APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Nov. 2005, p. 6831–6837 Vol. 71, No. 11
0099-2240/05/$08.00+0 doi:10.1128/AEM.71.11.6831–6837.2005
Copyright © 2005, American Society for Microbiology. All Rights Reserved.

# Monitoring Stress-Related Genes during the Process of Biomass Propagation of *Saccharomyces cerevisiae* Strains Used for Wine Making

Roberto Pérez-Torrado,[1,2]† Jose M. Bruno-Bárcena,[3]‡ and Emilia Matallana[1,2]*

*Departamento Bioquímica y Biología Molecular, Facultad de Ciencias Biológicas, Universitat de Valencia, Apartado 73, E-46100 Burjassot, Spain[1]; Departamento de Biotecnología, Instituto de Agroquímica y Tecnología de Alimentos, CSIC, Apartado 73, E-46100 Burjassot, Spain[2]; and Planta Piloto de Biotecnología, Instituto de Agroquímica y Tecnología de Alimentos, CSIC, Apartado 73, E-46100 Burjassot, Spain[3]*

Received 11 March 2005/Accepted 20 July 2005

**Physiological capabilities and fermentation performance of *Saccharomyces cerevisiae* strains to be employed during industrial wine fermentations are critical for the quality of the final product. During the process of biomass propagation, yeast cells are dynamically exposed to a mixed and interrelated group of known stresses such as osmotic, oxidative, thermic, and/or starvation. These stressing conditions can dramatically affect the parameters of the fermentation process and the technological abilities of the yeast, e.g., the biomass yield and its fermentative capacity. Although a good knowledge exists of the behavior of *S. cerevisiae* under laboratory conditions, insufficient knowledge is available about yeast stress responses under the specific media and growth conditions during industrial processes. We performed growth experiments using bench-top fermentors and employed a molecular marker approach (changes in expression levels of five stress-related genes) to investigate how the cells respond to environmental changes during the process of yeast biomass production. The data show that in addition to the general stress response pathway, using the *HSP12* gene as a marker, other specific stress response pathways were induced, as indicated by the changes detected in the mRNA levels of two stress-related genes, *GPD1* and *TRX2*. These results suggest that the cells were affected by osmotic and oxidative stresses, demonstrating that these are the major causes of the stress response throughout the process of wine yeast biomass production.**

Production of *Saccharomyces cerevisiae* biomass is an economically important process. Industrial strains of *S. cerevisiae* are used by many food companies as starters for fermentative processes in the making of bread, wine, beer, and other alcoholic beverages. The technological characteristics of commercially produced yeast determine, to a high extent, the quality and fermentation performance of those processes. Several studies have evaluated the energetic, kinetic, and yield parameters of the yeast biomass production process (6, 8, 28, 29). However, the evaluation of the yeast transient response to various environmental challenges which occurs during stressful conditions in the propagation process and how, ultimately, the cells alleviate or remedy them remains to be addressed.

In the wine and baker's yeast production processes, aerated molasses supplemented with nutrients is inoculated with selected strains. Then cells grow through various transient stages during the batch and fed-batch phases of the process. In a sequence of consecutive fermentations, yeast biomass grown in small fermentors is used to inoculate larger tanks (6, 8, 28, 29). In the initial batch phase, cells are exposed to increased osmotic pressure due to the high concentration of sugars present in the molasses. Also, high aeration and aerobic metabolism can lead to oxidative stress. During the fed-batch phase, the feed rate is set to limit the sugar concentration to maintain respiratory metabolism and increase the biomass yield. In the case of wine yeast, the biomass is concentrated and dehydrated at the end of the process to obtain active dry wine yeasts that can be stored for long periods of time (8, 28, 29). During this process, wine yeast cells endure various stressful situations that induce multiple intracellular changes (1, 24). Subsequently, in a period of several hours during maturation and final processing, yeast cells suffer nutrient limitation and a complex mix of different stresses (thermic and osmotic, etc.) caused by the drying process. As a result, these dynamic environmental injuries affect the biomass yield, the fermentative capacity, the vitality, and the viability of the cells (1, 24).

Eukaryotic cells have developed molecular mechanisms to sense stressful situations, transfer information to the nucleus, and adapt to the new conditions (9, 14, 15). Protective molecules are rapidly synthesized and transcriptional factors are activated, thus changing the transcriptional profile of the cells. Many stress response genes are induced by several adverse conditions through the sequence element STRE (general stress responsive element), which targets the transcriptional factors Msn2p and Msn4p (17, 20). This pathway, also known as the "general stress response pathway," increases the expression of many different genes, including the well-studied *HSP12* and *GSY2* genes which are involved in protein folding and glycogen metabolism, respectively (2, 9). Furthermore, yeasts

* Corresponding author. Mailing address: Departamento de Biotecnología, Instituto de Agroquímica y Tecnología de Alimentos, CSIC, Apartado 73, E-46100 Burjassot, Spain. Phone: 34 96 390 0022. Fax: 34 96 363 63 01. E-mail: emilia.matallana@uv.es.

† Present address: Laboratory of Nuclear Dynamics and Genome Plasticity, Curie Institute/CNRS, Paris, France.

‡ Present address: Department of Microbiology and Biomanufacturing Training and Education Center (BTEC), North Carolina State University, Box 7615, Raleigh, NC 27695-7615.



