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High hydrostatic pressure activates gene expression that leads to ethanol production enhancement in a *Saccharomyces cerevisiae* distillery strain

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Abstract

High hydrostatic pressure (HHP) is a stress that exerts broad effects on microorganisms with characteristics similar to those of common environmental stresses. In this study, we aimed to identify genetic mechanisms that can enhance alcoholic fermentation of wild *Saccharomyces*

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cerevisiae isolated from Brazilian spirit fermentation vats. Accordingly, we performed a time course microarray analysis on a *S. cerevisiae* strain submitted to mild sublethal pressure treatment of 50 MPa for 30 min at room temperature, followed by incubation for 5, 10 and 15 min without pressure treatment. The obtained transcriptional profiles demonstrate the importance of post-pressurisation period on the activation of several genes related to cell recovery and stress tolerance. Based on these results, we over-expressed genes strongly induced by HHP in the same wild yeast strain and identified genes, particularly *SYM1*, whose over-expression results in enhanced ethanol production and stress tolerance upon fermentation. The present study validates the use of HHP as a biotechnological tool for the fermentative industries.

Keywords

Saccharomyces cerevisiae; High hydrostatic pressure; Fermentative process; Ethanol production; Microarray; Stress response

Introduction

The world faces an energy crisis and numerous efforts are underway to generate sustainable energy from renewable resources to minimise the dependence on fossil fuels. The production of ethanol from renewable biomass, or bioethanol, has gained recent popularity for its economic viability and preservation of rapidly decreasing non-renewable resources (Maiti et al. 2011). Sugar cane molasses is an inexpensive substrate with a substantial concentration of sucrose that has been used for industrial production of ethanol in Brazil for over 20 years. Brazil is the largest ethanol exporter in the world, and from 2010 to 2011, Brazilian sugar cane crop produced 27.6 billion litres of ethanol (8 billion litres of anhydrous and 19.6 of hydrous). In 2010, Brazil exported 1.9 billion litres of alcohol, totalling US\$1 billion in domestic income (MIDIC 2010).

The global market for spirits is estimated to be 14 billion L/ year (Trejo-Pech et al. 2010). The total worldwide annual consumption of pure alcohol is approximately 5.1 L/adult, of which beer accounts for 1.9 L, spirits for 1.7 L and wine for 1.3 L (WHO 2004). Fermentation process control and the yeast strain used are highlighted as the most important factors on distillery beverage quality (Oliveira et al. 2005). Cachaça, the Brazilian sugar cane spirit, is the third most frequently consumed distilled beverage in the world (Tfouni et al. 2007), and several recent efforts undertaken to improve cachaça quality have added to its increased exportation volume.

Saccharomyces cerevisiae, a budding yeast with high ethanol productivity and tolerance, is an efficient microorganism for producing ethanol from hexose sugars. However, during the fermentation process, yeast cells are exposed to numerous environmental stresses, leading to myriad intracellular changes that affect biomass production, fermentation efficiency and cell viability (Teixeira et al. 2011). Yeast populations undergoing fermentation are challenged with osmotic stress, high temperature and high ethanol concentration (Pataro et al. 2000). Accordingly, economically optimal fermentation requires identification of *S. cerevisiae* strains that are stress tolerant while preserving efficiency in ethanol production.

High hydrostatic pressure (HHP) is a stress that exerts broad effects on microorganisms with characteristics similar to those of other environmental stresses, such as high temperature, ethanol and oxidative stresses (Fernandes 2008). The HHP response of wild *S. cerevisiae* shows high correlation with that resulting from increased ethanol concentration or high-temperature stresses, suggesting that HHP may be a useful stress model to employ for selecting suitable *S. cerevisiae* strains for industrial applications (Bravim et al. 2010). Moreover, HHP treatment can elicit responses that provide cross-protection from multi-stresses. *S. cerevisiae* cells submitted to a mild sublethal pressure treatment (50 MPa for 30 min) followed by a short recovery at atmospheric pressure (0.1 MPa) acquire increased tolerance to heat, ultra-cold shock and high-pressure treatments (Palhano et al. 2004a).

In order to understand the molecular basis underlying HHP stress response, microarray analysis of *S. cerevisiae* submitted to various intensities and durations of hydrostatic pressure were performed (Fernandes et al. 2004; Iwahashi et al. 2003; Iwahashi et al. 2005). Gene expression profiling after a harsh treatment of 200 MPa for 30 min showed that the majority of the upregulated genes are involved in stress defence and carbohydrate metabolism while most of the repressed genes are involved in cell cycle progression and protein synthesis (Fernandes et al. 2004). A milder treatment of 30 MPa for 16 h, which results in growth delay without cell death, resulted in induction of genes involved in lipid synthesis and aminoacid metabolism (Iwahashi et al. 2005).

In this study, we explored whether comprehensive analysis of transcriptomic responses of wild yeast to HHP stress might provide valuable information applicable to the broader biotechnology industries. We carried out fermentation using a wild yeast isolate and measured ethanol production before and after pressure treatment. We observed that treatment with hydrostatic pressure led to an increase in ethanol content upon fermentation. In parallel, we conducted global transcriptional analysis to identify genes induced by hydrostatic pressure and demonstrated that at least for one gene related to metabolism and stress response, its over-expression in the wild yeast strain enhanced ethanol production capacity, likely by increasing its tolerance to stress. These results validate the utility of transcriptome profiling of yeast cells subjected to hydrostatic pressure as a method for identifying routes for enhanced ethanol production through selective genetic manipulations.

Material and methods

Yeast strain

The yeast strain used in this study was previously isolated from fermentation vats of two cachaça (Brazilian spirit) distilleries in the State of Espírito Santo, Brazil, and identified as described in Bravim et al. (2010). This strain (URM 6670, *S. cerevisiae* URM 604) was selected for its flocculation ability, tolerance to ethanol, osmotic and heat shock stresses and also for its high fermentation rates. URM 6670 is stored at the Federal University of Pernambuco Culture Collection (URM604) and in this work is coded as BT0510.

HHP treatment

Cells were grown at 28 °C with aeration (150 rpm) in liquid YEPD medium (1 % yeast extract, 2 % peptone and 2 % glucose) to exponential growth phase ($OD_{600nm}=1.0$). Yeast suspensions were placed inside a 4-mL Teflon tube without air bubbles at room temperature. External hydrostatic pressure was applied to the BeCu piston cylinder and measured with a calibrated mechanical manometer, as previously described (Bravim et al. 2010). Samples were submitted to four treatments: (1) 50 MPa for 30 min at room temperature and (2) 50 MPa for 30 min followed by incubation at room pressure with aeration for 5, (3) 10 and (4) 15 min. Experiments were performed twice in duplicate.

Microarray analyses

RNA preparation, amplification, microarray hybridisation and analysis were performed as described previously (Lippman and Broach 2009). Briefly, total RNA was extracted using the Qiagen RNeasy Mini Kit (Valencia, CA). cRNA was synthesised using the standard protocol of the Agilent Low RNA Input Linear Amplification Kit (Agilent Technologies, Palo Alto, CA), including an additional DNase I purification step (Agilent Technologies, Palo Alto, CA). Briefly, 100 ng of total RNA was used as a template for first- and second-strand cDNA synthesis with reverse transcriptase using a primer containing poly dT and T7 polymerase promoter. Labelled cRNA was synthesised from cDNA using T7 RNA polymerase and cyanine (Cy)3- or Cy5-labelled CTP (PerkinElmer Life and Analytical Sciences, Boston, MA). The amount of cRNA synthesised and incorporation of Cy3- and Cy5-CTP into cRNA were measured using a NanoDrop (NanoDrop Technologies, Wilmington, DE). Equal amounts of Cy3- and Cy5-labelled cRNA were combined, mixed with the control target and fragmented for 30 min. Each sample was then hybridised to an Agilent Yeast Oligo Microarray (V1, 4x44K G2519F) or (V2, 8x15K G4813A) for 17 h at 60 ° C. The arrays were washed and scanned using Agilent Microarray Scanner (Agilent Technologies) at 100 % photo-multiplier tube for red and green channels and at 5- μ m resolution. The feature information was extracted from the microarrays using Agilent Feature Extraction Software version 9.5 with Linear Lowess dye normalisation and no background subtraction and submitted to the Princeton University Microarray database for storage and analysis. Dye normalisation for each array was determined by the rank consistency method and then spot intensities were calculated by the LOWESS method (Zaman et al. 2009). Spots were retained for further analysis only if both the Cy3 and Cy5 channels were greater than 2.6σ of mean background intensity and were uniform in intensity. Only those genes for which 80 % of the arrays yielded good data were retained for analysis. R^2 values between experimental duplicate were greater than 0.99. Agilent Yeast Microarray with part numbers G2519F and G4813A are assigned accession numbers GPL7542 and GPL13340, respectively on the Gene Expression Omnibus. All data described in this study can be publicly viewed and downloaded from the PUMAdb website http://puma.princeton.edu/cgi-bin/publication/viewPublication.pl?pub_no=542.

