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# STUDY OF GROWTH, KINETICS OF PRODUCTION AND PARTIAL CHARACTERIZATION OF BIOSURFACTANT PRODUCED BY A *ACHROMOBACTER SPANIUS* STRAIN ISOLATED FROM SOIL CONTAMINATED WITH ENGINE OIL

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***Abstract:*** Biosurfactants (BSs) are molecules produced by microorganisms that are surface-active and amphiphilic in nature. BS can reduce the surface tension and interfacial tension. A bacterial isolate from soil contaminated with engine oil in a garage in Kalamboli, Navi Mumbai was found to be efficient biosurfactant producer. This isolate was identified as *Achromobacter spanius* through morphological, biochemical, MALDI-TOF mass spectrometry, 16S rRNA sequence analysis. The present work is investigation on the isolation and partial structural characterization of the BS produced by *Achromobacter spanius.* The isolate showed maximum emulsification index of 87.30%. The surface tension of the culture medium dropped from 71.18 N/cm to 30.38 N/cm. The biosurfactant production was studied using 2% engine oil in the mineral salt medium. Maximum production of biosurfactant as well as biomass was observed at 120 hours of incubation. Structural characterization of BS was done by using a biochemical test (Anthrone assay) and Fourier transform infrared spectroscopy, which confirmed the glycolipid character of BS. The extracted BS mostly contained lipid and sugar components. The biosurfactant was identified as Rhamnolipid, which is a type of glycolipid. The BS showed high emulsification activity and surface activity that makes it suitable for various industrial and environmental uses.

***IndexTerms*** **- Biosurfactants, Emulsification index, Surface tension, Rhamnolipid, Growth kinetics, Production kinetics**

## I. INTRODUCTION:

Biosurfactants from renewable resources are produced by a variety of microorganisms, including bacteria, fungi and yeasts (1). Due to their superior properties like higher surface activity, reduced toxicity, improved biodegradability and environmental compatibility, these compounds have been given a lot of attention in recent years as compared with synthetic surfactants (2). According to the reports, glycolipids have a significantly higher yield than other groups of biosurfactants [3]. Rhamnolipids (type of glycolipids), are commonly produced by *Pseudomonas species*. This organism produces a mixture of mono-rhamnolipid and di-rhamnolipid (4). Rhamnolipids are used in bioremediation, food, cosmetics, and pharmaceutical industries (5). Compared to chemical surfactants, biosurfactants are highly valued for their antibacterial action and lower toxicity (6,7).

The composition of the rhamnolipid mixture in the biosurfactant produced by the microorganism depends on bacterial isolate used, type of the carbon source and the purification method (6). In rhamnolipid homologues, the number of rhamnose units, chain length, and saturated fatty acid concentrations differ; thus, their physicochemical and biological characteristics are affected by these differences (8). Hence rhamnolipids are important in the research for a prospective biosurfactant with better properties. Utilising engine oil as one of the carbon source, an effort was put forth to identify a new bacterial isolate that produces rhamnolipid in the crude oil waste site. This research focuses on the production, extraction, and partial chemical analysis of rhamnolipids.

## II. MATERIALS AND METHODS:

**Enrichment of biosurfactant-producing micro-organisms:**

Soil samples were collected in a sterile plastic container from a site contaminated with engine oil and transported to the lab. In 10 ml of sterile water, one gram of soil was added and mixed thoroughly. Under sterile conditions, 1 ml of this soil mixture was inoculated into two separate 250-milliliter Erlenmeyer flasks, each containing 100 ml of either Nutrient broth medium (NB) or mineral salt medium (MSM). Both NB or MSM were supplemented with 2% (v/v) engine oil to enrich the biosurfactant-producing micro-organisms. These flasks were incubated at 37°C for 5 days in a temperature-controlled shaker at the shaking speed of 135 rpm. After incubation, samples of the enrichment culture were further used for the screening of the biosurfactant producers.

