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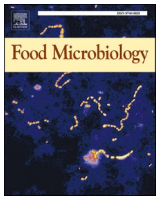

Exploring the suitability of *Saccharomyces cerevisiae* strains for winemaking under aerobic conditions

Jordi Tronchoni [a,b], Ramon Gonzalez [a], Andrea M. Guindal [a], Elena Calleja [a], Pilar Morales [a,*]

[a] *Instituto de Ciencias de La Vid y Del Vino (CSIC, Gobierno de La Rioja, Universidad de La Rioja), Finca La Grajera, Carretera de Burgos Km 6, 26007, Logroño, La Rioja, Spain*

[b] *Universidad Internacional de Valencia – VIU, C/ Pintor Sorolla 21, 46002, Valencia, Spain*

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ABSTRACT

Aerobic fermentation was previously proposed to reduce the ethanol content of wine. The main constraint found for *Saccharomyces cerevisiae* to be used under these conditions was the high levels of acetic acid produced by all *S. cerevisiae* strains previously tested. This work addressed the identification of *S. cerevisiae* wine yeast strains suitable for aerobic fermentation and the optimization of fermentation conditions to obtain a reduced ethanol yield with acceptable volatile acidity. This approach unveiled a great diversity in acetic acid yield for different *S. cerevisiae* strains under aerobic conditions, with some strains showing very low volatile acidity. Three strains were selected for further characterization in bioreactors, with natural grape must, under aerobic and anaerobic conditions. Ethanol yields were lower under aerobic than under anaerobic conditions for all strains, and acetic acid levels were low for two of them. Strain-dependent changes in volatile compounds were also observed between aerobic and anaerobic conditions. Finally, the process was optimized at laboratory scale for one strain. This is the first report of *S. cerevisiae* wine strains showing low acetic acid production under aerobic conditions and paves the way for simplified aerobic fermentation protocols aimed to reducing the alcohol content of wines.

1. Introduction

One of the main concerns of the wine industry in the last decades is the steady increase in the alcohol level of wines, which is directly related to the sugar content of grapes at harvest. A major factor driving this problem is the imbalance between the technological, phenolic and aromatic maturity of the grapes due to global climate change (Mira de Orduña 2010). Furthermore, there is a trend toward longer hang times due to a greater consideration of aroma and phenolic ripeness (Mira de Orduña 2010). Rising ethanol levels are detrimental for the wine industry in several respects, including public health concerns, a negative impact on the sensory quality of wines, and a growing tax burden in many countries.

Researchers are working on different strategies to reduce the ethanol content of wine, spanning from selection of plant material to wine physical dealcoholisation, and including vineyard and microbial fermentation management (Goold et al., 2017). Among them, microbiological approaches are a relevant part of the toolkit currently under consideration; mainly focusing on reducing alcohol yield during fermentation (Ciani et al., 2016; Kutyna et al., 2010). *Saccharomyces cerevisiae* is widely regarded as the best adapted yeast species for wine fermentation; it is the main species that drives the process in non-inoculated fermentations, and for many decades it was almost the only yeast species marketed as a wine starter culture. Phenotypic variability in ethanol yield for this species is low (Camarasa et al., 2011; Palacios et al., 2007); probably due to its high specialization for alcoholic fermentation. Accordingly, biotechnological strategies based on *S. cerevisiae* to lower ethanol yield do not focus on new natural isolates, but on modifying carbon metabolism of strains already in use. The objective is diverting sugar carbon to the production of other metabolites, mainly through metabolic engineering, but also by adaptive laboratory evolution (reviewed in Tilloy et al., 2015).

One way to circumvent the metabolic rigidity of *S. cerevisiae* in terms of ethanol yield is using strains of other yeast species. In order to ensure complete fermentation of grape must, these new species should typically be used in combination with *S. cerevisiae*, either in a simultaneous or sequential inoculation (Ciani et al., 2016). Several authors have indeed explored inter and intraspecific variability in ethanol yields among

* Corresponding author.
E-mail addresses: jtronchoni@universidadviu.com (J. Tronchoni), rgonzalez@icvv.es (R. Gonzalez), andrea.martin@icvv.es (A.M. Guindal), ecalleja@hotmail.es (E. Calleja), pilar.morales@icvv.es (P. Morales).

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different species of wine yeasts (Arroyo-Lopez et al., 2010; Castellari et al., 1994; Contreras et al., 2014; Gobbi et al., 2014). Although carbon fate is not usually analysed in depth, the variability of ethanol yield in these experiments can be explained mainly by differences in biomass production and fermentation by-products.

In order to further divert the yeast metabolism away from ethanol production, our research group proposed a few years ago to switch to sugar respiration (Gonzalez et al., 2013). The proposed procedure involved using aerobic conditions during the first days of fermentation, to provide the required oxygen, and relied on non-*Saccharomyces* strains to overcome the Crabtree positive feature of *S. cerevisiae*. Initial strain screening and laboratory trials showed that, despite the Crabtree effect, the ethanol yield of *S. cerevisiae* was considerably lower under aerobic conditions than under standard wine fermentation conditions (Morales et al., 2015; Quirós et al., 2014); which would open the possibility of using *S. cerevisiae* for alcohol level reduction by respiration. However, their high acetic acid yield under aerobic conditions rendered these strains of *S. cerevisiae* unsuitable for the process.

While the procedure using some non-*Saccharomyces* strains has been developed with acceptable success (Tronchoni et al., 2018), the exclusive use of *S. cerevisiae* would offer some advantages concerning simplicity, yeast strain dominance, and ultimately process control. Similar to the procedure with non-*Saccharomyces* strains, the industrial process with a suitable *S. cerevisiae* strain would be two-step. An initial aerobic step will allow sugar respiration, until a sufficient reduction in the expected ethanol yield is achieved. To reach total sugar consumption fermentation would be finished under standard anaerobic conditions. As described above, our perception about the main bottleneck for using *S. cerevisiae* strains for this purpose shifted from the Crabtree effect to volatile acidity (i.e. from ethanol yield to acetic acid yield). Therefore, in this work we explored the acetic acid yield of several *S. cerevisiae* strains under aerobic conditions. *Saccharomyces* strains showing the lowest aerobic acetic acid yields would be good candidates to develop aerobic fermentation processes in order to reduce the ethanol content of wine. At least two *S. cerevisiae* strains potentially useful for lowering ethanol content in wine by aerobic respiration have been found in this work. The process has been adjusted at laboratory scale for one of them.

