Antibacterial Activity of Sophorolipids Produced by *Candida bombicola* on Gram-positive and Gram-negative Bacteria Isolated from Salted Hides

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Abstract

Salted hides and soaked hides treated with certain antibacterial agents, may still contain different proteolytic and lipolytic Gram-positive and Gram-negative bacteria that affect the quality of leather adversely. The prevalence of bacteria resistant to antimicrobial agents in the leather industry has drawn attention of scientists to search new and effective antimicrobial agents. Examination of antimicrobial glycolipids such as sophorolipids for their effectiveness against proteolytic and lipolytic hide-degrading microorganisms, may offer important information. Hence, we describe a research that evaluates the susceptibility of various hide-degrading bacteria to sophorolipids. These extracellular glycolipids were produced by fermentation using *Candida bombicola* ATCC 22214. Palmitic acid, stearic acid, and oleic acid were used respectively to produce SL-p, SL-s, and SL-o. The minimal inhibitory concentrations (MICs) of SL-p, SL-s, and SL-o against Gram-positive endospore-forming bacteria (*Bacillus licheniformis*, *B. pumilus* and *B. mycoides*), Gram-positive bacteria (*Enterococcus faecium*, *Aerococcus viridans*, *Staphylococcus xylosus*, *S. cohnii* and *S. equorum*), Gram-negative bacteria (*Pseudomonas luteola*, *Enterobacter cloacae*, *E. sakazakii* and *Vibrio fluvialis*), and mixed culture of these isolates were examined using an agar dilution method. The MICs of both SL-p and SL-o against the test bacteria were determined as 19.5 µg/mL, with an exception that *E. cloacae* was inhibited by SL-o at a MIC of 9.76 µg/mL. Although MICs of SL-p did not change against the test bacteria, the MICs of SL-s (ranging from 4.88 µg/mL to 19.5 µg/mL) changed according to species of the test bacteria. The lowest MICs of SL-s were found to be 4.88 µg/mL against *B. licheniformis*, *B. pumilus, P. luteola, S. xylosus* and *B. mycoides*. The MICs of SL-p, SL-s, and SL-o against the mixed bacterial culture were detected as the same (19.5 µg/mL). In conclusion, SL-p, SL-s, and SL-o inhibited the growth of 12 different hide bacteria and their mixed culture, and have broad-spectrum activity. The results obtained in the present study may be valuable for the development of SL-p, SL-s, and SL-o as antimicrobial surfactants in the preservation and soaking processes of hides and skins.

Introduction

The leather industry is a significant commercial sector contributing to the world economy. Almost 23 billion square feet of leather is produced annually, with an estimated total value of more than 100 billion USD.[1-3] Ineffective preservation techniques therefore lead to important substantial financial losses in the industry and World economy. Raw hides and skins are preserved with salt, boric acid and antibacterial agents such as didecyldimethylammonium chloride, isothiazolinone, glutaraldehyde, 2-bromo-2-nitropropane-1,3-diol, naphthalene and 1,2-dichlorobenzene, trichloro-s-triazinetrione and sodium sulphate, methylene bis(thiocyanate) and 2-(thiocyanomethylthio)benzothiazole to prevent bacterial growth and subsequent damage on hides or skins during storage and transportation.[4-6] Despite preservation of hides or skins with salt, salted hides/skins may still contain a wide variety of Gram-positive and Gram-negative bacteria originating from faeces, soil, air, water, manure, feeds, and extraneous filth.[7-9] We previously reported that a diverse range of Gram-positive bacteria (12 genera, 47 different species 396 isolates) and Gram-negative bacteria (21 genera, 46 different species and 256 isolates) were isolated from 10 salted hides belonging to different

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Manuscript received Februry 17, 2016, accepted for publication May 13, 2016.
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countries.[10,11] Due to the economic importance of hides and skins, in the present study we wanted to examine the antibacterial effect of sophorolipids against different species of bacteria isolated from the salted hides.

