YEAST STRAIN SELECTION AND ITS TRACKING DURING BIOETHANOL PRODUCTION

Bianca C. G. M. Peixoto[1], Patrícia G. P. Barbosa[1], Mariana F. G. de Albuquerque[1], Margarete A. F. Saraiva[2], Ana M. dos Santos[2], Julia C. Romanello[1, 2], Maria J. M. Trópia[1], Izinara R. da Cruz[1], Aureliano C. da Cunha[1] & Rogelio L. Brandão[1, 2, *]

[1] *Laboratório de Biologia Celular e Molecular, Departamento de Farmácia, Universidade Federal de Ouro Preto, Ouro Preto, Brazil*
[2] *CERLEV- Projetos e Inovação Tecnológica na Biotecnologia da Fermentação, Ouro Preto, Brazil*
** Corresponding author's email address: rlbrand@ufop.edu.br*

ABSTRACT

In Brazil, a leading global producer of bioethanol, the utilization of *Saccharomyces cerevisiae* strains resistant to adverse environmental conditions is crucial during the production process. The adaptation and fermentation efficiency of these yeasts will impact the final ethanol yield. Therefore, the Bio28 strain was selected from isolates from an industrial plant after exposure to conditions similar to those of the mill. Studies have addressed the replacement of inoculated yeasts with wild strains during the harvest. The prevalence of the selected strain was monitored throughout the 2022 harvest, demonstrating its persistence despite a gradual decline.

Keywords: Flocculation. Acid recycle. Fermentation. Polymorphism.

1 INTRODUCTION

Brazil stands out as one of the world's largest producers of sugarcane and ethanol, with the Southeast region playing a leading role in this scenario.[1,2] Together with the United States, the global leader, these two countries account for 80% of the total production of ethanol consumed worldwide.[3,4] The sugarcane is main raw material used in ethanol production in Brazil, and through the action of the yeast *Saccharomyces cerevisiae*, sucrose is converted into ethanol and carbon dioxide (CO_2).[5] Sudden temperature changes, high alcohol concentrations, acidic pH, molasses and contaminants are just a few of the obstacles that these yeasts will be subjected to and that can impact the final ethanol yield.[6] The use of selected stress-tolerant strains that present good production efficiency can be tools to improve productivity.[5]

Genotyping and karyotyping studies have revealed that commercial yeasts inoculated at the beginning of the harvest season in the production process are replaced by other wild strains in the fermentation tanks, leading to increased flocculation and foam production, which causes losses.[3,5] In this context, the project aimed to produce bioethanol using a selected strain and monitor the prevalence of this strain during the harvest season in the fermentation vats of the industrial plant.

2 MATERIAL & METHODS

The research group at the Cellular and Molecular Biology Laboratory of the Universidade Federal de Ouro Preto, where this work was developed, has extensive experience and knowledge acquired through previous studies that allowed them to conduct the yeast isolation and selection methodology presented here. Samples were collected from fermentation vats of an industrial plant located in São Paulo and stored under refrigeration until isolation.[7,8,9]

For isolation, serial dilutions were performed up to 10^{-8}, from which 200 µL aliquots were distributed onto sterile Petri dishes containing Yeast Peptone Sucralose 8% (YPS 8%) medium supplemented with 100 µg/mL chloramphenicol. Isolates were transferred to 96 well plates and designated as master plates. The plates were incubated at 30 °C for 24 to 48 hours and then stored under refrigeration. Knowing that *Saccharomyces cerevisiae* does not grow on certain carbon and nitrogen sources, growth was tested on 96 well plates in minimal medium with 0.14% Lysine, Yeast Peptone Lactose 2% (YPL 2%), and Yeast Peptone Mannitol 2% (YPM 2%). After incubation and visual inspection of growth, isolates that did not grow in any of the three media were considered potential *S. cerevisiae*.[10,11]

Potential *S. cerevisiae* strains were subjected to stress resistance tests in the first selection step, including osmotic stress (Yeast Peptone Dextrose 33% and Yeast Peptone Sucralose 20%), thermotolerance (growth at 37°C), ethanol tolerance (ethanol 10, 15, and 17%), and acidic pH (pH = 2.5). The tests were evaluated visually after incubation and growth in 96 well plates. An overlay of the growth results from all stress tests in this step was performed, and the resistant, selected isolates were transferred to new 96 well plates containing YPS 8% medium and 100 µg/µL chloramphenicol. The resulting new plates were subjected to aconitic acid resistance tests in minimal medium (1 mM, 5 mM, 10 mM, and 20 mM) and molasses growth (15 and 30%), both in 96 well plates, in the second step. In this step, growth in Al^{+3} (10.9; 16.3, and 18.5 mM) on solid YPS 2% medium in Petri dishes was also evaluated, for both undiluted and 10^{-4} diluted isolates. As in the previous step, the results were overlaid, and the resistant strains were transferred to a new plate under the same conditions. In the third and final selection step, a flocculation test (qualitative and quantitative)[12] and an evaluation of the estimated ethanol and foam production on a 50 mL

laboratory scale of YPS 15% with acid recycle were performed.[13] The selected strain was multiplied in a 10 L fermenter, sent to the industrial plant, and its prevalence was monitored during the harvest season by polymorphism analysis (COX1-PCR).[8]