**Screening for biosurfactant-producing micro-organism:**

Primary screening of biosurfactant producers was done by blood agar assay. The serially diluted enriched medium was spread on the blood agar plates and incubated at 37°C. Several haemolytic colonies appeared on the plate. Colonies showing clear zone (hemolytic colonies) were selected for further studies. Each morphologically distinct colony was transferred separately on the new plates to obtain pure cultures. Cultures were preserved on agar slants and stored in a refrigerator for further use as described by Saravanan & Vijayakumar (9). Flasks containing 100 ml of MSM were inoculated with 24 hour old cultures (1% v/v) and incubated for 24 hours in a shaker incubator (37°C and 135 rpm). Culture supernatants were obtained by centrifugation at 10000 rpm for 10 min, and were tested to detect the presence of surface-active substances using oil spreading assay, emulsification assay and surface tension measurement assays. Distilled water and sodium lauryl sulphate were used as a negative and positive controls, respectively. Uninoculated MSM was used as additional control to check false positive outcomes resulting from components within the medium.

**Qualitative oil spreading test:** The test was carried out as described by Nwaguma et al (10). Briefly, distilled water (20 ml) was taken in a petri plate and hydrocarbon source (here 2.5 ml engine oil) was added making a thin layer of oil on the surface of water. Culture supernatant (10 µl) was delivered onto the oil. Distilled water, 10 % Sodium Lauryl Sulfate and sterile MSM were used as negative control, positive control, and media control respectively. Positive result was indicated by the displacement of oil.

**Emulsification activity:** As described by Nwaguma et al (10), following formula was used to determine emulsifying capability of the biosurfactant. The culture supernatants and the oil were mixed in equal proportions (v/v) followed by vortexing for 15 min. and then allowing to stabilize for 24 h at room temperature.

$$\% \text{ of } E_{24} = h_{emulsion} / h_{total} * 100$$

($h_{emulsion}$ = height of the emulsion, and $h_{total}$ = total height of the liquid)

**Surface tension measurement:** The capillary rise method described by Mahalingam & Sampath (11) was used to measure the surface tension of the culture supernatant. A capillary tube, with a diameter of 1 mm, was immersed into the solution. Surface tension was measured by determining the height of the water column within the capillary tube, and using the following equation.

$$\text{surface tension (S.T)} = (h \rho g r)/2\text{Cos } ø.$$

where g (Gravity) is 980 cm/ second squared, r is 0.05 cm, $\rho$ (Density), ø is zero. Height was measured using the equation: Total reading of meniscus – Total reading of pin or tip.

The isolate that showed maximum emulsification index and the best surface tension reduction was selected for the further studies. The isolate was preserved on a nutrient agar slant at a temperature of 4°C and sub culturing was done once every month.

**Identification of the biosurfactant-producing isolate:**

The selected isolate was primarily characterized using Gram staining and biochemical tests. All essential biochemical tests were done to find the closest match with known bacterial genus and assign bacterial signature according to Bergey's manual (12). Colony of the isolate was taken and analysis was carried out based on their protein and peptide registered information using Matrix Assisted Laser Desorption/Ionization Time of Flight (MALDI-TOF) Mass spectroscopy. Identification was done by comparing the peptide mass fingerprint (PMF) of test organisms with the PMFs contained in the database (13). The 16 S rRNA sequencing was used to confirm the identification.

For **16S rRNA sequencing**, the bacterial 16S rRNA gene was used as a target and the amplification of the purified DNA from the isolate was carried out using the universal primers 16S27F (5'-CCA GAG TTT GAT CMT GGC TCA G-3') and 16S1492R (5'-TAC GGY TAC CTT GTT ACG ACT T-3') (14). The amplified PCR product (1446 bp) was further purified by salt-precipitation. Agarose gel electrophoresis was carried out to determine the quality of PCR amplicons. Purified amplicons were then subjected to cycle sequencing using BDT v3.1 chemistry and subsequently sequenced on an ABI 3500XL Genetic Analyzer. The sequence was further analysed for homology using BLAST.

