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Increasing Ethanol Tolerance and Ethanol Production in an Industrial Fuel Ethanol *Saccharomyces cerevisiae* Strain

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Abstract: The stress imposed by ethanol to *Saccharomyces cerevisiae* cells are one of the most challenging limiting factors in industrial fuel ethanol production. Consequently, the toxicity and tolerance to high ethanol concentrations has been the subject of extensive research, allowing the identification of several genes important for increasing the tolerance to this stress factor. However, most studies were performed with well-characterized laboratory strains, and how the results obtained with these strains work in industrial strains remains unknown. In the present work, we have tested three different strategies known to increase ethanol tolerance by laboratory strains in an industrial fuel–ethanol producing strain: the overexpression of the *TRP1* or *MSN2* genes, or the overexpression of a truncated version of the *MSN2* gene. Our results show that the industrial CAT-1 strain tolerates up to 14% ethanol, and indeed the three strategies increased its tolerance to ethanol. When these strains were subjected to fermentations with high sugar content and cell recycle, simulating the industrial conditions used in Brazilian distilleries, only the strain with overexpression of the truncated *MSN2* gene showed improved fermentation performance, allowing the production of 16% ethanol from 33% of total reducing sugars present in sugarcane molasses. Our results highlight the importance of testing genetic modifications in industrial yeast strains under industrial conditions in order to improve the production of industrial fuel ethanol by *S. cerevisiae*.

Keywords: ethanol stress; ethanol tolerance; industrial yeast strains; high-gravity fermentation; *TRP1*; *MSN2*

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1. Introduction

Fuel ethanol production has been intensively investigated as a result of increasing concerns on energy security, sustainability, and global climate change, and consequently the fuel ethanol market reached 124 billion liters in 2021 [1,2]. Yeasts of the *Saccharomyces* genus (including hybrids) are used in many industrial fermentation processes, but *S. cerevisiae* is the predominant yeast species responsible for fuel ethanol production worldwide [3,4]. *S. cerevisiae*, and few other yeasts, are able to ferment sugars even in the presence of oxygen, a phenomenon known as the "Crabtree effect" [5–7]. This fermentation capacity reduces the cell biomass production but provides a tool to out-compete other microorganisms due to the toxicity of high levels of ethanol. However, the increased concentration of ethanol produced during fermentation will influence the membrane fluidity, and thus is also toxic to the yeast cells producing ethanol, leading to growth inhibition and even death. Consequently, there have been several investigations dealing with ethanol tolerance and the stress response that yeasts have when submitted to high ethanol concentrations (reviewed in [8–12]). A better understanding of the molecular mechanisms underlying yeast tolerance to these

fermentation-associated stresses is essential for the improvement of yeast cell performance during ethanol stress by genetic engineering approaches [13,14].

Properties such as membrane lipid composition, chaperone protein expression, vacuolar and peroxisome function, and trehalose content are important determinants of ethanol tolerance. In *S. cerevisiae*, signal transduction pathways triggered by the most common types of stress (the general stress response), allowing cells exposed to a mild stress to develop tolerance, not only to higher doses of the same stress, but also to other stresses [15]. This system regulates the induction of many stress genes through a common cis element in their promoter (also known as stress response element, STRE), which is recognized by the two redundant *MSN2*/*MSN4* transcription factors [16,17]. Upon exposure to a stressful environment (e.g., high ethanol levels), these transcription factors migrate to the nucleus to promote the expression of hundreds of genes, the majority of them through the activity of *MSN2* [18]. The protein encoded by *MSN2* has several functional regions, including the C-terminal zinc finger DNA-binding domain, the region containing the nuclear localization signal and the nuclear export signal, and the essential *MSN2* transcriptional-activating domain (TDA), located in the N-terminal region of the protein, that is also involved in the nuclear localization of this transcription factor [19,20]. Finally, overexpression of *MSN2*, or a truncated version of the protein lacking the first 48 amino acids, allows increased ethanol tolerance and productivity by yeast cells [21,22]. Besides this general stress response, other publications have shown that some amino acids (particularly tryptophan) are involved in yeast tolerance to ethanol and other stresses. Genome-wide transcriptomics and screening of the collection of deletion mutants revealed many genes required for tolerance to ethanol, including genes involved in tryptophan biosynthesis that promoted an increased sensitivity to ethanol when deleted. Indeed, an ethanol-tolerant sake brewing yeast showed higher expression levels of tryptophan biosynthesis genes under ethanol stress when compared with a laboratory strain. These studies also showed that the overexpression of any of the tryptophan biosynthesis genes (*TRP1*–*TRP5*, particularly *TRP1*), or the overexpression of the tryptophan permease gene (*TAT2*), together with tryptophan supplementation on the culture medium, improved ethanol tolerance by a laboratory yeast strain [22–26].

The Brazilian sugarcane fuel ethanol industry is highly competitive when compared with other ethanol production processes (e.g., from corn or sugarbeet), as it shows the lowest production costs and the highest energy balance and yields per hectare, as well as the highest percentage of greenhouse gas emissions reduction [27]. The productivity of the Brazilian fuel ethanol industry has increased steadily due to several improvements, including the amount of ethanol produced from each ton of sugarcane, as the fermentation process has also reached high industrial efficiency [28,29]. More than 70% of the fuel ethanol facilities in Brazil perform the Melle–Boinot process, a fed-batch fermentation with high cell density that allows for very short fermentation times and higher production stability when compared to continuous fermentations [30]. At the end of each fermentation, the yeast cells are centrifuged, treated with diluted sulfuric acid for 1–2 h to reduce the bacterial contamination, and over 90% of the yeast biomass is reused from one fermentation to the next so to ensure the high cell density that contributes to the improved productivity of the process [28,29,31]. However, as in many industrial nonsterile processes, a continuous input of contaminant microorganisms is observed, leading to decreased efficiency and stuck fermentations [32–34]. Studies on the microbiological dynamics of the industrial fermenters revealed a very rapid succession of yeast strains, and consequently the original starter yeast can be completely replaced by other strains in a matter of weeks [35,36]. Nevertheless, some yeast strains tend to dominate the fermenters, allowing the selection of suitable industrial fuel ethanol strains (e.g., strain CAT-1) with high fermentative capacity and viability able to withstand the stressful industrial conditions, which include high ethanol concentrations [36–38].

