ANALYSIS OF SURFACTIN FROM *BACILLUS VELEZENSIS* H2O-1 IN MOLASSES MEDIUM

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ABSTRACT

Biosurfactants, natural compounds produced by microorganisms, lower surface tension between gases and liquids due to their amphiphilic nature, which includes both polar and nonpolar groups. Unlike synthetic petroleum-derived surfactants, biosurfactants offer ecological benefits such as low toxicity, high biodegradability, and the use of renewable resource. Surfactin, a prominent biosurfactant, demonstrates stability across varied physicochemical conditions, making it suitable for industrial and environmental applications. However, its widespread use is hindered by high production costs, prompting ongoing research into optimizing production processes and using cost-effective raw materials like industrial residues, to enhance sustainability in biotechnology. This study focused on surfactin production by *Bacillus velezensis* H2O-1 in a molasses medium, achieving surfactin concentrations of 220 mg/L in 72 hours. Surface tension measurements below 72 mN/m confirmed surfactin's efficacy. The critical micelle concentration (CMC) for surfactin in molasses medium was determined to be 5.11 mg/L, indicating high efficiency. Freeze and lyophilization methods maintained surfactin's surface tension properties over time, suggesting viable preservation methods for industrial applications.

Keywords: Surfactin. Molasses. Biosurfactants. *Bacillus velezensis* H2O-1.

1 INTRODUCTION

Biosurfactants are secondary metabolites produced by microorganisms that exhibit surfactant properties, reducing interfacial and surface tension between gases and liquids due to their amphiphilic nature, including polar and nonpolar groups. Unlike synthetic petroleum-derived surfactants, biosurfactants offer ecological advantages such as low environmental toxicity, high biodegradability, and using renewable substrates for their production[1]. There are different classes of biosurfactants distinguished by the hydrophilic group of the molecule, including glycolipids, phospholipids, fatty acids, polymeric surfactants, and lipopeptides, with the latter being one of the most extensively studied[2]. Among lipopeptides, surfactin is well-characterized and increasingly recognized for its diverse biotechnological applications[3].

Surfactin is an anionic lipopeptide composed of a heptapeptide ring and a fatty acid chain. Bacteria such as *Bacillus*, including species like *Bacillus subtilis*, *Bacillus amyloliquefaciens*, and *Bacillus velezensis*, are prominent producers of these biosurfactants. Surfactin is considered one of the most potent biosurfactants, capable of reducing water surface tension from 72 mN/m to 27 mN/m[4]. It exhibits multiple active properties including emulsification, interfacial and surface tension reduction, antimicrobial, antibiofilm, antiadhesive, and anticorrosive activities. Due to these characteristics, surfactin is a significant focus of industrial research and patent applications. It remains stable under various physicochemical conditions such as pH, temperature, and salinity, maintaining its biocidal and anti-biofilm activities even under boiling and autoclaving conditions[5;6]. In industrial settings, these molecules are potential substitutes for their chemical counterparts and find applications across environmental processes, cosmetics, food production, agriculture, pharmaceuticals, and primarily in petroleum industries[6].

To control biocorrosion, the oil industry employs chemical biocides and synthetic surfactants to inhibit the adhesion and metabolic activities of microorganisms associated with corrosion, thereby preventing the growth of these species[7]. However, biocide use is associated with environmental toxicity and the potential development of resistance mechanisms[8]. Chemical surfactants, meanwhile, are largely non-biodegradable and harmful to the environment and certain organisms[9]. Thus, biosurfactants like surfactin offer environmentally safer alternatives, potentially reducing reliance on synthetic surfactants and chemical biocides[6].

Certain strains that produce surfactin have been reported in the literature to have considerable potential as inhibitors of microorganisms that cause biocorrosion in production water and to prevent the formation of microbial biofilms. Previous studies demonstrated the positive antimicrobial activity of surfactin produced by *Bacillus velezensis* H2O-1 against sulfate-reducing bacteria[10]. Therefore, given the impact of sulfate-reducing bacteria on biocorrosion in the petroleum industry, surfactin from *B. velezensis* H2O-1 holds promise as an effective inhibitor of biocorrosive microorganisms in production water. Guimarães and colleagues (2019)[5] evaluated biofilm formation and consequent biocorrosion on carbon steel coupons conditioned with surfactin produced by *B. velezensis* H2O-1, exposed to production water in a bioreactor. Conditioned coupons showed minimal adhered cells and debris on the surface compared to unconditioned coupons, which exhibited structures consistent with mature biofilms and areas of corrosion. Importantly, corrosion areas observed in conditioned coupons were significantly smaller than those in unconditioned ones[5].