2. Materials and methods

2.1. Yeast strains and media

Twenty-five *S. cerevisiae* strains were used in the present work. Nine of them are commercial wine yeast starters, and 16 of them are wild isolates from the private laboratory collection (PRICVV) (Table S1). Yeasts were maintained at 4 °C on YPD plates (2% glucose, 2% peptone, 1% yeast extract and 2% agar), or as glycerol stocks at −80 °C. Inocula were grown on YPD for 48 h at 25 °C, washed and suspended in water. Natural white must from the 2019 harvest was kept frozen. This must contained 220 g/L of sugar and 186 mg/L yeast assimilable nitrogen, pH 3.45. Enough volume for each experiment was thawed and pasteurized. Pasteurization consisted of heating to 105 °C for less than 1 min and then allowing it to cool down inside the closed autoclave.

2.2. Aerobic fermentation of grape must

All experiments were performed in triplicate. For fermentation in shake flasks (100 mL nominal volume) these were filled with 20 mL pasteurized grape must, inoculated at 0.2 final OD_{600}, covered with aluminium foil allowing gas exchange, and incubated at 25 °C 180 rpm. After 4 days, samples were withdrawn, centrifuged, and supernatants were kept frozen for HPLC analysis. This time was enough to have an important amount of consumed sugars without sugar exhaustion, to avoid further ethanol consumption by respiration.

For bioreactor assays, batch cultures were performed using Applikon MiniBio bioreactors (250 mL nominal volume), equipped with Peltier refrigerated gas condensers. Bioreactors were filled with 150 mL of pasteurized natural white grape must, and 200 μL of antifoam 204 (Sigma-Aldrich, Spain). Temperature was set to 25 °C and stirring to 1000 rpm. The cultures were sparged with compressed air (condition A) or pure nitrogen (condition B) at 25 mL/min (10 VVH; volumes of gas/volume culture/hour) (Table S2). Gas flow was controlled with MFC17 mass flow controllers (Aalborg Instruments and Controls, Inc., Orangeburg, NY), whose calibration was regularly verified with an electronic flowmeter (Agilent Technologies, Santa Clara, CA). Bioreactors were inoculated to 0.2 final OD_{600} with independent inocula prepared as described above. During the experiment, 1 mL samples were collected at the times indicated in the different figures for HPLC analysis; as well as an additional 10 mL final sample for the analysis of volatile compounds. Samples were centrifuged and supernatants were kept frozen for chromatographic analyses. Dissolved oxygen was measured by polarographic sensors (Applikon).

In the optimization experiments, the gas flow was adjusted at 25 mL/min (10 VVH), and, when indicated dissolved oxygen was set to 50% (conditions D and F) and 80% (condition G) saturation and maintained by automatic on/off control of the gas valve. Aeration was stopped suddenly (condition D) after 4 days, or gradually (conditions F and G) decreased in 24 h to 0% dissolved oxygen. Agitation (1000 rpm) was interrupted when aeration was permanently stopped. Continuous air and nitrogen flow for 4 days were also run as control conditions (C and E). See Table S2 and Section 3.4 for complete details on aeration conditions.

2.3. Determination of metabolite concentration

The concentration of glucose, fructose, glycerol, ethanol, and acetic 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 PL Hi-Plex H^+ (8 μm particle size) column (Agilent Technologies, Santa Clara, CA) and 4 × 3 mm ID Carbo-H guard (Phenomenex, Torrance, CA). 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. Prior to injection in duplicate, the samples were filtered through 0.22 μm pore size nylon filters (Micron Analitica).

2.4. Analysis of volatile compounds

Samples for gas chromatography-mass spectrometry (GC-MS) analysis contained 1800 μl of the original sample in a final volume of 2000 μl, completed with ethanol and water to get the same amount of ethanol in all samples (up to 12% ethanol), 1 g NaCl, and 20 μl internal standard, in 20 mL flasks. Internal standard contained 1000 ppm each of 4-methyl 2-pentanol and heptanoic acid, and 100 ppm each of ethyl nonanoate and 1-nonanol, in water, prepared from 10,000 ppm individual solutions in ethanol. Sample was preincubated for 10 min at 45 °C, followed by 30 min at 45 °C with 50/30 μm DBV/CAR/PDMS SPME fiber (Stableflex, SUPELCO, Bellefonte, PA). Fiber was desorbed for 5 min at 250 °C.

GC-MS was carried out in a Thermo TRACE GC Ultra apparatus coupled to a Thermo ISQ mass detector, equipped with a Thermo TriPlus autosampler. Gas chromatography was carried in a Thermo Scientific fused-silica capillary column TG-WAXMS A (30 m long; 0.25 mm OD; 0.25 μm film thickness). Chromatographic conditions were as follows: 5 min at 40 °C, 3 °C/min up to 200 °C, 15 °C/min up to 240 °C, 10 min at 240 °C. Helium was used as carrier gas at a flow rate of 1 mL/min, operating in split mode (ratio 30). Total analysis time was 71 min. Detection was performed with the mass spectrometer operating in the Full Scan mode (dwell time 500 ms), with 70 eV ionization energy, and source and quadrupole temperatures of 250 °C. Detection was stopped during the time interval for ethanol elution. Peak identification was made by comparison of ion spectra with NIST mass spectral library. For each compound, including internal standards, the sum of the areas of the

peaks of selected characteristic ions was obtained.

2.5. Statistical analysis

The levels and yield on substrate of the main fermentation metabolites and volatile compounds were compared by one-way analysis of variance. Comparison of means was carried by Tukey test. All statistical analyses, including PCA, were performed using SPSS Statistics v. 25 program (IBM, Armonk, NY, United States).

