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Antibacterial Activity of Sophorolipids from *Candida bombicola* Against Human Pathogens

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HIGHLIGHTS

- A high productivity of sophorolipids was observed in a bioreactor with a poultry fat-based medium.
- The sophorolipids were monoacetylated C18:2 acidic and diacetylated C16:0 lactonic forms.
- The sophorolipids present high percentages of the C18:2 monoacetylated acidic form.
- Antimicrobial activity of sophorolipids was greater against Gram-positive bacteria.

Abstract: Sophorolipids are glycolipids that have natural antimicrobial properties and present great potential in the pharmaceutical field. The present study aimed to produce sophorolipids from *Candida bombicola* using a chicken fat-based medium and evaluate the antimicrobial activity against Gram-negative (*Proteus mirabilis, Escherichia coli, Salmonella enterica* subsp. *enterica*) and Gram-positive bacteria (*Enterococcus faecium, Staphylococcus aureus* and *Streptococcus mutans*). The production of sophorolipids reached 27.86 g L^{-1}. Based on the structural characterization, 73.55% of the sophorolipids present a mixture of acidic monoacetylated C18:2 and lactonic diacetylated C16:0, and 26.45% were present in the diacetylated C18:1 lactonic form. Bacteria submitted to sophorolipid exposure showed a reduction in viability at doses of 500 µg mL^{-1} and 2,000 µg mL^{-1} against Gram-positive and Gram-negative bacteria, respectively. These results suggest that sophorolipids produced in chicken fat medium may be used as antimicrobial agents to prevent or eliminate contamination by different pathogens.

Keywords: sophorolipids; *Candida bombicola*; biosurfactant; lipid residue; antibacterial properties

INTRODUCTION

Globally, bacterial infections are the main causes of chronic diseases and death [1]. The main causes of mortality are the resistance of several pathogens, such as *Escherichia coli, Klebsiella pneumoniae* and

Staphylococcus aureus, to commonly used drugs and the scarcity of new classes of drugs with antibacterial properties, mainly for Gram-negative bacteria [2].

Thus, research for new natural compounds for application in the treatment and/or prevention of human diseases has been increasing over the years [3,4]. The search for these molecules has focused on the structural diversity of biofilm components, which aggravates the development of antibiotic resistance and makes it difficult to eradicate [5].

Sophorolipids are especially produced by *Candida bombicola*. The production of sophorolipids depends on the strain, culture conditions, components of the medium, and other factors. *Candida bombicola* is the main producer of sophorolipids presenting the high yield of sophorolipids and absence of pathogenicity due to its inability to colonize tissues. Therefore, this strain is largely used in food, pharmaceutical and cosmetic industry. This yeast is considered safe for human health and possess the generally recognized as safe (GRAS) status [6,7].

Sophorolipids have anti-adhesive and biocidal properties and could be used as antimicrobial agents to prevent or eliminate contamination by different pathogens [6,7]. The antimicrobial activity of sophorolipids depends on several factors such as the producing microorganism, the pathogen tested and the chemical structure of the molecule [8].

Sophorolipids belong to the class of extracellular glycolipids [9,10] and are structurally composed of a sophorose disaccharide (2´-O-β-D-glucopyranosyl-1-β-D-glucose) linked by a β-glycosidic bond to a long-chain fatty acid, especially palmitic (C16) or stearic (C18) acids. They may be deacetylated, monoacetylated or diacetylated at the 6' and/or 6'' position of the sophorose [11]. The fatty acid carboxyl group may be esterified at the C4', C6' or C6'' position, resulting in lactone forms or in the carboxyl group of the free fatty acid resulting in the acid form [11].

The sophorolipids exhibit various properties as emulsifier, lubricant, micelle formation, detergency, dispersibility, and wettability foaming [12]. The antimicrobial properties of sophorolipids were already described against some strains of Gram-positive and Gram-negative bacteria [6,7,13]. However, the literature still lacks of more consistent data exploring the sophorolipids antimicrobial activity.

Moreover, the production of sophorolipids is costly and the development of less expensive production processes is still necessary. In this context, the use of industrial wastes and its efficient and sustainable conversion into high added-value products through fermentation is a currently addressed in several studies [14-20]. The residual chicken fat, a cheap and abundant byproduct produced by the Brazilian industries, is usually applied for the production of animal food, soap and biofuel. However, due to their high content of fat acids, the chicken fat could be used in microbial fermentation processes to generate sophorolipids [21]. In a preliminary study, our group already showed the possibility of using chicken fat to produced sophorolipids [21].

