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Production and Characterization of a Novel Glycolipid Biosurfactant from *Bradyrhizobium* sp.

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Abstract

Biosurfactants (BS) are surface-active compounds synthesized by microorganisms with broad industrial applications. Although BS-producing strains are widely reported, little is known about their production by diazotrophic bacteria. This study investigated, for the first time, the BS produced by *Bradyrhizobium* sp. ESA 81, a diazotrophic bacterium isolated from the Brazilian semiarid region. The strain was cultivated in the mineral medium using sunflower oil and ammonium nitrate as carbon and nitrogen sources. The compound was chemically characterized using TLC, FAME, FTIR, and mass spectrometry (MALDI-TOF). The results revealed a mixture of glycolipids composed of trehalose linked to fatty acid chains ranging from C9 to C18. The BS exhibited a surface tension of 31.8 mN/m, a critical micelle concentration of 61.2 mg/L, and an interfacial tension of 22.1 mN/m. The BS also showed an emulsification index (EI24) of 55.0%. High stability was observed under extreme conditions of temperature (−20 to 121 °C), pH (2–12), NaCl (5–20%), and sucrose (1–5%). These findings indicate that the trehalolipid BS produced by *Bradyrhizobium* sp. ESA 81 is a stable and efficient surface-active agent, with promising potential for use in biotechnological and industrial processes.

Keywords: Biosurfactant; *Bradyrhizobium*; trehalolipid; production; rhizobia

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1. Introduction

Surfactants are organic substances with surface-active properties, most commonly produced through chemical synthesis using petroleum-based precursors [1]. These compounds hold significant commercial importance, being widely used in numerous industrial formulations of cosmetic, pharmaceutical, agricultural, and food products. However, from production to disposal, synthetic surfactants are associated with numerous environmental harms and adverse effects on human health due to their low biocompatibility, bioaccumulation potential, high toxicity, and limited biodegradability, among other characteristics that raise concerns from both ecological and consumer safety perspectives [2]. Consequently, the negative impacts caused by these agents have garnered growing attention in recent decades, driving the search for more sustainable alternatives that satisfy both technological performance and environmental safety requirements [3,4].

Microbial surfactants, or biosurfactants (BS), are a class of multifunctional surfactant molecules produced by a wide variety of bacteria, fungi, and yeasts [5]. BS are analogous

to their synthetic counterparts [6]; however, they are notable due to better environmental compatibility, lower toxicity, higher biodegradability, broad structural diversity, and functional stability under extreme conditions of pH, salinity, pressure, and temperature. Additionally, these biomolecules can be produced from renewable substrates and low-cost raw materials such as vegetable oils and industrial, domestic, or agricultural waste, meeting the criteria of sustainability and the circular bioeconomy. Due to these advantages, BS gathered significant attention as an efficient alternative to traditional surfactants in various industrial fields [4,7].

The search for microorganisms capable of producing natural surfactants has been intensively explored in recent years [8]. Despite significant advances in identifying BS-producing isolates, there is still a scarcity of information regarding the production of these compounds by certain bacterial groups, such as diazotrophic bacteria. These microorganisms constitute an important class, as they play a fundamental role in the process of biological nitrogen fixation [9]. The structural diversity and biotechnological potential of natural products derived from these microsymbionts represent a field that remains largely underexplored yet promising for the discovery of new commercially valuable molecules [10,11].

The genus *Bradyrhizobium* is widely recognized for its role in biological nitrogen fixation. In addition to this diazotrophic capability, some species of this genus have been associated with hydrocarbon emulsification and degradation, suggesting potential for BS biosynthesis [12,13]. However, despite some results regarding these properties, the study of biosurfactant production by *Bradyrhizobium* remains limited.

In the present work, we report for the first time the production of biosurfactant by the diazotrophic bacterium *Bradyrhizobium* sp. ESA 81, isolated from peanut (*Arachis hypogaea* L.) in the semiarid region of Brazil. The strain was cultured in sunflower oil as the sole carbon source, and the produced BS was characterized in terms of physicochemical properties, stability, and structural composition.

2. Materials and Methods

2.1. Microorganism and Culture Conditions

A preliminary screening was carried out with five diazotrophic isolates to evaluate their BS production potential. For this assay, isolates were cultured in a mineral medium supplemented with sunflower oil, composed of: sunflower oil (10.0 g/L), NH_4Cl (4.0 g/L), NaCl (0.5 g/L), KH_2PO_4 (0.5 g/L), $Na_2HPO_4{\cdot}7H_2O$ (1.0 g/L), and $MgSO_4{\cdot}7H_2O$ (0.5 g/L) at pH 7 [14] (modified). Cultures were incubated at 30 °C and 200 rpm for 120 h in a shaking incubator (MaxQ 6000, Thermo Scientific, Waltham, MA, USA). After centrifugation (10,000× *g* for 20 min at 4 °C) to remove biomass, the cell-free supernatant was collected. The performance of each isolate was determined based on the reduction in surface tension (ST) in the supernatant (~25 °C), using the Du Noüy ring method [15]. As controls, the ST of distilled water (~72.8 mN/m) and the uninoculated mineral medium (68.8 mN/m) were used. The strain *Bradyrhizobium* sp. ESA 81 showed the best result and was therefore selected for further investigation (Table S1).

Bradyrhizobium sp. ESA 81 belongs to the Collection of Microorganisms of Agricultural Interest of Embrapa Semiárido (CMISA). This strain was isolated from root nodules of peanut (*Arachis hypogaea* L.) from the semiarid region of Brazil and was identified in a previous study (Santos et al., 2017) [16]. Partial sequencing of its 16S rRNA gene is available in the GenBank database (https://www.ncbi.nlm.nih.gov/nuccore/1185580050, accessed on 9 July 2025) (accession number KY978637).

Bradyrhizobium sp. ESA 81 was grown in Yeast Malt (YM) medium containing: K_2HPO_4 (0.5 g/L), $MgSO_4.7H_2O$ (0.2 g/L), NaCl (0.1 g/L), Yeast extract (0.4 g/L), Agar (15.0 g/L), Mannitol (10.0 g/L), and 0.5% bromothymol blue (5 mL/L) at pH 7. Cultures were incu-

bated at 30 °C for 96 h [17]. Colonies were selected, and stock cultures were prepared in YM medium supplemented with 20% glycerol and stored at −80 °C.

