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I CONGRESSO BRASILEIRO DE BIOTECNOLOGIA INDUSTRIAL
XXIV SINAFERM ·· XV SHEB ·· XV ENZITEC FLORIANÓPOLIS ·· 2024
Creating connections between biotechnology and industrial sustainability
August 25 to 28, 2024
Costão do Santinho Resort, Florianópolis, SC, Brazil
INDUSTRIAL MICROBIOLOGY: PROSPECTING AND APPLIED MOLECULAR BIOLOGY

BIOPROSPECTING BRAZILIAN SAVANNA *BACILLUS SPP.* FOR BIOTECHNOLOGICAL PRODUCTION OF SUCCINIC ACID

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ABSTRACT

Succinic acid (SA) is a crucial precursor in many industries. Presently, it is derived from petrochemical sources, which raises environmental concerns. Since it could be synthesized biologically, it is one of the 12 building blocks with high-added value pivotal for the bioeconomy establishment. The fermentative production routes have important advances in the last decades, but challenges are still faced with the current microorganisms applied. In this work, we evaluate *Bacillus spp*. strains isolated from the Brazilian Savanna biome for enhanced SA production. Initial screening identified potential producers, notably strain 100, which exhibited the better growth and the higher SA yields under anaerobic conditions. However, other organic acids are produced together with SA, especially lactic acid, suggesting a metabolic competition. Redirecting bacterial metabolic flux through molecular biology tools could be promising in improving SA production in *Bacillus spp*. Strain 100 emerges as a candidate for further improvements, including the evaluation of alternative nitrogen sources and electrons supply by some co-substrates.

Keywords: Succinic Acid. *Bacillus spp*. Fermentation. Brazilian Savanna. Biotechnology.

1 INTRODUCTION

Succinic acid (SA) is a vital precursor in the food, pharmaceutical, chemical, and agricultural industries[1]. It is typically derived from petrochemical sources[2], posing environmental concerns due to its non-renewable nature as well as carbon dioxide emissions. It is also one of the 12 biological building blocks with high-added value[1] pivotal for the bioeconomy establishment. The fermentative production routes offer a more sustainable alternative, in which microorganisms potentially fix carbon dioxide[3] (Figure 1). Despite this promise, current microorganisms used for bio-succinic acid production face challenges such as degeneration tendencies, sensitivity to pH changes, high auxotrophy and complex growth media requirements[4,5].

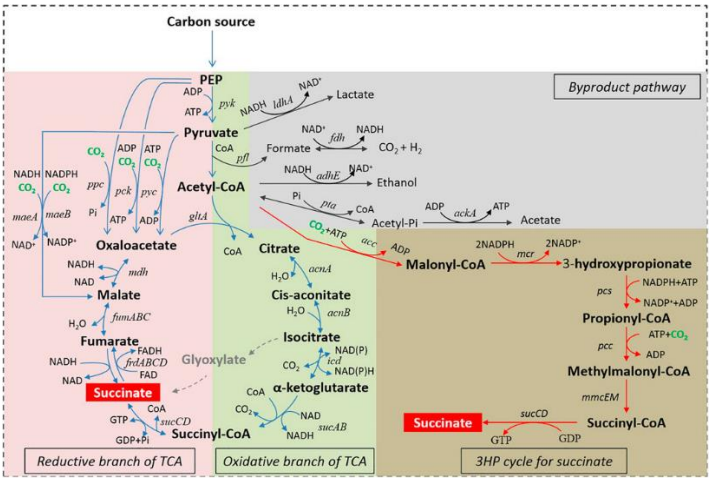

Figure 1. Biosynthetic pathways for bio-succinic acid production. Adapted from LIU et al., 2022.

Bacillus spp., known for high adaptability in industrial applications[6], presents a promising avenue for enhancing SA production. Thriving in diverse environmental conditions and demanding simple nutritional needs[7], *Bacillus spp*. hold potential for efficient and cost-effective bioprocesses. In Brazil, the Savanna biome's under-explored microbial diversity[8] offers a resource for identifying novel strains with SA-producing potential. Studies indicate that *B. megaterium* can produce it as a secondary metabolite in Czapek medium[9]. Furthermore, the expression of pyruvate carboxylase enzyme from *B. subtilis* in genetically modified *E. coli* increased the production of SA by up to 170%[10]. However, detailed analysis of SA production by *Bacillus* is still limited in the literature. Thus, this work aims to enhance the fermentative bioprocess of SA production, focusing on high efficiency, low operational costs, and visibility for industrial application, using microorganism from a *Bacillus spp.* collection from Cerrado.

