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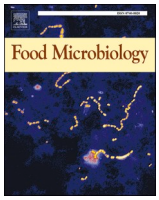

Directed evolution of *Saccharomyces cerevisiae* for low volatile acidity during winemaking under aerobic conditions

Andrea M. Guindal [a], Ramon Gonzalez [a], Jordi Tronchoni [a,b], Jorik S. Roodink [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

The use of yeast respiratory metabolism has been proposed as a promising approach to solve the problem of increasing ethanol content in wine, which is largely due to climate change. The use of *S. cerevisiae* for this purpose is mostly hampered by acetic acid overproduction generated under the necessary aerobic conditions. However, it was previously shown that a *reg1* mutant, alleviated for carbon catabolite repression (CCR), showed low acetic acid production under aerobic conditions. In this work directed evolution of three wine yeast strains was performed to recover CCR-alleviated strains, expecting they will also be improved concerning volatile acidity. This was done by subculturing strains on galactose, in the presence of 2-deoxyglucose for around 140 generations. As expected, all evolved yeast populations released less acetic acid than their parental strains in grape juice, under aerobic conditions. Single clones were isolated from the evolved populations, either directly or after one cycle of aerobic fermentation. Only some clones from one of three original strains showed lower acetic acid production than their parental strain. Most clones isolated from EC1118 showed slower growth. However, even the most promising clones failed to reduce acetic acid production under aerobic conditions in bioreactors. Therefore, despite the concept of selecting low acetic acid producers by using 2-deoxyglucose as selective agent was found to be correct, especially at the population level, the recovery of strains with potential industrial utility by this experimental approach remains a challenge.

1. Introduction

Over the last decades, the average global temperature has increased at the fastest rate in recorded history (NOAA Global Climate report for 2020), thus affecting the production of many crops, including grapevines. Global warming has increased the gap between the technological maturity (sugar and organic acid content) and the aromatic and phenolic maturity of grapes in many growing regions. Furthermore, market trends point to a greater appreciation of the aroma strength and phenolic maturity of the wines. As a consequence, the sugar content of the grapes at harvest has also risen steadily (Mira de Orduña, 2010). This excess sugar is converted into alcohol by the yeast *Saccharomyces cerevisiae* during wine fermentation, and may negatively affect wine quality, recommended wine intake values (on public health or responsible driving grounds), and international wine trade (Mira de Orduña, 2010).

In the attempt to limit the increase in the alcohol content of wines, research efforts cover all stages of the production process, from grapevine breeding (Delrot et al., 2020) to partial dealcoholisation by physical means (Sam et al., 2021). Advances are also taking place in vineyard management (Santos et al., 2020) and winemaking practices (Piccardo et al., 2019). Biotechnological approaches to reduce ethanol yield during wine fermentation have explored both *S. cerevisiae* species and non-*Saccharomyces* species, and both wild isolates and genetic improvement (reviewed by Ciani et al., 2016; Gonzalez et al., 2021).

Aerobic fermentation conditions have been proposed as a way to reduce the ethanol content of wine. This would allow yeast respiro-fermentative metabolism and thus reduce the ethanol yield compared to pure fermentation of the grape juice sugars (Gonzalez et al., 2013). A screening for yeast strains, belonging to different species that could be useful for this process indicated that the three most relevant parameters to take into account were, first, the respiratory capacity under high sugar concentration; second, the kinetics of sugar consumption; and

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

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third, the yield of acetic acid (Quirós et al., 2014). An unexpected finding was that the *S. cerevisiae* strains used in that study could reduce ethanol yield under aerobic conditions, despite the Crabtree-positive character of the species (De Deken, 1966). However, under these conditions they produced high amounts of acetic acid, which precluded their applicability for this purpose (according to OIV, 2022, the maximum acceptable limit for volatile acidity is 1.2 g/L of acetic acid equivalents, but most quality wines should remain far below this limit for consumer acceptance). In contrast, several strains of different non-*Saccharomyces* species showed suitable sugar consumption kinetics, with low ethanol yield and low volatile acidity. One of these strains was successfully used, in combination with a strain of *S. cerevisiae*, to test the concept of reducing alcohol levels by respiration (Morales et al., 2015). The use of *S. cerevisiae* was necessary to ensure that fermentation was complete. Due to the many effects that oxygen can have on wine constituents, the process was scaled up with some non-*Saccharomyces* strains in order to test for the sensory impact of aerobic fermentation conditions (Tronchoni et al., 2018). No major defects were found, but it was concluded that process improvement and strain selection must consider both major compounds (like ethanol or acetic acid) and minor ones, as well as sensory validation. In practice, the need for two different starter cultures makes the industrial implementation of the process more cumbersome. It requires special attention to yeast nutrition in sequential inoculation processes, to inoculum ratio in simultaneous inoculation, and to strain compatibility in any case (Jolly et al., 2014; Morales et al., 2015). The availability of *S. cerevisiae* strains that do not increase volatile acidity under aerobic fermentation conditions is expected to simplify the process and ease its scale-up.

Previous screenings of *S. cerevisiae* strains under standard winemaking conditions have shown high uniformity in ethanol yield, but a large diversity in acetic acid yield (Camarasa et al., 2011; Palacios et al., 2007). A similar result was obtained by Tronchoni et al. (2022) under aerobic fermentation conditions, i.e. similar ethanol yield values for the different *S. cerevisiae* strains, but high diversity in acetic acid yield. Interestingly, some of these strains showed low acetic acid production even under aerobic conditions. One of them was used to establish a single-strain aerobic fermentation process for alcohol level reduction on a laboratory scale (Tronchoni et al., 2022). However, a technology that relies on a single yeast strain worldwide is unlikely to bring a real impact to the industry. The implementation of industrial processes based on *S. cerevisiae* for alcohol reduction by respiration would require the availability of additional suitable yeast genotypes.

S. cerevisiae strains that show low acetic acid production under aerobic conditions have also been derived by genetic engineering. Even though they are unlikely to reach the market, these strains provide valuable information for strain development. For example, recombinant *reg1*-defective strains obtained in an industrial genetic background showed similarly low acetic acid production under aerobic or anaerobic fermentation conditions (Curiel et al., 2016). Reg1 is involved in glucose repression and defective mutants are carbon catabolite derepressed (Matsumoto et al., 1983). Reg1 is a regulatory subunit for the Glc7 protein phosphatase (Jiang et al., 2000) and directs phosphatase activity to proteins involved not only in glucose repression, but also cell growth or glycogen accumulation (Cui et al., 2004). This upstream position of Reg1 in several signal transduction pathways results in pleiotropic effects of reg1 loss-of-function mutations. Therefore, the low acetic acid production under aerobic fermentation conditions shown by *reg1* strains is not directly linked to carbon catabolite repression (CCR). Indeed, other CCR defective mutants did not show this behaviour (Curiel et al., 2016). Even though indirect, this link between CCR alleviation and low acetic acid production in some mutant strains might be exploited for wine yeast genetic improvement. CCR defective mutants can use galactose as carbon source, in the presence of 2-deoxyglucose (2DG), a non-useable glucose analogue, whereas normal strains cannot. 2DG has been widely used to isolate *S. cerevisiae* mutants defective in CCR (Neigeborn and Carlson, 1987). In this work, experimental evolution was conducted, using galactose as carbon source in the presence of 2DG, to select strains that resemble the previously characterized recombinant *reg1* strain.

