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Fermentative capacity of dry active wine yeast requires a specific oxidative stress response during industrial biomass growth

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Abstract Induction of the oxidative stress response has been described under many physiological conditions in *Saccharomyces cerevisiae*, including industrial fermentation for wine yeast biomass production where cells are grown through several batch and fed-batch cultures on molasses. Here, we investigate the influence of aeration on the expression changes of different gene markers for oxidative stress and compare the induction profiles to the accumulation of several intracellular metabolites in order to correlate the molecular response to physiological and metabolic changes. We also demonstrate that this specific oxidative response is relevant for wine yeast performance by construction of a genetically engineered wine yeast strain overexpressing the *TRX2* gene that codifies a thioredoxin, one of the most important cellular defenses against oxidative damage. This modified strain displays an improved fermentative capacity and lower levels of oxidative cellular damages than its parental strain after dry biomass production.

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Introduction

The industry of yeast biomass production represents the largest bulk production of any single-celled microorganism in the world. In addition to the classical fresh pressed yeasts for baking, new products, as dry active yeasts, have been developed to allow a long-term storage of the starters (Rosen 1989). The stability of the starter is particularly relevant for wine industry due to its seasonal use, just after grapes harvest. Although new technologies have been implemented, the main stage of yeast biomass production, i.e., the growth of yeasts, follows the same principles that baker's yeasts manufacture (Beudeker et al. 1990; Nagodawithana and Trivedi 1990; Pereira et al. 2001; Rose and Vijaylakshimi 1993). Several batch stages in increasing culture volumes on aerated molasses are followed by several fed-batch stages where highest yield of biomass is achieved, because the low input of sugars promotes a respiratory metabolism and ethanol formation is avoided. Although technologically optimized for the highest biomass yields, these industrial processes are poorly characterized from the point of view of yeast molecular adaptation to the adverse growth conditions. This aspect is, however, critical for a good performance of the final product (Walker 1998). Difficulties in the study of yeasts under real industrial conditions can be overcome by the use of bench-top trials reproducing both industrial biomass yield and growth rate, which allow the application of molecular tools for the understanding and improvement of yeast behavior (Pérez-Torrado et al. 2005).

The molecular responses of laboratory strains of *Saccharomyces cerevisiae* to different stresses have been deeply

studied, and a great body of knowledge is already available (Hohmann and Mager 2003). Also, several approaches to the characterization of stress response under industrial conditions have been carried out, and some correlations have been found between stress resistance of several yeast strains and their suitability for industrial processes (Beudeker et al. 1990; Jamieson 1998; Ivorra et al. 1990; Aranda et al. 2002; Pérez-Torrado et al. 2002a, b; Zuzuarregui and del Olmo 2004a, b; Zuzuarregui et al. 2005). Recently, we developed experiments for the selection of specific stress gene markers, and then we showed the gene expression profiles for several of them along bench-top trials of the wine yeast biomass production (Pérez-Torrado et al. 2005). The expression analyses pointed to the induction of *TRX2*, the gene coding for yeast thioredoxin and a good marker for oxidative stress, as a major response. Induction of this marker occurs during the initial batch phase of growth, when cell metabolism experiences a transition from mainly fermentative to fully respiratory.

The response of *S. cerevisiae* to oxidative stress has been studied in different conditions, including both the addition of a variety of reactive oxygen species (ROS) generating agents (such as hydrogen peroxide, menadione, and several metallic ions) in laboratory growth conditions and also the study of the physiological conditions in which the endogenous metabolism and its transitions cause harmful changes in the redox state of the cell (Costa and Moradas-Ferreira 2001; Gibson et al. 2008; Godon et al. 1998; Koerkamp et al. 2002; Monje-Casas et al. 2004; Moye-Rowley 2003; Pereira et al. 2001). The vital importance of keeping the intracellular redox balance makes the molecular processes against those stress conditions one of the most complex adaptation mechanisms; therefore, there is no full picture of the cellular responses to redox challenges, particularly under industrial conditions. The key role of the transcriptional factor Yap1p has been clearly established (Rodrigues-Pousada et al. 2004). It mediates the induction of a battery of genes involved in redox protection. However, this is not the only pathway activated by redox stress. The general stress response pathway, working through the transcriptional factor Hsf1p and its homologue Skn7p, also play a role in the defense against oxidative agents (Moye-Rowley 2003). There are many genes whose expression levels are regulated by those transcriptional factors, including genes coding for enzymatic activities that detoxify oxygen reactive species (as catalase and superoxide dismutase) and antioxidant systems like glutathione/glutaredoxin and thioredoxin (Grant 2001).

Glutaredoxins and thioredoxins are small oxidoreductases acting as hydrogen donors for metabolic enzymes that form a disulphide as part of their catalytic cycle. They are structurally similar, but their redox state is regulated by different systems, thioredoxin being directly reduced by NADPH and thioredoxin reductase, whereas glutaredoxin reduction uses electrons donated by NADPH through glutathione (Grant 2001). Both redox control systems are linked as indicated by the requirement of a functional glutathione reductase Glr1p for viability of double *trx1trx2* mutants (Muller 1996). Moreover, these mutants display elevated levels of oxidized glutathione, which again shows the link between glutathione and thioredoxin systems.