3. Results

3.1. Aerobic growth of S. cerevisiae strains

Twenty-five *S. cerevisiae* strains were grown in shake flasks for 4 days in natural grape must under aerobic conditions, in triplicate. For operational reasons (incubation space), the strains had to be distributed into two batches. Strain PR50 (EC1118 from Lallemand Inc, Canada) was included in both batches and was treated as two different strains for the analyses (noted as PR50R1 and PR50R2 in the figures). Statistical analysis showed no differences between repetitions of PR50 for any of the parameters studied (Fig. 1).

Sugar consumption after four days ranged from 52% to 75% of the original content. The three strains showing the lowest sugar consumption (PR699, PR705, and PR1156) were wild isolates, whereas the four strains showing the highest consumption values (PR543, PR1269, PR1270, and PR1272) were commercial wine strains (Fig. 1). The ethanol yield (g of ethanol produced per g of sugars consumed) determined under these experimental conditions ranged from 0.28 g/g for strain PR699 to 0.40 g/g for strain PR49 (Fig. 1). Significant differences were found only between the strain with minimal value (PR699) and the two strains with maximal values. The highest value is far below 0.46 g/g, a typical value for anaerobic conditions (Camarasa et al., 2011). This could be partly due to alcohol stripping, but also probably to yeast respiratory metabolism under aerobic conditions. Anyway, the ethanol yield found in this experiment involves an important reduction in wine alcohol content compared to anaerobic conditions. This result agrees with previous experiments, controlled for ethanol stripping, that showed a clear impact of aeration on ethanol yield of *S. cerevisiae* (Morales et al., 2015). On the other side, glycerol yields ranged from 31 to 79 mg/g (Fig. 1), with the wild isolate PR699 appearing as the largest glycerol producer. The difference in glycerol yield partly but not fully explain those in ethanol yields between the lowest and highest ethanol producing strains (PR699 and PR49, respectively). A relation of 2.5x was found between the highest and the lowest values of glycerol yield, similar to the variability found for glycerol production under anaerobic conditions in a screening involving *S. cerevisiae* strains of different origins (Camarasa et al., 2011). Since glycerol production is a common selection criterion for starter yeasts, this variability should be kept in mind for strain selection, also in the context of aerobic fermentation.

The most variable parameter in this analysis was acetic acid yield, spanning almost one log unit, between 0.93 and 8.64 mg/g, for strains PR117 and PR221 respectively (Fig. 1). Interestingly, some of the strains show very low acetic acid yield under these experimental conditions, similar (or lower) to those more frequently found for commercial *S. cerevisiae* strains under anaerobic conditions, 0.6 ± 0.1 g/L (around 2.5 mg/g) according to Camarasa et al. (2011). However, most strains showed much higher acetic acid yields than expected for anaerobic conditions. Indeed, in many cases, this would result in unacceptable volatile acidity (above 0.8 g/L), and therefore wine spoilage, if fermentation conditions were kept aerobic. Among the four strains showing the lowest acetic acid yields, we found one commercial wine yeast strain and three wild isolates. The reference strain used in this work, PR50, which was known to produce a large amount of acetic acid under aerobic conditions (Morales et al., 2015; Quirós et al., 2014) showed an intermediate value among all the strains analysed in this experiment.

3.2. Main fermentation parameters under aerobic and anaerobic conditions

The three strains with lower acetic acid production, one commercial

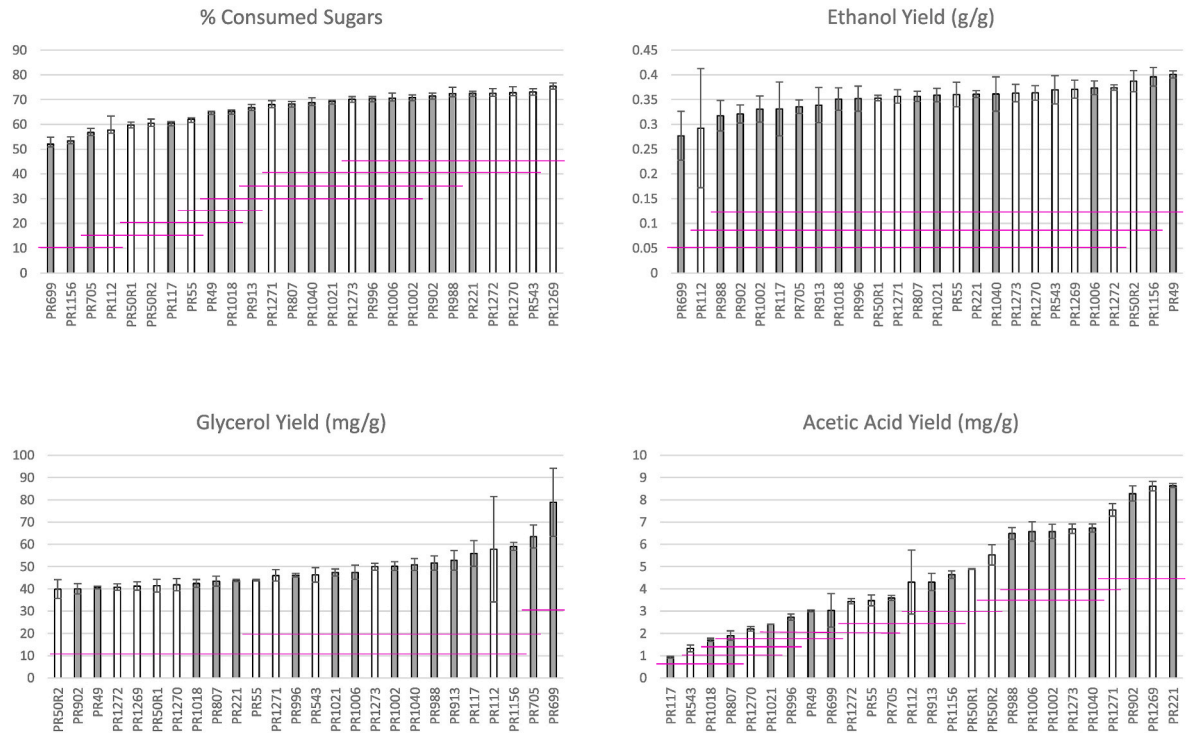

Fig. 1. Measure of main fermentation parameters of cultures of *Saccharomyces cerevisiae* after 4 days of growth on sterilised natural white grape must (sugars 220 g/L) in shake flasks. Grey bars, wild isolates; white bars, commercial strains. Horizontal magenta lines indicate groups of significance ($p < 0.05$).

strain and two wild isolates, were selected for further characterization. Strain PR50 was used as a reference showing elevated acetic acid production under aerobic conditions (Morales et al., 2015). Fermentation experiments were carried out in bioreactors with a constant gas flow (N_2 or air). Using the same gas flow for aerobic and anaerobic conditions contributed to equalize ethanol stripping and allowed for a better comparison of ethanol yields between both conditions.

