Tolerance and response of *Saccharomyces cerevisiae* wine strains to stress treatments

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ABSTRACT This study evaluated the stress tolerance and stress response of three indigenous *Saccharomyces cerevisiae* strains and one reference strain against temperature (45, 50, and 55 °C) and ethanol (12 and 14 % v/v) stresses over time by assessing the yeast counts and intracellular trehalose content. The maximum decrease in the yeast counts was at 55 °C and 14 % ethanol. At 45 °C and 14 % ethanol the highest intracellular trehalose content was induced for all strains ($P < 0.05$). The correlation between the trehalose content and Log N levels of the strains was significant with the correlation coefficient (r) 0.62 and 0.39 for strains 2 and 7 against temperature stress. A strong and significant correlation was also determined with r values for strains 2,7, 47 in response to the ethanol stress, respectively ($P < 0.05$).

KEYWORDS *Saccharomyces cerevisiae*; Temperature stress; Ethanol stress; Intacellular trehalose

1. Introduction

Wine fermentation is a dynamic and complex process, during which yeasts are exposed to several possible stress factors (Varela et al., 2005). During the entire process of fermentation, from strain storage to the product formation, microorganisms are exposed to several stress factors, such as freeze–thaw treatment (Park et al., 1997), high temperature (Lin et al., 2012), high ethanol concentration, osmotic pressure (Auesukaree et al., 2009), oxidative stress (Lewis et al., 1997), and high SO_2 concentration (Cocolin et al., 2004).

High temperature leads to some modifications of the physical properties of the cellular membrane. The membrane becomes hyperfluid, and the lamellar phase may destabilize, which ultimately results in the loss of bilayer integrity (Guyot et al., 2015). An increase in the temperature beyond the tolerance limit of the organism may denatured cell membrane proteins, increase membrane blebbing, and induce cell lysis (Ancín-Azpilicueta et al., 2012). On the other hand, high ethanol concentration influences the composition, structure and permeability of the cell membrane. Ethanol accelerates the passive entrance of protons from the medium to the cytoplasmic space and dissipates the proton motive force necessary for amino acid transport into the cell (Cartwright et al., 1986). The presence of ethanol in growth media may influence the redistribution of fatty acids in structural lipids and other processes of homeoviscous adaptation (Šajbidor et al., 1995).

Yeasts can respond to various stresses through the reprogramming of cellular activities to ensure survival in challenging conditions; they protect essential cell components or increase the content of some cellular components that facilitate "physiological" cellular activities during recovery (absence of stress factors). One such component is trehalose, a nonreducing sugar that contains two glucose units linked via α, α-1, and 1-glycosidic bonds. Trehalose is a white, odorless powder with a relative sweetness of nearly 45 % of that of sucrose—this sugar is present in several species of bacteria, yeasts, molds, fungi, plants, and invertebrates (Wang et al., 2013).

When yeast cells are subjected to high temperature (Gomes et al., 2002; Nakamura et al., 2009; Cai et al., 2018; Magalhães et al., 2018) and ethanol concentration (Vianna et al., 2008; Ding et al., 2009; Mahmud et al., 2010; Viana et al., 2014; Wang et al., 2014; Divate et al., 2016; Elbakush and Güven, 2021) their cellular trehalose content increases as a self-protection strategy. The cell-protective effect of trehalose is still unclear at the molecular level, and hypotheses regarding water replacement and vitrification have been proposed (Trevisol et al., 2011). According to the first hypothesis, trehalose replaces the water molecules that interact with polar head groups of lipids, thus maintaining the membrane integrity, or it may act as a potent stabilizer when a cellular injury occurs, such as the aggregation of denatured proteins (Crowe et al., 1984). The vitrification hypothesis states that trehalose forms a glassy matrix that resembles the glassy form of ice beyond a glass transition temperature, thus encapsulating the biomolecules (Wang et al., 2013).

This study evaluated the tolerance level of four *Saccharomyces cerevisiae* strains against temperature (45 °C, 50 °C, and 55 °C) and ethanol (12.14 %) stress factors by determining the yeast counts and stress response measured in terms of cellular trehalose content. The correlation between the change in the yeast count and cellular trehalose content of each strain under stress was also determined.

2. Methods

2.1 Yeast strains and culture conditions

Three indigenous *S. cerevisiae* wine strains—*S. cerevisiae* Kalecik II (strain 2), *S. cerevisiae* Narince 3 (strain 7),

DOI: 10.52292/j.laar.2024.1147
Received: August 16, 2022.
Accepted: March 18, 2023.
Recommended by Diego Lomonaco.

and *S. cerevisiae* 1A (strain 47)—and one commercial wine strain (reference)—Fermicru AR2 *S. cerevisiae* No. LO122 (strain S122)—were used in the study. Among them, the indigenous *S. cerevisiae* strains were isolated from Turkish wines and obtained from the Ankara University culture collection. Yeasts were isolated and identified based on a previous study (Bağder Elmacı et al., 2014).