Gene over-expression

Escherichia coli XL1-Blue was used for plasmid construction and propagation. The multi-copy expression vector pYEG-not (Omura et al. 2007) contains ampicillin and geneticin

(G-418) resistance genes markers and the promoter and terminator sequences of the yeast glyceraldehyde 3-phosphate dehydrogenase gene (*TDHI*). *E. coli* strains were grown in LB medium (1 % tryptone, 1 % NaCl and 0.5 % yeast extract) and selected on LB plates with 100 µg/mL ampicillin. Restriction endonucleases sites used for cloning were *NotI* and *BamHI*.

Primers used for amplification are listed in Table 1. The PCR program for amplification of both *HSP12* and *STF2* fragment was 95 °C for 10 min, 30 cycles of 95 °C for 30 s, 55 °C for 30 s and 72 °C for 45 s, followed by incubation at 72 °C for 10 min. *SYM1* fragments were amplified using the same program except for the extension time of 2 min and 18 s.

For *S. cerevisiae* transformation, the modified lithium acetate method was used (Gietz and Schiestl 2007). Transformants over-expressing genes of interest were selected on YPD plates containing 100 µg/mL geneticin.

Gene expression confirmation

Total RNA was extracted from yeast cells using phenol/ chloroform and precipitated with 3 M sodium acetate/absolute ethanol. Nucleic acid pellets were washed in 70 % ethanol and resuspended in DEPC-treated water. Extracted RNA samples were treated for 10 min with 0.5 U of RNase-free DNase I/µg RNA at 37 °C to remove any residual genomic DNA. cDNA was synthesised using High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA). Real-time RT-PCR experiments were carried out to determine the changes in the mRNA levels of several genes and to confirm over-expression of the cloned gene. Primers used for amplification are shown in Table 2. Reactions were carried out in an Applied Biosystems StepOnePlus™ Real-Time PCR (ABI 6.200, Applied Biosystems). For each gene, calibration curves with 10-fold serial dilutions of cDNAs from the selected strain were obtained to determine amplification efficiency (Table 2). All the analyses were performed in duplicate. Relative expression levels were obtained through the calculation of 2^{-Ct} , where $Ct = C_{t\text{ treatment}} - C_{t\text{ control}}$ (Livak and Schmittgen 2001). The acquired data were normalised to *ALG9* or *TAF10* expression levels (Teste et al. 2009). As the results for both genes were similar, just *ALG9* data were used for the expression level calculation.

Fermentation and ethanol measurement after stress treatments

Wild yeast cells were grown and pressurised as described above. The *S. cerevisiae*-engineered strains were grown at 28 °C with aeration (150 rpm) in liquid YEPD medium (1 % yeast extract, 2 % peptone and 2 % glucose) supplemented with geneticin (100 µg/mL) to exponential growth phase ($OD_{600nm}=1.0$). Fermentation was carried out in 250-mL flasks containing 100 mL of sterile sugar cane juice at 16 ° Brix (soluble solids content). The flasks were incubated at 28 °C for 24 h without stirring throughout the entire fermentation process. Samples were taken at 4, 8, 12 and 24 h, harvested, and the fermented media from each time point was distilled. Ethanol concentration was determined by the potassium dichromate method (Vicente et al. 2006). Exponentially growing *S. cerevisiae*-engineered cells on YEPD geneticin were submitted to the following stresses: (1) ethanol stress by incubation with 12 and 15 % ethanol for up to 120 min at 150 rpm at 28 °C. Erlenmeyer

flasks were tightly sealed to avoid ethanol evaporation. (2) Heat shock treatment at 45 °C for up to 120 min at 150 rpm and (3) HHP treatment (50–200 MPa) for 30 min. Viability was determined by plating the appropriate dilution of cells on YEPD plus 2 % agar. After incubation for 48 h at 28 °C, cell survival was calculated by comparing the colony forming units of treated versus untreated samples. All experiments were performed in duplicate and reproduced at least three times; mean values were calculated. Statistical analysis of ethanol productivity after HHP treatment was performed using a one-way analysis of variance and Tukey's test ($P<0.05$).

Results

Ethanol production after pressure treatment

In order to investigate the physiological consequences of pressure treatment on ethanol production, wild *S. cerevisiae* strain BT0510 was submitted to two different pressure treatments prior to fermentative process: 50 MPa for 30 min followed by incubation at room pressure for 15 min or 50 MPa for 30 min without incubation. Fermentation efficiency was compared between cells submitted to both pressure treatments and untreated cells. Ethanol production at the beginning of the fermentation process was higher with HHP treated cells than with untreated cells. As shown in Fig. 1, after 4 h of fermentation, ethanol production reached 0.3 % in pressurised cells culture while unpressurised cells did not produce ethanol during this period. After 10 h of fermentation, pressurised cells yielded ethanol concentration of approximately 0.8 %, whereas non-pressurised cells produced 0.6 % (Fig. 1). Table 3 shows the glucose consumption (in moles per litre) and ethanol production (in moles per litre) after both pressure treatments compared with control (non-pressurised samples). Thus, hydrostatic pressure increases the potential of wild-type cells to produce higher levels of ethanol in subsequent fermentation.

Global gene expression analysis

To understand the molecular mechanism underlying increased fermentation capacity as a result of pressure treatment, we investigated the transcriptional profiles of cells immediately after 50 MPa pressure treatment for 30 min and after a time-course of recovery periods following pressurisation. Global microarray analysis revealed modest transcriptional reprogramming in the cells during the 30 min of pressure treatment. Among 6,200 known or predicted genes in yeast, mRNA levels for 128 genes increased greater than 4-fold immediately after release from pressure, while expression levels for 20 genes decreased over 4-fold. These limited transcriptional effects contrast with those observed in response to mild heat shock. For instance, elevating the temperature of yeast culture from 23 to 37 °C for 15 min resulted in over 4-fold induction of almost 300 genes and repression of 400 genes (Gasch et al. 2000). However, the minor changes in expression levels observed immediately after pressure treatment expanded substantially during the post-treatment incubation period, affecting various classes of genes described below. Thus, while pressure treatment per se does not result in robust transcriptional restructuring, it likely primes the cell for subsequent transcriptional changes upon release from pressure.

Hydrostatic pressure-responsive genes were classified in distinct categories according to the MIPS functional database (<http://mips.helmholtz-muenchen.de/genre/proj/yeast/>). A schematic representation of the global gene expression profile in functional categories is shown in Fig. 2. Although only modest changes in global gene expression were observed immediately following pressure treatment (Fig. 2a), the mRNA levels of genes representing various functional categories increased during the post-treatment incubation of 5 to 15 min (Fig. 2b–d). As shown in Fig. 2, genes classified as “cell rescue, defense and virulence” (Fig. 2, class 4) showed increases in transcript levels, particularly those involved in oxidative and osmotic stress. Similarly, transcript levels were elevated for genes associated with functions in the category “energy” (Fig. 2, class 9), specifically those associated with the glycolytic pathway, tricarboxylic acid (TCA) cycle, fermentation and oxidative phosphorylation. Moreover, genes related to methionine and glutamate biosynthesis, as well as those involved in membrane and cell wall structure displayed increases in mRNA abundance. Among the functional categories negatively responsive to pressurisation, genes involved in cellular “development” (Fig. 2, class 8), “transcription” (Fig. 2, class 16) and “transposable elements, viral and plasmids” (Fig. 2, class 17) were down-regulated after the treatment. Table 4 lists the relative transcript measurements of representative genes whose expression levels are responsive to pressurisation.

Stress response—Yeast cells subjected to pressure treatment mounted a weaker global transcriptional response than elicited by other environmental stresses, such as heat shock, as observed by Gasch et al. (2000). While the intensity of expression changes and the range of affected genes increased throughout the post-treatment incubation, their robustness never achieved the level of response elicited by other stresses. As shown in Fig. 3, genes previously identified to be positively regulated by environmental stress (Gasch et al. 2000) showed a mild induction immediately after pressurisation. By 15-min post-treatment, elevation of expression levels of these genes was comparable as observed in cells subjected to heat shock. In contrast, the set of genes previously shown to be negatively regulated by environmental stress (Gasch et al. 2000) was only partially repressed even after the post-treatment incubation. Thus, pressure treatment appears to engage only a portion of the environmental stress response (ESR), a common response utilised by yeast cells when challenged with many the stressful conditions (Gasch et al. 2000; Causton et al. 2001).

