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Yeast Strains Inhabiting an Ethanol Production Process with Cell Recycling: Own Dynamics?

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***Abstract*—Yeast cell recycling in ethanol production processes is a recurring practice at industrial units installed in Brazil. Units usually use yeasts selected from other industrial processes to start up the season. These yeasts are acquired in large amounts to guarantee a quick start-up by yeast with good fermentative performance. This work examined yeast population dynamics during an entire season at an industrial unit using a select yeast strain (CAT) as inoculum to start up the process. The unit also artificially introduced, in the course of the season, two other yeasts, one select (FT 858) and the other isolated from its own process in previous seasons. Yeast identification was carried out using the karyotyping technique. The results suggest that none of the select strains was able to remain until the end of the season. Strain SM584, introduced during the final months of operation, was the one that remained at the end of the season. The data suggest that each fermentation process has its own dynamics with regards to the yeast inhabiting the process. Biotic and abiotic factors, translated into facilities associated to the type of yeast naturally inhabiting the feedstock, favor the installation of yeast capable of floculating at the unit studied, since out of the 21 yeasts installed during the process, 18 presented some type of floculating characteristic.**

***Index Terms*—Alcoholic fermentation, bioethanol, Saccharomyces sensu stricto, Yeast.**

I. Introduction

Brazil is one of the countries with greatest presence of liquid bioenergy in the country's transportation matrix. In 2020, renewable energy represented 48.4% of the Brazilian energy matrix, while in the rest of the world only 14%, undoubtedly a source of pride for the country [1]. The advance towards the use of renewable sources in the Brazilian energy matrix was a result of the introduction of the Proálcool Program created by the government to replace oil by ethanol in view of the worldwide crisis of this fossil fuel.

Today there are 422 industrial units processing sugar cane in Brazil [2], with the greatest majority of them producing both sugar and ethanol. In the 2021 season, Brazil had produced 27,000 thousand m^3 of ethanol by November [2].

The most widely used ethanol manufacturing process at the Brazilian units, regardless of the operation method (continuous or batch-fed) is based on yeast cell recycling. Each fermenation cycle, the fermented must passes through a centrifugation unit that separates yeast cells from the fermented wine. The yeastless wine is sent to distillation and the yeast cream goes through acid treatment to control bacterial contamination, returning to the process to start a new fermentation cycle [3]. The industrial units start the season with large amounts of yeast cells measured according to the unit size and crush volume for yeasts beloning to the *Saccharomyces* genus. In addition to a quick start-up, this strategy ensures that yeast not adapted to fermentation conditions will be able to install in the process. Yeast used in the start-up process originates from baker's yeast or the sugar and ethanol process itself. At present, select yeast sold in large amounts are those of the *Saccharomyces* strains PE-2; CAT-1; BG-1; SA-1; FT 858L and Fermel. Some industrial units, to start up the season, propagate the yeast in their own laboratories.

Since the must to be fermented does not suffer any treatment to eliminate the native microbiote carried into the process, the yeast used as inoculum, regardless of its origin, will need to compete with yeasts and bacteria found in the must. The presence of non-*Saccharomyces* yeasts in ethanol processing line has been extensively reported [4], [5], [6], [7]. Even though the presence of these yeasts is undesirable in the process, since they can cause a series of problems – including yield drops, foam formation and inhibiting formation of products – , they are not the ones that cause the greatest concern. Other yeast strains, belonging to the *Saccharomyces* genus with some specific characteristic unsuitable to the process, are the ones that may cause significant losses to fermentation. Since they are of the *Saccharomyces* genus, they are able to withstand extreme process conditions, competing on equal terms with the strain inoculated at the beginning of the season.

Among the special characteristics found in the *Saccaromyces* strains is the flocculation capacity, which is highly relevant. Yeast flocculation can be defined as a nonsexual, homotypic (involving only one type of cell in the interaction), reversible (flocs can be reversible dispersed by the action of EDTA or specific sugar like mannose) and multivalent process of aggregation of yeast cells into multicellular masses called flocs, with the subsequent rapid sedimentation from the medium in which they are suspended [8]. This characteristic does not disqualify the *Saccharomyces* strains in terms of their ability to ferment, as many researchers explore this sedimentation characteristic of the yeasts as an alternative to replace the centrifuges for the cell separation process of the fermented wine [9], [10], [11], [12]. The problem associated with the presence of this yeast is in the absence of homogeneity due to floc formation in the fermented must. In traditional processes, must needs to be centrifuged so that yeast cells separate from wine and return to the process after an acid treatment. The heterogeneity in the liquid resulting from the flocs compromises centrifugation. It is rare to identify the presence of flocculating yeasts, which are introduced into the process by the must and end up cohabiting

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the process with yeasts that do not essentially have this property. Within this context, this work assessed the incident of essentially flocculent strains during the 2014 season at an industrial fermentation unit that started the season with two select strains (FT 584 and CAT), which were introduced in the process at distinct

periods.

