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Use of Native Yeast Strain (*Saccharomyces cerevisiae*) as Inoculum in Bioethanol Fermentation

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***Abstract*—For several years, Brazilian distilleries have used native yeasts selected from their own processes as starter cultures for bioethanol production in the beginning of the crop season. This study aimed to monitor the stability of native yeasts selected for their good fermentation ability in the previous season and used as inoculum in the two following seasons. The data presented here refer to three different distilleries and two distinct crop seasons. The findings demonstrated the permanence of native yeasts throughout the crop season, indicating that native strains remained dominant during most of the time, thereby providing great stability to bioethanol production. These results show that, even in the presence of other yeasts originated from raw materials, native yeasts predominate in fermentation tanks at all times during the crop season.**

***Index Terms*— Bioethanol, Yeast, Saccharomyces sensu stricto, alcoholic fermentation.**

I. Introduction

The United States of America and Brazil are the major producers of biofuels worldwide, with Brazil accounting for 30% of the global bioethanol production and ranking first among sugarcane bioethanol producers [1]. After the oil crisis of the 1970s, the Brazilian government created the National Alcohol Program (Proálcool), culminating in the development of the most successful fossil fuel replacement program. Such a success was facilitated by the fact that the target raw material, sugarcane, had always been abundant in the country, owing to its history of sugar production. Most sugar plants already had fermentation units for treating molasses, which, at the time, was considered an agroindustrial waste. The Brazilian National Alcohol Program also benefited from important advances in the agricultural sector, such as the development of new sugarcane varieties and agricultural technologies, the selection of suitable yeast strains, and improvements in fermentation processes as well as in the monitoring and control of contaminants [2], [3]. In the 2020/2021 season, according to ÚNICA [4], Brazil had a production of 23.99 billion liters of bioethanol, consolidating its position as the second largest producer in the world.

The use of ethanol as a sustainable fuel provides major environmental gains, including reduced emission of greenhouse gases and high efficiency of CO_2 capture and fixation by sugarcane crops [5]. Given this encouraging scenario, several technologies have been tested with the aim of further increasing ethanol production in Brazil. However, there are still some barriers to be overcome, the major of which are related to the selection of yeast strains.

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Most fermentation processes currently used in Brazil were designed for cell recycling [6]. At the beginning of the season, industries start their fermentation processes using large amounts of *Saccharomyces sensu stricto* cells. This taxonomic group includes yeasts relevant for industrial applications as well as for basic research [7]. Starter cultures are either Baker's yeast or selected strains. Baker's yeast, a term encompassing different strains developed for bread fermentation, is used in bioethanol production because of its wide availability and low cost. Selected yeasts are commonly isolated from industrial alcohol fermentation processes [8], grown on a large scale, and then sold as fermentation inoculum. Before the introduction of karyotype analysis [9], it was believed that the yeasts added to fermentation tanks as starters were the same as those found in the tanks at the end of the season. Now, it is known that yeasts used at the beginning of the season might not be present in fermentation tanks at the end of the season, as shown by Basso and colleagues [9]. Based on this finding, studies aimed to investigate native yeasts from industrial plants to identify strains adapted to the fermentation tank environment that could be used in other ethanol-producing industries [10], [11], [12].

A considerable part of industrial units in Brazil uses selected strains of *Saccharomyces sensu stricto* to start the fermentation process. Four strains, namely CAT1, PE2, SA1, and BG1, are produced on a large scale and sold as inoculum for the initiation of alcoholic fermentation processes [10]. The use of selected yeasts as inoculum has been recommended as a strategy to enhance fermentation efficiency [10]. However, a recent study found that selected yeast strains added as fermentation starters might be replaced by native strains [8], [12].

As demonstrated by Steckelberg [8], the probability of a selected strain persisting in a fermentation tank throughout the crop season is extremely low. The authors concluded that there are some advantages in using selected yeasts to start the fermentation process because these cells are highly adapted to the extreme conditions of tanks (i.e., high acidity, high alcohol content, high temperatures), but it is necessary to evaluate the cost of this strategy compared with the use of Baker's yeast. An alternative found by industries is to isolate the most well-adapted native strains and use them to initiate the next fermentation cycle [13], [3]. Selection depends on several characteristics of the fermentation process. It is important to consider not only ethanol yields but also characteristics that enhance cell survival under stress conditions. Dominance (abundance of one strain over others) and persistence (presence of yeast strains over time) are influenced by factors such as cell population kinetics, specific growth rate, and stress resistance [14].

This study aimed to assess the dominance and permanence of native strains isolated from an industrial process over two

crop seasons in three different industrial units to understand whether these yeast populations are able to survive until the end of the bioethanol production process.