Downloaded from http://aem.asm.org/ on December 2, 2020 by guest

cells have been shown to respond specifically to certain stresses. During thermic stress, the transcriptional factor Hsf1p activates transcription of genes, such as *STI1*, which code for proteins that counteract protein denaturation and aggregation (18, 30). Aerobic growth and prooxidants generate reactive oxygen species (ROS), leading to oxidative damage on the cells. To neutralize the harmful effects of oxidative stress, proteins are generated that participate in two major functions: antioxidants (such as *GSH1*, *TRX2*, *CUP1*, and *CTT1*) to reduce proteins and eliminate ROS damage and metabolic enzymes (such as *PGM1* and *TDH2*) that redirect metabolic fluxes to synthesize NADPH, slowing down catabolic pathways like glycolysis (12). Another well-known specific stress response is the high-osmolarity glycerol response pathway (3), which induces genes involved in glycerol synthesis (*GPD1*, *GPP2*) and methylglyoxal detoxification (*GLO1*). Intracellular accumulation of glycerol counteracts hyperosmotic pressure to avoid water loss (14, 15). There also are some stress response pathways still poorly understood, such as those involved in the adaptation to nutrient limitation. Large groups of glucose-repressed genes, nitrogen-regulated genes, and others of unknown function, such as *YGP1*, are induced after exposing cells to nutrient limitation (14). During the study of the stress response systems, it has been demonstrated that exposure to one kind of stress can activate protective mechanisms against other different stresses, a phenomenon known as cross-protection (7, 23, 32, 33).

Previous studies have demonstrated the suitability of marker gene expression as a tool for the study of yeast stress responses in industrial processes (13, 16, 21). In this work, we performed stress experiments and bench-top growth trials of wine yeast biomass production to demonstrate that the induction of specific stress-related genes may enable us to determine the environmental disturbances to which the cells are dynamically exposed. The data indicate that osmotic and oxidative stresses are two of the main adverse conditions that *Saccharomyces cerevisiae* senses during this process.

## MATERIALS AND METHODS

**Organisms and cultivation media.** The strains used in this study were *S. cerevisiae* industrial strain T73 (CECT1894), a natural diploid strain isolated from Alicante (Spain) musts (26), which has been commercialized by Lallemand, Inc. (Montreal, Canada). This strain has been previously used in several studies and has proven to be a good wine yeast model (11, 21, 22, 25). The *S. cerevisiae* strain IFI87 is a natural wine strain isolated from Montilla (Spain) (supplied by the Instituto de Fermentaciones Industriales). Cultures and precultures were prepared in YPD medium (1% yeast extract, 2% bacteriological peptone, 2% glucose) or SD medium (0.17% yeast nitrogen base, 0.5% ammonium sulfate, 2% glucose) and incubated at 30°C with shaking (250 rpm). Culture growth was monitored by measuring the optical density at 600 nm ($OD_{600}$). YPD precultures were used to inoculate ($OD_{600}$ = 0.1) industrial media. Molasses medium (diluted to 60 g of sucrose liter$^{-1}$ for batch phase or 100 g of sucrose liter$^{-1}$ for fed-batch phase) was supplemented with 7.5 ml of $(NH_4)_2SO_2$ liter$^{-1}$, 3.5 g of $KH_2PO_4$ liter$^{-1}$, 0.75 g of $MgSO_4 \cdot 7H_2O$ liter$^{-1}$, 10 ml of vitamin solution liter$^{-1}$, and 1 ml of antifoam 204 (Sigma, St. Louis, Mo.) liter$^{-1}$. Molasses and mineral solutions were autoclaved separately. The vitamin solution containing 50 mg of D-biotin liter$^{-1}$, 1 g of calcium pantothenate liter$^{-1}$, and 1 g of thiamine hydrochloride liter$^{-1}$ was filter sterilized (0.2-μm pore size) prior to use in the molasses medium.

**Stress time course experiments.** Cultures were inoculated to an initial optical density of 0.1 ($OD_{600}$ = 0.1) from overnight YPD precultures, harvested after they reached the exponential phase of growth ($OD_{600}$ = 0.5 to 0.7), washed with cold distilled water, and transferred to 30°C prewarmed YPD medium containing 0.5 M KCl or 1 M sorbitol (osmotic stress) or 39°C prewarmed YPD medium (thermic stress). In glucose deprivation experiments, cells were transferred from SD medium (0.17% yeast nitrogen base, 0.5% ammonium sulfate, 2% glucose) to S medium (0.17% yeast nitrogen base, 0.5% ammonium sulfate). In oxidative stress experiments, 2 mM hydrogen peroxide ($H_2O_2$) or 0.5 mM menadione (final concentrations) were added to the YPD medium after the cells reached the exponential phase of growth. For nutrient limitation stress experiments, SD medium or SDn medium (0.17% yeast nitrogen base, 0.05% ammonium sulfate, 10% glucose) for glucose or nitrogen limitation stresses, respectively, were inoculated (OD = 0.1) from SD medium overnight precultures. Aliquots were taken at several time points for RNA analysis. Experiments were carried out at 39°C for thermic stress and 30°C for other stress experiments.

TABLE 1. Genes and primers used for the amplification of DNA probes

| Gene | Primer | Sequence (5′–3′) |
|---|---|---|
| *TRX2* | TRX2-1 | AAATCCGCTTCTGAATAC |
| | TRX2-2 | CTATACGTTGGAAGCAATAG |
| *GSH1* | GSH1-1 | CCCGATGAAGTCATTAACA |
| | GSH1-2 | GGAAAAGGTCAAAATGCT |
| *CUP1* | CUP1-1 | TGTTCAGCGAATTAATTAAC |
| | CUP1-2 | CATTTCCCAGAGCAGCA |
| *GLO1* | GLO1-1 | GAACACTTCGGTATGAAG |
| | GLO1-2 | GGTATTCCTGACCCTCTC |
| *YGP1* | YGP1-1 | ACTTTGCCGGCATGGGATG |
| | YGP1-2 | GTGAAGACACCGGAGTAC |
| *GPD1* | GPD1-1 | TTGAATGCTGGTAGAAAG |
| | GPD1-2 | TGACCGAATCTGATGATC |

**Apparatus and operation.** Propagation growth experiments were conducted in a 5-liter reactor model BIOFLO III equipped with proportional, integral, and derivative (PID) control units for pH, temperature, oxygen, and agitation speed (NBS, New Jersey). Experiments started with a working volume of 2,000 ml at 30°C. The initial pH was 4.5, and it was allowed to freely vary between 4 and 5 during the batch phase. During the fed-batch phase, the reactor pH was maintained at 4.5 by the automatic addition of 42.5% $H_3PO_3$ or 1 M NaOH. The reactor was continuously fed with medium by a type 501 peristaltic pump (Watson-Marlow, Falmouth, United Kingdom) at the desired flow rate. Dissolved oxygen, measured with an electrode (Mettler-Toledo), was maintained above 20% by a PID control system that allowed the automatic modification of the agitation speed between the range limits of 300 to 500 rpm. Cell growth was monitored by measuring the $OD_{600}$. Cell dry weight determination was carried out by cell centrifugation at 5,000 × *g* for 10 min, washing the cells with distilled water, and drying the cells at 80°C until they reached a constant weight.

