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Population dynamics of *Saccharomyces cerevisiae* PE-2 and CAT-1 in CO-culture for the production of ethanol

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ABSTRACT. In the Brazilian industries, the inoculum used throughout the harvest of ethanol production consists of a combination of two or more yeast strains. The combination of yeasts may influence in the metabolic pathways of microorganisms and increase the yields and production rates of some compounds. In biotechnological processes with co-culture, one microorganism can prevail over the other. Therefore, the knowledge about how the population dynamics occurs during fermentation allows modifications in the process in order to obtain higher yields and to achieve greater fermentative efficiency. The aim of this study was to investigate the fermentation with synthetic sugar cane broth in co-culture of *Saccharomyces cerevisiae* strains CAT-1 and PE-2 followed by molecular fermentation monitoring. The concentration of biomass, ethanol, glycerol, acetic acid and residual sucrose were monitored to verify the influence of different combinations during the fermentation. The mixture of CAT-1 and PE-2 presented the highest ethanol production, with higher performance of fermentative parameters than pure cultures.

Keywords: fermentation conditions; mitochondrial DNA; mixed culture; yeast competence; yeast skills.

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Introduction

In Brazil, the main yeasts used in distilleries are CAT-1, PE-2, BG-1, SA-1 and Y904 (Basso, Amorim, Oliveira, & Lopes, 2008).Choosing the appropriate lineage for the process is crucial for the alcoholic fermentation success. Lineages that present fermentative characteristics, such as good fermentative efficiency, high fermentation speed, resistance to ethanol, conversion efficiency, resistance to low pH, resistance to antiseptics, yield and genetic stability characterize better yeastsss (Menezes, 1980; Santos, Borém, & Caldas, 2012).

The use of these lineages may be the way to increase ethanol yield with improvements in the performance of inoculated yeast cells, increasing cell robustness (Santos et al., 2017), or/and implementing a combination between the different strains. The inoculum used throughout the harvest period is composed of a combination of two or more yeast strains. This association allows the combination of metabolic pathways of different microorganisms that can lead to higher yields and production rates (Abate, Callieri, Rodríguez, & Garro, 1996). However, there are few studies about the relation between the population dynamics and metabolites produced by *Saccharomyces cerevisiae*.

Recently, research groups have carried out fermentations with yeast combinations from Brazilian distilleries in an attempt to explicit the population dynamics that exist at the molecular level (Basso et al., 2008, Carvalho-Netto et al., 2013, Santos et al., 2017). In the research by Santos et al. (2017), the competition for nutrients in co-culture between CAT-1 and PE-2 was analyzed and the potential characteristics which affect their performance were compared. This research identified a better fermentation performance and robustness of CAT-1 strain due to the better concentration of response proteins to oxidative stress (Sod1 and Trx1) and trehalose synthesis (Tps3). Although some studies have addressed the use of yeast combination in ethanol processing, there is still a lack of research that describes the kinetics of yeast growth and the production of its metabolites with data referring to the population during fermentation.

Thus, this study investigated the fermentation with synthetic sugarcane broth in co-cultivation of *Saccharomyces cerevisiae* (CAT-1 and PE-2) in order to understand the kinetic parameters and to monitor the population dynamics of yeasts during fermentation.

Material and methods

PE-2 and CAT-1, which are high performance yeasts in the Brazilian alcohol industry, were kindly provided by the alcoholic company *Anicuns S/A Álcool e Derivados*. The yeast was maintained in GPY medium (5 yeast extract, 5 peptone, 20 glucose, 20 agar) and all reagents had analytical grade provided by Sigma-Aldrich® and Merck Chemicals, Valencia, Spain.

Media and fermentation conditions

GPY medium was used for inoculum preparation without agar addition. In the preparation of the inoculum, cultures were scraped and placed in 50 mL medium for 24 hours at 28°C with initial pH of 5.4 and shaking at 150 rpm.

