LWT - Food Science and Technology 58 (2014) 166–172

Contents lists available at ScienceDirect

LWT - Food Science and Technology

journal homepage: www.elsevier.com/locate/lwt

Improved fermentation kinetics by wine yeast strains evolved under ethanol stress

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ARTICLE INFO

Article history:
Received 30 July 2013
Received in revised form
27 February 2014
Accepted 2 March 2014

Keywords:
Adaptive laboratory evolution
Wine yeast
Fermentation kinetics
Ethanol tolerance

ABSTRACT

Adaptive laboratory evolution works on the principle that populations of cells adapt to their environment over time by natural selection. In this work, we assess adaptive laboratory evolution as a tool to improve the performance of winemaking yeast strains, in order to cope with the challenges of global climate change. Specifically we addressed ethanol tolerance as a way to ensure good fermentation kinetics despite increasing sugar content in musts.

Two industrial wine yeast strains were subjected to adaptive laboratory evolution in continuous culture in the presence of 6–8% ethanol. Evolved strains showed improved fermentation kinetics and reduced residual sugar in synthetic must. Some of these strains also showed improved fermentation kinetics in pilot scale fermentation of natural white and rosé grape must. Levels of acetic acid, glycerol, and some volatile compounds were also different between the original and evolved strains.

This work proves that adaptive laboratory evolution is a promising method for the improvement of industrial wine yeast strains. However, both the genotype of the original strains and the particular conditions in which selective pressure is applied can have great influence on the usefulness of the final outcome.

1. Introduction

The current trends in oenological practices, involving an increased attention to the aromatic and polyphenolic ripeness of grape berries at harvest, combined with the effect of climate change on grape maturation (Mira de Orduña, 2010), result in the extremely high sugar concentrations reached at harvest nowadays, especially in warm climates. These high sugar concentrations often result in premature fermentation arrest, probably because high alcohol levels are reached far before complete sugar consumption. Ethanol is widely recognized as one of the causes of stuck or sluggish alcoholic fermentation (Bisson, 1999). The occurrence of such problems dramatically increases in hot years (Coulter, Henschke, Simos, & Pretorius, 2008), and represents a significant trouble for the wine industry. Currently available industrial yeast strains have been selected according to a number of criteria, including technological performance and quality of the final wine (Gonzalez, Muñoz, & Carrascosa, 2011), but they seem to be working close to their limits when it comes to fermenting grape musts with exceptionally high sugar contents.

Strain improvement can be approached by Adaptive Laboratory Evolution (ALE). It works on the principle that populations of cells adapt to their environment over time by natural selection (Stanley, Fraser, Chambers, Rogers, & Stanley, 2010). ALE usually requires hundreds of generations in order to give rise to clearly improved strains. Selective pressure for such long periods of time can be maintained by either repeated batch cultivation or in continuous culture. Some applications include improved yeast tolerance to freeze–thaw (Takagi, Iwamoto, & Nakamori, 1997), temperature (Wati, Dhamija, Singh, Nigam, & Marchant, 1996), salt concentration (Matsutani, Fukuda, Mutrata, Kimura, & Yajima, 1992), or acetic acid concentration (Aarnio, Suihko, & Kauppinen, 1991). Concerning ethanol tolerance, Brown and Oliver (1982), selected yeast strains with increased fermentation rates by continuous culture of a *Saccharomyces uvarum* haploid strain. Jimenez and Benitez (1988)

Abbreviations: ABV, alcohol by volume; ALE, adaptive laboratory evolution; YAN, yeast assimilable nitrogen.

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http://dx.doi.org/10.1016/j.lwt.2014.03.004
0023-6438/

used a pH regulated continuous culture (dilution rate controlled by pH) with ethanol in the feed to select, within a few days, the most tolerant strains among a mixture of *Saccharomyces cerevisiae* hybrid strains. Dinh, Nagahisa, Hirasawa, Furusawa, and Shimizu (2008) also recovered ethanol adapted yeast strains from a haploid laboratory strain after a stepwise increase in ethanol concentration from 0 to 10% during repetitive cultivation. Stanley, Fraser, et al. (2010) obtained mutants with increased growth rates in sub-lethal ethanol concentrations by continuous culture of a haploid strain in YEPD supplemented with increasing amounts of ethanol.

In these examples, ALE was employed on laboratory strains, either as a proof of concept (Brown & Oliver, 1982), or as a tool to study mechanisms involved in ethanol tolerance by yeasts. However, genetic improvement of industrial yeast strains might prove, in principle, more difficult given their higher ploidy and their better adaptation to industrial fermentation conditions than laboratory strains.