Considering that most studies regarding the genes involved in ethanol tolerance were performed using laboratory yeast strains resistant to relatively low (5–8% w/v) ethanol concentrations, in the present report we tested if the above approaches (overexpression of

TRP1, *MSN2*, or a N-terminal truncated *MSN2*) could improve the ethanol tolerance and production of the industrial strain CAT-1. Our results indicate that strain CAT-1 can tolerate up to 14% ethanol, and while the three strategies indeed increased its tolerance to ethanol, when these strains were subjected to fermentations with a high sugar content, simulating the industrial conditions used in Brazilian distilleries, only the strain with the overexpression of the truncated *MSN2* gene showed an improved fermentation performance.

2. Materials and Methods

2.1. Strains, Media, and Growth Conditions

The *S. cerevisiae* strains analyzed (Table 1) were all derived from the industrial fuel ethanol strain CAT-1 [38]. Yeasts were cultivated on rich YP medium (20 g/L peptone, 10 g/L yeast extract) containing 20 g/L glucose or sucrose. The pH of the medium was adjusted to pH 5.0 with HCl. When required, 20 g/L agar and 200 mg/L geneticin (G-418) sulfate (Invitrogen, Thermo Fisher Scientific Inc., Sinapse Biotecnologia, São Paulo, SP, Brazil) were added to the medium. Cells were grown aerobically at 28 °C with shaking (160 rpm) in cotton-plugged Erlenmeyer flasks filled to 1/5 of the volume with medium, and cellular growth was followed by turbidity measurements at 570 nm (OD_{570nm}).

Table 1. Yeast strains and primers used in this study.

Strains and Primers	Relevant Features, Genotype or Sequence (5′→3′)	Source
Yeast strains:		
CAT-1	Industrial strain isolated by Fementec Ltd.a. in 1998/1999 from Usina VO Catanduva, located in the State of São Paulo, Brazil.	[38]
CAT1-TRP1oe	Isogenic to CAT-1, but *KanMX*-P_{ADH1}::*TRP1*	This work
CAT1-MSN2oe	Isogenic to CAT-1, but *KanMX*-P_{ADH1}::*MSN2*	This work
CAT1-NΔMSN2oe	Isogenic to CAT-1, but *KanMX*-P_{ADH1}::NΔ(1-153)*MSN2*	This work
Primers:		
TRP1-Kanr-F	GAGAGGGCCAAGAGGGAGGGCATTGGTGACTATTGAGCACCCAGCTGAAGCTTCGTACGC	This work
TRP1-PADH1-R	TCACCAATGGACCAGAACTACCTGTGAAATTAATAACAGACATTGTATATGAGATAGTTG	This work
MSN2-Kanr-F	CGGGAAGATCACAACAGTAGTAGCAAGGTATTTCATACGCCCAGCTGAAGCTTCGTACG	This work
MSN2-PADH1-R	CATGGTCGACCGTCATTTTAGATCTAGTTCTTCTATGAGCCCAATGGACCAGAACTACCTG	This work
NΔMSN2-PADH1-R	CAGTGAAGTTTCTTGATTTTGAATGTCATTGAGATCCGCCAATGGACCAGAACTACCTG	This work
V-kan^r-F	CCGGTTGCATTCGATTCC	This work
VRT-TRP1-R	GTAAGCTTTCGGGGCTCTCT	This work
VRT-MSN2-R	TGAAGGTACCGGAAAAATGG	This work
RT-ACT1-F	TGGATTCCGGTGATGGTGTT	This work
RT-ACT1-R	CGGCCAAATCGATTCTCAA	This work
RT-TRP1-F	GTTCCTCGGTTTGCCAGTTA	This work
RT-MSN2-F	CGCGATGCAAGAACTATTGA	This work

2.2. Molecular Biology Techniques

Standard methods for bacterial transformation, DNA manipulation, and analysis were employed [39]. To overexpress the *TRP1* gene in *S. cerevisiae* CAT-1, the promoter region of this gene was modified according to the polymerase chain reaction (PCR)-based gene modification procedure [40]. Briefly, the *kanMX*-P_{ADH1} module from plasmid pFA6a-*kanMX*6-P_{ADH1} [40] was amplified with primers TRP1-Kanr-F and TRP1-PADH1-R (Table 1). These primers contain ~40 bp of homology to the promoter and start regions of the *TRP1* gene at their 5′ end, and ~20 bp to amplify the *kanMX*-P_{ADH1} module at their 3′ end. The resulting PCR product of 2394 bp containing the constitutive promoter of the *ADH1* gene was used to transform competent yeast cells by the lithium acetate method [41]. After 2 h cultivation on YP-20 g/L glucose, the transformed cells were plated on the same medium containing G-418 and incubated at 28 °C. The G-418-resistant isolates were tested for proper genomic integration of the *kanMX*-P_{ADH1} cassette at the *TRP1* locus by diagnostic colony PCR using primers V-kan^r-F and VRT-TRP1-R (Table 1), producing strain CAT1-TRP1oe (*KanMX*-P_{ADH1}::*TRP1*). A similar approach was used to overexpress the *MSN2* gene, but in this case, the *kanMX*-P_{ADH1} module was amplified directly from the genomic DNA of strain CAT1-TRP1oe using primers MSN2-Kanr-F and MSN-PADH1-R (Table 1), while the correct genomic integration of the *kanMX*-P_{ADH1} cassette at the *MSN2*