In this work, we have focused the gene expression analysis during bench-top trials of wine yeast biomass propagation in two other oxidative stress markers, in addition to *TRX2*, the *GSH1* gene coding for the enzyme catalyzing the first step in glutathione biosynthesis, and the *GRE2* gene, coding for a stress-related reductase selected as the most induced gene from published data of global gene expression analysis under oxidative stress (Gasch et al. 2000) and not related to the glutathione and thioredoxin systems. In order to study the influence of oxygen on the oxidative stress response, we also used aerated as well as non-aerated conditions. Determinations of the levels of several metabolites along the batch and fed-batch phases of growth were also performed to correlate the gene expression response with the biochemical status of the cells. Both approaches point to the metabolic transition from fermentation to respiration as the cause of a redox unbalance, which produces an oxygen-dependent induction of *TRX2*. The relevance of this specific oxidative response for the quality of dry active wine yeast is demonstrated by the enhanced fermentative capacity of a genetically modified wine strain overexpressing the *TRX2* gene.

Materials and methods

Yeast strains and plasmids

S. cerevisiae industrial strain T73 (CECT1894) is a natural diploid strain isolated from Alicante (Spain) musts (Querol et al. 1992) and has been commercialized by Lallemand (Montreal, Canada). This strain has been previously used in several studies (Gimeno-Alcañiz and Matallana 2001; Pérez-Torrado et al. 2002a, b; Pérez-Torrado et al. 2005; Puig and Pérez-Ortín 2000) and has proven to be a good wine yeast model.

The YEp-TRX2 plasmid was obtained by subcloning a 0.7-kb *Eco*RI fragment containing the yeast *TRX2* gene into the Yep352 vector, an episomal yeast plasmid carrying the selectable marker *URA3*. T73TRX2 strain is a genetically modified T73 strain obtained by transformation with YEp-TRX2 following the lithium acetate procedure as modified by Gietz et al. (1995).

Industrial production conditions

Experiments were carried out as previously described (Pérez-Torrado et al. 2005). YPD precultures were used to

inoculate (OD_{600} 0.1) industrial media. Molasses medium (2 L diluted to 60 g L^{-1} sucrose for batch or 2 L diluted to 100 g L^{-1} sucrose for fed batch) was supplemented with 7.5 g L^{-1} $(NH_4)_2SO_2$, 3.5 g L^{-1} KH_2PO_4, 0.75 g L^{-1} $MgSO_4 \cdot 7\ H_2O$, 10 mL L^{-1} vitamin solution (50 mg L^{-1} D-biotin; 1 g L^{-1} calcium pantothenate; 1 g L^{-1} thiamine hydrochloride), 1 mL L^{-1} antifoam 204 (Sigma). Molasses and mineral solutions were autoclaved separately, and the vitamin solution was filter-sterilized (0.2 μm) prior to use in the molasses medium.

Fermentations were performed in a bioreactor BIOFLO III (NBS, New Jersey, USA). Initial pH was 4.5, and it was allowed to freely vary between 4 and 5 during the batch step. In the fed-batch process, pH was automatically maintained at 4.5 with 42.5% H_3PO_4 and 1 M NaOH. Cell growth was followed by measuring the OD_{600} and the cell dry weight. Air flux of 0.5–1.5 kg/cm^3 was maintained constantly in normal conditions or started after sugar consumption in experiments without aeration. Dissolved O_2 was followed with an O_2 electrode (Mettler, Toledo, USA).

Dehydration and measurement of fermentative capacity

Yeast biomass was dehydrated overnight under air flux in an oven at 39°C. For determination of fermentative capacity, 10^7 cells/mL was inoculated in bottles with YPGF medium (1% yeast extract, 2% peptone, 10% glucose, 10% fructose) and incubated with gentle shaking (65 rpm) at 30°C. The exact number of cells was determined by recounting in a Neubauer camera. CO_2 production was measured every 20 min for 3 h in a Chittick instrument (American association of cereal Chemist, 12-10). The fermentative capacity for all strains in the freshly produced biomass (control condition) was higher than 0.15 mL CO_2 $(10^7\ \text{cells})^{-1}\ \text{min}^{-1}$, a similar value than that produced by baker yeasts (Panadero et al. 2007). Experiments were carried out in triplicate, and results were expressed as a percentage of the control condition.

Sucrose and ethanol determinations

For sucrose determination, diluted samples were incubated 10 min at 30°C in 200 μL of 50 mM sodium acetate pH 5.0 and 2.5 U invertase (Sigma). After adding 100 μL of 0.4 M K_2HPO_4, the reactions were stopped by boiling for 3 min. Samples were centrifuged 1 min at 12,000 rpm, and glucose concentration was determined in supernatants with a glucose oxidase/peroxidase assay. Ethanol was enzymatically determined in supernatants with a commercial kit (Sigma).

Analysis and quantification of mRNA

Total RNA from 10 mg of yeast cells was obtained with an automated device for multisample processing (Fast-Prep, Savant, USA) and then analyzed by electrophoresis in formaldehyde-containing agarose gels and by Northern blotting. Specific primers were used for PCR to synthesize probes shown in Table 1. Probes obtained by PCR or restriction were labeled by random priming (Rediprime, Amersham; High Prime, Roche) using [α-^{32}P]dCTP. High stringency conditions were used both for hybridizations and washes. Messenger RNA (mRNA) quantification was carried out by direct measurement of radioactivity on the filters with an Instant Imager FLA-3000 and the Image Gauge software (FujiFilm, USA). All sample data were normalized to 28S ribosomal RNA (rRNA) levels and to the probe-specific radioactivity. Gene expression experiments were performed twice.