**Determination of glucose, ammonium, and sucrose contents.** Residual glucose was determined using a glucose oxidase-peroxidase assay (Boehringer Mannheim GmbH). Extracellular ammonium was determined with a commercial kit (Boehringer Mannheim GmbH). Samples were centrifuged for 1 min at 13,000 × *g*, and the glucose and ammonium concentrations were determined in the supernatants. For sucrose determination, diluted samples were incubated for 10 min at 30°C in 200 μl of 50 mM sodium acetate (pH 5.0) and 2.5 U of invertase (Sigma). The reactions were stopped by adding 100 μl of 0.4 M $K_2HPO_4$ and boiling the reaction mixtures for 3 min. Samples were centrifuged for 1 min at 13,000 × *g*, and the glucose concentration was determined in supernatants with a glucose oxidase-peroxidase assay. In all of the determinations, the final concentrations were obtained by interpolation of the sample results using the slope of the fitting linear regression from the respective standards.

**Analysis and quantification of mRNA.** Total RNA from standardized yeast cell pellets (10 mg) obtained during either stress or fed-batch time course experiments was extracted with an automated device for multisample processing (FastPrep; Savant), separated by electrophoresis in formaldehyde-containing agarose gels, and analyzed by Northern blotting. Restriction fragments from the *STI1* gene (a 1.5-kb KpnI-SacI fragment), from the *HSP12* gene (a 0.6-kb EcoRI fragment), from the *CTT1* gene (1.4-kb and 1.1-kb EcoRI fragments), and from the *GSY2* gene (a 1.7-kb BanII fragment) were used as probes. Specific primers used in PCRs to synthesize all the other probes are shown in Table 1. Probes obtained by restriction digestion or PCR were labeled by random priming using [α-$^{32}$P]dCTP. Finally, the quantification of each specific mRNA was carried out by direct measurement of radioactivity on the filters with an Instant Imager FLA-3000 (FujiFilm). The results of gene transcription were normalized to rRNA levels, thus allowing the comparison of the different mRNA levels be-

Downloaded from http://aem.asm.org/ on December 2, 2020 by guest

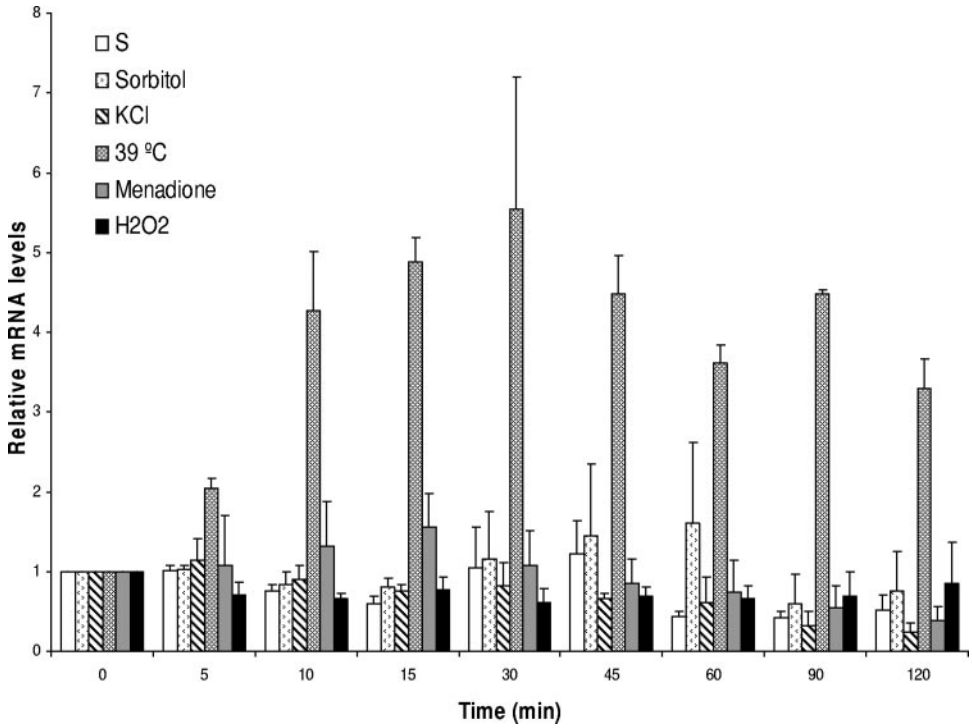


FIG. 1. Induction of one selected gene marker, *STI1*, after individual stress treatments. mRNA levels were determined under the following stress conditions: glucose deprivation in S medium, 1 M sorbitol, 0.5 M KCl, 39°C, 0.5 mM menadione, and 2 mM $H_2O_2$. Results were normalized with mRNA and with a common gene probe for different hybridizations of the same filter.

tween successive hybridizations. Normalization was also performed versus probe-specific radioactivity and internal hybridization controls. Results were expressed and presented as the relative mRNA gene levels. All experiments were carried out at least twice, and the results differed by less than 20% on the median coefficient of variation.

## RESULTS

**Specificity study of stress responsive genes.** To identify genes transcribed differentially after cell exposure to stress and validate them for their use as signal molecules along bench-top growth trials, we cultivated *S. cerevisiae* T73 and performed time course experiments to evaluate gene induction in response to different stress conditions (heat shock, oxidative, osmotic, and nutrient limitation). Subsequently, the transient responses evaluated included those of the following genes: *TRX2*, *STI1*, *GPD1*, *CUP1*, *GLO1*, *CTT1*, *GSH1*, *YGP1*, and *GSY2*. Figure 1 shows the averaged data of normalized mRNA induction levels for *STI1* as an example of the results. The normalization of the mRNA induction data allowed us to compare the results of gene transcription between independent experiments. Data normalizations increase the background noise; therefore, to avoid interpretation artifacts, we have considered that inductions below fourfold were not significant.

