RESEARCH ARTICLE

Transcriptional profiling of Brazilian *Saccharomyces cerevisiae* strains selected for semi-continuous fermentation of sugarcane must

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Abstract

Brazil played a pioneering role in the global establishment of the sugarcane bioethanol industry. The bioethanol fermentation process currently used in Brazil is unique due to the acid wash and recycling of yeast cells. Two, industrially adopted, wild yeast strains, CAT-1 and PE-2, have become the most widely used in Brazil. How these strains respond to the unique fermentation process is poorly understood. The improved performance of CAT-1 and PE-2 is hypothesised to be related to enhanced stress tolerance. This study presents a genome-wide analysis of the CAT-1 and PE-2 transcriptomes during a small-scale fermentation process that mimicked the industrial conditions. The common and unique transcriptional responses of the two strains to the Brazilian fermentation process were identified. Environmental stress response genes were up-regulated postfermenter feeding, demonstrating the impact of the prior acid wash and high glucose environment. Cell wall and oxidative stress tolerance were subsequently demonstrated to be enhanced for the industrial strains. Conversely, numerous genes involved in protein synthesis were down-regulated at the end of fermentation revealing the later impact of ethanol-induced stress. Subsequently, the industrial strains demonstrated a greater tolerance of ethanol and the disruption of endoplasmic reticulum homoeostasis. This increased ethanol tolerance was finally correlated with an increased unfolded protein response and increased *HAC1* splicing.

Introduction

The desire of governments worldwide to reduce their dependence on finite fossil fuels and to limit greenhouse gas emissions has increased the prevalence of biofuels. Brazil has played a pioneering role in the global establishment of the sugarcane bioethanol industry through the successful interaction between agrobusiness and biotechnology. In addition, Brazil is at the forefront in the use of ethanol powered vehicles. Currently, Brazilian bioethanol production is derived from first-generation technologies that utilise sucrose extracted from sugarcane. This simple sugar source is efficiently fermented by yeast (*Saccharomyces cerevisiae*) into bioethanol. Despite many countries implementing sugarcane fermentation, the Brazilian bioethanol industry remains different, due to the use of a unique semi-continuous fermentation process. This system utilises sugarcane juice and/or dilute substrates (150–200 g L^{-1} of total sugar) as a feedstock and yields 9–12% (v/v) ethanol at an efficiency of 90–92% (Basso *et al.*, 2008). At the end of each fermentation cycle, the yeast cells are collected and washed with dilute sulphuric acid. The

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objective of this acid treatment is to reduce bacterial contamination. After each acid treatment, the yeast cells are returned to the fermenters to start the next cycle. This process is repeated throughout the whole sugarcane cropping season, which lasts for 6–9 months of the year. The acid treatment and re-initiation of the next fermentation cycle with the acid-cleaned yeast is what makes the Brazilian bioethanol industry unique.

The development of the industrial yeast strains responsible for the efficient production of bioethanol in Brazil was based upon the study of the microbial populations within fermenters (Silva-Filho *et al.*, 2005; Basso *et al.*, 2008). Traditionally, the fermentation process was started with Baker's yeast. However, the aforementioned studies demonstrated that indigenous yeast strains quickly contaminated the fermenter and that only these wild strains survived the acid treatment. This was hypothesised to reflect the wild strains higher tolerance of the industrial conditions. Two of these wild strains, CAT-1 and PE-2, that contaminated and repopulated the fermenter have been industrialised, replacing Baker's yeast in 60% of Brazilian distilleries. Despite the extensive use of these superior strains, the molecular determinants of their enhanced performance, how they respond to the different phases of the fermentation process and how this response differs between the two strains, are poorly understood.

Recently, a molecular analysis of the diploid PE-2 strain and the complete genome sequence of a haploid derivative of PE-2, JAY291, showed extensive structural differences and a high level of single-nucleotide polymorphisms (approximately 2 SNPs kb^{-1}) compared with other sequenced yeast strains, with the majority of differences residing in the telomeric regions (Argueso *et al.*, 2009). In addition, chromosomal rearrangements were demonstrated to amplify a number of genes involved in environmental stress responses (ESRs). Microarray-based comparative genome hybridisations identified the variations in gene copy number, which were common to five industrially important fuel ethanol yeast strains, including CAT-1 and PE-2 (Stambuk *et al.*, 2009). These strains demonstrated an increased number of SNO and SNZ gene copies, which are involved in vitamins B1 (thiamin) and B6 (pyridoxine) biosynthesis. Increased SNO and SNZ copy number was shown to confer the ability to grow more efficiently under the repressing effects of thiamin, especially in high sugar medium lacking pyridoxine (Stambuk *et al.*, 2009). It remains to be verified whether these genetic changes are required for the efficient bioethanol fermentation of sugarcane substrates.

The Brazilian fermentation process starts when a mixture of sugarcane juice and molasses, called sugarcane must, is fed into the fermenter. In response to a rise in sugar content, yeast cell metabolism is diverted to ethanol production. After the feeding phase, the sugar concentration and pH level within the fermenter drop. During fermentation, an inverse relationship exists between the rate of yeast growth and the ethanol concentration within the medium. After 9 h of fermentation, cell growth and the increase in ethanol concentration plateau. This represents the end of the fermentation cycle, and the yeast cells are separated from the must via centrifugation, with only the must going into the distillation process. The concentrated yeast cells are then exposed to a low-sugar, acidic environment prior to being returned to the fermenter to restart the next cycle. Therefore, during the subsequent cycles of the aforementioned process carried out for the entire cropping season, the yeast cells are exposed to at least three distinct environments consisting of; (1) the low sugar and low ethanol concentrations during the acid wash, (2) the high sugar and low ethanol concentrations during fermenter feeding and (3) the low sugar and high ethanol concentrations at the end of fermentation process, as depicted in Fig. 1.

How these wild yeast cells, naturally adapted to the industrial conditions, respond to the altering environment within the fermenter, and the biological properties that keep these yeast populations productive throughout the entire cropping season, is of major interest to the

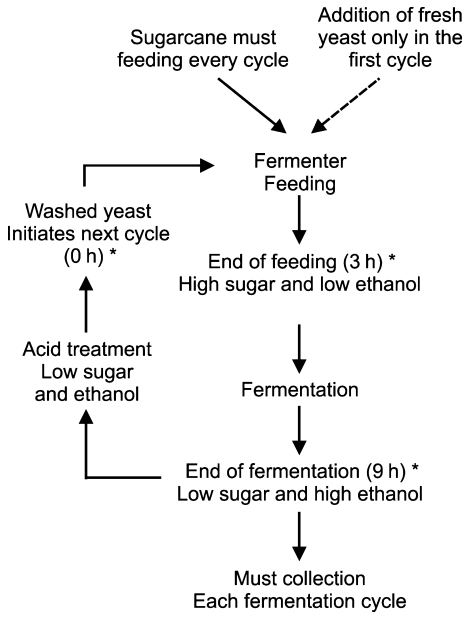

Fig. 1. The Brazilian semi-continuous fermentation process. Fresh yeast cells are only added during the first fermentation cycle of the sugarcane cropping season. At the end of each fermentation cycle, the yeast cells are treated with an acid wash prior to being reintroduced to the fermenter to start the following fermentation cycle. This process is repeated throughout the sugarcane cropping season. * Time points sampled for microarray analysis.

