International Journal of New Technology and Research (IJNTR)
ISSN:2454-4116, Volume-3, Issue-6, July 2017 Pages 69-71

Dominance and Persistence of PE strains (*Saccharomyces* Sensu Stricto) in Brazilian Bioethanol Fermentation Tanks (One Unit Four Seasons)

Claudia Steckelberg, Patricia R. Kitaka, Silvio R. Andrietta, Maria G.S. Andrietta

***Abstract* -The use of select yeasts as inoculum at the start of the season of bioethanol production has been a recurring practice in Brazilian distilleries. The Saccharomyces sensu stricto strain mostly used to start the process in distilleries is know as Pedra (PE). In this work, we assessed four different seasons (2009, 2010, 2011, 2012) at the same unit, which started the process with the PE strain in all years. The dominance and persistency capacity of the PE strain varied from season to season, but in none of the seasons we noted the presence of this strain the end of the activities, even though this strain was found to have considerable capacity to remain in the process. The data presented in this study elucidate that one selected strain is replaced by the other, or even by more than one originated from feedstock. We have also noted that yeast dynamics behavior in tanks is variable from season to season, suggesting the influence of feedstock in yeast selection.**

***Index Terms* - Alcoholic Fermentation, Bioethanol, Saccharomyces Sensu Stricto, PE Select Yeast.**

I. Introduction

Bioethanol and biodiesel are the main biofuels presently used worldwide, with the first occupying a privileged position in Brazilian economy [1]. This position is due to the creation, in the 1970s, of a program aimed at replacing gasoline with ethanol, as a result of the gas crisis at the time. The former National Alcohol Program – Proalcool - placed Brazil at the forefront, and ethanol was seen as an alternative for fueling the Brazilian fleet. Ethanol production as a sustainable fuel should also be highlighted, since it promoted major environmental gains, including reduction in emission of greenhouse gases and high efficiency of CO_2 capture and fixation from the environment by sugarcane itself [2].

The largest ethanol producing region in Brazil, the South Central, reached a production of approximately 26 billion liters of ethanol in 2016 according to UNICA [3], standing as the world's second largest producer in terms of amount produced, but as the world's leader in ethanol production from sugarcane. This prominent position in the international scenario has led the Brazilian government to invest significantly in research that meets the demands of the sugar and energy sector. From this research, it has been possible to understand the dynamics of yeast population inhabiting fermentation tanks. Presently, it is known that the yeast used to start the season is rapidly replaced by yeasts found in feedstock [4], [5]. This finding was possible only thanks to the introduction of molecular biology techniques in the sugar and ethanol sector [6], [4], [5], [7]. Among these techniques, we highlight karyotyping, which is capable of separating different yeast strains from the same species. Professor Luiz Carlos Basso (ESALQ-USP) was a pioneer in the use of this technique and the main person responsible for the understanding that the yeast inhabiting ethanol production processes is not the same introduced as inoculum at the start of the season. Drawing on this knowledge that yeasts inhabiting fermentation tanks are indigenous, a new strategy was adopted in the 1990s: identifying *Saccharomyces cerevisiae* strain isolates in fermentation tanks presenting combinations of various characteristics of commercial interest. Among them were high fermentation efficiency, high ability for completion and survival in industrial environment [8], [4]. Now yeasts referred to as indigenous for their feedstock origin are known as select. Select yeasts are understood as being those isolated from industrial fermentation processes. These yeasts belong to the *Saccharomyces* genus, which allows us to assume that they are comprised in the *Saccharomyces sensu stricto* group, created to encompass the majority of relevant yeasts in the fermentation industry as well as those used in basic science [9]. Initially, select yeasts were those first isolated, such as strains CAT1 PE2, SA1 e BG1 [5]. Strain PE-2 is currently known only as PE.

In the first decade of the 21st century, the PE-2 strain is estimated to have been responsible for the production of almost half of all Brazilian ethanol and about 10% of the world's ethanol [10]. The PE strain, isolated from an industrial unit named Usina da Pedra, thus the reference PE, was identified in the beginning of the 1990 through the separation technique of intact chromosomal DNA by electrophoresis, also known as karyotyping [1].

