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Selection of non-*Saccharomyces* yeast strains for reducing alcohol levels in wine by sugar respiration

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ABSTRACT

Respiration of sugars by non-*Saccharomyces* yeasts has been recently proposed for lowering alcohol levels in wine. Development of industrial fermentation processes based on such an approach requires, amongst other steps, the identification of yeast strains which are able to grow and respire under the relatively harsh conditions found in grape must. This work describes the characterization of a collection of non-*Saccharomyces* yeast strains in order to identify candidate yeast strains for this specific application. It involved the estimation of respiratory quotient (RQ) values under aerated conditions, at low pH and high sugar concentrations, calculation of yields of ethanol and other relevant metabolites, and characterization of growth responses to the main stress factors found during the first stages of alcoholic fermentation. Physiological features of some strains of *Metschnikowia pulcherrima* or two species of *Kluyveromyces*, suggest they are suitable for lowering ethanol yields by respiration. The unsuitability of *Saccharomyces cerevisiae* strains for this purpose was not due to ethanol yields (under aerated conditions they are low enough for a significant reduction in final ethanol content), but to the high acetic acid yields under these growth conditions. According to results from controlled aeration fermentations with one strain of *M. pulcherrima*, design of an aeration regime allowing for lowering ethanol yields though preserving grape must components from excessive oxidation, would be conceivable.

1. Introduction

There are two main drivers for the current interest in lowering alcohol levels in wine. One is trying to compensate for the effects of the increase in the global average temperature on viticulture, which include lower acidity, altered phenolic maturation and tannin content, and notably higher sugar levels by the time of harvest, especially in warm climates (Jones et al., 2005). Early harvest is not a good alternative to avoid high sugar content in grape must, since it would prevent the optimal phenolic maturity and aromatic complexity required to produce the well-structured and full body wines currently demanded by consumers (Kontoudakis et al., 2011a). Consumer demand is indeed the other driver, since excess ethanol compromises perception of wine aromatic complexity (Goldner et al., 2009; Pickering et al., 1998), as well as rejection by health conscious consumers, road safety considerations, or trade barriers and taxes.

Different approaches and strategies, targeting all stages of winemaking, have been proposed to reduce current alcohol levels in wine. These include the selection of grapevine clones, tailored agronomical methods (Intrigliolo and Castel, 2009), winemaking practices adapted to unripe grapes (Kontoudakis et al., 2011b), selection and engineering of yeast strains with lower ethanol yields (Loira et al., 2012) or partial dealcoholisation by physical means (Aguera et al., 2010; Belisario-Sánchez et al., 2009; Catarino and Mendes, 2011; Chanukya and Rastogi, 2013; Gambuti et al., 2011). However, several of these approaches have little impact on final ethanol content, compromise wine quality due to altered abundance of non-target metabolites, or are not feasible in the current market and regulatory scenario (e.g. GMO approaches).

Our research group recently proposed using the respiratory metabolism of non-*Saccharomyces* yeasts as a tool for reducing the alcoholic content of wine (Gonzalez et al., 2013). Some of these non-conventional species, such as representatives of the genera *Hanseniaspora* (anamorph *Kloeckera*), *Torulaspora* or *Metschnikowia*, constitute the main part of the microbiota of sound ripe grapes and are known to predominate during the initial stages of wine fermentation (Fleet, 2003; Tamang and Fleet, 2009). Additionally, several research lines have demonstrated that some non-*Saccharomyces* yeasts can positively contribute to the aroma profile, sensory complexity and colour stability of the resulting product (Andorrà et al., 2012; Comitini et al., 2011; Gobbi et al., 2013; Renault et al., 2009; Rojas et al., 2003; Sadoudi et al., 2012; Viana et al., 2008, 2011). However, data describing sugar catabolism in these species, especially in winemaking conditions, are still scarce.

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Table 1
Yeast strains used in this work.