The fermentations were carried out in 100 mL glass bottles with 50 mL in volume. Synthetic sugar cane broth was used as culture medium, consisting of (g L^{-1}): glucose, fructose 10, sucrose 210, malic acid 1.80, citric acid 8, KH_2PO_4 0.75, K_2SO_4 0.50, $MgSO_4.7H_2O$ 0.25, $CaCl_2.2H_2O$ 0.15 and NaCl 0.2. In this solution, it was also added 36.8 mL of vitamin solution [composed of (µg L^{-1}) d-biotin 37.5, calcium-d-pantothenate 750, nicotinic acid 750, myo-inositol 18750, thiamine hydrochloride 750, pyridoxal hydrochloride 750 and P-Aminobenzoic acid 150], 1 mL of amino acid solution [composed of (g L^{-1}) tyrosine 0.74, isoleucine 1.73, aspartic acid 4.11, glutamic acid 4.4, arginine 1.80, leucine 3.37, l-threonine 0.16, glycine 3.18, alanine 2.93, valine 2.84, methionine 0.36, phenylalanine 1.73, serine 2.2, histidine 0.66 and lysine 1.18] and 10 mL of trace element solution [composed of (mg L^{-1}) EDTA disodium salt 5.05, $ZnSO_4{\cdot}7H_2O$ 1.52, $MnCl_2$ 0.34, $CoCl_2.6H_2O$ 0.10, $CuSO_4$ 0.10, $Na_2MoO_4{\cdot}2H_2O$ 0.13, $CaCl_2.2H_2O$ 1.52, $FeSO_4$ 1.01, boric acid 0.34 and KI 0.03]. The fermentation was conducted in a co-culture with CAT-1 and PE-2 in a ratio of 1:1 (inoculum concentration of 0.09448 g L^{-1}) in duplicate, with sucrose concentration of 210 g L^{-1}, initial pH of 5.4 and 150 rpm shaking for 120 hours, with sample withdrawals at 0, 15, 24, 48, 72, 96 and 120 hours to evaluate the amount of biomass, ethanol, glycerol, sucrose and acetic acid, aiming to observe the phases of the fermentation and to better understand the fermentative process.

Analytical determinations

Biomass growth was quantified through optical density at 600 nm and converted to dry biomass X (g L^{-1}) through the equation that relates them ($X = 0.3693*DO_{600nm} + 0.0217$). This equation was obtained after verifying, in a not published study, that the sizes and quantity of yeasts per microliter did not differ among the strains used.

Supernatants were analyzed by HPLC on a Thermo chromatograph (Thermo Fisher Scientific, Waltham, MA) equipped with an ultraviolet refractive index detector. The column used was HyperREZTM XP Carbohydrate H + 8µm (Thermo Fisher Scientific), which was protected by HyperREZTM XP Carbohydrates (Thermo Fisher Scientific). The conditions used in the analyzes were: 1.5 mM eluent of H_2SO_4; flow rate of 0.6 mL $min.^{-1}$ and temperature of 50°C. The samples were diluted five times, filtered on a 0.45 micron nylon filter (Symta, Madrid, Spain) and analyzed in duplicate. Ethanol, sucrose, glycerol and acetic acid were analyzed at the end of the fermentation at 120 hours.

Evaluation of the competence of the selected yeasts

In order to study the competence of the yeasts, we collected a sample of 0.5 mL and added sterile water to reach 0.2 of absorbance. Then, in sterile micro tubes, dilutions of 10^{-1}, 10^{-2} and 10^{-3} were made and 100 µL of each dilution was spread with the aid of a Drigalski loop in Petri dish with solid GPY medium for colonization. After spreading, the plates were incubated at 28°C for 48 hours. At each collection time, 20 colonies were selected and identified by restriction fragment length polymorphism in the mitochondrial DNA (Querol, Barrio, Huerta, & Ramón, 1992), so that it was possible to observe the yeast competences within the fermentation process.

Restriction fragment length polymorphism of the mitochondrial DNA

Restriction fragment length polymorphism of the mitochondrial DNA was performed in order to characterize the strains during the fermentation, as well as to evaluate the competences of the yeast. To characterize CAT-1 and PE-2, they were inoculated into plates with GPY medium and incubated for 24 hours. DNA extraction was performed according to the methodology described by Querol et al. (1992). For

enzymatic digestion of the DNA, we used the endonuclease Hinf I (Roche Molecular Biochemicals, Mannheim, Germany), according to the protocol described by Querol et al. (1992). The fragments were visualized with UV light and photographed in comparison to the PstI-digested λ markers (247 to 11.501 base pairs at a concentration of 0.1 μg μL^{-1}).

