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Genetic Improvement of *Torulaspora delbrueckii* for Wine Fermentation †

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Abstract: The use of *Torulaspora delbrueckii* has been repeatedly proposed to improve wine's organoleptic quality. However, this yeast has lower efficiency in completing wine fermentation than *Saccharomyces cerevisiae* since it has less fermentation capability and greater sensitivity to SO_2, ethanol, and CO_2 pressure. Therefore, the completion of fermentation is not guaranteed when must or wine is single-inoculated with *T. delbrueckii*. To solve this problem, new strains of *T. delbrueckii* with enhanced resistance to winemaking conditions were obtained. A genetic study of four wine *T. delbrueckii* strains was done. Spore clones free of possible recessive growth-retarding alleles were obtained from these yeasts. These spore clones were used to successively isolate mutants resistant to SO_2, then those resistant to ethanol, and finally those resistant to high CO_2 pressure. Most of these mutants showed better fermentation capability in base wine than the parental strain, and some of them approached the fermentation capability of *S. cerevisiae*.

Keywords: *Torulaspora delbrueckii*; wine fermentation; sporeclone; sparkling wine; ethanol resistance; SO_2 resistance; pressure resistance

1. Introduction

Among non-*Saccharomyces* yeasts, *Torulaspora delbrueckii* is probably the one with a wine-fermentation performance closest to *Saccharomyces cerevisiae*, and therefore the most suitable for winemaking. The features of this yeast specie may improve wine quality or complexity [1,2] and displays higher rates of CO_2 production and O_2 consumption than *S. cerevisiae*. On the other hand *T. delbrueckii* grows more slowly than *S. cerevisiae* under strict anaerobic conditions [3,4]. As a consequence, *T. delbrueckii* has less fermentation vigor than *S. cerevisiae* under usual wine fermentation conditions, and has serious difficulties in dominating wine fermentation even when initially inoculated at a high proportion (above 10^7 CFU/mL) [2,5,6]. *T. delbrueckii* is also less resistant to other stressing conditions closely related to winemaking than *S. cerevisiae*, such as the rapid increase of ethanol concentration, the presence of SO_2 and high CO_2 pressure. These circumstances negatively affect the fermentation efficiency of *T. delbrueckii* during still or sparkling wine making. The present work describes sequential isolations of spontaneous mutants resistant to different stressful conditions related to still and sparkling wine making. The main aim was to improve the overall fermentation performance of this yeast species to bring it as close as possible to that usually shown by *S. cerevisiae* wine yeasts.

2. Materials and Methods

S. cerevisiae (*Sc*) EX229 (Klus-killer wine strain that kills other *S. cerevisiae* and *T. delbrueckii* yeasts [7]) and *Sc* 85R4A (non-killer, cycloheximide-resistant (cyh^R) spore clone obtained from the *Sc* EX85R (originally named JP85R; [8]) wine yeast) were used in this study as reference yeasts for still and sparkling wine fermentation. *T. delbrueckii* (*Td*) Kbarr EX1180 and *Td* EX1257 are prototrophic wine yeasts that kill all known types of *S. cerevisiae* killer and non-killer strains and non-killer *T. delbrueckii* strains. *Td* EX1180-11C4 and *Td* EX1257-CYH5 are cyh^R spontaneous mutants from *Td* EX1180 and *Td* EX1257, respectively. These strains had previously been selected for winemaking [9,10,11]. The genetic marker cyh^R allows easy traceability of the new mutants obtained from these yeasts. Standard culture media were used for yeast growth [12]: YEPD broth, YEPD agar, YEPD + EtOH, SD agar, SD+SO_2, YEPD + CYH. Standard procedures were used for the sporulation of yeast cultures and dissection of asci [13]. For base-wine making, Macabeo grape must and synthetic base wine were used. The density, ºBrix, yeast growth (total and viable yeast cells), and dead cells were monitored. Cava-type sparkling wine was made using the traditional method in our experimental winery as previously described [11]. The identity of these possible HPR mutants was verified by analysis of cell morphology, killer phenotype, resistance to cycloheximide, presence of viral dsRNA, RFLPs of mtDNA, and sequencing of Internal Transcribed Spacer of ribosomal DNA (ITS). Two different commercial base wines were used, one from Garnacha red grapes (pH 3.20, 4.93 g/L total acidity, 0.87 g/L reducing sugars, 10.9% alcohol *v*/*v*) and another from Macabeo white grapes (pH 3.18, 5.7 g/L total acidity, 1.2 g/L reducing sugars, 10.8% alcohol *v*/*v*). Degrees Brix (Brix) were measured using a digital refractometer. Alcohol content, pH, total acidity, volatile acidity, glucose and fructose, and density were determined using European Commission (EC) recommended methods [14]. Sparkling wine pressure was measured at room temperature using an aphrometer, and values were then corrected to 20 °C by using Henry's law constant.