2. Materials and methods

2.1. Yeast strains

Three wine yeast strains were used, EC1118 (Lalvin EC1118, Lallemand Inc.), T73 (Lalvin T73, Lallemand Inc.), and IFI473 (CECT 12658). Two of them are commercial wine yeast strains, while the IFI473 was shown useful for industrial use in sparkling wine production (Martínez-Rodríguez et al., 2001).

2.2. Natural and synthetic juices

White grape juice (2019 harvest; 220 g/L of sugar and 186 mg/L yeast assimilable nitrogen) and rosé juice (2015 harvest; 200 g/L of sugar and 153 mg/L yeast assimilable nitrogen) were kept frozen. Before use they were thawed and pasteurized (heated until completing less than 1 min at 105 °C and allowed to cool down inside the closed autoclave).

Jellified synthetic juice (MS300a) was based on MS300 (Bely et al., 1990) and solidified with 20 g/L agar.

2.3. Experimental evolution

Strains were grown in 100 mL shake flasks, containing 20 mL of culture medium. Culture medium, YPGal, contained per litre, 20 g peptone, 10 g yeast extract, and 20 g galactose. Flasks were covered with aluminium foil, allowing gas exchange, and incubated at 25 °C, 180 rpm. Once the culture had reached maximal OD, 10% of the volume was used to inoculate the next batch culture. The first culture was inoculated with cells grown in YPGal, without 2DG addition. Initial 2DG concentration was 50 mg/L (3 passages). 2DG content was first increased as shorter times were required to reach stationary phase to 100 mg/L (1 passage), then to 150 mg/L (1 passage) and 200 mg/L (43 passages). Most subcultures were done with 200 mg/L 2DG. Experimental evolution was run for around 140 generations, in triplicate for each wine yeast strain. Part of the final evolved population was used to inoculate fermentation experiments with grape juice (see below) and to prepare glycerol stocks (20% glycerol) kept at −80 °C (Supplementary Figure S1). Absence of external contamination was verified every 10 generations by interdelta PCR analysis (Legras and Karst, 2003). All single clones discussed in this work had also been confirmed as derivatives of the parental wine yeast strain by interdelta PCR analysis.

2.4. Assessment of CCR responses

Precultures in 5 mL YPD of evolved populations were prepared from frozen stock, in 50-mL Falcon tubes. After 40 h at 25 °C, serial dilutions (from 10^0 to 10^{-6}) of the precultures were prepared, and 1.5 μl were used to inoculate drop tests in different solid media. Media contained per litre, 20 g agar, 20 g peptone, 10 g yeast extract, and one of these carbon sources: 20 g glucose, 20 g galactose, 20 g raffinose, 20 g maltose, 20 g glycerol, 20 mL ethanol, 0.2 mL acetic acid. When indicated, 200 mg/L 2DG or 200 mg/L glucosamine (GA) were added.

2.5. Growth in grape juice

Shake flasks (100 mL nominal volume) were filled with 20 mL pasteurized rosé grape juice, inoculated to 0.2 final OD_{600}, covered with aluminium foil allowing for gas exchange, and incubated at 25 °C, 180 rpm for 4 days. Experiments were run in triplicate for each evolved population analysed. Cultures were centrifuged, and supernatants were kept frozen for HPLC analysis. Single clones selected from these fermentation experiments (see below) were grown in the same way, also

in triplicate.

Clones directly isolated from evolved cultures were grown in white grape juice in the same aerobic conditions for 4 days. After this time, a 1 mL sample was used for HPLC analysis, and then, the culture was transferred to 50-mL Falcon tubes, capped with an airlock, and incubated for 7 additional days at 25 °C, without agitation.

Selected EC1118 clones were grown in white grape juice at 25 °C for 11 days, in shake flasks at 180 rpm for aerobic conditions, and in Falcon tubes, capped with an airlock (Actylab, Logroño, Spain) for anaerobic conditions. Samples from shake flasks were taken on day 4 and 11, and from Falcon tubes on day 11.

2.6. Selection of evolved clones

Isolated strains were obtained from evolved populations at two different times, either directly from the final population, or after four days of aerobic fermentation of that evolved population in grape juice. The latter option involved an additional step of selection of strains able to withstand wine fermentation conditions. In the first case, evolved cultures (kept at −80 °C) were grown for two successive passages in liquid YPD for 48 h at 25 °C. Then, cultures were streaked on solid selective medium (containing galactose and 2DG). Five colonies from each evolved culture were picked, re-streaked on the same medium, and reisolated. For clones selected after a fermentation step, the biomass of four days fermentation experiments was plated onto YPD agar, in the appropriate dilutions, and three single colonies were picked from each culture. Supplementary Figure S1 shows the genealogy of each single clone analysed in this work, including information on the experimental evolution population and the post-evolution steps.

2.7. Stress resistance of isolated clones

Strains were grown in YPD at 25 °C for 48 h, and 1.5 μl were used to inoculate jellified synthetic juice with different stressors (10 mM H_2O_2, or 6% ethanol, or 10% ethanol, or 120 mg/L $K_2S_2O_5$). Plates were incubated at 25 °C. Growth at 12 °C and 37 °C in jellified synthetic juice was also checked. Each clone was inoculated in triplicate (Supplementary Figure S2).

2.8. Aerobic growth in bioreactors

For bioreactor assays, batch cultures were performed using Applikon MiniBio bioreactors (250 mL nominal volume). Bioreactors were filled with 150 mL of pasteurized white grape juice, 200 μL of antifoam 204 (Sigma-Aldrich, Spain) and inoculated to 0.2 final OD_{600} with the strains grown in liquid YPD for 48 h at 25 °C. Experiments were run in triplicate at 25 °C and 1000 rpm. The cultures were sparged with compressed air at 25 mL/min (10 vvh). 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). During the experiment, 1 mL samples were collected daily, centrifuged, and supernatants kept frozen for chromatographic analyses. Dissolved oxygen was measured by polarographic sensors (Applikon).

2.9. 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 refractive 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).

2.10. Statistical analysis

All fermentation experiments described in this work (either in shake flasks, Falcon tubes, or bioreactors) were performed at least in biological triplicates. Statistical analyses were performed using SPSS Statistics v. 28.0 program (IBM, Armonk, NY, United States). The levels and yield on substrate of the main fermentation metabolites were compared by one-way analysis of variance. Comparison of means was carried by Tukey test or bilateral Dunnet Test.

3. Results and discussion

3.1. Experimental evolution and properties of the evolved populations

Three wine yeast strains, two of them commercial, were grown with galactose as the sole carbon source, in the presence of the non-metabolizable glucose analogue 2DG. During the first rounds of subculture, the concentration of 2DG was set to 50 mg/L, and transfers (10% of the culture volume) were performed every 72 h. As the cultures reached stationary phase faster (maximum OD reached after 48 h), increasing amounts of 2DG were used, up to 200 mg/L, the concentration used for most of the evolution experiment. After reaching 140 generations under selective pressure, samples from each evolution replicate were used to inoculate grape juice, for characterisation of CCR features, or for the isolation of pure evolved strains.