2.2. Kinetics of Biosurfactant Production

Following initial cultivation in YM medium, an aliquot of 1 mL of *Bradyrhizobium* sp. ESA 81 culture (≅ 10^8 CFU/mL), used as pre-inoculum, was transferred to 125 mL Erlenmeyer flasks containing 35 mL of the mineral medium supplemented with sunflower oil, composed of: $Na_2HPO_4 \cdot 7H_2O$ (20.0 g/L), $NaH_2PO_4 \cdot H_2O$ (3.39 g/L), sunflower oil (10.0 g/L), NH_4NO_3 (4.0 g/L), NaCl (0.5 g/L), KH_2PO_4 (0.5 g/L), and $MgSO_4 \cdot 7H_2O$ (0.5 g/L), adjusted to pH 7 [14] (modified). Cultures were then incubated at 30 °C and 200 rpm in a rotary shaker (MaxQ 6000, Thermo Scientific, Waltham, MA, USA). Samples were collected at predetermined time intervals and subjected to the analyses described in the following sections.

2.2.1. Cell Growth and pH

The microbial growth was monitored using the colony count method (CFU/mL) [18].

The pH of the culture broth was determined with a benchtop digital potentiometer (Bel Engineering PHS3BW, Monza, Italy), equipped with a glass electrode. Following biomass separation (10,000× *g* for 20 min at 4 °C) (Thermo Sorvall Legend RT+ Thermo Scientific), the cell-free supernatant was used for the following analyses:

2.2.2. Determination of Sunflower Oil

The oil consumption by the bacteria was assessed using the cold lipid extraction method (Bligh-Dyer) [19].

2.2.3. Biosurfactant Concentration

BS concentration in the broth was estimated based on critical micelle dilution (CMD), through ST measurements (Du Noüy method) [15] of the supernatant diluted 10-fold (CMD^{-1}) and 100-fold (CMD^{-2}) in distilled water.

2.2.4. Biosurfactant Mass

The crude biosurfactant (cBS) content was estimated by determining the dry weight of the recovered material. Initially, the pH of the supernatant was adjusted to 2.0 using 6.0 M HCl and kept at 4 °C for 24 h to allow precipitation. The precipitate was then collected by centrifugation at 10,000× *g* for 20 min at 4 °C and redissolved in 10 mL of distilled water. The pH of this suspension was subsequently neutralized to 7.0 with 1 M NaOH. After confirming surfactant activity, the solution was incubated at 60 °C for 24 h and further dried for an additional 24 h in a desiccator containing silica. The final dry mass was weighed, and the concentration of cBS (g/L) was calculated using the formula: $[(P_2 - P^1) \times 100]$, where P_2 is the weight of the container with the dried BS and P_1 is the weight of the empty container.

2.3. Recovery and Purification of the Biosurfactant

Approximately 250 mL of cell-free broth was mixed with 300 g of Amberlite XAD-2 resin (Sigma-Aldrich, St. Louis, MO, USA) in a 1000 mL Erlenmeyer flask. After stirring at 150 rpm for 12 h at 37 °C, the supernatant was filtered, and the resin was washed with distilled water. It was then immersed in 250 mL of methanol, stirred for 20 min, filtered, and subjected to a second extraction. The resulting liquid fractions were dried using a rotary evaporator to obtain the BS [20]. The concentrated product was resuspended in distilled water and centrifuged (10,000× *g* for 10 min at 4 °C) (Thermo Sorvall Legend RT+ Thermo Scientific). The recovered aqueous fraction was tested for surfactant activity and

then dried at 60 °C. The brown solid obtained at the end of the process was considered the purified BS (pBS).

2.4. Physicochemical Characterization

2.4.1. Emulsification Index (E24)

The emulsification index was used as a qualitative assay to indicate the presence of BS in the culture medium. Due to limitations in the yield of the pBS, the test was performed using the cell-free supernatant. To determine E24, equal volumes (3 mL) of the supernatant and sunflower oil were added to test tubes and vigorously mixed using a vortex mixer for 2 min (VWR VM3000, Radnor, PA, USA) [21]. The samples were then left undisturbed at room temperature for 24 h. Distilled water was used as a negative control in place of the supernatant. After incubation, the height of the emulsion layer (EL) and the total height (TH) of the liquid column in each tube were measured. E24 was calculated using the following equation: E24 (%) = (EL/TH) × 100 [22].

2.4.2. Critical Micelle Concentration (CMC)

The CMC was determined by measuring ST using an Attension Sigma 700 tensiometer (Biolin Scientific, Espoo, Finland) coupled with an automatic titrator (Titronic® Universal, SI Analytics GmbH, Mainz, Germany). ST measurements, based on the Du Noüy ring method [15], were performed through consecutive automatic dilutions of a 0.5% pBS solution. The CMC value was calculated using the software provided with the Attension Sigma 700 (version 3.0).

2.4.3. Interfacial Tension (IFT)

The IFT measurements were performed using an Attension Sigma 700 tensiometer (Biolin Scientific, Espoo, Finland) following the Du Noüy ring method [15]. A 0.5% pBS standard solution (10 mL) was tested against an equal volume of *n*-hexadecane.

2.5. Biosurfactant Stability

The stability of the BS was determined by measuring the ST of 0.5% pBS solutions under different conditions of pH (2 to 12, adjusted with 1 M HCl and 1 M NaOH), temperature (−20 to 121 °C), NaCl concentration (5 to 20%), and sucrose concentration (1 to 5%). To evaluate thermal stability, the solutions were incubated for 1 h at various temperatures (−20 °C to 100 °C) and for 20 min in an autoclave at 121 °C (1 atm), followed by cooling to room temperature. ST measurements were performed using the Du Noüy method as described above.

