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RESEARCH ARTICLE

Selection of non-*Saccharomyces* yeasts to be used in grape musts with high alcoholic potential: a strategy to obtain wines with reduced ethanol content

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One sentence summary: An integrated selection strategy is proposed in order to select non-*Saccharomyces* yeasts to obtain reduced ethanol wines with positive oenological traits.

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ABSTRACT

Ethanol content of wine has increased over the last decades as consequence of searching phenolic maturity, requiring increased grape maturity. This may result in the production of wines with excessive alcohol levels (sometimes more than 15% (v/v)), sluggish and stuck fermentations and excessive volatile acidity. Many strategies to reduce ethanol in wines are being studied, and microbial methods have some additional advantages. However, because of the broad intra- and interspecies variability, new selection criteria should be included. Therefore, the goal of the present work was to design and evaluate a simple and integral procedure for non-*Saccharomyces* yeast selection. This strategy allowed selection of yeasts that presented successful implantation in grape must with high alcohol potential and their use in co-cultures could reduce the ethanol in wines. A total of 114 native non-*Saccharomyces* yeasts were assayed to determine their respiratory, fermentative and physiological characteristics of enological interest. *Hanseniaspora uvarum* BHu9 and BHu11, *H. osmophila* BHo51, *Starmerella bacillaris* BSb55 and *Candida membranaefaciens* BCm71 were selected as candidates to design co-culture starters.

Keywords: oxidative-fermentative metabolism; enological traits; non-*Saccharomyces* yeasts; reduced ethanol wines

INTRODUCTION

In the last few decades, consumer preferences toward well-structured, full-body wines have driven the requirement for late harvests in order to ensure an optimal phenolic maturity of grapes (Morales *et al.* 2015). This practice results in a noticeable increase in the sugar content of the berries at harvest which in turn could produce wines with a high alcohol percentage (Mira

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de Orduña 2010). In addition, the concentration of fructose in overripe grapes exceeds that of glucose because glucose is more susceptible to cellular plant respiration at higher temperatures prevailing in warm zones (Loira 2014). High fructose levels have been associated with sluggish and stuck fermentations (Berthels *et al.* 2004; Tronchoni *et al.* 2009).

High levels of alcohol can have several adverse effects. First, it can alter the sensorial quality of wines because of an increase in the perception of bitterness, astringency and hotness and it can mask certain volatile aromatic compounds (Fischer and Noble 1994; Wilkinson and Jiranck 2013). Second, high ethanol content can lead to stuck and sluggish fermentations (Gil *et al.* 2013). Third, it is well known that beverages with high alcohol content can have negative psychological and physiological effects on human health (Grønbæk 2009). Lastly, wines with high ethanol levels can raise economic issues because some countries impose taxes, which can considerably increase the final price (Gil *et al.* 2013; Contreras *et al.* 2014).

In this context, several viticultural and engineering methods have been developed to decrease sugar accumulation in grapes and reduce ethanol in wine, respectively. However these strategies can be expensive and can have negative effects on the organoleptic quality of the final product. An inexpensive and simple option would be the use of adequate wine yeasts to reduce the alcohol content (Tilloy, Ortiz-Julien and Dequin 2014; Contreras, Curtin and Varela 2015).

Over the last few decades, major advances have been made in the understanding of the ecology, physiology, biochemistry and molecular biology of yeasts involved in the fermentation process, including *Saccharomyces* and non-*Saccharomyces* species. Several studies have reported the impact of non-*Saccharomyces* yeasts on the composition, sensory properties and flavor of wines (Swiegers *et al.* 2005; Domizio *et al.* 2007; Fleet 2008). Controlled inoculation of *Saccharomyces cerevisiae* and non-*Saccharomyces* yeasts has been carried out (both in mixed and sequential fermentations) in order to improve the quality and aromatic complexity of wines (Toro and Vazquez 2002; Ciani and Comitini 2011; Giovani, Rosi and Bertuccioli 2012; Maturano *et al.* 2012, 2015). In particular, recent studies have reported a reduction in ethanol using yeast co-cultures, compared to the ethanol concentration obtained with a single *S. cerevisiae* inoculum (Ciani *et al.* 2014; Contreras *et al.* 2014; Contreras, Curtin and Varela 2015; Englezos *et al.* 2016; Varela *et al.* 2016). However, due to the broad intra- and interspecies variability of non-*Saccharomyces* yeasts, new selection criteria should be included.

Some important features of non-*Saccharomyces* yeasts could be used for ethanol reduction. It is known that several non-*Saccharomyces* yeasts have different respiro-fermentative regulatory mechanisms compared to *S. cerevisiae*. They can divert consumption of carbon sources toward products other than ethanol. Under aerobic conditions during the first stages of winemaking, certain yeasts are able to consume sugar by respiratory metabolism (negative Crabtree Effect), while *S. cerevisiae* is able to ferment sugar despite oxygen availability (positive Crabtree Effect) (Quirós *et al.* 2014; Ciani *et al.* 2016). In addition, during the fermentation process several non-*Saccharomyces* yeasts show lower ethanol yield and fermentative efficiency than *S. cerevisiae* (Ciani and Ferraro 1996). Therefore, fermentations of non-*Saccharomyces* yeasts together with *S. cerevisiae* could be attractive to reduce ethanol in winemaking. However, prior to co-inoculation in grape musts, it is very important to know certain metabolic characteristics for selection of non-*Saccharomyces* yeasts. They should be able to settle in the grape must, and in order to assure their successful implantation/persistence, several physiological features should be included in the selection process: resistance to high initial sugar, SO_2 and ethanol concentrations as well as the ability to ferment at low temperature. The microorganisms should also present a fructophilic character, tolerance to oxidative stress and they should be less efficient in the transformation of grape sugars into ethanol. Finally, appropriate screenings should not only focus on the absence of undesirable characteristics (such as low production of acetic acid and hydrogen sulfide), but also on a positive impact on the winemaking (e.g. high production of glycerol and hydrolytic enzymes).

