Food Microbiology 51 (2015) 1–9

Contents lists available at ScienceDirect

Food Microbiology

journal homepage: www.elsevier.com/locate/fm

Proteins involved in wine aroma compounds metabolism by a *Saccharomyces cerevisiae* flor-velum yeast strain grown in two conditions

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ARTICLE INFO

Article history:
Received 9 September 2014
Received in revised form
19 March 2015
Accepted 13 April 2015
Available online 24 April 2015

Chemical compounds studied in this article:
Acetaldehyde
(PubChem CID: 177)
Acetic acid
(PubChem CID: 176)
Benzaldehyde
(PubChem CID: 240)
Decanoic acid
(PubChem CID: 2969)
1,1-Diethoxyethane
(PubChem CID: 7765)
Ethanol
(PubChem CID: 702)
Ethyl decanoate
(PubChem CID: 8048)
Ethyl octanoate
(PubChem CID: 7799)
Glycerol
(PubChem CID: 753)
Isoamyl acetate
(PubChem CID: 31276)
2-Phenethyl acetate
(PubChem CID: 7654)

Keywords:
Saccharomyces cerevisiae
Flor yeast
Biofilm
Proteome
Exometabolome

ABSTRACT

A proteomic and exometabolomic study was conducted on *Saccharomyces cerevisiae* flor yeast strain growing under biofilm formation condition (BFC) with ethanol and glycerol as carbon sources and results were compared with those obtained under no biofilm formation condition (NBFC) containing glucose as carbon source. By using modern techniques, OFFGEL fractionator and LTQ-Orbitrap for proteome and SBSE-TD-GC-MS for metabolite analysis, we quantified 84 proteins including 33 directly involved in the metabolism of glycerol, ethanol and 17 aroma compounds. Contents in acetaldehyde, acetic acid, decanoic acid, 1,1-diethoxyethane, benzaldehyde and 2-phenethyl acetate, changed above their odor thresholds under BFC, and those of decanoic acid, ethyl octanoate, ethyl decanoate and isoamyl acetate under NBFC.

Of the twenty proteins involved in the metabolism of ethanol, acetaldehyde, acetoin, 2,3-butanediol, 1,1-diethoxyethane, benzaldehyde, organic acids and ethyl esters, only Adh2p, Ald4p, Cys4p, Fas3p, Met2p and Plb1p were detected under BFC and as many Acs2p, Ald3p, Cem1p, Ilv2p, Ilv6p and Pox1p, only under NBFC. Of the eight proteins involved in glycerol metabolism, Gut2p was detected only under BFC while Pgs1p and Rhr2p were under NBFC. Finally, of the five proteins involved in the metabolism of higher alcohols, Thi3p was present under BFC, and Aro8p and Bat2p were under NBFC.

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http://dx.doi.org/10.1016/j.fm.2015.04.005
0740-0020/

1. Introduction

Some special dry white wines produced in Spain (particularly in the Jerez, Sanlúcar de Barrameda and Montilla–Moriles regions), Italy (Sardinia and Sicily), France (Jura), USA (California) and various South African and Australian regions, are subjected to a characteristic aging process effected by selected *Saccharomyces cerevisiae* yeast strains capable of growing on the wine surface, where they form a thick biofilm. The main result of metabolic activity in these yeasts is a decrease in the contents in ethanol and glycerol —their major carbon sources—and an increase in that of acetaldehyde —the major metabolite released into the wine. In addition, ethanol metabolism increases the concentrations of acetic acid, 2,3-butanediol and acetoin, and facilitates their incorporation as carbohydrates, lipids and proteins into yeast cells via the tricarboxylic acid cycle (García-Maiquez, 1988, 1995; Domecq, 1989; Martínez et al., 1998; Zara et al., 2010; Moreno and Peinado, 2012; Moreno-García et al., 2013).

Although changes in flavor-related substances such as acetaldehyde and its derivatives, ethanol, some benzyl derivatives, organic acids, higher alcohols, esters, lactones and nitrogen compounds resulting from the metabolism of flor yeasts, and their associated sensory properties, have been examined in depth (Pham et al., 1995; Cortés et al., 1998; Mauricio et al., 2001; Mesa et al., 2000; Muñoz et al., 2005, 2007; Peinado and Mauricio, 2009; Villamiel et al., 2008; Zea et al., 2001, 2007; Alexandre, 2013), the proteome of flor yeasts remains poorly known (Moreno-García et al., 2014). In fact, although proteome analyses for enological purposes have expanded substantially in recent years, particularly as regards fermentative yeasts (Zuzuarregui et al., 2006; Salvadó et al., 2008; Rossignol et al., 2009), the relationship between changes in yeast proteome and exometabolome, and its influence on the organoleptic properties of wine, remain unexplored.

