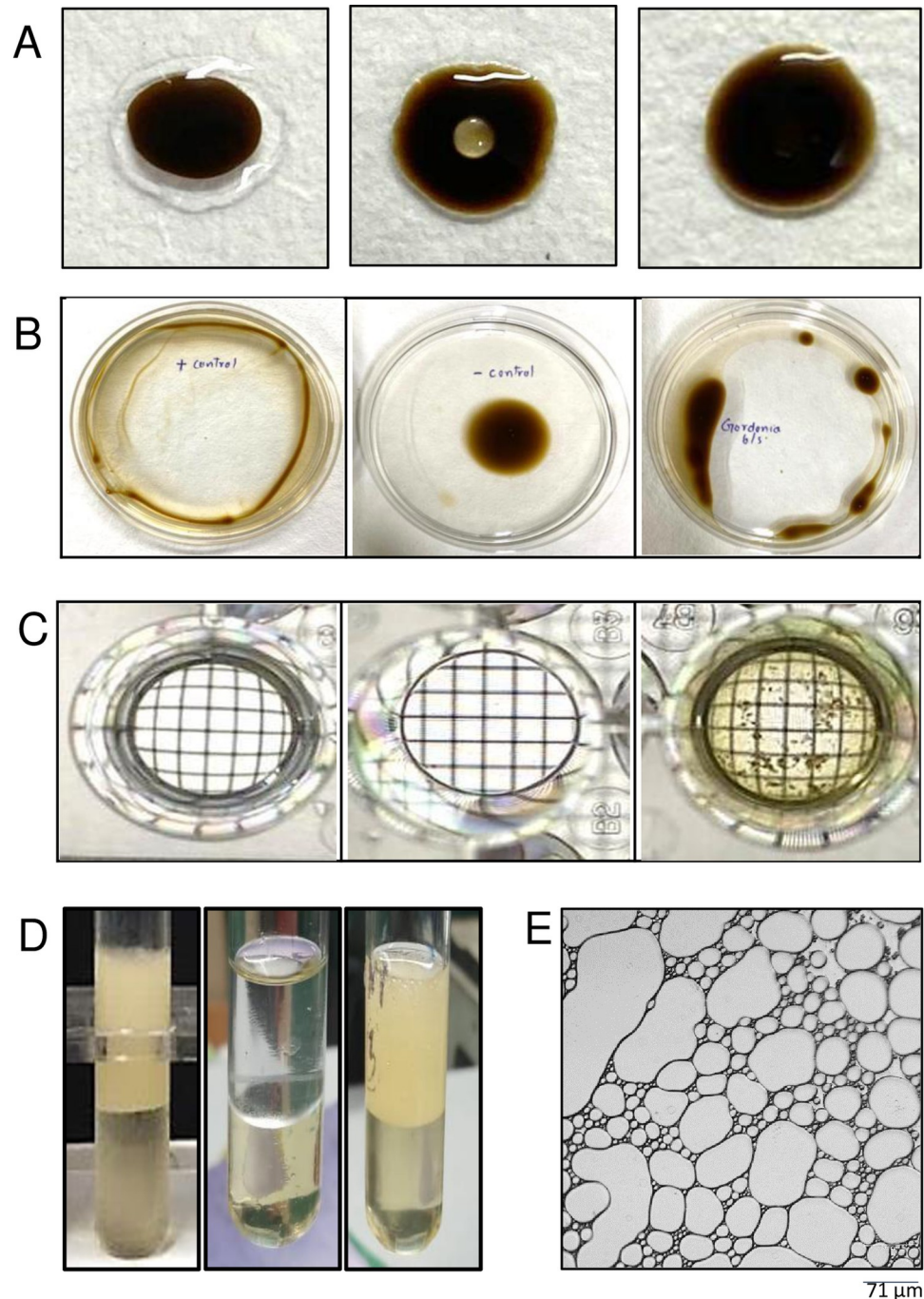
A similar mechanism for the biosynthesis of other glycolipids may occur in *Gordonia* sp. IITR100. Genes involved in the biosynthesis of C18 fatty acid chains which form the hydrophobic part of the biosurfactant produced by *Gordonia* sp. IITR100 were studied using KEGG pathways. The pathway was specified for different species of *Gordonia*, including *Gordonia terrae* and *Gordonia alkanivorans*. Similarly, genes involved in the synthesis of L-rhamnose and other carbohydrate moieties of biosurfactants, as well as those involved in linking carbohydrate moieties to lipid chains to form glycolipid biosurfactants, were studied using KEGG pathways and previous literature reports. Then the genome of *Gordonia* sp. IITR100 was screened for the presence of these genes to confirm the pathway for biosynthesis of the identified biosurfactant.

## Results

### Biosurfactant production assays

All the biosurfactant screening assays gave positive results. In the drop collapse assay (Fig 1A), drop collapse was observed after addition of cell-free culture and 10% SDS and other positive controls (cell-free extracts of *Rhodococcus* sp. IITD102 and *Paenibacillus* sp. IITD108) while no drop collapse was observed when water (negative control) was added to the oil-coated well. In the oil spreading assay, a clear zone of displaced oil was formed when crude biosurfactant-containing cell-free broths from *Gordonia* sp. IITR100, *Rhodococcus* sp. IITD102 and *Paenibacillus* sp. IITD108 were added with 10% SDS, while no oil spreading was observed for water (Fig 1B). In the microplate assay, grid distortion was observed for the crude biosurfactant from *Gordonia* sp. IITR100 and positive controls, while no grid distortion was observed in the case of water (Fig 1C).  $E_{24}$  assay also showed the production of biosurfactant with an emulsion index of approximately 47% with petrol (Fig 1D) and 52.2% with mineral oil. An emulsion index of 38.9% was obtained when diesel was used in the study. Microscopic imaging of the emulsion showed that emulsion formed was water in oil microemulsion. The droplets of size  $> 50\ \mu\text{m}$  correspond to oil droplets while the droplets of size  $< 15\ \mu\text{m}$  are water droplets.

Biosurfactant production was confirmed by determining the surface tension of the culture supernatant and comparing it with that of the control (uninoculated medium). The surface tension of the milliQ water and the control medium were found to be 72 mN/m and 61.07 mN/m, respectively, and that of the culture supernatant was found to be 36.82 mN/m.



**Fig 1.** Biosurfactant screening assays: A. Drop collapse assay on glass slide, B. Oil spreading assay, C. Microplate assay, D. Emulsion index assay, E. Microscopic image of the emulsion formed by cell free supernatant of *Gordonia* sp. IITR100. (Left panel) 10% SDS (Middle panel) water, and (Right panel) cell free extract of *Gordonia* sp. IITR100.

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Therefore, the biosurfactant was successful in reducing the surface tension of the medium by 40%. This decrease in the surface tension of the medium served as a confirmatory test for the production of biosurfactant by the bacteria. *Rhodococcus* sp. IITD102 and *Paenibacillus* sp. IITD108 were successful in reducing the surface tension of the culture medium to 41.41 mN/m and 28.43 mN/m, respectively.



Crude biosurfactant at a concentration of 4.02 g/l was produced by *Gordonia* sp. IITR100 during growth in Luria broth.

### Emulsion stability studies

Emulsion stability studies revealed that the emulsion was stable over a temperature range of 4°C to 30°C (Fig 2A). At 40°C the emulsion index reduced from 47.9 to 23.8% which further reduced to 15% at 50°C and 60°C. Microscopic imaging of the emulsion showed that at higher temperature (60°C), the number of water droplets trapped between oil droplets was significantly lower than that at lower temperature (30°C) (Fig 3). Emulsion stability was found to be minimum under acidic conditions. Maximum stability was obtained at neutral pH. Under alkaline conditions, the emulsion was found to be relatively stable with the emulsion index decreasing from 47.9% at pH 7 to 34.7% at pH 10 (Fig 2B). Salt concentration higher than 2% was found to decrease the stability of the emulsion with the emulsion index reducing from 47.9% at 0% salt concentration to 22% at 4 to 8% salt concentration (Fig 2C). Salt concentration and pH did not have a significant effect on the microstructure of the emulsions formed (Fig 3). Therefore, the ideal conditions for stability and function of biosurfactant produced by *Gordonia* sp. IITR100 are temperatures up to 30°C, pH of 7 and salt concentration below 2%.

### BATH assay

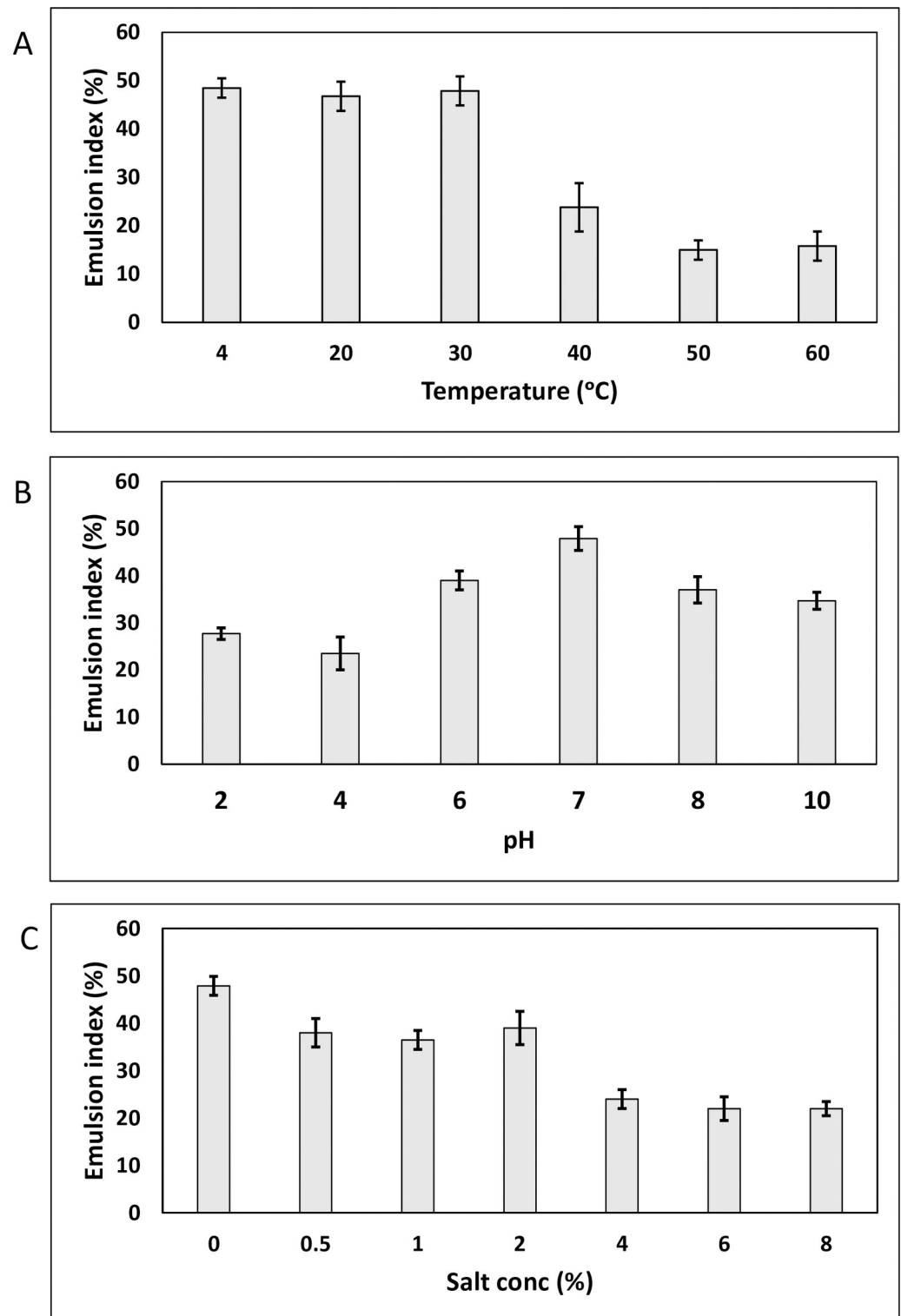
Cell surface hydrophobicity was determined with petrol and hexadecane. The bacterial strain showed lower adherence to hydrocarbons. Percentage BATH with petrol and hexadecane were found to be 13.44% and 18.7%, respectively. This indicates that the bacterial surfaces are hydrophilic in nature and are not responsible for the emulsion formation. Therefore, emulsion formation is a result of biosurfactant production and not surface characteristics of the bacterial cell.