Intracellular metabolite determination

Cell samples were extracted by a trichloroacetic acid method previously described (Gustafsson 1979). Enzymatic assays were used to analyze several metabolites (glucose-6-phosphate, fructose-1,6-bisphosphate, pyruvate, NAD^+, AMP, ADP, ATP) in yeast extracts as described by de Koning and van Dam (1992). Concentrations were calculated assuming an intracellular volume of 2 mL/mg. Averages of three independent experiments are shown.

Lipid peroxidation

Quantification of lipid peroxidation were carried out as described in Buege and Aust (1978) and modified by Espindola et al. (2003). This method is based on the reaction of thiobarbituric acid with the malondialdehyde, which is the product of oxidized fatty acid breakage. Cells (50 mg) were collected, washed twice with distilled water, and then extracted by vortexing with 0.3 g glass beads in 0.5 mL of 50 mM sodium phosphate buffer, pH 6.0, 10% TCA, in three series of 1 min alternated with 1-min incubation on ice. After 10-min centrifugation at 13,000 rpm, 300 μL of supernatants was mixed to 100 μL of 0.1 M EDTA and 600 μL 1% thiobarbituric acid in 0.05 M NaOH and then incubated at 100°C 15 min. After cooling on ice and centrifugation to eliminate precipitates, red species in supernatants were measured by the absorbance at 535 nm, and the

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
GRE2	*GRE1*-1	GTTTCAGGTGCTAACGGG
	GRE1-2	CGACACTGCCTCCCAAAT

molar absorptivity of MDA (1.56×10^5 M^{-1} cm^{-1}) was used to express concentration as picomoles of MDA per milligram of cells.

Results

Expression of several oxidative stress gene markers is differentially affected by aeration

In order to study thoroughly the oxidative response previously described during the propagation of wine yeast biomass (Pérez-Torrado et al. 2005), the analysis of changes in gene expression was extended to other oxidation markers, and the influence of oxygen on these gene expression response was investigated by carrying out bench-top trials of biomass propagation cultures with or without aeration. Figure 1 shows different parameters of fermentations in batch using T73 strain, both in the presence and in the absence of aeration. In experiments without aeration, the air flux was connected after sugar consumption in order to allow the progress of the fermentation to the fed-batch stage (see Fig. 1a and b). As can be seen in Fig. 1b, sucrose consumption rate was slightly slower, and the total ethanol production is higher when cells grew without aeration (oxygen level dropped below 2%; Fig. 1a). The growth profile shown in Fig. 1c, indicates a growth arrest during the transition between ethanol production and consumption in the experiment without aeration, although cells recovered after that period, and the final biomass yield at the end of the batch was identical (data not shown).

In addition to the previously analyzed *TRX2* gene, coding for the main yeast thioredoxin, two new markers, were included in these batch experiments. Due to the abovementioned connections between thioredoxins and glutathione systems, the *GSH1* gene, coding for γ-glutamylcysteine synthetase, was also analyzed along with the *GRE2* gene, one of the most induced genes under oxidative stress conditions (Gasch et al. 2000), but unrelated to the glutathione and thioredoxin systems. As can be seen in Fig. 2, all three genes were induced along growth in both conditions; however, clear differences can be observed in their mRNA level and patterns, with *TRX2* being the most induced gene in normal cultures with oxygen (Fig. 2a). *GSH1* displayed similar profiles in both conditions and also similar levels of induction, even higher without aeration (Fig. 2b). However, the relative levels of both *GRE2* and *TRX2* (Fig. 2b) were significantly reduced when no aeration was supplied. In the case of *TRX2*, the main peak during the batch phase, coincident with the metabolic transition from sucrose fermentation to ethanol respiration, is almost completely absent in growth without aeration, as well as another peak at time 10 coincident to the highest sucrose consumption rate.