Our results indicate that differential transcription of *STI1* (Fig. 1) occurred during heat shock stress but not in response to oxidative, osmotic, or starvation stresses. These results are in agreement with previous findings (16). As can be seen in the results summarized in Table 2, *TRX2* showed induction in yeast grown on YPD medium containing 2 mM $H_2O_2$. However, there was no response when cells were challenged with 0.5 mM menadione in YPD, during heat shock at 39°C, under osmotic stress (0.5 mM KCl or 1 M sorbitol), or during glucose deple-

TABLE 2. Summary of gene expression under laboratory conditions

| Stress challenge | Result for gene[a]: | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | *CUP1* | *GSH1* | *CTT1* | *TRX2* | *GLO1* | *GPD1* | *STI1* | *YGP1* | *GSY2* |
| 0.5 mM menadione | + | − | + | − | − | − | − | − | − |
| 2.0 mM $H_2O_2$ | − | − | ++ | + | − | + | − | − | − |
| 1 M sorbitol | − | − | + | − | ++ | + | − | − | − |
| 0.5 M KCl | − | − | +++ | − | + | +++ | − | + | − |
| 39°C | − | − | − | − | + | − | + | + | + |
| Glucose depletion | − | − | +++ | − | + | − | − | + | + |
| Glucose exhaustion | − | − | ++ | − | − | − | − | +++ | +++ |
| Nitrogen exhaustion | − | − | − | − | − | − | − | + | − |

[a] −, no induction; +, 4 to 8 times induction; ++, 8 to 12 times induction; +++, ≥12 times induction.

Downloaded from http://aem.asm.org/ on December 2, 2020 by guest

tion (transferring cells from SD minimal medium to S medium). Glucose (SD medium) or nitrogen (SDn medium) exhaustion did not increase *TRX2* expression levels. *CUP1* appears to be specifically induced in response to 0.5 mM menadione (Table 2). As opposed to *TRX2*, *CUP1* was not significantly induced by the presence of 2 mM $H_2O_2$. Other stress conditions did not affect *CUP1* gene expression in wine yeasts. We did not see induction throughout glucose or nitrogen exhaustion experiments. Data on *GPD1* expression in the presence of the same stressors are shown in Table 2. During osmotic stress, *GPD1* revealed the strongest response of all genes analyzed in the presence of any stress condition. *GPD1* mRNA levels increased 16-fold in response to the presence of 0.5 mM KCl, while an 8-fold induction was seen in response to 1 M sorbitol. A smaller response (~4-fold) was also observed in response to 2 mM $H_2O_2$. Other stress conditions did not induce *GPD1* expression.

Other genes analyzed in this study were less specifically induced (Table 2). *GSY2* expression increased in response to heat shock and glucose exhaustion. *YGP1* was induced by glucose depletion and by 0.5 mM KCl osmotic stress. *GLO1* increases its mRNA levels in response to osmotic stress, heat shock, and glucose depletion. Finally, gene expression analysis showed that *CTT1* is the most pleiotropic stress response gene, responding to every condition studied, except nitrogen exhaustion and high temperatures.

**Bench-top trials of yeast biomass production.** The yeast proliferation method used during industrial processes is a combination of two growth configurations: a batch stage followed by a fed-batch stage. Here, we performed bench-top trials of yeast biomass production by scaling down the process and reproducing the environmental conditions (temperature, pH, aeration, growth medium) usually employed during the industrial processes. Under these conditions, two independent experiments were performed for each of the two chosen wine yeast strains (T73 and IFI87). Figure 2 shows the evolution of critical variables in one of the experiments cultivating *S. cerevisiae* T73. The turbidity, cell dry weight, and parameters that define strain growth were determined during the experiments and are summarized in Table 3.

During the batch stage, *S. cerevisiae* carried out an alcoholic fermentation when grown in an excess carbon source. Immediately afterwards and in the presence of oxygen, cells produced carbon dioxide and water by aerobic respiration from the generated ethanol. These differential stages could be monitored by monitoring the pH, dissolved oxygen consumption, and required agitation speed during the process (Fig. 2). Growth kinetics (not shown) and ethanol accumulation

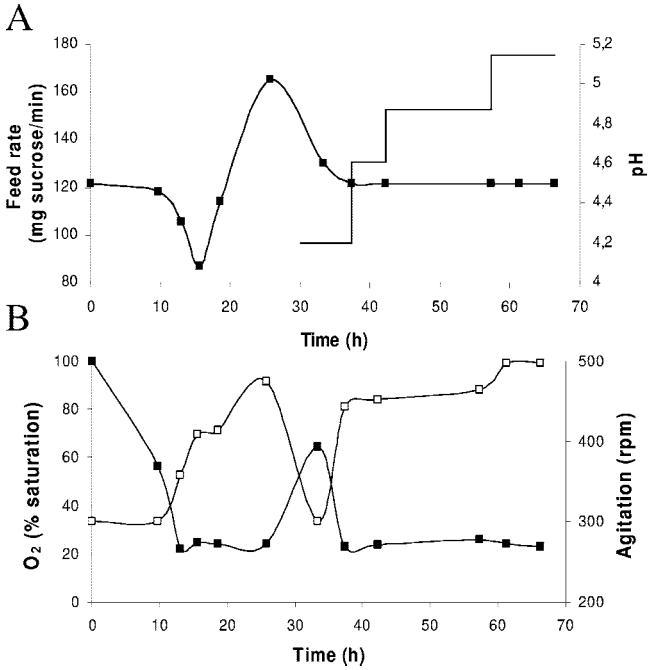


FIG. 2. Critical parameters during a typical bench-top scale experiment of *Saccharomyces cerevisiae* biomass production. (A) Feed rate (—) and pH evolution (■); (B) percentage of $O_2$ saturation (■) and the coupled agitation (□).

