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Yeast enhancement by mass mating and selective pressure for the integrated production process of first and second-generation ethanol

Aprimoramento de leveduras por cruzamento massal e pressão seletiva para o processo integrado de produção de etanol de primeira e segunda gerações

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ABSTRACT

Second-generation ethanol production is a worldwide applicable technology with the potential to replace fossil fuels and contribute to sustainability. The incorporation of second-generation ethanol production in Brazilian biorefineries, besides the technological advantages, adds to the abundance of feedstock derived from the sugar and alcohol industry itself. However, developing yeast strains that resist the inhibitory conditions of the new substrate, potentiated by cellular recycling, is extremely necessary. The aim of the present work was to develop yeast strains by hybridization and selective pressure techniques, with multi-tolerant profile for the fed-batch fermentation process using a mixture of molasses and bagasse hydrolysate as substrate. Therefore, the mass crossing technique was carried out involving five strains of *Saccharomyces cerevisiae*, previously selected, for demonstrating high tolerance to fermentation from mixed-must composed of lignocellulosic hydrolysate and sugarcane molasses. The culture resulting from the mass mating was followed by a selective pressure during 51 generations, generating enrichment of more tolerant strains. Employing microplate growth evaluation (optical density [DO] 600 nm), ten evolved isolates were selected, which were submitted to lab scale fermentation, simulating industrial conditions to the maximum. In the end, it was possible to highlight a lineage (C8E1-13T) presenting trehalose reserve content significantly higher than the other lineages evaluated, thus demonstrating the generation of an improved phenotype.

Keywords: biofuel; hybridization; inhibitors; lignocellulosic hydrolysate; tolerance.

RESUMO

A produção de etanol de segunda geração é uma tecnologia de aplicação mundial com potencial para substituir os combustíveis fósseis e contribuir para a sustentabilidade. A incorporação da produção de etanol de segunda geração nas biorrefinarias brasileiras, além das vantagens tecnológicas, acrescenta-se a abundância de matéria-prima proveniente da própria indústria sucroalcooleira. Porém, o desenvolvimento de cepas de leveduras que resistam às condições inibitórias do novo substrato, potencializadas pela reciclagem celular, é extremamente necessário. O objetivo do presente estudo foi o desenvolvimento de linhagens de leveduras por técnicas de hibridização e pressão seletiva, com perfil multitolerante para o processo de fermentação em batelada alimentada, utilizando uma mistura de melaço e hidrolisado de bagaço como substrato. Portanto, foi realizada a técnica de cruzamento massal envolvendo cinco cepas de *Saccharomyces cerevisiae*, previamente selecionadas, por demonstrarem alta tolerância à fermentação a partir de mosto misto composto por hidrolisado lignocelulósico e melaço de cana-de-açúcar. O cultivo resultante do cruzamento massal foi acompanhado de pressão seletiva durante 51 gerações, gerando enriquecimento de linhagens mais tolerantes. Por meio da avaliação do crescimento em microplacas (densidade óptica [DO] 600 nm), foram selecionados dez isolados evoluídos, os quais foram submetidos à fermentação em escala laboratorial, simulando ao máximo as condições industriais. Ao final, foi possível destacar uma linhagem (C8E1-13T) apresentando teor de reserva de trealose significativamente maior que as demais linhagens avaliadas, demonstrando assim, a geração de um fenótipo melhorado.

Palavras-chave: biocombustíveis; hibridização; inibidores; hidrolisado lignocelulósico; tolerância.

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Introduction

Sustainable solutions are being explored to reduce dependence on fossil fuels due to global warming and climate change concerns. Biofuels have gained increasing attention as a way to mitigate environmental impacts (Khan et al., 2021; Li et al., 2022). First-generation (1G) bioethanol has been the most widely produced biofuel in the world; however, second-generation (2G) bioethanol is becoming an important renewable strategy because it is a carbon-neutral energy source (Dionísio et al., 2021; Phillips, 2022).

In Brazil, 2G ethanol can be produced from sugarcane bagasse hydrolysates and sugarcane molasses (coproducts from the sugar industry) as raw materials. The use of these nutrients, combined with the structure and knowledge already existing in the 1G ethanol process, could result in fermentation with higher ethanol content. This approach contributes to a favorable energy balance in distilleries and provides minerals and organic nutrients for the yeast (Andrade et al., 2013; Moonsamy et al., 2022).

Nevertheless, 2G ethanol production requires a pre-treatment process that results in the formation of toxic compounds, such as acetic acid, furfural, hydroxymethylfurfural, and phenolic compounds, which inhibit yeast growth (Bhavana et al., 2022; Qi et al., 2023; Yao1 et al., 2024). Furthermore, yeast is exposed to unfavorable conditions that compromise its viability and performance in cell recycling fermentations. Therefore, yeasts that accumulate reserve carbohydrates, such as trehalose, that protect cells from multiple stresses, can perform effectively in fermentation (Elbakush and Güven, 2021).

Therefore, several strategies have been developed to improve yeast strains for industrial applications based on multi-tolerance performance. Methods such as mass mating and selective pressure are well-known and applied to improve yeast in industrial settings (Steensels et al., 2014). The mass-mating technique produces a large number of haploid cells. Although their crossing is random, it is very efficient for analyzing complex quantitative phenotypes controlled by multiple genes (Naseeb et al., 2021). Under selective pressure, each new cycle is inoculated from an exponentially growing culture, under constant or increasing selective pressure, which allows for the selection of mutants with higher maximum specific growth rates (μmax) (Mans et al., 2018).

