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Viability Enhancement of *Schizosaccharomyces pombe* Cells During Desiccation Stress

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Abstract In winemaking, the sequential inoculation of grape must with different oenological yeasts has been widely used due to the accessibility of different types of active dry yeast (ADY). *Schizosaccharomyces pombe* is of special interest for winemakers due to its ability to metabolize L-malic acid. Therefore, the availability of *Schiz. pombe* as an ADY will provide a new biotechnological tool with the same effectiveness as current commercially available oenological yeasts. In the present study, the features of metabolites were analyzed during the dehydration-rehydration process for different *Schiz. pombe* strains to determine whether these metabolites might play a positive role in ensuring cell viability before inoculation into the must. Our results show that the viability of cells dried in the presence of 10% trehalose and rehydrated in a solution complemented with 5 mM $MgSO_4$ was enhanced by up to 70% for certain strains. No significant change in fermentation behavior and main volatile compounds were detected in the wines obtained with *Schiz. pombe* ADY in sequential- and co-inoculated with *Saccharomyces cerevisiae* grape must fermentation at laboratory scale.

Keywords *Schizosaccharomyces pombe*, Active Dry Yeast, Cell viability, Wine

1. Introduction

At present, most of the wine produced worldwide is made from musts inoculated from selected wine yeasts called *active dry wine yeasts* (ADWY) that are often traded in dried form [1]. The ability of *Saccharomyces cerevisiae* to withstand dehydration and subsequent rehydration allows for the production of ADWY inocula, ensures that fermentation will start by increasing confidence in the strain identity and genetic stability at room temperature, and reduces the costs of transportation and storage [2]. ADWY production involves dehydration of the yeast biomass to a final product with a residual moisture percentage of below 8% [3]. Many *S. cerevisiae* strains with an optimum oenological profile are excluded from commercial catalogues due to the sensitivity to the drying treatment. Studies have investigated the dehydration behavior exhibited by sensitive strains [4, 5]. In contrast, few studies have investigated increases in dehydration tolerance to drying and rehydration by wine yeast strains; most of these studies were performed on representatives of the genus *Saccharomyces* [3, 6-9]. Previously, only non-*Saccharomyces* yeast strains had been considered responsible for organoleptic defects in wine, but studies have reported that the sequential growth of different genera of non-*Saccharomyces* wine yeasts, such as *Hanseniaspora*, *Kloeckera*, *Candida*, *Pichia*, *Zygosaccharomyces*, *Schizosaccharomyces* and *Torulaspora,* may provide a greater aromatic complexity that is attributable to the production of secondary metabolites such as glycerol, 2-phenylethyl acetate and isoamyl acetate [10-13]. To date, a few non-*Saccharomyces* ADWYs, such as the *Torulaspora delbrueckii* 291 and *Metschnikowia pulcherrima* L1781 strains (Lallemand), have achieved sufficient quality to ensure good proliferation under wine-making conditions [14, 15]. Winemakers have a special interest in *Schiz. pombe* because of its high fermentative power, capacity to reduce the gluconic acid content of must and ability to efficiently metabolize L-malic acid into ethanol and carbon dioxide; these characteristics differentiate it from the other *Saccharomyces* strains [16-18]. Additionally, co-inoculation with *S. cerevisiae* has been reported to prevent the off flavors generated by the production of H_2S, acetaldehyde, acetoin and ethyl acetate [19, 20]. In this process, fresh, immobilized *Schiz. pombe* cells are used for the partial or total consumption of L-malic acid before being removed to prevent off-flavor production [21]. The availability of active dry yeast of *Schiz. pombe* strains has provided winemakers with a new biotechnological tool with the same effectiveness as commercial ADWYs.

In the present study, a protocol that transforms dry and rehydrated *Schiz. pombe* strains into active dry yeast was developed. Our results showed that considering the cellular

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physiological state before drying and initiating the rehydration process in a physiological solution improved the viability of *Schiz. pombe* strains isolated from grape juice by up to 70%. The consequences of drying a *Schiz. pombe* strain for fermentative performance was investigated. The results obtained showed that the treatment to obtain ADWY of *Schiz. pombe* improved cell viability without affecting fermentation efficiency and metabolic behavior.

2. Materials and Methods

2.1. Yeast Strains and Growth Conditions

Table 1 summarizes the *Schizosaccharomyces pombe* strains used in this study. The yeast strains were grown in shaker flasks at 100 rpm in EMM (Edinburgh Minimal Medium) at 32°C, inoculated with an overnight liquid culture at an initial OD_{600} of 0.25, and measured by microscope cell counting.

Table 1. *Schiz. pombe* strains used in this study

Strain	Source	Origin
Sp1	[a]CECT137	Grape must
Sp2	CECT11197	Sulphated grape juice
Sp3	CECT12622	Grape juice
Sp4	CECT12773	Castelli collection, Italy
Sp5	CECT12821	Concentrate grape juice
Sp6	CECT12918	Concentrate grape juice
Sp7	[b]CBS10395	Grape must
Sp8	CBS10498	Beverage industry
Sp9	CBS10500	Cape wines

[a]Spanish Type Culture Collection (http://www.cect.org/)
[b]Centraalbureau voor Schimmelcultures (http://www.cbs.knaw.nl/)

2.2. Desiccation-Rehydration Process

Growth curves were determined, and 5 x 10^7 yeast cell suspensions, measured by microscope cell counting during the stationary phase, were desiccated in the presence of several trehalose concentrations (5%, 10%, 20% and 30%) by exposure to dry air at 28°C for ~20 h [22]. In all cases, 5 x 10^7 cells were rehydrated into a 1-ml final volume of water. Various rehydration temperatures (25°C, 30°C, 37°C and 40°C) and times (5 min, 15 min, 30 min and 45 min) were tested using pure water as a control. The effect of rehydration solutions on dry *Schiz. pombe* cells after rehydration was studied by adding each compound individually to the pure water-based condition.