2.6. Structural Characterization

2.6.1. Thin-Layer Chromatography (TLC) Analysis

Thin-layer chromatography (TLC) was performed to obtain information about the chemical nature of the BS. For the TLC analysis, pBS was dissolved in methanol and collected using a capillary tube. Approximately 2 µL of the solution was applied to silica gel plates (Merck, Darmstadt, Germany). The mobile phase used to separate the compounds was chloroform/methanol/water (65/15/2, *v*/*v*/*v*). Iodine, ninhydrin, and α-naphthol solutions were used to detect the presence of lipids, peptides, and sugars, respectively. The plates were incubated at 120 °C until the spot bands became visible [23].

2.6.2. Fatty Acid Methyl Ester Analysis (FAME) Analysis

A 10 mg sample of pBS was dissolved in 1 mL of methanol, acidified with concentrated H_2SO_4, and heated at 60 °C for 30 min. After cooling, 2 mL of 10% NaCl was added. The fatty acid methyl esters (FAMEs) were extracted with 1 mL of *n*-hexane and analyzed by gas

chromatography-mass spectrometry (GC-MS) using a Shimadzu GC-2010 Plus (Shimadzu Corporation, Kyoto, Japan) equipped with an AOC-5000 injector and an MS2010 Plus mass detector (70 eV) (Shimadzu Corporation, Kyoto, Japan). Separations were performed on an SLB-5MS capillary column (Supelco®, Merck). The analysis conditions included a flow rate of 0.94 mL/min, a pressure of 67.7 kPa, helium as the carrier gas, an injector temperature of 250 °C, and an initial column temperature of 100 °C (held for 1 min), followed by a gradual increase of 10 °C/min up to 300 °C, with isothermal conditions maintained for 29 min [20]. The total analysis time was 50 min, with a 1 µL injection and a split ratio of 10.0. The ion source temperature was set at 200 °C, and the interface temperature at 270 °C. Ions were monitored from 4 to 50 min within the *m/z* range of 40 to 600.

2.6.3. Fourier Transform Infrared Spectroscopy (FTIR) Analysis

FTIR analyses were performed to characterize the functional groups of the BS. The infrared absorption spectra were measured using an FTIR-Affinity 1 spectrophotometer (Shimadzu, Kyoto, Japan), operating in attenuated total reflection (ATR) mode over a wavenumber range of 4000–400 cm^{-1}, with a resolution of 4 cm^{-1} [24].

2.6.4. Mass Spectrometry (MALDI-TOF) Analysis

The pBS sample was analyzed by mass spectrometry using MALDI-TOF with the AutoFlex Max instrument (Bruker Daltonics®, Billerica, MA, USA). The smartbeam-II laser was used in positive mode, covering the *m/z* range of 250 to 3500 with a reflector. FlexControl software was used for data acquisition, and FlexAnalysis software was used for data processing. For MS/MS, the LIFT mode was employed, allowing fragmentation of the precursor ion. For the analysis, a 1 µL aliquot of the sample, prepared at a concentration of 1 mg/mL in TA50 (acetonitrile/0.1% trifluoroacetic acid, 50/50, *v*/*v*), was mixed with 1 µL of α-cyano-4-hydroxycinnamic acid (HCCA) at a concentration of 10 mg/mL in TA50. This mixture was deposited onto a stainless-steel MALDI plate and dried at room temperature (dried droplet method) prior to analysis.

2.7. Statistical Analysis

All experiments were conducted in triplicate. The results of all experimental analyses are presented as the mean ± standard deviation (SD), obtained from at least three independent replicates. OriginPro 9.9 software (OriginLab, Northampton, MA, USA) was used for data processing.

3. Results

3.1. Biosurfactant Production

A preliminary assay was conducted to determine the best carbon and nitrogen sources for BS production by *Bradyrhizobium* sp. ESA 81. The ST values of the cell-free supernatant were used as the selection criterion (Tables S2 and S3). Sunflower oil and NH_4NO_3 showed the best results (68.8 mN/m to 30.2 mN/m) and were selected for the formulation of the BS production medium.

The kinetic profile of BS production is shown in Figure 1A–C. Figure 1A illustrates bacterial growth, oil consumption, and medium pH throughout the experiment. The pH of the production medium decreased from 6.9 to 6.4 after 192 h (Figure 1A). A reduction in the viable cell population was observed between 24 and 48 h. Sunflower oil was completely consumed after 120 h of cultivation.

