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Enhanced arginine biosynthesis and lower proteolytic profile as indicators of *Saccharomyces cerevisiae* stress in stationary phase during fermentation of high sugar grape must: a proteomic evidence.

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ABSTRACT

A strain of *Saccharomyces (S) cerevisiae* (ISE19), which displayed an initial good adaptation to a high sugar medium with increased acetate and glycerol production but weak overall growth/fermentation performances, was selected during the alcoholic fermentation of Cortese grape must. To obtain insights into the metabolic changes that occur in the must during growth in particular conditions (high ethanol, high residual sugars and low nitrogen availability) leading to a sluggish fermentation or even fermentation arrest, comparative in-gel proteomic analyses were performed on cells grown in media containing 200 g/L and 260 g/L of glucose, respectively, while the YAN (Yeast Assimilable Nitrogen) concentration was maintained as it was. Two post-translationally different arginine synthases (pI_s 5.6 and 5.8) were found in higher abundances in the high glucose-grown cells, together with an increased abundance of a glycosyltransferase involved in cell-wall mannans synthesis, and of two regulatory proteins (K7_Bmh1p and K7_Bmh2p) that control membrane transport. In parallel, a proteinase K-like proteolytic enzyme and three other protein fragments (Indolepyruvate decarboxylase 1, Fba1p and Eno1p) were present in lower abundances in the high glucose condition, where oxidative stress and cell cycle involved enzymes were also found to be less abundant. The overall results suggest that in stationary phase stress conditions, leading to stuck fermentation, *S. cerevisiae* ISE19 decreases cell replication, oxidative stress responses and proteolytic activity, while induces other metabolic modifications that are mainly based on cell-wall renewal, regulation of the solute transport across the cell membrane and *de novo* arginine synthesis.

Key words: *in-gel*-proteomics, glycolytic enzymes, cell-wall mannans, proteins controlling stress-induced apoptosis, K7_Bmh.

HIGHLIGHTS

- The paper describes what happens at a proteomic level when *S. cerevisiae* ISE19 is grown in high sugar grape musts in a condition leading to a fermentation arrest.
- A decreased abundance of proteolytic enzymes and enzyme fragments was observed in a high sugar condition, in agreement with recent literature data that have reported how the low expression of proteases in glucose-rich musts can cause the arrest of alcoholic fermentation.
- An increased abundance of cell-wall renewal enzymes and of the proteins that regulate membrane transport underline an effective stress response concerning envelope structures.
- Apart from proline, arginine could also play a direct protective role against osmotic stress in *S. cerevisiae* ISE19 (as previously demonstrated for cold stress), since its biosynthetic pathway seems to be activated.

INTRODUCTION

70 During industrial processes, and in particular during alcoholic fermentation, microorganisms
71 encounter several stressors that can compromise their life, their performances and the achievement of
72 satisfactory amounts of the desired end-product. The most common environmental factors that can
73 affect yeasts growth and end-product biosynthesis are must pH, temperature, redox potential and
74 osmolarity (Auesukaree, 2017; Matallana and Aranda, 2017). *Saccharomyces cerevisiae* is the most
75 prominent microorganism involved in wine production. During alcoholic fermentation, the microbial
76 growth phases are characterized by different metabolic events. During the lag-phase, yeast cells adjust
77 their metabolism to adapt to the high initial glucose/fructose concentrations and to be able to grow
78 and convert these sugars into ethanol. One-third of the total ethanol and most of the glycerol amounts
79 are produced in the exponential phase, in parallel with the/an increase in biomass. The remaining two
80 thirds of ethanol are generated in the stationary phase, together with the aromatic compounds that
81 determine the final sensory profile and quality of the wine (Salmon and Barre, 1998). In all these
82 stages, yeast cells undergo stress such as: I) low temperatures (Pizarro et al, 2008), II) osmotic stress
83 (Yale and Bohnert, 2001), III) anaerobiosis (Kwast et al., 2002) IV) lack of nutrients (Boer et al.,
84 2003) and V) ethanol stress (Alexandre et al., 2001).

85 It is well known that a too high osmotic strength (high solute-low solvent concentration) can
86 damage microbial physiology, above all by subtracting the solvation water that stabilizes the tertiary
87 and quaternary structures of proteins (Goldbeck et al., 2001). A salty or sugar-rich environment
88 prevents or delays growth (Hohmann, 2002). Some aspects of microbial responses to osmotic stress
89 have long been elucidated: for instance, salt damage is linked to an altered ionic strength, whereas a
90 high sugar content can cause osmotic stress, without altering the surface charges of the
91 macromolecules (Lages et al., 1999).

92 As far as yeasts are concerned, it has been established that cells exposed to hyperosmotic NaCl
93 concentrations adapt by increasing glycerol uptake inside the cell (Lages et al, 1999), or they die
94 through an apoptotic process (Huh et al, 2003). However, this apoptotic process is the consequence

of an ion disequilibrium rather than of hyperosmotic stress. It has been demonstrated that sugar hyperosmotic stress (70% glucose or sorbitol w/w) can induce both morphological (chromatin condensation along the nuclear envelope, mitochondria swelling, DNA strand breaks) and biochemical (reactive oxygen species (ROS) production, meta-caspase activation) events that lead to cell apoptosis. Cell death is growth-phase dependent, with stationary cells displaying the highest death rate (Silva et al., 2005).

Ethanol stress affects cell viability to a great extent at the final phase of batch fermentation. Toxicity is mainly due to the interaction of ethanol with the cytoplasmic membrane (and, to a lesser extent, to cytosolic enzyme damage). The action of ethanol on both the phospholipid heads and membrane proteins is counteracted by the cell, primarily through membrane-level modifications. Increases in the content of unsaturated fatty acids and ergosterol and reductions in the membrane protein content are the best known yeast responses to ethanol stress (Vanegas et al, 2012). These changes lead to consequences such as: i) variation of the proton flow, which in turn impairs pH homeostasis, ii) reduced capability of Ca^{++} and Mg^{++} translocation, iii) impaired activity of active membrane transporters (Bisson, 1999). The overall result is an alteration of membrane fluidity that strongly affects the trafficking capability of the cell.

In a previous investigation, conducted in the very early stages of fermentation, metabolites and transcripts were evaluated for several yeasts strains grown in a hyperosmotic medium (Noti et al., 2015). A high glycerol and acetate producer, that is *S. cerevisiae (*strain ISE19*)*, was detected. This phenotypic trait correlates with an initial good growth and alcohol production in a sugar rich environment, but then with weaker performances when the simultaneous effects of prolonged hyperosmosis, an increasing ethanol concentration and a low nitrogen content are present. These stressing conditions, which occur in must fermentations with a high sugar content, frequently lead to stuck or sluggish fermentations (Bisson, 1999). The present research is aimed at shedding light on the effects triggered by the late fermentation phase stress on the protein profiles of *S. cerevisiae*

ISE19. An in-gel approach was applied to compare the proteomes of high sugar grown cells and control cells both harvested in stationary phase, in order to identify metabolic indicators of cell stress in the incoming sluggish or arrested fermentation

MATERIALS AND METHODS

Overall strategy

Comparative proteomic analyses of a high glycerol and acetate producer, namely, the *S. cerevisiae* strain ISE 19, which belongs to the CREA-Centro di Ricerca Viticoltura ed Enologia culture collection (Asti, Italy), and which was grown in natural Cortese must with both a regular and a high sugar content, in the advanced phases of fermentation, have been performed.