A number of transcriptional activators, including Cin5p, Yap1p, Hsf1p, Xbp1p, Rpn4p, Msn2p and Msn4p, are responsible for stressed-induced expression of genes associated with ESR. Consistent with our observations, genes encoding these transcription factors were as up-regulated in response to pressure treatment as they were seen to be under heat shock. Actually, transcription factors are regulated mostly at the level of cellular localisation but they are also regulated at the level of expression (Mai and Breeden 1997; Gasch et al. 2000; Igual and Estruch 2000; Hahn et al. 2006; Hanlon et al. 2011). The mechanism of how cells integrate environmental stress to coordinate gene repression is less well understood. Stress generally results in a temporary cessation in growth as well as rapid repression of hundreds of genes required for mass accumulation, which is synthesis of biomass to allow cell size increase and cell cycle progression, particularly genes encoding ribosomal proteins and those required for ribosome biogenesis. However, the exact causal relationship between

stress-induced growth arrest and reduced expression of the mass accumulation genes remains unclear. Relevant to this question, we previously showed that pressure caused a more substantial growth arrest than did heat shock, as yeast cells reach a minimum value of unbudded cells 45 min after pressure and are fully active only 2 h after the piezotreatment, while cells halt growth after 30 min at 40 °C and take 90 min to recover growth (Palhano et al. 2004a). Therefore, after the yeast cells have been relieved from the pressure stress, they still suffer metabolic changes and are responding to this stress (Fernandes 2005). Corroborating to this observation is our results of elevation of gene expression levels during the post-pressurisation time. Accordingly, our results provide evidence that the change in transcription does not derive from growth arrest but rather that stress directly influences gene expression, with different stresses having different effects.

Microarray analysis showed that genes involved in oxidative stress response, namely those encoding catalase (*CTT1*), mitochondrial superoxide dismutase (*SOD2*) and metallothionein (*CUP1-1*) were induced post-pressurisation, and this was confirmed by qRT-PCR (Fig. 4). Moreover, genes associated with biosynthesis and transport of methionine (*SAM3*, *MET14*, *MET16*, *MUP3*, *MET2* and *MXR1*) were up-regulated immediately after pressure treatment. Additional genes induced immediately after pressure treatment, and post-pressurisation recovery period include genes required for glutamate transformation, such as *GAD1*, *UGA1* and *UGA2*. Both of these gene groups necessary for methionine and glutamate production and metabolism have been shown to contribute to the yeast cell's oxidative stress response (Coleman et al. 2001; Luo and Levine 2009). However, the identified methionine and glutamate biosynthetic genes in this study do not constitute the ESR defined by Gasch et al. (2000), and thus may reflect a cellular response specific to pressure treatment.

Metabolism—Yeast cells subjected to 50 MPa of pressure exhibited significant changes in their transcriptional program involved in cellular metabolism and energy (Fig. 2, Table 4). Hydrostatic pressure induced expression of both synthetic and catabolic enzymes associated with glycogen and trehalose metabolism, such as *TPS1* and *GSY2*, encoding trehalose 6-P synthase and glycogen synthase, respectively (Table 4). Moreover, transcript levels of genes involved in trehalose hydrolysis, including *NTH1* and *NTH2* which encode neutral trehalase I and II, respectively, increased approximately 3-fold post-pressurisation (Table 4).

Similarly, expression of genes associated with carbon assimilation and metabolism were highly sensitive to pressure stress. For example, genes encoding high-affinity hexose transporters, *HXT6* and *HXT7*, and genes encoding glycolytic enzymes, such as hexose kinases *HXX1* and *GLK1*, were up-regulated 5-fold at 15-min post-pressurisation (Table 4; Fig. S1 in the Electronic supplementary material (ESM)). Microarray analysis also showed an increase in mRNA abundance of *PFK26*, whose product catalyzes the synthesis of fructose-2,6-bisphosphate, a strong allosteric effector of the glycolytic enzyme phosphofructokinase (Fig. S1 in the ESM). In addition, it was observed that immediately after pressure treatment the *ADH1* gene (alcohol dehydrogenase), required for the reduction of acetaldehyde to ethanol, was down-regulated and 15-min post-pressurisation an increase in expression could be observed (Table 4). Moreover, enhanced expression profiles of mRNA encoding enzymes driving the TCA cycle were observed following pressure treatment, including *ACO1*, *KGD2*, *CIT1* and genes encoding the subunits of the succinate

dehydrogenase complex (Sdh1p, Sdh2p, Sdh3p and Sdh4p) (Table 4). These results are discussed below.

Genetic modification of the wild yeast strain

Our analysis demonstrated that pressure treatment resulted in up-regulation of a number of genes with concomitant increase in ethanol production. Accordingly, we investigated which specific set of genes can directly be attributed to increased ethanol production. We selected ten genes (*CTT1*, *GAC1*, *HSP12*, *HSP26*, *HSP30*, *HSP104*, *SSE2*, *SOD2*, *SYM1* and *STF2*) to examine based on the criteria of being positively regulated in response to pressure treatment and belonging to the yeast transcriptional stress response program. Over-expressing seven of the ten genes using a strong-expression promoter driven vector into BT0510 resulted in inviability of the transformed cells. While we have not directly investigated the molecular mechanism underlying lethality, the results suggest a growth inhibition upon over-expression of these seven genes. However, we were able to generate transformants over-expressing *HSP12*, *SYM1* or *STF2*, and evaluated fermentative efficiency and/or fermentation stress tolerance associated with over-induction of these genes.

We first examined the effects of high ethanol concentration (Fig. 5a) and heat shock (Fig. 5b) on the viability of *S. cerevisiae* strains over-producing Hsp12, Sym1 or Stf2. BT0510-pSYM1 exhibited increased tolerance to ethanol stress relative to the control strain or the two strains over-expressing either Hsp12 or Stf2. Whereas over 30 % of the BT0510-pSYM1 survived 2 h treatment of 12 % of ethanol, only 7.5–11 % of the other mutant strains survived the treatment. BT0510-pHSP12 displayed slightly increased resistance to heat shock of 45 °C for 60 min relative to the other two over-producing strains (Fig. 5b), with a survival rate of approximately 90 % while the other two strains showed a survival rate of 62–69 %. In contrast, over-expression of *STF2* did not result in any enhanced tolerance to ethanol nor heat stress (Fig. 5a, b).

In order to compare physiological responses elicited by individual stresses, we employed a previously developed strategy (Bravim et al. 2010), which constructs correlation plots of sensitivity measurements of different genetic background to various stress treatments (Fig. 5c–e). For instance, a straight line on the generated plot indicates that the compared stresses are equivalent and correlated ($Y=X$). The proximity of symbols to the correlation line as shown in Fig. 5c suggests that treatment of cells to 15 % ethanol for 120 min results in a comparable cellular response to exposure to hydrostatic pressure of 150 MPa for 30 min. In contrast, the strains over-inducing *HSP12*, *SYM1* or *STF2* did not display correlated responses between heat shock (45 °C for 120 min) and pressure treatment (150 MPa for 30 min) or when comparing heat shock versus ethanol treatment (Fig. 5d, e).

Since the main goal of this study was to identify genetic mechanisms to increase alcoholic fermentation, we investigated if the three over-expression strains displayed enhanced fermentative efficiency. As shown in Fig. 6, higher ethanol production was observed in all three strains at 24 h: BT0510-pSYM1 with 19 % ethanol output over the control and BT0510-pHSP12 and BT0510-pSTF2 strains with 4 % and approximately 3.5 %, respectively. At 48 h of fermentation, over-induction of *SYM1*, *STF2* or *HSP12* resulted in higher ethanol production of 15, 11 and 5 %, respectively, over the control strain.

Interestingly, overall ethanol production by the BT0510-pSYM1 strain decreased after 48 h, suggesting that it had reached the maximum productivity before 48 h (Fig. 6). After 72 h, only BT0510-pSTF2 attained 8.5 % ethanol production, similar to BT0510-pSYM1 at 48 h; while ethanol concentrations were approximately 7.5 % for the other strains (Fig. 6).