locus was verified using primers V-kan^r-F and VRT-MSN2-R (Table 1), producing strain CAT1-MSN2oe (*KanMX*-P_{ADH1}::*MSN2*). To overexpress the truncated version of the *MSN2* gene, the reverse primer (NΔMSN2-PADH1-R, Table 1) was designed to have 40 bp of homology to the sequence downstream of nucleotide 153 of the open reading frame, and thus the first 51 amino acids of the protein were deleted. When the G-418-resistant isolates were tested for proper genomic integration of the *kanMX*-P_{ADH1} cassette at the *MSN2* locus (using primers V-kan^r-F and VRT-MSN2-R, Table 1), a ~153 bp shorter amplicon was obtained when compared with the product obtained with strain CAT1-MSN2oe, indicating that strain CAT1-NΔMSN2oe was correctly constructed.

2.3. Quantitative RT-PCR Analysis

Quantitative RT-PCR (qRT-PCR) was conducted to verify the overexpression of the *TRP1*, *MSN2* and NΔ(1-153)*MSN2* genes in strains CAT1-TRP1oe, CAT1-MSN2oe, and CAT1-NΔMSN2oe, respectively, when compared to the expression of these genes in strain CAT-1. The yeast strains were grown in YP-20 g/L glucose medium to mid-log phase, centrifuged (5000× *g*, 4 min at 4 °C), washed with cold distilled water, and, according to the manufacturer's protocols, the total RNA of the cell pellets was extracted using the RNeasy® Mini Kit (Qiagen Brazil, São Paulo, Brazil). The total RNA of each sample (1 ug) was reverse transcribed to cDNA using the QuantiTect® Reverse Transcription Kit (Qiagen). The qRT-PCR reactions were performed with the QuantiFast® Sybr Green PCR Kit and the Rotor-Gene® Q equipment (Qiagen) using the primers for the *TRP1* gene (primers RT-TRP1-F and VRT-TRP1-R, Table 1), the *MSN2* and NΔ(1-153)*MSN2* genes (primers RT-MSN2-F and VRT-MSN2-R, Table 1), and, for the actin gene (*ACT1*), that was selected as the endogenous reference gene (primers RT-ACT1-F and RT-ACT1-R, Table 1). A dissociation curve was generated for each assay in order to confirm the amplification of only one product. The $2^{-\Delta\Delta CT}$ method [42] was used to calculate the relative expression levels of the *TRP1*, *MSN2*, and NΔ(1-153)*MSN2* genes relative to the *ACT1* gene for each yeast strain, in triplicate.

2.4. Analysis of the Ethanol Tolerance of Yeast Strains

Yeast cells were pregrown overnight in 3 mL of YP-20 g/L sucrose, and 1:100 dilutions of these precultures were used to inoculate 100 µL of YP-20 g/L sucrose medium containing different concentrations of ethanol (10–16%, *w*/*v*) in 96-well plates in a TECAN Infinite® M200 Pro microplate reader (Tecan Group Ltd., Grödig, Salzburg, Áustria) to determine the growth of the yeast strains at 28 °C. All wells in the plate were tightly sealed with AccuClear Sealing Film for qPCR (E & K Scientific, Santa Clara, CA, USA), and growth of each culture was monitored by measuring the OD_{570nm} every 15–60 min, with high intensity orbital shaking between measurements. Under the conditions used (100 µL of medium in 96-well plates), the maximum OD_{570nm} measured when the cells reached the stationary phase of growth was 0.8–0.9 [37]. The yeast growth data in the presence of different ethanol concentrations was analyzed with the PRECOG software [43] to extract the fitness components affected by the ethanol stress in the different yeast strains, including the length of the lag phase (in h), growth rate (doubling time, in h), and growth efficiency (ΔOD_{570nm} from the beginning to the stationary phase of growth).

2.5. Measurement of Intracellular Oxidation Level and Cell Viability

The intracellular oxidation level due to reactive oxygen species (ROS) trigged by exposure of the yeast cells to 20% (*v*/*v*) ethanol for 6 h was measured using the oxidant-sensitive fluorescent probe 2′,7′-dichlorofluoresein (DCF-DA) (Molecular Probes, Eugene, OR, USA), as previously described [44]. The fluorescence intensity of the strains before exposure to the ethanol stress (no stress) was relatively taken as 100%. Cell viability was estimated by microscopy in a Neubauer chamber with a 40× objective by differential cell staining using a solution of erythrosine in phosphate buffer, as previously described [45]. Viability was expressed in percentage (%) of viable cells with respect to the total cell counting.

