VALORIZATION OF CASSAVA WASTEWATER FOR THE BIOSYNTHESIS OF SURFACTIN USING HYDROPHOBIC INDUCERS

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ABSTRACT

Biosurfactants, valuable in health and environmental applications, are hindered by high production costs. In order to reduce the production costs, it is possible to replace the synthetic media by agro-industrial residues and hydrophobic inducers. Hydrophobic inducer (HI) enhances biosurfactant productivity by promoting microbial growth and activating production metabolism. Inducers such as soybean oil and fatty acids affect surfactin production by *Bacillus subtilis* ATCC6633. Nevertheless, their specific impacts on biosurfactant structure and production are unexplored. This study explores various concentrations (1, 2, 5, and 10%) of HI (soybean oil, palmitic, and oleic acids) using cassava as a carbon source in submerged fermentation. Parameters analyzed included bacterial growth, pH, surface tension (ST), sugar consumption, and surfactin production effects. Results showed that 5% palmitic acid yielded the highest surfactin concentration (≈1.3 g/L), reducing ST to approximately 26.5 mN/m, and oleic acid led to diverse surfactin homologues, suggesting potential for novel biological activities. The analysis of MALDI-TOF reveals clusters with m/z ≈1003-1134 corresponding to surfactin with fatty acid side chains (C13-C16). Suggesting that the inducers have effectively stimulated the production of novel surfactin homologues. This study corroborates that the strategic application of specific inducers can substantially influence both the yield and characteristics of surfactin.

Keywords: Lipopeptides. Palmitic acid. Oleic acid. biosurfactant inducers.

1 INTRODUCTION

Surfactin is a notable biosurfactant primarily produced by various *Bacillus* species, including, *B. licheniformis*, *B. amyloliquefaciens*, and especially *B. subtilis.* This compound is celebrated for its wide range of biological activities - anti-inflammatory, antifungal, antiviral, and as a biostimulant - coupled with outstanding surfactant qualities[1]. These features spotlight its potential across diverse sectors, including environmental, agriculture, oil, and pharmaceutical industries. However, the economic feasibility of surfactin production is hindered by high costs associated with its raw materials - glucose, sucrose, and mineral salts - which account for over half of the total cost of biosurfactant production. As a result, there's a growing imperative in biosurfactant research to adopt methodologies that are both high-yield and cost-effective[2].

An innovative strategy to curb the production expenses of surfactin involves leveraging agro-industrial by-products, such as the effluents from cassava flour manufacturing. The cassava industry produces significant amounts of waste, roughly 0.65 kg of solids and 25.3 liters of effluent per kilogram of cassava processed. Acchar & da Silva (2021) point out that beyond its environmental pollution implications, the toxicity associated with cassava residues - mainly due to cyanogenic compounds - can pose health risks to both humans and animals. Hence, devising eco-friendly and cost-efficient methods for either treating or repurposing cassava wastewater is crucial. Nitschke and Pastore (2006) identified cassava wastewater (CW) as a rich carbon source and essential nutrients, advantageous for surfactin production. Their research demonstrated the successful use of CW to produce surfactin, achieving yields of about 3.0 g of crude surfactin per liter using *B. subtilis* strains. Similarly, Andrade et al. (2016) reported producing approximately 1.01 g/L of pure surfactin using *B. subtilis* LB5a and cassava wastewater. Despite challenges such as substrate standardization, storage, transport, and purification costs, it's feasible to establish controlled surfactin production processes with defined chemical properties by incorporating hydrophobic inducers.

At the biochemical level, the addition of inducers to the culture medium can significantly enhance biosurfactant productivity by promoting microbial growth and stimulating the biosurfactant producing metabolism. These inducers, typically hydrophobic molecules like those found in soy and olive oil (including a variety of saturated and unsaturated fatty acids, proteins, and vitamins), can affect the surfactant's molecular size, leading to improved stability and efficiency. However, detailed information on the impact of specific molecules, such as oleic and palmitic acids, on the production and chemical structure of surfactin remains scarce. This study is the first examination of soybean oil and these fatty acids as hydrophobic inducers for surfactin production by *Bacillus subtilis* ATCC 6633, utilizing CW as the growth medium. This innovative approach marks a significant advancement in the field, offering a dual benefit of waste valorization and enhanced biosurfactant production.