II - MATERIAL AND METHODS

A. Samples

The samples were collected from an ethanol production process from sugarcane and byproducts in a unit operating continuously with cell recycling in the State of São Paulo, Brazil. The unit started the season with the CAT strain and injected the FT858 strain over the season (around 90 days into the season). Both strains used are selected from ethanol production units and representatives of the *Saccharomyces sensu stricto*. Samples were collected at non-regular intervals, not longer than 40 days during the 2014 season.The samples were previously diluted in 0.9% saline solution and cultivated in WLN differential medium (DIFCO # 0424) supplemented with 100 ppm of monensin for inhibition of bacteria found in the samples. The surface-spreading technique was used. Plates were incubated at 32°C for seven days for selection of different colony morphologies. The distinction of biotypes was made based on the morphological differentiation of the colony. The parameters used were size, color and texture. Different biotypes were, in duplicate, purified and maintained in PDA slant (Potato dextrose agar).

B. Yeast Identification

Yeasts were identified molecularly through the karyotyping technique. Chromosome isolation was made by modifying a protocol proposed by Blond and Vezinhét [13]. Chromosomes were spread using agarose gel in pulsed-field electrophoresis in CHEF III (Bio-Rad) equipment. The gel was colored with ethidium bromide prepared in a TAFE solution (0.5 l/ml) and analyzed under ultraviolet light (UVP BioImagem System). The chromosomal profile was made in duplicate for one of the different biotypes (colony morphology)

Assessment of the flocculation capacity. Strains were cultivated in synthetic medium with the following composition: 160 g/L sucrose, 6.0 g/L yeast extract, 5.0 g/L monobasic potassium phosphate, 1.5 g/L ammonium chloride, 1.0 g/L hepta hydrated magnesium sulphate and 1.0 g/L potassium chloride. Cultivation conditions were 24h/32°C/150rpm. Yeast behavior in terms of growth was visually observed and described as follows: (a) no flocculation; (v) plume flocculation (similar to cotton lint in suspension); (c) light flocculation (similar to grains of sand, forming small pellets when in suspension); and (d) heavy flocculation (aggregation of cells in a way that the liquid becomes translucid with large pellets).

III. RESULTS AND DISCUSSIONS

Table 1 presents the total concentration of yeasts present in the fermentation tanks during the 2014 season, which lasted approximately 192 days from May to November. The concentrations found were expected for fermentation tanks, that is, between 10^8 and 10^9 yeast cells.

Table 1 - Total yeast cell population (CFU/ml) during the 2014 season

Population (CFU/ml)	Season day				
	10	**38**	**60**	**79**	**92**
	2.0×10^8	9.0×10^8	1.6×10^9	2.6×10^9	1.3×10^9
	Season day				
	96	**120**	**145**	**176**	**192**
	1.0×10^9	1.0×10^9	2.2×10^8	1.1×10^9	2.0×10^9

Figure 1 presents the yeast population dynamics from May to December, when ethanol was produced. A total of 24 strains inhabited the process during the season, of which 21 were native strains. The season started with the select CAT strain, which remained as the sole strain for 60 days in the season (until June). At 79 days into the season (July), CAT represented 46.2% of the yeast population. Three different native strains L2, L3 and L4, inhabited the tank with CAT, respectively representing 30.8, 15.4 and 7.9% of the yeast population in the tanks. All native yeasts presented flocculating characteristics, with L2 presenting a heavy flocculation profile and L3 and L4, a light flocculation profile. At 92 days into the season (July), the configuration of yeasts inhabiting the tanks changed completely. After only thirteen days, it was not possible to identify any of the yeasts previously present. At that point, four new yeast strains cohabited the tanks with the following distribution: 38.5% of L5, 26.9% of L6, 26.9% of L7 and 7.7% of L8, all of which with heavy flocculation characteristics. Since 100% of the population constituted of heavily flocculating yeasts, the industrial unit, aiming to tackle operational problems, artificially introduced select yeast FT858 at 96 days into the season and this strain represented 80.8% of the yeast population, sharing the tank with L6 strain, which started to represent 19.2% of the population (in previous collection it represented 26.9%). At 120 days into the season (August), FT858 was no longer found in the tanks. The unit made another interference in yeast dynamics by introducing a select yeast from previous seasons at the unit, referred to as SM584.

