FLOR YEAST CELLULAR COMPONENTS

COMPONENTELE CELULARE A LEVURILOR PELICULARE

MORENO-GARCÍA J.[1], DUMITRIU Georgiana-Diana[2], GARCÍA-MARTÍNEZ Teresa[1], MILLÁN M. Carmen[1], MAURICIO J.C.[1], MORENO J.[3]
e-mail: jaime41@hotmail.com

Abstract. *Flor yeast develops a biofilm on the wine surface after the alcoholic fermentation of grape which remains during the "biological aging" process in the elaboration of Sherry wines. The aim of this study is to identify cellular components which role might be essential for the yeast survival under this process. A proteome analysis was carried out for a flor yeast grown in a biofilm forming and in a reference non-biofilm forming conditions and proteins annotations to cellular components were performed by using the SGD database. Higher abundant proteins in the biological aging condition localized mostly in the cell wall, extracellular region and peroxisome; in the reference condition higher abundant proteins belong to the cellular bud and site of polarized growth. Further works dealing with genetics, and also utilization of different flor yeast strains could be considered and aimed to improve the quality of Sherry wines in a near future.*
Key words: *Flor yeast, proteome, cellular components*

Rezumat. *Levurile peliculare (Flor yeasts) formează un biofilm pe suprafaţa vinului după fermentaţia alcoolică, biofilm care rămâne în timpul procesului de "maturare biologică" in elaborarea vinurilor de tip Sherry. Scopul acestui studiu este de a identifica componentele celulare care ar putea avea un rol esential pentru supravietuirea levurile sub acest proces. O analiză proteomică a fost efectuată pentru o levură pelliculară crescută într-un mediu sintetic de maturare biologică şi în condiţii fermentative de referinţă. [4]Adnotarea proteinelor la componentele celulare a fost efectuata utilizând baza de date SGD. Cantitati mai mari de proteine au fost localizate în regiunea extracelulară, peretele celular şi peroxizomi in conditii de maturare biologica; în condiţii fermentative de referinţă cantitati mai mari de proteine aparţin veziculelor legate de membrana citoplasmatica, citoschelet şi cortexul celulei. Studii ulterioare legate de genetica, precum si utilizarea a diferite suse de levuri peliculare pot fi considerate pentru îmbunătăţirea calitatii vinurilor de tip Sherry.*
Cuvinte cheie: *Levuri peliculare, proteomul, componente celulare.*

INTRODUCTION

Flor yeasts are *Saccharomyces cerevisiae* strains whose interest within the enological field lies in their influence on the sensorial properties of a special type

[1] Department of Microbiology, University of Córdoba, Spain
[2] University of Agricultural Sciences and Veterinary Medicine of Iaşi, Romania
[3] Department of Agricultural Chemistry, University of Córdoba, Spain

of wine, so-called “Sherry wines”. This organoleptic change takes place during a process known as “biological aging” which is carried in many different areas around the world (Spain, France, Italy, South Africa, Armenia, California and southern Australia).

During the biological aging process harsh conditions prevail (low oxygen concentration, high ethanol concentration, low pH, etc.) and most organisms aside from flor yeasts are not able to survive and proliferate.

It is known that the flor yeast ability to float on the surface of the wine and form a biofilm or “flor” is essential for its survival. It allows the yeast to reach an oxygen-rich zone where it is possible to catalyze ethanol or glycerol. This property was first attributed to a high cell surface hydrophobicity (Martinez et al., 1997c) which would be due to a specific cell wall composition. Reynolds and Fink (2001) among other authors showed that the Flo11p cell wall protein is involved in yeast biofilm formation.

Another feature that needs to be mentioned is an efficient proteomic antioxidant defense system. In aerobic conditions such as the biological aging, ethanol affects respiratory chain function in yeast mitochondria, leading to substantial increases in the amounts of reactive oxygen species (ROS).