### Determination of critical micelle concentration (CMC)

The concentration of the biosurfactant at which the lowest value of surface tension is achieved is referred to as the CMC. The surface tension of pure milliQ water (free of biosurfactants) was measured to be 72 mN/m. Addition of the biosurfactant to milliQ resulted in the decrease in its surface tension. The surface tension of water decreased as a function of the concentration of biosurfactant added to it and reached a minimum of 36 mN/m when 90 mg/l of biosurfactant was added to it (Fig 4). As the concentration of the biosurfactant was further increased, no further change in the surface tension was observed suggesting that the CMC of the biosurfactant is 90 mg/l.

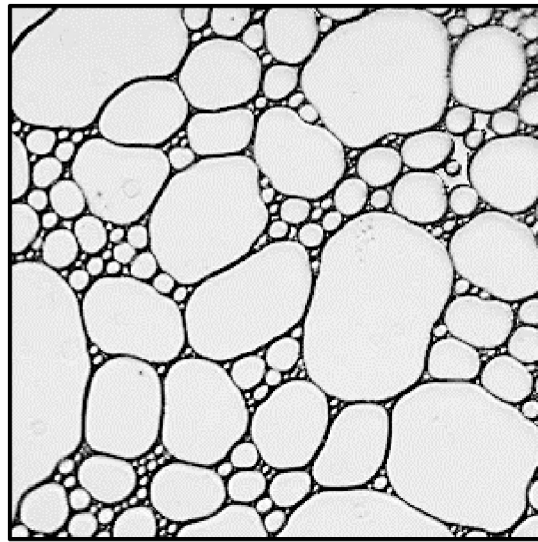
### Kinetics of biosurfactant production

Kinetics of the biosurfactant production was studied in a 5 l batch reactor with a working volume of 3.5 l (Fig 5). After inoculation, a lag phase of 16 hours was observed during which no biosurfactant production was observed. Log phase was observed after 16<sup>th</sup> hour as the microbes started growing exponentially till the culture reached an OD<sub>600</sub> of 6.5 (biomass concentration of 9.2 g/l). During the exponential growth phase, sucrose was rapidly utilized by the growing culture and completely consumed after 80 hours of batch run. This marked the start of the stationary phase. Biosurfactant production was found to be growth associated since the microbes started producing the biosurfactant during early growth phase and reached a maximum concentration of 4.7 g/l at the end of exponential phase. Stationary phase was observed after 90<sup>th</sup> hour of the run and no further biosurfactant production was observed during this

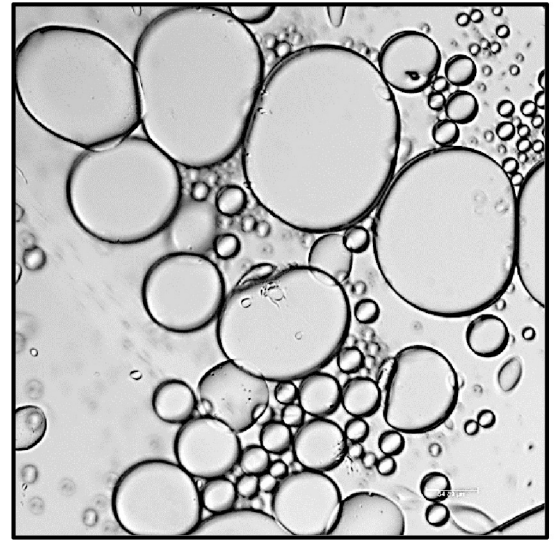


**Fig 2.** Emulsion stability studies: Effect of temperature (A), pH (B) and salt concentration (C) on emulsion stability. Statistical analysis of the results was performed using single factor ANOVA and p-values for temperature, pH and salt concentration profiles < 0.0001.

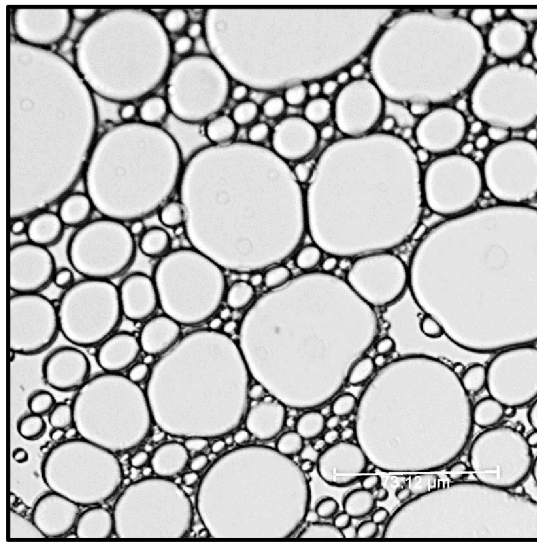
<https://doi.org/10.1371/journal.pone.0264202.g002>



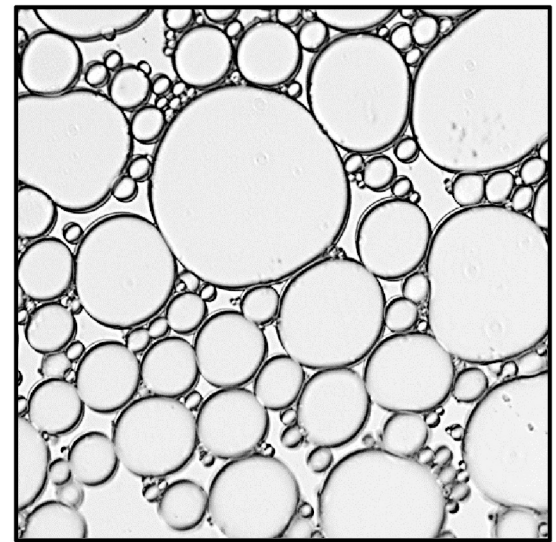
T = 30 °C, pH = 7 and salt conc = 0%



T = 60 °C, pH = 7 and salt conc = 0%



T = 30 °C, pH = 10 and salt conc = 0 %



T = 30 °C, pH = 7 and salt conc = 8 %

70  $\mu$ m

**Fig 3. Effect of temperature, pH and salt concentration on the microstructure of emulsion.**

<https://doi.org/10.1371/journal.pone.0264202.g003>

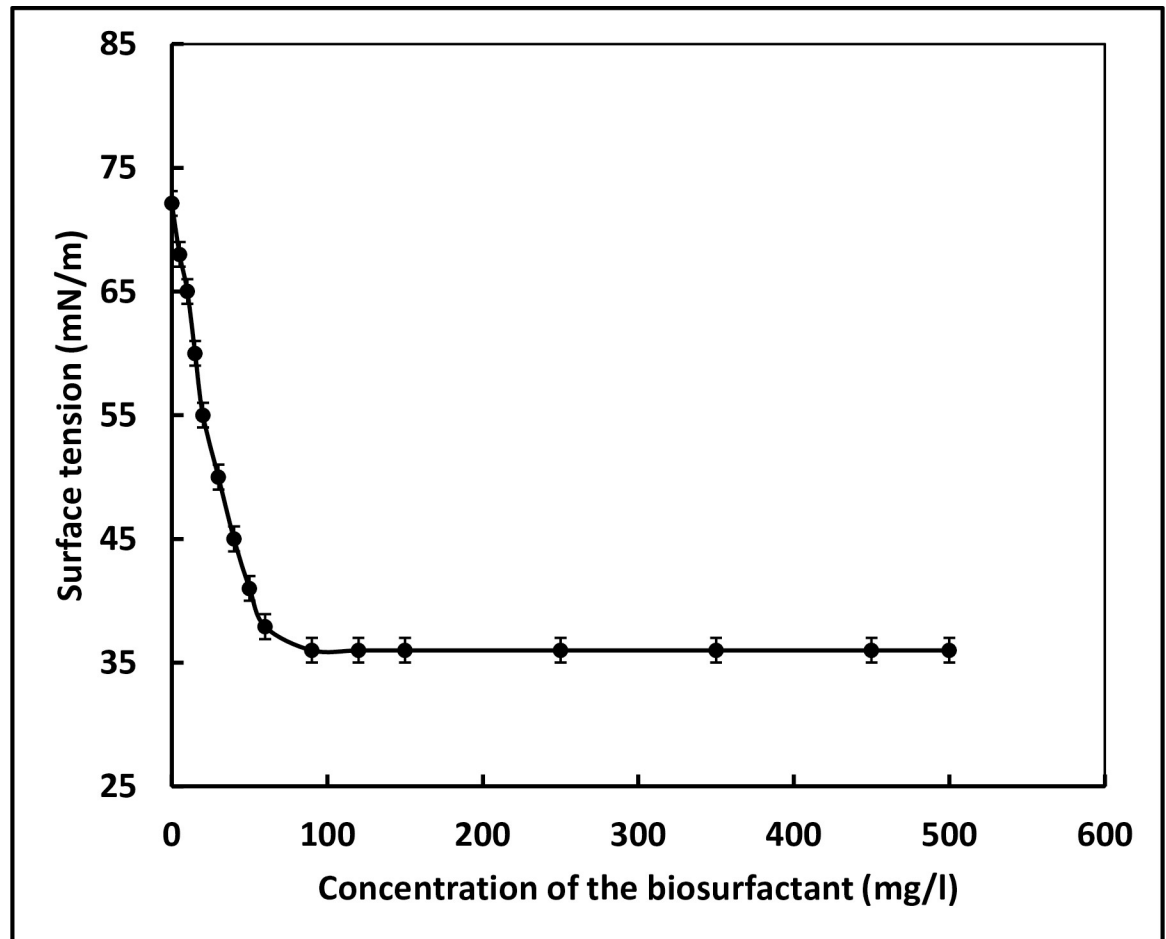


Fig 4. Critical micelle concentration of the biosurfactant.

<https://doi.org/10.1371/journal.pone.0264202.g004>

phase. At the end of the fermentation run, an average dry biomass concentration of 9.2 g/l and biosurfactant concentration of 4.7 g/l were obtained. The yield of biomass ( $Y_{X/S}$ ) and biosurfactant ( $Y_{P/S}$ ) with respect to sucrose were 0.54 g/g and 0.27 g/g respectively. During the exponential phase biosurfactant was produced at a rate 0.07 g/lh while biomass was produced at a rate 0.14 g/lh.

### Characterization of biosurfactant

Staining the TLC plate with iodine vapor resulted in the development of yellow spots as soon as it was placed in the iodine-saturated chamber (Fig 6A and 6E), showing the presence of lipid in the crude biosurfactant. Staining of TLC plate with ninhydrin did not result in any blue colour development (Fig 6B), showing that the crude biosurfactant does not contain any peptide or amino acid. The presence of a carbohydrate moiety in the crude biosurfactant was confirmed by development of light blue spots when the TLC plate was stained with anisaldehyde reagent (Fig 6C), Therefore, the crude biosurfactant contains a lipid part and a carbohydrate part and, as such, is glycolipid in nature.