Fermentation conditions

S. cerevisiae ISE 19 was pre-cultured in YPD at 26°C for 48 h. It was subsequently inoculated, at a concentration of $5*10^6$ cell/mL (total cell), in 0.22 µm filter sterilized Cortese white grape must (800 mL of culture). The main parameters of the must were: pH 3.30, 200 g/L of reducing sugar and 200 mg/L of Yeast Assimilable Nitrogen (YAN). Two different conditions were tested in the comparative proteomic study. The former (ISE19g-) was tested with a regular amount of sugar (200 g/L), and the latter (ISE19g+) was tested by adding glucose/fructose 50% w/w to the must to reach 260 g/L of sugar and by restoring the YAN to 200 mg/L. Fermentations were performed at 20°C without stirring, and three biological replicates were set up. Samples were harvested during fermentation: growth was estimated by optical density (OD) at 600 nm calculating cell/mL by calibration curve done with Bürker chamber count, ethanol and acetate were determined by enzymatic analysis (R-Biopharm AG, Darmstadt, Germany), the residual sugar content (glucose and fructose) and glycerol were evaluated by means of an HPLC equipped with a refractometric detector, using a Rezex RCM-Monosaccharide column (dimension: 300 x 7.8 mm; particle size 8 µm; Phenomenex, Torrance, USA). The adopted conditions were the following: eluent: water; column temperature: 85°C; flow: 0.35 mL/min; injection volume: 20 µL. Samples for the proteomic analyses were

harvested at 175 g/L of consumed sugar(s), at 11 days of fermentation for ISE19g+ and 14 days of fermentation for the ISE19g- test. Samples were pelleted and immediately frozen. At this fermentation stage, residual sugar contents of 25 g/l and of 85 g/l were present in the low and high sugar media, respectively.

Preparation of the protein extracts

Yeast cells (50 mL of about $9*10^7$ total cells/mL) were washed with 50 mL of a 0.85% NaCl solution, centrifuged (5000xg for 20 min) and the supernatant was discarded. Cell lysis was obtained by re-suspending the pellet with 3 mL of lysis buffer (Tris-HCl 50 mM, pH 7.3, EDTA 1mM), and by adding an equal volume of 0.5 mm glass beads to break the cells (10 cycles of 20 minutes on ice, vortexing at maximum speed). At the end of the procedure, centrifugation was carried out (5000xg for 20 minutes at 4°C), and the supernatant was transferred to a new vial for total protein quantification.

Protein quantification was performed using the "2D Quant kit" (GE Healthcare) (UV analysis at 480 nm), according to the manufacturer's instructions. After the preliminary preparation, protein samples were ultra-centrifuged (100000xg for 1 hr. at 4°C) in a Beckman L8-60M Ultracentrifuge (Type 60 rotor). The supernatant containing the soluble proteins was recovered, supplemented with 10 µL/mL of Nuclease Mix (GE Healthcare) and dialyzed/concentrated against four volumes of bidistilled water in 10 KDa membrane cutoff tubes (Amicon), at a final volume of 1 mL. A second quantification was performed, followed by methanol/chloroform protein precipitation, according to Wessels and Flugge (1984). The obtained pellet was then solubilized in a rehydration solution (6.5 M urea, 2.2 M thiourea, 4% w/v CHAPS, 5 mM Tris-HCl, pH 8.8, 0.5% IPG buffer (GE Healthcare), 100 mM DTT). The protein concentration was evaluated again by using the 2D Quant kit (GE Healthcare).

Two dimensional electrophoresis

Isoelectric focusing

Thirteen cm long strips were used, over a 4-7 pH range, for Isolectric Focusing (IEF). Three technical replicates were performed for each of the three biological replicates in the two different fermentation conditions (glucose-enriched and control). About 275 µg of extracted proteins was loaded for each strip. IEF was performed using IPGphor (GE Healthcare) at 20°C, with 66.000 Vh, at 8000 V, after 10 h of active rehydration (50 V).

The IEF strips were then incubated at room temperature for 15 min in a reduction buffer (6 M urea, 30% v/v glycerol, 2% w/v SDS, 50 mM Tris-HCl pH 8.6), and enriched with 10 mM DTT. They were subsequently incubated for 15 min in an alkylation buffer (6 M urea, 30% v/v glycerol, 2 w/v SDS, 50 mM Tris-HCl pH 8.6), and enriched with iodoacetamide 4.5% w/v to alkylate the sulfide groups and prevent re-oxidation during electrophoresis.

SDS-PAGE

After equilibration, the strips were sealed at the top of 1.00 mm vertical second dimension gels with 0.5% of boiling agarose. SDS-PAGE was carried out for each sample on 11.5% T and 3.3% C acrylamide (Biorad) on homogeneous gels. The running buffer was 25 mM Tris, 192 mM glycine, 0.1% SDS, pH 8.3. The running conditions were: 600 V constant voltage, 20 mA/gel, 60 W for 15 min at 15°C, 600 V constant voltage, 40 mA/gel, and 80 W for about 2.5 h at 15°C. The used molecular weight marker was a Low M_r Electrophoresis Calibration Kit (GE Healthcare).

The gels were automatically stained, using Processor Plus (Amersham Biosciences), with freshly prepared Colloidal Comassie Blue stain (Neuhoff et al., 1988). After 12 hours of staining, the gels were dried in a GD 2000 Vacuum Gel Drier System (GE Healthcare).

Image analysis

2-DE gel images were acquired using a SI Molecular Dynamics Personal Densitometer (Amersham Biosciences). Image analysis was performed using the Progenesis PG 220 software (Non Linear Dynamics). Spot detection was automatically performed using the 2005 detection software algorithm.

Statistical analysis

Nine replicates were performed for each 2-DE gel, and for each condition (high and normal sugar contents in the culture media): three analytical replicates were conducted for each biological replicate. Spot intensities were measured, *via* normalized spot volumes, using the "total quantity in valid spot" standardization system. The spot volumes were statistically analyzed by means of the Student's *T-test*: the mean values were considered significantly different when $p<0.05$. Protein spots with a fold change ≥ 2 and $p<0.05$ were selected for MS analysis.

Mass spectrometry analysis and protein identification

The selected spots were identified by means of MALDI-TOF/TOF mass spectrometry, using an Ultraflex III MALDI TOF/TOF instrument (Bruker Daltonics, Bremen). The spots withdrawn from the 2DE gels were destained overnight (with a 50 mM ammonium bicarbonate and 40% v/v ethanol solution), washed three times for 10 min with acetonitrile and then dried in a Speedvac device. The proteins were *in gel* digested with trypsin (Promega, Madison, USA), and their spectra were acquired as described by Zava *et al*. (2009). The MS-Fit software package (http://prospector.ucsf.edu) was used to search against the NCBI 2015.3.10 database, using the peptide mass fingerprinting method (PMF) (Pappin *et al.*, 1993). The following parameters were set for the searches: S-carbamidomethyl derivate on cysteine as a fixed modification, oxidation on methionine as a variable modification and two missed cleavage sites for trypsin digestion. The peptide mass tolerance was 20 ppm.