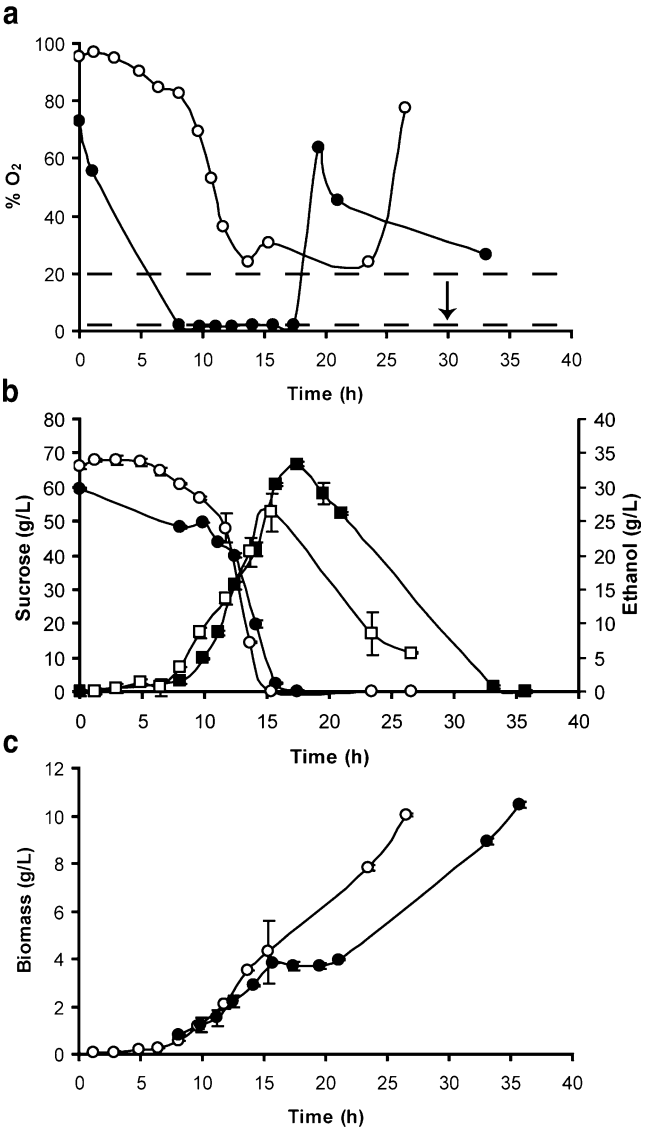

Fig. 1 Fermentation parameters for the biomass propagation bench-top trials with and without aeration. **a** The percentage of dissolved O_2 along the aerated (*white circles*) and non-aerated (*black circles*) fermentations. The *arrow* indicates the batch to fed-batch transition. **b** Sucrose (*circles*) and ethanol (*squares*) profiles in aerated (*white*) and non-aerated (*black*) conditions. **c** Changes in biomass content along aerated (*white circles*) and non-aerated (*black circles*) fermentations. Average of two independent experiments and standard deviations are shown

Intracellular metabolite profiles suggest metabolic transitions coincident with the oxygen-dependent transcriptional inductions of *TRX2*

The oxygen requirement for the molecular changes we observed and the sucrose and ethanol profiles shown in Fig. 1b suggested that the metabolic transition from the

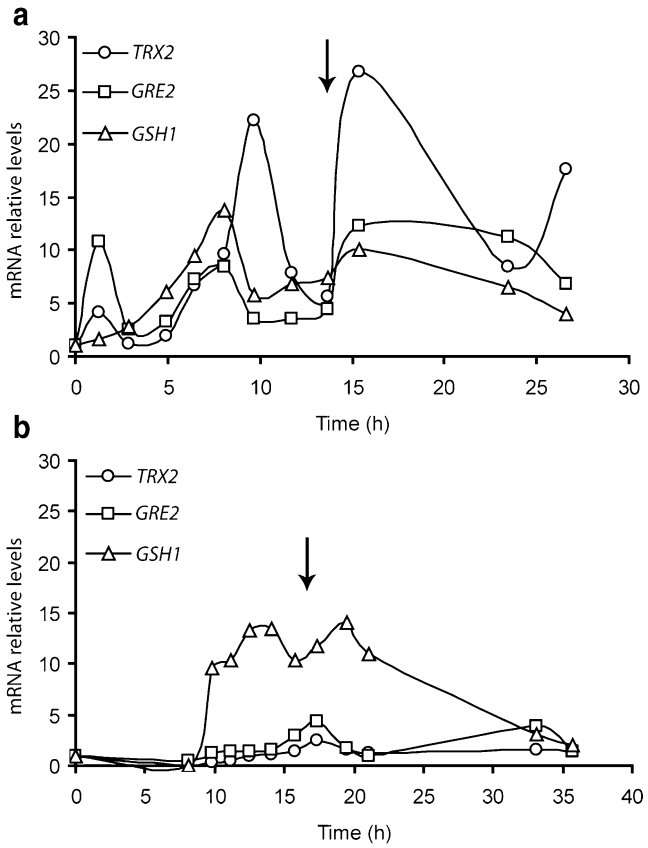

Fig. 2 Analysis of oxidative stress gene markers along the batch growth stage of biomass propagation bench-top trials. mRNA relative levels for *TRX2* (*circles*), *GRE2* (*squares*), and *GSH1* (*triangles*) oxidative stress gene markers analyzed along the batch phase of growth in experiments with (**a**) or without (**b**) aeration. *Arrows* indicate sugar to ethanol consumption transitions. Data from two independent experiments were normalized to 28S rRNA levels and to the probe-specific radioactivity

mainly fermentative growth at the beginning of the batch fermentation to the fully respiratory phase after sugar exhaustion could be the reason for the oxidative stress. To gain biochemical information about the physiological state of the cells, we undertook the profile determination of several relevant intracellular metabolites in samples from both batch and fed-batch stages. We selected three metabolites related to the glycolytic flux and four metabolites indicative of the cellular energy charge and redox state. Enzymatic assays were used as referenced in the "Materials and methods" for the determination of glucose-6-phosphate (G6P), fructose-1,6-bisphosphate (FBP), pyruvate (Pyr), ATP, ADP, AMP, and NAD^+. Figure 3 shows the evolution in the level of these metabolites along the batch stage of growth (Fig. 3a and b) and the calculated ATP/ADP and ATP/(ADP+AMP) ratios (Fig. 3c). Also, the sucrose consumption and ethanol levels (Fig. 3d) are shown for a better correlation between the metabolite profiles and the physiological state of the cells. Accumulation of the