Discussion

Global transcriptional restructuring in response to pressure treatment

To identify genes that can be utilised to optimise the fermentative process, we conducted a global transcriptional analysis of yeast cells following pressure treatment. The results of this analysis highlighted that pressure-treated cells exhibit a modified version of the environmental stress response: while genes normally induced by stress were also induced by pressure treatment only a partial set of the genes repressed by stress were repressed by pressure treatment (Fig. 3). Interestingly, HHP resulted in more sustained growth inhibition than that induced by other environmental stresses (Palhano et al. 2004a). Based on transcriptional profiling, pressure treatment appears to disentangle growth inhibition from repression of genes associated with mass accumulation, though the mechanistic basis of this disassociation is unclear. These results suggest that pressure treatment provides yeast cells with stress protection comparable to that elicited by environmental stress without the associated inhibition of mass accumulation. Moreover, the disconnection in the regulation of mass accumulation and growth inhibition could explain the enhanced ethanol production afforded by pressure treatment.

Genes induced by pressure treatment include those encoding a set of heat shock proteins (HSPs). Many of the conserved HSPs play essential roles in promoting the folding and unfolding of other proteins, the assembly and disassembly of proteins in oligomeric structures and the degradation of proteins that are improperly assembled or denatured (Estruch 2000). *HSP12* and *HSP26* encode two small Hsps associated with stresses that cause severe membrane damage, especially osmotic and ethanol stresses (Yale and Bohnert 2001; Sahara et al. 2002). The transcript levels of these genes increased significantly following pressure treatment (Table 4; Fig. S1 in the ESM), consistent with earlier studies (Iwahashi et al. 2003; Iwahashi et al. 2005). *Hsp12* promotes survival under a variety of stress conditions, for instance by increasing membrane fluidity and stability, thereby stabilising the membrane systems of *S. cerevisiae* (Motshwene et al. 2004). *HSP12* also has an effect on cell morphology; *hsp12* cells have rough and irregular surfaces after stress treatments (Welker et al. 2010). HHP induces alterations of membrane structure (Fernandes et al. 2001), suggesting that the induction of small HSPs, mainly *HSP12*, is related to membrane destabilisation after piezotreatment (Fernandes et al. 2004).

A previous analysis examining global transcriptional responses to a treatment of 30 MPa for 16 h revealed significant induction of genes involved in methionine biosynthesis, suggesting a role of this amino acid in cell survival during an extended piezotreatment (Iwahashi et al. 2005). Our results also showed a substantial induction of genes related to methionine metabolism, such as *MET17*, *MET6*, *MET10*, *MXR1*, *MET14* and *MET10* immediately after pressure treatment, which decreased during post-pressurisation, suggesting that these genes may play a role in the immediate cellular response to stress (Table 4). Methionine, both free

and incorporated into proteins, is an important target of reactive oxygen species (Schöneich 2005; Weissbach et al. 2002). Methionine plays an important role in defence against oxidative stress (Luo and Levine 2009) and functions as an important cellular sensor to stress (Hoshi and Heinemann 2001). Moreover, since methionine feeds into lipids biosynthesis, an increase in the methionine content could lead to protection of the cell plasma membrane to pressure stress (Murata et al. 2003).

GAD1, *UGA1* and *UGA2* were highly up-regulated after pressure treatment (Table 4). The products of these genes convert glutamate into gamma-aminobutyric acid (GABA). GABA plays a role in protection against oxidative stress, and deletion of *GAD1* has been shown to reduce stress tolerance of yeast cells subjected to H₂O₂ treatment (Coleman et al. 2001).

The oxidative stress subgroup exhibited the most pronounced response to pressure treatment. Previous microarray analyses have demonstrated that 131 genes associated with oxidative stress are >2-fold up-regulated after a treatment of 200 MPa for 30 min (Fernandes et al. 2004). Moreover, yeast cells submitted to HHP of 220 MPa (a lethal treatment) in the presence of glutathione exhibited piezoresistance (Palhano et al. 2004b). In bacteria, an alternative model has been proposed, in which excess pressure treatment inactivates specific enzymes, resulting in a state of metabolic imbalance and a lethal burst of reactive oxygen species (Aertsen et al. 2004; Aldsworth et al. 1999).

Additionally, under diverse environmental stress conditions, yeast cells induce a variety of genes that affect glucose metabolism, including those encoding glucose transporters and hexose kinases. In response to stress, glucose is funneled into trehalose synthesis and glycogen storage, adenosine-5'-triphosphate (ATP) is synthesised through glycolysis, and nicotinamide adenine dinucleotide phosphate is regenerated by the pentose phosphate shuttle (Gasch 2003). Our current results demonstrate that hydrostatic pressure has a similar effect, inducing genes involved in glycolysis, TCA cycle and trehalose metabolism (Table 4; Fig. S1 in the ESM).

Glycogen and trehalose accumulate in yeast under nutrient starvation and trehalose provides protection against many environmental stresses (Bandara et al. 2009; Parrou et al. 1997). Accordingly, *tps1* mutants, which are unable to accumulate trehalose, are more sensitive to hydrostatic pressure than wild-type cells, underscoring the link between trehalose accumulation and cell survival in hydrostatic pressure (Fernandes et al. 1997). Surprisingly, *NTH1*, which encodes trehalase responsible for degrading trehalose, is necessary for thermotolerance and its loss results in decreased piezotolerance of *S. cerevisiae* (Iwahashi et al. 2000). One explanation for this observation is that, while trehalose provides protection during pressure treatment, it interferes with recovery after the treatment. During cell recovery, molecular chaperones promote reactivation of denatured proteins that have been destabilised and prevented from aggregation by trehalose. Nevertheless, the persistence of high levels of trehalose interferes with the reactivation of denatured substrates and thus needs to be eliminated by trehalase-dependent hydrolysis (Singer and Lindquist 1998). This may explain why the *TPS1* gene, required for trehalose synthesis, is induced immediately upon pressure treatment while *NTH1* and *NTH2*, coding for neutral trehalase I and II, respectively, are induced primarily post-pressurisation (Table 4).

Ethanol production after pressure treatment

We observed that pressure treatment decreased the latency period for ethanol fermentation as well as overall yield of ethanol after 10 h of fermentation. The ability to optimise the efficiency of the fermentation process with a short latent phase is a desired physiological trait of *S. cerevisiae* strains for biotechnological purposes, such as beverages and ethanol for biofuel production (Schuller and Casal 2005; Schwan et al. 2001; Silva et al. 2009). While the biological mechanisms underlying reduced ethanol yield after pressurisation remain unclear, our results suggest that genes induced by HHP treatment contribute to the adaptation of yeast cells to the high sugar content fermentation medium. Furthermore, strong induction of *CIN5* transcription factor, which has been shown to mediate salt tolerance and regulate oxidative stress response (Table 4), suggests that the cells are in a reduced condition. Thus, increase in ethanol content after pressure treatment could be related to a cell effort to establish a redox balance by transforming acetaldehyde into ethanol and NAD^+ through the fermentation process (Bakker et al. 2001). Moreover, an up-regulation of genes related to hexose transporters (*HXT6* and *HXT7*) and genes encoding glycolytic enzymes, such as hexose kinases was observed (Table 4; Fig. S1 in the ESM). Also, even though the *ADH1* gene was down-regulated immediately after pressure treatment, its expression was increased after 15 min at room pressure following high pressure treatment (Table 4), suggesting that during cellular recovery from piezotreatment anaerobic metabolism could be activated, contributing to ethanol production.

We further explored the mechanism of pressure-induced fermentation enhancement by testing the fermentation capacity of a strain over-expressing individual genes whose transcript levels increased upon pressure treatment and whose functions were related to yeast stress response and cell metabolism. Over-expression of *SYM1*, involved both in metabolism and tolerance to ethanol under high temperature, did in fact enhance the fermentative capacity of our wild-type strain. Trott and Morano (2004) previously reported that cells lacking *SYM1* displayed attenuated growth under the combined debilitating effects of both high temperature and high ethanol concentration. Thus, we speculate that over-expression of *SYM1* in the wild *S. cerevisiae* strain may help, to some degree, to boost tolerance to ethanol toxicity.

Recently, Cao et al. (2010) showed that cells deleted for *GPD2*, which encodes glycerol-3-phosphate dehydrogenase, but simultaneously over-expressing glutamate synthase (*GLT1*), glutamine synthetase (*GLN1*) and *SYM1*, produce approximately 14 % more ethanol than the wild-type strain. The *gpd2* strain is deficient in glycerol biosynthetic pathway and redirects carbon source flow from glycerol to ethanol synthesis; *GLT1* and *GLN1* over-expression reduces surplus nicotinamide adenine dinucleotide (NADH) and increases consumption of excess ATP in the ammonia assimilation pathway. Nevertheless, the result of the combined effects of over-expression of *GLT1*, *GLN1* and *SYM1* concomitant with deletion of *GPD2* in ethanol production (4 % after 24 h) was lower than that reached by the BT0510-pSYM1 strain in our present study (5 % after 24 h) (Fig. 6). Furthermore, considering that Sym1 is an integral membrane protein of the inner mitochondrial membrane (Trott and Morano 2004), we would expect that its over-expression affects respiratory metabolism but not fermentative metabolism. Rather, we speculate that *SYM1* over-expression might cause a

general defect in NADH oxidation (NADH/NAD⁺—ethanol/acetaldehyde shuttle), which occurs on the mitochondrial matrix (Bakker et al. 2001). To compensate for this impairment, NADH could be oxidised by formation of ethanol in the cytoplasm during fermentative metabolism to increase ethanol production (Fig. 6).