Sophorolipid (SL) is a class of microbial glycolipids that are synthesized by certain yeast species.[12] Among the reported SL-synthesizing organisms, *C. bombicola* is by far the most studied yeast in term of SL production chiefly because of the ability to achieve high production yields.[13] *C. bombicola* requires the simultaneous presence of carbohydrate and fatty acid substrates in order to produce large quantities of SLs.[14] The most commonly used fermentation co-substrates for SL production by *C. bombicola* are glucose and oleic acid, which leads to predominantly the synthesis of SLs having the structures of 17-L-[(2′-*O*-*β*-glucopyranosyl-*β*-D-glucopyranosyl)-oxy]-9-octadecenoic acid 1′,4′′-lactone 6′,6′′-diacetate (*SL-1*; Figure 1) and its free-acid form (*SL-1A*; Figure 1).[14]

Ashby et al. (2008)[15] subsequently showed that by substituting palmitic acid or stearic acid for the oleic acid in the fermentation, the fatty acid moiety of the SLs can be altered. Thus, the use of palmitic acid substrate resulted in SLs containing 15- and 16-hydroxyhexadecanoic acids as the hydrophobic moiety, while stearic acid yielded SLs having 17-hydroxydecanoic acid as the hydrophobic moiety.[15] Since SLs are amphiphilic compounds, they possess good surface active properties that make them potentially useful as biobased surfactants in many industrial applications.[16-18] More importantly, SL was found to have strong antimicrobial activity either in solution[19-22] or when embedded in films.[23] Based on the surface-active and antimicrobial properties of SLs, Ramos et al. (2011, 2012)[24,25] had studied the application of these molecules in the treatment and preservation of animal hides. SL is advantageous over the traditional surfactants and antimicrobial agents. Because SLs are biosynthesized through microbial fermentation process using renewable feedstocks, it is a preferred "green" biobased material that can be manufactured in a sustainable manner. Furthermore, unlike commonly used antimicrobial agents in medical field against which many bacteria have developed resistance,[26] SL is an emerging antimicrobial agents that are potentially effective against many antibiotic-resistant bacteria.

Hence, the objective of the present study was to determine MICs of SL-Glu/Oleic, SL-Glu/Stearic and SL-Glu/Palmatic against *B. licheniformis*, *B. pumilus*, *B. mycoides*, *E. faecium*, *S. xylosus*, *A. viridans*, *S. cohnii*, *S. equorum*, *P. luteola*, *E. cloacae*, *V. fluvialis*, *E. sakazakii*, and mixed culture of these isolates. These microorganisms were isolated from salted hides in previous studies.[10,11,27,28]

Experimental

Test Microorganisms

C. bombicola ATCC 22214 was acquired from American Type Culture Collection (Manassas, VA). Endospore-forming Gram-positive bacteria (*B. licheniformis*, *B. pumilus* and *B. mycoides*), Gram-positive bacteria (*E. faecium*, *S. xylosus*, *A. viridans*, *S. cohnii* and *S. equorum*), Gram-negative bacteria (*P. luteola*, *E. cloacae*, *V. fluvialis* and *E. sakazakii*), and mixed culture of these isolates were used as test bacteria in this study. These bacteria were obtained from the culture collections of Division of Plant Diseases and Microbiology, Department of Biology, Faculty of Arts and Sciences, Marmara University. Test microorganisms were isolated from salted hides and identified using API test kits (BioMérieux, Inc, France) in the previous studies. [6,10,11,27,28]

