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Outlining a future for non-*Saccharomyces* yeasts: Selection of putative spoilage wine strains to be used in association with *Saccharomyces cerevisiae* for grape juice fermentation

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ABSTRACT

The use of non-*Saccharomyces* yeasts that are generally considered as spoilage yeasts, in association with *Saccharomyces cerevisiae* for grape must fermentation was here evaluated. Analysis of the main oenological characteristics of pure cultures of 55 yeasts belonging to the genera *Hanseniaspora*, *Pichia, Saccharomycodes* and *Zygosaccharomyces* revealed wide biodiversity within each genus. Moreover, many of these non-*Saccharomyces* strains had interesting oenological properties in terms of fermentation purity, and ethanol and secondary metabolite production. The use of four non-*Saccharomyces* yeasts (one per genus) in mixed cultures with a commercial *S. cerevisiae* strain at different *S. cerevisiae*/non-*Saccharomyces* inoculum ratios was investigated. This revealed that most of the compounds normally produced at high concentrations by pure cultures of non-*Saccharomyces*, and which are considered detrimental to wine quality, do not reach threshold taste levels in these mixed fermentations. On the other hand, the analytical profiles of the wines produced by these mixed cultures indicated that depending on the yeast species and the *S. cerevisiae*/non-*Saccharomyces* inoculum ratio, these non-*Saccharomyces* yeasts can be used to increase production of polysaccharides and to modulate the final concentrations of acetic acid and volatile compounds, such as ethyl acetate, phenyl-ethyl acetate, 2-phenyl ethanol, and 2-methyl 1-butanol.

1. Introduction

Yeasts belonging to the genera *Hanseniaspora*, *Pichia*, *Saccharomycodes* and *Zygosaccharomyces* are generally believed to be wine spoilage yeasts, as they are often isolated during stuck or sluggish fermentations, or from wines with anomalous analytical and sensorial profiles. However, different studies have indicated that most of the yeasts belonging to these genera produce enzymes involved in the release of aromatic compounds from non-aromatic precursors in the grapes (Fernandez et al., 2000), and they might therefore enhance the wine aroma (Ferreira et al., 2001). Also, the production of acetate esters by these yeasts has been investigated in depth (Ciani et al., 2006; Kurita, 2008; Moreira et al., 2005; Plata et al., 2003; Rojas et al., 2001; Rojas et al., 2003; Viana et al., 2008). These studies have indicated that although they can produce high amounts of ethyl acetate, non-*Saccharomyces* wine yeasts are good producers of esters, such as isoamyl acetate (banana-like aroma) and 2-phenyl-ethyl acetate (fruity and flowery flavour), which contribute to the fruity flavour of wines. In particular, Viana et al. (2008) found that among 38 non-*Saccharomyces* yeasts screened as pure cultures, those belonging to the genus *Hanseniaspora* were the best producers of acetate esters, and especially of 2-phenyl-ethyl acetate. Similar to *Hanseniaspora*, the genus *Pichia* also showed high production of ethyl acetate, and was the second-best producer of isobutyl acetate and isoamyl acetate. However, while fermentations carried out with pure cultures of these yeasts often lead to the production of wines with mainly negative features, their presence in mixed cultures with *Saccharomyces* might result in the production of wines with particular sensorial characteristics. Indeed, over the last few years, different studies have investigated some interactions between *Saccharomyces* and non-*Saccharomyces* yeasts in spontaneous fermentations, and they have shown that the *Saccharomyces* yeasts can modulate the expression of some of the oenological traits of the non-*Saccharomyces* yeasts (Anfang et al., 2009; Bely et al., 2008). Several examples regard the production/reduction of acetaldehyde and acetoin (Cheraiti et al.,

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2005; Ciani and Ferraro, 1998; Ciani et al., 2006; Vriesekoop et al., 2007) and the significant variations in the production of esters and ethyl acetate (Ciani et al., 2006; Gil et al., 1996; Moreira et al., 2008) in mixed fermentations, in comparison to pure cultures of *S. cerevisiae*. Also, the growth and death kinetics of the yeasts in mixed cultures are mutually influential. In particular, different studies have shown that mixed fermentations can result in lower biomass production and increased persistence of non-*Saccharomyces* yeasts particularly at low temperature (Moreira et al., 2005; Ciani et al., 2006; Mendoza et al., 2007).

Based on these evidences, and with the aim to increase the complexity of the final wines, we evaluated the use of wild non-*Saccharomyces* yeasts in association with *S. cerevisiae* for grape-must fermentation. Initially, the main oenological characteristics and the fermentative performances of 55 yeasts belonging to the genera *Hanseniaspora*, *Pichia, Saccharomycodes* and *Zygosaccharomyces* were determined. In a second step, one yeast from each genus was selected and used in mixed cultures with a commercial *S. cerevisiae* strain, at different inoculum ratios.

