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

Manuel Quirós [a,c], Virginia Rojas [a,b], Ramon Gonzalez [a,*], Pilar Morales [a]

[a] Instituto de Ciencias de la Vid y del Vino (CSIC, Universidad de la Rioja, Gobierno de La Rioja), C/Madre de Dios, 51, Logroño, 26006 La Rioja, Spain
[b] Facultad de Ciencias Químicas, Universidad de Autónoma de San Luis Potosí, Mexico
[c] Evolva Biotech A/S, Copenhagen, Denmark

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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.

* Corresponding author.
E-mail address: rgonzalez@icvv.es (R. Gonzalez).

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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

(BLAST) available at http://blast.ncbi.nlm.nih.gov/Blast.cgi. Verification of the correct identification of *Debaryomyces fabryi* PR66 strain was performed by PCR-RFLP of the IGS region of the rDNA according to the protocol described by Quirós et al. (2006) and Romero et al. (2005).

2.3. RQ determination

To facilitate a reliable determination of respiratory quotients, experimental conditions involved high cell densities and relatively low gas flow in order to maximize sensitivity in the quantification of O_2 consumption. Yeast inocula were incubated in 25 mL YPD broth at 25 °C and 250 rpm orbital shaking for 48 h. Cells were then washed twice with sterile deionized water and the OD_{600} determined. Five hundred OD_{600} units were suspended in 25 mL of a 2X chemically defined medium previously described by Herwig et al. (2001) with two modifications: glucose was omitted, and pH was adjusted to 3.5. The suspension was then transferred to 250 mL shake flasks, and incubated in the resulting resting conditions for 24 h at 25 °C. After that time, 25 mL of a 400 g/L glucose solution was added to resting cultures in order to reach a final concentration of 200 g/L glucose, similar to the sugar content of a standard grape must. Cells were then incubated for 24 h at 25 °C and 250 rpm orbital shaking. Immediately after that, cultures were transferred to sterile 100 mL flasks and supplemented with 200 μL of antifoam 204 (Sigma Aldrich) for the determination of the respiratory quotient (RQ) in fully aerobic conditions. To this end, cultures were sparged with air at a flow rate of 0.6 L/h (0.2 vvm) and magnetically stirred at 250 rpm. Fermentation off-gas was led through silica gel filters and the concentration of O_2 and CO_2 determined every 10 s in a GA4 gas analyzer (DASGIP AG, Jülich, Germany) for 90 min. Concentration of both gases was used to calculate RQ values as the ratio between released CO_2 and consumed O_2 for each time point.

2.4. Determination of yields on substrate

Yeast strains selected on the basis of RQ values were used to conduct additional batch fermentations in a chemically defined medium roughly mimicking the composition of grape must. The formulation of this synthetic must, expressed in g/L, was as follows: glucose, 100.0; fructose, 100.0; citric acid, 6.0; malic acid, 6.0; YNB w/o amino acids w/o ammonium sulphate (Difco™, Becton Dickinson, New Jersey, USA), 0.67; ammonium chloride, 0.764; myo-inositol, 0.018; and pH 3.5.

These batch fermentations, performed in duplicate, were inoculated with cells prepared as described above, to an initial OD_{600} of 0.2. Incubation was performed at 25 °C and 250 rpm orbital shaking in 250 mL shake flasks containing 50 mL of the medium. Samples were taken every 24 h for 4 days in order to monitor the evolution of biomass and the concentration of the main metabolites.

2.4.1. Determination of metabolite concentration

The concentration of glucose, glycerol, ethanol, acetic and succinic acid was determined using a Surveyor Plus liquid chromatograph (Thermo Fisher Scientific, Waltham, MA) equipped with a refraction index and a photodiode array detector (Surveyor RI Plus and Surveyor PDA Plus, respectively) on a 300 × 7.7 mm HyperREZ™ XP Carbohydrate H+ (8 μm particle size) column and guard (Thermo Fisher Scientific). The column was maintained at 50 °C and 1.5 mM H_2SO_4 was used as the mobile phase at a flow rate of 0.6 mL min^{-1}. Prior to injection in duplicate, the samples were filtered through 0.22 μm pore size nylon filters (Micron Analitica) and diluted 10-fold.