The aim of the present work was to improve yeast strains by hybridization and selective pressure techniques and evaluate the multi-tolerant profile of hybrids generated through the fed-batch fermentation process with cell recycling for the integrated production of 1G+2G ethanol.

Materials and methods

Yeast strains and inoculum preparation

Five strains of *Saccharomyces cerevisiae* (365, 430, CD68, CD132, and D10), isolated from Brazilian bioethanol distilleries, which exhibit high tolerance in lignocellulosic hydrolysate and molasses-based fermentation media, were analyzed. These were deposited in the collection of the Laboratory of Biochemistry and Yeast Technology of the Department of Biological Sciences (Luiz de Queiroz College of Agriculture - University of São Paulo, Brazil). All yeast strains were reactivated in yeast extract peptone dextrose (YPD) medium (2% peptone, 1% yeast extract, and 2% glucose) and incubated at 30°C for 48 hours.

Bagasse hydrolysate

Sugarcane bagasse was pretreated by a steam explosion with diluted phosphoric acid (9.5 mg H_3PO_4/g dry solids) at 180°C for 5 minutes. Subsequently, the pretreated material (both liquor and solid cellulose-lignin) was digested with Cellic® CTec3 (233 mg protein/mL; 170 FPU/mL) at 50°C for 72 h (Novozymes Latin America Ltda.).

Molasses-hydrolysate fermentation medium

The fermentation medium was prepared by mixing molasses with sugarcane bagasse lignocellulosic hydrolysate in a way that 20% of the total reducing sugars (TRS) were obtained by the hydrolysate. The medium was centrifuged at 800×g for 20 min. The pellet was then discarded, and the supernatant was sterilized (121°C for 25 min) in hermetic condition to avoid loss of acetic acid and aldehydes. Concentrations of TRS, acetic acid, furfural, hydroxymethylfurfural, and/or ethanol in the medium were adjusted at each experimental stage to increase cell stress and were described in the respective sections.

Sporulation, mass mating, selective pressure, and pre-screening of hybrids

Sporulation and spore purification

The selected yeast strains were streaked on a raffinose-acetate sporulation medium (0.02% raffinose, 0.3% potassium acetate, and 2% agar) and incubated at 30°C for 7 days until sporulation was reached. The purification protocol was developed by adapting the methodologies described (Treco and Winston, 2008; Hou, 2010; Pinel et al., 2011) regarding the amount of enzyme used, sonication time, and vortex agitation with glass beads.

A 2 mL aliquot of each sporulated culture was centrifuged (5000 rpm/10 min), replaced with 800 L of micromanipulation buffer (1 M sorbitol; 10 mM TRIS-HCl pH 7.5; 10 mM NaH_2PO_4; 10 mM EDTA pH 8.0), and 100 units of lyticase from *Arthrobacter luteus* Sigma Aldrich. Asci were digested at 37°C for 12 h. After this period, the cells were centrifuged, the supernatant was discarded, and 2 mL of Triton X-100 (1%) was added. Sonication was performed for 30 min in a Cristófoli Ultrasonic Cuba, followed by vortex agitation with glass beads for 60 seconds. Distilled water was used to wash the cultures after shaking.

Mass mating

A volume of 2 mL of each properly sporulated and purified parental culture with 9.2 x 107 cells/mL was transferred to an Erlenmey-

er flask containing 100 mL of YPD liquid medium. The germination and random crossing were conducted for 72 h at 30°C and 110 rpm. Cell concentration and viability were evaluated after this period in optical microscopy (Lindgren and Lindgren, 1943). A total of 60 mL of the culture was designated for selective pressure (30 mL for each evolution line), while the remaining 30 mL were stored (ultrafreezer at -80°C in 20% glycerol).

Selective pressure

The culture resulting from mass mating was subjected to consecutive batch growth in mixed must based on molasses and lignocellulosic hydrolysate, whose composition was altered several times by varying the mixture's proportions and adding furfural and ethanol during transfers. The growths were performed in Erlenmeyer flasks at 30°C using two parallel lines of selective pressure and selection—evolutions 1 (E1) and 2 (E2).

Growth analysis of hybrids on microplates

Progenitor lineages and haploids generated were evaluated by growth under microculture conditions in molasses-based and lignocellulosic hydrolysate medium (15.7% TRS, 5 g/L acetic acid, 1.5 g/L furfural, 4% ethanol, and pH 5.0) using 96-well flat-bottomed microplates. The microplate was set up in three replicates with 90 µL of medium and 10 µL of fresh cell suspension (pre-grown overnight in 3 mL YPD), followed by incubation at 30°C in a multifunctional thermostat microplate reader (Tecan, model Infinite M200). Optical density (OD 600 nm) readings were collected every 2 h for 24 h with prior agitation of 10 min before readings. Maximum specific growth rate (µmax) was calculated (Tahara et al., 2013).

