Oxidative stress responses and lipid peroxidation damage are induced during dehydration in the production of dry active wine yeasts

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ABSTRACT

The tolerance of the yeast *Saccharomyces cerevisiae* to desiccation is important for the use of this microorganism in the wine industry, since active dry wine yeast is routinely used as starter for must fermentations. Many studies have shown the complexity of the cellular effects caused by water loss, including oxidative injuries on macromolecular components. However the technological interest of yeast drying was not addressed in those studies, and the dehydration conditions were far from the industrial practice. In the present study a molecular approach was used to characterize the relevant injuring conditions during pilot plant dehydrations under two different drying temperatures (i.e., 35 and 41ºC). We have analyzed expression changes for several stress gene markers and we have determined two biochemical redox indicators (glutathione and lipid peroxidation levels) during pilot plant dehydrations to produce active dry biomass, according to the standard practice in industry. The main gene expression response involves the induction of genes *TRR1* and *GRX5*, corresponding to the two main redox balance systems, thioredoxins and glutathione/glutaredoxins. Elevated glutathione content and significant lipid peroxidation damage indicate the physiological impact of the oxidative stress on cellular components. The comparison between commercial stocks and pilot plant samples demonstrate the suitability of the molecular approach at the pilot plant scale to study physiological traits of industrial yeast products.

1. Introduction

Must inoculation with selected yeast strains is nowadays a general winemaking practice because the use of starters reduces the risk of sluggish fermentations and contributes to reproducible sensorial properties and quality in wine. Often, these starters are commercialized in active dry yeast form (ADY). The performance of dry yeast products, including their fermentation capacity and flavour release, depends on the genetic constitution of the selected yeast strain, but the industrial practice during biomass propagation and desiccation is also important due to the presence of environmental adverse conditions (Attfield, 1997; Pretorius, 2000). The technological parameters of the biomass production process, such as energetic, kinetic and yield, have been extensively evaluated and optimized. However, increasing numbers of molecular studies are showing good correlations between the characteristic stress resistance of a particular yeast strain and its performance to complete wine fermentations. Evaluations of the yeast transient response to environmental challenges during the yeast biomass propagation process and also during must fermentation have been performed and have shed light on critical points of those processes (Gibson et al., 2008; Pérez-Torrado et al., 2005, 2009; Zuzuarregui et al., 2005; Zuzuarregui and del Olmo, 2004a, 2004b). However, molecular analysis of yeast adaptation during the handling and drying steps after wine yeast biomass propagation remains to be addressed.

ADY production begins with the propagation of yeast biomass in a multiple-stage process (Chen and Chiger, 1985; Degre, 1993). The selected strain is inoculated in aerated nutrient-supplemented molasses and then cultivated in a sequence of consecutive batch and fed-batch fermentations in increasing volumes that ends with the ‘commercial’ fermentation. Along this process wine yeast cells suffer multiple environmental challenges. In the initial batch phase, yeast cells are exposed to high

osmotic pressure, due to the elevated sugars concentration in the molasses, that elicits a molecular stress response that activates glycerol synthesis (Pérez-Torrado et al., 2005). Also, aeration leads to important oxidative stress and induces expression of genes involved in ROS (reactive oxygen species) scavenging. During the fed-batch phase, the feed rate is set to limit the sugar concentration in order to ensure respiratory metabolism and increase the biomass yield. The respiratory metabolism causes oxidative stress and the response to this injuring condition seems to be the most relevant molecular adaptation (Pérez-Torrado et al., 2005, 2009; Shima et al., 2005). At the end of biomass propagation, wine yeast cells are separated from the fermented media by centrifugation. The resulting yeast cream is processed through a filter press or rotary vacuum filters to obtain a product with the highest solids content. Usually, the filtered biomass is mixed with emulsifiers and then it is extruded into strands. Finally, the extruded yeast strands are dehydrated to obtain a product with less than 8 % residual moisture that is packed in vacuum or inert atmosphere and stored for long time periods (Chen and Chiger, 1985). Yeast cells endure various injuring environmental conditions, such as nutrient limitation during several hours of maturation, and potentially a complex mix of different stresses during the drying process. Dehydration is known to cause cell growth arrest and severe damage to membranes and proteins (Potts, 1994; Singh et al., 2005). The removal of water molecules causes protein denaturalization, aggregation, and loss of activity in an irreversible manner (Prestrelski et al., 1993). Additionally, at the membrane level, desiccation is associated with an increased package of polar groups of phospholipids and formation of endovesicles leading to cell lysis during rehydration (Crowe et al.,1992; Simonin et al., 2007). Recently, free radical damage has also been suggested as one of the most important injuries during dehydration. Several studies with laboratory yeast strains have shown an important accumulation of ROS during

dehydration resulting in denaturation of proteins, nucleic acid damage and lipid peroxidation (Espindola et al., 2003; Pereira et al., 2003; França et al., 2005, 2007). As a result, these environmental injuries affect negatively the fermentative capacity, the viability and the vitality of cells.

In this work we have analyzed the molecular response of wine yeast strains during dehydration experiments simulating the industrial desiccation process at the pilot plant scale, by determining the expression profiles of several stress gene markers. The predominant oxidative stress response has been more deeply defined by studying several genes for specific oxidative defences and by determining biochemical indicators of redox unbalance, such as glutathione content, and lipid peroxidation damages. The relevance of these results in the industrial production of dry wine yeast biomass has been confirmed by comparison to real commercial stocks from an ADY company.

2. Materials and methods

2.1. Strains

The industrial *Saccharomyces cerevisiae* strain T73 (CECT1894) has been used in this study. It is a natural diploid wine strain isolated from Alicante (Spain) musts (Querol et al., 1992), which has been commercialized by Lallemand, Inc. (Montreal, Canada).