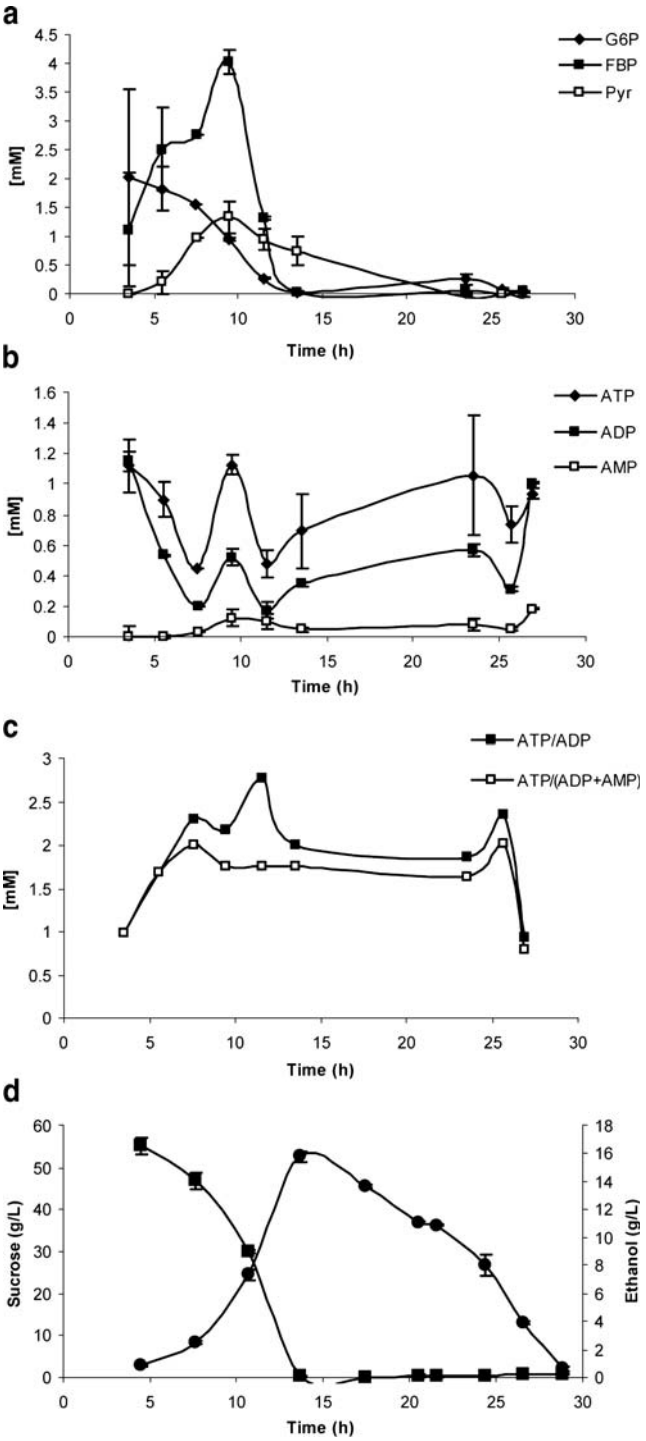

Fig. 3 Analysis of intracellular metabolites along the batch growth stage of biomass propagation bench-top trials. The profiles of glycolytic intermediates (**a**) glucose-6-phosphate (*black diamond*), fructose-6-phosphate (*black square*), and pyruvate (*white square*) and nucleotides (**b**) ATP (*black diamond*), ADP (*black square*), and AMP (*white square*) were determined along the batch phase of growth, and ratios ATP/ADP (*black square*) and ATP/(ADP+AMP) (*white square*) were calculated (**c**). The sugar consumption (*black square*) and ethanol production (*black circle*) curves (**d**) are shown for a better correlation of metabolite profiles to physiological state. Average of three independent experiments and standard deviations are shown

glycolytic intermediates G6P, FBP, and pyruvate was observed as long as the fermentable sugar sucrose was available, but their concentrations decreased when sucrose was depleted (Fig. 3a). With respect to the nucleotides phosphate, all three compounds displayed accumulation peaks when sucrose and ethanol consumption proceed at maximal rates, 9.5 and 23.5 h, respectively (Fig. 3b). AMP concentrations were always very low. More interesting is the behavior of the ATP/ADP ratio, close to 1 at the beginning of growth and continuously increasing to peak just after sucrose consumption reached its maximal rate (11.6 h). A second peak in the ATP/ADP ratio is observed also after the high rate of ethanol consumption phase (25.7 h). A similar profile showed the ATP/(ADP+AMP) ratio, although the first peak appeared earlier during the initial phase of sucrose consumption. The analysis of oxidized NAD^+ (Fig. 4) indicated that the content in this redox-related molecule was very low, showing peaks just at the beginning (7.5) and at the end (13.5) of the high rate of sucrose consumption period. In a different set of experiments, we measured NAD^+ levels at time 16 and found even higher levels (0.58±0.18), showing that the second peak is prolonged.

During the fed-batch stage of growth, the levels of all the metabolites were present at very low concentrations. The ATP/ADP and ATP/(ADP+AMP) ratios increased continuously with the biomass density, and the levels of NAD^+ showed little change (data not shown).

