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RESEARCH

Production, characterization, and antifungal action of a biosurfactant obtained from diazotrophic *Paenibacillus* sp.

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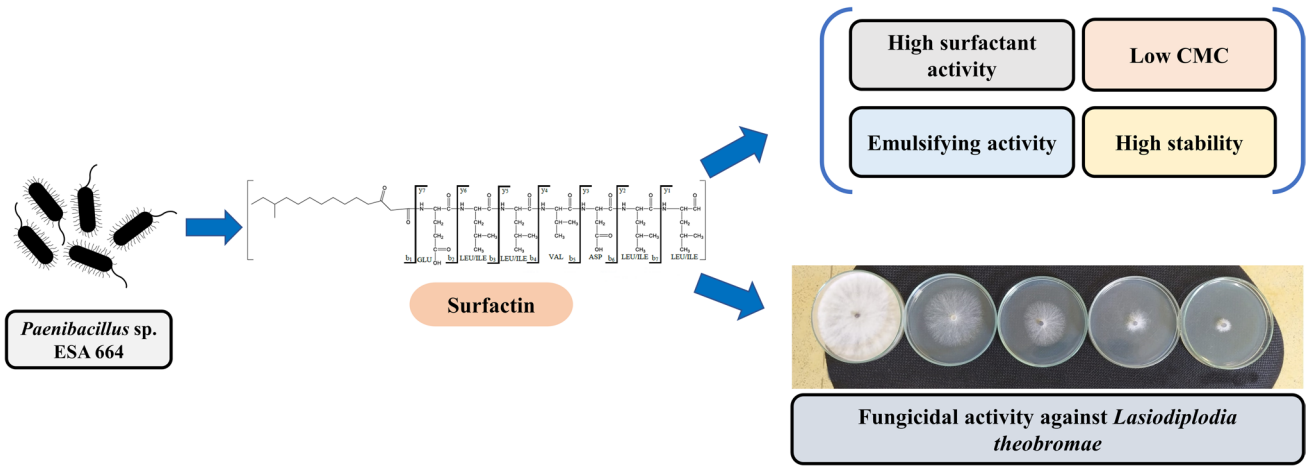
Abstract

Biosurfactants (BS) are surface-active agents derived from microbes, garnering increasing industrial interest due to their environmentally friendly and sustainable nature. Although several bacterial isolates capable of producing biosurfactants have been reported, their production by diazotrophic strains remains underexplored. This study investigated the production, physicochemical properties, chemical composition and antifungal activity of BS produced by the diazotrophic bacteria *Paenibacillus* sp. ESA 664, isolated from the semi-arid region of Brazil. The results showed that the isolate was able to grow and produce BS in mineral media using glucose and ammonium chloride as carbon and nitrogen sources, respectively. The chemical structure of the surfactant was elucidated using liquid chromatography-mass spectrometry (LC–MS) and Fourier transform infrared spectroscopy (FTIR). Results revealed a lipopeptide structure similar to surfactin with the C15 isoform being the most abundant. The surfactin obtained showed surface tension of 24.4 mN/m, critical micellar concentration (CMC) of 3.88 mg/L, interfacial tension of 2.87 mN/m and emulsification index of 62.0%. In addition, the BS presented stability over a wide range of temperatures (-20 to121°C), pH (2 to 12), NaCl (1 to 20%), and sucrose (1 to 5%), characterizing the product as a highly effective surfactant with potential for application in various industrial fields. Moreover, the compound exhibited significant fungicidal activity against the phytopathogen *Lasiodiplodia theobromae*, suggesting its use in agriculture as a biocontrol agent. Future investigations should address the development of field-applicable formulations and the elucidation of its antifungal mode of action.

Graphical Abstract



Keywords Antifungal activity · Biosurfactant · Endophytic strain · *Paenibacillus* · Surfactin

Extended author information available on the last page of the article

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Introduction

Surfactants are fundamental chemical compounds in industry, essential in the formulation of countless products owing to their detergency, emulsification, lubrication, foaming, wettability, solubilization and dispersion properties (Sharma et al. 2022). In agriculture, they play multiple roles acting as wetting and spreading agents, emulsifiers and dispersants (Abd-Elhalem et al. 2015). However, their production process, as well as their by-products, which are considered hazardous from an occupational and environmental point of view, have resulted in a search for more sustainable alternatives (Sen et al. 2017; Bhadani et al. 2020; Antonioli Júnior et al. 2022).

Microbial biosurfactants (BS) are surfactant compounds consisting of metabolic by-products from a wide variety of bacteria, fungi, microalgae and yeasts (Sanches et al. 2021). BS share similar characteristics to their synthetic counterparts (Moldes et al. 2021), but stand out for their lower toxicity, greater biodegradability and high chemical diversity, as well as their ability to be produced from renewable and cheap resources, which contributes to promoting sustainability and the circular bioeconomy (López-Prieto et al. 2019; Mohy Eldin and Hossam 2023). The desirable functional properties of BS have attracted great attention in recent decades because of their ecological and sustainable characteristics, which make them an interesting alternative to synthetic surfactants in various sectors such as oil, agriculture, food, biomedical, cosmetics, pharmaceuticals, cleaning, textiles, chemicals and nanotechnology (Johnson et al. 2021; Mishra et al. 2021).

BS are classified into four main groups that vary according to the producing species and the chemical nature as lipopeptides and lipoproteins, phospholipids, polymeric surfactants, and glycolipids (Twigg et al. 2021; Mohy Eldin and Hossam 2023). Lipopeptides are a well-known class of BS that have excellent surface activity and low CMC values, along with antimicrobial, antitumor, antiadhesive, antiviral and immunomodulatory activity (Carolin et al. 2021; Markande et al. 2021). Surfactin is the most notable cyclic lipopeptide, capable of reducing the surface tension of water to 26 mN/m while demonstrating high stability that can be exploited for various applications (Carolin et al. 2021; Wu et al. 2023).

Currently, the studies on BS production available in the literature are limited to a few well-known bacterial groups (Dias and Nitschke 2023). In most studies involving surfactin-producing strains for example, the genus *Bacillus* spp. prevails (Gudiña and Teixeira 2022; Wang et al. 2023). The Norine platform (https://norine.univ-lille.fr/norine) is a database dedicated to non-ribosomal peptides (NRPS), with information on the structure of a large number of peptides, together with data on their biological activity and producing organism (Flissi et al. 2020, 2023). Based on a database survey, approximately 263 different lipopeptides produced by 11 microbial genera have been described, with *Bacillus* spp. being the most common (Coutte et al. 2017; Geissler et al. 2019). However, the searching for BS produced by different strains of microorganisms is advantageous as it can reveal new surfactant molecules with unique properties.

Diazotrophic bacteria comprise a group of microorganisms usually associated with plants (Pankievicz et al. 2021). Due to their close relationship with the vegetal, diazotrophic and endophytic strains are very promising and their biotechnological potential is still underexploited. Moreover, the natural products from these microorganisms are structurally diverse and potentially active, which encourages their bioprospecting (Soumare et al. 2020; Raimi and Adeleke. 2021). Despite many research reports are available concerning BS produced by bacterial isolates, there is still a great scarcity of data on their production by diazotrophic bacteria. The genus *Paenibacillus* is known for its potential to produce BS of the lipopeptide class (Jimoh and Lin 2019, 2020; Kannan et al. 2021) but there are no reports in the literature about the synthesis of these BS by diazotrophic *Paenibacillus* sp.