2.2. Media and industrial cultivation

Industrial cultivation was performed according to the Laboratory of Research and Development (Lallemand S.A.S.) protocol. Precultures and batch growth were performed in MALT media, pH 4.80, containing 80 g sugars/L. Precultures were incubated at 30 ºC over-night with shaking. Culture growth was monitored by measuring the optical density at 600 nm (OD_{600}) and cell counting in a Neubauer camera. Precultures were used to inoculated $1–2 \times 10^6$ cells/mL in 10 L of MALT

media in a bioreactor. Batch and fed-batch cultivations were performed in a BIOFLO 4500 Bioreactor (New Brunswick Scientific). The first step of industrial cultivation was a 24 hours batch growth. Then, a part of the biomass and media was removed and the remaining biomass (33.75 g) was used as inoculum for the fed-batch step. Before starting the feeding, the volume was adjusted to 11 L with sterile water. During the fed-batch growth, the culture was fed with molasses media, pH 4.80, and containing 300 g sugars/L and supplemented with NH_4OH 5%, vitamins and minerals (pantothenic acid, biotin, thiamine, zinc sulphate, magnesium sulphate, phosphoric acid and ammonium hydroxide). Along the process, temperature (30 ºC), pH (4.80) and aeration (1 vvm) were kept constant. The stirrer speed was 250 rpm for the first 7 hours and 400 rpm for the remaining batch growth. During the fed-batch stage the stirrer speed was fluctuating between 400 and 650 rpm depending on cells oxygen request. The minimum level of dissolved oxygen was set to 15 %. At the end of the process, the fed was stopped for 1–2 hours before recovering the produced biomass.

2.3. Drying

The drying process was performed according to the Laboratory of Research and Development (Lallemand S.A.S.) protocol. At the end of the fed-batch fermentation, biomass was centrifugally separated (Westfalia Separator AG) from the fermented media and subjected to several washing steps. A 3-fold concentrated yeast cream was obtained, and a mix of sorbitan monoestearate and NaOH was added. Filtration of the yeast cream was performed in a Büchner funnel and a vacuum pump to obtain a cake. Then, the yeast cake was extruded through a perforate plate obtaining strands (1 mm diameter). Extruded yeast strands were dried in a fluidized-bed dryer (Versa-Glatt GPCC Type 1, USA) for 20 to 30 min. Air temperature was 60 ºC at the beginning of the drying period, keeping the temperature of the biomass at 35 ºC or 41 ºC. The final

moisture content of the ADY was beneath 8 %. Some samples were taken along handling and drying of biomass and its moisture contents were determined with a moisture analyser (Sartorius MA30).

2.4. Determination of fermentative capacity

Samples containing 2.5 g (dry weight) of cells were rehydrated into 50 mL of warm water (37 ºC) for 20 min (10 min statically and 10 min shaking). The rehydrated yeast was used to inoculate 1×10^7 cells/mL in 100 mL of YPGF media (10 % fructose, 10 % glucose, 2 % peptone, 1 % yeast extract) and incubated at 30 ºC with shaking (140 rpm). The exact number of cells was determined by recounting in a Neubauer camera. The production of CO_2 was measured in a Chittick Apparatus (American association of cereal chemists, 12-10) during 4 hours. The fermentative capacity was expressed as the mean of last nine instantaneous rates. The instantaneous rate is the CO_2 increment (mL) by number of cells and by elapsed time between two consecutive measures (20 min).

2.5. RNA extraction and cDNA synthesis

Total RNA from 50 - 70 mg yeast cells was extracted with cycles of vigorous agitation on a vortex in 0.5 mL LETS buffer (LiCl 0.1 M, EDTA 0.01 M pH 8.0, Tris-HCl 0.01 M pH 7.4, SDS 0.2 % (p/v)), 0.5 mL phenol pH 4.3 (AMRESCO) and 0.5 mL glass beads. Supernatants were extracted with phenol:chloroform (5:1) (v:v) and phenol:chloroform:isoamylic alcohol (25:24:1) (v:v:v). RNA precipitate was obtained after incubation with 1 volume of LiCl 5 M at -20 ºC during at least 3 hours. The quantity and the quality of the extracted RNA were checked spectrophotometrically and by electrophoresis, respectively. 1 μg of total RNA was used for the synthesis of first strand cDNA using the Transcriptor First Strand cDNA Synthesis Kit (Roche) in the presence of random hexamer primers. The obtained product was RNA free and suitable to be used in a quantitative real-time PCR.

169 *2.6. Gene expression analysis by quantitative real-time PCR*

170 PCR primers (Table 1) were designed with the available Gene Bank sequence data and
171 the Primer Express software (PE Applied Biosystems, Cheshire, UK) according to the
172 Roche Applied Science and the Bio-Rad Laboratories guidelines. Quantitative real-time
173 PCR was performed in a LightCycler® 2.0 apparatus (Roche) using the LightCycler®
174 FastStart DNA Master^{PLUS} SYBR Green I kit (Roche) for fluorescent labeling. 2.5 µL
175 cDNA was added to each reaction in a final volume of 10 µL. Real-time PCR reactions
176 using 1 pmol /µL of the corresponding oligonucleotides were performed under the
177 following conditions: 95 ºC for 10 min, followed by 40 cycles of 20 s at 95 ºC, 5 s at 55
178 ºC and 6 s at 72 ºC. At the end of the amplification cycles, a melting analysis was
179 conducted to verify the specificity of the reaction. This was carried out by heating the
180 amplification products from 65 °C to 95 °C at 0.1 °C/s and monitoring the decrease in
181 fluorescence. For each analyzed gene, a negative control was included and a standard
182 curve was made with serial dilutions of a pool of representative samples from each step
183 of the drying process (2×10^{-1}, 1×10^{-1}, 4×10^{-2}, 2×10^{-2} and 1×10^{-2}, except for the
184 *RDN18* gene that were 4×10^{-2}, 2×10^{-2}, 1×10^{-2}, 5×10^{-3}, 2×10^{-3} and 1×10^{-3}). The
185 2nd Derivative Maximum Method of LightCycler® software was used to identify the Cp
186 (crossing point) of a sample and to transform it to cDNA values. As recently suggested
187 by different authors, several reference gene were used (Bustin et al., 2009) and the
188 results were normalized by using the normalization factor obtained from geNorm VBA
189 applet (Vandesompele et al., 2002). The expression of two standard reference genes
190 (*ACT1, RDN18)* and *HSP12*, a stress induced gene with a high and relatively constant
191 expression during dehydration (not shown).

