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ANTIMICROBIAL ACTIVITY OF SURFACTANTS PRODUCED BY *BACILLUS SUBTILIS* R14 AGAINST MULTIDRUG-RESISTANT BACTERIA

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ABSTRACT

Lipopeptides represent a class of microbial surfactants with increasing scientific, therapeutic and biotechnological interests. The genus *Bacillus* is a producer of these active compounds, and among them *B. subtilis* produces surfactin, the most potent biosurfactant known. These compounds can act as antibiotics, antivirals, antitumorals, immunomodulators and enzyme inhibitors. In this work, the antimicrobial activity of biosurfactants obtained by cultivation of *B. subtilis* R14 was investigated against multidrug-resistant bacteria. During cultivation in defined medium, the surface tension of the medium was reduced from 54 mN/m in the beginning of the microbial growth to 30 mN/m after 20 hours. A crude surfactant concentration of 2.0 g/L was obtained after 40 hours of cultivation. A preliminary characterization suggested that two surfactants were produced. The evaluation of the antimicrobial activity of these compounds was carried out against 29 bacteria. *Enterococcus faecalis* (11 strains), *Staphylococcus aureus* (6 strains) and *Pseudomonas aeruginosa* (7 strains) and *Escherichia coli* CI 18 (1 strain) displayed a profile of well defined drug resistance. All strains were sensitive to the surfactants, in particular *Enterococcus faecalis*. The results demonstrated that lipopeptides have a broad spectrum of action, including antimicrobial activity against microorganisms with multidrug-resistant profiles.

Key words: Biosurfactant, *Bacillus subtilis*, Lipopeptides, Antimicrobial activity

INTRODUCTION

Surfactants are amphipathic molecules consisting of both hydrophilic and hydrophobic moieties. Partition occurs preferentially at the interface between fluid phases with different degrees of polarity (3). The surface and interfacial tension reducing properties of surfactants present excellent detergency and emulsifying, foaming and dispersing traits, making them some of the most versatile products for use in chemical processes (10).

Microbial compounds which exhibit pronounced surface activity are classified as biosurfactants. Microbial biosurfactants include a wide variety of surface active compounds, such as glycolipids, lipopeptides, polysaccharide-protein complexes, phospholipids, fatty acids, and neutral lipids (15). Biosurfactants commonly have the advantages of biodegradability, low toxicity, and biocompatibility over chemically synthesized surfactants (18). In particular, the most suitable agent for a selected application may be adapted by the choice of the producer organism, the composition of the nutrient broth, and the culture conditions (23).

Among the genus *Bacillus*, *B. subtilis* produces a broad spectrum of bioactive lipopeptides which have a great potential

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for biotechnological and biopharmaceutical applications. The characteristic structure of lipopeptides is a fatty acid combined with an amino-acid moiety. Several lipopeptides have potent antibiotic activity and have been the subject of several studies on the discovery of new antibiotics. The list includes surfactin, produced by *B. subtilis*, the most powerful biosurfactant known to date (17). These compounds have many pharmacological activities: antibacterial, antifungal, antiviral, and antimycoplasma properties; inhibition of the fibrin clot formation and hemolysis (3); formation of ion channels in lipid bilayer membranes (19); antitumour activity against Ehrlich's ascites carcinoma cells (3); and inhibition of the cyclic adenosine 3,5-monophosphate phosphodiesterase (11).

B. subtilis R14 strain has been investigated previously for agricultural application and has proven to be an effective biocontrol agent against important phytopathogens (13). In this paper the activity of surfactants produced by *B. subtilis* R14 against multidrug-resistant bacteria was investigated.

MATERIALS AND METHODS

Microorganisms

B. subtilis R14, isolated from the surface of kale leaves, was used in this work. The microorganism belongs to the Culture Collection of the Laboratory of Phytobacteriology of the Federal Rural University of Pernambuco (Brazil). From the lyophilized culture, subcultures were done on nutrient agar, consisted of meat extract 3.0g/L, meat peptone 10.0g/L and agar 15.0g/L.