RESULTS

Fermentation performance

Figure 1 shows the fermentation profiles of the two tested conditions (with or without added sugars), which were obtained by monitoring sugar consumption and ethanol production. The fermentation of Cortese must with the added sugars, initially shows a better performance with a higher daily rate of ethanol production. The conversion of the sugars into ethanol slowed down from the eleventh day of fermentation onwards, until it reached a complete stop on the eighteenth day of fermentation. Stuck fermentation occurred at an alcohol content of about 13.40 vol%, with 36 g/L of residual sugar, mainly consisting of fructose. The fermentation profile of the must not supplemented with sugars was regular, with complete exhaustion of the residual sugars and a final ethanol concentration of about 12% (v/v). The growth kinetics in the two conditions (Fig. 2) showed a higher yeast population in the supplemented must, which reached a maximum of about 120 x 10^6 cell/mL after six days of fermentation, whereas the must without any sugar addition underwent a maximum growth of 100 x 10^6 cell/mL, which was reached after seven days of fermentation. From the 14^{th} day onwards, the yeast population tended to decrease in both sugar conditions reaching similar values on day 21.

Glycerol and acetate production differed in the two tests in relation to the initial sugar content (Fig. 3). In grape must with higher sugar concentrations, yeasts showed an acetic acid production rate constantly higher as compared to the fermentation with regular sugar, reaching 0.60 ± 0.03 (SD) g/L after 7 days. Later, acetate accumulation proceeded slower, showing, at the end of fermentation, concentration of 0.68 ± 0.03 g/L (Fig. 3). Regular sugar test showed a decrease in the acetate production rate after 3 days and reached a maximum of 0.5 ± 0.02 g/L acetate at the end of the fermentation. Similarly, the test with added sugar exhibited a higher concentration also for glycerol that occurs since the beginning of the fermentation and reached 7.0 ± 0.12 compared to a max of 6.30 ± 0.28 in fermentation with regular sugar. However, glycerol showed a more regular biosynthetic rate as compared to the production of acetate and its concentration remains stable under both conditions (high and low sugar) after 9 days of fermentation (Fig. 3)

240 **Comparative proteomic studies**

241 Protein profiles of the high glycerol and acetate producer *S. cerevisiae* ISE 19 grown in regular
242 (ISE19g-) and high (ISE19g+) sugar contents, respectively, have been analyzed at a critical moment
243 of the cell growth, that is, when the simultaneous presence of osmotic stress, ethanol stress and
244 nitrogen depletion occurs. Samples were harvested at the stationary phase (Fig. 2), when 10.5%
245 vol/vol of ethanol was produced in both conditions, whereas the remaining residual sugars were 25
246 and 85 g/L in ISE19g- and ISE19g+, respectively (Fig. 1). Protein concentrations at the end of the
247 extraction process were 9.97±0.71 µg/µL in ISE19g- and 11.78±0.75 µg/µL in ISE19g+.
248 Comparative proteomic analyses were performed on the total cell protein extract (*in toto* proteome)
249 in the acidic pI range (pH 4-7), where most yeast proteins can be found, in order to detect the presence
250 of the differentially abundant proteins.

251 **Protein identification**

252 The 2DE allowed a good protein separation degree to be obtained for each considered sample.
253 Image analysis of the 2DE gels revealed differences in spot intensity in the two experimental
254 conditions. (Figure 4).

255 At first sight, a greater total protein pool abundance in gels loaded with extracts from cells
256 grown in high sugar condition is detectable. Image analysis revealed 28 statistically significant
257 different abundant spots. Three spots were absent in the high sugar condition and four spots were
258 present exclusively in the high sugar condition. Of the remaining spots, 7 were present in higher
259 abundances and 14 in lower abundances in the condition stimulated with a high sugar content. The
260 spot identifications and assignment of the proteins to specific metabolic pathways are shown in Table
261 1.

262 **DISCUSSION**

263 The protein expression profiles of *S. cerevisiae* ISE19, in an advanced stage of must fermentation
264 with different initial sugar concentrations, have been analyzed in this work. The imbalance condition

between the must sugar content and the presence of Yeast Assimilable Nitrogen (YAN) has been recognized as being the primary cause of fermentative arrests, even though the molecular mechanisms involved in sluggish fermentation and the arrest of fermentation are still unclear (Bisson 1999).

In the present study, the fermentation with the higher sugar content initially showed a better performance, both in terms of ethanol production and cell growth, with respect to the control conditions. The difference of 60 g/l in the initial juice sugar load did not result in any extension of the lag phase, thus demonstrating strain response capacity toward the high initial osmotic pressure. At this early stage, the ISE19 strain was probably favored by a higher carbon source availability, whereas the nitrogen concentration was not yet a limiting factor. The stuck fermentation, which occurred at 13.40% ethanol at the end of the stationary phase, was presumably due to the low nitrogen availability, which is known to be a strong limiting factor (Christ et al., 2015). Actually, owing to the higher sugar concentration and the good osmotolerance of this strain, the biomass yield was higher. In this condition, there is an increased need of nitrogen to support growth. Moreover, the concomitant presence of several stressors, that is, a high ethanol content, the osmotic pressure of the residual sugars and the accumulation of toxic compounds, such as medium-chain fatty acids, may have contributed to the slowing down of the fermentative efficiency, as previously suggested by other authors (Lafon-Lafourcade et al., 1984, Borrull et al., 2015). When fermentation arrest was observed, the main sugar present was fructose (data not shown), due to the lower affinity of the hexose transporters (HXT) with fructose and the differences in the efficiency of the hexokinases (HXK) activity toward this sugar observed in yeasts (Bisson, 1999, Berthels et al., 2008).

S. cerevisiae cells react to stress through general and/or specific response mechanisms. Global changes in the majority of transcripts have been observed during the fermentation process of wine making, thus highlighting a general adaptive response to the typical stressors of alcoholic fermentation (Marks et al., 2008). In the early fermentation stages, the greatest environmental constraint is osmotic stress, which generates a water flow from inside to outside the cytoplasmic

membrane. Later, the yeast cell adapts itself through multi-level specific response mechanisms (Hohmann, 2002; Yanget al., 2006, Zuzuarregui et al., 2006; Tapia et al., 2015). However, the main response of yeast cells to high-osmolarity is the production of glycerol, the best osmotic balance stabilizer (Hohmann, 2002). Glycerol accumulation has been shown to account for 95% of the internal osmolarity recovery (Reed et al., 1987).