2 MATERIAL & METHODS

Approximately 50 L of effluents from cassava flour processing were collected at a flour industry (Santa Catarina - Brazil) and transported under refrigeration. The CW was boiled at 100 °C for 3 min to facilitate the removal of insoluble solid material and then centrifuged. The natural pH of the medium was 5.8 and was not adjusted. The substrate was characterized by the analysis of mineral fraction by inductively coupled plasma optical emission spectrometry, and the quantification of major sugars was performed by high-performance liquid chromatography.
The same batch of CW was used for all experiments. The fermentative assays were performed in 250 mL Erlenmeyer flasks containing 150 mL of culture medium composed of CW supplemented with different concentrations (1, 2, 5, and 10% (w/v)) of inducers (palmitic acid (PA), oleic acid (OA), and soybean oil (SO), separately) and previously sterilized at 120 °C, 1 atm for 20 min. The kinetic fermentation profile was assessed over 72 h (inoculum 7%, 0.5 absorbance, OD600 nm). Sugar consumption, pH variation, biomass, and the reduction of ST of the medium were monitored during the fermentation period. At the end of cultivation, the samples were centrifuged to separate biomass. The pH of the supernatant was adjusted to 2 using HCl solution (3 and 1 M) and maintained under decantation (5 °C for 24 h). After this period, the precipitate was centrifuged, neutralized (pH 7) with a NaOH solution (3 and 1 M), and lyophilized. The solid obtained was termed crude surfactin, and its final yield was analyzed. To quantify surfactin production, 20 mg of the sample was diluted in 1 mL methanol, filtered, diluted at a 1:5 ratio, and subjected to HPLC and MALDI-TOF analysis.

3 RESULTS & DISCUSSION

It was concluded that the culture medium composed of CW and hydrophobic inducers increased surfactin production by *B. subtilis* ATCC 6633. For the first time, fatty acids were used in their pure form for surfactin production. The surfactin produced by the inducers reduced the ST (≈26.5 mN/m) (Figure 1A) and achieved a maximum yield of ≈1.3 ± 0.08 g/L (5% PA) (Figure 1B). The analysis of the results from all fermentations using the inducers SO, PA, and OA in all concentrations indicated the possible formation of different surfactin homologs, increasing their yields by up to ≈70, 17, and 98%, respectively. There was an observed increase (≈2 times) in biosurfactant production. No significant changes in pH were observed. Surface tension decreased by 40% in 12 h, indicating surfactin production.

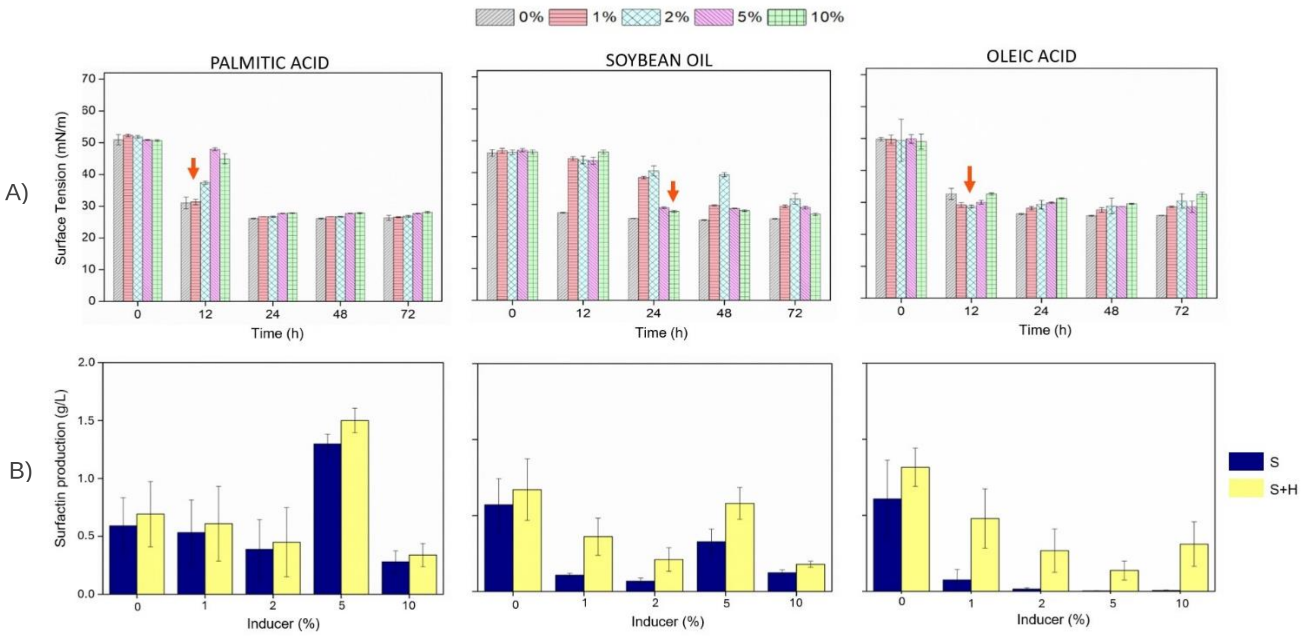

Figure 1 Surface tension (**A**), surfactin (S) produced in this study (**B**) using palmitic acid, soybean oil, and oleic acid as hydrophobic inducers (S+H) in different concentrations (1, 2, 3, 5 e 10%).