Some fungal species of the genus *Lasiodiplodia*, especially *L. theobromae*, are pathogens that cause post-harvest diseases in mangoes (*Mangifera indica* L), resulting in significant losses in productivity and fruit quality (Coelho et al. 2020). With the increasing exigence of some markets worldwide to fruits without agrochemical residues, there is a growing search for technologies to improve mango preservation (Silva et al. 2023). Recently, research has focused on the antimicrobial and biocontrol potential of natural compounds which can reduce agricultural losses caused by phytopathogens without the need for chemical pesticides (Sani et al. 2024).

In this context, this study describes the production and characterization of a biosurfactant by a diazotrophic and endophytic strain of *Paenibacillus* sp. ESA 664 isolated from maize. Potential application of the biosurfactant as a novel controlling agent towards *L. theobromae* is also discussed.

Materials and methods

Microorganism and culture condition

The bacterium used in this work belongs to the Collection of Microorganisms of Agricultural Interest of Embrapa Semiárido (CMISA). *Paenibacillus* sp. ESA 664 is a seed-borne bacterium isolated from the shoots of maize (*Zea mays* L.) grown under axenic conditions, and identified by

the partial 16S rRNA gene sequencing (GenBank Accession MT482579.1) (Bomfim et al. 2020). The strain was first spread on DYGS agar plates containing: Glucose (2.0 g/L); Malic acid (2.0 g/L); Bacteriological peptone (1.5 g/L); Yeast extract (2.0 g/L); K_2HPO_4 (0.5 g/L); $MgSO_4.7H_2O$ (0.5 g/L); Glutamic acid (1.5 g/L); Agar–agar (15 g/L) (pH = 6.5), and incubated at 28ºC for 48 h (Rodrigues Neto 1986). Colonies were selected and stock cultures prepared in DYGS broth with 20% glycerol were kept at −80 °C.

BS production

After initial growth in DYGS medium, bacterial colonies were screened for BS production using mineral medium containing glucose (MMG), composed of: $Na_2HPO_4.7H_2O$ (20.1 g/L); $NaH_2PO_4.H_2O$ (3.39 g/L); glucose (13.0 g/L); KH_2PO_4 (1.5 g/L); NH_4Cl (4.0 g/L); $MgSO_4.7H_2O$ (0.2 g/L); $CaCl_2.2H_2O$ (0.01 g/L); $Fe_2(SO_4)_3.7\ H_2O$ (0.005 g/L), pH 7 (Wattanaphon et al. 2008). The screening assay was conducted at 28ºC and 180 rpm for 120 h in a shaker (MaxQ 6000—Thermo Scientific). After biomass separation (10,000 g for 20 min at 4 °C), the cell-free supernatant (± 25 °C) was subjected to surface tension (ST) measurements to verify BS production. Measurements were carried out using an Attension Sigma 700 tensiometer (Biolin Scientific, Espoo, Finland) employing the Du Noüy ring method (Bodour and Miller-Maier 1998). The ST values of distilled water (~ 72.8 mN/m) and uninoculated conventional medium (66.9 mN/m) were used as controls.

Further, the kinetics of BS production in liquid medium was carried out. An aliquot of 1 mL of *Paenibacillus* sp. ESA 664 (≅ 10^8 CFU/mL) inoculum was transferred to 125 mL Erlenmeyer flasks containing 35 mL of MMG medium and incubated at 28ºC, 180 rpm on a rotary shaker (MaxQ 6000—Thermo Scientific, USA). Samples were collected at defined time intervals and subjected to the tests described below.

Cell growth and pH, Glucose consumption and BS concentration

Microbial growth was monitored by measuring the optical density (OD) at 600 nm using a spectrophotometer (Spectronic Genesys 10—Thermo Scientific). The pH of the culture broth was determined with a benchtop digital potentiometer (Bel Engineering W3B, Brazil), equipped with a glass electrode. Following biomass separation (10,000 g for 20 min at 4 °C) (Thermo Sorval Legend RT +—Thermo Scientific), the cell-free supernatant was used for the following analyses:

Residual glucose content was determined using a commercial enzymatic glucose kit (AA, Wiener Lab), following the manufacturer's instructions, and the results were expressed in g/L.

BS concentration in the broth was estimated based on critical micelle dilution (CMD), through surface tension (ST) measurements of the supernatant diluted tenfold (CMD^{-1}), 100-fold (CMD^{-2}), and 1000–fold (CMD^{-3}) in distilled water.

Crude BS mass

The quantity of crude biosurfactant (cBS) was monitored by measuring the dry mass of the product. The pH of the supernatant was adjusted to 2.0 with 6.0 M HCl and maintained at 4 °C for 24 h. After centrifugation (10,000 g for 20 min at 4 °C), the pellet was resuspended in 10 mL of distilled water and adjusted to pH 7.0 with 1 M NaOH. The surfactant activity was verified, and the solution was incubated at 60 °C for 24 h, followed by an additional 24 h in a desiccator with silica. The samples were weighed to calculate the cBS quantity (g/L) according to the equation: $[(P2 - P1) \times 100]$, where P2 = mass of the container + cBS and P1 = mass of the container.

Recovery and purification of the BS

The culture was grown for 48 h at 28ºC in 500 mL Erlenmeyer flasks containing 100 mL of MMG medium (pH = 7), on a rotary shaker at 180 rpm. The medium was centrifuged (10,000 g for 20 min. at 4 °C) (Thermo Sorval Legend RT +—Thermo Scientific) to separate the cell biomass. The cell-free supernatant was acidified with 6 M HCl to pH 2.0 and kept at 4 °C overnight and the BS was separated by centrifugation (10,000 g for 30 min at 4 °C). The precipitate was suspended in distilled water and adjusted to pH 7.0 using 1 M NaOH. The solution was dried at 60ºC to obtain the crude biosurfactant (cBS).

The cBS was extracted twice with organic solvents. The first extraction was carried out by three washes in methanol. The mixture was stirred for 20 min in a Schott flask and left for the same time to separate the phases. The liquid phase was collected, and the solvent was evaporated using a rotary evaporator. The dark brown product obtained at the bottom of the flask was resuspended with distilled water and dried at 60ºC. The solid was subjected to further extraction with absolute ethanol, following the same procedures described above. The product obtained at the end of the process was considered to be semi-purified BS (spBS).

The spBS was purified using a chromatographic column. A 150 mg sample of the compound was dissolved in 5 mL of methanol/chloroform (60:40 v/v) and transferred to a chromatographic column (25 × 2.0 cm) packed with silica gel (230–400 Mesh, Neon, Brazil). Elution was carried out with methanol/chloroform (60:40 v/v) (Bezza and Chirwa

2015a; Zargar et al. 2022). Fractions of approximately 10 mL were collected, and the presence of BS was monitored by TLC. Fractions (1–5) were selected based on TLC analysis, which indicated the presence of the BS. These fractions were pooled, and the solvents were removed by rotary evaporation. The purified BS (pBS) obtained was used for critical micelle concentration (CMC) analysis, interfacial tension, stability, structural characterization, and antifungal evaluation.