In this study, we selected to focus on the over-expression of the genes in BT0510 described above based on global transcriptional readouts following pressure treatment, and these genes reflect a cellular stress response of yeast cells to pressure. We propose that the cellular response to ethanol and pressure treatment can be correlated if ethanol and pressure treatment converge on the plasma membrane as the stressors' main target (Fig. 5c).

In conclusion, our results bring additional information about the effect of HHP on *S. cerevisiae* cells, and demonstrate the importance of post-pressurisation incubation for the activation of several important genes related to the cell recovery and stress tolerance. Moreover, we show that the over-expression of *SYM1* leads to an increase of 18.4 % on ethanol production. Therefore, if we consider ethanol production at industrial level, BT050-pSYM1 would produce 16.6 mL more ethanol than the control. Considering that for each 8 L of sugar cane juice we have 1 L of cachaça, BT050-pSYM1 could produce 1.16 L of cachaça to 8 L of sugarcane. Our evidence for enhanced ethanol production after HHP treatment presented in this study may be of strong interest for fermentation industries, both cachaça production and other beverages, such as wine and beer, as well as for biofuel production.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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References

- Aertsen A, De Spiegeleer P, Vanoirbeek K, Lavilla M, Michiels CW. Induction of oxidative stress by high hydrostatic pressure in *Escherichia coli*. Appl Environ Microbiol. 2004; 71:2226–2231.10.1128/AEM.71.5.2226-2231.2005
- Aldsworth TG, Sharman RL, Dodd CER. Bacterial suicide through stress. Cell Mol Life Sci. 1999; 56:378–383.10.1007/s000180050439
- Bakker BM, Overkamp KM, van Maris AJ, Kötter P, Luttik MA, van Dijken JP, Pronk JT. Stoichiometry and compartmentation of NADH metabolism in *Saccharomyces cerevisiae*. FEMS Microbiol Rev. 2001; 25:15–37.
- Bandara A, Fraser S, Chambers PJ, Stanley GA. Trehalose promotes the survival of *Saccharomyces cerevisiae* during lethal ethanol stress, but does not influence growth under sublethal ethanol stress. FEMS Yeast Res. 2009; 9:1208–1216.10.1111/j.1567-1364.2009.00569.x
- Bravim F, Palhano FL, Fernandes AA, Fernandes PM. Biotechnological properties of distillery and laboratory yeasts in response to industrial stresses. J Ind Microbiol Biotechnol. 2010; 37:1071–1079.10.1007/s10295-010-0755-0

- Cao L, Kong Q, Zhang A, Chen X. Overexpression of *SYM1* in a *gpdDelta* mutant of *Saccharomyces cerevisiae* with modified ammonium assimilation for optimization of ethanol production. *J Taiwan Inst Chem Eng.* 2010; 41:2–7.10.1016/j.jtice.2009.04.012
- Causton HC, Ren B, Koh SS, Harbison CT, Kanin E, Jennings EG, Lee TI, True HL, Lander ES, Young RA. Remodeling of yeast genome expression in response to environmental changes. *Mol Biol Cell.* 2001; 12:323–337.
- Coleman ST, Fang TK, Rovinsky SA, Turano FJ, Moye-Rowley WS. Expression of a glutamate decarboxylase homologue is required for normal oxidative stress tolerance in *Saccharomyces cerevisiae*. *J Biol Chem.* 2001; 276:244–2450.10.1074/jbc.M007103200
- Estruch F. Stress-controlled transcription factors, stress-induced genes and stress tolerance in budding yeast. *FEMS Microbiol Rev.* 2000; 24:469–486.10.1111/j.1574-6976.2000.tb00551.x
- Fernandes PMB. How does yeast respond to pressure? *Braz J Med Biol Res.* 2005; 38:1239–1245.
- Fernandes, PMB. *Saccharomyces cerevisiae* response to high hydrostatic pressure. In: Michiels, C.; Bartlett, DH.; Aertsen, A., editors. High-pressure microbiology. American Society of Microbiology; Washington: 2008. p. 145-166.
- Fernandes PM, Panek AD, Kurtenbach E. Effects of hydrostatic pressure on a mutant of *Saccharomyces cerevisiae* deleted in trehalose-6-phosphate synthase gene. *FEMS Microbiol Lett.* 1997; 152:17–21.10.1111/j.1574-6968
- Fernandes PM, Farina M, Kurtenbach E. Effect of hydrostatic pressure on the morphology and ultrastructure of wild-type and trehalose synthase mutant cells of *Saccharomyces cerevisiae*. *Lett Appl Microbiol.* 2001; 32:42–46.10.1111/j.1472-765X.2001.00853.x
- Fernandes PMB, Domitrovic T, Kao CM, Kurtenbach E. Genome expression pattern in *Saccharomyces cerevisiae* cells in response to high hydrostatic pressure. *FEBS Lett.* 2004; 556:153–160.10.1016/S0014-5793(03)01396-6
- Gasch, AP. The environmental stress response: a common yeast response to diverse environmental stresses. In: Hohmann, S.; Mager, WH., editors. Yeast stress response. 2. Springer; Berlin: 2003. p. 11-70.
- Gasch AP, Spellman PT, Kao CM, Carmel-Harel O, Eisen MB, Storz G, Botstein D, Brown PO. Genomic expression programs in the response of yeast cells to environmental changes. *Mol Biol Cell.* 2000; 11:4241–4257.
- Gietz RD, Schiestl RH. High-efficiency yeast transformation using the LiAc/SS carrier DNA/PEG method. *Nat Protoc.* 2007; 2:31–34.10.1038/nprot.2007.13
- Hahn JS, Neef DW, Thiele DJ. A stress regulatory network for co-ordinated activation of proteasome expression mediated by yeast heat shock transcription factor. *Mol Microbiol.* 2006; 60:240–251.10.1111/j.1365-2958.2006.05097.x
- Hanlon SE, Rizzo JM, Tatome DC, Lieb JD, Buck MJ. The stress response factors Yap6, Cin5, Phd1, and Skn7 direct targeting of the conserved co-repressor Tup1-Ssn6 in *S. cerevisiae*. *PLoS One.* 2011; 6:e19060.10.1371/journal.pone.0019060
- Hoshi T, Heinemann S. Regulation of cell function by methionine oxidation and reduction. *J Physiol.* 2001; 531:1–11.10.1111/j.1469-7793.2001.0001j.x
- Igual JC, Estruch F. Signalling stress in yeast. *Food Technol Biotechnol.* 2000; 38:263–276.
- Iwahashi H, Nwaka S, Obuchi K. Evidence for contribution of neutral trehalase in barotolerance of *Saccharomyces cerevisiae*. *Appl Environ Microbiol.* 2000; 66:5182–5185.
- Iwahashi H, Shimizu H, Odani M, Komatsu Y. Piezophysiology of genome wide gene expression levels in the yeast *Saccharomyces cerevisiae*. *Extremophiles.* 2003; 7:291–298.10.1007/s00792-003-0322-y
- Iwahashi H, Odani M, Ishidou E, Kitagawa E. Adaptation of *Saccharomyces cerevisiae* to high hydrostatic pressure causing growth inhibition. *FEBS Lett.* 2005; 579:2847–2852.10.1016/j.febslet.2005.03.100
- Lippman SI, Broach JR. Protein kinase A and *TORC1* activate genes for ribosomal biogenesis by inactivating repressors encoded by Dot6 and its homolog Tod6. *Proc Natl Acad Sci U S A.* 2009; 106:19928–19933.10.1073/pnas.0907027106
- Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2^{-CT} method. *Methods.* 2001; 25:402–408.10.1006/meth.2001.1262