192 *2.7. Glutathione determination*

100 mg of cells were resuspended in 8 mM HCl, 1.3 % (w/v) 5-sulphosalicylic acid (4 ºC). Cells were broken with 0.6 g glass beads in four cycles of 1 min agitation on a vortex mixer, followed by 1 min on ice, and then incubated on ice for 15 min to precipitate proteins. The supernatants obtained after centrifugation were used to determine total and oxidized glutathione (GSSG) by a colorimetric assay (Griffith, 1980; Tietze, 1969). 200 µL of extract were added to 120 µL of enzymatic cocktail (0.4 mg/mL $NADP^+$, 0.16 mg/mL glucose-6-phosphate, 3 µg/mL glucose-6-phosphate dehydrogenase, 1 mU glutathione reductase, 0.2 M MES, 2 mM EDTA, 0.1 M sodium phosphate buffer, pH 6.0) and 480 µL of 200 µM DTNB (5, 5'-dithiobis-(2-nitrobenzoic acid)). The reaction mixture was incubated with shaking at room temperature in the dark for 20 min and the absorbance at 412 nm was measured. To determine oxidized glutathione, cell extracts were previously incubated with 1 M 2-vinylpyridine for 1 hour. A standard curve from 0 to 16 µM GSSG was prepared and processed as the samples. Reduced glutathione (GSH) was calculated by subtraction between total and oxidized glutathione. Results are expressed as nmoles of glutathione/mg of dry cell weight.

2.8. Measurement of lipid peroxidation

The method based on the reaction of thiobarbituric acid with reactive species derived from lipid peroxidation, particularly malondialdehyde (MDA), was used. Detection of thiobarbituric acid reactive species (TBARS) was carried out by a colorimetric assay described by Buege and Aust (1978) with some modifications (Espindola et al., 2003). 50 mg of cells were resuspended in 500 µL of 50 mM phosphate buffer, pH 6.0, containing 10 % trichloroacetic acid, and 0.3 g glass beads were added. The samples were broken by three cycles of 1 min agitation on a vortex mixer followed by 1 min on ice. After centrifugation, supernatants were mixed with 0.1 mL of 0.1 M EDTA and 0.6

mL of 1 % (w/v) thiobarbituric acid in 0.05 M NaOH. The reaction mixture was incubated at 100 ºC for 15 min and then cooled on ice for 5 min. The absorbance at 532 nm was measured. Lipid peroxidation is expressed as pmoles of malondialdehyde/mg of dry cell weight.

2.9. Intracellular trehalose determination

Cell free extracts from 10 mg cells were obtained according to Parrou and François, (1997). Trehalose was measured by enzymatic degradation with commercial trehalase (Sigma). Released glucose was determined by the glucose oxidase/peroxidase assay. The amount of trehalose is given in µg of trehalose/mg of dry cell weight.

3. Results

3.1. Residual moisture and physiological parameters in pilot plant and commercial ADY products

Yeast biomass was obtained in pilot plant scale simulations of industrial process with a biomass yield of 0.47 ± 0.01 g dry weight of cells/g of sucrose. Sorbitan monoestearate and NaOH were added to the concentrated yeast cream obtained after centrifugation and washing, and this initial product was used as reference for the parameters analyzed during the dehydration experiments. The residual moisture was determined after every step and the results are shown in Figure 1. The stabilized yeast cream was filtered to remove about 30 % of residual moisture, and subsequently, the yeast cake was extruded into fine strands to easier the drying. The water content did not change at this step. After that, extruded yeast strands were fed into an air-lift dryer and hot air was blown through the biomass at a sufficient power to keep yeast particles in suspension. During the drying process, biomass maximal temperatures reached 35 ºC or 41 ºC. After 8 minutes of drying, the water content diminished to 40 % at both temperatures (Fig. 1). At a

drying temperature of 41 ºC, the biomass showed a residual moisture beneath 8 % after 18 minutes of drying, and the moisture content in the final product was 4.77 % ± 0.26 %. In the drying at 35 ºC, the biomass reached the same level of dehydration after 28 minutes of drying, and the moisture content in the final product was 5.51 % ± 0.38 % (Fig. 1).

The quality of final products (ADY) obtained in a pilot plant scale was compared to three T73 commercial stocks supplied by Lallemand S.A.S. Trehalose content and fermentative rate were analyzed as physiological parameters for pilot plant yeast creams and ADY from 35 and 41 ºC drying protocols, and also for ADY commercial products. The results of those determinations are shown in Table 2. The trehalose content in the pilot plant yeast cream was 180.8 ± 16.5 μg of trehalose/mg of dry cell weight, it slightly varied along the handling and dehydration processes, and it was not significantly affected by the drying temperature (Table 2). However, trehalose accumulated to higher levels in the three industrial stocks (A, B and C) with relatively small differences between them (less than 10 %). No significant differences in fermentative capacity were detected between pilot plant ADY samples, and their CO_2 production rate was similar to the rate displayed by commercial stocks.