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Brazilian bioethanol industry. Due to the nature of the breeding programme that isolated the CAT-1 and PE-2 strains from contaminated industrial fermenters and the fact that the original starting Baker's yeast culture is not able to survive fermentation recycling, there was a lack of a suitable parental control strain that could proliferate for successive generations under the stressful conditions used in this study. Thus, a direct transcriptomic comparison was not applicable as quality RNA could not be produced for microarray hybridisation. Subsequently, this investigation developed and utilised a small-scale sugarcane-fed fermentation process that mimicked the Brazilian industry. The common and unique transcriptional response of the CAT-1 and PE-2 strains to this unique fermentation process was determined and subsequently related to the improved stress tolerance of these industrially adopted strains, revealing the contribution of an enhanced unfolded protein response (UPR).

Methods

Yeast strains

The two industrial yeast strains CAT-1 and PE-2 developed by Fermentec, Piracicaba, Brazil (Basso *et al.*, 2008), were used for the fermentation assays. These strains are available upon request. Laboratory yeast strains BY4743 (MATa/α his3Δ1/his3Δ1 leu2Δ0/leu2Δ0 LYS2/lys2Δ0 met15Δ0/MET15 ura3Δ0/ura3Δ0) and S288c (MATα SUC2 gal2 mal mel flo1 flo8-1 hap1 ho bio1 bio6) were also used. All strains were cultivated on solid YPD media (yeast extract 10 g, peptone 20 g, glucose 20 g, water 1 L, agar 20 g) at 30 °C for 2 days, and then, single colonies were subsequently incubated at 33 °C in 50 mL liquid YPD and mounted on a rotary shaker (180 r.p.m.) until the exponential growth phase was reached ($OD_{600\ nm} \sim 0.5$), prior to cell counting and inoculum preparation.

Fermentation recycles

Small-scale batch-fed fermentations with recycled yeast cells were performed in triplicate and represented the typical Brazilian industrial process, as depicted in Fig. 1. Yeast cells were collected from the liquid YPD medium via centrifugation and resuspended [10% (w/v) approximately 10^9 cells mL^{-1}] in 50 mL sugarcane must containing 18% sugar (50% originating from the sugarcane juice and 50% the molasses) to start the first fermentation cycle. Each fermentation cycle represented 9 h of incubation, without agitation, at 33 °C. At the end of the first cycle, the yeast cells were collected via centrifugation (4000 ***g*** for 5 min) and treated with sulphuric acid (pH 2.5) for 1.5 h at room temperature. Postacid treatment, the washed yeast cells were collected via centrifugation and fed sugarcane must, for a second time, to restart the fermentation process. The first fermentation cycle represents yeast adaptation, and therefore, the data are generally regarded as highly variable (Argueso *et al.*, 2009). Accordingly, yeast cells were only sampled from the second fermentative cycle. Samples were collected from the three distinct phases of the fermentation process: postacid treatment (before feeding, 0 h), after sugarcane must feeding (3 h) and at the end of the fermentation cycle (9 h). During sampling, 1 mL of the yeast cell suspension was collected from the three fermentation flasks per strain, under sterile condition, frozen in liquid nitrogen and stored at −70 °C.

Analysis of fermentation process during the second cycle

Yeast cell viability was determined by light microscopy (Nikkon E200) and erythrosine viability staining (Bonneu *et al.*, 1991). The rate of fermentation was assessed by measuring the loss of weight from the fermentation flasks, due to CO_2 mass evolution, expressed as a function of time (Cherubin, 2003). Residual sugar (glucose, fructose and sucrose) and glycerol concentration, in the fermented sugarcane must at the end of the second fermentation cycle, were evaluated by high-performance anion-exchange chromatography with pulse amperometric detection (HPAEC-PAD) (Dionex BioLC equipped with a PA-1 column) according to Eggleston & Clarke (1997). The alcohol content in the fermented sugarcane must was measured by determining the density of the distilled vapour extraction with a densimeter (Anton Paar DMA 4500) as described by Amorim *et al.* (1982). Finally, the amount of ethanol produced in relation to the sugars in the fermented sugarcane must was calculated according to Cherubin *et al.*, 2003.

RNA isolation

Yeast cells collected from the different phases of the second fermentation cycle were disrupted by vortexing with glass beads, and total RNA was extracted using Trizol, according to manufacturer's instructions (Invitrogen). Total RNA was purified, postDNAse treatment (Promega), using the RNeasy® Mini Kit (Qiagen). The concentration of the purified RNA was measured on a NanoDrop® 2000 (Thermo Scientific, Uniscience).

Microarray hybridisation

The Agilent *S. cerevisiae* microarray (Yeast (V2) gene expression microarray, 8 × 15K) was used to evaluate

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gene expression. The microarray slides contain 15 208 probes for *S. cerevisiae* (S288c strain). cRNA labelling was performed according to the standard protocol described by Agilent using the two-colour microarray-based gene expression system (Agilent Technologies). The RNA isolated from the three different phases of the second fermentation cycle, including two biological replicates, was utilised for the microarray hybridisations. The RNA isolated at 0 h, postacid wash, was used as a reference and labelled with Cy3, while the RNA isolated at 3 or 9 h into the fermentation process was labelled with Cy5. Briefly, 4–5 μg of total RNA was used to synthesis labelled cRNA. Then, 300 ng of Cy3- and Cy5-labelled cRNAs (specific activity >8.0 pmol Cy3-Cy5/μg cRNA) was fragmented at 60 °C for 30 min in 25 μL, containing 1× Agilent fragmentation buffer and 2× Agilent blocking agent. Postfragmentation, 25 μL of 2× Agilent hybridisation buffer was added to the fragmentation mixture and hybridised to the *S. cerevisiae* microarray slides for 17 h at 65 °C, on Agilent rotator rack, in an Agilent G2545A hybridisation oven. Subsequently, microarrays were sequentially washed: 1 min at room temperature with GE wash buffer 1 (Agilent) and 1 min at 37 °C with GE wash buffer 2 (Agilent), and then 10 s with acetonitrile (Agilent) followed by 30 s with a stabilisation and drying solution (Agilent). Slides were immediately subjected to fluorescence detection using GenePix 4000B (Molecular Devices) that simultaneously scans for the Cy3 and Cy5 channels at a resolution of 5 μm. The laser was set at 100%, and PMT gain was adjusted automatically for each slide, according to the signal intensity of each array, via GenePix Pro (Molecular Device).

