Proteins involved in flor yeast carbon metabolism under biofilm formation conditions

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A B S T R A C T

A lack of sugars during the production of biologically aged wines after fermentation of grape must causes flor yeasts to metabolize other carbon molecules formed during fermentation (ethanol and glycerol, mainly). In this work, a proteome analysis involving OFFGEL fractionation prior to LC/MS detection was used to elucidate the carbon metabolism of a flor yeast strain under biofilm formation conditions (BFC). The results were compared with those obtained under non-biofilm formation conditions (NBFC). Proteins associated to processes such as non-fermentable carbon uptake, the glyoxylate and TCA cycles, cellular respiration and inositol metabolism were detected at higher concentrations under BFC than under the reference conditions (NBFC). This study constitutes the first attempt at identifying the flor yeast proteins responsible for the peculiar sensory profile of biologically aged wines. A better metabolic knowledge of flor yeasts might facilitate the development of effective strategies for improved production of these special wines.

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1. Introduction

Biologically aged wines require special production methods. Fino wine from grapes grown in the Sherry or Montilla–Moriles region, the production process for which is described in detail elsewhere (Peinado and Mauricio, 2009), is probably the best known type of wine obtained by biological ageing. Following fermentation of the starting must, biologically aged wines spontaneously develops a natural biofilm known as “flor velum”. The biofilm is formed by aggregated yeasts called “flor yeasts”, available knowledge about which was recently reviewed by Alexandre (2013).

Although biologically aged wines undergo changes similar to those in all aged wines including chemical reactions between compounds, extraction of specific compounds from the container wood and salt precipitation, most of the changes affecting their composition and sensory properties are associated to the metabolism of flor yeasts (Martínez et al., 1998; Mesa et al., 2000; Moyano et al., 2002; Munoz et al., 2005). Flor yeasts in biologically ageing wines use previously fermented must—which contains high concentrations of fermentative products such as ethanol and glycerol but no fermentable sugars—as substrate. Composition changes in the medium cause flor yeasts to metabolize other molecules formed during fermentation, as substrate. Composition changes in the medium cause flor yeasts to metabolize other molecules formed during fermentation (ethanol and glycerol, mainly). In this work, a proteome analysis involving OFFGEL fractionation prior to LC/MS detection was used to elucidate the carbon metabolism of a flor yeast strain under biofilm formation conditions (BFC). The results were compared with those obtained under non-biofilm formation conditions (NBFC). Proteins associated to processes such as non-fermentable carbon uptake, the glyoxylate and TCA cycles, cellular respiration and inositol metabolism were detected at higher concentrations under BFC than under the reference conditions (NBFC). This study constitutes the first attempt at identifying the flor yeast proteins responsible for the peculiar sensory profile of biologically aged wines. A better metabolic knowledge of flor yeasts might facilitate the development of effective strategies for improved production of these special wines.

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ethanol by alcohol dehydrogenase II or Adh2p. After ethanol, glycerol is the second most abundant carbon source in wine (6–10 g/L initially and around 1 g/L at the end of the ageing process). The metabolism of flor yeasts during biological ageing of wine affects the contents in ethanol, glycerol and acetaldehyde of the wine mainly.

The primary aim of this work was to go deeper into the elucidation of carbon metabolism in flor yeasts forming a biofilm by using controlled and standardized conditions. For this purpose, the flor yeast S. cerevisiae G1 was subjected to proteome analysis in the first stage of the biofilm formation process. The proteome results were compared with those obtained by cultivating a synthetic medium with a high glucose concentration under non-biofilm formation conditions (viz., the reference medium) for 12 h—time by which S. cerevisiae is known to have produced a large number of proteins (Salvado et al., 2008).

2. Materials and methods

2.1. Microorganism, inocula and cultivation conditions

The microorganism used was S. cerevisiae G1 (ATCC: MYA-2451), a wild type of industrial wine flor yeast from the collection of the Department of Microbiology of the University of Cordoba (Spain). This strain forms a thick velum about 30 days after inoculation with a cell viability higher than 90% (Mauroci et al., 1997). Yeast cells were cultivated in a pre-inoculum medium consisting of 200 mL YPD (1% yeast extract, 2% peptone and 2% glucose) for 24 h, after which cells were separated by centrifugation and washed under aseptic conditions. A solution of 1 × 10^6 cells/mL was inoculated to each synthetic medium under biofilm formation conditions (BFC) and non-biofilm formation conditions (NBFC).

The biofilm formation medium consisted of 0.67% (w/v) YNB without amino acids (Difco), 1% w/v glycerol, 10 mM glutamic acid (Henschke and Jiranek, 1993) and 10% (v/v) ethanol. Volumes of 250 mL of the medium were cultivated in Erlenmeyer flasks at 21 °C without shaking for 29 days. The NBFC (reference) medium contained 0.67% (w/v) YNB without amino acids (Difco), 17% glucose and 10 mM glutamic acid (Henschke and Jiranek, 1993). Cell suspensions were incubated at 21 °C with slight agitation according to Salvadó et al. (2008) in order to ensure homogeneous nutrient distribution.

All media were autoclaved at 120 °C for 20 min. Glucose was sterilized by autoclaving separately from the reference medium. Following autoclaving and cooling, the BFC medium was supplied with sterilized glucose and NBFC medium with pure ethanol (Merck). The number of yeast cells in each culture was determined in a Beckman Coulter particle counter Z2 according to the manufacturer’s instructions. Cell viability was verified on plating. All experiments were carried out in triplicate, using 250 mL flasks stoppered with hydrophobic cotton. The initial dissolved oxygen concentration (8 mg/L) was measured with a Cronox OXY-92 oxygen sensor.