2.4.2. Determination of biomass

To determine yeast growth during the course of batch fermentations, the OD_{600} was monitored using a Shimadzu UV-1800 Spectrophotometer (Shimadzu Europe GmbH, Duisberg, Germany). When necessary, samples were diluted with deionised water to obtain readings in the 0.1–0.4 range. Samples were always maintained at 4 °C until OD_{600} and dry cell weight (DCW) measurements were performed. Final (96 h) DCW was determined in triplicate by filtering appropriate volumes of the cultures (4 to 10 mL, depending on the OD_{600} values previously determined) followed by 5 mL deionized water through a 25 mm, 0.45 μm pore size pre-dried and pre-weighed nitrocellulose filter (Millipore, Billerica, USA). Filters were heat dried at 70 °C until constant weight (12–24 h).

2.5. Stress tolerance assays

Phenotypic tests were performed in microtiter plates. Sensitivity of the selected strains to stress factors representative of the early stages of wine fermentation, osmotic stress by glucose or moderate ethanol levels, was assessed.

YPD broth was employed as the control medium in this set of experiments. This medium was supplemented with 220 g/L glucose, or 4% (v/v) ethanol, for sensitivity assays. Yeasts were grown in 5 mL YPD at 25 °C and 250 rpm for 48 h, washed twice, and resuspended in 2 mL deionized water. 5 μL of these cultures were used to inoculate 195 μL of the above mentioned media in quadruplicate, reaching an initial OD_{600} of approximately 0.2. Growth was monitored at 600 nm every 30 min during 48 h in a SPECTROstar Omega instrument (BMG Labtech, Offenburg, Germany). The agitation regime was set to one minute shaking periods alternated by one minute resting periods. Uninoculated wells were used in every experimental series in order to determine and subtract the noise signal. OD_{600} data were additionally corrected with a saturation equation previously determined.

2.6. Bioreactor assays

Batch cultures were performed in duplicate, using Applikon MiniBio bioreactors (250 mL nominal volume), equipped with Peltier-refrigerated gas condensers. Bioreactors were filled with 150 mL of a filter-sterilized natural white grape must, mixture of Malvasia and Viura varieties (260 g/L of sugars), and 200 μL of antifoam 204. Temperature was set to 25 °C, stirring to 1000 rpm and inoculation to 0.2 final OD_{600}. The cultures were sparged with air 1.2 L/h. CO_2 and O_2 contents in the off-gas were continuously monitored with BlueInOne Cell sensors (Bluesens). Samples were withdrawn after 24 and 48 h for determination of metabolite concentrations.

3. Results

The initial strain selection had been designed to include, whenever possible, at least three different isolates from each species, especially those frequently isolated from the winemaking environment. In order to broaden the initial metabolic diversity, the screening also included some isolates from species not commonly found in this milieu, but described as poorly or non-fermenting (Kurtzman et al., 2011). However, after molecular identification at the species level, 18 strains were assigned to a different yeast species. As a result, the actual range of species was found to be wider than expected, but they were represented by a variable number of strains (Table 1). Nevertheless, the physiological diversity represented by these strains was considered appropriate for the purposes of the study.

3.1. Respiratory quotient

In order to investigate the extent of respiro-fermentative metabolism of the different yeast strains included in this study (Table 1), the RQ was determined under fully aerobic conditions, in the presence of about 200 g/L of sugar and pH 3.5, as described in the Materials and methods section. A simple stoichiometric calculation (Fig. S1), just taking energy metabolism into account, indicates that the percentage of sugar metabolised by respiration would be equivalent to 100/(3RQ-2). Therefore, RQ values around 1.0, as those measured for a typical

Table 2
Yields on substrate, consumption of sugars, and RQ values obtained for selected yeast strains. Results are expressed as the average ± standard deviation of two biological replicates.

