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Structural Characterization and Biological Properties of a Lipopeptide Surfactant Produced by *Bacillus subtilis* on Cassava Wastewater Medium

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Abstract Surface-active compound produced by *Bacillus subtilis* LB5a using cassava wastewater as substrate was isolated, purified, and chemically characterized, and its infrared spectrum revealed characteristic of lipopeptide. Peptide analysis revealed presence of glutamic acid, aspartic acid, valine, and leucine at 1:1:1.2:3.4, respectively. Electrospray ionization mass spectrometry showed biosurfactant was composed of homologous lipopeptide mixture, with that of molecular weight 1035 being the most abundant. Tandem mass spectrometry of each protonated molecules established the peptide sequence as R-Glu-Leu-Leu-Val-Asp-Leu-Leu. Isolated surfactant showed similar chemical composition and peptide sequence as that of commercial surfactin, although some differences in homologous distribution and presence of dimer molecules were detected. Preliminary biological evaluation demonstrated surfactant has potential as antiviral agent with broad spectrum of antibacterial activity.

Key words: biosurfactant, *B. subtilis*, cassava wastewater, lipopeptide, surfactin

Introduction

Many microorganisms, particularly bacteria, produce biosurfactants which exhibit surface-active properties. Among the main classes of microbial surfactants are the glycolipids, lipopeptides, phospholipids, neutral lipids, fatty acids, and lipopolysaccharides (1, 2). Most of the biologically produced surfactants have notable advantages such as biodegradability and low toxicity when compared to their synthetic counterparts (3). Biosurfactants are also of great interest because of their physicochemical and biological properties, which can be exploited in food, oil, and pharmaceutical industries (1, 3).

In addition to their properties as detergents, some of these compounds exhibit other interesting activities, such as surfactin, a cyclic lipopeptide surfactant from *B. subtilis*, which has been investigated as an antitumoral, antibacterial, antiviral and antimycoplasma agent (4-6). Despite the many advantages of biosurfactants, their commercial production is not yet competitive in terms of chemical synthesis. Different ways could be explored to reduce production costs through high yields and product accumulation, economical engineering processes, and the use of cost-free or cost-credit feedstock for microorganism growth and surfactant production (7). We recently have reported the production of a biosurfactant by *Bacillus subtilis* LB5a using cassava wastewater as an alternative substrate (8), showing that the cassava residue is a suitable medium for surfactant production. The elucidation of the structure of the surfactant produced on alternative substrates is very important, since the properties of surface-active lipopeptides depend on their chemical composition, which is influenced by the carbon source and culture conditions (9). In this paper we report the isolation, structural characterization, and biological activity of a biosurfactant produced by *B. subtilis* LB5a growing on cassava wastewater substrate.

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Materials and Methods

Strain *Bacillus subtilis* LB5a was selected based on its ability to grow and produce surfactant from cassava waste (8).

Substrate preparation Cassava effluent obtained through the manufacturing of cassava flour was collected and stored at -18°C until needed. The medium was prepared by heating the waste until boiling to remove solids. After cooling, the substrate was centrifuged at 8,000 × g for 20 min using a Beckman J2-21 centrifuge (Beckman Instruments Inc., Fullerton, CA, USA). The supernatant was distributed in flasks and sterilized in an autoclave at 1 atm, 121°C for 15 min. The resulting pH of the medium was 5.9 and was not adjusted.

Inoculum and culture conditions The bacterial strains were streaked on a nutrient agar slant and incubated at 30°C for 24 hr. Two loops of culture were inoculated in 20 mL of nutrient broth (Difco, Sparks, MD, USA) in a 50-mL Erlenmeyer flask and incubated in a rotary shaker (New Brunswick Scientific Co., Edison, NJ, USA) at 150

rpm, 30°C for 7-8 hr until cell numbers reached 10^8 cfu/mL. An aliquot with 5 mL of inoculum was transferred to 75 mL of cassava effluent medium contained in a 250-mL Erlenmeyer flask and incubated at 150 rpm, 30°C in the rotary shaker for 48 hr.