2.2. Proteome analysis

Cells from the reference medium were harvested at the initial exponential growth phase (after 12 h), when glucose still remained at high concentrations and a level of 27.2 × 10^6 cells/mL was reached. Cells from the biofilm were collected by suction from the surface of each Erlenmeyer flask once the velum was fully formed (viz., after 29 days). Cells from both types of media were centrifuged on a Rotina-38 apparatus at 4500 × g for 10 min and washed in sterile distilled water at 4 °C. The cell pellets thus obtained were resuspended in 1 mL of extraction buffer (100 mM Tris—HCl at pH 8, 0.1 mM EDTA, 2 mM DTT and 1 mM PMSF) supplemented with Protease Inhibitor Cocktail tablets (Roche) according to the manufacturer’s instructions, and cell walls were broken by vortexing in a Vibrogen Cell Mill V6 (Edmund Bühler), using a volume of glass beads equivalent to that of the extraction buffer with the pellet (viz., 15 pulses of 1 min followed by 30 s on ice). Glass beads and cell debris were discarded by centrifugation at 500 × g for 5 min. Proteins were precipitated by overnight incubation at −20 °C after addition of 10% w/v trichloroacetic acid (TCA) and 4 volumes of ice-cold acetone to the supernatant. After incubation, samples were centrifuged at 16,000 × g for 30 min, and the resulting protein pellets vacuum dried and resuspended in solubilization buffer (8 M urea, 2 M thiourea, 4% CHAPS and 1% DTT). Protein concentrations were estimated via the assay of Bradford (1976) and samples stored at −80 °C prior to analysis.

The OFFGEL High Resolution kit pH 3–10 (Agilent Technologies, Palo Alto, CA) was used for protein preparative isoelectric focussing (IEF) in solution. Protein samples (ca. 450 μg) were solubilized in Protein OFFGEL fractionation buffer containing urea, thiourea, dithiothreitol (DTT), glycerol and buffer with ampholytes (Agilent Technologies, Part number 5186–6444), and aliquots evenly distributed in a 12-well 3100 OFFGEL Fractionator tray, also from Agilent Technologies, according to the supplier’s instructions. For separation, program OG12PR00 separation limits were used as per the manufacturer’s recommendations, namely: 4500 V, 200 mW and 50 μA; starting voltage 200–1500 V; ending voltage 5000–8000 V; and a constant voltage for protein separation zones after application of 20 kV.

Peptides from each well were scanned and fragmented on an LTQ Orbitrap XL mass spectrometer (Thermo Fisher Scientific, San Jose, CA, USA) equipped with a nano LC Ultimate 3000 system ( Dionex, Germany). The electrospray voltage was set to 1300 V and the capillary voltage to 50 V at 190 °C. The LTQ Orbitrap was operated in the parallel mode, which enabled accurate measurement of the precursor survey scan (400–1500 m/z) in the Orbitrap selection and afforded 60,000 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 LT 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 sequencing and quantitation. The normalized collision energy used was 40% for HCD and 35% for CID. The maximum injection times for MS and MS/MS were set to 50 and 500 ms, respectively. The precursor isolation width was 3 Da and the exclusion mass width 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.

A database search with Proteome Discoverer v. 1.0 (Thermo Fisher Scientific Software, San Jose, CA, USA) against Uniprot including fixed modification (viz., carbamidomethylation in Cys) was performed and the proteome results were statistically analysed with the same software. After identification, proteins were sorted into “carbon source metabolism”, “carbohydrate metabolic process” and “generation of precursor metabolites and energy” in accordance with GO terminology by using the Gene Ontology section on the Saccharomyces genome database (http://www.yeastgenome.org). These terms gather chemical reactions and pathways involving carbon source and carbohydrate metabolism as follows: consumption of carbon source, polysaccharide metabolism, regulation of carbohydrate metabolism, carbohydrate catabolism, formation of precursor metabolites and any process involved in the release of energy from these metabolites. Also, they include the formation of carbohydrate derivatives by addition of a carbohydrate residue to another molecule. The SGD manually
The amounts of proteins measured under both types of conditions were compared via the exponentially modified protein abundance index (emPAI; Ishihama et al., 2005), which is calculated as follows:

$\text{emPAI} = \frac{10^{\text{PAI}}}{C_0} - 1$

The PAI value for each specific protein was obtained by dividing the number of peptides observed—with provision for charge state and missed cleavages—into that of observable peptides, which was estimated by using the software MS Digest. Fragmentation spectra matching the same peptide sequence but with a different charge or modification state, or containing a maximum of 3 missed cleavage sites, were counted separately. Protein relative contents under each condition were calculated from:

$$\text{Protein content (weight %)} = \frac{\text{emPAI} \times \text{Mr}}{\sum (\text{emPAI} \times \text{Mr})} \times 100$$

where $\text{Mr}$ is the protein molecular weight.

### 3. Results and discussion

A total of 94 proteins related to carbon source metabolism, carbohydrate metabolic processes, and production of precursor metabolites and energy were examined (see Table 1). Detected proteins taking part in these processes included 31 from yeast cells growing under BFC, 30 from yeast cells growing under NBFC for 12 h, and 33 from cells found under both conditions. Cell viability was higher than 90% under both BFC and NBFC.

The score value for each protein was calculated by combining the XCorr values of its peptides. Table 1 shows the observed and observable peptides, and molecular weight, for each protein, which were used to calculate the relative protein content (wt %) from the equations under Materials and methods; then, the calculated contents were divided into those for BFC and NBFC for comparison. Those proteins that were only detected under either condition or had a relative abundance more than 2 times higher or lower than under the reference condition (NBFC) are highlighted.

The proteins involved in carbon metabolism were found to be associated to more specific processes such as non-fermentable carbon source metabolism (8 under BFC and 7 under NBFC), the glyoxylate cycle (3 vs 1), the tricarboxylic acid cycle (12 vs 1), cellular respiration (10 vs 8), glycolysis/gluconeogenesis (14 vs 17), reserve carbohydrate metabolism (5 vs 6), pentose phosphate metabolism (6 vs 9), inositol metabolism (1 under both conditions), cell wall biosynthesis (4 vs 5), protein glycosylation (2 vs 5), pentose metabolism (3 vs 1) and disaccharide metabolism (1 under both conditions).