Chromatographic analysis of the purified biosurfactant showed a single spot on a TLC which confirmed the removal of impurities from the biosurfactant mixture (Fig 6F and 6I). The control rhamnolipid (Fig 6D and 6G) and the purified biosurfactant (Fig 6F and 6I) both

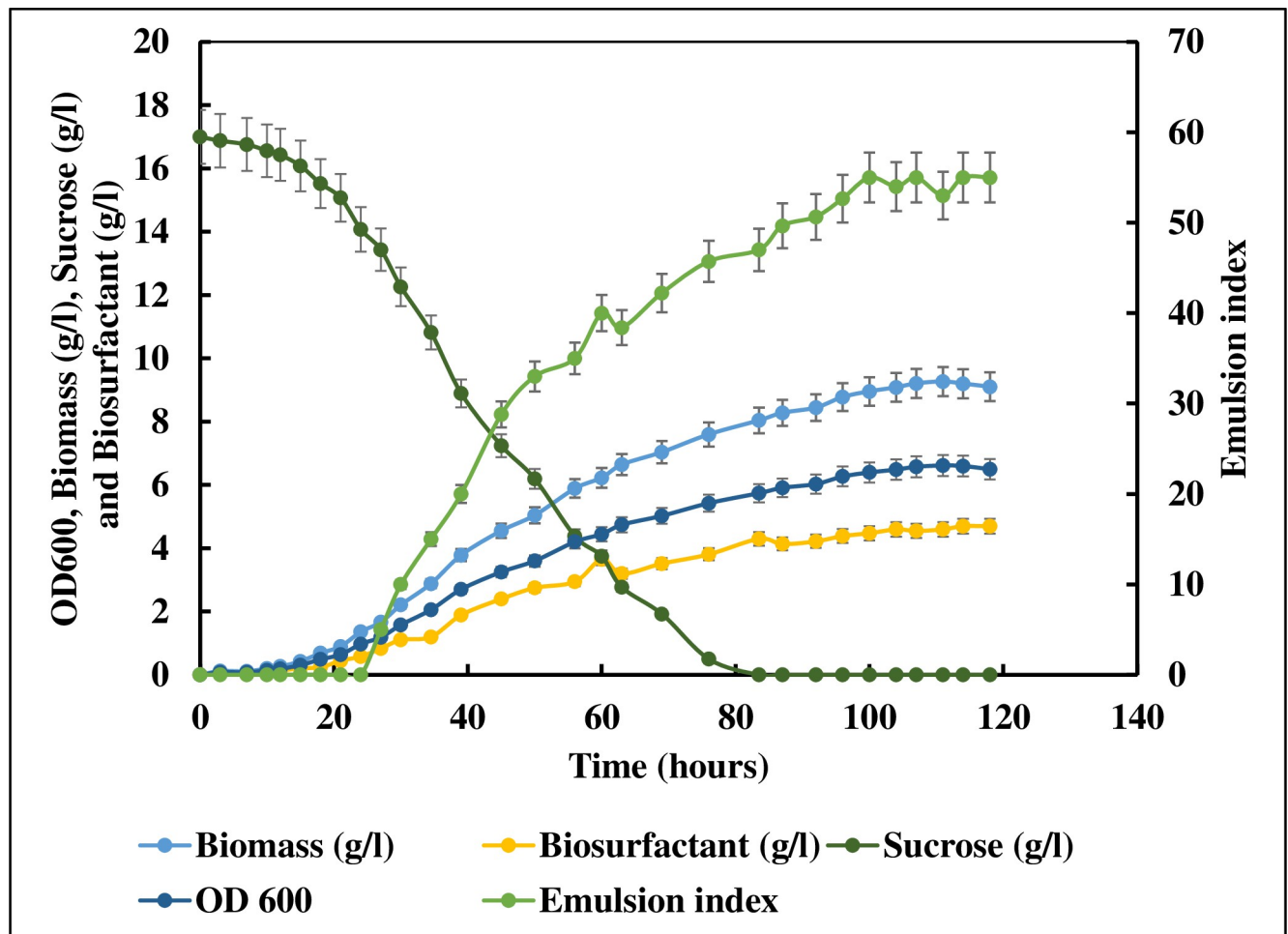


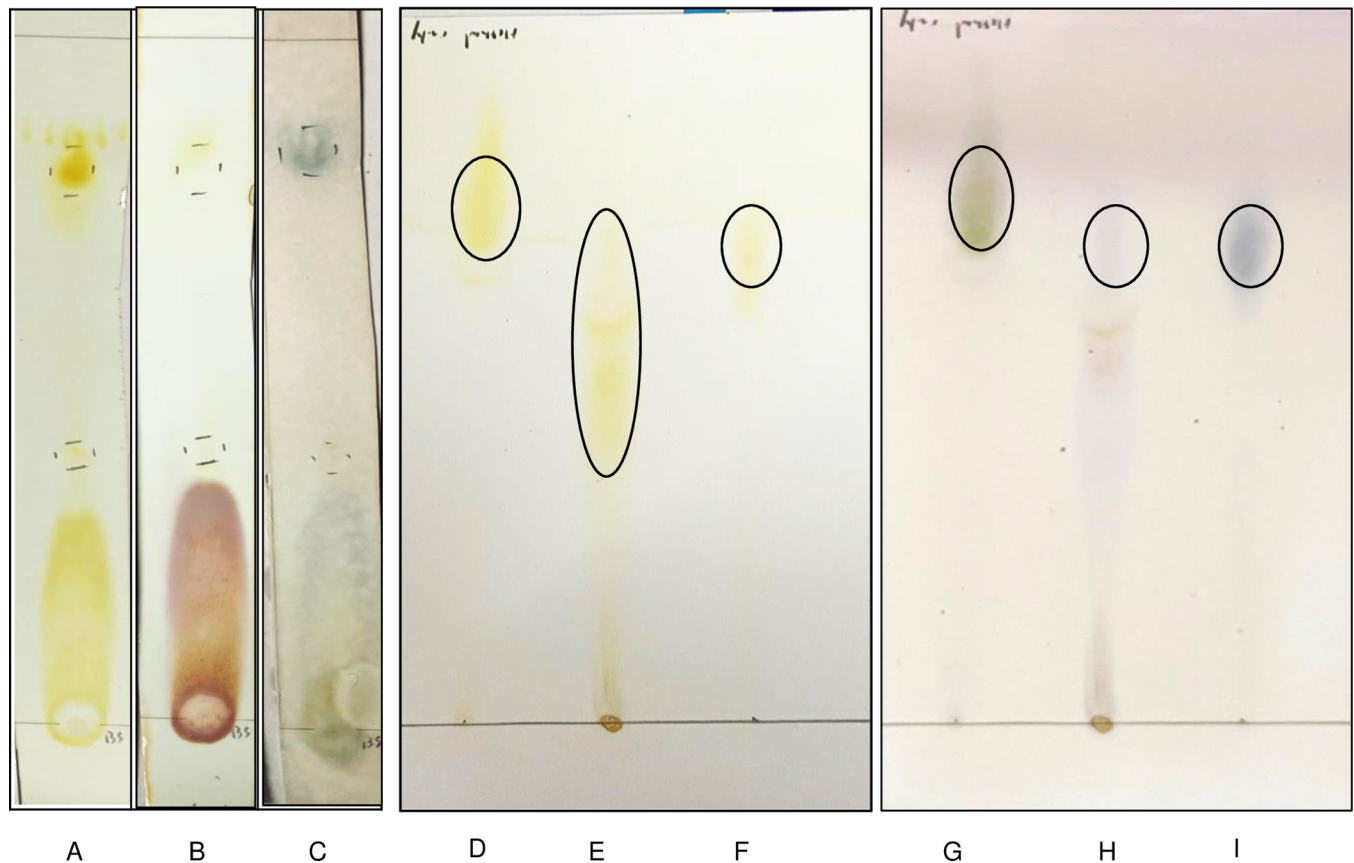
Fig 5. Biosurfactant production in a 5 L bioreactor.

<https://doi.org/10.1371/journal.pone.0264202.g005>

stained positive for the presence of lipid and the carbohydrate and showed similar  $R_f$  values.  $R_f$  value for the commercial rhamnolipid (control) was 0.74 while that for the biosurfactant produced by *Gordonia* sp. IITR100 was found to be 0.70. This confirmed that the purified biosurfactant is a glycolipid.

### Chemical identification of the biosurfactant

LCMS data obtained was analysed using METLIN software at an accuracy of 10 ppm. The biosurfactant was identified as a glycolipid containing a hydrophobic tail of 18C octadecanoic acid (molecular weight 285 g/mol) and  $\beta$ -D-glucopyranose, 4-O- $\beta$ -D-glucopyranosyl (345 g/mol) as a hydrophilic moiety. In LCMS these fragments were detected at a ppm accuracy of 9 and 6, respectively. In the GCMS spectrum (Fig 7), fragments of biosurfactant, octadecanoic acid methyl ester ( $m/z$  value 296) and  $\beta$ -D-glucopyranose, 4-O- $\beta$ -D-glucopyranosyl ( $m/z$  value 345) were detected at retention time of 12.9 min and 7.9 min, respectively (Fig 8). MS breakdown fragments of both parts were also detected in LCMS and GCMS spectra e.g., in GCMS, an analysis peak for 3-deoxyglucose ( $m/z$  value 164.16) was obtained at a retention time of 4.58 min, while the peak for octadecanal ( $m/z$  value 268) was obtained at retention



**Fig 6. TLC characterization of biosurfactant.** Left panel: Crude biosurfactant (A) stained with iodine (B) stained with ninhydrin (C) stained with p-anisaldehyde. Middle panel: Iodine staining for lipid detection (D) control rhamnolipid (E) crude biosurfactant (F) purified biosurfactant. Right panel: p-anisaldehyde staining for carbohydrate detection (G) control rhamnolipid (H) crude biosurfactant (I) purified biosurfactant.