Surfactant compounds were tested against a group of multiresistant bacteria isolated from clinical specimens: *Pseudomonas aeruginosa* (CI1, CI3, CI5, CI6, CI7, CI10, CI15); *Escherichia coli* CI18; *Staphylococcus aureus* (CI15, CI16, CI155, CI247, CI311, CI404) and *Enteroccoccus faecalis* (CI 55671, CI55918, CI144, CI068, CI56671, CI56354, CI55995, CI295, CI222, CI55195, CI56288) (Table 1). Four microorganisms from culture collections, representing each bacterial genus, were included in the study: *Pseudomonas aeruginosa* UFPEDA 39 and *Staphylococcus aureus* UFPEDA 22 (microorganisms belonging to the Culture Collection of the Antibiotic Departament of the Federal University of Pernambuco, Brazil), and *Enterococcus faecalis* ATCC 29212 and *Escherichia coli* ATCC 8739. The microorganisms were maintained on Mueller-Hinton agar slants at refrigerated temperature.

Culture medium

A defined medium described by Carvalho (4) was used for the production of surfactants and consisted of (per liter of distilled water): glucose, 40.0g; $(NH_4)_2SO_4$, 8.5g; $NaNO_3$, 8.5g; K_2HPO_4, 13.6g; KH_2PO_4, 4.0g; $MgSO_4.7H_2O$, 0.5g; and in this medium 10mL of the solution consisting of (per liter of distilled water): $CaCl_2$, 0.42g; $FeSO_4 \cdot 7H_2O$, 2.29g; $MnCl_2 \cdot 4H_2O$, 0.10g; $ZnCl_2$, 0.17g; $CuCl_2$, 0.03g; $CoCl_2 \cdot 6H_2O$, 0.06g and $Na_2MoO_4 \cdot 2H_2O$, 0.06g was added. The broth pH was adjusted to 7.

Inoculum and culture conditions

B. subtilis R14 was streaked on a nutrient agar slant and incubated at 37ºC (± 0.5). After 24 h, two loops of cells were inoculated in 25 mL of nutrient broth in a 250 mL Erlenmeyer flask and incubated in a rotatory shaker (New Brunswick Scientific, model C25KC) at 250 rpm and 37ºC (± 0.5) until absorbance around 3, measured spectrophotometrically at 600 nm (Hewlett Packard 8453) was reached. The culture from the Erlenmeyer flask was then transferred to 225 mL of production medium in a Fernbach flask and incubated in the rotatory shaker at 150 rpm and 30ºC (± 0.5) for 52 h. Samples were collected at time-defined intervals and submitted to analysis for determination of biomass production, glucose consumption, nitrite production, and changes in surface tension, emulsification activity and biosurfactant concentration. All experiments were performed in triplicate.

Analytical measurements

Biomass concentration was determinated spectrophotometrically during the cultivation at 600 nm (Hewlett Packard 8453) and by determination of dry weight at 80ºC for 24h after filtration of the samples through 0.22 μm membranes (4). With these results, a curve was plotted for the bacterial dry weight as a function of the optical density.

The concentration of glucose was assayed by the Trinder method, based on the enzymatic oxidation of glucose to gluconic acid and hydrogen peroxide (22).

Nitrite concentration was measured spectrophotometrically (Hewlett Packard 8453) at 543 nm. This method was based on reduction of nitrate to nitrite, which reacts with sulfanilic acid and α-naftilamine resulting in p-benzensulfonic-azo-*p*-α-naftilamine. The product formed is directly proportional to the nitrite concentration in the sample (8,12).

Culture samples were centrifuged at 11.000 xg for 20 minutes for cell removal and the supernatant was submitted to surface tension measurement with a tensiometer (model KSV Sigma 70, Hl, Finland) using Wilhelmy plate method. The emulsification activity was determined according to Cooper and Goldenberg (5): 1.5 mL of hexane was added to 1mL of the supernatant in a screw cap tube and vortexed at high speed for 2 minutes. The emulsion stability was determined after 24h, and the emulsification index (E_{24}) was calculated by dividing the measured height of emulsion layer by the mixture's total height and multiplying by 100.

Precipitation and Extraction of Biosurfactant

The surfactant was precipitated from cell-free broth of the culture of 52h by adjusting the broth pH to 2.0 using 6N HCl and keeping it at 4ºC overnight (5,17). Precipitated material was

Table 1. Susceptibility profile of the microorganisms used in the antimicrobial tests.

