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9. Lichenysin production and application in the pharmaceutical field

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Abstract. Lipopeptides such as lichenysin are biosurfactants of great interest, due to the demand for natural surface-active agents with low toxicity. *Bacillus licheniformis* AL 1.1 produces a lipopeptide characterized as lichenysin ($Lch_{AL1.1}$), which acts as a powerful surfactant, able to reduce surface tension to 28.5 mN m^{-1} and with a critical micelle concentration of 15 mg L^{-1}. $Lch_{AL1.1}$ is particularly effective in preventing biofilm formation by pathogenic strains, has an emulsifying capacity and permeabilizes membranes by a colloid-osmotic process. The production of lipopeptides from agro-industrial residues, particularly molasses, is a sustainable process of great potential for the development of economic bioprocesses.

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Introduction

Surfactants are amphiphilic compounds with a hydrophobic and hydrophilic domain. They have the ability to concentrate at interfaces and increase the aqueous solubility of non-aqueous liquids. Bio-surfactants (BS) are produced by microorganisms on surfaces to enhance access to nutrients or facilitate growth in the environment, BS have many advantages compared with their chemically synthesized counterparts, including biodegradability, low toxicity, availability from renewable resources, resistance to environmental factors, and high surface and interfacial activity. In short, they are molecules with a promising future [1].

BS can be classified based on molecular weight. Those of a low molecular weight, such as glycolipids and lipopetides, effectively reduce surface and interfacial tension. High molecular weight BS, or bioemulsifiers, are more effective at stabilizing oil in water emulsions and include polymeric surfactants such as polysaccharides, proteins, lipopolysaccharides and complex mixtures [2]. According to their polar group, BS are anionic or neutral [1].

BS are also grouped according to the chemical structure of the hydrophilic moiety. (i) Glycolipids are carbohydrates combined with long-chain aliphatic or hydroxyaliphatic acids. This group includes the most studied BS, the rhamnolipids, trehaloselipids, sophorolipids, cellobioselipids and mannosylerytroil lipids. (ii) Lipopeptides consist of cyclic peptides or unattached lipidic chains and are characterized by remarkable surfactant and antimicrobial properties. Examples are gramicidin, surfactin, polymixin, subtilisin, iturin, mycosubtilin, fengycin, and viscosin. (iii) Phospholipids and fatty acids with surfactant activity, such as phosphatidylethanolamine, are overproduced by several bacteria during growth on n-alkanes [3]. (iv) Polymeric surfactants consist of heteropolysaccharides combined with proteins. Commercially important compounds included in this group are emulsan, liposan, biodispersan, alasan, and manoprotein. (v) Particulate BS formed by extracellular membrane vesicles are able to form stable emulsions, important for microbial alkane uptake [1,4].

Microbial BS are secreted or attached to cellular walls. They are usually produced in the presence of water-insoluble substrates, but not always, which is an impediment in explaining the bacterial benefits associated with their production. Numerous bacteria and yeasts of diverse

genera produce BS of varying chemical nature. Cyclic lipopetides, produced as secondary metabolites by different species of *Bacillus*, are remarkable surfactants with high surface activity and antimicrobial properties. Considering the high demand for new products with health applications, the lipopeptide lichenysin, with its surface activity, emulsifying capacity, and anti-adherent and antiproliferative properties, is of particular interest.

1. *Bacillus licheniformis*

Bacillus licheniformis is an endospore-forming bacterium widespread in soils and other environments, including food and clinical and veterinary samples. It grows in a wide range of temperatures, from 15ºC to a maximum of 68ºC (strain AL1.1 was isolated from a geothermal zone in the Antarctic). Its rapid growth, low nutritional requirements, resistance and capacity to produce enzymes (proteases and amylases), polysaccharides and biosurfactants, make this bacterial species interesting for the fermentation industry as a productive microorganism or probiotic [5,6,7].