3.2. Transcriptional response of stress-related genes during the drying process at the pilot plant scale

We have analyzed a collection of gene markers based on their specific induction by a single stress condition in order to study the physiological adaptation of wine yeasts to dehydration, the final industrial process in ADY production. Figure 2 shows the results of the expression analysis for some of those gene markers along handling and drying of wine yeast biomass in pilot plant scale. The analyzed gene markers were: *STI1*,

transcriptionally activated by the transcriptional factor Hsf1p during thermic stress, *GSH1* and *TRX2*, coding for proteins participating in antioxidants functions, *GPD1*, involved in the high-osmolarity glycerol (HOG) response pathway, and *HSP12*, regulated by the general stress response pathway through Msn2p/4p transcriptional factors. Figure 2 shows the quantification of the mRNA levels of these stress genes along drying processes at two different temperatures. As some differences were observed in the expression of the marker genes in the cream samples, data along drying were normalized to their corresponding expression in the cream. As can be seen, only very small changes in the mRNA level of the analyzed genes were observed along the first steps of the process, including filtration and extrusion of the yeast biomass. Higher expression changes were observed mainly in samples obtained at different time points during the step of drying at 35 ºC (Panel A) and 41 ºC (Panel B), and also in the active dry yeast products. A 2-fold increase after 28 min of drying at 35 ºC was observed in the mRNA levels of the *GSH1* gene and more than 3-fold in the final ADY product. Also the transcript level of the *GPD1* gene was up to 2-fold higher in ADY (Fig. 2, Panel A). A similar expression pattern was observed in biomass samples obtained along drying at 41 ºC, but transcriptional changes were already detected after 18 min of drying (Fig. 2, Panel B). Interestingly the expression of the additional gene marker *STI1* increased at 41 ºC. The mRNA level in the ADY final product was 2-fold that in the cream (Fig. 2, Panel B).

An equivalent study of gene marker expression was applied to the commercial ADY A, B and C stocks. Because biomass yield and fermentative capacity of the ADY pilot and industrial plants were similar, the cream yeast of the pilot plant was used to normalize the mRNA levels of industrial stocks. Then, these levels were compared to those obtained during the pilot plant desiccation process. As shown in Figure 3 (Panel A)

mRNA levels for the induced stress gene markers in industrial stocks were higher than in pilot plant ADY (see Fig. 2), and some gene induction differences were detected among the three industrial stocks. Interestingly, we observed more than 4-fold increase of *GPD1* gene expression in all three industrial stocks, a 6-fold increase of *GSH1* gene in stocks A and B, similarly to pilot plant, and 5-fold induction in *STI1* gene but only in stock C.

3.3. Transcriptional response of oxidative stress genes during the drying process

The reproducible induction of *GSH1* gene in pilot plant desiccation experiments pointed to oxidative stress response as the main molecular adaptation process along wine yeast biomass drying. In order to study thoroughly this stress response, the analysis of changes in gene expression was extended to additional oxidation gene markers, as well as to other biochemical markers for redox control, such as glutathione levels, or oxidative cellular damage, and lipid peroxidation.

Four oxidative stress gene markers representing both the glutathione and thioredoxin systems were analyzed along pilot plant drying: *GRX2* and *GRX5* genes, coding for dithiol and monothiol glutaredoxins, respectively; and *TRR1* and *TSA1* genes, coding for cytosolic thioredoxin reductase and a thioredoxin peroxidase. As can be seen in Figure 4, the mRNA level increases were detected mainly along drying with hot air and also in final ADY product, similarly to the gene marker expression patterns showed previously (see Fig. 2). The gene displaying a maximal induction was *TRR1*, reaching 5-fold higher mRNA level in ADY obtained by drying at 35 ºC biomass final temperature (Fig. 4, Panel A). Also the *GRX5* gene showed transcriptional changes, near to 2-fold increase was observed in the final product obtained by drying at 35 ºC and 3-fold for ADY obtained by drying at 41 ºC (Fig. 4,). The expression analysis of

318 oxidative stress markers was also carried out for the industrial stocks A, B and C (Fig.
319 3, Panel B), and the elevated expression of the *TRR1* gene was again the most important
320 induction, close to a 8-fold rise in stock B.
321 The *GSH1* gene codifies for γ-glutamylcysteine sinthetase, the first enzyme involved in
322 glutathione synthesis. As elevated mRNA levels of this gene was observed during the
323 drying process, intracellular levels in glutathione were determined in order to assess
324 correlation between gene expression and physiological adaptation. As can be seen in
325 Figure 5, the total glutathione levels started to increase after 8 min of hot air drying in
326 the two tested conditions, 35 ºC and 41 ºC (Panels A and B, respectively), and not
327 during previous handling. The higher content of total glutathione in both dehydration
328 conditions was similar, about 60 nmol of glutathione/mg of dry cell weight, but this
329 highest level was observed after 28 min of drying at 35 ºC (Fig. 5, Panel A), while it
330 was reached after 18 min at 41 ºC (Fig. 5, Panel B). When both the oxidized and the
331 reduced forms of glutathione were quantified, we found that the elevated glutathione
332 levels were due with a high content of the reduced form. After 8 min in the air-lift dryer,
333 there was a significant increase in the intracellular oxidized GSSG in the two drying
334 temperatures tested, and then it decreased quickly in the following 10 min. As expected,
335 the GSH/GSSG ratio displayed an oxidation peak at the beginning of drying but a
336 reduced state was recovered along drying and the final ADY product showed a
337 reduced/oxidized glutathione ratio even higher than the initial yeast cream (Table 3).
338 Desiccation has been described as causing multiple molecular stresses including
339 oxidation that can affect all kinds of macromolecules, proteins, nucleic acids and lipids.
340 Membrane structure and function are damaged by lipid peroxidation under cell
341 dehydration. Therefore the extent of oxidative injury during dehydration was evaluated
342 by determining the level of this chemical modification (Fig. 5, Panel C). The level of