For the growth and cultivation of the strains, the laboratory temperature was maintained at 30 °C and yeast peptone dextrose (YPD) growth medium (Sigma Aldrich, Steinheim, Germany) was used.

2.2 Stress treatments

Yeast cells were pregrown in YPD broth for 18 h; these pregrown strains were taken (1 mL) and inoculated into 10 mL YPD broth and incubated until they reached the mid-log phase. The growth curve of the strains in the YPD broth was drawn with the data points gathered using a spectrophotometer (Optima, SP 300, Japan) at 600 nm, based on previous studies (data not shown). When the cells reached the mid-log phase (approximately 4 h), they were exposed to various stress treatments. Strains grown until the mid-log phase were maintained at 30 °C and sampled at regular intervals, which served as control.

For temperature treatment, the temperatures of 45, 50, and 55 °C were selected; the cells were maintained at these three temperatures for different times (max. 360 min). During the highest temperature treatments, the strains were monitored at close intervals because high temperature may cause the rapid inactivation of the strains, resulting in considerably high inactivation rates. The process was as follows: yeast suspensions grown for 4 h were placed in a water bath set at 60–80 °C (temperature of the water bath depends on the desired treatment temperature: for lower temperature treatments, lower water bath temperatures were used) under shaking conditions to quickly reach the desired temperature. The temperature was controlled with a 10 mL YPD broth uninoculated control tube. When the control tube reached the desired temperature (approximately in 1–2 min), suspensions were placed into another water bath set at the desired temperature and incubated with occasional stirring.

For ethanol treatment, the concentrations of 12 % and 14 % (v/v) were selected; cells were treated for 240 min at 30 °C with vigorous shaking. Ethanol pretreatments were as follows: ethanol was added to the 4 h cell suspension to reach the desired final concentration of the suspensions. Analyses were performed by obtaining samples aseptically at different time points. All stress treatment experiments were performed in triplicates.

2.3 Determination of colony numbers

The survival of each yeast strain after stress treatments was measured in terms of colony-forming units by plating the appropriate decimal dilution of the cell suspension on YPD agar immediately after each stress treatment. The culture was incubated at 30 °C for 48 h, and the colonies that grew on the Petri dish were counted.

2.4 Determination of the cellular trehalose content

The intracellular trehalose content was determined by dividing the concentrations (mg/mL) calculated from the trehalose standard curve, which was generated using the data on absorbance measured against different trehalose concentrations, by the cellular dry weight at the end of each treatment; the results were presented as mg/g of cellular dry weight. The equation of the standard curve was as follows:

$$y = 1.8033 \cdot x + 0.023 \quad (1)$$

and the R^2 value was 0.998. Sample preparation and trehalose content determination were performed following the sulfuric acid–anthrone method suggested by Mahmud et al. (2010).

To determine the cellular dry weight during stress treatments, 1 mL of strain suspensions were sampled at specified time intervals and filtered through polyvinylidene difluoride filters (PVDF, 0.45 µm), which were previously dried in a vacuum oven (OV 11, Jeio Tech, Korea). The initial weights were recorded, and then the filters were stored in a vacuum oven at 70 °C for approximately 24 h. Sufficiently dry filters were weighed after keeping them in a desiccator for a while. The final weight was then subtracted from the initial weight, and the dry weight of the strains was thus determined in mg/mL. Experiments were performed in triplicates.

2.5 Statistical analysis

The effect of different factors on dependent variables was determined via the analysis of variance (ANOVA) test using SPSS Statistics for Windows (version 16.0; IBM Corp., Armonk, NY, USA) program at 95 % confidence interval (CI). The stress treatments that triggered the differences observed in the ANOVA analyses were assessed via Tukey's test using the same program. Correlation analyses were performed using the Statistica package program (1995; StatSoft, Tulsa, OK, USA).

3. Results and discussion

In this study, the time-dependent tolerance levels and responses of three indigenous *S. cerevisiae* isolated from Turkish wines and one reference strain against different stress factors were determined. Stress conditions can not only reduce the number of cells but also simultaneously increase the content of vital cellular components to improve the chances of survival.

3.1 Responses of yeasts against temperature and ethanol stresses.

3.1.1 Temperature stresses

To evaluate the effects of temperature stress on strains coded 2, 7, 47, and S122, a "full factorial" study design was planned. However, the fact that temperatures of 45 and 50 °C critically reduced the yeast count in the culture medium over time, shorter intervals were used compared with control (30 °C) treatments. Because the strains showed a faster inactivation rate at 55 °C, the monitoring interval between two time points was shorter. Therefore,

during statistical analyses, data obtained at 30 °C, 45 °C and 50 °C, and 55 °C were evaluated separately. Table 1 presents the colony count of tested strains during the different temperature treatment period. As expected, the cell count increased over time at the optimum growth temperature of 30 °C. The counts of strains 2, 7, 47, and S122 after 360 min were determined as the log values of 7.37, 7.49, 7.17, and 7.48 log cfu/mL, respectively. In the ANOVA analysis, the strain type, treatment duration, and bilateral interaction exhibited significant effect on the counts of yeasts grown at 30 °C ($P < 0.05$). When data were analyzed using Tukey's test, strain S122 exhibited the highest yeast count at the earliest time point (120 min) compared with the other strains ($P < 0.05$). The count of yeasts exposed to the high temperatures decreased over time (Table 1). In particular, although the counts of all strains at 45 °C at 0 min did not differ much from those of the control groups, their values decreased after 120 min, to reach values of 3.75, 3.75, 3.75, and 3.94 log cfu/mL, respectively.