Despite their potential, biosurfactants are not widely used due to production costs. Current research focuses on optimizing production processes and utilizing cheaper raw materials such as industrial residues. The utilization of residual fractions of raw materials for synthesizing bioproducts like surfactin is crucial for reducing production costs and promoting sustainability in the biotechnological industry[11]. Large-scale production of biosurfactants faces challenges primarily due to high cultivation costs[12]. This study aims to address this limitation by exploring the substitution of glucose, a conventional carbon source in established culture media, with molasses. Molasses, a low-value industrial byproduct widely available in Brazil, was chosen for its abundant production[13]. Generally, biosurfactants like lipopeptides are produced as a mixture of homologs whose composition depends on

the substrate, strain, and cultivation conditions[14; 15]. Therefore, biosurfactants with varying amino acid/homolog compositions can exhibit diverse physicochemical properties[14; 16]. The objective is to evaluate the feasibility of this substitution strategy to reduce the production costs of surfactin. The surfactin used is produced by the *B. velezensis* H2O-1 strain, known for its excellent surfactin production capabilities[5; 17].

2 MATERIAL & METHODS

In the present study, the *B. velezensis* H2O-1 strain, kindly provided by the Molecular Genetics Laboratory - UFRJ, was used. As a comparison, the MM2 medium (%, w/v: glucose 1.0, NaCl 1.0, Na_2HPO_4 0.5, KH_2PO_4 0.2, $MgSO_4$ 0.02, $(NH_4)_2SO_4$ 0.2)[5] was used as the standard medium for surfactin production. To evaluate the use of a carbon source derived from agro-industrial residues, molasses (2.0% w/v) (referred to as Molasses Medium) was added as a substitute for glucose. The cryopreserved bacterium was reactivated on a Petri dish containing Luria Bertani medium for 24 hours at 30°C. Subsequently, the strain was inoculated into the pre-inoculum medium (standard medium supplemented with 0.05% w/v yeast extract). The sample was incubated with shaking at 30°C and 170 rpm for 14 hours. Then, an equivalent volume of 20 mg/L of cells from the pre-inoculum medium was added to the Molasses Medium for surfactin production. The sample was incubated with shaking at 30°C and 170 rpm for 24, 48 and 76 hours the duration required for each experiment. Following incubation, the *B. velezensis* H2O-1 culture was centrifuged to obtain the cell-free supernatant containing the surfactin produced. The obtained supernatant was autoclaved and stored under refrigeration until testing.

Surface tension reduction was measured using a goniometer, employing the pendant drop method. To determine the critical micelle concentration (CMC) of the surfactant present in the obtained supernatant, a series of serial dilutions (2, 5, 10, 20, 50, 100, 200, 500, and 1000 times) were performed. The dilutions were evaluated using the goniometer to calculate the CMC value (mg/L). Additionally, to quantify the surfactin present in the supernatant, an acid precipitation and a liquid-liquid extraction were performed[18]. The extracted surfactin was then quantified by HPLC[17]. Finally, to assess the stability of the surfactin produced in Molasses Medium against conservation methods, the supernatant was frozen and lyophilized. Subsequent measurements of surface tension were taken periodically.

3 RESULTS & DISCUSSION

To prove that *B. velezensis* H2O-1 can produce biosurfactants in the molasses medium, a surface tension measurement was performed. The surface tension data obtained from the goniometer allow for a comparative evaluation of the surface tensions of the surfactin-containing supernatants obtained from the cultivation of *B. velezensis* H2O-1 in conventional and alternative medium at different periods. Surfactin was evaluated after 24, 48, and 72 hours of cultivation (Figure 1). In all assays, all samples showed surface tension values lower than ultrapure water (≅ 72 mN/m). In the case of the supernatant obtained in molasses medium, the lowest surface tension values were recorded at 24 and 72 hours, both averaging around 26.7 mN/m. In comparison with the conventional medium, the lowest surface tension values were observed at 72 hours, averaging 24.3 mN/m. The surface tension values between the biosurfactant produced in the standard medium and the molasses medium were similar, showing no significant difference.

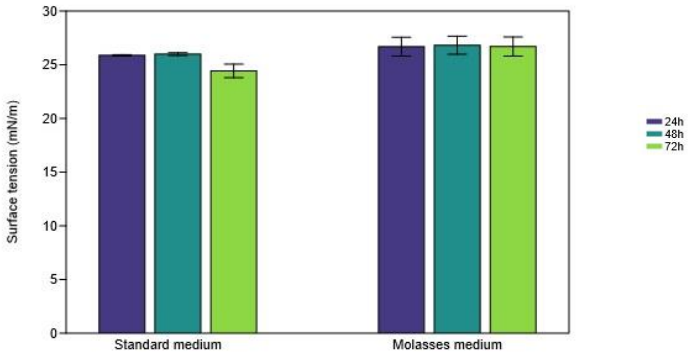

Figure 1 - Surface tension values of surfactin produced by *B. velezensis* H2O-1 in molasses and standard media at different cultivation times.