Evolution on 2DG was presumed to result in the selection of carbon catabolite derepressed strains, with the expectation that at least some of them would also produce less acetic acid under aerobic conditions. To confirm that the evolved populations were indeed affected in CCR, they were tested for growth on different carbon sources in the presence of 2DG or glucosamine (GA; another non-metabolizable analogue of glucose) (Fig. 1). Like 2DG, GA has been previously used to test the CCR properties of yeast cultures and for the characterization of the prion-like element [GAR^+] (Brown and Lindquist, 2009).

As anticipated, growth on glucose was not affected by 2DG or GA, neither for the original nor for the evolved populations. Growth on galactose in the presence of 2DG was clearly improved in the evolved cultures compared to their parental strain. This was also to be expected, as this was the main selective pressure in the evolution experiments. Growth on galactose was also affected by GA in the original strains, although to a lesser extent than 2DG. The evolved populations were also improved for growth on galactose-GA medium, indicating that evolution had probably affected CCR signalling, and not simply 2DG tolerance by some alternative mechanism (Schmidt and O'Donnell, 2021). Growth on raffinose, with or without glucose analogues, paralleled the results on galactose. Similarly, although T73 was the only original strain clearly affected by 2DG or GA for growth on maltose, the evolved populations showed better growth than the starting T73 strain on maltose-GA or maltose-2DG media. Derepression for the use of non-fermentable carbon sources was more heterogeneous (Fig. 1). Growth on acetic acid was not affected by GA in the original strains, and was only slightly impaired by 2DG, with no obvious improvement for the evolved populations. Growth on glycerol or ethanol in the presence of 2DG or GA was impaired for all three starting wine yeast strains. The improvement in growth of the evolved populations was not uniform between replicates; it was absent for all IF473-derived cultures in glycerol-2DG; and it was generally less pronounced than for fermentable carbon sources. Taken together, this analysis indicates that galactose-2DG evolution generally affected CCR. The genes ultimately affected by the genetic modification appear to be slightly different depending on the experimental replicate and the genetic background, and this probably reflects subtle strain differences in the wiring of the CCR signalling.

Samples of the evolved cultures were also used to inoculate rosé grape juice and incubated under aerobic conditions. To avoid the

		Glucose			Galactose			Maltose			Raffinose			Glycerol			Ethanol			Acetic acid		
		Ctrl	2DG	GA	Ctrl	2DG	GA	Ctrl	2DG	GA	Ctrl	2DG	GA	Ctrl	2DG	GA	Ctrl	2DG	GA	Ctrl	2DG	GA
EC1118	Wt	-5	-3	-4	-5	0	-2	-5	-2	-4	-6	0	-1	-5	0	0	-6	0	0	-5	-2	-5
	EC1	-5	-4	-5	-5	-3	-5	-4	-4	-4	-5	-1	-4	-4	0	-3	-4	-1	-3	-5	-3	-4
	EC2	-5	-5	-4	-6	-5	-5	-5	-5	-5	-4	-5	-6	-5	-3	-6	-5	-3	-6	-4	-3	-4
	EC3	-5	-5	-4	-3	-4	-5	-5	-5	-4	-5	-4	-4	-3	-4	-5	-4	-3	-5	-5	-4	-5
IFI473	Wt	-6	-5	-5	-6	0	-2	-6	-3	-5	-6	0	0	-4	0	0	-5	0	0	-5	-3	-6
	IC1	-5	-6	-4	-6	-4	-6	-6	-5	-6	-6	-4	-5	-6	0	-2	-6	0	0	-5	-2	-6
	IC2	-6	-6	-5	-6	-6	-6	-6	-5	-5	-6	-4	-4	-5	0	-2	-5	-2	-4	-4	-2	-6
	IC3	-5	-4	-4	-6	-5	-5	-5	-5	-5	-6	-3	-4	-5	0	-2	-5	-2	-4	-6	-2	-6
T73	Wt	-5	-4	-3	-5	0	-2	-4	0	-3	-5	0	-3	-3	0	-2	-5	0	-2	-4	-2	-4
	TC1	-4	-4	-4	-5	-4	-5	-5	-5	-5	-5	-5	-4	-3	-3	-2	-5	-3	-2	-5	-4	-4
	TC2	-4	-4	-4	-5	-5	-5	-4	-5	-4	-4	-4	-3	-5	-3	-3	-5	-3	-2	-3	-3	-3
	TC3	-6	-6	-5	-6	-6	-5	-5	-5	-5	-6	-6	-6	-6	-4	-3	-6	-4	-2	-4	-3	-3

Fig. 1. Drop-tests of evolved populations and their parental strains on complete medium containing different carbon sources alone (Ctrl), or in the presence of 2-deoxyglucose (2DG) or glucosamine (GA). Numbers indicate lowest decimal dilution showing growth (up to 10^{-6}). Colour intensity is related to the dilution. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

potentially confounding impact of acetic acid consumption under aerobic conditions (after depletion of sugars), sugar consumption and main fermentation yields were measured after 4 days (Fig. 2A, Table 1). At this sampling point, the fermentations inoculated with the original strains had consumed more than 98% of the initial sugars. All fermentations inoculated with the evolved populations contained more residual sugars than the initial strains. Populations derived from T73 were only slightly affected, while EC2, derived from EC1118, was strongly affected, with only 26.9% of the initial sugars consumed after four days. Ethanol yields were similar in the different fermentation experiments, with no statistically significant differences between the parent strains and the evolved populations (Table 1). This result is in agreement with the fact, reported by several authors, that ethanol yield is a robust physiological trait of *S. cerevisiae*, it is lower under aerobic conditions, but there are few differences between strains (Camarasa et al., 2011; Palacios et al., 2007; Tronchoni et al., 2022). In turn, glycerol yield was impacted by evolution in a strain-specific way; it was not significantly affected for the EC1118 evolved populations, significantly (but slightly) increased for T73, and clearly improved for the IFI473 ones. In the case of EC1118, the high dispersion observed in EC2 replicates could be related to the intrinsic instability of the evolved populations (which are a heterogeneous mixture of strains). This could be masking a real impact of evolution on glycerol yield for these evolved populations, as suggested by the results with some isolated clones (see below). These

