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Performance of a Customized Yeast Strain Selected from an Industrial Process and Used as Inoculum: A Case of Success

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***Abstract*—This study monitored the population dynamics of yeast strains in industrial ethanol fermentation tanks throughout the 2019 crop season. The aim was to select a native strain with good fermentative performance for use as inoculum in the 2020 season. The custom strain RAA5 showed favorable characteristics for fermentation (ethanol yield > 0.465, conversion >90%, biomass yield > 0.045%) as well as the ability to persist throughout the entire process. Monitoring of fermentation in the 2020 season confirmed that the custom yeast strain is robust, being detected in virtuallyall collections and dominating the tank in some months. It was concluded that the custom yeast selection strategy enhances the stability of alcohol fermentation by ensuring the permanence of the yeast strain or its reintroduction by raw materials.**

***Index Terms*— yeast, bioethanol, fermentative capacity, alcoholic fermentation**

I. Introduction

Brazil was the world's leading producer of bioethanol until 2005, when it lost the top position to the United States of America. Currently, Brazil is the second largest ethanol producer, owing to advances in production technology, extensive arable lands, and favorable climatic conditions. Brazil is also the world's largest producer of sugarcane, which is the most efficient raw material for ethanol fermentation. Ethanol production from sugarcane juice is well-established in the country. These factors, together with the growing global demand for ethanol, make Brazil highly competitive in the international market. Furthermore, the Brazilian energy matrix is a prominent example of sustainability: 42.9% of the primary energy produced in Brazil comes from renewable sources [1].

Global biofuel production has grown steadily over the past decade, from 16 billion liters in 2000 to 27 billion liters in 2021. Fossil fuels are predicted to be increasingly replaced by biofuels. By 2050, it is estimated that 25% of the world's transportation energy will come from renewable sources [2], [3].

A diversity of species from the genus *Saccharomyces*are used as fermentation agents in Brazilian distilleries. Of these, the most commonly used strains belong to the species *Saccharomyces cerevisiae*.According to Del Rio [4], yeasts must exhibit certain characteristics to be efficient ethanol fermenters, such as a high rate of sugar fermentation, determined from the amount of sugar converted to ethanol in a given time. The higher the rate of fermentation, the higher the productivity, which leads to increased daily production, reduced costs, and reduced risks of process contamination by undesirable microorganisms. Another interesting characteristic of fermentative yeasts is tolerance to alcohol concentrations above 10% (w/v), given that low tolerance results in low ethanol yields and productivity during industrial fermentation. In addition to these factors, resistance to contaminants, population dominance, and physiological stability to withstand variations in process conditions are fundamental [5].

Yeast strains isolated from Brazilian industrial plants have been widely applied in sugar and alcohol production, such as the *S. cerevisiae* strains BG-1, SA-1, CAT-1, PE-2, and Y-904 [6], [7]. Combinations of two or more of these strains are used as starter cultures at the beginning of the crop cycle. At the end of the season, the prevailing population consists of the best adapted yeast cells, which might be either wild or commercial yeasts.

Monitoring of yeast population dynamics during alcohol fermentation can provide valuable information for process optimization. Collected data can be used for the selection of native yeasts with high fermentative potential as well as to adapt process conditions to the requirements of fermentative microorganisms through process design, improvement projects, and correction of operational procedures [8].

Andrietta [9] proposed a differentiation system for yeast strains based on their fermentative potential or fermentative capacity [10]. The fermentative capacity of yeasts is determined from kinetic studies conducted in synthetic medium. From the results of ethanol production, fermentable sugar consumption, and cell biomass production, it is possible to calculate the following parameters: $Y_{x/s}$, cell yield (g dry weightg^{-1} fermentable sugar consumed); $Y_{p/s}$, ethanol yield (g dry weightg^{-1} fermentable sugar consumed); P, productivity (g ethanolL^{-1}h^{-1}); k, rate of substrate consumption (g fermentable sugar consumedL^{-1}h^{-1}); C, conversion (%);P_{sp}, specific productivity in relation to cell biomass production; and k_{sp}, rate of substrate consumption per unit weight of cell biomass production. Although all these parameters can provide a better understanding of the performance of yeast strains, two are crucial for yeast selection in distilleries, namely ethanol yield ($Y_{p/s}$) and conversion (C). Cell yield ($Y_{x/s}$) also has significant importance because the higher the value of this parameter, the greater the chance of a yeast strain dominating the process. Yeast strains are considered to have superior fermentative capacity when they achieve an ethanol yield greater than 0.465 and conversion greater than 90%. As for biomass yield, values above 0.045 indicate that the strain is likely to survive or become dominant during the process.