McBryde, Gardner, de Barros Lopes, and Jiranek (2006) used sequential batch fermentation for adaptive evolution of a wine yeast strain. While the original strain gave rise to sluggish fermentations in a 200 g/L sugars synthetic must, the final mixed culture obtained from adaptive evolution was able to complete total sugar consumption. Cadière, Ortiz-Julien, Camarasa, and Dequin (2011) using sequential batch cultivation on gluconate as carbon source of a wine commercial strain obtained evolved strains with increased flux towards the pentose phosphate pathway.

In this work we have used adaptive laboratory evolution in continuous culture, with ethanol as the challenging agent, in order to obtain derivatives of two industrial wine yeast strains. Some of these evolved strains show improved fermentation kinetics under harsh environmental conditions, among other phenotypic modifications.

2. Materials and methods

2.1. Strains and culture conditions

The two original *S. cerevisiae* yeast strains were isolated from two different regions in Catalonia, Spain. L76 from a Parellada grape sample from “Pontons”, and L78 from a Cabernet Sauvignon grape sample from “Conca de Barberà”. They had been selected for industrial use on the basis of fermentation power, fermentation kinetics, and technological properties. Yeast strains were kept at −80 °C in 20% glycerol. Pre-cultures used for the inoculation of fermentation experiments were grown on YPD (2% glucose, 1% yeast extract, 2% peptone) for 48 h at 28 °C and 150 rpm.

2.2. Determination of the ethanol concentration to be used for adaptive evolution

Growth of yeast strains at 28 °C in YPD5 (5% glucose, 1% yeast extract, 2% peptone) containing 4%, 6% and 8% ethanol was monitored at 600 nm using a SPECTROstar Nano instrument (BMG Labtech, Offenburg, Germany). The wells of the microplate were filled in with 0.01 mL of inoculum and 0.19 mL of medium. Measurements were taken every 30 min. The agitation regime was set to 1 min shaking periods alternated by 1 min rest periods. The experiments were run for 24 h with four replicates per strain and alcohol concentration. Growth parameters were calculated by directly fitting OD measurements versus time to the reparametrized Gompertz equation as proposed by Zwietering, Jongenburger, Rombouts, and van’t Riet (1990).

2.3. Continuous culture in the presence of ethanol

Continuous culture conditions were established in homemade bioreactors. Briefly, each reactor consisted of a 50 mL Erlenmeyer flask closed with screw cap with hole and silicone stopper. In and out media were pushed with peristaltic pumps through the silicone stopper by means of needles. A connexion between the headspace of flask and external air was provided by another needle connected to a 0.20 μm filter in order to allow for pressure normalization. Working volume was fixed to 40 mL by adjusting the position of the out needle. Homogenization was achieved by magnetic stirring. The whole system was mounted on a Medilow temperature controlled chamber (JP Selecta, Abrera, Barcelona, Spain) maintained at 28 °C. After 24 h of batch culture on YPD, feeding was turned on. YPD5 plus 6% ethanol was used initially as feed medium, and plus 8% for L76 after 225 generations. The OD_{600} of the out medium was controlled daily and the speed of the feed pump adjusted in order to keep it constant. Sugar consumption, ethanol content, and pH were also daily measured to detect any deviation. The final cell population from each evolution experiment was platted on YPD plates containing 8% ethanol, and isolated colonies recovered for further characterization.

2.4. Fermentation experiments

Fermentation experiments on 15 mL synthetic must with high sugar content (140 g/L glucose, 140 g/L fructose, 1.7 g/L Yeast Nitrogen Base without ammonium sulphate and amino acids, 6 g/L citric acid, 6 g/L malic acid, 1.15 g/L ammonium chloride, 30 mg/L potassium disulphite, pH adjusted at 3.5 with KOH) were carried out at 25 °C in 50 mL Falcon tubes capped with fermentation locks filled with mineral oil. Each tube was inoculated with 1% volume of a fresh culture in YPD (grown for 48 h at 28 °C and 180 rpm). Fermentation kinetics was monitored daily by quantifying weight loss due to CO_2 release. Experiments were performed in duplicate. HPLC analysis of the main fermentation metabolites (glucose, fructose, glycerol, ethanol and acetic acid) was carried out as previously described (Quirós, González-Ramos, Tabera, & Gonzalez, 2010), with two determinations for each fermentation replicate.

