SCREENING OF IN-HOUSE YEAST STRAINS BEFORE AND AFTER HEAT TREATMENT IN BIOETHANOL DISTILLERIES

Mariana F. G. de Albuquerque[1], Julia C. Romanello[1,2], Ana M. dos Santos[2], Bianca C. G. M. Peixoto[1], Margarete A. F. Saraiva[2], Patrícia G. P. Barbosa[1], 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

The research focused on bioethanol production in Brazil highlights its global significance, with a process involving open sugarcane fermentation using *Saccharomyces cerevisiae* and some peculiar conditions like fed-batch fermentation, yeast cells recycling, and acid washing to try to keep the process productivity. Some challenges include microbial diversity and strain dynamics in industrial settings, such as when the starter strain is quickly replaced by other strains that often present characteristics that are harmful to the process, such as high flocculation and high foam production. In this work, 13 yeast strains were selected from samples collected before and after the heat treatment to sterilize the feedstock in four Brazilian bioethanol plants. The results showed that the heat treatment efficiently eliminated yeast strains that do not belong to the genus *Saccharomyces*. Furthermore, the screening protocol directed the selection toward the *S. cerevisiae* species and obtained yeast strains resistant to various conditions inherent to bioethanol production and with varying flocculation rates and fermentative performance.

Keywords: Bioethanol. Fermentation. *Saccharomyces cerevisiae*. Selected yeasts.

1 INTRODUCTION

Brazil is the second largest producer of bioethanol in the world, responsible for around 30% of the global production of this biofuel.[1,2] Most Brazilian sugarcane first-generation bioethanol plants employ a fed-batch fermentation process characterized by high cell density, ensuring superior production stability compared to continuous mode operations. After each fermentation cycle, yeast cells are centrifuged and treated with diluted sulfuric acid. More than 90% of these cells are recycled for subsequent fermentations, maintaining high cell density crucial for short fermentation times and high process productivity.[3]

Producing bioethanol from sugarcane involves several key steps: harvesting and cleaning sugarcane, sugar extraction, juice treatment, concentration and sterilization, fermentation, distillation, and dehydration. Thermal treatment is carried out by heating the treated juice to increase the concentration of sucrose and eliminate microorganisms from the previous steps.[4] However, the fermentation of sugarcane feedstock into fuel ethanol on a large scale and in non-aseptic conditions in biorefineries creates a distinctive ecological environment where the yeast *Saccharomyces cerevisiae* reigns as the dominant organism.[2] This fermentative process presents some characteristics that make it very peculiar: the foot-of vat is formed by almost 30% (v/v) of yeasts that work in an open fermentative process for 8-9 months/year; yeast cells are recycled each 12 h after the acid treatment combined or not with specific antimicrobials to control the bacterial contamination and to try to keep the fermentation performance.[2,5]

Research into the microbiological changes within industrial fermenters has shown a swift turnover of yeast strains during bioethanol production. Consequently, the initial starter yeast is entirely supplanted by other strains within weeks, and many contaminant yeast strains able to survive inside fuel ethanol industrial vats can present detrimental cell surface phenotypes, such as filamentation, invasive growth, flocculation, biofilm formation, and excessive foam production.[3] Conversely, specific yeast strains exhibit dominance within the fermenters. This dominance enables the identification of appropriate industrial strains possessing robust fermentative capabilities and resilience against the demanding conditions of industrial settings. These conditions encompass high temperatures and osmotic pressures, elevated ethanol concentrations, low pH levels, the presence of industrial antifoam agents, and potential process interruptions, among other stresses.[3]

Specialized starter strains have been employed to start the harvest in bioethanol fermentation plants. They have contributed to improving the industry's overall performance. However, invasion by foreign yeast strains is still quite common due to the significant variation between one plant and another, related to raw materials, industrial practices, environmental conditions, and even possible evolutionary changes in yeasts throughout a fermentation season.[2] Therefore, the present study aimed to select yeast strains from samples collected before and after the heat treatment applied to sterilize the feedstock in four Brazilian bioethanol plants. Thus, the results presented here allow future investigations into the origin of the yeast strains present in the fermentation vats for bioethanol production and contribute to a better elucidation of the microbial dynamics involved in this process.

2 MATERIAL & METHODS

The yeast strains were isolated from samples supplied by different bioethanol plants: three localized in Goiás and one in Minas Gerais, Brazil. Each of them provided one sample obtained before the thermal treatment and about six samples obtained after

the heat treatment. All samples were collected in sterile sampling bags, placed on ice, transported to the laboratory, and then stored under refrigeration (4 °C) until the conclusion of experiments.