Microarray analysis

Merged Cy3 and Cy5 TIFF images generated by the GenePix Pro were used in the analysis of gene expression using the Agilent feature extraction software (version 9.5.3.1, Agilent). The linear Lowess algorithm was used to obtain background subtracted and normalised intensity values. The normalised values generated were uploaded into the software Express Converter (version 2.1, TM4 available at http://www.tm4.org/utilities.html) that converts the Agilent file format to mev (Multi Experiment Viewer) file compatible with the TM4 software for microarray analysis (available at http://www.tm4.org/). The mev files were uploaded into the MIDAS software where experimental and biological means were determined using the tools 'flip dye consistency checking' and 'in slides replicates analysis'. The mev files generated were then loaded into the MEV software for further analysis. Genome-wide correlations between strains and/or fermentation phases were determined via linear regression (Origin version 6.1). A *t*-test was used to determine all the genes that were differentially expressed, in either time point (3 or 9 h) when compared to the reference (0 h) in both strains. A LIMMA analysis was used to identify the genes differentially expressed between the different phases of the fermentation process, for each strain. A SAM analysis was used to reveal the genes that were differentially expressed between the two strains irrespective of the time point. The *P* value for the aforementioned statistical tests is specified in the relevant text. Differentially expressed genes were functionally classified using MIPS FunCats (http://mips.helmholtz-muenchen.de/genre/proj/yeast/). Over-represented FunCats were identified as having a greater than twofold increase in representation within the data set compared to the genome, and the significance was assessed via Fisher's exact test ($P < 0.01$).

Cell wall, oxidative, ER and ethanol stress assays

Laboratory haploid and diploid strains, S288c and BY4743, and the two diploid industrial CAT-1 and PE-2 strains were assessed for resistance to cell wall, oxidative and ER stresses. Three biological replicates of the following stress assays were assessed. Spot dilution assays were performed on solid YPD media supplemented with either 2 or 10% glucose and in the presence of agents that elicit cell wall perturbation (CFW 30 mM) or oxidative stress (paraquat 0.1 mM). Optical density experiments were performed in YPD liquid media, in the presence of varying concentrations (10, 5, 2.5, 1 and 0.5 mM) of ER stress inducer dithiothreitol (DTT). The impact on the growth of 10^5 cells was evaluated via absorbance at $OD_{600\ nm}$ after 16 h of incubation at 30 °C. To determine the role of the UPR and ethanol tolerance, the different yeast strains were incubated overnight at 30 °C in the presence and absence of 10% ethanol, and subsequently, cell viability was verified (Bonneu *et al.*, 1991).

Real-time PCR evaluation of the UPR

The RNA isolated from the three different phases of the fermentation cycle, including three biological replicates, was utilised for the evaluation of the UPR. Purified RNA was used for cDNA synthesis using Superscript II (Invitrogen) according to manufactures instructions. Real-time polymerase chain reaction (RT-PCR) was performed as previously described (Semighini *et al.*, 2006). The following primers and Lux probes were used: SRP21 (SRP21_386RL, 5′-cggcGTTCTGTAGCAGTTTTCTTAGCcG-3′ and SRP21_386RL/365FU, 5′-GCCACCTTAATTGTCAACACAGA-3′), TRX1 (TRX1_171RL, 5′-cgatgaGCAACATCACCCAATTCATcG-3′ and TRX1_171RL/152FU, 5′-TCTGAACAATACC

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CACAAGCTGA-3′), and SSS1 (SSS1_183RL, 5′-cggtaATGG CGTAACCAATGATACcG-3′ and SSS1_183RL/165FU, 5′-C AAGATTGTCAAGGCTGTTGGT-3′), TAF10_300RL(TAF1 0 cggtCGTACATCTGCTACGTTAAACCcG) and TAF10_30 0RL/272FU (CTCCTCCTATCATTCCCGATGC).

RT-PCR detection of HAC1 splicing

The purified RNA isolated from the three different phases of the fermentation cycle, including three biological replicates, was utilised for cDNA synthesis and the evaluation of *HAC1* splicing. RT-PCR was used to detect splicing of the *HAC1* mRNA in the S288c, CAT-1 and PE-2 strains, using *HAC1* primers that annealed 343 bp upstream (5′-TCAAGGGATTTCCAGAGC-3′) and 56 bp downstream (5′-TCATGAAGTGATGAAGAAATCATT-3′) of an intron. The unspliced *HAC1* amplicon was 651 bp ($HAC1^u$), while the spliced *HAC1* amplicon was 399 bp ($HAC1^i$) (Bicknell *et al.*, 2007). Amplicon quantification was determined via measuring pixel intensity, using the ImageJ software (http://rsbweb.nih.gov/ij/index.html), and normalised by the intensity of the TAF10 reference gene.

Results

Evaluation of the small-scale bioethanol fermentation process

The first fermentation cycle (adaption phases) is known to be highly variable (Argueso *et al.*, 2009). Therefore, only the second fermentation cycle was evaluated. To validate the efficiency of the small-scale fermentation process and the material used for the subsequent analyses, several parameters representative of the fermentation process were measured at the end of the second 9-h fermentation cycle (Table 1). The concentration of residual sugars (g/100 mL) in the media postfermentation was consistently lower for PE2 (0.030 ± 0.001) and CAT1 (0.031 ± 0.001) when compared to the laboratory strain (0.090 ± 0.021). Industrial processes of alcoholic fermentation in Brazil work with residual sugars as low as 0.08 g/100 mL. The rate of fermentation (CO_2 loss) was faster for the industrial strains, which produced 1.5 g of CO_2 compared with S288c that released 1.2 g after 6 h of fermentation (Fig. 2). The industrial yeast strains fermented at a rate of 0.25 g CO_2 h^{-1} compared with 0.2 g CO_2 h^{-1} for S288c. This demonstrates that the industrial strains required 20% less time than S288c to complete fermentation (Table 1 and Fig. 2). At the end of the second fermentation cycle, the final concentration of ethanol was similar for the industrial and laboratory strains (Table 1). The same kinetics in ethanol production (during a single fermentation cycle) was previously observed in a comparison between PE-2 and S288c. However, after five successive fermentation cycles, a substantial increase in final ethanol concentration was achieved (Argueso *et al.*, 2009). Despite the final differences in CO_2 loss, between the laboratory and industrial strains, appearing similar at the end of the second cycle, in a typical distillery running a fed batch fermentation process, with seven million litre tanks and two fermentation cycles per day, the difference in the rate of sugar utilisation between the industrial and laboratory strains represents a fermentation loss of 8.4 tons of sugars per day. The observed higher production of glycerol by the industrial yeast strains may represent a greater production of cellular biomass during fermentation (Oura, 1973). These parameters therefore indicated that the ethanol fermentation process was occurring successfully and also showed the superior ability of these industrial strains to convert sugars derived from sugarcane into ethanol at a faster rate.

Table 1. Fermentation parameters of the yeast strains CAT-1, PE-2 and S288c. Fermentation parameter were measured after a single, 9 h fermentation of a mixed must consisting of 50% sugarcane juice and 50% molasses. The data is presented as the mean (±standard deviation) of three biological replicates

Strain	Ethanol (%)	Residual sugar (g/100 mL)	Glycerol (g/100 mL)	H_2SO_4 (g L^{-1})
PE-2	9.28 ± 0.03	0.031 ± 0.001	0.501 ± 0.001	1.02 ± 0.07
CAT-1	9.27 ± 0.14	0.030 ± 0.001	0.513 ± 0.004	1.00 ± 0.04
S288c	9.38 ± 0.08	0.090 ± 0.021	0.328 ± 0.002	0.98 ± 0.00

The transcriptional response of CAT-1 and PE-2 to the fermentation process

The three phases that represented the different conditions within the fermenter were selected for the microarray analysis: (1) the start of the second cycle, postacid treatment (0 h), (2) after sugarcane must feeding (3 h) and (3) at the end of the second fermentation cycle (9 h). The start of the second cycle (0 h) was used as a reference. The genome-wide correlation in transcription was greater between strains in the same fermentation phase than for the individual strain between the different fermentation phases (Supporting Information, Fig. S1). This demonstrated that globally both strains underwent a similar transcriptional response to the altering conditions. The full data set was deposited in the Gene Expression Omnibus (GEO) from the National Center of Biotechnology Information (NCBI) with the number GSE26619 (http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE 26619).