Two types of natural must were used for pilot scale experiments, a rosé one, from red Grenache grapes, and a white one, from Chardonnay grapes. Grapes from the 2011 harvest session were collected, destemmed and pressed, and grape juice was treated with Velcorin® (150 ppm) and sulphited (30 mg/L SO_2, final concentration). Sugar content was estimated by FTIR and corrected with sucrose when necessary, in order to get the desired estimated final alcohol by volume (predicted ABV). The Boehringer Mannheim Ammonia Kit (R-Biopharm AG, Darmstadt) and the method of Dukes and Butzke (1998) were used for determination of ammonia and amino acids respectively. Yeast Assimilable Nitrogen (YAN) content was calculated as the sum of ammonia and amino acids contents, and corrected with diammonium phosphate and organic commercial products when necessary. Starters were prepared in commercial must, grown for 2 days at 22 °C, and inoculated to an initial concentration of 10^6 cells/mL. Fermentation was carried out at 16–17 °C in 15 L vats. Fermentation kinetics was monitored daily by density determination and samples were taken up to quantify sugar consumption and fermentation products. Dominance of the inoculated strains was verified by restriction analysis of mitochondrial DNA (Querol, Barrio, Huerta, & Ramon, 1992) of 20 random isolates from the appropriate dilutions grown on YPD plates.

2.5. Analysis of volatile compounds

The content in volatile compounds of wines derived from pilot scale fermentation experiments was analysed by Gas Chromatography coupled to Mass Spectrometry. Wine samples, 1200 μl, were placed in a 20 mL flask with 600 mg NaCl and 300 μl of internal standards solution containing 25 mg/L of each of following

compounds: 2-butanol, 4-methyl 2-pentanol, ethyl pentanoate, 2-octanol, 2-ethylhexanol, ethyl nonanoate, 1-nonanol, and heptanoic acid. Flasks were tightly capped with a PTFE/silicon cap. Prior to injection, each flask was shaken for 10 min at 40 °C, and then a Supelco 100 μm PDMS fibre was exposed to the headspace for 30 min in the same conditions, and desorbed in the GC injection port for 5 min. The SPME liner was held at 180 °C.

The analysis 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 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 retention times and ion spectra from real standards (Sigma–Aldrich Química) and spectra from the NIST mass spectral library. For each compound, including internal standards, the sum of the areas of the peaks of up to five characteristic ions was obtained. The relative abundance of a particular compound was calculated as 1000 times the addition of the areas of the peaks of its characteristic ions divided by the addition of the areas of the peaks of the characteristic ions of its selected internal standard.

2.6. Statistical analysis

One way analysis of variance was carried out by means of IBM SPSS Statistics v. 20 program on the main fermentation metabolites with yeast strain as main effect, white must ABV 14%, white must ABV 15%, red must YAN 210 and red must YAN 295 wine samples being separately treated.

Analysis of variance was carried on the relative abundances of 14 individual volatile compounds and main fermentation metabolites with yeast strain and must composition (YAN for rosé wine and ABV for white wine) as main effects.

Means were compared using Tukey's test, with significance level at 5%.

3. Results and discussion

According to the main causes described for premature fermentation arrest (Bisson, 1999), in this work we chose ethanol tolerance as the main improvement target in order to obtain evolved strains able to bring about complete fermentation of grape must under harsh environmental conditions. As an additional factor, since premature arrest of fermentation always means the presence of still substantial amounts of sugar, the evolution medium contained 5% sugar instead of the 2% usually found in standard laboratory media.

3.1. Determination of ethanol concentration to be used for adaptive laboratory evolution

The aim of these experiments was to identify an ethanol concentration having a clear influence in the growth kinetics of the original yeast strains (i.e. constituting a selective pressure), while still maintaining a dilution rate that would allow to get hundreds of generations in just a few weeks. The strains were cultivated in YPD5 medium supplemented with different amounts of ethanol, and growth kinetics was monitored as described in Materials and Methods. Both strains were able to grow in the presence of each of the ethanol concentrations assayed (4%, 6% and 8%) following a similar growth pattern. Growth inhibition increased with increasing ethanol concentrations (Fig. 1). Control growth condition (YPD5 without ethanol) showed a μ_{max} of 0.54 h^{-1} for L76 and L78 and that value was reduced to 0.41 h^{-1} for L76 and 0.42 h^{-1} for L78 already for 4% ethanol. The intermediate ethanol concentration (6%) was chosen as initial concentration for the adaptive evolution experiment, as a good compromise between growth rate and inhibitory effect, according to the above mentioned criteria.