3. Results and Discussion

3.1. Isolation and Characterization of New T. delbrueckii Mutants Resistant to SO_2 and Ethanol

Several *Td* EX1180-11C4 (27) and *Td* EX1257-CYH5 (18) spore clones were plated onto YEPD plates supplemented with 250 mg/L SO_2. Resistant papillae were isolated only from the *Td* EX1180-11C4-5B and -6A spore clones. A purified colony was selected from Td EX1180-11C4-5B and -6A papillae: *Td* Mut5B-SO2R and *Td* Mut6A-SO2R, respectively. The fermentation capability in synthetic must of the SO_2 resistant mutants *Td* Mut5B-SO2R and *Td* Mut6A-SO2R was similar to that of their parental yeast *Td* EX1180-11C4. However, a slight improvement was seen in the SO_2 + ethanol resistant mutants (named *Td* Mut6A-SO2R-EtOHR-31 and *Td* Mut6A-SO2R-EtOHR-33), although this improvement became irrelevant after 14 days of fermentation (Figure 1A). The fermentation capability of *Td* Mut5B-SO2R and Mut6A-SO2R in synthetic must supplemented with 50 mg/L SO_2 was also slightly better than that of the parental strain during the first days of fermentation, but this improvement also became irrelevant after the sixth day of fermentation, when approximately 5% ethanol was reached.

However, an evident and relevant improvement was observed in *Td* Mut6A-SO2R-EtOHR-31 and *Td* Mut6A-SO2R-EtOHR-33 that was maintained throughout fermentation (Figure 1B).

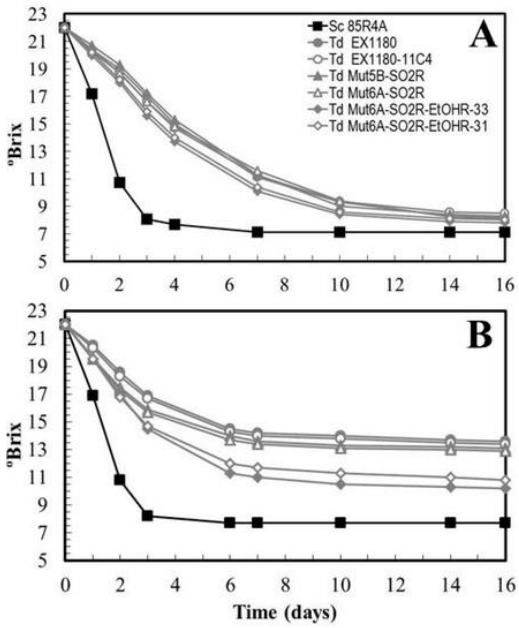

Figure 1. Fermentation kinetics of *T. delbrueckii* SO_2 resistant mutants (*Td* Mut5B-SO2R and *Td* Mut6ASO2R) and SO_2 + ethanol resistant mutants (*Td* Mut6A-SO2R-EtOHR-31 and *Td* Mut6A-SO2R-EtOHR-33) in synthetic must (**A**) and synthetic must containing 50 mg/L SO_2 (**B**). Data are the mean values of three fermentations inoculated with each yeast strain. Standard deviations were less than 10% of the means. The degree of dominance throughout fermentation of each inoculated yeast strain was 100%.