This work was carried out to assess the behavior of the PE *Saccharomyces sensu stricto* strain with regard to its dominance and persistence ability at a Brazilian bioethanol unit, which used this yeast as process inoculum during four consecutive seasons.

Claudia Steckelberg, CPQBA, Universidade de Campinas, São Paulo, Brasil.
Patricia Kitaka, CPQBA, Universidade de Campinas, São Paulo, Brasil.
Silvio R. Andrietta, Biocontal, Engenharia de Bioprocessos, São Paulo, Brasil.
Maria da Graça S. Andrietta, CPQBA, Universidade de Campinas, São Paulo, Brasil.

II - MATERIAL AND METHODS

A. Samples

The work was carried out with samples from a fermentation process using sugarcane must. The unit under study routinely starts fermentation using the PE strain as inoculum. This same unit was assessed during four seasons (2009, 2010, 2011 e 2012). The sample number varied based on season duration of the year in question. To wit: ten samples for the 2009 season and nine samples for the 2010, 2011 and 2012 seasons. 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 [11]. 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, made in duplicate for one of the different biotypes (colony morphology) isolated in each collection was compared with the PE strain profile, as well as between themselves. Indigenous yeasts in different seasons were not compared in this work.

III. RESULTS AND DISCUSSIONS

Figures 1, 2, 3 and 4 present the dynamics of the yeast population in the studied seasons.

In the 2009 season (Figure 1), the PE strain was able to remain as the only strain until July. In August, it is possible to note the installation of an indigenous strain (Indigenous 1), which increased its population gradually and, by October, it represented 50% of the yeast population in the tanks. In November, the PE is completely eliminated from the process, likely due to the emergence of a second indigenous strain (Indigenous 2), which dominated in the month of November, but was not able to dominate the process until the end of the season. Even though Indigenous 2 was present at the end of the season, Indigenous 1 dominated the process at the end of the 2009 season.

In the 2010 season (Figure 2) the PE strain remained as the only strain for approximately 60 days (March to April). As of May, an indigenous yeast (1) was able to install itself in the process. In August, indigenous strain 1 matched PE in concentration, representing, as PE, 50% of the population.

In September, two other indigenous yeast strains (2 and 3) are found. With the entry of these new strains, PE regains dominance of the process, representing 70% of the yeast population in the process. In October, the two indigenous yeasts are eliminated from the process, resuming the same situation in September (50% PE and 50% indigenous 1). A fourth indigenous strain appears at the end of the season and it seems to be responsible for the complete elimination of PE from the fermentation tank, starting to cohabit the tank with indigenous 1 at the same proportion.

Fig. 1: Yeast distribution in fermentation tanks during the 2009 season at an industrial unit

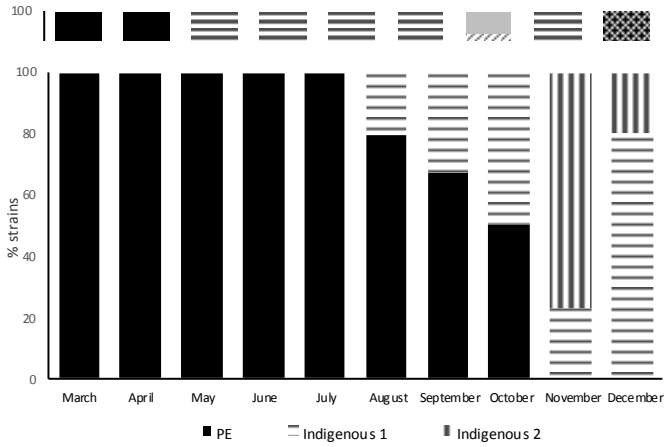

Fig. 2: Yeast distribution in fermentation tanks during the 2010 season at an industrial unit.

