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Quantitative proteomic analysis of the *Saccharomyces cerevisiae* industrial strains CAT-1 and PE-2

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ABSTRACT

Brazilian ethanol fermentation process commonly uses baker's yeast as inoculum. In recent years, wild type yeast strains have been widely adopted. The two more successful examples are PE-2 and CAT-1, currently employed in Brazilian distilleries. In the present study, we analyzed how these strains compete for nutrients in the same environment and compared the potential characteristics which affect their performance by applying quantitative proteomics methods. Through the use of isobaric tagging, it was possible to compare protein abundances between both strains during the fermentation process. Our results revealed a better fermentation performance and robustness of CAT-1 strain. The proteomic results demonstrated many possible features that may be linked to the improved fermentation traits of the CAT-1. Proteins involved in response to oxidative stress (Sod1 and Trx1) and trehalose synthesis (Tps3) were more abundant in CAT-1 than in PE-2 after a fermentation batch. Tolerance to oxidative stress and trehalose accumulation were subsequently demonstrated to be enhanced for CAT-1, corroborating the comparative proteomic results. The importance of trehalose and the antioxidant system was confirmed by using mutant stains deleted in Sod1, Trx1 or Tps3. These deletions impaired fermentation performance, strengthening the idea that the abilities of accumulating high levels of trehalose and coping with oxidative stress are crucial for improving fermentation.

Significance: The importance of the present works emerges from the necessity to better understand the peculiar biological features from two important bioethanol industrial strains of *Saccharomyces cerevisiae* during batch fermentation. We applied an iTRAQ-based quantitative proteomics analysis to compare these two important strains during batch fermentation and identified possible features involved in the fermentation performance. The results provided by this work will serve as an initial framework for future investigations on the biology of both strains.

1. Introduction

Bioethanol is the main type of biofuel that is currently commercially produced. World ethanol production stands at around 83 billion litters and comes mainly from USA (2/3) and Brazil (1/3) [1]. Bioethanol is produced from sugar cane in Brazil and from corn in USA; both processes use the yeast *Saccharomyces cerevisiae* as fermentative agent [2]. By using more accurate control techniques, efficient inputs and process automation, bioethanol yields have increased significantly in recent years. However, how much bioethanol the world can sustainably produce is a crucial question. The increase of ethanol productivity without expansion of cultivated fields depends on, among other factors, improvements in the robustness and performance of yeast cells.

The yeast faces different kinds of stress during the fermentation process, such as changes in the sugar, acidity and ethanol concentration [3,4]. The Brazilian fermentation process uses high cell concentrations (10–17% w, wet weight/v) to ferment cane juice and/or molasses, containing up to 20% (w/v) of total sugar, producing high ethanol concentrations (around 10% v/v) within a short period (on average, 8 h). Both high cell densities, obtained by cell recycling, and elevated ethanol concentrations reduce yeast growth, resulting to high ethanol yields (90%–92% of the theoretical maximum). In Brazil, yeast cells are intensively recycled [2], increasing aged cell levels in the sequential fermentations. After each batch, cells are separated by centrifugation, exposed to sulfuric acid at pH 2.0 for 1–2 h and reintroduced into a fresh medium for a new cycle of fermentation. This is repeated throughout the sugar cane crop, which lasts about 6 to 8 months [2,4].

Most of the Brazilian distilleries use baker's yeast as inoculum, because of its lower cost and greater availability [2]. However, since the

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culture medium is not sterilized and the fermentations occur under non-aseptic conditions, soon, the cultures become contaminated by wild yeasts, which arrive to the process through the cane [5,6]. These robust contaminant wild yeasts stand out for higher ethanol yield, low glycerol formation and higher viability during cell recycle when compared to baker's yeast, combined with the prolonged persistence in the process [2]. CAT-1 and PE-2 are two of these wild strains of *S. cerevisiae*. They have being widely used in Brazilian distilleries [5]. CAT-1 and PE-2 genomes were sequenced [7,8] and their transcriptomes during fermentation process were determined [9]. According to these studies, in both wild yeasts were identified genes related to a greater adaptation to the industrial environment.

In this work, the fermentative performance and competition assays between CAT-1 and PE-2 were evaluated in order to find the most robust strain. Quantitative proteomics analysis (iTRAQ) was used to compare both wild yeast strains during fermentation and identify possible features involved in the better performance.

2. Material and methods

2.1. Yeast strains and grown conditions

The diploid strains of *S. cerevisiae* PE-2 and CAT-1, isolated from ethanol plants, were acquired from Fermentec, Piracicaba, Brazil [2]. Stocks of the wild yeasts were maintained on solid YPD (1% yeast extract, 2% glucose, 2% peptone and 2% agar). Cells were grown in conical flasks filled with liquid YPD medium, with a ratio of flask: medium volume of 5:1, at 28 °C, 160 rpm, until mid-log phase of growth (1.0 mg cell dry weight/ml).

2.2. Fermentation conditions

The fermentation was carried out at 30 °C/90 rpm, using 50 ml of flasks filled with 25 ml medium containing 5% glucose, 0.4% $(NH_4)_2SO_4$, 0.4% KH_2PO_4, pH 5.0. The cells were previously grown on YPD, harvested by centrifugation and inoculated in the fermentation medium, at a concentration of 1.5 mg cell/ml. After fermentation, yeast cells were collected by centrifugation and reused in the subsequent fermentation. At the beginning and after each fermentation cycle, aliquots were harvested, centrifuged, and the supernatants were used to determine the level of glucose and ethanol trough HPLC [10]. Cells were used for trehalose [11] and lipid peroxidation determinations [12] as well as for proteomic analysis.