2.6. Fermentations Mimicking the Brazilian Sugarcane Biorefinery

The fermentation assays were conducted simulating the industrial conditions of Brazilian distilleries, with a cell recycle as described by Raghavendran and coworkers [31] using sugarcane molasses from São Manuel mill (São Paulo, Brazil) as the substrate. The molasses was diluted with water to obtain a desirable percentage of total reducing sugars (TRS) for both the propagation of the strains (with 10% TRS) and the fermentation assays (18–33% TRS). The molasses' must was centrifuged (2000× *g*, 20 °C, 20 min) to remove the suspended solids and autoclaved in Erlenmeyer flasks. The yeast strains were propagated in 10% TRS molasses at 30 °C with shaking (100 rpm) for 48 h. After centrifugation, 3 g of wet biomass was placed in 50 mL conical bottom tubes and mixed with 2 mL of the fermented medium (from the propagation step) without yeast, plus 6 mL of distilled water to start the first fermentation cycle by adding 28 mL (divided in three equal portions, each added at 2 h intervals to simulate the fed-batch process) of molasses' must containing 18% TRS. The temperature was controlled at 30 °C in a static incubator, and the tubes were weighed at regular intervals to determine weight loss due to CO_2 release by fermentation. At the end of fermentation, the tubes were homogenized and a 1 mL sample was taken for cell viability and metabolites analysis. The yeasts were separated from the fermented substrate by centrifugation (2000× *g*, 10 min) and weighed for determination of biomass content. For the subsequent fermentation cycle, 2 mL of the fermented substrate without yeasts (from the previous cycle) was added to the cell pellet, simulating a yeast cream in an industrial centrifuge, and diluted in 6 mL of distilled water, which was acidified with sulfuric acid to pH 2.5 during 1 h under static conditions to simulate the acid treatment performed in the industry. Subsequently, 28 mL of molasses' must containing 22% TRS were added as previously described, and fermentation conducted under the same conditions. This procedure was repeated for each of the next two fermentation recycles, increasing the concentration of the molasses´ must to 28% TRS (3rd cycle) and 33% TRS (4th cycle). The molasses' must used had a pH variation of 5.1–5.4 during all cycles. All variables analyzed were submitted to ANOVA and Tukey pairwise comparison tests with R software (https://www.R-project.org, accessed on 21 August 2021). Triplicates of each strain were used to calculate the means, statistically compared within each cycle. Treatments were considered different at a significance level of 5% ($p < 0.05$).

2.7. Residual Sugars, Glycerol, and Ethanol Determination

The residual TRS (sucrose, glucose, and fructose) and glycerol production were determined by high-performance liquid chromatography [36] using a chromatograph ion exchange Dionex DX-300 HPAEC system (Thermo Fisher Scientific Inc., Sunnyvale, CA, USA) containing a CarboPac PA-1 column (4 × 250 mm) and pulse amperometric detector. The mobile phase used was 100 mM NaOH at a flow rate of 0.9 mL/min. The centrifuged fermented must without yeasts (10 or 25 mL) was transferred to a Kjeldahl microdistiller (Tecnal Equipamentos Cientificos, Piracicaba, Brazil) for steam distillation, and distilled samples were transferred to a digital Anton Paar DMA-48 densitometer (Anton Paar GmbH, Graz, Austria) to estimate the ethanol content (%, *v*/*v*) [36].

3. Results

3.1. Ethanol Tolerance of the Industrial Yeast Strains

The ethanol tolerance of the industrial fuel ethanol strain CAT-1 was determined by the capacity to grow in liquid YP-20 g/L sucrose media, supplemented with increased concentrations (10% to 16%) of ethanol. As can be seen in Figure 1A, this strain could grow with up to 14% ethanol present in the medium, while 16% inhibited completely its growth. This result is in accordance with other reports that have shown that this strain could tolerate up to 13% (*v*/*v*) ethanol in YP-20 g/L glucose agar plates [46]. The analysis of the fitness components (length of the lag phase, growth rate, and growth efficiency) affected by the ethanol stress revealed that increasing concentrations of ethanol extended the duration of the lag phase (Figure 2A) from ~2 h in the absence of ethanol to more than 43 h in the

presence of 16% ethanol (note that growth was significantly inhibited under this condition), reflecting the time required by the cells to adapt to the stressful environment promoted by the presence of high ethanol concentrations. Once the yeast cells started to grow, the growth rate (determined as doubling time, in hours) was not affected by the presence of a high ethanol concentration (doubling times of 0.5–0.7 h both in the presence or absence of ethanol), but the other fitness component, growth efficiency (ΔOD_{570nm} between the smallest to the largest population sizes at the stationary phase of growth), was also affected by the presence of high ethanol concentrations, with a 90% drop in ΔOD_{570nm} (Figure 2B) at the highest ethanol concentration in relation to the absence of ethanol in the media.

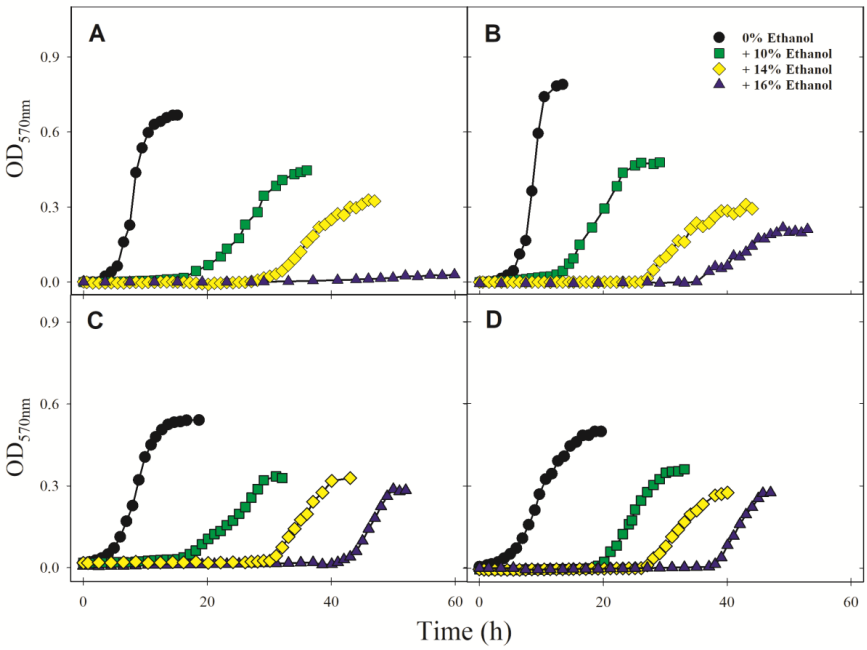

Figure 1. Ethanol tolerance of the industrial fuel ethanol strain CAT-1 (**A**) and the recombinant strains CAT1-TRP1oe (**B**), CAT1-MSN2oe (**C**), and CAT1-NΔMSN2oe (**D**), determined by the capacity to grow in liquid YP-20 g/L sucrose media in the presence of the indicated concentrations of ethanol.