**Growth and Product formation kinetics:**

To study biosurfactant production by the selected organism, MSM (300 ml) containing 2% engine oil as carbon source was inoculated with 24 hour old culture (1% v/v) of the isolate and incubated in a shaker incubator at 37º C, for 10 days. The culture sample 10 ml was aliquoted at every 24 hours for 10 days. Absorbance of the culture sample was taken at 600 nm to measure the growth and then it was used to find the concentration of biosurfactant. The anthrone assay was used to quantify the amount of glycolipid present in the culture supernatant. Rhamnose was used as standard at concentration range 10-100 µg/ml as described earlier (15).

**Purification of biosurfactant:** Mineral Salt medium (300 ml) containing 2% engine oil as carbon source was inoculated with 24 hours old culture (1% v/v) of the isolate in flask and incubated in a shaker incubator at 37º C, for 5 days. Culture supernatant was obtained by removing bacterial cells by centrifugation (10,000 rpm for 20 min). HCL (6N) was added to the culture supernatant to adjust its pH to 2.0. The precipitate formed was allowed to settle at 4°C and was collected by centrifugation at 12,000 rpm for 20 min as described earlier (16). Precipitate was dissolved in sodium bicarbonate solution (0.1M) then extracted with equal volume of chloroform- methanol mixture (2:1 v/v). The organic phase from the separating funnel was collected in a glass petri dish and dried as described earlier (17).

**Characterization of biosurfactant:**

Structural analysis of glycolipid biosurfactant was done using colorimetric detection by Anthrone assay & Fourier transform infrared spectroscopy (FTIR).

**Colorimetric Detection by Anthrone Assay**

Biosurfactant detection in the extract or the culture supernatant was carried out using colorimetric techniques. Presence of glycolipid was detected and quantified using the anthrone assay as described earlier by Smyth et al (18). Briefly, culture supernatant (200 µl) and anthrone (1,000 µl) reagent were mixed and heated in boiling water for 9 min., cooled and the absorbance of the green-coloured complex was measured at 625 nm.

**Fourier transform infrared spectroscopy (FTIR):**

FTIR spectra of the extracted biosurfactant produced by the bacterial isolate was analyzed to determine the functional groups and types of bonds that are present by using FTIR instrument as described by Khademolhosseini et al (15).

## III. RESULTS AND DISCUSSION:

**Isolation of biosurfactant-producing micro-organisms:**

Soil samples contaminated with engine oil & diesel oil and marine water samples were used to isolate organisms by enrichment technique. Isolates obtained from the enriched culture were primarily screened for their biosurfactant producing ability using hemolysis assay as described by Mulligan et al (19,20). Isolates showed both alpha and beta hemolytic activities (Figure 1). We could identify 11 different bacterial isolates (based on colony characteristics) that showed hemolytic activity. These isolates were used for the further screening.

**Screening for biosurfactant-producing micro-organism:**

**Oil displacement assay:**

Concentration of the surface-active compound in the solution is directly correlated with the oil displacement area, hence qualitative analysis of the culture supernatants of the isolates was carried out to determine presence of biosurfactant in them (Figure 2). Supernatants of all the 11 isolates (IS1, IS2, IS3, IS5, IS6, IS7, IS8, IS9, IS10, IS11, IS12) showed clearing zone on the oil surface (Figure 2c).

**Emulsification activity:**

The emulsification assay serves as an indirect method of screening to evaluate the biosurfactant production. Presence of biosurfactant in the culture supernatant is likely to emulsify the hydrocarbons. Hence, the selection of biosurfactant producers were done based on the results of this screening. To test the emulsification activity of the biosurfactant in the culture supernatant, engine oil was used as the hydrophobic substrate. The $E_{24}$ of the culture supernatants of all the 11 isolates is shown in Table 1. Out of these 11 Isolates, IS3 showed maximum emulsification index viz. 87.30% (Table 1) with engine oil.