2.3. Evaluation of robustness and dominance: Competition assay

To discriminate between CAT-1 and PE-2, both were transformed with the episomal plasmid pYC230, containing the *kanMX4* gene, using the lithium acetate method [13]. The same amount of CAT-1 and PE-2 *kanMX4* or PE-2 and CAT-1 *kanMX4* cells was inoculated in a fresh fermentation medium. Cell viability was determined at the beginning and end of a fermentation cycle by standard dilution plate counts on YPD. Cells were also plated on YPD containing geneticin. Colonies were counted after growth at 28 °C for 3 days. The number of PE-2 or CAT-1 colonies was obtained from the difference between the number of colonies from the plates without geneticin and the plates with geneticin (only PE-2 *kanMx4* and CAT-1 *kanMx4* mutants were able to grow on both plates).

2.4. Proteomics analysis

2.4.1. Cell extract

Fifty mg of cells were harvested by centrifugation before and after 8 h of fermentation, washed twice with cold water, and then resuspended in sodium phosphate buffer 50 mM, pH 7.0, containing protease inhibitors (complete, EDTA-free protease inhibition cocktail tablets, Roche; 1 tablet per 50 ml). Cells were disrupted by vortexing with glass beads [11]. Cell lysates were cleared at 13,000 rpm/3 min in a microcentrifuge. Protein concentration was determined by fluorometry (Qubit® 2.0, Invitrogen).

2.4.2. Protein digestion

Proteins (300 μg) were precipitated with 10% TCA in acetone (overnight at −20 °C), followed by cold acetone washing. Pellets were resuspended in 7 M urea/2 M thiourea. Disulfide bonds were reduced using 10 mM dithiothreitol, then alkylated using 40 mM iodoacetamide. For trypsin digestion, protein samples were diluted 9 times in 50 mM TEAB (triethylammonium bicarbonate) buffer and hydrolyzed with trypsin (Sequencing Grade Modified Trypsin - Promega) 1:50 (w/w) at 30 °C/16 h. The protein digestion was stopped adding formic acid at final concentration of 0.1%.

2.4.3. iTRAQ labeling

Tryptic peptides were measured using Qubit assay (Invitrogen), concentrated and desalted using homemade stage-tips with Poros® 50R2 resin (Applied Biosystems) [14]. iTRAQ labeling of 25 μg of peptide was performed according to the manufacturer's recommendations. Each iTRAQ™ 4-plex reagent vial (114 to 117) was resuspended in 70 μl of ethanol and incubated at room temperature for 1 h with the respective peptide sample, as follows: 114 – CAT-1 0 h (peptides from CAT-1 at the beginning of a batch fermentation); 115 – CAT-1 8 h (from CAT-1 at the end of a batch fermentation); 116 – PE-2 0 h (from PE-2 at the beginning of a batch fermentation); 117 – PE-2 8 h (from PE-2 at the end of a batch fermentation). Subsequently, each sample and reagent mixtures were mixed. Four biological replicates were labeled as described above. To remove the excess of iTRAQ reagent, it was performed a strong cation exchange (SCX) chromatography using the Macro Spin Columns (Harvard Apparatus). Mix of iTRAQ labeled peptides were dried under vacuum centrifugation and re-suspended in 10 mM KH_2PO_4/25% acetonitrile (ACN), pH 3 (sample buffer). The sample was incubated with the SCX Spin Column previously equilibrated with the sample buffer. The Macro Spin Column was centrifuged at 500 rpm for 2 min. The non-retained fraction was called flow through (FT) and the retained sample was eluted in steps using different salt concentrations (50, 150, 300 and 500 mM KCl) in the sample buffer. Afterwards, all fractions were cleaned using homemade stage-tips prepared with R2 Poros® 50 R2 resin as previously described [15].

2.4.4. Analysis by mass spectrometry

The mixture of peptides was quantified by fluorometry and resuspended in 0.1% formic acid and analyzed in triplicate using a nano-LC Proxeon EASY-nLCII coupled to a LTQ-Orbitrap Velos mass spectrometer (Thermo Scientific). iTRAQ labeled peptides were loaded in a pre-column (2 cm length, 200 μm inner diameter, packed in-house with ReproSil-Pur C18-AQ 5 μm resin – Dr. Maisch GmbH HPLC) and fractionated in a column (15 cm length, 75 μm inner diameter, packed in-house with ReproSil-Pur C18-AQ 3 μm resin – Dr. Maisch GmbH HPLC). The gradient used was 95% of solvent A (95% H_2O, 5% ACN, 0.1% formic acid) until 40% of solvent B (95% ACN, 5% H_2O, 0.1%formic acid) for 167 min; 40% to 95% of solvent B for 5 min; and 95% of solvent B for 8 min). The full scan and MS/MS acquisition was made in a positive mode applying a Data Dependent Acquisition (DDA). The operating parameters of the mass spectrometer were: spray voltage at 2.5 kV, zero flow of sheath and auxiliary gas and 235 °C in the heated capillary. MS full scan was acquired in 60.000 resolution at m/z 400 in the Orbitrap analyzer, 10^6 AGC, and 500 ms maximum ion injection time. The 10 most intense ions were selected for higher-energy collision dissociation (HCD) fragmentation and fragments acquisition in the Orbitrap using the parameters: 7500 resolution at m/z 400, 30,000 signal threshold, 5-ms activation time at 40 normalized collision energy, and dynamic exclusion enabled for 30 s with a repeat count of 1. A 5% ammonia–water

solution was used to avoid the supercharge effect, as previously described [16].

