Flor Yeast Proteomics under a Biofilm and under a Non-Biofilm Forming Conditions: Biological Processes in which more abundant Proteins are involved

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Abstract

In the Sherry wine elaboration process carried out in specific regions around the world, *Saccharomyces cerevisiae* "flor" yeast develops a velum (biofilm) on the wine surface. This velum remains along several years during the so called "biological aging" process and it contributes to the typical wine organoleptic properties. Besides biofilm formation, some others biological processes are regarded as essentials for the flor yeasts to survive. The aim of this work is to identify other biological processes apart from the biofilm formation which can be required for the flor yeast to survive in a biological aging condition. OFFGEL fractionator coupled to LTQ Orbitrap XL MS equipment were used trying to identify the maximum possible number of proteins in a biofilm forming condition (BFC) and under a reference non-biofilm forming condition (NBFC). Further, proteins were quantified and sorted by biological processes GO Terms by using the SGD database. Higher abundant proteins related with nucleus organization, cellular respiration, oligosaccharide metabolic process were found in the BFC. In the NBFC, proteins related with RNA splicing, ribosomal subunit export from nucleus and protein dephosphorylation were more abundant. Biological processes mentioned in this study may be essential for the flor yeast to survive in a BFC such as wines biologically aged. Genetic experiments are needed to definitively confirm the necessity of these proteins synthesis. All together may lead to the genetic improvement of flor yeast strains aimed to enhance their survival during the biological aging process and hence the whole Sherry wine elaboration process.

Keywords: *biological processes, flor yeast, proteome.*

INTRODUCTION

Biological aging is a wine elaboration process carried in many different areas around the world. The production process of Fino wine from grapes grown in the Sherry or Montilla-Moriles region, is probably the best known type of wine obtained by biological aging. During its course, the color, texture, flavor and taste properties change due to the metabolism of a kind of yeast, commonly known as "flor yeasts", resulting in a special type of wine so called "Sherry wine" (Martínez *et al.*, 1998; Mesa *et al.*, 2000; Moyano *et al.*, 2002; Muñoz *et al.*, 2005; Peinado and Mauricio, 2009).

Flor yeasts have to face several harsh conditions for survival (low oxygen concentration, high ethanol concentration, low pH, etc.) during biological aging where few organisms can survive. Consequently, more than 95% of the flor over the wines may be composed of *Saccharomyces cerevisiae* as this is the most adapted yeast to grow in this environment (Pozo-Bayón and Moreno-Arribas, 2011). Until now, some features have been suggested that make these yeasts survive

(Alexandre, 2013). Biological processes like buoyancy and biofilm formation in the wine-air area allowing the yeast to reach an oxygen-rich zone where it is possible to catalyze ethanol or glycerol (Farris *et al.*, 1993; Purevdorj-Gage *et al.*, 2007); or an efficient antioxidant defense system that protect the yeast cells against reactive oxygen species (ROS) which come from the oxidative metabolism of non-fermentable carbon sources as ethanol or glycerol (Castrejon *et al.*, 2002; Penate *et al.*, 2001); are both examples of features that make flor yeast survival and proliferation possible under a condition such as biological aging.

Taking into account that proteins like other biological macromolecules such as polysaccharides and nucleic acids, are essential parts of organisms and participate in virtually every biological process within cells, a proteome analysis have been carried out in order to detect another biological processes important for the flor yeast to survive under the biological aging environment. Here, proteomics has been applied to the same flor yeast strain growth under two different conditions: a biofilm forming condition (BFC), as is the biological aging medium, and in a non-biofilm forming condition (NBFC) as a reference. A database search has been performed for the relation of proteins with the biological processes in which they are involved.

MATERIALS AND METHODS

Flor yeast *Saccharomyces cerevisiae* G1 strain (ATCC: MYA-2451), was used in this work. A population of 1×10^6 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 which was added after sterilization, 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 during shaking 12 hours). 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 an 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/ Σ (emPAI x Mr))x100.

Proteins detected have been submitted to biological process GO (Gene Ontology) terms aggrupation through SGD database. Biological processes 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 biological processes with higher number of proteins in BFC than in NBFC have been highlighted.

