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Relationship between ethanol and oxidative stress in laboratory and brewing yeast strains

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Ethanol is a chemical stress factor that inhibits cellular growth and determines metabolic changes leading to reduction of cell viability during fermentation and yeast storage. To determine the effect of time, temperature and ethanol during storage of brewing yeasts we have monitored viability of cells stored for 72 h, at 6 °C or 12 °C, in the presence of various ethanol concentrations. Under the conditions tested, 6 °C is the most favourable temperature to store brewing yeast creams emphasizing the importance of a tight temperature control in the storage vessels. Because W210 is less resistant to storage in the presence of ethanol than W34/70, the optimal storage parameters obtained under our laboratory conditions vary significantly. The ale strain is sensitive to storage under ethanol concentrations higher than 5% (v/v) for more than 48 h at 6 °C whereas at the same temperature the lager strain tolerates ethanol up to 7.5% (v/v) for 72 h. Also, the viability assays indicate that the antioxidant protein Yap1 is an important factor to storage resistance of BY4741 laboratory strain. To investigate the molecular mechanisms underlying tolerance of brewing yeast strains to ethanol, we have performed phenotypic analysis, localization studies and have monitored the activation of antioxidant and protection genes as well as the intracellular contents of glycogen and trehalose. Overall, our data suggest that the ale strain W210 has a defective antioxidant defence system and that ethanol may induce the antioxidant defences as well as glycogen and trehalose protection mechanisms in laboratory and brewing yeast strains.

[**Key words:** W210; W34/70; Yeast storage; Yap1; Antioxidant responses; Trehalose; Glycogen; *SOD1*; *TPS1*; *GPH1*]

Beer is one of the most widely consumed alcoholic beverages in the world (1). During its manufacturing, brewing yeasts catalyze the conversion of wort sugars to ethanol, carbon dioxide and other minor metabolites.

Depending on the *Saccharomyces* brewing strains used in the fermentation process, beers can be classified as ales or lagers. Ales are the oldest beers in the world and, due to its reduced fermentation time and low cost production process, most of today's craft brews are ales. The strains used to produce ale beers belong to *Saccharomyces cerevisiae* and include microorganisms displaying multiple ploidy and consequently a great genome variability (2). Lagers represent the largest part of beer market. It was recently proposed that brewing *lager* strains were originated from a cross between an *S. cerevisiae* ale strain and the wild *Saccharomyces eubayanus* yeast strain (3). W34/70 is one of the most used strains in European lager beer brewing. The complete sequencing of its genome was deciphered revealing that it is an allopolyploid interspecies hybrid between *S. cerevisiae* and *Saccharomyces bayanus* (1).

Ethanol stress is one of the major environmental stresses generated during the brewing process. Ethanol primarily targets membranes, increasing their fluidity and permeability, thus affecting the transport system of essential compounds such as amino acids and glucose. Its accumulation compromises a wide range of cellular functions leading to the reduction of cell metabolic rate, growth and viability, ultimately promoting a sluggish fermentation (4,5).

The cellular response to the pleiotropic effects of ethanol stress is mediated by the reprogramming of gene expression, including the down-regulation of genes involved in cellular biogenesis/cell growth and the activation of those related to the cell integrity pathway/ergosterol biosynthesis, whose products are required to recover plasma membrane rigidity (6). Moreover, the activation of HSF1 and Msn2/Msn4 transcriptional factors leads to the upregulation of genes encoding the heat shock proteins and controlling trehalose accumulation, such as *TPS1* (4,6–9). Together, both mechanisms are essential to stabilize and prevent aggregation of denatured and misfolded proteins (6,10,11). Besides the regulation of trehalose cytoplasmic contents, Msn2/Msn4 also stimulate

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ethanol-dependent activation of genes encoding enzymes of the glycogen metabolic pathway such as the glycogen phosphorylase (4,12). Furthermore, it has been reported that under mild ethanol stress conditions there is an increase of membrane-bound insoluble glycogen pools, which in turn exerts a protective role on cell wall and plasma membrane (13).

A correlation between ethanol and oxidative stress has been extensively reported in yeasts (4,14–20). Indeed, Qi and Ge have reported the key role of Yap1 in the stress response during the alcoholic fermentation process (21). Yap1 is the prototype member of the Yap family of bZIP transcription factors and the master regulator of oxidative stress responses in yeasts. Its activity is induced in cells exposed to various stress conditions, including H_2O_2, thiol-reactive compounds and metals (22,23). Under these conditions Yap1, which is commonly shuttled between the cytoplasm and the nucleus, is rapidly accumulated in the nucleus, thus triggering the expression of many antioxidant and defence genes including *SOD1*, *TRX2* and *GSH1* (24).