Surfactant isolation and purification The biosurfactant was isolated from cell-free broth by acid precipitation after adjusting the broth pH to 2.0 using 6N HCl and keeping it at 4°C overnight. The precipitate thus obtained was pelleted at 8,000 × g for 20 min, redissolved in distilled water, adjusted to pH 7.0 and dried at 50°C for 24 hr. The dry surfactant was ground and extracted with $CHCl_3/CH_3OH$ (65:15). The solvent was filtered using a Whatman 42 filter paper (Whatman Int. Ltd., Maidstone, UK) and evaporated at 50°C. Resulting crude product was used for further purification steps.

Adsorption chromatography Approximately 0.4 g of crude surfactant was dissolved in 5 mL of $CHCl_3/CH_3OH$ (65:15) mixture and loaded on a silica gel 60 (Merck, Darmstadt, Germany) column (25 × 2 cm). The product was eluted using 3 bed volumes of solvents by subsequently increasing the polarity: $CHCl_3$, $CHCl_3/CH_3OH$ (2:1) and CH_3OH. Fractions of 10 mL were collected, and the surfactant detected by measuring the absorbance of each collected fraction at 220 nm using a DU 640 Beckman spectrophotometer (Beckman Instruments Inc., Fullerton, CA, USA). The biosurfactant-detected fractions were pooled and dried at 50°C. The product obtained was used for further analysis.

Infrared spectroscopy Fourier Transform Infrared absorption spectra of LB5a biosurfactant were obtained using a Spectrum One Perkin-Elmer spectrophotometer (Perkin-Elmer Inc., Boston, MA, USA). Samples were dissolved in chloroform, and a drop of solution was dispersed in NaCl pellet and maintained at dry atmosphere until solvent evaporation. The spectra were generated from 500 to 4,000 cm^{-1} with a 4 cm^{-1} resolution.

Amino acid analysis The amino acid composition of surfactant was determined on an amino acid analyzer (Pickering Laboratories Inc., Mountain View, CA, USA) after total hydrolysis of the sample in 6N HCl at 105°C for 24 hr.

Mass spectrometry MS was performed using a hybrid quadrupole time-of-flight (TOF) high resolution (7.000) and high accuracy (5 ppm) Q-Tof mass spectrometer (Micromass, Manchester, UK) equipped with an electrospray ion source (ESI). The working conditions for positive ESI were as follows: desolvatation gas (nitrogen) was heated at 100°C; capillary was held at a potential of 3.5Kv, and the cone voltage was 35 Kv. MS/MS tandem mass spectra were acquired by mass-selecting the target ion using the quadrupole mass analyzer followed by 25eV, collision induced dissociation using argon in the quadrupole collision cell and mass analysis by TOF. The lipopeptide material was dissolved in a mixture of acetonitrile and water 1:1 (v/v) and 0.01% formic acid for analysis in positive ion mode. The sample was introduced into the source at 15 µL/min with a syringe pump.

Antimicrobial activity The biosurfactant obtained and a commercial standard surfactin (Sigma Chemical Co., St. Louis, MO, USA) were tested against bacteria, yeast, and fungi using the agar diffusion method. A solution was prepared in chloroform/methanol mixture (65:15) at 20 µg/µL, and a filter-paper disk containing 200 µg compound was assayed on the surface of an agar medium containing the tested microorganism. The bacterial strains were grown on nutrient agar at 37°C; and the yeasts and fungi on Sabouraud dextrose agar at 30°C. Growth inhibition was measured in terms of diameters (mm) of halos around papers disks. The experiments were independently conducted in triplicates.

Antiviral and cytotoxicity assays Antiviral activity of the surfactant was assessed using the following virus/cell systems: VSIV (*Vesicular Stomatitis Indian Virus*)/ BHK-21 and MHV-3 (*Murine Hepatitis Virus*) / L929. A stock solution of surfactant was prepared in Eagle's medium at a final concentration of 500 µM, filtered through a 0.22 µm, and stored at -18°C. The adherent cell lines were grown in Eagle's medium supplemented with 5% bovine fetal serum, transferred to a microplate (96 wells) and maintained at 37 °C in a CO_2 incubator (5%) until a confluent cell monolayer was attained. The virus titres were determined by a standard plaque assay (10), where the virus solutions were serially diluted and evaluated microscopically for cytophatic effects. Aliquots (50 µL) of media containing various concentrations of the test compound were poured into wells of the microplate with the cell monolayers, and, to each well, 50 µL of medium containing $100TCID_{50}$ of virus was added. For the cytotoxicity test, a similar set of microplates without the addition of the virus solution was prepared. After 48 hr of incubation at 37°C under 5% CO_2, the concentration of surfactant that causes inhibition of virus action (cytophatic effect) as well as that at which cytotoxic effect on cell lines was induced were determined. The experiments were independently replicated four times.

