Ethanol tolerance of sugar transport, and the rectification of stuck wine fermentations

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The incomplete consumption of sugar resulting from stuck wine fermentation is associated with important economic losses. One of the solutions to this serious problem consists of reinoculating the brew with a yeast starter culture that is both alcohol tolerant and a vigorous fructose fermenter. The present work aimed to select yeast strains capable of restarting stuck wine fermentations, and identify key parameters that contribute to the efficiency of the strains. Commercial and non-commercial Saccharomyces wine strains were tested, as well as strains of the fermentative non-Saccharomyces species Zygosaccharomyces bailii and Torulaspora delbrueckii. Although the latter species were shown to be more resistant to a combination of ethanol- and acetic-acid-induced cell death, commercial Saccharomyces cerevisiae strains were the most efficient fructose consumers in medium simulating a stuck fermentation. Stationary-phase S. cerevisiae cells performed better than inocula prepared from exponentially growing cultures, which correlates with the higher resistance to ethanol of non-growing populations. Stationary-phase cells pre-adapted to ethanol did not improve fructose consumption rates; this was in contrast to exponential-phase cells that benefited from prior incubation in ethanol-containing medium. Notably, a correlation was observed between yeast fructose consumption capacity and glucose (or fructose) transport. Our results challenge the current belief that ethanol tolerance, expressed in terms of cell viability, is a reliable criterion for the selection of yeast strains to restart stuck fermentations. Instead, this capacity seems to be based on sugar transport and its resistance to ethanol. In an attempt to further improve cell viability in the presence of high ethanol concentrations, hybrid strains of T. delbrueckii and S. cerevisiae were produced, and they showed high potential as restarter strains. The present work opens perspectives for the application of innovative strategies in the wine-making industry.

INTRODUCTION

Incomplete wine fermentations, known as sluggish and stuck fermentations, are relatively common, and they have an important economic impact. Various factors, including nutrient limitation, excessive (high or low) temperatures, oenological practices, occurrence of spoilage micro-organisms, and presence of toxic compounds, such as fungicides or ethanol, are potential causes of incomplete fermentations (Alexandre & Charpentier, 1998; Bisson, 1999; Bisson & Butzke, 2000; Varela et al., 2004). A controlled vinification process helps to avoid this problem, but reinoculation with a selected yeast strain is often required to accomplish complete sugar consumption. In this situation, the yeast sensitivity to ethanol, which is usually present in high concentrations in stuck musts, is recognized as one of the main obstacles to reactivating the fermentation (Cavazza et al., 2004). In fact, high ethanol concentrations act on yeast cells by damaging cell membranes (Leão and van Uden, 1984; Alexandre et al., 1994; Swan & Watson, 1997; You et al., 2003) and inactivating transport systems (Salmon, 1989; Cardoso & Leão, 1992a), and this culminates in cell death and an inability to metabolize. In addition, under these fermentation conditions, acetic acid, another by-product of alcoholic fermentation, may also be present in high concentrations, and act synergistically with ethanol (Cardoso & Leão, 1992b). During grape must-fermentation, the most physiologically relevant hexose transporters (Hxt1p–Hxt4p, and Hxt6/7p), which accept both glucose and fructose as substrates, show
distinct expression patterns in accordance with their regulatory and kinetic properties (Reifenberger et al., 1997; Perez et al., 2005). In general, the lower affinity of hexose transporters for fructose compared with glucose explains the prevalence of fructose towards the end of fermentation. The role of sugar transport in yeast strains used to relaunch the fermentation process remains to be elucidated.

Recently, because of the increasing demand for better quality wines, the winemaking industry has turned its attention to the non-\textit{Saccharomyces} yeast species that are usually present in the grape-must microflora (Fleet, 2003). Conceivably, the non-\textit{Saccharomyces} yeasts might also be useful for the reinoculation of stuck fermentations. Among these yeasts, \textit{Zygosaccharomyces bailii} emerges as a possible good candidate, as it is known for its frutophilic character, and for high resistance to both ethanol and acetic acid (Sousa et al., 1996; Pina et al., 2004b). On the other hand, \textit{Torulaspora delbrueckii}, a yeast species closely related to \textit{Saccharomyces cerevisiae}, is already used to improve aroma development in wine production (Ciani & Maccarelli, 1998; Plata et al., 2003). Since genetically modified wine yeasts cannot be marketed in most wine-producing countries, a combination of desired traits to enhance fermentation performance, and avoid stuck fermentations, may be achieved by traditional strain breeding of wine-associated yeasts.

