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The application of non-*Saccharomyces* yeast in fermentations with limited aeration as a strategy for the production of wine with reduced alcohol content

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ABSTRACT

High alcohol concentrations reduce the complexity of wine sensory properties. In addition, health and economic drivers have the wine industry actively seeking technologies that facilitate the production of wines with lower alcohol content. One of the simplest approaches to achieve this aim would be the use of wine yeast strains which are less efficient at transforming grape sugars into ethanol, however commercially available wine yeasts produce very similar ethanol yields. Non-conventional yeast, in particular non-*Saccharomyces* species, have shown potential for producing wines with lower alcohol content. These yeasts are naturally present in the early stages of fermentation but in general are not capable of completing alcoholic fermentation. We have evaluated 48 non-*Saccharomyces* isolates to identify strains that, with limited aeration and in sequential inoculation regimes with *S. cerevisiae*, could be used for the production of wine with lower ethanol concentration. Two of these, *Torulaspora delbrueckii* AWRI1152 and *Zygosaccharomyces bailii* AWRI1578, enabled the production of wine with reduced ethanol concentration under limited aerobic conditions. Depending on the aeration regime *T. delbrueckii* AWRI1152 and *Z. bailii* AWRI1578 showed a reduction in ethanol concentration of 1.5% (v/v) and 2.0% (v/v) respectively, compared to the *S. cerevisiae* anaerobic control.

1. Introduction

Microbial strategies to produce wine with less ethanol are required to reverse a trend over recent decades where the average ethanol concentration of wine has increased (Godden and Muhlack, 2010; Robinson, 2012). This trend has been driven largely by consumer preference for wine styles dependent upon sugar-rich 'flavour-ripe' grapes. Resultant wines with high levels of ethanol can be perceived negatively due to health concerns linked to excessive alcohol consumption (Grant, 2010; MacAvoy, 2010), compromised wine quality (Athes et al., 2004; Guth and Sies, 2001) and taxation levied according to alcohol content. Whilst a number of viticultural and engineering strategies have been pursued to achieve this goal (Belisario-Sanchez et al., 2009; Bindon et al., 2013; Schmidtke et al., 2012; Stoll et al., 2010), using wine yeast to produce wine with reduced alcohol content remains one of the simplest potential approaches for winemakers to implement. However, commercially available *S. cerevisiae* wine strains exhibit similar ethanol yields when fermenting the same must (Palacios et al., 2007; Varela et al., 2008), therefore research has focused on generating new *S. cerevisiae* strains that produce less alcohol than traditional wine yeast during fermentation (Ehsani et al., 2009; Kutyna et al., 2010; Tilloy et al., 2014; Varela et al., 2012).

Non-*Saccharomyces* yeast are part of the natural microbiota present on grapes, and harvesting and winemaking equipment, and are present at least during the early stages of fermentation (Fleet and Heard, 1993; Renouf et al., 2005, 2007). While generally incapable of completing alcoholic fermentation, their application in co-inoculation or sequential inoculation with *S. cerevisiae* is increasingly popular (Ciani et al., 2006; Ciani and Maccarelli, 1998; Comitini et al., 2011; Jolly et al., 2006; Soden et al., 2000), particularly for their effects on wine composition, flavour and aroma (Benito et al., 2011; Ciani et al., 2006; Comitini et al., 2011; Cordero Otero et al., 2003; Di Maio et al., 2012; Domizio et al., 2011a; Ehsani et al., 2012; Garcia et al., 2002; Jolly et al., 2006, 2014; Magyar and Toth, 2011; Morata et al., 2012; Soden et al., 2000; Toro and Vazquez, 2002). Recently, a specific strain of the non-*Saccharomyces* species *Metschnikowia pulcherrima* was shown to produce wines with 0.9-1.6% (v/v) less ethanol when used in sequential inoculation (Contreras et al., 2015), with more substantial decreases possible when combined with a strain of *Saccharomyces uvarum* (Contreras et al., 2014).