This yeast represented 19.4% of the population. The other portion of the population is represented by four native yeast strains: L5 (2.9%), L8 (9.7%), L9 (48.6%) and L10 (19.4%). Among these only L5 had been present in previous collections (38.5% at 120 days into the season), with the other four (L8, L9, L10 and L11) present for the first time, with all of them presenting heavy flocculation characteristics. At 145 days into the season (September), the yeast population configuration shows the presence of eight different strains. Only one among them, L6, had been present previously. At 92 and 96 days, it represented 26.9% and 19.2%, respectively. In that period (145 days into the season) there was no dominant strain, since none of the strains was found at concentrations higher than 20%. The concentrations found for the strains were: 13.7; 18.2; 18.2; 18.2; 13.7; 9.0; 4.5 and 4.5 for strains L6, L11, L12, L13, L14, L15, L16 and L17, respectively. It is possible to notice that in that period (145 days into the season) 72.8% of the yeast population presented flocculation characteristics, since strains L6, L12, L13, L14, L16 and L17 grow in a flocculated way, with only L17 presenting plume flocculation characteristics, while the others had heavy flocculation. Strains L11 and L15, which were not flocculating, represented together 27.2% of the total yeast population.

At 176 days into the season (October), yeast population was completely different from that found thirty days before. The population now consisted of five strains. Three of them have showed up for the first time (L18, L19 and L20), while the others had already been present at some point in the season. L5, representing 10% of the population, was detected at 92 days into the season, where it represented the majority of the population (38.5%) and, at 120 days into the season, when it had an unsignificant presence, that is 2.9%, and L8, which was the dominant strain in this period, since it represented 40% of the population. It was present at 92 days into the season (7.7%) and at 120 days (9.7%). Even though the inhabiting yeasts at this point (176 days) were different from those found in the previous collection (145 days), the percentage of flocculating yeasts is similar. Yeast L18, representing 30% of the population did not have flocculating characteristics, whereas the other 70% of yeasts in the tanks had the following distribution: L5 – 10%; L8-40%, L19-10% and L20-10%, all of them heavy flocculent strains.

The season ends at 192 days (November) with the indisputable presence of SM 584 strain, introduced in August and that came up again in the tanks as dominant, representing 70% of total yeast inhabiting the fermentation. The other 30% are represented by heavy flocculating yeasts that appeared in the process for the first time. L21 represents 25% of the population and L22 represents 5% of the total.

Figure 2 presents the flocculation distribution during the 2014 season. It is possible to notice that only at the beginning of the season, when CAT was used as inoculum, the presence of flocculating yeasts was not identified. From the collection at 79 days into the season, we note the presence of flocculating yeast strains, which become the majority in the process, except when FT858 is artificially introduced in the process (96 days). The season ends with the dominance of SM584, the non-flocculent strain.