<https://doi.org/10.1371/journal.pone.0264202.g006>

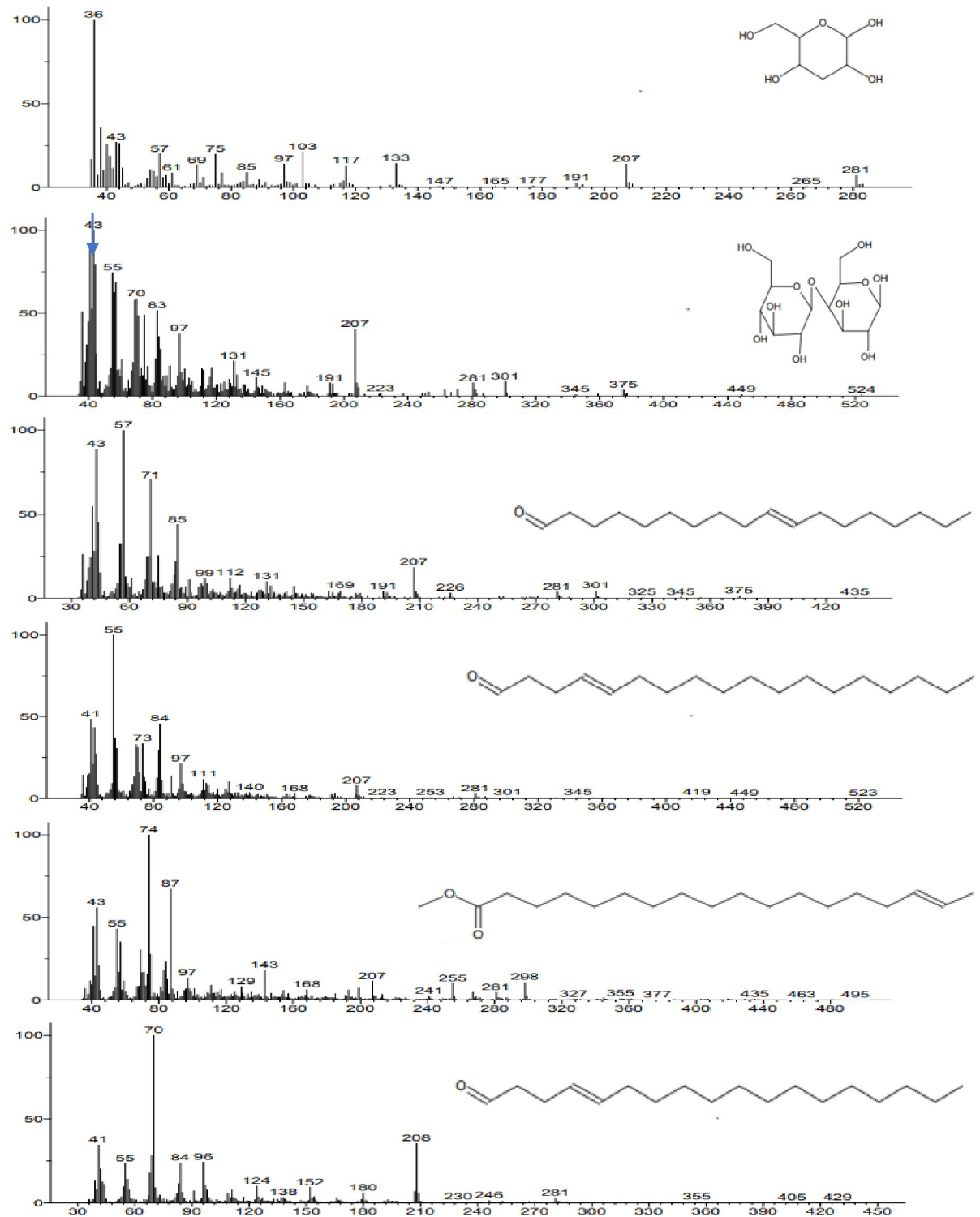
time of 12.9 min (Fig 8). From LCMS and GCMS studies, the overall molecular weight of the biosurfactant was estimated to be approximately 630 g/mol.

FTIR analysis of the extracted biosurfactant (Fig 9) showed a strong peak at  $3292\text{ cm}^{-1}$  which corresponds to OH stretching. Small peaks at  $2933\text{ cm}^{-1}$  and  $2832\text{ cm}^{-1}$  were obtained, these designate the presence of CH bonds. Peaks corresponding to C-O were obtained at wave numbers  $1218\text{ cm}^{-1}$  and  $1020\text{ cm}^{-1}$ . Another peak corresponding to C = O was obtained at  $1649\text{ cm}^{-1}$ . This spectrum perfectly matches the compound identified through LCMS analysis.

$^1\text{H}$  NMR of the extracted biosurfactant (Fig 10A) showed peaks at chemical shifts 0.8 which confirmed the presence of methyl hydrogens (R-CH<sub>3</sub>). A peak obtained at 1.1 shows the presence of alkyl hydrogens (R-CH<sub>2</sub>-R). Peaks obtained in the range 3.1–3.7 confirm the presence of hydrogens associated with a C-O bond. Peaks within the range 3.0 to 3.7 also correspond to hydrogens of alcohol and ester bonds.

$^{13}\text{C}$  NMR of the extracted biosurfactant (Fig 10B) showed peaks at chemical shift 13.9 which correspond to C of R-CH<sub>3</sub>. Peaks between 18 to 30 designate C of R-CH<sub>2</sub>-R. Carbon attached to oxygen is designated by peaks between shifts 50 to 78 ppm.

All three spectra confirm the extracted surfactant as a glycolipid containing a hydrophilic disaccharide moiety and a hydrophobic octadecanoic acid. The structure of the biosurfactant has been shown in Fig 11.



**Fig 7. GC-MS spectrum of the extracted biosurfactant.**

<https://doi.org/10.1371/journal.pone.0264202.g007>

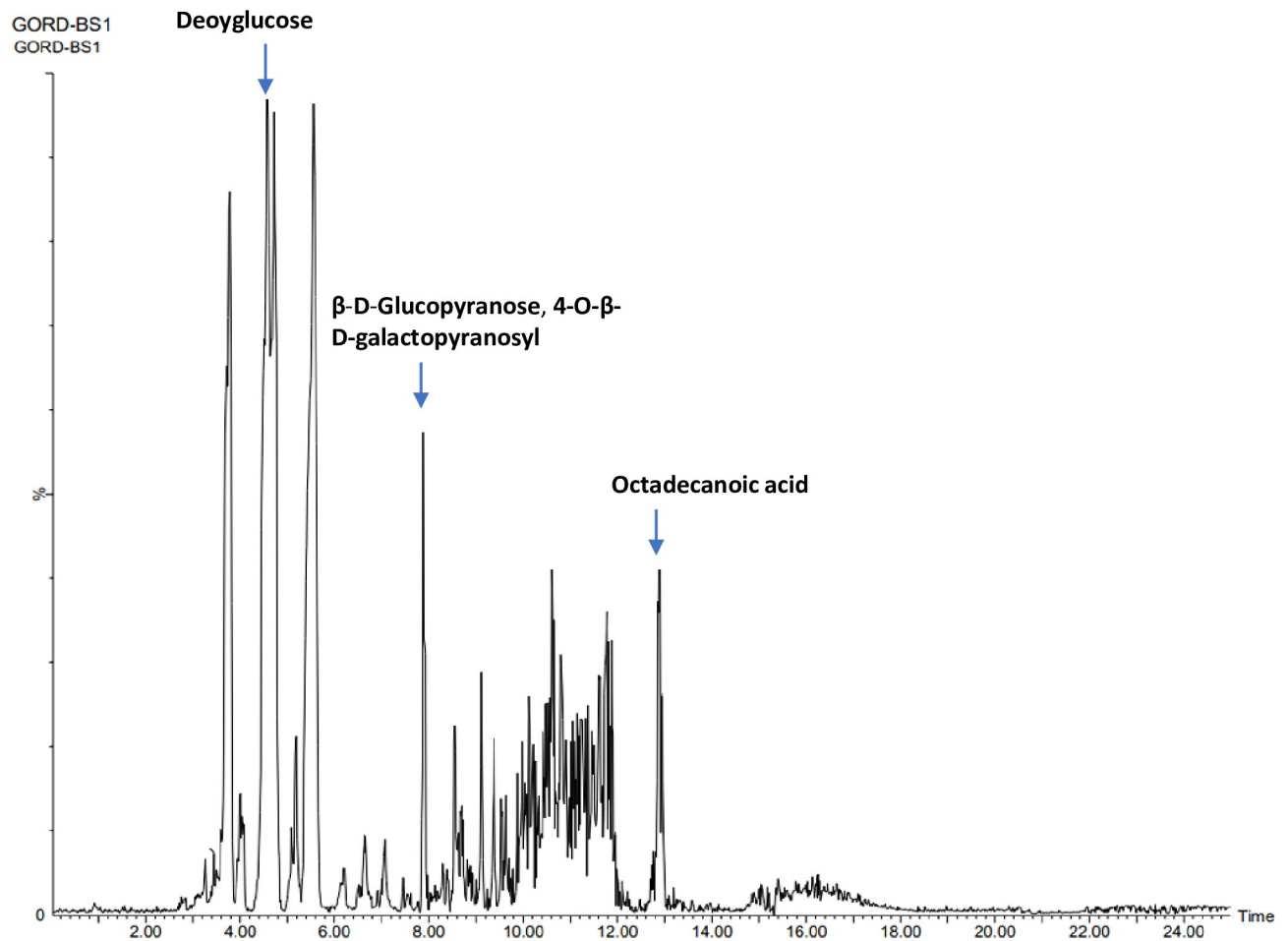


Fig 8. GC-MS chromatogram.

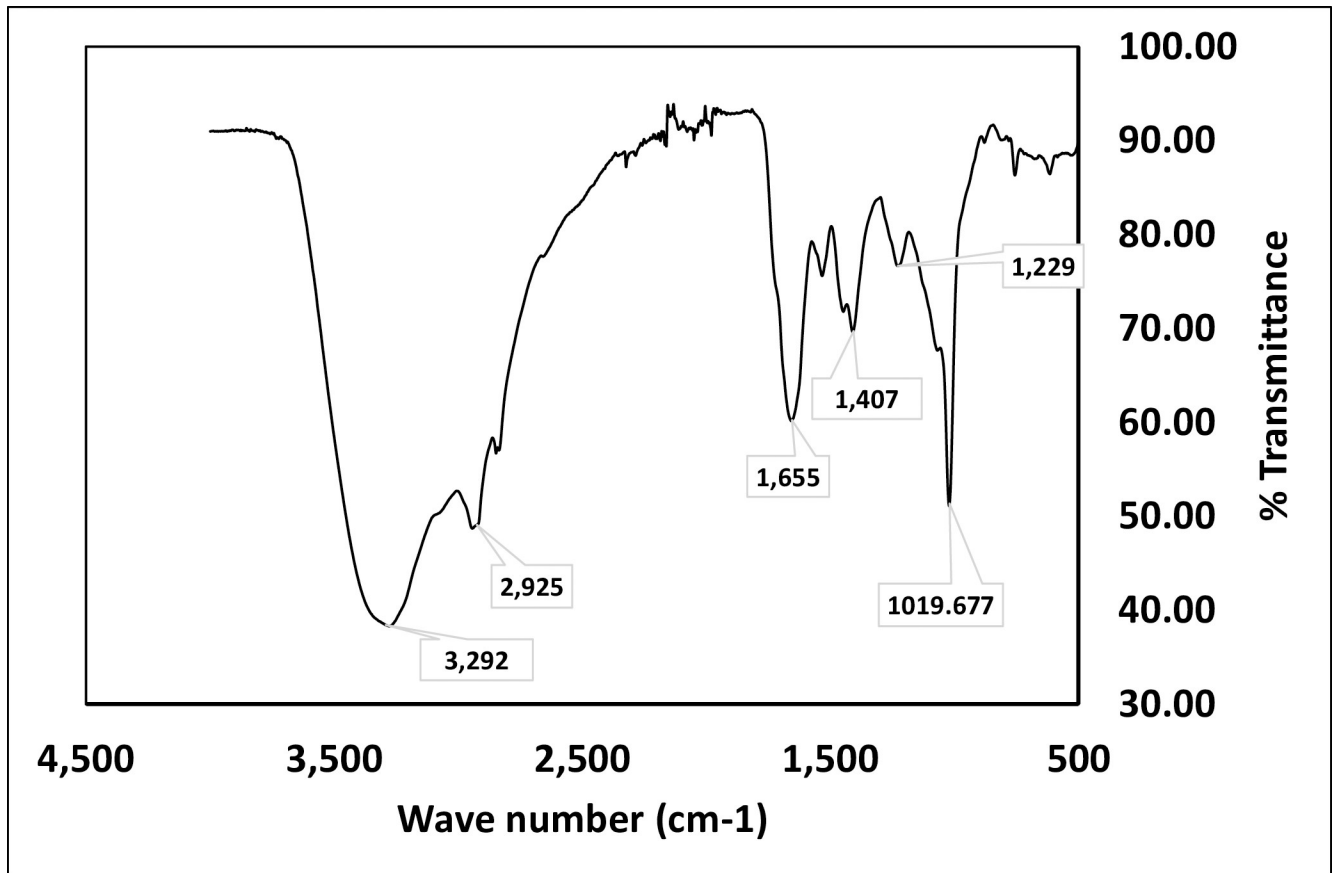
<https://doi.org/10.1371/journal.pone.0264202.g008>

