Food Microbiology 43 (2014) 5–15

Contents lists available at ScienceDirect

Food Microbiology

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

Use of non-*Saccharomyces* wine yeasts as novel sources of mannoproteins in wine

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ARTICLE INFO

Article history:
Received 12 February 2014
Received in revised form
22 March 2014
Accepted 14 April 2014
Available online 30 April 2014

Keywords:
Wine
Non-*Saccharomyces*
Yeast
Mannoprotein
Polysaccharide
N-glycan
MALDI-TOF

ABSTRACT

Eight non-*Saccharomyces* wine strains, previously selected for their ability to modulate the final concentrations of various volatile compounds and to persist with *Saccharomyces cerevisiae* in mixed inocula fermentations of grape juice, have been analyzed in the present work to test their ability to release mannoproteins. The eight strains were members of different genera originally isolated from grape: *Hansensiaspora osmophila*, *Lachancea thermotolerans*, *Metschnikowia pulcherrima*, *Pichia fermentans*, *Saccharomycodes ludwigii*, *Starmerella bacillaris*, *Torulaspora delbrueckii* and *Zygosaccharomyces florentinus*. A synthetic polysaccharide-free grape juice, was used to characterize the mannoproteins released during the alcoholic fermentation. Mannoproteins profiles were characterized by gel electrophoresis and carbohydrate composition was analyzed both by HPLC and by mass spectrometry. The eight non-*Saccharomyces* yeasts demonstrated a higher capacity to release polysaccharides compared to *S. cerevisiae*. The proteins released by the eight yeast strains showed a wide variety of protein sizes, ranging from 25 kDa to greater than 250 kDa. The mass spectrometric profile of the *N*-glycans ranged from 1600 to 4000 Da and was characteristic for each strain. Detailed investigation of the degree of polymerization of released *N*-glycans revealed variable composition from 8 to 15 units of monosaccharides.

1. Introduction

Mannoproteins are released from the yeast cell wall during the alcoholic fermentation and wine aging processes (Boivin et al., 1998; Charpentier and Feuillat, 1993; Charpentier et al., 2004a; Llaubères et al., 1987) and represent one of the major polysaccharides found in wine. The mannoproteins have been recognized to have many positive enological properties such as improving mouth-feel and fullness (Vidal et al., 2004), decreasing astringency (Carvalho et al., 2006), adding complexity and aromatic persistence (Chalier et al., 2007), increasing sweetness and roundness (Guadalupe et al., 2007), and reducing protein and tartrate instability (Gonzalez-Ramos et al., 2008). Moreover, mannoproteins can stimulate malolactic fermentation by lactic acid bacteria (Guilloux-Benatier et al., 1995; Rosi et al., 2000), adsorb some toxic compounds possibly present in the wine such as ochratoxin A (Caridi, 2007; Moruno et al., 2005), and improve the foam quality of sparkling wines (Moreno-Arribas et al., 2000; Nunez et al., 2006).

In order to increase the amount of mannoproteins released during the alcoholic fermentation, some researchers have developed autolytic thermosensitive mutants (Giovani and Rosi, 2007) or genetically engineered wine yeast strains of *Saccharomyces cerevisiae* (Brown et al., 2007; Gonzalez-Ramos and Gonzalez, 2006; Gonzalez-Ramos et al., 2008). However, these organisms are likely to be viewed as releasing components not normally present in wine.

Recently, some authors (Comitini et al., 2011; Domizio et al., 2010, 2011; Gobbi et al., 2013; Romani et al., 2010) have shown that other non-*Saccharomyces* wine yeasts found in grape and wine making environments have a high capacity to release important

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http://dx.doi.org/10.1016/j.fm.2014.04.005
0740-0020/

polysaccharides (including mannoproteins) into wine during alcoholic fermentation and these polysaccharides and mannoproteins would be naturally present in wine.

The possibility to increase the content of mannoproteins naturally by the use of non-*Saccharomyces* yeasts could represent an added value to the already heightened interest towards these wine yeasts for flavor modification (Ciani et al., 2010). Indeed, there are many reports indicating that the use of non-*Saccharomyces* yeasts leads to a more complex aroma and an improved wine quality (Anfang et al., 2009; Domizio et al., 2007; Jolly et al., 2003, 2006; Renouf et al., 2007; Rojas et al., 2001; Swiegers et al., 2005). The use of non-*Saccharomyces* wine yeasts in association with *Saccharomyces* strains has been suggested to also enhance the glycerol content (Ciani and Ferraro, 1996, 1998; Soden et al., 2000), deacidify the grape juice or wine (Ciani, 1995; Magyar and Panyik, 1989), reduce the acetic acid content (Bely et al., 2008; Rantsiou et al., 2012), and enhance the total acidity of wines (Gobbi et al., 2013; Kapsopoulou et al., 2007; Mora et al., 1990). The presence on the market of new yeast formulas of mixed starter cultures containing both *Saccharomyces* and non-*Saccharomyces* strains is evidence of the strong commercial interest in mixed fermentations.