Microorganisms	Origin	Resistance to	Sensitivity to
Enterococcus faecalis CI 55671	Urine	CL; GEN; TET; ATM	TEIC
Enterococcus faecalis CI 55918	Hemoculture	CL; GEN; TET; ATM	TEIC; VAN
Enterococcus faecalis CI 144	Urine	CL; TET; ATM	TEIC
Enterococcus faecalis CI 068	Urine	CL; ERI; TET; ATM	TEIC
Enterococcus faecalis CI 56671	Urine	CL; ERI; TET; ATM	TEIC
Enterococcus faecalis CI 56354	Urine	CL; ERI; TET; ATM	TEIC
Enterococcus faecalis CI 55995	Urine	CL; ERI; TET; ATM	TEIC
Enterococcus faecalis CI 295	Urine	CL; ERI; TET; ATM	TEIC
Enterococcus faecalis CI 222	Urine	CL; ERI; TET; ATM	TEIC
Enterococcus faecalis CI 55195	Hemoculture	CL; GEN; TET; ATM; AMP	TEIC; VAN
Enterococcus faecalis CI 56288	Hemoculture	CL; GEN; TET; ATM; AMP	TEIC; VAN
Staphylococcus aureus CI15	Unknown	AMP; CFO; ERI; AMOX	IMP; SZT
Staphylococcus aureus CI16	Unknown	AMI; AMP; CFO	IMP; CL; AZI
Staphylococcus aureus CI155	Vaginal secrection	GEN; AMI; CFO; CTX; CL; SZT; ERI	CIP; IMP; MER
Staphylococcus aureus CI247	Nose wound	AMI; CL; ERI	CIP; IMP
Staphylococcus aureus CI311	Finger wound	GEN; AMI; CFO; CTX; CL; SZT; ERI; TET; PEN	CIP
Staphylococcus aureus CI404	Orofaringe secrection	GEN; AMI; CFO; CTX; CL; ERI; TET	CIP
Pseudomonas aeruginosa CI 1	Hemoculture	CIP; GAT; GEN; AMI; CFL; CFO; CTX; CPM; CL; IMP; MER	ATM
Pseudomonas aeruginosa CI 3	Traqueal secrection	CIP; GAT; GEN; AMI; CFL; CFO; CTX; CPM; CL; IMP; MER	ATM
Pseudomonas aeruginosa CI 5	Hemoculture	CIP; GAT; GEN; AMI; CFL; CFO; CTX; CPM; CL; ATM; MER; AMP+SUB	not found
Pseudomonas aeruginosa CI 6	Traqueal secrection	CIP; GAT; GEN; AMI; CFL; CFO; CTX; CPM; CL; MER; SZT; AMP+SUB	not found
Pseudomonas aeruginosa CI 7	Traqueal secrection	CIP; GEN; AMI; CFL; CFO; CTX; CPM; ATM; IMP; TOB; MER	not found
Pseudomonas aeruginosa CI 10	Abdominal secrection	CIP; GEN; AMI; CFL; CFO; CTX; CPM; TOB; ATM; IMP; MER	not found
Pseudomonas aeruginosa CI 15	Hemoculture	CIP; GAT; GEN; AMI; CFL; CFO; CTX; CPM; CL; ATM; MER; AMP+SUB	not found
Escherichia coli CI 18	Urine	AMP; CFO	CTX

AMI = Amikacin 30 µg; AMP = Ampicillin 10 µg; AMOX = Amoxicillin 10 µg; ATM = Aztreonam 30 µg; AMP+SUB = Ampicillin + Sulbactam 10/10 µg; AZI = Azithomycin 15 µg; CFO = Cefoxitin 30 µg; CFL = Cephalothin 30 µg; CIP = Ciprofloxacin 5 µg; CL = Chloramphenicol 30 µg; CPM = Cefepime 30 µg; CTX = Cefotaxime 30 µg; ERI = Erytromycin 15 µg; GEN = Gentamycin 10 µg; GAT = Gatifloxacin 10 µg; IPM = Imipenem 10 µg, MER = Meropenem 10 µg; PEN = Penicillin G 10 IU; SZT = Sulfazotrim 25 µg; TET = Tetracycline 30 µg; TEIC = Teicoplanin 30 µg; TOB = Tobramycin 10 µg; VAN = Vancomycin 30 µg; CI= Clinical Isolate.

colleted by centrifugation, 11.000xg for 20 min. The crude surfactant was lyophilized and weighed for quantification. For the extraction of biosurfactant compounds, 50 mL of chloroform-methanol (2:1v/v) was added to 500mg of the dry product and incubated in a rotatory shaker (New Brunswick Scientific, model C25KC) at 250 rpm, 30ºC (± 0.5) for 15 minutes. The mixture was filtrated using a 0.45 mm Millipore membrane (4). The filtrate (50mL) was used for thin layer chromatography (TLC) analysis and antimicrobial activity tests.