In this context, the goal of the present work was to examine the oxidative-fermentative metabolism and enological traits of non-*Saccharomyces* yeasts in order to allow their implantation in grape must with high alcoholic potential and evaluate their positive impact on winemaking. Based on this study, the non-*Saccharomyces* selected yeasts will employed in future assays to carry out co-cultures with *S. cerevisiae* that achieve to reduce ethanol content of wines.

MATERIALS AND METHODS

Yeast strains

This study assayed 114 non-*Saccharomyces* yeasts (Table 1). Yeasts had previously been isolated from clusters and grape musts at different stages of spontaneous fermentations during 1992, 1994, 2004 and 2011 (from mayor viticulture regions in San Juan and Mendoza, Argentina). *Saccharomyces cerevisiae* BSc114 was used as negative control regarding the respiratory activities assayed and as positive control concerning fermentative performance. Strains were identified using conventional morphological, physiological and biochemical assays according to Kurtzman, Fell and Boekhout (2011). Molecular identification was carried out by polymerase chain reaction–restriction fragment length polymorphism of internal transcribed spacers, as described by Esteve-Zarzoso *et al.* (1999). All isolates were obtained from the Culture Collection of Autochthonous Microorganisms of the Institute of Biotechnology, School of Engineering – UNSJ, San Juan, Argentina. Microorganisms were cryogenically preserved–80°C).

Yeast respiratory activity

Cytochrome C oxidase activity

Yeast strains were spot-inoculated on Petri dishes with YEPD-agar (g L^{-1}): Yeast extract 10, Peptone 20, Glucose 20, agar-agar 20; and incubated at 26 ± 1°C for 7 days. Ten microliters of an aqueous solution of 1% tetramethyl-ρ-phenylenediamine dihydrochloride were poured on the yeast colonies, in order to detect cytochrome C oxidase activity. This reagent stains colonies blue, and the change in color of the colonies was measured over time. Yeasts that changed the color of colonies between 0 and 60 s were considered cytochrome oxidase positive. Those that changed the color of colonies in more than 60 s were considered cytochrome oxidase negative (Steel 1961; McEwen, Cameron and Poyton 1985; both methods were slightly modified).

Oxidative stress tolerance

Yeast strains were grown aerobically in YEPD broth. Twenty milliliters of molten YEPD agar were inoculated with 1×10^6 cells mL^{-1} of each strain and poured into Petri dishes. Sensitivity to hydrogen peroxide was assayed by pouring 70 μL of

Table 1. Non-*Saccharomyces* yeast isolates assayed.

Species	Total no of isolates	Nomenclature
Hanseniaspora uvarum	28	BHu1, BHu2, BHu3, BHu4, BHu5, BHu8, BHu9, BHu10, BHu11, BHu12, BHu13, BHu16, BHu17, BHu18, BHu19, BHu20, BHu21, BHu22, BHu23, BHu24, BHu27, BHu28, BHu29, BHu30, BHu31a, BHu31b, BHu38, BHu40
Hanseniaspora guilliermondii	5	BHg42, BHg44, BHg46, BHg47, BHg48
Hanseniaspora vineae	3	BHv43, BHv49, BHv50
Hanseniaspora osmophila	1	BHo51
Starmerella bacillaris	13	BSb 52, BSb53, BSb54, BSb55, BSb56, BSb57a, BSb57b, BSb58,BSb59, BSb62, BSb63, BSb66, BSb67
Candida stellata	1	BCst68
Candida membranaefaciens	3	BCm69, BCm70a, BCm71
Candida pararugosa	1	BCp73
Candida sake	5	BCsk74, BCsk83, BCsk86, BCsk88, BCsk95
Candida diversa	1	BCd75
Candida steatolytica	1	BCs76
Candida intermedia	2	BCi77, BCi85
Candida cantarelli	1	BCcn78
Candida catenulata	1	BCct79
Candida apis	1	BCa80
Candida rugosa	1	BCr81
Candida famata	4	BCf84a, BCf84b, BCf90, BCf91
Pichia occidentalis	17	Bpo96a, BPo96b, BPo101, BPo102, BPo106, BPo108, BPo110, BPo111, BPo112, BPo113, BPo114a, BPo115, BPo116, BPo118, BPo120a, BPo120b, BPo123
Pichia manshurica	1	BPm125
Pichia fabianii	1	BPf127
Pichia kudriavzevii	3	BPku128, BPku129, BPku134
Pichia kluyveri	2	BPk130, BPK131
Pichia membranaefaciens	1	BPmf136
Pichia guilliermondii	1	BPg138
Metschnikowia pulcherrima	3	BMp141, BMp144, BMp145
Torulaspora delbrueckii	2	BTd147, BTd148
Debaryomyces hansenii	4	BDh150a, BDh150b, BDh154, BDh155
Clavispora lusitaniae	1	BCl157
Cryptococcus albidus	1	BCrya156
Cryptococcus laurentii	3	BCryl161, BCryl163, BCryl164
Issatchenkia orientalis	1	BIo160
Wickerhamomyces anomalus	1	BWa158
TOTAL	114	
Saccharomyces cerevisiae	1	BSc114 (control)