Cell-recycling fed-batch fermentation

Ten strains were selected from previous fermentation screening. Fermentations were performed by simulating the fed-batch process, called MelleBoinot (Neitzel et al., 2020). Fermentations were carried out at 30°C in 15 mL conical tubes, initially containing 0.8 g of wet centrifuged biomass. An 8 mL molasses-hydrolysate fermentation medium (15.5% total sugar, acetic acid 6.6 g/L, furfural 1.8 g/L, 1% ethanol, and pH 5.0) was added. At the end of each fermentation cycle, the yeast biomass was collected by centrifugation (800×g for 20 min), weighed, and reused to inoculate the subsequent fermentation cycle (three cycles total). At the end of each cycle, cell viability was estimated by erythrosine differential cell staining (Oliveira et al., 1996). Wet centrifuged biomass was determined by weighing the pelleted cells after centrifugation, and ethanol concentration was determined in a digital densitometer (ANTON PAAR DMA48) after steam distillation of the centrifuged fermented medium in a micro Kjeldahl distillation apparatus (Zago et al., 1989). Glycerol and residual sugars (glucose, fructose, sucrose) were measured by high-performance anion-exchange chromatography (HPAEC) using an ion exchange chromatography (DIONEX DX-300) equipped with a CarboPac PA-1 column 4 × 250 mm and a pulsed amperometric detector, as described by Basso et al. (2008). At the end of the last cycle, yeast cell trehalose was assessed (Trevelyan and Harrison, 1956). Biomass was washed with ice-cold distilled water and precipitated by centrifugation (3500 rpm for 10 min). Trehalose was extracted using 2 mL of trichloroacetic acid (0.5 M) in an ice bath for 20 min. After centrifugation, the supernatant extract was collected and stored in 1.5 mL microtubes. The sample was measured by HPAEC using DIONEX DX-300, equipped with a CarboPac PA-1 column 4 × 250 mm, and a pulsed amperometric detector (Basso et al., 2008).

Karyotyping

Five progenitor lineages and ten hybrids selected in cell-recycling fed-batch fermentation were plated on YPD agar medium and incubated at 30°C for 42 h. After growth, isolated colonies from each sample were individually treated according to the protocol of Blondin e Vézinhet (1988), with some modifications as described by Basso et al. (2008). For each colony, 2–4 mg of wet biomass were suspended in 30 µl of lytic enzyme solution and 40 µl of agarose 1.3% at 60°C contained in a mold. The blocks obtained after agarose gelation were incubated and subjected to pulsed-field gel electrophoresis at 14°C, CHEF mode, using Bio-Rad model DR®III equipment, programmed for 6 V/cm for 9 h with a 5-sec pulse, both blocks configured at a 120° angle. The gels were stained with ethidium bromide and photographed under ultraviolet lighting to obtain intact chromosome banding profiles.

Statistical analyzes

Mean comparison tests were conducted to verify the significant difference between the selected lines in terms of viability (%), trehalose content (%), biomass content (g), ethanol production (%), and yield (%). The means from the triplicates of each lineage were obtained using R Statistical Software, version 3.3.2. Significant differences were considered at a 5% significance level (p*0.05).

Results and discussion

Sporulation and spore purification

S. cerevisiae diploid cells, in the absence of a nitrogen source and in the presence of a non-fermentable carbon source, can be induced to enter meiosis during spore formation (Tomova et al., 2019).

As part of this approach, sporulated cultures were enzymatically treated and physically separated to ensure spore survival. Based on the experimental results, the strains evaluated achieved a sporulation rate close to 90%, and the enzymatic action followed by mechanical action in the spore purification process resulted in their release and dispersion, allowing a more significant number of random crossings between cells of different lineages (Figure 1).

Mass mating

The mass mating technique, using haploid suspensions of yeasts 365, 430, CD68, CD132, and D10, produced a culture containing 8.9 × 107 cells/mL and 95.2% viability. Recombination within a population can enhance gene diversity by creating new combinations of lineages, therefore improving individual performance (Phillips, 2022).

Most studies that used mass mating found strains that were more tolerant to industrially relevant stress factors, including higher tolerance to ethanol (Lairón-Peris et al., 2020), hybrid vigor (Catallo et al., 2021), tolerance to acetic acid (Ko et al., 2020) and high cell viability rates. In this way, such strategies can contribute to optimizing strains for 2G bioethanol production.

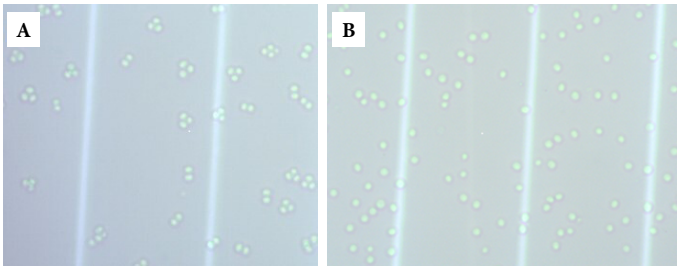

Figure 1 – Culture of sporulated cells in a 40x objective; (A) Strains sporulated in raffinose acetate sporulation medium; (B) Strains sporulated in raffinose acetate sporulation medium after the purification protocol.

Selective pressure

The biodiversity generated by mass mating was evaluated through selective pressure, and the lines most resistant to the inhibitors were selected. During selective pressure in line 1 (E1), ethanolic stress was prioritized, contributing to a rigorous selection of biodiversity generated by mass mating. In line two (E2) of selective pressure, stresses due to acetic acid and furfural, the main inhibitors in the lignocellulosic hydrolysate, were prioritized, as demonstrated in Tables 1 and 2.

Ethanol was used to enhance the hydrolysate's inhibitory effects, simulating cell reuse conditions (in the recycling process), thus producing a phenotype with multi-tolerance.

The selective pressure process resulted in the isolation of 98 colonies. From selective pressure E1, 49 products were obtained (29 from the 13th transfer, 20 from the 16th transfer), and 49 isolated products from selective pressure E2 were obtained (29 from the 11th transfer, 20 from the 14th transfer).