Chemical characterization of biosurfactant

The chemical characterization of the isolated compounds was done by TLC analysis. The components of the chloroform/methanol extract were separated on silica gel (Si 60 F_{254}, 0.25

mm, Merck) using chloroform-methanol-water (65:25:4v/v/v) as the solvent system. Spots were revealed by spraying with: a) distilled water and heating at 110ºC for 5 min, for detection of hydrophilic compounds; b) ninhydrin 0.05%w/v (in methanol/water, 1:1 v/v) and heating at 100ºC for 4-5 min, for detection of compound with free amino groups; and c) rhodamine B 0.25%w/v (in absolute ethanol) for detection of the presence of lipids under ultraviolet light (24). Surfactin (Sigma) was used as a standard.

The components of the chloroform/methanol extract were submitted to acid hydrolysis in 6 N HCl at 105ºC for 24 h for detection of free amino groups by ninhydrin 0.05% w/v by TLC (17).

Furthermore, the crude surfactant obtained was submitted to biochemical analysis of protein (9) and lipid (1) content.

Antimicrobial activity

The antimicrobial activity of the biosurfactant was evaluated using the agar diffusion method proposed by Bauer *et al*. (2). In order to produce an appropriate inoculum an overnight culture (grown at 37ºC ±1.0) of bacteria in Mueller-Hinton broth was standardized to an opacity equivalent to 0.5 on the McFarland scale (10^8 CFU/mL). The resulting suspension was diluted to yield a cellular concentration of 10^7CFU/mL. Two mL of standardized suspensions of the microorganisms were deposited in Petri dishes (diameter 90 mm) and 18 mL of Mueller-Hinton agar at 45ºC was added. Aliquots of 20 mL of the filtrate were applied to paper disks (6mm in diameter, Whatman Nº 1), which resulted in disks containing 200 µg of the product. After evaporation of the loading solvent, each disk was placed at the centre of the Petri dishes containing previously inoculated Mueller-Hinton medium and incubated at 37ºC for 24 h. At the end of the incubation time, the diameter of microbial growth inhibition halo was measured in millimeters using a ruler with a sliding caliper.