3.2. Adaptive laboratory evolution experiments

Strains L76 and L78 were separately cultivated in continuous culture with 6% ethanol in the feed medium. The initial dilution rate (D) was set to 0.23 h^{-1} for both strains. Dilution rates were manually increased to keep a similar steady state biomass content. Culture of the L78 strain was arrested after 225 generations, having reached a dilution rate of 0.35 h^{-1}. At this time, the ethanol content in the feed medium for L76 was increased to 8%, and continuous culture kept to reach a total of 340 generations and a dilution rate of 0.46 h^{-1}. Appropriate dilutions of samples from the final steady states were plated in YPD containing 8% ethanol and colonies were randomly picked for phenotypic characterization. After selection, strains were grown under non-selective conditions (YPD), single colonies isolated and glycerol stocks prepared. Strains were regularly recovered from the original glycerol stocks for preparing inocula for the experiments (under non-selective pressure). Comparing with other reports of adaptive laboratory evolution the most similar previous report would be that by Stanley, Fraser, et al. (2010), except they maintained a low dilution rate and modulated the selective pressure by modifying ethanol content in the feed, while we used a faster dilution rate and modulated the selective pressure by increasing the dilution rate. The feasibility of the relatively high dilution rates in the presence of ethanol in our experiment may be related with the genotype of the industrial strains we used, and the fact they are naturally adapted to ethanol. The drawback, as mentioned in the Introduction, is that further

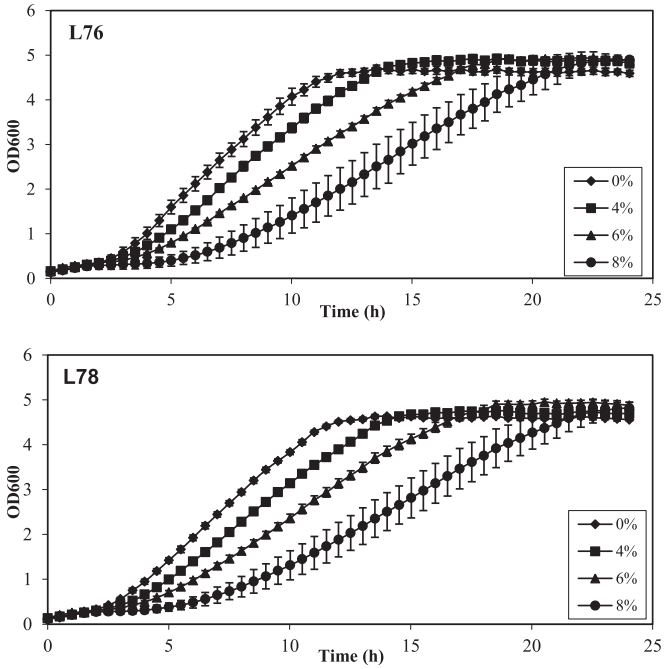

Fig. 1. Growth kinetics of yeast strains L76 and L78 at different ethanol concentrations (% v/v).