Yeast species	Strain designation	Substrate of isolation (country)
Candida sake	AQ1944	Sake-moto (Japan)
	CBS1939	Worth pipe in a brewery (Sweden)
	CBS4076	Grape must (Japan)
	CBS5093	Grape juice (France)
Candida stellata	AQ656	Wine grapes (Germany)
Candida vanderwaltii	AQ275	Winery (Unknown)
Candida vinaria	AQ283	Grape must (Japan)
Candida zemplinina	BC60[a]	Grape juice (Italy)
	AQ247	Wine (Italy)
	AQ232	Grape juice (France)
	FC54[a]	Grapes (Italy)
Debaryomyces hansenii	AQ426	Tomato (Spain)
	IFI866	Sausage (Spain)
	CECT11369[T]	Beer (Denmark)
Debaryomyces fabryi	PR66	Fermented dry sausage (Spain)
Hanseniaspora guilliermondii	CBS465[T]	Infect nail (South Africa)
	CBS1972	Grape juice (Italy)
	CBS2567	Grape must (Israel)
Hanseniaspora uvarum	AQ185	Grape juice (Spain)
	AQ245	Grapes affected by sour rot (Italy)
Hanseniaspora vineae	BC115[a]	Fermentation wine (Italy)
Hansenula polymorpha	IFI1128	Black olive (Spain)
Kazachstania exigua	DBVPG6354	Fermenting cucumber brine (USA)
	DBVPG6749	Grape must (Ex. Czechoslovakia)
Kloeckera apiculata	ICVV249[b]	Fermentation must (Spain)
	ICVV250[b]	Winery (Spain)
Kluyveromyces lactis	AQ2166	*Quercus robur* (Hungary)
Kluyveromyces lactis/marxianus	IFI1329	Blue cheese (Spain)
	AQ1101	Winery (South Africa)
Kluyveromyces marxianus	AQ184	Grapes (Spain)
Kluyveromyces nonfermentans	CBS8778[T]	Marine sediment (Japan)
Kregervanrija fluxuum	AQ2279	Tokaj wine (Hungary)
Lanchancea cidrii	AQ208	Cider (France)
Lanchancea thermotolerans	IFI1142	Red wine (Spain)
Metschnikowia pulcherrima	AQ158	Date (Egypt)
	IFI1459	Grape must (Spain)
	IFI1240	Cherry (Spain)
	IFI1244	Unknown (Spain)
Pichia anomala	ICVV244[b]	Winery (Spain)
	ICVV245[b]	Winery (Spain)
	ICVV246[b]	Winery (Spain)
	ICVV247[b]	Winery (Spain)
Pichia membranifaciens	AQ165	Red wine (Spain)
	AQ166	Red wine (Spain)
	AQ169	Grapes (Spain)
	IFI1334	Black olives (Spain)
Saccharomyces cerevisiae	EC1118	Commercial yeast, Lallemand, Inc.
	IFI707	Castelli Collection (Italy)
	IFI1148	Concentrate must (Spain)
	UCD522	Commercial yeast, Oenofrance
Scheffersomyces stipitis	CBS5773[T]	Insect larva, on fruit tree (France)
	CBS5775	Insect larva, on fruit tree (France)
	CBS5776	Insect larva, on fruit tree (France)
	CBS7124	Soil (unknown)
Starmerella bombicola	AQ1751	Unknown (unknown)
	CBS6009[T]	Honey of *Bombus sp.* (unknown)
	CBS8451	Flower of *C. sepium* (Canada)
	CBS9711	Nectar of *Knautia longifolia* (Germany)
Torulaspora delbrueckii	AQ200	Grape must (Spain)
	AQ216	Grapes (France)
	AQ249	Wine grapes (Germany)
Zygosaccharomyces bailii	AQ229	Grape must (Italy)
Zygosaccharomyces rouxii	AQ251	Wine grape (Germany)

CECT: Colección Española de Cultivos Tipo (Spanish Type Culture Collection, Valencia, Spain); CBS: Centraalbureau voor Schimmelcultures (Delft, The Netherlands); IFI: Instituto de Fermentaciones Industriales (CSIC, Madrid, Spain); DBVPG: Industrial Yeast Collection, Dipartimento di Biologia Vegetale (Perugia, Italy); AQ: kindly provided by Dr. Amparo Querol (Instituto de Agroquímica y Tecnología de Alimentos, IATA (CSIC), Paterna, Spain); ICVV: Instituto de Ciencias de la Vid y del Vino Yeast Collection (Logroño, Spain); [T] Type strain; [a] Previously included in the study by Rantsiou et al. (2012); [b] Previously included in the study by Ocón et al. (2010).

The metabolic pathways involved in central carbon metabolism are essentially conserved amongst yeast species. Nevertheless, mechanisms for nutrient uptake and, most importantly, those involved in the regulation of respiro-fermentative metabolism significantly differ (Flores et al., 2000). The Crabtree effect, first described by Herbert G. Crabtree as a decrease in sugar respiration after glucose addition in tumour cells (Crabtree, 1928), represents a distinctive physiological phenomenon for the classification of yeasts. However, a clear consensus on the precise definition of this effect is currently lacking (Rodrigues et al., 2006). It is accepted that whilst Crabtree-positive yeasts, such as *Saccharomyces cerevisiae*, still ferment under aerobic conditions, provided that sugar is above a certain threshold, the extent of fermentative metabolism in species reported to be Crabtree-negative, such as *Scheffersomyces stipitis* or *Candida utilis*, would be very limited, provided that enough oxygen is available. Although the concentration of molecular oxygen is particularly low during wine fermentation, mainly due to CO_2 release, several practices employed during the first stages of winemaking such as pumping over, *délestage*, or microoxigenation, can transiently but significantly increase O_2 concentration. These, or ad hoc oxygenation practices, would allow for the partial respiration of grape sugars by the appropriate yeast strains.

The present work aims to identify candidate strains, from non-conventional yeast species, to be used in the reduction of the alcohol level in wine by means of their sugar respiratory catabolism. Physiological characterization involved, in the first instance, the determination of respiratory quotient (RQ). Other relevant physiological parameters were determined, including the yield on substrate of biomass and key metabolites (ethanol, glycerol, acetic acid, and succinic acid), as well as strain sensitivity to high sugar concentration and ethanol.

2. Materials and methods

2.1. Yeast strains

Sixty three yeast strains belonging to twenty nine different ascomycetous yeast species were used in the present work (Table 1). Yeasts were maintained at 4 °C on YPD plates (2% glucose, 2% peptone, 1% yeast extract and 2% agar), as well as in glycerol stocks at −80 °C.

2.2. Molecular identification of yeast strains

The identification of all the strains was confirmed or updated by sequencing of the D1/D2 domain of the large subunit rRNA (Kurtzman and Robnett, 1998). Genomic DNA was isolated from one single colony grown on a YPD plate using the protocol described by Lõoke et al. (2011). The D1/D2 domain of the 26S rRNA gene was amplified using primers NL1 and NL4 (O'Donnell, 1993) and treated with ExoSAP-IT (Affymetrix, Santa Clara, USA) prior to sequencing (both strands). The consensus double-strand sequence was compared to sequences available at the Genbank database of the National Center for Biotechnology Information (NCBI) using the basic local alignment search tool