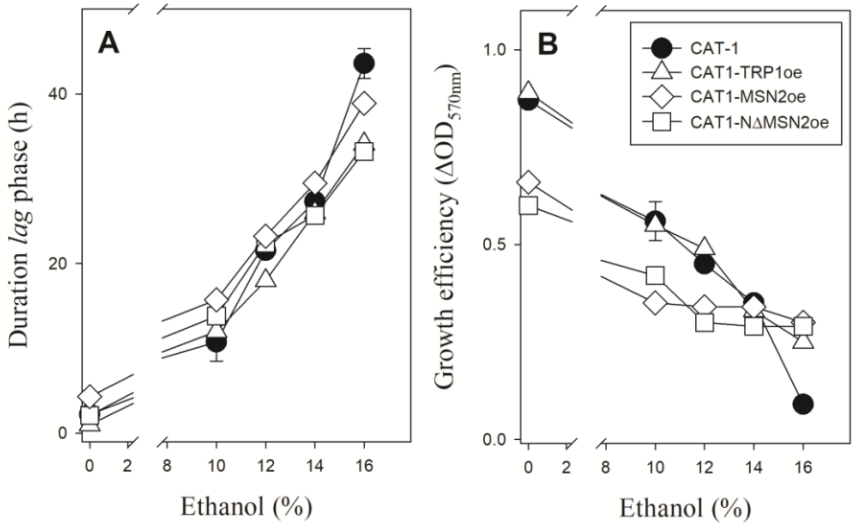

Figure 2. Fitness components ((**A**), duration of the lag phase, and (**B**), growth efficiency) affected by the presence of the indicated concentrations of ethanol during growth in YP-20 g/L sucrose by the industrial fuel ethanol strain CAT-1, and the recombinant strains CAT1-TRP1oe, CAT1-MSN2oe, and CAT1-NΔMSN2oe.

3.2. Overexpression of the TRP1, MSN2, or Truncated MSN2 Genes in Strain CAT-1

The results shown above indicate that the industrial CAT-1 strain is clearly more resistant to the ethanol stress when compared to laboratory strains, which usually do not tolerate more than 8–10% ethanol (e.g., ref. [22,25,26]). Aiming at increasing the ethanol tolerance of the industrial strain, three strategies known to improve growth of laboratory strains in the presence of ethanol were tested with strain CAT-1: (i) overexpression of the *TRP1* gene; (ii) overexpression of the *MSN2* gene; (iii) overexpression of a N-terminal truncated *MSN2* gene. Figure 3 shows that the genomic engineering approach employed (see Materials and Methods section), using a *kanMX*-P_{ADH1} module to replace the promoters of the target genes, succeeded in the overexpression of the *TRP1* (approximately 12-fold, Figure 3A) and *MSN2* and NΔ(1-153)*MSN2* genes (approximately five- and seven-fold, respectively, Figure 3B) when compared to the expression of these genes in strain CAT-1, as determined by qRT-PCR. It should be stressed that only one copy of the target genes had its promoter region modified, and the other copy present in the diploid genome of strain CAT-1 retained its original promoter region, as determined by diagnostic colony PCR with a different set of primers (data not shown).

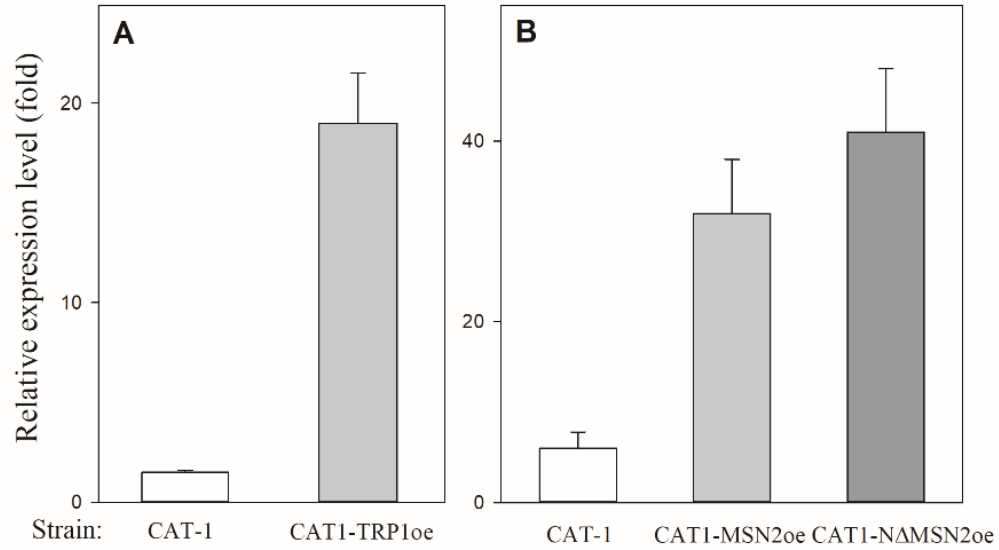

Figure 3. Relative expression levels of *TRP1* (**A**), and *MSN2*, and NΔ(1-153)*MSN2* genes (**B**). Yeast cells were cultured to the midexponential growth phase in YP-20 g/L glucose medium. The total RNA was prepared and reverse transcribed. Then, qRT-PCR analysis was performed. The cycle thresholds for each gene were normalized to the *ACT1* gene and the relative induction fold of the recombinant overexpressing strains compared with the control strain CAT-1 is shown.