The general aim of the present work was to assess different yeast species for their ability to restart stuck fermentations, and to evaluate environmental factors conditioning an efficient performance. To that end, we compared commercial and non-commercial \textit{Saccharomyces} wine-associated strains, and two fermentative non-\textit{Saccharomyces} species, under conditions prevailing in stuck wine fermentations. Special attention was given to relationships between fructose consumption rates and the resistance of sugar transport to ethanol. Furthermore, a hybrid strain combining the high ethanol and acetic acid tolerance of \textit{T. delbrueckii}, and the high fructose consumption capacity of \textit{S. cerevisiae}, was obtained, and compared with its parental strains.

METHODS

Micro-organisms and growth conditions. Throughout this work the following strains were used. Commercial strains \textit{S. cerevisiae} PYCC 5792, PYCC 5793, PYCC 5794, PYCC 5795 and PYCC 4072, and \textit{T. delbrueckii} PYCC 5321, were all supplied by the Portuguese Yeast Culture Collection, Faculdade de Ciências e Tecnologia, Universidade Nova de Lisboa, Caparica, Portugal. \textit{Z. bailii} ISA 1307 was obtained from the Instituto Superior de Agronomia, Lisboa, Portugal. \textit{S. cerevisiae} Bio-PF and \textit{T. delbrueckii} Bio-J32 are both maintained at the Department of Biology, Universidade do Minho, Portugal. A series of \textit{S. cerevisiae} hxt mutants derived from strains \textit{S. cerevisiae} MC996A (MATa ura3-52 his3-11,15 leu2-3,112 MAL2 SUC2 GAL MEL) and \textit{S. cerevisiae} MC996B (MATa ura3-52 his3-11,15 leu2-3,112 MAL2 SUC2 GAL MEL) by deleting all but one of the \textit{HXT1–HXT7} genes (Reifenberger et al., 1997) were kindly provided by Eckhard Boles, University of Frankfurt, Germany; these were strains RE601A HXT1+, RE602B HXT2+, RE603A HXT3+, RE604A HXT4+, RE605 HXT5+, RE606A HXT6+ and RE607A HXT7+.

Stock cultures were maintained at 4 °C on YPD medium (2 % glucose, 0.5 % yeast extract, 1 % peptone and 2 % agar; all w/v). For the experimental work, yeast strains were grown in YPD medium (2 % glucose, 1 % peptone and 0.5 % yeast extract; all w/v) up to the exponential (OD_{660} approx. 0.4) or stationary growth phase (OD_{660} approx. 4.0), at 26 °C and 140 r.p.m. Ethanol-adapted cells were obtained either after growth for different periods (at 26 °C and 140 r.p.m.) in YPD medium supplemented with the desired ethanol concentration, or after serial cultivation of the yeast (at 16 °C and 140 r.p.m.) in SF synthetic medium [2.5 % (w/v) fructose, 1 % (w/v) yeast extract, 2 % (w/v) peptone, 12 % (v/v) ethanol and 0.06 % (v/v) acetic acid, pH 3.7] simulating a stuck fermentation, until fructose was exhausted from the medium.

Cell viability assays. Yeast cells in exponential growth phase (OD_{660} approx. 0.4) in YPD medium were harvested by centrifugation, and aseptically transferred to YPD containing 18 % (v/v) ethanol and 0.06 % (v/v) acetic acid, and incubated at 20 °C and 140 r.p.m. Samples were taken periodically for 2 h, and plated in duplicate on YPD medium. The number of c.f.u. was determined after incubation for 2–3 days at 30 °C. Survival curves are represented as the percentage of viable cells as a function of incubation time.

Fermentations. Fermentations were carried out at 20 °C and 120 r.p.m., under oxygen limitation, in 250 ml rubber-stopped flasks containing 150 ml SF medium. The fermentation medium was inoculated with yeast strain (4 × 10^6 cells ml^{-1}) grown under the conditions described above.