lipid peroxidation increased when wine yeast biomass was subjected to hot air drying in both procedures at different temperatures. In the drying at 35 ºC, the raise in lipid peroxidation occurred after 18 min reaching similar levels than in the ADY final product. Similarly but in a shorter time period, yeast cells dried at 41 ºC also displayed an increase in MDA levels which reached the maximal level in only 8 min. The lipid peroxidation level in both ADY final products were similar, about 15 – 16 pmoles of MDA/mg of cells, representing a 30-40 % increment with respect to the initial yeast cream.

Both glutathione content and lipid peroxidation analysis were also performed with samples of industrial stocks A, B and C. The GSH/GSSG ratio was higher for these ADY stocks (29.28, 23.13 and 22.43, respectively) than for the pilot plant ADY samples (Table 3). The three stocks showed 20-to-30 fold more reduced form than oxidized glutathione form. Accordingly to the GSH/GSSG ratio, the level of lipid peroxidation in the three commercial stocks was lower (4.29, 10.22 and 6.27 for A, B and C stocks respectively) than in the pilot plant ADY samples, although there was variability between them and the stock B had the highest level of lipid peroxidation damage.

4. Discussion

The industrial process of wine yeast biomass dehydration involves damaging environmental changes. As biomass is being concentrated, water molecules are removed and the temperature is increased, all affecting cell viability and vitality (Matthews and Webb, 1991). In this work we have approached the study of the cellular state during all the processing steps by applying a useful tool, the expression changes for a set of gene stress markers in pilot plant simulations of industrial dehydrations. This kind of analysis

368 has been often used to study the physiological state and yeast stress responses in
369 industrial processes (Higgins et al., 2003, Pérez-Torrado et al., 2002a, 2005, 2009; Riou
370 et al., 1997). In previous gene markers studies, an important osmotic stress was decribed
371 at the beginning of vinification, showed by the transcriptional induction of the *GPD1*
372 gene marker (Pérez-Torrado et al., 2002a; Zuzuarregui et al., 2005). The correlation
373 between stress resistance, risk of sluggish fermentation and *HSP12* gene expression in
374 different wine yeast strains (Ivorra et al., 1999); and also the existence of oxidative
375 stress during wine yeast biomass production, indicated by *TRX2* gene induction
376 depended on the strong aeration (Pérez-Torrado et al., 2005, 2009).
377 Recent studies on dehydration conditions in lab strains have also showed the existence
378 of oxidation changes and sensitivity to drying was correlated to oxidative stress
379 (Espindola et al., 2003; França et al., 2005, 2007; Pereira et al., 2003). In the present
380 study, molecular markers of this oxidative stress response along handling and drying
381 conditions simulating the industrial desiccation process of the wine yeast strain T73,
382 were described. This approach was relevant for assessing the performance of ADY. Due
383 to the particular conditions of desiccation, in which no cell growth occurs, the choice of
384 reference gene for normalization of mRNA level is difficult. Three genes were selected
385 for this purpose, two generally accepted reference genes (*ACT1* and *RDN18*) and
386 *HSP12*, a stress response gene which expression is high and relatively constant along
387 dehydration (data not shown). By using this triple normalization quantitative differences
388 in transcriptional induction are reduced with respect to the values obtained when a
389 single normalizing gene is used. The inductions of gene marker expression in the wine
390 yeast strain T73 during desiccation were generally moderate although statistically
391 significant in some steps, such as hot air drying and final product. These results were
392 expected as fresh yeast biomass was obtained simulating the industrial growth

393 conditions, where cells are subjected to batch and fed-batch cultivation steps and then to
394 a maturation step. The purpose of this maturation step is the complete consumption of
395 residual glucose by yeasts, that end up in a stationary growth state where the expression
396 of several stress genes is induced, enhancing the resistance of yeast cells to stressful
397 conditions (Causton et al., 2001; De Risi et al., 1997; Gasch et al., 2000). The gene
398 marker expression patterns observed were similar in both drying conditions (35 and 41
399 ºC), except for the induction of the *STI1* gene, detected only when yeast biomass
400 reached 41 ºC during drying. This differential induction of the *STI1* gene was expected
401 because the induction of this gene was already detected at 39 ºC in previous analysis
402 with the same strain in laboratory conditions (Pérez-Torrado et al., 2005). Another
403 expected result was the induction of the osmotic stress marker *GPD1*, due to the water
404 loss. However, despite that yeast biomass lost approximately 95 % of water content
405 during this dehydration process, *GPD1* induction was not as important as previously
406 observed in lab yeast strains under osmotic stress (Pérez-Torrado et al., 2002a). These
407 data are in agreement with the robustness of industrial yeasts strains compared to
408 laboratory strains (Querol et al., 2003), and also with the well-known relevance of
409 biomass propagation conditions to confer resistance to subsequent suboptimal
410 conditions (Bisson et al., 2007). Unexpectedly, the highest induction in our first
411 expression analysis was displayed by the stress marker *GSH1*, pointing out the
412 relevance of the oxidative stress response along wine yeast drying to obtain ADY. This
413 observation was supported i/ by significant inductions of other genes involved in the
414 oxidative stress response (as *TRR1* and *GRX5*), ii/ by the rise in the level of cellular
415 lipid peroxidation, iii/ by increased intracellular glutathione accumulation, and iv/ by a
416 peak of its oxidized form GSSG during the first minutes of drying.
417 The expression profiles of *GSH1* and *TRR1* genes were similar in the two drying