In this way, the aim of this work was to produce sophorolipids using chicken fat medium in bioreactor and evaluate their antibacterial activity against Gram-positive (*Enterococcus faecium*, *Staphylococcus aureus* and *Streptococcus mutans*) and Gram-negative bacteria (*Proteus mirabilis*, *Escherichia coli*, and *Salmonella enterica* subsp. *enterica*).

MATERIAL AND METHODS

Microorganisms and preservation medium

Candida bombicola (ATCC 22214™) was obtained from the American Type Culture Collection (ATCC), Manassas, USA (2013) and maintained in preservation medium containing (g L^{-1}): glucose (10), yeast extract (3), peptone (5), malt extract (3), and agar (20) at 4 °C. For the antibacterial activity, three gram-negative strains were used: *P. mirabilis* ATCC 7002, *E. coli* ATCC 8739, *S. enterica* subsp. *enterica* ATCC 14028; and three gram-positive strains: *E. faecium* ATCC 6569, *S. aureus* ATCC 6336 and *S. mutans* ATCC 25175. Strains were preserved in brain and heart infusion broth medium at 37 g L^{-1} with 20 to 30% glycerol at -80 °C.

Production of sophorolipid in chicken fat

The fermentation was conducted in a 5.0 L bioreactor (FerMac 320 - Electrolab Biotech Ltda. in United Kingdom) containing 4 L of the fermentation medium consisting of the following (g L^{-1}): glucose (77.5), yeast extract (2.5), and chicken fat (75). The composition of the chicken fat was 0.65% myristic acid, 23.9% palmitic acid, 6.69% stearic acid, 38.46% oleic acid, 28.21% linoleic acid and 2.06% linolenic acid [21]. Fermentation was performed at 30 ºC while rotating at 450 rpm and with an aeration rate of 1.0 vvm. The inoculum was

standardized to 10% (v/v). Samples were collected every 12 h to evaluate the production of sophorolipids and biomass as well as sugar and lipid consumption. Chicken fat were extracted from residual tissue from the chicken industry from Londrina-Paraná region [21].

Quantification of sophorolipids

After centrifugation for the removal of biomass, the sophorolipids were submitted to three consecutive extractions with ethyl acetate (1:1 v/v) in a separation funnel. In the organic phase, methanol water (4:1 v/v) and hexane with methanol and water (1:1 v/v) were added, which formed two phases, the upper (hexane) phase containing the residual fat and the lower (methanolic) phase containing the sophorolipids. In the methanolic phase, another three washes were conducted with ethyl acetate and water (1:3 v /v) with intervals of 40 min each at 4 °C. The sophorolipids were monitored by thin layer chromatography. The biomass and the residual fat were quantified by gravimetry at 70 °C. The total sugars were determined by sulfuric phenol [22]. The experiments were conducted in triplicate.

Characterization of sophorolipids

High-Performance Liquid Chromatography (HPLC) with an iodine arrangement detector

For the characterization of sophorolipids, a Shimadzu CLC-ODS (M) ® C18 Shim-pack column (4.6 × 250 mm; 4.6 µm; 12 nm) was used with an iodine arrangement detector at 207 nm and with a gradient mobile phase composed of 30% acetonitrile and 70% water for 5 minutes before increasing to 80% acetonitrile and 20% water for 50 minutes [23]. 1',4''-Sophorolactone 6',6''-diacetate (Sigma Aldrich, USA) were used as the sophorolipid standard (Sigma Aldrich).

Mass spectrometry (MS)

MS analysis was performed with a hybrid quadrupole/time-of-flight mass spectrometer (Bruker, Cologne, Germany)–Compact Triple-Quadrupole ESI-OTFO II Model. Methanol was the solvent system, and the samples were analysed by direct injection. Analysis parameters were electrospray negative ionization (ESI) in a positive mode of 3200 V, 200°C of capillary temperature, 4 L min^{-1} of gas flow in the cone of and nebulizer pressure at 4 bars. Data were analysed by Bruker Data Analysis 4.2 software.

Antibacterial activity

The bacterial strains were grown in tryptone soy broth (30.0 L^{-1}) for 18 to 24 h at 37 °C. Gram-negative bacteria were transferred to MacConkey agar (50.0 g L^{-1}), and gram-positive bacteria were transferred to Müller-Hinton medium (38.0 g L^{-1}), and the cultures were maintained for 24 h at 37 °C.