3.2. Isolation and Fermentation Capability of New T. delbrueckii Mutants Resistant to High CO_2 Pressure (HPR) from Mutants already Resistant to SO_2 and Ethanol

One mutant of each type was selected to make rosé sparkling wine (cava) under cellar conditions: *Td* Mut5B-SO2R (resistant to SO_2) and *Td* Mut6A-SO2R-EtOHR-33 (resistant to SO_2 and ethanol). Following the trend displayed by the parental strain *Td* EX1180-11C4, no *Torulaspora delbrueckii* mutant was able to dominate the entire process to the end and complete the second in-bottle fermentation, while this was accomplished successfully by the reference yeast *Sc* EX229. *Td* Mut6A-SO2R-EtOHR-33 was better than the parental strain and *Td* Mut5B-SO2R during the first 40 days of fermentation (Figure 2A,B). Yeast colonies were isolated on YEPD agar inoculated with samples from the sparkling wines that were single inoculated with *T. delbrueckii* yeasts taken at 30, 40, and 60 days of fermentation. After 60 days, when 4.5 atm pressure had been surpassed (Figure 2C), no viable *T. delbrueckii* yeasts were isolated. Subsequently, this pre-selection was restricted to eighteen HPR mutants, these were inoculated into Macabeo grape must, synthetic must, and synthetic must with 100 mg/L SO_2. Fermentative vigor and the ability to complete fermentation were analyzed. Some improvement was observed for some HPR mutants (such as *Td* MutHP41 and *Td* MutHP42) with respect to their parents in fresh grape must fermentations (Figure 3A), but this improvement was less clear in synthetic must (Figure 3B). The HPR mutants had faster fermentation kinetics than their parental yeast *Td* Mut6A-SO2R-EtOHR-33, being less affected by the presence of SO_2 (Figure 3C).

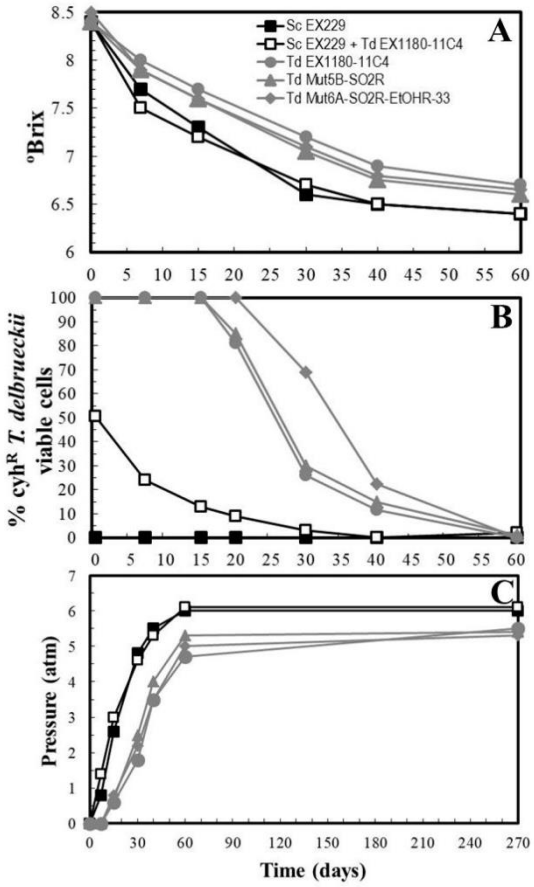

Figure 2. Fermentation kinetics and yeast-population dynamics during sparkling wine second-fermentations inoculated with *T. delbrueckii* mutants resistant to SO_2 and ethanol. (**A**) Evolution of sugar consumption (ºBrix). (**B**) Percentage of cyhR yeast cells in each fermentation. Note that the cyhR *T. delbrueckii* viable cells tended to disappear as CO_2 pressure increased. (**C**) Pressure inside the bottle. Data are the mean values of three fermentations inoculated with each yeast strain. Standard deviations were less than 13% of the means.