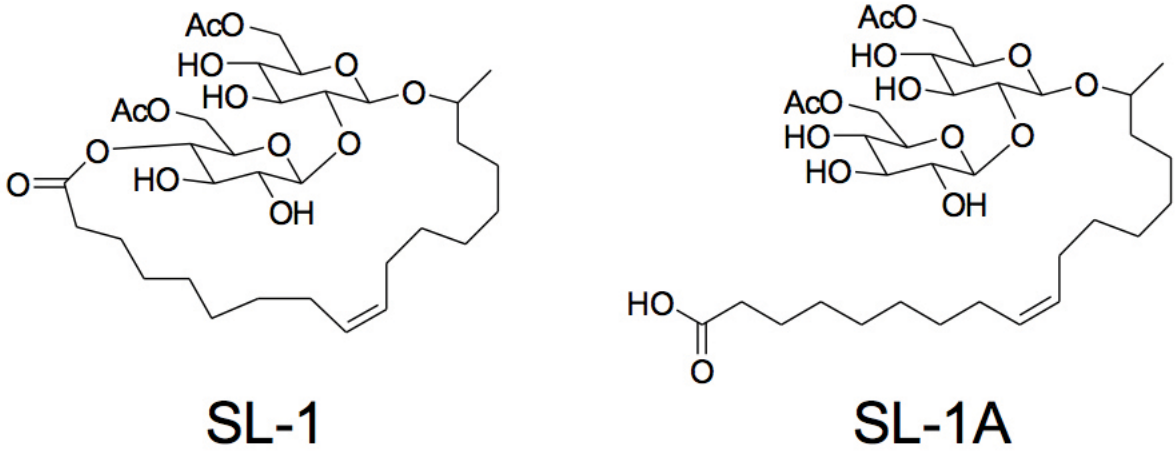

Figure 1. Structures of sophorolipids predominantly produced by *C. bombicola* grown on glucose+oleic acid. The 17-L-[(2′-*O*-*β*-glucopyranosyl-*β*-D-glucopyranosyl)-oxy]-9-octadecenoic acid 1′,4′′-lactone 6′,6′′-diacetate (SL-1) and its free-acid form (SL-1A).

Production of Sophorolipids

Sophorolipids (SLs) were produced by fermentation using *C. bombicola* ATCC 22214 as the producing organism, and glucose + fatty acid of choice as co-substrates according to a previously described protocol.[15] For the present study, we used palmitic acid, stearic acid, and oleic acid to produce SL-p, SL-s, and SL-o, respectively. Briefly, fermentations were performed in a 12-L capacity vessel containing 10 L of culture medium in a bench-top fermenter (Bioflo 3000 Batch/Continuous Bioreactor, New Brunswick, NJ). The basal Candida Growth Media (CGM) consists of glucose (10%, w/v), yeast extract (1%, w/v), urea (0.1%, w/v). An amount of the appropriate fatty acid co-substrate was then added to the basal CGM to attain a concentration of 2% (w/v). The 10-L CGM+fatty acid medium was inoculated with 50-mL *C. bombicola* inoculum culture that had been previously prepared in bulk and stored in -80°C freezer in the form of 15% (v/v) glycerol-containing stocks. All fermentations were performed using the following settings: temperature, 26°C; impeller speed, 700 rpm; aeration rate, 2 L/min; and no pH control. Additional substrates were added on day 2 [glucose, to 7.5% (w/v); fatty acid, to 2% (w/v)] and day 5 [fatty acid only, to 1% (w/v)]. On day 7, the culture as a whole (i.e., cells and medium) was distributed in several 2-L capacity lyophilization jars and subsequent subjected to lyophilization. The lyophilized dry-culture was transferred to Erlenmeyer flasks containing ethyl acetate in excess to extract the SLs. Extraction was carried out at room temperature for 2 days with constant shaking in a shaker-incubator. The extraction mixture was filtered through Whatman No. 2 filter paper, and the solids were returned to the Erlenmeyer flasks for two additional extractions. The combined ethyl acetate extracts containing the SLs were concentrated by evaporation and precipitated in 1-L aliquots of hexane to obtain pure SLs. The SLs were recovered on a Whatman No. 2 filter paper and vacuum-dried in a desiccator. The structures of the SLs were confirmed by LC-MS as described previously.[29]