418 conditions, thus suggesting that these genes were specifically induced by the drying step
419 and not by previous biomass handling. Global expression studies in response to
420 environmental changes have shown that expression of *GSH1* and *TRR1* genes is not
421 induced by sugar exhaustion and stationary phase, being *TRR1* gene expression even
422 repressed under those conditions (Causton et al., 2001; Gasch et al., 2000). Therefore,
423 the increased mRNA levels detected during drying seem not to be related to growth
424 arrest. In the present study, the induction of these two markers was detected when yeast
425 biomass was introduced in the fluidized-bed dryer and not in previous filtration and
426 extrusion steps. This is in contrast to other industrial processes, such as wine yeast
427 biomass propagation (Pérez-Torrado et al., 2005) and lager brewing yeast industrial
428 propagation and fermentation (Gibson et al., 2008), for which the oxidative stress
429 response was related to the growth state and the presence of glucose or other sugars in
430 the medium. Whereas *GSH1* gene expression can be affected by ROS and high
431 temperatures (Sugiyama et al., 2000b), *TRR1* induction has been detected only in
432 response to oxidative challenge (Kim et al., 2006), making this gene a good maker for
433 oxidative stress along dehydration process. The notable induction in the expression of
434 the *TRR1* gene could be related to the redox state of thioredoxins and the Yap1-
435 dependent oxidative stress response along drying. The oxidation of the Yap1p
436 transcriptional factor prevents its nucleus export, where it can activate the expression of
437 several oxidative stress genes, such as *TRR1* and *TRX2*. Trr1p, the cytosolic thioredoxin
438 reductase, is the key enzyme for reducing cytosolic thioredoxins Trx1p and Trx2p
439 (Trotter and Grant, 2003), that are key elements in Yap1p-dependent transcriptional
440 regulation and proper oxidative stress response (Carmel-Harel et al., 2001). The
441 thioredoxin system is responsible for reduction, and then inactivation, of Yap1p causing
442 the end of the oxidative stress response (Delaunay et al., 2000; Izawa et al., 1999;

Temple et al., 2005). The increase in Trr1p levels during the dehydrated state could guarantee the recovery of oxidized cytosolic thioredoxins for a fast Yap1p reduction when yeasts are rehydrated, allowing the redox equilibrium state to be restored. Moreover, thioredoxins participate in other cellular functions which could be related to growth recovery after rehydration, such as DNA replication (as donor for ribonucleotide reductase) or biosynthesis of sulphur amino acids (as hydrogen donor for 3'-phosphoadenosin-5'-phosphosulfat reductase) (Toledano et al., 2007).

Glutathione has been described as a fundamental molecule for dehydration tolerance in many organisms, from bacteria to small vertebrates, including yeast for which it acts as cofactor for antioxidant enzymes. Glutathione can react with ROS and protein thiol groups, and it has been related to membrane protection in anhydrobiosis conditions (Espindola et al., 2003; França et al., 2007; Pereira et al., 2003). It is an abundant metabolite reaching between 0.1% and 1% yeast dry cell weight (Pocsi et al., 2004). In our simulation of industrial growth conditions, yeast cells accumulated more than 1 % of glutathione and this percentage increased along drying, reaching approximately 2 %. The high glutathione content in T73 industrial yeast strain could explain the low level of lipid peroxidation detected both in fresh and dry biomass, 5-to-10 fold lower than previously described for laboratory yeast strains (Espindola et al., 2003). Glutathione accumulation started just after introduction of the yeast biomass into the fluidized-bed dryer and correlated with the increased expression of *GSH1*, the gene coding for the γ-glutamylcysteine synthetase, the first enzyme of glutathione biosynthesis (Ohtake and Yabuuchi, 1991). On the other hand, postranscriptional regulation of the γ-glutamylcysteine synthetase activity can also contribute to the increase in reduced glutathione (GSH) content in response to the accumulation of the oxidized form GSSG during the first 8 drying minutes, as it has been described in mutants affected in

NADPH production and unable to reduce GSSG (Ng et al., 2008). As previously mentioned, *GSH1* gene expression, and therefore glutathione synthesis, can be induced by heat (Sugiyama et al., 2000b). This cross-linked response between heat and oxidative stresses has been extensively described in the literature (Moraitis and Curran, 2004, 2007): it causes the induction of *HSP* genes by exposition to oxidant agents, and also the accumulation of antioxidant proteins by exposition to high temperatures (Kim et al., 2006; Pereira et al., 2001; Sugiyama et al., 2000a, 2000b). It is difficult to assess the causes for glutathione biosynthesis during the industrial drying process to obtain ADY, because yeast biomass is subjected to the elimination of water molecules by high temperatures, so both the factors could affect the accumulation of ROS. ROS accumulation is likely due to high temperature because it increases mitochondrial respiration whereas water loss favours free radicals formation and increases their concentration (França et al., 2007; Leprince et al., 1994; Sugiyama et al., 2000b). In addition to glutathione biosynthesis, ROS accumulation along drying can be deduced from the increase of GSSG and lipid peroxidation levels (Espindola et al., 2003; França et al., 2005; Pereira et al., 2003).