Organelles or cellular components such as the flor yeast cell wall or mitochondria, play an important role in their survival under a harsh condition like biological aging. In order to detect another cellular components important to survive under this environment, a proteome analysis have been carried out of a flor yeast grown in a biofilm forming condition (BFC) and in a non-biofilm forming condition (NBFC) for comparison and a database search has been performed in order to relate proteins with the cellular component where they are localized.

MATERIAL AND METHOD

Flor yeast *Saccharomyces cerevisiae* G1 strain (ATCC: MYA-2451), was used in this work. A population of 1 x 10^6 cells/mL was inoculated in a biofilm formation medium or BFC (0,67% (w/v) YNB without amino acids (Difco), 1% w/v glycerol, 10 mM of glutamic acid and 10% (v/v) ethanol without shaking during 29 days) and in the non-biofilm formation medium or NBFC (0,67% (w/v) YNB without amino acids (Difco), 17% glucose and 10 mM of glutamic acid). The process was carried out at 21 ºC. All media were autoclaved at 120 ºC for 20 minutes. Cells from the biofilm were harvested by suction and from the non-biofilm culture by centrifugation both in a initial phase when the cell viability was higher than 90%. The resulting cellular pellet from each condition was resuspended in 1 mL extraction buffer supplemented with Protease Inhibitor Cocktail tablets, and cell wall was 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) 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 190 Cº. 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 60000 full-width at half-maximum (FWHM) resolution at m/z 400 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 m/z 400 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.

To compare quantity of proteins detected in both conditions, a relative quantification has been carried by using the exponentially modified protein abundance index or emPAI (Ishihama et al., 2005): emPAI = $10^{PAI} - 1$. The PAI index is obtained by dividing the observed peptides (taking into account the charge state and missed cleavages) of a specific protein in the analysis by the observable peptides. The observable peptides were determined by using the “MS Digest” software”. The protein relative contents in each condition were calculated using the next equation, being Mr the protein molecular weight: Protein content (weight %)= (emPAI x Mr/ □ (emPAI x Mr))*100. Proteins average content were: 0,24 in BFC and 0,16 in NBFC.

Proteins detected have been submitted to organelles or cellular components aggrupation through SGD database. Cellular components with higher differences among conditions, depending on the number of specific proteins in the condition but also on the number of proteins more abundant in this condition, have been considered in depth. Moreover those cellular components with higher number of proteins in BFC than in NBFC have been highlighted.

RESULTS AND DISCUSSIONS

416 proteins were detected under BFC and 611 under NBFC from which 208 were common in both conditions (Fig. 1).

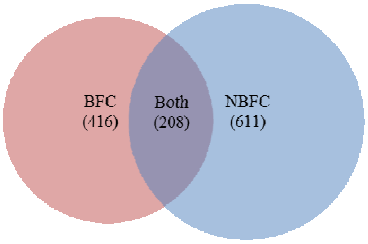

Fig. 1 - Venn diagram showing proteins detected in BFC, in NBFC and detected in both conditions.

With protein identification it was only possible to define which proteins are BFC specific, NBFC specific and proteins detected under both conditions. By using the protein content (weight %) from PAI, it is also possible to detect proteins that are present with more abundance (>2-fold) in one condition or the other and proteins with similar contents (<2-fold) (Table 1). In this way, a more refined search for relevant proteins can be acquired.

Table 1.

Number of proteins detected specifically in BFC and in NBFC, in both conditions, those detected in higher abundance in BFC (BFC > NBFC) and in NBFC (NBFC>BFC) and with similar conditions (BFC ≈ NBFC).

	BFC specific	NBFC specific	Both conditions	BFC > NBFC	NBFC>BFC	BFC≈NBFC
No. proteins	208	403	208	65	46	97

After protein agrupation within cellular components, highest proportion of protein belong to cytoplasmic proteins (59,4 and 54%, in BFC and NBFC, respectively), nucleus (20,2 and 23,6%), mitochondrion (19,5 and 13,6%) and membrane (17,8 and 16,5%) (Fig. 2). These organelles coincide with those which shelter most part of the total proteins so no conclusion can be afforded. As it was expected a higher protein frecuency was detected under mitochondrion under BFC than under NBFC as it is necessary a oxydative metabolism rather than an efficient proteomic antioxidant defense system (Moreno-García et al., 2014). If considering both specific and more abundant proteins, more notable differences were found in other organelles: cell wall, extracellular region or peroxisome under the BFC; and cellular bud and site of polarized growth in NBFC.