B. licheniformis has occasionally been reported as an opportunistic pathogen in man and animals and a cause of food poisoning, with large amounts being associated with intoxication in a few cases. *B. licheniformis* has been described as a contaminant of dairy products, and toxin-producing isolates have been found in raw milk and baby food [8]. Lichenysin has been proposed as a virulence factor, although the mechanism of action is unknown [7].

In contrast, *Bacillus* species have been used as probiotics, or live microbes, which when administered confer a health benefit to the host. Spore probiotics are being used in humans (dietary supplements), animals (competitive exclusion agents) and in aquaculture (to increase disease resistance). *B. licheniformis* is used in combination with *B. subtilis* in two commercial products, the animal feed BioPlus®2B in the Ukraine and the medicine Biosporin in Russia. Its probiotic effect is associated with Amicoumacin production, with activity against *Helicobacter pylori*. The easy production and stability of spores and their immune stimulation, antimicrobial and competitive exclusion properties suggest potential application as probiotic dietary supplements, although more clinical studies are required to confirm the absence of adverse effects [9, 10].

Exopolysaccarides with antioxidant and anti-aging activity produced by *B. licheniformis* KS-17 and KS-20 may be used as functional ingredients in novel probiotics [11]. However, results suggest the toxigenic potential of *Bacillus* species used in nutrition needs to be revised [7].

2. Lichenysin characterization and production

Lipopeptides are a class of microbial surfactants with a growing attraction for the therapeutic, cosmetic and food industries. They occur across the whole spectra of microorganisms, but above all in *Bacillus* sp. The basic structure of lipopeptides consists of a specific fatty acid combined with an amino acid moiety. They are usually found in mixtures of closely related compounds with slight variations in their lipid part and amino acid composition. Lipopeptide activities include antibiotic, antiviral, antitumor, inmunomodulator and inhibition of specific toxins and enzymes. These properties make them potential agents for therapeutic applications [12, 13].

The first lipopeptide to be isolated was surfactin [14]. Produced by *Bacillus subtilis*, it is among the most powerful surfactants, along with iturin, fengycin and lichenysin, whose exceptional surface activity endows them with powerful biological effects [15]. The mechanisms of action of lipopetides have not been clarified in detail, but their different activities are clearly due to their surface and membrane properties. Surfactin, as the first lipopetide described, is the most studied. Produced as a mixture of isoforms, it has a molecular weight of 1007-1035 Da and is formed by one heptapeptide with the amino acid sequence Glu-Leu-Leu-Val-Asp-Leu-Leu [16]. Lichenysin is the most potent anionic cyclic lipoheptapeptide BS reported to date [17]. It is produced by most, if not all, *B. licheniformis* strains on media containing glucose as the carbon source [16, 18]. Lichenysin production has recently been described in *B. lichenyformis* AL 1.1, isolated from an extreme Antarctica environment [19]. Lichenysin consists of a peptide moiety with seven amino acids and a β-hydroxy fatty acid of 12-17 carbon atoms, with normal iso and anteiso branching. Several lichenysin isoforms and homologues are found in nature, due to modifications in the length and branching of the fatty acid chain and amino acid substitutions. Six variations are accepted, named lichenysin A, B, C, D, G and surfactant BL86, lichenysin A being the most abundant isoform. Lichenysins are anionic surfactants due to the presence of Asp and/or Glu residues. Lichenysin$_{AL1.1}$ (Lch$_{AL1.1}$) is a mixture of lipopeptide homologues,

with a molecular weight between 1006 and 1034. The peptide moiety consists of glutamine as the N-terminal amino acid, two leucines, valine, aspartic acid and iso-leucine as the C-terminal amino acid. The lipid moiety is formed by β-hydroxy fatty acids ranging in size from C_{14} to C_{16}, with high similarity to lichenysin groups A, D, and G [19]. In conclusion, lichenysin A is very similar to surfactin, differing only by 1 Da in molecular mass, a consequence of the substitution of glutamic acid for glutamine in the first amino acid position. This small difference significantly modifies the physicochemical properties of lichenysin, notably the surface tension [7].