## Discussion

The biosurfactant production for *Gordonia* sp. IITR100 was confirmed by positive results in drop-collapse assay, oil spreading assay, microplate assay,  $E_{24}$  assay and surface tension reduction. Emulsion stability assays at different temperatures, pH values and salt concentrations were performed to determine optimum conditions for biosurfactant action. Higher temperature significantly reduced the stability of the emulsion. It is well known that higher temperature affects the physical properties of the oil (especially viscosity), interfacial films and surfactant properties [46]. The thermal energy of the droplets increases as the temperature rises, as does the frequency of drop collisions. This lowers the interfacial tension, which raises the film drainage rate and promotes faster drop coalescence, lowering emulsion stability [47]. Effect of pH and salt concentration is a result of the change in the concentration of inorganic ions on emulsion stability [48]. The attractive (Van der Waals forces) and repulsive (electrostatic forces) forces that are engaged during particle interaction determine whether colloids coalesce or separate [49]. When salt crystals dissolve in water, they generate their own electrical charges, which adsorb onto the emulsion droplets. The expansion and repulsion of the second layer change as the salt concentration changes, affecting the emulsion's stability.

*Gordonia* sp. IITR 100 produced a surface-active agent that gave positive results when the TLC plate was stained with iodine and p-anisaldehyde, which confirms the presence of lipids

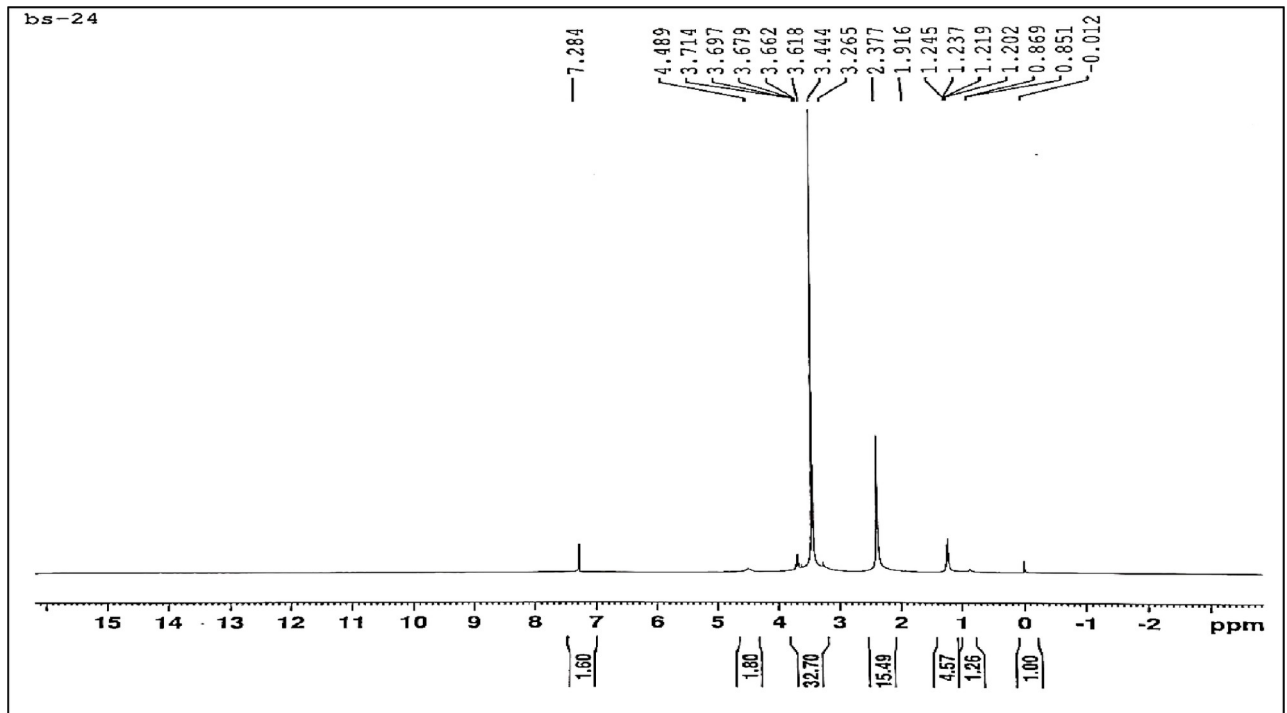




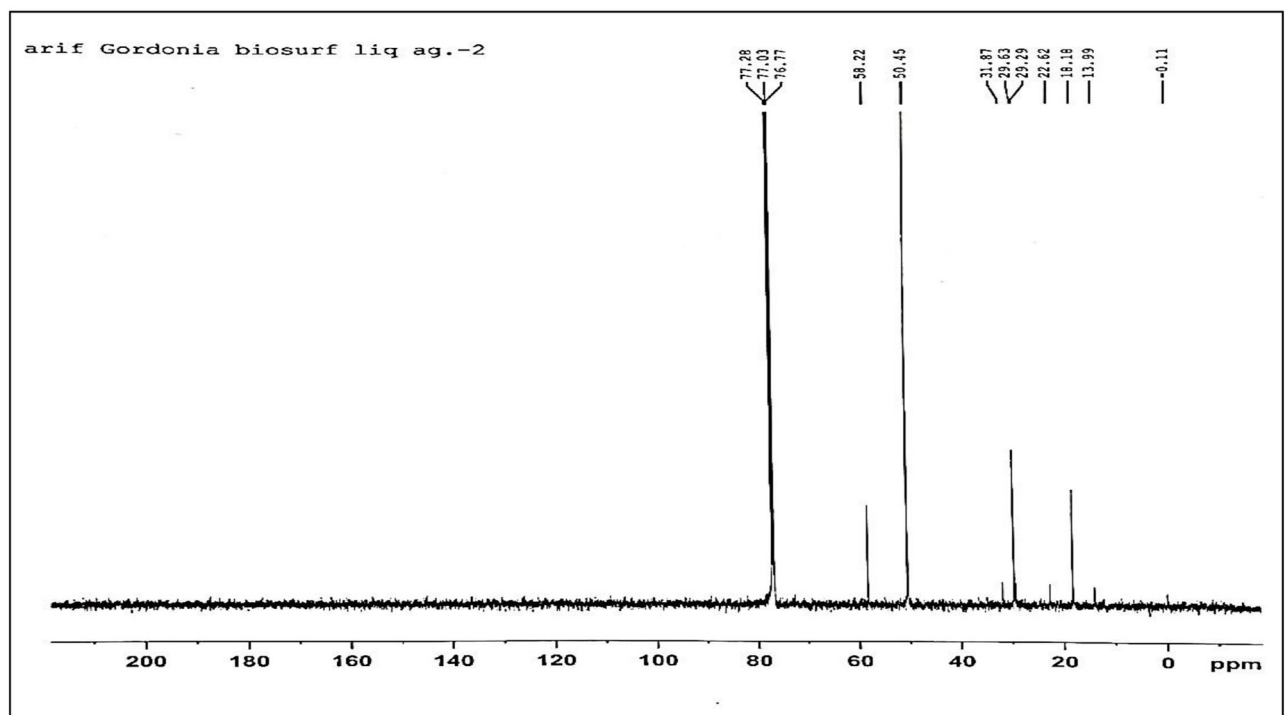
**Fig 9.** FTIR of biosurfactant produced by *Gordonia* sp. IITR 100.

<https://doi.org/10.1371/journal.pone.0264202.g009>

and carbohydrates in the biosurfactant. It gave negative results when stained with ninhydrin which shows the absence of amino acids. LCMS and GCMS studies of the extracted biosurfactant identified it to be a glycolipid with a hydrophilic  $\beta$ -D-glucopyranose, 4-O- $\beta$ -D-glucopyranosyl group and a hydrophobic octadecanoic acid tail containing an 18-carbon chain. The chemical formula is  $C_{24}H_{46}O_7$  and the mass is equal to 630 g/mol. The identity of this compound was confirmed by FTIR,  $H^1$  NMR and  $C^{13}$  NMR of the extracted biosurfactant. Various reports on microbial production of fatty acid esters containing hexoses have indicated they are superior surfactants compared to anionic surfactants such as sodium bis(2-ethylhexyl) sulfosuccinate [1]. While a number of studies report the isolation and application of biosurfactants, there are only limited reports on the chemical identification of biosurfactants produced by microorganisms. This becomes important for field applications where a mixture of different types of biosurfactants would be desirable. The biosurfactant was successful in reducing the surface tension of the media from 61.07 mN/m to 36.82 mN/m. Since the biosurfactant reduced the surface tension of the medium below 45 mN/m, the strain can be considered to be a favourable biosurfactant-producing strain. Biosurfactant production has been reported for other members of *Gordonia*. A glycolipid biosurfactant has been identified from *Gordonia westfalica* [39]. *Gordonia* sp. BS29 has been reported to produce two different surface-active agents; a cell bound glycolipid biosurfactant capable of reducing the surface tension of the medium and another extracellular bioemulsifier capable of producing the stable emulsion but incapable of reducing the surface tension of the medium [50]. Other species of *Gordonia*



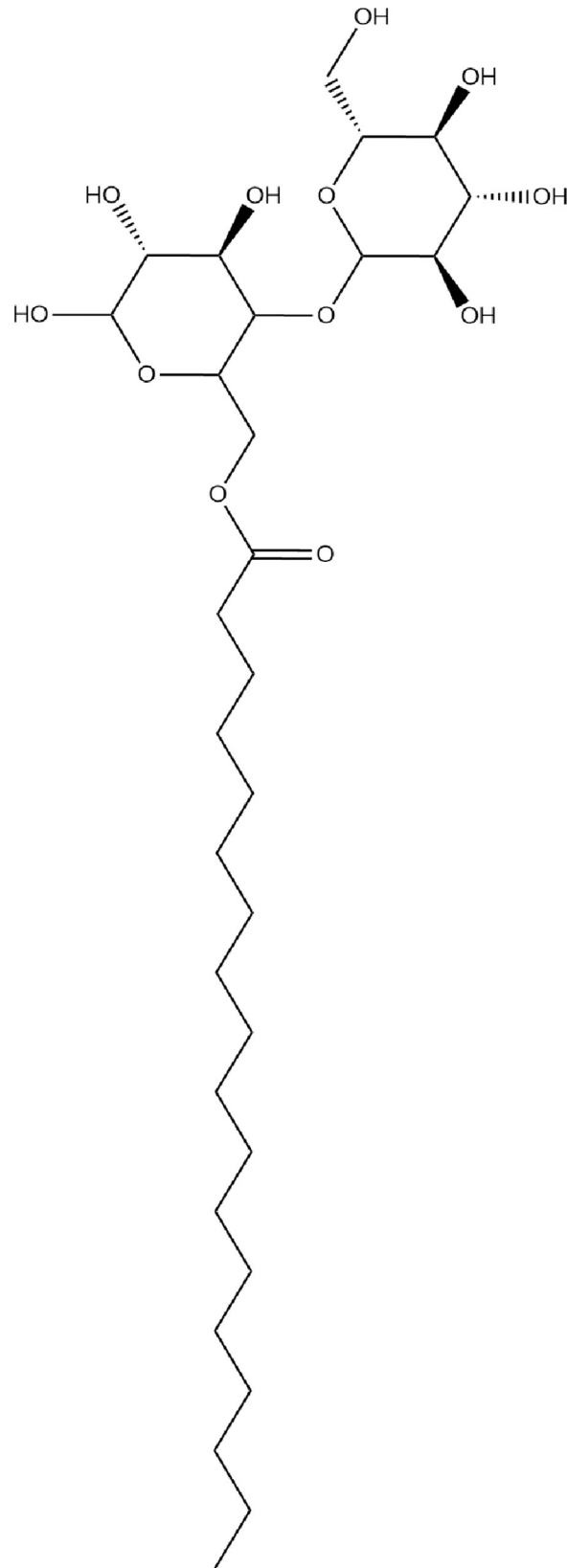
(A)



(B)

Fig 10. NMR of biosurfactant produced by *Gordonia* sp. ITR 100. (A)  $H^1$  NMR (B)  $C^{13}$  NMR.