Strain	YE/S (g/g)	YG/S (g/g)	YSUC/S (mg/g)	YACE/S (mg/g)	YBM/S (g/g)	Consumed sugar (%)	RQ
S. cerevisiae EC1118	0.25 ± 0.01	0.05 ± 0.01	5.95 ± 0.29	3.85 ± 0.45	0.05 ± 0.01	48.06	1.94 ± 0.50
S. cerevisiae UCD 522	0.30 ± 0.01	0.03 ± 0.00	6.08 ± 0.61	6.63 ± 0.56	0.03 ± 0.00	69.54	1.99 ± 0.00
C. sake CBS1939	0.11 ± 0.01	0.02 ± 0.00	11.56 ± 0.31	0.48 ± 0.18	0.22 ± 0.01	13.35	1.66 ± 0.02
C. sake CBS5093	0.18 ± 0.00	0.04 ± 0.00	12.34 ± 0.22	0.96 ± 0.42	0.07 ± 0.01	44.69	2.31 ± 0.24
D. fabryi PR66	0.01 ± 0.00	0.11 ± 0.00	2.90 ± 0.01	0.00 ± 0.00	0.61 ± 0.02	10.85	0.97 ± 0.03
D. hansenii IFI866	0.01 ± 0.00	0.1 ± 0.01	2.92 ± 0.19	0.00 ± 0.00	0.43 ± 0.01	11.74	0.89 ± 0.12
K. exigua DBPVG6354	0.39 ± 0.00	0.09 ± 0.01	4.08 ± 0.3	6.88 ± 1.8	0.05 ± 0.01	35.23	1.41 ± 0.10
K. lactis AQ2166	0.27 ± 0.00	0.05 ± 0.00	9.67 ± 0.09	0.37 ± 0.05	0.12 ± 0.01	32.79	0.8 ± 0.04
K. lactis/marxianus AQ1101	0.16 ± 0.03	0.06 ± 0.01	4.57 ± 0.49	0.42 ± 0.10	0.20 ± 0.00	23.34	1.25 ± 0.17
M. pulcherrima IFI1459	0.25 ± 0.07	0.05 ± 0.01	8.44 ± 3.07	0.54 ± 0.07	0.15 ± 0.02	29.78	1.04 ± 0.13
M. pulcherrima IFI1240	0.24 ± 0.03	0.02 ± 0.00	10.09 ± 1.13	2.02 ± 0.13	0.07 ± 0.00	49.21	1.21 ± 0.07
M. pulcherrima IFI1244	0.26 ± 0.00	0.03 ± 0.00	11.33 ± 0.18	1.71 ± 0.23	0.10 ± 0.00	43.19	1.26 ± 0.14
P. membranifaciens AQ166	0.00 ± 0.00	0.08 ± 0.00	11.44 ± 0.27	0.18 ± 0.25	0.72 ± 0.06	4.65	0.97 ± 0.13
P. membranifaciens AQ169	0.00 ± 0.00	0.28 ± 0.00	1.10 ± 0.11	1.86 ± 0.16	0.42 ± 0.11	13.86	0.93 ± 0.11
S. stipitis CBS 5776	0.11 ± 0.01	0.00 ± 0.00	7.55 ± 6.51	0.00 ± 0.00	0.69 ± 0.04	6.85	1.05 ± 0.00
T. delbrueckii AQ216	0.35 ± 0.01	0.03 ± 0.00	7.53 ± 1.02	1.71 ± 0.08	0.04 ± 0.00	69.75	1.39 ± 0.16
T. delbrueckii AQ249	0.3 ± 0.02	0.03 ± 0.01	5.01 ± 1.37	4.18 ± 0.29	0.04 ± 0.00	69.75	1.42 ± 0.06
S. bombicola CBS8451	N/A	N/A	N/A	N/A	N/A	N/A	1.34 ± 0.05
S. bombicola CBS9711	N/A	N/A	N/A	N/A	N/A	N/A	1.84 ± 0.03

YE/S, ethanol yield on glucose; YG/S, glycerol yield on glucose; YSUC/S, succinic acid yield on glucose; YACE/S, acetic acid yield on glucose; YBM/S, biomass yield on glucose. N/A, not available (*S. bombicola* strains did not grow in the medium used to calculate these yields). RQ values were obtained under different experimental conditions than the other parameters (see Materials and methods).

Crabtree-negative species such as *S. stipitis* (formerly *Pichia stipitis*), indicate a fully respiratory metabolism of glucose; whilst RQ values around 2.0, like those determined for *S. cerevisiae* strains (Table 2), indicate that, under the fully aerobic conditions employed, about 25% of the sugar consumed was respired. Non-*Saccharomyces* strains presenting RQs clearly higher than those of the two control *S. cerevisiae* strains were discarded for further analysis (Table S1). In addition, some strains showing very high acetic acid levels in this preliminary analysis, or showing very low metabolic activity under these assay conditions (as judged by CO_2 production rates), were also discarded (Table S1). The remaining strains, up to a maximum of three per species, were analyzed in an additional set of experiments in order to confirm RQ values.