Growth assessment of hybrids on microplates

Five strains of *S. cerevisiae* (365, 430, CD68, CD132, and D10) with greater tolerance to lignocellulosic hydrolysate and sugar cane molasses fermentations were evaluated for growth in microplates. The D10 strain showed more significant growth and a higher maximum specific growth rate (μmax) than the other parental strains. For this reason, it was selected as a control strain for the other evaluations (Figure 2).

Table 1 – Evolution 1 monitoring parameters, containing a concentration of inhibitors, total reducing sugars content, initial and final ethanol content, initial and final cell concentration, cell viability, number of generations, and yield in each transfer.

Transfer	Acetic acid (g/l)	Furfural (g/l)	Initial ethanol (%)	TRS (%)	Initial cell concentration (cell/mL)	Final cell concentration (cell/mL)	Number of generations	Viability (%)	Final ethanol (%)	Yield (%)
1	0.88	0.63	5.0	8.6	2.9E+07	9.9E+07	1.8	93.8	8.7	66.4
2	1.10	0.78	5.0	8.6	3.0E+07	8.8E+07	1.4	92.6	9.0	71.8
3	1.47	1.05	5.0	8.6	2.2E+07	8.7E+07	2.0	93.0	9.0	71.8
4	2.20	1.57	5.0	8.6	2.2E+07	6.3E+07	1.5	94.9	8.9	70.0
5	2.20	1.57	5.0	8.6	2.2E+07	8.4E+07	2.0	96.3	9.0	71.8
6	2.93	2.10	3.0	8.6	8.4E+06	6.1E+07	2.9	93.1	7.0	71.8
7	2.93	2.10	3.0	8.6	8.4E+06	8.0E+07	3.3	97.1	7.1	73.6
8	3.52	2.52	3.0	8.6	8.0E+06	4.7E+07	2.6	94.8	6.5	62.9
9	3.52	2.52	3.0	8.6	8.0E+06	5.9E+07	2.9	98.3	6.9	70.0
10	3.52	2.52	3.0	8.6	8.0E+06	5.0E+07	2.6	98.4	6.8	68.2
11	3.52	2.52	3.0	8.6	4.0E+06	4.7E+07	3.6	97.8	6.7	66.4
12	3.52	2.52	3.0	8.6	4.0E+06	4.3E+07	3.4	98.1	6.3	59.3
13	3.52	2.52	3.0	8.6	4.0E+06	7.0E+07	4.1	98.5	7.1	73.6
14	3.52	2.52	3.0	8.6	2.0E+06	7.3E+07	5.2	98.9	7.0	71.8
15	3.52	2.52	3.0	8.6	1.0E+06	5.3E+07	5.7	98.1	7.1	73.6
16	3.52	2.52	3.0	8.6	5.0E+05	5.8E+07	6.9	97.6	7.0	71.8

TRS: total reducing sugars.

Table 2 – Evolution 2 monitoring parameters, containing a concentration of inhibitors, total reducing sugars content, initial and final ethanol content, initial and final cell concentration, cell viability, number of generations, and yield in each transfer.

Transfer	Acetic acid (g/l)	Furfural (g/l)	Initial ethanol (%)	TRS (%)	Initial cell concentration (cell/mL)	Final cell concentration (cell/mL)	Number of generations	Viability (%)	Final ethanol (%)	Yield (%)
1	2.50	1.42	0.96	5.92	2.9E+07	6.3E+07	1.1	91.3	3.95	78.0
2	2.50	1.42	0.96	5.92	2.9E+07	8.3E+07	1.5	96.1	4.00	79.3
3	3.00	1.70	1.16	7.10	8.3E+06	5.4E+07	2.7	88.5	4.80	79.2
4	3.00	1.70	1.16	7.10	8.3E+06	7.3E+07	3.1	98.7	4.65	75.9
5	3.00	1.70	1.16	7.10	8.3E+06	8.3E+07	3.3	96.9	4.60	74.8
6	3.74	2.12	1.44	8.87	8.3E+06	6.1E+07	2.9	98.7	5.70	74.2
7	3.74	2.12	1.44	8.87	8.3E+06	6.7E+07	3.0	98.8	5.45	69.8
8	3.74	2.12	1.44	8.87	8.3E+06	5.9E+07	2.8	97.8	5.60	72.4
9	3.74	2.12	1.44	8.87	4.2E+06	5.2E+07	3.6	98.5	5.55	71.6
10	3.74	2.12	1.44	8.87	4.2E+06	6.7E+07	4.0	98.4	5.65	73.3
11	3.74	2.12	1.44	8.87	4.2E+06	8.1E+07	4.3	98.7	5.60	72.4
12	3.74	2.12	1.44	8.87	2.1E+06	6.9E+07	5.0	98.6	5.65	73.3
13	3.74	2.12	1.44	8.87	1.0E+06	5.9E+07	5.9	98.2	5.40	68.9
14	3.74	2.12	1.44	8.87	5.2E+05	6.4E+07	6.9	91.2	5.70	74.2

TRS: total reducing sugars.

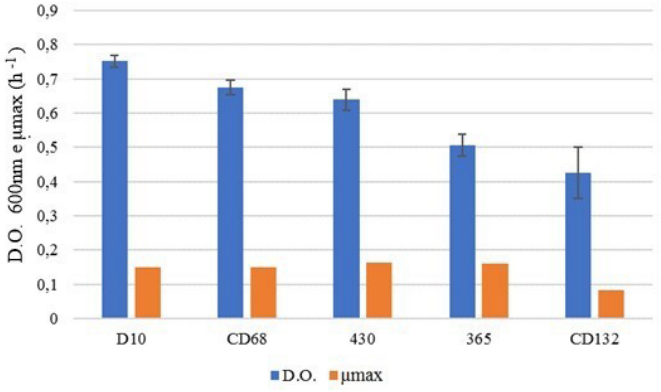

Figure 2 – Growth in microplates of five parental strains on hydrolysate and molasses (15.7% total reducing sugars, 5.0 g/L acetic acid, 1.5 g/L furfural, 4.0 g/L ethanol, pH 5.0).