Overall, 244 of 610 genes, or 4 of 10% of genes on the *S. cerevisiae* microarrays, were identified as being differentially regulated between the feeding and the end of

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Table 2. MIPS FunCats overrepresented at the end of the fermentation process, within different subsets of genes. The data presented represents the *P* values from a Fisher's exact test of the MIPS functional categories (defined in Table S9) with a greater than twofold increase in proportional representation within the data set compared to the yeast genome. *represents MIPS functional categories with a significant (P < 0.05) overrepresentation

	CAT-1		PE-2		Both strains	
MIPS FunCat	Up	Down	Up	Down	Up	Down
1.05			0.0005*		< 0.0001*	
1.05.25			0.2434		0.0027*	
2	0.0106*		< 0.0001*		< 0.0001*	
2.01			0.5085		0.0118*	0.3584
2.01.3					0.0412*	
2.07		0.0658	0.8164	0.9167		0.7057
2.10					0.1722	
2.11	0.0051*		0.0083*		< 0.0001*	
2.13	0.0426*		0.0185*		0.0007*	
2.13.1						0.0685
2.13.3	0.0280*		0.0024*		0.0490*	
2.16	0.1700		0.0363*		0.0026*	
2.19			< 0.0001*		0.5381	0.3248
11		< 0.0001*				
12		< 0.0001*		< 0.0001*		< 0.0001*
14.1						0.2008
16		< 0.0001*				

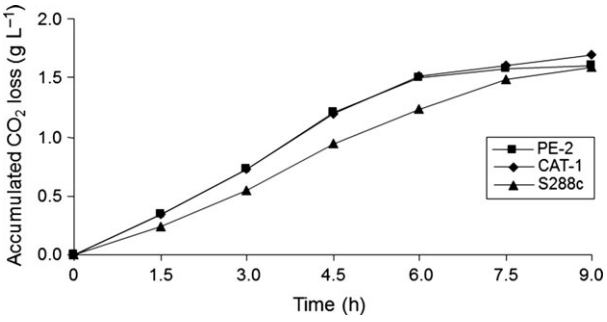

Fig. 2. Validation of the small-scale fermentation process. The graph depicts the fermentation rate of the two industrial (CAT-1 and PE-2) and a single laboratory (S288c) yeast strain over 9 h.

fermentation phases, for the CAT-1 and PE-2 strains, respectively (LIMMA analysis $P < 0.01$ for CAT-1 or $P < 0.04$ for PE-2). This shows that CAT-1 demonstrated a greater transcriptional response than PE-2 to the changing conditions during fermentation. These genes represent the transcriptional difference between the two active fermentation phases. At the end of the fermentation process, 105 of 226 genes were up-regulated and 139 of 384 genes down-regulated in CAT-1 and PE-2, respectively, when compared to the feeding phase at the start of the fermentation process (Tables S1 and S2). The genes within the differentially regulated subsets were functionally classified using MIPS (http://mips.helmholtz-muenchen.de/genre/proj/yeast/). This analysis created a functional profile of the transcriptional response and revealed that for both the CAT-1 and PE-2 strains, there was a transcriptional up-regulation of genes involved in energy production at the end of the fermentation process (Table 2). This included genes that play a role in glycolysis, the tricarboxylic acid cycle (TCA) cycle, the electron transport chain (ETC), fermentation and aerobic respiration (Fig. 3a and c). Conversely, the MIPS analysis of the genes down-regulated at the end of the fermentation process revealed that the majority, 37% and 51% in CAT-1 and PE-2, respectively, were involved in protein synthesis (Fig. 3b and d). In addition, the transcription of genes in the pentose phosphate pathway (PPP) was down-regulated in both strains. Genes involved in amino acid transport biosynthesis and metabolism were coordinately up-regulated. Different subsets of amino acid biosynthesis genes were down-regulated in CAT-1 only, while no amino acid-related genes were down-regulated in PE-2 at the end of fermentation.

The common and unique transcriptional responses of the industrial strains to the fermentation process

A comparison of the genes that were differentially expressed in the two industrial strains in the response to the fermentation process (0 h compared with 3 h and 9 h, *t*-test $P < 0.01$) identified 1789 genes, or approximately 28.4% of the microarray, as being differentially expressed (Table S3). The MIPS functional profiles of the genes differentially expressed between the feeding and the end of the fermentation phases were similar for both stains (Fig. 3). Only a relatively small number of genes (156), accounting for approximately 2.48% of the

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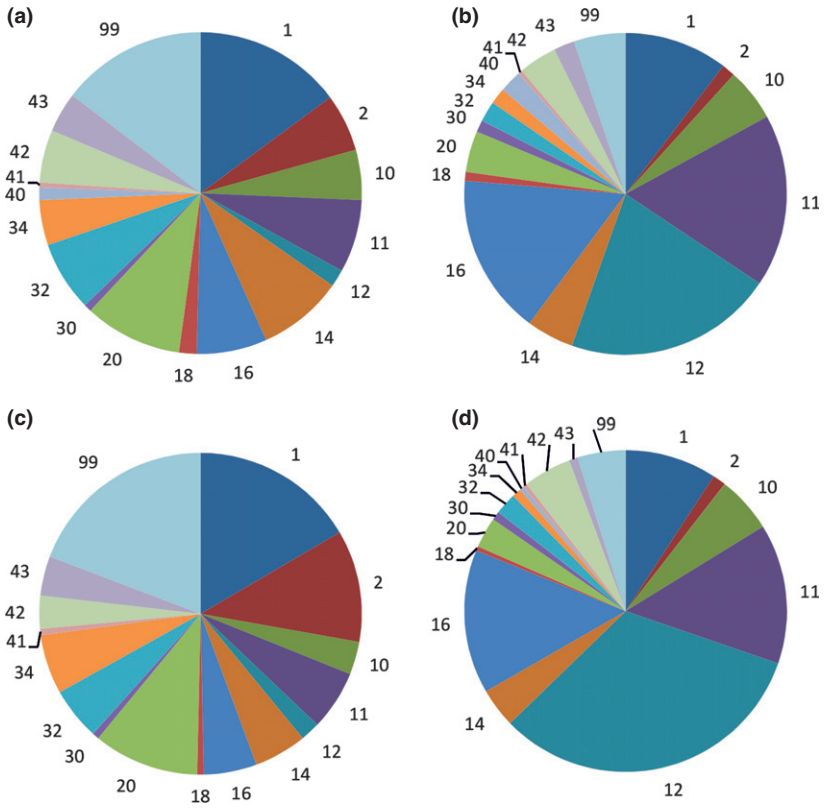

Fig. 3. The representation of the MIPS FunCats in different subsets of genes. Significantly up (a and c)- or down (b and d)-regulated genes in the CAT-1 and PE-2, respectively, at the end of the fermentation process when compared with postfermenter feeding. For the definitions of MIPS FunCats, refer to Table S9.

microarray, were differentially regulated (SAM $P < 0.01$) between the two industrial strains during the fermentation process. This represents the unique transcriptional responses to fermentation by the individual strains. These two data sets and the genome-wide correlations therefore demonstrate that the two industrial strains functionally respond in a similar manner to the changing conditions. However, the small number of genes differentially regulated between strains represents an exciting subset with possible biotechnological implications. In total, 136 genes demonstrated a higher transcriptional response in CAT-1 compared to PE-2, while in contrast, only 20 genes showed a lower transcriptional response in PE-2 (Tables S4 and S5).