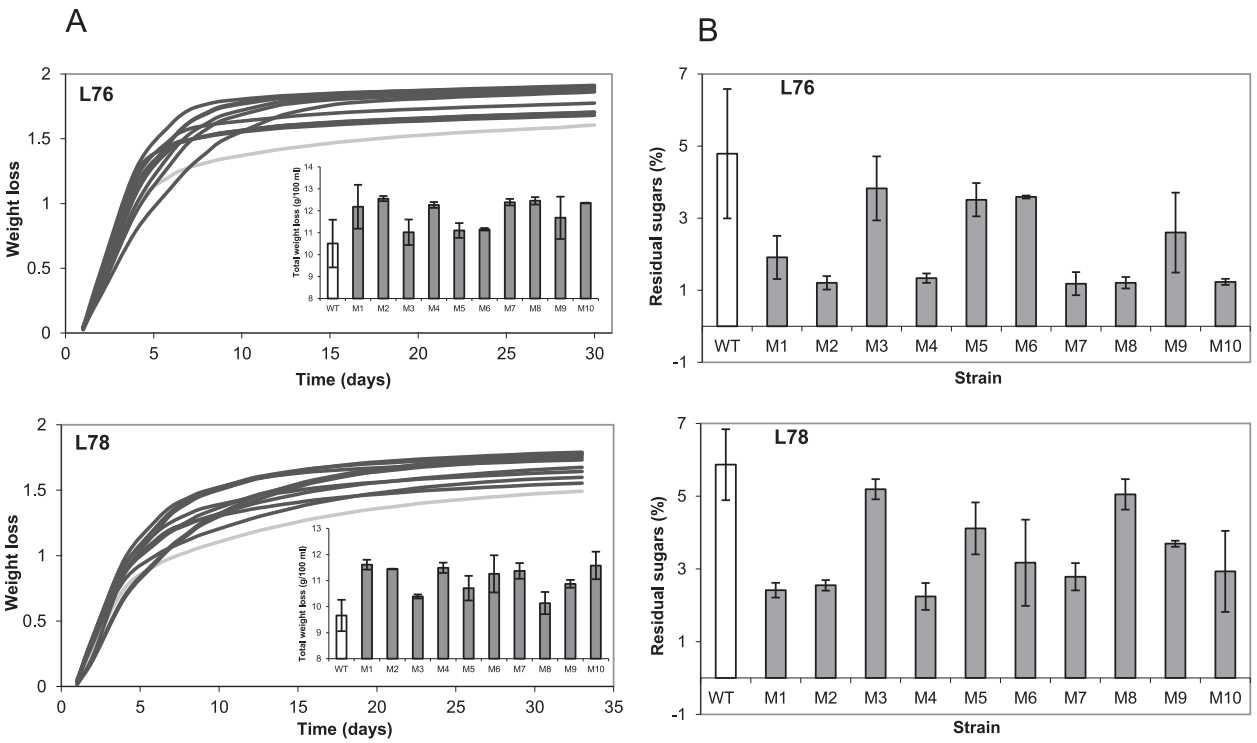

Fig. 2. (A) Fermentation kinetics of evolved strains (black lines) compared with the original strain (grey line) in a synthetic must containing 28% sugars. Embedded graph: Total weight loss of evolved strains (grey bars) compared to the original strain (white bars). (B) Residual sugars left in wine by evolved strains (deep grey bars) compared to the original strain (light grey bars).

adaptation of these strains to ethanol stress might prove more difficult. A first indication of the adaptation to ethanol of the evolving population was indeed the increasing dilution rate the culture was able to withstand.

3.3. Fermentation of synthetic must

Ten independent isolates from each evolution experiment were used for comparing fermentation performance with the original strain. For these experiments, synthetic must sugar concentration was set to 28%, in order to be able to show technological advantages of the evolved strains under conditions that are expected to result in a stuck fermentation. Indeed, all the strains left considerable amounts of residual sugars, but the trend for all the evolved strains was for faster fermentation kinetics, higher weight loss (CO_2 production), and lower residual sugar content, than the original industrial strains (Fig. 2). The differences among the strains indicate that the final population of the evolution experiments was not homogeneous, but it was rather a mixed culture of a number of strains, a result in agreement with the observation by Brown and Oliver (1982). Four strains among those leaving the lowest amounts of residual sugars, L76M2, L76M4, L76M8 and L78M4, were chosen for further characterization in pilot scale assays in Bodegas Torres S.A.

3.4. Fermentation of natural grape must

A comparison of capacities of mutant strains against their original strain was made in natural grape must. Variations in two important factors affecting fermentation performance were assayed, sugar concentration and nitrogen availability.

Strain L76 and its derivatives were grown in Chardonnay must adjusted to 337 mg/L YAN, and two different sugar concentrations to get a predicted ABV of 14% and 15%, respectively. Fermentation kinetics in musts with 14% predicted ABV were very similar among strains (data not shown), and all them completed fermentation (see Table 1). However, fermentation kinetics in musts with 15% predicted ABV of the evolved strains L76M2 and L76M4 was better than the original strain (Fig. 3A): the levels of residual sugars 14 days after inoculation for L76M2 (14.27 g/L) were significantly lower than for L76 (38.05 g/L), and this latter was not significantly different from

Table 1
Metabolites found in wines elaborated with evolved strains on natural musts of Chardonnay variety (white) and Priorat (Grenache) variety (red). Nitrogen and sugar contents of musts were corrected to the values indicated for the YAN and final predicted ABV indicated.

Must	Strain	Glucose (% w/v)	Fructose (% w/v)	Glycerol (% w/v)	Ethanol (% w/v)	Acetic acid (mg/L)
Chardonnay	L76WT	0.00	0.15 A	0.61 B	13.85	494.47 A
ABV 14%	L76M2	0.00	0.18 A	0.77 D	14.22	887.84.B
337 YAN	L76M4	0.00	0.08 A	0.70 C	13.88	927.82.B
	L76M8	0.00	0.33 B	0.57 A	13.76	462.71 A
Chardonnay	L76WT	0.01 A	0.51 A	0.65 A	14.40	584.58 A
ABV 15%	L76M2	0.00 A	0.23 A	0.84 D	14.81	963.51 B
337 YAN	L76M4	0.00 A	0.40 A	0.81 C	14.36	1068.59 C
	L76M8	0.08 B	1.57 B	0.67 B	14.50	612.67 A
Grenache	L76WT	0.00	0.16 A	0.72 A	15.34 B	177.80 A
ABV 15%	L76M2	0.00	0.22 B	0.72 A	14.68 A	214.52 AB
210 YAN	L76M4	0.00	0.26 B	0.79 B	14.93 AB	243.09 B
	L76M8	0.00	0.13 A	0.68 A	15.04 AB	189.80 A
	L78WT	0.00	0.11	0.74 B	14.40	160.08
	L78M4	0.00	0.20	0.59 A	14.72	163.94
Grenache	L76WT	0.00	0.09 A	0.81 B	15.21	289.48 A
ABV 15%	L76M2	0.00	0.21 B	0.81 B	15.00	483.25 B
295 YAN	L76M4	0.00	0.18 B	0.85 B	14.78	723.82 C
	L76M8	0.00	0.17 B	0.72 A	14.87	353.98 A
	L78WT	0.00	0.07 A	0.80	14.82 A	244.90 A
	L78M4	0.00	0.14 B	0.58	15.42 B	378.53 B