Physicochemical characterization

Emulsification index (E24)

The emulsification activity (E24) (Wei et al. 2005), was estimated by adding 3 mL of cell-free supernatant to an equivalent volume of sunflower oil in test tubes. The tubes were vortexed (VM3000—Vixar Mixer) for 2 min and then allowed to stand for 24 h at room temperature. The control test was performed using distilled water. After this period, measurements were taken of the height of the emulsion layer (EL) and the total height (TH) of the liquids in the tubes, and E24 index was calculated using the formula: E24 (%) = (EL/TH) × 100 (Cooper and Goldenberg 1987).

Critical micelle concentration (CMC)

The determination of the CMC was carried out using an Attension Sigma 700 tensiometer (Biolin Scientific, Espoo, Finland) equipped with an automatic titrator (Titronic® universal). Surface tension measurements (Du Noüy ring method) were performed through successive automatic dilutions of a 0.05% pBS solution. The CMC was calculated using the Attension Sigma 700 software (version 3.0).

Interfacial tension (IFT)

The IFT was measured using an Attension Sigma 700 tensiometer (Biolin Scientific, Espoo, Finland) by the Du Noüy ring method using 10 mL of a 0,1% pBS standard solution against an equal volume of n-hexadecane.

BS stability

The ability of the BS to remain stable under varying conditions of temperature, pH, salinity, and sugar content is closely related to its applicability across various industrial sectors (Zhu et al. 2016; Umar et al. 2021). The stability of pBS in our study was determined by measuring the surface tension of 0.1% pBS solutions under different concentrations of NaCl (5 to 20%), sucrose (1 to 5%), pH (2 to 12) (using 1 M HCl and 1 M NaOH), and temperature (−20 to 121 °C). To analyze thermal stability, the solutions were incubated for 1 h in the different conditions (−20 to 100ºC) and for 20 min in an autoclave (1 atm) at 121ºC, then cooled to room temperature. Distilled water was used as a control, and was subjected to the same conditions as the pBS solutions.

Structural characterization

Preliminary characterization of the BS

The BS was preliminarily characterized by thin layer chromatography (TLC). The pBS was dissolved in methanol, and 2 µL of the solution were applied to silica gel 60 F254 plates (Merck, Darmstadt, Germany) using a capillary tube. The mobile phase consisted of chloroform: methanol: water (65:15:2, v/v/v). Lipids and amino acids were detected using iodine vapor and 0.35% ninhydrin solution (w/v in acetone), respectively. Plates sprayed with ninhydrin were incubated at 120 °C until spot development (Bezza and Chirwa 2015b). Protein presence was assessed by the biuret test (Gornall et al. 1949).

Fourier transform infrared spectroscopy (FTIR)

To characterize the functional groups, 2 mg samples of pBS and standard surfactin (Sigma-Aldrich) were mixed separately with 200 mg of KBr and pressed to obtain translucent pellets. The infrared absorption spectra of the sample and the standard were measured using an FT-IRAffinity 1 spectrophotometer (Shimadzu, Kyoto, Japan) in the 4,000–500 cm^{-1} wave number range with a resolution of 4 cm^{-1} (Vilela et al. 2014).

Mass spectrometry (LC–MS)

The pBS and the surfactin standard (Sigma-Aldrich) were analyzed by electrospray ionization mass spectrometry using an LTQ Velos mass spectrometer (Thermo Fisher Scientific, Bremen, Germany) with a linear ion trap analyzer. The experiments were carried out in positive ionization mode. Spectra were acquired from the *m/z* range 50 to 2000. For MS^2-type experiments, fragmentation experiments were carried out using collision-induced dissociation (CID) with an energy of 25 eV.

Antifungal activity against Lasiodiplodia theobromae

The isolate of *L. theobromae* from post-harvest rot on mango was obtained from the collection of the Phytopathology Laboratory at Embrapa Semiárido. To demonstrate the activity of pBS on *L. theobromae*, aqueous solutions were prepared containing different concentrations of pBS (0, 250, 500, 1000, and 2000 ppm). The solutions were previously autoclaved (1 atm, 121ºC, 20 min) to ensure sterility of the

treatment prior to the antifungal assay. Then, 200 µL of each solution was deposited on the surface of Petri dishes containing Potato Dextrose Agar (PDA) culture medium and spread across the plate. Subsequently, 0.3 cm diameter discs of the fungi mycelium, prepared from two-day-old cultures in PDA medium, were placed in the center of the plates. The plates were incubated in a growth chamber at 25ºC and a 12-h photoperiod. After 96 h of incubation, the radial diameter of the colony was measured along two orthogonal axes (modified from Regnier et al. 2008).

Statistical analysis

All experiments were carried out in triplicate. The results of all the experimental data analyzed were expressed as arithmetic mean values ± standard deviation (SD) of the measurements, derived from at least three independent repetitions. Origin Pro 9.9 software (OriginLab) was used for data processing.

Results

BS production

Initially, the screening assay confirmed the BS-producing ability of the *Paenibacillus* sp. ESA 664 isolate. The ST of the cell-free supernatant was measured using the Du Noüy ring method, resulting in a mean value of 26.1 ± 0.01 mN/m, indicating significant BS production. A preliminary trial was conducted to determine the most effective combination of carbon and nitrogen for the production of BS by *Paenibacillus* sp. ESA 664. The ST values of the cell-free supernatant were used as a criterion for this determination. In this study, glucose (13.0 g/L) and NH_4Cl (4.0 g/L) proved to be the most effective C/N combination for BS production by *Paenibacillus* sp. ESA 664 compared to the other substrates tested (Table S1).

The kinetic profile demonstrated the relationship between cell growth, glucose consumption, and BS accumulation, making it possible to determine the optimum cultivation time at which the product concentration was maximum (Fig. 1).

Figure 1A shows that the pH remained relatively stable, ranging from 6.9 to 6.5, throughout the 72 h of cultivation. Optical density measurements indicated that microbial growth followed a typical bacterial growth curve. An initial lag phase was observed during the first few hours, followed by a sharp increase in OD values between 8 and 16 h, corresponding to the exponential (log) growth phase. This was accompanied by active glucose consumption, as nearly 100% of the available carbon source was depleted by the end of this period. After 24 h, the OD values stabilized, suggesting the onset of the stationary phase, likely due to nutrient depletion or accumulation of metabolic by-products.

The accumulation of the BS was estimated through CMD as shown in Fig. 1B. The BS has the ability to reduce the surface tension of the culture medium from 62.8 mN/m to 25.3 mN/m within the first 12 h of cultivation, demonstrating its surfactant power. The ST of the medium decreased within the first 8 h (from 62.8 mN/m to 27.7 mN/m), reaching a lowest value of 25.2 mN/m after 16 h. The CMD is an indirect measure of BS concentration through the surface tension measurement of the supernatant diluted multiple times. It is possible to observe that the surface tension curves, CMD^{-1} and CMD^{-2}, exhibit similar behaviors and close values.

