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Selected non-*Saccharomyces* wine yeasts in controlled multistarter fermentations with *Saccharomyces cerevisiae*

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ABSTRACT

Non-*Saccharomyces* yeasts are metabolically active during spontaneous and inoculated must fermentations, and by producing a plethora of by-products, they can contribute to the definition of the wine aroma. Thus, use of *Saccharomyces* and non-*Saccharomyces* yeasts as mixed starter cultures for inoculation of wine fermentations is of increasing interest for quality enhancement and improved complexity of wines. We initially characterized 34 non-*Saccharomyces* yeasts of the genera *Candida*, *Lachancea* (*Kluyveromyces*), *Metschnikowia* and *Torulaspora*, and evaluated their enological potential. This confirmed that non-*Saccharomyces* yeasts from wine-related environments represent a rich sink of unexplored biodiversity for the winemaking industry. From these, we selected four non-*Saccharomyces* yeasts to combine with starter cultures of *Saccharomyces cerevisiae* in mixed fermentation trials. The kinetics of growth and fermentation, and the analytical profiles of the wines produced indicate that these non-*Saccharomyces* strains can be used with *S. cerevisiae* starter cultures to increase polysaccharide, glycerol and volatile compound production, to reduce volatile acidity, and to increase or reduce the total acidity of the final wines, depending on yeast species and inoculum ratio used. The overall effects of the non-*Saccharomyces* yeasts on fermentation and wine quality were strictly dependent on the *Saccharomyces*/non-*Saccharomyces* inoculum ratio that mimicked the differences of fermentation conditions (natural or simultaneous inoculated fermentation).

1. Introduction

One of the most important technological advances in winemaking has been the inoculation of grape juice with selected cultures of *Saccharomyces cerevisiae*. This has been based on the evidence that microbiological control of the fermentation process allows better management of this alcoholic fermentation (Barre and Vezinhet, 1984; Bisson and Kunkee, 1993; Pretorius, 2000; Ranieri and Pretorius, 2000; Fleet, 2008). It is believed that a selected and inoculated strain of *S. cerevisiae* will suppress any 'indigenous' non-*Saccharomyces* species and dominate the fermentation process. Although this expectation has been widely accepted by winemakers, several studies have revealed that non-*Saccharomyces* yeasts can indeed persist during the various stages of fermentations that are inoculated with pure cultures of *S. cerevisiae* (Bouix et al., 1981; Heard and Fleet, 1985; Martinez et al., 1989; Mora et al., 1990).

The use of non-*Saccharomyces* wine yeasts in pure cultures as fermentation starters has indicated that these have some beneficial and several negative fermentation characteristics. Among the latter, there is the production of acetic acid, ethyl acetate, acetaldehyde and acetoin at high concentrations, which generally prevents the use of such strains as starter cultures. Moreover, most of the non-*Saccharomyces* species coming from wine-related environments have limited fermentation potential, such as low fermentation power and rates, as well as low SO_2 resistance (Herraiz et al., 1990; Ciani, 1997; Ciani and Maccarelli, 1998; Ferreira et al., 2001; Farkas et al., 2005; Garde-Cerdán and Ancín-Azpilicueta, 2006; Howell et al., 2006; Jolly et al., 2006). However, over the last decade, several studies have been revaluating the involvement of non-*Saccharomyces* yeasts during alcoholic fermentation and their role on the metabolic impact and aroma complexity of the final product (Lema et al., 1996; Egli et al., 1998; Henick-Kling et al., 1998; Rojas et al., 2001; Zohre and Erten, 2002; Fleet, 2003; Jolly et al., 2003; Domizio et al., 2007).