In aerobic cultures, dissolved oxygen (DO) dropped after inoculation for all four yeast strains (Fig. 2). After reaching a minimal value (between 15 and 30 h from the time of inoculation), the DO values gradually increased over time. However, each strain showed a distinctive pattern in the evolution of DO, which was consistent among replicates. Strain PR1018 was the one showing a faster oxygen consumption in the initial stages of fermentation, with a sharp V-shape curve during the first day of culture, after which its behaviour was closer to the other strains. Sustained oxygen consumption confirms that all four strains were deploying respiro-fermentative metabolism under aerobic conditions.

Respiro-fermentative metabolism under these conditions was also supported by comparing ethanol yields between aerobic and anaerobic conditions for each yeast strain (Fig. 2). The ethanol yield of all four strains was consistently lower under aerobic than under anaerobic conditions. For the same growth condition, all strains showed very similar values. Under aerobic conditions, ethanol can be respired by *S. cerevisiae* after sugar exhaustion. To avoid interferences due to ethanol respiration, data from day 5 were selected for the comparisons shown in Table 1. For this sample point, ethanol yield under anaerobiosis was 0.38–0.40 g/g (Table 1). This value is lower than 0.45 g/g, typical value for anaerobic cultures, probably due to ethanol stripping through nitrogen flow. Ethanol yield in aerobiosis was 0.27–0.30 g/g for the same sample point (Table 1). This value is significantly lower (about a quarter) than for anaerobic conditions, indicating that a substantial fraction of sugar metabolism was diverted from ethanol to other carbon sinks, presumably through respiratory metabolism.

The response of sugar consumption to differences in oxygen availability was opposite for the two wild strains (PR117 and PR1018), showing faster sugar consumption under aerobic conditions; or the commercial strains (PR50 and PR543), which consumed it faster under anaerobic conditions (Fig. 2). Strain PR117 consumed sugars more slowly than the others, and after 11 days residual sugars for this strain were 2.67 g/L in aerobiosis but 22.48 g/L in anaerobiosis. Strains PR1018 and PR50 finished sugars in 7 days in anaerobiosis; whereas in aerobiosis PR1018 took only 6 days, and PR50 took longer than in anaerobiosis. Strain PR543 depleted sugars in 6 days in anaerobiosis, while in aerobiosis sugar consumption was arrested after day 5, leaving more than 43 g/L residual sugars (Fig. 2).

For each strain, the glycerol yield was higher in anaerobiosis than in aerobiosis (Table 1). In all cases, it decreased with time till sugar exhaustion (Fig. 2). This is most probably due to glycerol being mostly produced during the first stages of fermentation under both aerobic and anaerobic conditions. There are great and significant differences between all four strains for glycerol yield, being the wild strain PR117 the one with higher glycerol yield under both growth conditions. Under anaerobic conditions, wild strains produced higher amounts of glycerol than commercial strains (Table 1).

Acetic acid yield under anaerobic conditions was low for all four strains over time (Fig. 2). By day 5, two strains produced clearly higher amounts of acetic acid under aerobic conditions than under anaerobic conditions (Table 1). Strain PR1018 had a remarkably low acetic acid yield during the first days of aerobic fermentation, but levels increased dramatically once the sugars were depleted (Fig. 2), indicating a major metabolic reprogramming (probably energy metabolism supported by ethanol respiration). Strain PR117 showed very low acetic acid yield under aerobic conditions throughout the experiment, but sugars had not been exhausted (Fig. 2). Strain PR50 showed high acetic acid yield under aerobic conditions, as we had determined in other experiments (Morales et al., 2015; Quirós et al., 2014), increasing moderately after sugar exhaustion. Strain PR543 was chosen for this experiment because of its low acetic acid yield in the screening in shake flasks. However, it showed a high acetic acid yield under aerobic conditions in bioreactors, probably due to differences in oxygen availability between both cultivation systems.