Agar Dilution Method

Firstly, the test microorganisms were streaked and grown on Mueller Hinton Agar (MHA) (Merck, Darmstadt, Germany) at 37°C for 24 h. Overnight cultures of these isolates were prepared by inoculating 10 mL Mueller Hinton Broth (MHB) with 2-3 colonies of each test microorganism taken from MHA. The inoculated broths were incubated 12 h at 37°C. A 0.5 McFarland standard was used to adjust each bacterial suspension to a density equivalent to 10^8 CFU/mL. Then, each bacterial suspension of test microorganisms was diluted in 0.85% saline to obtain 10^7 CFU/mL. In addition, the mixed culture of the test isolates was prepared from these bacterial suspensions.[30,31] Then, each of the test agents (SL-o, SL-s and SL-p) was separately dissolved in 70% ethanol to a final concentration of 10.000 µg/mL. Series of two-fold dilutions of these test agents, ranging from 10.000 µg/mL to 0.076 µg/mL, were prepared in MHA containing 3% NaCl. One mL volumes of 17 different concentrations of SL-p, SL-s, and SL-o solutions were separately added to 19 mL volumes of molten MHA. Next, these agar media were mixed thoroughly and poured into sterile petri dishes. One µL (an inoculum of 10^4 CFU/spot) of diluted bacterial suspension of each test isolate and their mixed culture was separately transfered to agar plates containing 17 different concentrations (10.000 µg/mL, 5000 µg/mL, 2500 µg/mL, 1250 µg/mL, 625 µg/mL, 312.5 µg/mL, 156 µg/mL, 78 µg/mL, 39 µg/mL, 19.5 µg/mL, 9.76 µg/mL, 4.88 µg/mL, 2.44 µg/mL, 1.22 µg/mL, 0.61 µg/mL, 0.30 µg/mL, 0.15 µg/mL, 0.076 µg/mL) of the test agents. SL-p, SL-s, and SL-o free control media were also used in all experiments. Later, the plates were incubated at 37 °C for 18 h, and MICs of antibacterial agents against the test bacteria were determined.[30-32] Inhibitory effects of 70% ethanol on the test bacteria and mixed culture were also examined on MHA containing the same volume of ethanol concentrations as other agar media containing antimicrobial agents. All test bacteria and the mixed culture grew in the series of ethanol found in MHA. Dilutions of three antimicrobial agents were prepared according to the method explained in the EUCAST Definitive Document E.Def 3.1 (2000).[30]

Results and Discussion

Proteolytic and lipolytic microorganisms on the salted hides and skins may provoke hair slip, discoloration, serious grain peeling, fiber disintegration, odor, looseness, weakness and holes in leather.[7-9] Research has shown that among the microorganisms found in animal hides and skins, *B. licheniformis*, *B. pumilus*, *E. faecium*, *P. luteola*, *A. viridans*, *B. mycoides* and *S. cohnii* were shown to exhibit both protease and lipase activities. Although *E. cloacae, V. fluvialis* and *S. equorum* were protease positive and lipase negative, *S. xylosus* and *E. sakazakii* were protease negative but lipase positive.[6,10,11, 27, 28]

Our test isolates in the present study were therefore the commonly found microorganisms on the salted hides that cover different Gram-positive and Gram-negative, proteolytic, and lipolytic isolates. While *B. licheniformis* and *B. pumilus* were isolated from 9 salted hide samples, *B. mycoides* were isolated from 4 hide samples. Both *E. faecium* and *A. viridans* were isolated from 10 salted hides, but *S. cohnii* and *S. xylosus* were isolated respectively from 8 and 7 salted hide samples. Although *E. cloacae* and *P. luteola* were isolated from 10 salted cured hide samples, *V. fluvialis* and *E. sakazakii* were found on 8 and 6 salted hide samples, respectively.[10,11]

Despite the salt curing process of hides, a wide variety of genera of *Aerococcus*, *Aneurinibacillus*, *Bacillus*, *Brevibacillus*, *Enterococcus*, *Geobacillus*, *Kocuira*, *Lactococcus*, *Paenibacillus*, *Streptococcus*, *Staphylococcus* and *Virgibacillus* belonging to