The CAT1-TRP1oe strain had its ethanol tolerance improved (Figure 1B), as shown by a shorter lag phase (~34 h, Figure 2A) and improved growth efficiency (Figure 2B) in the presence of 16% ethanol, with a ΔOD_{570nm} that corresponds to one third of the value obtained in the absence of ethanol. Similar results of improved growth in the presence of 16% ethanol, with a shorter lag phase (Figures 1C,D and 2A) and improved growth efficiency (Figures 1C,D and 2B), were obtained with the CAT1-MSN2oe and CAT1-NΔMSN2oe strains when compared to strain CAT-1, although it should be noted that the overexpression of the *MSN2* and NΔ(1-153)*MSN2* genes reduced the growth efficiency of the industrial strain at lower ethanol concentrations, or even in the absence of ethanol (Figures 1C,D and 2B).

3.3. Intracellular Oxidation Levels in Yeast Cells Trigged by Exposure to 20% Ethanol

Given the relationship between ethanol and oxidative stress in *S. cerevisiae* [47], and the involvement of the *MSN2* transcription factor in the expression of some ROS-degrading enzymes [48], we also determined the intracellular oxidation levels in the CAT-1, CAT1-

TRP1oe, CAT1-MSN2oe, and CAT1-NΔMSN2oe strains trigged by exposure of the yeast cells to 20% (*v*/*v*) ethanol for 6 h. As can be seen in Figure 4, crude extracts from strain CAT-1 showed a nine-fold increase in fluorescence after exposure of the cells to the ethanol stress, indicating that ethanol induced intracellular ROS generation. Strain CAT1-TRP1oe also had high intracellular ROS generation trigged by the ethanol stress, not different from strain CAT-1. However, we observed a 24 to 38% decrease in the fluorescence when the cells of the CAT1-MNS2oe and CAT1-NΔMNS2oe strains were exposed to the ethanol stress, indicating that overexpression of *MSN2*, and particularly the truncated NΔ(1-153)*MSN2* version of the gene, reduced the intracellular level of ROS in the presence of ethanol, probably due to the higher expression of antioxidant enzymes.

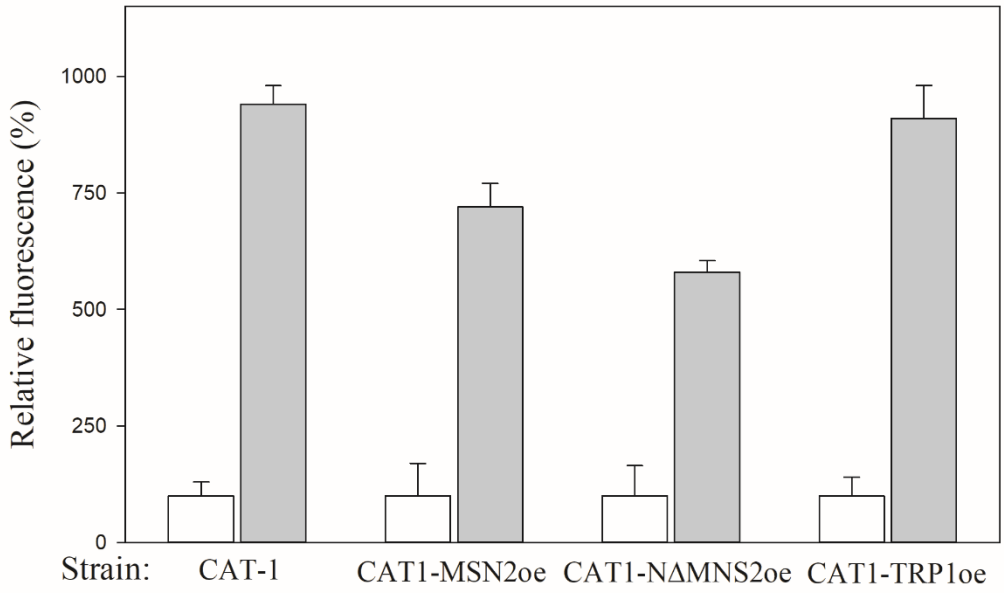

Figure 4. Intracellular oxidation level in strains CAT-1, CAT1-MSN2oe, CAT1-NΔMSN2oe, and CAT1-TRP1oe in the presence of ethanol. The fluorescence intensity due to the presence of ROS in the strains was determined before (white bars) and after 6 h of exposure to 20% ethanol (grey bars).

3.4. Fermentations Mimicking the Brazilian Sugarcane Ethanol Production Process

We next tested the fermentation performance of the fuel ethanol strains overexpressing the *TRP1*, *MSN2*, and truncated *MSN2* genes under conditions that simulate the current Brazilian sugarcane ethanol production process: use of sugarcane molasses as substrate (with no nitrogen supplementation), high cell densities, recycling of the cell biomass, and acid treatment after each fermentation cycle. Four fermentative cycles were performed imposing drastic stressful conditions to the yeasts by increasing the sugar concentration of the molasses' must from 18% TRS in the first cycle to 22% in the second, 28% in the third, and 33% in the last cycle. Figure 5 shows that, compared to the parental strain CAT-1, the strain overexpressing the *TRP1* gene (strain CAT1-TRP1oe) had a significantly lower performance, producing less ethanol (Figure 5A) and less biomass (Figure 5B) during practically all fermentative cycles. The strain overexpressing the *MNS2* gene (strain CAT1-MSN2oe) had a fermentative performance (both for ethanol and biomass produced) that did not differ significantly from strain CAT-1 (Figure 5A,B). The best results in terms of ethanol production were obtained with the strain overexpressing the truncated *MSN2* gene (strain CAT1-NΔMSN2oe), as this strain produced more ethanol during all fermentation cycles (when compared to the other three strains), reaching almost 16% ethanol in the last cycle (Figure 5A), while maintaining the same amount of biomass as the CAT-1 and CAT1-MSN2oe strains during practically all cycles (Figure 5B). While the strain CAT1-TRP1oe produced less ethanol, because it failed to consume all the sugars (TRS) from the molasses medium, especially in the last cycle (Figure 5C), the strain overexpressing the truncated *MSN2* gene (CAT1-NΔMSN2oe) was the strain that in all cycles analyzed consumed all the

sugar from the medium, leaving less than ~0.5% of TRS at the end of the fermentations (Figure 5C). These results indicate that the CAT1-NΔMSN2oe strain consumed almost all sugar present in the medium and was positively correlated to the results of ethanol production under high sugar and high ethanol stress by this strain.