different H_2O_2 concentrations (25, 50, 100, 250 mM) in wells (5 mm diameter) punched in the YEPD agar. Petri dishes were incubated at 25 ± 1°C for 24–48 h. The average diameter (mm) of the inhibition zone surrounding the wells was determined and tolerance to oxidative stress (hydrogen peroxide) was defined by the size of the inhibition zone for each yeast, according to Stephen, Rivers and Jamieson (1995; modified).

Catalase activity

Catalase activity of yeasts was evaluated according to the method described by Whittenbury (1964). Hydrogen peroxide at 3% (v/v) was directly added to 48-h-old yeast colonies. Catalase activity was evidenced by the formation of bubbles. Results are expressed in terms of absence or presence of this activity.

Yeast sugar metabolism under fermentative conditions

This assay provides information about the yeast sugar consumption and the main secondary metabolites produced by yeasts under fermentative conditions in a pure culture.

Pre-inocula of all strains were obtained by growing yeasts in sterile 13 °Bx grape juice at 26 ± 1°C during 24 h under aerobic conditions (130 rpm). The cultures were subsequently inoculated at 1×10^6 cells mL^{-1} in Erlenmeyer flasks with 175 mL of sterile grape juice (21 °Bx, pH 3.8, total acidity: 5.5 g L^{-1}, fructose: 105 g L^{-1} and glucose: 98.3 g L^{-1}) and incubated at 24 ± 1°C under static conditions. Fermentation performance was monitored through the release of CO_2, by measuring weight loss every 24 h during 21 days. At the end of this period, ethanol (% v/v), pH, residual fructose, residual glucose, glycerol, titratable acidity, total sugars and acetic acid (g L^{-1}) were determined with an ALPHA FT-IR Wine Analyzer (Bruker Optics, Ettlingen, Germany). This equipment analyzes samples by Fourier Transform Infrared spectroscopy with Attenuated Total Reflection (ATR-FT-IR). Opus software (v. 7.0, Bruker Optics) was used for spectral acquisition, instrument control and preliminary file manipulation. The instrument allows simultaneous analysis of different parameters with one measurement. Furthermore, it is an easy, fast and non-destructive method and does not require any sample preparation. ATR-FT-IR spectroscopy is a powerful tool used to monitor and assess the composition and quality of numerous food

samples such as grape must and wine (de Villiers *et al.* 2012; Friedel, Patz and Dietrich 2013).

Two metabolic parameters were calculated from the analytical data:

(i) The glucose-fructose consumption ratio to determine the fructophilic character of the yeasts:

$$\frac{\text{glucose consumption (\%)}}{\text{fructose consumption (\%)}}$$

Values <1 indicate higher fructose than glucose consumption and vice versa.

(ii) The ratio between sugar required (g L^{-1}) and percentage of ethanol produced (% v/v):

$$\frac{\text{initial sugars} - \text{final sugars } (gL^{-1})}{\text{final ethanol concentration } (\%\ ^{v}/_{v})}$$

Values ≥ 19 were considered inefficient sugar-ethanol conversion.

Sterilized non-inoculated must was used as negative control under the same assay conditions.

Physiological characteristics of enological interest

Ability to start fermentation at low temperature, and at high concentrations of initial sugar, ethanol and sulfur dioxide

Yeasts ability to start fermentation at high sugar concentration (30 °Bx), at low temperature (15°C), at different ethanol concentrations (8, 10, 12 and 14% v/v) and sulfur dioxide concentrations(50, 100, 200, 300 mg L^{-1}) was determined according to Vazquez, de Figueroa and Toro (2001) with modifications. Fermentation assays were performed under static conditions in test tubes with 5 mL of grape must (21 °Bx, 0.1% yeast extract, pH 3.8, except for the high sugar concentration assay). An inverted micro tube (Durham tube) was placed in test tubes, and it allowed collection of gas produced by yeasts during the fermentation process. Isolates were inoculated at 1×10^6 cells mL^{-1} and incubated at 25°C (except low temperature assay: 15°C). Fermentations were controlled daily up to the third day post-inoculation. Entrapment of gas in inverted Durham micro tubes indicated fermentative activity (absence of gas in Durham's tubes indicated no fermentative activity).

H_2S production

The ability of the isolates to produce different levels of hydrogen sulfide (H_2S) was assayed by using BD BIGGY agar. The medium was spot-inoculated and plates were incubated at 25°C for 48 h. An arbitrary scale from 1 (white color = no production) to 6 (dark brown = high production) was used to define H_2S production (Comitini *et al.* 2011).

Enzyme activities

Enzymatic activities of enological relevance such as β-glucosidase, pectinase and protease were analyzed and assayed under optimal conditions. It is well known that these enzymes can improve the winemaking process and enhance wine quality (Charoenchai *et al.* 1997).