In mixed fermentations, such as natural fermentations, some negative enological characters of non-*Saccharomyces* yeasts might

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not be expressed, or might be modified by *S. cerevisiae* cultures (Howell et al., 2006; Anfang et al., 2009; Varela et al., 2009). Different studies have shown that in natural fermentations, *Saccharomyces* and non-*Saccharomyces* yeasts do not passively coexist; instead, they appear to interact. Under these conditions, some enological traits of the non-*Saccharomyces* yeasts are not expressed, or they can be modulated by the *S. cerevisiae* yeast cultures (Ciani and Ferraro, 1998; Ciani et al., 2006; Bely et al., 2008; Anfang et al., 2009). In this context, the use of controlled mixed fermentations of *Saccharomyces* and non-*Saccharomyces* yeast species isolated from the wine environment has been proposed (Soden et al., 2000; Toro and Vazquez, 2002; Ciani et al., 2006, 2010; Jolly et al., 2006; Mendoza et al., 2007; Moreira et al., 2008; Anfang et al., 2009). Indeed, the use of non-*Saccharomyces* wine yeasts together with *Saccharomyces* strains in mixed fermentations might be recommended as a tool to obtain the advantages of spontaneous fermentation, while avoiding the risks of stuck fermentation (Romano et al., 2003; Rojas et al., 2003; Jolly et al., 2006; Ciani et al., 2010). Furthermore, non-*Saccharomyces* wine yeasts have some specific enological characteristics that are absent in *S. cerevisiae* species, and these can have additive effects on wine flavour and aroma (Languet et al., 2005; Strauss et al., 2001; Viana et al., 2008).

In the present study, we initially characterized 34 non-*Saccharomyces* yeasts isolated from wine-related environments, with the aim of determining their enological potential. On the basis of this characterization, four of these non-*Saccharomyces* yeasts were selected to be used together with starter cultures of *S. cerevisiae*. The fermentation behavior of these mixed cultures and the analytical profiles of the final wines were then evaluated. Here, we show that in winemaking, the use of controlled mixed cultures of selected non-*Saccharomyces* and *Saccharomyces* strains can have advantages over fermentations inoculated with pure cultures of *S. cerevisiae*. This can thus lead to the production of wines with more predictable and desirable characteristics.

2. Materials and methods

2.1. Microorganisms and media

Thirty-four non-*Saccharomyces* strains from the Yeast Culture Collection of the SAIFET Department of the Polytechnic University of Marche (Ancona, Italy) and belonging to the genera *Candida*, *Torulaspora*, *Lachancea* (formerly known as *Kluyveromyces*) and *Metschnikowia* were used. All of the strains had been previously identified by PCR-RFLP analysis of their rDNA internal transcribed spacers (Stringini et al., 2009). The Lalvin EC1118 *S. cerevisiae* commercial strain (purchased as active dry yeast by Lallemand Inc.,) and three further *S. cerevisiae* strains (#44, #49, #102) belonging to the Yeast Culture Collection of SAIFET were used as the selected starters.

The yeast strains were sub-cultured on YPD (10 g l^{-1} yeast extract, 20 g l^{-1} peptone, 20 g l^{-1} glucose, 20 g l^{-1} agar) at six-month intervals, and maintained at 6 °C. The media used were WL Nutrient Agar (Oxoid, Hampshire, UK), for differentiation of wild yeasts from must samples (Pallman et al., 2001), and Lysine Agar (Oxoid, Hampshire, UK), for the differentiation of non-*Saccharomyces* yeast populations from the *S. cerevisiae* starter strains.

2.2. Screening for enological characteristics

2.2.1. Screening on agar plates

The yeast SO_2 resistances were evaluated on sterile YPD medium buffered at pH 3 with citrate-phosphate buffer. The different concentrations of SO_2 were obtained from a 100× concentrated, filter-sterilized (pore-size, 0.45 μm), stock solution of $K_2S_2O_5$, which was prepared and added to the medium as needed. An arbitrary scale was used that had five levels (0–4) that corresponded to 0, 10, 20, 40, 60 mg l^{-1} free SO_2, respectively. The medium was poured into Petri dishes and the SO_2 stock solution was added at 40 °C. The strains were inoculated onto the medium using a multipoint inoculator, and incubated at 25 °C for 3 days. *S. cerevisiae* strains (#44, #49, #102) belonging to the Yeast Culture Collection of SAIFET were used as positive control.