Gram-positive bacteria and genera of *Acinetobacter*, *Aeromonas*, *Alcaligenes*, *Burkholderia*, *Citrobacter*, *Comamonas*, *Edwardsiella*, *Enterobacter*, *Escherichia*, *Hafnia*, *Klebsiella*, *Mannheimia*, *Pasteurella*, *Proteus*, *Pseudomonas*, *Salmonella*, *Serratia*, *Sphingomonas*, *Stenotrophomonas*, *Vibrio* and *Yersinia* belonging to Gram-negative bacteria were isolated from ten salted hides.[10,11] In addition, a high percentage of these isolates showed proteolytic and lipolytic activities.[10,11]

Our test microorganisms such as *E. sakazakii*, *E. cloacae*, *P. luteola*, *E. faecium*, *A. viridans*, *S. xylosus*, and *S. cohnii* were also isolated from main soak liquor treated with antibacterial agent containing 0.8 g/L of didecyldimethylammonium chloride.[6,27] These studies proved that proteolytic and lipolytic bacterial populations on salted and soaked hides cannot be effectively prevented by commonly used antibacterial agents. [6,10,11,27] Microbial spoilage of hide usually results from the failure to eliminate these microorganisms during salt and brine curing or soaking processes. Careful selection of antimicrobial agents facilitates high quality leather production. Hence, in this study we concentrated on finding an antibacterial agent that may prove potent against proteolytic and lipolytic hide bacteria.

Antibacterial activities of SL-p, SL-s and SL-o against the test bacteria are presented in Table I. The MICs were determined as the lowest concentration of antibacterial inhibiting the visible growth of each microorganism on the agar media containing 3% NaCl. The MICs of SL-p and SL-o against all test bacteria and their mixed culture were found as 19.5 µg/mL, except *E. cloacae*. Although the MIC of SL-p against *E. cloacae* was 19.5 µg/mL, the MIC of SL-o against this test microorganism was 9.76 µg/mL. The MICs of SL-s against each test bacteria were lower than those of SL-p and SL-o, with the exception that *E. cloacae* and *S. equorum* were inhibited by SL-o at MICs of 9.76 µg/mL and 19.5 µg/mL, respectively. The MICs of SL-s against *B. licheniformis*, *B. pumilus*, *P. luteola*, *S. xylosus* and *B. mycoides* were found to be 4.88 µg/mL, while the MICs of SL-s against *E. faecium*, *E. cloacae*, *V. fluvialis*, *A. viridans*, *S. cohnii* and *E. sakazakii* were 9.76 µg/mL. However, the MICs of SL-s against both *S. equorum* and the mixed culture (19.5 µg/mL) were found higher than those of the other test bacteria. Among the test bacteria, the highest MIC values belonging to three sophorolipids were detected against *S. equorum* (19.5 µg/mL). The MICs of SL-p, SL-s and SL-o against the mixed culture were found to be same (19.5 µg/mL) (Table I).

These three sophorolipids were fairly active against Gram-positive endospore-forming bacteria, Gram-positive bacteria, Gram-negative bacteria and their mixed culture. In comparison, Shah et. al. (2007)[33] reported that SLs were more effective against Gram-positive bacteria than Gram-negative bacteria. In our study, the variance in antibacterial activity of sophorolipids between Gram-positive and Gram-negative bacteria was negligible except for *S. equorum* (Table I). Although further studies are needed to find the exact reason(s), the precise culture environment such as the presence of NaCl in our study might be a factor. In the leather processing such as brine curing [25% (w/v)] and soaking [3% (w/v)], NaCl is always added into these processes. Our results showed that SL-p, SL-s and SL-o were highly effective to inhibit proteolytic and lipolytic hide bacteria even in the test media containing organic substances and 3% NaCl (w/v).