β-Glucosidase activity was determined as described by Rosi, Vinella and Domizio (1994), using a medium containing (g L^{-1}): arbutin (hydroquinone β-D-glucopyranoside, Sigma) 5, YNB with amino acids 6.7 and agar-agar 20. Prior to sterilization, the pH of the medium was adjusted to 5.0. Two milliliters of sterile ferric ammonium citrate (1%) were added to 100 mL of the medium before it was poured into the plates. Strains were spot-inoculated on the medium and plates were incubated at 25°C for 3 days. β-Glucosidase activity was determined by the medium turning brown.

Pectinase activity was assayed according to Fernandez-Salomao *et al.* (1996) and Merín *et al.* (2011). The medium contained (g L^{-1}): citrus pectin 2; yeast extract 1, KH_2PO_4 0.2, $CaCl_2$ 0.05, $(NH_4)_2SO_4$ 1, $MgSO_4.7H_2O$ 0.8, $MnSO_4$ 0.05, agar-agar 20; pH 4.5. Isolates were spot-inoculated and incubated at 30°C for 3 days. After this time, Lugol solution was added, and pectin degradation was determined as a clear halo around yeast colonies.

Protease activity was detected using the method described by Comitini *et al.* (2011). The medium was prepared as follows (g L^{-1}): malt extract 3, yeast extract 3, bacteriological peptone 5, glucose 10, NaCl 5, agar-agar 20. Separately, an equal volume of skim milk solution (10% w/v) was prepared using sterile water. After sterilization, both solutions were mixed and the pH was adjusted to 3.5 with 0.1 M HCl. The medium was subsequently poured into sterile Petri dishes. Isolates were spot-inoculated and then incubated at 25°C for 3 days. A clear zone around yeast colonies indicated protease activity.

Data analysis

Each assay was performed independently in triplicate and the results represent the average of the three determinations with the corresponding standard deviation (± SD).

Experimental data obtained during fermentations were analyzed by repeated analysis of variance measurements. Significant differences were determined by Tukey's test and results were considered significant if the associated *P* value was <0.05. Spearman's correlation coefficients were calculated to determine statistical significance between ethanol, glycerol and acetic acid values of fermentation trials. SPSS version 19.0 was used for all tests.

Results obtained from each physiological characterization assay were converted into a binary code matrix and presence or absence is indicated by '1' and '0', respectively. In the case of H_2S production, isolates that registered low productivity (1, 2 or 3 on the arbitrary scale) were assigned '1' and isolates that presented high productivity were assigned '0'. Furthermore, the ability to start fermentation in 14% v/v ethanol and at a sulfur dioxide concentration of 300 mg L^{-1} were assigned '1', whereas remaining concentrations were assigned '0'.

RESULTS

A total of 114 different native non-*Saccharomyces* yeasts covering 33 species and belonging to 12 different genera (Table 1) were screened for their respiratory activity and ability to assimilate sugars under fermentative conditions. According to the parameters employed, a subgroup of selected yeasts was subjected to enological characterization.

Yeast respiratory activity

The respiratory activity of 114 non-*Saccharomyces* yeasts is given in Table 2. Three different assays were used to obtain

Table 2. Respiratory activity of 114 non-*Saccharomyces* yeast isolates.

	Cytocrome C oxidase activity		Stress tolerance		Catalase activity	
Species	Yes	No	High	Low	Yes	No
Hanseniaspora uvarum (28)	19	9	24	4	20	8
Others *Hanseniaspora (9)*	7	2	6	3	8	1
Starmerella bacillaris (13)	9	4	8	5	8	5
Candida sp *(22)*	10	12	11	11	16	6
Pichia occidentalis (17)	9	8	10	7	10	7
Others *Pichia (9)*	3	6	2	7	7	2
Metschniowia pulcherrima (3)	1	2	3	0	1	2
Torulaspora delbrueckii (2)	1	1	1	1	0	2
Debaryomyces hansenii (4)	3	1	2	2	2	2
Cryptococcus albidus (1)	1	0	1	0	1	0
Clavispora lusitaniae (1)	1	0	0	1	0	1
Wickerhamomyces anomalus (1)	1	0	1	0	0	1
Issatchenkia orientalis (1)	0	1	1	0	0	1
Cryptococcus laurentii (3)	0	3	1	2	0	3
Saccharomyces cerevisiae (control)		*		*		*
	65	**49**	**71**	**43**	**73**	**41**

Saccharomyces cerevisiae BSc114 was used as negative control.
*Respiratory activity of the control yeast.
The total number of isolates corresponding to each species is given in brackets.

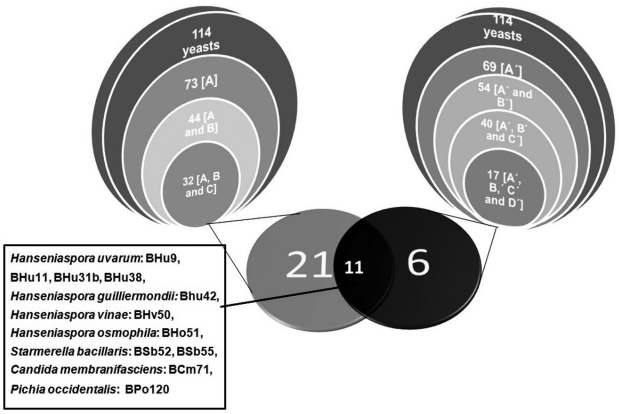

Figure 1 . Stacked Venn diagrams I and II represent the oxidative capacity and fermentative performance, respectively, of the 114 non-*Saccharomyces* yeast isolates assayed. The classic Venn diagram composed of diagram III and IV represents isolates that presented all characteristics required. References: A: yeast isolates with high levels of cytochrome C oxidase activity; B: catalase-positive yeast isolates; C: yeast isolates tolerant to oxidative stress. A´: yeast isolates that required ≥19 g of sugar L^{-1} to produce 1% of ethanol; B´: yeast isolates able to produce ≥5.2 g of glycerol L^{-1}; C´: yeast isolates that produced ≤0.7 g of acetic acid L^{-1}; D´: yeast isolates able to consume ≥50% of initial fructose from grape must.

