IMPACT OF GROWTH MEDIA SUPPLEMENTATION ON SURFACTIN PRODUCTION BY *Bacillus subtilis*

Fabiane O. Barban[1*], Vitória F. B. Zocca[1] & Danielle B. Pedrolli[1]

[1] *Bioprocess and Biotechnology Engineering/ Department of Bioprocess Engineering and Biotechnology, Faculty of Pharmaceutical Sciences/ Unesp, Araraquara, São Paulo, Brazil.*

**fabiane.barban@unesp.br*

ABSTRACT

One of the major challenges in current research is to find methods for producing biomolecules with minimal environmental impact, and Synthetic Biology can serve as an ally in this issue. Some of these molecules are cyclic lipopeptides, which have high industrial interest due to the various applications of their surfactant properties. Among them, surfactin is noteworthy, as it can be naturally produced by *Bacillus* species, although with a low yield. To address this limitation, it is possible to optimize the metabolic state of the bacterium by supplementing the fermentation media with amino acids and employing the Synthetic Biology toolbox to promote the overproduction of surfactin in a manner that is both green, environmentally sustainable, high-yielding, and faster. For this purpose, this study proposed a series of Bacillus subtilis cultures in diverse media supplemented with amino acids to evaluate the impact of this supplementation on surfactin production. The results were positive for supplementation with leucine and glutamine, reaching 5 and 4.2 g/L of surfactin respectively, while the negative control remained between 3 and 4 g/L of surfactin. Furthermore, these two supplements also had a positive impact on microbial growth. The other supplementations with different amino acids did not show positive results, resulting in production and growth equal to or lower than the control. Therefore, it is possible to conclude that there are gaps in the production of these amino acids for surfactin biosynthesis, which can be corrected using genomic tools.

Keywords: Surfactin 1. Biosynthesis 2. *Bacillus* 3.

1 INTRODUCTION

Bacillus subtilis is a Gram-positive bacterium belonging to the Bacillaceae family, *Bacillus* genus, and Eubacteria order widely recognized for its ability to produce various molecules at industrial scale such as enzymes (amylases, proteases, and laccases) and vitamins like riboflavin (vitamin B_2). This microorganism has become the focus of recent studies due to its ability to produce different types of cyclic lipopeptides such as fengycins, iturins, polymyxins, and surfactins, albeit with low yields [1].

The applicability of these biosurfactants depends on their isoform, as the physicochemical properties also vary with structure. Surfactins have a wide range of applications [2], ensuring their use in pharmaceutical industries as antitumor, antiviral, and antimicrobial agents [3], besides presenting apoptotic, hemolytic, and anti-adhesive activities [4]. From an environmental perspective, surfactins can be applied in processes such as bioremediation and the production of biopesticides and fertilizers. Finally, this molecule can also function in the cosmetics and food industries as an emulsion component [1].

The synthesis of surfactin by *Bacillus* cells occurs through non-ribosomal pathways and requires different amino acids and fatty acids as precursor molecules. As these molecules are needed in many other pathways, their availability may be a bottleneck for the high production of surfactins. Microbial biosynthesis offers numerous advantages such as fast production and low costs [5], moreover presenting lower toxicity to the environment when compared to synthetic molecules [4]. Thus, it is evident that the production of biologically derived surfactin is advantageous in various aspects and that it is possible to identify gaps in this synthesis for subsequent improvement using synthetic biology tools. Considering this, this study aimed to identify bottlenecks in the amino acids supply for surfactin biosynthesis through medium supplementation with the different amino acids that constitute the surfactin structure.

2 MATERIAL & METHODS

The tests were conducted using cultures of a *B. subtilis* under identical conditions in triplicates: in test tubes with a final volume of 5 mL, with an initial optical density (OD_{600}) of 0.05, and incubated at 37°C and 220 rpm. Inoculum was prepared after two pre-cultures of 18 h each, the first in Luria Bertani (LB) medium and the second in PW fermentation media [6]. Samples were taken at 24 and 42 h for OD_{600} and surfactin quantification. OD_{600} quantification was carried out at 600 nm in a microplate reader using a transparent 96-well plate. Additionally, surfactin quantification was carried out using the colorimetric CPC-BTB method [6], where the presence of surfactin generates a chromatic response detected at 600 nm.

The PW (high sugar base medium) was formulated without and with amino acid supplementation. Supplementation was carried out with the amino acids that form the peptide part in the surfactin structure: leucine, valine, glutamate, and valine. Additionally, glutamine was tested due to its high conversion rate to glutamate in the cell. All these amino acids were added to the medium at

a concentration of 0.1% (m/v), except for aspartate, used in the concentration of 0.01% (m/v) due to its low solubility in water. All supplementations were carried out separately for each amino acid.

3 RESULTS & DISCUSSION

Supplementation with valine reduced surfactin production, reaching approximately 3 g/L, while in the control (non-supplemented medium - PW), surfactin production reached approximately 4 g/L (Fig. 1). Growth was also poorer, reaching an OD_{600} of 3, whereas in the control (PW) it reached approximately 3.7 absorbance unite (AU). Supplementation with aspartate caused a loss in growth and surfactin production, resulting in a maximum OD_{600} of 2.7 AU and approximately 2.7 g/L of surfactin. Glutamine, glutamate, and leucine supplementation resulted in higher surfactin production, reaching approximately 5 g/L, 4 g/L, and 4.2 g/L respectively. Media supplemented with leucine and glutamine resulted in the best growth performance, reaching a maximum OD_{600} close to 4 AU. Noteworthy, the growth curve for glutamine continued to rise after 24 hours, a behavior not observed before. Finally, growth with glutamate did not exceed the control, reaching a maximum OD_{600} of 3 AU.