<https://doi.org/10.1371/journal.pone.0264202.g010>



**Fig 11. Structure of biosurfactant produced by *Gordonia* sp. IITR100.**

<https://doi.org/10.1371/journal.pone.0264202.g011>

reported to produce biosurfactants include *Gordonia amicalis* HS-11 [51], *Gordonia nitida* strain LE31 [52], *Gordonia amarae* [9,53,54], *Gordonia alkanivorans* [55], *Gordonia amicalis* LH3 [56], *Gordonia* sp. strain JE-1058 [57], *Gordonia polyisoprenivorans* [58].

The results from emulsion stability tests show that the emulsion is stable at lower temperatures (4°C to 30°C). Lower pH adversely affects the stability of the emulsion, but the emulsion is relatively stable at higher pH up to 10. The emulsion was found to be stable up to a salt concentration of 2%, above which the emulsion index dropped to half. Emulsion stability results indicate that this biosurfactant can be useful in applications like desludging or in oil bioremediation studies. Also, since this strain was isolated from a petroleum-contaminated soil sample, it can be used in a co-culture with other oil-degrading microorganisms to ameliorate the access of the substrate (hydrocarbon mixture) to the microorganisms.

A comparison of the biosurfactant production by *Gordonia* sp. IITR100 with other reported strains of *Gordonia* has been presented in Table 1. All the strains of *Gordonia* have been reported to produce glycolipids. Among the available reports, with the exception of *Gordonia* sp. strain JE-1058, *Gordonia* sp. IITR100 produced the highest concentration of biosurfactant up to 4.01 g/l. The highest concentration of biosurfactant produced was reported by Saeki et al. 2008 and was found out to be equal to 69 g/l [57]. Our studies have reported the second highest concentration of glycolipid biosurfactant. Other strains of *Gordonia* (*Gordonia westfalica* GY40, *Gordonia amicalis*) have been reported to produce as high as 1.85 g/l and 0.53 g/l of biosurfactants [39,58].

**Table 1. Comparison of biosurfactants produced by various strains of *Gordonia*.**

S. No	Microbe	Type of BS	Emulsion stability	Surface tension reduced to	Yield of crude biosurfactant	Reference
1	<i>Gordonia amarae</i>	Trehalose lipid	-	40 mN/m	68 mg/l	[59]
2	<i>Gordonia bronchialis</i>	Lipoglycan	-	-	-	[60]
3	<i>Gordonia</i> sp. APB	-	-	20.4% reduction	-	[61]
4	<i>Gordonia alkanivorans</i>	-	-	33 mN/m	-	[55]
5	<i>Gordonia rubropertincta</i>	Lipoglycan	-	-	-	[62]
6	<i>Gordonia amarae</i>	Trehalose lipid	-	-	96 mg/l	[53]
7	<i>Gordonia</i> sp. M22, BS25, BS29	Glycolipids	-	29.7 mN/m	-	[50]
8	<i>Gordonia amarae</i>	-	-	55 mN/m	-	[9]
9	<i>Gordonia amicalis</i> LH3	Rhamnolipid	Salt conc. < 5%, Temp = 40°C	38.4 mN/m	-	[56]
10	<i>Gordonia</i> sp. strain JE-1058	Glycolipid	-	-	69 g/l	[57]
11	<i>Gordonia</i> sp. S14-10	Glycolipid	-	31.6 mN/m	-	[63]
12	<i>Gordonia</i> sp. strain BS29	Lipopolysaccharide	-	-	-	[50]
13	<i>Gordonia</i> sp. strain JE-1058.	-	-	-	-	[58]
14	<i>Gordonia amicalis</i>	Novel class of Lipopolysaccharides	-	37 mN/m and 55 mN/m	0.53 g/l and 0.11 g/l	[64]
15	<i>Gordonia cholesterolivorans</i> AMP 10	-	-	24.7 mN/m	-	[65]
16	<i>Gordonia westfalica</i> GY40	Glycolipid	Salt conc. 2–10 % Temp 50–121 °C pH value 4–6	35 mN m <sup>-1</sup>	1.85 g/l	[39]
17	<i>Gordonia amicalis</i> HS-11	Glycolipid	Temp 30°C pH 7	40 mN/m	0.48 g/l	[51]
18	<i>Gordonia</i> sp. 1D (VKM Ac-2720D)	Trehalose lipid	-	35 mN/m	-	[66]
19	<i>Gordonia</i> sp. IITR100	Glycolipid	30°C pH 7	36.83 mN/m	4.01 g/l	This study

<https://doi.org/10.1371/journal.pone.0264202.t001>

The capacity of the biosurfactants produced by different species of *Gordonia* in reducing the surface tension of the culture medium has been widely reported by various research groups. Table 1 also evaluates the performance of various biosurfactants produced by different species of *Gordonia* in terms of their reduction in surface tension of the medium. Biosurfactants from *Gordonia cholesterolivorans* AMP 10 and *Gordonia* sp. BS29 have been reported to reduce the surface tension of the culture medium to 24.7 mN/m and 29.7 mN/m, respectively [50,65]. This is the maximum reduction in surface tension reported for any of the biosurfactants produced by *Gordonia*. Most of the biosurfactants from *Gordonia* have been reported to reduce the surface tension of the culture medium to between 30 mN/m and 40 mN/m [39,50,51,55,56,58,63]. In our study, we found *Gordonia* sp. IITR100 to be successful in reducing the surface tension of the culture medium from 61.06 mN/m to 36.82 mN/m.

Bioinformatic analysis of the genome sequence of *Gordonia* sp. IITR100 confirmed the presence of all the genes required for biosynthesis of the glycolipids. Similar to rhamnolipid biosynthesis, glycolipid biosynthesis in *Gordonia* sp. IITR100 appears to occur in three steps: synthesis of a long fatty acid backbone from acyl-CoA, formation of a carbohydrate moiety from D-glucose and coupling of the carbohydrate moiety with the fatty acid backbone to form the glycolipid biosurfactant. KEGG pathway analysis showed that biosynthesis of C18 fatty acid chains from acetyl Co-A in *Gordonia terrae* and *Gordonia alkanivorans* is carried out by various enzymes which include acetyl-CoA ligase, ACP S-malonyl-transferase, acetyl-CoA-acetyltransferase, acyltransferase, NADP<sup>+</sup> oxidoreductase, beta-hydroxyacyl-ACP dehydratase and NAD<sup>+</sup> oxidoreductase. The enzymes involved in conversion of dTDP D-glucose to form the carbohydrate moiety of the biosurfactant (e.g., dTDP L-rhamnose) include glucose-1-phosphate thymidyltransferase, dTDPD-glucose 4,6-dehydratase, dTDP-6-deoxyD-xylo-4-hexulose 3,5-epimerase and dTDP-6-deoxy-L-lyxo-4-hexulose reductase [67]. The last steps in glycolipid biosynthesis- transfer of the carbohydrate moiety to the fatty acid backbone- is carried out by various transferases like rhamnosyltransferases and glycosyltransferases. Analysis of the genome sequence of *Gordonia* sp. IITR100 revealed the presence of genes for all the important enzymes required for glycolipid biosynthesis. The details of the genes and their gene numbers are given in Tables 2–4.

**Table 2. Genes involved in biosynthesis of C18 fatty acid chain.**

S No.	Sequence name	Sequence description
1	Gene_311	fatty-acid—ligase
2	Gene_617	long-chain-fatty-acid—ligase
3	Gene_1405	long-chain-fatty-acid—ligase
4	Gene_1849	long-chain-fatty-acid—ligase
5	Gene_2122	acyl—ligase
6	Gene_2401	long-chain-fatty-acid—ligase
7	Gene_2869	long-chain-fatty-acid—ligase
8	Gene_2959	long-chain-fatty-acid—ligase
9	Gene_3734	fatty acid—ligase
10	Gene_3780	fatty-acid—ligase
11	Gene_4000	fatty-acid—ligase
12	Gene_4419	fatty-acid—ligase
13	Gene_71	MULTISPECIES: long-chain-fatty-acid—ligase
14	Gene_2952	ACP S-malonyltransferase
15	Gene_465	acetyl- acetyltransferase
16	Gene_1403	acetyl- acetyltransferase

(Continued)

Table 2. (Continued)

S No.	Sequence name	Sequence description
17	Gene_1408	acetyl- acetyltransferase
18	Gene_1515	acetyl- acetyltransferase
19	Gene_1815	acetyl- acetyltransferase
20	Gene_2508	acetyl- acetyltransferase
21	Gene_3181	acetyl- acetyltransferase
22	Gene_3925	acetyl- acetyltransferase
23	Gene_4178	acetyl- acetyltransferase
24	Gene_4234	acetyl- acetyltransferase
25	Gene_4414	acetyl- acetyltransferase
26	Gene_212	acyltransferase
27	Gene_291	acyltransferase
28	Gene_292	acyltransferase
29	Gene_1256	acyltransferase
30	Gene_1963	acyltransferase
31	Gene_2346	acyltransferase
32	Gene_2351	acyltransferase
33	Gene_2436	acyltransferase
34	Gene_2644	acyltransferase
35	Gene_2798	acyltransferase
36	Gene_3383	acyltransferase
37	Gene_3599	acyltransferase
38	Gene_4067	acyltransferase
39	Gene_1724	NAD(P)-dependent oxidoreductase
40	Gene_2967	NAD(P)-dependent oxidoreductase
41	Gene_3944	NAD(P)-dependent oxidoreductase
42	Gene_4108	NADP oxidoreductase
43	Gene_4423	NADP-dependent oxidoreductase
44	Gene_53	oxidoreductase
45	Gene_435	oxidoreductase
46	Gene_519	oxidoreductase
47	Gene_702	oxidoreductase
48	Gene_810	oxidoreductase
49	Gene_1227	oxidoreductase
50	Gene_1241	oxidoreductase
51	Gene_2121	oxidoreductase
52	Gene_2215	oxidoreductase
53	Gene_2310	oxidoreductase
54	Gene_2407	oxidoreductase
55	Gene_2420	oxidoreductase
56	Gene_2588	oxidoreductase
57	Gene_2712	oxidoreductase
58	Gene_3186	oxidoreductase
59	Gene_3213	oxidoreductase
60	Gene_3487	oxidoreductase
61	Gene_3738	oxidoreductase
62	Gene_3745	oxidoreductase
63	Gene_3920	oxidoreductase