Overexpression of the *TRX2* gene causes increased enhanced fermentative capacity of dry active wine biomass and reduced levels of lipid peroxidation

The good correlation of the gene expression analysis and the metabolic information from the biochemical determinations support the existence of the hypothesized oxidative stress along the batch stage of growth during wine yeast industrial biomass propagation. In order to check the physiological relevance of this oxidative stress response, we decided to genetically modify our model T73 strain in the expression level of the most induced oxidative stress marker, the thioredoxin coding gene *TRX2*. The comparison of these strains can provide us information on the suitability and benefits of this modification for an industrial application. Increased expression was achieved by multicopy subcloning of the *TRX2* gene under the control of its own promoter to maintain the physiological transcriptional regulation. To check this fact, *TRX2* induction was tested in the overexpressing strain T73*TRX2* after oxidative challenge with 5 mM H_2O_2. As shown in Fig. 5a and b, the overexpressing strain displayed up to fivefold more *TRX2* mRNA than its parental strain, and transcriptional induction by oxidative stress was still detected. Once the *TRX2* overexpression and induction by oxidative stress were confirmed, the modified strain, together with the parental strain, was used for standard experiments of biomass propagation (Pérez-Torrado et al. 2005) followed by dehydration in order to assay the fermentative capacity of dry yeasts. No growth differences were found between the modified and the reference strains (data not shown). Assays were performed for two kinds of samples, 5-day-old moist biomass and dry yeast, and the fermentative capacity of freshly obtained biomass was taken as 100%. Figure 5c shows the percentage of fermentative capacity for both the modified and the parental strains. As can be seen, the loss of fermentative capacity was bigger for dry cells in both strains, but the *TRX2* overexpressing strain maintained a higher percentage of activity than the reference strain in both states, being the difference more evident for dry cells.

The enhanced performance of the T73*TRX2* strain suggested that overexpression of the main yeast cytoplasmic thioredoxin can somehow improve the resistance of wine yeast to oxidative challenges during industrial biomass propagation. In order to gain additional information on the physiological state of the modified strain, we decided to determine the level of lipid peroxidation as a biochemical marker of oxidative damage. New biomass propagation experiments were performed with both the control and the overexpressing strain, and several samples were taken along the batch stage of the growth to analyze lipid peroxidation by the reaction of breakage products from peroxidized fatty acids with thiobarbituric acid. Figure 5d shows the quantification of malondialdehyde-derived species accumulation during the batch phase and at the end of biomass propagation for both strains, indicating a higher level of lipid oxidation damage in the control strain during the batch growth, correlating with the induction of the *TRX2* gene and the metabolic transition from mainly fermentative to fully respiratory metabolism, whereas other points present lower differences. The level of lipid

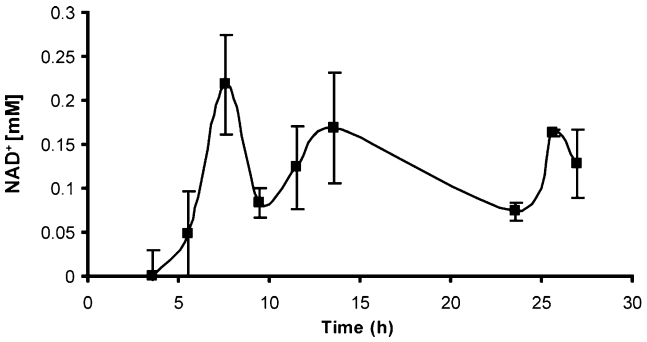

Fig. 4 Analysis of intracellular NAD^+ along the batch growth stage of biomass propagation bench-top trials. The profile of NAD^+ was determined along the batch phase of growth in the same set of experiments of Fig. 3. Average of three independent experiments and standard deviations are shown