information about oxidative stress tolerance and catalase and cytochrome C oxidase activity. *Saccharomyces cerevisiae* BSc114 was used as negative control; it shows delayed cytochrome C oxidase activity, absence of catalase activity and did not tolerate the H_2O_2 concentrations assayed (Table 2).

More than half of the isolates, 56.14%, showed cytochrome C oxidase activity, 64.91% demonstrated catalase activity and 62.28% of the isolates tolerated the increasing H_2O_2 concentrations assayed (Table 2). A total of 32 yeast isolates showed all three features assayed regarding oxidative activity (venn diagram I in Fig. 1).

Sugar metabolism of yeasts under fermentative conditions

To evaluate the performance of each non-*Saccharomyces* yeast under fermentative conditions, laboratory-scale fermentations were carried out and chemical and metabolic parameters were assayed after 21 days (Table 3).

From the 114 native non-*Saccharomyces* yeasts assayed, 72 consumed more than 50% of available sugars in the grape must. A total of 49 of them, mainly species belonging to *Hanseniaspora* (24 isolates) and *Starmerella bacillaris* (11 isolates), consumed more than half of the initial fructose concentration. Eight of the 49 isolates consumed more fructose than glucose (4 *Hanseniaspora uvarum*: BHu9, BHu29, BHu31b, BHu40; 1 *H. guilliermondii*: BHg42; 1 *H. vineae*: BHv50; 1 *H. osmophila*: BHo51; and 1 *S. bacillaris*: BSb67), reflected in the glucose/fructose consumption index (<1) (Table 3).

Metabolic parameters were calculated from the fermentative assays (detailed in Materials and Methods section). The native non-*Saccharomyces* isolates assayed registered ethanol values between 0.8 ± 0.05% v/v and 11.8 ± 0.08% v/v, while *S. cerevisiae* BSc114 produced 12.6 ± 0.2% v/v. The conversion rate of sugars into ethanol was also considered important to select yeast strains. From the total of 114 isolates, 69 consumed more than 19 g of sugar L^{-1} to produce 1% v/v of ethanol. It is important to highlight that 84% of the *Pichia* isolates registered low conversion efficiency, whereas only 19% of the *Candida* isolates required more than 19 g of sugar L^{-1} to produce 1% v/v of ethanol (Table 3). The control yeast (BSc114) showed a conversion rate of 16.04 g of sugar L^{-1} to produce 1% of ethanol (v/v).

Other important metabolites that affect the quality of the final product, glycerol and acetic acid were also produced during the fermentative process. The taste threshold of glycerol is 5.2 g L^{-1} (Noble and Bursick 1984), whereas the maximum acceptable level is 25 g L^{-1} (Scanes, Hohmann and Prior 1998). Therefore, native non-*Saccharomyces* yeasts that produced between 5.2 and 25 g of glycerol L^{-1} (99/114 yeasts) were selected. *Starmerella bacillaris* (92.30%), *Pichia* (*P. occidentalis*: 100% and other *Pichia* strains: 77.7%), *Hanseniaspora* (*H. uvarum*: 85.71% and other

Table 3. Chemical analysis and metabolic parameters of 114 non-*Saccharomyces* yeast isolates under fermentative conditions.

	Sugar consumption ($g\ L^{-1}$)		Fructose assimilated ($g\ L^{-1}$)		Glucose/ fructose consumption (%)		Sugar required ($g\ L^{-1}$)/ethanol (%) ratio		Glycerol production ($g\ L^{-1}$)		Volatile acidity production ($g\ L^{-1}$ acetic acid)		Ethanol production (% v/v)	
Species	<50%	>50%	<50%	>50%	<1%	>1%	<19 $g\ L^{-1}$	>19 $g\ L^{-1}$	<5.2	>5.2	<0.7	>0.7	<10	>10
Hanseniaspora uvarum (28)	8	20	10	18	4	24	12	16	4	24	23	5	17	11
other *Hanseniaspora (9)*	3	6	2	6	3	6	3	6	1	8	7	1	4	5
Starmerella bacillaris (13)	2	11	2	11	1	12	5	8	1	12	9	4	10	3
Candida sp (22)	13	9	18	5	0	22	18	4	1	21	18	5	22	0
Pichia occidentalis (17)	5	12	12	5	0	17	3	14	0	17	11	6	16	1
other *Pichia (9)*	3	6	7	2	0	9	1	8	2	7	7	2	9	0
Metschniowia pulcherrima (3)	0	3	2	1	0	3	1	2	1	2	2	1	3	0
Torulaspora delbrueckii (2)	0	2	2	0	0	2	0	2	0	2	1	1	2	0
Debaryomyces hansenii (4)	2	2	4	0	0	4	2	2	1	3	4	0	4	0
Cryptococcus albidus (1)	0	1	0	1	0	1	0	1	0	1	1	0	1	0
Clavispora lusitaniae (1)	1	0	1	0	0	1	0	1	1	0	1	0	1	0
Wickerhamomyces anomalus(1)	1	0	1	0	0	1	0	1	1	0	1	0	1	0
Issatchenkia orientalis (1)	1	0	1	0	0	1	0	1	1	0	1	0	1	0
Cryptococcus laurentii (3)	3	0	3	0	0	3	0	3	1	2	3	0	3	0
Saccharomyces cerevisiae		*		*		*	*			*		*		*
	42	**72**	**65**	**49**	**8**	**106**	**45**	**69**	**15**	**99**	**89**	**25**	**94**	**20**

