SELECTION OF OSMOTIC-STRESS TOLERANT STRAINS FOR BRAZILIAN 1G BIOETHANOL PRODUCTION

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ABSTRACT

Due to high sugar and salt concentrations in sugarcane molasses, osmotic stress is a significant challenge in Brazilian bioethanol production. This study explores the potential of indigenous Brazilian yeast strains to tolerate extreme osmotic conditions, offering alternatives to the traditionally used *Saccharomyces cerevisiae*. Twenty yeast strains isolated from diverse Brazilian environments were evaluated for their growth and fermentation capabilities in high-osmolarity media. Among these, *S. cerevisiae* and *Zygosaccharomyces sp*. exhibited robust performance, with higher ethanol and biomass production than other investigated strains, including other *S. cerevisiae*. Both strains demonstrated superior fermentation rates and ethanol yields, close to 75% of the theoretical maximum, even under stressful industrial conditions in a scaled-down system mimicking industrial bioethanol production. This work demonstrated the potential of indigenous strains with specific characteristics suitable for the Brazilian bioethanol process as a platform for developing new industrial candidates. Additionally, the study showcased the use of other species besides *S. cerevisiae*, emphasizing the importance of strain selection for enhancing ethanol yield and sustainability in the bioethanol industry.

Keywords: Yeast. Fermentation. Osmotic stress. Sugarcane molasses. Bioethanol.

1 INTRODUCTION

Molasses is a byproduct of the sugar industry. After sugarcane is pressed and its juice is obtained, it undergoes clarification and is subsequently submitted to a concentration process by evaporation for sucrose crystallization. Once sugar crystals are harvested, a thick and dark brown liquid comprising a high content of polysaccharides is obtained, denominated molasses. The latter feature presents molasses as a highly competitive feedstock for the ethanol industry, representing decreased production costs due to their relatively abundant input[1]. However, high sugar levels lead to high osmotic pressure, i.e., causing cellular water to passively diffuse along the concentration gradient, leading to hyperosmotic stress[2]. Additionally, molasses is a byproduct that underwent concentration for sugar crystallization, resulting in a highly salt-concentrated feedstock that contributes to the hyperosmotic stress[1]. Such conditions may be unsuitable for some yeast strains, decreasing cell viability and production rates, leading to adverse physiological effects[3].

The current Brazilian ethanol production process relies on *Saccharomyces cerevisiae*. Although all yeasts are potentially capable of fermentation, the biodiversity of indigenous yeast strains from diverse environments may offer a potential alternative to the osmolarity challenge. Adapted to unique conditions such as pH, salinity, and temperature, these indigenous yeasts may prove more robust and adaptable, potentially overcoming the osmolarity obstacle[4].

Here, we aim to explore the potential of Brazilian biodiversity in the context of first-generation bioethanol production. Thus, we isolated and selected microorganism species from a plurality of distinct environments, investigating their potential to grow and ferment in extreme osmotic conditions. The selection of new species and strains may pave the way to potential applications in the ethanol and food industries. We also demonstrate the potential use of non-*S. cerevisiae* microorganisms in bioethanol production

2 MATERIAL & METHODS

Yeast isolation and identification

Microorganism samples were collected from the environment in Parque Nacional do Iguaçu (Paraná, Brazil) and Barracão (Paraná), in a brewery in Cascavel (Paraná), and from native beehive in São Paulo (São Paulo, Brazil). Collected samples were diluted in NaCl 0.9% in sterile falcon tubes and 50 uL of the solution were plated on RBC medium. After 3 days at 30°C, white creamy colonies were selected. Isolated yeasts were identified by MALDI-TOF MS.

Yeast cultivation

A loopful of yeast colony was inoculated into 25 mL of YPS (1% Yeast extract, 2% Peptone, and 2% Sucrose) for 24 h. Cells were centrifuged (10,000 g, 15 minutes), and the supernatant was removed. 25 mL of fresh YPS (5% Sucrose) was added to the cells for 24 h. This procedure was repeated 3 times, yielding at least 1 g of wet biomass for each strain. Cells were stored in a 0.9% NaCl solution until use.