(Fig. 3C and 4C) were used to double-check the achievement of this condition. Overall biomass yield and kinetic parameters of cell growth obtained in these bench-top scale experiments (Table 3) were close to the expected theoretical values and those values obtained in the industrial production of wine yeasts (8, 28, 29).

During the fed-batch stage, the medium feed rate is a critical parameter. Each biomass factory develops its own feed rate pattern depending on the yeast strain and other factors to achieve the desired biomass yield. Based on data from various sources, we set a feed rate pattern for the wine yeast strains T73 and IFI87 tested in this research. The feed rate pattern used in our experiments (Fig. 2) enabled sucrose aerobic respiration to yield carbon dioxide and water. Thus, limiting the feed of sucrose to the fermentor allowed us to maintain the value of dissolved oxygen above 20% and the agitation at a maximum of 500 rpm. Therefore, we were able to achieve this critical value, avoiding cell shear due to agitation, oxygen limitation, and the fermentative metabolism of sucrose. The data

TABLE 3. Kinetic and yield parameters during bench-top trials of *S. cerevisiae* biomass production

| Strain | Result for stage[a]: | | | |
|---|---|---|---|---|
| | Batch | | Fed batch | |
| | μ | Y | μ | Y |
| T73 | $0.37 \pm 0.02$ | $0.076 \pm 0.016$ | $0.025 \pm 4.1 \times 10^{-4}$ | $0.18 \pm 0.017$ |
| IFI87 | $0.42 \pm 5.0 \times 10^{-3}$ | $0.093 \pm 4.1 \times 10^{-4}$ | $0.048 \pm 5.0 \times 10^{-3}$ | $0.15 \pm 7.6 \times 10^{-3}$ |

[a] μ, maximum specific growth rate in $h^{-1}$; Y, biomass yield in g (dry weight) of cells g of $sucrose^{-1}$. Averages ± standard deviations of the results from at least two experiments are shown.

Downloaded from http://aem.asm.org/ on December 2, 2020 by guest

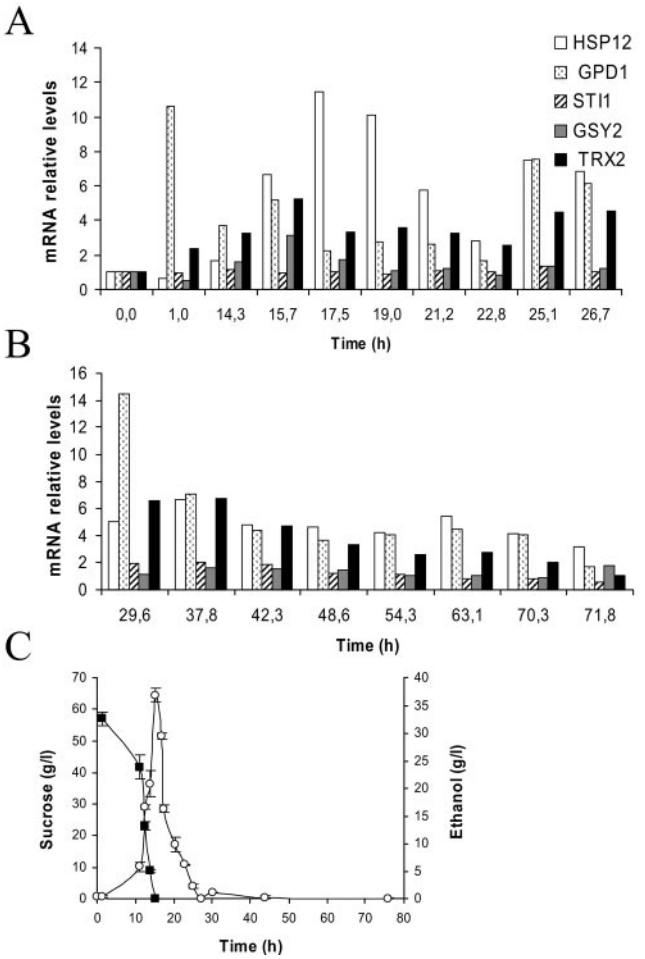


FIG. 3. Pattern of gene expression during a typical bench-top scale experiment of growth for *Saccharomyces cerevisiae* T73 industrial strain. The expression of the gene markers *HSP12*, *GPD1*, *STI1*, *GSY2*, and *TRX2* is shown during the batch (A) and fed-batch (B) stages of growth. Sucrose consumption (■) and ethanol concentration (○) profiles are shown in panel C.