A total of 98 hybrids were evaluated for growth capacity (OD 600nm) under microculture conditions in a hydrolyzed molasses medium and compared with lineage D10. Of these, ten were selected for their greater growth capacity for evaluation in fermentative cycles (Figure 3).

Assessment through testing with fermentative cycles

Tolerance and fermentative potential of ten hybrids with rapid growth in microplate assay were evaluated in simulated fed-batch process, also known as MelleBoinot (Neitzel et al., 2020), in an intentionally stressful fermentation medium (15, 5% TRS, 6.6 g/L acetic acid, 1.8 g/L furfural, 1.0% ethanol).

During fermentation, yeasts are subjected to many stress conditions that decrease cell viability. The acetic acid in the substrate used for evaluation is one of the most limiting factors in yeast growth during alcoholic fermentation (Ko et al., 2020).

According to this study, the reference strain D10 and the hybrid strains C8E1-13T and C14E1-13T maintained viability over 80% after the third fermentation cycle, which indicates their ability to survive a competitive fermentation environment (Figure 4). However, only the C8E1-13T hybrid showed high reserves of trehalose (Figure 5). Trehalose is a cellular reserve carbohydrate that helps maintain cell viability under stressful conditions.

Several researchers have reported the protective effects of trehalose, including the survival of yeast under high temperatures (Péter et al., 2021) and severe osmotic and oxidative stress (Santos et al., 2017).

Ethanol production was significantly lower in the third fermentation cycle (Figure 6), and consequently, yield declined (Figure 7), reflecting the sharp drop in cell viability in the second cycle. Despite the stressful effects caused by the inhibitors in the hydrolysate, all strains achieved greater than 80% ethanol in the first and second cycles. Notably, the C8E1-13T hybrid showed higher ethanol production and increased fermentative yield than most of the hybrids evaluated, demonstrating improved stress tolerance and ethanol production.

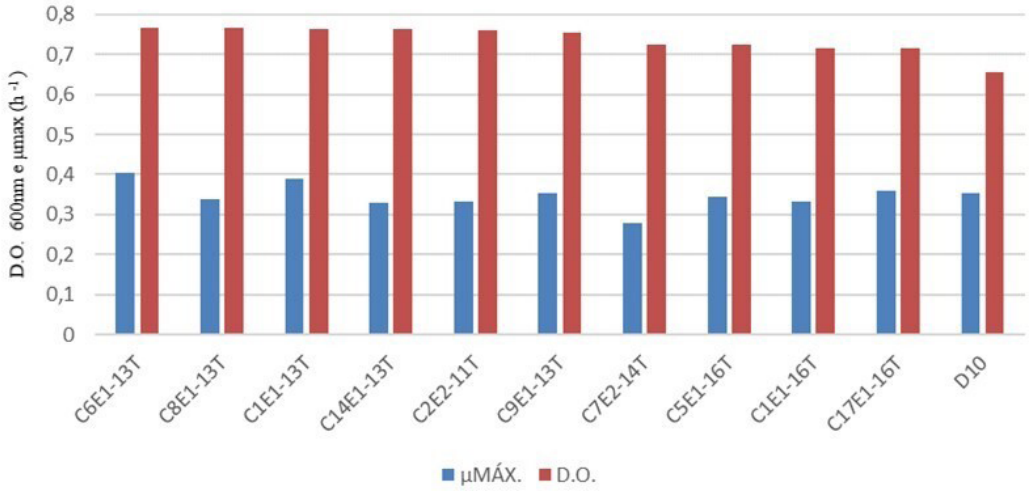

Figure 3 – Hybrids showing more significant growth in microplates using molasses-hydrolyzed substrate (15.7% total reducing sugars, 5.0 g/L acetic acid, 1.5 g/L furfural, 4.0% ethanol, pH 5.0) compared to the parental line D10.