The first exploratory experiment was carried out with a modified version of YPS containing 100 $g.L^{-1}$ of sucrose (10%), 160 $g.L^{-1}$ of $MgSO_4.H_2O$, and 9.6 $g.L^{-1}$ of KCl. After propagation, the salt-rich YPS was added to the tubes, resulting in a cell concentration of approximately 10 $g.L^{-1}$ (dry basis). Volume was adjusted to the microorganism's corresponding weight. The tubes were weighted periodically to monitor CO_2 loss, and a sample was taken at the end for HPLC analysis. Additionally, initial and final cell mass were used to determine biomass variation.

Tubes from YPS cultivation were centrifuged again, and the supernatant was removed. The biomass was used in a sequential experiment using 40°Brix sugarcane molasses (c.a. 400 $g.L^{-1}$ of total reducing sugars, TRS). Medium was added to the tubes, yielding an initial concentration of 10 $g.L^{-1}$ of dry biomass. The CO_2 loss and ethanol production were monitored.

The results of the previous cultivations were used to select two microorganisms with the best performance in terms of cellular growth and fermentative capacity in this harsh osmotic stressful media. Selected strains were used in a scaled-down system to mimic Brazilian bioethanol production[5]. The protocol includes cell recycling, relatively high temperature (34°C), acid treatment between fermentation cycles, and non-aseptic conditions. Biomass variation, ethanol and glycerol production, and CO_2 loss were evaluated in a total of 3 cycles.

HPLC analysis

An HPLC system (Shimadzu Prominence LC-20AB, Japan) with a refractive index detector equipped with a Bio-Rad HPX-87H column at 60°C was used to determine sugar and metabolite concentrations in fermentation experiments. A 5 mM H2SO4 solution (0.6 $mL.min^{-1}$) was used as an eluant.

3 RESULTS & DISCUSSION

The concentrations of the three most abundant compounds in sugarcane molasses, in addition to sugars, are potassium, sulfur, and magnesium, with an average of 4, 2, and 1 $g.L^{-1}$, respectively. Thus, the proposed modified YPS medium with the addition of magnesium sulfate and potassium chloride resulted in concentrations up to 5 times higher of each compound than 20°Brix molasses[1], generating high osmotic stress for the yeasts. The cultivations using this medium were quite distinct (Figure 1). Some strains presented cellular growth but produced low ethanol titers, while others presented high ethanol concentrations with decreased cellular growth. Despite ethanol being the main product of interest in the biofuel industry, cellular growth ensures process viability throughout the sugarcane season, as it renews cells, maintains high cell viability, and compensates for losses during recycling steps. Two strains (8 and 18) showed balanced performance (Figure 1A) in these two selection criteria: strain 8 was identified by MALDI/TOF as *S. cerevisiae*, whereas strain 18 was *Zygosaccharomyces* sp.. Another critical factor besides biomass variation and ethanol concentration for these two strains was the fermentation rate, observed in Figure 1B through CO_2 loss. Both strains showed higher losses than others, indicating the ability to rapidly consume sugars and reduce production time in future stages.