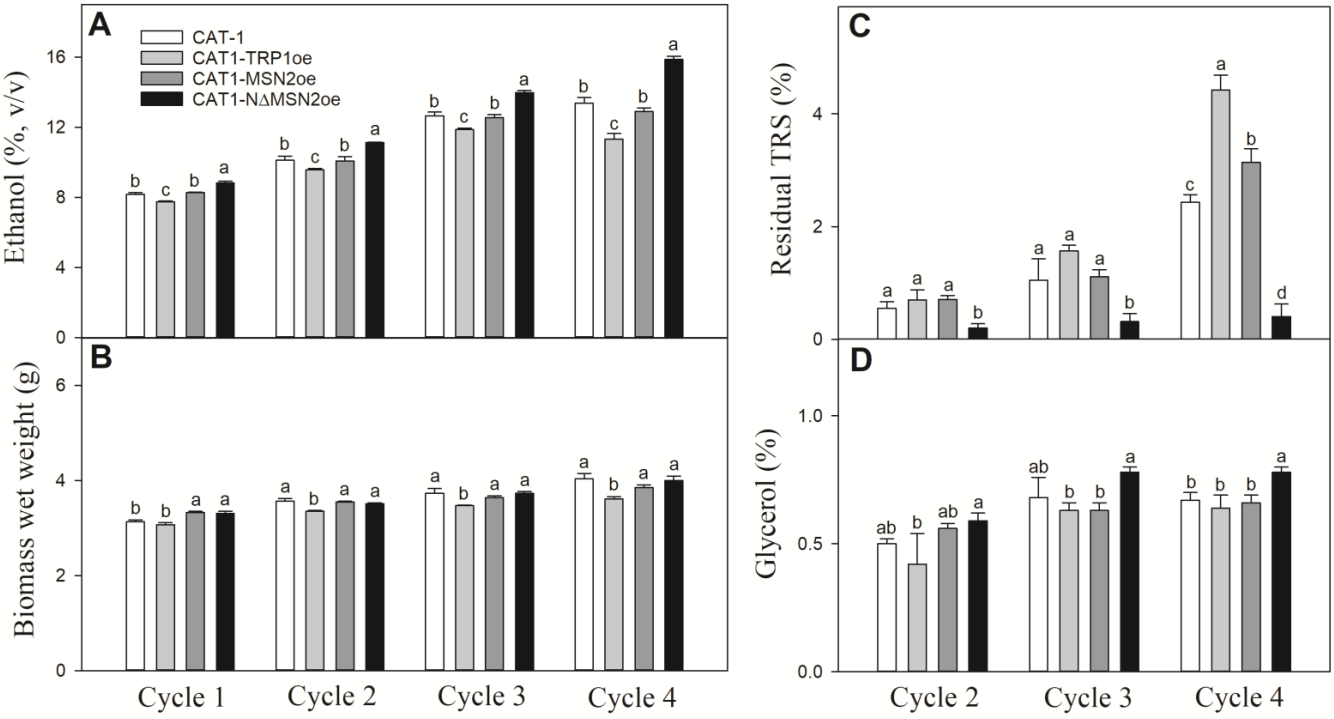

Figure 5. Fermentation performance under scaled-down and mimicked process resembling Brazilian sugarcane biorefineries by strains CAT-1 (white bars), CAT1-TRP1oe (gray bars), CAT1-MSN2oe (dark gray bars), and CAT1-NΔMSN2oe (black bars). (**A**) Ethanol produced during each cycle; (**B**) Wet biomass at the end of each cycle; (**C**) Residual TRS left at the three last cycles; (**D**) Glycerol produced during the three last cycles. Within each cycle, bars with different lowercase letters indicate significant differences between strains ($p < 0.05$).

Regarding glycerol production by the yeast strains (Figure 5D), again strain CAT1-NΔMSN2oe withstands as the best strain producing this fermentation product (albeit still at very low levels), reflecting the better performance of the industrial strain overexpressing the truncated *MSN2* gene under the employed conditions. Finally, all strains during all the fermentation cycles had viabilities of over ≥99% of viable cells, with the exception of strain CAT1-NΔMSN2oe, which only at the end of the fourth cycle presented a viability of 93.5 ± 1.9%, probably reflecting the higher amount of produced ethanol in this last cycle (Figure 5A), which certainly impacted cell viability.

4. Discussion

The fermentation of very-high-gravity (VHG) mediums has long received considerable attention, as it can increase the fermentation rate and the amount of ethanol produced, reducing capital costs and the risk of bacterial contamination [49,50]. VHG technology promotes less process water and energy requirements, specially promoting considerable savings on the energy for distillation, one of the most energy consuming steps in fuel ethanol production, as well as reducing the volume of vinasse (the resulting residue after distillation of ethanol), which can have significant impacts in the environmental water supply [28,29,51]. However, VHG fermentations, using sugar levels over 250 g/L, enabling to achieve more than 15% (*v*/*v*) ethanol, certainly also expose yeast cells to increasing stressful conditions, including a very high ethanol stress. The present report not only characterized the ethanol tolerance of a Brazilian industrial fuel ethanol yeast strain, but

also aimed at analyzing if previous strategies developed with laboratory yeast strains (tolerant to significantly less ethanol concentrations) could also work with the fuel ethanol strain CAT-1. The stress tolerance from different Brazilian industrial yeast strains have been characterized and compared to laboratory yeast strains previously, revealing, for example, significant tolerance to high temperatures (e.g., 40 °C), a common reality in the Brazilian sugarcane ethanol mills [52,53]. Certainly, each bioethanol process selects for strains with improved fermentation performance under industrial conditions, strains with interesting genome adaptations [4,37,38,53,54].