Based on our research group's extensive experience in screening yeast strains with desirable phenotypes for cachaça production[6], we developed a protocol for selecting yeast strains for bioethanol production. Initially, to obtain yeast strains isolated, the samples were diluted in sterile distilled water (1:10 until 1:1000000), and 0.2 mL of each dilution was spread on plates containing agar 1.5% (w/v), peptone 2% (w/v), and yeast extract 1% (w/v) supplemented with sucrose 8% (w/v) and chloramphenicol 0.1% (w/v). Plates were incubated at 30 °C for three days. The isolated strains obtained were transferred to the 96-well culture plate containing 0.2 mL of YP (peptone 2% (w/v) and yeast extract 1% (w/v)) supplemented with sucrose 8% (w/v) and chloramphenicol 0.1% (w/v) in each well.[7] These isolates were submitted to biochemical and molecular methods for obtaining yeast strains with desirable phenotypes for bioethanol production.

The first step was based on the utilization of carbon and nitrogen sources, using YP mannitol 2%(w/v), YP lactose 2%(w/v), and yeast nitrogen base (YNB) supplemented with lysin 0.14% (w/v) and glucose 1% (w/v) as culture mediums to grow the isolates at 30 °C for 48h.[6,7,8] The isolates that did not grow in Step 1 were evaluated concerning resistance to different stress conditions (Step 2).[7] To simulate high-temperature stress, the selected previously isolates were cultured in YP supplemented with sucrose 8% (w/v) at 37 °C for 48h. The high osmotic pressure was applied using YP supplemented with sucrose 20% (w/v) and glucose 33% (w/v) to grow the previously selected isolates at 30°C for 48h. The low pH level resistance test was performed using YP supplemented with sucrose 8% (w/v) at pH 2.5, 30°C for 48h. The resistance to elevated ethanol concentrations was evaluated by using YP supplemented with sucrose 8% (w/v) and 10% (v/v), 15% (v/v), and 17% (v/v) of ethanol to culture the selected previously isolates at 30°C for 48h. Isolates resistant to all or most stressful conditions were assigned to Step 3 to evaluate tolerance to aconitic acid, molasses, and aluminum ions.[7,9,10] For this, the isolates selected in the previous step were cultivated at 30°C for 48h on three different mediums: YNB supplemented with sucrose 2% (w/v) and aconitic acid (10 mM and 20 mM); YP supplemented with molasses (15° and 30° Brix); YP agar supplemented with sucrose 8% (w/v) and aluminum ions (16.3 mM and 18.5 mM). Those tolerating most conditions tested proceeded to Step 4 to evaluate the flocculation. The qualitative flocculation assay was carried out after growing the pre-selected isolates in YP supplemented with sucrose 4% (w/v) at 30°C for 48h. Observation of flake formation was made visually at 5-minute intervals for 30 minutes. The quantitative flocculation test (Step 5) also was performed.[11] In both flocculation assays, the yeast strains LBCM761 and Ethanol Red were used as positive and negative controls, respectively.

In the molecular analysis, in Step 6, the pre-selected isolates were submitted to the yeast identification based on the amplification of the ribosomal DNA internal transcribed spacer region (ITS) and digestion of the ITS-PCR product with the *Hae III* restriction enzyme, using the *S. cerevisiae* strain S288c as positive control.[6,12] Finally, in Step 7, the isolates identified as *Saccharomyces cerevisiae* were applied in the fermentation experiment to estimate the bioethanol and foam production. For this, the yeast strains pre-selected were incubated in fermentation tubes containing 50 mL of YP supplemented with sucrose 15% (w/v) at 30 °C, 120 rpm for 24h, using the commercial strain Pe-2 as control. The bioethanol production was estimated by weight loss, and the foam formation was measured using a ruler. The yeast strains with the best fermentative performance of each sample were stored in the ultra-freezer for later analysis.

3 RESULTS & DISCUSSION

The yeast isolates were obtained from samples collected before and after the heat pretreatment applied in the bioethanol plants. These isolates were screened in 7 stages, and the results of each sample are described in Table 1.

Table 1 Results, by stage, of yeast strain selection from samples collected before (BHT) and after (AHT) heat treatment applied in four Brazilian bioethanol plants.