It should be underlined that, from a metabolic standpoint, glycerol can originate from glycolysis, at the expense of the biomass, by diverting dihydroxyacetonephosphate (DHAP) from the glycolytic flux after sensing altered osmolarity (Remize et al., 2003; Capaldi et al., 2008), thus performing the so-called glyceropyruvic fermentation (Fig 5). However, during hyperosmotic stress, yeasts can also enhance the intracellular glycerol content by limiting the glycerol flux outside the cytoplasmic membrane with closure of aquaglyceroporin Fps1 channels (Oliveira et al., 2003) and activating synthesis of glycerol transporters encoded by the *STL1* gene (Petelenz-Kurdziel et al., 2013). These proton symport systems can enhance the active uptake of external glycerol, which in turn is used both as a carbon source and during adaption to osmotic stress (Ferreira et al., 2005) and temperature (Tulha et al., 2010). Transcriptional and non-transcriptional mechanisms are involved in these flux regulations (Hohmann, 2009; Ahmadpour et al., 2014). Along with the osmo-adaptation mechanism, glycerol production during fermentation is the result of the redox balance control mechanism set up to oxidize the excess of NADH produced in the formation of 1,3-diphosphoglycerate starting from the 3-phospate glyceraldehyde in glycolysis (Ansell et al., 1997). On the other hand, in order to balance the excess of NAD+ generated by the initial formation of the osmo-protective glycerol, the cell utilizes NAD + dependent *aldehyde dehydrogenases*, synthesizing acetate (Navarro-Aviño et al., 1999, Pigeau and Inglis, 2005). In the present investigation, the higher glycerol and acetate production observed in must with added sugar reflects the mechanism for adaptation to hyperosmosis and the successive redox regulation during alcoholic fermentation according to the mechanisms outlined above.

No apparent modifications concerning the glyceropyruvic pathway (Fig 4) were observed, at a proteomic level (Tab.1), after prolonged exposure of *S. cerevisiae* ISE 19 to high sugar environment in a condition of incoming sluggish and stuck fermentation. As far as the functional state (open or closed) of the aquaglyceroporin Fps1 channels is concerned, no conclusion can be reached from the proteomic studies. However, the regulatory proteins K7_Bmh2p (spot 1687B) and K7_Bmh1p (spot 1689B), which control the formation of the vesicles involved in transport and exocytosis, were found to be present, but only in the hyperosmotic condition. These proteins, which belong to the 14-3-3 gene family, are also involved in the protection against stress-induced apoptosis, and they play an important role in the post-transcriptional control of yeast proteins (Clapp et al., 2012).

Peculiar results concerning glycolysis are worth discussing. Three enzymes of the alcoholic fermentation pathway were present, with higher abundance in the sugar-stimulated condition: fructose biphosphate aldolase (Fba1p) (spot 1415B), phosphopyruvate hydratase (Eno 2 p) (spot 1378B), and pyruvate decarboxylase (Pdc1p) (spots 1331B, 1564B and 1569B). The latter enzyme, converting pyruvate into acetaldehyde and having TPP as a cofactor, was present in three spots displaying different isoelectric points (6.1, 5.8 and 5.9 respectively) and the same molecular weight, thus suggesting that they are the result of post-translational modifications of the same gene product. As far as the other two enzymes are concerned, the higher abundance of Fba1p can be exploited to supply more DHAP for glycerol biosynthesis whose greater production has actually been observed in yeasts during high sugar fermentation (fig. 3b). The higher abundance of Eno2p is in agreement with the fact that Eno2p is the true glycolytic enzyme, and that glucose can act as an inducer of its synthesis, as previously observed (Kornblatt et al., 2013).

Other enzymes involved in glycolysis/alcoholic fermentation are present in lower abundances in high osmotic condition-grown yeasts. The low level of enolase Eno1p (spot 1434A) (25 fold less abundant in high glucose) is consistent with the known repression on the encoding gene exerted by glucose (Kornblatt et al., 2013). Previous studies showed that Eno1p (as well as the oxidative alcohol

dehydrogenase ADH_2) is more abundant in yeast cells grown in media containing ethanol, thus suggesting a gluconeogenic rather than a glycolytic role (Futcher et al., 1999). Two triose-phosphate isomerase Tpi1 (spot 1519A) and Tpi1p (spot 1515A) enzymes, with different entry codes (NP_010335 and EGA87556 respectively) (probably the products of two different genes), were present in low abundance. Triose phosphate isomerase has the function of maintaining a correct balance between glyceraldehyde phosphate (GAP) and DHAP (derived from fructose 1.6 biphosphate), and of supplying glycolysis with GAP. A part of the DHAP is probably necessary, in the hyperosmotic condition, to feed the glyceropyruvic route (Fig.5), hence triose phosphate isomerase has to work at a basal level to prevent all the DAPH from being converted into GAP. Three spots (1396A 1400A and 1419A), corresponding to oxidative alcohol dehydrogenases (ADHs), were found to be of low abundance in high glucose-medium grown cells. The inhibition of oxidative ADHs, together with the enhanced abundance of pyruvate decarboxylase, suggests the activation of alcoholic fermentation.

The overall results concerning glycolysis (in the cells grown in the hyperosmotic medium) suggest: i) an initial activation of the glycolytic flux, due to an enhanced abundance of fructose 1,2 biphosphate aldolase, ii) a slight slowing-down of glycolysis, due to a decreased abundance of triose-phosphate isomerase, which probably favors glycerol synthesis and iii) an activation of the conversion of 2-phosphoglycerate to PEP through the enhanced biosynthesis of Eno2p (the glycolytic enzyme) and a decreased abundance of Eno1p (the gluconeogenic enzyme). A stimulation of the alcoholic fermentation in the hyperosmotic medium was obtained, as proved by the enhanced abundance of pyruvate decarboxylase and lower abundance of oxidative alcohol dehydrogenases. It is possible to hypothesize that alcoholic fermentation proceeds, even in a relatively lowered glycolytic flux, by exploiting also the pyruvate that comes from other paths.

Three fragments originating from Pdc1p (spot 1354A), Fba1p (1500A) and Eno1p (1507A) were found to be less abundant or totally lacking in a hyperosmotic medium. Since these fragments

365 are the result of a proteolytic action over the native protein, this finding suggests that proteolysis
366 occurs to a lesser extent in high glucose conditions. As a support to this, the only proteolytic enzyme,
367 Proteinase K (spot 1494A) was found in a lower abundance in the high-sugar medium grown cells.
368 These results are in agreement with a recent report that suggests that the low expression of proteases
369 in glucose-rich musts can cause fermentation arrests, due to the lack of nitrogen scavenger activity
370 exerted by proteolytic enzymes (Szopinska et al., 2016). This is probably what also occurs for *S.*
371 *cerevisiae* ISE 19.

372 Erasmus et al. (2003) reported an up-regulation of genes for glycolysis and for the pentose
373 phosphate pathway, whereas genes involved in the *de novo* biosynthesis of purines, pyrimidines,
374 histidine and lysine were down-regulated during hyperosmotic stress. These findings have also been
375 confirmed at a proteomic level (Pham and Wright, 2002).