Data obtained at 50 °C (Table 1) revealed that the yeast count began to decline at beginning of stress treatment. After 240 min, the counts of strains 2, 7, 47, and S122 in the medium were as the log values of 1.69, 1.42, 1.25, and 1.68 log cfu/mL, respectively. For these four strains, the effects of strain type, temperature, and treatment duration on the strains at 45 and 50 °C were analyzed using ANOVA. The effect of temperature stress on the count of strains was significantly influenced by all factors as well as all double and triple interactions ($P < 0.05$). Tukey's test was used to identify the stress treatment with considerable effect. The counts obtained at 0 min at 45 °C were higher and different from those obtained in all other treatments evaluated together ($P < 0.05$). In addition, the yeast counts of all strains decreased rapidly at 120 min at 45 °C and remained unaltered after this period ($P > 0.05$). At 50 °C, the yeast count of all strains at 0 min was identical to that at 45 °C at $\geq$ 120 min ($P < 0.05$); however, the counts of the strains 2, 7, and 47 showed a significant decrease ($P < 0.05$) at 120 min, similar to that observed at 45 °C ($P < 0.05$).

The strain S122 was found more stable under both 45 °C and 50 °C depending on treatment duration. The maximum decrease in the yeast count was at 55°C. After 155 min at 55 °C, the counts of strains 2, 47, and S122 were 0.64, 1.75, 1.19 log cfu/mL, respectively; however, no growth was detected for strain 7. The effect of strain type and treatment duration on the yeast count at 55 °C at various time points was analyzed using ANOVA. Significant effect of each factor and bilateral interaction on the yeast count was significant ($P < 0.05$). As shown in Table 1, when the differences due to various treatments were analyzed using Tukey's test, the time-dependent counts of strains 2, 7, and 47 at 45 min were similar to that of strain S122 at 125 min ($P < 0.05$).

These data suggest that strain S122 remained more stable also at 55 °C over time than the other strains. Ivorra et al. (1999) assessed the effect of temperature treatment at 45 °C for 2 h on wine-producing strains of yeasts and reported that the lowest survival rate was 0.45 % (6–8 times lower than that of other strains). Gomes et al. (2002) stated that the survival rates of two different strains of *Schizosaccharomyces pombe* after incubation at 48 °C for 30 min were 0.37 % and 12.5 %, respectively.

In the present study, strains 2, 7, 47, and S122 were incubated at 45 °C for 2 h for to compare with the findings of the previously mentioned studies Ivorra et al. (1999) and Gomes et al. (2002). The viability rates were higher, 60.48 %, 60.38, 60.28 and 60.06, respectively. Thus, the strains used in this study may be more resistant to temperature stress than those used in the studies by Ivorra et al. (1999) and Gomes et al. (2002). Lin et al. (2012) evaluated the effect of high temperature on the fermentation ability of *S. cerevisiae* BY4742 and demonstrated that the growth and ethanol production of strains decreased drastically at 50 °C owing to the inactivation of the strain at the high temperature. They reported that high temperature may negatively affect cellular exchange; reduce the amount of soluble components in the cell or increase the level of toxic compounds in the cell, including ethanol. They further indicated that high temperature has a negative effect on the denaturation of ribosomes and enzymes on the permeability of the cell membrane.

The counts determined immediately before the stress treatments of the strains growing until the mid-log phase under optimum conditions (30 °C, 0 h) were 6.20, 6.21, 6.22, and 6.56 log cfu/mL for strains 2, 7, 47, and S122, respectively (Table 1). Considering ethanol treatments, the counts of strains 2, 7, 47, and S122 were the log values of 4.81, 5.31, 4.96, and 5.67 log cfu/mL, respectively, at 0 min (at the time of stress exposure) and 3.60, 3.42, 2.21, and 3.34 log cfu/mL, respectively, at 240 min in the media containing 12 % ethanol.

3.1.2 Ethanol stresses

Applying 14 % ethanol, these values were 3.79, 3.00, 3.20, and 4.37 log cfu/mL at 0 min as well as 1.66, 1.28, 1.84 and 1.92 log cfu/mL at 240 min, respectively (Table 2). As expected, 14 % ethanol concentration resulted in a higher rate of strain inactivation than 12 % ethanol at the time of first exposure to ethanol stress.