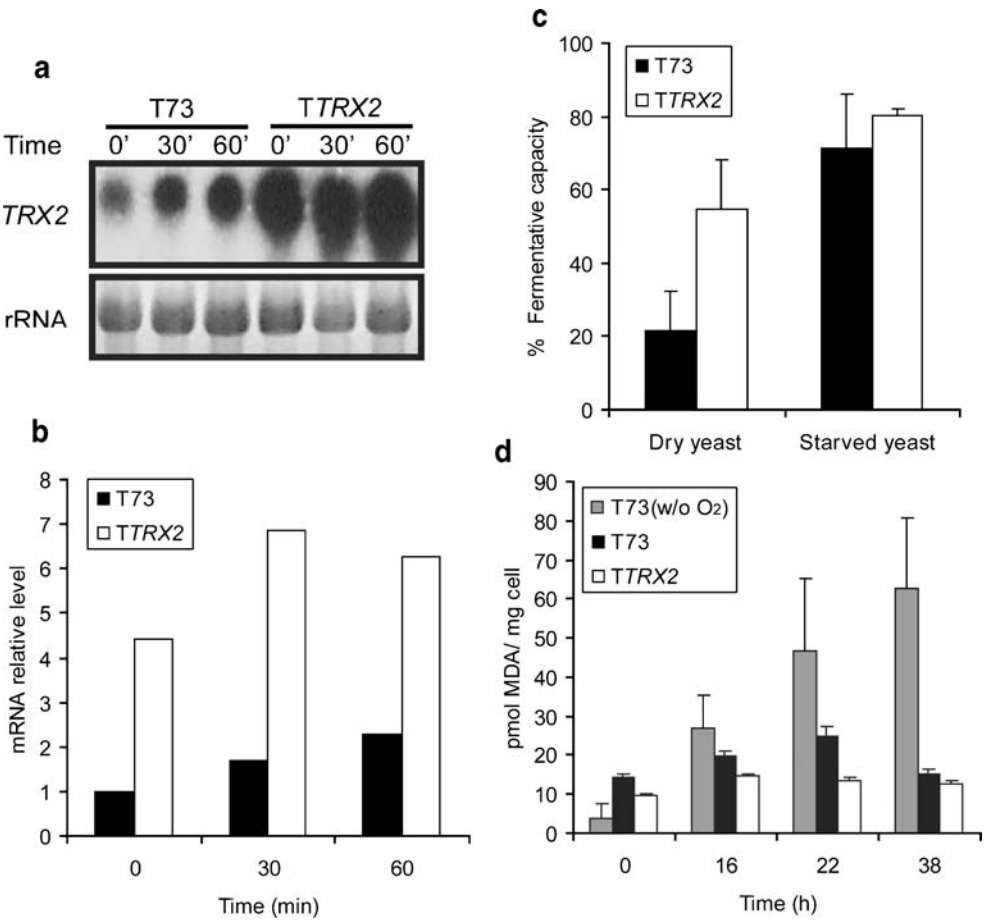

Fig. 5 Enhancement of fermentative capacity and reduction of oxidative damage in the genetically modified wine yeast strain overproducing thioredoxin. Overexpression of *TRX2* gene in the genetically modified strain was confirmed by northern blot analysis (**a**) and normalization with 28S rRNA of quantified radioactivity (**b**) after shocking cells from the modified and parental strain for 30 min with 5 mM H_2O_2. Starved and dried biomass from wild-type T73 (*black bars*) and modified strain (*white bars*) were analyzed for fermentative capacity with respect to the freshly obtained product of bench-top trial fermentations (**c**). **d** The lipid peroxidation profile of strain T73 obtained in aerated (*black bars*) or non-aerated (*gray bars*) conditions and modified strain T73*TRX2* in aerated conditions (*white bars*). Average of three independent experiments and standard deviations are shown

peroxidation was also tested for the parental T73 strain along growth in batch conditions without oxygen, where the oxidative damage of lipids was higher as can be seen in Fig. 5d.

Discussion

The process of yeast biomass propagation to produce starters for different food fermentation is a traditional, well-established industry, which has been optimized for high biomass yields and good reserve and protection carbohydrate accumulation, allowing complete consumption of nutrients by cells before entering the stationary phase (Jorgensen et al. 2002). However, very little information is available about the physiology of yeast cells during the growth and the molecular mechanisms involved in their adaptation to adverse conditions, although these aspects are critical for the performance of the starter (Walker 1998). The study of cell behavior has been limited for the difficulty in controlling experimental conditions at the large industrial scale processes and the unsuccessful reproducibility of industrial conditions in classical laboratory stress experiment. We previously described the suitability of bench-top simulation of yeast biomass propagation in combination with an analytic strategy based on the use of known stress gene markers to define specific injuring effects in the complex and changing industrial environment (Pérez-Torrado et al. 2005). The strongest gene induction we found affected specifically the thioredoxin coding gene *TRX2* and, unexpectedly, it happened early during the batch stage of growth, when yeast cells are growing on sucrose fermentation, no exogenous ROS-generating agents exists, and no general stress response is detected, so we decided to undertake a deeper characterization of this oxidative gene stress response and its biochemical, physiological, and industrial relevance.

Although the mechanisms of stress response in yeasts operate through cross-talking pathways and many genes may be induced by a specific stress agent due to the activation of transcriptional factors targeted to *cis* acting elements present at their promoters (see Hohmann and Mager 2003, for a review), the *TRX2* induction we described is accompanied by lower, but significant induction of other genes coding for products also related to the maintenance of cellular redox balance and Yap1p-regulated, as *GSH1* (Wu and Moye-Rowley 1994), or previously identified in global transcriptomic analysis as oxidative stress induced, as *GRE2* (Gasch et al. 2000). However, the general stress response marker *HSP12* is not induced,

indicating the specificity of the oxidative response in contrast to general stress inductions described in other industrial processes (Gibson et al. 2008). The different induction profiles for the different oxidative response genes also points to non-artifactual events but an ordered series of adaptation steps. Further support for the existence of an oxidative intracellular signal came from the experiments of biomass propagation through a non-aerated batch phase, where the inductions of *GRE2* and, specially *TRX2* were almost completely abolished, suggesting that intracellular processes requiring oxygen, probably respiratory metabolism, are causing the molecular response. In the industry, aeration of the fermentation tanks is initiated at the beginning of the batch stage of growth due to the importance of oxygen for biosynthesis of ergosterol (Dimster-Denk and Rine 1996; Servouse and Karst 1986), an essential molecule for membrane fluidity and permeability (Parks and Casey 1995). When sucrose is becoming limiting, the presence of oxygen allows a gradual metabolic change to ethanol respiration, and this causes ROS accumulation and the induction of the oxidative stress response. In experiments without aeration during the batch phase, no induction of the *TRX2* gene is observed, and a lag phase occurs until oxygen is supplied at the end of sucrose fermentation. This lag phase is likely due to the time needed for the synthesis and activation of enzymes participation in respiration and also occurs during the diauxic shift (Haarasilta and Oura 1975). The presence of oxygen seems then to induce a protection response, which favors the metabolic transition. The main response at the transcriptional level involves thioredoxin, and the machinery for glutathione biosynthesis does not respond to the oxidative signals under these conditions, as the *GSH1* gene transcription is not dependent on oxygen supply. There are evidence of differential oxidative stress transcriptional induction of these two genes, being the *GSH1* gene regulated through the Yap1 transcriptional factor, whereas both Yap1p and Skn7p are involved in the regulation of *TRX2* (Grant 2001).