376 In the present work, no modifications have been observed in the biosynthetic pathway of
377 histidine. However, the enzymes involved in amino acid biosynthesis displayed fold changes in the
378 high glucose-grown cells. In fact, diaminopimelate epimerase (Yhi9p) (spots 1490A and 1493A), a
379 piridoxalphosphate (PLP)-dependent isomerase involved in the so-called aspartate pathway leading
380 to the synthesis of lysine, displayed a low abundance in the hyperosmotic medium, in agreement with
381 the data reported above (Pham and Wright, 2002). On the contrary, two post-translationally different
382 arginine-succinate synthases (Arg1p) (spot 1358B, pI 5.8 and spot 1577B pI 5.6) were more abundant
383 (up to 10 fold) in the high sugar condition. Xu and co-workers (2011) demonstrated that arginine
384 could protect the yeast *Candida glabrata* during hyperosmotic stress. Both imported (extracellular)
385 arginine and *de novo* synthesized arginine lead to a higher biomass production (measured as dry cell
386 weight). However, these authors also observed the enhanced transcription of genes encoding enzymes
387 for arginine biosynthesis, thus suggesting that the second mechanism (*i.e. de novo* synthesis) is
388 prevalent. Although a cryoprotective effect of arginine on the freezing stress of *S. cerevisiae* has been
389 reported (Morita et al., 2002), the present proteomic results are the only ones that have found that

arginine is a good osmoprotectant in *S. cerevisiae* ISE19 as well as in *Candida glabrata*. Furthermore, our results support the observations of Gutierrez et al. (2012, 2015), who demonstrated that arginine exerted a positive effect (compared to other amino acids) on the growth and fermentation rate of *S. cerevisiae.* It is possible that arginine exerts this beneficial effect on growth not only because of its nutritional role as a nitrogen source, but also because of its osmoprotectant effect. On the other hand, arginine is one of the most abundant amino acid present in must (Gutierrez *et al*., 2015), and it could have contributed to an evolutionary selection of *S. cerevisiae* strains in this ecological niche.

A glycosyltransferase, involved in the synthesis of cell-wall mannans (YJR075Wp-like protein) (spot 1688B), was only present in the high glucose concentration, thus suggesting the need of cell-wall processing and renewal during hyperosmotic stress. This is to be expected, since the cell-wall of *S. cerevisiae* is an elastic and dynamic structure responsive to changes in the external environment, which provides osmotic and physical protection to the yeast cell. Changes the cell-wall mannan structures under osmotic stress have been previously observed in yeasts (Koyama et al. 2009). On the other hand, a unique oligosaccharide modification, mannosylphosphorylation, has been demonstrated in the cell-wall mannoproteins of *S. cerevisiae* during cellular response to a variety of stresses, among which osmotic stress, which strongly enhances mannosyl phosphorylation (Odani et al., 1997). These authors hypothesized that mannosylphosphate transfer, which gives a net negative charge to the cell-wall, allows the formation of a hydration shell on the cell surface, resulting in yeast cell protection from high solute stress. Whether this mechanism is also operative in *S. cerevisiae* ISE19 during osmotic stress still has to be elucidated.

An inverse correlation was found between osmotic stress and the abundance of oxidative-stress related enzymes, such as catalase T (Ctt1p) (spot 1326A and spot 1743A). Szopinska et al. (2016) have recently reported that the highest transcription rate of genes involved in antioxidant activities can be found when all the fermentable sugars have been depleted. The present findings are in agreement with these observations, and confirm that oxidative stress is a statistically rare event when sugars are abundant and hence not completely consumed.

CONCLUSIONS

Alcoholic and glyceropyruvic fermentation are closely linked throughout winemaking. On average, 8% of must sugars undergoes glyceropyruvic fermentation, while the remaining part is converted to ethanol. The ability to produce glycerol varies according to the yeast strain, and can therefore affect the percentage of glycerol and ethanol formed by the same amount of fermented sugars. The present proteomic results on *S. cerevisiae* ISE 19 have not revealed any direct activation of the glyceropyruvic pathway at stationary phase after prolonged exposure to a hyperosmotic environment, in a condition leading to stuck fermentation, although the catalytic activation of the enzymes involved in glycerol synthesis cannot be excluded. On the other hand, an overall enhancement of the abundance of the proteins involved in alcoholic fermentation, cell-wall synthesis, aromatic amino acid as well as arginine biosynthesis has been detected. No apparent increase in abundance of stress proteins has been observed and proteolysis seems to be negatively modulated in hyperosmotic conditions.

Understanding how osmotolerant yeasts are able to survive and adapt to critical conditions will allow a better biomass yield and an overall better alcoholic fermentation to be obtained. Moreover, the elucidation of the cytotoxic effects, induced by high glucose concentrations, could contribute to the optimization of the industrial fermentative yeast performance under hyperosmotic stress.

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CONFLICTS OF INTEREST/ETHICAL CONCERNS

441 The authors declare that there are no conflicts of interest and no ethical issues (no animal or human
442 involvement) in the experiments performed in the present investigation.
443

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589 **CAPTIONS TO FIGURES**

590 Figure 1. Fermentation performance, referring to ethanol production versus glucose consumption, in
591 the ISE19 strain on Cortese grape must with regular (black line) and high (grey line) sugar contents.
592 Error bars represent standard errors of the mean (n=3).

593 Figure 2. Growth kinetics of the ISE19 strain in Cortese grape must with regular (black line) and high
594 (grey line) sugar contents. Error bars represent standard errors of the mean (n=3).

595 Figure 3. Acetate and glycerol production during fermentation by strain ISE19 in Cortese grape must
596 with regular (black line) and high (grey line) sugar content. Error bars represent standard error of the
597 mean (n=3).

598 Figure 4. Representative 2DE images of intracellular soluble proteins of *Saccharomyces cerevisiae*
599 ISE 19 grown under a regular sugar condition (200 g/L) (on the left) and with the addition of
600 supplementary sugar (260g/L) (on the right).

601 Figure 5. Schematic representation of the glyceropyruvic fermentation pathway.

602

603 **Tab.1. Protein identification.** B: proteins more abundant in the high glucose medium. A: proteins more abundant in the

604 regular glucose medium

Spot n°	Fold changes	Name of the protein and acronym (if it exists)	Protein Entry (NCBI 2015.3.10)	Number of peptides/number of total signals	Sequence coverage by PMF	Molecular weight (Da) (Theoretical/experimental)	pI (Theoretical/experimental)
1331B	4	Pyruvate decarboxylase isozyme 1, Pdc1p	EGA57763	10/56	24%	61542/56000	5.8/6.1
1358B	2	Argininosuccinate synthase, Arg1p	EGA76938	14/51	36.6%	46455/47000	5.7/5.8
1378B	2.5	Phosphopyruvate hydratase, Eno2p	NP_012044	16/55	44.4%	46915/45000	5.7/6.2
1415B	3.5	Fructose-bisphosphate aldolase, Fba1p	EGA61531	12/58	43.5%	39607/40000	5.5/5.9
1564B	5	Pyruvate decarboxylase isozyme 1, Pdc1p	EGA57763	15/63	33%	61542/53000	5.8/5.8
1569B	2	Pyruvate decarboxylase isozyme 1, Pdc1p	EGA57763	18/74	41.7%	61496/58000	5.8/5.9
1577B	10	Argininosuccinate synthase, Arg1p	EGA76938	17/56	45.8%	46455/46500	5.7/5.6