Regarding the amount of crude BS produced by the strain in MMG medium (Fig. 1C), it is possible to observe a maximum value of 16.3 g/L after 48 h where the lowest ST and CMD values were also obtained.

Physicochemical characterization

After 24 h, the EI greater than 62.0% ± 0.01 was observed against sunflower oil (Fig. 2). Thus, the BS exhibited significant emulsifying capacity, suggesting its potential to emulsify other hydrocarbons. The CMC value of BS isolated from *Paenibacillus* sp. ESA 664 was estimated as 3.88 ± 0.15 mg/L. The interfacial tension (IFT) of the water/n-hexadecane system was considerably reduced in the presence of BS, dropping from 46.2 ± 0.07 to 2.87 ± 0.04 mN/m. The ST (26 mN/m), IFT (2.87 mN/m) and CMC (3.88 mg/L) obtained in our study characterize the product as an effective surfactant agent.

BS stability

The stability of the BS under various conditions was monitored by measuring ST, with results displayed in Fig. 3A-D. The ST values of the 0.1% pBS solution remained relatively constant across the tested temperatures (−20 to 121ºC), with minimum values of 24.4 ± 0.03 mN/m, 26.0 ± 0.02 mN/m for CMD^{-1}, and 29 ± 0.02 mN/m for CMD^{-2}. A significant reduction in surface activity was noted only at 1000 × dilution (CMD^{-3}), demonstrating the thermal stability of the compound even after autoclaving. The ST showed the best activity at pH 6, with values of 26.2 ± 0.02 mN/m undiluted and 38.1 ± 0.91 mN/m in CMD^{-3}. Gradual increases in ST were noted at pH 2 (30.9 ± 0.16 mN/m) and pH 12 (29.2 ± 0.01 mN/m). A pronounced increase in ST at pH 2 in CMD^{-3} (70.1 ± 1.56 mN/m) suggests precipitation of surfactin. The BS maintained ST stability with salinity, ranging from 24.1 ± 0.31 mN/m at 5% salt to 26.4 ± 0.15 mN/m at 20%. A significant ST increase occurred only in diluted samples. The presence of 1–5% sucrose did not significantly affect the ST of the BS in undiluted solutions (27 ± 0.06

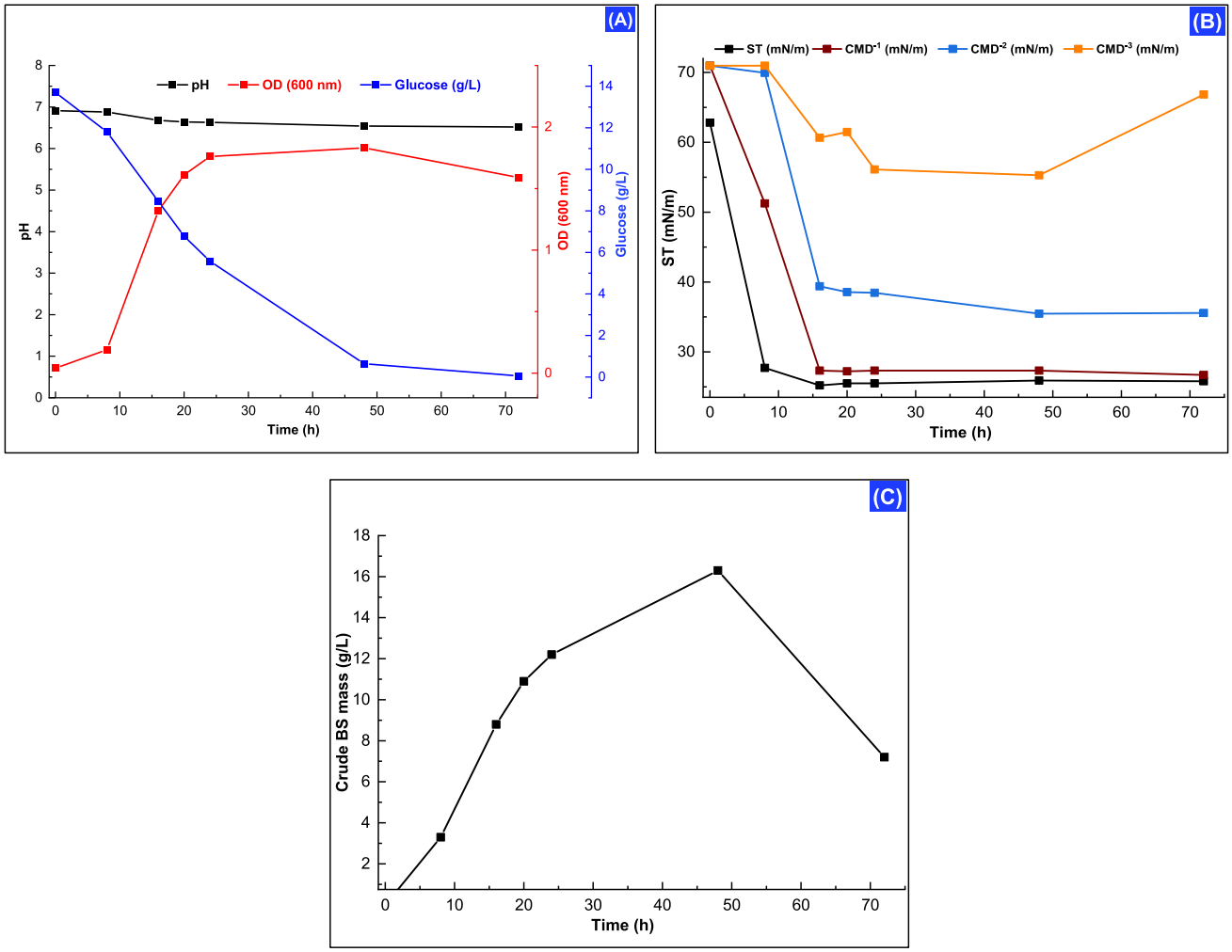

Fig. 1 Kinetic profiles of biosurfactant production by *Paenibacillus* sp.: (**A**) cell growth, medium pH and glucose consumption; (**B**) surface tension and critical micelle dilution (CMD) and (**C**) crude BS production

mN/m) or 10 × dilutions (CMD^{-1}) with a value of 30.1 ± 0.25 mN/m, which also showed no variation.

Structural characterization

The preliminary characterization of the BS by TLC revealed bands indicative of amino acids and lipids (Fig. S4A and B), while the biuret test confirmed the presence of protein (Fig. S5), suggesting that the BS belongs to the lipopeptide class. Further analysis were conducted to elucidate the BS chemical structure.

Fourier transform infrared spectroscopy (FTIR)

Figure 4A shows the presence of a broad band characteristic of peptides in the region of 3298 cm^{-1}, resulting from the stretching vibration of the N–H bond. The absorption bands between the frequencies of 2958 to 2850 cm^{-1} and 1467 to 1380 cm^{-1} indicate the presence of aliphatic bonds. The absorption at 1734 cm^{-1} is associated with the presence of the carbonyl group. At 1652 cm^{-1}, an intense peak corresponding to the stretching of the C = O bond can be noted, while at 1559 cm^{-1}, the angular deformation of N–H occurs combined with the stretching of the C-N bond.