The majority of genes that demonstrated a higher transcriptional response to fermentation in CAT-1, when compared to PE-2, are still to be assigned a function. However, there was a high representation of genes involved in cell defence, virulence and rescue that was unique to CAT-1 (Fig. 4a). The annotated genes within this subset included genes involved in stress responses including oxidative stress, heat shock, pH maintenance and DNA damage responses. Multiple genes involved in cell wall biogenesis and protein folding demonstrated a higher transcriptional response in CAT-1, suggesting a response to cell wall and endoplasmic reticulum stress. The transcription of genes involved in energy production also responded to a greater extent in CAT-1, including several hexose transporters *HXT1* and *HXT5*, the high-affinity iron transporter *FET3* and the metabolic enzymes benzyl alcohol dehydrogenase AAD14, the aldehyde dehydrogenase ALD4, glycogen synthase GSY1 and the transcription factor PHO4.

The majority of genes that demonstrated a greater transcriptional response to fermentation in the PE-2, when compared to CAT-1, are yet to be assigned function (Fig. 4b). Among the limited number of functionally annotated genes were two maltose fermentation genes *MAL12* and *MAL32* that are also able to hydrolyse sucrose. In addition, genes involved in nitrogen stress including the amino acid/nitrogen starvation response transcription factor GCN4 and a low-affinity amino acid permease AGP3 that under poor nitrogen conditions supplies amino acids as a source of nitrogen were differentially transcribed at a higher level in PE-2.

The induction of environmental and fermentation-specific stress responses

The observed reduction in the transcription of genes involved in protein synthesis at the end of the fermentation process, by both of the industrial strains, suggested

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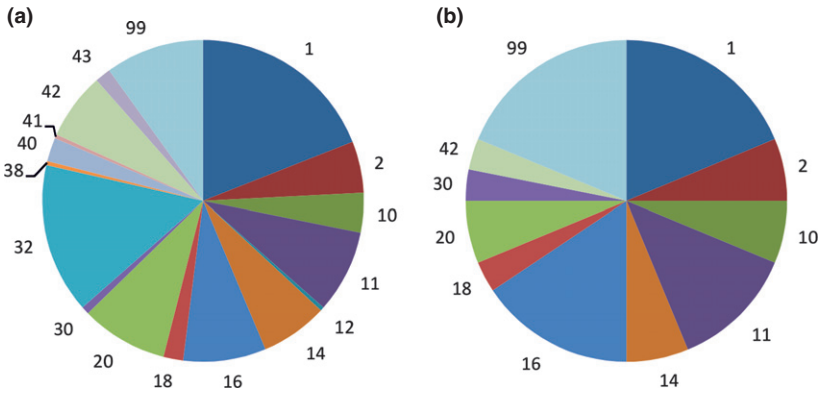

Fig. 4. The representation of the MIPS FunCats of the genes differentially regulated between the two strains. Genes up-regulated in CAT-1 (a) and PE-2 (b) during the entire fermentation process. For the definitions of MIPS FunCats, refer to Table S9.

that these strains were undergoing a stress response. Subsequently, the transcription of genes known to be involved in the response to environmental and fermentation-specific stresses was analysed. Environmental stress response genes include those defined to be involved in the oxidative, osmotic, heat, pH and starvation stress responses (Gasch *et al.*, 2000). At the end of the fermentation process, only 38 and 12 of a possible 868 ESR genes were up-regulated in CAT-1 and PE-2 strains, respectively, when compared to the feeding phase. These up-regulated ESR genes were involved in alternative energy production such as the YAK1 kinase that inhibits growth in response to the absence of glucose, the xylulokinase XKS1 required for xylose fermentation and the hexose transporter HXT5 that is induced in the presence of nonfermentable carbon sources. A far greater number of genes, 250 and 93 in the respective strains, were down-regulated at the end of fermentation (Table S6). The numerous down-regulated genes were involved in protein synthesis, transcription, cell wall biogenesis and amino acid metabolism.

A comparatively greater number of the 273 HOG1-dependent genes (Capaldi *et al.*, 2008), 15 of 48 genes in CAT-1 and PE-2 respectively, were up-regulated at the end of the fermentation process, compared to only one gene being up-regulated postfermenter feeding (Table S7). Similarly, of the possible 224 fermentation stress response (FSR) genes (Marks *et al.*, 2008), 12 of 48 were up-regulated at the end of the fermentation process, when compared to the feeding phase, in CAT-1 and PE-2, respectively (Table S8), demonstrating that for these specific stresses CAT-1 mounted a greater transcriptional response.

CAT-1 and PE-2 demonstrate enhanced environmental stress resistance

Growth assays were performed to determine whether stress tolerance contributed to the enhanced ability of the two industrial strains to survive fermentation recycling and ferment at a faster rate than the original starting strain, Baker's yeast. Laboratory haploid and diploid strains, S288c and BY4743, respectively, were chosen to represent Baker's yeast, which was originally used to start the fermentation cycle, but was quickly outcompeted and replaced by the wild strains CAT-1 and PE-2. The resistance of these four strains to cell wall and oxidative stresses induced by calcoflour white (CFW) and paraquat, respectively (Balzan *et al.*, 1995; Nobel *et al.*, 2000), were assessed (Fig. 5). The CAT-1 and PE-2 strains showed much higher tolerance to cell wall perturbing CFW (30 mM) than the haploid and diploid laboratory strains

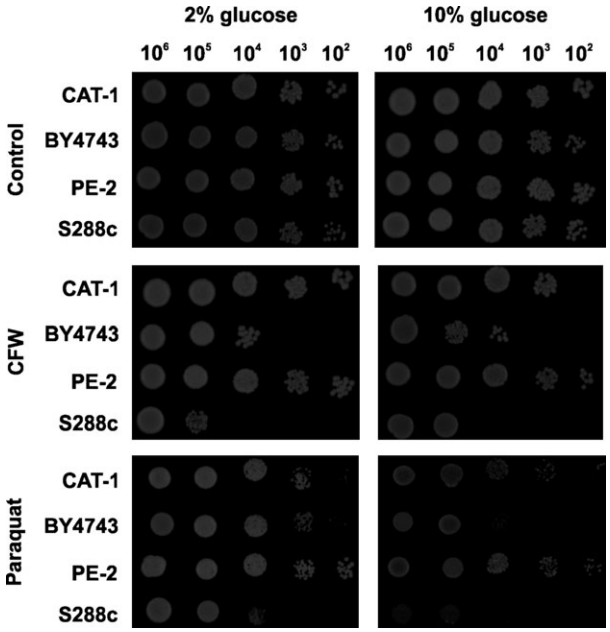

Fig. 5. The enhanced tolerance of the industrial strains to environmental stresses. Including oxidative and cell wall stresses induced by exposure to paraquat (0.1 mM) and calcoflour white (30 mM), respectively, in the presence of 2 or 10% glucose, compared with a nonstressed control.