Unlike surfactin, lichenysin is synthesized during growth under an aerobic or anaerobic atmosphere. It is synthesized by lichenysin synthetase, a multiple enzyme complex, encoded by lichenysin operon lchA (26.6 Kb). The structure of lichenysin and its operon indicate a nonribosomal biosynthesis with the same multifunctional modular arrangement as observed in surfactin synthetase SrfA [16]. The nature of the peptide and fatty acids dictate the activity of BS, which can be tailor-made to have the desired attributes using engineered synthetases. The industrial production of environmentally friendly BS remains a pending subject, due to factors such as low-yield, high cost of raw materials, and inefficient purification processes [17, 21]. A reduction in production cost could be achieved by two approaches: i) the development of hyper-producer microbial strains and ii) the design of the production medium and optimization of the culture conditions with a highly efficient recovery process, or combining different strategies. Generally, *Bacillus* species co-produce various families of lipopeptides with different homologues and isoforms [21, 22]. When lichenysin production was qualitatively examined in 53 different *B. licheniformis* strains, all of them produced the same isoforms but in varying ratios. Rønning et al. [7] reported that lichenysin production is more dependent on growth conditions (physical or chemical) than genotypes. It was demonstrated by Coronel et al. [19] that environmental factors such as temperature, pH and aeration are very important for product yield. *B. licheniformis* strain AL1.1, a fast-growing thermophilic isolate with an optimal growth temperature of 65ºC, shows visible colonies in TSA after 3-4 h incubation; nevertheless, at this temperature BS production is inhibited, being optimum at 30-37ºC, when growth is much lower.

The nature of the carbon and nitrogen sources and other micronutrients can influence the amount of BS produced, as well as the cost of the

process. Pure carbon sources such as glucose, sucrose, and glycerol, and above all hydrophobic compounds such as n-alkanes, vegetal oils are used for BS production. When *B. lichenyformis* AL 1.1 was studied, neither growth nor BS production were obtained when oils were used as the carbon source, but carbohydrates or glycerol gave a remarkable BS production. In contrast, microorganisms such as *Rhodococcus erythropolis* [23] or *Sphingobacterium detergens* need n-alkanes, alone or in combination with carbohydrates, as a carbon source for BS production [24]. To improve BS yields and reduce the initial costs of raw materials, the use of local and cheap agro-industrial wastes is proposed. The use of residual substrates can have a double benefit, providing a solution to an environmental problem while allowing the development of a new product with added value. Various substrates, including frying oil, peanut oil cake, molasses, whey, sugarcane bagasse, potato peel and rice straw, have been tested for BS production [25, 26, 27, 28, 29, 30, 31]. *B. licheniformis* AL1.1 growth and lichenysin production are supported by cassava water, cassava starch and whey. Regarding the nitrogen source, pure compounds such as nitrate, ammonium salts and urea can be used since they are inexpensive [19, 32].

Downstream processing is an important step in biomolecule production processes, accounting for 50-80% of the total production cost. Its study and optimization is an important stage in the overall optimization process and constitutes an obstacle to a reasonable economical production [33]. When lichenysin is produced in a bulk medium, the first crucial step to obtain a highly pure compound is often acid precipitation, followed by solvent extraction. Alternative systems involve foam fractionation and membrane filtration, the choice depending on cost and effectiveness [34].

Response surface methodology (RSM), which includes factorial experimental design and regression analysis, is suitable for multifactor experiments, such as kinetics studies of microbial production, since it avoids having to consider one variable at a time. RSM with a central composite rotatable design (CCRD) constitutes a simple and economical method for designing experiments and evaluating the effect of factors and desirable responses. The production of $Lch_{AL1.1}$ using sugar-cane molasses was optimized using RSM. Molasses is a cheap substrate with a high content in sugars; its complex composition includes a variety of micronutrients, allowing the development of a medium that only requires

the addition of a low amount of nitrogen and phosphorous sources. To optimize the medium composition for $Lch_{AL1.1}$ production and bacterial growth, different concentrations of molasses, nitrate and phosphates were tested. The two variables examined were biomass formation (Equation 1) and $Lch_{AL1.1}$ production (Equation 2). The design matrix of the variables allowed the construction of an empirical second-order polynomial model for biomass and $Lch_{AL1.1}$ production [32]. The functional form of the models for the two response variables is:

Equation 1

$\mathbf{Y_1}$**=** $8.32-2.15\ x_1^2 + 2.03\ x_2 + 2.64\ x_1\ x_2 + 1.39\ x_1 - 0.59\ x_3^2 - 0.46\ x_2^2 - 0.48 x_2 x_3 - 0.13 x_3$

Equation 2

$\mathbf{Y_2}$**=** $3.14 - 0.78 x_1^2 - 0.50\ x_3^2 - 0.45 x_2^2 - + 0.33 x_3 + 0.13 x_1 - 0.17\ x_1 x_3 - 0.16 x_x x_3$

An F-test (ANOVA) was used to check the statistical significance of the second-order model equations. Table 1 shows the results of the ANOVA for both models. As can be seen, there is no significant lack of fit of the regression models. The results of Fisher's F test for the regression models were highly significant ($p<0.05$). Besides, the R^2 of the biomass polynomial model and $Lch_{AL1.1}$ polynomial model was calculated to be 0.998 and 0.97 [32], respectively, indicating that 99.8 and 97%, respectively, of the variability in the responses could be explained by the second-order polynomial prediction equations given above (Equations 1 and 2).

In Figure 1, contour plots show the effect of the concentrations of molasses (x_1), nitrate (x_2) and phosphates (x_3) on biomass production (Y_1) and $Lch_{AL1.1}$ accumulation (Y_2). The horizontal and vertical axes correspond to two significant factors for response variables x_1, x_2, and x_3, and the other axes are equal to response variables Y_1 and Y_2, respectively.

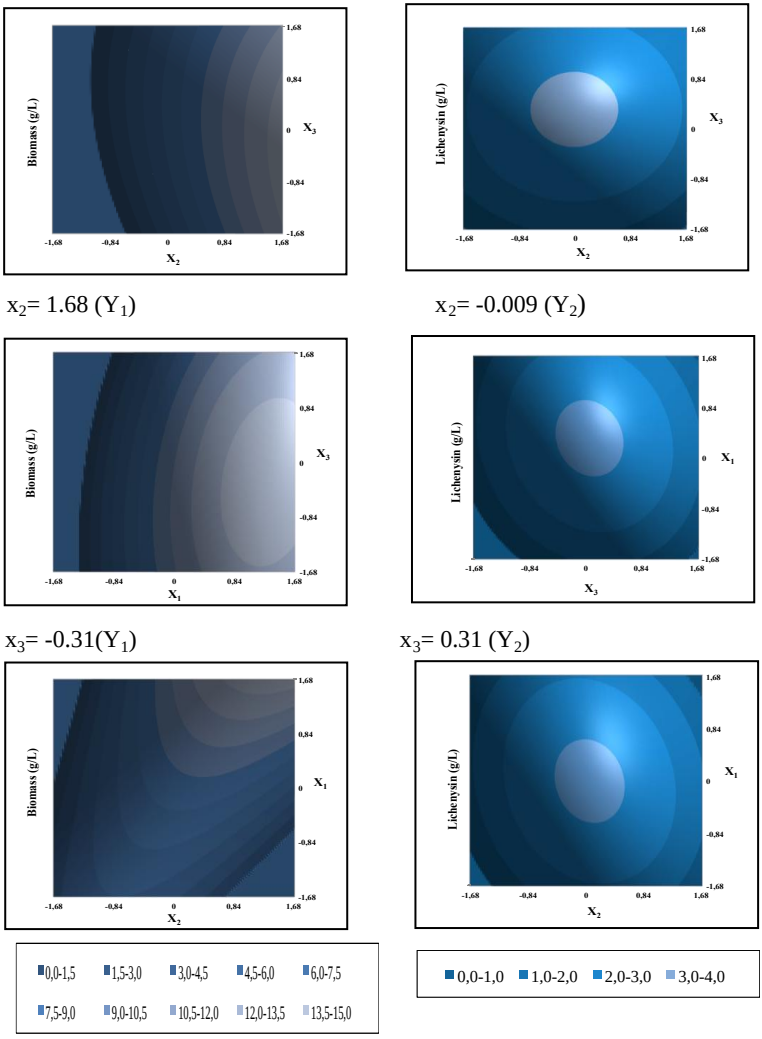
The optimum components for biomass production (g l^{-1}) were molasses 180.2, nitrate 12 and phosphate 7.5. The predicted maximum production value for biomass corresponding to these values was 14.5 g L^{-1} and the obtained production was 13.7 g L^{-1}, after 72 h of incubation, whereas in the initial non-optimized conditions, biomass production was found to be 3.5 g L^{-1}.