Results and Discussion

Cassava wastewater is a residue generated in a high quantity during the manufacture of cassava flour, a very common ingredient used in Brazilian cookery. The major nutrients present on cassava waste are sugars (sucrose, glucose, fructose, and maltose), nitrogen, and mineral salts. Although disposal of this residue is an increasing problem due to its high organic load, it is also a very attractive substrate for biotechnological processes including biosurfactant production. In a previous work, we established that *B. subtilis* LB5a was able to produce a biosurfactant when grown in a cassava wastewater medium (8). Therefore, as a continuing work, it is indispensable to evaluate the composition of the product obtained.

Infrared spectrum IR spectrum of the biosurfactant isolated from cassava wastewater (Fig. 1) showed strong bands characteristics of peptides at 3298 cm^{-1} (A) resulting from the N-H stretching mode. At 1652 cm^{-1} (F), the stretching mode of C=O bond was observed, and at 1559 cm^{-1} (G) the deformation mode of NH combined with C-N stretch-

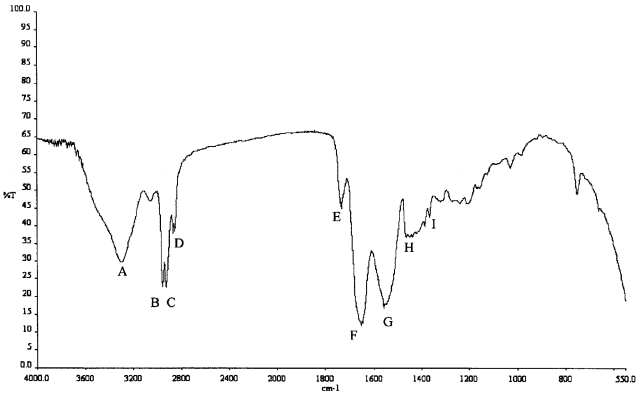

Fig. 1. Infrared spectrum of the surfactant obtained from cassava wastewater. Letters represent the bands explained on text.

ing mode occurred. The bands from 2958 to 2850 cm^{-1} (B-C-D) and 1467 to 1380 cm^{-1} (H-I) reflected aliphatic chains. The band at 1734 cm^{-1} (E) was assigned to the lactone carbonyl absorption. These results indicate that the isolated product contains a lipid aliphatic hydrocarbon chain as well as a peptide moiety.

Mass spectrometry of surfactant ESI mass spectrum in the positive ion mode of the biosurfactant (M) showed a homologous series of lipopeptides of molecular weight (MW) 1007, 1021, and 1035 detected by their protonated molecules $[M+H]^+$ of *m/z* 1008,1022 and 1036; as well as their sodium and potassium adducts, that is $[M+Na]^+$ of *m/z* 1030, 1044, and 1058 and $[M+K]^+$ of *m/z* 1046, 1060, and 1074 (Fig. 2). Interestingly, doubly charged clusters of isotopomeric ions of *m/z* 1034, 1041, 1048 and 1055, interpreted as homogenous or mixed dimers, ionized by both protonation and by sodium or potassium cation addition, $[2M+Na(K)+H]^{+2}$ or $[MM'+Na(K)+H]^{+2}$, were also detected. Because we are working with a homologous series with close chemical properties, the ion relative intensities should closely reflect the composition of homologous lipopeptides in the sample analyzed. The predominant ions were that of *m/z* 1022, 1036, and 1048 and the more abundant one was that of *m/z* 1036, which corresponds to the protonated lipopeptide with a C15 lipid carbon chain. The other ions with 14 and 28 *m/z* units lower (1008 and 1022) are the homologous protonated molecules with C13 and C14 lipid carbon chains, respectively. The doubly charged dimers $[2M+Na(K)+H]^{+2}$ or $[MM'+Na(K)+H]^{+2}$ are formed likely due to the relatively high salt content of the isolated biosurfactant.