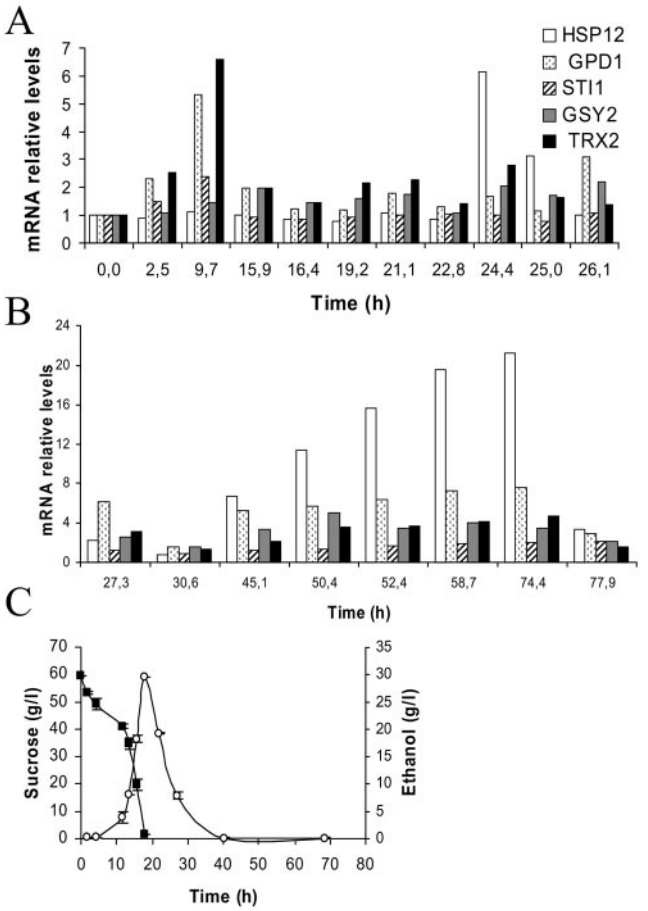


FIG. 4. Pattern of gene expression during a typical bench-top scale experiment of growth for *Saccharomyces cerevisiae* IFI87 industrial strain. The expression of the gene markers *HSP12*, *GPD1*, *STI1*, *GSY2*, and *TRX2* is shown during the batch (A) and fed-batch (B) stages of growth. Sucrose consumption (■) and ethanol concentration (○) profiles are shown in panel C.

(Table 3) do not show significant variations in the biomass yield and in the kinetic parameters of cell growth. The reproducibility of our growth experiment results allowed us to analyze the specific cellular stress responses in the obtained biomass by applying molecular tools as described below.

**Differential transcriptional response of stress-related genes of wine yeast during biomass proliferation.** The transcriptional time course response of five stress-related genes during yeast propagation was evaluated using bench-top fermentation experiments as described above. The *TRX2*, *STI1*, and *GPD1* genes were used to analyze the yeast stress-specific responses to oxidative, thermic, and osmotic stresses, respectively. Expression of the Msn2p/Msn4p-regulated genes *HSP12* and *GSY2* were also studied to distinguish between stress-specific changes in gene expression and the induction of the general stress response. The averages of the data from the proliferation experiments are shown in Fig. 3 and 4. These figures display the gene induction profiles during the batch stage (Fig. 3A and 4A) and from the start of the fed-batch stage (Fig. 3B and 4B) for *S. cerevisiae* T73 (Fig. 3) and *S. cerevisiae* IFI87 (Fig. 4). Panels C in both figures show sucrose consumption and ethanol concentration profiles for the corresponding strains.

As seen in Fig. 3A, there is a clear response to osmotic stress in the first hour of fermentation in strain T73. The high sugar content in molasses (60 g of sucrose liter$^{-1}$) provokes an increase in mRNA levels for the osmotic stress marker *GPD1*. The same results were also observed in strain IFI87 at these early times (data not shown). In Fig. 4A, the first time point in the experiment was taken after 2.5 h, so only the end of this induction peak was observed. After the lag phase, sugar consumption reaches its highest rate (Fig. 3C and 4C). Subsequently, a second stress response was triggered, and yeasts started to express the oxidative stress marker *TRX2*. Both strains show this oxidative response but at different times. While IFI87 responds during the first part of sucrose consumption (Fig. 4A and C), T73 reacts close to the point of sucrose exhaustion (Fig. 3A and C). Interestingly, as expected from the stress experiments, the osmotic stress marker *GPD1*, weakly expressed during oxidative stress conditions (Table 2), was also transcribed in parallel to *TRX2*.

Downloaded from http://aem.asm.org/ on December 2, 2020 by guest

Coinciding with ethanol exhaustion, *HSP12* transcription reaches its maximum for both wine strains (at 24 h for IFI87 and 25 h for T73) 4 h before the end of the batch stage (Fig. 3A and 4A). At this point, a new oxidative stress response is observed as well. After sucrose exhaustion, a strain-specific response of *HSP12* is observed for strain T73 compared to strain IFI87.

During the fed-batch process, just after sucrose was supplied to the medium, a clear osmotic stress response was observed in both strains, while only strain T73 showed a link to the oxidative stress response (Fig. 3B and 4B). *GPD1* and *TRX2* were significantly transcribed in strain T73; however, only *GPD1* was induced in strain IFI87. *HSP12* expression increases continuously in strain IFI87 until the end of the entire process and coincides with *GPD1* expression. *TRX2* shares this pattern, but it only reaches significant levels near the end of the fed-batch stage. In strain T73, the same genes are induced but with different patterns, e.g., there is an initial induction of *GPD1*, *HSP12*, and *TRX2* and a slow decrease in the mRNA levels during the fed-batch stage. A significant induction in the general stress response gene *GSY2* and the specific heat shock marker *STI1* was not observed under our experimental conditions.

## DISCUSSION

Living cells respond to environmental challenges with global changes in gene expression. Extensive data on stress-associated genes in *Saccharomyces cerevisiae* are available in the literature, and a few studies have analyzed and compared the expression of each one of these genes in response to exposure to specific kinds of stresses. A remarkable study by Gasch et al. (10) provides important information concerning the transitory responses of laboratory *Saccharomyces* strains developed during the very early stages of the growth cycle. However, limited information is available about cell responses to stress during the propagation process of polyploid wine-making strains. Moreover, similar studies have shown important differences in the patterns of gene expression between industrial and laboratory *Saccharomyces* strains (4, 5, 25, 27).

Our results indicate that four of the nine analyzed genes can be used as specific stress response markers (Table 2). *STI1* was selected as a heat shock gene marker because the mRNA levels increased after heat shock, but a significant induction was not found in response to osmotic, oxidative, or nutrient limitation stresses. Ivorra et al. (16) reported increased expression of *STI1* near the end of microvinification experiments using *S. cerevisiae* T73 when a mix of nutrient limitation and ethanol stress occurred during the late stationary phase of growth. An equivalent situation was not present under the conditions evaluated in our research; therefore, *STI1* was selected as a heat shock marker. *TRX2* is another good gene marker in wine yeasts. Expression of this gene increases only after exposure to oxidative stress in 2 mM $H_2O_2$. Similarly, *CUP1* is significantly induced only in the presence of the superoxide radical generator menadione (0.5 mM) and could also be used as an oxidative marker. However, low expression levels were obtained during our studies, while previous results of other researchers have indicated stress cross-induction (19, 31). Therefore, *TRX2* was selected as the best oxidative stress marker. Our results agree with published data regarding differential expression of oxidative response genes depending on the oxidative agent (19). Another useful gene marker is *GPD1*, which has been shown to be induced in response to osmotic stress in wine yeast (21). In our study, *GPD1* mRNA reaches high levels in tested osmotic stress conditions and also responds to 2 mM $H_2O_2$. However, its expression under oxidative conditions is lower than in the osmotic stress conditions shown in Table 2. Thus, *GPD1* was selected as a suitable marker for osmotic stress using an oxidative marker in parallel. Other analyzed genes do not exhibit specific induction and respond to more than two of the stress tested situations. Consequently, they were not considered as adequate markers for monitoring processes where mixed stresses could occur.