Non-*Saccharomyces* yeasts could influence not only the polysaccharide concentration in the wine, as previously reported, but could also influence the final chemical composition of these macromolecules, leading to functional differences in the resultant wine. Previous analyses of mannoproteins released in wine during alcoholic fermentation have been conducted mainly using strains belonging to *Saccharomyces* (Escot et al., 2001; Llaubères et al., 1987). The resulting macromolecule composition was found to be similar to that of the yeast cell wall, with a molecular mass between 50 and 500 kDa (Villetaz et al., 1980).

The *Saccharomyces* mannoproteins are found in the outmost layer of yeast cell wall (Fleet, 1991; Klis et al., 2006), and consist of 85–90% carbohydrates, mainly mannose, and 10–15% proteins. The release of these macromolecules during the growth of yeast cells (Guilloux-Benatier et al., 1995; Llaubères et al., 1987) is due to a controlled hydrolysis of the mother cell wall to allow bud emergence (Charpentier et al., 1986; Fleet, 1991).

The glycans are attached to the protein via an asparagine residue; these *N*-linked glycans represent one form of glycosylation previously described in yeasts (Ballou, 1976). Previous works have shown that *S. cerevisiae* *N*-linked glycans are composed of a core of $Man_{8-14}GlcNAc_2$, (Mannose, *N*-acetyl glucosamine) and a highly branched outer chain (50–200 mannose units) of α-linked mannose and mannose-phosphate residues (Ballou, 1990; Trimble and Atkinson, 1992).

Glycans and glyco-conjugates are an interesting and diverse class of molecules with many important bioactive functions; however their inherent structural complexity and diversity renders them challenging to study in a comprehensive manner and requires the use of intensive purification prior to analytical characterization.

The recent use of accurate mass spectrometry techniques such as the soft ionization method known as MALDI (Matrix-assisted laser desorption ionization) as well as the ESI (electrospray ionization) method, in combination with microchip-based nano-liquid chromatographic separation, have allowed isolation and characterization of new oligosaccharides in both red and white wine (Bordiga et al., 2012; Ducasse et al., 2010).

In the present study we evaluated eight non-*Saccharomyces* strains, selected because of desirable enological attributes, for their ability to release mannoproteins into a synthetic grape juice. We report the chemical characterization of these components in terms of carbohydrate composition analyzed by HPLC, protein profile by gel electrophoresis as well as detailed investigation of the degree of polymerization of released *N*-glycans (8–15 units) by mass spectrometry.

2. Materials and methods

2.1. Yeast strains

Eight non-*Saccharomyces* strains from the yeast culture collection of the Department of Agricultural, Food and Forestry Systems (GESAAF, University of Florence, Italy) and of the Department of the Polytechnic University of Marche SAIFET (Ancona, Italy) were used. These yeast strains, all belonging to different genera (Table 1), have been isolated from grape and must of different origins, and already were selected for their enological attributes in mixed fermentation in grape juice at the laboratory scale (Comitini et al., 2011; Domizio et al., 2011). A commercial strain, Lalvin EC1118 (Lallemand Inc., Montreal, Canada), was used as reference strain for *Saccharomyces* and for comparison determination.

2.2. Fermentation trials

The fermentations were carried out in duplicate at 28 °C in 200 mL Erlenmeyer flasks containing 150 mL of a synthetic grape juice medium "Minimal Must Medium" (MMM) (Spiropoulos et al., 2000). The medium was sterilized by filtration. The flasks were inoculated at optical density of 0.1 ($OD_{600\ nm}$), with 48-h precultures grown in 10 mL of YPD medium (10 g/L yeast extract, 20 g/L peptone, 20 g/L glucose) (Oxoid Unipath Ltd, Hampshire, UK), at 25 °C in a roller drum.

The levels of sugar and total assimilable nitrogen were 220 g/L and 208 mg/L, respectively. The assimilable nitrogen concentration was obtained by using 0.2 g/L of L-arginine and 0.5 g/L of ammonium phosphate. The flasks, continuously agitated at 150 rpm, were stoppered with silicone vent bungs (Ferm-Rite), allowing the CO_2 to "burp" out and escape. The flasks were weighed every day until the end of fermentation (as a constant weight for two consecutive days) to monitor the fermentation kinetics.

2.3. Analysis

2.3.1. Biomass determination

Samples were taken from each flask during the alcoholic fermentation to monitor the growth kinetics by $OD_{600\ nm}$. The cell viability, expressed as number of colony forming units (cfu), of *S. cerevisiae* and non-*Saccharomyces* yeast strains, was measured at the beginning and at the end of the alcoholic fermentation by

Table 1
Origin of the eight non-*Saccharomyces* strains and the one commercial strain of *S. cerevisiae* used in this study.

Strain	Species	Origin
# EC1118	*Saccharomyces cerevisiae*	Lallemand[a]
# 4	*Pichia fermentans*	SAIFET[b]
# 22	*Starmerella bacillaris* (formerly *Candida zemplinina*)	GESAAF[c]
# 32	*Hanseniaspora osmophila*	GESAAF[c]
# 42	*Zygosaccharomyces florentinus*	GESAAF[c]
# 46	*Metschnikowia pulcherrima*	SAIFET[b]
# 64	*Saccharomycodes ludwigii*	GESAAF[c]
# 92	*Torulaspora delbrueckii*	GESAAF[c]
# 101	*Lachancea thermotolerans*	SAIFET[b]

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