- Luo S, Levine RL. Methionine in proteins defends against oxidative stress. *FASEB J*. 2009; 23:464–472.10.1096/fj.08-118414
- Mai B, Breeden L. Xbp1, a stress-induced transcriptional repressor of the *Saccharomyces cerevisiae* Swi4/Mbp1 family. *Mol Cell Biol*. 1997; 17:6491–6501.
- Maiti B, Rathore A, Srivastava S, Shekhawat M, Srivastava P. Optimization of process parameters for ethanol production from sugar cane molasses by *Zymomonas mobilis* using response surface methodology and genetic algorithm. *Appl Microbiol Biotechnol*. 2011; 90:385–395.10.1007/s00253-011-3158-x
- MIDIC. [Assessed 16 Sept] Ministério do Desenvolvimento, Indústria e Comércio Exterior. 2010. <http://www.mdic.gov.br/sitio/>
- Motshwene P, Karreman R, Kgari G, Brandt W, Lindsey G. LEA (late embryonic abundant)-like protein Hsp 12 (heat-shock protein 12) is present in the cell wall and enhances the barotolerance of the yeast *Saccharomyces cerevisiae*. *Biochem J*. 2004; 377:769–774.10.1042/BJ20031301
- Murata Y, Watanabe T, Sato M, Momose Y, Nakahara T, Oka S, Iwahashi H. Dimethyl sulfoxide exposure facilitates phospholipid biosynthesis and cellular membrane proliferation in yeast cells. *J Biol Chem*. 2003; 278:33185–33193.10.1074/jbc.M300450200
- Oliveira ES, Cardello HMAB, Jeronimo EM, Souza ELR, Serra GE. The influence of different yeasts on the fermentation, composition and sensory quality of cachaça. *World J Microb Biot*. 2005; 21:707–715.10.1007/s11274-004-4490-4
- Omura F, Hatanaka H, Nakao Y. Characterization of a novel tyrosine permease of lager brewing yeast shared by *Saccharomyces cerevisiae* strain RM11-1a. *FEMS Yeast Res*. 2007; 7:1350–1361.10.1111/j.1567-1364.2007.00310.x
- Palhano FL, Gomes HL, Orlando MT, Kurtenbach E, Fernandes PM. Pressure response in the yeast *Saccharomyces cerevisiae*: from cellular to molecular approaches. *Cell Mol Biol*. 2004a; 50:447–457.10.1170/T533
- Palhano FL, Orlando MT, Fernandes PM. Induction of baroresistance by hydrogen peroxide, ethanol and cold-shock in *Saccharomyces cerevisiae*. *FEMS Microbiol Lett*. 2004b; 233:139–145.10.1016/j.femsle.2004.02.002
- Parrou JL, Teste MA, François J. Effects of various types of stress on the metabolism of reserve carbohydrates in *Saccharomyces cerevisiae*: genetic evidence for a stress-induced recycling of glycogen and trehalose. *Microbiology*. 1997; 143:1891–1900.10.1099/00221287-143-6-1891
- Pataro C, Guerra JB, Petrillo-Peixoto ML, Mendonça-Hagler LC, Linardi VR, Rosa CA. Yeast communities and genetic polymorphism of *Saccharomyces cerevisiae* strains associated with artisanal fermentation in Brazil. *J App Microbiol*. 2000; 89:24–31.10.1046/j.1365-2672.2000.01092.x
- Sahara T, Goda T, Ohgiya S. Comprehensive expression analysis of time-dependent genetic responses in yeast cells to low temperature. *J Biol Chem*. 2002; 277:50015–50021.10.1074/jbc.M209258200
- Schöneich C. Methionine oxidation by reactive oxygen species: reaction mechanisms and relevance to Alzheimer's disease. *Biochim Biophys Acta*. 2005; 1703:111–119.10.1016/j.bbapap.2004.09.009
- Schuller D, Casal M. The use of genetically modified *Saccharomyces cerevisiae* strains in the wine industry. *Appl Microbiol Biotechnol*. 2005; 68:292–304.10.1007/s00253-005-1994-2
- Schwan RF, Mendonça AT, da Silva JJ Jr, Rodrigues V, Wheals AE. Microbiology and physiology of cachaça (Aguardente) fermentations. *Antonie van Leeuwenhoek*. 2001; 79:89–96.10.1023/A:1010225117654
- Silva CLC, Vianna CR, Cadete RM, Santos RO, Gomes FC, Oliveira ES, Rosa CA. Selection, growth, and chemosensory evaluation of flocculent starter culture strains of *Saccharomyces cerevisiae* in the large-scale production of traditional Brazilian cachaça. *Int J Food Microbiol*. 2009; 131:203–210.10.1016/j.ijfoodmicro.2009.02.027
- Singer MA, Lindquist S. Thermotolerance in *Saccharomyces cerevisiae*: the yin and yang of trehalose. *Trends Biotechnol*. 1998; 16:460–468.10.1016/S0167-7799(98)01251-7
- Teixeira MC, Mira NP, Sá-Correia I. A genome-wide perspective on the response and tolerance to food-relevant stresses in *Saccharomyces cerevisiae*. *Curr Opin Biotechnol*. 2011; 22:150–156.10.1016/j.copbio.2010.10.011

- Teste MA, Duquenne M, François JM, Parrou JL. Validation of reference genes for quantitative expression analysis by real-time RT-PCR in *Saccharomyces cerevisiae*. BMC Mol Biol. 2009; 10:99–114.10.1186/1471-2199-10-99
- Tfouni SAV, Machado RMD, Camargo MCR, Vitorino SHP, Vicente E, Toledo MCF. Determination of polycyclic aromatic hydrocarbons in cachaça by HPLC with fluorescence detection. Food Chem. 2007; 101:334–338.10.1016/j.foodchem.2006.01.040
- Trejo-Pech CO, López-Reyna MC, House LA, Messina W. Appellation of origin status and economic development: a case study of the Mezcal industry. Int Food Agrib Man Rev. 2010; 13:117–136.
- Trott A, Morano KA. *SYM1* is the stress-induced *Saccharomyces cerevisiae* ortholog of the mammalian kidney disease gene *Mpv17* and is required for ethanol metabolism and tolerance during heat shock. Eukaryot Cell. 2004; 3:620–631.10.1128/EC.3.3.620-631.2004
- Vicente MA, Fietto LG, Castro IM, dos Santos AN, Coutrim MX, Brandão RL. Isolation of *Saccharomyces cerevisiae* strains producing higher levels of flavoring compounds for production of “cachaça” the Brazilian sugarcane spirit. Int J Food Microbiol. 2006; 108:51–59.10.1128/AEM.01729-07
- Weissbach H, Etienne F, Hoshi T, Heinemann SH, Lowther WT, Matthews B, St John G, Nathan C, Brot N. Peptide methionine sulfoxide reductase: structure, mechanism of action, and biological function. Arch Biochem Biophys. 2002; 397:172–178.10.1006/abbi.2001.2664
- Welker S, Rudolph B, Frenzel E, Hagn F, Liebisch G, Schmitz G, Scheuring J, Kerth A, Blume A, Weinkauff S, Haslbeck M, Kessler H, Buchner J. Hsp12 is an intrinsically unstructured stress protein that folds upon membrane association and modulates membrane function. Mol Cell. 2010; 39:507–520.10.1016/j.molcel.2010.08.001
- World Health Organization—WHO. [Assessed 07 Nov 2011] WHO global status report on alcohol 2004. 2004. Available from http://www.who.int/substance_abuse/publications/global_status_report_2004_overview.pdf
- Yale J, Bohnert HJ. Transcript expression in *Saccharomyces cerevisiae* at high salinity. J Biol Chem. 2001; 276:15996–16007.10.1074/jbc.M008209200
- Zaman S, Lippman SI, Schnepher L, Slonim N, Broach JR. Glucose regulates transcription in yeast through a network of signaling pathways. Mol Syst Biol. 2009; 5:245.10.1038/msb.2009.2

Fig. 1.

BT0510 ethanol production (in percent) after pressure treatment. *S. cerevisiae* cells in logarithmic phase (1×10^7 cells mL⁻¹) were submitted to a hydrostatic pressure of 50 MPa for 30 min (*empty bars*) and 50 MPa for 30 min and then incubated at room pressure (0.1 MPa) for 15 min (*filled bars*), and after that, the fermentative efficiency of this strain was evaluated. A non-pressurised sample was used as a control (*striped bars*). *Error bars* represent the SD of three measurements

Fig. 2.

Boxplot of global gene expression profile. *Bars* represent the percentage of induced genes after HHP treatment of 50 MPa for 30 min at room temperature (**a**) and 50 MPa for 30 min and then incubated at room pressure with aeration for 5 (**b**), 10 (**c**) and 15 min (**d**). The classification is based on the MIPS database available on the Web, following: 1, biogenesis of cellular components; 2, cell cycle and processing; 3, cell fate; 4, cell rescue, defense, and virulence; 5, cell type differentiation; 6, cellular communication/signal transduction mechanism; 7, cellular transport, transport facilities, and transport routes; 8, development; 9, energy; 10, interaction with the environmental; 11, metabolism; 12, protein fate; 13, protein synthesis; 14, protein with binding function or cofactor requirement; 15, regulation of metabolism and protein function; 16, transcription; 17, transposable elements, viral, and plasmid proteins; and 18, unclassified protein. *Boxes* represent 50 % of the gene population, with the remainder scattered along the upper and lower boundaries. *Thick lines* indicate means; the *thin line within the box* marks the median. *Circles* represent the outlier genes

Fig. 3.