To determine the sugar concentrations in the growth medium, the cultures were sampled, and immediately centrifuged at 16 100 g for 3 min. The supernatant was frozen, and stored at -20 °C until it was analysed. Quantitative analysis of sugar was based on the 3,5-dinitrosalicylic acid assay method (Miller, 1959).

Glucose-uptake assays. For glucose-uptake assays, [U-^{14}C]glucose, with a specific activity of 317 mCi mmol^{-1} (11.73 GBq mmol^{-1}; Amersham), was used. \textit{S. cerevisiae} strains were grown as described above. The cells were subsequently harvested by centrifugation, washed twice with cold water, suspended in water to a cell density of 35–45 mg mL^{-1} (dry wt), and kept on ice. Zero-trans influx of labelled glucose was determined at 26 °C as follows. A 10 μL volume of cell suspension was mixed with 30 μL 0.1 M potassium phosphate buffer (pH 5.0). For the determination of the effect of ethanol on glucose transport, 12 % (v/v) ethanol was added to the buffer. The cell suspension was incubated for 5 min, and the reaction was started by adding 10 μL of an aqueous solution of [U-^{14}C]glucose at the desired concentration. After a 5 s incubation, 4.5 mL chilled water was added, and the mixture was immediately filtered through glass-fibre filters (GF/C filters; Whatman). The cells on the filter were washed with 15 mL chilled water, and the filter was immediately transferred into a vial containing 5 mL scintillation OptiPhase HiSafe II liquid (LKB Scintillation Products). The radioactivity was counted with a Packard Tri-Carb 2200 CA liquid scintillation counter (Packard Instrument), with correction for disintegrations per minute. Non-specific binding of radiolabelled sugar to the yeast cells and filter was determined in parallel by adding ice-cold water immediately before the addition of the labelled sugar. For each sugar concentration, the reaction was performed in triplicate, and standard deviation values were below 10 %.

Generation of hybrids. Hybrids were generated by protoplast fusion between \textit{S. cerevisiae} PYCC 5792 and \textit{T. delbrueckii} Bio-J32. Each parental yeast strain was grown to stationary growth phase (OD_{660} approx. 4.0), harvested by centrifugation (4500 g for 5 min), washed
twice with ice-cold water, and pelleted cells were suspended in PT solution (0.1 M Tris/HCl, pH 7, 0.01 M EDTA, 1 M KCl, and 0.1 M β-mercaptoethanol) to a final concentration of $10^6$ cells ml$^{-1}$. The cells were incubated at 30 °C for 30 min, with gentle agitation (70 r.p.m.), and then harvested by centrifugation (4500 g for 5 min), washed once with 1 M KCl, and suspended in P solution [0.05 M potassium phosphate buffer, pH 7.5; 1 M KCl and 200 U Lyticase (Sigma) ml$^{-1}$], followed by an incubation period of 120 min at 30 °C, with gentle agitation (70 r.p.m.). After at least 80 % protoplast formation, determined by microscope observation, the suspension was centrifuged for 10 min at 6000 g, washed three times with 1 M KCl, and suspended in 0.4 M CaCl$_2$ to a final concentration of $5 \times 10^2$ cells ml$^{-1}$. For protoplast fusion, 1 ml suspensions of each strain (S. cerevisiae PYCC 5792 and T. delbrueckii Bio-J32) were mixed, and centrifuged at 4500 g for 10 min. The supernatant was discarded, and the pellet was suspended in the residual volume (about 0.2 ml), followed by the addition of 2 ml 35 % PEG containing 15 % DMSO. After 30 min incubation at 30 °C, 200 μl of the suspension was combined with 8 ml agar (1.5 %, w/v) and 1 M KCl, at 45 °C, and quickly poured onto YPDA plates containing 18 % (v/v) ethanol. The plates were then incubated for 4–5 days, at 30 °C, until colony formation.

