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Proteomic yeast stress response to pressure in a final stage in the second fermentation during sparkling wine elaboration

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Abstract. Spanish sparkling wine or cava (Certified Brand of Origin) elaborated following the "champenoise" method undergoes a second fermentation in closed bottles of base wine, followed by aging of wines with lees of at least 9 months. Both processes are considered as important factors contributing to the quality of cava. During the second fermentation, yeasts are subjected to pressure in which the response of the yeast cells have not been still clearly elucidated. The objective of this study is to identify proteins that may participate in the response to pressure. OFFGEL fractionator coupled to LTQ Orbitrap XL MS equipment were used trying to detect the maximum possible number of proteins in yeasts grown in a traditional second fermentation condition and under a reference condition not subjected to pressure. The obtained proteomic profiles show 679 proteins detected under the first condition while 979 under the reference condition. From the total number of proteins identified under the second fermentation with pressure, 251 were just detected under it being mainly ribosomal and extracellular; and involved in biological processes such as ribosome assembly, cytoplasmic translation or organelle assembly. The cellular components and biological processes mentioned in this study may be essential for the fermenting yeast survival in a condition such as second fermentation during sparkling wine elaboration. Genetic experiments are needed to definitively confirm the necessity of these proteins synthesis under pressure.

1. Introduction

Sparkling wine elaboration by the champenoise or traditional method (like champagne in France and cava in Spain) involves two main steps. First, a fermentation where the grape must is converted to wine and second, a process called "prise de mousse", that take place in closed bottles, which comprises a second fermentation and a period of aging when yeast cells die and undergo autolysis. "Prise de mousse" process results in the production of a range of compounds that characterize the organoleptic quality of these types of wines: increases alcohol content, carbon dioxide pressure, minor secondary products and yeast intracellular compounds [1–5].

Under second fermentation conditions yeast growth and metabolism are affected by several stress factors, either common to all wine-making styles (high ethanol content of the base wine, nitrogen starvation and low pH) or specific to second fermentation like low temperature and CO_2 overpressure [6].

A transcription profile performed by Penacho et al. (2012) [6] did not point out CO_2 overpressure stress factors as relevant constraints for the adaptation of wine yeast cells to sparkling wine production. However, Debs-Louka et al. (1999) [7] who studied the effect of compressed carbon dioxide on microbial cell viability, reported a linear correlation between microbial inactivation and CO_2 pressure and exposure time.

For the first time, in this work a yeast proteome analysis was performed in order to characterize the stress response to CO_2 gas overpressure in an isolated way within a sparkling wine second fermentation context. A better knowledge of yeast stress response might facilitate the development of effective strategies for improve yeast survival and further organoleptic quality of these special wines.

2. Materials and methods

Saccharomyces cerevisiae P29 strain (CECT 11770), was used as starter culture. This strain was inoculated in a pasteurized must as fermentation substratum. Later, when high cell concentration and high viability were attained, the "tirage" was made to carry out the second fermentation. Standard 750 ml sparkling wine bottles were filled with the fermenting mixture: a base wine consisting in a mixture of Macabeo and Chardonnay (6:4) plus sugar at 22 g/L and 1.5×10^6 cells/mL.

Half of the bottles were closed with a plastic lid (bidule) and metal overcap (pressure condition or PC) while in the remaining were placed a perforated bidule (non-pressure condition or NPC) in order to avoid CO_2 accumulation and overpressure. PC bottles were positioned horizontally while NPC bottles vertically, both in a thermostated chamber at 14°C.

Control fermentation kinetics was performed by recording the increase in pressure inside the bottles with an aphrometer. Once the pressure reached the maximum (between 6 and 7 bars) and stabilized, bottles were opened and processed to obtain samples for proteome analysis. For counting the viable population, three bottles were randomly drawn and after homogenizing the content, appropriate dilutions with Ringer solution were made and plated with Sabouraud-chloramphenicol agar medium. Incubation was performed at 28°C for 48 h after which the colony counting was performed.

Samples for proteome analysis were sedimented and cleaned 2 times with abundant cold sterile distilled water. Resulting pellets were resuspended in 1 mL extraction buffer supplemented with Protease Inhibitor Cocktail tablets, and cell walls were broken by vortexing in a Vibrogen Cell Mill. Glass beads as well as cell debris were discarded by centrifugation. Protein precipitation was carried out by overnight incubation at –20°C after addition of 10% w/v of trichloroacetic acid (TCA) and 4 volumes of ice-cold acetone to the supernatant. After incubation, samples were centrifuged and the protein pellet was vacuum dried and then resuspended in solubilization buffer. Protein concentration was estimated by Bradford assay (1976) [8] and samples stored at –80°C until proteins analysis.