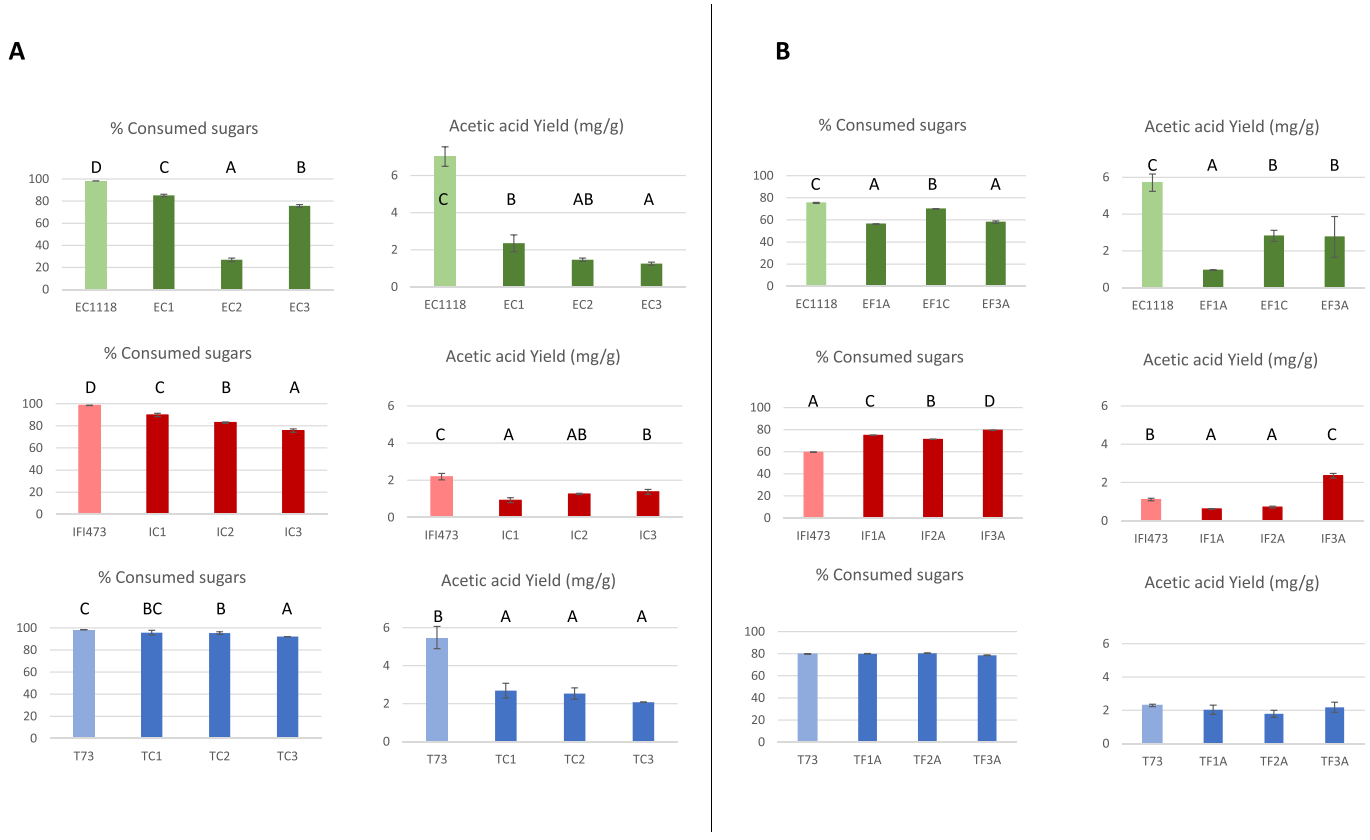

Fig. 2. Sugar consumption and yields in acetic acid after 4 days of growth on sterilised rosé grape juice (200 g/L initial sugar content), under aerobic conditions. A: evolved populations; B: selected clones from fermentation of evolved cultures. Capital letters indicate statistical differences ($p < 0.05$) between populations or strains.

Table 1
Main fermentation parameters of evolved cultures after 4 days of growth on sterilised rosé grape must (200 g/L initial sugar content), under aerobic conditions. Capital letters indicate statistical differences ($p \leq 0.05$) for that group of strains.

	Consumed sugars (%)	Ethanol Yield (g/g)	Glycerol Yield (mg/g)	Acetic Acid Yield (mg/g)
	Mean ± ds	Mean ± ds	Mean ± ds	Mean ± ds
EC1118WT	98.30 ± 0.03 D	0.355 ± 0.007	27.67 ± 0.90	7.01 ± 0.52 C
EC1	85.17 ± 1.03 C	0.377 ± 0.016	28.72 ± 0.63	2.35 ± 0.45 B
EC2	26.90 ± 1.57 A	0.346 ± 0.057	45.40 ± 15.63	1.46 ± 0.09 AB
EC3	75.74 ± 1.16 B	0.362 ± 0.034	41.51 ± 1.99	1.25 ± 0.08 A
IFI473WT	98.54 ± 0.14 D	0.325 ± 0.002	31.65 ± 0.73 A	2.19 ± 0.17 C
IC1	89.91 ± 1.55 C	0.381 ± 0.046	43.94 ± 3.43 B	0.93 ± 0.14 A
IC2	82.99 ± 0.65 B	0.365 ± 0.051	47.07 ± 3.03 B	1.26 ± 0.04 AB
IC3	75.65 ± 1.50 A	0.382 ± 0.009	54.16 ± 2.70 C	1.38 ± 0.13 B
T73WT	98.42 ± 0.02 C	0.353 ± 0.024	33.60 ± 0.39 A	5.48 ± 0.58 B
TC1	95.74 ± 2.05 BC	0.382 ± 0.045	37.98 ± 0.70 B	2.68 ± 0.39 A
TC2	95.31 ± 1.12 B	0.395 ± 0.025	37.97 ± 0.32 B	2.53 ±0.30 A
TC3	92.17 ± 0.04 A	0.412 ± 0.002	39.26 ± 0.28 C	2.07 ±0.01 A

differences might be casual or respond to differences in CCR signalling between the parental strains.

Finally, acetic acid yield was significantly lower in all evolved cultures compared to their parent strain. In a different context, Mizuno et al. (2006) found that spontaneous 2DG-resistant mutants produced less acetic acid than the parental strain during high-gravity beer fermentation. However, these authors targeted anaerobic fermentation conditions and did not use a specific carbon source together with 2DG. Whether due to the effect on CCR, or for another reason, it seems that 2DG allows selection of populations with low acetic acid production.

3.2. Characterization of clones isolated from fermentation experiments

The results with the evolved populations were promising, and supported the initial hypothesis of this work, i.e., that it would be possible to derive strains with a lower aerobic yield of acetic acid by selecting for a weaker CCR response. However, these populations are expected to contain an unstable mixture of competing sibling strains (Mangado et al., 2018), and would be unmanageable in an industrial setting. In addition, adaptation to a relatively constant environment, such as that commonly used in experimental evolution, could lead to the selection of strains that showed poor performance when returned to industrially relevant conditions. This may be due to genetic drift or trade-offs of the selected genetic modifications (Elena and Lenski, 2003).

Since tolerance to fermentation stress factors is a key feature of wine yeast strains, without which no industrial application could be envisaged (Matallana and Aranda, 2017), nine different clones were isolated from each population after fermentation of rosé juice and tested for relevant winemaking stress factors in jellified synthetic juice (Supplementary Figure S2): high ethanol concentration (6%–10% v/v), low and high temperature (12 °C and 37 °C), oxidative stress (10 mM H_2O_2), and sulphite treatment (120 mg/L $K_2S_2O_5$). The three isolates of the EC1118 EC2 population showed slower growth than the parent strain, both in the jellified synthetic juice and under the different stress conditions. Also, one isolate of EC1118 EC3 showed poor growth at 37 °C. Otherwise, most strains seemed to retain the stress tolerance of their respective original strains. Three clones derived from each initial strain (one from each evolution experiment) were taken for further characterisation. Because all clones coming from the EC1118 EC2 population had been discarded due to poor growth, two strains from EC1118 EC1 were selected, EF1A and EF1C.