2.4.5. Data analysis

The reference proteome set of S288c haploid, composed of 6.720 sequences, was downloaded from the Uniprot consortium. PatternLab was used for generating a target-decoy database by first grouping subset sequences, then including the sequences of 127 common mass spectrometry contaminants, and, finally, for each sequence, including a reversed version of it. The final database used for peptide spectrum matching (PSM) contained. The Comet 2014 rev. 1 search engine [17], which is embedded into PatternLab for proteomics 3.2 [18,19], was used to compare experimental MS2 against those theoretically generated from our sequence database and select the most likely peptide sequence candidate for each spectrum. Briefly, the search was limited to fully and semi-tryptic peptide candidates; we imposed carbamidomethylation of cysteine and the iTRAQ-4 modification (n-terminus and K) as fixed. The search engine accepted peptide candidates within a 40-ppm tolerance from the measured precursor m/z, up to two missed-cleavages and used the XCorr as the primary search engine score with bins of 1.0005. PSM validity was assessed using the search engine processor (SEPro), which is embedded in PatternLab version 3.2 [20]. Briefly, identifications were grouped by charge state (+2 and ≥+3) and then by tryptic status (fully tryptic, semi-tryptic), resulting in four distinct subgroups. For each result, the Comet XCorr, DeltaCN, DeltaPPM, and Peaks Matched values were used to generate a Bayesian discriminator. The identifications were sorted in non-decreasing order according to the discriminator score. A cutoff score was established to accept a false-discovery rate (FDR) of 1% at the peptide level based on the number of labeled decoys. This procedure was independently performed on each data subset, resulting in an FDR that was independent of tryptic status or charge state. Additionally, a minimum sequence length of six amino-acid residues was required. Results were post-processed to only accept PSMs with <6 ppm from the global identification average. One-hit wonders (i.e., proteins identified with only one mass spectrum) were only considered if an XCorr > 2.5 was obtained. These criteria led to FDRs, now at the protein level, to be lower than 1% for all search results. PatternLab's isobaric module was then used to extract the iTRAQ reporter ion intensities, apply the purity correction as indicated in the manufacturer's instructions and then normalize the signal from each isobaric marker according to the total ion current for that respective reporter ion mass. The proteomic analysis identified and quantitated a total of 1013 proteins considering redundancy, and by maximum parsimony [21]. To evaluate differences in protein expression between CAT-1 and PE-2 along a fermentation, it was determined Log_e of the ratio of the ratios [CAT-1 8 h (115)/CAT-1 0 h (114)]/[PE-2 8 h (117)/PE-2 0 h(116)]. Ratio of ratios pointing to proteins that diverge was singled-out according to the single-sample student *t*-test ($p < 0.05$).

3. Results

3.1. Comparative performance of CAT-1 and PE-2

First, the fermentative performance of each strain was analyzed (Fig. 1). As expected, CAT-1 and PE-2 had similar profiles in terms of glucose consumption and ethanol production. They consumed almost all sugar, showing increased ethanol yields (the ratio between ethanol production and glucose consumption) were very close to the maximum theoretical yield (51%). When recycled back for a subsequent fermentation, cells consumed less glucose and, consequently, produced less ethanol, although the yields remained roughly unchanged. However, during the second fermentation, CAT-1 performance was little better than PE-2, indicating that CAT-1 could be more adapted to the fermentation process than PE-2. Despite the small difference in ethanol production between CAT-1 and PE-2, this difference sums to an increase of millions of liters of ethanol in a medium-capacity distillery, which can reach around 34.3 billion liters.

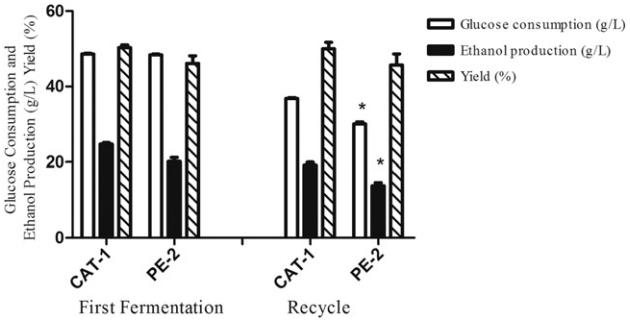

Fig. 1. Sugar consumption (white bars), ethanol production (black bars), and yield (hatched bars) after subsequent fermentations. Cells were grown in YPD medium until mid-log phase. They were collected by centrifugation, washed with distilled water and transferred to a fermentation medium containing 5% glucose. At the end of the first fermentation, cells were recycled to a new batch fermentation. Each fermentation cycle lasted 24 h. Yield of ethanol from glucose was expressed as [grams of ethanol produced/grams of glucose consumed] × 100. Values are mean ± standard deviation (SD) of three independent experiments.* means statistically different results at $p < 0.05$ (CAT-1 vs. PE-2, for each parameter).

To better compare the fitness of the wild yeasts, equal amounts of cells of CAT-1 and PE-2 were mixed in a fresh medium and the percentage of living cells of both strains was monitored. The gene was used to discriminate between the two strains; *kanMX4* confers resistance to geneticin. According to Fig. 2, irrespective if *KanMX4* would be introduced in CAT-1 or in PE-2, when PE-2 had to compete against CAT-1, >70% of cells that survived to fermentation was CAT-1. Taken together, these results led us to conclude that CAT-1 shows a greater robustness to fermentation conditions than PE-2.