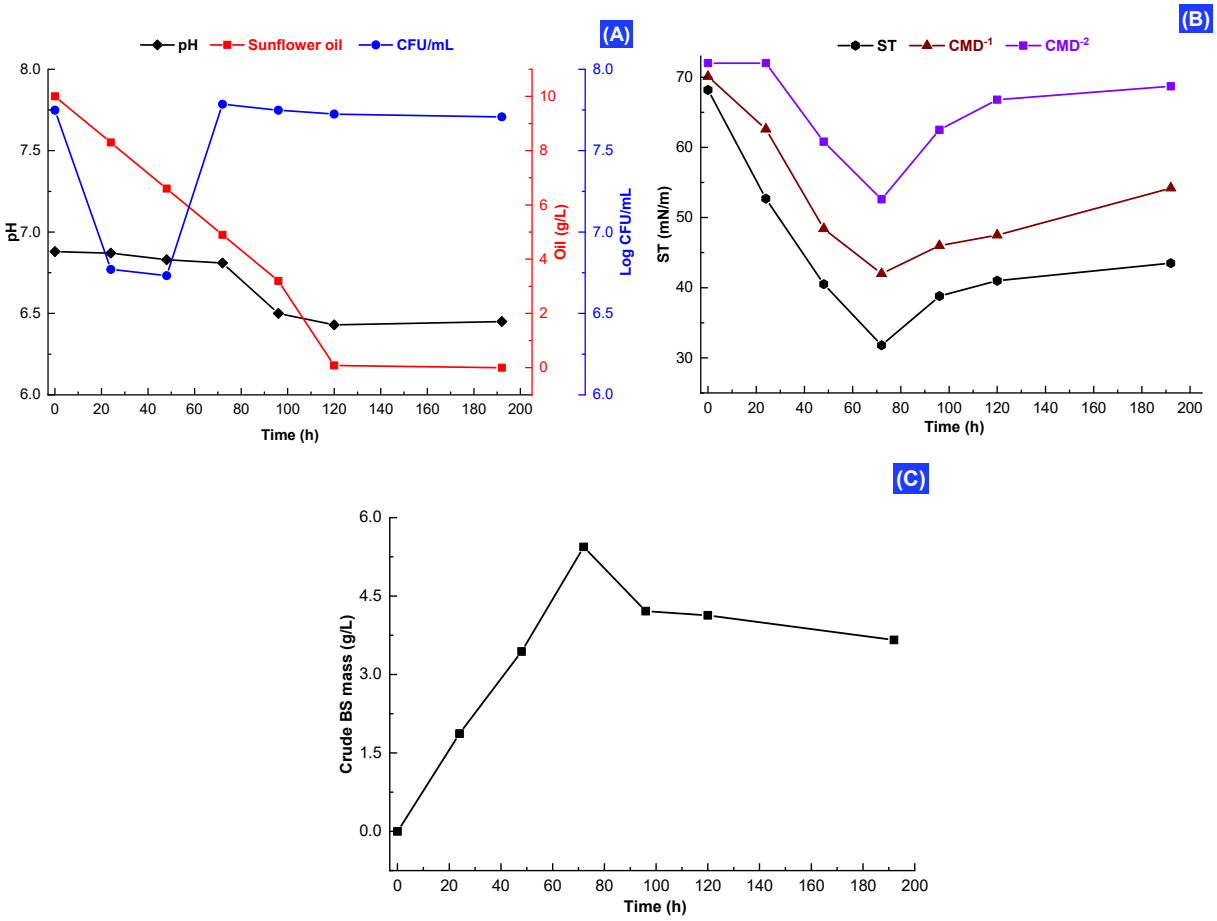

Figure 1. Kinetic profiles of BS production by *Bradyrhizobium* sp. ESA 81 in the mineral medium: Viable cell growth, pH, and sunflower oil consumption (**A**); surface tension and critical micelle dilution (CMD) (**B**); and crude BS production (**C**).

BS production was estimated by ST and CMD measurements. As shown in Figure 1B, a reduction in ST from 68.8 mN/m to 31.8 mN/m was observed after 72 h, with CMD^{-1} and CMD^{-2} values of 42.0 mN/m and 52.6 mN/m, respectively. After 72 h, both surface tension and CMD values increased.

The amount of cBS produced is presented in Figure 1C. Accumulation reached a maximum of 5.4 g/L at 72 h, followed by a decrease to 4.2 g/L at 96 h and to 3.7 g/L at 192 h. This maximum corresponds to a substrate conversion yield of 0.54 g of cBS per gram of oil consumed (54%), considering the total consumption of 10 g/L sunflower oil within 120 h.

After purification, 1.37 g/L of pBS was obtained, corresponding to a recovery of 25.4% from the crude extract. The substrate conversion yield for pBS was estimated at 0.137 g per gram of oil consumed.

3.2. Physicochemical Properties

The compound showed an emulsification index (EI) of 55.0 ± 0.04 in sunflower oil, and the resulting emulsion remained stable at the end of the test.

The CMC of the BS was estimated at 61.2 mg/L, with a minimum ST of 31.1 mN/m.

The IFT of the water/*n*-hexadecane system was reduced from 46.2 mN/m to 22.1 mN/m in the presence of BS, representing a decrease of approximately 52.16% compared to the control.

3.3. Stability of Biosurfactant

The stability of the compound was evaluated by monitoring ST under different conditions of temperature, pH, salinity, and sugar concentration (Figure 2A–D).