**Surface tension measurement:**

The efficiency of a biosurfactant was estimated by its ability to lower the surface tension (ST) of the medium. The culture supernatant of all the isolates were tested. Maximum surface tension reduction was seen with the culture supernatant of the isolate IS3. The surface tension reduced upto 30.38 dynes/cm (Table 2). This isolate was selected for further studies.

Emulsification index and Surface tension measurement assay are sensitive and easy methods to test for biosurfactant production (21). We found that all the 11 isolates showed positive results for all the 4 screening methods viz., haemolytic test, oil spreading, emulsification index and surface tension measurement assay.

**Identification of potent isolates by biophysical methods:**

Out of all 11 isolates, IS3 showed maximum ability to produce biosurfactant. Hence, it was used for further identification.

**Biochemical tests:**

Gram staining of the selected isolate IS3 showed it to be Gram negative, rod shaped. It was cultured and characterized through biochemical tests using Bergey's manual (12). Isolate was positive for Catalase (strong reaction), Oxidase, Urease, Bile esculin and Citrate test. Also, it was found to ferment mannitol and galactose. These results suggested that the isolate was likely to be *Achromobacter spp.*

**MALDI-TOF (Matrix Assisted Laser Desorption/Ionization) mass spectrometry:**

Identification of the isolate was further performed by using MALDI-TOF mass spectrometry by examining the peptide mass fingerprint of the bacterial protein and comparing it with the proteome database. The culture was confirmed as *Achromobacter spp.* Further identification was performed using 16 S rRNA sequencing.

**16 S rRNA sequencing**

The 16S rRNA sequence analysis was performed for the culture identification. PCR amplification of the 16SrRNA gene of the organism was carried out followed by its sequencing. The sequence (Figure 3) obtained was used for the comparison using GenBank sequence utility. Phylogenetic tree drawn based on the sequence homology (Figure 4) showed *Achromobacter spp.* (isolate IS3 denoted as 23N110 022 in the tree) was closest to *Achromobacter spanius*. These sequence data, under **accession number PP446700,** has been submitted to the GenBank database.

There are many reports on biosurfactant production by *Achromobacter* but the reports on *Achromobacter spanius* are limited. Earlier work has shown that the *Achromobacter spanius,* isolate from the soil from detonation field with ability to transform Trinitrotoluene, produced biosurfactant (22). The Emulsification Index at 24 hours for this culture was reported to be 83±14% which was similar to what we observed (87.3±0.0%).

**Growth and Product formation kinetics:**

The biosurfactant production was studied using 2% engine oil in the mineral salt medium. As shown in figure 5, biosurfactant production as well as the growth of the culture by *Achromobacter spanius* (Absorbance at 600nm) peaked at 120 hours of incubation. The maximum production reached 0.99+0.13 dg/ml.

Anthrone assay is commonly used to estimate the production of biosurfactant in relation with the growth of the organism. Studies have shown that the biosurfactant production is positively influenced by the time. There was an increase in the absorbance values at 620 nm (growth) with increase in the biosurfactant production indicating that the growth and biosurfactant production are associated with each other. It was observed that after 120 hours, growth as well as production decreased suggesting the production to be growth associated. The absorbance values for the growth increased till 120 hours which subsequently followed downward trend indicating the decline phase of the bacterial growth. There was direct correlation between the production of biosurfactant and the growth of the bacteria. With the start of the decline phase there was reduction in biosurfactant production. Biosurfactant

production was primarily noted during the exponential growth phase, indicating its likely association with primary metabolism and its contribution to biomass development, suggesting kinetics linked to growth. Abbasi et al have reported that maximum rhamnolipids production by *Pseudomonas aeruginosa* was at the 10th day and decreased thereafter (23). Tabatabaee et al have also reported that there is positive correlation between the growth and the biosurfactant production (24). Similar trends were observed in the studies conducted earlier (25-27). This characteristic suggests that the biosurfactant production can be carried out under chemostat conditions or by immobilized cells (28).