RQ values of the selected strains are shown in Table 2. RQ values of the selected strains varied from 0.80 ± 0.04, for *Kluyveromyces lactis* AQ2166, to 2.31 ± 0.24 for *Candida sake* CBS5093. RQ values below 1.0 might indicate the concomitant consumption of the ethanol or other metabolites produced during the previous incubation step.

An important intra-specific variability was observed for respiratory metabolism under these assay conditions (Table 2 and data not shown). In summary, 15 strains from 10 different yeast species, showing RQs below 1.5, and close to 1.0 in several instances, were identified in this screening. These include some species commonly reported in enological environments, like *Metschnikowia pulcherrima*, *Starmerella bombicola*, or *Torulaspora delbrueckii*, as well as species from other origins. Under suitable aeration conditions, 40 to 100% of the sugar consumed by these strains is expected to be respired.

3.2. Yields on substrate of the main fermentation metabolites

A low RQ value was expected to be a positive indication of the strain potential for the reduction of ethanol content in wine by the previously proposed strategy (Gonzalez et al., 2013). Yields of ethanol ($Y_{E/S}$) and other fermentation metabolites on glucose were determined by growing the different yeast strains in synthetic must (Table 2). In order to give an indication of the performance of each strain for growth in synthetic must, data on global sugar consumption after four days have also been included in Table 2.

A positive correlation was indeed found for RQ and ethanol yield. It was weak, but statistically significant (correlation coefficient 0.470; Table 3 and Fig. S2). Other parameters, like acetic acid yield or global sugar consumption, were also positively correlated to RQ and ethanol yield, whilst glycerol and biomass yields were negatively correlated to

Table 3
Correlation (Spearman coefficient) between different physiological parameters for 15 strains non-*Saccharomyces* stains and two *S. cerevisiae* strains.

		Ethanol	Glycerol	Succinic acid	Acetic acid	Biomass	Sugar consumed	RQ
Ethanol	Rho	1.000	−0.365	0.039	0.666	−0.880	0.797	0.470
	Significance		0.075	0.441	0.002	0.000	0.000	0.029
Glycerol	Rho	−0.365	1.000	−0.615	−0.162	0.358	−0.411	−0.462
	Significance	0.075		0.004	0.267	0.079	0.051	0.031
Succinic acid	Rho	0.039	−0.615	1.000	−0.098	−0.020	0.077	0.286
	Significance	0.441	0.004		0.354	0.470	0.384	0.133
Acetic acid	Rho	0.666	−0.162	−0.098	1.000	−0.850	0.797	0.620
	Significance	0.002	0.267	0.354		0.000	0.000	0.004
Biomass	Rho	−0.880	0.358	−0.020	−0.850	1.000	−0.963	−0.665
	Significance	0.000	0.079	0.470	0.000		0.000	0.002
Sugar consumed	Rho	0.797	−0.411	0.077	0.797	−0.963	1.000	0.609
	Significance	0.000	0.051	0.384	0.000	0.000		0.005
RQ	Rho	0.470	−0.462	0.286	0.620	−0.665	0.609	1.000
	Significance	0.029	0.031	0.133	0.004	0.002	0.005	