Batch and fed-batch processes have been traditionally evaluated from the point of view of energy-yielding metabolism. Here, we have studied the cell response by analyzing the changes in expression levels of stress-related genes during a scaled-down version of the industrial yeast production process. Although other studies have been performed to identify molecular markers in industrial yeast strains (13, 16, 21), this is the first report of application to the analysis of the stress response in bench-top trials of yeast biomass production. The technological relevance of these results greatly depends on the similarity of the bench scale experiments to the real industrial process. Therefore, we have developed a two-stage experiment that fits the industrial parameters of media composition, feed rate, aeration, and pH (6, 8, 28). Growth and biomass yield were close to those theoretically expected during batch industrial propagation processes (8). Interestingly, during the mainly respiratory fed-batch phase, the results show a lower yield than that described for baker's yeast (6, 8, 28). This may indicate a strong adaptation of wine yeast strains for wine making, where they perform mainly a fermentative metabolic role.

The expression of stress gene markers was analyzed during the batch and fed-batch stages of the production experiments with two yeast wine strains. Besides the specific gene markers selected in this work, two general stress gene markers (*HSP12* and *GSY2*) were also included. The study of these additional genes allowed us to follow the induction of the general stress response pathway and helped to identify stress-specific responses. Clear differences between strains can be seen in the level and in the timing of gene induction. However, several conclusions can be taken from these studies. The induction of *HSP12* shows that yeast sense stress during different stages of yeast production, even under the controlled environmental proliferation conditions. Induction is stronger for *HSP12* than for other stress genes in wine yeasts, as has been described previously (16, 21). As expected, *STI1* expression analysis shows that heat shock stress does not occur during this well-controlled fermentation process. The inoculation of yeasts into the molasses medium generates an osmotic shock, as is shown by changes in the *GPD1* expression. The osmotic stress response of *GPD1* to the high sugar content in the medium has been previously described for laboratory strains (14, 15) as well as for wine yeast strains during wine fermentations (21). More interesting is the yeast response to oxidative stress during the initial 20 h of the batch stage. It is well known that respiratory metabolism of sugars produces the generation of ROS. Therefore, during yeast growth and in the presence of a high aera-

Downloaded from http://aem.asm.org/ on December 2, 2020 by guest

tion, respiratory metabolism is facilitated and may increase intracellular oxidation of proteins and lipids. This subsequently leads to cellular damage and may halt the cellular cycle. As a result, an oxidative stress response is required, as was observed by the increased expression of the *TRX2* molecular marker in both strains, especially when sucrose consumption is reaching its maximal rate for strain IFI87 and at sucrose exhaustion for strain T73. This situation may also be present during the ethanol consumption phase and during most of the respiratory fed-batch stage. This indicates the relevance of oxidative stress resistance in wine yeast for its endurance along the industrial process of biomass production. The oxidative stress response is more intense for strain T73 than for IFI87. This difference might be related to the adaptation of the commercial strain T73 to industrial growth.

A better understanding of the adaptation to oxidative stress may allow biomass producers to improve cell survival, vitality, and performance by controlling oxidative conditions and also by using selected strains with increased resistance to oxidative stress.

## ACKNOWLEDGMENTS

We thank Pierre-Antoine Defossez for critical reading of and comments on the manuscript.

This work was supported by grants from the "Generalitat Valenciana" (GV99-105-1-13 and GRUPOS03/012) and the "Ministerio de Ciencia y Tecnología" (CICYT ALI99-1224-002-02 and CICYT AGL2002-01109). R.P-T. was the recipient of an F.P.I. fellowship from "Generalitat Valenciana."