3.2. iTRAQ-based quantitative proteomic analysis of CAT-1 and PE-2 before and after fermentation

Fig. 3 shows the main steps of the proteomic strategy used in this study. It allowed us to identify 1013 proteins from both strains which were expressed in both conditions, at the beginning (0 h) and at end of a fermentation (8 h). These proteins are listed in (Table S1) and were classified according to their biological activity category using

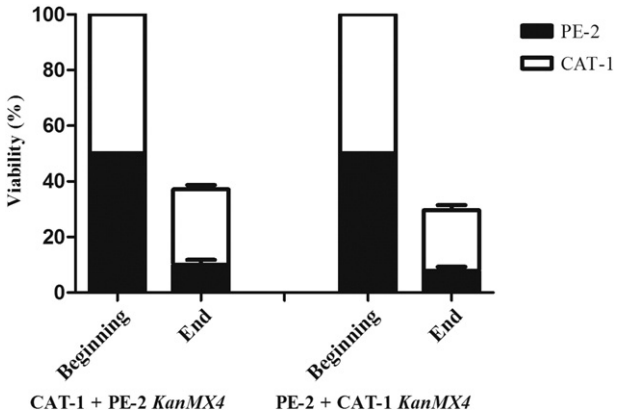

Fig. 2. Fitness analysis of mixed cultures (CAT-1 + PE-2 *kanMX4* or CAT-1 + PE-2 *kanMX4*) during 24 h fermentation. The composition of viable cells at the beginning and end of a batch fermentation was obtained from the difference between the number of colonies from the plates without geneticin and the plates with geneticin (only PE-2 *kanMX4* and CAT-1 *kanMX4* are able to grow on both plates). Values are mean ± standard deviation (SD) of three independent experiments. The statistical analysis showed that the results were non-different at $p < 0.05$ (CAT-1 + PE-2 *kanMx4* mixed culture vs. PE-2 + CAT-1 *kanMx4* mixed culture, for each time).

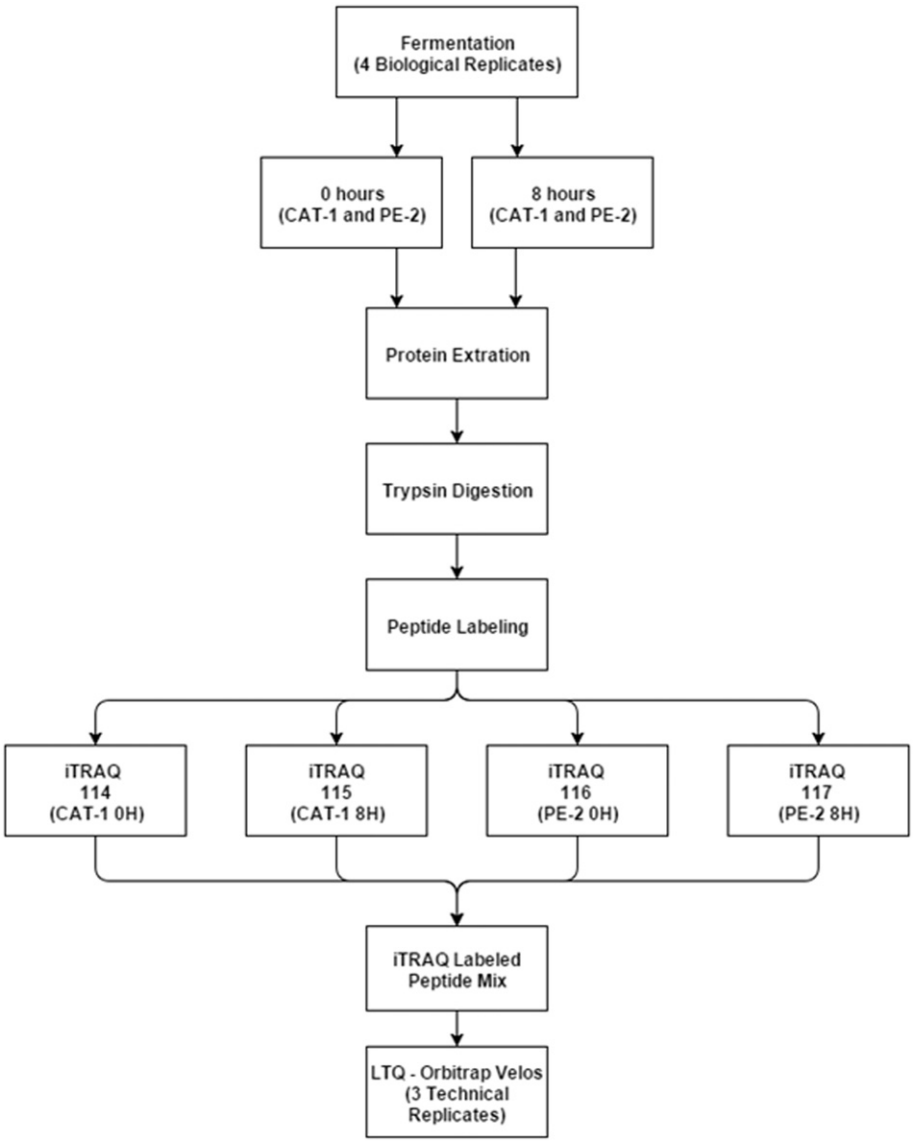

Fig. 3. Experimental workflow. Proteins expressed after a batch fermentation of CAT-1 or PE-2 were digest with trypsin and the same amount of peptides from each stage was labeled with iTRAQ, generating four different samples. After cleaning, the samples were analyzed in four replicates by an EASY-LC coupled with a LTQ-Orbitrap Velos mass spectrometer.