RESULTS AND DISCUSSION

A total of 413 proteins were identified under BFC, 611 under NBFC and 207 under both (Fig. 1).
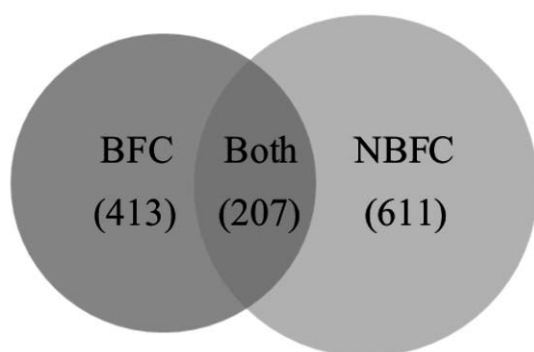

Fig. 1. Venn diagram showing proteins detected in the biofilm forming condition (BFC), in the non-biofilm forming condition (NBFC) and detected in both conditions.

These proteins were subjected to relative quantification through the EmPAI value and protein average content values of 0,24 in BFC and 0,16 in NBFC were calculated. There were reporting 65 more abundant under BFC, 45 under NBFC and 97 with similar quantities under both conditions. After that, a GO Term searching for biological processes was performed for the specific proteins detected under BFC and under NBFC (206 and 404, respectively) and for the proteins with higher quantity under BFC and under NBFC (65 and 45, respectively). The following table shows the frecuency of proteins (comparing with the total number of proteins identified under each condition) annotated in a specific biological process. The highest differences among conditions were reported in the following biological processes: nuclear organization, cellular respiration and oligosaccharide metabolic process in the case of BFC (Fig. 2); and RNA splicing, ribosomal subunit export from nucleus and protein dephosphorylation in the case of NBFC.

Nucleus organization is a process that is carried out at the cellular level which results in the assembly, arrangement of constituent parts, or disassembly of the nucleus. This may explain the fact that in flor yeast has been regarded a genetic heterogeneity which at the same time has been associated with adaptation to a wine medium which is depleted in fermentable sugars and has high levels of ethanol and acetaldehyde (Martínez *et al.*, 1998). This biological process could moreover be related to the flor yeast features as high heterozygosis degree (Budroni *et al.*, 2000), aneuploidy (Guijo *et al.*, 1997) and the high frequency of heterothallic life cycle up to 10% reported by Mortimer *et al.*, 1994. Kar2p was detected with a high content under the biofilm condition, this protein plays a role in the karyogamy or creation of a single nucleus from multiple nuclei, involved in conjugation with cellular fusion indicating that under this condition, sexual reproduction might be taking place. Furthermore, Kar3p participating as well in this process in an essential manner was detected only under BFC.

On the other hand, as it was expected, a high proportion of proteins were detected in BFC involved in the cellular respiration. This is explained since flor yeast metabolism is purely oxidative in biological aging conditions (Mauricio

Fig. 2 .More abundant proteins involved in biological processes under BFC (red) and under NBFC (blue). In the case of BFC, proteins have been shown from biological processes that presented highest differences among conditions (more under BFC).

et al., 1997). In this type of environment lacking fermentable carbon sources, the only remaining carbon sources, ethanol and glycerol, can only be catabolized through an oxidative metabolism in which cellular respiration plays an important role. Aco1p detected with an abundance of up to 0,80 under BFC, apart of having a role in the cellular respiration, plays also an essential role in mtDNA maintenance which may be damaged by the reactive oxygen specied produced during cellular respiration (Moradas-Ferreira *et al*., 1996). Another protein detected with a high content under this condition was Mdh1p (0,98 in BFC and not under NBFC) that is a mitochondrial malate dehydrogenase that catalyzes interconversion of malate and oxaloacetate and involved in the tricarboxylic acid .

Not as expectedly, a high proportion of more abundant proteins were found to be involved in the oligosaccharide metabolic process. This could be regarded as a yeast starvation response to a medium in which there are not hexoses as carbon sources. Moreover a protein that is implicated in the consumption of sucrose was detected in a very high content. This was Suc2p.

Table 1 shows the proteins found in more abundance under the biofilm formation that are involved in the biological process mentioned. It is also indicated in which processes they participate specifically.

Much less abundant proteins were detected in BFC involved in RNA splicing, ribosomal subunit export from nucleus or protein dephosphorylation. The two first processes are important for organisms which are facing a new condition and need to build a new proteomic machinery as an adaptation response to the new environment. As in BFC, yeasts had more time to adapt to the

Tab. 1. More abundant proteins detected under BFC involved in the biological processes: nucleus organization, cellular respiration and oligosaccharide metabolic process. Isoelectric point (IP), molecular weight in Dalton (Mw) and Uniprot entry have been included.