Number of genes related to environmental stress response (ESR) induced (*black lines*) or repressed (*gray lines*) after 37 °C for 15 min (*continuous lines*), 50 MPa for 30 min (*dotted lines*) and 50 MPa for 30 min followed by incubation at room pressure for 15 min (*dashed lines*) treatments

Fig. 4.

Relative expression of *CTT1*, *HSP12*, *HSP26*, *HSP30*, *HSP42*, *HSP104*, *SOD2*, *SSA1*, *SSE2*, *STF2* and *SYM1* genes after pressure treatment. The yeast cells in logarithmic phase (1×10^7 cells mL^{-1}) were submitted to a hydrostatic pressure of 50 MPa for 30 min (white bars) and 50 MPa for 30 min and then incubated at room pressure (0.1 MPa) for 15 min (black bars). The data have been normalised with the *ALG9* gene. Error bars represent the SD of two measurements

Fig. 5.

Behaviour of different yeast transformants. The yeast transformants cells BT0510-control (white symbols), BT510-pHSP12 (light gray symbols), BT05010-pSTF2 (dark gray symbols) and BT0510-pSYM1 (black symbols) in logarithmic phase growth (1×10^7 cells mL⁻¹) were submitted to an ethanol treatment of 12 and 15 % for 120 min (a) and heat shock treatment of 45 °C for 120 min (b). Correlation of tolerance to ethanol×hydrostatic pressure (c), heat shock×hydrostatic pressure (d) and ethanol×heat shock (e) among modified strains.

Numbers on both axes to correlation analyses represent cell survival (in cells per milliliters) after 150 MPa for 30 min (HHP), 15 % ethanol for 120 min (EC) or 45 °C for 120 min (HS)

Fig. 6.

Ethanol production (in per cent) of different yeast transformants. BT0510-control (*white columns*), BT510-pHSP12 (*light gray columns*), BT05010-pSTF2 (*dark gray columns*) and BT0510-pSYM1 (*black columns*) were pre-grown in YEPD medium (supplemented with geneticin) until they reached 2×10^8 cellmL⁻¹ and incubated at 28 °C in sugar cane juice medium for 72 h. Ethanol production in the culture supernatants were determined at various time points. *Error bars* represent the SD of three measurements

Table 1

Oligonucleotides used as primers for PCR amplification of the interest gene

Target mRNA	Primer sequence 5'-3'	Amplicon size (bp)
<i>HSP12</i>	Forward, 5'TAGCGGCCGCATGTCTGACGCAGGTAGAAAAGG3' Reverse, 5'CGGGATCCTTACTTCTTGGTTGGGTCTTCTT3'	329
<i>STF2</i>	Forward, 5'TAGCGGCCGCATGACGAGAACAAACAAGTGGAC3' Reverse, 5'CGGGATCCTCATTCTTTTGGACGTTTCA3'	254
<i>SYM1</i>	Forward, 5'TAGCGGCCGCATGAAGTTATTGCATTTATATGAAGC3' Reverse, 5'CGGGATCCTTATTCGACCACGGGTGGATA3'	593

Table 2

Oligonucleotides used as primers for qRT-PCR analysis

Target mRNA	Primer sequence 5'-3'	Amplicon size (bp)	PCR efficiency ^a (%)
<i>CTT1</i>	Foward, 5'ACGGCCCTATCTTACTGCAA3' Reverse, 5'TACACGCTCCGGAACCTCTTT3'	79	95
<i>GAC1</i>	Foward, 5'TAGCAATACCGCTGCTGATG3' Reverse, 5'GCAAGTTTGTGACCAACGAA3'	82	98
<i>HSP12</i>	Foward, 5'CGCAGGTAGAAAAGGATTTCG3' Reverse, 5'TCAGCGTTATCCTTGCCCTTT3'	194	95
<i>HSP26</i>	Foward, 5'ATGCTGGCGCTCTTTATGAT3' Reverse, 5'TTCTAGGGAAACCGAAACCA3'	95	98
<i>HSP30</i>	Foward, 5'TGGCCTGGATATGCACATTA3' Reverse, 5'GACTGCAAACACTGCCCATTA3'	60	97
<i>HSP42</i>	Foward, 5'TGAACGCATTATCCAACCAA3' Reverse, 5'TTGTCATAATGGGGATGGT3'	94	97
<i>HSP104</i>	Foward, 5'ATTGGCTTCGGATCATCAAC3' Reverse, 5'AAGGACTTTCCCCAAAGCAT3'	228	90
<i>SOD2</i>	Foward, 5'AACCAGGATACCGTCACAGG3' Reverse, 5'TTCCAGTTGACCACATTCCA3'	130	97
<i>SSA1</i>	Foward, 5'TGGTACCATTGCTGGTTTGA3' Reverse, 5'CGGCGGTAGGTTTCGTTAATA3'	52	98
<i>SSE2</i>	Foward, 5'CACTGGGGTCAAGGTTCTTA3' Reverse, 5'GGTAAAGGCACTGGCTCTTG3'	137	94
<i>STF2</i>	Foward, 5'CGGTGAATCTCAAATCACA3' Reverse, 5'CACTGGGGGTATTTACCAT3'	108	96
<i>SYM1</i>	Foward, 5'ACGGGTAGCTGTGATCAAT3' Reverse, 5'AGGCCACCATGCTCTTTTA3'	126	99
<i>TAF10</i>	Foward, 5'GCTAGGCAGCTATTGCAAGG3' Reverse, 5'CAACAGCGCTACTGAGATCG3'	129	96
<i>ALG9</i>	Foward, 5'ACATCGTCGCCCCAATAAAT3' Reverse, 5'GATTGGCTCCGGTACGTAAA3'	145	92

^aThe PCR efficiency of each primer was evaluated by the dilution series method using a mix of sample cDNAs as the template and the formula $10^{(-1/\text{slope})}$. For the calculations, the base of the exponential amplification function was used (Teste et al. 2009)

Table 3

Glucose consumption (in moles per litre) and ethanol production (in moles per litre) during fermentative process after pressure treatment. *Saccharomyces cerevisiae* cells in logarithmic phase (1×10^7 cells/mL⁻¹) were submitted to a hydrostatic pressure of 50 MPa for 30 min (50) and 50 MPa for 30 min and then incubated at room pressure for 15 min (50+0.1). A non-pressurised sample was used as a control (0.1)

Fermentation time (h)	Treatment (MPa)	Glucose (mol/L)	Ethanol (mol/L)
0	0.1	0.83±0.02	0
	50	0.83±0.02	0
	50+0.1	0.83±0.02	0
4	0.1	0.48±0.02	0.008±0.02
	50	0.35±0.05	0.243±0.02
	50+0.1	0.40±0.03	0.080±0.08
8	0.1	0.37±0.10	0.156±0.02
	50	0.33±0.08	0.226±0.01
	50+0.1	0.30±0.03	0.175±0.02
10	0.1	0.28±0.06	0.559±0.03
	50	0.27±0.05	0.597±0.05
	50+0.1	0.19±0.02	0.726±0.08

Means±standard deviation

Table 4

Relative transcriptional level of genes affected after pressure treatment (expressed as log₂ value) of 50 MPa for 30 min at room temperature (0') followed by incubation at room pressure with aeration for 5 (5'), 10 (10') and 15 min (15')