3.3. Grape juice fermentation with selected clones

The selected clones were grown in rosé grape juice for 4 days under aerobic conditions. The results obtained with these individual clones differ from those obtained previously with the whole evolved populations under the same fermentation conditions, especially regarding acetic acid production (Fig. 2B). None of the T73 derivatives showed a statistically significant reduction in acetic acid yield, while one of the IFI473 derivatives, IF3A, was even more productive than the original yeast strain. The results with EC1118 derivatives were the most consistent ones, with a clear decrease in acetic acid yield for all three clones. EC1118 derivatives were also affected in fermentation time, showing slower sugar consumption than the original strain, while IFI473 derivatives seemed to ferment faster and T73 derivatives did not seem to be affected in this parameter.

As described above, the strains shown in Fig. 2B had been selected after passaging the respective evolved population through a cycle of fermentation of grape juice. This step aimed to reduce the risk of selecting strains showing impaired growth in grape juice. However, when comparing the results of the IFI473 and T73 derivatives with those of the population they came from (Fig. 2), it appeared that this step had somewhat counterselected the main trait of interest (reduced acetic acid production under aerobic conditions). Therefore, new clones were selected from the evolved populations (five clones of each) after two passages in non-selective medium followed by two passages in selective medium (galactose-2DG) (Sup Figure S1). These isolates were used in the aerobic fermentation of white grape juice for four days (Fig. 3). After this time, only one out of 15 derivatives of T73, TM12, showed lower acetic acid levels than the original strain. However, although the acetic acid yield shown by strain TM12 was significantly lower than that of T73, it was still too high (above 5 mg/g) to be suitable for winemaking under aerobic conditions. No improvement was observed either among the new isolates from the experimental evolution of IFI473. In fact, all IFI473 IC3 isolates produced higher amounts than the initial IFI473 strain. However, most of the EC1118 derivatives showed a significant reduction in volatile acidity production, and some of them showed less than half the yield of EC1118 in acetic acid (below 2 mg/g). They also showed a negative impact of experimental evolution on aerobic fermentation duration that was not observed for the other genetic backgrounds. Thus, despite skipping the fermentation step before strain isolation, a similar trend was observed, with no clear improvement for T73 and IFI473 derivatives and some interesting clones among the EC1118 derivatives, albeit these showed slightly impaired aerobic fermentation (less sugar consumed in four days).

After four days under aerobic conditions, these fermentation experiments were transferred to anaerobic conditions for an additional seven days, for a total of eleven days (Fig. 3). By then, sugar consumption was complete for the three parental strains and most of the evolved derivatives, but fermentation was apparently arrested for all clones from two out of the three EC1118 evolution experiments (with less than 80% of the initial sugar consumed).

In *S. cerevisiae* a dramatic increase in acetic acid production following a sudden change from aerobic to anaerobic conditions has previously been described (Tronchoni et al., 2022). This was also observed for the parental strains used in this work (Fig. 3). As for the evolved clones, sudden change to anaerobic conditions also triggered a shift in acetic acid yield, but the final yield was still lower than that of the control strain for all EC1118 derivatives (below 6 mg/g). As with

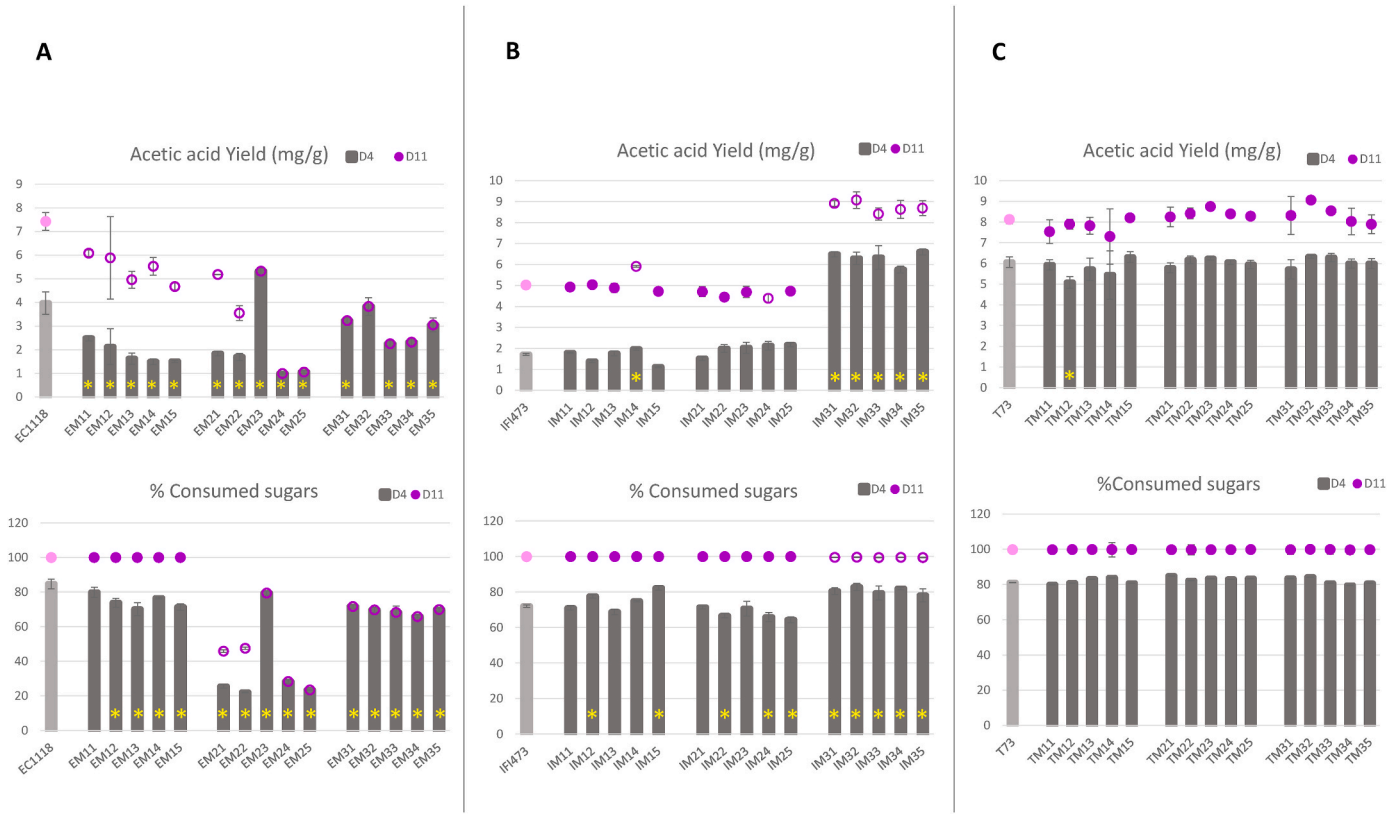

Fig. 3. Sugar consumption and yields in acetic acid of clones isolated from evolved populations on sterilised white grape juice (220 g/L initial sugar content). Grey bars: 4 days of growth under aerobic conditions; asterisks indicate statistical differences ($p < 0.05$) with the parental strain. Magenta circles: 11 days of growth (+7 days under anaerobic conditions). Open circles indicate statistical differences ($p < 0.05$) with the parental strain.