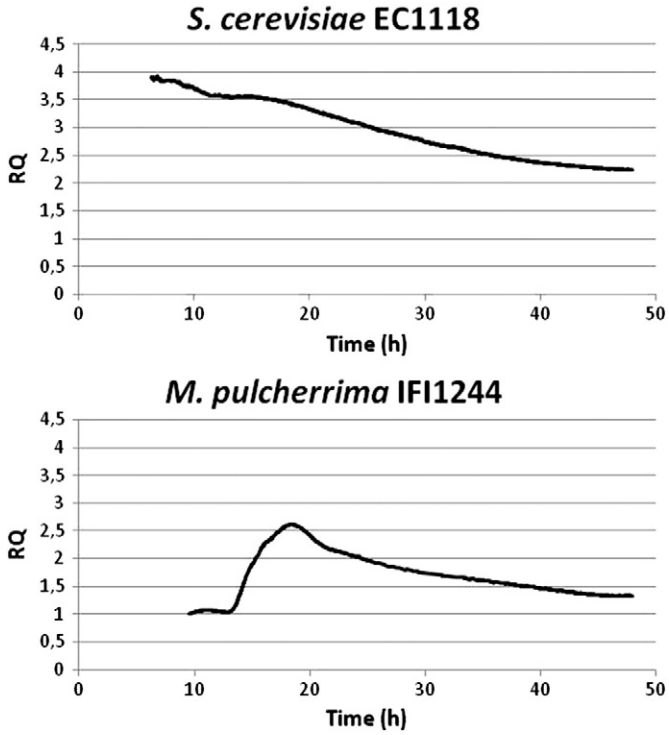

Fig. 1. Evolution of RQ values during the fermentation of natural grape must under controlled aeration conditions. Results are the average of two biological replicates. Data were recorded every minute. Only values with RSD below 5% are shown.

RQ (Tables 2 and 3). Interestingly, the correlation of RQ with acetic acid yield was stronger than with ethanol yield. Other noteworthy correlations are the negative ones between biomass yield and either ethanol yield, acetic acid yield or global sugar consumption.

However, in spite of these general trends, when examined at the single strain level, RQ values had a low predictive power on ethanol yields. For example, despite low RQ values, *Kazachstania exigua* DBVPG6354, *K. lactis* AQ2166, and all the *M. pulcherrima*, or *T. delbrueckii* strains showed ethanol yields similar or higher than *S. cerevisiae* (Table 2). This poor agreement between RQ and ethanol yield for some strains is probably due to the differences in the experimental setup used to calculate each parameter, including medium composition, aeration regime, metabolic state of the cells, and incubation time. In contrast, ethanol yields for strains of *C. sake* (both strains), *D. fabryi* PR66, *Debaryomyces hansenii* IFI866, both strains of *Pichia membranifaciens*, or *S. stipitis* CBS5776 were clearly below *S. cerevisiae* (Table 2).

Noteworthy, ethanol yields on sugar for *S. cerevisiae* strains under these experimental conditions were quite below normal values under anaerobic conditions (about 0.45 g/g). This was only partly explained by alcohol stripping (data not shown), and indicates a significant contribution of respiratory metabolism. However, the acetic acid yield for both *S. cerevisiae* strains was extremely high under these aerated conditions (Table 2), and the resulting increase in volatile acidity would preclude any practical application of the respiratory capacity of these *S. cerevisiae* strains. Indeed, these strains showed some of the highest $Y_{ACE/S}$ values amongst the strains analysed, together with *T. delbrueckii* AQ249, and *K. exigua* DBVPG6354. Acetic acid yield for the other yeast strains in Table 2 was lower or much lower than for the above mentioned strains. The correlation between ethanol and acetic acid yields has some noteworthy and interesting exceptions, like *K. lactis* AQ2166, or *M. pulcherrima* IFI1459.

3.3. Tolerance to winemaking related stress factors

Global sugar consumption during the first four days of fermentation in synthetic must (Table 2) already gave an indication of the potential of the selected strains to overcome some fermentation related stress factors. However, a more detailed analysis was performed considering the two main growth limiting conditions found by yeasts during the first stages of wine fermentation, osmotic stress and ethanol stress.

High glucose level resulted in stronger growth impairment than moderate ethanol content. *S. cerevisiae* strains experienced a moderate growth inhibition by osmotic stress as compared to the control condition (Fig. S3). The effect of this stress factor was also moderate for several other yeast strains, which reached OD_{600} values similar or higher than the control after 24 to 48 h of incubation. Growth of some strains to higher OD_{600} levels under glucose stress is due to the higher availability of glucose as carbon source. Strains of *S. stipitis*, *C. sake* and *P. membranifaciens*, were especially sensitive to osmotic stress by glucose, whilst *S. bombicola* and *Debaryomyces* strains showed an intermediate sensitivity.

Concerning alcohol, 4% ethanol was well tolerated by most of the strains (Fig. S4). Again, some of the strains reached higher OD_{600} levels under the stress condition than under the control one, probably due to the use of ethanol as carbon source. *C. sake* CBS1939 and *D. hansenii* IFI866 were amongst the strains showing the higher sensitivity to ethanol.