3 RESULTS & DISCUSSION

The total of 960 colonies were isolated from the collected sample, resulting in 10 master plates. Bioethanol plants conventionally use selected industrial *S. cerevisiae* yeasts due to their efficiency and safety.[14] Knowing that this species does not use Lysine as a nitrogen source or Mannitol and Lactose as carbon sources, almost 80% of the isolates were found to be potential *S. cerevisae*.[10,11] Given this still high number of isolates, the 10 master plates were used to perform the first phase of the selection tests. In the industrial plant, yeasts face various stressful environmental conditions that will activate different responses. The response to these conditions can affect the efficiency and ethanol yield. In this context, the use of isolated yeasts that are more adapted to the process is essential for improving fermentation performance.[15,5]

Following the overlay of the first-stage tests (thermal, osmotic, alcoholic, and acidic pH), 155 isolates grew under the tested stress conditions, and 15 of these withstood up to 17% ethanol. Only the potential *S. cerevisiae* strains that resisted up to 15% ethanol and the other tests were selected for the second phase of selection, totaling 127 isolates. All selected yeasts were resistant to the tested concentrations of aconitic acid, and therefore the 127 were subjected to the aluminum resistance test. A total of 22 isolates grew in the three aluminum concentrations and in at least one of the dilutions, thus being selected for the third phase. Only 5 of the 22 isolates were pre-selected in the qualitative and quantitative flocculation tests, as they presented the lowest flocculation percentages compared to the other isolates. The fermentation test was performed on a laboratory scale with the 5 isolates, applying acid recycle daily under the conditions used by the plant. During fermentation, foam production and estimated ethanol production were evaluated, calculated by the stoichiometry of the reaction and weight loss. Pedra-2 (PE2), an industrial strain commonly used in the plants, was used as a positive control for the experiments in this last stage. Despite its average flocculation, Bio28 was the selected strain due to its excellent fermentation performance, with an estimated ethanol production (>70 $g.L^{-1}$, after the fifth acid recycle) close to or even higher than PE2 at times, in addition to tolerating stress conditions, including 17% ethanol.

The Bio28 strain was sent for industrial plant testing during the 2022 harvest season, and its prevalence was evaluated by isolating 50 colonies from three distinct periods (May, July, and September), resulting in 150 isolates for analysis. Employing the DNA polymorphism analysis method (COX-PCR) enabled the differentiation and comparison of the profile of the isolates from each period with Bio28. This differentiation was achieved by amplifying the variations in the introns of the COX1 mitochondrial gene, which is responsible for encoding subunit 1 of cytochrome oxidase.[8] The prevalence of the selected strain following multiple acid treatments and fermentation cycles will depend on the strain's robustness and adaptability throughout the process.[15]

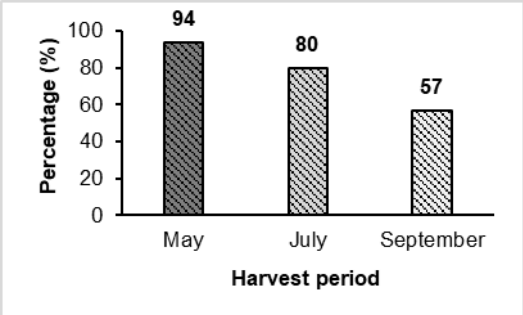

Figure 1 Percentage of Bio28 prevalence during the 2022 harvest.

Throughout the harvest periods, an increase in polymorphism was observed, generating band patterns different from Bio28. According to Figure 1, despite a gradual decrease, the selected strain remained in the vats until the end of the harvest, corresponding to 57% of the analyzed isolates. The use of strains that prevail until the end of the harvest can be another tool for improving ethanol productivity and final yield.[5]

4 CONCLUSION

A selection strategy, the stress conditions that yeasts face during the production process were considered, as well as the fermentation efficiency that will impact the final yield of bioethanol. *Saccharomyces cerevisiae*, due to the high percentage, proved to be the predominant species in the collected sample. The Bio28 strain was selected due to its tolerance to stressful conditions and high ethanol production estimated. The molecular characterization technique of COX1-PCR polymorphism analysis has proven to be a useful tool for strain differentiation. Knowing that yeasts are reused from the beginning to the end of the harvest, Bio28, in addition to its robustness, possibly has good adaptability to the process, since it remained and accounted for 57% of the isolates identified in the September period. Therefore, the prevalence of the selected strain until the end of the harvest will have a positive impact on bioethanol yield.

REFERENCES

[1] KLEIN, B. C., DE MESQUITA SAMPAIO, I. L., MANTELATTO, P. E., FILHO, R. M., & BONOMI, A. 2019. Beyond ethanol, sugar, and electricity: a critical review of product diversification in Brazilian sugarcane mills. Biofuels, Bioproducts and Biorefining, 13(3), 809-821.