PatternLab's Gene Ontology Explorer module [22] and the gene ontology consortium obo database [23] (Fig. S1).

The majority of these proteins is associated with stress response, including proteins involved in response to stimulus (44%), oxidative stress (5%) and protein folding and assembly (9.7%). During fermentation, yeasts are exposed to various environmental changes, such as nutrient availability, ethanol concentration and change in pH. Therefore, cells must maintain their homeostasis, which is achieved through a highly coordinated molecular response for sense, repair and adaptation [24]. A considerable proportion of the stress proteins participates in the antioxidant system (5%). Ethanol causes dehydration, leakage of iron from iron-sulfur clusters and inactivation of antioxidant enzymes, among other toxic effects [25]. These effects increase the levels of reactive oxygen species (ROS), explaining the cellular antioxidant response induction during fermentation.

The iTRAQ-based quantitative proteomic strategy employed to identify relative expression changes of proteins from CAT-1 and PE-2 might explain the better fermentative fitness of CAT-1. The proteins present in both wild yeast (CAT-1 and PE-2) as well as in both conditions (beginning, 0 h, and end, 8 h, of a fermentation cycle) were distributed according to their p-value and the Log_e (ratio of ratios) (Fig. S2). The criteria used to select the proteins which represent a significant difference between CAT-1 and PE-2 along a batch fermentation were: i) at least 2 peptides identified per protein; ii) Log_e (ratio of ratios) >0.5 or <−0.5, meaning that the ratio [CAT-1 8 h/CAT-1 0 h] was at least 65% higher than the ratio [PE-2 8 h/PE-2 0 h], in the first condition, and the ratio [PE-2 8 h/PE-2 0 h] was at least 65% higher than the ratio [CAT-1 8 h/CAT-1 0 h], in the second condition; iii) p-value <0.05. As can be seen in (Table S2), 141 proteins among the detected 1013 proteins met the three criteria. From those, we selected possible candidates which might explain the supremacy of CAT-1 over PE-2 (Table 1).

In CAT-1 fermenting cells, proteins involved in stress response and longevity were expressed more strongly than in PE-2 cells. The cellular stress response is conserved, a major role being attributed to the induced heat-shock proteins (Hsps) and other molecules that confer stress protection. The majority of Hsps acts as molecular chaperones in protein refolding and protecting them from aggregation. As pointed out at Table 1, CAT-1 showed a higher abundance of the chaperones Ssa1, Hsp26 and Hsp104.

Mbf1 (Multiprotein Bridging Factor) was also up-regulated in CAT-1 fermenting cells. It is a suppressor of frameshift mutations, whose abundance increases in response to DNA damage [26]. Involvement of Mbf1

Table 1
Selected proteins differentially expressed after a batch fermentation of CAT-1 or PE-2.

Proteins	Number of peptides identified	LOG_e CAT-1 8h/CAT-1 0h PE-2 8h/ PE-2 0h	P-value	Description of the protein
Up-regulation proteins in CAT-1				
Ssa1	3	0.62	0.043	Heat Shock Protein
Hsp104	20	0.57	4.4E-19	Heat Shock Protein 104
Hsp26	2	1.51	8.00E- 06	Heat Shock Protein 26
Ino1	9	0.70	4.4E- 07	Inositol-3 Phosphate Syntase
Hor7	2	1.06	0.0007	Hyperosmolarity-Responsive
Mbf1	3	1.42	0.005	Multiprotein Bridging factor1
Tps3	3	0.56	0.011	Trehalose Synthase Complex Regulatory Subunit
Sod1	15	0.67	0.003	Superoxide Dismutase [Cu-Zn]
Trx1	5	0.67	0.006	Thioredoxin-1
Trx2	5	0.56	0.003	Thioredoxin-2
Cwp1	4	1.16	2.08E- 09	Cell Wall Protein
Cwp2	6	0.85	3.9E- 14	Cell Wall Protein
Kre6	4	0.87	0.0003	Type II Integral Membrane Protein
Ccw12	2	0.75	0.001	Cell Wall Protein
Ccw22	2	0.75	0.001	Cell Wall Protein
Top3	3	1.11	0.014	DNA Topoisomerase III
Up- regulation proteins in PE-2				
Ndi1	4	- 0.55	0.008	NADH- Dehydrogenase Internal
Nde1	3	- 0.45	0.040	NADH-Dehydrogenase External
Ubp6	5	- 0.69	0.050	Ubiquitin Specific Protease
Sod2	8	- 0.64	3.22E- 16	Superoxide dismutase [Mn], mitochondrial

in the DNA replicative stress response may be correlated with CAT-1 robustness. Ethanol was reported to cause DNA damage and genomic instability in yeast, such as chromatin condensation, fragmentation, and DNA cleavage [27].

Higher levels of Hor7 (HyperOsmolarity-Responsive) and Ino1 (inositol-3-phosphate synthase) were also detected in fermenting CAT-1 cells. *HOR7* codes for a protein of unknown function, whose transcription is induced under hyperosmotic stress. Hor7 overexpression suppresses Ca^{2+} sensitivity of mutants lacking inositol phosphorylceramide mannosyltransferases, which links its role to membrane protection [28]. Ino1 catalyzes the conversion of glucose 6-phosphate to inositol 3-phosphate, a precursor for the synthesis of inositol-containing glycerophospholipids in the yeast membrane [29]. It was reported that Ino1 overexpression lowers sensitivity to ethanol [30]. Thus, Hor7 and Ino1 upregulation might contribute to CAT-1 performance by conferring protection to the cell membranes, a target of ethanol toxicity [3].