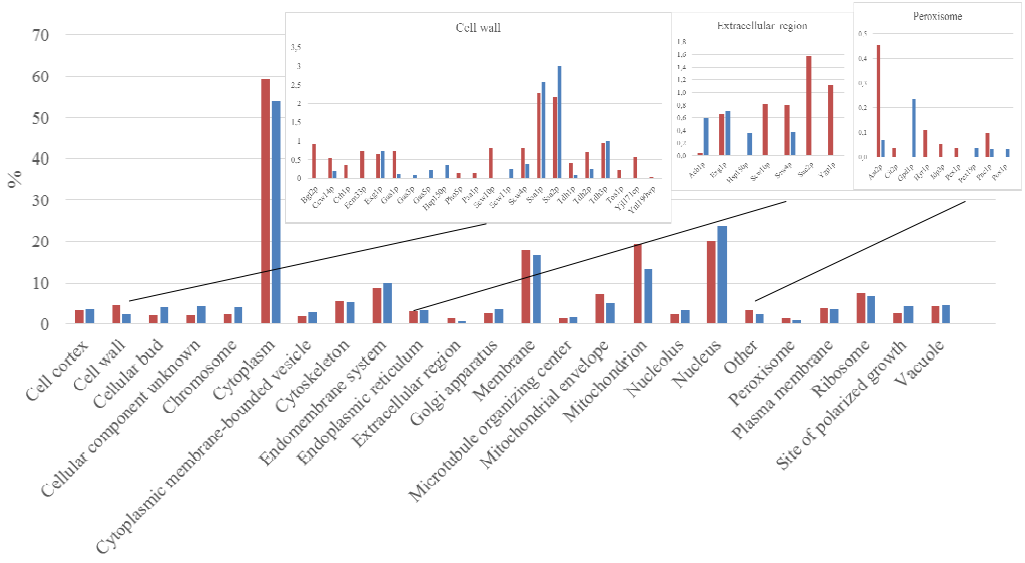

Fig. 2 - Percentage of proteins sorted by organelles or cellular in BFC (red) and NBFC (blue). GO Terms that gather much more proteins (>2-fold) than NBFC condition, taking into account proteins only found in the BFC condition or with more abundance in this condition (>2-fold), have been represented.

From the 22 cell wall proteins detected in the study, 14 were more abundant or specific in BFC, some of them related to the conjugation process (Scw4p and Scw10p), protein glycosylation (Psa1p) which is linked to the cell surface hydrophobicity (Alexandre et al., 2000) and to the resistance to ethanol (Ecm33p). Although not detected Flo11p, probably because is mostly expressed during the stationary phase (Aragon et al., 2008), other cell wall proteins involved in the biofilm formation were detected, Tdh1p (4-fold higher in BFC) and Ssa2p (similar contents in both conditions). Extracellular proteins, Suc2p and Ygp1p, were found in BFC with a very elevated content and none under NBFC. Suc2p is related to oligosaccharide consumption as glucose starvation response (Taussig and Carlson, 1983) while Ygp1p, a cell wall-related secretory glycoprotein, is involved in the cell wall assembly process (Pardo et al., 1999). At last, peroxisome, a small organelle that contains peroxidases and other enzymes involved in a variety of metabolic processes including free radical detoxification and hydrogen peroxide metabolism, also shelter a high number of BFC proteins specific or with more abundance in comparation with NBFC. In BFC, the role of this organelle is important as degradation of ROS are required for the yeast to survive in such an oxidative metabolism condition (Mauricio et al., 1997). Two proteins related to the resistance to oxidative stress, Hyr1p and Pnc1p, were quantified under BFC specifically and with a higher content than in NBFC, respectively. Aat2p (6-fold higher under BFC) and Pex1p (BFC exclusive) increase ethanol resistance. This second protein has also been related to the biofilm formation whereas no peroxisome proteins were related to this phenotype in NBFC. In NBFC, another ethanol resistance-related protein was detected only under this condition, Pex19p. Cit2p only in BFC is implicated in the glyoxilate cycle, essential pathway for a growing yeast in a two-carbon compounds such as ethanol or glycerol.