## REFERENCES

1. **Attfield, P. V.** 1997. Stress tolerance: the key to effective strains of industrial baker's yeast. Nat. Biotechnol. **15:**1351–1357.
2. **Boy-Marcote, E., M. Perrot, F. Bussereau, H. Boucherie, and M. Jacquet.** 1998. Msn2p and Msn4p control a large number of genes induced at the diauxic transition which are repressed by cyclic AMP in *Saccharomyces cerevisiae*. J. Bacteriol. **180:**1044–1052.
3. **Brewster, J. L., T. de Valoir, N. D. Dwyer, E. Winter, and M. C. Gustin.** 1993. An osmosensing signal transduction pathway in yeast. Science **259:** 1760–1763.
4. **Carrasco, P., A. Querol, and M. del Olmo.** 2001. Analysis of the stress resistance of commercial wine yeast strains. Arch. Microbiol. **175:**450–457.
5. **Cavalieri, D., J. P. Townsend, and D. L. Hartl.** 2000. Manifold anomalies in gene expression in a vineyard isolate of *Saccharomyces cerevisiae* revealed by DNA microarray analysis. Proc. Natl. Acad. Sci. USA **97:**12369–12374.
6. **Chen, S. L., and M. Chiger.** 1985. Production of baker yeast. *In* M. Moo-Young (ed.), Comprehensive biotechnology. Pergamon Press Oxford, United Kingdom.
7. **Coote, P. J., M. B. Cole, and M. V. Jones.** 1991. Induction of increased thermotolerance in *Saccharomyces cerevisiae* may be triggered by a mechanism involving intracellular pH. J. Gen. Microbiol. **137:**1701–1708.
8. **Degre, R.** 1993. Selection and commercial cultivation of wine yeast and bacteria, p. 421–447. *In* G. H. Fleet (ed.), Wine microbiology and biotechnology. Harwood Academic Publishers, Chur, Switzerland.
9. **Estruch, F.** 2000. Stress-controlled transcription factors, stress-induced genes and stress tolerance in budding yeast. FEMS Microbiol. Rev. **24:**469–486.
10. **Gasch. A. P., P. T. Spellman, C. M. Kao, O. Carmel-Harel, M. B. Eisen, G. Storz, D. Botstein, and P. O. Brown.** 2000. Genomic expression programs in the response of yeast cells to environmental changes. Mol. Biol. Cell **11:** 4241–4257.
11. **Gimeno-Alcañiz, J. V., and E. Matallana.** 2001. Performance of industrial strains of *Saccharomyces cerevisiae* during wine fermentation is affected by manipulation strategies based on sporulation. Syst. Appl. Microbiol. **24:** 639–644.
12. **Godon, C., G. Lagniel, J. Lee, J. M. Buhler, S. Kieffer, M. Perrot, H. Boucherie, M. B. Toledano, and J. Labarre.** 1998. The $H_2O_2$ stimulon in *Saccharomyces cerevisiae*. J. Biol. Chem. **273:**22480–22489.
13. **Higgins, V. J., P. J. Rogers, and I. W. Dawes.** 2003. Application of genome-wide expression analysis to identify molecular markers useful in monitoring industrial fermentations. Appl. Environ. Microbiol. **69:**7535–7540.
14. **Hohmann, S., and W. H. Mager.** 1997. Yeast stress responses. RG Landes Company and Chapman & Hall, Austin, Tex.
15. **Hohmann, S.** 2002. Osmotic stress signalling and osmoadaptation in yeasts. Microbiol. Mol. Biol. Rev. **66:**300–372.
16. **Ivorra, C., J. E. Perez-Ortin, and M. del Olmo.** 1999. An inverse correlation between stress resistance and stuck fermentations in wine yeasts. A molecular study. Biotechnol. Bioeng. **64:**698–708.
17. **Kobayashi, N., and K. McEntee.** 1993. Identification of cis and trans components of a novel heat shock stress regulatory pathway in *Saccharomyces cerevisiae*. Mol. Cell. Biol. **13:**248–256.
18. **Lindquist, S., and E. A. Craig.** 1988. The heat-shock proteins. Annu. Rev. Genet. **22:**631–677.
19. **Liu, X. D., and D. J. Thiele.** 1996. Oxidative stress induced heat shock factor phosphorylation and HSF-dependent activation of yeast metallothionein gene transcription. Genes. Dev. **10:**592–603.
20. **Martinez-Pastor, M. T., G. Marchler, C. Schuller, A. Marchler-Bauer, H. Ruis, and F. Estruch.** 1996. The *Saccharomyces cerevisiae* zinc finger proteins Msn2p and Msn4p are required for transcriptional induction through the stress response element (STRE). EMBO J. **15:**2227–2235.
21. **Pérez-Torrado, R., P. Carrasco, A. Aranda, J. Gimeno-Alcañiz, J. E. Pérez-Ortin, E. Matallana, and M. del Olmo.** 2002. Study of the first hours of microvinification by the use of osmotic stress-response genes as probes. Syst. Appl. Microbiol. **25:**153–161.
22. **Pérez-Torrado, R., J. V. Gimeno-Alcañiz, and E. Matallana.** 2002. Wine yeast strains engineered for glycogen overproduction display enhanced viability under glucose deprivation conditions. Appl. Environ. Microbiol. **68:** 3339–3344.
23. **Piper, P. W.** 1995. The heat shock and ethanol stress responses of yeast exhibit extensive similarity and functional overlap. FEMS Microbiol. Lett. **134:**121–127.
24. **Pretorius, I. S.** 2003. The genetic analysis and tailoring of wine yeasts, p. 99–134. *In* J. H. de Winde (ed.), Functional genetics of industrial yeasts. Springer Verlag, Berlin, Germany.
25. **Puig, S., and J. E. Pérez-Ortin.** 2000. Expression levels and patterns of glycolytic yeast genes during wine fermentation. Syst. Appl. Microbiol. **23:** 300–303.
26. **Querol, A., T. Huerta, E. Barrio, and D. Ramón.** 1992. Dry yeast strain for use in fermentation of Alicante wines: selection and DNA patterns. J. Food Sci. **57:**183–185.
27. **Rachidi, N., P. Barre, and B. Blondin.** 2000. Examination of the transcriptional specificity of an enological yeast. A pilot experiment on the chromosome-III right arm. Curr. Genet. **37:**1–11.
28. **Reed, G., and T. W. Nagodawithana.** 1991. Yeast technology. Van Nostrand Reinhold, New York, N.Y.
29. **Reed, G.** 1982. Production of baker's yeast. *In* G. Reed (ed.), Prescott and Dunn's industrial microbiology, 4th ed. Avi Publishing Company, Westport, Conn.
30. **Sorger, P. K.** 1991. Heat shock factor and the heat shock response. Cell **65:**363–366.
31. **Tamai, K. T., X. Liu, P. Silar, T. Sosinowski, and D. J. Thiele.** 1994. Heat shock transcription factor activates yeast metallothionein gene expression in response to heat and glucose starvation via distinct signalling pathways. Mol. Cell. Biol. **14:**8155–8165.
32. **Trollmo, C., L. André, A. Bloomberg, and L. Adler.** 1998. Physiological overlap between osmotolerance and thermotolerance in *Saccharomyces cerevisiae*. FEMS Microbiol. Lett. **56:**321–326.
33. **Varela, J. C. S., C. A. van Beekvelt, R. J. Planta, and W. H. Mager.** 1992. Osmostress-induced changes in yeast gene expression. Mol. Microbiol. **6:**2183–2190.

Downloaded from http://aem.asm.org/ on December 2, 2020 by guest