#### 3.1. Non-fermentable carbon source metabolism

Some proteins related to the consumption of ethanol and glycerol were detected. Adh1p was found only under BFC (relative content 0.15). This enzyme catalyses the conversion of ethanol into acetaldehyde; thus, Moreno-García et al. (2014) previously observed an ethanol decrease of 359.6 ± 0.1 mM and an acetaldehyde increase of 3.68 ± 0.02 mM. This is the initial step in the uptake of ethanol when glucose in the medium is depleted (Ciriacy, 1975; Mauricio et al., 1997). Another protein directly involved in ethanol assimilation is Ald6p, which was also detected under BFC only; this is a mitochondrial aldehyde dehydrogenase that converts acetaldehyde into acetate and expression of which is glucose-repressed (Wang et al., 1998; Remize et al., 2000). Cytosolic aldehyde dehydrogenase isozyme 6 (Ald6p), which is constitutively expressed, was detected under both conditions. Aranda and del Olmo (2003) previously found aldehyde dehydrogenase activity in flor yeasts to exceed that in fermentative yeasts. The presence of Acs2p, a protein that synthesizes acetyl CoA from acetate, was detected under NBFC only. According to van den Berg et al. (1996) Acs2p is the “anaerobic” isozyme of acetyl-coenzyme A synthetase and required for growth on fermentable carbon sources, so it is non-functional under BFC.

The following proteins involved in ethanol biosynthesis through glucose fermentation were also detected: Adh1p, Pdc1p and Pdc5p, all with a higher relative abundance under NBFC.

Glycerol degradation proteins were also identified. Gut2p, which is involved in glycerol catabolism (Romnow and Kielland-Brandt, 1993), was only detected under BFC (relative abundance 0.14). According to Zara et al. (2010), flor yeasts use glycerol preferentially over ethanol to form a biofilm when non-fermentable carbon is the sole carbon source present in the growing medium. The protein Dak1p or dihydroxyacetone kinase, which is involved in glycerol catabolism (Norbeck and Blomberg, 1997), was also detected under BFC, albeit with a very similar relative abundance as under NBFC (0.08).

Rhr2p, which is related to the synthesis of glycerol as a byproduct of glycolysis was only detected under NBFC (Pahlman et al., 2001).

Ethanol uptake prevailed over glycerol uptake under BFC (Moreno-García et al., 2014). Martínez et al. (1998) previously found ethanol uptake in a “solaera and criaderas” industrial system used to obtain Fino wine to be higher in the “sobretablas” (viz., the barrel row containing young wine); by contrast, other compounds such as acetic acid or glycerol were steadily consumed throughout the ageing period.

#### 3.2. Glyoxylate cycle

This pathway is essential for yeast growth on two-carbon compounds such as ethanol or acetate and plays an anaplerotic role in the provision of precursors for biosynthesis. The glyoxylate cycle is a modified version of the TCA cycle excluding its two decarboxylation reactions. As a result, two-carbon substrates, which enter the cycle as acetyl-CoA, can be converted to four-carbon compounds which in turn can be further metabolized to produce sugars and other essential organic compounds. Specific proteins related to this pathway were detected under both conditions. Thus, Cat8p, which takes part in the positive regulation of glyoxylate cycle (Haurie et al., 2001), and Cit2p (McAlister-Henn and Small, 1997), were detected only under BFC, whereas Leu2p (Branduardi et al., 2013) was detected under both conditions, but in greater amounts under NBFC. Efficient use of two-carbon compounds by yeasts also requires the TCA cycle and gluconeogenesis, which are coordinately regulated with the glyoxylate cycle. As can be seen from Fig. 1 in the Supplementary material, succinate provided by the glyoxylate cycle enters the TCA cycle and is converted into malate that re-enters the cytoplasm for conversion into oxaloacetate and, eventually, into glucose by gluconeogenesis.

#### 3.3. Tricarboxylic acid cycle

All TCA cycle proteins detected here (Aco1p, Cit1p, Fum1p, Idh1p, Idh2p, Kg1d1p, Kg1d2p, Lpd1p, Lsc1p, Mdh1p, Sdh1p and Pdh1p) were much more abundant under BFC than under NBFC. The TCA cycle constitutes a nearly universal metabolic pathway that results in the oxidation of acetyl groups from acetyl coenzyme A.
Table 1
Proteins related to carbon source metabolism, carbohydrate metabolic process and generation of precursor metabolites and energy in biofilm forming condition (BFC) and non-biofilm forming condition (NBFC). Score value for each protein was calculated by summing XCorr values of all the peptides.