Commercial preparations of surfactin were also evaluated by ESI-MS, and the mass spectrum was found to be quite similar to that acquired for our sample (Fig. 2B), showing the same homologous series of lipopeptides. Note, however, that in the commercial surfactin the most abundant lipopeptide is that of MW 1021 (*m/z* 1022 in the ESI-MS spectrum), whereas in the isolated lipopeptide mixture the most abundant one is that of MW 1035 (ion of *m/z* 1036). Dimer molecules, although present, are less abundant than in our sample. The fragmentation patterns observed in the ESI-MS/MS tandem mass spectra of the main protonated molecules of 1008, 1022, and 1036 were identical to those obtained with the *B. subtilis* LB5a surfactant.

Amino acid composition The analysis indicated the presence of four amino acid residues in the biosurfactant, determined to be glutamic acid, aspartic acid, valine, and leucine at 1:1:1.2:3.4.

Peptide sequence The peptide sequence was deduced by interpreting the ESI-MS/MS spectra of main protonated lipopeptides, that is, those of *m/z* 1036, 1022, and 1048. Figure 3 shows the tandem mass spectrum of the protonated lipopeptide of *m/z* 1036. Assuming preferential cleavage of the lactone ring, the following sequence was determined: Glu-Leu-Leu-Val-Asp-Leu-Leu. The same sequence was

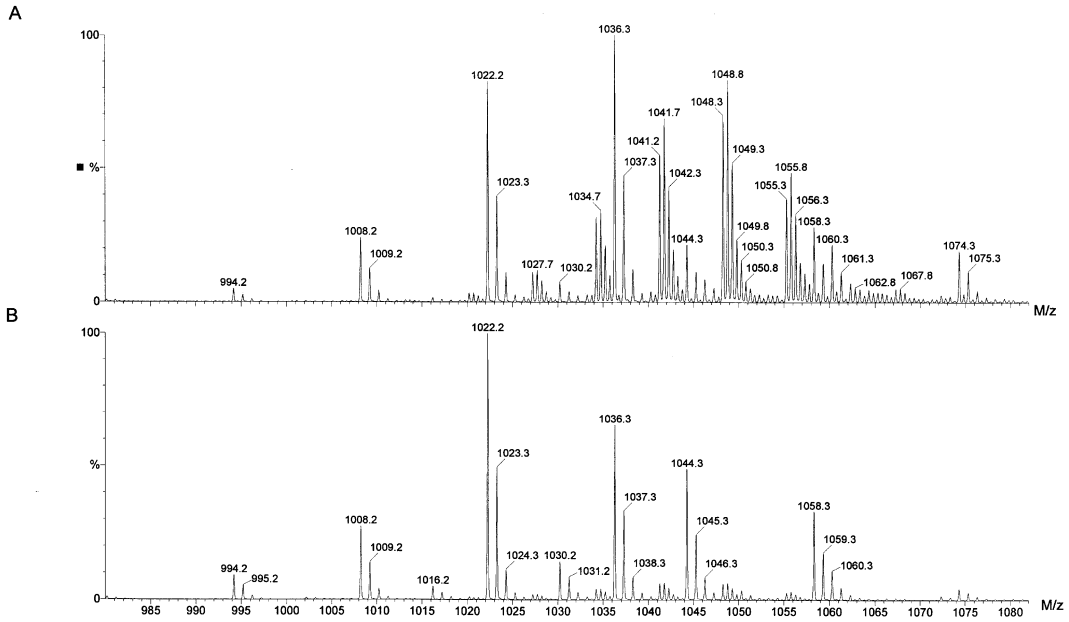

Fig. 2. ESI-MS spectrum of A) isolated biosurfactant and B) commercial sample of surfactin.