Differences among related strains for the same compound ($P < 0.05$) are indicated in capital letters.

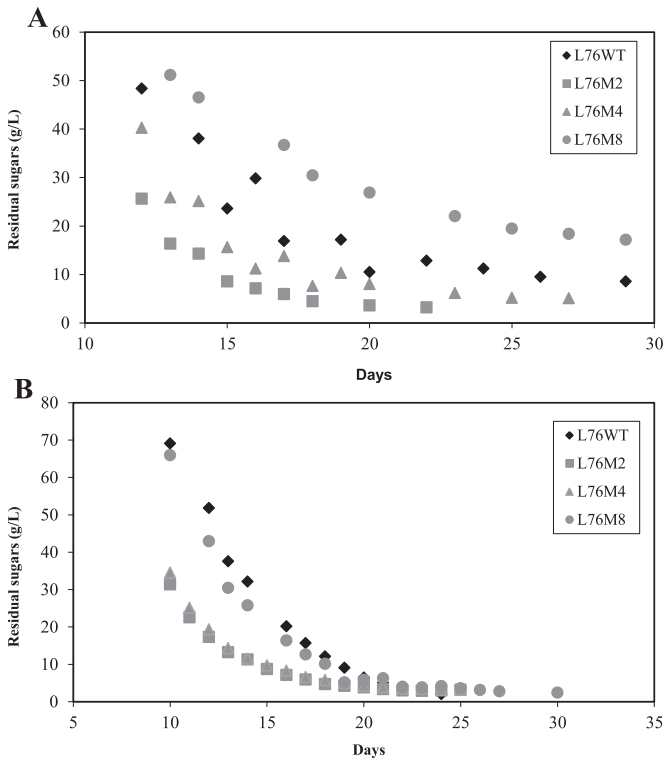

Fig. 3. Fermentation of natural musts by parental and evolved strains of L76. (A) Chardonnay must, 15% expected ABV and 337 ppm available N. (B) Grenache must, 15% expected ABV and 210 ppm available N.

L76M8 (46.50 g/L) or L76M4 (25.13 g/L). According to these data and to results shown in Fig. 1, the effect of adaptive evolution on fermentation kinetics seems to be operating already when the inhibitory effect of ethanol is still mild. Alternatively, adaptation to ethanol stress might have induced cross tolerance to other fermentation related stress factors. Indeed, a small overlapping between ethanol and osmotic stress responses has been described for *S. cerevisiae* (Stanley, Bandara, Fraser, Chambers, & Stanley, 2010). In spite of faster fermentation kinetics, final residual sugar was not significantly reduced for L76M2 or L76M4 fermentations as compared to that by L76 (Table 1), and it was in all cases better than L76M8. Final alcohol degrees were similar for all the strains, but an increased yield in glycerol was observed for the two fastest fermenting strains L76M2 and L76M4 (Table 1). Unfortunately, this increase in glycerol content was accompanied by a parallel increase in the amount of acetic acid produced. These results seem to confirm a relationship between stress tolerance and glycerol overproduction, even though this relationship has been mostly studied in relation with osmotic stress. Stanley, Fraser, et al. (2010) also found ethanol tolerant strains to overproduce glycerol and acetic acid; the later only in the presence of ethanol, but this would be the case after some hours of fermentation during winemaking. McBryde et al. (2006) evolved a wine strain not able to finish fermentation on a 20% sugars synthetic must and producing high levels of acetic acid (1.7 g/L), and the evolved mixed culture also overproduced glycerol (112%) and slightly more acetic acid (106%).

A sensory analysis was carried out on wines made with lower sugar addition by a four-taster panel, at bottling and 6 months after bottling. Wine made with L76M2 was the preferred one. Wines made with the higher sugar content were not tasted due to differences found in final sugar concentration that would bias the result.