II. Materials and Methods

Samples

Samples of fermented broth were collected during two consecutive seasons from three industrial units that produce bioethanol from sugarcane and its byproducts. Samples were collected at non-regular intervals, spaced no more than 40 days apart, during the 2018 and 2019 seasons. Samples were previously diluted in 0.9% saline solution and cultured on WLN differential medium (DIFCO no. 0424) supplemented with 100 ppm monensin for inhibition of bacterial growth. Plates were prepared by the spread-plate method and incubated at 32 °C for 7 days for selection of different colony morphologies. Biotype identification was based on the morphological characteristics of colonies (size, color, and texture). Different biotypes were subcultured, in duplicate, purified, and maintained on PDA (potato–dextrose agar) slants.

Yeast identification

Yeasts were identified molecularly by karyotyping. Chromosome isolation was performed by modifying the protocol proposed by Blond and Vezinhét [15]. Chromosomes were separated by pulsed-field agarose gel electrophoresis using a Bio-Rad CHEF III equipment. The gel was stained with ethidium bromide in TAFE solution (0.5 mL/L) and analyzed under ultraviolet light (UVP BioImagem System). The chromosomal profile was analyzed in duplicate for each biotype (colony morphology).

III. Results and Discussion

Unit A

Figures 1 and 2 show the dynamics of the yeast population in Unit A during the two crop seasons. Fermentation was started in the 2018 season using a native yeast strain isolated from the process in the previous year, labeled as SM584. This strain persisted in the tanks until the end of the season and showed interesting technological characteristics.

In the 2018 season (Figure 1), 19 different yeast strains were identified at some point of the fermentation process, including strain SM584 and 18 native yeasts. As can be seen in Figure 1, SM584 was the only strain detected in the first 50 days of the season and remained dominant until day 180. New native strains were identified in samples collected on days 75, 100, 120, 150, 180, and 235, but none was detected in more than one sampling. After 235 days, only SM584 and SM18 were present, the former in higher concentration.

Unit A started the 2019 fermentation process using native yeast isolated in the previous year (SM584). This strain persisted until the end of the season, demonstrating efficient technological characteristics and high persistence. In addition to SM584, other five strains inhabited the tank during 2019 (Figure 2). SM18 and SM12, which had been detected in the 2018 season, appeared after 180 and 210 days of fermentation in 2019, respectively, but at low concentrations. SM584 was the only yeast present up to 120 days of fermentation, remaining as the dominant strain until the end of the process. On day 150, SM584 and SM19 were detected in the tanks, the latter at a concentration of 37.5%. SM19 was detected on day 240, albeit at a lower concentration (12.2%). After 270 days, at the end of fermentation, only SM584 was found inhabiting the tank.

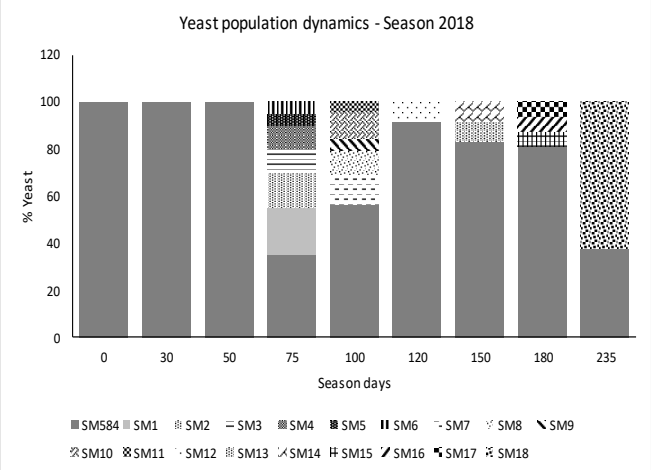

Figure 1: Yeast population dynamics in the fermentation process of Unit A during the 2018 season.

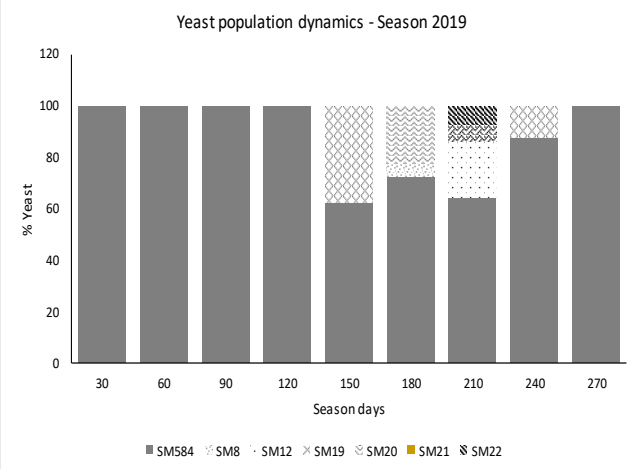

Figure 2: Yeast population dynamics in the fermentation process of Unit A during the 2019 season.