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Given the vast potential for diverse wine relevant phenotypes amongst non-*Saccharomyces* yeast, it has been proposed that strains able to utilise oxygen to oxidise grape sugars could be used to decrease ethanol concentration in wine (Gonzalez et al., 2013). Unlike *S. cerevisiae*, which favours fermentative metabolism over aerobic respiration when sugar concentration exceeds 10 g/L (due to the Crabtree effect), many non-*Saccharomyces* yeast are able to use oxygen for growth regardless of sugar concentration (Alexander and Jeffries, 1990; de Deken, 1966) and thus, divert carbon into other metabolites and therefore away from ethanol formation. The most studied of these Crabtree-negative yeast include *Hanseniaspora uvarum*, *Kluyveromyces lactis and K. marxianus* (Bellaver et al., 2004; González Siso et al., 1996; Postma et al., 1989; Venturin et al., 1995; Verduyn et al., 1991; Zeeman et al., 2000).

Although aeration is a common practice in commercial wine production, performed through 'pump-overs' or macro-oxygenation techniques, data on the impact of air addition on ethanol production by non-*Saccharomyces* yeast is scarce. While some studies have reported reduced ethanol production (Brandam et al., 2013; Erten and Tanguler, 2010; Quiros et al., 2014) the culture and aeration conditions tested (*e.g.* agitation up to 1000 rpm, aeration rate up to 1 volume of air per volume of culture per minute - VVM) were considerably different to what might reasonably be applied during winemaking.

In the present work, we evaluated 48 non-*Saccharomyces* isolates, covering 33 species belonging to 21 different genera, with the aim of identifying yeasts that, with limited aeration and in sequential inoculation with *S. cerevisiae*, could be used for the reduction of alcohol concentration in wine. A chemically defined grape juice medium was used in order to provide reproducible fermentation conditions.

2. Materials and methods

2.1. Microorganisms and media

Forty-eight non-*Saccharomyces* isolates were obtained from the Australian Wine Research Institute (AWRI) Wine Microorganism Culture Collection (WMCC) (Table 1). Cryogenically preserved strains (-80°C) were cultured and maintained on YM plates (3 g/L malt extract, 3 g/L yeast extract, 5 g/L peptone, 10 g/L glucose, 16 g/L agar) and stored at 4°C. Defined medium used in screening and confirmation analyses consisted of 75 g/L glucose, 75 g/L fructose, 3 g/L tartaric acid and 6.76 g/L Yeast Nitrogen Base adjusted to pH 3.5. Chemically Defined Grape Juice (CDGJ) medium consisted of (per litre): glucose 100 g, fructose 100 g, citric acid 0.2 g, malic acid 3 g, potassium hydrogen tartrate 2.5 g, K_2HPO_4 1.1 g, $MgSO_47H_2O$ 1.5 g, $CaCl_22H_2O$ 0.4 g, H_3BO_3 0.04 g, proline 0.84 g, nitrogen mix solution 20 mL, trace elements stock solution 1 mL, vitamins solution 1 mL, fatty acids stock solution 1 mL, and sterol stock solution 1 mL (Schmidt et al., 2011). CDGJ contained 307 mg N/L of yeast assimilable nitrogen (YAN).

2.2. Screening of non-Saccharomyces yeasts

Strains were screened individually in small-scale (250 mL) bioreactors (Medicel Oy, Finland) that enabled control of sparging gas flow and composition. Starter cultures of all yeast strains were grown overnight in YM medium under semi-aerobic conditions at 22°C, shaking at 200 rpm. These cultures were then used to inoculate 200 mL of defined medium at a final optical density of 0.1 (OD_{600} nm). Ferments were performed at 22°C (200 rpm) under semi-aerobic conditions in bioreactors. Semi-aerobic conditions were attained by sparging sterile filtered (0.22 μm, Millipore) air continuously into the medium at 5 mL/min (0.025 VVM) with a stainless steel diffuser. Aeration rate for each bioreactor was controlled by using a flow meter (Medicel Oy, Finland). The acronym VVM corresponds to the volume of air sparged (in litres) per volume of culture (in litres) per minute. After four days, fermentations were stopped and samples taken for analysis, including ethanol concentration and sugar consumption. Fermentations inoculated with *S. cerevisiae* AWRI1631 were used as controls.

Table 1
Ethanol yield and percentage sugar consumed for non-*Saccharomyces* yeasts evaluated under aerobic conditions.