3.3. Volatile compounds produced under aerobic and anaerobic conditions

The profile of volatile compounds was rather different between

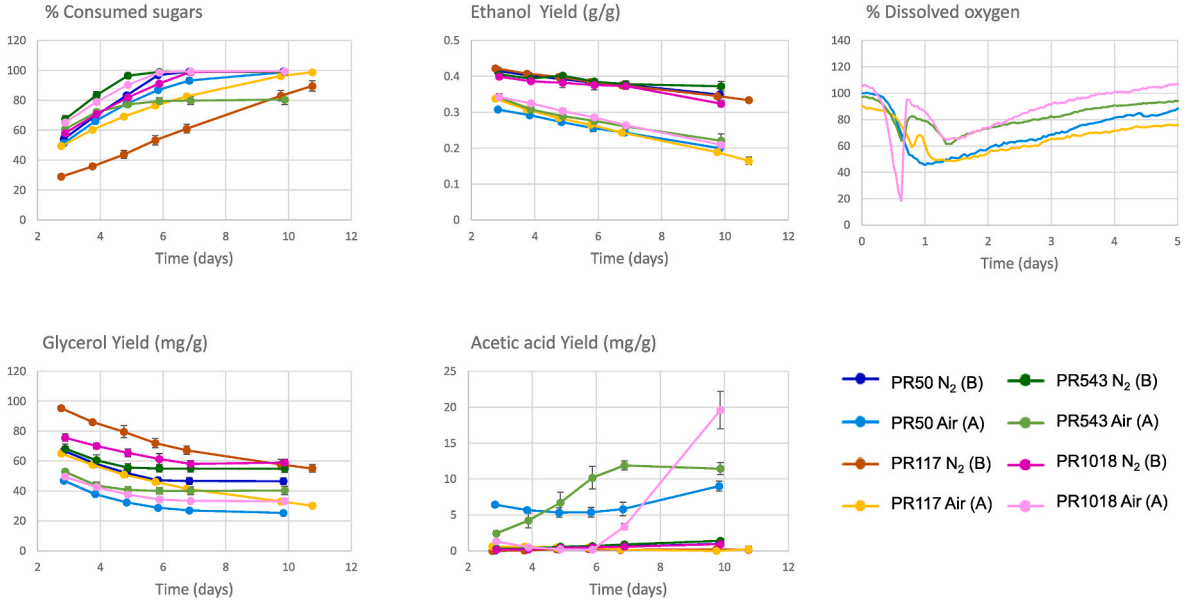

Fig. 2. Measure of dissolved oxygen and main fermentation parameters of selected strains of *Saccharomyces cerevisiae* on sterilised natural grape must (sugars 220 g/L) in bioreactors, in aerobiosis and in anaerobiosis. Colour codes for strains and aeration are included. Bioreactor conditions, A or B, according to Table S2 are indicated between brackets in the graphical legend. PR50 and PR543 are commercial wine yeast strains. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

Table 1
Consumed sugars and yields of main fermentation metabolites on consumed sugars on day 5 after inoculation, in sterilised natural must in anaerobic (10 VVH N2) and in aerobic (10 VVH air) conditions of strains grown in bioreactors. Capital letters in the same row indicate statistical differences ($p \leq 0.05$).

		PR50			PR117			PR543			PR1018		
		mean	±	std dev	mean	±	std dev	mean	±	std dev	mean	±	std dev
% Consumed Sugars	N_2	83.34	±	0.95 B	43.84	±	2.63 A	96.49	±	1.64 C	81.93	±	0.72 B
	Air	77.70	±	1.23 B	69.20	±	1.03 A	77.37	±	1.44 B	90.40	±	0.63 C
Glycerol Yield (mg/g)	N_2	52.09	±	2.01 A	79.54	±	4.08 C	55.62	±	2.59 A	65.47	±	2.63 B
	Air	32.39	±	1.08 A	50.96	±	0.50 D	40.75	±	1.98 C	37.64	±	0.39 B
Acetic Acid Yield (mg/g)	N_2	0.22	±	0.02 A	0.24	±	0.01 A	0.58	±	0.05 B	0.41	±	0.17 AB
	Air	5.34	±	0.65 B	0.47	±	0.02 A	6.70	±	1.47 B	0.23	±	0.01 A
Ethanol Yield (g/g)	N_2	0.39	±	0.00 AB	0.40	±	0.00 AB	0.40	±	0.00 B	0.38	±	0.01 A
	Air	0.27	±	0.01 A	0.28	±	0.00 AB	0.29	±	0.00 B	0.30	±	0.01 C

aerobic and anaerobic conditions for all four strains (Fig. 3). The groups of compounds shown on top of Fig. 3 were more abundant under aerobic conditions. Other groups of compounds did not respond homogenously to oxygenation, with compound concentrations varying in one direction or the other. There were also compounds showing a different response to aeration, depending on the yeast strain.

The production of acetaldehyde and its acetal derivatives was higher under aerobic conditions (Fig. 3); and the same was observed for dioxolanes and dioxanes. Interestingly, under aerobic conditions, levels of most of these compounds were significantly lower for strain PR1018 than for the other three strains.

The production of diacetyl, acetoin, and 2,3-butanediol was also increased in aerobic conditions. Again, the levels of acetoin and diacetyl in aerobic conditions were lower for PR1018 than for the other strains. In contrast, this strain (PR1018) showed the highest (but not significant) levels of 2,3-butanediol among the four strains under aerobic conditions. This polyol is considered odourless, with neutral organoleptic impact (Tilloy et al., 2015).

Ethyl acetate levels also increased with aeration. The highest values for this compound were found for strain PR543 (Fig. 3). Three additional acetate esters were quantified from these fermentation experiments. Isobutyl acetate was detected only under aerobic conditions, and only for strains PR50, PR543 and PR1018 (Fig. 3). Isoamyl acetate and phenylethyl acetate were more abundant under anaerobic conditions for all strains. The highest levels of isobutyl acetate, isoamyl acetate, and phenylethyl acetate under aerobic conditions were found for the PR1018 strain, and the lowest ones for the PR117 strain. PR543 was the strain showing the highest concentration of ethyl esters, both under aerobic and anaerobic fermentation conditions.

Isobutanol and linear alcohols showed their highest levels in aerobiosis, while isoamyl alcohols, phenylethanol, and methionol were mostly produced in anaerobiosis (Fig. 3). Strain PR1018 showed the highest levels of 2-phenylethanol, under both fermentation conditions. Levels of octanoic acid and isovaleric acid were lower under aerobic conditions, and levels of isobutyric acid higher. The highest values of ethyl lactate were found for strain PR543 under aerobic conditions (Fig. 3). In summary, the impact of oxygenation on the production of these families of compounds depended on the yeast strain and the specific compound, with instances of both higher and lower production under aerobic conditions.

A PCA analysis using the data in Fig. 3 was performed. A total of 7 PCs were obtained. PC1 explained 50%, and PC2 a 18% of the variance. The coefficients of each compound for PC1 and PC2 are shown in Fig. 3. A representation of samples in the plane defined by PC1 and PC2 as variables shows that PC1 clearly separates aerobic from anaerobic samples (Fig. 4). According to this plot, PR117 and PR1018 show the closest distance between aerobic and anaerobic conditions. Furthermore, aerobic cultures of strain PR1018 are closer to the bulk of anaerobic cultures in PC1 than any other strain, mostly due to the low levels of acetaldehyde and related compounds, including dioxane/dioxolane ring molecules. Other molecules defining aerobic fermentations, like propanol and butanol are also lower for PR1118 aerobic cultures (but not isobutanol). PC2 is apparently capturing strain-specific features, since aerobic and anaerobic samples from each strain show relatively similar values for this component.