In the present study we evaluated the effect of temperature and various doses of ethanol upon the survival of laboratory, ale and lager brewing yeasts strains stored for 72 h. Also, we have assessed the contribution of Yap1 to this process as well as to the tolerance of brewing yeast growth in the presence of ethanol stress. Besides reporting the most favourable conditions for storing ale and lager brewing strains, our results suggests that Yap1 may be required for ethanol tolerance during storage and growth under laboratory conditions. Moreover, the data here presented indicate that the antioxidant defence mechanisms of W210 may be deficient, as concluded by the similarities displayed by the ale W210 and the *YAP1*-deleted strains. At last, this work also reports the contribution of the antioxidant defences, as well as trehalose and glycogen protection mechanisms, to ethanol stress tolerance of ale and lager brewing yeast strains.

MATERIALS AND METHODS

Bacterial strains and growth media The *Escherichia coli* strain XL1-Blue *recA1 endA1 gyrA96 thi-1 hsdR17 supE44 relA1 lac [F proAB laclqZΔM15 Tn10 (Tetr)]* (Stratagene, Agilent Technologies, USA) was used as the host for routine cloning purposes. Outgrowth was performed in SOC medium [0.5% (w/v) yeast extract, 2% (w/v) tryptone, 10 mM NaCl, 2.5 mM KCl, 10 mM $MgCl_2$, 10 mM $MgSO_4$ and 20 mM glucose] at 37 °C in an orbital shaker at 200 rpm. Cellular growth was monitored by measuring the OD_{600} nm. Transformed *E. coli* cells were selected on LB plates [0.5% (w/v) yeast extract, 1% (w/v) tryptone, 1% (w/v) NaCl, 1.5% (w/v) agar pH 7] supplemented with 100 mg/L ampicillin. Plasmids were recovered from bacterial cells using the NZYMiniprep kit (NZYTech Lda.)

Yeast strains and growth conditions A list of laboratory and industrial yeast strains used in this work is presented in Table 1. Strains were grown in complete YPD [1% (w/v) yeast extract, 2% (w/v) bactopeptone and 2% (w/v) glucose] or synthetic complete media [SC - 0.67% (w/v) ammonium sulfate/yeast nitrogen base without amino acids (Difco), 2% (w/v) glucose, supplemented with the following amino acids/nitrogen bases: Lα-tryptophane (200 mg/L), Lα-histidine HCl monohydrate (200 mg/L), Lα-lysine HCl (300 mg/L) and Lα-leucine (1000 mg/L), Lα-arginine-HCl (20 mg/L), Lα-methionine (200 mg/L), Lα-tyrosine (300 mg/L), Lα-isoleucine (300 mg/L), Lα-phenylalanine, (500 mg/L), Lα-valine (1500 mg/L), Lα-threonine (2000 mg/L), Lα-serine (4000 mg/L), Lα-adenine hemisulfate (20 mg/L) and Lα-uracil (200 mg/L)]. When required, 200 mg/L geneticin (G418) and/or 1.5% (w/v) agar were added to the media. Exponential phase cells were attained through the dilution of overnight cultures to an OD_{600} of 0.1 in fresh SC, followed by incubation at 30 °C (in an orbital shaker at 200 rpm) to an OD_{600} of 0.4. Cells were treated by the addition of ethanol or hydrogen peroxide at the concentrations indicated and samples were collected at the indicated time points in the respective figures. Phenotypic growth assays were carried out by spotting 5 μl of sequentially diluted cultures (approx. 10^3–10^1cells) in synthetic complete media containing up to 10% (v/v) ethanol or 3 mM H_2O_2. Growth was recorded after 2 days at 30 °C. To ensure an accurate ethanol concentration in plates, ethanol was added to liquefied agar medium (approximately at 40 °C) using a fresh 40% (v/v) ethanol stock solution. Ethanol-containing plates were incubated in a lidded container enclosing a beaker with the highest concentration of ethanol tested. To monitor cell growth in liquid media early exponential phase cultures (OD_{600} 0.5) were diluted to 0.1 and were treated with 1 mM H_2O_2. The cultures were incubated during 24 h at 30 °C with orbital agitation (200 rpm) and the OD_{600} was monitored in intervals of 2 h. Prior to each experiment, the strains used in this work were tested to assure that cells did not form *petites*, a common mitochondrial mutation that appears in brewing yeast cells due to prolonged storage and continuous reuse (25). For this purpose, cultures were grown on YPD plates and after 48 h at 30 °C they were replica-plated onto YPG [1% (w/v) yeast extract, 2% (w/v) bactopeptone and 3% (v/v) glycerol]. The cultures unable to grow under non-fermentable conditions were not used.