Table 1. Analysis of variance (ANOVA) for the nine-term equation for biomass (Y_1) production and for the seven-term equation 2 for $Lch_{AL1.1}$ production (Y_2).

Analysis of Variance for Biomass Production (Y_1)					
Source	Sum squares	Degrees freedom	Men square	F_0	Probability P(>F)
Regression	224.57	9	24.95	1025.8	$4.81e^{-17}$
Residual	0.316	13	0.243		
LOF error	0.147	5	0.029	1.39	0.323
Pure error	0.169	8	0.021		
Total	224.89	22			
Analysis of variance for $lichenysin_{AL1.1}$ production (Y_2)					
Regression	19.17	7	2.739	79.3	$1.04\ e^{-10}$
Residual	0.518	15	0.034		
LOF error	0.189	6	0.031	0.864	0.555
Pure error	0.329	9	0.036		
Total	19.69	22			

Thus, optimizing the medium composition using RSM increased the biomass yield 4-fold. Unlike the study of BS production, the concentrations of molasses and nitrate were the most important factors for bacterial growth, with high levels favoring biomass production. In contrast, the phosphate concentration had little influence. When *B. licheniformis* AL1.1 was grown under optimal production conditions, it was possible to enhance biomass from 3.5 g L^{-1} to 13.7 gL^{-1} . Previously published data on biomass production show that increasing the concentration of molasses from 10 to 100 g l^{-1} (1% to 10% (w/v)) leads to a gradual increase in biomass production for strains of *B. licheniformis* TR7 and *B. subtilis* SA9 [27]. This is consistent with the results obtained in our study, in which biomass production was favored by an increase in the molasses concentration up to the optimal value of 180 g L^{-1}, above which microbial growth declined. It is also important to note that at molasses concentrations greater than 107.8 g L^{-1}, $Lch_{AL1.1}$ production by *B. licheniformis* AL1.1 was inhibited.

a) x_1= 1.33 (Y_1) x_1= 0.0515 (Y_2)



b) x_2= 1.68 (Y_1) x_2= -0.009 (Y_2)

c) x_3= -0.31(Y_1) x_3= 0.31 (Y_2)

Figure 1. Contour plot graphs showing the effect of the molasses concentration (x_1), nitrate concentration (x_2) and phosphate concentration (x_3) at the optimum conditions for biomass (Y_1, left column) and lichenysin$_{AL1.1}$ production (Y_2, right column).

On the other hand, optimum components (g L^{-1}) for $Lch_{AL1.1}$ production (Y_2) were molasses 107.8, nitrate 6.5 and phosphate 9.7. The predicted and obtained maximum production of $Lch_{AL1.1}$ was 3.2 g L^{-1}, after 72 h of incubation, 4.5-fold higher than the initial production, 0.7 g L^{-1}. It is noteworthy that the production of $Lch_{AL1.1}$ (Y_2) was not affected by the concentration of sodium nitrate (x_2) added to the medium. This suggests that the nitrogen content of the raw material was enough to support bacterial growth (Y_1) and production. In contrast, phosphate (x_3) addition was crucial, being essential for growth and production, and its buffering effect was necessary for the BS yield [32].