[2] CONAB (Companhia Nacional de Abastecimento). Análise mensal de cana-de-açúcar. Fevereiro/Março 2023. In: < https://www.conab.gov.br/info-agro/analises-do-mercado-agropecuario-e-extrativista/analises-do-mercado/historico-mensal-de-cana-de-acucar/item/20227-cana-de-acucar-analise-mensal-fevereiro-2023>.

[3] VIANA, N. C., DO NASCIMENTO, D. D., PRADA, M., & DA CRUZ, S. H. 2020. Molecular characterization of isolated dominant yeast strains during ethanol fermentation process. Brazilian Journal of Agriculture, 95(1), 48-58.

[4] KUNWER, R., PASUPULETI, S. R., BHURAT, S. S., GUGULOTHU, S. K., & KUMAR JOSHI, S. 2022. Economic assessment and production of ethanol-A review. Materials Today: Proceedings, 69, 543-548.
VIANA et al., 2020; KUNWER et al., 2022

[5] DE SOUZA, J. P., DO PRADO, C. D., ELEUTHERIO, E. C., BONATTO, D., MALAVAZI, I., & DA CUNHA, A. F. 2018. Improvement of Brazilian bioethanol production–Challenges and perspectives on the identification and genetic modification of new strains of Saccharomyces cerevisiae yeasts isolated during ethanol process. Fungal biology, 122(6), 583-591.

[6] EARDLEY, J., & TIMSON, D. J. 2020. Yeast cellular stress: impacts on bioethanol production. Fermentation, 6(4), 109.

[7] VICENTE, M. A., FIETTO, L. G., DE MIRANDA CASTRO, I., DOS SANTOS, A. N. G., COUTRIM, M. X., & BRANDAO, R. L. 2006. Isolation of Saccharomyces cerevisiae strains producing higher levels of flavoring compounds for production of “cachaça” the Brazilian sugarcane spirit. International journal of food microbiology, 108(1), 51-59.

[8] OLIVEIRA, V. A., VICENTE, M. A., FIETTO, L. G., DE MIRANDA CASTRO, I., COUTRIM, M. X., SCHÜLLER, D., ... & BRANDAO, R. L. 2008. Biochemical and molecular characterization of Saccharomyces cerevisiae strains obtained from sugar-cane juice fermentations and their impact in cachaca production. Applied and Environmental Microbiology, 74(3), 693-701.

[9] CAMPOS, A. C. S., ARAÚJO, T. M., MORAES, L., CORRÊA DOS SANTOS, R. A., GOLDMAN, G. H., RIANO-PACHON, D. M., ... & BRANDÃO, R. L. 2024. Selected cachaça yeast strains share a genomic profile related to traits relevant to industrial fermentation processes. Applied and Environmental Microbiology, 90(1), e01759-23.

[10] SANNI, A. I., & LÖNNER, C. 1993. Identification of yeasts isolated from Nigerian traditional alcoholic beverages. Food Microbiology, 10(6), 517-523.

[11] VAUGHAN-MARTINI, A., & MARTINI, A. 1993. A taxonomic key for the genus Saccharomyces. Systematic and Applied Microbiology, 16(1), 113-119.

[12] ALVAREZ, F., DA MATA CORREA, L. F., ARAÚJO, T. M., MOTA, B. E. F., DA CONCEIÇÃO, L. E. F. R., DE MIRANDA CASTRO, I., & BRANDÃO, R. L. 2014. Variable flocculation profiles of yeast strains isolated from cachaça distilleries. International journal of food microbiology, 190, 97-104.

[13] DA CONCEIÇÃO, L. E. F. R., SARAIVA, M. A. F., DINIZ, R. H. S., OLIVEIRA, J., BARBOSA, G. D., ALVAREZ, F., ... & BRANDÃO, R. L. 2015. Biotechnological potential of yeast isolates from cachaça: the Brazilian spirit. Journal of Industrial Microbiology and Biotechnology, *42*(2), 237-246.

[14] MULLER, G., DE GODOY, V. R., DÁRIO, M. G., DUVAL, E. H., ALVES-JR, S. L., BÜCKER, A., ... & STAMBUK, B. U. 2023. Improved Sugarcane-Based Fermentation Processes by an Industrial Fuel-Ethanol Yeast Strain. Journal of Fungi, 9(8), 803.

[15] RIBEIRO, R. A., BOURBON-MELO, N., & SÁ-CORREIA, I. 2022. The cell wall and the response and tolerance to stresses of biotechnological relevance in yeasts. Frontiers in Microbiology, 13, 953479.

[16] AMORIM, H. V., DE AMORIM NETO, H. B., LOPES, M. L., & DE LIMA PAULILLO, S. C. 2017. Evolution of yeast selection for fuel ethanol: breaking paradigms. Diversity and Benefits of Microorganisms from the Tropics, 391-401.

ACKNOWLEDGEMENTS

This work was carried out with the support of the Coordination for the Improvement of Higher Education Personnel - Brazil (CAPES) - Financing Code 001. The authors would like to thank the support of UFOP (Universidade Federal de Ouro Preto) of Catalisa-ICT and the agencies funding FAPEMIG (Foundation for Research Support of the State of Minas Gerais) and CNPq (National Council for Scientific Development).