1663 B	Only in B	phenylalanyl-tRNA synthetase beta subunit , Frs1p	AAT92797	9/25	20.3%	67332/70000	5.5/5.8
1687 B	Only in B	14-3-3 family protein, K7_Bmh2p	GAA22335	9/22	38.6%	30933/35000	4.8/4.5
1688B	Only in B	YJR075Wp-like protein	EDZ71169	4/33	11.7%	30721/32000	6.1/6.0
1689B	Only in B	14-3-3 family protein, K7_Bmh1p	GAA23007	13/30	47.9%	30205/35000	4.9/4.6
1306A	3	Methionine synthase, Met6p	EDN63067	27/58	39.4%	85831/85000	6.1/6.5
1326A	62	Catalase T, Ctt1p	AJP38866	9/76	21.4%	64560/64000	6.2/6.3
1354A	2.5	Pyruvate decarboxylase isozyme 1, Pdc1p (fragment)	EGA57763	12/54	24.7%	61496 /51000	5.8/5.9
1396A	2	Alcohol dehydrogenase, ADH1	AAA34410	10/35	31.3%	36823 /40000	6.3/6.5
1400A	2	Alcohol dehydrogenase, ADH1	AAA34410	9/61	24.4%	36823/40000	6.3/6.3
1419A	4	Alcohol dehydrogenase, ADH1	AAA34410	6/41	18%	36837/40000	5.9/5.6
1434A	25	Enolase, Eno1p	EGA82740	11/27	40.1%	41709/38000	6.2/6.5
1485A	Only in A	S-adenosylmethionine synthetase, Sam1p	EGA73728	5/49	29.5%	28481/33000	6.0/6.0
1490A	Only in A	Diaminopimelate epimerase, Yhi9p	EGA58492	7/32	31.6%	32186/31000	5.5/6.2

		Ssf2p	EGA83416	4/32	18.2%	32878/31000	6.2/6.2
1493A	8.5	Inorganic pyrophosphatase, Ipp1p Diaminopimelate epimerase,Yhi9p	AJP83924 EGA58492	8/43 5/43	37.3% 24.7%	32284/31000 32186/31000	5.4/5.8 5.5/5.8
1494A	2.5	Peptidases_S8_PCSK9_ProteinaseK_like (from YEL060Cp-like protein aa 130-406)	EDZ72718	6/34	12.8%	29297/31000	6.8/6.6
1500A	3.5	Fructose-bisphosphate aldolase, Fba1p fragment (starting from aa 1)	EGA61531	7/42	20.3%	39607/30000	5.5/5.5
1507A	Only in A	Enolase, Eno1p fragment (starting from aa 116)	EGA82740	7/50	20%	29715/30000	5.3/5.1
1513A	5	YAL034W-Ap-like protein	EDZ74019	6/42	32.9%	29537/28000	6.1/5.8
1515A	2	Triose-phosphate isomerase, Tpi1p	EGA87556	3/39	18.3%	27305/27000	6.3/6.3
1519A	2	Triose-phosphate isomerase, Tpi1	NP_010335	10/48	49.2%	26796/27000	5.7/5.9
1743A	5.5	Catalase T, Ctt1p	EDV10159	13/44	27.4%	65696/63000	6.2/6.6

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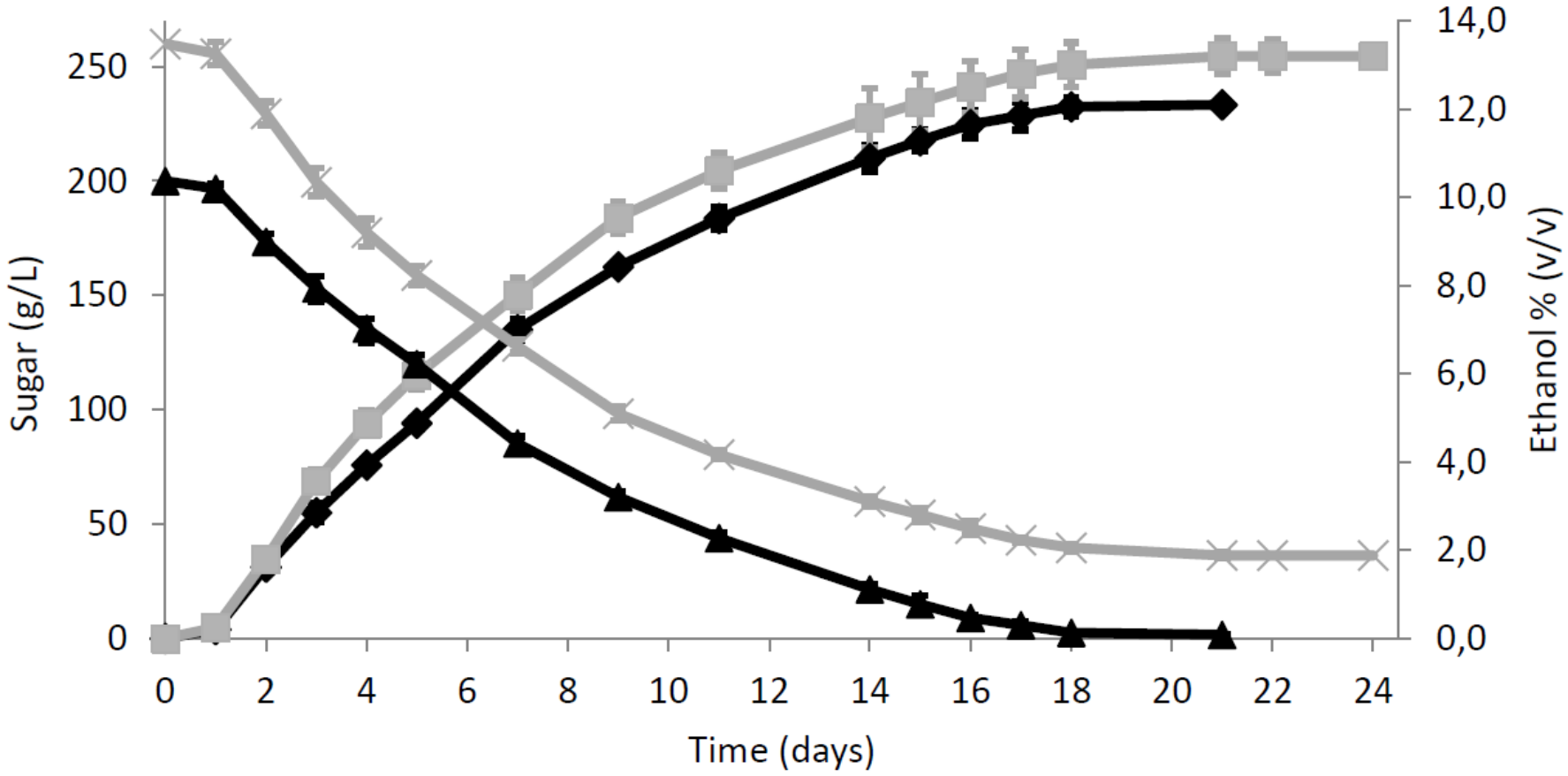
607 Fig. 1