The results were evaluated through One-Way - ANOVA (Analysis of Variance) statistical analysis using R software, version 2.0.0 (Core Team, Goiania, Brazil) and the averages were compared by Tukey's test ($p = 0.05$) in order to determine any significant difference between treatments.

Results and discussion

In a previous research made by our group, it was possible to identify two strains with higher production of ethanol, PE-2 and CAT-1. In this study, co-culture and pure cultures were compared to understand kinetic and physiological behavior. Figure 1 shows yeast fermentation time curves obtained from the individual yeast cultures PE-2, CAT-1 and co-culture. Figure 1A shows yeast growth kinetics for 120 hours of fermentation.

The fermentation presented a lag phase and an exponential phase, in which respiration and intense production of biomass occured during the first 24 hours of fermentation with specific speed of growth (μXmax) (see Table 1). After 24 up to 48 hours of culture, a growth deceleration phase is observed, and finally, a stationary phase from 48 to 120 hours. The maximum biomass concentration was noticed at 120 hours for the co-culture, as well as for the pure cultures, with mean values ranging from 8.25 to 9.58 g L^{-1}, and substrate conversion factor in cells ($Y_{X/S}$) as shown in Table 1.

A small increase of biomass was observed in the co-culture fermentation when compared with the pure cultures. From these results and according to the definition of Lopitz-Otsoa, Rementeria, Elguezabal, and Garaizar (2006) and Amorim, Lopes, Oliveira, Buckeridge, and Goldman (2011), a synergistic effect is observed when two yeasts grow together, providing a positive interaction between the microorganisms, which allows the balanced survival of both strains in the same environment.

The average ethanol production at 120 hours of pure culture fermentation and in co-culture (Figure 1B) were between 10.34 and 11.17% (v v^{-1}), which did not differ statistically from each other by Tukey's test at 5% probability. Although the results were not statistically significant, there were some positive trends in which ethanol concentration increases for both yeasts when compared to pure cultures. It was also observed that yield and fermentative efficiency of the co-cultures were slightly higher than the pure cultures. When compared to pure cultures, this small increase can generate a 9% increase in ethanol production. This difference could result in the gain of millions of ethanol liters in a medium capacity distillery (Santos et al., 2017).

Figure 1C shows the concentration of sucrose during the fermentation process. For all tests, the residual sugar showed a decline. However, none of them could consume the sugars completely. The interaction between PE-2 and CAT-1 led to lower consumption of sucrose when compared to pure cultures, which may be related to a higher fermentative efficiency. This fact may lead the tests to have a higher fermentative efficiency.

For acetic acid (Figure 1D), co-cultivation resulted in an increase in the production of this acid when compared to pure cultures. In *S. cerevisiae*, the additional metabolism of acetic acid is extremely important since it produces acetic acid as an essential component of fatty acid biosynthesis for yeasts through acetyl-CoA synthetase, which is the only source of cytosolic acetyl-CoA (Van Den Berg & Steensma, 1995). In the present work, a biomass increase was observed in the co-culture, which presented the highest concentration of acetic acid.

However, when acetaldehyde is converted to acetic acid, a reducing surplus is produced. The formation of such product raises an additional problem in the redox balance that is solved by the production of glycerol (Péter & Rosa, 2006). However, in the present study, glycerol (Figure 1E) presented the lowest concentration with higher production of biomass and acetic acid in the co-culture. The reduction of glycerol during fermentation may allow an increase in ethanol yield (Basso, Basso, & Rocha, 2011).

Regarding the study of competence in co-culture, the dominant molecular profile (band standard) was obtained. Figure 2a refers to PE-2 and Figure 2b to CAT-1.

Restriction enzymes that cleave the DNA in specific regions create a characteristic number of fragments that is comparable to the markers. The differences in the number of DNA bands are the homologous regions of different sizes. In some cases, the polymorphism is so high that each lineage can be identified by its specific region pattern (Benítez & Codón, 2002).