Of the three strategies tested, although the overexpression of the *TRP1* gene allowed growth of the CAT1-TRP1oe strain on media with 16% ethanol, this strain had a lower performance when tested under conditions that simulate the Brazilian sugarcane ethanol production process using high concentration of sugars. High ethanol levels was shown to lower the active uptake of several amino acids and other nutrients across the plasma membrane, compromising the amount of nutrients required to fulfill growth requirements under ethanol stress [55,56]. While *TRP1* overexpression improves the growth of the cells in the rich YP medium with 16% ethanol, the molasses medium used in the fermentation assays is certainly more complex, although previous data has shown that supplementation with different amino acids (including tryptophan) does not improve the growth of strain CAT-1 in this industrial substrate [45]. Although it is generally believed that strains with higher tolerance to ethanol can produce more ethanol, the maximal ethanol production capacity and the ethanol tolerance of cell growth was shown to have a weak correlation, and these two phenotypes have a partially different genetic basis [57]. The two other strategies tested, the overexpression of the *MSN2* or the N-truncated *MSN2* gene, showed not only improved growth in the presence of 16% ethanol, and reduced the intracellular level of ROS in the presence of ethanol, but in the case of the CAT1-NΔMSN2 strain, also improved fermentation performance under conditions that simulate industrial fermentations with high sugar concentrations (33% TRS), producing higher concentrations of ethanol (~16%) due to efficient consumption of all sugars when compared with the parental strain CAT-1. While the three overexpression strategies were tested individually in the industrial yeast strain, it would be interesting to verify if combining two of them simultaneously (e.g., *TRP1* and *MNS2* overexpression) would improve the fermentation performance of the industrial strain.

A structural analysis of the *MNS2* transcriptional activator revealed that this protein has an intrinsically disordered structure with two short structural motifs in its transcriptional-activating domain (TAD, the N-terminal residues 1–264), one located within the first 50 residues and another located at the end of the TAD (residues 252–268) [20]. While the motif located at the end of the TAD is functionally conserved in several yeast species, and is essential for proper stress-induced nuclear localization of *MSN2* upon exposure to several stresses [18], the deletion of the motif involving the first 50 amino acids of the TAD had no significant effect on *MSN2* nuclear localization following yeast exposure to stress, although it reduced the transcriptional activation of a reporter gene by 15–30% when compared to the activity of the full length *MSN2* protein [20]. Moreover, deletion of the first 50 amino acids abolished the interaction of *MSN2* with *GAL11*, a subunit of the RNA polymerase II mediator complex that promotes the hyperphosphorylation and degradation of *MSN2* during the stress response [20,58]. These results explain why an N-terminal truncated version of *MSN2* improved the ethanol tolerance of a laboratory yeast strain [22], and our results indicate that it also improves the ethanol tolerance and ethanol production by the industrial strain CAT-1.

Recent reports have started to show that the phenotypic consequences of genomic modifications (e.g., gene deletions) can vary considerably between different yeast backgrounds [59], an issue that can have significant implications in metabolic engineering applications for generating optimized yeast cell factories [60]. For example, it was shown that the fermentation performance of laboratory and sake yeast can be enhanced by inhibiting mitophagy (by deleting the *ATG32* gene) [61]. However, when this strategy was tested in the Brazilian fuel ethanol PE-2 strain under the same fermentation conditions employed

in this work, no significant enhancement of ethanol production could be observed [62]. Another example is the deletion of the *ECM33* gene, which was shown to improve the fermentation performance of a haploid strain derived from a wine yeast strain [63], but a recent report showed that deletion of this gene failed to improve the fermentation performance of another commercial (diploid) wine strain [64]. Thus, it is highly recommended that the phenotypic consequences of a genetic modification should be characterized in the appropriate strain background and substrate used if the goal is to implement such technology in an industrial process.

In the Brazilian fuel ethanol mills, the substrate used is sugarcane juice and/or molasses, which does not have enough nitrogen for cell growth [65,66], and indeed there is little increase in biomass proliferation from one fermentative cycle to the next one. The supplementation of urea has been shown to improve the fermentation of this substrate by yeasts [67], including strain CAT-1 that could produce up to 17% (*v*/*v*) ethanol from a 30°Brix concentrated sugarcane juice medium with urea supplementation [68]. Another approach that reduces ethanol toxicity and enhances ethanol production by both laboratory and industrial yeast strains is elevating the concentration of potassium (by KCl and KOH supplementation) and the pH (to pH 6.0) of the medium, indicating that enhancing the K^+ and lowering the H^+ gradients across the plasma membrane may be responsible for intrinsically higher ethanol tolerance by yeasts [69]. While these last supplementations were not tested in sugarcane juice and/or molasses medium, it would be interesting to see if the strain CAT1-NΔMSN2oe would produce more ethanol when the industrial molasses medium is supplemented with urea, or with KCl and KOH at a higher pH. Nevertheless, our findings contribute to the improved performance and efficiency of bioethanol production and also provide a valuable insight for the breeding of bioethanol yeast strains.

5. Conclusions

From the three different strategies known to increase the ethanol tolerance by laboratory strains, tested in the industrial fuel-ethanol-producing strain CAT-1 (already resistant to 14% ethanol), the overexpression of a truncated version of the *MSN2* gene not only increased its tolerance to ethanol, but also improved the performance of the yeast strain under fermentations with a high sugar content, cell recycle, and acid treatment between cycles, simulating the industrial conditions used in Brazilian distilleries.

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