FLOR YEAST RESISTANCE TO ETHANOL AND ACETALDEHYDE HIGH CONTENTS

REZISTENȚA LA CONȚINUTURI RIDICATE DE ETANOL ȘI ACETALDEHIDĂ A LEVURILOR PELICULARE DE "FLOR"

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Abstract. *In the elaboration of Sherry wines, flor yeast develops a biofilm on the wine surface after the alcoholic fermentation of grape which remains during the "biological aging" process. The aim of this study is to identify proteins that respond to high content of ethanol and acetaldehyde. A proteome analysis was carried for a flor yeast grown in a synthetic biological aging and in a reference fermentative condition. 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. 35 proteins were detected under the biological aging condition higher in abundance with respect to the reference condition related to ethanol resistance and 3 related to acetaldehyde resistance. This study together with genetic experiments may lead to the genetic improvement of flor yeast strains aimed to enhance the wine elaboration process.*
Key words: *Flor yeast, proteome, resistance, ethanol, acetaldehyde.*

Rezumat. *În elaborarea vinurilor de tip Sherry, levurile peliculare (Flor yeasts) formează un biofilm pe suprafața vinului după fermentația alcoolică, biofilm care rămâne în timpul procesului de "maturare biologică". Scopul acestui studiu este de a identifica proteinele care răspund la conținut ridicat de etanol și acetaldehidă. O analiză proteomică a fost efectuată pentru o levură peliculară crescută într-un mediu sintetic de maturare biologică și în condiții fermentative de referință. Lucrări ulterioare legate de genetica levurilor și de utilizarea a diferite tulpini de levuri peliculare ar putea fi luate în considerare în scopul de a îmbunătăți calitatea vinurilor Sherry într-un viitor apropiat. 35 de proteine au înregistrat abundențe mai mari în cazul condițiilor de maturare biologică față de condițiile de referință în ceea ce privește rezistența la etanol și 3 proteine în ceea ce privește rezistența la acetaldehidă. Acest studiu împreună cu experimențe genetice poate duce la ameliorarea genetică a tulpinilor de levuri peliculare menite să îmbunătățească procesul de elaborare a vinului.*
***Cuvinte cheie*:** *Levuri peliculare, proteomul, rezistența, etanol, acetaldehida*

INTRODUCTION

In some wine-producing regions around the world biological aging method is used for the elaboration of some special white types of wine known as Sherry wines. During this process, the final organoleptic properties of the

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oenological product are modified principally due to the metabolism performed by peculiar yeast strains, so-called flor yeasts (Peinado et al., 2009).

Biological aging takes place after fermentation when flor yeasts become predominant (Esteve-Zaroso et al., 2001). In this moment, the medium is characterized by high ethanol content that result from a previous fermentative metabolism but also by a high acetaldehyde concentration due to a non-fermentable carbon metabolism developed by the yeasts when fermentation is over. In order to survive in the mentioned conditions, flor yeasts have the capacity to develop biological systems that ensure survival in this medium.

Protein alcohol dehydrogenase suggested by Mauricio et al. (1997) to fulfil a redox balance maintanance role in this type of medium and Hsp12p, both respond to ethanol and acetaldehyde high concentration meanwhile superoxide dismutases (Sod1p and Sod2p) are known to be implicated in the response to ethanol and oxidative stresses (Ma and Liu, 2010).

Until now, genes that codify stress response proteins have been used as targets for the genetic improvement of wine yeast strains to enhance the fermentation performance. Also, in the biological aging process, a higher quality in velum and a higher cell viability have been attained by the overexpression of response to stress genes.

In this study, we used a novel proteomic technique OFFGEL fractionator, coupled to LTQ Orbitrap XL MS to detect as much proteins as possible. All proteins related to acetaldehyde and ethanol stresses have been selected for the comparison with those in a reference fermentative condition and discussed subsequently. This knowledge alongside that provided by experiments aimed to definitively confirm the necessity of these proteins synthesis in the biological aging, can serve for the selection of target genes in order to construct flor yeast strains by biotechnological engineering and hence prolongs in time the yeast survival in the biological aging elaboration process.

MATERIAL AND METHOD

Inoculum. During 24 hours flor yeast *Saccharomyces cerevisiae* G1(ATCC: MYA-2451) was cultivated in a preinoculum medium composed by 200 mL of YPD (1% yeast extract: 2% peptone and 2% glucose). A population of 1 x 10^6 cells/mL was inoculated in the synthetic biological aging medium and in the fermentative medium.