Broth microdilution assay

A Broth microdilution assay was used to determine the minimal inhibitory concentration (MIC) according to the methodology described in CLSI [24]. Bacterial suspensions were adjusted to 10^6 CFU.mL^{-1} (0.9% NaCl) using a 0.5 McFarland scale. Sophorolipids (samples produced in this study and a commercial standard) were solubilized in 2% chloroform and methanol (1: 1) with a final concentration of 2,000 µg mL^{-1} in tryptone soya medium. Sophorolipids (50 µL) were inoculated with 50 µL of different bacterial suspensions to reach a final concentration of 5 × 10^5 CFU.mL^{-1}. A series of two-fold dilutions of these test agents ranged from 2,000 µg mL^{-1} to 7.8 µg mL^{-1}. The samples were incubated at 37 °C for 18 to 24 hours. The inhibitory effect was detected by the turbidity of the medium. When necessary for confirmation, 10 µL of the well solution were plated on Müller-Hinton Agar (38 g L^{-1}), and after 18-24 h, the colony forming units were counted. The experiments were conducted in triplicate.

Disc diffusion assay

The bacterial suspensions were plated by swab on Müeller-Hinton agar medium. Disks (6 mm) containing 2.0 µg of sophorolipids were placed on the plates and incubated at 37 °C for 24 h. The antibacterial activity of the sophorolipids was verified by the formation of a halo around the disc, which indicated the inhibition zone. The assay was determined according to the methodology described in CLSI [24]. The experiments were conducted in triplicate.

Statistical analysis

The profile of sophorolipids production and biomass synthesis were modeled using the non-linear logistic regression (Equation 1) by Software R [24].

$$P = P_{max}/(1 + \exp[(x_0 - x)/k]) \quad \text{(Equation 1)}$$

Where Pmax is the maximum asymptotic value of sophorolipids and biomass production (g L^{-1}), k is the numerical scale parameter on the input axis, x_0 is the value x at the inflection point of the curve. For the significance of the parameters, the t-test was used. The fit quality of the models was expressed by the coefficient R^2 and its statistical significance was verified by the F-test. The assumptions of the models, normality and homogeneity of residues variance were determined by the Shapiro-Wilk test [25].

RESULTS AND DISCUSSION

Production of sophorolipids in chicken fat

Using bioreactor, the production of sophorolipids reached 27.86 g L^{-1} at 120 h. The maximum asymptotic production according to the statistical model was 33.42 with a variation of 19% and demonstrated consistent results. The consumption of glucose and chicken fat increased progressively according to the fermentation time and reached 72.8% and 63.8%, respectively (Figure 1). The microbial growth reached 8.48 g L^{-1} in 48 h. The predicted maximum production was 8.68 g L^{-1} according to the statistical model.

The proposed model (Equation 1) showed high agreement between predicted and observed values. The R^2 for the responses of the variables in the study was 0.97. The residue analysis showed homogeneity of variance and normality (Shapiro-Wilk test, $p>0.05$) of the residual when considering the assumptions of the model. The kinetic parameters defined by equation 1 were statistically significant ($p<0.001$) for sophorolipids and biomass production [26].

The production of sophorolipids is directly affected by the culture medium composition and alternative sources can lead to the reduction of costs and provide sustainability to the production of biosurfactants [7]. The use of residual fat from the poultry industry resulted in 39.81 g L^{-1} of sophorolipids in Erlenmeyer flasks [21]. The high production costs of sophorolipids is related to the complexity of the culture media, which can reach 60% of the total costs of the fermentation process [27]. Choosing a proper residue is necessary to verify if its composition of nutrients attends to the needs of microorganism growth and the production of the desired metabolite. Then, it may be difficult to choose agroindustry residues as alternative substrates for the fermentative processes [28].