Viability assays Laboratory and industrial yeast cells were grown on YPD for 48 h at 27 °C and biomass was centrifuged at 5000 g for 10 min. Cells were washed four times with cold water and the resulting yeast biomass was used for the storage experiments. For this purpose a yeast cream containing 4–5 × 10^{11} cells/mL was resuspended in 100 mM acetate buffer pH 4.2 and ethanol was added to a final concentration of 5%, 7.5% or 9% (v/v). Yeast viability was monitored before cell storage and after 24 h, 48 h and 72 h of storage at 6 °C or 12 °C, using the pour plate technique (26). Briefly, the yeast biomass was serially diluted, the samples were pitched in two sterile Petri plates and were mixed with liquefied YPD agar previously cooled to 45 °C. After the agar has hardened, the plates were incubated at 27 °C for two days and the number of colonies from each plate was counted (ISO 4833:2003, Microbiology of food and animal feeding stuffs - Horizontal method for the enumeration of microorganisms; ISO 21527-1:2009). For calculating the number of viable cells in each original sample, plates with a number of colonies between 20 and 200 were selected and values were obtained through the equation ($CFU/mL = \Sigma N/(n_1 + 0.1n_2)^*k$), where N corresponds to the number of colonies grown in the selected plates; n_1 is the number of plates from the first dilution; n_2 is the number of plates from the second dilution; k refers to the dilution coefficient, corresponding to the first dilution chosen for counting (for a dilution Dn, the dilution coefficient is 10^n). The results were expressed as the number of colony forming units (CFU) per mL and interpreted in terms of order of magnitude (27,28).

Cloning of *ScGFP-YAP1* To express *GFP-YAP1* in industrial yeast strains lacking auxotrophic markers, the respective recombinant fusion was first cloned in the centromeric vector pLEJ009 (29), encoding the *KANMX4* gene. For this purpose, the plasmid *cp-GFP-HA-YAP1* (30) was double digested with *Sac*I and *Eco*RI to isolate the fusion *CUP1* promoter-*GFP-HA-YAP1*. The purified fragment was subjected to a DNA polymerase-klenow fragment (Fermentas molecular biology tools, Thermo Fisher Scientific Inc.) fill in of the protruding ends and was subsequently ligated into pLEJ009, previously digested with *Hinc*II. Standard methods were used for cloning (31) and yeast transformation procedures (32).

Fluorescence microscopy To monitor *S. cerevisiae* GFP-(*Sc*)Yap1 subcellular distribution in the industrial and laboratory yeast strains expressing the recombinant plasmid pLEJ-*GFP-YAP1*, cells were grown to early exponential growth phase in YPD medium supplemented with G418 and were induced with 7.5% (v/v) ethanol or 1 mM H_2O_2 for 20 min. DAPI (4′,6-diamidino-2-phenylindole) was added as a DNA marker to a final concentration of 5 μg/ml, 5 min before microscopy. After washing with PBS, cells were resuspended in a solution of 200 mM DABCO (1,4-diazadicyclo [2.2.2]octane) dissolved in 7.5 % (v/v) glycerol and 0.25× PBS (Sigma–Aldrich). GFP signals were analysed in living cells using a Leica DMRXA fluorescent microscope equipped with a Roper Scientific Micro-Max cooled charge-coupled device (CCD) camera and using the MetaMorph software (Universal Imaging Inc.).

Real-time PCR Total RNA isolation and quantitative PCR were performed as described (33). In brief, RNA was extracted from early exponential phase cultures

TABLE 1. List of the laboratory and brewing yeast strains used in this work.

Strain	Taxonomic classification	Genotype	Source
BY 4741	*S. cerevisiae*	*MATa his3Δ1 leu2 Δ0 met15 Δ0 ura3 Δ0*	EUROSCARF
BY4741 *yap1*	*S. cerevisiae*	*MATa his3 Δ1 leu2v0 met15 Δ0 ura3 Δ0 YML007w::kanMX4*	EUROSCARF
W210	*S. cerevisiae*	Polyploid	Weihenstephan Hefebank, Germany
W34/70	*S. pastorianus*	Allotetraploid interspecies hybrid between *S. cerevisiae* and *S. bayanus* 1	Weihenstephan Hefebank, Germany