(Continued)

**Table 2.** (Continued)

S No.	Sequence name	Sequence description
64	Gene_4003	oxidoreductase
65	Gene_4029	oxidoreductase
66	Gene_4384	oxidoreductase
67	Gene_4405	oxidoreductase
68	Gene_4531	oxidoreductase
69	Gene_4578	oxidoreductase
70	Gene_3936	acyl dehydratase
71	Gene_818	beta-hydroxyacyl-ACP dehydratase

<https://doi.org/10.1371/journal.pone.0264202.t002>

**Table 3. Genes involved in biosynthesis of carbohydrate moiety.**

S No.	Sequence name	Sequence description
1	Gene_2014	polyribonucleotide nucleotidyltransferase
2	Gene_581	glucose-1-phosphate thymidyltransferase
3	Gene_1077	glucose-1-phosphate thymidyltransferase
4	Gene_1078	dTDP-glucose 4,6-dehydratase
5	Gene_580	dTDP-glucose 4,6-dehydratase
6	Gene_2199	ribulose-phosphate 3-epimerase
7	Gene_2427	nucleoside-diphosphate sugar epimerase
8	Gene_3845	ribulose phosphate epimerase
9	Gene_579	UDP-glucose 4-epimerase
10	Gene_582	dTDP-4-dehydrorhamnose 3,5-epimerase
11	Gene_236	Glucose dehydrogenase sorbosone dehydrogenase
12	Gene_1081	UDP-glucose 6-dehydrogenase

<https://doi.org/10.1371/journal.pone.0264202.t003>

**Table 4. Genes involved in linking lipid chain to carbohydrate moiety to form glycolipid.**

S No.	Sequence name	Sequence description
1	Gene_1725	N-acetylglucosaminyl-diphospho-decaprenol L-rhamnosyltransferase
2	Gene_1764	glycosyl transferase
3	Gene_2044	glycosyl transferase
4	Gene_2127	glycosyl transferase
5	Gene_2338	mannosyltransferase
6	Gene_2345	glycosyl transferase family 1
7	Gene_2704	glycosyl transferase
8	Gene_2862	mannosyltransferase
9	Gene_3450	glycosyl transferase
10	Gene_3452	glycosyl transferase
11	Gene_3455	glycosyl transferase family 1
12	Gene_3499	glycosyl transferase
13	Gene_3500	phosphoribosyltransferase
14	Gene_3618	glycosyl transferase family 1
15	Gene_3948	glycosyl transferase family 1
16	Gene_4260	glycosyl transferase
17	Gene_4264	glycosyl transferase
18	Gene_4497	glycosyl transferase

(Continued)

**Table 4.** (Continued)

S No.	Sequence name	Sequence description
19	Gene_300	glycosyl transferase
20	Gene_302	glycosyl transferase
21	Gene_360	galactofuranosyl transferase
22	Gene_445	glycosyl transferase
23	Gene_588	glycosyl transferase
24	Gene_589	glycosyl transferase family 2
25	Gene_771	glycosyl transferase
26	Gene_1084	glycosyl transferase
27	Gene_1085	glycosyl transferase
28	Gene_1087	glycosyl transferase
29	Gene_1099	glycosyl transferase
30	Gene_1100	glycosyl transferase
31	Gene_1759	glycosyl transferase
32	Gene_1760	glycosyl transferase
33	Gene_1761	glycosyl transferase

<https://doi.org/10.1371/journal.pone.0264202.t004>

Based on the yield and the surface tension reduction capacity, the biosurfactant produced by *Gordonia* sp. IITR100 can be useful in a wide range of applications like oil solubilization, desludging of oil containers and enhancement of oil biodesulfurization and biodegradation.

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## Author Contributions

**Conceptualization:** Manoj Kumar, Preeti Srivastava.

**Data curation:** Arif Nissar Zargar, Sarthak Mishra.

**Formal analysis:** Arif Nissar Zargar, Sarthak Mishra, Manoj Kumar, Preeti Srivastava.

**Funding acquisition:** Manoj Kumar, Preeti Srivastava.

**Investigation:** Arif Nissar Zargar, Manoj Kumar, Preeti Srivastava.

**Methodology:** Arif Nissar Zargar, Sarthak Mishra.

**Project administration:** Manoj Kumar, Preeti Srivastava.

**Resources:** Arif Nissar Zargar, Manoj Kumar, Preeti Srivastava.

**Software:** Arif Nissar Zargar.

**Supervision:** Manoj Kumar, Preeti Srivastava.

**Validation:** Arif Nissar Zargar.

**Visualization:** Arif Nissar Zargar, Manoj Kumar, Preeti Srivastava.

**Writing – original draft:** Arif Nissar Zargar.

**Writing – review & editing:** Manoj Kumar, Preeti Srivastava.



## References

1. Desai JD, Banat IM. Microbial production of surfactants and their commercial potential. *Microbiol Mol Biol Rev*. 1997; 61(1):47–64. <https://doi.org/10.1128/mmb.61.1.47-64.1997> PMID: 9106364
2. Youssef NH, Duncan KE, Nagle DP, Savage KN, Knapp RM, McInerney MJ. Comparison of methods to detect biosurfactant production by diverse microorganisms. *J Microbiol Methods*. 2004; 56(3):339–47. <https://doi.org/10.1016/j.mimet.2003.11.001> PMID: 14967225
3. Banat IM. Biosurfactants production and possible uses in microbial enhanced oil recovery and oil pollution remediation: a review. *Bioresour Technol*. 1995; 51(1):1–12.
4. Cooper DG, Goldenberg BG. Surface-active agents from two *Bacillus* species. *Appl Environ Microbiol*. 1987; 53(2):224–9. <https://doi.org/10.1128/aem.53.2.224-229.1987> PMID: 16347271
5. Banat IM, Satpute SK, Cameotra SS, Patil R, Nyayanit NV. Cost effective technologies and renewable substrates for biosurfactants' production. *Front Microbiol*. 2014; 5:697. Epub 2015/01/08. <https://doi.org/10.3389/fmicb.2014.00697> PMID: 25566213; PubMed Central PMCID: PMC4264478.
6. Velikonja J, Kosaric N. Biosurfactants in food applications. *Surfactant Science Series*. 1993:419–.
7. Razafindralambo H, Paquot M, Baniel A, Popineau Y, Hbid C, Jacques P, et al. Foaming properties of surfactin, a lipopeptide biosurfactant from *Bacillus subtilis*. *J Am Oil Chem Soc*. 1996; 73(1):149–51.
8. Kretschner A, Bock H, Wagnee F. Chemical and physical characterization of interfacial-active lipids from *Rhodococcus erythropolis* grown on n-alkane. *Appl Environ Microbiol*. 1982; 44:864–70. <https://doi.org/10.1128/aem.44.4.864-870.1982> PMID: 16346110
9. Heard J, Harvey E, Johnson BB, Wells JD, Angove MJ. The effect of filamentous bacteria on foam production and stability. *Colloids Surf B Biointerfaces*. 2008; 63(1):21–6. Epub 2007/12/11. <https://doi.org/10.1016/j.colsurfb.2007.10.011> PMID: 18068338.
10. Sachdev DP, Cameotra SS. Biosurfactants in agriculture. *Appl Microbiol Biotechnol*. 2013; 97(3):1005–16. <https://doi.org/10.1007/s00253-012-4641-8> PMID: 23280539
11. Singh P, Cameotra SS. Potential applications of microbial surfactants in biomedical sciences. *Trends Biotechnol*. 2004; 22(3):142–6. <https://doi.org/10.1016/j.tibtech.2004.01.010> PMID: 15036865
12. Rodrigues L, Moldes A, Teixeira J, Oliveira R. Kinetic study of fermentative biosurfactant production by *Lactobacillus* strains. *Biochem Eng J*. 2006; 28(2):109–16.
13. Shepherd R, Rockey J, Sutherland IW, Roller S. Novel bioemulsifiers from microorganisms for use in foods. *J Biotechnol*. 1995; 40(3):207–17. [https://doi.org/10.1016/0168-1656\(95\)00053-s](https://doi.org/10.1016/0168-1656(95)00053-s) PMID: 7632394
14. Nitschke M, Silva SSe. Recent food applications of microbial surfactants. *Crit Rev Food Sci Nutr*. 2018; 58(4):631–8. <https://doi.org/10.1080/10408398.2016.1208635> PMID: 27437564
15. Geetha S, Banat IM, Joshi SJ. Biosurfactants: Production and potential applications in microbial enhanced oil recovery (MEOR). *Biocatal Agric Biotechnol*. 2018; 14:23–32.
16. Fiechter A. Biosurfactants: moving towards industrial application. *Trends Food Sci Technol*. 1992; 3:286–93. [https://doi.org/10.1016/0167-7799\(92\)90215-h](https://doi.org/10.1016/0167-7799(92)90215-h) PMID: 1368395
17. Sharma D, Gupta E, Singh J, Vyas P, Dhanjal DS. Microbial biosurfactants in food sanitation. *Sustainable Food Systems from Agriculture to Industry*: Elsevier; 2018. p. 341–68.
18. Peters A, Otter J, Moldovan A, Parneix P, Voss A, Pittet D. Keeping hospitals clean and safe without breaking the bank; summary of the healthcare cleaning forum 2018. Springer; 2018.
19. Singh P, Patil Y, Rale V. Biosurfactant production: emerging trends and promising strategies. *J Appl Microbiol*. 2019; 126(1):2–13. <https://doi.org/10.1111/jam.14057> PMID: 30066414
20. Christopher FC, Ponnusamy SK, Ganesan JJ, Ramamurthy R. Investigating the prospects of bacterial biosurfactants for metal nanoparticle synthesis—a comprehensive review. *IET Nanobiotechnol*. 2019; 13(3):243–9. <https://doi.org/10.1049/iet-nbt.2018.5184> PMID: 31053685
21. Le Guenic S, Chaveriat L, Lequart V, Joly N, Martin P. Renewable surfactants for biochemical applications and nanotechnology. *J Surfactants Deterg*. 2019; 22(1):5–21.
22. Chandra R, Sharma P, Yadav S, Tripathi S. Biodegradation of Endocrine-Disrupting Chemicals and Residual Organic Pollutants of Pulp and Paper Mill Effluent by Biostimulation. *Front Microbiol*. 2018; 9:960. <https://doi.org/10.3389/fmicb.2018.00960> PMID: 29867864
23. Xu G, Chen Y, Eksteen J, Xu J. Surfactant-aided coal dust suppression: A review of evaluation methods and influencing factors. *Sci Total Environ*. 2018; 639:1060–76. <https://doi.org/10.1016/j.scitotenv.2018.05.182> PMID: 29929275
24. Tortora F, Innocenzi V, Prisciandaro M, De Michelis I, Vegliò F, Mazziotti di Celso G. Removal of tetramethyl ammonium hydroxide from synthetic liquid wastes of electronic industry through micellar enhanced ultrafiltration. *J Dispersion Sci Technol*. 2018; 39(2):207–13.