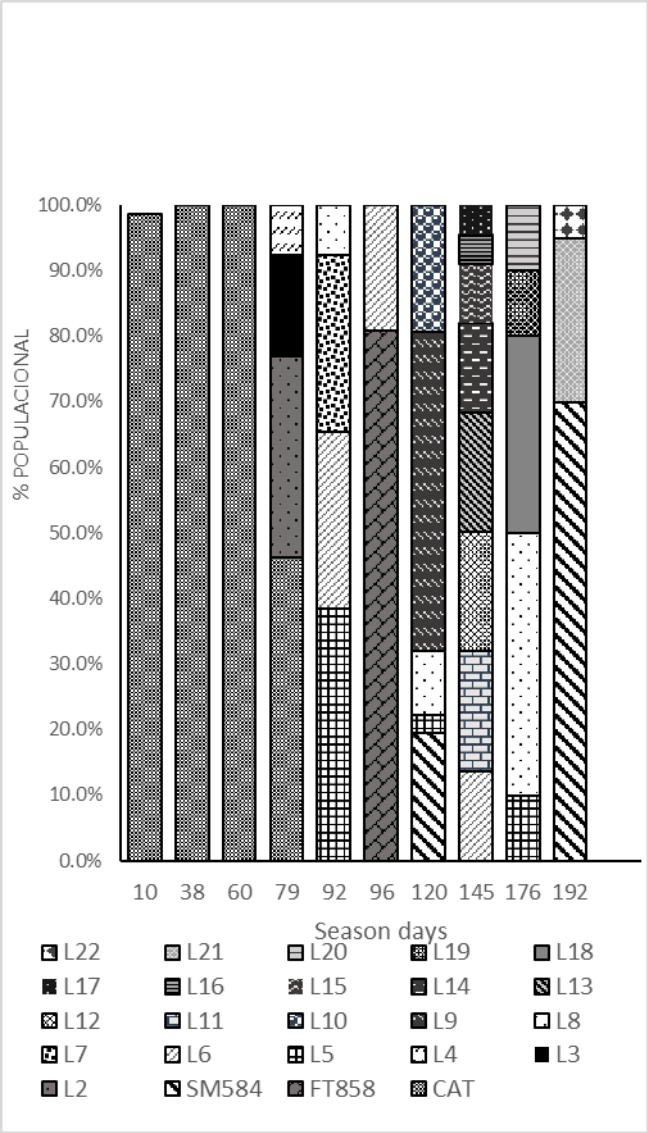

Figure 1: Figure 1 – Yeast strain population dynamics during the 2014 season

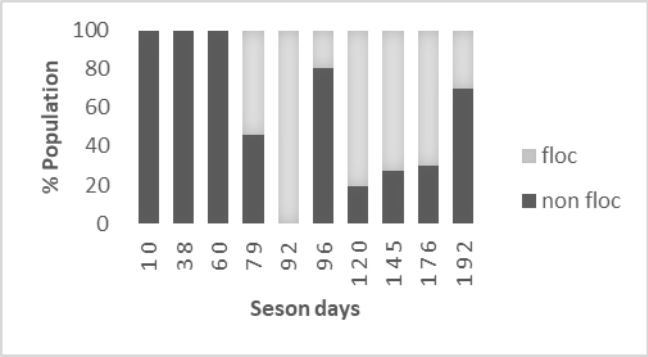

Figure 2: Flocculation profile during the 2014 season

IV. RESULTS AND DISCUSSION

The data suggest that each fermentation process has its own dynamics with regards to the yeast inhabiting the process. In this case, biotic and abiotic factors, translated into facilities associated to the type of yeast naturally inhabiting the feedstock favor the installation of yeast capable of flocculating. At the start of the season, when sugar cane is not

yet in its ideal condition, thus carrying less diversity in terms of native yeasts, the select CAT yeast was capable to remain as the sole yeast in the process. From the moment native yeasts started to install in the process, the CAT strain was completely eliminated and replaced by native strains with emphasis in flocculating yeasts. The unit intervened twice in the microbiote of the tanks expecting to eliminate flocculating yeast population, since its presence is a factor that significantly interferes with centrifugation. The first intervention was with the introduction of the select strain FT858, which, even though it once represented 80% of the population when inserted in the process (96 days into the season), it was unable to remain, even at low concentration in the subsequent collection (120 days). In the second intervention, the strain introduced was an isolated yeast from the tanks at the unit in previous seasons, and is referred as SM584 and does not have flocculating capacity. This yeast, introduced at 120 days into the season, it was not detected in the following two collections, but it reappears as dominant in the last collection, representing 70% of the population. It is not possible to state whether this yeast remained in the process at lower concentrations to those detected by the method used in this work, since this is a native yeast that could have been naturally introduced in the process carried by the feedstock.

Native yeasts L5, L6 and L8 appear in one more collection, but intermittently. Even though this is possible, since yeasts originate from the feedstock and may be introduced in different periods, the same consideration needs to be made for the discontinuous detection of SM584 strain. These yeasts may be present at other collections, but due to the method employed, they were not detected. In spite of this deficiency in this method, it is the safest among those available, since it is able to detect the presence of yeasts at 4% concentrations. The presence of yeasts at concentrations lower than 10% is not relevant as control parameter.

One particularity found for this industrial unit is the diversity of the yeasts found during the season. At total of 21 native strains were identified, with only 14% not presenting flocculating characteristics (L5, L6 and L8). Flocculating yeasts represent 86% of native yeasts present in the tanks, with emphasis to heavy flocculation. Out of the 18 that grow in a flocculating way, 3 have plume flocculation and the other 15 heavy flocculation. Several interferences made during the season, to replace the flocculating native strains by select yeasts may have also contributed for the high number of native strains inhabiting the process in the season.

The search to eliminate the flocculating native yeasts at this unit included the use of SM584 yeast at the start of the subsequent seasons. The results, not yet published, suggest that the unit achieved higher stability with the use of this strain in the process, since this yeast remained until the end of the season.

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