**Purification and characterization of biosurfactant:**

Characterization of the biosurfactant from this *Achromobacter spanius* has not yet been reported.

**Qualitative Anthrone Assay:**

The anthrone assay is utilized to detect and provide an approximate measurement of the quantity of glycolipid present in the culture supernatant (29). In this study, the test showed formation of green coloured complex that indicated glycolipid nature of the biosurfactant produced by *Achromobacter spanius*. Anthrone test showed formation of green coloured complex that indicated glycolipid type of the biosurfactant produced by *Achromobacter spanius*. For further confirmation FTIR was performed.

**Fourier transform infrared spectroscopy (FTIR):**

Characterization of biosurfactant produced by microorganism was done using FTIR. Functional groups in FTIR spectra revealed O-H group at 3282.86, CH-stretching at 2954.96, 2853.14 and 2922.16, C=O ester bond at 1652.67, ether at 1231.72 and Rhamnose sugar at 1051.0 (Figure. 6). Results indicated that *Achromobacter spanius* produced Rhamnolipids, type of glycolipid as a biosurfactant.

For further confirmation FTIR revealed that *Achromobacter spanius* produced Rhamnolipids, type of glycolipid as a biosurfactant. Earlier study by Jing et al (30) showed that *Achromobacter spp*. produced lipopeptide as biosurfactant. Glycolipid biosurfactant production is reported in *Achromobacter kerstersii* (31).

The biosurfactants generated by *Pseudomonas aeruginosa*, specifically rhamnolipids, garnered significant attention in research due to their extensive potential applications across various industries and their notably high production levels (32). Rhamnolipids, consisting of either one or two molecules of rhamnose connected to one or two molecules of β-hydroxy-decanoic acid, stand out as the most extensively studied glycolipids. Rhamnose-containing glycolipid production was first reported in *Pseudomonas aeruginosa* (33). Results of this study were similar to the ones reported by Rahman et al (34)) who reported biosurfactant production by *P. aeruginosa* DS10-129. Da Rosa et al (35) also showed that *P. aeruginosa* LBM10 produced rhamnolipid type biosurfactant.

## IV. CONCLUSION

This study demonstrated the biosurfactant activity of bacterial strains isolated from soil contaminated with engine oil at petrol station and garage environments. From results it is obvious that the environment has an influence on the metabolism of the tested microbes. *Achromobacter spanius* isolated from engine oil contaminated soil showed very good biosurfactant producing ability. Biosurfactant produced by *Achromobacter spanius* was identified as Rhamnolipid, a type of glycolipid. The biosurfactant showed high emulsification activity and surface activity. This makes it suitable for various industrial and environmental applications.

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**Table 1:** Percent emulsification index results of 11 isolates for the production biosurfactant

| Isolates | Emulsification activity Mean±SE |
|---|---|
| IS 1 | 74.07 ± 1.6% |
| IS 2 | 76.72 ± 1.6% |
| IS 3 | 87.30 ± 0.0% |
| IS 5 | 84.66 ± 1.6% |
| IS 6 | 58.20 ± 1.6% |
| IS 7 | 74.07 ± 1.6% |
| IS 8 | 82.01 ± 1.6% |
| IS 9 | 58.20 ± 1.6 |
| IS 10 | 82.01% ± 1.6 |
| IS 11 | 82.01% ± 1.6 |
| IS 12 | 58.20% ± 1.6 |

**Table 2:** Surface tension results of 11 isolates for the production biosurfactant

| Isolates | Surface Tension (Dynes/cm) Mean ±SE |
|---|---|
| IS 1 | 33.55 ± 0.5 |
| IS 2 | 33.37 ± 0.0 |
| IS 3 | 30.38 ± 0.1 |
| IS 5 | 31.16 ± 0.0 |
| IS 6 | 33.43 ± 0.4 |
| IS 7 | 33.37 ± 0.0 |
| IS 8 | 30.53 ± 0.0 |
| IS 9 | 33.43 ± 0.5 |
| IS 10 | 30.76 ± 0.0 |
| IS 11 | 30.76 ± 0.0 |
| IS 12 | 33.43 ± 0.4 |