Strain	Species	Ethanol yield [g ethanol/g sugar]	Consumed sugar [%]
AWRI1631	*Saccharomyces cerevisiae*	**0.46**	**99.6**
AWRI1199	*Pichia fermentans*	0.04	5.5
AWRI1047	*Wickerhamomyces subpelliculosus*	0.06	10.5
AWRI1095	*Pichia membranifaciens*	0.08	10.8
AWRI1006	*Yamadazyma mexicana*	0.08	7.8
AWRI743	*Rhodotorula glutinis*	0.09	2.6
AWRI1165	*Candida diversa*	0.10	10.7
AWRI69	*Sporobolomyces roseus*	0.12	1.5
AWRI1124	*Pichia terricola*	0.19	6.0
AWRI1127	*Dekkera bruxellensis*	0.21	16.0
AWRI258	*Hanseniaspora valbyensis*	0.24	19.1
AWRI2053	*Pichia spp*	0.25	4.5
AWRI1051*	*Wickerhamomyces anomalus*	0.25	46.3
AWRI1045*	*Cyberlindnera mrakii*	0.25	37.8
AWRI60	*Zygopichia spp*	0.26	9.2
AWRI1656*	*Metschnikowia pulcherrima*	0.28	52.0
AWRI1159*	*Candida stellata*	0.29	33.9
AWRI1152*	*Torulaspora delbrueckii*	0.30	39.0
AWRI747	*Torulaspora pretoriensis*	0.30	24.8
AWRI141*	*Schizosaccharomyces pombe*	0.31	33.5
AWRI1578*	*Zygosaccharomyces bailii*	0.31	83.5
AWRI1046*	*Cyberlindnera saturnus*	0.33	42.5
AWRI1220*	*Pichia kudriavzevii*	0.33	75.6
AWRI1043	*Wickerhamomyces ciferrii*	0.34	43.6
AWRI1665	*Schwanniomyces capriottii*	0.34	77.9
AWRI1164	*Trigonopsis cantarellii*	0.34	53.0
AWRI1157	*Debaryomyces hansenii*	0.35	33.7
AWRI1499	*Dekkera bruxellensis*	0.35	23.7
AWRI1181*	*Kluyveromyces lactis*#	0.36	64.0
AWRI1098	*Schizosaccharomyces japonicus*	0.36	27.5
AWRI1149	*Metschnikowia pulcherrima*	0.36	21.2
AWRI1821	*Pichia kluyveri*	0.36	48.1
AWRI1161	*Candida sake*	0.37	15.6
AWRI1552	*Meyerozyma guilliermondii*	0.38	40.5
AWRI861	*Lachancea thermotolerans*	0.40	9.7
AWRI1094	*Pachysolen tannophilus*	0.40	47.7
AWRI863*	*Hanseniaspora uvarum*#	0.41	15.1
AWRI58	*Kluyveromyces marxianus*#	0.41	33.6
AWRI1101	*Dekkera anomala*	0.42	21.4
AWRI442	*Schizosaccharomyces pombe*	0.42	52.2
AWRI1044	*Pichia holstii*	0.43	54.7
AWRI1005*	*Kluyveromyces marxianus*#	0.44	97.6
AWRI1128	*Dekkera anomala*	0.45	67.9
AWRI1103	*Dekkera bruxellensis*	0.47	19.1
AWRI1425	*Hanseniaspora uvarum*#	0.50	4.7
AWRI1032	*Schwanniomyces occidentalis*	0.50	11.9
AWRI1158	*Hanseniaspora uvarum*#	[a]	2.1
AWRI1053	*Schwanniomyces vanrijiae var. vanrijiae*	[a]	0.3
AWRI950	*Dekkera custersiana*	[a]	3.9

Initial sugar concentration was 150 g/L.
* Candidate, 'low-ethanol', non-*Saccharomyces* yeast strains chosen to be trialled in subsequent sequential fermentation experiments.
[a] Ethanol produced below limit of detection.
Species previously described as Crabtree-negative yeasts.

2.3. Evaluation of candidate 'low-ethanol' strains in sequential inoculation trials in defined medium

Non-*Saccharomyces* strains identified from the above screening as having reduced ethanol yields relative to *S. cerevisiae* were evaluated in triplicate using small-scale bioreactors as described above. After four days of fermentation, *S. cerevisiae* AWRI1631 was inoculated into each bioreactor (OD_{600} equivalent to 0.1) to ensure completion of fermentation. Samples were taken for analysis 7 days after initial inoculation with non-*Saccharomyces* strains.