The OFFGEL High Resolution kit pH 3–10 was used for protein preparative isoelectric focusing (IEF) in solution. Protein samples (450 ± 50 µg) were solubilized in Protein OFFGEL fractionation buffer, glycerol, and buffer with ampholytes and aliquots evenly distributed in a 12-well 3100 OFFGEL Fractionator tray. Preset program OG12PR00 separation limits were used following recommendations of the manufacturer: 4500 V, 200 mW, and 50 µA; starting voltage, 200–1500 V; ending voltage, 5000–8000 V; after the application of 20 kVh, the protein separation zones were maintained at constant voltage. Peptides from each well were scanned and fragmented with the LTQ Orbitrap XL mass spectrometer equipped with a nano LC Ultimate 3000 system. The electrospray voltage was set to 1300 V and the capillary voltage to 50 V at 190ºC. The LTQ Orbitrap was operated in the parallel mode, allowing for the accurate measurement of the precursor survey scan (400–1500 m/z) in the Orbitrap selection, a 60,000 full-width at half-maximum (FWHM) resolution at 400 *m/z* concurrent with the acquisition of three CID Data-Dependent MS/MS scans in the LIT for peptide sequence, followed by three Data-Dependent HCD MS/MS scans (100–2000 *m/z*) with 7500 FWHM resolution at 400 *m/z* for peptide sequence and quantification. The normalized collision energies used were 40% for HCD and 35% for CID. The maximum injection times for MS and MS/MS were set to 50 ms and 500 ms, respectively. The precursor isolation width was 3 Da and the exclusion mass width was set to 5 ppm. Monoisotopic precursor selection was allowed and singly charged species were excluded. The minimum intensity threshold for MS/MS was 500 counts for the linear ion trap and 8000 counts for the Orbitrap. Database search was performed with Proteome Discoverer 1.0 (Thermo Fisher Scientific software, San José, CA, USA) against Uniprot including fixed modification Carbamidomethylation in Cys and proteome results were statistically analyzed with the Proteome Discoverer program.

In order to describe in an overall view proteins specifically detected under the pressure condition in terms of their localization and processes in which they participate, a GO Term analysis was performed by using SGD (`http://www.yeastgenome.org/`). Frequency values have been calculated by dividing the number of proteins annotated with a GO Term by the total number of PC specific proteins. If a GO Term which frequency value overpass the frequency value of that GO Term within the *S. cerevisiae* total number of proteins, it is considered as significant. At last, the tool "GO Term finder" from SGD was used to determine the *p*-value for each annotation, which is the probability or chance of seeing at least "*x*" number of genes out of the total "*n*" genes in the list annotated to a particular GO term, given the proportion of genes in the whole genome that are annotated to that GO Term.

3. Results and discussion

At the sampling time, the gas pressure attained under PC were 6.5 atm when yeast viability reached $3.33 \times 10^4 \pm 1 \times 10^4$ cells/mL while in the reference non-pressure condition this value was up to $1.14 \times 10^6 \pm 4 \times 10^5$ cells/mL. Further, a high qualitative difference could be observed in terms of the proteome response (Fig. 1).

High frequencies of PC specific proteins were ribosome and extracellular region-localized proteins, overpassing by 3.25- and 3-fold *S. cerevisiae* frequencies, respectively. With regards to biological processes, "ribosome assembly", "cytoplasmic translation" or "organelle assembly" GO Terms were those that gathered highest frequencies of proteins. All these commented concepts besides "extracellular region" are directly related to the synthesis of proteins (Table 1).

Both cytosolic and mitochondrial ribosomal proteins (RPs) were identified, 33 and 9, respectively. Taking into accout *p*-values, these proteins may participate in processes like cytoplasmic translation, ribosome biogenesis as well as mitochondrial translation (*p*-values

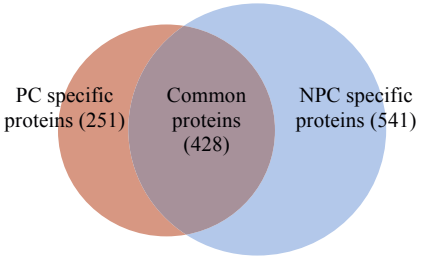

Figure 1. Venn diagram showing proteins detected under the pressure condition (PC), under the reference non-pressure condition (NPC) and detected under both conditions.

Table 1. GO Terms of PC specific proteins which frequency value overpass the frequency value of that GO term within the *S. cerevisiae* total number of proteins.