**RESULTS**

**Survival in ethanol and acetic acid, and its relation to yeast fructose consumption capacity**

In a search for adequate yeast strains to rectify stuck wine fermentations, different yeast strains, comprising commercial and non-commercial *Saccharomyces* wine strains, as well as fermentative non-*Saccharomyces* species, were first evaluated for their resistance (expressed as the capacity to remain viable) to high ethanol (18 %, v/v) and acetic acid (0.06 %, v/v) concentrations. The acetic acid concentration is similar to that frequently found in stuck musts. Under these aggressive conditions, *T. delbrueckii* and *Z. bailii* strains exhibited a survival of about 100 % after a 2 h incubation (Fig. 1), in contrast to the commercial *S. cerevisiae* strains, which showed 10 %, or less, survival at the end of the same period.

Using a synthetic medium that simulates a stuck wine fermentation (SF medium), we then investigated whether the resistance to ethanol and acetic acid would be reflected by a superior capacity of the non-*Saccharomyces* strains to consume fructose, which is the prevalent residual sugar at the final fermentation stage. The results showed that commercial *S. cerevisiae* strains PYCC 5792 and PYCC 5793, despite their increased cell death rates in the presence of ethanol and acetic acid, performed better, and consumed fructose faster (Fig. 2). The fructophilic species *Z. bailii*, which is known to prefer fructose over glucose in a mixed-sugar environment (Pina et al., 2004b), was slower to consume fructose. Moreover, strains *T. delbrueckii* Bio-J32 and *S. cerevisiae* Bio-PF, though differing considerably in their sensitivity to ethanol and acetic acid (Fig. 1), showed behaviour that was similar to each other in SF medium, and were not able to complete the fermentation during the 16 day assay period (Fig. 2). Overall, these results show that cell survival in ethanol (18 %, v/v) and acetic acid (0.06 %, v/v) does not reflect the capacity of the yeast to consume fructose in a medium simulating a stuck wine fermentation.

![Fig. 1. Survival capacity of *Saccharomyces* and non-*Saccharomyces* strains in YPD medium with 18 % (v/v) ethanol and 0.06 % (v/v) acetic acid, at 20 °C. The inoculum consisted of exponential-phase cells harvested from YPD medium. Values are means of duplicate measurements. ◆, *S. cerevisiae* PYCC 5792; ■, *S. cerevisiae* PYCC 5793; ▲, *S. cerevisiae* PYCC 5794; ▼, *S. cerevisiae* PYCC 5795; □, *S. cerevisiae* PYCC 4072; ○, *S. cerevisiae* Bio-PF; △, *Z. bailii* ISA 1307; ○, *T. delbrueckii* PYCC 5321; ●, *T. delbrueckii* Bio-J32.](image1)

![Fig. 2. Fructose consumption by *Saccharomyces* and non-*Saccharomyces* strains in SF medium, at 20 °C. The inoculum (4×10$^7$ cells ml$^{-1}$) consisted of exponential-phase cells harvested from YPD medium. ◆, *S. cerevisiae* PYCC 5792; ■, *S. cerevisiae* PYCC 5793; ○, *S. cerevisiae* Bio-PF; △, *Z. bailii* ISA 1307; ●, *T. delbrueckii* Bio-J32. Points on the curves are from one representative experiment, from at least three independent replicates.](image2)
Pre-adaptation to ethanol, and its relation to the fructose consumption capacity

Aiming to optimize fructose fermentation rates, the strain that showed the best rate of fructose consumption in SF medium (S. cerevisiae PYCC 5792, Fig. 2) was tested by preparing inocula in different ways. The effects of the growth phase and pre-adaptation to ethanol [alcohol concentrations ranging from 6 to 14 % (v/v), and adaptation periods ranging from 1 to 24 h] were examined. As expected, without previous ethanol adaptation, stationary-phase cells were much more efficient than exponential-phase cells; the latter cells took twice as long as the former to achieve total fermentation (Fig. 3). However, ethanol pre-adapted stationary-phase cells did not increase their fructose consumption rate (results not shown), whereas exponential-phase cells adapted for 2 h in YPD medium containing 6 % (v/v) ethanol displayed a fructose consumption capacity similar to that observed for non-adapted stationary-phase cells (Fig. 3). Gradual adaptation to the stress conditions prevailing in stuck wine fermentations was also carried out by serially transferring a commercial S. cerevisiae strain (PYCC 5792) to fresh SF medium whenever fructose was totally consumed in the inoculated medium. No improvement in fructose consumption rate was observed after three successive transfers (results not shown).