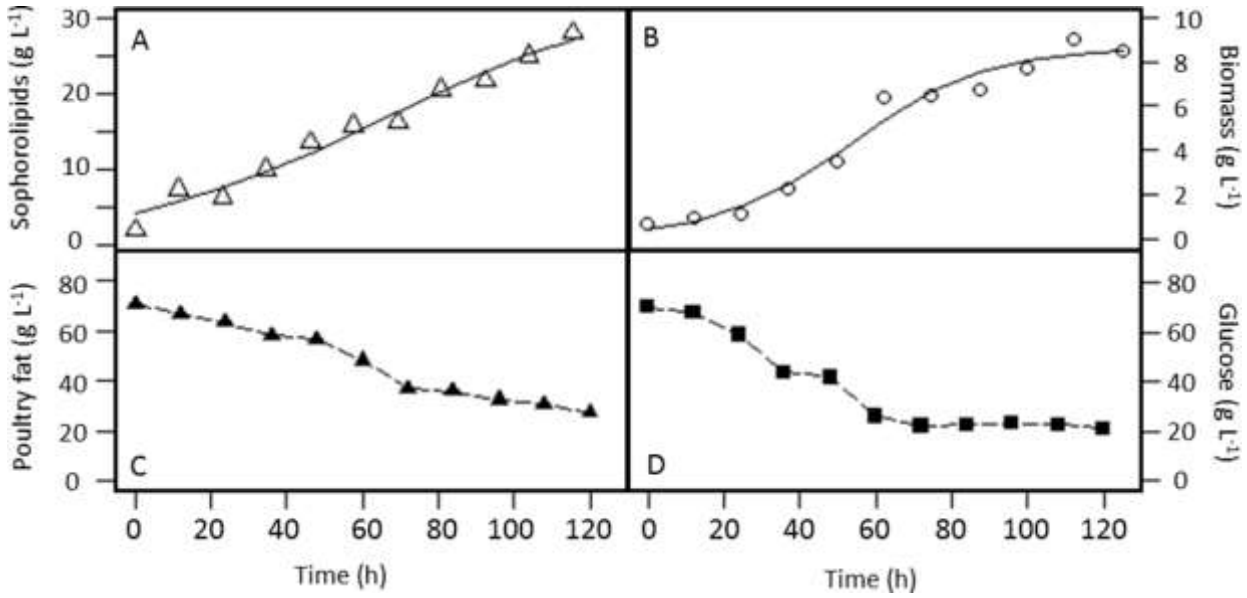

Figure 1. Production of sophorolipids (A), biomass (B), consumption of chicken fat (C) and sugar (D) by *C. bombicola* at 30 °C, 450 rpm, 1.0 vvm in bioreactor.

Sophorolipids from *C. bombicola* were studied in sugarcane molasses and coconut oil (10%) for 168 hours at 180 rpm. The maximum yield was 10 g L^{-1} [29]. Using castor oil, the production of sophorolipids

reached 6.1 g L^{-1}. This low production may be due to the low concentration of palmitic acid (9.9%), which may have reduced the production of sophorolipids [30]. Using chicken fat, in this study we achieve the production of 27.86 g L^{-1} in a bioreactor over 120 h of fermentation. This greater production may have been influenced by the higher amount of palmitic acid (23.9%) of produced sophorolipids. In studies of the production of sophorolipids by *C. bombicola* using a conventional lipid substrate (oleic acid), the production was 199 g L^{-1}, which corresponded to a yield of 0.9 g L h^{-1} [31]. When comparing these data to the production obtained in the present study, the use of the lipid substrate as chicken industry may represent an interesting strategy to produce this biosurfactant.

Characterization of sophorolipids

HPLC and direct injection MS were used to separate and identify the sophorolipids produced by *C. bombicola*. The main types of sophorolipids found in the chromatographic profile were the acidic C18:2 monoacetylated and diacetylated C16:0 lactonic forms (with a retention time of 39.56 min). At 43.39 min, the lactonic C18:1 diacetylated form was detected (Figure 2). The results were confirmed by spectroscopy with a mass m/z of 685 ([M + Na^+]) and 711 ([M + Na^+]) for the C18:2 monoacetylated and C18:1 diacetylated forms, respectively. Therefore, 73.55% of the sophorolipids were a mixture of acidic monoacetylated C18:2 and lactonic diacetylated C16:0, and 26.45% were diacetylated C18:1 lactonic form (Figure 3). Mass spectrum of standard sophorolipid (1',4''-Sophorolactone 6',6''-diacetate) is showed in Figure 4 Minucelli and co-workers [21], showed that sophorolipid obtained from chicken fat by *C. bombicola* using Erlenmeyer flasks was predominantly diacetylated lactone form. Although the authors used similar medium components, this different could be explaining by the interactions of the microorganism with the fermentation medium promoted by the bioreactor environment.