Media. The reference fermentative medium was composed by 0,67% (w/v) YNB without amino acids (Difco), 17% (w/v) glucose and 10 mM of glutamic acid filled with distilled water until a total volume of 250 mL. The fermentations were performed at 21ºC. Cells were gathered centrifugally from broth cultures at 12 hours or middle of the exponential phase of growth when a population of 27,2 x 10^6 cells/mL was reached. This medium was used as reference trying to avoid typical biological aging stresses: high content of glucose (17%), no ethanol content and fermentable carbon metabolism through Crabtree effect. From now, the condition related to this medium will be referred as RFC (reference fermentative condition). The Biological aging medium was composed by 0.67% (w/v) YNB without amino acids (Difco), 1% w/v glycerol, 10 mM of glutamic acid

and 10% (v/v) ethanol in Erlenmeyer flasks until a volume of 250 mL cultivated at a temperature of 21 °C without shaking during 29 days when cells viability is about 90% and the velum completely formed (Mauricio et al., 1997). From now, the condition related to this medium, the biological aging condition will be referred as BAC. All media were autoclaved at 120ºC for 20 mins before the experiment. The number of yeast cells was determined in a Beckman Coulter particle counter Z2 performing previously the appropriate dilution and then mixing with a ratio 0.1:39.9 of Isoton dilution solution according to the recommendations. All experiments were carried out by triplicate in flasks closed with hydrophobic cotton.

Samples and proteome analysis. Cells from the fermentation culture were harvested by centrifugation and cells from the flor velum were collected by a suction system from the surface of each Erlenmeyer flask when the velum was fully formed. Final cell pellet obtained from each condition was resuspended in 10 mL of lysis buffer supplemented with Protease Inhibitor Cocktail tablets, and cells wall broken by vortexing in a Vibrogen Cell Mill V6 using a volume of glass beads equivalent to that of the cell pellet. 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 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. OFFGEL High Resolution kit pH 3−10 was used for protein preparative isoelectric focusing (IEF) in solution.

Protein samples (500 µg approx. of fermentation yeast protein and 400 µg approx. of biological aging yeast) were solubilized in Protein OFFGEL fractionation buffer and aliquots were evenly distributed in a 12-well 3100 OFFGEL Fractionator tray according to the supplier instructions.

Peptides from each OFFGEL 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. Trypsin missed cleavages in peptides with a maxiumum value of 3 were detected.

Database search was performed with Proteome Discoverer 1.0 against Uniprot including fixed modification Carbamidomethylation in Cys and proteome results were statistically analyzed with the Proteome Discoverer program. The score of proteins was calculated by summing those from each peptide.

After identification, proteins related to the response to biological aging stresses (ethanol and acetaldehyde) were selected and sorted from both conditions attending to mutant phenotypes, gene ontology terms (GO Terms) or references using YeastMine tool from SGD and Uniprot databases.

In order to compare quantity of proteins detected in within and among conditions, a relative quantification has been carried by using the emPAI index or exponentially modified protein abundance index. The emPAI is calculated as follows: $emPAI = 10^{PAI}-1$. The PAI index is obtained by dividing the observed peptides of a specific protein in the analysis by the observable peptides.

The observable peptides were determined by using the "MS Digest" software. Fragmentation spectra matching the same peptide sequence but with different charge, modification state or containing missed cleavage (max. 3) sites were counted separately. The protein contents in each condition were calculated using the next equation: Protein content (% weigh)= $[(emPAI \times Mr)/(\Sigma(emPAI \times Mr))] \times 100$.

RESULTS AND DISCUSSIONS

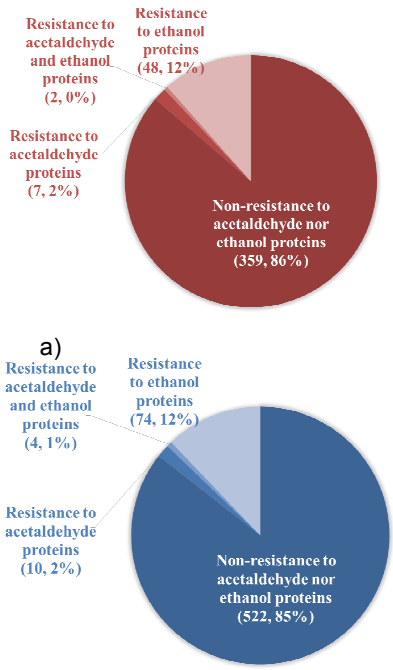
416 proteins were detected under the BAC condition and 611 under RFC.

Similar percentages in proteins related to acetaldehyde and ethanol resistances have been reported in both conditions (2,2% of acetaldehyde resistance proteins in BAC and 2,4% in RFC; 12,0% of ethanol resistance proteins in BAC and 12,9% in RFC) although proteins in each of them are different (Fig. 1).

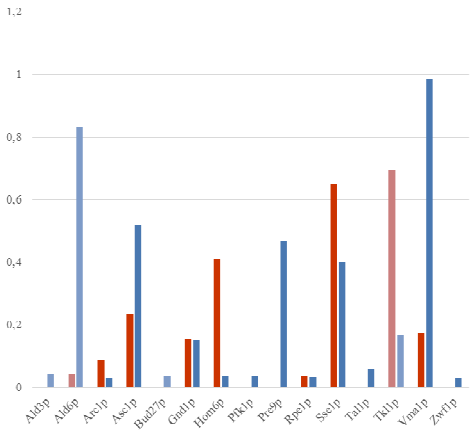
With regard to acetaldehyde, in a condition such as BAC, this molecule is produced throguh the ethanol degradation while it can also be produced as a sub-product of the glucose fermentation in RFC.