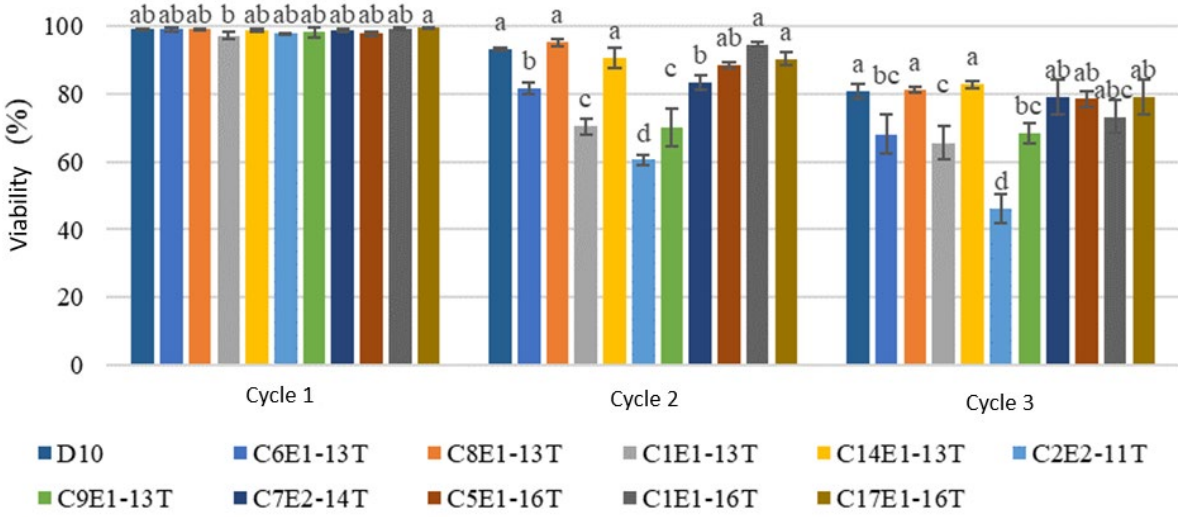

Figure 4 – Cell viability (%) during three cycles of cell reuse, fed-batch fermentation at 30°C using molasses hydrolyzed medium (15.5% total reducing sugars, 6.6 g/L acetic acid, 1.8 g/L L furfural, 1.0% ethanol). Mean values with different letters are significantly different (p<0.05).

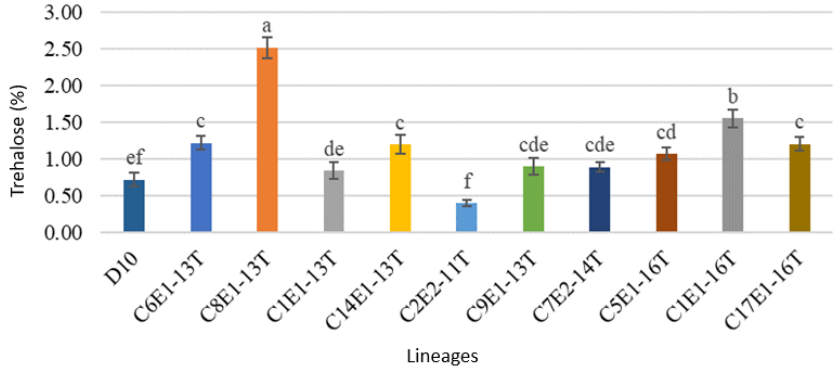

Figure 5 – Cellular trehalose content (% dry weight) at the end of the third cycle of fed-batch fermentation with cell reuse at 30°C using hydrolyzed molasses medium (15.5% total reducing sugars, 6.6 g/L acetic acid, 1.8 g/L furfural, 1.0% ethanol). Mean values with different letters are significantly different (p<0.05).

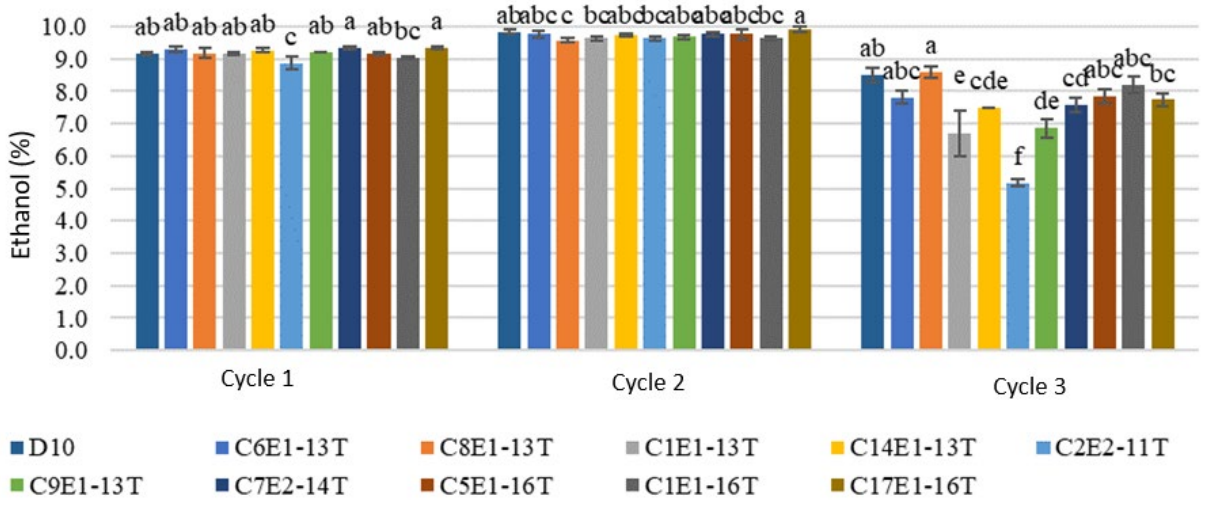

Figure 6 – Comparison of ethanol production of yeast strains (% v/v) during three cycles of fed-batch fermentation with cell reuse at 30°C using hydrolyzed molasses medium (15.5% total reducing sugars, 6.6 g/ L acetic acid, 1.8 g/L furfural, 1.0% ethanol). Mean values with different letters are significantly different ($p<0.05$).