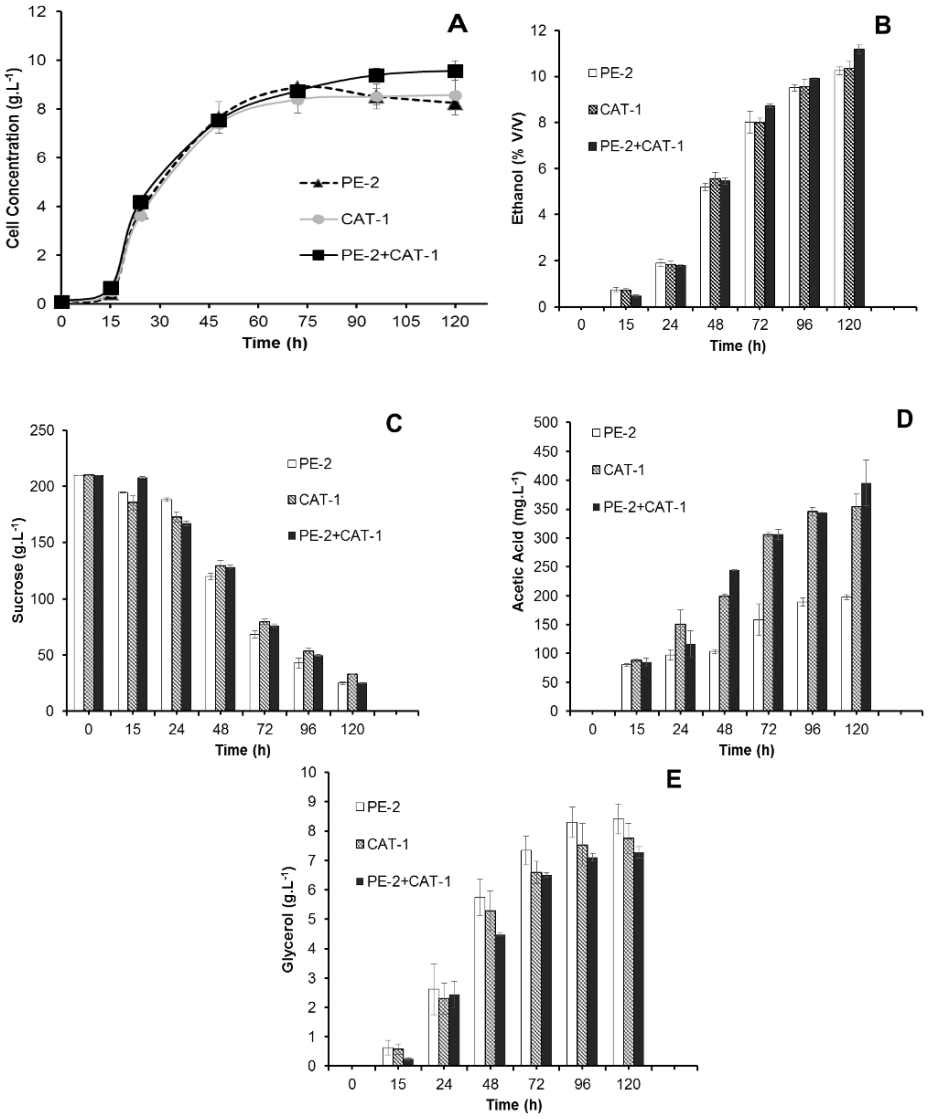

Figure 1. Result of cell growth, residual sugar and ethanol, acetic acid and glycerol from pure yeast fermentations PE-2, CAT-1 and co-culture of PE-2 + CAT-1 for 120 hours. (A) refers to cellular concentrations, (B) ethanol, (C) sucrose, (D) acetic acid and (E) glycerol. The error bar refers to the standard deviation of the averages (N = 2, mean ± SEM, dependent).

Table 1. Kinetic parameters in pure cultures and co-culture (PE-2 and CAT-1) during 120 hours.