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S288c and BY4743. Little difference was observed between CAT-1 and PE-2. The CAT-1 and PE-2 strains were more tolerant to the oxidative agent paraquat (0.1 mM) than the laboratory strain S288c at 2% glucose, while the two industrial strains were more tolerant than both laboratory strains at 10% glucose.

CAT-1 and PE-2 demonstrated an enhanced UPR during fermentation

The accumulation of misfolded proteins in the lumen of the ER disrupts organelle homoeostasis and induces the UPR, which in turn stops protein synthesis, increases folding capacity and degrades salvageable proteins (Ron & Walter, 2007). The dramatic down-regulation of protein synthesis at the end of the fermentation process suggested that protein synthesis may have been halted in the initial phase of the UPR. Dithiothreitol interferes with protein disulphide bond formation, generating misfolded proteins and therefore inducing the UPR. In addition to the UPR, ethanol-induced stress has also been linked to decreased protein synthesis during fermentation. Subsequently, DTT-induced ER stress tolerance of the CAT-1, PE-2 and laboratory strains, which reflects the capacity of the UPR, and ethanol tolerance were assessed. The industrial strains showed comparatively higher growth than the laboratory strains at the higher glucose concentration in the absence of DTT stress (Fig. 6). The CAT-1 and PE-2 strains demonstrated increased resistance to DTT in the presence of 2 or 10% glucose when compared to S288c. The haploid S288c strain demonstrated the least tolerance to DTT in the presence of both 2 and 10% glucose (Fig. 6). Ethanol tolerance was also enhanced in the industrial strains. The viability of CAT-1 and PE-2 cells postincubation with 10% ethanol ranged between 40 and 50%, while the laboratory strains S288c and BY4743 demonstrated only 28–32% viability.

Three genes that are induced by the UPR were examined by RT-PCR, including *SRP21* that encodes a subunit of the signal recognition particle, which targets proteins to the ER membrane, *TRX1* that encodes a cytoplasmic thioredoxin isoenzyme, which protects the cell against oxidative/reductive stresses, as well as being required for ER to Golgi transport and vacuole inheritance, and *SSS1* that encodes a subunit of the Sec61p translocation complex (Sec61p-Sss1p-Sbh1p), which forms a channel for the passage of secretory proteins through the ER membrane, while also forming part of the Ssh1p complex (Ssh1p-Sbh2p-Sss1p). The *TAF10* gene that encodes a subunit of TFIID and SAGA complexes, involved in RNA polymerase II transcription initiation and in chromatin modification, was shown as an appropriate reference gene for quantitative gene expression analysis by RT-PCR (Teste *et al.*, 2009). In addition, *TAF10* was not shown to be differentially expressed in the presented microarray analysis (data not shown). The RT-PCR analysis demonstrated that these three genes were transcribed at a higher level in both the CAT-1 and PE-2 strains (Fig. 7), predominantly in the postfeeding (3 h) phase.

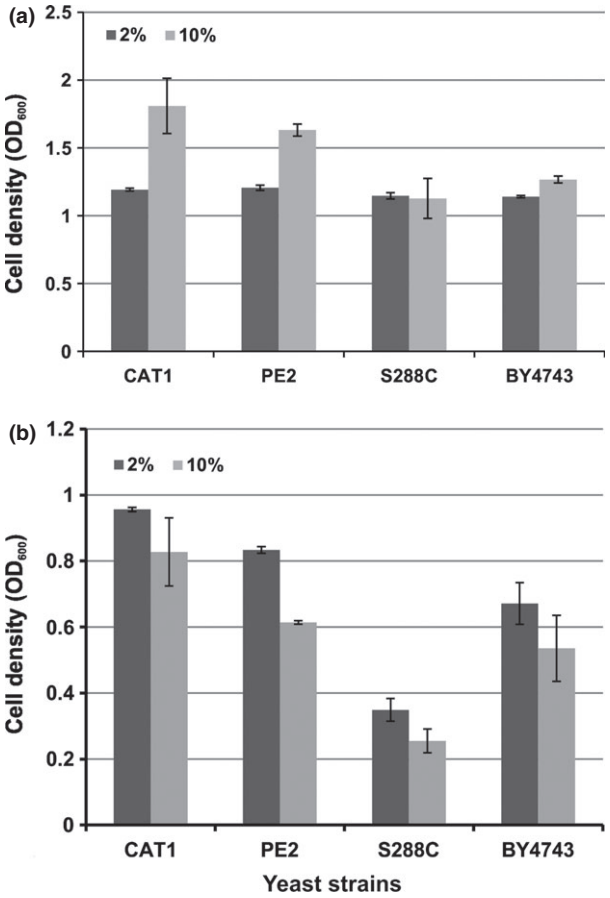

Fig. 6. The enhanced tolerance of the industrial strains to ER stress. The industrial yeast strains outperformed the laboratory strains in the presence of high glucose (a). Subsequent cell densities when cultured in the presence of 2.5 mM DTT at 2 or 10% glucose (b) was normalised to the growth of the untreated control (a).

The accumulation of aberrant folded proteins in the ER activates the bifunctional transmembrane kinase/endoribonuclease, IRE1, in yeast (Korennykh *et al.*, 2009). IRE1 excises an intron from the transcript of the *HAC1* cytoplasmic precursor ($HAC1^u$, uninduced). The removal of the intron generates the induced ($HAC1^i$) form of the transcript (Cox & Walter, 1996). The IRE1p-mediated splicing of the 252-nucleotide intron from the $HAC1^u$ mRNA relieves a translational block (Kawahara *et al.*, 1997) and creates a frameshift that allows for the translation of a transcription factor

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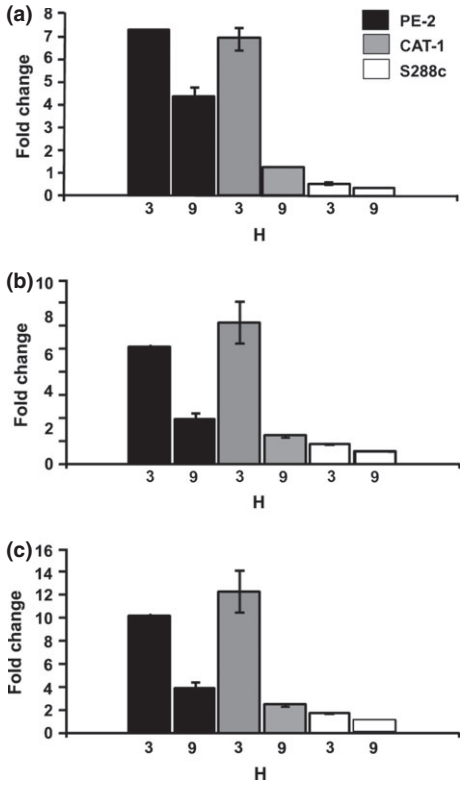

Fig. 7. The elevated transcription of UPR genes in the industrial strains compared to the laboratory strain. Gene expression, presented as fold change compared to the endogenous control gene *TAF10*, was determined by RT-PCR at 3 and 9 h into the fermentation cycle (labelled on *x*-axis), representing the postfeeding and end of fermentation phases, respectively. UPR genes included *SRP21* (a), *SSS1* (b) and *TRX1* (c).