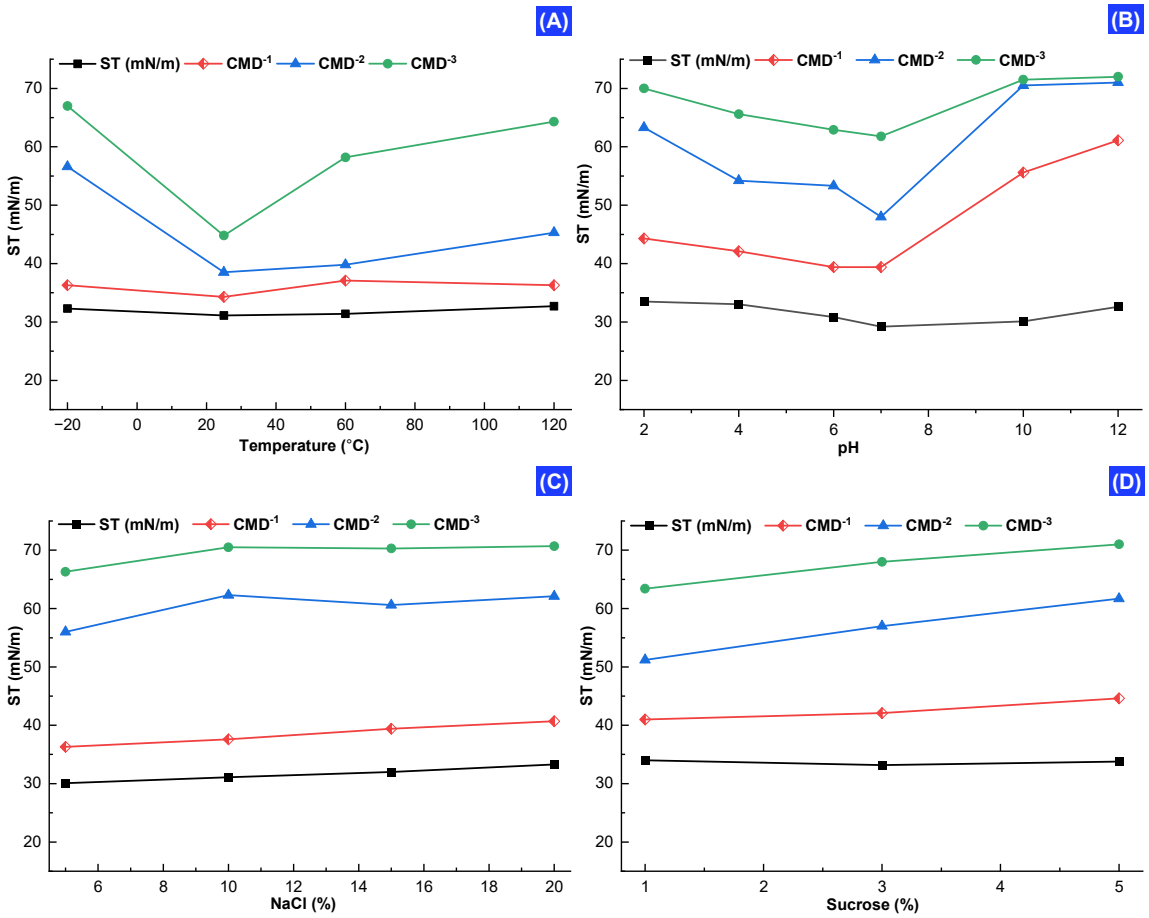

Figure 2. Stability of *Bradyrhizobium* sp. ESA 81 BS under different conditions of temperature (**A**), pH (**B**), NaCl (**C**) and sucrose (**D**).

The ST values of the BS solution remained relatively constant across the tested temperatures (25 °C to 121 °C), ranging from 31.1 ± 0.07 mN/m at 25 °C to 32.7 ± 0.08 mN/m after autoclaving (Figure 2A). Among all samples, the lowest ST values were recorded at 25 °C, with a maximum value of 44.8 ± 0.12 mN/m observed for the 1000 × dilution (CMD^{-3}).

As shown in Figure 2B, the BS exhibited optimal surfactant activity at pH values between 6 and 7, with an ST of 29.2 ± 0.18 mN/m. Slight increases in ST were observed under more acidic or alkaline conditions, suggesting possible structural modifications of the surfactant.

The ST-reducing capacity of the BS showed little variation in response to salinity and sugar gradients (Figure 2C,D). ST values ranged from 30.1 ± 0.28 mN/m at 5% NaCl to 33.3 ± 0.19 mN/m at 20%. A significant increase in ST was only observed in CMD^{-2} and CMD^{-3} dilutions. The test with sucrose (Figure 2D) revealed no significant adverse effect on BS stability in the presence of 1% to 5% sugar, with ST maintained at 33.8 ± 0.17 mN/m. A noticeable increase in ST was only observed after successive dilutions of the compound, with values reaching 41.0 ± 0.19 mN/m at 1% sucrose and 44.6 ± 0.29 mN/m at 5% for CMD^{-1}.

3.4. Characterization of Biosurfactant Structure

3.4.1. Thin-Layer Chromatography (TLC)

TLC analysis provided preliminary insights into the chemical composition of the BS. When the plate was incubated in a chamber saturated with iodine vapor (Figure 3A), a yellow spot with an Rf value of 0.82 was observed, indicating the presence of lipids. The presence of sugars was confirmed by a dark spot with an Rf value of 0.85 after staining with α-naphthol solution (Figure 3B). No spots were detected after staining with ninhydrin solution (Figure 3C), suggesting the absence of peptides or amino acids.

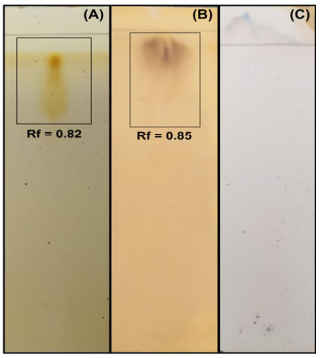

Figure 3. TLC of the BS produced by *Bradyrhizobium* sp. ESA 81, stained with iodine (**A**), α-naphthol (**B**), and ninhydrin (**C**).

3.4.2. Fatty Acid Methyl Ester Analysis (FAME)

The GC–MS profile revealed the presence of six distinct FAMEs, with retention times ranging from 6.1 to 19.3 min. Based on the GC–MS library, the identified fatty acids included nonanoic acid (C9), pentadecanoic acid (C15), octadecadienoic acid (C18:2), 9-hydroxyoctadecadienoic acid (C18:2), octadecenoic acid (C18:1), and 9,10-dihydroxyoctadecanoic acid (C18:0). These compounds corresponded to *m/z* values of 158, 242, 280, 282, and 316, respectively (Table S4).

3.4.3. Fourier Transform Infrared Spectroscopy (FTIR)

FTIR analysis of the BS (Figure 4) revealed a broad band at 3454 cm^{-1}, attributed to the stretching vibration of hydroxyl (–OH) groups, indicating the presence of carbohydrates. The absorption bands between 2926 and 2854 cm^{-1} correspond to the stretching of aliphatic C–H bonds, suggesting the presence of long hydrocarbon chains. The absorption band at 1710 cm^{-1} is associated with the stretching of ester carbonyl (C=O) groups.

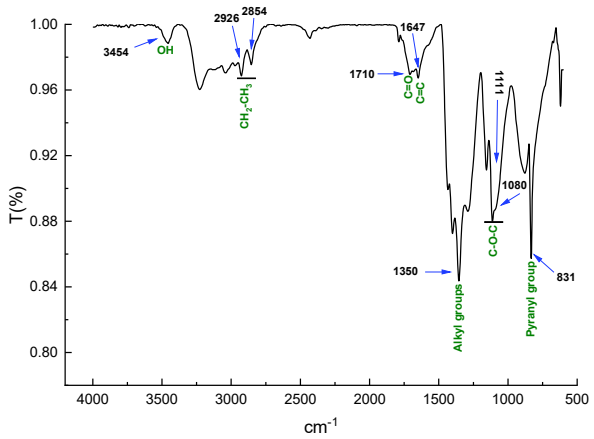

Figure 4. FTIR spectrum of BS produced by *Bradyrhizobium* sp. ESA 81.

The band observed at 1647 cm^{-1} corresponds to C=C stretching vibrations, indicating the presence of conjugated carbon–carbon double bonds. The intense band at 1350 cm^{-1} suggests the presence of alkyl groups, specifically indicating bonds between hydroxyl groups and carbon atoms in the glycosidic moiety [25]. The absorption bands at 1111 cm^{-1} and 1080 cm^{-1} were assigned to symmetric and asymmetric C–O–C stretching vibrations, respectively. The presence of carbohydrate rings was further supported by the band observed at 831 cm^{-1} [26].

