Characterization and Stability of Biosurfactant Produced by *Xanthomonas campestris* Isolated from Tannery Effluent

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Abstract: The structural characterization of biosurfactants produced by Xanthomonas campestris isolated from tannery effluents was performed using Fourier Transform Infrared Spectroscopy (FT-IR) and Gas Chromatography–Mass Spectrometry (GC–MS). FT-IR analysis revealed distinct functional groups consistent with amphiphilic biosurfactants. A broad band at 3339 cm^{-1} indicated O–H stretching associated with hydroxyl groups, while a strong absorption at 1640 cm^{-1} corresponded to amide carbonyl groups. Peaks at 1220 cm^{-1} and 1019 cm^{-1} confirmed C–O and C–O–C vibrations typical of sugar moieties, whereas low-frequency peaks at 750 cm^{-1} and 672 cm^{-1} represented C–H bending of long hydrocarbon chains. These results support the presence of both hydrophilic and hydrophobic domains, consistent with glycolipid-type biosurfactants. GC–MS analysis further confirmed this classification, detecting fatty acid fragments (57.1–257.3 m/z) that constitute the hydrophobic region, alongside a diagnostic sugar fragment at 183.2 m/z indicative of rhamnose. High molecular weight fragments (467.6–593.7 m/z) corresponded to intact or partially fragmented glycolipid conjugates. The combined evidence strongly suggests the biosurfactant is a rhamnolipid-like glycolipid with structural complexity that explains its emulsifying and surface-active properties and the crude biosurfactant from Xanthomonas campestris showed stability at pH 8, 45 °C, and 10% NaCl. These findings highlight the potential of X. campestris from tannery effluents as a promising source of glycolipid biosurfactants for environmental and industrial applications.

Key word: Biosurfactant, FT-IR; GC–MS, tannery effluents, *Xanthomonas campestris*

INTRODUCTION

Biosurfactants are Surface-active metabolites of microbial origin, that have broad biotechnological significance because of their capability to emulsify hydrophobic substances, lower surface and interfacial tension, and uphold their activity in the face of severe temperature, pH, and salinity conditions (Dini *et al*., 2024).They are structurally varied compounds that include high molecular weight polymeric emulsifiers like polysaccharides and lipopolysaccharides as well as low molecular weight glycolipids and lipopeptides (Castor *et al.*, 2024). Because of their low toxicity, biodegradability, and multifunctionality, biosurfactants are preferred in industrial and environmental applications over synthetic ones. This makes them attractive for use in pharmaceuticals, food processing, cosmetics, petroleum, and environmental remediation (Arumanatharayil, 2024).

Tannery effluents are one of the most hazardous types of industrial wastewater because they contains high levels of organic matter, dyes, sulfides, and especially heavy metals like chromium, which can be harmful to the environment and human health (Meena, *et al*., 2025). These effluents provide a selective environment that supports microbial communities that can endure hazardous environments (Yang *et al*., 2024). Because strains from polluted niches frequently show improved metabolic versatility, stress tolerance, and the capacity to produce robust biomolecules appropriate for industrial application, such environments are therefore promising reservoirs for isolating potent biosurfactant-producing microorganisms (Sepe, *et al*., 2025).

Xanthomonas campestris is a well-known producer of xanthan gum, an extracellular anionic heteropolysaccharide with special rheological and bioemulsifying qualities (Mouro *et al.,* 2024). Structurally, xanthan is composed of a β-(1→4)-D-glucan backbone

with trisaccharide side chains of mannose–glucuronic acid–mannose, variably substituted with acetyl and pyruvate groups (Berezina *et al.*, 3034). The viscosity, emulsifying ability, and interaction with metal ions are all significantly impacted by these structural changes, indicating a possible dual function in bioremediation and emulsification (Ahuekwe *et al.*, 2016). While the industrial significance of xanthan gum in food and pharmaceutical industries is well established, fewer studies have investigated its structural variability and biosurfactant properties when produced by *X. campestris* isolated from industrial effluents such as tanneries (Zahović *et al*., 2024).