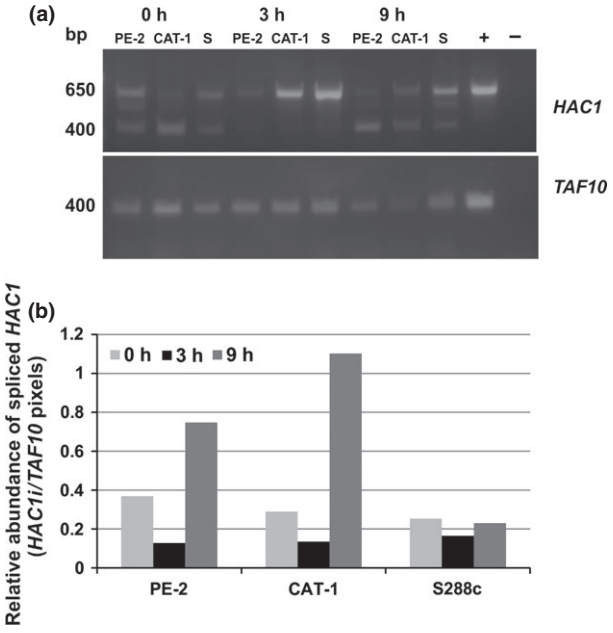

Fig. 8. The industrial strains demonstrated enhanced accumulation of spliced *HAC1* mRNA during the fermentation cycle. Processing of the 252-bp intron from *HAC1*^{u} (uninduced 651 bp) to the *HAC1*^{i} (induced 399 bp) mRNA (a). The densitometric analysis of pixel intensity, using the ImageJ software (http://rsbweb.nih.gov/ij/index.html) of the spliced *HAC1*^{i} product divided by *TAF10* demonstrated the dramatic increase in splicing only in the industrial CAT-1 and PE-2 at the end of the fermentation process (9 h) (b).

that moves to the nucleus where it regulates the expression of UPR target genes (Travers *et al.*, 2000; Kimata *et al.*, 2006). A RT-PCR assay adapted from Bicknell *et al.* (2007) distinguished between the two forms of the *HAC1* transcript. To investigate *HAC1* splicing, the laboratory and industrial yeast strains underwent bioethanol fermentation in conditions identical to those used for the microarray analysis. At the start (0 h) and 3 h into fermenter feeding, *HAC1*^{i} transcript abundance was similar for all strain. After 9 h of fermentation, the *HAC1*^{i} transcript levels were significantly higher in CAT-1 and PE-2, 4.8- and 3.2-fold, respectively, when compared to S288c (Fig. 8). Accordingly, CAT-1 and PE-2 comparatively demonstrated a higher level of *HAC1* splicing, during exposure to ethanol (2.5 and 3.5-times, respectively), while there was no major alteration in the accumulation of spliced *HAC1* in the S288c strain (Fig. 9). Therefore, the UPR appears to contribute to the enhanced tolerance of the industrial strains to ethanol during the fermentation process.

Discussion

Originally, the unique Brazilian semi-continuous fermentation process commenced with the addition of sugarcane must and Baker's yeast to the fermenter. Postfermentation, the must was harvested and the yeast cells washed with acid prior to starting the second fermentation cycle. However, fermenters were rapidly contaminated with wild yeast strains that, over successive cycles, outcompeted Baker's yeast and repopulated the fermenter. Two of these wild strains, CAT-1 and PE-2, have since been widely adopted by the Brazilian bioethanol industry. The molecular determinants for the efficient, semi-continuous fermentation of sugarcane must by the industrially adopted CAT-1 and PE-2 strains are of great interest. How these strains are better adapted to the harsh conditions, specifically the recycling process, is poorly understood and is hypothesised to be a key to their enhanced performance.

The viability of the small-scale fermentation process used in this study was confirmed, with CAT-1 and PE-2 demonstrating a faster rate of sugar utilisation, fermentation and gain in biomass than the laboratory strain, as previously observed for a single fermentation cycle (Argueso *et al.*, 2009). Similar to Baker's yeast, the laboratory strain was unable to successfully proliferate in successive

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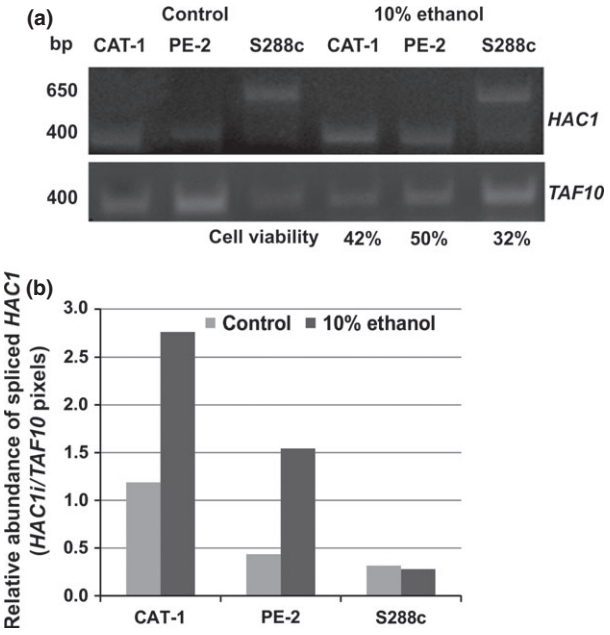

Fig. 9. Accumulation of spliced *HAC1*^{i} is induced by ethanol stress in the industrial strains only. Processing of the 252-bp intron from *HAC1*^{u} (uninduced 651 bp) to the *HAC1*^{i} (induced 399 bp) mRNA (a). Below the top panel, the viability of each strain, after incubation with ethanol, is indicated. Cell viability was determined by plating appropriated cell concentrations and counting the number of grown colonies. The densitometric analysis of pixel intensity, using the ImageJ software (http://rsbweb.nih.gov/ij/index.html) of the spliced *HAC1*^{i} product, divided by *TAF10* demonstrated that spliced *HAC1*^{i} mRNA accumulation was greater during the exposure to ethanol stress only in the industrial CAT-1 and PE-2 strains (b).

cycles. Over the many fermentation cycles carried out in a cropping season, the difference in fermentation rate and sugar utilisation would have a substantial economic impact. This investigation described the transcriptional response of these two industrial Brazilian strains to a small-scale fermentation process representative of the industrial process. The analysis of genome-wide gene expression during the different phases of the fermentation process indicated the pressures exerted upon the yeast cells and led to an evaluation of how these strains were better adapted to the industrial conditions.