3.2 Changes in intracellular trehalose level against temperature and ethanol stresses

3.2.1 Temperature stresses

The 50 °C treatment is among the high temperature treatments that lead to strain inactivation. Under the 50 °C treatment, the intracellular trehalose content of strains 2, 47, and S122 reached the highest value at 60 min, while no significant change was observed for strain 7 (Table S1, Supplementary Material). The trehalose contents of strains 2, 7, 47, and S122 at 0 and 240 min were 17.94 – 25.78, 11.71 – 14.29, 7.26 – 8.07, and 11.23 – 9.62 mg/g of cellular dry weight, respectively.

The strains were most rapidly inactivated at 55 °C (Table 1). The intracellular trehalose content increased during incubation, similarly to the finding of other temperature treatments (Fig. 1). The intracellular trehalose contents of strains 2, 7, 47, and S122 under the 55 °C treatment at

Table 1: Yeast counts (Log cfu/mL) of strains incubated at 30 °C (control) or subjected to different temperature treatments.

Temperature (°C)	Time (min)	Strain codes Log N (cfu/mL)[1]			
		2	7	47	S122
30 (control)	2	6.20 ± 0.05^{d}	6.21 ± 0.08^{d}	6.22 ± 0.24^{d}	6.56 ± 0.02^{cd}
	120	6.51 ± 0.08^{cd}	6.56 ± 0.28^{cd}	6.57 ± 0.26^{cd}	7.18 ± 0.01^{ab}
	240	7.18 ± 0.06^{a}	7.12 ± 0.08^{ab}	6.83 ± 0.24^{bc}	7.49 ± 0.03^{a}
	360	7.37 ± 0.09^{a}	7.49 ± 0.02^{a}	7.17 ± 0.27^{ab}	7.48 ± 0.02^{a}
45	3	6.05 ± 0.16^{a}	6.30 ± 0.10^{a}	5.80 ± 0.27^{ab}	6.13 ± 0.20^{a}
	120	3.75 ± 0.44^{cdef}	3.75 ± 0.27^{cdef}	3.75 ± 0.53^{cdef}	3.94 ± 0.20^{cde}
	180	3.30 ± 0.63^{cdefgh}	2.05 ± 0.10^{cdefghij}	3.35 ± 0.65^{cdefgh}	3.63 ± 0.13^{cdefg}
	240	2.72 ± 0.60^{cdefghij}	2.10 ± 1.37^{fghij}	2.33 ± 0.89^{cdefghij}	2.98 ± 0.03^{cdefghij}
50	3	4.30 ± 0.72^{bc}	3.77 ± 0.47^{cdef}	4.02 ± 0.35^{cd}	3.44 ± 0.69^{cdefgh}
	120	2.03 ± 1.10^{ghij}	1.76 ± 1.49^{hij}	1.98 ± 0.88^{hij}	2.36 ± 0.95^{defghij}
	180	1.69 ± 0.26^{ij}	2.42 ± 0.62^{defghij}	ND[4k]	2.39 ± 1.42^{efghij}
	240	1.69 ± 0.26^{ij}	1.42 ± 1.31^{jk}	1.25 ± 0.3^{jk}	1.68 ± 0.80^{ij}
55	3	4.00 ± 0.38^{a}	4.25 ± 0.04^{a}	3.73 ± 0.42^{ab}	3.78 ± 0.47^{ab}
	45	1.00 ± 0.70^{fg}	1.53 ± 1.26^{def}	2.38 ± 1.02^{cde}	2.34 ± 0.54^{ab}
	80	1.51 ± 0.56^{def}	1.35 ± 0.22^{def}	1.77 ± 0.21^{def}	2.55 ± 0.08^{ab}
	125	0.66 ± 0.40^{fg}	0.73 ± 0.34^{fg}	3.16 ± 0.84^{abc}	1.25 ± 0.88^{ef}
	155	0.64 ± 0.74^{fg}	ND[4g]	1.75 ± 1.32^{def}	1.19 ± 0.32^{efg}

* 1 Mean ± standard deviation of three repetitons. Data obtained at 30 °C, 45 °C–50 °C, and 55 °C were evaluated separately; within these data sets, no significant difference was found between the values indicated with similar letters ($P < 0.05$). 2 Data of strains grown in Yeast Extract–Peptone–Glycerol medium after 4 h. 3 Count of strains grown in YPG medium at 30 °C for 4 h before the temperature treatment. 4 ND: Colony growth was not observed in direct inoculation from suspensions.

Table 2: Yeast counts (Log cfu/mL) of strains subjected to different ethanol treatments.

