ENHANCING SURFACTIN PRODUCTION BY *Bacillus subtilis* ATCC 6633 THROUGH HYDROPHILIC GROWTH INDUCERS

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ABSTRACT

The production of biocompounds, such as biosurfactants, is directly influenced by the availability of nutrients and energetic metabolic pathways. While the primary carbon source supports cellular growth and enzyme synthesis, micronutrients - e.g. hydrophilic inducers - act as enzyme cofactors, aiding in cellular growth and thereby facilitating biosurfactant production. Considering the gap in available data on how hydrophilic inducers impact both biosurfactant productivity and chemical structure, this study investigated the use of cassava wastewater as an alternative culture medium, enriched with hydrophilic inducers - $FeSO_4$, $MnSO_4$, and $MgSO_4$, at concentrations of 2.0, 0.2, and 0.8 mM, respectively -, to enhance surfactin production by *Bacillus subtilis*. The results indicated that the tested salts were promising, significantly reducing surface tension from 72 $mN.m^{-1}$ to 28.36, 29.86, and 29.92 $mN.m^{-1}$, respectively.

Keywords: Biosurfactant inducers. Hydrophilic inducers. Cassava wastewater. Surfactin.

1 INTRODUCTION

Bacteria, when subjected to adverse conditions, increase interactions among themselves to create a favorable environment. This phenomenon is known as quorum sensing and explains why biosurfactants are produced, even though it requires high levels of resources and energy.[1,2] Surfactin, produced by *Bacillus subtilis*, is among the most well-known biosurfactants. Its widespread use in various industries is attributed to its multifaceted properties, including antifungal, antiviral, and biostimulant characteristics.[3] While the production method is well-established, current studies are mainly focused on cost reduction, using low-cost culture media (such as cassava wastewater), and on increasing productivity.[4,5] Biosurfactant inducers, whether hydrophilic (such as metals) or hydrophobic (such as vegetable oils), play a crucial role in increasing the productivity of these compounds. They assist in microbial development, contributing to the production of biosurfactants with desirable characteristics such as biodegradability, low toxicity, and stability under different conditions.[6] The nature of the inducers influences their function: hydrophilic ones act as cofactors for microbial growth, while hydrophobic ones are involved in the synthesis of hydrocarbons, modifying the structure and applications of biosurfactants.[7,8,9] Until now, hydrophilic inducers have been essentially treated as supplementary carbon sources. However, it's becoming increasingly evident that these compounds hold significant potential for technological implementation. This is underscored by their ability to ensure a 3.6-fold increase in biomass production through the addition of iron, manganese, and magnesium salts.[10] Given the essential need to focus studies on bacterial metabolism to optimize the process in question and make it commercially more viable, the objective of this work is to evaluate the production of surfactin by *Bacillus subtilis* ATTCC 6633 using cassava wastewater as an alternative, low-cost, residual culture medium supplemented with hydrophilic growth inducers (ferrous, manganese, and magnesium sulfates) at different concentrations.

2 MATERIAL & METHODS

As the objective of the study was to evaluate the impact of inducers on surfactin productivity, $FeSO_4$, $MnSO_4$, and $MgSO_4$ were chosen, at concentrations of 2.0, 0.2, and 0.8 mM, respectively. The selection of these hydrophilic inducers was guided by previous studies [11,12.13], which highlighted iron, manganese, and magnesium as crucial micronutrients for both - cell growth and surfactin production.

The growth kinetics of *Bacillus subtilis* and the resulting surfactin production in a medium containing cassava wastewater and inducers were assessed by monitoring cell growth and the reduction in surface tension. Samples were collected periodically, every 4 hours for the initial 12 hours, and subsequently every 12 hours, over a total fermentation period of 72 hours, resulting in 18 collection points. As shown in Figure 1.

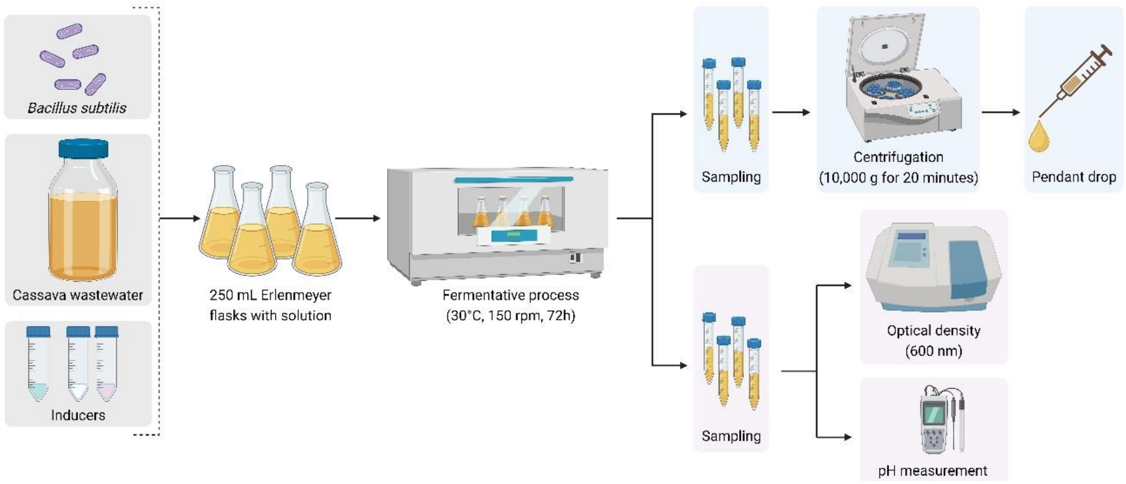

Figure 1 Methodology applied in current work.