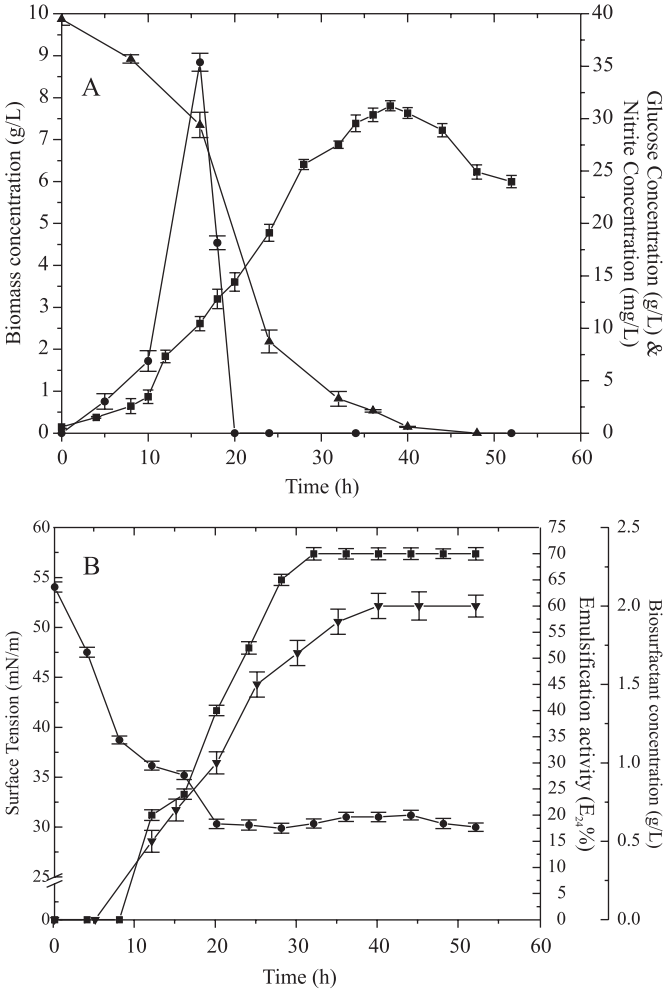

Figure 1. Growth and biosurfactant production by *Bacillus subtilis* R14. **(A)**- Biomass concentration (■), nitrite concentration (●), glucose concentration (▲) **(B)-** biosurfactant concentration (▼), surface tension (○) and emulsification activity (□) were monitored along the microbial growth.

RESULTS AND DISCUSSION

Biosurfactant Production

The results of growth and biosurfactant production of *B. subtilis* R14 are presented in Fig. 1.

A direct relationship between microbial growth and biosurfactant production was observed, as had been previously observed for this strain (4) and *B. subtilis* C9 (15) in oxygen limited conditions. After 40 hours of cultivation, the complete consumption of glucose occurred coinciding with the maximum production of the biosurfactant. The maximum concentrations of biomass and crude biosurfactant obtained were 7.8g/L and 2.0g/L, respectively (Fig. 1A).

Using the indirect method, it was shown that nitrate was reduced to nitrite. Davis *et al*. (6) demonstrated that in cultures under oxygen absence *B. subtilis* is capable of using nitrate as a terminal electron acceptor. A rapid increase in nitrite concentration was observed between 5 and 16h, reaching the maximum concentration of 35.38 mg/L after 16h, suggesting that a restriction of oxygen throughout the culture of *B. subtilis* R14 had occurred.

The surface tension of the culture medium was reduced from 54mN/m at the beginning of growth to 30 mN/m after 20 hours of cultivation (Fig. 1B). According to Mulligan (14), a good biosurfactant must reduce the surface tension of the water from 72 to 35 mN/m, characterizing the product as a powerful surface-active agent.

The highest emulsification activity was 70%, and was reached after 32 hours of cultivation.

Characterization of the Compounds

Thin-layer chromatography revealed two white spots when the plate was sprayed with water, with R_f values 0.55 and 0.37. The standard surfactin presented a retention index of 0.55. The same spots were revealed with rhodamine B, indicating the presence of lipids in the compounds. No spot was revealed when the plate was sprayed with ninhydrin. However, when the biosurfactant was submited to hydrolisis, a spot could be observed when ninhydrin was used, indicating the presence of peptides, but the absence of free amino groups in the molecule. Similar results were observed by Yu *et al.* (24).

The biochemical analysis of the crude surfactant extract showed a lipid content of 49.4% and a protein content of 35.9%, confirming that the surfactant had a lipopeptidic composition, similar to results reported by Nitschke and Pastore (16) for another strain of *B. subtilis*.

Antimicrobial Activity

The results of the antimicrobial activity of the lipopeptides produced by *B. subtilis* R14 against pathogenic bacteria are shown in Table 2.

The compounds showed higher activity against Gram-positive cocci than against Gram-negative bacilli. For *Enterococcus faecalis*, the halos presented a mean value of 14.6mm. These results are important since these microorganisms have natural resistance to aztreonam, co-trimoxazole, cephalosporins, chloramphenicol and clindamycin and low sensibility to aminoglycosides and penicillin G as discussed by Tavares (21) and Furtado (7).

The halos indicating the antimicrobial activity of the compounds against *Staphylococcus aureus* presented a mean value of 14.2 mm. The *S. aureus* 311 isolate was the most sensitive, *Staphylococcus* with a halo diameter of 28.1 mm. It is important to note that some of these strains are resistant to at least two β-lactams.

For *Escherichia coli* the mean halo diameter was 13.8 mm and for *Pseudomonas aeruginosa* the diameter varied from 9.8 to 12.1 mm.

The activity against Gram-negative bacteria was lower when compared to Gram-positive bacteria. Singh and Cameotra (20) have also observed that lipopeptide N1, produced by *B. subtilis* C1, was active against several microorganisms, especially *S. aureus* and *Mycobacterium* sp.

The lipopeptide surfactants produced by *Bacillus* genus present a great potential for biotechnological and biopharmaceutical applications due their biological properties. Future work should be done in order to investigate the chemical structure and cellular toxicity of these compounds.