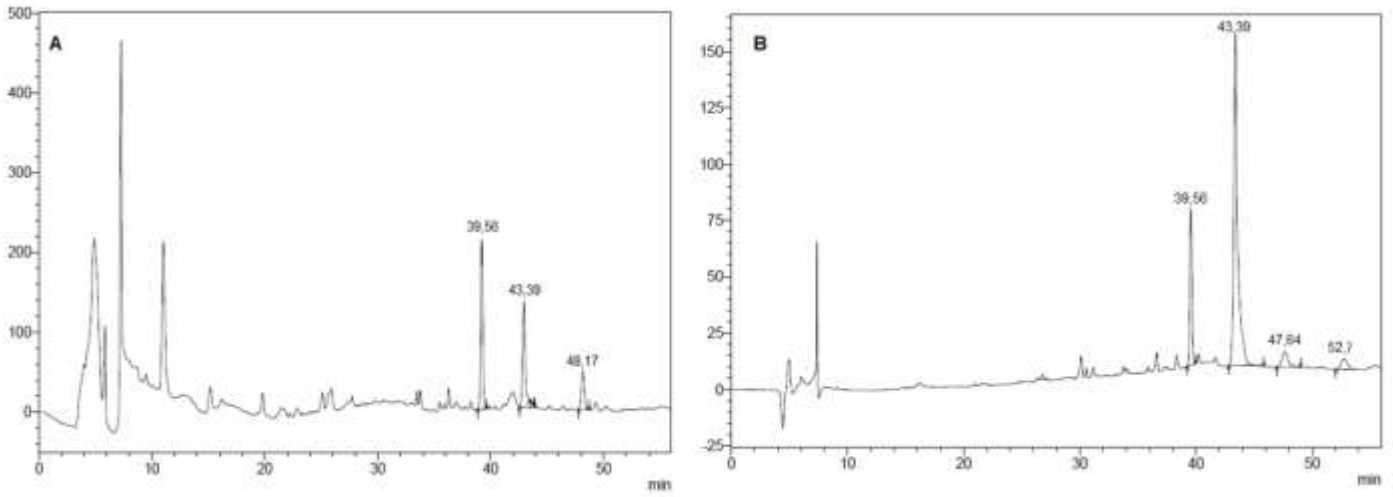

Figure 2. Chromatogram of sophorolipids produced by *C. bombicola* (A) and of sophorolipid standard (1',4''-Sophorolactone 6',6''- diacetylated) (B).