The optimal concentration of molasses for lichenysin production varies with the microorganisms studied, being 4% for *B. licheniformis* TR7 and *B. subtilis* SA9 [29], 7% for *B. subtilis* and *Bacillus* HS3 20B [28], and 10% for *B. licheniformis* AL 1.1 [32]. This variation might be due to the molasses composition, which can depend on the cultivation conditions and treatment of the sugarcane. When glucose was used as a carbon source, the maximum $Lch_{AL1.1}$ production, which was linked with bacterial growth, was 0.86 g L^{-1} after 24 h incubation [19]. In contrast, when molasses were used as the carbon source, production peaked after 72h and was only partially associated with bacterial growth (Figure 2). Accumulation began during the exponential phase and continued after growth ceased. Under these conditions, the 3.2g L^{-1} obtained represented a remarkable increase over the initial production [32]. Similar results (3.3 g L^{-1} BS) have been reported for *B. licheniformis* TR7 when using a molasses medium [27].

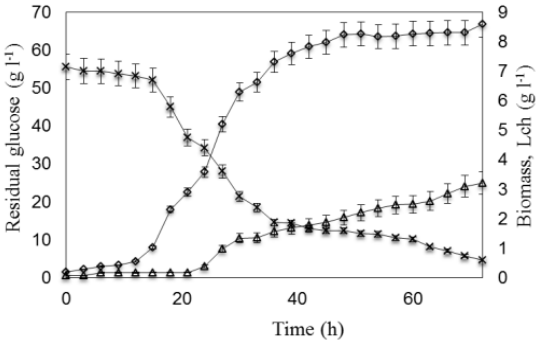


Figure 2. Time course of growth and $lichenysin_{AL1.1}$ production by *B. licheniformis.* (Δ) lichenysin; (◊) biomass; (x) residual glucose.

This work thus demonstrates the feasibility of using molasses as a component of a minimal mineral medium to produce BS (Table 2). This substrate has also been successfully used for the production of surfactin in *B. subtilis* B20 [35] and, diluted in tap water, in different strains of *Bacillus* [28]. Molasses, a by-product of the sugar industry with little commercial value, therefore has potential as a cost-saving tool, not only for the production of BS, but also for the development of other biotechnological processes, allowing the pursuit of sustainable development.

Table 2. Production data of the media used in the optimization experiments for $Lch_{AL1.1}$. Cellular yield of product formation ($Y_{P/X}$); volumetric productivity of product formation (P).

Parameter	Initial medium	Developed medium	
		Theoretical	Experimental
Lichenysin (g L^{-1})	0.73	3.20	3.20
Biomass (g L^{-1})	3.52	8.50	8.40
$Y_{p/x}$ (g g^{-1})	0.20	0.37	0.38
P (mgL^{-1} h^{-1})	10.13	44.3	44.4

Having confirmed the excellent qualities of molasses as a culture medium, and acquired knowledge about the effect of two other medium components, will be explored different strategies with the goal of fully exploiting the potential of this raw material, and if possible increasing $Lch_{AL1.1}$ production.

3. Physiological role of lichenysin

Many physiological roles are attributed to BS, which are produced by microorganisms living in a wide range of environments. Their most important function seems to be a capacity to produce emulsions to enhance the accessibility of non-water-soluble substrates. Yet the production of high surface-active compounds, like lichenysin by *B. licheniformis* AL 1.1, has been achieved with soluble nutrients, but not hydrophobic carbon sources such as n-alkanes and olive oil [19]. Further research is required to explain these results. Microbial surfactants also play an important role in the regulation of attachment-detachment of microorganisms from surfaces in natural environments. Adhesion is a physiological mechanism for growth and survival on abiotic surfaces or water-insoluble hydrocarbons

affecting bacterial transport. Other advantageous properties associated with BS are their antimicrobial activities associated with defense mechanisms and virulence factors. The advantages of BS over synthetic surfactants lie in their activity, specificity, versatility and biodegradability [13].