It is not easy to quantify the intracellular content of metabolic intermediates and molecules involved in the energy and redox cellular state as nucleotides phosphate or NAD^+ due to their fast turn-over and generally low levels. However, the enzymatic determinations carried out in this work provided a good picture of the metabolic fate during industrial biomass propagation, coherent to external fermentation parameters, and the results are similar to other related metabolic studies (Rizzi et al. 1997; Theobald et al. 1997). Also, the metabolite profiles are consistent with external fermentation parameters indicative of the metabolic changes. Glycolytic intermediates glucose-6-phosphate, fructose-1,6-bisphosphate, and pyruvate displayed high levels during sucrose fermentation, and their levels drops down when the fermentable sugar disappears and metabolism turns to ethanol respiration. ATP and ADP concentrations decrease when cells begin to grow due to need of molecules for anabolic processes (Theobald et al. 1997). However, the ATP/ADP and the ATP/(ADP+AMP) ratios increase during the first hours and display the highest value coincident with the highest sucrose consumption rate. NAD^+ is practically undetectable during the first hours probably due to its high consumption under fermentative metabolic growth. Regeneration of the oxidized cofactor needed for glycolytic flux maintenance is accomplished by the alcohol dehydrogenase reaction and helped by the glycerol shuttle (Larsson et al. 1998). Accordingly, NAD^+ level raise after the first hours was accompanied by the appearance of extracellular glycerol (data not shown). Together, the analysis of the metabolite profiles, although limited to a reduced set of them, reinforces the idea of the oxidative response as consequence of the metabolic transition, as main peaks of *TRX2* mRNA can be correlated to the pattern of NAD^+ along the batch phase. The use of NAD^+ reduction equivalents may protect cell structures from oxidative damage by the action of antioxidant defense systems as thioredoxins or glutaredoxins. The relevance of transcriptional changes on *TRX2* has been corroborated by genetic manipulation of its gene expression through multicopy overexpression and testing the fitness of the modified strain. No growth differences were observed between both strains, suggesting that genetic manipulation did not alter the ability of the T73 strain to propagate in our experimental conditions. Yeast thioredoxin, together with glutathione, is the main non-enzymatic component of the complex redox maintenance cellular system (Grant 2001; Toledano et al. 2003). Thioredoxins can directly neutralize hydrogen peroxide and other peroxides and also protect thiol groups in proteins (Holmgren 1989). Their implication in oxidative stress protection has been revealed by gene overexpression and deletion in laboratory strains (Garrido and Grant 2002). Overexpression of *TRX1* or *TRX2*, the genes coding for the two cytosolic yeast thioredoxins, increases resistance to hydrogen peroxide, whereas *Δtrx2* mutants are unable to grow on 4 mM H_2O_2. It was then likely that our modified wine yeast strain displayed enhanced resistance to exogenous oxidative stresses as we checked (data not shown), but it was not that evident that it might display a better performance along simulations of the industrial propagation process due to the early stages where the oxidative stress responses take place. However, the diminished loss of fermentative capacity of the T73*TRX2* strain suggests a long-term effect of the improved response to endogenous oxidative challenges. The fermentative efficiency of the modified strain with respect to the parental strain is particularly improved when it is assayed in dry biomass from both strains, suggesting that additional oxidative stresses may occur during this last stage of wine yeast production and that elevated thioredoxin levels can also

yield additional protection. There are many different functional and structural damages driven by high production of ROS, affecting mainly to nucleic acids, proteins, and membrane lipids (Jamieson 1998; Toledano et al. 2003). The occurrence of such damages correlates with low cell survival, and it is indicative of severe redox unbalance. Here, we have tested one of these parameters, lipid peroxidation, in order to get biochemical support for the physiological behavior of the modified strain overexpressing the thioredoxin gene. Our results suggest that the increased expression of one of the main redox protection systems can efficiently reduce the peroxidation of lipids during metabolic oxidative stress and likely also other molecular damages, as thioredoxin is not directly involved in lipid protection. The appearance of lipid peroxidation damage is associated to the oxygen-dependent metabolic transition taking place during yeast growth in batch conditions, as it is clearly increased when the control strain is grown without aeration, reinforcing our previous hypothesis about the advantageous effects of oxygen supply by preparing cells for the metabolic transition. The complex cellular machinery for the maintenance of the redox balance includes a wide variety of mechanism working synergistically to reduce and eliminate ROS, to protect cellular structures from their oxidant effects, and to eliminate oxidized and non-functional proteins and lipids (Toledano et al. 2007). All these systems are intimately connected through two antioxidant molecules, thioredoxin and glutathione, and the enzymatic machinery coupling their redox conversions to the cellular redox necessities. In this scenario, it is not surprising that by improving one component of the redox maintenance machinery, benefits can be extended to the whole system.

In addition to the potential industrial applicability of the T73*TRX2* strain, its improved fermentative capacity and the good correlation with diminished cellular damage make it a very interesting tool for further analysis of the biochemical connections helping to resist metabolic oxidative damage, a general injury in many microbial industrial uses. Experiments are underway in order to extend the physiological and molecular characterization of the oxidative stress resistance of this strain along the production of wine yeast dry biomass and to define the biochemical basis of its improved fermentative capacity.

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