After fermentation, Tps3 levels, involved in trehalose synthesis, were 75% higher in CAT-1 than in PE-2. Several studies have demonstrated a positive correlation between trehalose levels of yeast strains and their ability to withstand fermentation stress [31]. Proteins involved in antioxidant protection, such as Sod1 (Cu/Zn superoxide dismutase), Trx1 (thioredoxin-1) and its paralog Trx2 were also more abundant in CAT-1, indicating that this strain copes better with the oxidative damages arising from fermentation [32].

After fermentation, CAT-1 also showed increased levels of proteins that play a role in stabilizing (Cwp1 and Cwp2) and biogenesis of the cell wall (Kre6, Ccw12 and its paralog Ccw22), which allow a higher maintenance of the cell wall integrity and, consequently, a higher cell robustness [33]. It was also observed higher levels of Top3 (DNA Topoisomerase III) in the proteome of fermenting CAT-1 cells when compared to PE-2. Top3 is involved in the stability of telomeres, preventing genomic instability and increasing longevity [34].

On the other hand, fermenting PE-2 cells showed higher levels of Ndi1 (Rotenone-insensitive NADH-ubiquinone oxidoreductase) and Nde1 (NADH-ubiquinone oxidoreductase External 1) than CAT-1. The up-regulation of these proteins of the electron transport chain (ETC) can lead to increased ROS production [35]. PE-2 cells showed increased levels of the Sod2 (Mn superoxide dismutase) (Table 1). This enzyme is confined to mitochondria, being related to elimination of superoxide radical produced by the ETC. However, Sod2 is an important antioxidant defense against ROS in respiratory metabolism and not fermentative metabolism, where the yeast is directed to the production of ethanol [36].

What draws attention is the largest abundance of Ubp6 (Ubiquitin-specific Protease) in PE-2 after fermentation, which might affect the degradation of ubiquitinated proteins [37]. Protein turnover plays an essential role in maintaining the life of an organism. Accumulation of altered proteins can cause a gradual degeneration of cellular functions, increasing the probability of death.

Taken together, these results point out how CAT-1 deals with the fluctuations of the environment during fermentation, explaining its prevalence and persistence over PE-2.

3.3. Lipid peroxidation and trehalose accumulation

To corroborate proteomic data, which suggested that CAT-1 is better adapted to fermentation than PE-2 because CAT-1 can cope better with stress than PE-2, the levels of oxidative damage and trehalose were determined.

Confirming that fermentation increases the level of oxidative stress [3] both strains showed increased levels of lipid peroxidation at the end of a fermentation cycle (Fig. 4). However, in PE-2 cells this increase was almost 30% higher than in CAT-1, probably due to the antioxidant defense system of PE-2 is less effective or/and PE-2 generates more ROS during fermentation. CAT-1 fermenting cells presented higher levels of the antioxidant enzymes Sod1, Trx1 and Trx2, while PE-2 showed increased expression of Ndi1 and Nde1 (Table 1).

CAT-1 also demonstrated a two-fold higher capacity of accumulating trehalose (Fig. 4). This sugar acts as a stress protector, stabilizing membranes and proteins during adverse conditions [31]. Higher levels of trehalose are also associated with protection against lipid peroxidation during fermentation [3]. In agreement with this, CAT-1 showed higher levels of trehalose and reduced levels of lipid oxidation. The higher levels of trehalose might be associated with the increased expression

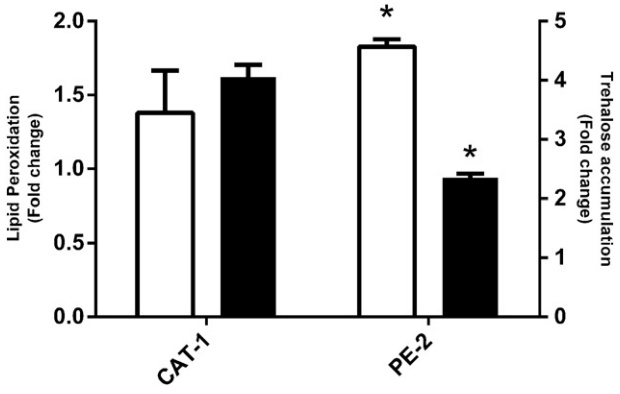

Fig. 4. Increase in lipid peroxidation (white bars) and in trehalose (black bars) levels in response to 24 h fermentation. The results were expressed as a ratio between the levels of lipid peroxidation or intracellular trehalose at the end of a fermentation and the levels of these parameters at the beginning of a fermentation. The experiments were done as described in "Material and methods". The results represent the mean ± SD of at least three independent experiments. * means statistically different results at $p < 0.05$ (CAT-1 vs. PE-2, for each parameter).