This study aimed to monitor and select custom yeasts from an ethanol fermentation process through analysis of population dynamics and fermentative performance in the 2019 season at an industrial unit located in the Midwest region and apply the selected strain as inoculum in this same unit in 2020 to assess its ability to survive throughout the crop season.

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II. Materials and Methods

Samples

Samples were collected from an industry that manufactures ethanol from sugarcane and byproducts, at a unit operating with continuous cell recycling in São Paulo State, Brazil. The unit started the season using a mixture of yeast strains as starters (CAT, FT1736, FT858, and PE). Seven samples were collected at 30-day intervals during the 2019 season. Samples were previously diluted in 0.9% saline solution and cultured in WLN differential medium (DIFCO # 0424) supplemented with 100 ppmmonensin for bacterial inhibition. Plates were prepared by the spread-plate method and then incubated at 32 °C for 7 days. Biotypes were identified based on colony morphology (size, color, and texture). Different biotypes were subcultured in duplicate, purified, and maintained on potato dextrose agar slants.

Yeast identification

Yeasts were molecularly identified by karyotype analysis. Chromosome isolation was performed according to the protocol proposed by Blond and Vezinhét [11], with modifications. Chromosomes were separatedby pulsed-field agarose gel electrophoresis using a CHEF III (Bio-Rad) equipment. The gel was stained with ethidium bromide in TAFE solution (0.5 LmL^{-1}) and analyzed under ultraviolet light (UVP Bioimaging System). The chromosomal profile was analyzed in duplicate for each biotype (colony morphology).

Fermentative capacity

The parameters were determined by mass balance calculations. Fermentation was carried out in 250 mL Erlenmeyer flasks containing 100 mL of sterile culture medium under controlled temperature (32 °C) and agitation (150 rpm) conditions for 24 h. The culture medium was composed of the following (per liter): 150 g of glucose, 5 g of KH_2PO_4, 5 g of NH_4CL, 1.0 g of $MgSO_4{\cdot}7H_2O$, 1 g of KCl, and 6 g of yeast extract. For parameter estimation, dry weight, ethanol content, and sugar contents were determined, as described below.

Dry weight determination

Briefly, a 10 mL aliquot of fermented brothwas centrifuged at 4000 rpm for 5 min. The material was washed with distilled water, re-centrifuged three times, weighed on a previously tarred plate, and oven-dried at 55 °C. Dry weight was determined as the difference between initial and final weights.

Determination of ethanol and sugar (sucrose, glucose, and fructose) contents

A 10 mL aliquot of fermented broth supernatant was diluted 1:10 for detection of sugars (2.5 to 50 g L^{-1}) and ethanol (0.2 to 2°GL). The sample was filtered through a PVDF membrane filter and analyzed by high-performance liquid chromatography (HPLC-IR). HPLC-IR was conducted in a Waters system connected to a refractive index detector and an Aminex HPX-87H analytical column (300 mm x 7.8 mm, 5 µm, Bio-Rad). The column temperature was 40^0C. The mobile phase consisted of 5mmol $L^{-1}H_2SO_4$ used at a flow of 0.6 ml min^{-1} using an injection volume of 15 µL. A calibration curve was obtained by using standard solutions at concentrations of 0.25, 1.25, 2.5, 3.75 and 5.0 g/l of sucrose, glucose and fructose with correlation coefficient (*R*) above 0.999.