<table>
<thead>
<tr>
<th>Protein standard name</th>
<th>Accession number (Uniprot)</th>
<th>Brief description</th>
<th>Score</th>
<th>BFC/NBFC</th>
<th>Protein content (weight %)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aco1p</td>
<td>P19414</td>
<td>Aconitase</td>
<td>180.21</td>
<td>0.80</td>
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<tr>
<td>Acs2p</td>
<td>P52910</td>
<td>Acetyl-CoA synthetase isoform</td>
<td>1.79</td>
<td>0.04</td>
<td></td>
</tr>
<tr>
<td>Ade16p</td>
<td>P54113</td>
<td>Enzyme of 'de novo' purine biosynthesis</td>
<td>3.35</td>
<td>0.04</td>
<td></td>
</tr>
<tr>
<td>Adh1p</td>
<td>P00330</td>
<td>Alcohol dehydrogenase</td>
<td>163.06</td>
<td>0.91</td>
<td>1.23 0.74</td>
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<tr>
<td>Adh2p</td>
<td>P00331</td>
<td>Glucose-repressible alcohol dehydrogenase II</td>
<td>8.91</td>
<td>0.15</td>
<td></td>
</tr>
<tr>
<td>Ald4p</td>
<td>P46367</td>
<td>Mitochondrial aldehyde dehydrogenase, required for growth on ethanol</td>
<td>3.93</td>
<td>0.08</td>
<td></td>
</tr>
<tr>
<td>Ald6p</td>
<td>P54115</td>
<td>Aldehyde dehydrogenase</td>
<td>1,19581</td>
<td>0.04</td>
<td>0.83 0.05</td>
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<tr>
<td>Bmh1p</td>
<td>P29311</td>
<td>14-3-3 protein, major isoform</td>
<td>26.73</td>
<td>0.24</td>
<td>6.24</td>
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<td>Bmh2p</td>
<td>P34730</td>
<td>14-3-3 protein, minor isoform</td>
<td>19.56</td>
<td>0.12</td>
<td></td>
</tr>
<tr>
<td>Cat8p</td>
<td>P39113</td>
<td>Zinc cluster transcriptional activator</td>
<td>1.68</td>
<td>0.04</td>
<td></td>
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<tr>
<td>Cdc19p</td>
<td>P00549</td>
<td>Pyruvate kinase</td>
<td>501.88</td>
<td>1.45</td>
<td>0.59 2.45</td>
</tr>
<tr>
<td>Chs5p</td>
<td>Q12114</td>
<td>Component of the exomer complex</td>
<td>2.86</td>
<td>0.05</td>
<td></td>
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<tr>
<td>Cit1p</td>
<td>P00890</td>
<td>Citrate synthase</td>
<td>42.66</td>
<td>0.27</td>
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<tr>
<td>Cit2p</td>
<td>P08679</td>
<td>Citrate synthase</td>
<td>1.52</td>
<td>0.04</td>
<td></td>
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<tr>
<td>Coq5p</td>
<td>P49017</td>
<td>2-hexaprenyl-6-methoxy-1,4-benzoquinone methyltransferase</td>
<td>1.62</td>
<td>0.04</td>
<td></td>
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<tr>
<td>Cpr1p</td>
<td>P00924</td>
<td>Enolase I, a phosphopyruvate hydratase</td>
<td>527.79</td>
<td>1.17</td>
<td>0.93 1.27</td>
</tr>
<tr>
<td>Cre3p</td>
<td>P32356</td>
<td>Neutral trehalase, degrades trehalose</td>
<td>1.57</td>
<td>0.03</td>
<td></td>
</tr>
<tr>
<td>Dap1p</td>
<td>P04717</td>
<td>Dihydroxyacetone kinase</td>
<td>3.22</td>
<td>0.08</td>
<td>0.80 0.10</td>
</tr>
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<td>Eno1p</td>
<td>P00925</td>
<td>Enolase I, a phosphopyruvate hydratase</td>
<td>527.79</td>
<td>1.17</td>
<td>0.93 1.27</td>
</tr>
<tr>
<td>Eno2p</td>
<td>P00926</td>
<td>Enolase II, a phosphopyruvate hydratase</td>
<td>293.57</td>
<td>0.87</td>
<td>1.21 0.72</td>
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<tr>
<td>Fba1p</td>
<td>P14540</td>
<td>Fructose 1,6-bisphosphate aldolase</td>
<td>105.93</td>
<td>0.69</td>
<td>0.97 0.71</td>
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<tr>
<td>Fum1p</td>
<td>P08417</td>
<td>Fumarase</td>
<td>106.30</td>
<td>0.62</td>
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<tr>
<td>Glk1p</td>
<td>P00928</td>
<td>Glucokinase</td>
<td>8.10</td>
<td>0.15</td>
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<tr>
<td>Gnd1p</td>
<td>P38720</td>
<td>6-phosphogluconate dehydrogenase (decarboxylating)</td>
<td>18.48</td>
<td>0.15</td>
<td>0.15 1.01</td>
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<tr>
<td>Gpm1p</td>
<td>P00950</td>
<td>Tetrameric phosphoglycerate mutase</td>
<td>77.52</td>
<td>0.28</td>
<td>0.22 1.31</td>
</tr>
<tr>
<td>Gsc2p</td>
<td>P40989</td>
<td>Catalytic subunit of 1,3-beta-glucan synthase</td>
<td>74.91</td>
<td>0.66</td>
<td>0.72 0.92</td>
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<tr>
<td>Gut2p</td>
<td>P32191</td>
<td>Mitochondrial glycerol-3-phosphate dehydrogenase</td>
<td>24.84</td>
<td>0.14</td>
<td></td>
</tr>
<tr>
<td>Hsp104p</td>
<td>P31539</td>
<td>Disaggregase</td>
<td>12.92</td>
<td>0.16</td>
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<td>Hxk1p</td>
<td>P04806</td>
<td>Hexokinase isoenzyme 1</td>
<td>7.37</td>
<td>0.19</td>
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<td>Hxk2p</td>
<td>P04807</td>
<td>Hexokinase isoenzyme 2</td>
<td>40.09</td>
<td>0.68</td>
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<tr>
<td>Idh1p</td>
<td>P20967</td>
<td>Subunit of mitochondrial NAD(+)-dependent isocitrate dehydrogenase</td>
<td>25.28</td>
<td>0.21</td>
<td></td>
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<tr>
<td>Idh2p</td>
<td>P28241</td>
<td>Subunit of mitochondrial NAD(+)-dependent isocitrate dehydrogenase</td>
<td>45.37</td>