3.4.4. Mass Spectrometry (MALDI-TOF)

Mass spectrometric analysis of the pBS extract (Figure 5) revealed the presence of sodium [M + Na^+] (*m/z* 656 and 699) and potassium [M + K^+] (*m/z* 672) adducts, which are commonly observed in samples containing carbohydrate derivatives [27].

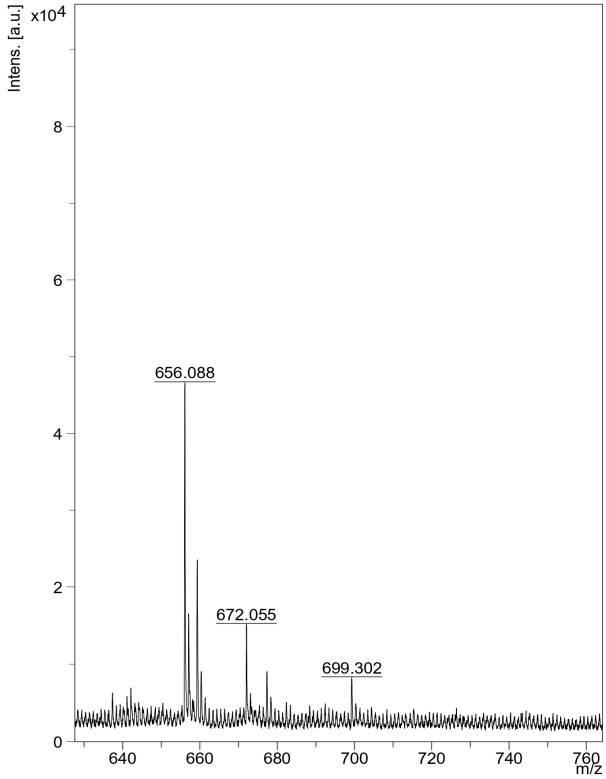

Figure 5. Mass spectrum (MALDI-TOF) of the BS sample from *Bradyrhizobium* sp. ESA 81.

The relative intensity of the ions provides insight into the proportional abundance of each molecule in the analyzed samples. In the obtained spectrum, the adducts at *m/z* 656.0 [M + Na^+] and *m/z* 672 [M + K^+] exhibited the highest intensities, indicating their predominance in the sample. Based on this relative abundance, the [M + Na^+] adduct at *m/z* 656.0 was selected for structural interpretation of the compound, which was identified as a trehalolipid (TL) BS.

The MS/MS fragmentation of the *m/z* 656.0 [M + Na^+] ion (Figure 6) showed a major peak at *m/z* 654, supporting the proposed molecular structure.

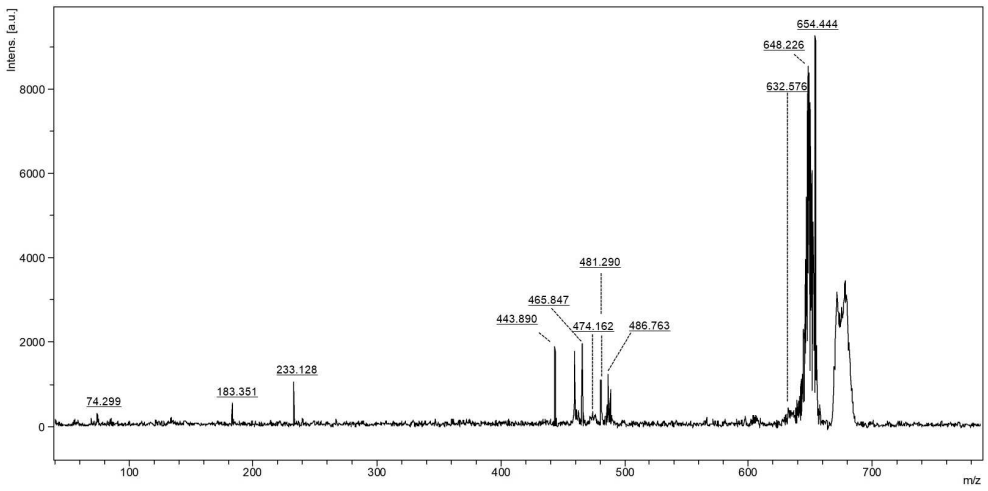

Figure 6. Mass spectrum of the fragment molecular ion [M + Na^+] at *m*/*z* 656 of the BS from *Bradyrhizobium* sp. ESA 81.

The 2 Da difference relative to the original peak was attributed to the loss of two hydrogen atoms (dehydrogenation) from the fatty acid chain of the [631 + Na^+] molecule. The fragment ions detected at *m*/*z* 465.8 and *m*/*z* 481 [M + Na^+] suggest cleavage of the α-D-glycopyranosyl-1,1-α-D-glycopyranoside bond within the trehalose moiety of the biosurfactant's polar region. These fragments correspond to a single glucose unit linked to the fatty acid chain, indicating the loss of one glucose residue from the trehalose disaccharide during fragmentation.

In the mass spectrometry technique, the selectivity of the ionization process favors the formation of ions from specific regions of the molecule, while others remain neutral. These neutral fragments can be indirectly detected by analyzing the mass differences between the observed peaks. In our analyses, non-ionized mass fractions of 180 Da were identified, as evidenced by the difference between the peaks at *m*/*z* 654 and *m*/*z* 474 (654 − 474 = 180), which corresponds to the molar mass of a glucose unit.