Linking a biosurfactant's chemical makeup to its functional characteristics requires structural characterization (Zompra *et al.,* 2022). Fourier Transform Isnfrared Spectroscopy (FTIR) is used to identify functional groups (Siddique, 2024). Mass spectrometric and chromatographic methods, such as GC-MS, and LC-MS, offer comprehensive information on the molecular weight distribution and monosaccharide content (Meyer *et al.,* 2022). Surface tension decrease, critical micelle concentration (CMC), emulsification index (E24), stability tests under varying pH, salinity, and temperature are examples of functional assays that further demonstrate the usefulness of these biosurfactants in industrial and environmental settings (Bjerk *et al.,* 2021;Satpute *et al*., 2010). Given the pressing environmental challenges associated with tannery effluents and the demand for eco-friendly surfactants, this study focuses on the production and structural characterization of biosurfactants from X. *campestris* isolated from tannery wastewater.

MATERIALS AND METHODS

Fourier Transform Infra-red Spectroscopy (FT-IR) analysis: FT-IR analysis FTIR (Fourier Transform Infra-red Spectroscopy) is a sensitive and primarily technique for identifying the functional groups present in the sample. KBr pellet of crude extracts will be made and the frequency range will be measured at wave numbers 4000 – 500 cm^{-1}(Enas *et al.,* 2014).

Gas Chromatography Mass Spectrometry (GC-MS) analysis: Gas Chromatography Mass Spectrometry analysis was performed on GC-(agilent technologies 7890B model) and MS- (agilent technologies 5977Å MSD model) to determine the active compounds that are present in the biosurfactants. The apparatus consists of a mass spectrometer interfaced with a gas chromatograph, The following conditions were used with the instruments:elite-1 fused silica capillary column infusion (30x0.25 mm IDx1 EM df, composed of 100% dimethyl polysiloxane), operating in electron impact mode at 70ev; helium (99-999%) were used as carrier gas at a constant flow of 1 ml/min an injection volume of 0.5 EI were employed (split ratio of 10:1 injector temperature 250 ºC .The oven temperature was programmed from 110º C (isothermal for 2 min) with an increase of 10ºC / min , to 200 ºC then 5 ºC / min 280 º C , ending with a 9 min isothermal at 280 º C Mass Spectra were taken at 70 Ev; a scan interval of 0.5s and fragments from 40 to 550 Da. Analysis of the mass spectrum The National Institute of Standards and Technology (NIST) database, which contains more than 62,000 patterns, were used for GC-MS analysis. The known components' spectra that were kept in the NIST library were compared to the unknown component's spectrum. The components of the test materials' names, molecular weights, and structures were determined (Enas *et al.,* 2014).

Biosurfactant Stability Test: Stability studies were carried out as described by Obayori *et al*. (2009). Cell free broth would be obtained by centrifuging the cultures at 5000rpm for 20 minutes. The stability of the biosurfactants against pH, temperature and salt (NaCl) would be determined. (i) The Temperature of the biosurfactant were kept under (45, 55, 65, 75 and 85°C), and also the pH of the biosurfactant were adjusted to acidic (2 using HCl) and alkaline (7, 9 and

11 using NaOH). Emulsification index (E_{24}) were later determined after 24 hours (Obayori *et al*. 2009).