3.4. Optimization of the aerobic fermentation process

Strains PR117 and PR1018 have been shown able to grow on grape must under aerobic conditions with a low acetic acid production. However, strain PR1018 produces a high amount of acetic acid when sugars are exhausted. This phenomenon has not been observed for PR117, perhaps because this strain had not consumed all sugars at the end of the experiment.

Taking advantage of the properties of these strains, the aim of this optimization was finding a combination of strain and growth conditions that would result in complete fermentation, reduced ethanol yield, and no overproduction of acetic acid. In a preliminary experiment, both strains were assayed under 3 different aeration conditions (2, 5 and 10 VVH) for 48 h, and then, aeration and agitation were stopped. In these conditions, sugar exhaustion was achieved in 7 days for PR117 and 8 days for PR1018 (data not shown). But a dramatic increase in acetic acid was observed after closing aeration, while the ethanol yield by that time point was intermediate between aerobic and anaerobic conditions.

We then tested the possibility of controlling aeration by dissolved oxygen control, rather than a continuous air flow. PR1018 was taken for this assay, considering it would be more reliable than PR117, attending to the poor fermentation kinetics of the later under fully aerobic or fully anaerobic conditions (Fig. 2). Three aeration conditions were assayed in triplicate, shown as conditions C, D, and E in Table S2. Under condition D dissolved oxygen level was set to 50% of saturation for four days and then aeration was stopped. Conditions C and E correspond to fully aerobic and fully anaerobic control conditions, also for four days, in order to equalize stripping for better comparisons. Sugars were exhausted by day 7 in all three aeration conditions (Fig. 5). Concerning ethanol and acetic acid production, no differences were observed between both aerobic conditions. A difference in ethanol levels above 2% (v/v) was achieved at the end of fermentation in aeration conditions (from 0.43 to 0.34 g/g ethanol yield on sugar). However, stop of aeration is followed by an increase in acetic acid, as observed in previous experiments, making the process still unsuitable for applied purposes (Fig. 5).

Finally, we explored the possibility of avoiding the boost in acetic acid production with two additional aeration regimes for PR1018 (Table S2). Dissolved oxygen was set to 50% saturation (condition F), or 80% (condition G) during the first 84 h (day 3,5), and then, dissolved oxygen setting was programmed to drop to 0% in 24 h. Stirring was stopped when aeration was completely shut down by day 4,5 (108 h). The profile of dissolved oxygen under conditions F and G is shown in

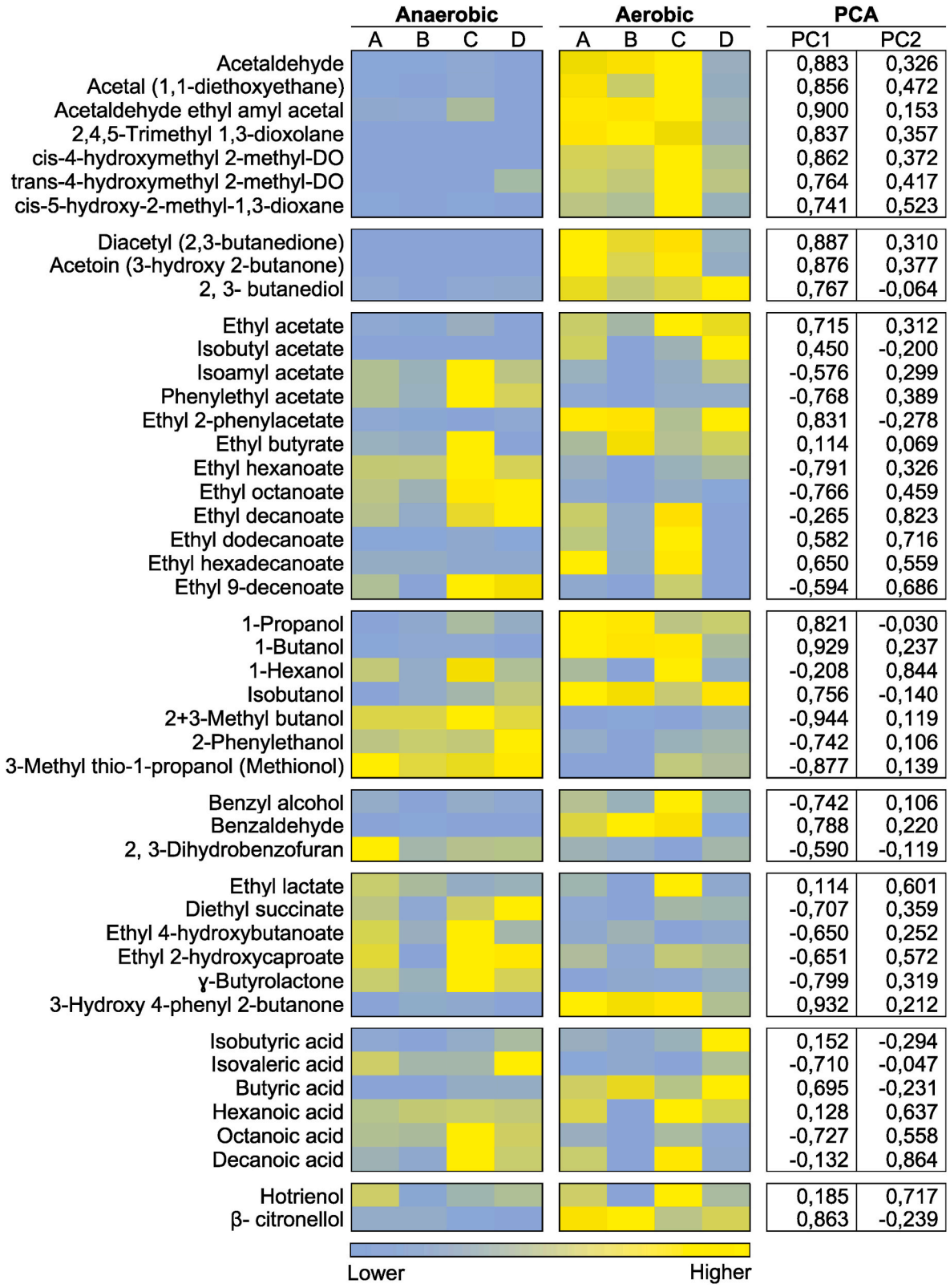

Fig. 3. Heatmap of yield of volatile compounds found in cultures in bioreactors. Colour graduation is stablished for each compound between maximal and minimal values. Coefficients of each compound for PC1 and PC2 of PCA are indicated. Strain code: A: PR50; B: PR117; C: PR543; D: PR1018. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