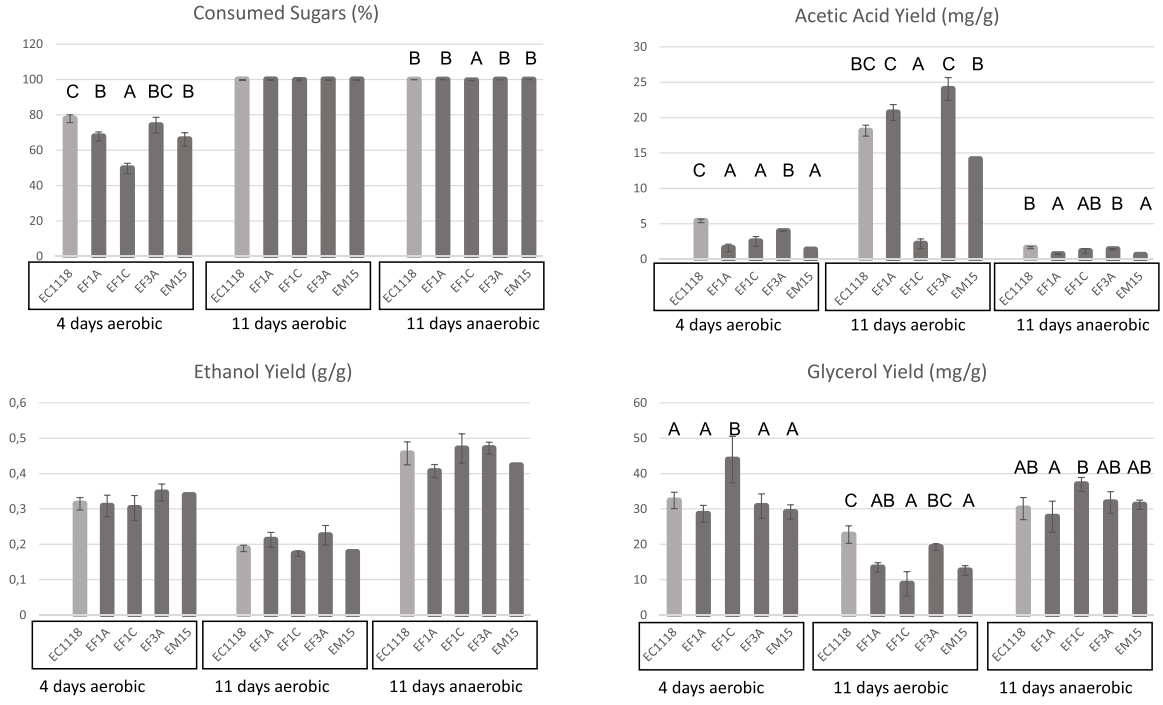

Fig. 4. Main fermentation parameters of EC1118 strain and four selected clones on day 4 and 11 of growth in aerobiosis, and on day 11 in anaerobiosis, on sterilised natural white grape juice (220 g/L initial sugar content). For each condition, capital letters indicate statistical differences ($p < 0.05$) between strains.

aerobic fermentation, no improvement in acetic acid yield was observed for strains derived from T73 or IFI473 by the end of the process (sometimes quite the opposite). As the process involved passage though non-selective medium, the replacement of evolved strains by less evolved ones suggests growth impairment to be associated to the desired phenotype.

Considering all the results presented in this section, four EC1118 derivatives were selected for further characterisation, attending to sugar consumption and aerobic acetic acid yield. Three of them were isolated from fermentation (EF1A, EF1B and EF3A), and one was isolated in the second round of selection (EM15).

3.4. Characterization of growth of selected derivatives of EC1118, under aerobic and anaerobic conditions

These four strains were grown in grape juice under constant aerobic or anaerobic conditions until complete fermentation (11 days). Samples from the aerobic cultures were also analysed by day four to compare to previous results. Indeed, the results of the fourth day of aerobic fermentation were consistent with those described above for each of the evolved strains, showing a moderate delay in sugar consumption compared to EC1118 and a statistically significant reduction in acetic acid yield for all of them (Fig. 4; compare to Figs. 2 and 3). No strain-dependent differences were observed for ethanol yield, but this was clearly lower under aerobic conditions, as expected (Quirós et al., 2014; Tronchoni et al., 2022). Reduction of ethanol yield under aerobic conditions has been previously demonstrated for several *S. cerevisiae* strains, and this result confirms the ability of all tested EC1118 derivatives to reduce ethanol yield through respiration during aerobic fermentation. By day eleven, all yeast strains had exhausted sugars under either aerobic or anaerobic conditions. Since yeasts can use ethanol as a carbon source under aerobic conditions (Hagman et al., 2013), aerobic ethanol yield data by day 11 might be an underestimate and should not be used for comparison purposes. As for the acetic acid yield, it was low in anaerobic fermentations, as expected. Although it was lower for the evolved strains by day four under aerobic conditions, by day eleven it had increased in most cases to values between 15 and 25 mg/g, similar to the control strain (Fig. 4). Only strain EF1C maintained a low acetic acid yield (around 2.5 mg/g) after 11 days under aerobic conditions. Interestingly, this strain showed the highest glycerol yield after four days of aerobic fermentation and the lowest one by the end of fermentation (Fig. 4).

3.5. Growth in bioreactors under aerobic conditions

Based on the results described so far, two strains, EM15 and EF1C, from the same evolved population (respectively selected before or after an intermediate fermentation step), were retained for a final characterisation of aerobic fermentation performance. Both were grown in white grape juice in bioreactors under aerobic conditions. After three days of fermentation, acetic acid values averaged 900 mg/L for EM15 and 485 mg/L for EF1C, reaching respectively 2000 mg/L and 1675 mg/L of acetic acid by day sixth (Fig. 5). These values are incompatible with the use of any of the strains for alcohol level reduction in an industrial setup. In addition, EM15 fermentation stalled between the fifth and sixth day of aerobic fermentation (Fig. 5). The behaviour of both strains in bioreactors was inconsistent with previous results (Fig. 5). Since this result does not fit with that obtained in shake flasks, both bioreactor and shake flasks experiments were repeated several times with EF1C, obtaining similar results. Acetic acid production was low in shake flaks fermentations for EF1C, compared to the parental strain, but it was not reduced in bioreactors (data not shown). The difference between the bioreactor and shake flask results confirms that control of aeration conditions is a critical element in driving *S. cerevisiae* metabolism. Under these circumstances it would be difficult to scale-up an industrial process using the strains developed in this work. In a previous work Tronchoni et al. (2022) also found inconsistencies between shake flasks and bioreactor results for a natural isolate of *S. cerevisiae*. In that work, it was possible to tune process conditions in bioreactors to get low acetic and low ethanol yields. Unfortunately, the solution of a stepwise reduction in