<td>0.23</td>
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<td>Ino1p</td>
<td>P11986</td>
<td>Inositol-3-phosphate synthase</td>
<td>20.61</td>
<td>0.45</td>
<td>0.09 5.19</td>
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<tr>
<td>Kgd1p</td>
<td>P20967</td>
<td>Subunit of the mitochondrial alpha-ketoglutarate</td>
<td>31.29</td>
<td>0.23</td>
<td></td>
</tr>
<tr>
<td>Kgd2p</td>
<td>P19262</td>
<td>Dihydroxyacetone kinase</td>
<td>12.68</td>
<td>0.19</td>
<td></td>
</tr>
<tr>
<td>Kre6p</td>
<td>P32486</td>
<td>Type II integral membrane protein</td>
<td>4.35</td>
<td>0.18</td>
<td></td>
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<tr>
<td>Ksr1p</td>
<td>P27810</td>
<td>Alpha-1,2-mannosyltransferase</td>
<td>1.62</td>
<td>0.03</td>
<td></td>
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<tr>
<td>Leu2p</td>
<td>P04173</td>
<td>Beta-isopropylmalate dehydrogenase (IMDH)</td>
<td>17.01</td>
<td>0.11</td>
<td>0.23 0.50</td>
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<td>Lrp1p</td>
<td>P32486</td>
<td>Putative positive regulator of mannosylphosphate transferase Mnn6p</td>
<td>5.59</td>
<td>0.04</td>
<td></td>
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<tr>
<td>Mmp1p</td>
<td>P41940</td>
<td>GDP-mannose pyrophosphorylase (mannose-1-phosphate guanylyltransferase)</td>
<td>19.81</td>
<td>0.12</td>
<td>0.36 0.33</td>
</tr>
<tr>
<td>Nth1p</td>
<td>P32356</td>
<td>Neutral trehalase, degrades trehalose</td>
<td>1.57</td>
<td>0.03</td>
<td></td>
</tr>
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<td>Pclp1p</td>
<td>P02473</td>
<td>E1 beta subunit of the pyruvate dehydrogenase (PDH) complex</td>
<td>12.71</td>
<td>0.32</td>
<td>0.18 1.81</td>
</tr>
<tr>
<td>Pdc1p</td>
<td>P06169</td>
<td>Major of three pyruvate decarboxylase isozymes</td>
<td>104.29</td>
<td>0.85</td>
<td>1.59 0.53</td>
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<tr>
<td>Psd5p</td>
<td>P16467</td>
<td>Minor isoform of pyruvate decarboxylase</td>
<td>29.95</td>
<td>0.31</td>
<td>0.49 0.64</td>
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<tr>
<td>Pet109p</td>
<td>P32522</td>
<td>Specific translational activator for the COX1 mRNA</td>
<td>3.59</td>
<td>0.03</td>
<td></td>
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<tr>
<td>Pet5p</td>
<td>P18239</td>
<td>Major ADP/ATP carrier of the mitochondrial inner membrane</td>
<td>14.46</td>
<td>0.08</td>
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<td>Pfr1p</td>
<td>P16861</td>
<td>Alpha subunit of heteroamiplastic phosphofructokinase</td>
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<td>Pgr1p</td>
<td>P12709</td>
<td>Glycolytic enzyme phosphoglucone isomerase</td>
<td>4.42</td>
<td>0.13</td>
<td>0.34 0.38</td>
</tr>
<tr>
<td>Pgc1p</td>
<td>P00360</td>
<td>3-Phosphoglycerate kinase</td>
<td>620.89</td>
<td>1.05</td>
<td>0.54 1.93</td>
</tr>
<tr>
<td>Pmi40p</td>
<td>P29952</td>
<td>Mannose-6-phosphate isomerase</td>
<td>8.36</td>
<td>0.10</td>
<td></td>
</tr>
<tr>
<td>Pyc2p</td>
<td>P32327</td>
<td>Pyruvate carboxylase isoform</td>
<td>2.42</td>
<td>0.03</td>
<td></td>
</tr>
<tr>
<td>Qcr6p</td>
<td>P00127</td>
<td>Subunit 6 of the ubiquinol cytochrome-c reductase complex</td>
<td>12.05</td>
<td>0.10</td>
<td>0.05 2.05</td>
</tr>
<tr>
<td>Rap1p</td>
<td>P11938</td>
<td>Essential DNA-binding transcription regulator that binds many loci</td>
<td>1.75</td>
<td>0.04</td>
<td></td>
</tr>
</tbody>
</table>
A, reduction of coenzymes—which are then used indirectly in the synthesis of ATP by oxidative phosphorylation or cellular respiration—and production of intermediates for many other biosynthetic processes (e.g., amino acids and fatty acids for gluconeogenesis). For this last reason, TCA proteins were expected to occur under both conditions, but were in fact much more abundant under BFC. Since this condition was characterized by the presence of non-fermentable carbon sources to be degraded through cellular respiration, and also by oxidative stress caused by the presence of reactive oxygen species (ROS) formed by incomplete reduction of oxygen during cellular respiration (Richter and Schweizer, 1997), it is reasonable to assume that TCA proteins should be more abundant under BFC because they provide reducing power for both oxidative phosphorylation and reduction of ROS. At the time it participates in the TCA cycle, the protein Mdh1p is a component of an NADH shuttle regulating the NAD/NADH ratio in the cytoplasm and mitochondria (Bakker et al., 2001); this protein was detected with a higher abundance under NBFC. Respiratory proteins were expected under oxidative metabolic conditions but not in a fermentation process; however, the 12 h sampling stage under NBFC was not a true fermentation process. The switch from respiration to fermentation cannot have occurred immediately because any mitochondrial proteins involved in oxidative metabolism are induced within the first few hours after inoculation (Salvadó et al., 2008); also, there is the presence of oxygen during fermentation by effect of the process occurring under “semi-anaerobic” conditions.