Ethanol (% v/v)	Time (min)	Log N (cfu/mL)[1]			
		2	7	47	S122
12	0	$4.81\pm0.27^{Ab\alpha}$	$5.31\pm0.33^{Ab\alpha}$	$4.96\pm0.13^{Aab\alpha}$	$5.67\pm0.14^{Aa\alpha}$
	60	$3.95\pm0.13^{Bb\alpha}$	$4.13\pm0.78^{Bb\alpha}$	$4.83\pm0.28^{Bab\alpha}$	$4.82\pm0.09^{Ba\alpha}$
	120	$3.47\pm1.11^{BCb\alpha}$	$3.68\pm0.37^{BCb\alpha}$	$3.35\pm1.20^{BCab\alpha}$	$4.19\pm0.02^{BCa\alpha}$
	180	$3.23\pm0.75^{CDb\alpha}$	$3.51\pm0.36^{CDv\alpha}$	$2.73\pm0.12^{CDab\alpha}$	$4.66\pm0.14^{CDa\alpha}$
	240	$3.60\pm0.08^{Db\alpha}$	$3.42\pm0.36^{Db\alpha}$	$2.21\pm0.13^{Dab\alpha}$	$3.34\pm0.18^{Da\alpha}$
14	0	$3.79\pm0.88^{Ab\beta}$	$3.00\pm0.49^{Ab\beta}$	$3.20\pm0.20^{Aab\beta}$	$4.37\pm0.67^{Aa\beta}$
	60	$2.28\pm0.45^{Bb\beta}$	$2.53\pm0.38^{Bb\beta}$	$2.56\pm0.39^{Bab\beta}$	$2.69\pm0.15^{Ba\beta}$
	120	$1.97\pm0.57^{BCb\beta}$	$2.06\pm0.49^{BCb\beta}$	$2.35\pm0.65^{BCab\beta}$	$2.36\pm0.08^{BCa\beta}$
	180	1.62 ± 0.28^{CDb}	$1.57\pm0.58^{CDb\beta}$	$2.07\pm0.84^{CDab\beta}$	$2.02\pm0.42^{CDa\beta}$
	240	$1.66\pm0.63^{Db\beta}$	$1.28\pm0.63^{Db\beta}$	1.84 ± 0.85^{Dab}	$1.92\pm0.1^{Da\beta}$

* 1 Mean ± standard deviation of three repetitons. Data obtained at 30 °C, 45 °C–50 °C, and 55 °C were evaluated separately; within these data sets, no significant difference was found between the values indicated with similar letters ($P < 0.05$). 2 Data of strains grown in Yeast Extract–Peptone–Glycerol medium after 4 h. 3 Count of strains grown in YPG medium at 30 °C for 4 h before the temperature treatment. 4 ND: Colony growth was not observed in direct inoculation from suspensions.

0 and 240 min were 13.59 – 30.18, 9.27 – 19.12, 22.89 – 16.13, and 20.18 – 15.46 mg/g of dry weight, respectively. The highest content of intracellular trehalose was detected at 0 and 240 min during the 55 °C treatment, whereas at other time points, the strains had lower contents. These results were in accordance with those reported at 55 °C at $\leq$ 180 min, (Lewis et al., 1995), in which the maximum value of 5 % (w/w cellular dry weight) cellular trehalose of *S. cerevisiae* at 30 min at 52 °C decreased within 180 min. However, unlike their study, the cellular trehalose contents of the strains increased again at 240 min in the present study.

The effect of several factors such as the strain type, temperature, treatment duration, and double and triple interactions on the time-dependent cellular trehalose contents after all temperature treatments were found to be significant based on the results of ANOVA ($P < 0.001$). No difference was observed between the cellular trehalose contents of strains 2, 7, and S122 during the 45 °C treatment, whereas the strain 47 reached the highest cellular

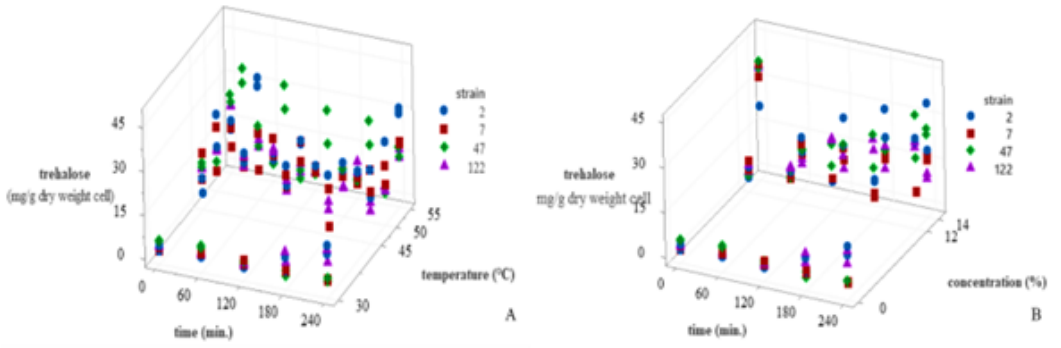

Figure 1: 3D Scatterplot of trehalose vs. temperature vs. time of different treatments (A: temperature, B: ethanol treatment).