Cell viability was determined for the following compounds: 1% proline; 1% glutamate; 1% ammonium; 0.25 and 1% raffinose; 0.25, 0.5 and 1% galactose; 0.25 and 0.5% glucose; 0.25 and 0.5% fructose; 5, 10, 20 and 30% trehalose; 5 mM calcium; 1, 5 and 10 mM Mg; 0.25, 0.5 and 1 mM $FeSO_4$; 0.5 mM $FeCl_3$; 1 mM $FeSO_4$; 5 $mg{\cdot}l^{-1}$ sulfur dioxide; 5 mM ascorbic acid; 10 mg l^{-1} thiamine; 1 mM hydrogen peroxide; 0.1 mM dimethyl sulfoxide (DMSO); 5 mM benzyl alcohol; 5 mM 2-phenyl-1-butanol; 5 mM 2-phenyl-1-propanol; and 7 mM 2-ethyl-phenol [3, 23-25].

2.3. Determining Yeast Viability

After the rehydration process, the viable cell count was calculated by spreading cell dilutions using a Whitley Automatic Spiral Plater (AES Laboratoire, France) on YPD agar medium. The plates were incubated at 32°C for 48 h, and the CFUs (colony-forming units) were quantified using the ProtoCOL SR/HR counting system software version 1.27 supplied by Symbiosis (Cambridge, UK).

2.4. Tests for Intracellular ROS Accumulation

Dihydroethidium (DHE) staining was performed as described in [26]. The samples were analyzed by fluorescence microscopy. To determine the frequencies of the morphological phenotypes revealed by the DHE staining, a minimum of 500 cells were evaluated from three independent experiments using a Leica fluorescence microscope (DM4000B, Germany). A digital camera (Leica DFC300FX) and Leica IM50 software were used for the image acquisition.

2.5. Determination of Biological Parameters

Growth in microplate wells was monitored at 600 nm every 10 min after 20 s of shaking for 24 h at 32°C in a POLARstar OMEGA instrument (BMG Labtech, Germany). Microplate wells filled with 190 µl of YPD medium were inoculated with 10 µl of rehydrated cell inoculum measured by flow cytometry cell counting to obtain an OD_{600} of 0.6, which is above the minimal detection limit previously established by calibration. Blanks were determined from five independent non-inoculated wells for each experimental 96-wells plate. Three independent cultures of each strain were evaluated (six times each). Growth data from plate counts were enumerated as $\log_{10}$ values. The biological parameters, duplication times (DT) and lag phase times (λ) were estimated by fitting the growth curves into the model using MicroFit software (Institute of Food Research, Norwich, UK) [27].

2.6. Must Preparation, Fermentation and Sampling

Sucrose was added to Tempranillo grape juice to raise the sugar content to 200 $g{\cdot}l^{-1}$. Before fermentations, the juice with a pH of 3.25 was complemented with diammonium phosphate (250 $mg{\cdot}l^{-1}$), L-malic (8 $g{\cdot}l^{-1}$) and 125 $mg{\cdot}l^{-1}$ dimethyl dicarbonate (DMDC). The juice was mixed and kept at 8°C for 24 h to give time for the DMDC to inhibit wild yeast and lactic acid bacteria. The effectiveness of this treatment was verified by plate counting. Fermentations were performed in 550 ml fermentation flasks filled with 500 ml of must. The must was initially inoculated with *S. cerevisiae* EC-1118 strain $1{\cdot}10^7$ $cells{\cdot}ml^{-1}$ -determined by using a Neubauer chamber- to ensure a complete fermentation of the sugars. The sequential- and co-inoculated must were inoculated with 1 ml of rehydrated

Schiz. pombe Sp2 strain (± $1 \cdot 10^8$ cfu·ml^{-1}) as determined by plate counting. All fermentations were performed in triplicated and conducted at 25°C without agitation. Samples were taken every day to test for L-malic acid, to determine sugar concentration and yeast population sizes determined by plate counting on media YPD and the selective media lysine obtained by replica plating. Volatile compounds were analysed when fermentations arrived to a containing less than 5 g l^{-1} of residual sugar. Major volatile wine compounds analyses were carried on by Lab for Flavour Analysis and Oenology (University of Zaragoza, Spain).

2.7. Statistical Analysis

Results were statistically analyzed by one-way ANOVA and the Scheffé test using the SPSS 15.1 statistical software package. The statistical significance was set at $p<0.05$.

3. Results and Discussion

3.1. Cell viability during Different Growth Phases

The desiccation tolerance capacity of nine *Schiz. pombe* strains was assessed during growth using a colony-counting assay as described by [6] (Figure 1). After re-suspending the cells in pure water, the CFU·ml^{-1} mean value for survival was calculated after taking into account the cells' viability before drying. Then, the 9 strains were ranked in ascending order based on the viability rate and divided them into three arrays: Sp3, Sp5 and Sp6, <8% viability; Sp1 Sp4, and Sp7, 8-17% viability; and Sp2, Sp8 and Sp9, >17% viability (Table 1). The viability timeframe evaluated for all strains showed that the highest tolerance to dehydration occurred during the early stationary phase, between 18 h and 20 h. These results differ from previous observations for *S. cerevisiae*, which reached its highest desiccation tolerance capacity during the late stationary phase, before the decline phase [2].