Mass spectrometry (LC–MS)

Figure 5A, B illustrates the mass spectra of the purified BS extract (pBS) from *Paenibacillus* sp. ESA 664 (A) and the surfactin standard (B). The pBS analysis revealed the presence of protonated molecules $[M + H^+]$ at *m/z* 994.9, 1008.8, 1022.8, and 1036.9, along with their sodium adducts $[M + Na^+]$ at *m/z* 1016.7, 1030.8, 1044.9, 1058.9, and potassium $[M + K^+]$ at *m/z* 1074.8. The relative intensity of the ions may reflect how each molecule is represented by percentage in the examined samples. The mass

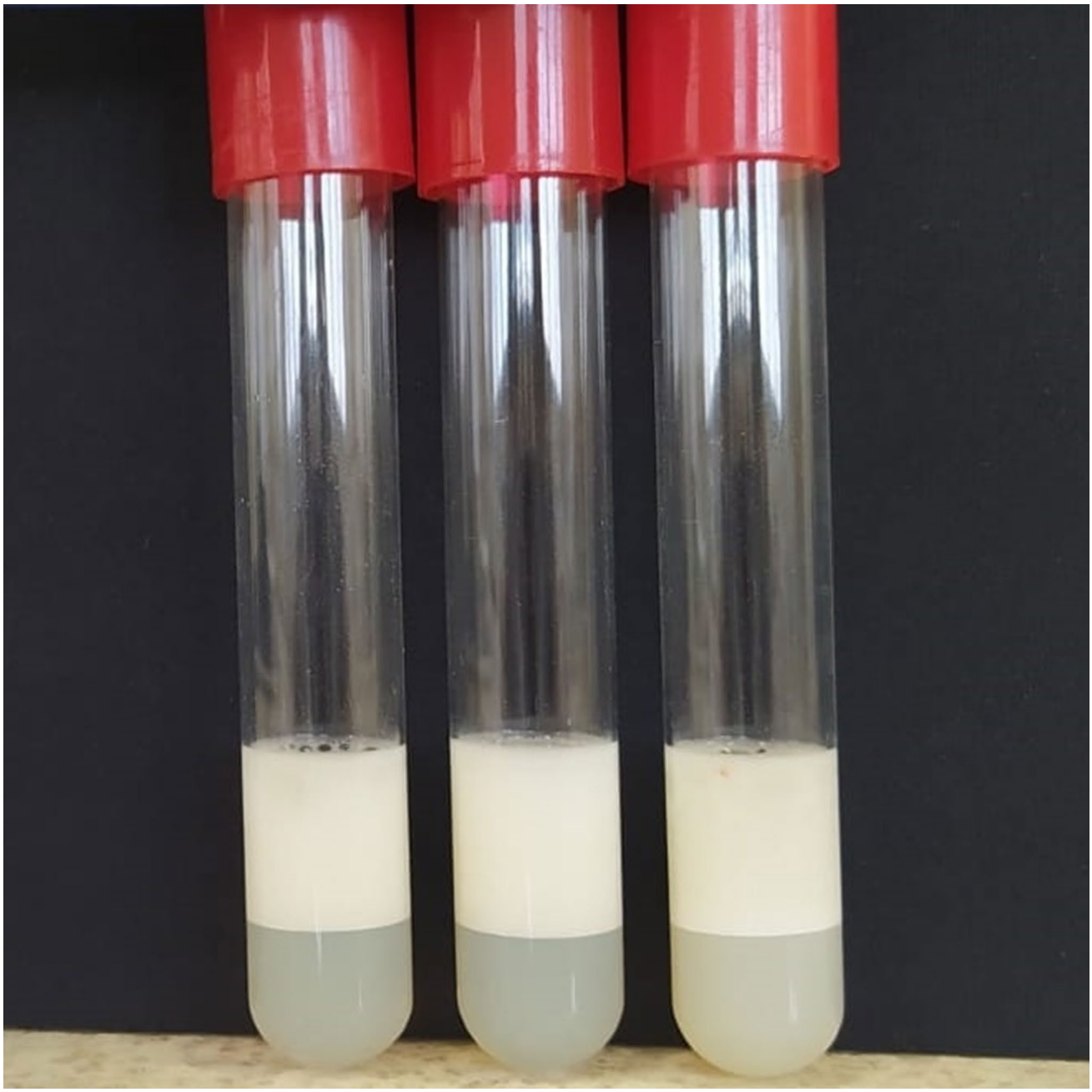

Fig. 2 Emulsification test of *Paenibacillus* sp. BS against sunflower oil after 24 h

spectrum showed that the predominant ions in the sample were those at *m/z* 1022.7, 1030.8, 1044.9, and 1058.9, with the most abundant ion being *m/z* 1058.9, corresponding to the sodium adduct of the lipopeptide with 15 carbons in its fatty acid chain (surfactin D isoform) (Patiño et al. 2021). The difference in the mass-to-charge ratio between each peak is 14 mass units, corresponding exactly to a CH_2 residue. Thus, the ions 994.9, 1008.9, and 1022.9 represent homologs with 12, 13, and 14 carbons, respectively, in the lipid chain, indicating that the analyzed compound is a mixture of lipopeptides with a series of different fatty acid chain lengths.

The surfactin standard was also analyzed, and the obtained mass spectrum showed a high similarity in the distribution of mass-to-charge ratios of its main ions compared to the sample from *Paenibacillus* sp. ESA 664 (Fig. 5B) with the presence of four main ions of *m/z* 1022.8, 1036.8, 1044.9, and 1058.9. The fragmentation