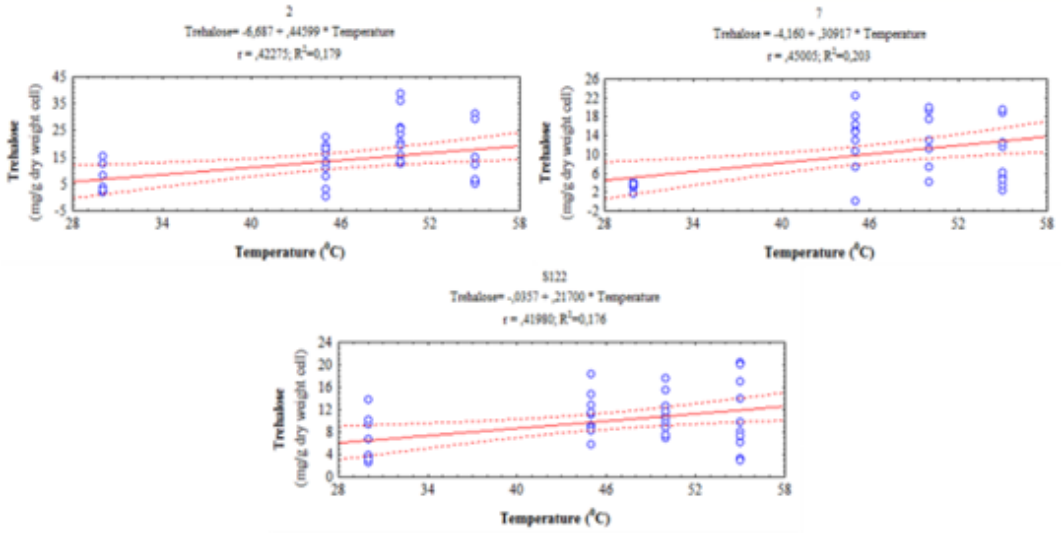

Figure 2: Correlation (95 % CI) between temperature and intracellular trehalose contents against temperature treatment for 2,7 and S122 strains.

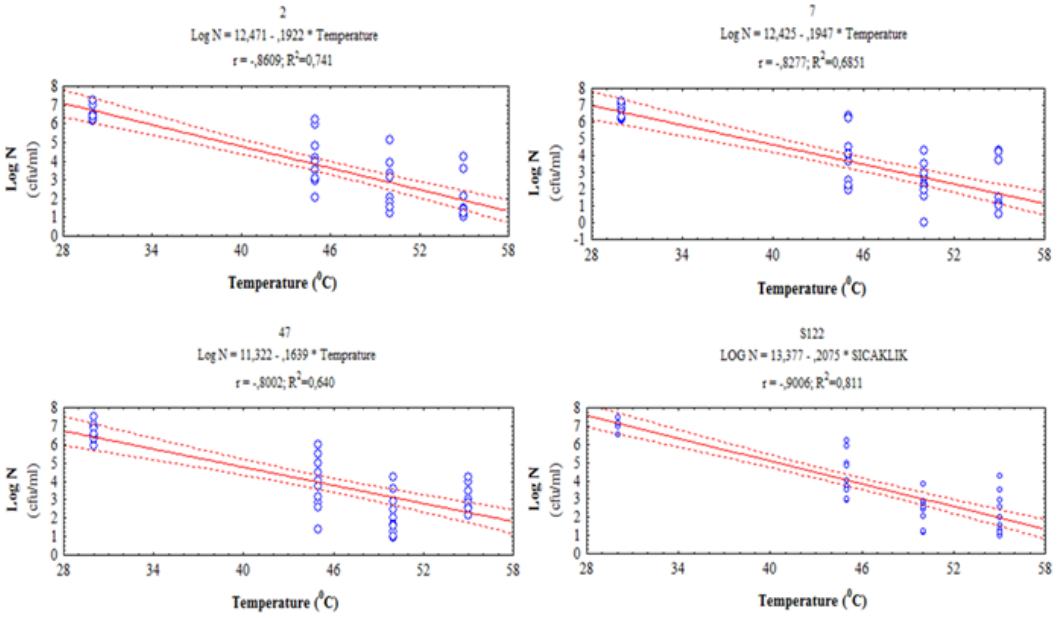

Figure 3: Correlation (95 % CI) between temperature and Log N levels against temperature treatment for all strains.