3.2. Evaluation of Temperature and Time during Cell Rehydration

The temperature and the time course have a direct effect on cell viability during rehydration. During this process, *S. cerevisiae* cells have shown a loss of up to 30% of soluble compounds due to the non-functionality of the cell membrane [3, 28]. Therefore, a more rapid functionality of the membrane may be beneficial for the viability of the rehydrated yeast cells. The temperature and time required to complete the rehydration process were correlated with desiccation tolerance. Cell viability was determined using a colony-counting assay for three *Schiz. pombe* strains (Sp2, Sp3 and Sp4) that demonstrated different viability rates. The viability at temperatures between 20°C and 60°C after 30 min of exposure to each temperature and at durations of exposure ranging from 5 min to 45 min at 37°C were assessed (data not shown). The incubation of cells at 55°C and 60°C resulted in a decrease of more than 80% for all strains, but no significant difference was observed between strains at temperatures between 20°C and 50°C. No statistically significant differences in the viability rate were observed for the three evaluated *Schiz. pombe* strains at any of the time points during incubation at 37°C. These results suggest that *Schiz. pombe* strains reach their best values for desiccation tolerance 10 min earlier than *S. cerevisiae* under similar conditions [3]. For the remainder of experiments, *Schiz. pombe* cells were rehydrated the by incubating them at 37°C for 5 min.

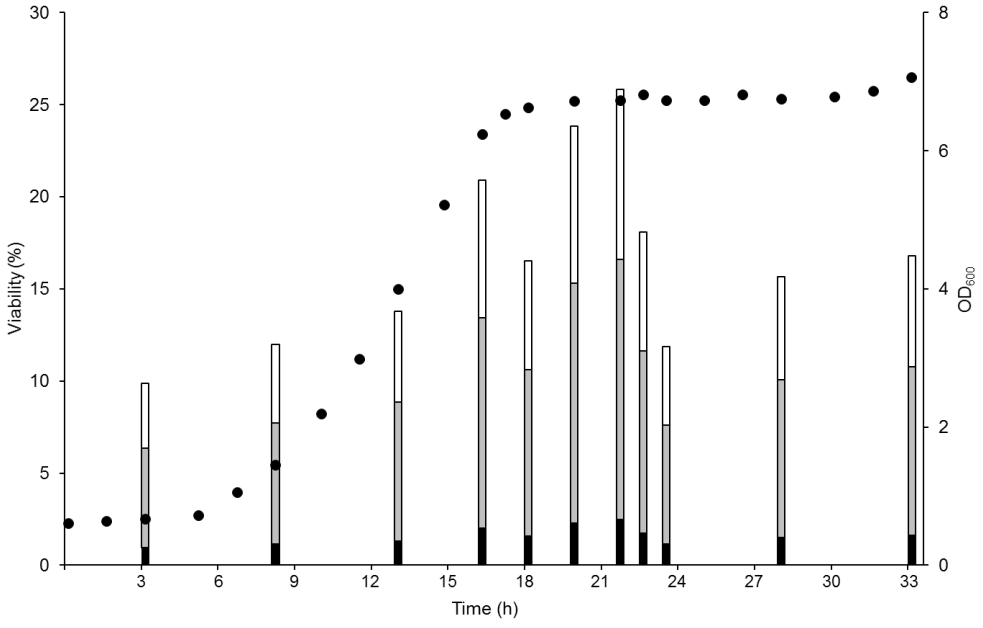

Figure 1. *Schiz. pombe* cell viability based on the cellular physiological state. Both the Sp2 (•) strain growth curve and the viabilities (%) obtained at different time points for the Sp3 (black bars), Sp4 (gray bars) and Sp2 (white bars) strains are provided as a representative example of the evaluated strains. Values shown are means of at least n=3 independent samples ± standard deviation (SD). The SD values were lower than 15%

3.3. Evaluation of Cell vitality under Several Rehydration Conditions

The first attempt to enhance dehydration stress tolerance was performed by re-suspending the cells for each *Schiz. pombe* strain prior to the drying process in 5%, 10%, 20% and 30% trehalose; deionized water was used as the reference condition. The survival rates of the Sp2, Sp3 and Sp4 strains in water and in 20% and 30% trehalose were very low, with none of the strains exhibiting greater than 30% viability (Figure 2). However, the Sp2 and Sp4 strains showed a significant increase in cell viability of approximately 30% and 20% when re-suspended in 5% and 10% trehalose, respectively, compared with the pure water control.

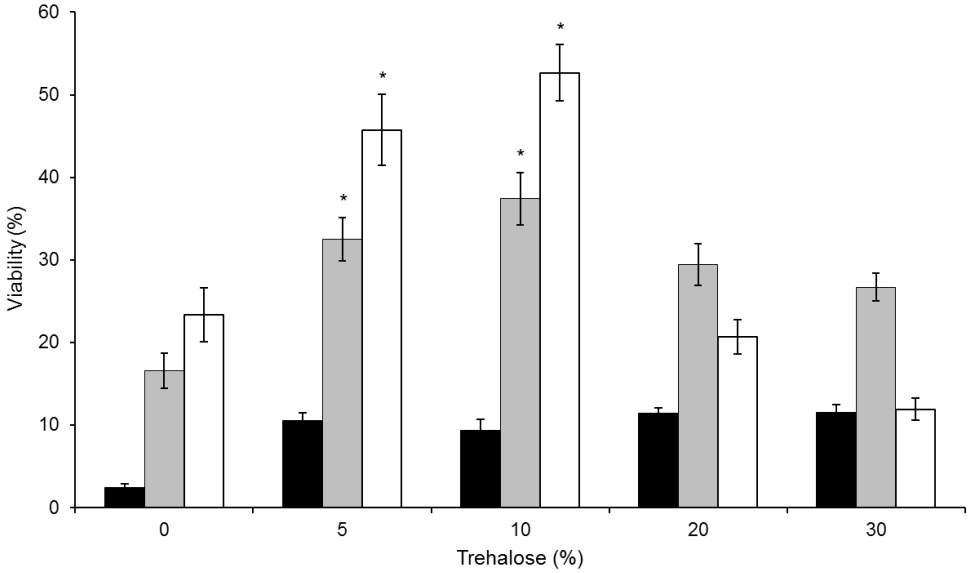

Figure 2. A Figure 2. Effect of trehalose treatment on *Schiz. pombe* cell viability following air drying and rehydration. The scale of viability (%) indicates the percentage of experimental values for the Sp3 (dark bars), Sp4 (gray bars) and Sp2 (white bars) strains. Values shown are means of at least n=3 independent samples ± SD. *Indicates $p<0.01$ compared to the 0% trehalose condition for each strain