### 3.5. Glycolysis/gluconeogenesis

Gluconeogenesis (i.e., the production of sugars for macromolecule biosynthesis) is required to enable yeast growth when only ethanol and glycerol are available as carbon sources. The oxidative metabolism of flor yeasts reduces the concentrations of ethanol, acetic acid, ethyl acetate, glycerol, amino acids and organic acids (Alexandre, 2013); in parallel, it increases the concentrations of acetaldehyde, higher alcohols, acetoin and 2,3-butanediol (Mauricio and Ortega, 1993; Martínez et al., 1998). As stated above, ethanol is the main source of energy for flor yeasts, which use alcohol dehydrogenase to convert it into acetaldehyde. Some acetaldehyde is oxidized to acetic acid and subsequently transformed into acetyl-CoA, which enters either the glyoxylate cycle to form succinic acid or the TCA cycle to be incorporated into gluconeogenesis through malate. Proteins involved in gluconeogenesis or glycolysis were detected under BFC. Eno1p or enolase I, which catalyzes the conversion of 2-phosphoglycerate into phosphoenolpyruvate during glycolysis and the reverse reaction during gluconeogenesis (Cohen et al., 1987; Wold and Ballou, 1957a, 1957b), was detected with a high relative abundance (1.17 under BFC vs 0.925 under NBFC). The direction of this reaction depends on the particular carbon source present—in the presence of non-fermentable carbon sources such as ethanol or glycerol, the reaction leads to gluconeogenesis. Expression of this enzyme is repressed in response to glucose (Entian et al., 1987). Eno2p was also detected. This enzyme catalyzes the same reaction as Eno1p but is induced by glucose (Entian et al., 1987). As expected, it was more abundant under NBFC.

Proteins such as enolases, which participate in both glycolysis and gluconeogenesis, were found under BFC. Gpm1p, Pgk1p, Tdh1p, and other enzymes involved in gluconeogenesis (e.g., amino acids and fatty acids for gluconeogenesis).
and Tdh2p were more abundant under BFC; Fba1p and Pgi1p under NBFC; and Tdh3p and Tpi1p similarly abundant under both conditions.

Proteins involved in irreversible gluconeogenesis and glycolysis reactions were also detected. Cdc19p, Glk1p and Hxk1, which lead to glycolysis, were found with a higher abundance under BFC. Cdc19p, with a relative abundance of 1.44 under BFC (2.4 times more than under NBFC), catalyses the conversion of phosphoenolpyruvate to pyruvate. This protein was not expected in such a high abundance in a medium containing no glucose. The substrate of Cdc19p, phosphoenolpyruvate, may have come from glucose degradation after glycogen accumulation and subsequent catabolism, non-oxidative pentose phosphate or degradation pathways of amino acids or glycerol. Glk1p, which was detected only under BFC, catalyses the phosphorylation of glucose in the first irreversible step of glycolysis. The reaction is also catalysed by the hexokinases Hxk1p and Hxk2p (Walsh et al., 1983; Clifton et al., 1993; Bianconi, 2003), of which the latter was only detected under NBFC. Although all three proteins are involved in glucose uptake (Bisson and Fraenkel, 1983), Hxk2p appears to play the main role during glucose phosphorylation in vivo because it is the predominant isoenzyme during growth on glucose (Walsh et al., 1983; Bianconi, 2003). The HXK2 gene is expressed when yeast cells are grown on a fermentable medium containing glucose, fructose or mannose as carbon source (Bisson and Fraenkel, 1983; De Winde et al., 1996). Moreno-Garcia et al. (2014) found 13 ± 5 mM of glucose to be consumed and 28 ± 3 mM of ethanol formed at the time of cell harvest under NBFC. When cells are shifted to a non-fermentable carbon source, the HXK2 gene is repressed and the HXK1 and GLK1 genes are rapidly de-repressed (Lobo and Maitra, 1977; Rodriguez et al., 2001). Pfk1p, which is involved in glycolysis irreversible reaction, and Pyc2p were detected under NBFC. The latter protein is involved in an irreversible step of gluconeogenesis (Brewster et al., 1994); therefore, in a medium such as this containing a large amount of glucose, Pyc2p may consume glucose from the medium as a response to osmotic stress or take part in other processes (e.g., biosynthesis of amino acids).

Several proteins that regulate glycolysis or gluconeogenesis were also detected. Thus, Cat8p (zinc cluster transcriptional activator), which was found with a relative abundance of 0.04 under BFC, is involved in the positive regulation of gluconeogenesis (Haurie et al., 2001). By contrast, Upb1p and Vid30p, which are known to take part in the negative regulation of gluconeogenesis (Regelmann et al., 2003), were only found under NBFC. Also, Rap1p, which regulates glycolysis via positive regulation of transcription from RNA polymerase II promoter (Mizuno et al., 2004), was only detected under NBFC, and so was Std1p, which interacts with glucose sensors to control glucose-regulated gene expression (Schmidt et al., 1999).

Glycolysis is known to be essential for yeast growth in any medium because it produces six starting materials for the biosynthesis of building blocks for macromolecules (viz., β-D-glucose-6-phosphate, D-fructose-6-phosphate, dihydroxyacetone phosphate, 3-phospho-D-glycerate, phosphoenolpyruvate and pyruvate) and other, small molecules (ATP and reducing agents). Because various degradation pathways feed into glycolysis at many different points, glycolysis or portions of the process run in the forward or reverse direction (gluconeogenesis) to produce hexoses depending on the particular carbon source being utilized in order to meet cellular requirements for precursor metabolites and energy.

### 3.6. Reserve carbohydrate metabolism

Yeasts can efficiently synthesize reserve molecules such as glycogen and trehalose provided glucose is either present in the medium or produced by gluconeogenesis. Bmh1p was detected with a 6.2 times higher abundance under BFC than under NBFC and Bmh2p was only found under the former condition; these proteins are both involved in glycogen metabolism (Roberts et al., 1997), Glc8p and Gsy2p, which are involved in glycogen synthesis (Farkas et al., 1991; Ramaswamy et al., 1998), were only detected under NBFC. Glc7p and Shp1p, which are required to store glycogen (Feng et al., 1991; Zhang et al., 1995), were also identified. Glycogen is a storage molecule the metabolism of which increases in response to a wide variety of environmental stresses including high levels of ethanol and nutrient starvation (François and Parrou, 2001) as observed under BFC; under NBFC, where the initial glucose content was 17%, the yeasts may have triggered a cell response to store this energetic molecule.