Biological process	Protein names		I. P.	Mw	Entry	Gene ontology (biological process)
Nucleus organization	Acc1p	Acetyl-CoA carboxylase	6.21	250318.7	Q00955	Acetyl-CoA carboxylase activity; biotin carboxylase activity; long-chain fatty acid biosynthetic process; malonyl-CoA biosynthetic process; nuclear envelope organization; protein import into nucleus
	Gsp1p	GTP-binding nuclear protein GSP1/CNR1	6.52	24808.6	P32835	Exonucleolytic trimming to generate mature 3'-end of 5.8S rRNA from tricistronic rRNA transcript (SSU-rRNA, 5.8S rRNA, LSU-rRNA); GTPase activity; intracellular protein transport; nucleocytoplasmic transport; nucleus organization; regulation of chromatin silencing at telomere; small GTPase mediated signal transduction
	Kar2p	78 kDa glucose-regulated protein homolog	4.53	74434.4	P16474	ATPase activity; ATP catabolic process; ER-associated ubiquitin-dependent protein catabolic process; karyogamy involved in conjugation with cellular fusion; posttranslational protein targeting to membrane, translocation; response to unfolded protein; SRP-dependent cotranslational protein targeting to membrane, translocation
	Kar3p	Kinesin-like protein KAR3 (Nuclear fusion protein)	9.24	84013.6	P17119	Karyogamy involved in conjugation with cellular fusion; meiotic nuclear division; metabolic process; minus-end-directed microtubule motor activity; mitotic nuclear division; mitotic sister chromatid cohesion; nuclear migration involved in conjugation with cellular fusion
	Net1p	Nucleolar protein NET1	8.13	128533.6	P47035	Chromatin silencing at rDNA; mitotic nuclear division; nucleolus organization; regulation of exit from mitosis

Tab. 1 (continued)

Biological process	Protein names	I. P.	Mw	Entry	Gene ontology (biological process)
Nucleus organization	Sec13p	Protein transport protein SEC13	5.54	33031.2	Q04491	COPII-coated vesicle budding; ER-associated ubiquitin-dependent protein catabolic process; mRNA transport; nuclear pore distribution; positive regulation of GTPase activity; positive regulation of transcription, DNA-templated; protein transport
Cellular respiration	Aco1p	Aconitate hydratase, mitochondrial	8.15	85368	P19414	Aconitate hydratase activity; mitochondrial genome maintenance; tricarboxylic acid cycle
	Cit1p	Citrate synthase, mitochondrial	8.86	53360	P00890	Acetyl-coa catabolic process; cellular carbohydrate metabolic process; citrate (Si)-synthase activity; citrate metabolic process; glutamate biosynthetic process; tricarboxylic acid cycle
	Cor1p	Cytochrome b-c1 complex subunit 1, mitochondrial	7.34	50227	P07256	Aerobic respiration; catalytic activity; hydrogen ion transmembrane transport
	Fum1p	Fumarate hydratase, mitochondrial	8.26	53151	P08417	Fumarate hydratase activity; fumarate metabolic process; tricarboxylic acid cycle
	Idh1p	Isocitrate dehydrogenase [NAD] subunit 1, mitochondrial	9.81	39324	P28834	Glutamate biosynthetic process; isocitrate dehydrogenase (NAD+) activity; isocitrate metabolic process; tricarboxylic acid cycle
	Idh2p	Isocitrate dehydrogenase [NAD] subunit 2, mitochondrial	9.15	39739	P28241	Glutamate biosynthetic process; isocitrate dehydrogenase (NAD+) activity; isocitrate metabolic process; tricarboxylic acid cycle
	Kgd1p	2-oxoglutarate dehydrogenase, mitochondrial	7.21	114415	P20967	2-oxoglutarate metabolic process; oxoglutarate dehydrogenase (succinyl-transferring) activity; tricarboxylic acid cycle
	Kgd2p	Dihydrolipoyllysine-residue succinyl-transferase component of 2-oxoglutarate dehydrogenase complex, mitochondrial	9.62	50430	P19262	2-oxoglutarate metabolic process; dihydrolipoyllysine-residue succinyltransferase activity; L-lysine catabolic process to acetyl-coa via saccharopine; mitochondrial genome maintenance; tricarboxylic acid cycle
	Lsc1p	Succinyl-CoA ligase [ADP-forming] subunit alpha, mitochondrial	8.73	35032	P53598	ATP citrate synthase activity; succinate-coa ligase (ADP-forming) activity; succinyl-coa metabolic process; tricarboxylic acid cycle
	Mam33p	Mitochondrial acidic protein MAM33	4.34	30132	P40513	Aerobic respiration
	Mdh1p	Malate dehydrogenase, mitochondrial	8.99	35650	P17505	Aerobic respiration; cellular carbohydrate metabolic process; chronological cell aging; L-malate dehydrogenase activity; malate metabolic process; replicative cell aging; tricarboxylic acid cycle
	Mic17p	Mitochondrial intermembrane space cysteine motif-containing protein MIC17	8.53	16669	Q03667	Aerobic respiration