RESULTS AND DISCUSSION

The FT-IR spectrum of the biosurfactant produced by *Xanthomonas campestris* (Figure 1) shows distinct peaks that correspond to both hydrophilic and hydrophobic functional groups, supporting its amphiphilic nature. The broad band at 3339 cm^{-1}, representing O–H and/or N–H stretching, suggests the presence of hydroxyl or amide groups, commonly associated with carbohydrate or peptide moieties. Similar findings were reported by Aranda *et al*., (2023), where glycolipid and lipopeptide biosurfactants exhibited broad absorption in this region due to hydrogen-bonded –OH groups. The C=O stretch at 1640 cm^{-1} is particularly indicative of amide carbonyl groups in lipopeptides or conjugated C=C bonds in unsaturated fatty acids. This correlates with the report of Alemie *et al*. (2025), and Bobroff, *et al*., (2016) who identified similar absorption bands in biosurfactants, attributing them to peptide linkages and fatty acid interactions. The medium peaks at 1220 cm^{-1} and strong peak at 1019 cm^{-1} correspond to C–O and C–O–C vibrations, characteristic of sugar residues in glycolipids. According to Liu *et al*., (2020), these absorptions are reliable indicators of glycolipid-type biosurfactants. The lower frequency bands at 750 cm^{-1} and 672 cm^{-1} indicate C–H bending vibrations of long hydrocarbon chains, which represent the hydrophobic tail of the molecule. Comparable findings have been reported in biosurfactants produced by *Pseudomonas* and *Bacillus*, where long-chain fatty acids constitute the non-polar portion of the amphiphile (Al-Seraih, *et al*., 2022). Based on these on this result, the peak at 3339 cm^{-1} could indicate N–H stretching from amide groups in peptide linkages and The C=O stretch at 1640 cm^{-1} corresponds to amide carbonyl groups which is the Hydrophilic Region of Biosurfactant produced. While the Peaks at 750 cm^{-1} and 672 cm^{-1} indicate fatty acid chains, which are typical of the hydrophobic portion of lipopeptides, this structural complexity may explain their dual functionality in emulsification and surface tension reduction. This spectrum it might fits well with Glycolipid, which has a characteristic amide bond from peptide chains and a fatty acid tail. These findings are supported by reference spectra in globally recognized repository (NIST Chemistry Web book), where similar functional group vibrations are consistently reported for rhamnose derivatives, peptides and biosurfactant standards.

Figure 2, shows the Gas Chromatography Mass Spectroscopy (GC-MS) chromatogram of Biosurfactants produced by *Xanthomonas campestris* and the result revealed a complex mixture of structural components characteristic of glycolipid-type surfactants. The GC–MS analysis detected fatty acid fragments at retention peaks 57.1, 98.1, 140.1, and 257.3 indicates the presence of aliphatic hydrocarbon chains, which form the hydrophobic tail of biosurfactants. Similar fatty acid derivatives have been reported in glycolipids and lipopeptides, where long-chain fatty acids contribute to emulsification and surface activity (Mnif *et al.*, 2018). The fragment detected at 183.2 (m/z) corresponds to sugar moieties, particularly rhamnose, which is a diagnostic marker for rhamnolipid-type glycolipids. This aligns with earlier reports that sugar residues of rhamnose which is critical hydrophilic components of biosurfactants (Darwiche *et al*., 2024). The identification of this sugar fragment strongly supports the classification of the biosurfactant as a glycolipid (rhamnolipid) rather than a pure lipopeptide. Furthermore, the presence of high molecular weight fragments at 467.6–593.7(m/z) suggests intact or partially fragmented glycolipid molecules. High-mass peaks of this nature have been reported for complex biosurfactants, where the intact sugar–lipid conjugates generate heavy molecular ion signals (Pascale *et al*., 2023). Such findings are consistent with the amphiphilic structure of glycolipids, which

typically comprise a sugar head group linked to long-chain fatty acids. The chromatograms were also in conformity with reference library standard of Glycolipid (rhamnolipid) and lipopeptide biosurfactants.

Table 1 shows the results of stability test on the crude biosurfactants produced by *Xanthomonas campestris* at different pH (2, 6, 8, 10 and 12), temperature (45, 55, 65, 75 and 85$^{\circ C}$) and NaCl salt concentration (2%, 4%, 6%, 8% and 10%). The highest emulsification activities were recorded at pH of 8 (70%), temperature of 45 ^{o}C (69%) and 10 percentage of NaCl (53%). The findings for temperature in this study are align with those for pH and NaCl (%) from Agarry *et al*. (2015) and Ibrahim *et al*. (2013). There are several reports on the stability of biosurfactants at extreme conditions of pH, temperature and NaCl that make biosurfactant stable and ideal for industrial application (Colla *et al.,* 2010), who worked on simultaneous production of lipases and biosurfactants by submerged and solid-state bioprocesses. Biosurfactants and their surface action are safe towards natural factors such as, temperature and pH.