Our research results were similar to the findings of Hommel et al. (1994) [34] Lactonic sophorolipids obtained from *Candida apicola* IMET 43747 were found effective against both Gram-positive bacteria (*Azotobacter chroococcum*, *B.subtilis*, *Micrococcus luteus*, *Mycobacterium rubrum* and *S. aureus*) and Gram-negative bacteria (*Escherichia coli, Proteus vulgaris* and *P. aeruginosa*, *Serratia marcescens*). MICs of lactonicsophorolipids against *A. chroococcum*, *B. subtilis*, *M. luteus*, *M. rubrum* and *S. aureus* were respectively 1.95 µg/mL, 0.12 µg/mL, 0.48 µg/mL, 0.12 µg/mL and >800 µg/mL, while MICs of lactonic sophorolipids against *E. coli*, *P. vulgaris*, *P.aeruginosa* and *S. marcescens* were 7.8 µg/mL, >31.3 µg/mL, 7.8 µg/mL and 1.95 µg/mL, respectively.[34]

It has been known that bacterial cells have diverse abilities to survive and grow in the presence of antibacterial agents.[35] Therefore, it is usually difficult to inactivate the mixed cultures of different species of bacteria using antibacterial agents. Our previous studies showed that total bacterial counts in 34 hide-soak liquors treated with different antibacterial agents were between 10^5-10^8 CFU/mL. Total proteolytic and lipolytic bacterial counts in these liquors were between 10^4-10^8 CFU/mL.[36] In another study, although the antibacterial agent containing didecyldimethylammonium chloride (0.4 g/L) was used in soaking process of hides for 8 h, we found total counts of all bacteria, proteolytic, and lipolytic bacteria to be $1x10^6$ CFU/mL, $1x10^6$ CFU/mL and $1x10^5$ CFU/mL, respectively. A total of 26 different bacterial species belonging to genera of *Enterobacter*, *Pseudomonas*, *Enterococcus*, *Lactococcus*, *Aerococcus*, *Vibrio*, *Kocuria*, *Staphylococcus* and *Micrococcus* were isolated from main soak liquors of hides treated with antibacterial agent containing didecyldimethylammonium chloride (0.8 g/L).[6]

Shah et al., (2007)[21] compared the antibacterial activities of sophorolipids obtained from *C. bombicola* grown in medium containing glucose, fructose, xylose, ribose, lactose, mannose, arabinose, or galactose against *Rhodococcus erythropolis, B. subtilis, S. epidermis, Streptococcus agalactiae, Moraxella* sp., *P. putida, E. aerogenes* and *E. coli.* These researchers observed that all sophorolipids obtained from different sugar-containing media were effective against the test bacteria, but they differ in the levels of their activities against the tested organisms. SLs obtained from arabinose-containing medium were found to be

more effective against *R. erythropolis, B. subtilis, S. agalactiae,* and *Moraxella* sp. than SLs obtained from glucose-containing medium. On the other hand, while sophorolipid obtained from arabinose medium did not prevent the growth of *E. coli,* sophorolipid obtained from lactose-containing medium were found to be the most effective against *B. subtilis.*

Kitamoto et al. (1993)[19] compared antimicrobial properties of two kinds of microbial glycolipids, i.e., mannosylerythritol lipids (MEL-A and B), produced by *Candida antarctica* T-34 , against *B. subtilis*, *M. luteus, M. rhodochrous, S. aureus, P. aeruginosa, P. rivoflavina* and *E. coli*. Unlike our study showing the antimicrobial activity of SLs against both the Gram-positive and Gram-negative test bacteria, their results showed that both MEL-A and B were fairly effective against Gram-positive bacteria but only mildly active against Gram-negative bacteria.