Upon confirming the presence of biosurfactant in the molasses medium culture supernatant, lipopeptide extraction was performed. Subsequently, surfactin in the cell-free supernatant was identified and quantified using HPLC. Based on the chromatographic profile observed in the chromatogram resulting from the surfactin standard run, the presence of the surfactant was confirmed. *B. velezensis* H2O-1 produced 220 mg/L of surfactin in 72 hours of cultivation in the molasses medium. In comparison, the optimized standard medium supernatant contained 294.26 mg/L of the surfactant. Although the surfactin concentration in the molasses medium was approximately 33% lower than in the standard medium, the result was considered satisfactory. It is worth noting that the molasses medium is not optimized for surfactin production, and potentially, with an optimization process, this concentration could increase. This finding suggests that molasses is a low-cost carbon source that promotes the production of this bacterial surfactant. Furthermore, the results align with the surface tension values considered good in the literature, observed, for example, in other *Bacillus* species producing surfactin[5; 10; 15].

A key factor in determining the efficiency of a surfactant is the CMC. The lower the CMC value of a surfactant, the higher its efficiency, as a lower concentration of surfactant will be needed to reduce surface tension. Therefore, the CMC of the surfactin produced by *B. velezensis* H2O-1 in a molasses medium was evaluated. The supernatant from the molasses medium achieved a CMC of 5.11 mg/L. Comparatively, the CMC of the supernatant from the standard medium was 6.52 mg/L.

To evaluate the stability of surfactin, the supernatant obtained from the molasses medium was subjected to different preservation methods: freezing and lyophilization (Figure 2). The supernatant from the molasses medium was frozen, and the surface tension was measured periodically (between 0 and 134 days). During the evaluated period, no significant difference was observed between the initial value (day 0) and the other days (mean value: 27,02 ± 1,1 mN/m) (Figure 2. A). When the supernatant was lyophilized, no significant change in surface tension was observed after 7 days (28.4±0.4 mN/m), compared to the value observed immediately after the lyophilization process (28.18±0.7 mN/m) (Figure 2. B). It will be necessary to measure the surface tension after a longer period to see if the activity of surfactin is maintained. However, these preservation methods for surfactin

produced in a molasses medium appear promising. This preservation method could reduce the cost of storing the molecule for applications that do not require purified surfactin, such as the application of the surfactant for the removal of microbial biofilms in pipelines during the oil extraction process.

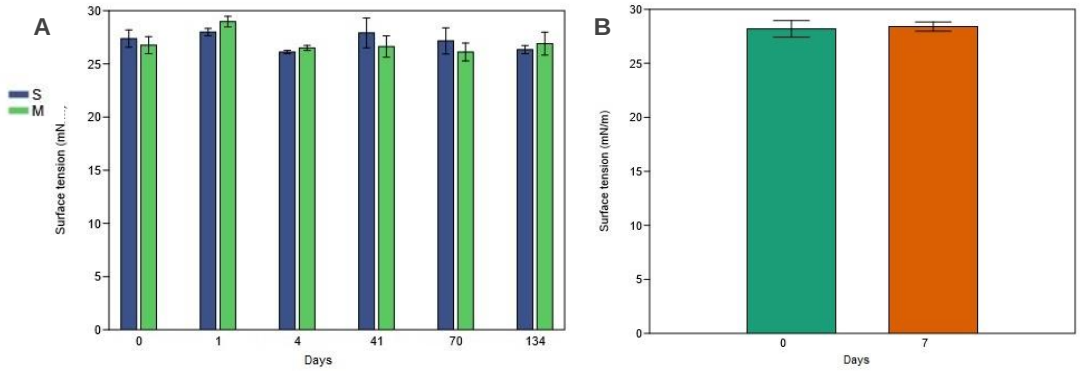

Figure 2 - Surface tension (ST) values of supernatants obtained after different periods of freezing and lyophilization. A: ST of supernatants from molasses and standard media after 138 hours of freezing. B: ST of lyophilized supernatant from molasses medium.

4 CONCLUSION

The use of alternative sources for surfactin production appears very promising, not hindering the synthesis of surfactin by *B. velezensis* H2O-1. Reducing the cost of the cultivation medium is crucial for achieving economically competitive values for large-scale surfactin production. Another essential aspect of the project is the stability of the synthesized bioproduct. It was observed that freezing the surfactin maintains or slightly reduces its physicochemical properties. Further tests will be conducted to study the efficiency of surfactin in reducing tension after longer periods of freezing and lyophilization. Additionally, it will be necessary to explore potential optimizations in the alternative cultivation medium to improve surfactin production, as well as perform a cost analysis to evaluate the economic feasibility of this production. Finally, the anti-adhesive, biocidal, and anticorrosive action of surfactin produced in a molasses medium will be evaluated. For this, tests will be conducted in bioreactors using production water from oil wells.

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