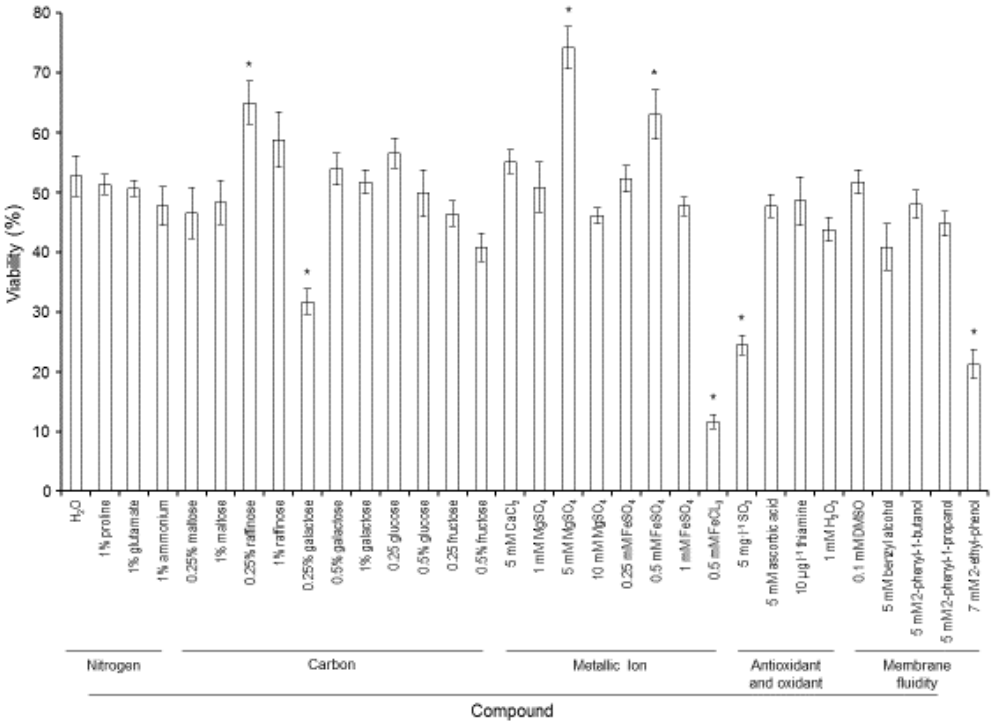

Figure 3. A sample line Figure 3. Effect of re-hydration treatments on cell vitality. Sp2 cells dried in the presence of 10% trehalose were incubated at 37°C for 5 min in pure water or in the presence of nitrogen, carbon, metallic ions, oxidants/antioxidants and membrane fluidity compounds. The values represent the means of three independent experiments ± SD. *Indicates $p<0.05$ compared to the reference condition (H_2O)

Trehalose, which is present during yeast drying, has been shown to act as a membrane protector that reduces the membrane phase-transition temperature during the rehydration process [29]. Our data coincide with previous observations for two different *S. cerevisiae* strains [6]. For the remainder of the experiments, the *Schiz. pombe* cells were re-suspended in 10% trehalose before drying. To overcome the drop in yeast viability during this process, several types of rehydration media were tested. Five groups of media were used for dried Sp2 cells: carbon, nitrogen, metallic ions, oxidants/antioxidants, and membrane fluidity compounds (Figure 3). It was suggested that doubling the intracellular proline levels of desiccated *S. cerevisiae* might lead to a five-fold increase in the survival rate [30]; however, this report does not agree with our findings for proline rehydration. Proline rehydration in the present study showed similar mean values of $CFU \cdot ml^{-1}$ compared to the control cells and the cells rehydrated in the presence of ammonium and monosodium glutamate. Our results are in agreement with previous studies in which supplementation during the rehydration process in the presence of nitrogen compounds did not improve *S. cerevisiae* survival rates [3].

Other studies that have investigated whether the response to rehydration involves regulation at the level of transcription and/or translation reported changes in sugar utilization [31]. The viability capacity of rehydrated Sp2 cells has been assessed in the presence of carbon sources that are metabolized more quickly (glucose and fructose) and more slowly (raffinose and maltose) than trehalose [32]. Raffinose is a trisaccharide composed of glucose, fructose and galactose, and increasing its concentration by 0.25% resulted in an increased cell viability of 15% (Figure 3). Considering that increasing the galactose, glucose and fructose concentrations did not provide any beneficial effects, this finding may suggest that raffinose plays a role as a carbon store rather than as a faster energy supplier. The availability of certain metal ions in the fermentation media has been reported to be a key factor in the performance of Schiz. *pombe* [33]. To assess any impact on yeast viability, the availability of several ions during the rehydration process were evaluated (Figure 3). The ferric (Fe^{+3}) ion had a harmful effect on yeast viability; however, there was a beneficial increase in cell viability when the medium was complemented with 5 mM Mg and 0.5 mM ferrous (Fe^{+2}) ion (74% and 65%, respectively, compared with the pure water control). Rehydrating conditions co-complemented with 5 and 10 mM calcium, 1 and 10 mM magnesium, or 0.25 and 1 mM ferrous ion exhibited profiles that were broadly similar to complementation with 1 and 10 mM calcium (data not shown). These results suggest that the ferric ion suppresses yeast viability via an antagonistic mechanism [34]. In [33] was revealed that magnesium was essential for re-establishing cell cycle progression in *Schiz. pombe* cells.