It is worth to note the fast reduction in the GSSG content after the initial peak, might be achieved by the glutathione reductase Glr1p, because the high temperatures used for drying can induce the expression of *GLR1* (Kim et al., 2006). In some organism, like lichens and plants, it has been observed that tolerance to dehydration is dependent on the GSH/GSSG ratio and the capability to recover the reduced GSH form during the process (Kranner, 2002; Kranner et al., 2002, 2006). Moreover, the apoptotic effect of ROS accumulation is accentuated in yeast mutants unable to synthesize glutathione (Madeo et al., 1999). Then, the great dehydration tolerance of the industrial T73 wine yeast strain could be related to its capability to quickly reduce GSSG.

As mentioned, biochemical or physiological differences were not observed in final biomass obtained by drying at 35 and 41 ºC, and fermentative capacity, trehalose and glutathione contents, and lipid peroxidation levels were similar. The main temperature-dependent differences were the induction of the heat stress marker *STI1* during dehydration at 41 ºC, and also a higher expression of *GRX5* at that temperature, in addition to the earlier appearance of the molecular response. *GRX5* expression is not induced by oxidative agents, nor by hyperosmotic stress, or high temperatures (Rodríguez-Manzaneque et al., 1999, 2002). Therefore, it is likely that the induction of *GRX5* described in the present study was not a direct consequence of the drying temperature. Instead, it could be related to several consequences of water loss, that is faster at 41 ºC than at 35 ºC. Water loss affects proteins and other cellular structures, such as membranes and organelles, and also metallic ion concentration, and might be driving the transcriptional induction of *GRX5*.

Pilot plant ADY production is a simplification of the real industrial process routinely used by companies to test the performance of new strains and technological changes before undertaking the high scale production. The comparison to real industrial stocks showed some differences, such as lower level of oxidative damage by lipid peroxidation, higher reduced glutathione content, and higher trehalose accumulation, suggesting that pilot plant and industrial scales are not identical. However, those differences are also found between different industrial stocks. When technologically relevant parameters are compared, such as biomass yield (Jorgensen et al., 2002) and fermentative capacity, similar results are obtained in both pilot plant and industrial scales, suggesting that processes are similar enough to yield physiologically equivalent products. Previous studies with baker's yeasts (Van Hoek et al., 2000) showed a correlation between the specific growth rate along the fed-batch stage and the

fermentative capacity of the final product suggesting that the performance of yeast biomass for fermentation is dependent on the growth conditions, then reinforcing the similarity between pilot plant and industrial ADY production scales.

Similarly, the stress gene expression analysis and the study of specific oxidative markers gave the same results for both processes, being the same genes (*GPD1*, *STI1*, *GSH1* and *TRR1*) induced in all the experiments. These results suggested that pilot plant scale experiments are suitable to study wine yeast biomass propagation and dehydration processes, in despite of their complexity and variability. Further analyses are underway in order to correlate the fitness of different wine yeast strains during ADY production to their oxidative stress response capability.

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724 **Figure legends**

725 Fig. 1**.** Moisture content in yeast biomass at different phases of handling and drying of
726 wine yeast T73 to obtain Active Dry Yeast (ADY). The yeast biomass was obtained and
727 handled in plant pilot production as described in Materials and Methods. Drying in air-
728 lift dryer was performed keeping the yeast temperature at 35 ºC (black bars) or 41 ºC
729 (white bars) to obtain the final ADY product with moisture content beneath 8 %. The
730 residual moisture was determined in function of weight loss into a moisture analyser at
731 110 ºC until constant weight was reached. The mean and the standard error correspond
732 to four independent experiments. Significantly different at $P < 0.01$ (a).

733 Fig. 2**.** Pattern of gene markers expression during handling and drying of wine yeast
734 T73 to obtain ADY at pilot plant level. The expression of gene markers *HSP12*, *STI1*,
735 *GPD1*, *GSH1* and *TRX2* is shown during drying processes keeping the yeast
736 temperature at 35 ºC (Panel A) or 41 ºC (Panel B). Error bars stand for standard errors.
737 Significantly different from the cream at P-value < 0.01 (a), at P-value < 0.05 (b).

Fig. 3**.** Pattern of gene markers expression (Panel A) and selected genes involved in oxidative stress response (Panel B) in ADY commercial stocks. This analysis was made in three different industrial stocks of T73 yeast (A, B, C).

Fig. 4. Induction of selected genes involved in oxidative stress response during handling and drying of wine yeast T73 to obtain ADY at pilot plant level. The expression of genes *GRX2*, *GRX5*, *TRR1* and *TSA1* is shown during drying processes keeping the yeast temperature at 35 ºC (Panel A) or 41 ºC (Panel B). Error bars stand for standard errors. Significantly different from the cream at P-value < 0.01 (a), at P-value < 0.05 (b).

Fig. 5. Glutathione content (Panels A and B) and lipid peroxidation (Panel C) in yeast biomass at different phases of handling and drying of wine yeast T73 to obtain ADY at pilot plant scale. Panel A shows glutathione data during drying processes keeping the yeast temperature at 35 ºC and Panel B at 41 ºC. Total (grey bars) and oxidized (line) glutathione levels were determined by DTNB reaction, and reduced glutathione (white bars) from the difference between these two values. Glutathione content was expressed in nmoles of glutathione/mg of dry weight cells. Panel C shows lipid peroxidation data with symbols as in Fig. 1. Error bars stand for standard errors. Significantly different from the cream at P-value < 0.01 (a), at P-value < 0.05 (b).

Table legends

TABLE 1. Genes and primers used for quantitative real-time PCR.

TABLE 2. Trehalose content and fermentative rate for T73 wine ADY obtained in pilot plant and different commercial stocks.

TABLE 3. GSH/GSSG ratio at different phases of handling and drying of wine yeast T73 to obtain ADY at pilot plant scale.