Tab. 1 (continued)

Biological process	Protein names		I. P.	Mw	Entry	Gene ontology (biological process)
Cellular respiration	Pet9p	ADP,ATP carrier protein 2	10.61	34426	P18239	ADP transport; aerobic respiration; anaerobic respiration; apoptotic process; ATP:ADP antiporter activity; ATP transport; heme transport; mitochondrial transport; transmembrane transport
	Qcr6p	Cytochrome b-c1 complex subunit 6	3.81	17257	P00127	Aerobic respiration; hydrogen ion transmembrane transport; mitochondrial electron transport, ubiquinol to cytochrome c; ubiquinol-cytochrome-c reductase activity
	Sdh1p	Succinate dehydrogenase [ubiquinone] flavoprotein subunit, mitochondrial	7.47	70229	Q00711	Cellular respiration; mitochondrial electron transport, succinate to ubiquinone; succinate dehydrogenase (ubiquinone) activity; tricarboxylic acid cycle
Oligosa-ccharide metabolic process	Hsp104p	Heat shock protein 104	5.2	102034	P31539	Atpase activity, coupled; ATP catabolic process; cellular heat acclimation; chaperone cofactor-dependent protein refolding; inheritance of oxidatively modified proteins involved in replicative cell aging; protein folding in endoplasmic reticulum; protein unfolding; trehalose metabolism in response to heat stress
	Nth1p	Neutral trehalase	7.82	85879	P32356	Alpha,alpha-trehalase activity; trehalose catabolic process
	Suc2p	Invertase 2	4.44	60639	P00724	Beta-fructofuranosidase activity; fructan catabolic process; inulinase activity; inulin catabolic process; raffinose catabolic process; sucrose alpha-glucosidase activity; sucrose catabolic process
	Swi3p	SWI/SNF complex subunit SWI3	4.6	92926	P32591	ATP catabolic process; ATP-dependent chromatin remodeling; carbon catabolite activation of transcription from RNA polymerase II promoter; cellular alcohol catabolic process; positive regulation of mating type switching; positive regulation of transcription from RNA polymerase II promoter; sucrose catabolic process; transcription, DNA-templated
	Tps2p	Trehalose-phosphatase	8.19	102976	P31688	Cellular response to heat; dephosphorylation; trehalose biosynthetic process; trehalose-phosphatase activity

new condition (29 days) these proteins were not as abundant as in the NBFC in which time from inoculation to sampling was 12 hours. With regard to protein dephosphorylation proteins in yeast they are mostly involved in the regulation of cell cycle, important in a yeast in an active reproducing stage which is not the case of BFC.

Nuclear organization proteins detected only or more abundant in BFC were specifically involved in processes like: karyogamy involved in conjugation with cellular fusion or nuclear migration involved in conjugation with cellular fusion; cellular respiration proteins in tricarboxylic acid cycle and oligosaccharide metabolic process proteins were involved in the sucrose catabolic process or trehalose biosynthetic process.

CONCLUSION

Biological processes in which the more abundant proteins in a biofilm formation condition and in a non-biofilm formation condition were reported. These processes could play an important role in the studied conditions taking into account that proteins like other biological macromolecules participate in virtually every biological process within cells. As conclusion, it could be said that processes like nuclear organization, cellular respiration or oligosaccharide metabolic process may play an important role in the survival of the flor yeast during the biological aging condition and not as important, processes like RNA splicing, ribosomal subunit export from nucleus or protein dephosphorylation at the time of the sampling.

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