Table 1: Stability test of the biosurfactant produced by *Xanthomonas campestris* at different temperature ($^{\circ}$C), salt concentration (%) and pH

Temp. ($^{\circ}$C)	Average E_{24} (%)	NaCl (%)	Average E_{24} (%)	pH	Average E_{24} (%)
45	69	2	50	2	57
55	61	4	52	6	65
65	58	6	50	8	70
75	50	8	48	10	68
85	55	10	53	12	56

KEY: **E_{24} (%)** = Emulsification index in percentage

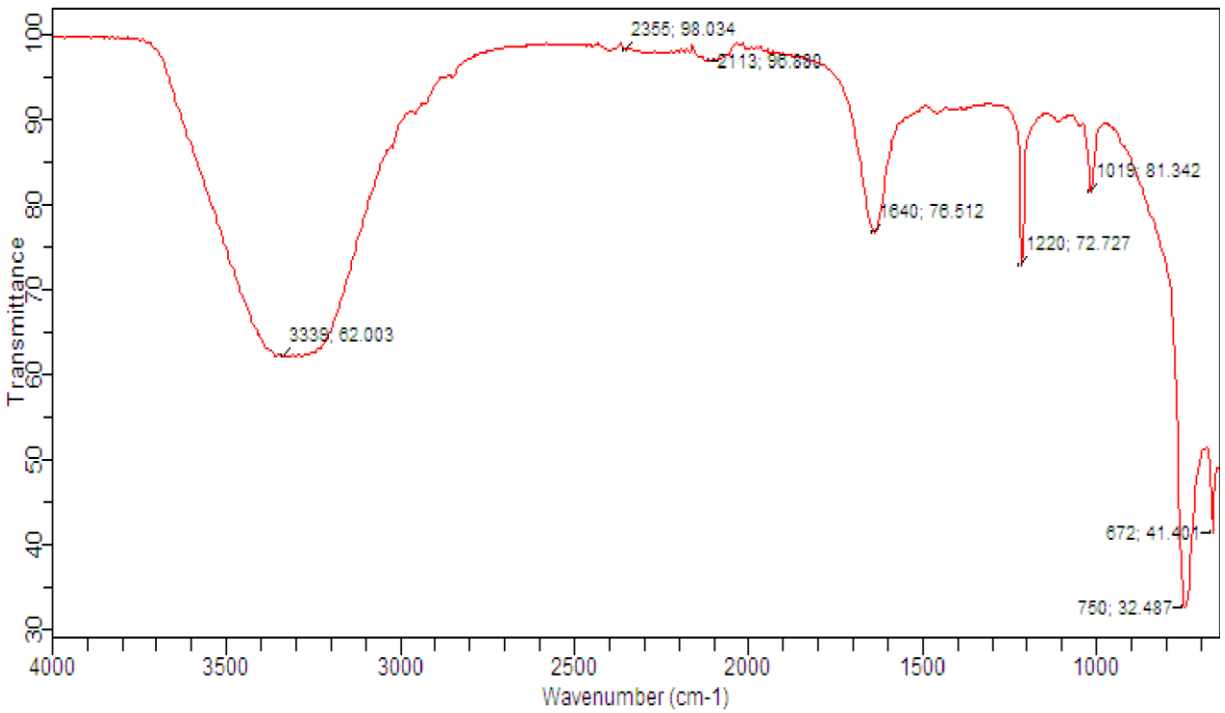

Figure 1: FT-IR spectrum of biosurfactants produced by *Xanthomonas campestris*

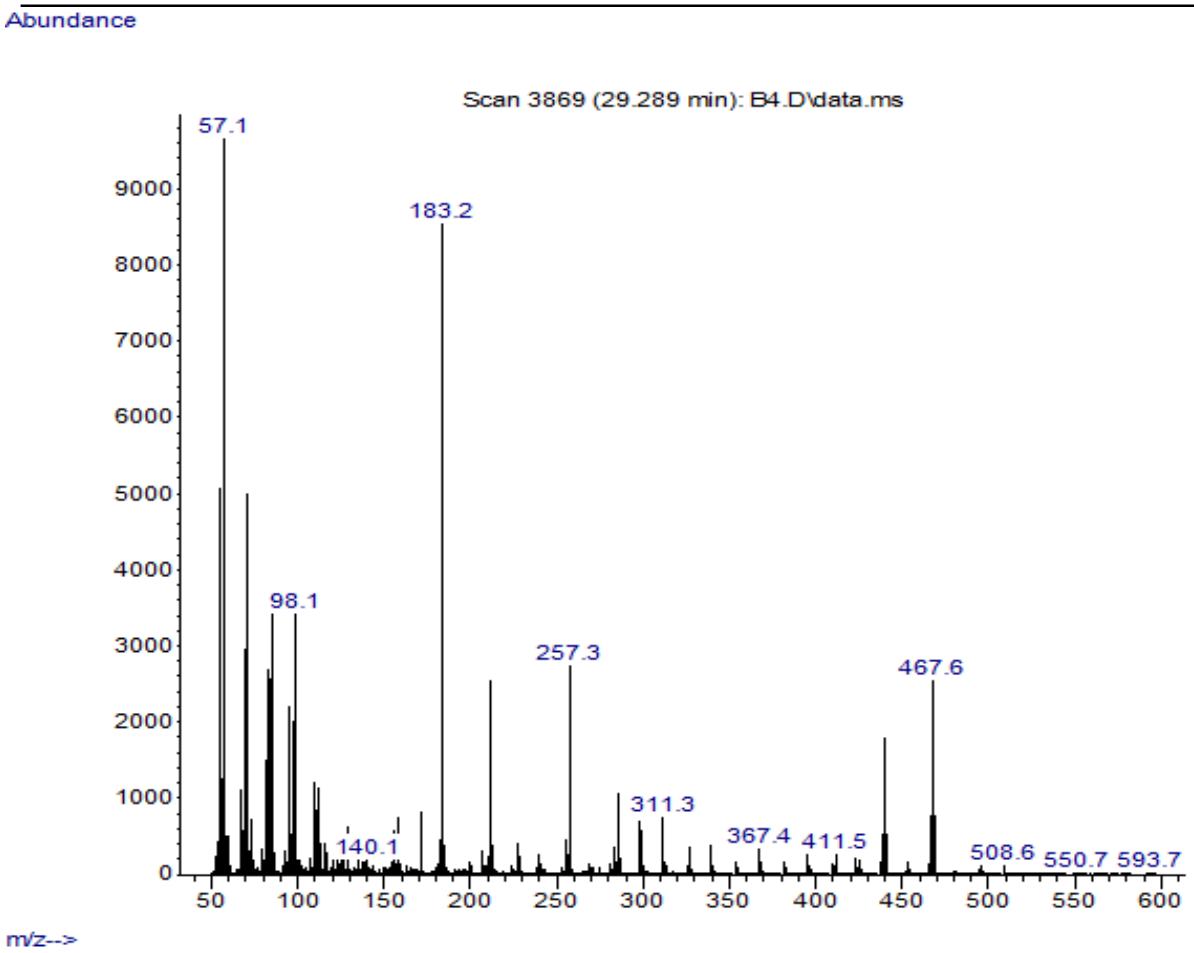

Figure 2: GC-MS spectrum of biosurfactants produced by *Xanthomonas campestris*

CONCLUSION

The FT-IR and GC–MS profile collectively demonstrate that the biosurfactant produced by Xanthomonas campestris possesses an amphiphilic structure with both hydrophilic and hydrophobic domains. The FT-IR spectra confirmed the presence of hydroxyl, amide, carbonyl, and carbohydrate functional groups, along with long-chain hydrocarbon vibrations, indicating structural features typical of glycolipids and lipopeptides. The GC–MS further validated these findings by revealing fatty acid fragments that constitute the hydrophobic moiety and sugar residues, particularly rhamnose, which serve as the hydrophilic head group. The detection of high molecular weight fragments corresponding to intact sugar–lipid conjugates strongly supports the classification of the biosurfactant as a rhamnolipid-type glycolipid. This structural complexity underpins its dual functionality in emulsification and surface tension reduction, highlighting its potential for diverse industrial and environmental applications, especially in bioremediation of tannery effluents. The stability of *Xanthomonas campestris*-produced crude biosurfactant had significant emulsification activity throughout a broad range of environmental conditions, with the best stability at pH 8, 45 °C, and 10% NaCl. This shows how resilient the biosurfactant is, which makes it appropriate for use in saline, alkaline, and moderately hot settings.

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