4. Discussion

Preliminary screening demonstrates that *Bradyrhizobium* sp. ESA 81 strain was able to grow and produce BS when cultivated in a mineral medium containing sunflower oil and NH_4NO_3 as carbon and nitrogen sources, respectively. The low reduction in pH during cultivation was probably due to the buffering effect of the phosphate salts present in the medium. The decrease in viable cell counts between 24 and 48 h was likely related to bacterial adaptation to the new substrate. The complete consumption of sunflower oil within 120 h demonstrated the strain's good adaptation to hydrophobic substrate, possibly facilitated by lipase activity [28]. The reduction in ST and CMD values after 72 h suggested a high concentration of BS in the medium, highlighting its role as an emulsifying agent that facilitates bacterial access to the oil substrate [29]. The CMD measurements serve as a qualitative indicator of BS effectiveness, providing an in situ assessment of its functional performance in the medium, with CMD^{-1} and CMD^{-2} values showing a significant decrease in ST, consistent with the observed reduction in ST to 31.8 mN/m. These values collectively reflect the enhanced emulsifying capacity and surface activity of the BS produced by *Bradyrhizobium* sp. ESA 81. Furthermore, the quantitative measurement of cBS concentration (reaching 5.4 g/L at 72 h) corroborates the CMD and ST data by confirming that a substantial amount of BS was produced at this time interval. This production profile suggests that BS synthesis by *Bradyrhizobium* sp. ESA 81 is associated

with the active growth phase, characteristic of primary metabolism [30]. The subsequent increase in ST and CMD values after 72 h (Figure 1B), along with the reduction in cBS concentration to 3.7 g/L at 192 h (Figure 1C), indicates a decline in BS availability or activity, likely due to degradation or consumption of the BS by the bacterium following carbon source depletion, as evidenced by the oil consumption profile in Figure 1A. This behavior is supported by the stable CFU counts from 72 h onwards, demonstrating that the changes are not due to cell lysis or dilution effects. This dynamic suggests that while the initial BS production enhances emulsification and substrate availability, nutrient limitation later reduces BS levels and activity. Complementary assessments of cell viability, such as dry biomass measurement, may contribute to a more robust understanding of growth dynamics in this system in future studies. Thus, the integrated analysis of ST, CMD, and cBS concentrations provides a comprehensive understanding of BS production kinetics and functionality in the culture system.

The emulsification test aims to assess the quality of a BS by its ability to form stable emulsions with hydrocarbons [31]. The percentage obtained in our study indicates significant emulsifying activity, demonstrating the effectiveness of the BS from *Bradyrhizobium* sp. ESA 81 in forming stable emulsions. Similar results were found by Zargar et al. (2022) [32], where a glycolipid BS isolated from *Gordonia* sp. exhibited good emulsifying capacity in mineral oil, with an EI of around 50%. Patel et al. (2021) [33] reported an IE of 55.89 for *n*-hexadecane using glycolipid produced by *Lactobacillus rhamnosus*.

The critical micellar concentration is the amount of surfactant required to achieve the maximum reduction in ST and the predominance of micelles [34]. Efficient surfactants exhibit low CMC values, indicating that smaller quantities are required to reduce ST, while efficacy refers to the extent of ST reduction achieved [35,36]. The CMC of the most effective BS ranges from 1 to 200 mg/L, and is influenced by the producing microorganism, carbon and nitrogen sources, cultivation conditions, pH and the presence of impurities [35,37]. The molecular structure, the proportion and composition of homologues, unsaturation, branching and the length of the aliphatic chain also affect the CMC of surfactants [38]. Various CMC values for glycolipid BS have been reported across a wide range. Zhao et al. (2018) found CMCs of 60 and 80 mg/L for rhamnolipids from *Pseudomonas aeruginosa* cultivated under different conditions [39]. Similarly, the rhamnolipid produced by *Franconibacter* sp. IITDAS19 had a CMC of 80 mg/L [40]. Joy et al. (2019) reported a CMC of 136 mg/L for rhamnolipids synthesized by *Achromobacter* sp. PS1 [27]. The glycolipid BS produced by *Gordonia* sp. IITR100 exhibited a CMC of 90 mg/L [32], while the trehalose lipid from *R. qingshengii* showed a CMC of 85 mg/L [41].

Interfacial tension (IFT) is another parameter used to evaluate BS quality; lower IFT values indicate higher efficiency [42]. The BS produced by *Bacillus subtilis* ITBCC30 was able to reduce IFT (water/xylene) to 22.35 mN/m [42]. In another study, a BS from a thermohalophilic strain isolated from a Brazilian oil reservoir reduced the IFT at the water/*n*-hexadecane interface to 17.6 mN/m [43]. IFT values between 24.3 and 29.6 mN/m have also been reported for enzymatic synthesis glycolipids [44].

High surface activity combined with a low CMC are essential characteristic of efficient surfactants. Considering the ST (31.1 mN/m), IFT (22.1 mN/m) and CMC (61.2 mg/L) values obtained, the BS from *Bradyrhizobium* sp. ESA 81 can be a promising candidate for industrial applications.

The stability towards temperature, pH and salinity is another important parameter to define BS applicability [27,45]. Our results indicate that the ability to reduce ST was maintained even after exposure to a wide temperature range, demonstrating the product's thermostability. Regarding pH stability, the observed increase in ST under extreme acidic or alkaline conditions suggests possible structural modifications or reduced solubility of

the BS. Under such conditions, surfactants tend to become less soluble, often leading to precipitation [38]. Additionally, the reduced efficiency may be related to BS concentration in the CMD dilutions. Overall, the BS showed good surface activity stability across a range of pH values. Salinity tolerance is also critical for potential use in processes involving high ionic strength, such as enhanced oil recovery. High salt concentrations interfere with the aggregation and solubilization properties of surfactants, reducing their effectiveness due to the presence of Na^+ and Cl^- ions. These ions can exert complex effects on the surfactant activity through "salting out", ion-dipole, and electrostatic interactions that alter micelle structure-essential for surfactant function-ultimately weakening their ability to disrupt water-water interactions [46].

The presence of sucrose did not significantly affect BS activity across the tested concentrations, reinforcing its potential for use in formulations containing sugars.

Stability is a key feature for BS intended for industrial use, given the variable conditions of temperature, pH, salinity, and sugar concentrations encountered in many processes. The BS obtained from *Bradyrhizobium* sp. ESA 81 exhibited high stability under all these conditions, significantly expanding its potential applications across various sectors.