The results indicate that supplementation with 0.01% (m/v) leucine and glutamine had a positive effect on growth, and glutamine had also a positive effect on surfactin production. This suggests a metabolic deficit in the bacteria and a potential target for genetic modification of metabolic pathways.

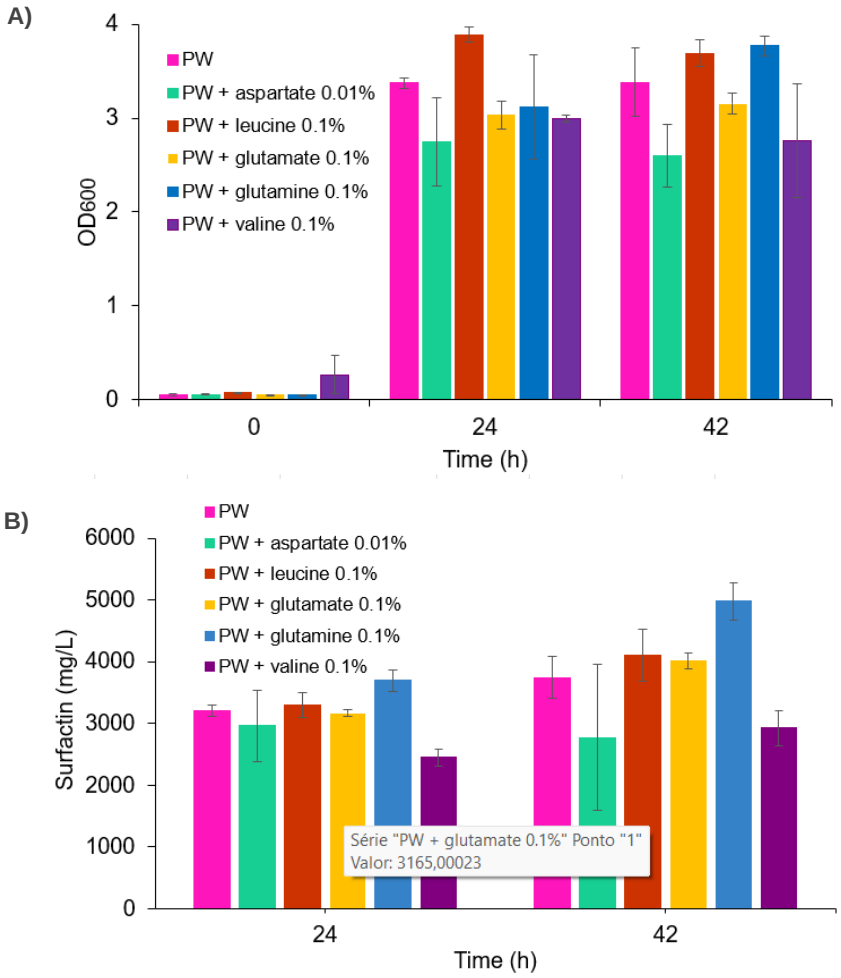

Figure 1. Groth and surfactin production in culture media supplemented with amino acids. A) Population growth as a function of time (hours) measured as optical density at 600 nm. B) Surfactin production (mg/L) as a function of time (hours).

4 CONCLUSION

After analyzing the supplementation of the culture medium with amino acids, we concluded that there were positive results for leucine and glutamine. In particular, glutamine may be a limiting precursor in surfactin biosynthesis in the conditions tested. Thus, the biosynthesis of both amino acids is a potential target for genetic modification, which could be achieved through the construction of vectors for the overexpression of these amino acids, eliminating the need for medium supplementation to improve surfactin production.

REFERENCES

[1] GEISSLER, M., HERAVI, K. M., HENKEL, M., & HAUSMANN, R. (2019). Lipopeptide Biosurfactants From Bacillus Species. In Biobased Surfactants: Synthesis, Properties, and Applications (pp. 205–240). Elsevier. https://doi.org/10.1016/B978-0-12-812705-6.00006-X.

[2] ARIMA, K.et al. Surfactin, a crystalline peptide lipid surfactant produced by Bacillus subtilis: isolation, characterization and its inhibition of fibrin clot formation. Biochem. Biophys. Res. Commun, v.31,p.488–494, 1968.

[3] MEENA, K.R.; KANWAr, S.S. Lipopeptides as the antifungal and antibacterial agents: applications in food safety and therapeutics. BioMed Res. Int. , Article ID 473050,p. 1– 9,2015. Doi: https://doi.org/10.1155/2015/473050.

[4] JAHAN, R.et al. Biosurfactants, natural alternatives to synthetic surfactants: Physicochemical properties and applications. Advances in Colloid and Interface Science, v.275,102061, 2020. Doi: https://doi.org/10.1016/j.cis.2019.102061.

[5] LIU, D.et al. Construction, Model-Based Analysis, and Characterization of a Promoter Library for Fine-Tuned Gene Expression in Bacillus subtilis. ACS Synth. Biol., v. 7, p. 1785−1797, 2018.

[6] YANG, H., YU, H., SHEN, Z. 2015. A novel high-throughput and quantitative method based on visible color shifts for screening Bacillus subtilis THY-15 for surfactin production. Journal of Industrial Microbiology and Biotechnology, 42(8), 1139–1147. https://doi.org/10.1007/s10295-015-1635-4.

ACKNOWLEDGEMENTS

This work is supported by the São Paulo Research Foundation (FAPESP) grant 2023/06568-0, and by the National Council for Scientific and Technological Development (CNPq) grants 405490/2021-6 and 310023/2020-3