Additionally, magnesium and iron are involved in numerous essential functions in yeast physiology, such as respirofermentive metabolism and the response to environmental stress [35, 36]. Industrial biomass production occurs in a high concentration of molasses and aeration, which induces *Schiz. pombe* to initiate an adaptive response to osmotic and oxidative stress [37]. However, the drying process makes the membranes porous, thus exposing the cell components to the harmful effect of environmental oxygen [38]. To address this issue, several rehydration solutions complemented with oxidant or antioxidant agents have been designed to evaluate the effects on cell viability [23]. Ascorbic acid, thiamine, and hydrogen peroxide showed no significant effects on cell viability; however, sulfur dioxide had a detrimental effect, reducing cell viability to 25%. A relationship between higher stress sensitivity of cells in the exponential phase compared to cells in the stationary phase was previously reported in [3]. This finding may explain the high tolerance of non-growing cells to hydrogen peroxide but not the harmful effect of sulfur dioxide. Additionally, a rehydrating mix containing 1 mM hydrogen peroxide and 0.5% glucose increased cell death to 70% (data not shown). This high sensitivity of *Schiz. pombe* cells to hydrogen peroxide in the presence of fermentable carbon sources is in agreement with a previous report [25]. In [39] was reported that in fermentation conditions *Schiz. pombe* strains showed a three-fold increase in sulfur dioxide survival rate compared to *S. cerevisiae*. Our results differ from previous observations in *S. cerevisiae*, which did not show any change in its capacity for desiccation tolerance under sulfur dioxide rehydration conditions [3]. The initial effects on the cell during the rehydration process are changes in membrane fluidity. Faster membrane activity allows the cells to proliferate after adapting to new conditions. Taking these observations into account, we explored whether changes in membrane fluidity had an effect on the vitality of rehydrated *Schiz. pombe* cells (Figure 3). Dimethyl sulfoxide and benzyl alcohol are routinely used to rigidify and fluidize membranes, respectively [40, 41]. Therefore, we evaluated membrane-fluidizer agents with hydrophobic properties, including 2-ethyl-phenol, 2-phenyl-1-propanol, and 2-phenyl-1-butanol. Our results showed that membrane fluidity agents did not enhance *Schiz. pombe* cell viability during the rehydration process. Moreover, in some cases complementation with agents such as in 2-ethyl-phenol could be detrimental; this finding is in agreement with previous results in *S. cerevisiae* [3]. Our results indicated that 0.25% raffinose, 5 mM magnesium and 0.5 mM ferrous ion showed a similar significant increase in cell viability after stress imposition.

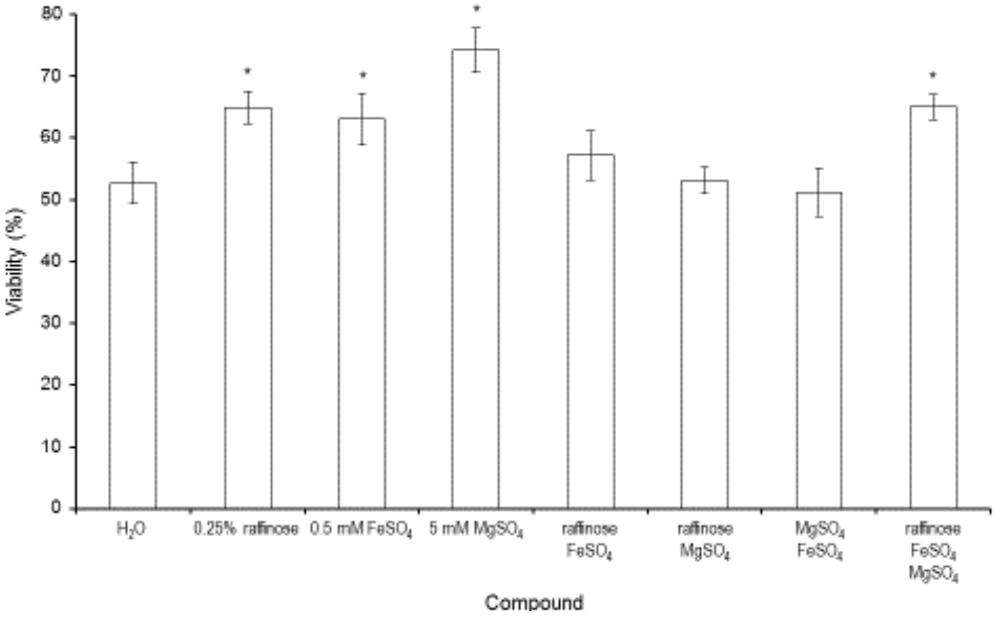

Figure 4. Effect of re-hydration treatments on cell vitality. Representative example of Sp2 cells dried in the presence of 10% trehalose and incubated at 37°C for 5 min in pure water or in the presence of 0.25% raffinose, 0.5 mM $FeSO_4$, 5 mM $MgSO_4$, 0.25% raffinose + 0.5 mM $FeSO_4$, 0.25% raffinose + 5 mM $MgSO_4$, 5 mM $MgSO_4$ + 0.5 mM $FeSO_4$ or 0.25% raffinose + 0.5 mM $FeSO_4$ + 5 mM $MgSO_4$. The values are the means of at least three independent experiments ± SD.*Indicates $p<0.05$ compared to the reference condition (H_2O)