The β-glucosidase activity was evaluated as reported by Rosi et al. (1994), on a medium containing 5 g l^{-1} arbutin (hydroquinone β-D-glucopyranoside), 6.7 g l^{-1} Yeast Nitrogen Base and 20 g l^{-1} agar. After autoclaving, 2 ml of sterile 1% ferric ammonium citrate solution was added to 100 ml melted medium, which was then poured into Petri dishes. The yeast strains were inoculated as above and incubated at 25 °C for 3 days. A dark brown halo around the colonies indicated the presence of β-glucosidase activity. Strains of *S. cerevisiae* #CO.8 and *Kodamenia laetipori* #N6 belonging to Yeast Culture Collection of SAIFET were used as negative and positive control, respectively.

The glycosidase activity was determined according to the method described by Hildebrand and Caesar (1989), using a plate assay on a medium containing 6.7 g l^{-1} Yeast Nitrogen Base, 1 g l^{-1} glucose, 2 g l^{-1} rutin (quercetin-3-rutinoside) and 20 g l^{-1} agar. The yeast strains were inoculated as above and incubated at 25 °C for 3 days. The glycosidase (α-L-rhamnosidase) activity was detected as a clear zone surrounding the seeded strains. *Debaryomyces polymorphus* DBVPG 3625 and *S. cerevisiae* DBVPG 1883 were used as positive and negative controls, respectively.

The protease activity assay was carried out on medium containing 3 g l^{-1} yeast extract, 3 g l^{-1} malt extract, 5 g l^{-1} peptone, 10 g l^{-1} glucose, 5 g l^{-1} NaCl, and 15 g l^{-1} agar. In a separate bottle, an equal volume of skimmed milk dissolved in sterile water was prepared. After sterilization, the two solutions were mixed and poured into sterile Petri dishes. The strains were inoculated as above and incubated at 25 °C for 3 days. The presence of a clear zone around the inoculum indicated the protease activity. *Kloeckera apiculata* DBVPG 3037 and *S. cerevisiae* DBVPG 1883 were used as positive and negative controls, respectively.

The ester–hydrolase activity was evaluated by adapting the procedure of Parkkinen et al. (1978) to a microplate screening using the following solutions: buffer solution, pH 6.25 (KH_2PO_4/Na_2HPO_4); pNPA solution, prepared by dissolving 18 mg p-nitrophenyl acetate (pNPA) in 10 ml methanol and adding one drop of 37% HCl. Just before the pNPA test solution was mixed with buffer solution 1:9 ratio. Ten minutes after the inoculation of the strains, the samples were assessed for esterase activity, which was revealed by the appearance of a yellow color. To determine the intensity of the color, the following arbitrary scale was used: 0, colorless (no activity); 1, light yellow; 2, dark yellow. *Dekkera bruxellensis* DBVPG 6710 and *S. cerevisiae* DBVPG 6497 were used as positive and negative controls, respectively.

The killer character was evaluated using the plate assay described by Rosini (1985), with positive activity recognized by inhibition of growth of the sensitive strain (*S. cerevisiae* DBVPG 6500), seen as a clear zone surrounding the seeded strain. The *S. cerevisiae* killer strain DBVPG 6499 was used as positive control.

The H_2S production was evaluated on Biggy Agar (Difco, Detroit, Mi, USA). On this medium, H_2S-negative strains show white colonies, while H_2S-producing colonies are a brown or dark brown color. The following arbitrary scale was used: 1, white color (no production); 2, light brown; 3, brown; 4, dark brown; 5, dark brown/black. The DBVPG 1883 wine strain of *S. cerevisiae* (H_2S non-producing strain) was used as negative control.