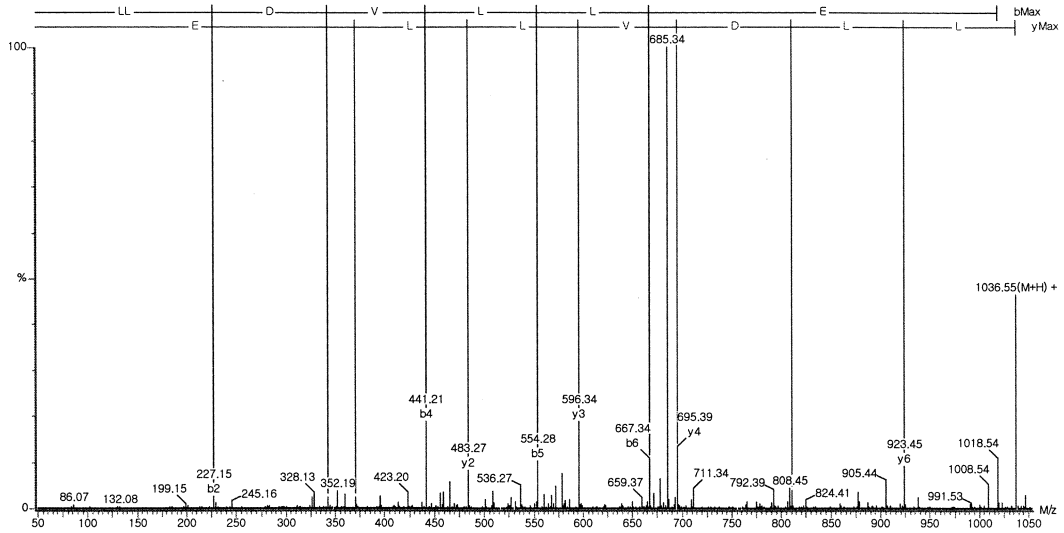

Fig. 3. ESI-MS/MS spectrum of the protonated molecule of *m/z* 1036. The fragment ions (with the corresponding amino acids residues) are marked by dashed lines.

also revealed by the tandem mass spectra of the two other homologous protonated lipopeptides (data not shown).

Biological activity Table 1 shows the antimicrobial spectra of LB5a lipopeptide and commercial surfactin. All bacterial strains tested, except *B. subtilis*, showed susceptibility to the compound produced by LB5a strain. *Pseudomonas aeruginosa* was the most sensitive Gram-negative bacteria, with inhibition of *E. coli*, *S. choterasius*, and *S. marcescens* also detected at lesser extents. LB5a lipopeptide also affected the growth of Gram-positive strains, mostly *M. luteus* and *B. cereus*. Commercial surfactin demonstrated a significantly different bacterial inhibition pattern in comparison to the surfactant from LB5a; only *B. cereus* showed some susceptibility to the surfactin. Growths of the yeast and fungi strains tested were not affected by the surfactants. The results of antiviral and cytotoxicity of *B. subtilis* LB5a surfactant are shown in Table 2. The minimum surfactin concentration that causes inhibition of virus-induced cytophatic effect was 12.5 μM for VSIV and 5.0 μM for MHV-3. Cytotoxic effects of surfactant were observed at concentrations above 15 μM on BHK-21 and 12.5 μM on L-929 cell lines.

Table 1. Antimicrobial activity of the lipopeptide from *B. subtilis* LB5a and standard surfactin

Microorganisms [b]	Sensitivity [a]	
	LB5a	surfactin
Bacillus subtilis ATCC 21332	-	-
Bacillus cereus ATCC 10876	++	+
Escherichia coli ATCC 11229	++	-
Micrococcus luteus ATCC 4698	+++	-
Pseudomonas aeruginosa ATCC 15442	++++	-
Rhodococcus equi ATCC 25729	++	-
Serratia marcescens ATCC1953	++	-
Staphylococcus aureus ATCC 25923	++	-
Salmonella choterasius ATCC 10708	++	-

[a]maximum diameter halos:< 5 mm (-), 5-8 mm (+), 8-12 mm (++), 12-18 mm (+++), >18 mm (++++).
The results represent an average of four independent replicates.
[b]no inhibition of growth was obtained with: *Candida glabrata, Saccharomyces cerevisae, Mucor michei, Aspergillus oryzae, Aspergillus niger, Colletotrichum sp, Alternaria macrospora, Rhizopus oligosporus, Fusarium oxisporium, Paecilomyces sp., Neurospora sp.*

Bacillus subtilis strains are known to produce a broad spectrum of lipopeptides, particularly iturin, surfactin and fengycin families. Some strains produce only one type of lipopeptide, while others coproduce two or three types of these molecules (11, 12). The production of these lipopeptides is dependent on the composition of the culture medium (13). The best characterized lipopeptide surfactant is surfactin, which occurs naturally as a mixture of closely related cyclic lipopeptides isoforms (homologous), which differ in the length and branching of the fatty acid side chains and in the amino acid substitutions in the peptide ring. These variations, rather than those of genetically determined, depend on the specific *B. subtilis* strain as well as the nutrition and environmental conditions (14, 15).