4. Physiochemical properties of lichenysin

Lipopeptides are characterized by high surface activity, an ability to effectively reduce surface tension, and a very low cmc. Surfactin produced by *B. subtilis* reduces surface tension of water to 27.9 - 29.5 mN m^{-1} with a cmc of 17 mg L^{-1} [36, 37], or 30 mg L^{-1} when using a molasses medium [27]. Lichenysin is more active, and has the capacity to lower the surface tension of water from 72 to 27- 28.5 mN m^{-1} [16, 19, 27, 38]. Acid precipitation of lichenysin B produces the lowest known interfacial tension against decane (0.006 mN m^{-1}) [16]. The cmc of $Lch_{AL1.1}$ is 12- 15 mg L^{-1}, while BL86 and lichenysin B have the lowest known cmc (10 mg L^{-1}) of any known surfactants under optimal conditions [16].

5. Biomedical and environmental applications of lichenysin

At the beginning of the XXI century, the world production of surfactants was 17 million metric tonnes, with an expected growth of 3-4% per year. Their most important application is in the cleaning industry (54%), followed by the textile, leather and paper industries. Only 10% of their usage is in pharmaceuticals and cosmetics, but recent studies have revealed interesting properties with potential new applications [1]. Research to find new products in order to develop new treatments has become a priority for the pharmaceutical industry. BS are considered relevant molecules with application in the treatment of many diseases. $Lch_{AL1.1}$ is stable under a wide pH range (6-11), high temperatures (up to 100ºC) and different salt concentrations (up to 20%), which are beneficial properties for exploitation in industrial and environmental processes [19], with potential applications in healthcare, cosmetics or food products with high added value.

Environment remediation. Oil remains a predominant source of energy and its transport causes accidents in marine environments. BS can be applied in environment bioremediation for oil dispersion and degradation after an accident at sea or for heavy metal mobilization after soil contamination. Saimmai et al. [27] reported that *B. lichenyformis* TR7 and

B. subtilis SA9 can enhance the solubility of polyaromatic hydrocarbons and therefore have the potential to remove oils from the environment. Surfactant production cost indicates that *in situ* production by microorganisms is a more economical strategy than the use of purified BS.

Antimicrobial activity. The use of BS as antimicrobial agents has been documented [39, 40, 41]. According to their structure, BS exert their toxicity on cell membrane permeability with a similar effect to that of detergents. The antimicrobial properties of Lichenysin A produced by *B. licheniformis* BAS50 and surfactin have been studied and compared. Surfactin is clearly more active against both Gram positive and Gram negative bacteria than lichenysin. A native form of lichenysin A presented antimicrobial activity against *Acinetobacter calcoaceticus*, *Alcaligenes eutrophus*, *Bacillus subtilis*, *Escherichia coli* and *Pseudomonas fluorescens* cells [42]. BS produced by *B. subtilis* SPB1 showed high antimicrobial activity against Gram negative cocci such as *Enterococcus faecalis* and *S. aureus*. These results are of interest, since these microorganisms are naturally resistant to many commonly used antibiotics. BS activity against Gram negative bacteria is lower. An important antifungal activity against *Penicillium notatum*, *Penicillium italicum*, *Aspergillus niger* and *Candida albicans* has also been observed [43]. Additionally, lichenysin has anti-inflammatory, antitumor and inmunosupressive properties, but its use is hampered by its hemolytic activity [44].

Emulsion capacity. Many promising new drug candidates, active components or food additives tend to have low water solubility, and consequently fail to enter industrial development processes. The incorporation of lipophilic compounds in O/W emulsions is an attractive solution to solubility problems. The capacity of $Lch_{AL1.1}$ to emulsify oils used in cosmetic preparations (isopropyl palmitate and myristate, octyldodecanol, cetearyl ethylhexaonate and caprylic triglycerides) has been demonstrated. The thermic resistance and stability of BS favors their application in industrial fabrication processes.