Trehalose metabolism proteins were also detected under both conditions. Nth1p or neutral trehalase, which degrades trehalose (Dong et al., 2013), was found under BFC (relative abundance 0.03). Other proteins associated to trehalose synthesis were also detected. Thus, Hsp104p, which was found under BFC (relative abundance 0.16), simultaneously boosts accumulation and degradation of trehalose during heat shock (Iwahashi et al., 1998). Tps2p, which was found under BFC only (relative abundance 0.034), takes part in trehalose biosynthesis (Sur et al., 1994); this process also involves Tps1p (Bell et al., 1992), which was detected under NBFC (relative abundance 0.04). Trehalose has proved significant with a view to surviving osmotic, ethanol and oxidative stresses (Pereira et al., 2001).

### 3.7. Pentose phosphate metabolism

Tkl1p was detected with a 4 times higher relative abundance under BFC than under NBFC and Tkl2p was found with an abundance of 0.13 under the former conditions and zero under the latter. Pgi1p, Rki1p, Sol3p, Tal1p and Zwf1p were detected with a higher abundance under NBFC; and Gnd1p, Rpe1p and Tip1p were detected in similar amounts under both conditions. These proteins are involved in pentose-phosphate metabolism, where glucose 6-P is oxidized to carbon dioxide (CO2), ribulose 5-P and NADPH; then, ribulose 5-P undergoes a series of interconversion reactions between sugar phosphates that yield a variety of sugar molecules required for the biosynthesis of nucleic acids and amino acids (Minard et al., 1998; Maaheimo et al., 2001; Lindqvist et al., 1992; Blank et al., 2005; Schaaff-Gerstenschlager et al., 1993; Kondo et al., 2004). This pathway is also important for protecting yeasts from oxidative stress; in fact, NADPH is an essential cofactor for glutathione- and thioredoxin-dependent enzymes defending cells against oxidative damage (Miosga and Zimmermann, 1996; Slekar et al., 1996; Minard and McAlister-Henn, 2001; Minard and McAlister-Henn, 2005).

### 3.8. Inositol metabolism

Inositol is an essential polyol in eukaryotes, where its phospholipid derivative phosphatidylinositol (PI) is a major constituent of phospholipid membranes. PI is cleaved by phospholipase C to form inositol phosphate (IP) and diacylglycerol, both of which are central cell signalling molecules. According to Zara et al. (2012), inositol availability affects biofilm formation, possibly through the key role of the polyol in the assembly of the GPI-anchor of Fló11p, which is a glycosyl-phosphatidylinositol (GPI)-anchored protein essential for cell rising and hydropobicity (Zara et al., 2005; Ishigani et al., 2006). Ino1p (inositol-3-phosphate synthase) was detected under both conditions, but at 5-fold relative concentrations under BFC. According to Donahue and Henry (1981), this protein is involved in inositol synthesis.
3.9. Cell wall biosynthesis

Bmh1p and Bmh2p, which are the major and minor isoform, respectively, of 14–3–3 protein, were both detected in BFC; the relative abundance of the former was 6 times higher under NBFC, and that of the latter was 0.12 under BFC but zero under NBFC. These proteins are involved in chitin biosynthesis in cell walls (Kaliuchti et al., 2007). Chs5p or chitin synthase-related protein was only detected under the reference conditions (NBFC, relative abundance 0.05). This protein has been associated to chitin catabolism in cell walls (Santos et al., 1997).

Additional proteins related to compounds in yeast cell walls other than chitin were also found. Thus, Crh1p, which was only detected under BFC (relative abundance 0.35), operates in the transfer of chitin to beta(1–6) and beta(1–3) glucans in cell walls; its expression is known to be induced under cell wall stress (Cabiib et al., 2007; Bermejo et al., 2008). Skn1p is involved in the (1→6)-beta-D-glucan biosynthetic process (Roemer et al., 1994) and was detected with a relative abundance twice higher under BFC than under the reference conditions. Chitin in cell walls is a linear polysaccharide consisting of P-1,4-linked N-acetyl-D-glucosamine residues and is typically found in the walls of fungal cells, whereas beta-glucans are polysaccharides consisting exclusively of glucose residues linked by beta-D-glycosidic bonds. Glucans constitute a major structural component of cell walls in fungi (Fleet and Phaff, 1981). The glucan component of cell walls is responsible for their tensile strength, rigidity and shape (Fleet and Phaff, 1981). Bradley et al. (2009) found SKN1 expression to increase as the carbon source of the medium was consumed. Proteins related to the beta-glucan metabolism (Gas5p, Gsc2p and kre6p) were only detected under NBFC.

3.10. Protein glycosylation

Rot1p, which is a molecular chaperone involved in protein folding in the endoplasmic reticulum, was only detected under BFC (relative abundance 0.14). This protein takes part in protein glycosylation—specifically, in mannosylation (Pasikowska et al., 2012). Glycosylation is an essential process and the most common protein modification in lower and higher eukaryotes. This process serves many intra- and extracellular functions including facilitating appropriate protein folding, stabilizing secreted glycoproteins and helping cell—cell adhesion (van den Berg et al., 2009), which can be useful for biofilm formation on the wine surface under BFC.

Several other proteins involved in glycosylation (viz., Mnn4p, Mpg1p, Ktr1p, Pmi40p and Yur1p) were detected, all with a higher relative abundance under NBFC.