763 TABLE 1. Genes and primers used for quantitative real-time PCR.
764

Primer	Sequence (5′−3′)	Gene	Amplicon size (bp)[a]	Amplification efficiency [b]
18S.F	TTGCGATAACGAACGAGACC	*RDN18*	95	1.919
18S.R	CATCGGCTTGAAACCGATAG			
ACT1.F	CATGTTCCCAGGTATTGCCG	*ACT1*	51	2.062
ACT1.R	GCCAAAGCGGTGATTTCCT			
GPD1.F	GGTGAGATCATCAGATTCGG	*GPD1*	129	1.959
GPD1.R	CCTAGCAACCTTGACGTTTC			
GRX2.F	AATCCAAGGCCCTTGTGTTG	*GRX2*	93	1.963
GRX2.R	GTACAGTTTTTTGGCCCGAG			
GRX5.F	GACCCAGAGCTACGTGAAG	*GRX5*	119	1.935
GRX5.R	CCAGAGCGTGCCATACTTG			
GSH1.F	CCGGACAAAAAGGATTCTCC	*GSH1*	88	1.838
GSH1.R	CGGAATACGCAGCGTTCTC			
HSP12.F	TGACAAGGCCGACAAGGTC	*HSP12*	153	2.010
HSP12.R	GCGGCTCCCATGTAATCTC			
STI1.F	CGGAGGCGTATGTTAACCC	*STI1*	84	2.108
STI1.R	CATTCGGCCAATCACTCTTG			
TRR1.F	GAAACCGATTTGCCAGTCAG	*TRR1*	92	1.959
TRR1.R	GCTTCATCAGTGTCGACTTG			
TRX2.F	GCTGAAGTTTCTTCCATGCC	*TRX2*	63	1.976
TRX2.R	GACTCTGGTAACCTCCTTAC			
TSA1.F	CCTTGAGATTGGTTGAAGCC	*TSA1*	76	2.012
TSA1.R	GCACCTGGAGTCCAGTTAC			

765 [a] bp, base. [b] Optimal theoretical efficiency value is 2.
766
767
768

TABLE 2. Trehalose content and fermentative rate for T73 wine ADY obtained in pilot plant and different commercial stocks.

	Samples	Trehalose[a]	Fermentative rate[b]
Pilot plant	35 ºC	171.9 ± 13.0[c]	0.121 ± 0.019 [c]
	41 ºC	180.5 ± 4.2 [c]	0.135 ± 0.020 [c]
Industrial stock	A	307.7	0.114 ± 0.011 [c]
	B	267.0	0.116 ± 0.001 [c,d]
	C	276.83	0.097 ± 0.009 [c,d]

[a] Trehalose content expressed in μg of trehalose (mg of dry weight cells)$^{-1}$. [b] Fermentative rate expressed in mL CO_2 (10^7 cells)$^{-1}$ min^{-1}. [c] Averages ± standard deviations of the results at least two independent experiments are shown. [d] Statistical analysis was performed by means of the Student t-test with P-value < 0.05.

778 TABLE 3. GSH/GSSG ratio at different phases of handling and drying of wine yeast
779 T73 to obtain ADY at pilot plant scale.
780

Process step		Drying temperature	
		35 ºC	41 ºC
Cream [a]		5.68 ± 1.71	
Filtered [a]		4.68 ± 0.47	
Extrusion [a]		4.99 ± 0.51	
Drying [b]	8 [b]	3.59 ± 0.62	3.89 ± 0.13
	18 [b]	5.69 ± 2.27	9.04 ± 4.58
	28 [b]	6.87 ± 1.49	N.D.[c]
ADY		7.57 ± 3.87	8.23 ± 3.02

781 [a] Process step previous to drying step. [b] Drying time (min) . [c] non-determined. Averages
782 ± standard deviations of the results at least two independent experiments are shown.
783
784

Fig. S1**. E**xpression of gene markers *HSP12*, *STI1*, *GPD1*, *GSH1* and *TRX2* during yeast biomass drying processes keeping the yeast temperature at 35 ºC (Panel A) or 41 ºC (Panel B) and using three different genes *RDN18*, *ACT1*, *HSP12* for normalization. The mean and the standard error correspond to, at least, two independent experiments.

Fig. S2. Expression of oxidative stress response genes *GRX2*, *GRX5*, *TRR1* and *TSA1* during yeast biomass drying processes keeping the yeast temperature at 35 ºC (Panel A) or 41 ºC (Panel B) and using three different genes *RDN18*, *ACT1*, *HSP12* for normalization. The mean and the standard error correspond to, at least, two independent experiments.

Fig. S3. Northern Blot analysis of *HSP12* gene expression during drying of wine yeast T73 biomass to obtain ADY in two independent experiments (Panels A and B). The yeast biomass was obtained through a lab scale simulation of the industrial propagation process (Pérez- Torrado et al, 2005). Batch and fed-batch cultivations on molasses were performed in a BIOFLO III bioreactor (New Brunswick Scientific) with 5 L of maximal capacity. The different dilutions of molasses media used at the batch and at the fed-batch were 6.1 % (p/v) sucrose and 10 % (p/v) respectively, and supplemented with $(NH_4)_2SO_4$ 0.75 % (p/v), KH_2PO_4 0.35 % (p/v), $MgSO_4 \cdot 7H_2O$ 0.075 % (p/v), biotin 0.5 mg/L, calcium pantothenate 1 mg/L, thiamine hydrochloride 1 mg/L. Desiccation was performed in a convection oven keeping the yeast temperature at 35 ºC to obtain the final ADY product with a moisture content beneath 8 %.