**Figure 1:** Blood agar assay: Zone of clearance around the colonies

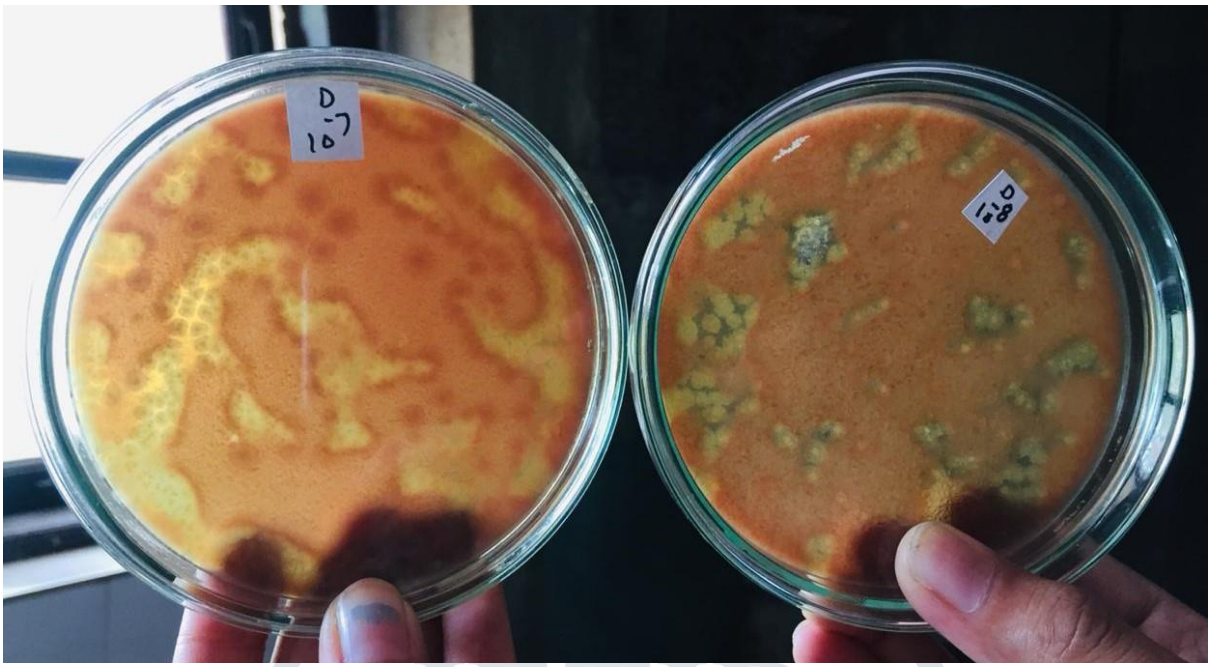

**Figure 2:** Oil displacement assay: negative control with Distilled water (2a), Medium control with MSM (2b), Culture supernatants of *Achromobacter spanius* (2c)