Glucose transport, and its relation to the fructose consumption capacity

In the selection of yeast strains able to rectify a stuck fermentation, the fructose consumption capacity under such adverse conditions is a key parameter. Therefore, transport across the plasma membrane was the next cell target investigated in our attempt to understand what determines an enhanced sugar consumption capacity. Since fructose is a weaker substrate of glucose transporters in both S. cerevisiae (Reifenberger et al., 1997) and T. delbrueckii (Alves-Araújo et al., 2005), and no fructose-specific transporter has been found in these species, sugar transport was evaluated by determining labelled-glucose-uptake rates in the presence and absence of ethanol and acetic acid. Exponential-phase cells of the commercial strain S. cerevisiae PYCC 5792, one of the best-performing strains in SF medium, presented a lower glucose transport capacity than either exponential-phase ethanol-pre-adapted cells or stationary-phase cells; the latter cells showed the highest V_max values (Table 1). In the presence of 12 % (v/v) ethanol, glucose transport was severely affected in exponential-phase cells (approx. 80 % inhibition). The presence of 0.06 % (v/v) acetic acid in the medium did not cause any additional significant effect on glucose-uptake rates (results not shown). Conversely, in stationary cells sugar transport was the least inhibited by ethanol, followed by exponential ethanol-pre-adapted cells (Fig. 4). These results fully agree with the fructose consumption rates observed under the same growth conditions and cultivation stages (Fig. 3). To elucidate whether these relationships could be generalized rather than be strain specific, all the other yeast strains analysed in the initial screening (Fig. 2) were tested using stationary-phase cells, which are less sensitive to ethanol. An inverse correlation between fructose consumption rate and the degree to which ethanol inhibited sugar transport was again found for all the seven yeast strains studied (Fig. 5).

Based on the above results, we decided to check whether the individual glucose transporters would respond differently to the presence of ethanol, and how the response was influenced by the growth phase. To clarify these aspects, glucose-uptake rates were determined, in the presence and in the absence of ethanol, in exponential- and stationary-phase S. cerevisiae cells expressing each of the major hexose transporters individually (Hxt1–Hxt7). As shown in Table 2, the parental strain MC996, carrying all the native Hxt transporters, was highly sensitive to ethanol in the stationary growth phase, and more resistant during the exponential phase, i.e. it behaved in a similar way to those strains that are inadequate to cure stuck fermentations. Transporters Hxt1 and Hxt3 were less sensitive to ethanol in exponential-phase cells than in stationary-phase cells, and seem to be responsible for the global performance of the yeast. In contrast, the intermediate- and high-affinity transporters Hxt4–Hxt7 exhibited a higher inhibition of

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**Fig. 3.** Fructose consumption by exponential- (▼) and stationary-phase (●) S. cerevisiae PYCC 5792 cells, and exponential-phase cells that had been pre-adapted to ethanol (△) by incubation for 2 h in YPD medium containing 6 % (v/v) ethanol. The experimental conditions were as described in the legend to Fig. 2, except for inoculum concentration (4x10^6 cells ml^-1). Points on the curves are from one representative experiment, from at least three independent replicates.
Table 1. Maximum glucose transport capacity of S. cerevisiae PYCC 5792 cells from different cultivation stages in YPD medium, and in exponential cells pre-adapted for 2 h in YPD with 6 % v/v ethanol

\(V_{\text{max}}\) values were calculated considering two transport components in Eadie–Hofstee plots. Values are means ± SD.