Bioethanol Plant	A		B		C		D	
Sample Type	**BHT**	**AHT**	**BHT**	**AHT**	**BHT**	**AHT**	**BHT**	**AHT**
Initial	480	1134	480	1920	480	1440	480	1446
Step 1	442	1100	247	1792	349	1291	432	1429
Step 2	87	172	34	45	153	248	294	575
Step 3	9	24	34	4	11	39	26	31
Step 4	6	10	6	3	11	11	2	20
Step 5	3	6	2	2	2	4	2	2
Step 6	3	6	1	2	1	2	1	2
Step 7	1	3	1	2	1	2	1	2

Yeasts of the genus *Saccharomyces* cannot metabolize lysine as a nitrogen source or mannitol and lactose as carbon sources.[8] Therefore, in Step 1, the isolated yeasts that grew in any of the conditions tested were excluded. The results indicate that most isolates in the samples collected after heat treatment (AHT) in the four plants belong to the genus *Saccharomyces* (89% to 99%). However, this percentage varies greatly (51% to 90%) between one plant and another when considering samples collected before heat treatment (BHT). Thus, it can be inferred that heat treatment appears to be efficient in eliminating contaminating yeasts, even in plants that initially presented almost 50% of isolates from other genera.

The stressful and demanding conditions of industrial environments were simulated in this study and have a high power to select yeasts with characteristics suitable for bioethanol production. Although in the BHT samples, the selection rigor was lower than that adopted in the AHT samples about alcoholic stress (resistant to 10% and 15% of ethanol for BHT and only resistant to 15% of ethanol for AHT) in both types of samples from most isolates (60% to 98%) to did not presented resistance to the conditions tested, except for the sample BHT from plant D, in which 61% of the isolates were selected for the next stage. These results indicate that even when contaminating strains originating from the raw material, not just any yeast can survive in the fermentation vats of bioethanol plants. All yeast isolates presented tolerance to aconitic acid and molasses. This result indicates that fermentation would not be harmed by the aconitic acid originating from sugar cane nor by compounds such as low molar mass organic acids, 5-hydroxymethyl-2-furfuraldehyde, and melanoidin, which may be present in molasses often used as a carbon source in bioethanol plants which also produce sugar.[7] On the other hand, the presence of aluminum in the must increases fermentation time and causes damage to the sugar and alcohol industry.[9] In Step 3, the tolerance to this ion allowed only about 0.2% to 7% of the initial number of isolates to be selected for the next stage.

Yeast flocculation is one of the worst problems presented by processes that recycle yeast by centrifugation.[5] In Step 4, the yeast isolates from BHT samples presented showed low or very low flocculation, except for the BHT sample from plant C, whose 11 yeast isolates showed very flocculating. Regarding the AHT samples, flocculation was very varied between different plants and isolates from the same plant. In step 5, yeast isolates from the BHT samples showed a low flocculation rate (4.4% to 27.3%), except for those from the BHT sample from plant C (≤ 60%). Concerning the AHT samples, the percentage varied considerably between the plants and isolates from the same plant, except for plant B (40.8% to 44.2%). These results may indicate that flocculation is associated with reusing yeast cells throughout the harvest.

In the molecular analysis (Step 6), of the 23 pre-selected isolates in the four plants, only five were not identified as *Saccharomyces cerevisiae*. These results demonstrate that the screening protocol was quite efficient in directing selection towards strains of the *S. cerevisiae* species. The final step allowed the selection of at least two yeast isolates per bioethanol plant, one of each type of sample (BHT and AHT). The best fermentative performances were those of the isolates A7-1 (70.12 ± 0.73 g/L, no foam) and H1-3 (69.84 ± g/L, no foam) of plant A; E1-1 (63.49 ± 2.85 g/L; no foam) and G9-1 (37.29 ± 1.94, no foam) of plant B; B11-1 (22.06 ± 0.44 g/L; no foam) and E8-2 (66.49 ± 1.05 g/L, no foam) of plant C; H12-1 (17.49 ± 0.53, little foam) and H2-8 (19.76 ± 0.74, little foam) of plant D, from samples BHT and AHT respectively.

4 CONCLUSION

Although the heat treatment applied in bioethanol plants is important for reducing contaminating yeast strains, the screening protocol developed by our research group and used in the present work is essential for obtaining yeast strains with desirable phenotypes for bioethanol production and a key element for better understanding the microbiological dynamics involved in the process.

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