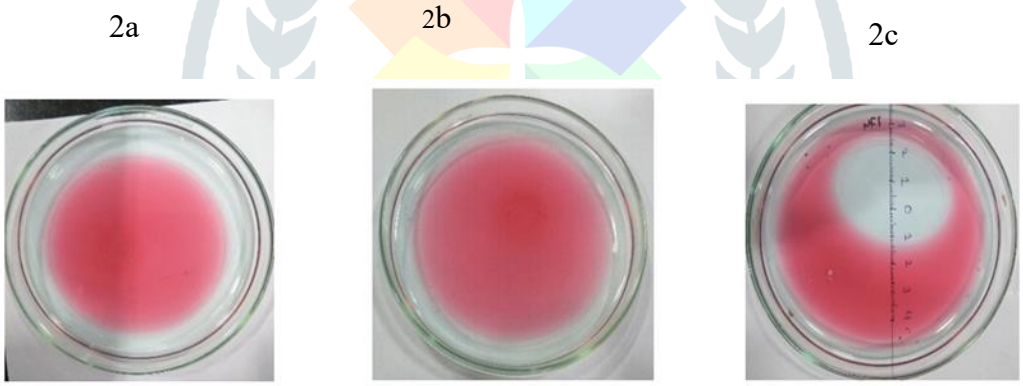

**Figure 3:** Sequence of the amplicon

>23N110_022_Achromobacter_spp

ACGCTAGCGGGATGCCTTACACATGCAAGTCGAACGGCAGCACGGACTTCGGTCTGGTGGCGAGTGGCGAACGGG
TGAGTAATGTATCGGAACGTGCCCAGTAGCGGGGGATAACTACGCGAAAGCGTAGCTAATACCGCATACGCCCTA
CGGGGGAAAGCAGGGGATCGCAAGACCTTGCACTATTGGAGCGGCCGATATCGGATTAGCTAGTTGGTGGGGTAA
CGGCTCACCAAGGCGACGATCCGTAGCTGGTTTGAGAGGACGACCAGCCACACTGGGACTGAGACACGGCCCAGA
CTCCTACGGGAGGCAGCAGTGGGGAATTTTGGACAATGGGGGAAACCCTGATCCAGCCATCCCGCGTGTGCGATG
AAGGCCTTCGGGTTGTAAAGCACTTTTGGCAGGAAAGAAACGTCGTGGGTTAATACCCCGCGAAACTGACGGTACC
TGCAGAATAAGCACCGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGGTGCAAGCGTTAATCGGAATTAC
TGGGCGTAAAGCGTGCGCAGGCGGTTCGGAAAGAAAGATGTGAAATCCCAGAGCTTAACTTTGGAACTGCATTTTT
AACTACCGAGCTAGAGTGTGTCAGAGGGAGGTGGAATTCCGCGTGTAGCAGTGAAATGCGTAGATATGCGGAGGA
ACACCGATGGCGAAGGCAGCCTCCTGGGATAACACTGACGCTCATGCACGAAAGCGTGGGGAGCAAACAGGATTA
GATACCCTGGTAGTCCACGCCCTAAACGATGTCAACTAGCTGTTGGGGCCTTCGGGCCTTGGTAGCGCAGCTAACG
CGTGAAGTTGACCGCCTGGGGAGTACGGTCGCAAGATTAAAACTCAAAGGAATTGACGGGGACCCGCACAAGCGG
TGGATGATGTGGATTAATTCGATGCAACGCGAAAAACCTTACCTACCCTTGACATGTCTGGAATTCCGAAGAGATT
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CTGCGTGAAGTCGGAATCGCTAGTAATCGCGGATCAGCATGTCGCGGTGAATACGTTCCCGGGTCTTGTACACACC
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ATTCATGACTGGGG