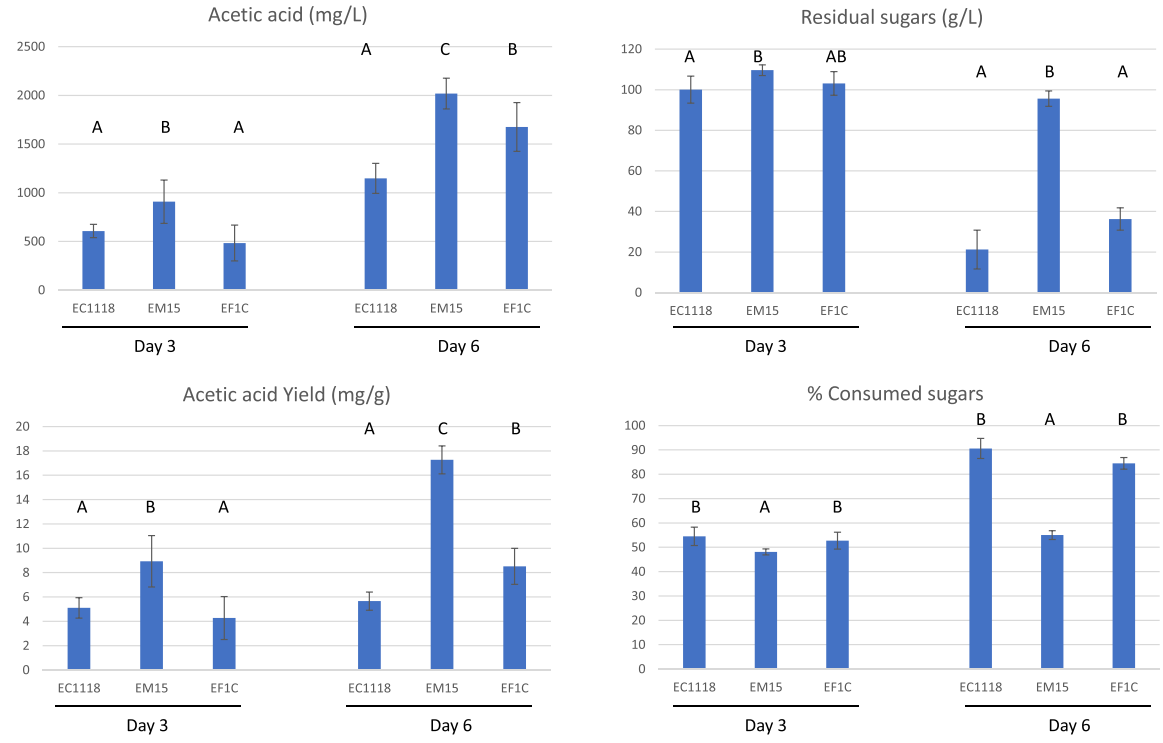

Fig. 5. Measure of fermentation parameters of interest for EC1118, EM15, and EF1C after three or six days of aerobic fermentation in bioreactors with sterilised grape juice (220 g/L initial sugar content). Capital letters indicate statistical differences ($p < 0.05$) between strains.

oxygen availability used in that work would not be practical in this case, since EF1C had already produced high amounts of acetic acid after only three days of fermentation (Fig. 5).

In addition, the initial genotype impacts the ability to obtain strains of interest. After isolation of individual clones, only one of the original genotypes, EC1118, consistently gave rise to strains showing reduced aerobic acetic acid yield (in shake flasks). The difficulties encountered in identifying individual evolved clones that mimic the behaviour of the evolved population were striking. As mentioned above, it was assumed that the fermentation step used in the first instance resulted in a counter-selection of the evolved traits. However, the second round of screening (avoiding that fermentation step) showed similar limitations. It could be concluded that strains displaying the target phenotypes are easily replaced by less evolved ones, once selective pressure was relieved. Some alternatives to solve this bottleneck are increasing the number of genomic backgrounds or the number of generations under selective pressure.

The purpose of the present work was to develop a methodology that would allow, by directed evolution, obtaining non-recombinant strains that could be used under aerobic fermentation conditions without the drawback of excess volatile acidity. Some EC1118 evolved populations as well as the EF1C clone confirm the success of the methodology, but the improvement was not good enough to be useful under more realistic operating conditions. The complex response of *S. cerevisiae* to oxygen availability in grape juice, together with the trade-offs associated to the selected mutations, seem to be at the basis of the difficulties encountered in this work.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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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.2023.104282.

References

Brown, J.C., Lindquist, S.A., 2009. Heritable switch in carbon source utilization driven by an unusual yeast prion. Genes Dev. 23, 2320–2332. https://doi.org/10.1101/gad.1839109.

Bely, M., Sablayrolles, J.M., Barre, P., 1990. Description of alcoholic fermentation kinetics: its variability and significance. Am. J. Enol. Vitic. 41, 319–324.

Camarasa, C., Sanchez, I., Brial, P., Bigey, F., Dequin, S., 2011. Phenotypic landscape of *Saccharomyces cerevisiae* during wine fermentation: evidence for origin-dependent metabolic traits. PLoS One 6, e25147. https://doi.org/10.1371/journal.pone.0025147.

Ciani, M., Morales, P., Comitini, F., Tronchoni, J., Canónico, L., Curiel, J.A., Oro, L., Rodrigues, A.J., Gonzalez, R., 2016. Non-conventional yeast species for lowering ethanol content of wines. Front. Microbiol. 7, 642. https://doi.org/10.3389/fmicb.2016.00642.

Cui, D.Y., Brown, C.R., Chiang, H.L., 2004. The type 1 phosphatase Reg1p-Glc7p is required for the glucose-induced degradation of fructose-1, 6-bisphosphatase in the vacuole. J. Biol. Chem. 279, 9713–9724. https://doi.org/10.1074/jbc.M310793200.

Curiel, J.A., Salvadó, Z., Tronchoni, J., Morales, P., Rodrigues, A.J., Quirós, M., Gonzalez, R., 2016. Identification of target genes to control acetate yield during aerobic fermentation with *Saccharomyces cerevisiae*. Microb. Cell Factories 15, 156. https://doi.org/10.1186/s12934-016-0555-y.

De Deken, R.H., 1966. The Crabtree effect: a regulatory system in yeast. J. Gen. Microbiol. 44, 149–156. https://doi.org/10.1099/00221287-44-2-149.

Delrot, S., Grimplet, J., Carbonell-Bejerano, P., Schwandner, A., Bert, P.F., Bavaresco, L., Dalla Costa, L., Di Gaspero, G., Duchêne, E., Hausmann, L., Malnoy, M., Morgante, M., Ollat, N., Pecile, M., Vezzulli, S., 2020. Genetic and genomic approaches for adaptation of grapevine to climate change. In: Kole, C. (Ed.), Genomic Designing of Climate-Smart Fruit Crops. Springer, Cham, pp. 157–270. https://doi.org/10.1007/978-3-319-97946-5_7.