Fermentation variables	PE-2	CAT-1	PE-2 + CAT-1
	Biomass		
Last (g L^{-1})	8.25 ± 0.26[a]	8.58 ± 0.82[a]	9.58 ± 0.01[a]
μx_{max} ($hour^{-1}$)	0.176 ± 0.005[a]	0.168 ± 0.006[a]	0.167±0.010[a]
$Y_{X/S}$ ($g_{Biomass}$ g^{-1})	0.0440 ± 0.0017[a]	0.04790 ± 0.047[a]	0.046 ± 0.002[a]
	Ethanol		
Last (%, v v^{-1})	10.24 ± 0.17[a]	10.34 ± 0.30[a]	11.17±0.20[a]
μ_{Pmax} (g g^{-1} $hour^{-1}$)	0.187 ± 0.007[a]	0.162 ± 005[a]	0.134±0.00[b]
$Y_{P/S}$ ($g_{Ethanol}$ g^{-1})	0.436 ± 0.010[a]	0.461 ± 0.014[a]	0.43 ± 0.01[a]
Productivity (g L^{-1} $hour^{-1}$)	0.673 ± 0.011[a]	0.680 ± 0.020[a]	0.734 ± 0.013[a]
Fermentation Efficiency (%)	81.20% ± 1.91[a]	85.74% ± 2.53[a]	88.66% ± 0.13[a]
	Sucrose		
Last (g L^{-1})	24.81 ± 1.29[b]	32.90 ± 0.14[a]	25.09 ± 0.76[b]
Last (%)	11.81% ± 0.61[b]	15.67% ± 0.07[a]	11.95% ± 0.36[b]
	Glycerol		
Last (mg L^{-1})	8.42 ± 0.51[a]	7.75 ± 0.51[a]	7.28 ± 0.20[a]
	Acetic Acid		
Last (mg L^{-1})	197.43 ± 4.4[b]	354.43 ± 22.3[a]	395.52 ± 39.6[a]

[a,b]: Averages followed by the same letter do not differ significantly among themselves, at 5% probability by the Tukey test. Data presented as standard deviation of the averages (N = 2, mean ± SEM, dependent).

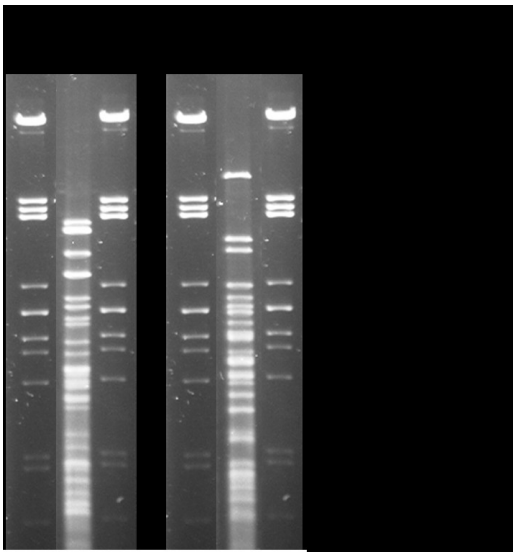

Figure 2. Molecular profiles of *S. cerevisiae* PE-2 (a) and CAT-1 (b).

Among the ways of identifying yeasts during fermentation, such as electrophoretic karyotyping, restricted regions analysis of ribosomal DNA (rDNA) and mitochondrial DNA (mtDNA), the analysis of restriction fragment length polymorphism (RFLP) allied to mtDNA has been successfully used as the best technique for characterization in mixed culture processes (Carrascosa, Muñoz, & González, 2011). Moreover, the method of identification of *S. cerevisiae* genotypes by mitochondrial DNA (DNAm) is distinguished from the others since DNAm presents a high degree of polymorphism and genetic stability during the vegetative multiplication (Ribéreau-Gayon, Dubourdieu, Donèche, & Lonvaud, 2006).

The existence of different microbial populations in a fermentative process causes interactions which can cause positive or negative effects for the culture. The study of the microbial population competence seeks to understand the population dynamics of microorganisms. Studies with this objective are of great value for application in industrial processes of ethanol production, since they provide information by describing what happens at the molecular level in the microbial population (Lima, Aquarone, Borzani, & Schmidell, 2001; Basso et al., 2008).