Saccharomyces cerevisiae BSc114 was used as positive control.

*Chemical analysis and metabolic parameters of the control yeast.

Total number of isolates corresponding to each species is given in brackets.

Hanseniaspora strains: 88.88%) and *Candida* sp. (95.45%) were the most representative isolates (Table 3). According to the 'Instituto Nacional de Vitivinicultura' (INV; National Viticulture Institute, San Juan, Argentina), the maximum acceptable level of volatile acidity for commercialization of wines is 0.7 g of acetic acid L^{-1}. It is interesting to emphasize that 78.07% of the yeast isolates assayed registered values below 0.7 g of acetic acid L^{-1} (Table 3).

Because ethanol, glycerol and acetic acid are simultaneously produced during glycerol-pyruvate and alcoholic fermentations (Ribereau Gayon *et al.* 2006), a simple correlation analysis was used to assess the correlation between the following metabolites after 21 days of fermentation: glycerol-acetic acid, glycerol-ethanol and acetic acid-ethanol. In all cases, a weak positive correlation was found (Spearman's coefficients of 0.50, 0.40 and 0.38, respectively).

In order to carry out a selection of non-*Saccharomyces* yeasts, isolates exhibiting the following characteristics were taken into consideration: production of glycerol and acetic acid >5.2 g L^{-1} and <0.7 g L^{-1}, respectively, fructose consumption ≥50% and consumption of ≥19 g of sugars to produce 1% of ethanol (v/v). Seventeen isolates met these requirements as they were inefficient ethanol and low acetic acid producers, good consumers of fructose and good glycerol producers (Fig. 1; stacked Venn diagram II).

Furthermore, as shown in Fig. 1, Venn diagram IV represents isolates that registered desired oxidative characteristics and highest performance under fermentative conditions. Where both circles overlap, 11 yeast isolates present all characteristics required: 7 *Hanseniaspora* isolates (4 *H. uvarum*: BHu9, BHu11, BHu31b, BHu38; 1 *H. guilliermondii*: BHu42; 1 *H. vineae*: BHv50; and 1 *H. osmophila*: BHo51), 2 *S. bacillaris* (BSb52 and BSb55), 1 *Candida membranaefaciens*: BCm71 and 1 *Pichia occidentalis*: BPo120b.

Physiological characteristics of enological interest of selected yeasts

Eleven non-*Saccharomyces* yeasts were preselected and assayed for the following enological criteria: ability to start fermentation at high sugar concentration (30 °Bx) and at low temperature (15°C), ability to tolerate increasing ethanol and SO_2 concentrations and H_2S production. In addition, analysis of certain enzyme activities of potential interest to winemaking was included in the yeast selection (Englezos *et al.* 2015) (Fig. 2). The control yeast *S. cerevisiae* BSc114 was able to ferment grape juice at 30 °Bx and 15°C and tolerated 14% ethanol and 300 mg of SO_2 L^{-1}. The control strain was also a low H_2S producer and did not register any of the enzymatic activities assayed.

During the first 3 days of fermentation, 7/11 isolates carried out fermentations at the highest sugar concentration assayed (30 °Bx), and 6 of the 11 isolates demonstrated the ability to ferment at low temperature (15°C). Seven and eleven isolates were able to ferment at 14% (v/v) of ethanol and 300 mg of SO_2 L^{-1}, respectively (Fig. 2).

From the 11 isolates selected, 6 (BHu9, BHu11, BHv50, BHo51, BSb55 and BCm71) registered low H_2S production (Fig. 2).

β-Glucosidase activity was observed in four isolates (Bhu31b, BHv50, BSb52 and BCm71) and protease activity in six isolates (BHu9, BHu11, BHu31b, BHo51, BSb55 and BCm71). Five yeast isolates (BHu11, BHu31b, BHc50, BHo51 and BPo120b) were positive for pectinase activity and two isolates (BHu38 and BHg42) did not have any of the three enzyme activities assayed (Fig. 2).

The non-*Saccharomyces* yeast isolates *H. uvarum* BHu9 and BHu11, *H. osmophila* BHo51, *S. bacillaris* BSb55 and *C. membranaefaciens* BCm71 presented the highest number of desirable physiological characteristics (above the arbitrary selection line, Fig. 2).