As demonstrated by the results, the native yeast isolated from the process was able to inhabit and dominate the fermentation tank throughout the season. It can be concluded that this type of strategy had a positive result for Unit A.

Unit B

Figures 3 and 4 show the dynamics of yeast populations in the studied seasons in Unit B. In the 2018 season, fermentation was started using native yeasts isolated from the process in the previous year, designated as RV2 and RV7. These strains remained until the end of the previous fermentation season, exhibiting efficient and persistent technological characteristics.

During the 2018 season (Figure 3), 12 different yeasts inhabited the tank, 2 of which originated from the inoculum itself (RV2 and RV7) and 10 of which were native. One strain was not of the genus *Saccharomyces*. RV2 and RV7

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remained as the only strains in the tank up to 30 days of fermentation, after which other yeasts became present; however, after 150 days, RV2 and RV7 were, once more, the only strains inhabiting the tank. At the end of the season, after 240 days of fermentation, RV2 was identified at a high concentration, sharing the tank with a non-*Saccharomyces* yeast. Of the 10 native yeasts that occupied the tank, only RV22 was detected on two sampling occasions. These findings indicate a large variation in yeast populations, which might not be interesting for fermentation processes.

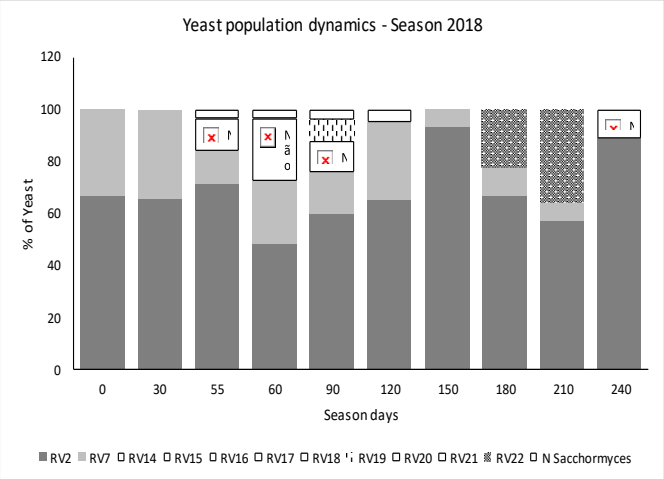


Figure 3: Yeast population dynamics in the fermentation process of Unit B during the 2018 season.

The unit started the 2019 fermentation process with native yeasts isolated from tanks (RV2 and RV7), which persisted until practically the end of the previous season. The strains showed efficient and persistent technological characteristics. In 2019, in addition to these strains, 14 other strains inhabited the fermentation tank. RV22, which was also detected in 2018, was identified on day 180 of fermentation at a concentration of 21.4%. RV2 and RV7 were unique for up to 30 days of fermentation, after which they shared the tank with other native yeasts. On day 180 of fermentation, of the two strains used as inoculum, only RV2 remained in the process with other native strains. On day 210, RV2 was detected at a very low concentration and RV31 began to dominate the tank.

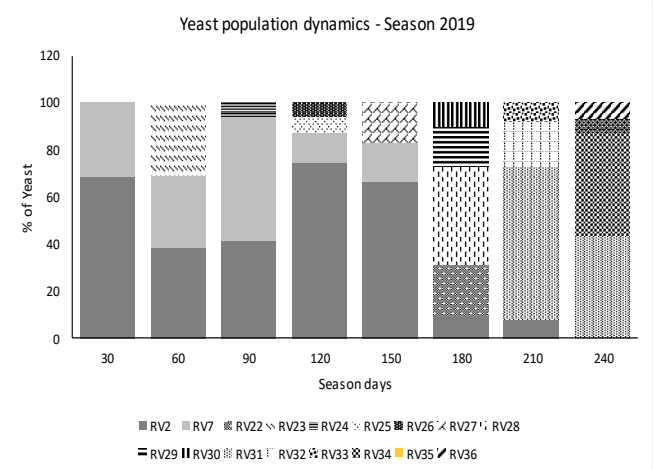


Figure 4: Yeast population dynamics in the fermentation process of Unit B during the 2019 season.

After 240 days of fermentation, RV2 and RV7 were extinguished from the process, and RV31, which appeared only after 210 days of fermentation, dominated the tank together with RV34.

In 2018, RV2 and RV7, which were used as inoculum, persisted throughout the entire season, with RV2 at higher concentrations. In 2019, however, both yeasts failed to remain in the tank until the end of the 240 days of fermentation. RV2 managed to inhabit the tank for the longest period (210 days), during most of which, the strain was predominant. RV7 persisted for 180 days, being dominant only on day 150; on day 180, it was no longer detected in the tank. The concentration of RV2 reduced drastically after 150 days of fermentation. It is concluded that, although yeasts used as inoculum (RV2 and RV7) did not remain until the end of fermentation, the strategy provided good results.