Biofilms. A biofilm is an organized ecosystem formed when microorganism growth is strongly adhered to a surface. The advantages of this ecosystem for the bacteria include more stability, synergism, and increased resistance to antibiotics and disinfectants. Biofilms may cause biodeterioration of materials and can act as a reservoir of contaminants with potential health problems. Among new approaches to the control of biofilm formation, BS application may be considered as a green strategy

because of their natural origin, simple production and biodegradability. The effect of surface pre- and post-treatment by $Lch_{AL1.1}$ on microbial adhesion has been studied by Coronel et al. [32]. When a polystyrene surface was covered with $Lch_{AL1.1}$, a decrease in microbial adhesion was observed in *Candida albicans* and *Staphylococcus aureus* (>60%). With *Escherichia coli*, *Yersinia enterocolytica*, *Listeria monocytogenes* and *Campylobacter jejuni*, an adhesion decrease of 40% was measured. The anionic nature of lichenysin may be responsible for the adhesion reduction in negatively charged surface microorganisms, due to forces of electrostatic repulsion. When the detergent effect of $Lch_{AL1.1}$ was studied, an adhesion decrease between 50-30% was observed. This result could be a consequence of BS penetration and absorption at the interface between the solid surface and the attached biofilm-forming bacteria, which reduced the interfacial tension and favored the bacterial detachment. According to these results, lichenysin could be an interesting alternative for controlling microbial biofilm growth on critical surfaces, including the protection of medical materials during use. Pathogen implantation in industrial and medical equipment or products is generally controlled by cleaning and disinfection procedures, but microorganisms possess a certain degree of resistance to the chemical-based products used [45].

Biomembranes. The molecular relationship established between $Lch_{AL1.1}$ and biomembranes has been explored in a recent interesting study. Hemolysis can be due to membrane permeabilization caused by pore formation or by disruption/solubilization of the membrane. In presence of human erythrocytes and $Lch_{AL1.1}$ at concentrations below its cmc, a slow process of hemolysis was developed. The release of K^+ before the hemoglobin leakage and hemolysis inhibition by PEG 3350 suggests that $Lch_{AL1.1}$ induced hemolysis by a colloid-osmotic mechanism, producing pores close to 34Å. These pores seem to be formed by clusters of lichenysin surrounded by phospholipids. Additionally, it was observed that the lipid membrane composition plays a role in the target membrane selectivity, since a high cholesterol ratio decreased the extent of leakage. The absence of cholesterol in bacterial membranes compared to eukaryotic membranes may be related with BS activity. The authors conclude that the presence of $Lch_{AL1.1}$ in the membrane increased the permeability to hydrophilic molecules, facilitating its flux across the lipid palisade [46]. Considering the interesting potential applications of BS in medicine as drug vehicles, as well as in the cosmetics and food industries, this study of BS hemolytic activity and behavior at membranes is of great importance.

6. Conclusion

$Lch_{AL1.1}$, the anionic BS produced by *B. licheniformis* AL1.1, has notable anti-adhesion activity, being able to prevent and eliminate biofilm formation by pathogenic strains. $Lch_{AL1.1}$ also produces colloid-osmotic hemolysis by pore induction and permeabilizes1-palmitoyl-2-oleoyl-*sn*-glycerol-3-hosphocholine (POPC) membranes to small-sized solutes, by the formation of lichenysin clusters surrounded by phospholipids. Notably, $Lch_{AL1.1}$ action is related with the presence of cholesterol, an important component of eukaryotic but not bacterial membranes. Optimizing the production of $Lch_{AL1.1}$ has confirmed that molasses can be regarded as a useful resource for biotechnological applications. The use of agro-industrial substrates has an important role in the sustainable and competitive development of several industrial sectors, as well as in industrial residues management. This new growth medium resulted in a 4-fold increase in production compared with the non-optimized medium. Nevertheless, despite their attractive properties for application in different fields, the commercial production of microbial surfactants such as lichenysin is still not a reality and more studies are necessary to explore their properties and disadvantages in more depth.

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