3.4. RQ in natural grape must for M. pulcherrima IFI1244

M. pulcherrima IFI1244 was chosen as a representative non-*Saccharomyces* strain in order to provide some additional clues on the feasibility of reducing alcohol content on wine by respiration. RQ values were continuously monitored during the first two days of growth in natural must. *S. cerevisiae* EC1118 was chosen as representative of this species for comparison purposes. According to the RQ profiles shown in Fig. 1, RQ values were always lower for the non-*Saccharomyces* strain than for *S. cerevisiae*. Initial RQ values were indeed around 1 (indicating pure respiratory metabolism) for *M. pulcherrima*, until oxygen becomes limiting after 12 h. At this time point dissolved oxygen decreased to about 0% saturation (data not shown and Table 4). Sugar consumption after 48 h was high for *M. pulcherrima*, about 100 g/L, even though lower than *S. cerevisiae*, 140 g/L (Table 4). Under these experimental conditions the differences in ethanol yields on sugar are clearly in favour of *M. pulcherrima*, 0.20 g/g instead of 0.35 g/g for *S. cerevisiae* (Table 4). Similarly, acetic acid yield after 48 h is almost null, in contrast to the 0.38 mg/g for *S. cerevisiae* (Table 4).

Table 4
Values measured for different physiological parameters during the fermentation of natural grape must under controlled aeration conditions. Results are expressed as the average ± standard deviation of two biological replicates.

Time (h)	Strain	YE/S (g/g)	YG/S (g/g)	YACE/S (mg/g)	Sugar consumed (g/L)	Dissolved oxygen (%)
24	*S. cerevisiae* EC1118	0.38 ± 0.01	0.06 ± 0.00	0.75 ± 0.01	37.8 ± 0.7	59.6 ± 0.6
24	*M. pulcherrima* IFI1244	0.27 ± 0.00	0.10 ± 0.00	0.00 ± 0.01	41.2 ± 1.3	0.1 ± 0.1
48	*S. cerevisiae* EC1118	0.35 ± 0.01	0.04 ± 0.00	3.78 ± 0.00	138.0 ± 0.2	41.9 ± 2.7
48	*M. pulcherrima* IFI1244	0.20 ± 0.00	0.11 ± 0.00	0.00 ± 0.00	99.7 ± 4.1	0.5 ± 0.7

YE/S, ethanol yield on glucose; YG/S, glycerol yield on glucose; YACE/S, acetic acid yield on glucose.

4. Discussion

The RQ value under aerated conditions was the first physiological feature used to ascertain the potential usefulness of non-*Saccharomyces* yeast strains for reducing alcohol levels in wine. It was, in turn, dependent on the Crabtree character of the strains. Several intermediate RQ values were determined, ranging between those of typical Crabtree-negative (*S. stipitis*) and typical Crabtree-positive (*S. cerevisiae*) species. In addition, the experimental setup (sugar availability, pH, aeration regime, cultivation time) has a great influence on the RQ value actually measured, as judged by the relatively poor (0.470), although statistically significant, correlation between RQ and ethanol yield (obtained using different setups; Table 3). Thus, classifying the strains as Crabtree-positive or negative was estimated to be of little help for the purpose of this work.

From the 59 non-*Saccharomyces* original strains, several of them were discarded due to high RQ values, high volatile acidity, or low metabolic activity in high sugar content media (Table S1). However, the already mentioned low correlation between RQ and ethanol yield suggests that it might be eventually interesting to re-evaluate, under different growth conditions, some of the discarded strains, namely those discarded on the basis of RQ value, or the *S. bombicola* strains that did not grow in synthetic must (Table 2). Other statistically significant correlations in Table 3 are stronger than this one, and support the expected link between reduced ethanol yield and increased sugar respiration (in turn associated to increased biomass yield), with a −0.880 correlation coefficient between ethanol and biomass yields. The correlation between global sugar consumption and ethanol (0.797) or biomass (−0.963) yield, suggests that most of the low RQ strains will perform poorly under winemaking conditions. Some clear examples of this behaviour are *C. sake* CBS1939, *D. fabryi* PR66, *D. hansenii* IFI866, both *P. membranifaciens* strains, or *S. stipitis* CBS5776 (Table 2). On the positive side, the correlation between acetic acid yield and RQ (0.620), ethanol (0.666), or biomass (−0.850) yields (Table 3) indicates that, as a trend, the contribution to volatile acidity of strains showing a mostly respiratory metabolism would be lower than for mainly fermentative strains.