In this work, we used an OFFGEL fractionator and an LTQ Orbitrap analyzer for proteome analysis in combination with the hyphenated techniques SBSE-TD-GC–MS and GC-FID for metabolome analysis with a view to distinguishing the proteome and exometabolome of *S. cerevisiae* flor yeast strain G1 growing under biofilm formation condition. For this purpose, *S. cerevisiae* G1 yeasts and their culture medium were examined at the first stage of the biofilm formation process and the results compared with those for a culture in a synthetic medium containing a high glucose concentration under non biofilm condition (viz., the reference condition).

2. Material and methods

2.1. Microorganism, inoculum and media

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 (University of Cordoba, Spain). This strain forms a thick film with up to 90% cell viability on plating about 30 days after inoculation in an appropriate medium (Mauricio et al., 1997).

The yeast was cultivated in 200 mL pre-inoculum medium YPD (1% yeast extract: 2% peptone and 2% glucose) for 24 h, after which cells were separated by centrifugation in a Rotina-38 centrifuge from Hettich GmbH (Tuttlingen, Germany) at 4500 g for 10 min and water-rinsed under aseptic conditions. A population of $1 \cdot 10^6$ cells/mL was inoculated to each synthetic medium under biofilm formation (BFC) and nonbiofilm formation conditions (NBFC).

The medium used to assess yeast growth under BFC consisted of 0.67% (w/v) YNB without amino acids (Difco), 10 mM glutamic acid, 1% (w/v) glycerol and 10% (v/v) ethanol as nonfermentable carbon sources plus Milli-Q water to a volume of 250 mL. The medium was incubated at 21 °C without shaking for 29 days, time by which its whole surface was covered by a yeast film. At that point, the film contained $83.5 \pm 30 \cdot 10^6$ yeast cells and exhibited a cell viability above 90%, which is consistent with the initial stage of a freshly formed biofilm (Mauricio et al., 1997).

The growing medium used as reference consisted 0.67% (w/v) YNB without amino acids (Difco), 10 mM glutamic acid and 17% (w/v) glucose as fermentable carbon source. Incubation was done at 21 °C under gentle shaking in order to ensure homogeneous distribution of nutrients (Salvadó et al., 2008). Yeast cells were gathered by centrifugation from the medium at the beginning of the exponential growth phase, when the yeast concentration was about $27 \pm 2 \cdot 10^6$ cells/mL and cell viability higher than 90%.

Both growing media were autoclaved at 120 °C for 20 min. Glucose was sterilized separately and ethanol was added to the biofilm formation medium after autoclaving and cooling. All tests were performed in synthetic media in order to facilitate monitoring and standardization. Yeast cell numbers were determined in a Beckman Coulter Z2 particle counter, using the manufacturer's recommended dilution. Cell viability was determined by plating and found to exceed 90% under both types of conditions. All experiments were performed in triplicate, using 250 mL Erlenmeyer flasks stoppered with hydrophobic cotton. The initial dissolved oxygen concentration (8 mg/L) was measured with a Crison OXY-92 oxygen meter.

2.2. Proteome analysis

Cells from the reference (NBFC) medium were collected by centrifugation within the first 12 h of the exponential growth stage, when the glucose concentration was still high and the number of detectable proteins large (Ghaemmaghami et al., 2003; Salvadó et al., 2008). In the BFC experiments, cells from the yeast film covering the surface of the medium were collected by siphonation with a sterilized glass tube from each Erlenmeyer flask after a flor film was fully formed (viz., after 29 days). Yeast cells from both media were centrifuged in a Rotina-38 centrifuge at 4500 g for 10 min and rinsed with sterile distilled water at 4 °C. The cell pellets thus obtained were subjected to protein extraction, followed by OFFGEL protein fractionation, LTQ Orbitrap MS analysis and quantitation in terms of the exponentially modified protein abundance index (emPAI; Ishihama et al., 2005). These procedures and methods are described in detail elsewhere (Moreno-García et al., 2015).

2.3. Metabolome analysis

Metabolites in the supernatant were analyzed following removal of yeast cells by centrifugation after 12 h of inoculation under NBFC or 29 days under BFC. Fermentable sugars, titratable acidity, volatile acidity, pH and ethanol were quantified by using the European Union's recommended methods (EEC, 1990). Exometabolites related to the sensory properties of wine and fermented beverages (e.g., major volatile organic compounds and polyols) were quantified by GC, using a model 6890 gas chromatograph from Agilent (Palo Alto, CA, USA) that was fitted with a CP-WAX 57-CB capillary column (60 m long × 0.25 mm i.d., 0.4 μm film thickness) and a flame ionization detector (FID), using direct injections of 0.5 μL aliquots of liquid sample. The procedure is described in detail elsewhere (Peinado et al., 2004).

Other major volatile compounds contributing to the sensory properties of wine despite their low concentrations —less than 10 mg/L— were determined by using the analytical platform SBSE-TD-GC–MS (Stir Bar Sorptive Extraction–Thermal Desorption–Gas Chromatography–Mass Spectrometry), which included a GC-