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609 Fig, 2

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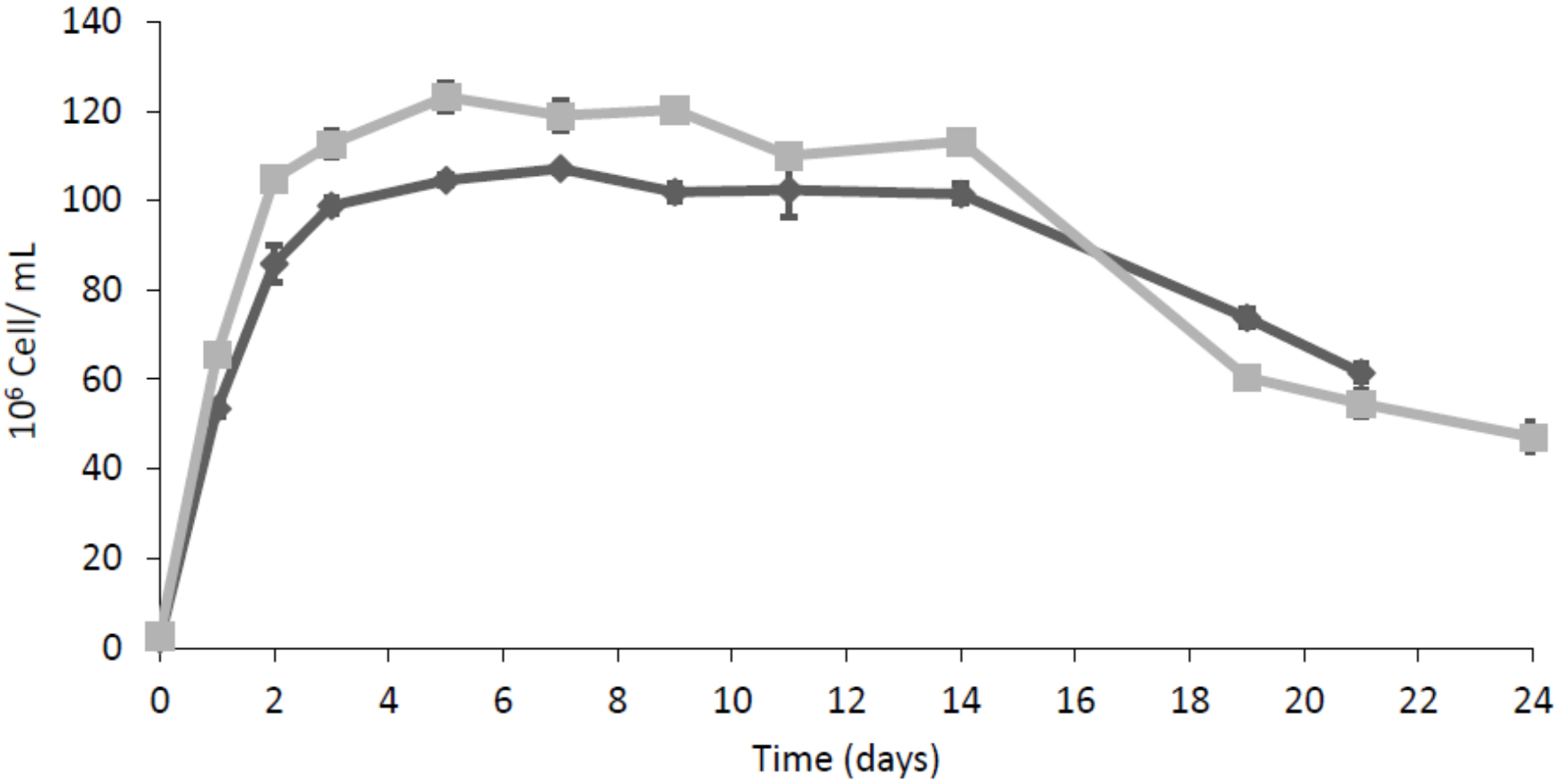