GO term	Frequency (%)	Genome frequency (%)	Frequency ratio	Protein(s)
Ribosome	17.9	5.5	3.25	Rps8ap, Rpl23ap, Rps9bp, Img2p, Tma17p, Rps11ap, Rsm24p, Mrp1p, Rpl12ap, Rpl30p, Rpl7ap, Lsg1p, Rps2p, Rpl26bp, Ygr054wp, Rpl34bp, Rsm25p, Rps21bp, Rpl39p, Rps22ap, Tma22p, Rpl14ap, Tef4p, Rps27ap, Rpl8bp, Rps31p, Rpl37ap, Rps28bp, Rps30ap, Rpl38p, Rps29ap, Rpl31bp, Rps1ap, Yml6p, Rpl6ap, Sis1p, Rpl9bp, Nam9p, Rps3p, Mrpl50p, Rsm19p, Rpl3p, Rpl5p, Mrpl40p, Rpl43ap
Extracellular region	1.2	0.4	3.00	Cis3p, Ape2p, Ygp1p
Ribosome assembly	4.8	0.9	5.33	Rps11ap, Rpl12ap, Lsg1p, Nsr1p, Rps27ap, Rps31p, Rpl6ap, Ria1p, Esf2p, Rex4p, Rpl3p, Rpl5p
Cytoplasmic translation	12.7	2.7	4.70	Rps8ap, Rpl23ap, Rps11ap, Gir2p, Tif35p, Rpl12ap, Rpl30p, Rpl7ap, Rpl26bp, Rpl34bp, Rps21bp, Rpl39p, Rps22ap, Rpl14ap, Rps27ap, Rpl8bp, Rps31p, Rpl37ap, Rps28bp, Rps30ap, Rpl38p, Rps29ap, Rpl31bp, Rps1ap, Rpl6ap, Rpm2p, Tif34p, Rpl9bp, Rps3p, Rpl3p, Rpl5p, Rpl43ap
Organelle assembly	6.8	1.9	3.58	Rps11ap, Skp1p, Rpl12ap, Lsg1p, Nsr1p, Rps27ap, Rps31p, Rpl6ap, Ria1p, Esf2p, Rex4p, Pkh2p, Sgt1p, Rpl3p, Caf20p, Rpl5p, Ipl1p

of 4.10×10^{-33}, 1.79×10^{-9} and 2.23×10^{-5}, respectively). Considering biological processes, highest differences were reported in ribosome and translation-related terms such as "ribosome assembly", "cytoplasmic translation", and "organelle assembly". These results are controversial with those obtained from a transcriptome analysis performed by Penacho et al. (2012) [6] who revealed a down-regulation of genes involved in translation during the first 19 days. Li et al. (1999) [9] demonstrated that a loss of RP mRNA after a stress condition expose such as a shift up in temperature, is due to the rapid transcriptional silencing of the RP genes, coupled to the naturally short lifetime of their transcripts. This might the case of the second fermentation in sparkling wine elaboration as there is a phase in which yeast are subjected to different stresses and followed by a cell proliferation step [6]. In this moment, RP genes might be transcribed and translated into proteins and later transcripts degraded while RPs remain. At the phenotype study level, there were detected a high frequency of RPs that influence autophagy. Two out of the 2 autophagy RPs in *S. cerevisiae* were detected just under the PC (Rpl14ap and Tef4p) reported to influence in this process which have been evidenced in fermenting yeast during sparkling wine elaboration [6,10,11]. Further, PC specific RPs influence in processes like replicative lifespan and stress resistance too (1 proteins out of 1, Rpl6ap; 2 out of 3, Rpl34bp and Rpl9bp; respectively).

Among the 28 extracellular region proteins registered in *S. cerevisiae*, 3 were detected only under PC (Ape2p, Cis3p and Ygp1p). All are specifically localized in the cell wall, being all glycosylated and one mannose-glycosylated (Cis3p). Recent studies have shown that glycoproteins together with mannoproteins (proteins glycosylated with mannose), rather than proteins are most prominent macromolecules responsible for the foam formation of sparkling wine and organoleptic properties [12–15]. They might be released during fermentation or aging on lees [16]. As cell wall of *S. cerevisiae* is an elastic structure that provides osmotic and physical protection to the cell in which heavily glycosylated mannoproteins are components. Besides protective function, these identified PC proteins participate in metabolism of peptide and amino acids (Ape2p and Ygp1p, respectively) [17], the cell wall organization and assembly (Cis3p and Ygp1p, respectively) [18,19].

4. Conclusion

CO_2 overpressure factor during the second fermentation of sparkling wine affects yeast viability. Moreover, differences among yeast proteome under the second fermentation condition subjected to CO_2 gas overpressure and that subjected to atmospheric pressure were reported. Among specific proteins identified under the pressure condition, high frequencies of proteins involved in the production of other proteins as well extracellular region glycoproteins were observed. A better knowledge of yeast stress response complemented with genetic studies that definitively confirm the role

of proteins identified, might facilitate the development of effective strategies for improved yeast survival and quality increase of these special wines.

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