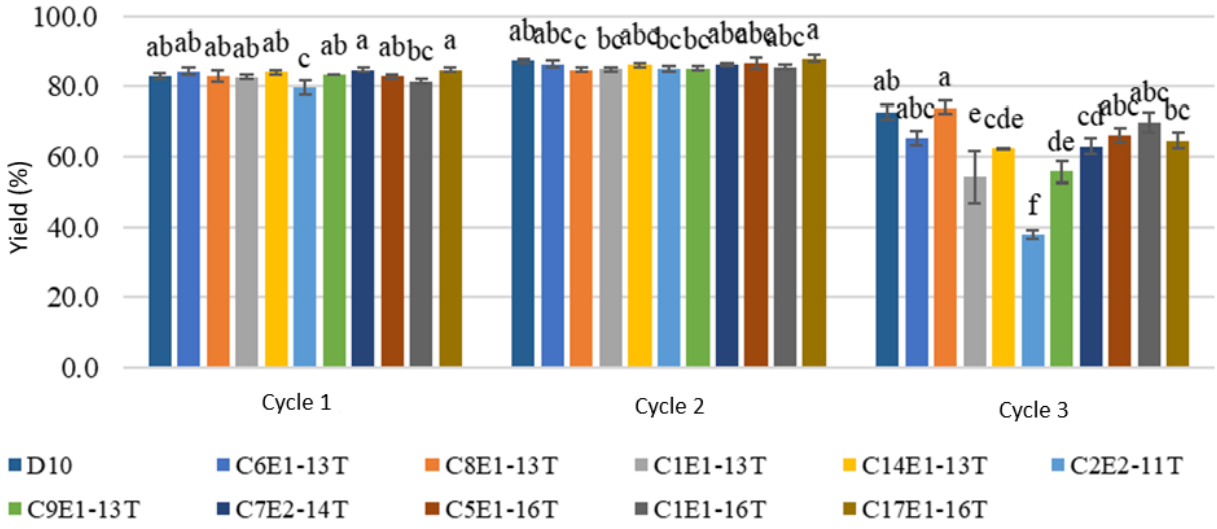

Figure 7 – Fermentative yield (%) of yeast strains (% v/v) during three cycles of fed-batch fermentation with cell reuse at 30°C using hydrolyzed molasses medium (15.5% total reducing sugars, 6.6 g/ L acetic acid, 1.8 g/L furfural, 1.0% ethanol). Mean values with different letters are significantly different ($p<0.05$).

Muynarsk et al. (2023) produced hybrids that accumulated more significant amounts of trehalose and had higher cell viability rates than their parental strains without compromising ethanol production through fed-batch fermentations with cell recycling based on molasses-bagasse hydrolysate. However, the substrate contained 6.1 g/L of acetic acid and 0.36 g/L of furfural, which was lower than those in the current study (6.6 and 1.8 g/L, respectively). Furfural damages mitochondrial and vacuolar membranes and inhibits yeast growth more pronouncedly than acetic acid (Hemansi et al., 2022).

The C8E1-13T line and the reference D10 showed a lower biomass drop after three cycles, 11 and 14%, respectively (Figure 8). Low cell biomass loss or its increase during fermentation cycles is of great interest to the ethanol industry. According to Carlos et al. (2011), the cellular reuse characteristic of the Brazilian industrial fermentation process reduces the need for intensive yeast propagation. Thus, less sugar is diverted to biomass production, which demonstrates an increase between 5 and 10% in the initial biomass during a fermentative cycle. Nevertheless, fermentations based on the first-generation process are considerably less harmful to yeast since the must derived from diluted molasses is not loaded with inhibitors like the hydrolysate from lignocellulosic materials.

It is important to emphasize that the strain D10 used in this study corresponds to a strain of *S. cerevisiae* that had previously been improved for a high tolerance phenotype. As a result, the obtained C8E1-13T hybrid exhibits high cell viability (significantly equal to D10 strain) and a high trehalose reserve, 3.5 times greater than the control strain (D10). Consequently, the C8E1-13T hybrid will be able to tolerate more stressful conditions than in this study or the same conditions for an extended period.

Karyotyping

The five progenitor lines and the ten cultures evaluated in the fermentative cycle assay were karyotyped using electrophoretic banding (Figure 9). In this technique, lineages of *S. cerevisiae* can be distinguished by separating intact chromosomal deoxyribonucleic acid (DNA) in an agarose gel according to its size (Zimmermann and Fournier, 1996). This gives each lineage a distinct pattern of bands in the gel.

The chromosomal profile of *S. cerevisiae* lineages ranges from 12 to 14 million bases spread across 16 chromosomes (Lopes et al., 2015). According to Figure 9, all colonies of the strains examined belonged to the *S. cerevisiae* species (with 16 chromosomes, the species exhibits a characteristic electrophoretic profile that differs from non-*Saccharomyces* yeasts).

Parental lineages refer to different lineages, each with a distinct electrophoretic profile (Figure 9A), in which specific bases can be observed in the gel, with red arrows indicating the most striking distinctions. These are regions referring to medium and low molecular weight chromosomes.