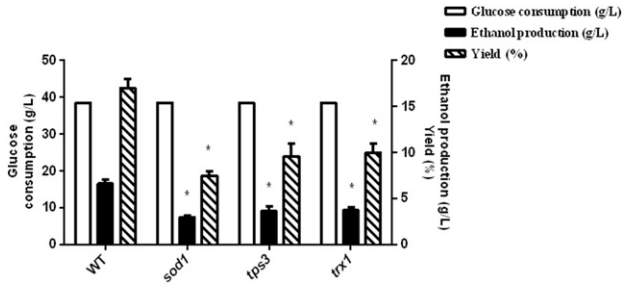

Fig. 5. Sugar consumption, ethanol production, and yield after 24 h fermentation of *sod1, trx1* and *tps3* mutant strains. Cells were grown in YPD medium until mid-log phase. They were collected by centrifugation, washed with distilled water and transferred to a fermentation medium. Yield of ethanol from glucose was expressed as grams of ethanol produced/grams of glucose consumed. Values are mean ± standard deviation (SD) of three independent experiments. * means statistically different results at $p < 0.05$ (WT vs. mutants, for each parameter).

of Tps3 found in CAT-1 (Table 1). This protein is involved in trehalose synthesis [11]. CAT-1 also showed higher levels of Tps1 and Tps2 (Table S1). In *S. cerevisiae*, trehalose synthesis involves two enzymes: trehalose-6-phosphate synthase (Tps1), which catalyzes the synthesis of trehalose-6-phosphate (T6P), and trehalose-phosphatase (Tps2), which dephosphorylates T6P to trehalose. The complex of synthesis in yeast also includes two other proteins, Tsl1 and Tps3, which seem to have regulatory functions. While Tsl1 is decisive for Tps1 activity, Tps3 seems to be an activator of Tps2 [11,31].

It is easy to study the biochemical function of gene products in yeast as libraries of deletion mutants of *S. cerevisiae* are commercially available. Thus, in order to confirm the significance of Sod1, Trx1 and Tps3 for an improved performance in fermentation., the control strain BY4741 - WT (*MATa*; *his3*; *leu2*; *met15*; *ura3*) and its isogenic strains *sod1*, *trx1* and *tps3*, harboring the genes *SOD1, TRX1*, and *TPS3*, respectively interrupted by *kanMX4*, all acquired from Euroscarf, Germany, were evaluated regarding their fermentation capacity. Considering the WT as a reference strain, all the mutant strains, *sod1*, *trx1* and *tps3*, produced less ethanol, exhibiting lower fermentation yields (Fig. 5). The mutants *sod1* and *trx1* showed a higher increase in the levels of lipid peroxidation than WT, corroborating the idea that an accumulation of oxidative damage impairs the cellular fermentative performance (Fig. 6A). As expected, the mutant *tps3* showed a lower capacity of accumulating trehalose when compared to the WT strain, which might explain its worse performance (Fig. 6B). In such a way, in the laboratory strain, BY4741, used as control to understand the biochemical roles of proteins, as well as in the wild yeast strains, CAT-1 and PE-2, used in industrial fermentations, the abilities to accumulate increased trehalose levels and to cope with the oxidative damage are important characteristics for improving fermentation. In this task, Tps3, Sod1 and Trx1 play crucial roles.

4. Discussion

CAT-1 and PE-2 strains, isolated from Brazilian distilleries, show remarkable fermentative performance, persistence and prevalence in the adverse environment found in the fuel ethanol industry. Together they are currently responsible for the production of >50% of the ethanol in Brazil [2]. The goal of this work was to analyze how CAT-1 and PE-2 cultures compete for nutrients in the same environment and compare these strains through proteomics to identify potential characteristics that caused the better performance of one of them.

According to our results, CAT-1 strain dominated the environment, overwhelming the PE-2 strain, leading us to conclude that CAT-1 shows a greater robustness to fermentation conditions than PE-2. Confirming CAT-1 superiority, CAT-1 showed a better fermentative performance than PE-2. During the first fermentation, there was no significant difference between the two strains. However, by recycling the cells at the end of the fermentation, and submitting them to another cycle, CAT-1 showed slightly better results than PE-2.

iTRAQ-mediated proteomics revealed possible characteristics which distinguish both wild yeasts with respect to fermentation adaptability, such as the increased expression of proteins involved with stress response shown by CAT-1. Yeast cells encounter a variety of environmental stresses during industrial fuel-ethanol fermentation. Thus, the identification of the factors that increase cell robustness is necessary to enhance the efficiency of the ethanol production process.

Industrial fermentations use high sugar concentrations, which can cause osmotic stress and lead to the production of high ethanol levels [38]. Even *S. cerevisiae* being considered highly adapted to alcoholic fermentation, ethanol inhibits yeast cell growth and reduces viability, limiting productivity and ethanol yields [3]. Exposure of yeast to ethanol increases plasma membrane fluidity, affecting its integrity; both, osmotic stress and ethanol, cause a reduction in water availability, destabilizing the structure of plasma membrane and denaturing proteins [39].

CAT-1 fermenting cells showed increased levels of the proteins from the trehalose synthesis complex, Tps1, Tps2 (Table S1) and Tps3 (Table 1), exhibiting a better capacity to accumulate trehalose (Fig. 4). According to Figs 5 and 6, Tps3 deficiency impaired trehalose accumulation as well as ethanol production. It is well known that trehalose increases cell robustness against stress because it stabilizes the structure of the lipid bilayer. This disaccharide also prevents protein denaturation and the aggregation of denatured proteins [40]. CAT-1 also showed increased levels of Ssa1, Hsp104 and Hsp26, which avoid protein aggregation and, consequently, increase cell longevity. Ssa1 belongs to the Ssa