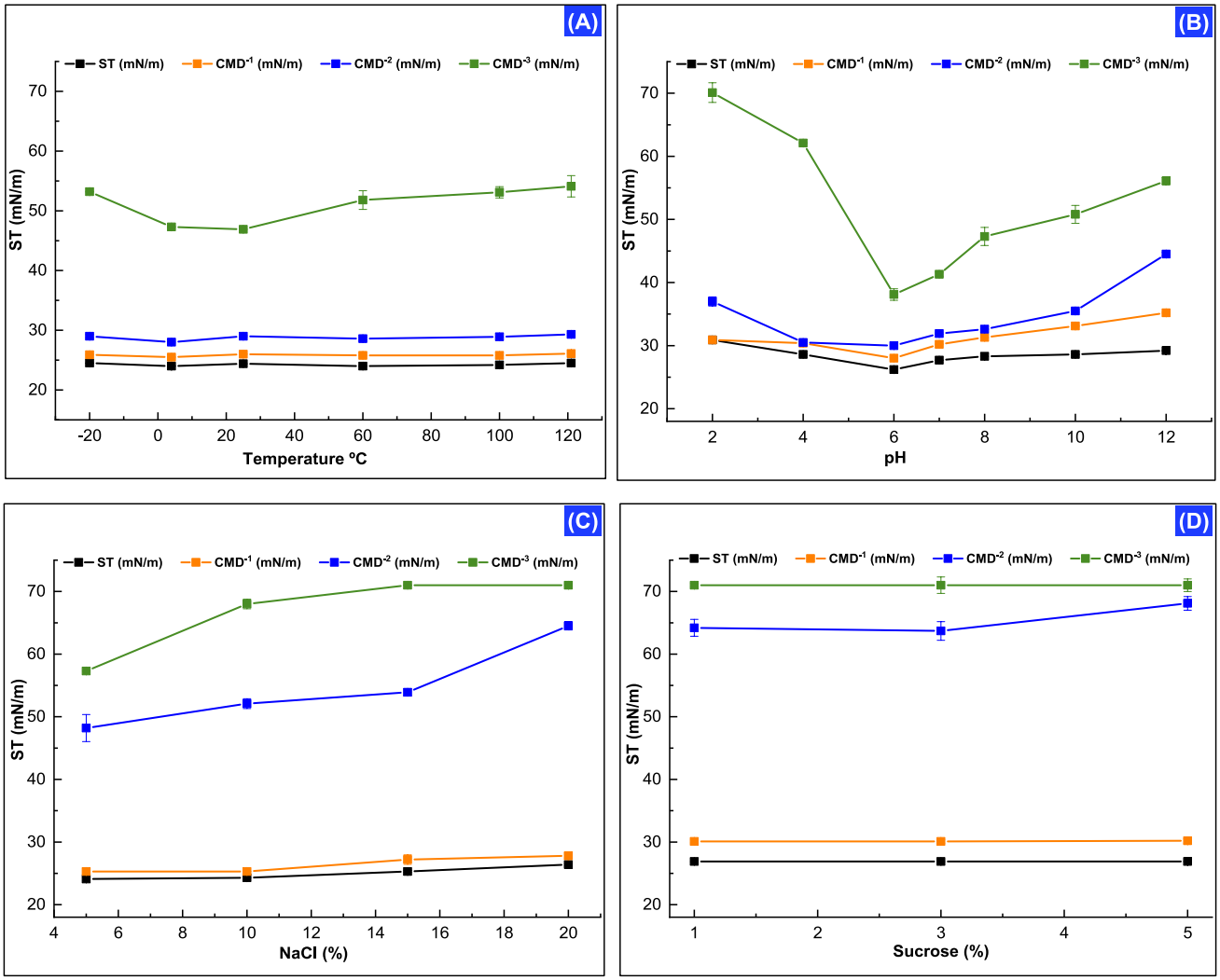

Fig. 3 Stability of pBS from *Paenibacillus* sp. under different conditions of temperature (**A**), pH (**B**), NaCl (**C**) and sucrose (**D**) concentrations

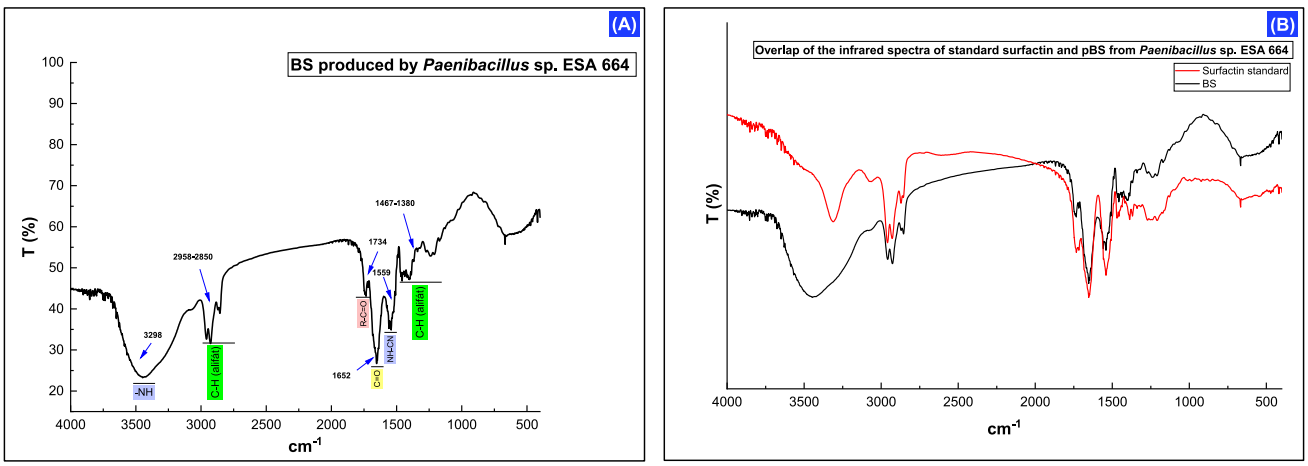

Fig. 4 FTIR spectra of BS produced by *Paenibacillus* sp. (**A**). Overlap of the infrared spectra of standard surfactin and the pBS from *Paenibacillus* sp. (**B**)