Sleiman et al. (2009)[22] reported that the MICs of the ethyl ester diacetate derivative of sophorolipid against *E. coli*, *S. aureus*, *Klebsiella pneumoniae*, *P. aeruginosa*, *S. pneumoniae*, and *Proteus mirabilis* were >128µg/mL, which is higher than the values we observed in our study. Interestingly, Joshi-Navare and Prabhune (2013)[37] determined that MICs of sophorolipid and tetracycline against *S. aureus* as 400 µg/mL and 150 µg/mL, respectively. They further found that a mixture of sophorolipid (300 µg/mL) and tetracycline (15 µg/mL) was effective to inhibit this microorganism in 6 h. It was also observed that this mixture formed pores and therefore caused damage to the bacterial cell membrane. Their results showed that a synergistic effect could result in the effective use of SLs and another antibiotic at low concentrations to achieve a similar or better antibacterial end-point.

In this study, we have successfully demonstrated that the "green" biosurfactant sophorolipids produced by *Candida bombicola* have antibacterial activity against a broad spectrum of bacteria found in hides. This opens up many research areas for the use of sophorolipids in the hides and leather industries. For example, aside from the SL-p, SL-s, and SL-o already tested here, we could compare their antibacterial activities with the sophorolipid produced by *Rhodotorula bogoriensis,* which has long hydroxy fatty acid chain of 22 carbons. Another area of important research is to determine the antimicrobial activities of sophorolipids against the extremely halophilic archaea often found on salt cured hides and skins ("red heat" condition). Furthermore, since sophorolipids are biosurfactants and therefore have washing/cleaning property, they can be used to develop washing and/or soaking bath that not only clean hides, skins, or leather but also help kill the bacteria. In summary, this study opens up exciting possibilities for us to further pursue development of applications of these "green" and antimicrobial biosurfactants sophorolipids in the hides and leather industries.

Table I
Minimum inhibitory concentrations of SL-p, SL-s and SL-o against 12 different hide bacteria and their mixed culture (µg/mL).

	Bacillus licheniformis	*Bacillus pumilus*	*Pseudomonas luteola*	*Enterococcus faecium*	*Staphylococcus xylosus*	*Enterobacter cloacae*	*Vibrio fluvialis*	*Bacillus mycoides*	*Aerococcus viridans*	*Staphylococcus cohnii*	*Enterobacter sakazakii*	*Staphylococcus equorum*	Mixed culture
SL-p	19.5	19.5	19.5	19.5	19.5	19.5	19.5	19.5	19.5	19.5	19.5	19.5	19.5
SL-o	19.5	19.5	19.5	19.5	19.5	9.76	19.5	19.5	19.5	19.5	19.5	19.5	19.5
SL-s	4.88	4.88	4.88	9.76	4.88	9.76	9.76	4.88	9.76	9.76	9.76	19.5	19.5

SL, Sophorolipids, p, Palmatic; o, Oleic; s, Stearic

Conclusion

This is the first study that determines the MICs of SL-p, SL-s, and SL-o against the hide isolates (*B. licheniformis*, *B. pumilus*, *B. mycoides*, *E. faecium*, *S. xylosus*, *A. viridans*, *S. cohnii*, *S. equorum*, *P. luteola*, *E. cloacae*, *V. fluvialis*, *E. sakazakii* and the mixed culture of all isolates) on MHA containing 3% (w/v) NaCl. The present study affirms that SL-p, SL-s, and SL-o inhibit the growth of Gram-positive endospore-forming bacteria, Gram-positive bacteria, Gram-negative bacteria and their mixed culture on MHA. MIC values of SL-p, SL-s, and SL-o against the mixed culture were found as 19.5 µg/mL. It was also determined that SL-p, SL-s, and SL-o have broad-spectrum activity. Sophorolipids are environmentally friendly, readily biodegradable, and ecologically benign to aquatic life.[12,-38-39] Therefore, these antimicrobial glycolipids may be used by the leather industry in preservation and soaking processes of hides and skins to prevent proteolytic and lipolytic activities.

Acknowledgements

The authors thank Nicole Crocker and Can Akpolat for technical assistance. We are grateful to Dr. Didem Berber and Dr. Emel Aslan Con for their wonderful studies on hide microorganisms.

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