In a different experiment, all the selected strains were used for the fermentation of a red Grenache must (15% expected ABV) with two different amounts of YAN, 210 and 295 mg/L. Fermentation kinetics was faster in high nitrogen content musts than in low nitrogen musts. In fact, 13 days after inoculation mean of residual sugars in musts of YAN 210 mg/L was 35.44 g/L, significantly higher than in musts of YAN 295 g/L (8.06 g/L). A multivariate analysis of variance carried on red Grenache wines with nitrogen content and strain as main effects, shows that levels of acetic acid in wines made with low nitrogen addition (mean = 191.53 mg/L) were significantly lower than in wines made with higher nitrogen addition (mean = 412.32 mg/L). This result shows that an excess of nitrogen in must can lead to a not desired increase in volatile acidity, and points to the importance of grape must nitrogen content on final wine quality, as already described (Bell & Henschke, 2005).

L76 M2 and M4 outperformed the original or M8 strains at both nitrogen content levels. Fig. 3B shows the fermentation kinetics of L76 and its derivatives in must with the lowest nitrogen content. A multivariate analysis of variance of data of residual sugars on day 13 after inoculation with nitrogen content and strain as variables shows that residual sugars for the mutant strains L76 M2 and M4 are significantly lower than for L76 and L76M8. But also as in Chardonnay wines, differences in fermentation kinetics are masked in final wine residual sugars due to the fact that all strains had finished fermentation (Table 1). Levels of residual sugars in these wines are under or close to the limits for a dry wine (2 g/L). Levels of glycerol found in wines made with mutants L76M2 and L76M4 are not higher than L76, as were in Chardonnay wines, and levels of

Table 2
Volatile compounds found in Chardonnay wines.

Compound	Sugar[a]	L76WT[b]	L76M2	L76M4	L76M8
Ethyl acetate	S	4821.74 C	4064.49 AB	4000.31 A	4683.80 BC
Isoamyl acetate	NS	78,083.67 B	32,393.55 A	33,288.54 A	62,084.56 B
Ethyl hexanoate	S	115.02 AB	101.35 A	111.82 A	129.34 B
Hexyl acetate	S	51.32 B	34.13 A	35.40 A	55.91 B
Ethyl octanoate	S	1788.40 B	1488.93 A	1687.82 AB	2093.00 C
Ethyl decanoate	NS	168.06 AB	132.14 A	129.71 A	186.44 B
Ethyl 9-decenoate	NS	215.82	112.20	129.30	170.69
Phenyl ethyl acetate	S	30.71 AB	26.28 A	26.41 A	31.75 B
2-Methyl propanol	NS	215.27 AB	242.14 B	226.48 AB	212.83 A
2 + 3-Methyl butanol	S	6308.95 AB	6455.61 AB	6608.72 B	5949.02 A
Hexanol	NS	15.13 A	21.63 B	21.29 B	16.15 A
Phenyl ethyl alcohol	NS	27.86 A	41.28 B	40.53 B	34.36 AB
Octanoic acid	S	1617.39 BC	1382.08 AB	1238.84 A	1706.06 C
Ditertbutylphenol	S	46.12	44.48	51.85	52.25

[a] Sugar: This column indicates if statistical differences ($P < 0.05$) have been found for each compound between wines made with two different sugar concentrations. S: significant; NS: not significant.
[b] Differences among related strains for the same compound ($P < 0.05$) are indicated in capital letters.

Table 3
Volatile compounds found in Grenache wines.

	L76				L78	
	L76WT	L76M2	L76M4	L76M8	L78WT	L78M4
Ethyl acetate	3389.91	3272.69	3431.51	3141.01	3289.92 B	2861.25 A
Isoamyl acetate	38,006.15	33,008.38	27,040.48	37,212.34	21,399.21 A	33,521.45 B
Ethyl hexanoate	53.15	53.92	47.20	78.44	32.15 A	88.70 B
Hexyl acetate	10.95	17.59	15.31	22.35	3.83 A	21.37 B
Ethyl octanoate	776.05	1063.47	860.76	1449.01	258.51 A	1805.59 B
Ethyl decanoate	172.07 A	167.97 A	126.15 A	398.47 B	59.91 A	380.70 B
Ethyl 9-decenoate	209.67	128.30	186.99	293.65	14.46 A	460.70 B
Phenyl ethyl acetate	36.10	30.90	29.57	43.83	10.40 A	45.06 B
2-Methyl propanol	769.62 B	512.45 A	562.19 AB	614.35 AB	836.12 B	636.52 A
2 + 3-Mehyl butanol	17,466.05 B	11,357.18 A	13,087.46 A	15,139.05 AB	21,421.60 B	16,549.60 A
Hexanol	30.97	27.24	28.97	26.70	31.21 B	25.50 A
Phenyl ethyl alcohol	249.44 B	176.83 A	183.67 A	288.27 B	289.44	284.98
Octanoic acid	883.98	727.15	704.79	1138.10	689.24 A	1591.25 B
Ditertbutylphenol	62.37 A	66.51 AB	81.90 B	79.35 AB	74.97	72.72

Differences among each original strain and its evolved strains for the same compound ($P < 0.05$) are indicated in capital letters.

acetic acid are higher with mutant strains than with the original one, but still far away from legal limits (1 g/L).