In order to identify the most interesting strains for the long-term objective of reducing wine alcohol levels it was judged necessary to find a compromise between ethanol yield, acetic acid production and growth performance in grape must. In some instances, the most interesting strains would be those behaving as exceptions to the general correlations found for these features (Table 3).

As pointed above, the low ethanol yields on sugar of *S. cerevisiae* strains under aerated conditions (0.25–0.30 g/g) were quite below the normal values under anaerobic conditions (i.e. 0.45 g/g). This would already involve a significant reduction in wine alcohol content if these growth conditions were reproduced under industrial conditions. Unfortunately, the acetic acid yield was extremely high, preventing any practical application of these *S. cerevisiae* strains under aerated conditions. In contrast, other yeast strains with similar or lower ethanol yields showed a better compromise between sugar consumption in synthetic must (performance) and acetic acid yield. This is the case of *C. sake* CBS5093, the *Kluyveromyces* strains AQ2166 and AQ1101, or *M. pulcherrima* strains (Table 2). Apart from *C. sake* CBS5093, none of the former strains seems to be severely affected by the stress factors present during the first stages of wine fermentation (high sugar or moderate ethanol content).

The results obtained for *M. pulcherrima* IFI1244 growing on natural grape must under controlled aeration conditions are encouraging and support the feasibility of the previously proposed strategy for alcohol level reduction in wine (Gonzalez et al., 2013). Ethanol and acetic acid yields, as well as the degree of sugar consumption after two days, are compatible with the use of this or similar strains for alcohol reduction. Interestingly, oxygen saturation was kept around 0% for most of the time, but the percentage of glucose respired was always above 17% (RQ values below 2.6). According to this observations, optimization of a procedure allowing for partial respiration of sugars (i.e. lowering final ethanol content), whilst avoiding excessive oxidation of grape must components, would be conceivable. Equipment intended to manage oxygen content at different stages of winemaking, already in use in some wineries, could probably be adapted for this purpose. In order to ensure complete fermentation, and to obtain a product with the familiar sensory profile of wine, the use of a *Saccharomyces* yeast strain would also be necessary. According to the results described above, winemakers will have to pay attention to a possible increase on volatile acidity if oxygenation was not interrupted after inoculation of *S. cerevisiae*. Obviously, industrial implementation of this strategy requires analysis and optimization of other variables such as compatibility between yeast strains, timing for co-inoculation, or nutrient availability for the *Saccharomyces* strain. Also, sensory analysis will be essential in order to rule out any organoleptic defects associated to the use of new yeast strains and fermentation conditions.

Recent studies found final alcohol yield reduction in sequential inoculation fermentations, with *S. cerevisiae* and strains of *Candida zemplinina* or *M. pulcherrima*. The decrease was up to 0.9° for *C. zemplinina* (Bely et al., 2013) and up to 1.6° for *M. pulcherrima* (Contreras et al., 2014). Probably alcohol reduction in the first case was not related to respiration, since at least the *C zemplinina* strains analysed by us, all showed high RQ values. In contrast, the use by Contreras et al. (2014) of either red-wine fermentation conditions, or stirring in the case of white wine, suggest that respiratory metabolism of *M. pulcherrima* might have played a role in lowering final ethanol yields, according to our results with other strains of this species. These results are also encouraging concerning the possibility of finding compatible strains between *S. cerevisiae* and some of the most promising species pinpointed by our work.

In summary, we have identified several non-*Saccharomyces* yeast strains with interesting features in order to reduce alcohol levels in wine, by taking advantage of their respiratory metabolism. Determination of RQ values using pH and glucose levels similar to grape must has been useful but not decisive in this identification. In contrast, we consider acetic acid yield on glucose, under aerobic conditions, and the ability to maintain a highly active metabolism in must, as especially relevant features for this purpose. At least for some of the non-*Saccharomyces* yeast strains, it would be possible to find a balance between yeast respiratory metabolism and dissolved oxygen to limit oxidation of grape must components.

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