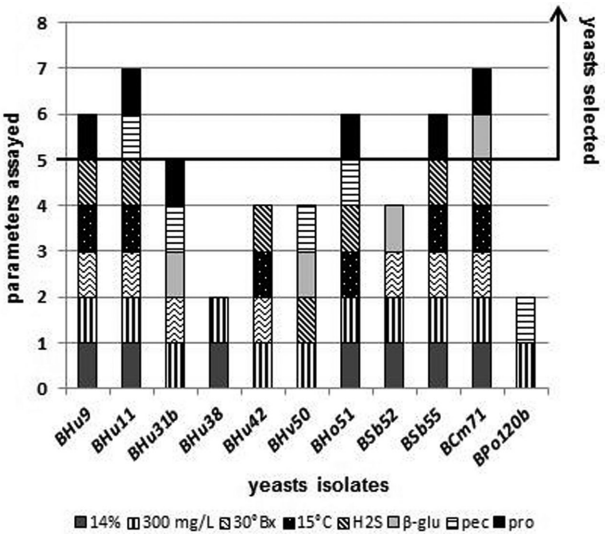

Figure 2 . Physiological characteristics with enological application of the 11 yeast isolates originally selected. References: yeast isolates: *H. uvarum* BHu9, BHu11, BHu31b, BHu38, BHu42; *H. vineae* BHv50; *H. osmophila* BHo51; *S. bacillaris* BSb52, BSb55; *C. membranaefaciens* BCm71; *P. occidentalis* BPo120b. Parameters assayed: 14%: ability to start fermentation at 14% (v/v) of ethanol. 300 mg L^{-1}: ability to start fermentation at 300 mg of SO_2 L^{-1}. 30 °Bx: ability to start fermentation at 30 °Bx. 15°C: ability to start fermentation at 15°C. H_2S: ability to produce H_2S. β-glu: production of β-glucosidase activity. pec: production of pectinase activity. pro: production of protease activity.

DISCUSSION

It is well known that non-*Saccharomyces* yeasts impacts on the final quality of wines (Fleet 2008). As a consequence, the winemaking process can be considerably influenced. Therefore, a more comprehensive understanding of the metabolism of the yeasts involved in the process is essential. The current study proposes (i) to select non- *Saccharomyces* yeasts able to growth in grape must with high alcohol potential at the beginning of the winemaking process (in order to ensure rapid and elevated sugar consumption); (ii) these yeasts have to produce low ethanol levels and to possess desirable enological traits. Five isolates belonging to *Hanseniaspora uvarum* (BHu9, BHu11), *H. osmophila* (BHo51), *Starmerella bacillaris* (BSb55) and *Candida membranaefaciens* (BCm71) were selected. It is important to highlight that *C. membranaefaciens* has not been reported in co-cultures yet, and the results obtained in this study could support its use.

The presence of non-*Saccharomyces* yeasts on grapes and during the first stages of winemaking is well known (Jolly, Varela and Pretorius 2014). During this period, the yeasts are exposed to aerobic conditions (enhanced by some practices such as pump-over operations, *délestage* or macro-oxygenation) at high sugar concentration (Jordão, Vilela and Cosme 2015). This situation enables species like *Saccharomyces cerevisiae* to carry out fermentation processes in the presence of oxygen (positive Crabtree effect). In contrast, certain yeast species of the genera *Pichia*, *Debaryomyces*, *Hanseniaspora*, *Kluyveromyces* and *Candida* are able to consume sugars by respiratory pathways (negative Crabtree effect) (Kregiel, Berlowska and Ambroziak 2008; Quirós *et al.* 2014).

In this study, the respiratory capacity of non-*Saccharomyces* yeasts was indirectly detected with the cytochrome C oxidase

(COX) assay. In general, two subgroups among isolates belonging to the same species were observed. Davies (1995) proposed that those microorganisms that present respiratory metabolism must challenge the 'oxygen paradox': respiratory catabolism is the most efficient way to obtain energy, but this process generates reactive oxygen species such as OH^-, O_2^- and mainly H_2O_2, which are toxic (Cadenas and Davies 2000). However, some authors have recommended the use of aerobic yeasts in order to oxide sugars during the early stage of winemaking and consequently reduce ethanol production (Erten and Campbell 2001; Quirós, *et al*. 2014; Rodrigues *et al*. 2016). To our knowledge, this is the first study demonstrating the tolerance of these wine yeasts to toxic products derived from sugar respiratory catabolism. The current results indicate that some non-*Saccharomyces* species were able to tolerate H_2O_2, which could be associated with the catalase activity expressed by these yeasts. Therefore, the non-*Saccharomyces* yeasts that presented COX activity and were able to tolerate H_2O_2 could be used during the first stages of fermentation. On the other hand, the fermentative performance of these non-*Saccharomyces* yeasts under anaerobic conditions should be different from *S. cerevisiae*, which has demonstrated a sugar consumption/ethanol production ratio that varied between 16.83 and 19 g L^{-1} (Ribéreau-Gayon *et al*. 2006; Loira 2014). In this study, 60% of the non-*Saccharomyces* yeasts used more than 19 g of sugar L^{-1} to produce 1% (v/v) ethanol and, consequently, these isolates could contribute to a reduction in ethanol in wines because of their low ethanol yield. Findings of this study are consistent with those by Contreras *et al*. (2014), who reported low fermentation efficiency in isolates belonging to the genera *Hanseniaspora*, *Pichia*, *Candida*, *Torulaspora*, *Metschnikowia* and *Issatchenkia*.