Finally, the effect of these agents during the dehydration and rehydration processes as simple and combined preparations for the *Schiz. pombe* strains that showed different stress tolerances were evaluated. The viability of Sp2, Sp3 and Sp4 cells increased by more than 10% when dried in the presence of 10% trehalose and rehydrated in the presence of 5 mM $MgSO_4$. However, there was no significant improvement in the viability values obtained for the rehydrated preparations in 0.25% raffinose, 0.5 mM $FeSO_4$, 5 mM $MgSO_4$, 0.5% raffinose + 0.5 mM $FeSO_4$, 0.5% raffinose + 5 mM $MgSO_4$, 5 mM $MgSO_4$ + 0.5 mM $FeSO_4$ or 0.5% raffinose + 0.5 mM $FeSO_4$ + 5 mM $MgSO_4$ (Figure 4). Moreover, co-complementation of the Sp2, Sp3 and Sp4 cells with the above-mentioned preparations before the dehydration process was somewhat detrimental to cell viability in comparison with the application of the same solutions during the rehydration process (data not shown). Our results indicate that *Schiz. pombe* cells dried in the presence of 10% trehalose and rehydrated in 5 mM magnesium exhibited enhanced viability; thus, these results might directly influence early fermentation of an over-active starter yeast.

3.4. $MgSO_4$ Prevents Cellular ROS Accumulation

Imposing dehydration stress by reducing intracellular ROS accumulation has been reported to enhance viability [42, 26]. Therefore, we investigated whether the higher viability rate of the cells rehydrated in the presence of $MgSO_4$ might be due to differences in ROS accumulation [43]. After 10 min of rehydration in H_2O, 5 mM ascorbic acid or 5 mM $MgSO_4$, Sp2, Sp3 and Sp4 cells were inoculated into YPD medium. ROS-accumulating cells were evaluated over time using the dihydroethidium (DHE) assay (Figure 5A). After 1 h of incubation, the rehydrated cells in H_2O exhibited approximately 40% DHE-positive fluorescence; after 2 h of incubation, 10% fewer cells were detected under intense intracellular DHE staining. A similar significant reduction in ROS accumulating-cells was observed for cells rehydrated in the presence of ascorbic acid, although this effect was observed 1 h earlier than under H_2O conditions. Unexpectedly, *Schiz. pombe* cells that were rehydrated in the presence of MgS_4O did not show any changes in fluorescence between 5 min and 2 h, with similar percentages of DHE-positive cells compared to those incubated for 1 h in ascorbic acid and 2 h in H_2O. Considering the lower intracellular ROS levels of the cells rehydrated in the presence of $MgSO_4$, our results suggest that $MgSO_4$ allows the cells to prevent the accumulation of ROS; this process seems to occur during rehydration and very early after incubation in the YEPD medium. Therefore, the faster scavenging of ROS by cells rehydrated in $MgSO_4$ compared to ascorbic acid might explain the 18% differences observed in cell viability (Figure 3).

3.5. Evaluation of Cell Vitality under Several Rehydration Conditions

Next, the relatively improved rehydration conditions were correlated with a shorter lag phase (λ) once the cells were inoculated into complete medium compared to the controls. The cells rehydrated in $MgSO_4$ and in ascorbic acid + $MgSO_4$ showed a λ that was 208 min and 86 min longer than the controls, respectively, whereas the lag time was 220 min shorter in cells rehydrated in ascorbic acid (Figure 5B). The ascorbic acid conditions showed a 1.53-fold increase in

doubling time DT compared to the controls, whereas the $MgSO_4$ condition showed 1.51-fold decrease in DT compared to the H_2O controls. However, rehydration in the presence of ascorbic acid + $MgSO_4$ did not result in any significant differences in DT. Cells rehydrated in raffinose and $FeSO_4$ did not exhibit any significant growth differences compared to the controls (data not shown). These results, together with the cell-accumulating ROS results, might confirm that there is no correlation between lower ROS values during stress imposition and rehydration conditions with a shorter λ phase (Figure 5A-B). Under magnesium conditions, there was an increase in the λ phase even though the accumulation of intracellular ROS stopped more quickly after stress induction. Similar variations in the λ phase were previously reported in [44], in which the reduction in the accumulation of intracellular ROS during dehydration stress was mediated by an over-expression of the encoding gene for *S. cerevisiae* hydrophilin.