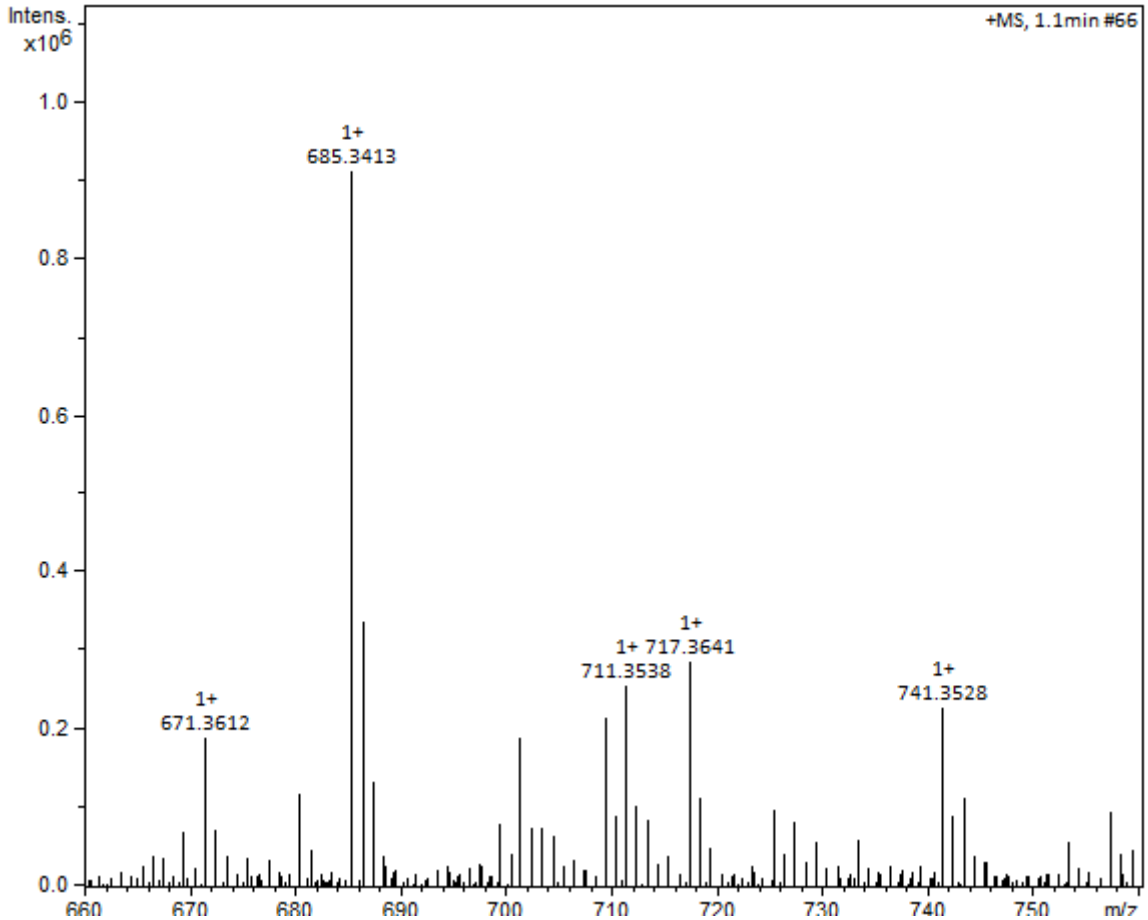

Figure 3. Mass spectrum of sophorolipids produced by *C. bombicola*. Note: Mass 685 ([M + Na+)] acidic C18:2 monoacetylated and lactonic C16:0 diacetylated forms.

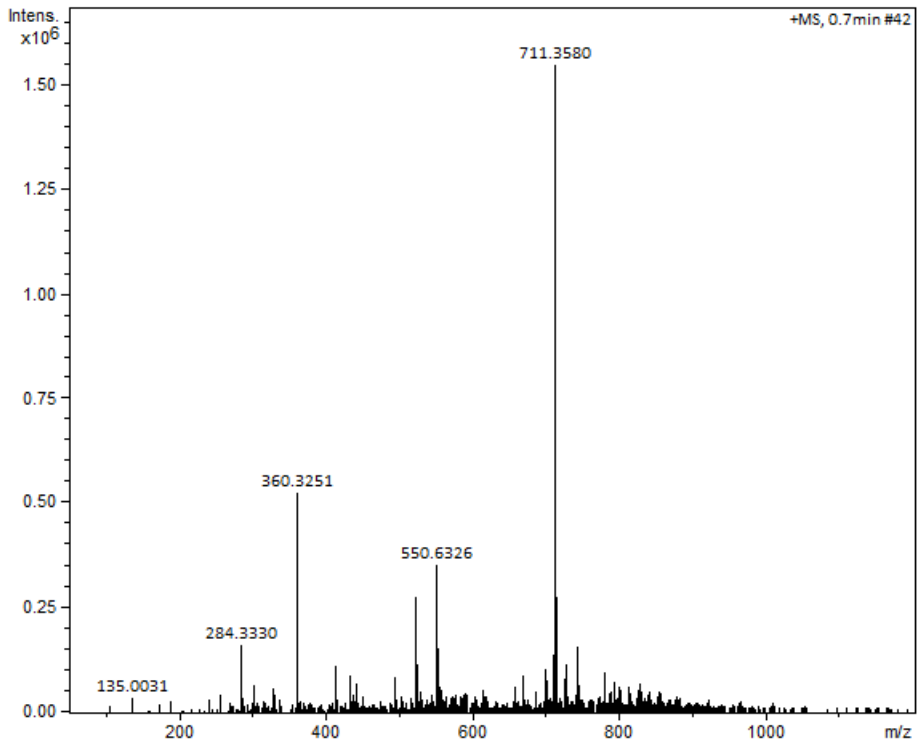

Figure 4. Mass spectrum of standard sophorolipid (1',4"-Sophorolactone 6',6"-diacetate).Note: Mass 711 ([M + Na+)] lactonic C18:1 diacetylated forms.