The consumption of soluble sugars was almost total for all study conditions, and oleic acid presented the greatest diversity of homologues (Figure 2).

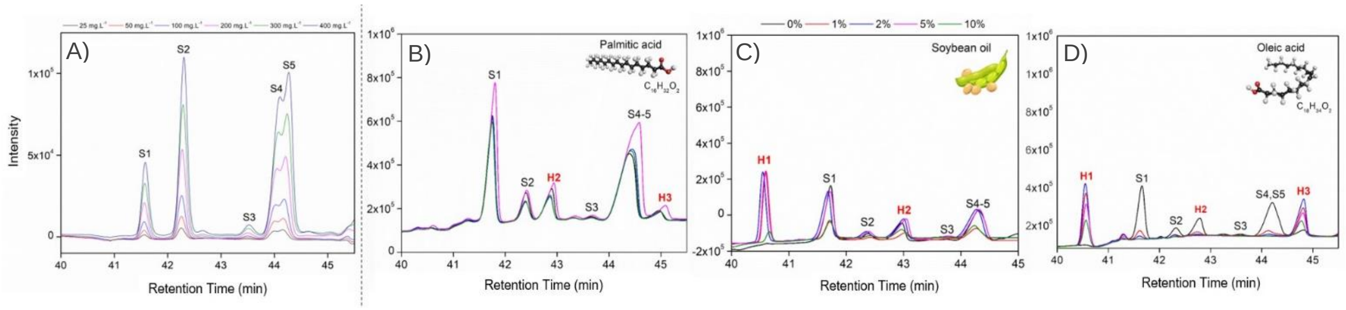

Figure 2 Chromatogram of surfactin standard (A), surfactin produced in this study using palmitic acid (B), soybean oil (C), and oleic acid as hydrophobic inducers (D).

Surfactin production was confirmed through MALDI-TOF MS analysis, revealing various clusters with m/z ≈1003-1134 in the tested cultures. These peaks correspond to surfactins with fatty acid side chains (C13-C16). No evidence of surfactin C16 was found in the Soybean oil-induced culture. Common contaminant peaks were identified, suggesting the presence of acyclic surfactins and ithurins. Despite the relative excess of some fatty acids, the side chain distribution indicates the inducers' complex biological role. Theoretical studies predicted the remaining macrocyclic amino acids in surfactin, with the most common being tyrosine (22%), tryptophan (15%), histidine (13%), arginine (12%), and phenylalanine (9%). Supplementation with these amino acids may be beneficial, as observed in previous studies with *Bacillus* spp [6]. These results refer to the valorization of agro-industrial residues, such as cassava, through new production strategies. Other suggestions to improve surfactin production include using hydrophilic inducers associated with hydrophobic inducers. However, further investigations should elucidate their correlation with the metabolic pathways of the biosurfactant.

4 CONCLUSION

The experimental results demonstrate that the culture medium formulation, incorporating CW with hydrophobic inducers, significantly enhanced the biosynthesis of surfactin by *B. subtilis* ATCC 6633. Notably, the introduction of fatty acids in their pure form as a substrate resulted in a marked reduction of surface tension to approximately 26.5 mN/m, achieving a maximum yield of about 1.3±0.08 g/L. Analysis via MALDI-TOF MS confirmed the production of surfactin, identifying peaks with m/z ranging from 1003-1134, corresponding to surfactin with fatty acid side chains (C13-C16). These findings provide a robust theoretical basis for the development of new production strategies that valorize agro-industrial residues, thereby contributing to the circular economy in cassava processing industries. considering the wide applicability of this biosurfactant.

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ACKNOWLEDGEMENTS

The authors acknowledge CAPES-PRINT, Project Number 88887.310560/2018-00, and FAPESC (Fundação de Amparo à Pesquisa e Inovação do Estado de Santa Catarina) PRONEM (Programa de Apoio a Núcleos Emergentes) 2020TR731 for the financial support and CAPES PROEX (Coordenação de Aperfeiçoamento Pessoal de Nível Superior-Programa de Excelência Acadêmica) for scholarship (Project Number 88887.388261/2019-00).