**Figure 4:** Phylogenetic tree

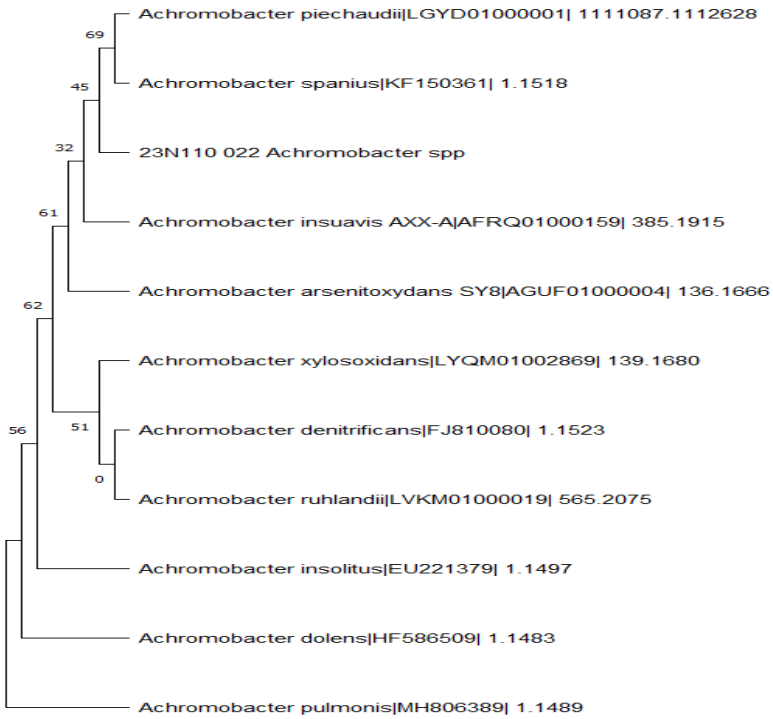

**Figure 5:** Growth Kinetics and Production Kinetics of *Achromobacter spanius*

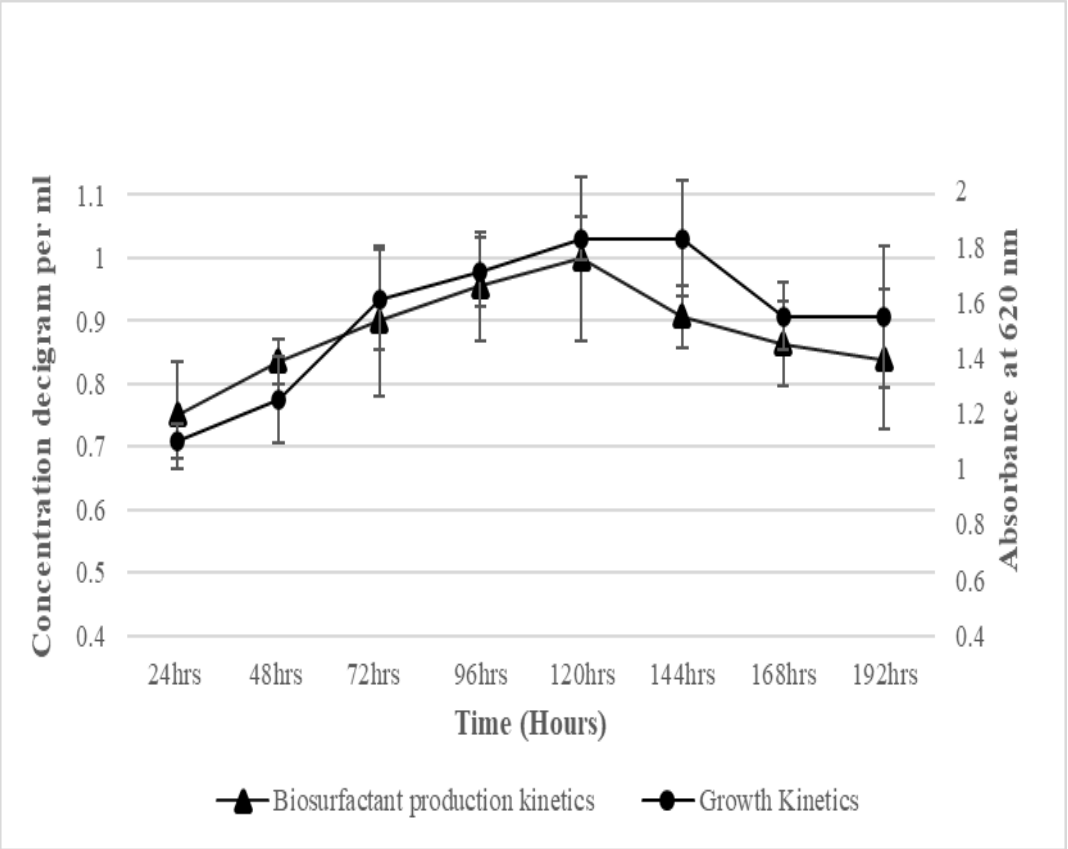


**Figure 6:** FTIR of partially purified biosurfactant produced by *Achromobacter spanius*