Antibacterial activity

The antibacterial activity was evaluated by MIC and by disk-diffusion test. As showed in Table 1, the lowest MIC values were found for Gram- positive bacteria using 250 μg mL^{-1} (sophorolipids standard) to 500 μg mL^{-1} (sophorolipids produced in this study). Gram-negative bacteria were shown to be less susceptible to these sophorolipids, which presented MIC higher than 2,000 μg mL^{-1} for both the sample and for the standard sophorolipids. No differences were observed by comparison of the activity of the standard with the sophorolipids produced in this study (predominantly acidic C18:2 monoacetylated). The sophorolipids at concentrations of 50 and 500 mg L^{-1} led to a reduction in the cell viability of Gram-positive (*E. faecalis*, *S. epidermidis*, and *S. pyogenes*) and Gram-negative bacteria (*E. coli*, *P. aeruginosa* and *S. typhimurium*) [13]. In this study, MIC values were shown to be more effective against Gram-positive bacteria (Table 1). Similar values were reported at concentrations of 50 μg mL^{-1} for *S. aureus* and above 750 μg mL^{-1} for *E. coli* [32].

The evidence of antimicrobial activity was verified by a disc diffusion test (Figure 5). For all strains tested, halos of inhibition were observed when 2 μg of sophorolipids were used. The disc diffusion assay is a presumptive test that indicates whether or not the antibacterial activity is present. In the case of the study of new molecules, the presence of halo (regardless of its size) is only an indication that the molecule tested has antibacterial activity. In this study, for all the bacteria tested there was halo formation, suggesting that sophorolipids produced by *C. bombicola* present antibacterial activity against Gram-negative and Gram-positive bacteria. This antimicrobial activity was also described for *B. subtilis* and *S. aureus* with a growth inhibition of 0.5% and 1.0%, respectively [33]. In films with 1.8 g of sophorolipids and polyhydroxy butyrate (PHB), the formation of halos occurred for *P. acnes* [23]. The data confirm antibacterial activity against Gram-positive and Gram-negative bacteria in the presence of sophorolipids. The mechanism of antibacterial action of sophorolipids has been mainly described by the extravasation of the cytoplasmic contents, which was proven by the release of malate-dehydrogenase enzyme. The loss of cell content decreases the possibility of bacteria becoming resistant to antibiotics [33].

Table 1. Minimum Inhibitory Concentration of *C. bombicola* sophorolipids against human pathogens

Pathogens	Minimum Inhibitory Concentration	
	Standard* (μg ml-1)	Sophorolipid (μg ml-1)
Escherichia coli (-)	2,000	2,000
Salmonela entérica (-)	2,000	2,000
Proteus mirabilis (-)	2,000	2,000
Staphylococcus aureus (+)	500	500
Streptococcus mutans (+)	500	500
Enterococcus faecium (+)	250	500

*1',4"-Sophorolactone 6', 6"–diacetylated

According to the literature, sophorolipids have been shown to be more effective for Gram-positive bacteria than Gram-negative bacteria [34], which suggests that the mode of action of sophorolipids can be related to the specific membrane composition of some strains of bacteria. For Gram-negative bacteria, the cell envelope is more complex because it is composed of an outer membrane of lipopolysaccharides and phospholipids before the peptidoglycan layer and the internal plasma membrane [13].

The differences between the cell envelope of gram-positive and gram-negative bacteria confer different charges. The charges and the adhesion are difficult to establish because they depend on the membrane constituents and the bacterial species [32]. At a neutral pH, the surface charge of *E. coli* is more negative than that of *S. aureus* because the gram-negative membrane presents a higher percentage of lipopolysaccharides. At a neutral pH, some molecules of sophorolipids are ionized and present negative charges, which can repel the gram-negative bacteria [32], possibly explaining the lower antibacterial potential of sophorolipids for gram-negative bacteria.

However, the authors report that sophorolipids may have less antibacterial activity against *E. coli* [32]. In this study, we verified that sophorolipids from *C. bombicola* required 2,000 μg mL^{-1} to inhibit the total growth

of *E. coli* (Table 1).The antimicrobial activity of sophorolipids produced by *Starmerella bombicola* was also tested. The lactonic and acidic forms of sophorolipids containing palmitic, stearic or oleic acid were tested at concentrations of 0.1%, 0.5% and 1.0% against 5 strains of *E. coli*. In periods of 1 to 2 hours, the lactonic forms composed of stearic and oleic acids were more effective against *E. coli*. All the strains of *E. coli* showed different susceptibilities for the three sophorolipids tested. The bacteria inactivated by sophorolipids suffered damage in the cell membrane and no differences were found between the action of the lactonic and acidic sophorolipids [8].

The mechanisms of antimicrobial action of sophorolipids are reported as the destabilization or alteration of the cell membrane permeability of the pathogen, modifications of the load-bearing properties, and the ability of the sophorose to damage the bacterial envelopes. Thus, sophorolipids are promising for the inhibition/rupture of biofilms formed by Gram-positive and negative microorganisms, and the type of fatty acid present in the molecule enhances their activity [13,33-35].