3 RESULTS & DISCUSSION

During the fermentation process of the bacterial solutions, a noticeable change in color occurred, as seen in Figure 2.

a) b)

Figure 2 Bacterial solutions during fermentation. a) initial time (0h) and b) final time (72h). From left to right: solutions with $MgSO_4$, $MnSO_4$, $FeSO_4$, standard and blank.

Initially, the solutions exhibited the characteristic color of cassava wastewater (orange yellow), except for the solution containing ferrous sulfate, which appeared darker. Over time, all inoculum solutions clarified, due to the consumption of nutrients from the medium and the subsequent production of compounds.

To analyze cell growth, measurements were taken using a spectrophotometer (wavelength of 600 nm) and a pH meter. The values obtained were used to construct graphs over time, as shown in Figure 3.

a) b)

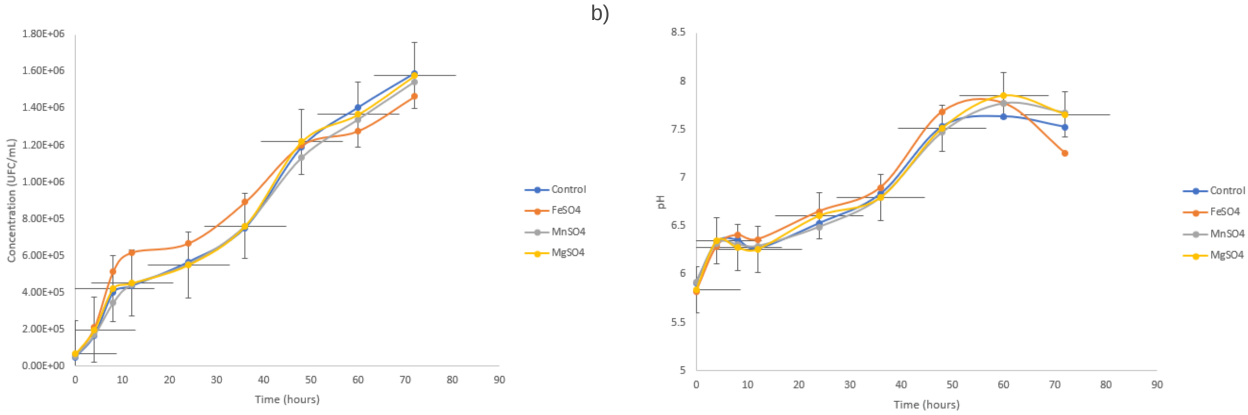

Figure 3 a) Bacterial growth and b) pH variation, in medium of cassava wastewater in the presence of hydrophilic inducers.

A good cell growth was observed across all solutions. Initially, the sample containing $FeSO_4$ exhibited high growth, exceeding the standard sample by 41% within the first 12 hours of fermentation. Following closely were the samples containing $MgSO_4$ and $MnSO_4$, showing 4% and 2%, respectively, more cells than the standard sample. By the end of the batch process, the sample supplemented with ferrous sulfate only reached $1.46x10^6$ CFU.mL^{-1}, whereas the other three samples approached concentrations nearing $1.59x10^6$ CFU.mL^{-1}. These results align with the notion that bacteria initially utilize micronutrients from the medium to grow and produce the polar portion of the biosurfactant.

Over time, a gradual increase in pH was observed across all solutions, with values ranging from approximately 5.9 to 7.6. Probably, the consumption of sugar from the environment rendered proteins targets of the cells, causing the release of alkaline compounds and, consequently, an increase in pH.

In addition, the hanging drop test, conducted using a goniometer, analyzed the shape of solution droplets. The equipment was initially tested with distilled water, boasting a surface tension of approximately 72 mN.m^{-1}. Employing a micro syringe, droplets were formed, and their shapes captured with a camera. Surface tension was then determined by applying the Young-Laplace equation to the droplet's morphology. The results indicated that the biosurfactant was produced due to the notable reduction in water tension from 72.31 mN.m^{-1} to 28.36, 29.86, and 29.92 mN.m^{-1} with the addition of hydrophilic inducers $FeSO_4$, $MnSO_4$, and $MgSO_4$, respectively.

4 CONCLUSION

Promising results were observed in reducing surface tension by the addition of hydrophilic inducers ($FeSO_4$, $MnSO_4$, and $MgSO_4$), with emphasis on ferrous sulfate, which not only achieved the most significant surface tension reduction (28.36 mN.m^{-1}), but also guaranteed the greatest cell growth in the first 48h of fermentation ($1.20x10^6$ CFU.mL^{-1}). Thus, hydrophilic inducers have proven to be excellent options for enhancing microbial biosurfactant productivity. To ensure the best conditions for growth and surfactin production, more tests must be carried out, exploring combinations of hydrophilic inducers and their correlation with hydrophobic inducers. In addition, analyses like HPLC, for quantifying surfactin production, and MALDI-TOF, for obtaining comprehensive structural insights throughout the process and its variations due to the addition of hydrophilic compounds, are recommended.

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