Unit C

Figures 5 and 6 show the dynamics of yeast populations in Unit C. The unit started fermentation with high concentrations of the selected yeasts PE2 and CAT1 as well as a native yeast isolated from the process in the previous year, designated as CEV1. The native yeast CEV1 remained in the process until the end of the previous season, as it had efficient technological characteristics and persistence.

In 2018 (Figure 5), a total of five yeasts inhabited the tank throughout the season, three originating from the inoculum and two native. CEV1 started at a low concentration, but, after 20 days of fermentation, it began to dominate the tank, persisting as the dominant strain until the end of the season. On days 85, 120, 145, 175, and 210, the native yeast (CEV1) was the only strain inhabiting the tank. At the end of the season, after 235 days, CEV1 was identified at a high concentration, sharing the tank with only one other strain. The selected yeasts, PE2 and CAT1, were completely eliminated from the process after 20 days of fermentation.

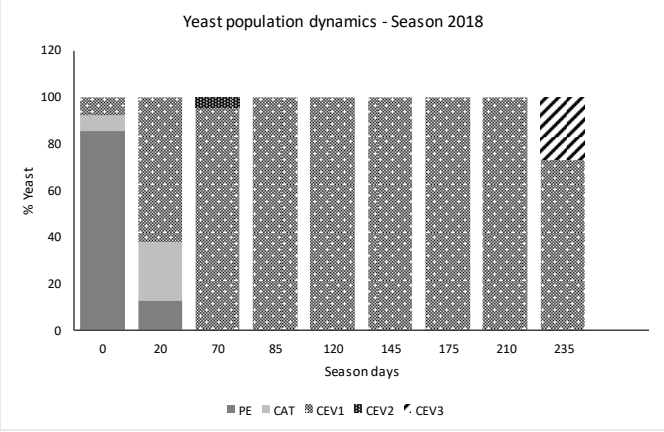
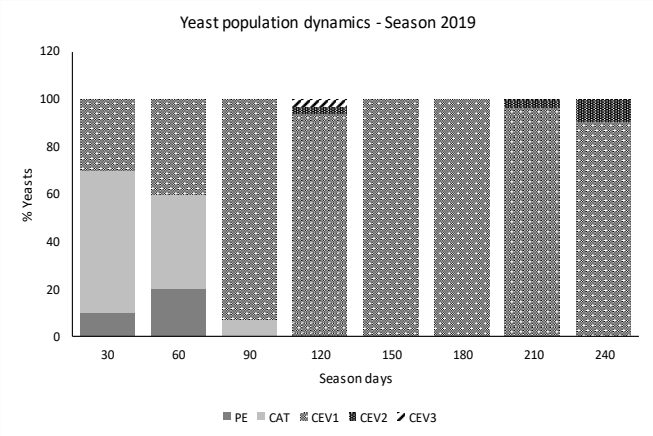


Figure 5: Yeast population dynamics in the fermentation process of Unit C during the 2018 season.

The 2019 fermentation process was started with selected yeasts PE2 and CAT1 and the native yeast CEV1. Similar to the previous year, five yeasts inhabited the tank, two native and three from the inoculum (Figure 6). After 60 days of fermentation, CEV1 had the same concentration as the

selected yeast CAT1. In 90 days of fermentation, CEV1 dominated the process, and, of the selected yeasts, only CAT1 was present. In 120 days of fermentation, the selected yeasts no longer inhabited the tank and CEV1 was the major strain. On days 150 and 180, only the native yeast CEV1 inhabited the tank. On days 210 and 240, CEV1 was the dominant strain, sharing the tank with CEV2 and CEV3, which were present at low concentrations. CEV2 and CEV3 were found



to inhabit the tank in the 2018 season.

Figure 6: Yeast population dynamics in the fermentation process of Unit C during the 2019 season.

As demonstrated by the results, the native yeast isolated from the process was able to inhabit and dominate the fermentation tank throughout the season. This strategy had a positive result in Unit C.

Starting fermentation with naturally selected yeasts (i.e., native yeasts) has some advantages compared with other methods, given that these yeasts are highly adapted to the austere conditions of fermentation tanks, such as high acidity, high alcohol content, and high temperatures.

IV. Conclusion

Using native yeast strains as inoculum to start the fermentation process is more promising than using selected, commercial strains, such as PE2 and CAT1. Selected yeasts have been recommended for industrial fermentations to ensure a more efficient fermentation process [10]. However, as argued by Steckelberg [8], the likelihood of a selected strain persisting throughout the fermentation season is low. The use of yeasts isolated from the process (native strains) is recommended [16], supported by the fact that the native yeasts in the three studied units were able to persist and remain dominant throughout the season. Yeast populations vary according to season, even when using strains isolated from fermentation tanks.

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