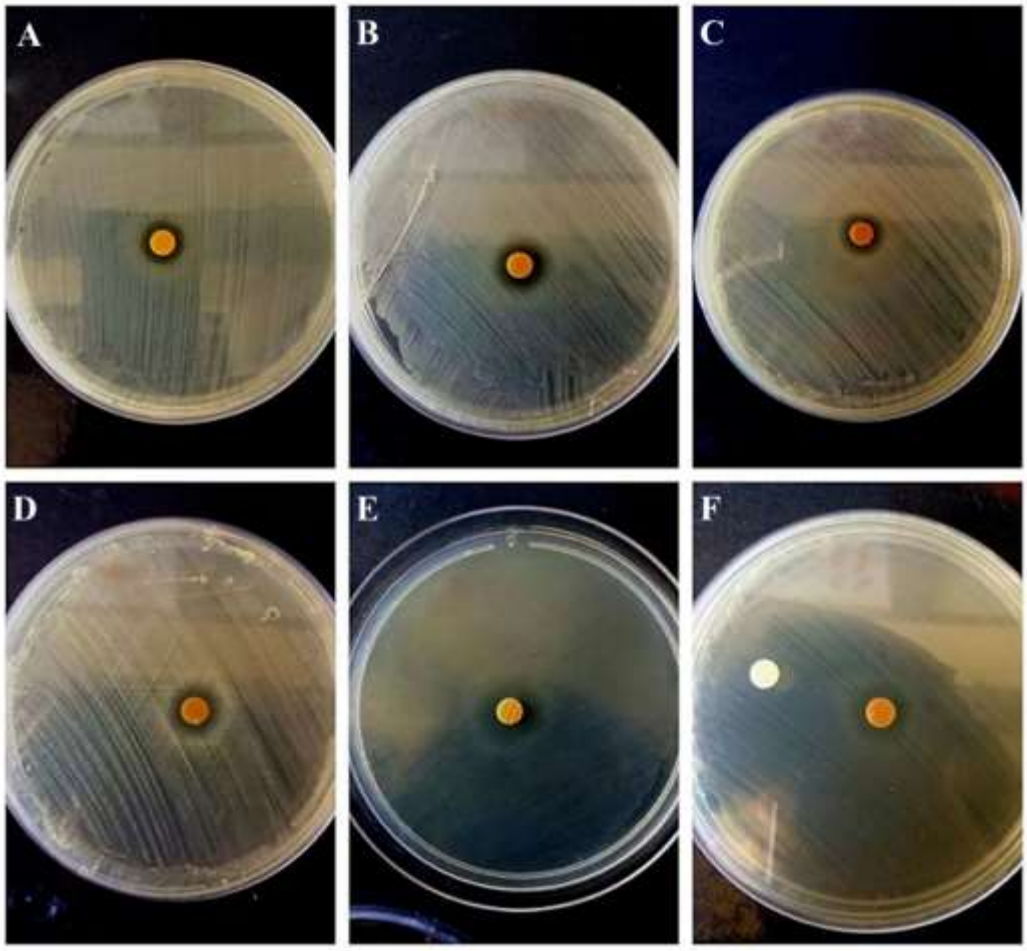

Figure 5. Disk diffusion test of the sophorolipid of *C. bombicola* against Gram-Negative (*A-Escherichia coli*, *B-Salmonella enterica subsp. enterica, C-Proteus mirabilis*) and Gram-Positive (*D-Staphylococcus aureus*, *E-Streptococcus mutans* and *F-Enterococcus faecium* and a white disk, control, without any antimicrobial).

CONCLUSION

This study clearly demonstrated the possibility of producing sophorolipids using a chicken fat residual substrate. In the bioreactor, the maximum production was 27.86 g L^{-1} at 120 h of fermentation. The predominant sophorolipids produced were the acidic C18:2 monoacetylated and lactonic C18:1 diacetylated forms. Antibacterial activity of 500 μg mL^{-1} and 2,000 μg mL^{-1} of sophorolipids was found for Gram-positive and Gram-negative bacteria, respectively. This study suggests that the sophorolipids produced in residual chicken fat medium present antimicrobial activity and can be a sustainable alternative for applications in healthcare area.

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Conflicts of Interest: The authors declare no conflict of interest.

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