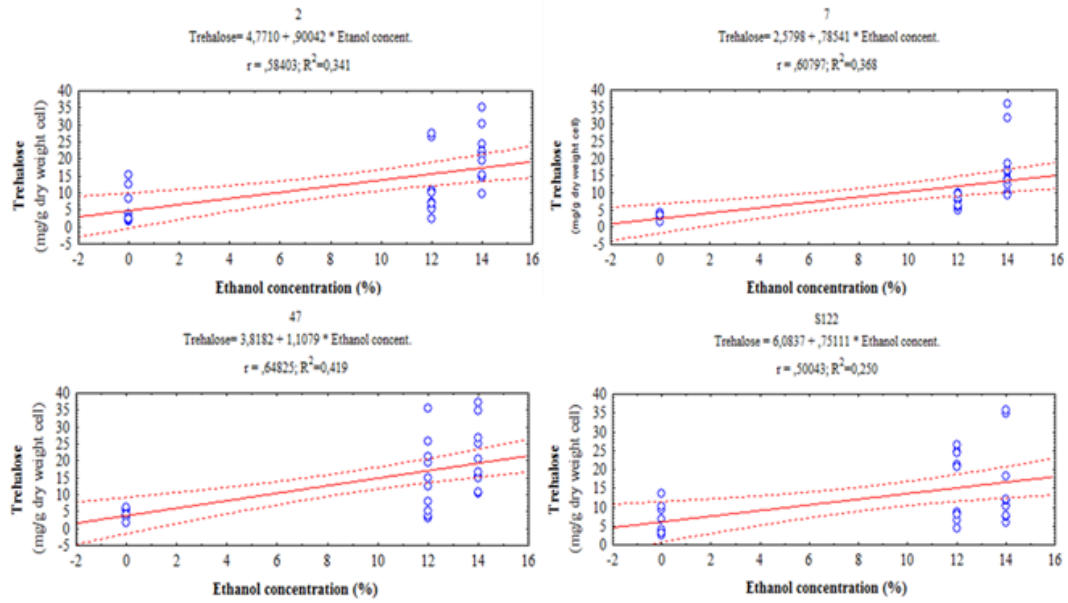

Figure 4: Correlation (95 % CI) between ethanol concentration and trehalose against ethanol treatment for all strains.

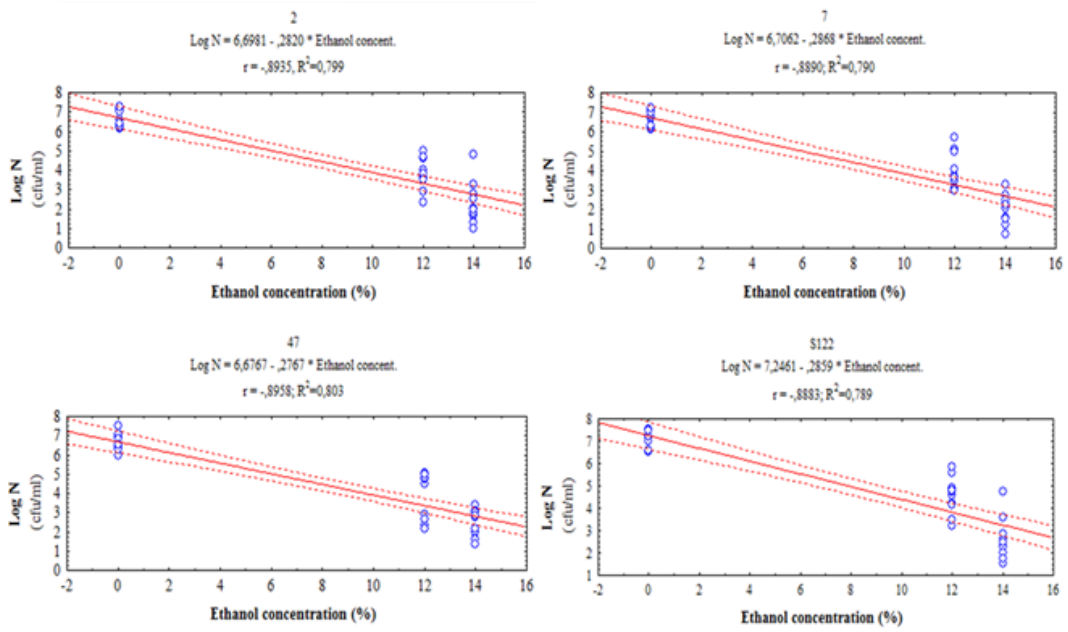

Figure 5: Correlation (95 % CI) between ethanol concentration and Log N levels against ethanol treatment for all strains.

Table 3: The relationship of each strain with the variables against of different stress factors.

Stress Factor	Depented Variables	Strain codes and the correlation coefficients							
		2		7		47		S122	
		Trehalose	Log N	Trehalose	Log N	Trehalose	Log N	Trehalose	Log N
Temperature	Temperature	0.42*	-0.86*	0.45*	-0.83*	0.18	-0.80*	0.42*	-0.90*
	Time	0.24	-0.32*	0.13	-0.36*	0.03	-0.25	-0.09	-0.25
Etanol	Trehalose	1	-0.42*	1	-0.39*	1	-0.25	1	-0.22
	Ethanol	0.58*	-0.89*	0.61*	-0.89*	0.65*	-0.90*	0.50*	-0.89*
	Time	0.49*	-0.16	-0.12	-0.16	0.2	-0.18	0.18	-0.19
	Trehalose	1	-0.62*	1	-0.65*	1	-0.74*	1	-0.37*

* $P < 0.05$

trehalose value ($P < 0.05$) at 60 min in all treatments and maintained the same value throughout the experimental period. The evaluation of all data revealed that significantly high cellular trehalose content was also obtained for strain 47 under the 45 °C treatment ($P < 0.05$; Fig. 1).