Elena, S., Lenski, R., 2003. Evolution experiments with microorganisms: the dynamics and genetic bases of adaptation. Nat. Rev. Genet. 4, 457–469. https://doi.org/10.1038/nrg1088.

Gonzalez, R., Guindal, A.M., Tronchoni, J., Morales, P., 2021. Biotechnological approaches to lowering the ethanol yield during wine fermentation. Biomolecules 11, 1569. https://doi.org/10.3390/biom11111569.

Gonzalez, R., Quirós, M., Morales, P., 2013. Yeast respiration of sugars by non-*Saccharomyces* yeast species: a promising and barely explored approach to lowering alcohol content of wines. Trends Food Sci. Technol. 29, 55–61. https://doi.org/10.1016/j.tifs.2012.06.015.

Hagman, A., Säll, T., Compagno, C., Piskur, J., 2013. Yeast "make-accumulate-consume" life strategy evolved as a multi-step process that predates the whole genome duplication. PLoS One 8, e68734. https://doi.org/10.1371/journal.pone.0068734.

Jiang, H., Tatchell, K., Liu, S., Michels, C.A., 2000. Protein phosphatase type-1 regulatory subunits Reg1p and Reg2p act as signal transducers in the glucose-induced inactivation of maltose permease in *Saccharomyces cerevisiae*. Mol. Gen. Genet. 263, 411–422. https://doi.org/10.1007/s004380051185.

Jolly, N.P., Varela, C., Pretorius, I.S., 2014. Not your ordinary yeast: non-*Saccharomyces* yeasts in wine production uncovered. FEMS Yeast Res. 14 (2), 215–237. https://doi.org/10.1111/1567-1364.12111.

Legras, J.L., Karst, F., 2003. Optimisation of interdelta analysis for *Saccharomyces cerevisiae* strain characterization. FEMS Microbiol. Lett. 221, 249–255. https://doi.org/10.1016/S0378-1097(03)00205-2.

Mangado, A., Morales, P., Gonzalez, R., Tronchoni, J., 2018. Evolution of a yeast with industrial background under winemaking conditions leads to diploidization and chromosomal copy number variation. Front. Microbiol. 9, 1816. https://doi.org/10.3389/fmicb.2018.01816.

Matallana, E., Aranda, A., 2017. Biotechnological impact of stress response on wine yeast. Lett. Appl. Microbiol. 64, 103–110. https://doi.org/10.1111/lam.12677.

Martnez-Rodriguez, A., Carrascosa, A.V., Barcenilla, J.M., Pozo-Bayon, M.A., Polo, M.C., 2001. Autolytic capacity and foam analysis as additional criteria for the selection of yeast strains for sparkling wine production. Food Microbiol. 18, 183–191. https://doi.org/10.1006/fmic.2000.0390.

Matsumoto, K., Yoshimatsu, T., Oshima, Y., 1983. Recessive mutations conferring resistance to carbon catabolite repression of galactokinase synthesis in *Saccharomyces cerevisiae*. J. Bacteriol. 153, 1405–1414.

Mira de Orduña, R., 2010. Climate change associated effects on grape and wine quality and production. Food Res. Int. 43, 1844–1855. https://doi.org/10.1016/j.foodres.2010.05.001.

Mizuno, A., Tabei, H., Iwahuti, M., 2006. Characterization of low-acetic-acid-producing yeast isolated from 2-deoxyglucose-resistant mutants and its application to high-gravity brewing. J. Biosci. Bioeng. 101, 31–37. https://doi.org/10.1263/jbb.101.31.

Morales, P., Rojas, V., Quirós, M., Gonzalez, R., 2015. The impact of oxygen on the final alcohol content of wine fermented by a mixed starter culture. Appl. Microbiol. Biotechnol. 99, 3993–4003. https://doi.org/10.1007/s00253-014-6321-3.

Neigeborn, L., Carlson, M., 1987. Mutations causing constitutive invertase synthesis in yeast: genetic interactions with snf mutations. Genetics 115, 247–253. https://doi.org/10.1093/genetics/115.2.247.

NOAA National Centers for Environmental Information, 2020. State of the Climate: monthly global climate report for annual. Published online January 2021, retrieved on May 26, 2022 from: https://www.ncei.noaa.gov/access/monitoring/monthly-report/global/202013.

OIV (International organisation of vine and wine), 2022. International code of oenological practices. OIV. Paris. France. https://www.oiv.int/public/medias/8630/code-2022-en.pdf.

Palacios, A., Raginel, F., Ortiz-Julien, A., 2007. Can the selection *Saccharomyces cerevisiae* yeast lead to variations in the final alcohol degree of wines? Aust. N.Z. Grapegrow. Winemak. 527, 71–75.

Piccardo, D., Favre Guzman, F., Pascual, O., Canals, J.M., Zamora, F., González-Neves, G., 2019. Influence of the use of unripe grapes to reduce ethanol content and pH on the color, polyphenol and polysaccharide composition of conventional and hot macerated Pinot Noir and Tannat wines. Eur. Food Res. Technol. 245, 1321–1335. https://doi.org/10.1007/s00217-019-03258-4.

Quirós, M., Rojas, V., Gonzalez, R., Morales, P., 2014. Selection of non-*Saccharomyces* yeast strains for reducing alcohol levels in wine by sugar respiration. Int. J. Food Microbiol. 181, 85–91. https://doi.org/10.1016/j.ijfoodmicro.2014.04.024.

Sam, F.E., Ma, T.-Z., Salifu, R., Wang, J., Jiang, Y.-M., Zhang, B., Han, S.-Y., 2021. Techniques for dealcoholization of wines: their impact on wine phenolic composition, volatile composition, and sensory characteristics. Foods 10, 2498. https://doi.org/10.3390/foods10102498.

Santos, J.A., Fraga, H., Malheiro, A.C., Moutinho-Pereira, J., Dinis, L.-T., Correia, C., Moriondo, M., Leolini, L., Dibari, C., Costafreda-Aumedes, S., Kartschall, T., Menz, C., Molitor, D., Junk, J., Beyer, M., Schultz, H.R., 2020. A review of the potential climate change impacts and adaptation options for European viticulture. Appl. Sci. 10, 3092. https://doi.org/10.3390/app10093092.

Schmidt, M.C., O'Donnell, A.F., 2021. 'Sugarcoating' 2-deoxyglucose: mechanisms that suppress its toxic effects. Curr. Genet. 67, 107–114. https://doi.org/10.1007/s00294-020-01122-7.

Tronchoni, J., Curiel, J.A., Sáenz-Navajas, M.P., Morales, P., de-la-Fuente-Blanco, A., Fernández-Zurbano, P., Ferreira, V., Gonzalez, R., 2018. Aroma profiling of an aerated fermentation of natural grape must with selected yeast strains at pilot scale. Food Microbiol. 70, 214–223. https://doi.org/10.1016/j.fm.2017.10.008.

Tronchoni, J., Gonzalez, R., Guindal, A.M., Calleja, E., Morales, P., 2022. Exploring the suitability of *Saccharomyces cerevisiae* strains for winemaking under aerobic conditions. Food Microbiol. 101, 103893 https://doi.org/10.1016/j.fm.2021.103893.