III. Results and Discussion

Fermentation was started using a mixture of CAT1, PE2, FT1736, and FT858 strains. In total, nine different yeasts inhabited the tank during fermentation, four of which were inoculum yeasts and five of which were native yeasts. The incidence of each strain, including inoculum yeasts, and the dynamics of yeast populations during the 2019 season are presented in Table 1 and Fig. 1, respectively.

Table 1. Incidence of yeast strains during fermentation in the 2019 season.

Day	No. of strains	Strains
0	3	FT1736//FT858/RAA5
30	4	CAT/FT1736/RAA5/RAA6
60	5	RAA5/CAT/RAA7/RAA8/FT1736
90	4	CAT/RAA5/PE/RAA9/
120	2	CAT/RAA5
150	2	CAT/RAA5
180	2	RAA5/CAT

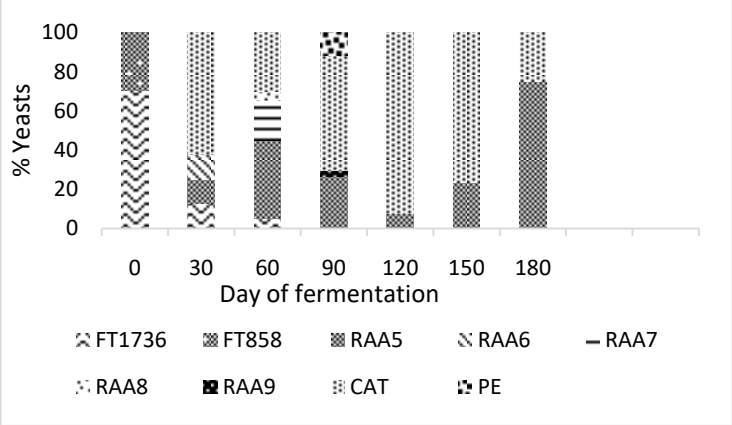

Fig. 1. Yeast population dynamics in the 2019 season.

Table 2 shows the incidence of native strains during fermentation. Wild yeasts were detected throughout the process, but, as shown in Table 1, RAA5 was detected in all samplings.

Table 2. Incidence of native yeasts in the 2019 season.

Yeast	Incidence	Day	%
		0	12.3
RAA5	7	30	12.3
		60	40.0
		90	26.5
		120	7.7
		150	23.5
		180	75.0
RAA6	1	30	12.5
RAA7	1	60	20.0
RAA8	1	60	5.0
RAA9	1	90	2.9

Table 3. Fermentative capacity of native yeasts.

Strain	$Y_{x/s}$	$Y_{p/s}$	P	k	C	P_{sp}	k_{sp}	Best parameters
RAA5	0.0480	0.4615	2.5442	5.8833	91.26	0.4008	0.8739	$Y_{x/s}$ and C
RAA6	0.0499	0.4522	2.3536	5.5548	88.62	0.3781	0.8415	$Y_{x/s}$
RAA7	0.0548	0.4486	2.3299	5.5421	86.31	0.3416	0.7660	$Y_{x/s}$
RAA8	0.0525	0.4508	2.3122	5.4732	85.31	0.3584	0.7999	$Y_{x/s}$
RAA9	0.0465	0.4592	2.4806	5.7648	89.55	0.4127	0.9041	$Y_{x/s}$

$Y_{x/s}$, cell yield (g dry weight g^{-1} fermentable sugar consumed); $Y_{p/s}$, ethanol yield (g dry weight g^{-1} fermentable sugar consumed); P, productivity (g ethanol L^{-1} h^{-1}); k, rate of substrate consumption (g fermentable sugar consumed L^{-1} h^{-1}); C, conversion (%); P_{sp}, specific productivity in relation to cell biomass production; and k_{sp}, rate of substrate consumption per unit weight of cell biomass production.

At the end of 2019, all yeasts inhabiting the tanks were subjected to fermentation for analysis of fermentative capacity. The results are described in Table 3.