A sensory analysis was carried out 3 months after bottling on wines made with lower nitrogen addition by a four-taster panel. Wines made with L76M2 and L76M8 were the preferred ones. The amount of glycerol and acetic acid of these wines was comparable to the levels in wine made with the original strain, while wine made with L76M2 had lower levels of ethanol than wine made with the original strain.

Concerning L78M4, it did not outperform the original L78 strain in either fermentation kinetics (data not shown) or wine composition (Table 1). The time to reach a sugar concentration of 20 g/L for L78 and L787M4 was 16 and 17 days respectively for must with 210 mg/L YAN, and 8 and 9 days respectively for 295 mg/L YAN. Indeed, fermentation with this evolved strain resulted in lower glycerol and higher acetic acid production than wines fermented with the original one.

3.5. Volatile compound profiles

We identified and quantified 27 different volatile compounds in the final wines, by GC–MS, as described in Materials and Methods. The relative abundance of all compounds was determined, but only 14 of them were considered in the analysis, because of the tiny amounts of the others in all wines studied. Although these wines contain acetic acid, it is not detected under these analysis conditions.

A multivariate analysis of variance on Chardonnay wines was carried out with initial sugar contents and yeast strain as the main effects. Results are shown in Table 2. Levels of five out of eight esters, one out of four alcohols, octanoic acid, and ditertbutyl phenol showed significant differences between wines coming from musts with different sugar contents. Only ethyl acetate was higher at lower sugar concentration. When comparing strains, 12 out of 14 compounds were statistically significant among them. The lowest levels for ester compounds and the highest for alcohols were found for the evolved strains L76M2 and/or L76M4.

Finally, a multivariate analysis of variance was carried on red Grenache wines with nitrogen contents and strain as main effects, on wines made with L76 and derivatives and separately on L78 and its derivative. Results are shown in Table 3. Only levels of three compounds, ethyl 9-decenoate in wines made with L76 and its derivatives and 2 + 3-methyl butanol and ethyl decanoate in wines made with L78 and its derivative, were different depending on nitrogen contents. The fusel alcohol increased with nitrogen and the esters decreased. Tukey test shows five compounds with different levels among L76 and its derivatives.

When comparing L78 and L78M4, all compounds but two are different between strains: levels of esters (except ethyl acetate) and octanoic acid are higher for L78M4, while levels of alcohols are higher in L78WT strain. These strong differences in volatile compounds could have an impact on wine flavour. According to the sensory impact expected for these compounds (Bartowsky & Pretorius, 2009), wine made with L78M4 would be more fruity and less spirituous than wine made with L78WT. L78M4 finishes sugars present in must for a final ABV of 15% and although levels of acetic acid are higher than with L78WT, they are still far away from limits of acceptance. This strain is an example of changes other than expected from adaptive evolution.

4. Conclusions

Among the strains developed in this work, L76M2 consistently gives improved results (concerning fermentation kinetics or completeness, glycerol production, or tasting) over the original strain for the fermentation of grape must with high sugar content and low nitrogen availability. Increased volatile acidity seems to be compensated by glycerol production or other metabolites, since it was always preferred in wine tasting.

In conclusion, adaptive laboratory evolution seems to be a feasible way to improve the technological properties of industrial wine yeast strains, even for features that would be expected to be extremely selected for during the history of fermented foods. In order to obtain the better results, further research would be required concerning specific growth conditions and media composition that would guide the evolution towards the most interesting outcomes.

Acknowledgements

This work was funded by the CDTI (Spanish Center for Technological Industrial Development) through the Ingenio 2010-CENIT Program (Project Deméter) and Project AGL 2012-32064 of the Spanish Ministry of Science and Innovation. M. Novo is recipient of a CSIC training JAE-Doc contract, co-funded by the European Social Fund of the EU. Authors wish to thank M.A. Fernández Recio for the analysis of volatile compounds, Z. Salvadó for the calculation of growth parameters, and L. López Bergés and C. Juez for their skillful technical assistance.

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