Sugar utilization pattern and respiratory fermentative metabolism of non-*Saccharomyces* yeasts are very important to know. It is also relevant to study their fructose affinity, because in overripe grapes, fructose is present in the major concentration (Amerine and Thoukis 1958). In turn, as previously mentioned, glucose is more susceptible to cellular plant respiration at higher temperatures (Loira 2014). In addition, glucose metabolism is prevalent in *S. cerevisiae* species (Berthels *et al*. 2004). In this study, yeast isolates belonging to the genera *Hanseniaspora* and *Starmerella* showed a slightly higher fructose than glucose consumption (<1%). In line with these results, several authors have reported a fructophilic character for *H. uvarum*, *H. guilliermondii* and *S. bacillaris* (Ciani and Fatichenti 1999; Englezos *et al*. 2015). While Granchi *et al*. (2002) described *H. osmophila* as a glucophilic yeast, in this study, *H. osmophila* BHo51 presented a fructophilic character. Regarding the current conditions of grapes must, evaluation of fructose consumption should be standardized in yeast selection programs because of intraspecific variations.

It is known that low temperatures affect sugar consumption and improve ethanol tolerance of yeasts. Consequently, low temperatures could allow a prolonged permanence of non-*Saccharomyces* yeasts during the fermentative process (Jolly, Augustyn and Pretorius 2006; Tronchoni *et al*. 2009). This condition would also enhance the wine quality by adding flavors. This study proposes new selection criteria for non-*Saccharomyces* yeasts: oxidative metabolism, low fermentative efficiency, and a fructophilic and cryotolerant character at the beginning of the alcoholic fermentation at 15°C. Co-cultures of these non-*Saccharomyces* with *S. cerevisiae* will possibly reduce ethanol in wines.

This study shows that selected non-*Saccharomyces* yeasts were able to start the fermentation at high concentrations of SO_2, ethanol and initial sugars, which would contribute to their successful implantation and good performance in the fermentation process. Furthermore, in order to rationally design co-cultures, parameters of enological interest such as production of glycerol, acetic acid, hydrogen sulfide and certain enzymatic activities should be considered.

Because glycerol, acetic acid (glycerol-pyruvate fermentation) and ethanol (alcoholic fermentation) are simultaneously produced, final concentrations of these metabolites could indicate the metabolic pathway used by the yeast to consume sugars (Ribéreau-Gayon *et al*. 2006). According to several authors, deviation of yeast metabolism toward increased glycerol production would result in a decreased ethanol concentration (Kutyna *et al*. 2010; Tilloy, Ortiz-Julien and Dequin 2014). On the other hand, overproduction of glycerol often results in a high production of acetic acid (Remize, Sablayrolles and Dequin 2000; Eglinton *et al*. 2002). However, this study revealed a weak correlation between final values of ethanol, glycerol and acetic acid. Therefore, the last two compounds were only considered as wine quality parameters for the selection process. It is well known that glycerol positively contributes to the quality of wine by providing body and sweetness at values between 5.2 and 25 g L^{-1} (Tilloy, Ortiz-Julien and Dequin 2014), while acetic acid is detrimental to wine when present at concentrations higher than 0.7 g L^{-1}. There is controversy over the levels of acetic acid produced by non-*Saccharomyces* strains. Romano *et al*. (2003) showed that some apiculate yeasts can produce considerable concentrations of acetic acid, whereas *S. bacillaris* was reported to have a low production of acetic acid (Magyar and Tóth 2011; Tofalo *et al*. 2012; Englezos *et al*. 2015). In contrast, the results in the current study indicate that most *Hanseniaspora* isolates (84%) produced low levels of acetic acid (<0.7 g L^{-1}), while 30% of *S. bacillaris* produced more than 0.7 g of acetic acid L^{-1}.

In accordance with findings by other authors, this study showed that non-*Saccharomyces* yeasts were able to produce a broad spectrum of extracellular hydrolytic enzymes (Fia, Giovani and Rosi 2005; Jolly, Augustyn and Pretorius 2006; Englezos *et al*. 2015). These enzymes can positively contribute to the fermentation process and improve the quality of the final product (Fia, Giovani and Rosi 2005). However, the potential production and effectiveness of these enzymes may be conditioned by factors such as high sugar content and low pH values of the grape must, as well as temperature and ethanol concentration throughout the fermentation process. The degree to which these factors inhibit enzyme production and activity is species and strain dependent (Padilla, Gil and Manzanares 2016). Therefore, further research is necessary to analyze enzyme activity under the real vinification conditions. Low production of H_2S by selected non-*Saccharomyces* yeasts is relevant because this compound imparts undesirable flavors to the wine (Cordente *et al*. 2009). Other authors have reported that H_2S production by non-*Saccharomyces* yeasts varies widely intraspecific (Strauss *et al*. 2001; Comitini *et al*. 2011) and this is in agreement with the results of this study. An important fact to highlight is that a low fermentation temperature favors low yeast H_2S production (Gardner 2016).

In conclusion, this study first proposes an integrated selection of non-*Saccharomyces* yeasts based on a detailed analysis of their physiological characteristics (respiratory-fermentation metabolism and enological traits). Second, screening of the yeasts should facilitate their successful implantation in co-cultures in grape juice with a high alcohol potential in order to reduce ethanol content of wine. Further studies are necessary to develop an effective strategy to co-inoculate strains (mixed or sequential cultures), and to establish whether there exist

potential interactions between selected non-*Saccharomyces* and *S. cerevisiae* yeasts. Finally, future research should assess fermentations at pilot scale and subsequently winery scale using the selected yeasts.

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