In the 2011 season (Figure 3) the PE strain is the only one found in the tanks until May. In June, two different strains appear (indigenous 1 and 2), which, added, represent 20% of the population. Even though the PE strain cohabits the tanks with two other strains, it still represents 80% of the population. In July, indigenous 1 is eliminated, with only indigenous 2 and PE remaining, the latter dominating the process (79%). In August, the situation reverses and indigenous 2 becomes dominant and represents 76% of the population. In September, it is no longer possible to detect the presence of PE. Indigenous 2 becomes the dominant strain, cohabiting the process with a third indigenous yeast (3). The same situation is observed in October. In November, when the season ends, a fourth strain (4) appears and aggressively dominates the process (approximately 56%), cohabiting the tank with indigenous 3.

In the 2012 season (Figure 4) the PE strain is capable of remaining in the process as the sole yeast strain until June. In July an indigenous strain (1) appears, representing only 13% of the yeast population in the tank. In August, we note the appearance of indigenous 2 strain, aggressively accounting for 58% of the yeast population in the tanks. PE is still present, but corresponding only to 25% of the population. Indigenous 1 remains, but at low concentration (16%), as in July. In September, indigenous 2 continues to dominate the process (72%), with PE found at a concentration lower than 10%. In this month, in addition to PE and indigenous 2, two other indigenous yeasts (3 and 4) appear, representing 20% of the population. Indigenous 1 is eliminated from the process in September. In October, PE is completely eliminated, with indigenous 2 remaining as dominant (80%) and indigenous 3 at 20% concentration. The season ends in November with the

same population distribution of the month of October, that is, 80% indigenous 2 and 20% indigenous 3.

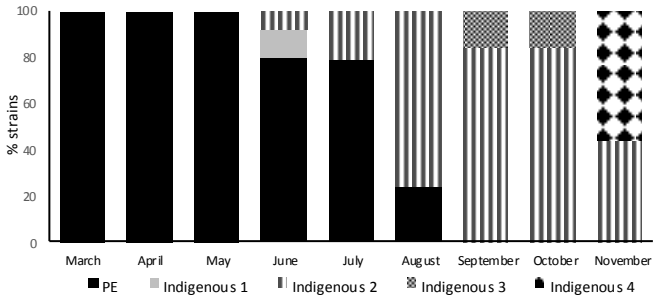

Fig. 3: Yeast distribution in fermentation tanks during the 2011 season at an industrial unit.

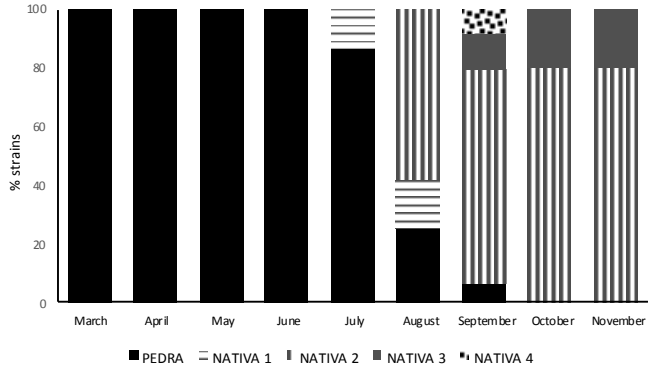

Fig. 4: Follow-up of yeast population inhabiting fermentation tanks during the 2011 season at an industrial unit.

IV. CONCLUSION

The use of select yeasts as inoculum for industrial fermentations has been recommended as a method to guarantee a more efficient fermentation [4]. Data obtained in this work suggest that the PE yeast was capable of remaining in the process for long periods, but it failed to remain at the end of the four assessed seasons. It was also possible to verify that no indigenous strains found in the four seasons were capable of remaining as the sole strain inhabiting the process, a fact observed for PE in the four seasons studied. In the 2009 season, it remained as the sole strain for 5 months, whereas it remained for two months in the 2010 season, for three months in the 2011 season, and for four months in the 2012 season. This fact allows us to state that this yeast is highly competitive with regard to the capacity of inhabiting fermentation tanks. This fact explains the choice of the PE strain as fermentation inoculum by industrial units. According to Basso et al. [4], 58% of distilleries starting their seasons with PE were able to end the operation with this yeast. The dominance capacity of PE is certainly associated with the cell mass amount this yeast is capable of producing. According to Andrietta et al. [5], PE strain presents higher $Y_{x/s}$ (cell mass yield) when compared with the value obtained for other select strains (CAT, SA, BG).