By the end of the second fermentation cycle, the yeast cells have been exposed to a nutrient starved acidic environment, followed by a high-sugar, low-ethanol environment, which subsequently alters as the cells ferment, concluding with a low-sugar, high-ethanol environment. A high genome-wide correlation between strains and identification of only a relatively small number of differentially expressed genes, between CAT-1 and PE-2, demonstrated that the overall transcriptional response to the fermentation process was similar. This similarity was reflected in the MIPS functional profile of the common transcriptional response genes, with both profiles revealing an up-regulation of genes involved in carbon compound metabolism and energy production including ETC, TCA, aerobic respiration and fermentation at the end of the fermentation process. The functional profiles of the strain-specific transcriptional responses to the different phases of the fermentation process were distinct.

Unexpected changes in the expression of genes that are regulated by carbon catabolite repression despite the presence of a high glucose content have previously been reported (Rossignol *et al.*, 2003; Zuzuarregui *et al.*, 2006; Mendes-Ferreira *et al.*, 2007). A transcriptomic analysis of fermenting yeast in the presence of 20 or 2% glucose showed the lower expression of genes related to respiration, ETC and both the TCA and the urea cycles at the higher glucose concentration (Jiménez-Martí *et al.*, 2011a). These results implied that the exposure to high glucose concentrations had additional repressing effects on the yeast cells besides carbon catabolite repression and that the up-regulation of such energy-producing processes at the end of Brazilian fermentation is likely to be the dropping glucose content in the fermenter. The increased transcription of high-affinity hexose transporters and the YAK1 kinase, which is part of the glucose sensing, nutrient-deprivation, signalling cascade (Moriya *et al.*, 2001) at the end of the fermentation process supports the hypothesis that the cells have sensed a drop in glucose content. The elevated transcription of genes involved in amino acid high-affinity transporters, biosynthesis and metabolism may also reflect the decreasing amount of nutrients in the media.

During industrial alcoholic fermentation, yeast cells are exposed to multiple stresses (Rossouw & Bauer, 2009). The yeast cells respond to changing oxygen/ethanol concentrations, oxidative/osmotic stresses, in addition to the altering availability of nutrient. The high sugar content at the start of any fermentation process exposes the cells to high osmotic stress (Attfield, 1997). This is reflected in the up-regulation of glycerol and trehalose biosynthesis in industrial strains (Erasmus *et al.*, 2003; Jiménez-Martí *et al.*, 2011b). The down-regulation of numerous ESR genes at the end of the described fermentation process reflected the stresses exerted upon the cells during the acid wash and osmotic stress caused via the high sugar concentration at the start of the cycle. The greater number of down-regulated ESR genes in CAT-1 compared with PE-2 suggests that the latter took longer to recover from the acid treatment or underwent greater stress in the fermenter. The greater down-regulation of ESR at the end of the fermentation process demonstrates that post-feeding, 3 h into the fermentation process, the yeast cells from both strain were still recovering from the stressful acid treatment. Conversely, the down-regulation of many

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genes involved in protein synthesis at the end of the fermentation process may have reflected the ethanol stress exerted upon the cells and a consequence of a loss of ER homoeostasis (Ron & Walter, 2007).

The induction of the oxidative stress response during continuous and batch-fed industrial bioethanol fermentation has been shown for various yeast strains (James *et al.*, 2003; Wu *et al.*, 2006; Cheng *et al.*, 2008; Hansen *et al.*, 2006; Pérez-Torrado *et al.*, 2009; Ma & Liu, 2010). An enhanced tolerance of fermentation associated stresses was hypothesised to have enabled CAT-1 and PE-2 to proliferate over successive cycles. The presented study confirmed that CAT-1 and PE-2 demonstrated increased tolerance to chemically induced cell wall and oxidative stresses. A yeast strain overexpressing *TRX2*, which encodes a thioredoxin, demonstrated improved fermentation capacity and lower levels of oxidative cellular damage (Pérez-Torrado *et al.*, 2009). In this study, another thioredoxin, *TRX1*, was shown to be transcribed at a higher level in the CAT-1/PE-2 strains, and this increase was greatest postfermenter feeding.

A comparison between continuous and feed batch fermentation of bioethanol undertaken on an industrial scale demonstrated the down-regulation of glycosylation and up-regulation of UPR in both processes, representing a disruption of ER homoeostasis (Li *et al.*, 2010). In the present study, there was an increase in the representation of fermentation stress–specific genes at the end of the cycle, while both strains also demonstrated a dramatic down-regulation of transcription- and protein synthesis-related genes at the end of the fermentation process. This reflected the impact of the increased ethanol content on the cells. Subsequently, ethanol tolerance, ER stress tolerance and the UPR were evaluated. Travers *et al.* (2000) demonstrated that there is an intimate coordination between ER-associated protein degradation and the UPR, while identifying 103 genes that were up-regulated when the laboratory S288c strain was exposed to agents, such as DTT, that disrupt ER homoeostasis and induce ER stress (Helenius & Aebi, 2004). It has also been noted that during fermentation, the UPR genes that demonstrated increased transcription in industrial strains encoded proteins involved in protein folding and were located to the ER (Li *et al.*, 2010). In the present study, CAT-1 and PE-2 demonstrated increased resistance to DTT and elevated transcription of several UPR genes. In addition, analysis of *HAC1* splicing demonstrated that the increased ethanol content at the end of the fermentation process caused a dramatic increase in active spliced *HAC1* transcripts that subsequently migrate to the nucleus and induces the UPR. Subsequently, the enhanced tolerance of cell wall, oxidative, ER and ethanol stress induced during this fermentation process enabled CAT-1 and PE-2 to thrive under the harsh industrial conditions, which is likely to contribute to the increased cell growth, fermentation rate and ethanol production over the sugarcane cropping season.

Conclusions

The large majority of the transcriptional responses, by CAT-1 and PE-2, to the different phase of this unique fermentation process were conserved and elucidated to the stresses exerted upon the yeast cells. Phenotypic analyses demonstrated the enhanced tolerance of these industrially adopted strains to multiple stresses, which in the case of ethanol tolerance was correlated with a higher activation of the UPR. This study showed the importance of environmental stress tolerance and the UPR for efficient fermentation and successful yeast cell proliferation, when the acid treatment and recycling of yeast cell are used. The functional profile of the common and strain-specific transcriptional responses provided an insight into the key metabolic processes behind the superior efficiency of these industrial strains including energy production, carbon compound metabolism and stress tolerance. Therefore, the identified gene sets and processes provide a key opportunity for industrial microbiology to genetically modify the native, naturally selected yeast strains to enhance their efficiency in the Brazilian bioethanol production process.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Fig. S1. The genome wide correlation in mean gene expression between the different datasets.

Table S1. Significant genes feeding vs. fermentation CAT1 (LIMMA $P < 0.01$).

Table S2. Significant genes feeding vs. fermentation PE2 (LIMMA $P < 0.04$).

Table S3. Significant genes, 0 h vs. 3 h + 9 h combined (t test $P < 0.01$).

Table S4. Up regulated in CAT-1 compared to PE-2 (SAM analysis of combined fermentation phases $P < 0.01$).

Table S5. Down regulated in CAT-1 compared to PE-2 (SAM analysis of combined fermentation phases $P < 0.01$).

Table S6. Representation of the environmental stress response genes within the different datasets (feeding vs. end of fermentation).

Table S7. HOG1 regulated genes detected within the different datasets.

Table S8. Representation of the fermentation stress response genes within the different datasets (feeding vs. end of fermentation).

Table S9. MIPS FunCat definitions.

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