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613 Fig. 3

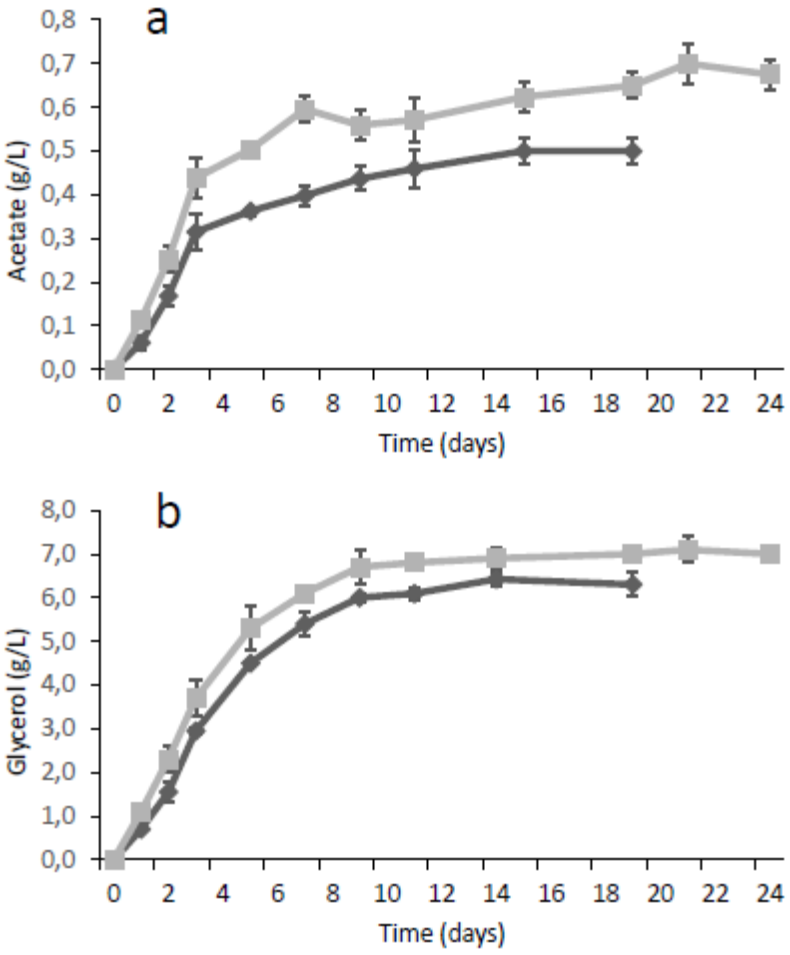

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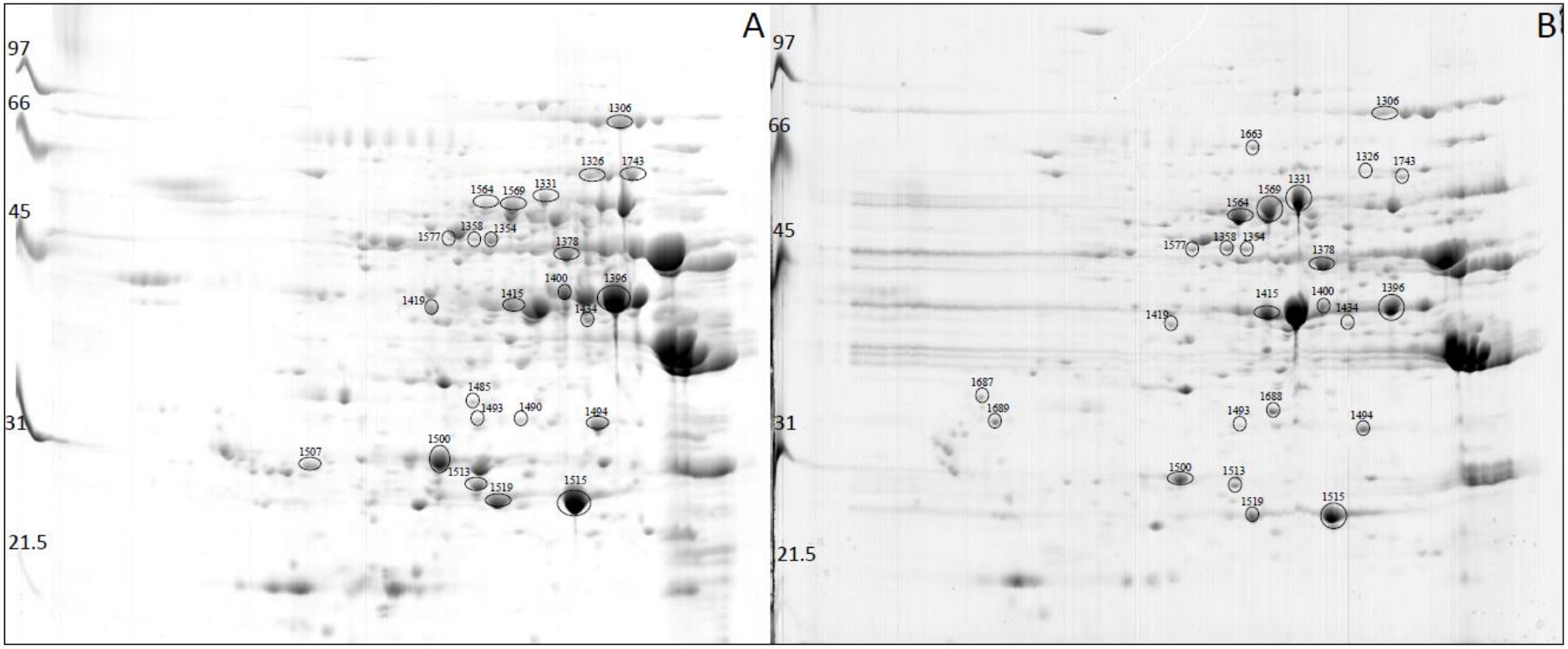
Fig. 4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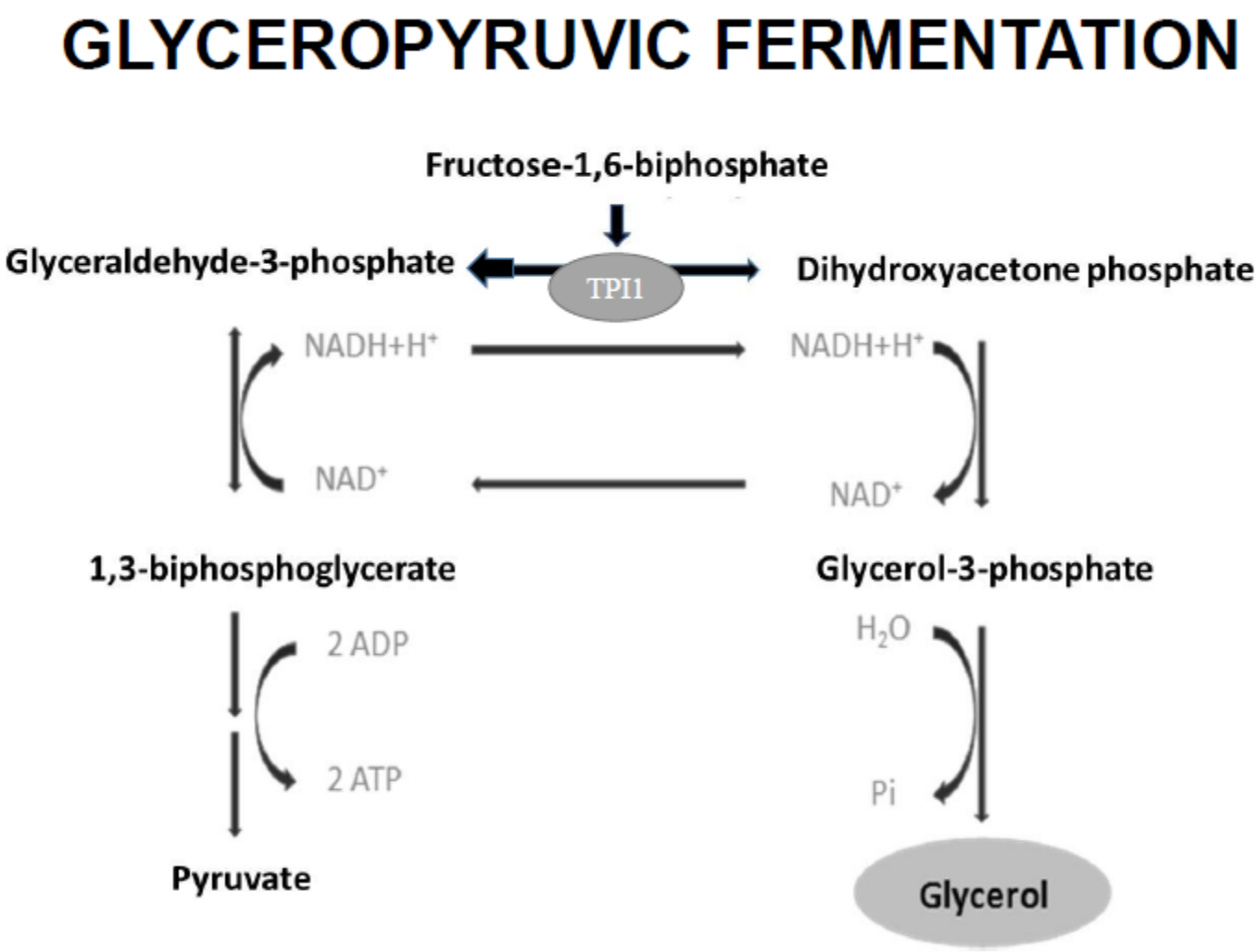
621 Fig. 5


GLYCEROPYRUVIC FERMENTATION
Fructose-1,6-biphosphate
Glyceraldehyde-3-phosphate
TPI1
Dihydroxyacetone phosphate
NADH+H+
NADH+H+
NAD+
NAD+
1,3-biphosphoglycerate
Glycerol-3-phosphate
2 ADP
H2O
2 ATP
Pi
Pyruvate
Glycerol

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HIGHLIGHTS

- The paper describes what happens at a proteomic level when *S. cerevisiae* ISE19 is grown in high sugar grape musts in a condition leading to a fermentation arrest.
- A decreased abundance of proteolytic enzymes and enzyme fragments was observed in a high sugar condition, in agreement with recent literature data that have reported how the low expression of proteases in glucose-rich musts can cause the arrest of alcoholic fermentation.
- An increased abundance of cell-wall renewal enzymes and of the proteins that regulate membrane transport underline an effective stress response concerning envelope structures.
- Apart from proline, arginine could also play a direct protective role against osmotic stress in *S. cerevisiae* ISE19 (as previously demonstrated for cold stress), since its biosynthetic pathway seems to be activated.