Systematic name	Standard name	Pressure treatments				Description
		0'	5'	10'	15'	
YLR304C	<i>ACO1</i>	0.84	0.58	2.25	2.75	Aconitase, required for the tricarboxylic acid (TCA) cycle
YOL086C	<i>ADHI</i>	-0.3	0.12	0.53	1.08	Alcohol dehydrogenase and fermentative isozyme active as homo- or heterotetramers, required for the reduction of acetaldehyde to ethanol, the last step in the glycolytic pathway
YOR028C	<i>CIN5</i>	0.52	4.27	4.70	3.90	Basic leucine zipper (bZIP) transcription factor of the yAP-1 family, required under reducing conditions
YNR001C	<i>CIT1</i>	4.24	3.94	4.98	5.26	Citrate synthase, catalyses the condensation of acetyl coenzyme A and oxaloacetate to form citrate
YGR088W	<i>CTT1</i>	2.36	6.64	6.96	7.05	Cytosolic catalase T, has a role in protection from oxidative damage by hydrogen peroxide
YHR053C	<i>CUP1-1</i>	5.32	5.70	5.66	5.66	Metallothionein, binds copper and mediates resistance to high concentrations of copper and cadmium
YMR250W	<i>GAD1</i>	-0.22	2.52	3.45	4.05	Glutamate decarboxylase, converts glutamate into γ -aminobutyric acid (GABA) during glutamate catabolism
YPL075W	<i>GCR1</i>	-0.32	0.84	1.76	1.77	Transcriptional activator of genes involved in glycolysis
YCL040W	<i>GLK1</i>	1.42	3.28	4.15	4.64	Glucokinase, catalyses the phosphorylation of glucose at C6 in the first irreversible step of glucose metabolism
YDL022W	<i>GPD1</i>	1.89	3.22	4.04	4.31	NAD-dependent glycerol-3-phosphate dehydrogenase, key enzyme of glycerol synthesis
YLR258W	<i>GSY2</i>	1.34	3.00	3.65	4.00	Glycogen synthase, similar to Gsy1p
YGL237C	<i>HAP2</i>	0.20	0.86	1.01	1.01	Subunit of the heme-activated, a transcriptional activator and global regulator of respiratory gene expression
YKL109W	<i>HAP4</i>	0.18	1.29	1.45	0.70	Subunit of the heme-activated, a transcriptional activator and global regulator of respiratory gene expression
YOR358W	<i>HAP5</i>	0.28	0.20	0.56	0.80	Subunit of the heme-activated, a transcriptional activator and global regulator of respiratory gene expression
YER062C	<i>HOR2</i>	0.42	0.67	1.90	2.44	1 of 2 redundant DL-glycerol-3-phosphatases involved in glycerol biosynthesis
YGL073W	<i>HSF1</i>	-0.08	0.55	1.13	1.27	Trimeric heat shock transcription factor
YLL026W	<i>HSP104</i>	3.18	5.37	5.70	5.79	Heat shock protein to refold and reactivate previously denatured
YFL014W	<i>HSP12</i>	-0.40	4.85	6.62	7.46	Plasma membrane protein involved in maintaining membrane organisation in stress conditions
YJL159W	<i>HSP150</i>	0.07	1.25	1.45	1.76	O-mannosylated heat shock protein that is secreted and covalently attached to the cell wall via beta-1,3-glucan and disulfide bridges
YBR072W	<i>HSP26</i>	0.53	2.76	3.39	3.65	Small heat shock protein (sHSP) with chaperone activity
YDR171W	<i>HSP42</i>	4.19	5.69	5.83	5.90	sHSP with chaperone activity
YFR053C	<i>HXK1</i>	4.27	5.42	6.01	6.14	A cytosolic protein that catalyses first irreversible step in the intracellular metabolism of glucose
YDR343C	<i>HXT6</i>	2.77	2.39	3.53	4.18	High-affinity glucose transporter
YDR342C	<i>HXT7</i>	2.72	2.49	3.78	4.52	High-affinity glucose transporter
YDR148C	<i>KGD2</i>	0.86	0.68	1.55	2.05	Dihydrolipoyl transsuccinylase, which catalyses the oxidative decarboxylation of α -ketoglutarate to succinyl-CoA in the TCA cycle

Systematic name	Standard name	Pressure treatments				Description
		0'	5'	10'	15'	
YFR030W	<i>MET10</i>	1.76	0.44	0.93	1.02	Subunit alpha of assimilatory sulfite reductase, related to methionine metabolism
YKL001C	<i>MET14</i>	2.29	1.68	1.34	1.40	Adenylylsulfate kinase, involved in methionine metabolism
YPR167C	<i>MET16</i>	1.94	0.60	0.49	0.22	3'-phosphoadenylylsulfate reductase, involved in methionine metabolism
YLR303W	<i>MET17</i>	3.59	-0.32	0.40	1.18	Methionine and cysteine synthase
YNL277W	<i>MET2</i>	4.19	1.81	1.73	2.48	L-homoserine- <i>O</i> -acetyltransferase, catalyses the first step of the methionine biosynthetic pathway
YER091C	<i>MET6</i>	2.13	-1.37	-0.70	-0.52	Cobalamin-independent methionine synthase, involved in methionine biosynthesis
YMR037C	<i>MSN2</i>	0.67	0.72	0.71	0.43	Transcriptional activator related to <i>Msn4p</i> , inducing gene expression
YKL062W	<i>MSN4</i>	1.36	0.64	0.51	0.36	Transcriptional activator related to <i>Msn2p</i> , activated in stress conditions
YHL036W	<i>MUP3</i>	3.50	2.32	2.65	2.77	Low-affinity methionine permease, similar to <i>Mup1p</i>
YER042W	<i>MXR1</i>	3.07	0.46	1.33	1.94	Methionine- <i>S</i> -sulfoxide reductase, involved in the response to oxidative stress
YML120C	<i>NDI1</i>	2.01	3.2	4.34	4.58	NADH/ubiquinone oxidoreductase, transfers electrons from NADH to ubiquinone in the respiratory chain
YDR043C	<i>NRG1</i>	2.22	2.23	0.13	0.58	Transcriptional repressor that mediates glucose
YBR066C	<i>NRG2</i>	2.07	2.59	1.62	0.69	Transcriptional repressor that mediates glucose
YDR001C	<i>NTH1</i>	0.41	2.07	2.78	3.14	Neutral trehalase, degrades trehalose
YBR001C	<i>NTH2</i>	1.07	2.23	3.21	3.73	Putative neutral trehalase
YIL107C	<i>PFK26</i>	1.40	1.87	2.14	2.29	6-phosphofructo-2-kinase, fructose-2,6-bisphosphatase activity; transcriptional regulation involves protein kinase A
YKL163W	<i>PIR3</i>	-0.84	1.91	3.15	3.71	<i>O</i> -glycosylated covalently bound cell wall protein required for cell wall stability
YDL020C	<i>RPN4</i>	0.27	1.64	1.11	0.75	Transcription factor, transcriptionally regulated by various stress responses
YPL274W	<i>SAM3</i>	3.37	0.10	0.78	1.26	High-affinity <i>S</i> -adenosylmethionine permease, required for utilisation of <i>S</i> -adenosylmethionine as a sulfur source
YKL148C	<i>SDH1</i>	1.93	1.48	2.82	3.40	Flavoprotein subunit of succinate dehydrogenase
YLL041C	<i>SDH2</i>	1.61	1.61	2.80	3.04	Iron-sulfur protein subunit of succinate dehydrogenase
YKL141W	<i>SDH3</i>	1.17	1.10	2.09	2.63	Cytochrome <i>b</i> subunit of succinate dehydrogenase
YDR178W	<i>SDH4</i>	1.34	1.81	2.92	3.53	Membrane anchor subunit of succinate dehydrogenase
YHR008C	<i>SOD2</i>	0.58	2.06	3.25	3.61	Mitochondrial manganese superoxide dismutase, protects cells against oxygen toxicity
YER150W	<i>SPI1</i>	-0.29	1.58	1.89	2.46	GPI-anchored cell wall protein, expression is induced under conditions of stress
YBR169C	<i>SSE2</i>	2.41	3.55	4.04	4.16	Member of the heat shock protein 70 (HSP70) family; may be involved in protein folding
YGR008C	<i>STF2</i>	3.94	5.10	5.43	5.82	Protein involved in regulation of the mitochondrial F1F0-ATP synthase
YLR251W	<i>SYM1</i>	0.64	1.89	2.61	3.13	Protein required for ethanol metabolism
YBR126C	<i>TPS1</i>	2.41	3.92	4.15	4.16	Synthase subunit of trehalose-6-phosphate synthase/phosphatase complex, which synthesises the storage carbohydrate trehalose

Systematic name	Standard name	Pressure treatments				Description
		0'	5'	10'	15'	
YGR019W	<i>UGA1</i>	2.50	3.10	3.51	3.59	GABA transaminase (4-aminobutyrate aminotransferase) involved in the 4-aminobutyrate and glutamate degradation pathways
YBR006W	<i>UGA2</i>	2.29	2.65	3.11	3.39	Succinate semialdehyde dehydrogenase involved in the utilisation of GABA as a nitrogen source
YIL101C	<i>XBP1</i>	NA	1.35	1.23	1.26	Transcriptional repressor that binds to promoter sequences of the cyclin genes, <i>CYS3</i> and <i>SMF2</i> , expression is induced by stress
YML007W	<i>YAP1</i>	0.46	0.45	0.93	1.22	bZIP transcription factor required for oxidative stress tolerance