2 MATERIAL & METHODS

About 200 bacterial isolates were cultured in solid LB (Lysogeny Broth) medium at 37°C for 24 hours. Aspects as color, luster, shape, margin, and elevation of the colonies were used to group the isolates. Cultures were conducted in two axenic stages for the 200 isolates. From isolated colonies, aerobic inoculum in liquid LB medium were prepared (37°C, overnight, 150 rpm). After growth, a 5% volume aliquot was transferred to 50 mL of Mineral Medium in 125 mL Erlenmeyer flasks plugged with either cotton or silicone plugs, representing the cultures named in this work as “aerobic” and “anaerobic”, respectively. The flasks were incubated in shaker (37°C, 72 hours, 150 rpm). The mineral medium was composed by: KH_2PO_4 1.5 g/L, Na_2HPO_4 3.51 g/L, $MgSO_4.7H_2O$ 0.2 g/L, $CaCl_2.2H_2O$ 0.01 g/L, $C_6H_5Na_3O_7.2H_2O$ 0.153 g/L, trace elements 1 ml/L ($FeSO_4.7H_2O$ 20 g/L, $MnCl_2.4H_2O$ 0.03 g/L, H_3BO_4 0.3 g/L, $CuSO_4.5H_2O$ 0.01 g/L, $CoCl_2.6H_2O$ 0.2 g/L, $(NH_4)_6Mo_7O_2$ 6.HaO 0.03 g/L, $ZnSO_4.7H_2O$ 0.03 g/L, $NiSO_4.7H_2O$ 0.03 g/L). Glucose (25 g/L) was used as the carbon source, while $(NH_4)_2SO_4$ 1g/L as the nitrogen source. The samples were harvested and centrifuged (8000xg, 10 minutes, 4°C); the supernatant pH was measured, filtered (0.22 µm) and analyzed for organic acids and residual carbon source through a high-performance liquid chromatograph equipped with an Aminex HPX-87H BioRad column (mobile phase 5 mM H_2SO_4, flow rate 0.5 mL/min, 55°C) as well as UV (214 nm) and RI (refractive index) detectors. The pellet was washed with 10% saline solution, lyophilized, and weighted to determine the dry biomass. Different carbon sources were tested for 2 strains, using the mineral medium as a base. Glucose, glycerol and xylose were evaluated at 20 g/L. The cultures were performed in biological quadruplicates and analytical duplicates.

3 RESULTS & DISCUSSION

The isolates library was repurified and carefully reisolated. The 200 isolates were distributed into 20 major phenotypic groups based on visual aspects of colonies as observed in Figure 2. We noted the prevalence of biofilm forming bacteria, a structure that confers resistance to harsh environmental conditions, such as acidic pH[6,7]. This was an interesting observation that corroborates the idea of *Bacillus spp*. as more resistant organisms for industrial application.

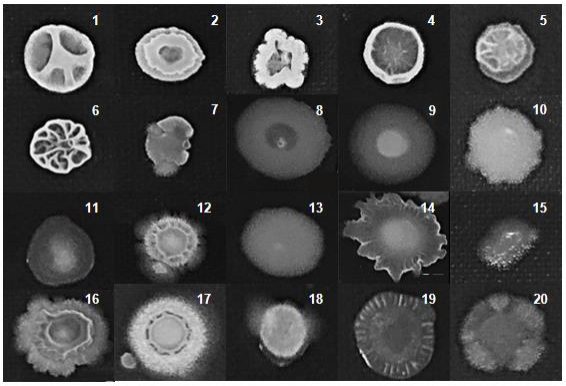

Figure 2. 20 morphological groups obtained after CFU classification.

Analysis of the supernatant allowed the identification of 43 bacterial strains that produced SA. The highest producers were strains 4, 19, 26, 100, and 150, yielding 0.233g/L, 0.296g/L, 0.241g/L, 0.765g/L, and 0.325g/L respectively. Cultures of *B. megaterium* under supercritical CO_2 and 1 atm CO_2 reported 0.001 g/L and 1 g/L succinate synthesis[11], while a maximum of 0.280 g/L of SA in Czapek medium supplemented with phosphate[9], which indicate our strains as interesting candidates for SA production.