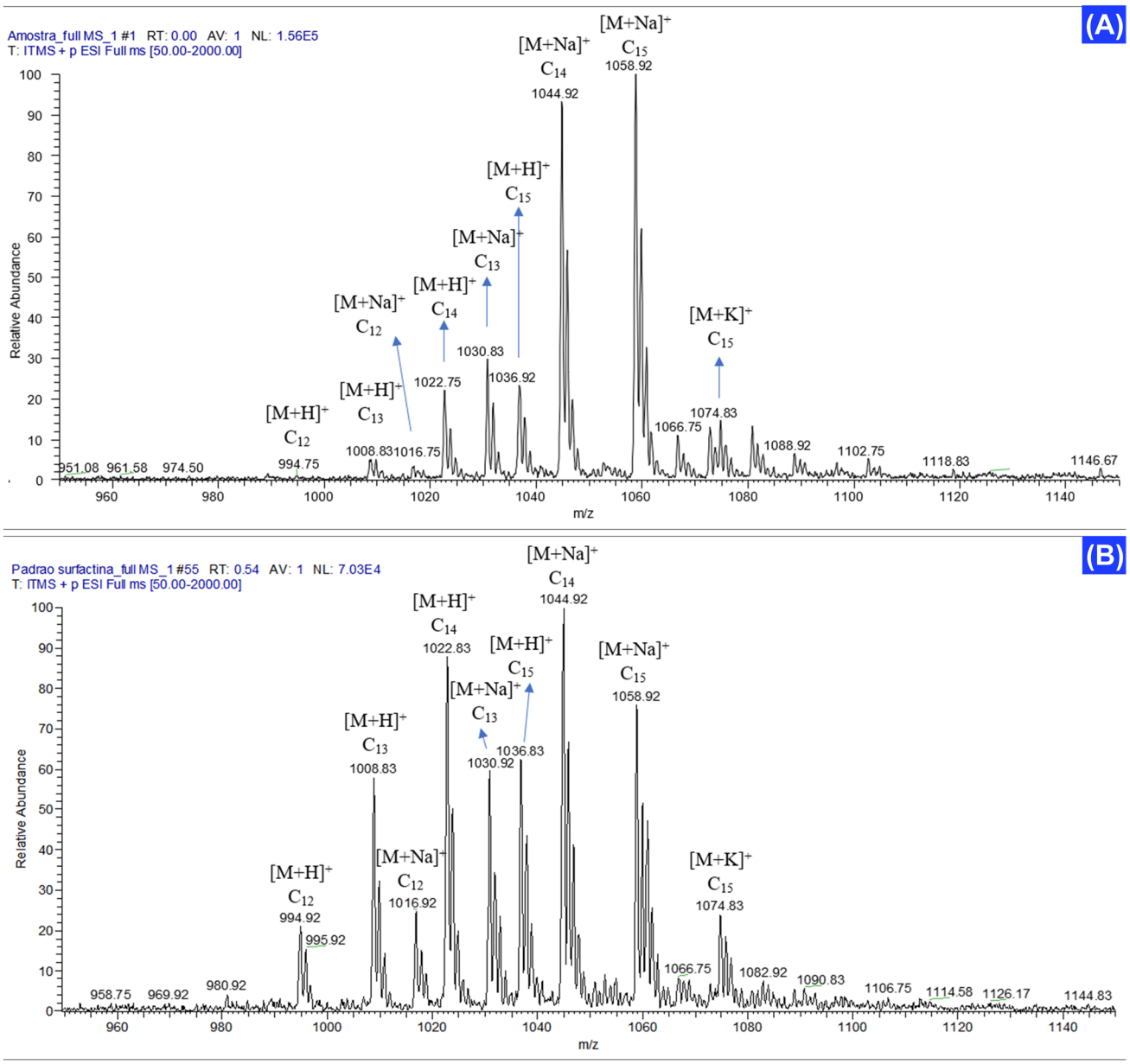

Fig. 5 Mass spectrometry (ESI) of the BS sample from *Paenibacillus* sp. ESA 664 (**A**) and surfactin standard (**B**)

of the predominant ion (*m/z* 1058.9) showed seven characteristic amino acid residues of surfactin: GLU-LEU/ILE-LEU/ILE-VAL-ASP-LEU/ILE-LEU/ILE (Fig. S1). The same peptide sequence was also inferred by analyzing other homologs in their protonated and sodium adduct forms (Fig. S2 and S3). For the same precursor ions present in the sample and the standard, the same fragmentation spectra (ESI–MS/MS) were obtained. Thus, based on our findings, the BS produced by *Paenibacillus* sp. ESA 664 was identified as a surfactin-like lipopeptide.

Table 1 Effect of the BS from *Paenibacillus* sp. ESA 664 on the growth of *L. theobromae*

Concentration (ppm)	Radial diameter (cm)	Fungal inhibition (%)
0	9	0
250	8	11.1
500	5.7	36.7
1000	2.7	70
2000	0.9	90

PPM = Parts per million

Antifungal activity against Lasiodiplodia theobromae

The in vitro antifungal activity of the BS produced by *Paenibacillus* sp. ESA 664 against *L. theobromae* was estimated based on the radial diameter of the fungal colony after 96 h of incubation (Table 1).

The radial diameters of the mycelium ranged between 8.0 and 0.9 cm for the treatments with BS, with inhibition percentages reaching up to 90% reduction compared to the control at a concentration of 2000 ppm. Figure 6 illustrates the concentration-dependent effect of the surfactant on preventing the mycelial growth of *L. theobromae*.

Discussion

Preliminary screening demonstrated that *Paenibacillus* sp. ESA 664 strain was able to grow and produce biosurfactant when cultivated in mineral medium containing glucose and ammonium chloride as carbon and nitrogen sources respectively. Further, the kinetic study revealed that maximum level of BS was detected after 48 h of cultivation showing also the lowest values of ST and CMD.

It worth noting that a reduction in the amount of BS observed after 48 h (Fig. 1C) was accompanied by a decline in cell growth and the depletion of glucose in the culture medium; possibly, the biosurfactant was utilized as a nutrient by bacterial cells in such deprived condition (Ciurko et al. 2023).

The ability to form and stabilize emulsions is a significant factor in determining the quality of a surfactant (Jimoh and Lin 2019). The effectiveness of BS is defined, among other things, by their ability to sustain the emulsion for 24 h with at least 50% of the total volume (Adiandri et al. 2023). A BS produced by *Paenibacillus popilliae* showed emulsification rates of 90.9% for crude oil, 81.8% for motor oil and 54.5% for kerosene, but did not emulsify sunflower oil (Mesbaiah et al. 2016) different to the observed in our work. Emulsification test are influenced by culture medium components and also by biosurfactant type and concentration in medium which in turns impact in the EI observed (Adiandri et al. 2023).

Efficient surfactants are generally characterized by low CMC values, as they require smaller concentrations to reduce ST (Behzadnia et al. 2020; El-Housseiny et al. 2020). The CMC is defined as the minimum concentration at which micelles begin to form in solution and ST reaches its lowest plateau (Zargar et al. 2022). The BS produced by *Paenibacillus* sp. ESA 664 exhibited a low CMC value comparatively to previous studies which have reported CMC values ranging from 185 to 200 mg/L for a BS from *Paenibacillus* spp (Bezza and Chirwa 2015a; Jimoh and Lin 2019). Similarly, a CMC of 500 mg/L was reported for a lipopeptide produced by *Bacillus subtilis* RSL2 (Sharma and Pandey 2020); however, these values were obtained using cell-free supernatants or crude extracts, which typically contain various impurities that can interfere with surface tension measurements conversely to our purified BS sample. On the other hand, lower CMC values have been described for semi-purified BS, such as 12.5 mg/L for *B. subtilis* LAMI005 and 21 mg/L for *B. licheniformis* Ali5 (Ali et al. 2019; Nogueira Felix et al. 2019).

The interfacial tension is a critical parameter for evaluating the effectiveness of biosurfactants (Bezerra et al. 2018). In this study, the purified BS produced by *Paenibacillus* sp. ESA 664 reduced the IFT to values comparable to those reported for other potent biosurfactants, such as the BS from *Bacillus licheniformis* W16, which decreased IFT to 2.47 $\pm$0.32 mN/m (Joshi et al. 2016). Similarly, surfactin produced by *Bacillus amyloliquefaciens* TSBSO 3.8 lowered the IFT (water/hexadecane) from 40.33 to 11.35 mN/m (Alvarez et al. 2015). These results demonstrate that the BS from our strain exhibits high surface activity, consistent with its low CMC, which reinforces the product's efficiency and its potential as an effective surfactant agent.