The amounts of the two strains varied throughout the fermentation process (Figure 3). In the exponential phase (from 0 to 24 hours), 47.06% of the cells in the culture presented molecular profiles of CAT-1 strain and 52.94% of PE-2 strain. From 48 to 96 hours (stationary phase), the CAT-1 concentration in the culture remained between 65 and 66.47%. As CAT-1 yeast has been identified as having the highest proportion in the mixture, it is attributed to it the responsibility for the increased respiration and production of biomass in that time interval. In 120 hours, the number of strains with similar molecular profiles to CAT-1 (57.15%) decreased and there was an increase in the amount of PE-2 cells (42.86%) (Carvalho-Netto et al., 2013).

In laboratory conditions with YPD medium, using fermentation with the mixture of CAT-1 and PE-2, Santos et al. (2017) observed that more than 70% of the cells that survived the fermentation were CAT-1, revealing its robustness. The researchers also demonstrated possible characteristics that may be linked to this resistance. Proteins involved in oxidative stress response (Sod1 and Trx1) and trehalose synthesis (Tps3) were more abundant in CAT-1 than in PE-2 after fermentation.

The behavior of the population dynamics found by Carvalho-Netto et al. (2013) verified that when inoculating equal amounts of PE-2 and CAT-1 at the beginning of the 2011 harvest, wild yeast and PE-2 were found in greater quantity, which indicates greater resistance to ethanol, inoculum treatment and greater competitive aggressiveness over other yeasts. Basso et al. (2008) reported similar behavior with Carvalho-Netto et al. (2013) who started in a distillery fermentation with 0.5 kg (wet weight) of each selected strain (PE-2, SA-1 and VR-1) mixed with 1 ton of pressed baker's yeast. After 29 days of recycling, baker's yeast was not found and all selected strains were present in different proportions. Only PE-2 was able to dominate the introduced strains and the contaminating yeasts, corresponding to the total existing biomass at the end of 193 days of recycling.

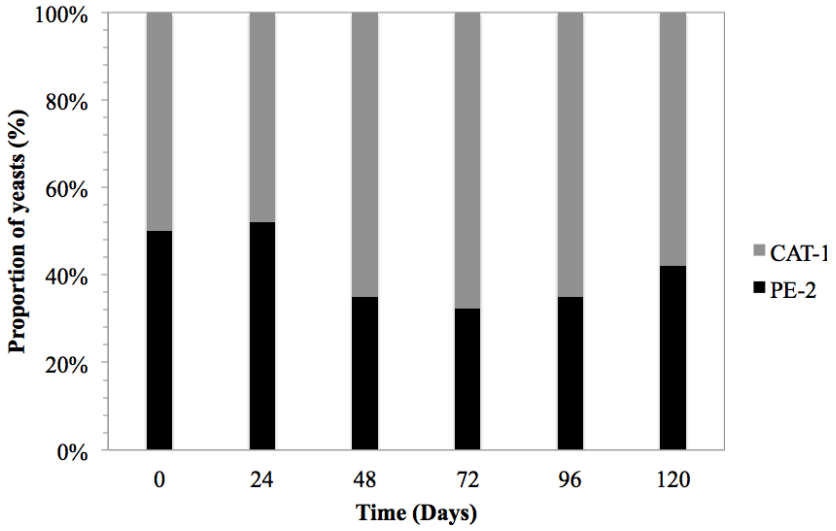

Figure 3. Quantification of PE-2 and CAT-1 strains during 120 hours of fermentation.

Conclusion

The fermentations carried out in this study showed great potential for the use of co-cultures of CAT-1 and PE-2 *Saccharomyces cerevisiae* in the ethanol industry, since it presented higher ethanol production, as well as yield and fermentative efficiency better than the pure cultures. It was also observed that the cell growth was higher in co-culture than in pure culture, evidencing synergistic effect among these lineages. The concentrations of glycerol reached lower values in the pure cultures. This study provides a scientific basis for a deeper research using co-cultivation with strains CAT-1 and PE-2 *Saccharomyces cerevisiae*, comparing the fermentative parameters against the commonly used cultures.

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