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Table 2. Antimicrobial activity of biosurfactant produced by *B. subtilis* R14.

	Microorganisms	Halo (mm) $\chi \pm \delta$
Gram positive cocci	*Enterococcus faecalis* CI 55671	12.1 ± 0.2
	Enterococcus faecalis CI 55918	15.9 ± 0.1
	Enterococcus faecalis CI 144	13.8 ± 0.1
	Enterococcus faecalis CI 068	17.4 ± 0.1
	Enterococcus faecalis CI 56671	13.2 ± 0.2
	Enterococcus faecalis CI 56354	12.4 ± 0.2
	Enterococcus faecalis CI 55995	14.7 ± 0.1
	Enterococcus faecalis CI 295591-	15.3 ± 0.1
	Enterococcus faecalis CI 222	16.2 ± 0.2
	Enterococcus faecalis CI 55195	16.7 ± 0.2
	Enterococcus faecalis CI 56288	12.5 ± 0.1
	Enterococcus faecalis ATCC 29212	15.9 ± 0.1
	Staphylococcus aureus CI155	13.7 ± 0.2
	Staphylococcus aureus CI311	28.1 ± 0.1
	Staphylococcus aureus CI15	12.9 ± 0.1
	Staphylococcus aureus CI404	10.7 ± 0.1
	Staphylococcus aureus CI247	10.4 ± 0.1
	Staphylococcus aureus CI16	10.7 ± 0.1
	Staphylococcus aureus UFPEDA22	13.2 ± 0.2
Gram negative bacilli	*Pseudomonas aeruginosa* CI 1	10.7 ± 0.1
	Pseudomonas aeruginosa CI 3	12.1 ± 0.1
	Pseudomonas aeruginosa CI 5	09.8 ± 0.1
	Pseudomonas aeruginosa CI 6	10.5 ± 0.1
	Pseudomonas aeruginosa CI 7	10.5 ± 0.1
	Pseudomonas aeruginosa CI 10	11.3 ± 0.2
	Pseudomonas aeruginosa CI 15	10.4 ± 0.2
	Pseudomonas aeruginosa UFPEDA 39	10.3 ± 0.1
	Escherichia coli CI18	14.4 ± 0.1
	Escherichia coli ATCC 8739	13.2 ± 0.2

ATCC- American Type Culture Collection; **UFPEDA**- Universidade Federal de Pernambuco - Departamento de Antibióticos; CI- Clinical Isolate; $\chi \pm \delta$ Mean ± Standard Deviation.

RESUMO

Atividade antimicrobiana de surfactantes produzidos por *Bacillus subtilis* R14 frente a bacterias multidroga-resistentes

Os lipopeptídeos representam uma classe de surfactantes microbiológicos com crescente interesse científico, terapêutico e biotecnológico. O gênero *Bacillus* é um dos maiores produtores destes compostos ativos. Dentre as espécies produtoras de biossurfactante, *B. subtilis* produz surfactina um dos mais conhecidos. Estes compostos atuam como

antibióticos, antivirais, agente antitumorais, imunomoduladores e inibidores enzimáticos. O objetivo deste trabalho foi determinar a atividade antimicrobiana de biossurfactantes, obtidos pelo cultivo de *B. subtilis* R14, frente a bactérias multidroga-resistentes. Durante o cultivo em meio quimicamente definido, a tensão superficial do meio foi reduzida de 54 mN/m no início do crescimento microbiano para 30 mN/m depois de 20 h. Uma concentração de surfactante bruto de 2 g/L foi obtida depois de 40h de cultivo. Uma caracterização preliminar sugeriu que dois surfactantes foram produzidos. A avaliação antimicrobiana destes compostos foi realizada frente a vinte e nove bactérias. O perfil de multidroga-resistência foi previamente definido para *Enterococcus faecalis* (11 cepas) *Staphylococcus aureus* (6 cepas), *Pseudomonas aeruginosa* (7 cepas) e *Escherichia coli* IC18. Todas as cepas foram sensíveis aos surfactantes, em particular *Enterococcus faecalis*. Os resultados demonstraram que os lipopeptídios têm um amplo espectro de ação, incluindo microrganismos multidroga-resistentes.

Palavras-chave: Biossurfactante, *Bacillus subtilis*, Lipopeptídios, Atividade antimicrobiana

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