Stability of the BS under varying conditions highlights its potential for diverse industrial applications. The lack of significant changes in ST across wide temperature ranges,

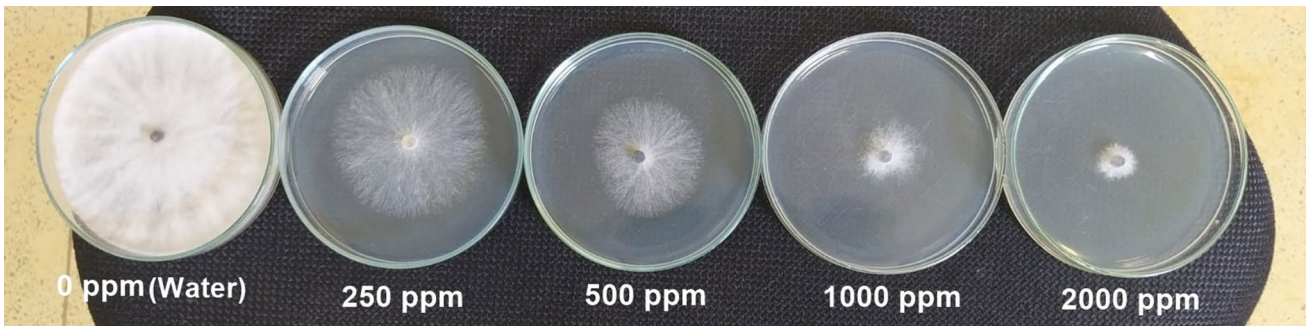

Fig. 6 Antifungal activity of surfactin produced by *Paenibacillus* sp. ESA 664 against *Lasiodiplodia theobromae*

including autoclaving, demonstrates the compound's thermal stability, making it suitable for industrial processes involving high temperatures (Umar et al. 2021). At neutral pH, the BS retained its surface-active properties, characteristic of surfactin molecules that remain solubilized in these conditions (Abdel-Mawgoud et al. 2008). However, at acidic pH, the observed ST increase is attributed to the precipitation of surfactin due to reduced solubility relative to changes in molecular charge. While acidic conditions impact solubility, they do not compromise the stability of the BS (Abdel-Mawgoud et al. 2008; Nogueira Felix et al. 2019; Umar et al. 2021).

The ability to maintain surface activity across salinity gradients suggests resilience in environments with varying ionic concentrations. However, the slight ST increase in diluted samples indicates that higher concentrations of Na^+ and Cl^- ions can reduce surfactant efficiency due to ion–dipole and electrostatic interactions (Nogueira Felix et al. 2019; Phulpoto et al. 2020). Finally, the stability of the BS in the presence of sucrose further underscores its robustness, allowing its application in food-related industries or other systems involving sugars. This versatility in stability across different conditions strengthens its applicability in diverse fields.

Structural characterization of the BS revealed the presence of a peptide portion and aliphatic groups evidenced by the appearance of N–H and C-H bond bands throughout the FTIR spectrum highlights the lipopeptidic nature of the BS. Similar FTIR absorption spectra have been reported in other studies that associated the obtained bands as characteristics of surfactin (Jemil et al. 2019; Nanjundan et al. 2019; Ali et al. 2022; Barale et al. 2022; Adiandri et al. 2023). Standard surfactin exhibited an FTIR absorption pattern very similar to that observed for the BS produced by our *Paenibacillus* sp. ESA 664 strain, as shown in Fig. 4B.

With regard to mass spectrometry, it can be observed that in the standard, the sodium adduct with *m/z* 1044.9 (14 carbons) predominates, while in the BS produced in our study, the predominant form is the sodium adduct with *m/z* 1058.9 (15 carbons). It was possible to determine the amino acid sequence of the peptidic portion of the surfactin sample by interpreting the ESI–MS/MS (+) spectrum of the primary precursor ions (*m/z* 1036.8, 1044.9, and 1058.9). Lipopeptide surfactants generally have aliphatic chains ranging from C9 to C19, and their peptide chains consist of 4 to 10 amino acids. This variation in structure is associated with several factors, such as the type of strain, the composition of the culture medium, and the cultivation conditions (Li et al. 2016).

Some species of fungi from the genus *Lasiodiplodia* have been associated with mango (*M. indica* L) diseases, and their ability to infect fruit places them among the most efficient seed-borne pathogens that cause post-harvest diseases (Coelho et al. 2020). In this way, this pathogen generates a negative economic impact on mango plantations since it can cause substantial losses in productivity and fruit quality (Coelho et al. 2020), reinforcing the need to control this fungal strain. Besides, the use of microbial metabolites to mitigate production losses in agriculture caused by phytopathogens can contribute to reducing the use of chemical pesticides, thus preserving the environment and health (Foysal and Lisa 2018; Bartal et al. 2023).

In recent years, there has been a significant increase in research focused on the antimicrobial and biocontrol potential of lipopeptide BS. Lipopeptides produced by endophytic isolates of *B. subtilis* have been effective in inhibiting the mycelial growth of *Moniliophthora roreri* and *M. perniciosa*, which cause rot in cocoa (*Theobroma cacao*). The BS exhibited potential as an ecological alternative in cocoa plants to prevent phytopathogen infection (Serrano et al. 2021). In another study, a lipopeptide BS produced by *B. velezensis* FJAT-46737, exhibited antifungal effect against three species of plant pathogenic *Fusarium oxysporum*, with inhibition rates ranging from 65.25% to 72.71% (Chen et al. 2020).

Surfactin showed a significant inhibitory power against *Fusarium graminearum*, which causes wheat head blight, showing a CE_{50} value of 102.1 µg/mL (Liang et al. 2023). In the study by Xiao et al. (2023), the authors investigated the antifungal effects of surfactin on the fungus *Botrytis cinerea*, which causes gray mold in winter jujube (*Ziziphus jujuba* Mill.). The results showed that the biosurfactant significantly inhibited the growth of *B. cinerea*, with a CE_{50} value of 46.42 mg/L after 5 days (Xiao et al. 2023).

The antimicrobial activity displayed by BS can be primarily attributed to the destabilization of the cell membrane, resulting in cytoplasmic changes that, in most cases, lead to cell rupture (Sani et al. 2024). The inhibitory effect of the BS from our *Paenibacillus* sp. against *L. theobromae* demonstrate the fungicidal capacity of the compound. Furthermore, the data also suggest the potential application of the surfactant in preserving the post-harvest quality of mangoes by preventing fruit rot caused by this microorganism. To the best of our knowledge, this is the first description of the antifungal activity of surfactin against *L. theobromae*, highlighting the promising nature of these findings regarding the development of new alternative strategies for controlling such fungal infections in mango cultivars.

The study of nitrogen-fixing strains for biosurfactant production is promising once they are associated with plant crops, opening perspectives to novel approaches in biological control and to a deep understanding of the roles of biosurfactants in such type of association.

Conclusion

The diazotrophic strain *Paenibacillus* sp. ESA 664 demonstrated the ability to grow and produce a biosurfactant identified as the lipopeptide surfactin. The compound exhibited surface and interfacial surfactant activity, as well as emulsifying effectiveness, in addition to a low critical micelle concentration and considerable stability across a wide range of temperatures, pH values, salinities, and sugar concentrations. These results indicate the potential of the product as an effective surfactant, with possible applications across various industrial sectors. Moreover, the antifungal activity observed against *Lasiodiplodia theobromae* highlights its promising use as a biocontrol agent for fungal infections in agriculture. Future studies should focus on developing field-applicable formulations and elucidating its antifungal mode of action.

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Authors contribution MAMD- Investigation, Writing – original draft, Formal analysis, Data curation, Conceptualization. ELR- Methodology, Investigation. DB- Methodology, Investigation. PMRJ- Methodology, Investigation. PIFJ- Methodology, Investigation. MN- Supervision, Validation, Resources, Project administration.

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Declarations

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