In the case of NBFC, organelles as cellular bud and site polarized growth grouped a high number of more abundant and specific proteins if compared with BFC. These two cellular components clearly play an important role in the yeast cells when dividing. This mean that this process is not as relevant in the BFC condition as in NBFC.

CONCLUSIONS

1. In a flor yeast proteome analysis, 416 proteins were detected under a biofilm forming condition and 611 under a reference non-biofilm forming condition.

2. Considering relevant those cellular components or organelles with an elevated number of proteins, mitochondrion, cell wall, extracellular region and peroxisome were found to perform an important role in the biofilm forming condition while not as important organelles like cellular bud or site polarized growth.

***Acknowledgments**: Spain's Ministry of Economy and Competitiveness and the European Community (FEDER), Grant RTA2011-00020-C02-02, MINECO-INIA-CCAA.*

REFERENCES

1. **Aragon A. D., Rodriguez A. L., Meirelles O., Roy S., Davidson G. S., Tapia P. H., Allen C., Joe R., Benn D., Werner-Washburne M., 2008 -** *Characterization of Differentiated Quiescent and Nonquiescent Cells in Yeast Stationary-Phase Cultures.* Mol Biol Cell, 3, pp. 1271-1280.
2. **Alexandre H., Blanchet S., Charpentier C., 2000 -** *Identification of a 49 Kda Hydrophobic Cell Wall Mannoprotein Present in Velum Yeast which may be Implicated in Velum Formation.* FEMS Microbiol, 185, pp. 147 - 150.
4. **Ishihama Y., Oda Y., Tabata T., Sato T., Nagasu T., Rappsilber J., Mann M., 2005 -** *Exponentially Modified Protein Abundance Index (Empai) for Estimation of Absolute Protein Amount in Proteomics by the Number of Sequenced Peptides per Protein.* Mol Cell Proteomics, 4.9, pp. 1265 - 1272.
5. **Martinez P., Perez-Rodriguez L., Benitez T., 1997 -** *Velum Formation by Flor Yeasts Isolated from Sherry Wines.* Am J Enol Viticult, 48, pp. 55 - 62.
6. **Mauricio J.C., Moreno J., Ortega J.M., 1997 -** *In Vitro Specific Activities of Alcohol and Aldehyde Dehydrogenases from Two Flor Yeasts during Controlled Wine Aging.* J. Agric. Food Chem, 45, pp. 1967–1971.
7. **Moreno-García J., García-Martínez T., Moreno J., Millán M. C., Mauricio J. C., 2014 -** *A Proteomic and Metabolomic Approach for Understanding the Role of the Flor Yeast Mitochondria in the Velum Formation.* Int J Food Microbiol, 172, pp. 21-29.
8. **Pardo M, Monteoliva L., Pla J., Sánchez M., Gil C., Nombela C., 1999 -** *Two-Dimensional Analysis of Proteins Secreted by Saccharomyces Cerevisiae Regenerating Protoplasts: a Novel Approach to Study the Cell Wall.* Yeast 15, pp. 459-472.
9. **T.B. Fink G.R., 2001 -** *Bakers' Yeast, a Model for Fungal Biofilm Formation.* Science 291**, p**p. 878 - 881.
10. **Taussig R., Carlson M., 1983 -** *Nucleotide sequence of the yeast SUC2 gene for invertase.* Nucleic Acids Res, 11, pp. 1943-1954.