Bacterial strains 19 and 100 (morphological classification, as group 12 and 13, respectively) were selected for further analysis. Strain 100 presented higher cellular growth than 19 in anaerobiosis (Figure 3A), which is essential for higher SA production. It also achieved lower pH levels with glucose (Figure 3B). This result is corroborated by the bioproducts profiling (Figure 3D), since strain 100 showed higher acids production, under anaerobic conditions, including SA. The most effectively consumed carbon source was glucose, while the least was xylose (Figure 3C). Even so xylose causes the beneficial impact of reducing LA and AA production, it also diminishes SA levels. Subsequently, glucose was defined as the ideal carbon source. Glycerol was set aside due to its contribution to relatively lower bioproducts yields.

SA synthesis is always accompanied by other organic acids, mainly lactic acid (Figure 3D). There is a competition between C4 pathway to produce SA and C3 pathways to produce AA, FA, and LA, caused by the change in redox potential and NADH level during fermentation[12]. Nevertheless, the redirection of metabolic flux through molecular biology tools seems to attenuate the synthesis of such organic acids and improve SA production[13], through lactate-dehydrogenase (LDH) deletion. This poses an interesting alternative for overcoming this imbalance, combined with NADH supplementation.

Despite the initial yield of 0.7 g/L SA, strain 100 presented later a 0.1g/L yield in the different carbon source cultures. Such decline remains in study, yet it still exceeds the medium SA yield for *B. megaterium*[9]. Additionally, another work indicated a relevant production of SA by *B. velezensis* at 6.62 g/L, however utilizing costly substrates as yeast extract[12], making industrial application

impractical. As the goal is a cost-effective bioprocess, further replicates will be conducted with the purpose of precisely determining the final yield for strain 100.

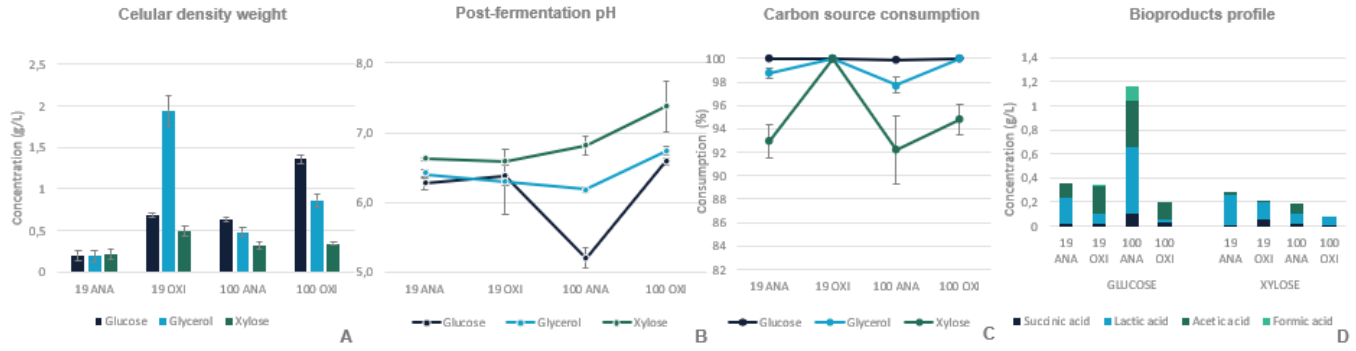

Figure 3. A. Dry biomass weight measured after pellet lyophilization. **B.** pH levels of supernatant after fermentation. **C.** Percentage of carbon source consumption by strains 19 and 100 **D.** Profile of organic acids produced. **Abbreviations:** OXI - aerobic cultures. ANA - anaerobic cultures.

4 CONCLUSION

While strain 100 emerged as the top candidate of the screening for further tests on alternative nitrogen sources and electron suppliers co-substrates, future experiments involving LDH pathway deletion in order to promote carbon and NADH flux redirection also hold promise. It stands as a potential microbial chassis candidate for SA production. Though initial yields are modest, such strategies might enhance SA production. Additionally, conducting 16S ribosomal RNA gene analysis of the bacteria is desired for deeper insights.

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ACKNOWLEDGEMENTS

This work was supported by CNPq/Braskem S.A. through the MAI-DAI Program (Processes 180206/2024-9). The contribution of Instituto Mato-Grossense do Algodão (IMA) is acknowledged for providing the *Bacillus spp*. collection.