3.11. Pentose metabolism

Gcy1p and Gre3p were only detected under BFC (relative abundance 0.32 and 0.06 respectively); these proteins are involved in pentose (α-xyllose, arabino3) catabolism (Traff et al., 2002). Ypr1p or NADPH-dependent aldo-keto reductase (Traff et al., 2002) was also detected, at concentrations almost 5 times higher under BFC. Pentoses are naturally occurring plant monosaccharides that cannot be synthesized by yeasts and were not added to the medium; hence, the previous proteins may have taken part in processes other than pentose catabolism. In fact, no proteins involved in this pathway were detected under NBFC.

3.12. Disaccharide metabolism

Suc2p (sucrose) was present with a relative abundance of 1.58 under BFC but absent under NBFC. This protein takes part in sucrose degradation; also, its non-glycosylated form is constitutively expressed, whereas its glycosylated form is regulated by glucose repression (Carlson and Botstein, 1982). Sucrose is the most abundant disaccharide in plants and can be used as an energy source by microorganisms when other nutrients are depleted (Gering and Bruckner, 1996; Bruckner et al., 1993). The absence of glucose from a medium such as that under BFC causes yeasts to synthesize the enzyme sucrase.

Ima3p, which was only detected under NBFC (relative abundance 0.03), is involved in disaccharide catabolism (Teste et al., 2010), where it catalyses the hydrolysis of (1→6)-α-D-glycosidic linkages in some oligosaccharides produced from glycogen by α-amylase—a potential reserve molecule.

4. Conclusions

Velum forming (flor) yeasts constitute a very special physiological variety of wine yeasts, which are excellent candidates for studies of cellular stress responses, genetic control of respiratory vs fermentative metabolism and other functions. This paper reports a comprehensive proteome analysis of a flor yeast with cellular viability above 90% under both biofilm-forming conditions (BFC) and non-biofilm-forming (reference) conditions (NBFC). Detected proteins related to non-fermentable carbon uptake (Adh2p, Ald4p, Ald6p, Dkg1p, Got2p and Rhr2p), the glycolyse cycle (Cat8p, Cit2p and Leu2p), TCA cycle (Aco1p, Cit1p, Fum1p, Idh1p, Idh2p, Kgd1p, Kgd2p, Lpd1p, Lsc1p, Mdh1p, Sdh1h and Pdh1p) and cellular respiration (Ade16p, Cog5p, Cor1p, Cox4p, Cox6p, Masm3p, Mdh1p, Mic17p, Pet309p, Pet59p, Qcr6p, Rib3p and Sod1p) were more abundant under BFC than under NBFC. On the other hand, the proteins related to glycolysis or gluconeogenesis (Cat8p, Cdc19p, Eno1p, Eno2p, Fba1p, Glk1p, Gpm1p, Hxk1p, Pfk1p, Pgo1p, Pyc2p, Rap1p, Tdh1p, Tdh2p, Tdh3p, Tpi1p, Ubp14p and Vid30p), reserve carbohydrate metabolism (Bmh1p, Bmh2p, Glc7p, Glc8p, Gsy2p, Hsp104p, Nth1p, Sph1p, Tps1p and Tps2p) and pentose phosphate metabolism (Gnd1p, Pgi1p, Rki1p, Rpe1p, Sol3p, Tal1p, Tkl1p, Tkl2p, Tpi1p, and Zwflp) were highly abundant under both conditions. One protein involved in inositol metabolism, Ino1p, was found in much greater amounts under BFC. On the other hand, proteins associated to cell wall biosynthesis (Bmh1p, Bmh2p, Chr1p, Gas5p, Gsc2p, Kre6p and Skn1p) or glycosylation (Ktr1p, Mnn4p, Mpg1p, Pmi40p and Yur1p) were abundant under both conditions. Proteins associated to pentose metabolism (Gcy1p, Gre3p and Ypr1p) and disaccharide metabolism (Ima3p and Suc2p) were also detected.

Fig. 1 in the Supplementary material summarizes the pathways behind production of the carbon source and subsequent metabolism of carbohydrates. The tricarboxylic acid cycle (TCA) is known to play a central role in producing amino acids and fatty acids, reducing power for cellular respiration and supplying molecules for gluconeogenesis. The proteins associated to this pathway were more abundant under BFC than under NBFC as a result of the prevailing process in the latter being fermentation rather than oxidative metabolism. Glucose 6-P, formed by gluconeogenesis or produced by degradation of previously stored reserve molecules, can be used to obtain building blocks for macromolecules, ATP and reductants, or diverted to the pentose-phosphate metabolism to obtain amino acids and nucleic acids. An additional protein involved in inositol formation (Ino1p) was found to be 5 times more abundant under BFC. Inositol, which can be obtained by combining two glucose molecules in a reaction catalysed by this protein, is used to anchor to the protein Flo11p—which is essential for velum formation—to the yeast cell surface. Proteins related to cell wall biosynthesis and involved in protein glycosylation were also detected. These processes can be important with a view to...
facilitating adhesion of yeast cells in the biofilm. Unexpectedly, no disaccharide or pentose degradation proteins, or any carbon sources not added to the medium or synthesized by the yeasts, were detected under biofilm formation conditions. This paper provides an overview of carbon source metabolism in the flor yeast S. cerevisiae GI. The proteins detected in the proteome analysis may play an important role in yeast survival under extreme conditions and influence the sensory properties of the resulting wine. However, further testing is required to unambiguously confirm the need for these proteins and others such as stress proteins with a view to planning effective strategies for improved production of biologically aged wines.

Acknowledgements
The authors wish to acknowledge co-funding of this work by Spain’s Ministry of Economy and Competitiveness (MINECO-INIA-CCAA) and the European Fund of Regional Development (FEDER, Grant RTA2011-00020-C02-02). The staff at the Central Service for Research Support (SCAI) of the University of Cordoba is also gratefully acknowledged for help with the analysis of the proteins.

Appendix A. Supplementary data
Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.fm.2014.07.001.

References