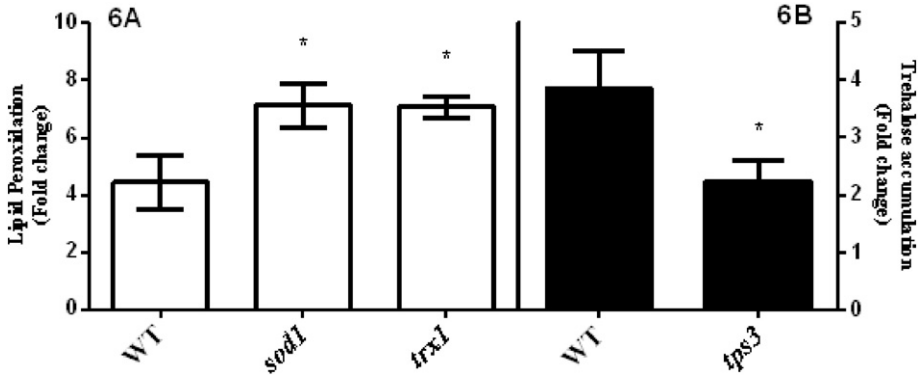

Fig. 6. A) Increase in lipid peroxidation (white bars) of *sod1*, *trx1* and WT (control) strains; B) change in trehalose levels (black bars) of WT and *tps3* mutant strain produced in response to fermentation. The results were expressed as a ratio between the levels of lipid peroxidation or intracellular trehalose at the end of a fermentation (24 h) and the levels of these parameters at the beginning of a fermentation. The experiments were done as described in "Material and methods". The results represent the mean ± SD of at least three independent experiments. * means statistically different results at $p < 0.05$ (WT vs. mutants, for each parameter).

subfamily of cytosolic Hsp70, whose main role is to serve as molecular chaperone, binding newly-translated proteins to assist in proper folding. Ssa1 in conjunction with the chaperone Hsp104 prevent aggregation/misfolding by disassembling aggregates of misfolded proteins [41]. Hsp26, which is a member of the small Hsps family, also prevent protein aggregation by binding and preventing unfolded proteins from irreversibly forming large aggregates. Stress-unfolded proteins bound to Hsp26 can be released and refolded in either a spontaneous or chaperone-assisted way [42].

Although oxidative stress has been traditionally associated with respiration, oxidative stress also occurs in fermenting yeasts [3]. Besides inactivating antioxidant enzymes, ethanol reduces the hydration shell of biomolecules, making them more prone to attack by ROS [25]. Confirming that oxidative stress takes place during ethanol production, both CAT-1 and PE-2 showed an increase in lipid oxidation after fermentation (Fig. 4). However, in CAT-1, the increase in the levels of lipid peroxidation was lower, which can be associated with the higher levels of the antioxidant enzymes Sod1, Trx1 and Trx2 observed in this strain. Sod1, which degrades superoxide anions, is one of the main enzymes involved in the homeostasis of the intracellular ROS levels. Deletion of *SOD1* gene may result in increased oxidative damage [43] . Trx1 and its paralog Trx2 are critical for antioxidant protection in maintaining the reduction of other proteins by catalyzing cysteine thiol-disulfide exchange reactions [44]. Corroborating proteomic analyses, other works demonstrated a correlation between Trx overexpression and increased fermentative performance [45]. An increased antioxidant activity attenuates the damaging effects of ROS and delay many events that contribute to cellular aging, extending life span [12]. By using mutant strains deficient in Sod1 or Trx1, we also demonstrated the importance of these antioxidant proteins for improving fermentation performance (Fig. 5).

The up-regulation of Top3 and Mbf1 might also be correlated with CAT-1 prevalence during fermentation, since both confer genomic stability. Top3 is involved in the stabilization of telomeres. Consequently, Top3 plays important roles in determining genomic integrity, favoring replication, transcription and segregation of chromosomes [46]. Each time a yeast cell divides, its telomeres shorten. When they get too short, the cell becomes senescent. Thus, telomere stabilization postpones replicative aging. It was previously reported that Mbf1 abundance increases genomic integrity during replicative stress [26].

CAT-1 also showed increased levels of Ino1 and Hor7, which are associated to inositol-containing phospholipids synthesis. In response to ethanol, lipid unsaturation decreases to counteract the less ordered, more 'fluid', state of membranes. Ethanol alters the permeability of the yeast membrane by perturbing the hydrophobic barrier [39]. Therefore, higher levels of Ino1 and Hor7 may have contributed to the best fermentative performance of CAT-1 by producing efficient phospholipid barriers against ethanol.

Another characteristic of outstanding good fermenting strains is cell wall robustness. The molecular architecture of the cell wall is not fixed. Considerable adjustments to the composition and structure of cell wall must be activated in response to environmental conditions to compensate for cell damage [47]. In sake yeast strains, it was observed that cells sensitive to ethanol also demonstrate a greater sensitivity to drugs that affect cell wall architecture, indicating that that the maintenance of cell wall integrity is of great importance for yeast ethanol tolerance [48]. CAT-1 showed increased levels of proteins involved in cell wall biogenesis and stabilization.

5. Conclusions

According to our results, CAT1-1 showed a fermentative supremacy over PE-2 strain. The proteomic profile obtained from industrial strains during fermentation allowed us to identify possible characteristics which might be causing the best performance of CAT-1, like Hsps, antioxidant enzymes, proteins involved with trehalose and inositol-containing phospholipids biosynthesis as well as telomere and cell wall stability. These data provides valuable knowledge on adaptability to alcoholic fermentation to improve bioethanol production process.

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Conflict of interest

All the authors declare no conflict of interest.

Transparency document

The Transparency document associated with this article can be found, in the online version.

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