The months of July and August seem to be the most favorable for replacement of the select strain used as inoculum at the start of the season. The best feedstock is obtained during these months, since sugarcane reaches ripening peak in this period, which means higher concentration of total reducing sugars and a fermentation process with higher alcoholic content. This condition seems to favor the installation of more robust strains in the process. The distinctive behavior of the PE strain in different seasons at the same industrial unit, which did not undergo any project change, strongly suggests the influence of feedstock in yeast population dynamics. It is known that there is no standard with regard to the content of the must to be fermented, since it is constituted based on numerous factors not controlled and for the most part unknown.

Starting the season with select yeast has some advantages compared with starting with other types of yeasts, which are highly adapted to the severe conditions of fermentation tanks (high acidity, high alcoholic content, high temperatures, among other adverse conditions). However, one should not expect that the strain used as inoculum at the start of the season will remain until the end, since during this period various changes are observed both in the running of the process and the feedstock used.

REFERENCES

[1] B.E. Della-Bianca, T.O. Basso, B.U. Stambuk, L. C. Basso, A.K. Gombert. "What do we know about the yeast strains from the Brazilian fuel ethanol industry?" *Applied Microbiology and Biotechnology*, 97:979-991, 2013.

[2] H.V. Amorim, K.M Gryschek, M.L.R. Vairo, P.M. Gambassi."The success and sustainability of the Brazilian sugarcane: Fuel Ethanol industry". *ACS Symposium Series*, Piracicaba, v.1058, n.1, p.73-82, 2010

[3] ÚNICA. Disponível http://www.unicadata.com.br/lista. Acesso em 20 de junho 2017.

[4] L.C. Basso, H.V. Amorim, A.J. Oliveira, M.L. Lopes. "Yeast selection for fuel ethanol production in Brazil". *FEMS Yeast Research*, v.8, n.7, p.1155-1163, Amsterdam, 2008.

[5] M.G.S. Andrietta, S.R. Andrietta, E.N.A. Stupiello E.N.A. "Bioethanol - What Has Brazil Learned about Yeasts Inhabiting the Ethanol Production Processes from Sugar Cane?" *Biofuel Production-Recent Developments and Prospects*. Ed: Bernardes, M.A.S., 596f., Intech, Set. 2011.

[6] L.C. Basso, A.J. Oliveira, A.A. Orelli, C.R. Campos, C.R. Gallo, H.V. Amorim. "Dominância das leveduras contaminantes sobre as linhagens industriais avaliada pela técnica de cariotipagem". Anais Congresso Nacional da STAB, v.5, n.1, p.246-250, Piracicaba, 1993.

[7] L.S. Lopes. "Caracterização molecular da Linhagem Pedra 2 de *Saccharomyces cerevisae* sob condições de alto etanol em fermentadores industriais**"**. (Tese de mestrado). Universidade de São Paulo, Escola Superior de Agricultura "Luiz de Queiroz", Piracicaba, 2015.

[8] S.R. Andrietta, M.G.S. Andrietta, C. Steckelberg, E.N.A. Stupiello. "Bioethanol – 30 years of Proalcool. International" *Sugar Journal*, v.109, n.1299, p.195-200, 2007.

[9] S. Rainieri, C. Zambonelli, Y. Kaneko. Review – "*Saccharomyces sensu stricto*: Systematics Genetic Diversity and Evolution". *Journal of Bioscience and Bioengineering*, v.96, n.1, p.1-9, 2003.

[10] J.L. Argueso et al. "Genoma structure of a *Saccharomyces cerevisaie* strain widely used in bioetanol production". *Genome Research*, v.19, n.12, p.2258-2270, 2009.

[11] B. Blondin, F. Vezinhet. "Identification de souches de levures oenologiques par leurs caryotypes obtenus en électrophorèse en champ pulse". *Revue Française D'Oenologie*, v.28, p.7–19, 1988.