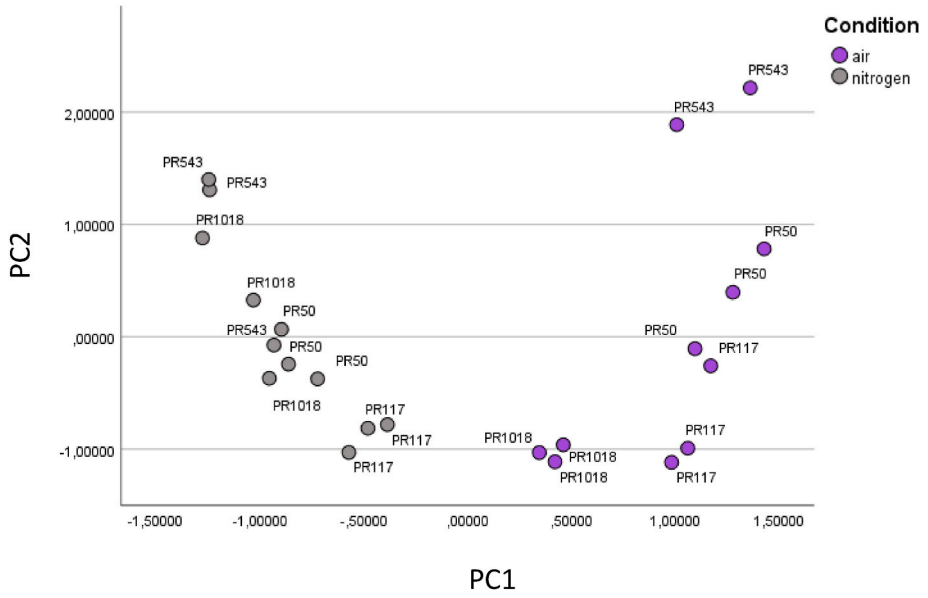

Fig. 4. Principal component analysis based on volatile compounds presented in Fig. 3 for *S. cerevisiae* strains under two different aeration conditions.

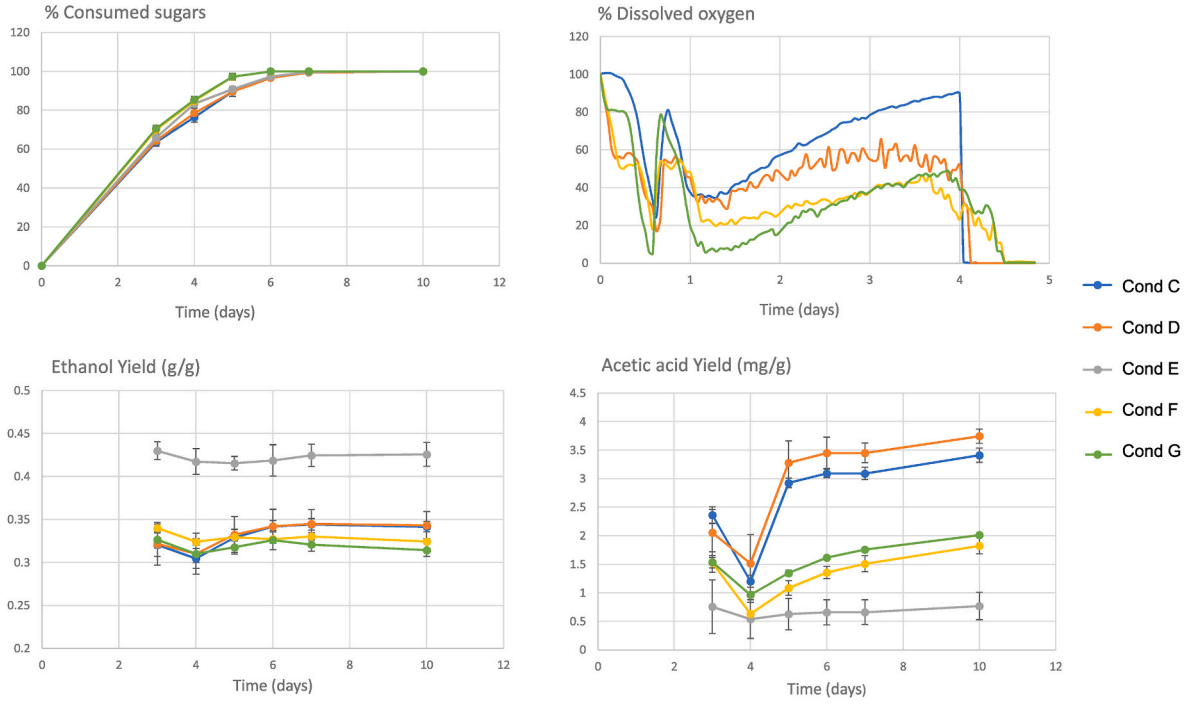

Fig. 5. Main fermentation parameters of *S. cerevisiae* PR1018 on sterilised natural grape must in bioreactors, under different aeration conditions. Colour codes for aeration conditions are included. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

Fig. 5. No differences in sugar consumption or in ethanol production can be seen between both aeration conditions, but acetic acid is lower in aeration limited to 50% dissolved oxygen (Fig. 5). Under these conditions, the increase in acetic acid after aeration stop is less pronounced, and levels of acetic acid remain low to the end of the process.