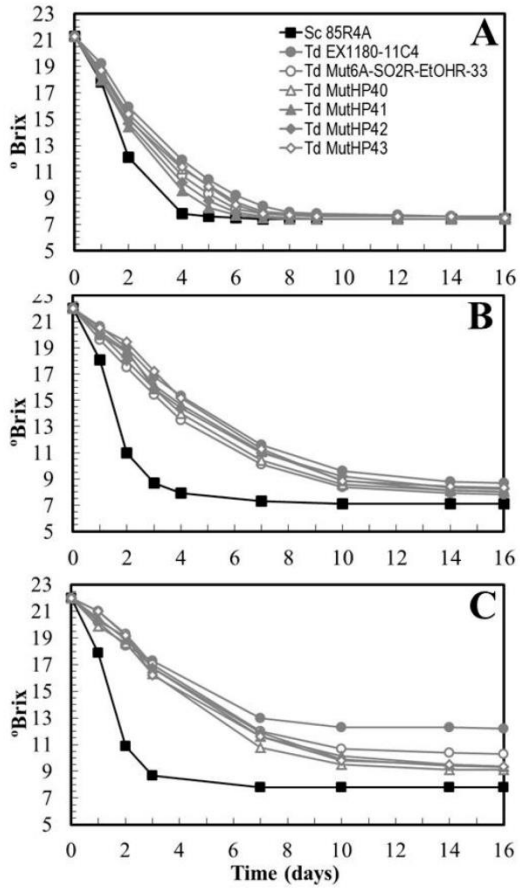

Figure 3. Fermentation kinetics of some *T. delbrueckii* high CO_2 pressure resistant (HPR) mutants inoculated in sterile fresh grape must (**A**), synthetic must (**B**), and synthetic must supplemented with 100 mg/L SO_2 (**C**). Data are the mean values of three fermentations inoculated with each yeast strain. Standard deviations were less than 11% of the means. The degree of dominance throughout fermentation of each inoculated yeast strain was 100%.

However, most HPR mutants showed a relevant improvement in synthetic base wine fermentations supplemented with 50 mg/L SO_2 (Figure 4A). Furthermore, they were able to complete the fermentation when the amount of SO_2 was reduced to 30 mg/L just 3–4 days after the reference yeast *Sc* 85R4A. Only one of the selected mutants, MutHP40, did not improve with respect to its direct parental strain Td Mut6A-SO2R-EtOHR-33 (Figure 4B). This SO_2 concentration is similar to that commonly used in the cava-type sparkling-wine industry (between 15 and 25 mg/L). Therefore, the two mutants with the best fermentation kinetics, *Td* MutHP41 and *Td* MutHP42, were selected.

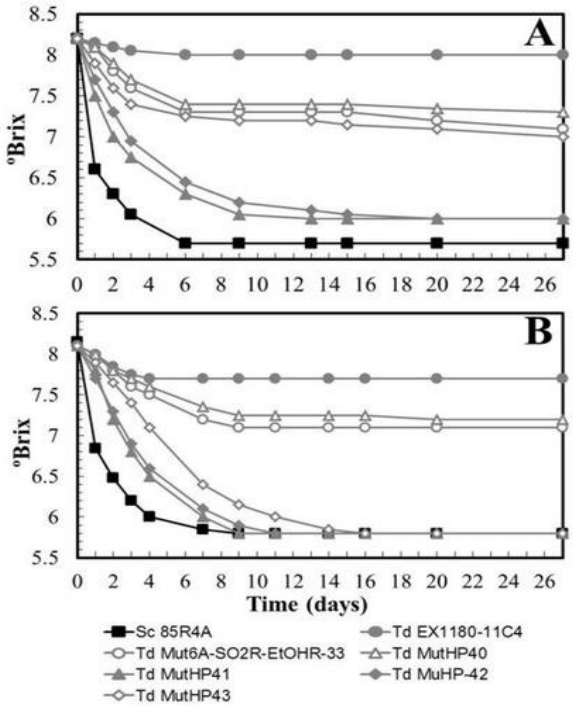

Figure 4. Fermentation kinetics of some *T. delbrueckii* HPR mutants inoculated in synthetic base wine supplemented with 50 mg/L (**A**) or 30 mg/L SO_2 (**B**). Data are the mean values of three fermentations inoculated with each yeast strain. Standard deviations were less than 8% of the means. The degree of dominance throughout fermentation of each inoculated yeast strain was 100%.

4. Conclusions

Isolation of spontaneous mutants resistant to SO_2 and ethanol seems to be a good strategy to slightly improve the fermentative efficiency of *T. delbrueckii* in must and base wine. Sequential isolation of HPR mutants from previously obtained mutants resistant to SO_2 and ethanol was required to obtain new mutants with significantly improved efficacy for the second fermentation of sparkling wine. These new mutants were genetically stable enough to be considered for industrial production.

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Conflicts of Interest: The authors declare no conflict of interest.

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