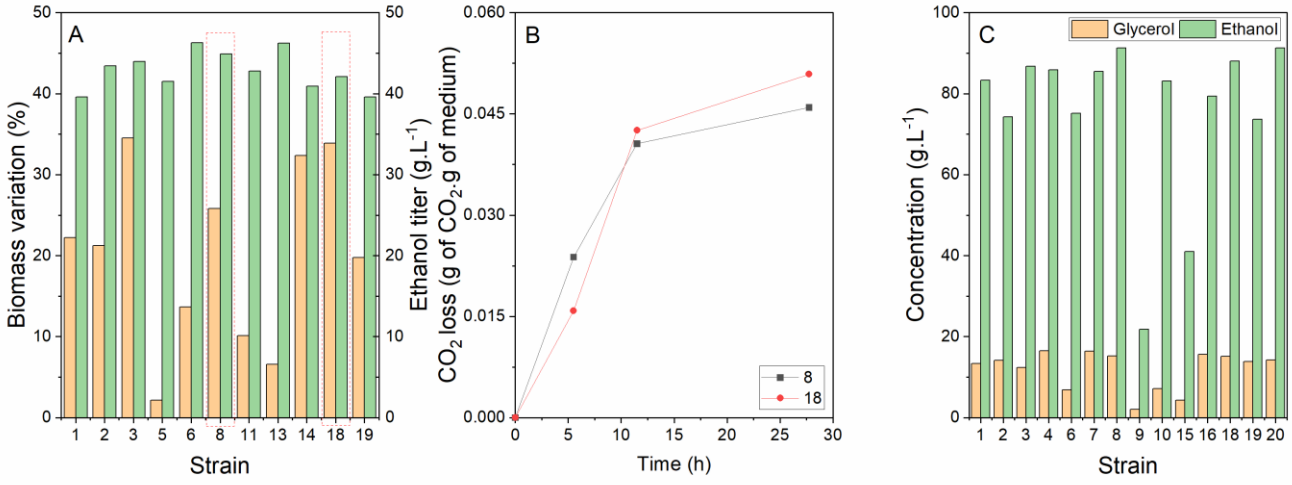

Figure 1 A: Biomass variation and ethanol titer in a modified YPS medium (Green: Ethanol titer; Orange: Biomass variation). B: CO2 loss along cultivation in modified YPS. C: Ethanol and glycerol concentrations in 40°Brix sugarcane molasses after 72 h.

After evaluating the physiology of the strains in YPD, more assays were performed using molasses containing high concentrations of total reducing sugars (400.07 $g.L^{-1}$). Strains 8 and 18 again indicated excellent performance, achieving ethanol concentrations (Figure 1C) of 91.23 and 88.02 $g.L^{-1}$ for 8 and 18, respectively. These strains exhibited two of the three highest ethanol concentrations; strain 20 produced 91.275 $g.L^{-1}$. HPLC analyses further indicated that most yeasts could not consume sugars below values close to 160 $g.L^{-1}$, possibly due to the high concentrations of ethanol produced. The selected strains showed TRS

at the end of fermentation of 146.16 and 156.15 $g.L^{-1}$, respectively. Glycerol concentrations were relatively close for both strains 15.175 and 15.150 $g.L^{-1}$.

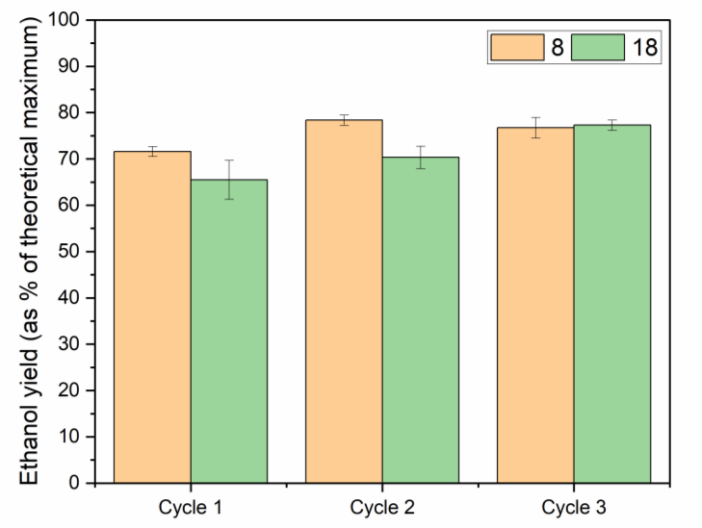

Figure 2 Ethanol yield as % of the theoretical maximum (0.511g of ethanol per g of total reducing sugar) in scaled-down system mimicking Brazilian ethanol production.

The performance of the two selected strains was evaluated in experiments simulating the Brazilian ethanol production process, following the protocol described by Raghavendran et al. (2017)[5]. The molasses used (190.04 $g.L^{-1}$ of TRS) is between 18-22°Brix, as reported in the literature. These assays also evaluated other stressing factors, such as acid treatment and non-aseptic conditions. The results in Figure 2 demonstrate the ethanol yields (as % of the theoretical maximum of 0.5111 g of ethanol per g of TRS) obtained in these assays. Despite the good performance of these strains, yields remained around 70% for strains 8 and 18, with slightly better performance for the *S. cerevisiae* strain. Literature results for dominant strains in the Brazilian production process with ethanol yield values above 90%[1] demonstrate that despite efforts, the evolution of naturally selected strains with unique characteristics, such as osmotic stress tolerance, is still necessary.

4 CONCLUSION

This work paves the way for the selection and development of strains that add essential characteristics to the Brazilian ethanol production process and demonstrates that new species may exhibit good fermentative performance, potentially competing with the dominant yeast *S. cerevisiae*. Additionally, this work can be used as a template for evaluating candidates through simple assays, further highlighting its potential for industrial application.

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