In this experiment, it reached a higher concentration under BAC than under RFC (3,7 mM and 0,3 mM, respectively) (Moreno-García et al., 2014). 3 proteins were detected with a higher acetaldehyde content under BAC and are known to be implicated in the following biological processes: cellular amino acid metabolic process (Arc1, Hom6p), pentose-phosphate shunt (Tkl1p) and tRNA aminoacylation for protein translation (Arc1p).

It has been reported that, acetaldehyde provokes mutations in the yeast chromosome DNA having a more deleterious effect than ethanol (Ristow et al., 1995) however much more proteins were related to ethanol resistance rather than acetaldehyde in the present study.



b) Resistance to acetaldehyde



c) Resistance to ethanol

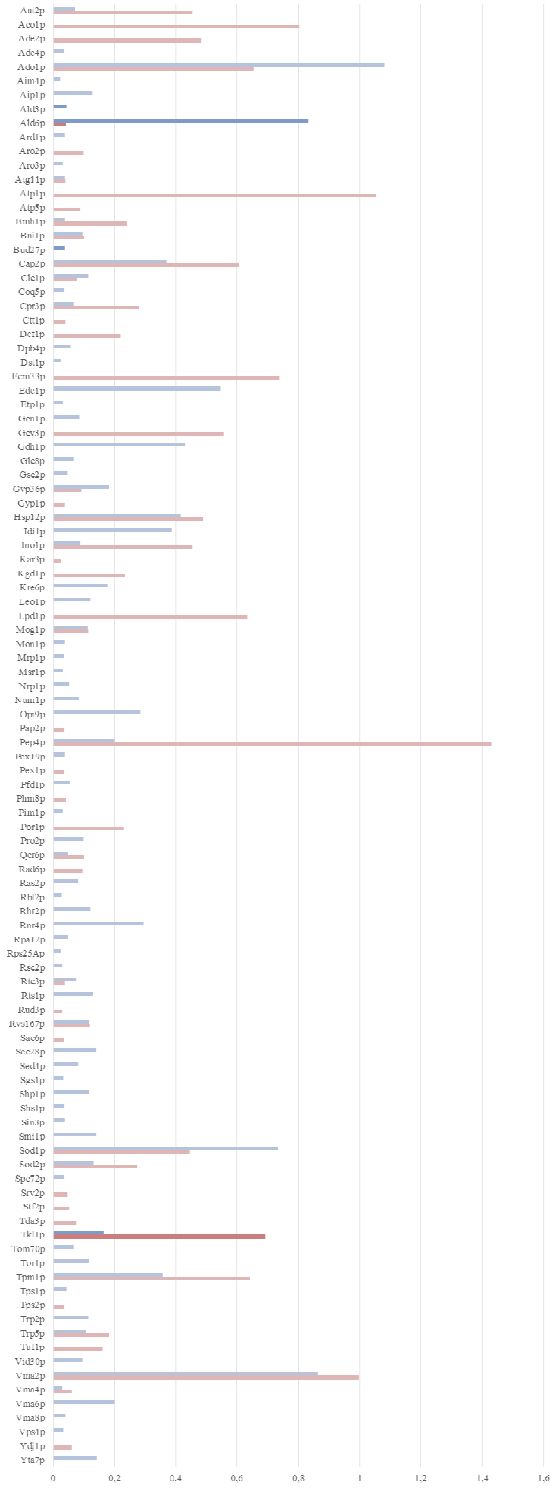


Fig. 1 - Cake diagrams showing the number and percentage of BAC (in red) and RFC (in blue) proteins related to acetaldehyde and ethanol stresses (a). Proteins related to the acetaldehyde resistance (b) and ethanol resistance (c).

Ethanol concentration reached a value of 1282 mM (7,8% v/v) at the time of sampling in BAC and 28 mM in RFC as result of the fermentative metabolism (equivalent to 0,16 % v/v) (Moreno-García et al., 2014). Although more proteins were identified under RFC, the protein content sum in BAC is higher than in RFC, this is 14,6 compared to 11,7, respectively. Etp1p or ethanol tolerance protein surprisingly was only found in RFC. It is known that this is needed to adapt efficiently to ethanol, either as sole carbon source or as cell stressor its gene expression is higher during the first stage of the stationary phase (Gasch et al., 2000). More abundant resistance to ethanol proteins in BAC were found to be involved mainly in biological processes like: nucleobase-containing small molecule metabolic process (Ade2p, Atp1p, Atp5p, Phm8p, Qcr6p and Tkl1p), cellular response to DNA damage stimulus (Bmh1p, Def1p, Pap2p and Rad6p) and carbohydrate metabolic process (Bmh1p, Ino1p, Tkl1p, Tps2p).

CONCLUSIONS

1. Although having acetaldehyde a more deleterious effect than ethanol, more ethanol resistance proteins were detected in the proteome analysis.

2. A similar percentage of proteins either related to the acetaldehyde and ethanol resistance were reported in BAC and in RFC.

3. The ethanol tolerance protein, Etp1p, was detected under RFC and not under BAC.

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