The results of the fermentation capacity assay suggest that both RAA5 and RAA9 may be used as inoculum, as they were classified as superior. However, it is necessary to investigate the persistence of yeasts during fermentation. RAA5 was found to have good fermentative performance as well as the ability to persist in tanks. The strain was identified throughout the season in all collections, even if not as the dominant yeast. Of note, RAA5 was found to have a light flocculation capacity. This form of growth has been constantly observed in industrial lineages, necessitating further evaluation. Although RAA6, RAA7, and RAA8 produced abundant biomass, they are not indicated to be used as inoculum because of their low conversion capacity.

The 2020 fermentation season was started using a mixture of FT1736 and the custom yeast RAA5. Throughout the season, a total of nine yeasts inhabited the tanks, two of which had been used as inoculum (FT1736 and RAA5) (Table 4).

Table 4. Incidence of yeasts during fermentation in the 2020 season.

Day	No. of strains	Strains
0	2	FT1736/RAA5
30	5	FT1736/RAA10/RAA11/RAA12/RAA13
60	1	FT1736
90	1	RAA5
120	1	RAA5
150	3	RAA5/RAA14/RAA15
180	1	RAA5
210	1	RAA5
240	2	RAA5/RAA16

RAA5 was detected on day 0 and then only after 90 days of fermentation. There are two hypotheses for this result. The strain might have been present at low concentrations, thereby not being detected on days 30 and 60,or it might have been reintroduced from raw materials. Given that the strain quickly dominated the tank, the second hypothesis seems to be the most likely.

Fig. 2 depicts the dynamics of yeast populations in the 2020 season.

From the results of Table 5, we can conclude that the custom yeast RAA5, selected in the 2019 season and used as inoculum in the 2020 season, is robust. Although the strain was not detected in two samplings (days 30 and 60), probably because it was at low concentrations, it reappeared and eventually completely dominated the tank, as observed on days 90, 120, 180, and 210. At the end of the season, it shared the tank with another wild yeast but continued to be dominant.

Table 5. Incidence of native yeasts in the 2020 season.

Strain	Incidence	Day	%
		0	25.0
RAA5	7	30	0.0
		60	0.0
		90	100.0
		120	100.0
		150	23.8
		180	100.0
		210	100.0
		240	80.0
RAA10	1	30	21,3
RAA11	1	30	6.3
RAA12	1	30	4.3
RAA13	1	30	4.3
RAA14	1	150	66.7
RAA15	1	150	9.5
RAA16	1	240	20.0

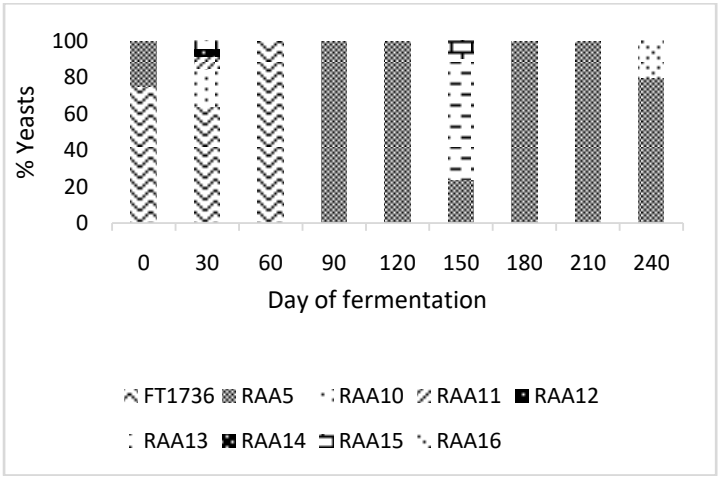

Fig. 2. Yeast population dynamics in the 2020 season.

The native yeasts found in the 2020 season had not been detected in 2019. No native yeast persisted for more than one sampling date in 2020.

Steckelberg [12] observed that using custom yeasts as inoculum is more promising than using selected strains available in the market, such as PE, CAT, and FT.

In view of the results, we conclude that selection of custom strains through monitoring and characterization of industrial units and subsequent use of these strains as inoculum in the same units contributes to high fermentation stability, given

that yeasts persist throughout the season or are reintroduced from raw materials.

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