<table>
<thead>
<tr>
<th>Transport component</th>
<th>Exponential-phase cells</th>
<th>Exponential-phase pre-adapted cells</th>
<th>Stationary-phase cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>Low-affinity</td>
<td>2.00 ± 0.46</td>
<td>3.19 ± 0.35</td>
<td>4.05 ± 0.27</td>
</tr>
<tr>
<td>High-affinity</td>
<td>0.33 ± 0.04</td>
<td>0.89 ± 0.12</td>
<td>1.16 ± 0.16</td>
</tr>
</tbody>
</table>

glucose transport by ethanol in exponential-phase cells than in stationary-phase cells. However, the Hxt2 transporter, also reported to have an intermediate affinity for glucose, was strongly inhibited in both growth phases. These results are puzzling, since high-affinity transporters Hxt6 and 7 are known to be mainly expressed in stationary-phase cells, and they suggest that the relative expression of the different hexose transporters tested (Hxt1–Hxt7) varies with the strain (Fig. 5). On the other hand, the analysis of individual transporters did not account for their active interplay during fermentation by process strains.

Performance of T. delbrueckii \(\times\) S. cerevisiae hybrid strains

As shown above (Figs 1 and 2), T. delbrueckii Bio-J32 maintained its viability in high ethanol and acetic acid for the first 2 h, but was unable to consume fructose in simulated stuck must (SF) medium. In contrast, S. cerevisiae PYCC 5792 was much less resistant to ethanol and acetic acid, but presented a high fructose-consumption capacity under the same conditions. In an attempt to combine the advantageous traits of these two yeasts, and slow down cell death under the harsh conditions present in stuck fermentations, hybrids between the two species were generated by protoplast fusion. The hybrids were recovered on solid YEPD medium with 18 % (v/v) ethanol; this concentration was chosen because it allowed growth of T. delbrueckii, but not S. cerevisiae. The clones selected, herein designated F1-11, F1-14, F1-15 and F1-16, displayed distinct fructose consumption patterns that were intermediate between those shown by the two parental strains.

Fig. 4. Inhibition of glucose transport by 12 % (v/v) ethanol in cells of S. cerevisiae PYCC 5792 from exponential and stationary growth phases, and from the exponential phase followed by adaptation for 2 h in YPD medium containing 6 % (v/v) ethanol. The SD values of glucose uptake determinations were below 10 %. Black bars, 1 mM glucose; white bars, 30 mM glucose.

Fig. 5. Degree of inhibition of glucose transport by 12 % (v/v) ethanol for selected strains, as a function of the residual fructose concentration in SF medium, at 20 °C, and 5 days after inoculation. Values are means; standard deviations of glucose uptake determinations were below 10 %. \(\bullet\), S. cerevisiae PYCC 5792; \(\square\), S. cerevisiae PYCC 5793; \(\triangle\), S. cerevisiae PYCC 5794; \(\downarrow\), S. cerevisiae PYCC 5795; \(\sqcap\), S. cerevisiae PYCC 4072; \(\diamond\), S. cerevisiae Bio-PF; \(\bullet\), T. delbrueckii Bio-J32.
Table 2. Inhibition of maximum glucose transport capacity by 12 % (v/v) ethanol in exponential-phase and stationary-phase glucose-grown cells of S. cerevisiae mutant strains

In strains RE602B, RE604A, RE606A and RE607A, $V_{\text{max}}$ was estimated by the determining initial uptake rates at a saturating glucose concentration of 30 mM. In strains MC966, RE601A, RE603A and RE605, which expressed low-affinity transporters, $V_{\text{max}}$ was estimated from initial uptake kinetics using glucose concentrations ranging from 5 to 60 mM. Values are means ± SD.

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Strain</th>
<th>$V_{\text{max}}$ inhibition (%)</th>
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<tbody>
<tr>
<td></td>
<td></td>
<td>Exponential-phase cells</td>
</tr>
<tr>
<td>HXT1−7+</td>
<td>MC996</td>
<td>56.8 ± 2.2</td>
</tr>
<tr>
<td>Low-affinity component</td>
<td></td>
<td>61.5 ± 0.6</td>
</tr>
<tr>
<td>High-affinity component</td>
<td></td>
<td></td>
</tr>
<tr>
<td>HXT1+</td>
<td>RE601A</td>
<td>49.0 ± 7.6</td>
</tr>
<tr>
<td>HXT2+</td>
<td>RE602B</td>
<td>68.2 ± 6.5</td>
</tr>
<tr>
<td>HXT3+</td>
<td>RE603A</td>
<td>59.4 ± 5.5</td>
</tr>
<tr>
<td>HXT4+</td>
<td>RE604A</td>
<td>71.4 ± 4.7</td>
</tr>
<tr>
<td>HXT5+</td>
<td>RE605</td>
<td>66.4 ± 2.8</td>
</tr>
<tr>
<td>HXT6+</td>
<td>RE606A</td>
<td>68.6 ± 4.6</td>
</tr>
<tr>
<td>HXT7+</td>
<td>RE607A</td>
<td>75.7 ± 5.8</td>
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</table>