25. Bai G, Brusseau ML, Miller RM. Influence of cation type, ionic strength, and pH on solubilization and mobilization of residual hydrocarbon by a biosurfactant. *J Contam Hydrol*. 1998; 30(3–4):265–79.
26. Cairns WL, Cooper DG, Zajic JE, Wood JM, Kosaric N. Characterization of *Nocardia amarae* as a potent biological coalescing agent of water-oil emulsions. *Appl Environ Microbiol*. 1982; 43(2):362–6. <https://doi.org/10.1128/aem.43.2.362-366.1982> PMID: 16345942
27. Mohanram R, Jagtap C, Kumar P. Isolation, screening, and characterization of surface-active agent-producing, oil-degrading marine bacteria of Mumbai Harbor. *Mar Pollut Bull*. 2016; 105(1):131–8. Epub 2016/02/26. <https://doi.org/10.1016/j.marpolbul.2016.02.040> PMID: 26912197.
28. Kosaric N, Sukan FV. *Biosurfactants: production: properties: applications*: CRC Press; 2010.
29. Thavasi R, Jayalakshmi S, Balasubramanian T, Banat IM. Biosurfactant production by *Corynebacterium kutscheri* from waste motor lubricant oil and peanut oil cake. *Lett Appl Microbiol*. 2007; 45(6):686–91. <https://doi.org/10.1111/j.1472-765X.2007.02256.x> PMID: 17944837
30. Sarkar A, Goursaud J, Sharma M, Georgiou G. A critical evaluation of MEOR processes. *Situ*. 1989; 13(4):207–38.
31. Nwinyi OC, Ajomiwe CG, Olawore YA. Morphological and Biochemical Characterization of Bio-surfactants Producing Bacteria from Diesel Contaminated Soil. *Int J Agric Biol*. 2018; 20(1):1–7.
32. Ahuja K, Singh S. *Biosurfactants market size by product (sophorolipids, rhamnolipids, alkyl polyglucosides [apg], methyl ethyl sulfonates [mes], sucrose esters, sorbitan esters), by application (household detergents, personal care, industrial cleaners, food processing, oil*. *Global Market Insights*. 2018.
33. Jaishankar J, Singh P, Srivastava P. Draft genome sequence of a biodesulfurizing bacterium, *Gordonia* sp. strain IITR100. *Genome Announc*. 2017; 5(17):e00230–17. <https://doi.org/10.1128/genomeA.00230-17> PMID: 28450509
34. Adlakha J, Singh P, Ram SK, Kumar M, Singh M, Singh D, et al. Optimization of conditions for deep desulfurization of heavy crude oil and hydrodesulfurized diesel by *Gordonia* sp. IITR100. *Fuel*. 2016; 184:761–9.
35. Arenskötter M, Bröker D, Steinbüchel A. Biology of the metabolically diverse genus *Gordonia*. *Appl Environ Microbiol*. 2004; 70(6):3195–204. <https://doi.org/10.1128/AEM.70.6.3195-3204.2004> PMID: 15184112
36. Youssef N, Simpson D, Duncan K, McInerney M, Folmsbee M, Fincher T, et al. In situ biosurfactant production by *Bacillus* strains injected into a limestone petroleum reservoir. *Appl Environ Microbiol*. 2007; 73(4):1239–47. <https://doi.org/10.1128/AEM.02264-06> PMID: 17172458
37. Vaux DJ, Cottingham M. Method and apparatus for measuring surface configuration. Google Patents; 2007.
38. Asadollahi L, Salehizadeh H, Yan N. Investigation of Biosurfactant Activity and Asphaltene Biodegradation by *Bacillus cereus*. *J Polym Environ*. 2016; 24(2):119–28. <https://doi.org/10.1007/s10924-016-0754-y>
39. Laorrattanasak S, Rongsayamanont W, Khondee N, Paorach N, Soonglerdsongpha S, Pinyakong O, et al. Production and application of *Gordonia westfalica* GY40 biosurfactant for remediation of fuel oil spill. *Water Air Soil Pollut*. 2016; 227(9):1–13.
40. Campos J, Stamford T, Sarubbo L. Characterization and application of a biosurfactant isolated from *Candida utilis* in salad dressings. *Biodegradation*. 2019; 30(4):313–24. <https://doi.org/10.1007/s10532-019-09877-8> PMID: 31089840
41. Zargar AN, Lymperatou A, Skiadas I, Kumar M, Srivastava P. Structural and functional characterization of a novel biosurfactant from *Bacillus* sp. IITD106. *Journal of Hazardous Materials*. 2022; 423:127201. <https://doi.org/10.1016/j.jhazmat.2021.127201> PMID: 34560483
42. Palumbo G, Zullo F. The use of iodine staining for the quantitative analysis of lipids separated by thin layer chromatography. *Lipids*. 1987; 22(3):201–5. <https://doi.org/10.1007/BF02537303> PMID: 2437429
43. Kamat S, Kawale G. Anthrone as a reagent for the detection of glycosides and saponins by thin layer chromatography. *Curr Sci*. 1987:818–21.
44. Wittgens A, Kovacic F, Müller MM, Gerlitzki M, Santiago-Schübel B, Hofmann D, et al. Novel insights into biosynthesis and uptake of rhamnolipids and their precursors. *Appl Microbiol Biotechnol*. 2017; 101(7):2865–78. <https://doi.org/10.1007/s00253-016-8041-3> PMID: 27988798
45. Dusane DH, Zinjarde SS, Venugopalan VP, Mclean RJ, Weber MM, Rahman PK. Quorum sensing: implications on rhamnolipid biosurfactant production. *Biotechnol Genet Eng Rev*. 2010; 27(1):159–84. <https://doi.org/10.1080/02648725.2010.10648149> PMID: 21415897
46. Boyd J, Parkinson C, Sherman P. Factors affecting emulsion stability, and the HLB concept. *Journal of Colloid and Interface Science*. 1972; 41(2):359–70.
47. Chen G, Tao D. An experimental study of stability of oil–water emulsion. *Fuel processing technology*. 2005; 86(5):499–508.

48. Shao P, Ma H, Zhu J, Qiu Q. Impact of ionic strength on physicochemical stability of o/w emulsions stabilized by *Ulva fasciata* polysaccharide. *Food hydrocolloids*. 2017; 69:202–9.
49. Kazemzadeh Y, Ismail I, Rezvani H, Sharifi M, Riazi M. Experimental investigation of stability of water in oil emulsions at reservoir conditions: Effect of ion type, ion concentration, and system pressure. *Fuel*. 2019; 243:15–27.
50. Franzetti A, Caredda P, La Colla P, Pintus M, Tamburini E, Papacchini M, et al. Cultural factors affecting biosurfactant production by *Gordonia* sp. BS29. *Int Biodeterior Biodegrad*. 2009; 63(7):943–7. <https://doi.org/10.1016/j.ibiod.2009.06.001>
51. Sowani H, Deshpande A, Gupta V, Kulkarni M, Zinjarde S. Biodegradation of squalene and n-hexadecane by *Gordonia amicalis* HS-11 with concomitant formation of biosurfactant and carotenoids. *Int Biodeterior Biodegrad*. 2019; 142:172–81. <https://doi.org/10.1016/j.ibiod.2019.05.005>
52. Lee M, Kim MK, Kwon MJ, Park BD, Kim MH, Goodfellow M, et al. Effect of the synthesized mycolic acid on the biodegradation of diesel oil by *Gordonia nitida* strain LE31. *J Biosci Bioeng*. 2005; 100(4):429–36. Epub 2005/11/29. <https://doi.org/10.1263/jbb.100.429> PMID: 16310733.
53. Dogan I, Pagilla KR, Webster DA, Stark BC. Expression of *Vitreoscilla* hemoglobin in *Gordonia amarae* enhances biosurfactant production. *J Ind Microbiol Biotechnol*. 2006; 33(8):693–700. Epub 2006/02/24. <https://doi.org/10.1007/s10295-006-0097-0> PMID: 16491354.
54. Iwahori K, Tokutomi T, Miyata N, Fujita M. Formation of stable foam by the cells and culture supernatant of *Gordonia* (*Nocardia*) *amarae*. *J Biosci Bioeng*. 2001; 92(1):77–9. <https://doi.org/10.1263/jbb.92.77> PMID: 16233062
55. Young C-C, Lin T-C, Yeh M-S, Shen F-T, Chang J-S. Identification and Kinetic Characteristics of an Indigenous Diesel-degrading *Gordonia* *alkanivorans* Strain. *World J Microbiol Biotechnol*. 2005; 21(8–9):1409–14. <https://doi.org/10.1007/s11274-005-5742-7>
56. Hao D-H, Lin J-Q, Song X, Lin J-Q, Su Y-J, Qu Y-B. Isolation, identification, and performance studies of a novel paraffin-degrading bacterium of *Gordonia amicalis* LH3. *Biotechnol Bioprocess Eng*. 2008; 13(1):61–8.
57. Saeki H, Sasaki M, Komatsu K, Miura A, Matsuda H. Oil spill remediation by using the remediation agent JE1058BS that contains a biosurfactant produced by *Gordonia* sp. strain JE-1058. *Bioresour Technol*. 2009; 100(2):572–7. Epub 2008/08/12. <https://doi.org/10.1016/j.biortech.2008.06.046> PMID: 18692393.
58. Matsui T, Namihira T, Mitsuta T, Saeki H. Removal of oil tank bottom sludge by novel biosurfactant, JE1058BS. *J Japan Pet Inst*. 2012; 55(2):138–41.
59. Kurniati TH, Rusmana I, Suryani A, Mubarak NR. Degradation of polycyclic aromatic hydrocarbon pyrene by biosurfactant-producing bacteria *Gordonia* *cholesterolivorans* AMP 10. *Biosaintifika*. 2016; 8(3):336–43.
60. Garton NJ, Sutcliffe IC. Identification of a lipoarabinomannan-like lipoglycan in the actinomycete *Gordonia* *bronchialis*. *Archives of microbiology*. 2006; 184(6):425–7. <https://doi.org/10.1007/s00203-005-0050-z> PMID: 16320036
61. Pizzul L, Pilar Castillo Md, Stenström J. Characterization of selected actinomycetes degrading polycyclic aromatic hydrocarbons in liquid culture and spiked soil. *World Journal of Microbiology and Biotechnology*. 2006; 22(7):745–52.
62. Flaherty C, Sutcliffe IC. Identification of a lipoarabinomannan-like lipoglycan in *Gordonia* *rubropertincta*. *Systematic and applied microbiology*. 1999; 22(4):530–3. [https://doi.org/10.1016/S0723-2020\(99\)80005-8](https://doi.org/10.1016/S0723-2020(99)80005-8) PMID: 10794140
63. Wang L, Liu Y, Shao Z. Isolation and identification of a hydrocarbon-degrading bacterium *Gordonia* sp. S14-10 from the surface water of Atlantic Ocean and analysis on its related characteristics. *Acta Microbiol Sin*. 2009; 49(12):1634–42. PMID: 20222450
64. Jackisch-Matsuura AB, Santos LS, Eberlin MN, Faria AFd, Matsuura T, Grossman MJ, et al. Production and characterization of surface-active compounds from *Gordonia amicalis*. *Brazilian Archives of Biology and Technology*. 2014; 57:138–44.
65. Pagilla K, Sood A, Kim H. *Gordonia* (*Nocardia*) *amarae* foaming due to biosurfactant production. *Water Science and Technology*. 2002; 46(1–2):519–24. PMID: 12216680
66. Deegan Y, Sargsyan A, Hovhannisyann N, Babayan B, Petrikov K, Vainstein M. Analysis of genome sequence and trehalose lipid production peculiarities of the thermotolerant *Gordonia* strain. *Journal of basic microbiology*. 2020; 60(1):14–21. <https://doi.org/10.1002/jobm.201900439> PMID: 31696960
67. Dong C, Beis K, Giraud M-F, Blankenfeldt W, Allard S, Major L, et al. A structural perspective on the enzymes that convert dTDP-d-glucose into dTDP-l-rhamnose. *Biochem Soc Trans*. 2003; 31(3):532–6.