Preliminary identification by TLC provided a quick and useful method to classify the BS before more detailed structural analyses. TLC results suggest that the BS produced by *Bradyrhizobium* sp. ESA 81 is a glycolipid, based on the detection of lipid and sugar components and the absence of peptide or amino acid indicators.

FAME analysis suggested the BS is a mixture of glycolipids with aliphatic chains ranging from C9 to C18 fatty acid lengths, commonly reported in glycolipid-type BS.

FTIR analysis was used to identify functional groups present in the compound. All major chemical groups detected are consistent with glycolipid structural characteristics. Similar FTIR spectra have been reported in other studies on BS glycolipids [26,32,47,48].

FAME analysis results, combined with the MS/MS fragmentation profile of the component at *m/z* 656, provided evidence of 9-hydroxyoctadecadienoic acid ($C_{18}H_{32}O_3$) forming an ester bond with the trehalose ring. The C=C stretching vibrations around 1647 cm^{-1} observed in FTIR are attributed to conjugated double bonds at positions 10 and 12 of this fatty acid.

The third pseudomolecular ion at *m/z* 699.302 [M + Na^+], present at a lower concentration in the sample, also corresponds to a TL-type BS, differing mainly in side-chain composition, which includes two fatty acids with C4 and C18 chains. The proposed structure of this homologue is shown in the Supplementary Material (Figure S1).

Mass spectrometry (MALDI-TOF) analysis of the major component (*m/z* 656), together with TLC, FAME, and FTIR, allowed the structural composition of the BS to be elucidated. Based on the data obtained, the BS is a TL containing a 9-hydroxy-octadecadienoic acid aliphatic chain esterified to the 4' carbon of the trehalose ring; the proposed chemical structure of the BS is illustrated in Figure 7.

Trehalose lipids have been investigated for a wide range of commercial applications, including bioremediation, enhanced oil recovery, and use in the biomedical, food, agricultural, and cosmetic industries, as well as for their antimicrobial activity [41]. Several microbially derived TLs have been described in the literature, differing in the structure and properties of the homologous mixtures they produce [49]. Most TLs reported to date have been produced by *Rhodococcus* spp. [41,49–52], although other bacterial genera, such as *Tsukamurella spumae*, *T. pseudospumae* [49], and *Nocardia farcinica* [53], have also been described. The pathogenicity of many TL-producing strains presents challenges for large-scale production and limits their applications in biomedical and pharmaceutical sectors. Therefore, the search for new non-pathogenic producers of these biocompounds is of great importance [54]. The proposed structural feature of the TL produced by *Bradyrhizobium*

sp. ESA 81 using sunflower oil includes a relatively short C18 acyl chain. Shorter acyl chains confer a more hydrophilic character to the BS, enhancing its suitability for various water-based applications [49].

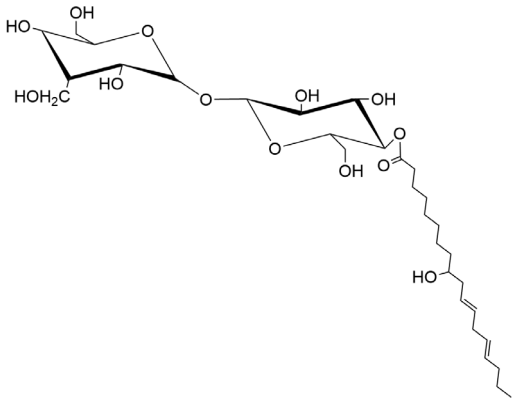

Figure 7. Proposed structure of the BS produced by *Bradyrhizobium* sp. ESA 81.

In this work, we report for the first time the production of a glycolipid biosurfactant by the non-pathogenic diazotrophic bacterium *Bradyrhizobium* sp. ESA 81. These findings highlight the promising potential of this strain for the production of novel TLs with properties of biotechnological interest. To the best of our knowledge, this is the first report of BS production by the *Bradyrhizobium* genus. Further investigations into the cytotoxicity of the compound, as well as its antimicrobial and bioremediation potential, could provide valuable insights into its practical applications.

5. Conclusions

This work describes, for the first time, the production of BS by the diazotrophic bacterium *Bradyrhizobium* sp. ESA 81. The compound was identified as a mixture of trehalose lipids. It exhibited significant surface-active and emulsifying properties, along with high stability across a wide range of temperatures, pH levels, salinity, and sugar concentrations. These characteristics suggest the potential applicability of the product in various industrial sectors. Additional studies exploring the biological effects and functional properties of this BS are essential to fully assess its practical value and expand its range of applications.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/fermentation11080471/s1, Table S1: Screening for BS-producing diazotrophic strains; Table S2: Effect of carbon source on BS production by *Bradyrhizobium* sp. ESA 81; Table S3: Effect of nitrogen source on BS production by *Bradyrhizobium* sp. ESA 81; Table S4: Fatty acid composition of BS produced by *Bradyrhizobium* sp. ESA 81; Figure S1: Proposed structure of the BS with m/z = 699 [M + K^+] produced by *Bradyrhizobium* sp. ESA 81.

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Data Availability Statement: The 16S rRNA gene sequence of the strain used in this study is available in the GenBank database under accession number KY978637 (https://www.ncbi.nlm.nih.gov/nuccore/1185580050). Additional data supporting the findings of this study are available from the corresponding author upon reasonable request.

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Conflicts of Interest: Author Douglas de Britto was employed by the Brazilian Agricultural Research Corporation (Embrapa). The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as potential conflicts of interest.

Abbreviations

The following abbreviations are used in this manuscript:

BS	Biosurfactant
YM	Yeast Malt
OD	Optical Density
CFU	Colony-Forming Unit
pH	Potential of Hydrogen
CMD	Critical Micelle Dilution
CMC	Critical Micelle Concentration
IFT	Interfacial Tension
TLC	Thin-Layer Chromatography
FAME	Fatty Acid Methyl Ester Analysis
FTIR	Fourier Transform Infrared Spectroscopy
MALDI- TOF	Matrix-Assisted Laser Desorption/Ionization—Time Of Flight
ST	Surface Tension
EI	Emulsification Index
TL	Trehalolipid

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