(Fig. 2 and Fig. 6a). The best-performing clone (F1-11), which showed a fructose consumption capacity similar to S. cerevisiae PYCC 5792, was further characterized physiologically. Cells of this strain displayed an increased resistance to ethanol and acetic acid in comparison with the S. cerevisiae parent strain (Fig. 6b). As for sugar transport in the presence of ethanol, F1-11 cells behaved in a similar way to S. cerevisiae PYCC 5792 (not shown); this supports the correlation between glucose transport in the presence of high ethanol concentrations and the capacity to consume fructose in the SF medium.

**DISCUSSION**

The present work aimed to identify relevant parameters for the selection and optimization of yeast strains suitable for an efficient restart of stuck wine fermentations. S. cerevisiae and potentially interesting wine-associated non-Saccharomyces species were studied regarding their capacity to survive, and their ability to consume fructose, under the stress conditions present in stuck musts. The non-Saccharomyces species Z. bailii and T. delbrueckii were shown to be more resistant than S. cerevisiae strains to ethanol- and acetic acid-induced cell death. A high resistance to these compounds has been described for Z. bailii (Souza et al., 1996; Fernandes et al., 1999), while T. delbrueckii is known to be weakly tolerant of high ethanol concentrations (Pina et al., 2004a, c). Such a discrepancy with our results may be ascribed to differences at the strain level. Actually, some T. delbrueckii strains have been described as highly freeze tolerant when compared with S. cerevisiae; this is a property that has been related to a better capacity to preserve membrane integrity (Almeida & Pais, 1996; Alves-Araújo et al., 2004). Such capacity may also be responsible for the observed higher resistance to ethanol and acetic acid. On the other hand, commercial oenological S. cerevisiae strains were the most efficient in fructose consumption in synthetic medium simulating a stuck fermentation, demonstrating that the capacity to survive in aggressive ethanol and acetic acid conditions is not a reliable criterion to select strains with a good capacity to speed up fructose consumption. The poor fructose consumption capacity of T. delbrueckii, which was unable to complete the fermentation (Fig. 2), was not due only to its high oxygen demand, and weak growth and fermentative capacity under anaerobic conditions (Mauricio et al., 1998; Holm Hansen et al., 2001; Hanl et al., 2005; Alves-Araújo et al., 2007), but it was also a result of the high sensitivity of glucose transport to ethanol inhibitory effects, as shown in the present work.

Another factor influencing the rate at which fructose was consumed under conditions mimicking stuck wine fermentations was the physiological state of the yeast. Stationary cells of S. cerevisiae PYCC 5792 performed as well as exponential-phase ethanol-pre-adapted cells, and adaptation of stationary-phase cells to ethanol did not improve their performance. Cells in the stationary phase of growth are well known for their capacity to accumulate trehalose, express heat-shock proteins and modulate membrane composition (Werner-Washburne et al., 1993); these factors have been described as contributing to the ethanol-stress response (Piper, 1995; Alexandre et al., 2001). Our results support the recommendation of wine yeast suppliers to pre-adapt the cells to ethanol before using them as an inoculum to restart stuck fermentations.