2. Materials and methods

2.1. Yeasts strains

Fifty-five non-*Saccharomyces* strains from the yeasts culture collection of the Department of Agricultural Biotechnologies (University of Florence, Italy) and belonging to the genera *Hanseniaspora*, *Pichia*, *Saccharomycodes* and *Zygosaccharomyces* were used. All the non-*Saccharomyces* strains utilized were previously isolated from grape must of different origins and were identified by means of polymerase chain reaction–restriction fragment length polymorphism (PCR-RFLP) of internal transcribed spacers (ITS), according to Esteve-Zarzoso et al. (1999) (Table 1). The *S. cerevisiae* strains used (Sc44, Sc49, Sc102) belong to the Yeast Culture Collection of the SAIFET Department of the Polytechnic University of Marche (Ancona, Italy); these were used as control strains for the species. All of these yeasts were maintained on slants in YPD medium (10 g/L yeast extract, 20 g/L peptone, 20 g/L glucose, 20 g/L agar) (Oxoid Unipath Ltd, Hampshire, UK), and kept at 4 °C. The *S. cerevisiae* commercial strain Lalvin EC1118 (purchased as active dry yeast by Lallemand Inc., Toulouse, France) was used as the selected starter, and as the control for the species in fermentation trials carried out with mixed cultures (Table 1).

Table 1
Strains used in the study.

Genus	Species	Strain code	Total strains
Hanseniaspora	*osmophila*	Ha25, Ha27, Ha32	3
	uvarum	Ha20, Ha21, Ha23, Ha30, Ha31	5
	valbyensis	Ha26, Ha28	2
	vineae	Ha24	1
Pichia	*anomala*	Pi9	1
	fermentans	Pi1, Pi2, Pi3, Pi4, Pi5	5
	fluxuum	Pi11, Pi13	2
	guilliermondii	Pi7	1
	kluyveri	Pi16	1
	membranifaciens	Pi8, Pi12, Pi15, Pi17	4
Saccharomycodes	*ludwigii*	Sd55, Sd56, Sd57, Sd58, Sd59, Sd60, Sd61, Sd62, Sd63, Sd64, Sd65, Sd66	12
Zygosaccharomyces	*bailii*	Zy80, Zy82, Zy84, Zy87, Zy88, Zy89	6
	bisporus	Zy83, Zy85, Zy86, Zy90, Zy97, Zy98	6
	florentinus	Zy41, Zy42, Zy43, Zy96	4
	fermentati	Zy91	1
	rouxii	Zy81	1
Saccharomyces	*cerevisiae*	Sc44, Sc49, Sc102, EC1118	4

2.2. Screening of non-Saccharomyces yeasts

2.2.1. Screening on agar plates

The SO_2 resistance was evaluated on YPD medium buffered at pH 3 (with citrate-phosphate buffer), added with increasing doses of $K_2S_2O_5$ An arbitrary scale was used, with five levels (0 to 4) that corresponded to 0, 10, 20, 40, 60 mg/L free SO_2, respectively. The strains were inoculated onto the medium and incubated at 25 °C. Yeast growth was observed daily up to the third day after the inoculation. *S. cerevisiae* strains (Sc44, Sc49, Sc102) belonging to the Yeast Culture Collection of SAIFET were used as positive control.

The H_2S production potential was determined by plating the yeasts onto Biggy Agar (Oxoid Unipath Ltd, Hampshire, UK). On this medium H_2S-positive strains colonies show brown or black colonies, while H_2S-negative colonies are white. The H_2S produced was evaluated after 48 h to 72 h of incubation at 26 °C, on the basis of the colours of the colonies. The following arbitrary scale was used: 1, white (no production); 2, light brown; 3, brown; 4, dark brown; 5, black. The DBVPG 1883 wine strain of *S. cerevisiae* (H_2S non-producing strain) was used as negative control. The ester-hydrolase activity was evaluated by inoculating a loop full of culture grown for 5 days on YPD medium into a solution containing 1 mM p-nitro-phenyl acetate (pNPA) in 0.33 M phosphate-buffered solution, pH 6.2. The ester-hydrolase activity was determined after 10 min by the appearance of a yellow colour with an increasing intensity proportional to the amount of p-nitrophenol (pNP) released after the hydrolysis of the p-nitro-phenyl acetate. To define the colour intensity, an arbitrary scale from 0 to 2 was adopted. *Dekkera bruxellensis* DBVPG 6710 and *S. cerevisiae* DBVPG 6497 were used as positive and negative controls, respectively.

The β-glucosidase activity was evaluated as described by Rosi et al. (1994) on agar plates containing arbutin (hydroquinone-β-D-glucopyranoside) as substrate. Strains with this activity hydrolyze the substrate, and dark brown colour develops in the agar. 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 protease activity was evaluated by streaking yeast strains onto a medium containing 0.17% (w/v) Yeast Nitrogen Base (YNB) (without amino acids and ammonium sulphate, Difco, Detroit, USA), 1% glucose (w/v), 0.002% L-histidine (w/v), 0.5% Hammarsten casein (w/v), 2% agar (w/v), pH 3.5. The plates were monitored for the appearance of clear zones surrounding the streak after 5–6 days of incubation at 26 °C. *Kloeckera apiculata* DBVPG 3037 and *S. cerevisiae* DBVPG 1883 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, showing K2 killer activity, was used as positive control.

2.2.2. Screening on grape juice (microfermentations)

Fermentations were carried out in 200 mL Erlenmeyer flasks containing 140 mL commercial white grape juice, not added with SO_2, supplemented with equimolar glucose and fructose to a final concentration of 26.3% (w/v) sugar. The flasks were inoculated with 48 h pre-cultures grown in the same medium at 25 °C, to obtain a cell concentration of 10^6 cell/mL. The flasks were equipped with Müller valves containing sulphuric acid (Ciani and Rosini, 1987) to allow only CO_2 to escape from the system and weighed every day until the end of fermentation (as a constant weight for two consecutive days). All the fermentations were carried out as two independent experiments, each carried out in duplicate, under static conditions.