4. Discussion

The framework of this research is the development of procedures to reduce the alcohol yield during wine fermentation based on the respiration of sugars by yeasts (Gonzalez et al., 2013); an approach that requires aerobic conditions. Contrary to initial expectations, it was found that the main drawback, of using *S. cerevisiae* for this purpose is not low respiration rates (due to the Crabtree effect), but excess acetic acid production (Quirós et al., 2014). Using genetically modified yeast strains to tackle this problem is feasible (Curiel et al., 2016) but has limited industrial application due to regulatory constraints. In turn, the use of Crabtree negative non-*Saccharomyces* yeasts requires optimization of yeast strain production, inoculation strategies, yeast nutrient management, and other process parameters (Morales et al., 2015; Tronchoni et al., 2018). Non-recombinant *S. cerevisiae* wine yeast strains with low acetic acid production would combine the advantages of some non-*Saccharomyces* strains with industrial know-how on *S. cerevisiae*, resulting in better process simplicity and robustness (single inoculation

step, no concerns about sulphite tolerances by non-*Saccharomyces* strains or yeast strain compatibility).

The phenotypic analysis under aerobic conditions of this collection of *S. cerevisiae* strains has shown a relative homogeneity of *S. cerevisiae* isolates in some fermentation parameters, with ethanol yield being the most homogeneous one. Indeed, ethanol yield in this species seems to be much more dependent on growth conditions than on the specific strain (Camarasa et al., 2011; Palacios et al., 2007). However, acetic acid yield showed the highest diversity, with almost one order of magnitude between the extreme values. This work also confirms, for all the *S. cerevisiae* strains, that despite the Crabtree effect, oxygenation of grape must during fermentation results in clearly reduced ethanol yields (about 25% lower for bioreactors flushed with air as compared to those flushed with nitrogen). This fact, combined with the identification of some *S. cerevisiae* strains showing very low volatile acidity during aerobic fermentation, opens the way to use wild *S. cerevisiae* strains for alcohol level reduction in wines.

The high impact of oxygen availability on yeast metabolism goes beyond respiration, or the yields of ethanol, glycerol, or acetic acid; and its influence on the profile of volatile compounds is especially relevant for winemaking. Accordingly, one selection criterion for yeast strains for reducing alcohol yields under oxygenation conditions should pay attention to the profile of volatile compounds (Tronchoni et al., 2018).

In the selection of novel *S. cerevisiae* wine yeasts, compatible with aerobic fermentation, it must be considered that differences in aeration regimes can lead to very significant differences in fermentation output. For example, fermentation in aerobic bioreactors by strain PR543 was incomplete, whereas it had shown promising results during the first screening in shake flasks. Furthermore, the low volatile acidity shown in shake flasks by this strain was not observed in the oxygenated bioreactors. Nevertheless, the behaviour of other strains, like the wild isolates PR117 or PR1018, was more consistent across different aerobic conditions, showing more promise for industrial application.

Indeed, each of these two later strains shows different features that make them interesting candidates for low alcohol winemaking (in the conditions mentioned above). The concentration of molecules typically related to oxidation processes is lower under aerobic conditions for PR1018 than for the other strains, including acetaldehyde, acetals or dioxan related molecules. Considering that acetaldehyde is a precursor of several compounds in this group (Fig. 3) the lower levels of acetaldehyde in aerobic cultures of PR1018 might be the cause of the general decrease observed for these compounds. Acetals increase during biological aging of sherry wines (Morales et al., 2020). Hydroxymethyl 2-methyl dioxanes/dioxolanes, formed by condensation of acetaldehyde and glycerol, have been shown to increase during oxidative aging of Porto wines (Silva Ferreira et al., 2002). Trimethyl dioxolane has been detected as an important odorant of oxidized wines (Escudero et al., 2000). This suggest that, by using this strain as starter, the impact of aeration on the aroma profile of wines might be minimized. However, it is necessary to pay attention to the sudden increase in volatile acidity, probably due to a metabolic switch for ethanol utilization once sugars are exhausted, to avoid reaching the critical point during the industrial fermentation process. On the other side, PR117 does not show this limitation (acetic acid yield remains low up to the end of fermentation), perhaps because fermentations in the conditions shown are slower and sugars have not been exhausted.

The aeration conditions have proven to exert a great effect on the levels of acetic acid produced. Ideally, considering we are dealing here with Crabtree-positive strains (not Crabtree-negative ones as proposed in Gonzalez et al. (2013)), the aeration time should last as long as possible to get a maximum reduction in ethanol levels, and aeration should be interrupted before sugar exhaustion to avoid acetic acid production. The combination of yeast strain and aeration conditions influences many different compounds, as described above. They have been assigned positive, neutral or negative attributes, but the different thresholds and interactions between them make it difficult to anticipate their actual sensory impact. Attention should also be paid to the impact of oxygen on other sensory active components directly derived from grape must, but a previous study did not indicate this should be a big concern, at least for aroma compounds (Tronchoni et al., 2018). In terms of wine colour, although no negative impact has been detected in this and previous work using comparable conditions, long-term stability should also be assessed. The moment of aeration stop has also proven crucial for acetic acid production, as a drastic increase in acetic acid release can be triggered, probably due to a metabolic unbalance caused by a sudden change from aerobic to anaerobic conditions, resulting in an excess of intermediaries of the respiration pathway. In this work a progressive decrease of oxygen levels resulted effective to keep these levels low, but we do not know whether a progressive decrease of oxygen is effective for other strains. The results obtained with PR1018 pave the way for process optimization with other strains. The procedure developed in this work, involving sugar respiration by a *S. cerevisiae* strain, constitutes an important improvement over the one involving non-*Saccharomyces* strains previously proposed by Gonzalez et al. (2013).

Declaration of competing interest

The authors of this manuscript declare that part of the results herein presented have been also included in a patent application whose co-inventors are Ramon Gonzalez, Pilar Morales, and Jordi Tronchoni.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.fm.2021.103893.

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