Our observations in the first stage of this work led us to assess the role of sugar transport on the fructose consumption capacity. Notably, a direct correlation
Fig. 6. Characterization of hybrids of *S. cerevisiae* PYCC 5792 and *T. delbrueckii* Bio-J32. (a) Comparative fructose consumption by stationary-growth-phase cells of *S. cerevisiae* PYCC 5792 and the different clones. Experimental conditions were as described in the legend to Fig. 3. (b) Survival capacity of *S. cerevisiae* PYCC 5792 and hybrid F1-11 in YPD medium with 18 % (v/v) ethanol and 0.06 % (v/v) acetic acid, at 20 °C. Experimental conditions were as described in the legend to Fig. 1. *S. cerevisiae* PYCC 5792: ■, F1-11; ▲, F1-14; ●, F1-15; ○, F1-16. Points on the curves are from one representative experiment, from at least three independent replicates.

between fructose-consumption and glucose (as indicative of fructose)-uptake rates was detected (Table 1, Fig. 3) when comparing cells in different physiological conditions (stationary-, exponential- and exponential-phase pre-adapted to ethanol). This correlation was reinforced by the observation that sugar transport systems less affected by high ethanol concentrations (12 %, v/v) are more likely to be associated with a better capacity of the yeast strains to act as restarters in stuck fermentations (Fig. 5), and by the results obtained with the F1-11 hybrid. The comparative analysis of individual *S. cerevisiae* Hxt transporters concerning their putative contribution to the overall sensitivity of the yeast towards ethanol was not very enlightening. The laboratory strain we used as a reference (Table 2) behaved differently from the best-performing commercial strain (Fig. 5). While glucose transport in the laboratory strain was more resistant to ethanol during the exponential phase of growth, exponential-phase cells of the best-performing commercial strains were significantly more sensitive than stationary cells (Fig. 4); this finding is more in line with present knowledge on this topic. Our results revealed that all the relevant glucose transporters (Hxt1p–Hxt7p), with the exception of Hxt2p, showed different sensitivities to ethanol as a function of the growth stage. Hxt3p and Hxt1p are known to be highly expressed during the exponential growth phase (Reifenberger *et al.*, 1997; Luyten *et al.*, 2002); mutants expressing only Hxt3p or Hxt1p displayed a lower level of ethanol inhibition, contradicting the general phenotype of oenological strains. A similar result was obtained with the high-affinity transporters Hxt6p and Hxt7p, which are known to be expressed in late-exponential phase and during stationary phase. Hxt6p and Hxt7p were found to be more sensitive to ethanol, in disagreement with the lower glucose-transport inhibition observed in stationary-phase cells of the commercial *S. cerevisiae* strains, but justifying the phenotype of the reference laboratory strain. It is noteworthy that stationary-phase cells of the *HXT5* strain were the most resistant to ethanol inhibition of glucose transport (Table 2). The *HXT5* gene is induced by stress conditions (Buziol *et al.*, 2002), and it is co-expressed with genes involved in reserve carbohydrate metabolism (Verwaal *et al.*, 2002). The transcript levels of *HXT5* increase upon glucose depletion from the growth medium (Diderich *et al.*, 2001). These data, together with our results, point to a potentially relevant contribution of the Hxt5 transporter to the lower sensitivity to ethanol found in stationary-phase cells of commercial *S. cerevisiae* strains. The results also suggest that manipulating the expression of a given transporter could have an impact on the ability of the yeast strain to remove fructose from media with high ethanol concentrations. In line with the crucial role of sugar transporters throughout vinification, it has been shown recently that the expression of a mutated form of the Hxt3 transporter could increase the rate of fructose consumption during a simulated wine fermentation (Guillaume *et al.*, 2007).

Two specific aspects of our work open new perspectives to the wine-making industry, and should be emphasized. One is the utilization of natural breeding programmes to combine desirable traits of wine-associated species and/or strains for the vinification process. The example given in this work is particularly relevant since mixed starter cultures containing *T. delbrueckii* and *S. cerevisiae* are already on the market to take advantage of the flavour-enhancing properties of *T. delbrueckii*. The generated hybrid, in addition to its potential to restart stuck fermentations, may also be useful to conduct an entire fermentation that benefits from its flavour properties. Our results also clearly demonstrate that cell survival in the presence of high concentrations of ethanol and acetic acid...
is not per se a good parameter to consider in a strain selection programme for efficient ethanolic fermentation processes; this is in contrast to currently accepted ideas. Instead, higher productivity seems to be associated with the ethanol tolerance of sugar transport. Therefore, we propose that the assessment of glucose transport in the presence of a high ethanol concentration is a good test for preliminary selection of yeast strains that are suitable to restart stuck wine fermentations.

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