IR spectrum of biosurfactant indicates the presence of aliphatic hydrocarbons, peptide, and lactone group. The amino acid analysis showed the presence of leucine, valine, aspartic acid, and glutamic acid; and the mass spectrometric sequencing confirmed the presence of these amino acids at the sequence RO-Glu-Leu-Leu-Val-Asp-Leu-Leu, forming a heptapeptide moiety as previously described for surfactin (16). The ESI-MS spectra showed a predominant protonated molecule of *m/z* 1036, representing the lipopeptide with a

Table 2. Antiviral activity and cytotoxicity of biosurfactant from *B. subtilis* LB5a.

	Biosurfactant concentration (μM)	
Virus/cell systems	Virus inhibition*	Cytotoxic effect**
VSIV/BHK-21	12.5	15
MHV-3/L929	5	12.5

*minimal surfactant concentration to inhibit virus cytophatic effect.
**maximal surfactant concentration that did not induce to cell toxicity.

C15 lipid carbon chain, an isoform called surfactin D (17). The ions of *m/z* 1008 and 1022 were the protonated forms of the C13 and C14 lipopeptide homologs, respectively. Surfactin standard was similarly analyzed, and the ESI-MS spectrum revealed a similar MW distribution and identical amino acid sequences with those of the isolated biosurfactant. The lipopeptide produced by *B. subtilis* LB5a on cassava wastewater is a surfactin with modified distribution and combination of the homologous components. For standard surfactin C14 is the most abundant form, whereas for the isolated biosurfactant C15 dominates.

Coproduction of other types of lipopeptides (iturins, fengycins) together with surfactin was not detected, suggesting that *B. subtilis* LB5a produces only one type of lipopeptide family when grown on the cassava wastewater medium. This selectivity could be an advantage, because it saves time and reduces production costs by simplifying the purification step.

Antiviral action of LB5a lipopeptide surfactant was observed against the two enveloped virus tested, and MHV-3 appeared to be more sensitive to the surfactant. Vollenbroich *et al*. (6) demonstrated the antiviral activity of surfactin against enveloped virus and postulated that the mechanism of action of this compound was related to its physicochemical interaction with the virus envelope. The composition (protein, glycoprotein, and lipid) of viral envelope influences the action of surfactin. This fact could explain the differences in the sensitivity observed for MHV and VSIV.

The cytotoxicity test revealed that surfactant affects cell lines at concentrations higher than those observed for virus inhibition, suggesting that this compound could be used for *"in vitro"* removal of viral contaminants from cell lines (6). Membrane barrier properties are likely to be damage when surfactin interacts with phospholipids. This might have caused structural fluctuations that may be the primary mode of antibiotic action and other biological effects of lipopeptide surfactants (18). Surfactin was also described as a potent agent against *Mycoplasma* (5) and *Mycobacterium* due to the unusual lipid composition of their cell walls; nevertheless, other pathogenic bacteria were not significantly affected (19). The LB5a surfactant showed an interesting antibacterial spectrum against pathogenic bacteria. The lack of activity against yeast and fungi also indicate the production of a unique type of lipopeptide surfactant, because iturins and fengycins are known to have high antifungal actions (13).

The biological activity of lipopeptides depends on the amino acid composition and the sequence of their peptide ring as well as on the nature of their lipid moiety (14). Therefore, modifications on culture medium could generate different lipopeptide compositions and novel biological properties. In fact, the surfactant produced on cassava medium was identified as a lipopeptide, analogous to surfactin, however, the differences in the relative intensities of homologous and the presence of dimer molecules on surfactant mixture possibly are the reason for its singular antibacterial action when compared to the standard surfactin (Table 2). It is important to point out that these slight modifications on surfactant composition were sufficient to modify some biological properties of this compound.

The surfactant components should be isolated to define the individual contribution of each molecule of the mixture on the biological activity. This study will help to better understand the mechanisms involved in lipopeptide surfactant action as well as help the future development of antimicrobial agents.

Bacillus subtilis LB5a was able to produce the lipopeptide surfactin when grown in a cassava wastewater medium. The isolated surfactant showed similar chemical composition and peptide sequence as that of a commercial surfactin, however, some differences in homologous distribution and the presence of dimer molecules were detected. Preliminary biological evaluation showed that the surfactant has interesting and unusual antibacterial activity when compared to the standard surfactin.

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