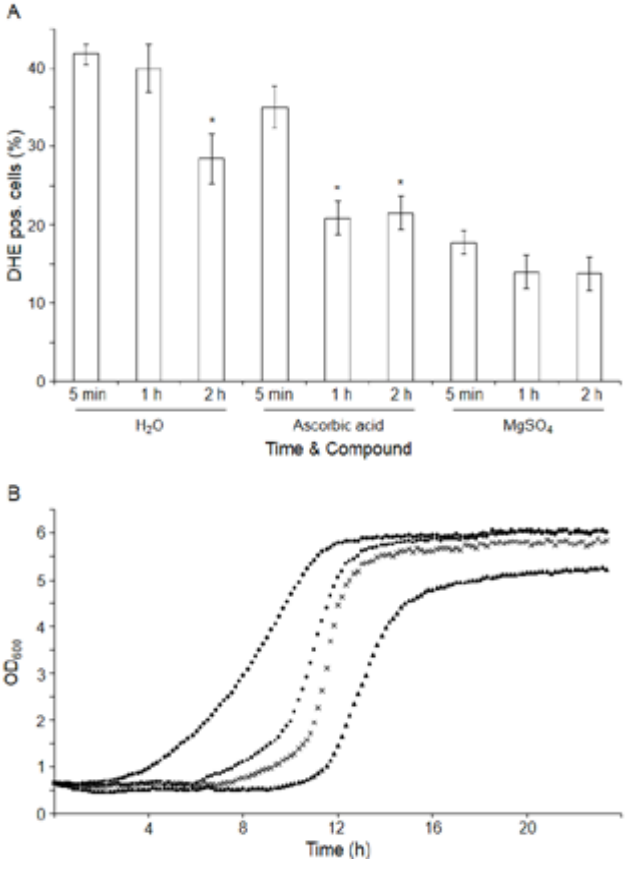

Figure 5. Chronology of ROS accumulation. A) Quantification of the accumulation of ROS by yeast cells after cell rehydration using DHE staining. Values are the means of n=4 determinations ± the SD. *Significant differences ($p \leq 0.05$) with respect to the 5 min step of each compound. B) Growth curves after rehydration in the presence of ascorbic acid (♦), H_2O (●), ascorbic acid + $MgSO_4$ (**x**) and $MgSO_4$ (▲). The curves are a representative example of growth experiments performed with yeast cells obtained from three independent compound-rehydration processes

3.6. Fermentations

The three treatments began soon after inoculation. The *S. cerevisiae* EC-1118 and the join and the sequential treatments (*Schiz. pombe* ADY-Sp2 and EC-1118) the wines reached values lower than 2 $g \cdot l^{-1}$ sugar in 6 days (Figure 6A). After 9 days fermentation, both double- and single-fermentations finished with an ethanol content of 12.7 ± 0.2% and 11.9 ± 0.3% $v \cdot v^{-1}$ ethanol, respectively. On day 6, prior to the addition of the rehydrated Sp2 cells to the sequential fermentations, the ethanol content was around 12% $v \cdot v^{-1}$ (data not shown). In the combined treatments, nearly 90% of the malic acid was metabolized 6 days after *Schz. pombe* inoculation (Figure 6B). Nevertheless, after day 9 in the EC-1118 single treatment only 12.5% of the total malic acid was metabolized. At the end of fermentation in the join and sequential treatments Sp2 cell viability was 2% and 10% respectively, while EC-1118 showed a viability of around 95% (data not shown). These data suggest that *Schiz. pombe* ADY is very efficient in malic acid utilization in sequential or join treatment, as previously observed using fresh inoculums [20].

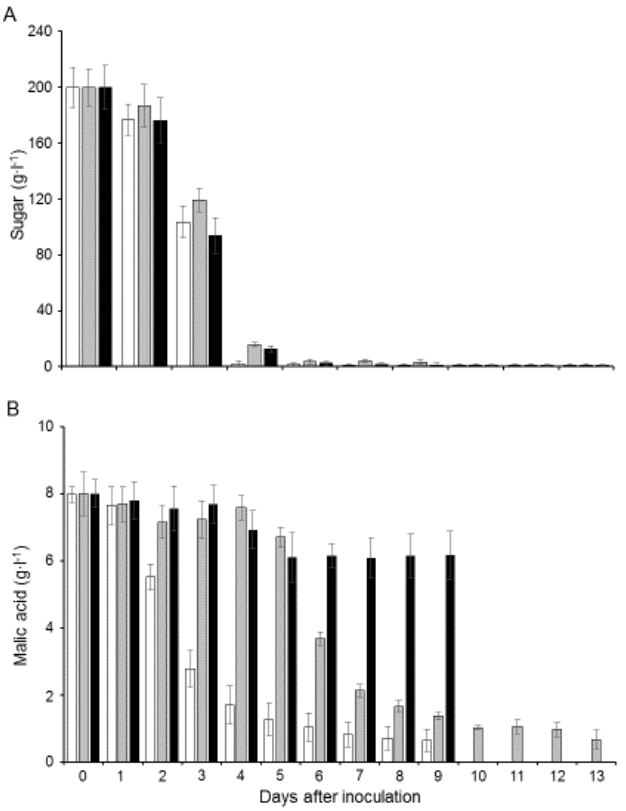

Figure 6. Consumption of sugars (A) and malic acid (B) during alcoholic fermentation in response to three yeast treatments: *S. cerevisiae* EC-1118 and *Schiz. pombe* Sp2 (white bars), EC-1118 + Sp2 at day 5 (grey bars) and EC-1118 (black bars). The values are the means of three independent experiments ± SD

3.7. *Schiz. pombe* ADY Metabolic Behavior was not Negatively Affected

The metabolic behavior of Sp2 ADY during fermentation was evaluated by comparing the amounts of the principal volatile compounds determined in the experimental wines after completion of alcoholic fermentation (Table 2). The three treatments did not exhibit significant differences of

total volatile compounds. In fact, only ten of the 24 evaluated compounds showed some differences among treatments. Furthermore, the join treatment (EC-1118 + Sp2) shows the higher values for most compounds such as ethyl acetate, isoamyl acetate, ethyl octanoate, isobutnol, butyric acid and isovaleric acid, suggesting a major fruited or floral profile.