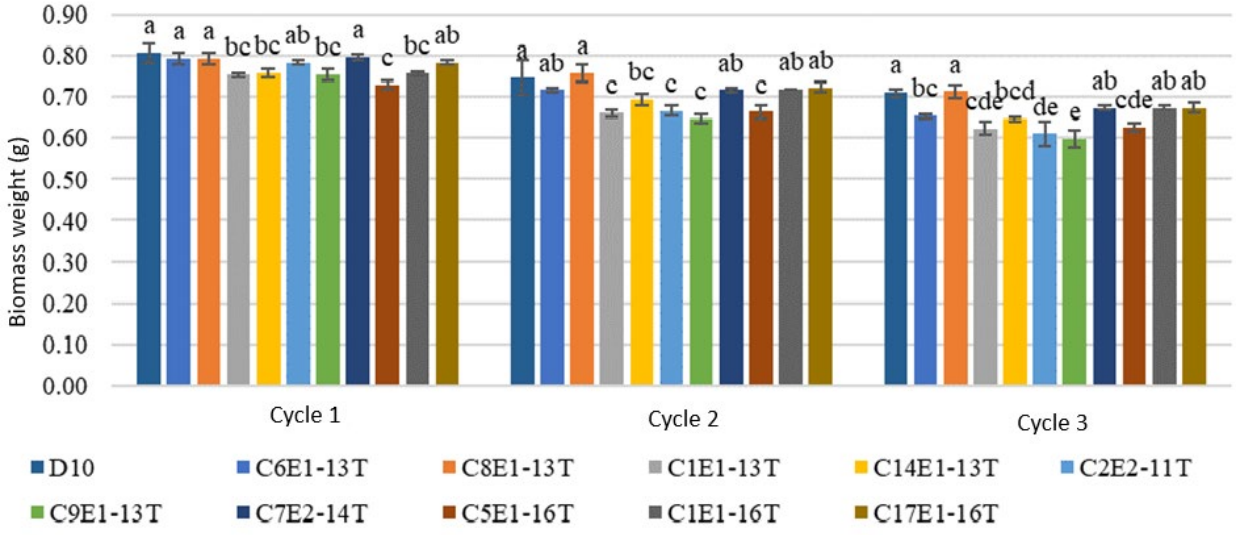

Figure 8 – Centrifuged wet biomass (g) during three cycles of cell reuse, fed-batch fermentation at 30°C using hydrolyzed molasses medium (15.5% total reducing sugars, 6.6 g/L acetic acid, 1.8 g/L L furfural, 1.0% ethanol). Mean values with different letters are significantly different ($p<0.05$).

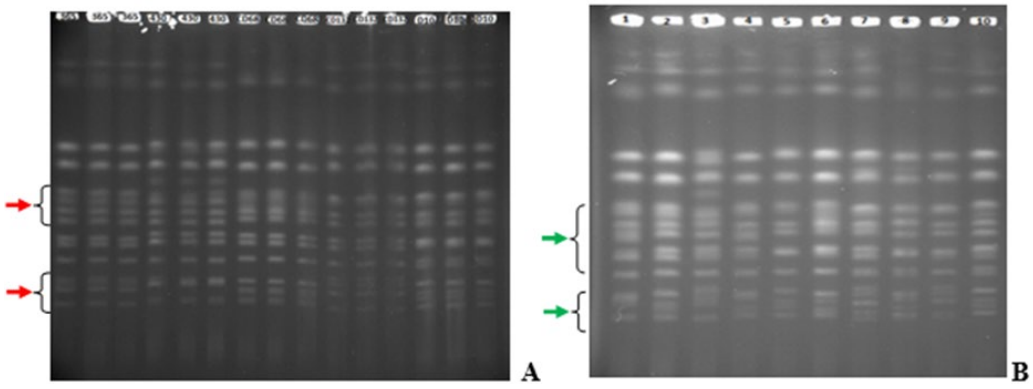

Figure 9 – Profiles of parental lines and hybrids generated by mass mating and selective pressure. (A) Progenitor strain electrophoretic profiles (365, 430, CD68, CD132, and D10) in triplicate. (B) Electrophoretic profiles of the ten lines selected after the mass crossing technique and selective pressure (1- C6E1-13T; 2- C8E1-13T; 3- C1E1-13T; 4- C14E1-13T; 5- C2E2-11T; 6- C9E1-13T; 7- C7E2-14T; 8- C5E1-16T; 9- C1E1-16T; 10- C17E1-16T).

Similarly, different electrophoretic profiles are observed for most isolates, with green arrows indicating regions of most evident differentiation (in which bands with different molecular weights appear). However, as expected, they share similarities with the parental lineages (upper region of the gel characterized by higher molecular weight chromosomal DNA).

It is known that *S. cerevisiae* sporulation results in populations with chromosomal rearrangements. These changes in chromosomal polymorphism are more intense in the region of smaller chromosomes (in the lower part of the gel), as documented by Lopes (2000). The diversity of electrophoretic profiles observed in both lines of evolution, compared with the original lines, demonstrates that the new genetic material was generated through mass crossing, which resulted in improved phenotypes. C8E1-13T hybrids showed a greater capacity for trehalose accumulation, which, in turn, was linked to a greater tolerance to fermentation stress.

Conclusion

The use of the mass crossing method and selective pressure combined with a selection methodology in fermentation, with recycling of cells in mixed must based on lignocellulosic hydrolysate and sugar cane molasses, allowed to obtain a hybrid strain (C8E1-13T) with a high trehalose content compared to a previously selected strain.

Strains with multi-tolerance attributes, such as the C8E1-13T, constitute valuable genetic material for the future insertion of metabolic attributes that promote pentose fermentation, favoring full use of sugars from lignocellulosic biomass. The use of hexoses/pentoses results in more excellent conversion into ethanol and, therefore, its use as an alternative/or replacement for fossil fuels, also bringing gains in favor of sustainability.

Authors' contributions

Muynarsk, E.S.M.: conceptualization, methodology, validation, investigation, formal analysis, writing – original draft, visualization. Christofoleti-Furlan, R.M.: conceptualization, methodology, validation, investigation, formal analysis, writing – original draft, visualization. Orozco Colonia, B.S.: investigation, writing – original draft. Belini Junior, E.: formal analysis, writing – review & editing, visualization. Silva, D.G.H.: formal analysis, writing – review & editing, visualization. Basso, L.C.: conceptualization, methodology, resources, writing – review & editing, supervision.

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