Table 2. Concentration of volatile compounds produced at the end of all fermentation processes

Volatile compounds	OTV	Fermentation treatment ($mg \cdot l^{-1}$)		
		Join	Sequential	EC-1118
Acetaldehyde	0.5	8.98±1.19[b]	7.90±1.30[b]	11.37±5.36[a]
Acetoin	150	1.80±0.13[b]	1.83±0.32[b]	3.20±0.18[a]
Ethyl acetate	12.3	30.14±1.02[c]	16.46±1.71[b]	21.28±2.91[a]
Isoamyl acetate	0.03	1.42±0.04[c]	0.38±0.09[b]	1.04±0.03[a]
Ethyl butyrate	0.125	0.16 ± 0.01[a]	0.13 ± 0.01[a]	0.13±0.01[a]
Ethyl hexanoate	0.062	0.16 ± 0.01[a]	0.10 ± 0.01[b]	0.16 ± 0.02[a]
Ethyl octanoate	0.58	0.03 ± 0.01[a]	0.10 ± 0.05[b]	0.02 ± 0.01[a]
Ethyl decanoate	0.2	0.04 ± 0.01[b]	0.06 ± 0.02[b]	0.13 ± 0.02[a]
Isobutanol	40	54.83 ± 4.84[a]	24.42 ± 2.02[b]	45.79 ± 7.80[a]
1-butanol	150	0.66 ± 0.06[b]	0.62 ± 0.11[b]	0.48 ± 0.14[a]
Isoamyl alcohol	30	131.57±7.58[a]	121.56 ± 3.72[a]	142.77±38.81[a]
1-hexanol	8	0.01 ± 0.00[a]	0.01 ± 0.00[a]	0.02 ± 0.00[a]
Methionol	1	4.66 ± 0.18[a]	5.15 ± 0.60[a]	5.42 ± 1.08[a]
2-phenylethanol	14	19.70 ± 0.15[a]	27.40 ± 3.85[b]	17.99 ± 6.56[a]
Ethyl lactate	154	1.47 ± 0.01[a]	0.64 ± 0.03[b]	1.68 ± 0.16[a]
Diethyl succinate	200	0.04 ± 0.01[a]	0.07 ± 0.01[b]	0.05 ± 0.02[a]
γ-Butirolactone	35	1.43 ± 0.07[a]	1.24 ± 0.04[a]	1.47 ± 0.29[a]
Acetic acid	300	80.97 ± 1.58[a]	98.83 ± 19.42[b]	72.60 ± 18.94[a]
Butyric acid	0.173	0.77 ± 0.02[b]	0.69 ± 0.05[b]	0.49 ± 0.03[a]
Isobutyric acid	2.3	1.47 ± 0.05[a]	1.69 ± 0.25[b]	1.23 ± 0.15[a]
Isovaleric acid	0.033	0.89 ± 0.01[b]	0.67 ± 0.01[a]	0.67 ± 0.17[a]
Hexanoic acid	0.42	2.58 ± 0.03[a]	2.26 ± 0.15[a]	2.31 ± 0.11[a]
Octanoic acid	0.5	7.39 ± 0.44[a]	6.10 ± 0.49[a]	7.60 ± 0.78[a]
Decanoic acid	1	0.39 ± 0.11[b]	0.31 ± 0.05[b]	0.61 ± 0.02[a]
Total		351.6 ± 31.8[a]	318.6 ± 34.3[a]	338.5 ± 48.7[a]

The EC-1118 single fermentation produced 20% higher acetaldehyde concentration (11.37 $mg \cdot l^{-1}$) than the combined treatments, while the sequential treatment showed the highest value for the acetic acid (99 $mg \cdot l^{-1}$). However, it must be highlighted that all of the tested compounds were present at acceptable levels. Our results agree with previous data of grape must treated with *Schiz. pombe* presenting a more complex volatile profile when compared to the one obtained with single-culture fermentation involving *S. cerevisiae* [16].

4. Conclusions

The desiccation tolerance of wine yeasts has enabled the wine industry to work with products with more stable microbiological characteristics. Nevertheless, such products exclude yeast strains that cannot cope with the cellular stress induced during dehydration and rehydration; this topic is of great interest for the beverage industry [6]. Non-*Saccharomyces* yeasts, especially those with interesting oenological properties, do not often survive under desiccation stress. This characteristic is a serious handicap for winemakers who are trained to use active dry yeasts. In the present study, different *Schiz. pombe* strains isolated from the beverage or concentration juice industries from different regions worldwide were characterized. Isolation of the *Schiz. pombe* strain occurred during spontaneous fermentations without prior selection based on oenological characteristics. Previous studies have aimed to improve the tolerance of *S. cerevisiae* to dehydration via compound supplementation before and after stress induction [3, 7, 8, 45]. This approach led us to characterize the cell viability and vitality of nine *Schiz. pombe* ADWYs under a set of physiological conditions. To the best of our knowledge, this is the first systematic study to establish a protocol for developing *Schiz. pombe* as an ADWY. Different techniques to evaluate the influence of the compounds during the dehydration-rehydration process in the studied strains were used. The 'fitness' of an ADWY is related to its ability to maintain cell viability and vitality during the yeast manufacture process, including desiccation and storage [1]. In our study, yeast viability was assessed both directly - by determining the loss of cells after stress imposition and indirectly by assessing the impact of different compounds on ROS accumulation by the cells, as previously reported in [42]. The influence on cell vitality was assessed by determining the biological parameters of optimal growth conditions of the rehydrated cells. The various methods used enabled us to evaluate the effect of several rehydration conditions by uncoupling cell vitality and viability. In conclusion, the presence of both trehalose during the drying process and magnesium during the rehydration process has a synergistic effect on the enhancement of cell viability. Nevertheless, the longer lag phase promoted by the magnesium, which moves into the cell during the rehydration process, promotes sluggish cell activity. Furthermore, no defects were found between wines obtained by single *S. cerevisiae* and *Schiz. pombe* combined treatments as regard to the fermentative performance and volatile compounds influencing wine aroma. On the other hand, the malic acid was nearly completely metabolized by *Schiz. pombe* in combined treatments. These findings indicate that the applied protocol to obtain a *Schiz. pombe* as ADW yeast does not affect its oenological behavior.

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