The 45 °C treatment results in an increase in the content of cellular trehalose without substantially reducing the yeast count compared with other temperatures. Cellular responses to mild stress treatments may have positive influence on the behavior of organisms.

Although the data from the present study show that the strains increased their intracellular trehalose content during temperature treatment, the trend is not clear and the trehalose content does not increase in parallel to the stress condition. Previous studies have shown that the cellular trehalose content in many organisms increases at the time of stress exposure and this trend decreases over time (Gomes et al., 2002; Rossouw et al., 2013; Lin et al., 2012; Liu et al., 2016; Cai et al., 2018). The rapid degradation of trehalose depending on the duration of stress is necessary for the cell to return to its normal activities (Gibson et al., 2007). An explanation of these findings may be that trehalose reportedly limits the activity of essential enzymes such as glutathione reductase, which plays a role in regulating cellular homeostasis and protecting the cell from oxidative damage (Sebollela et al., 2004).

3.2.2 Ethanol stresses

As regards ethanol treatment, trehalose contents of strains 2, 7, 47, and S122 at 0 and 240 min were 13.59–30.18, 9.27–19.12, 22.28–16.13, 20.18–15.46 mg/g of cellular dry weight, respectively, in the media containing 12 % ethanol (Table S2, Supplementary Material).

As detected in the high temperature treatment of 55 °C, a significant increase in the trehalose content of the strains at 0 min was noted in the 14 % ethanol treatment. A decrease in the trehalose content was observed at 60 min, but it increased gradually. The cellular trehalose contents of strains 2, 7, 47, and S122 at 0 and 240 min were 22.11 – 27.41, 34.01 – 17.49, 36.05 – 25.94, and 35.30 – 11.11 mg/g of dry weight, respectively, in the media containing 14 % ethanol (Fig. 1).

The factors influencing the change in the cellular trehalose contents under 12 % and 14 % ethanol treatments revealed that the strain type, ethanol concentration and treatment duration as well as all double and triple interactions exerted significant effects on the cellular trehalose contents ($P \leq 0.001$).

The evaluation of the effect of the 12 % ethanol treatment on the time-dependent cellular trehalose contents of the strains indicated that strain 7 did not show any significant increase at any time points; however, the difference with the control samples was not significant ($P > 0.05$). Strain 2 showed a significant increase only at 240 min, and strains 47 and S122 from 120 min ($P < 0.05$; Table 2). The assessment of the effects of 14 % ethanol treatment showed that strains 7 and S122 exhibited significant increase only at 0 min, strain 47 coded strain at 0 and 240 min, and strain 2 at 120 and 240 min ($P < 0.05$). Accordingly, strains 7, 47, and S122 had the highest cellular trehalose content in the 14 % ethanol treatment at 0 min. It was determined that the 47, 14 % ethanol concentrations among the concentrations, the 240th and 0th minutes were the factors with the highest cellular trehalose amount ($P < 0.05$).

Although strains increase their cellular trehalose contents when exposed to high ethanol concentration, no specific trend was identified after stress as seen in the high temperature treatments. Thus, the triple interaction of the strain type, concentration difference, and treatment period influences this trend. The results of a previous study Vianna et al. (2008) suggested that trehalose accumulation is strain-dependent and indicates a remarkable physiological diversity among the populations of *S. cerevisiae* involved in cachaça fermentation. Our previous study, in which we evaluated the glucose and fructose consumption behaviors of the same four strains in the yeast nitrogen base medium containing grape juice–like components, showed that pretreatment at a mild stress (temperature and ethanol) increases in glucose-consumption rate of strains 2, 7, and 47 (t90 reduced by approximately 8 %–10 % compared with control) (Karaoglan et al., 2021). Other previous studies showed that mild stress treatments of *S. cerevisiae* induce greater tolerance to what would otherwise be lethal temperature; H_2O_2 stress (Steels et al., 1994); osmotic stress (Trollmo et al., 1989) and ethanol, sorbic acid, or low external pH stress (Coote et al., 1991). Wang et al. (2013) reported that wild and mutant strains of *Zygosaccharomyces rouxii* and *Torulopsis versatilis* had the highest cellular trehalose content at 9 % and 12 % salt concentrations, respectively, when salt-containing media at different concentrations ranging from 0 % to 15 % were used.

Organisms exposed to stress increase their cellular trehalose content, but the exact mechanism has not been elucidated yet. Trehalose metabolism of *S. cerevisiae* has been studied in detail. Trehalose is synthesized by many enzyme complexes, including trehalose-6P synthase encoded as TPS1, trehalose-6P phosphatase encoded as TPP, and two regulatory subunits encoded by TSL1 and TSL3 (Eleutherio et al., 2015). TPP is synthesized by TPS from uridine-diphospho-glucose (UDP-glucose) and glucose-6-phosphate and degrades into trehalose-6-phosphate, trehalose, and inorganic phosphate (Miranda et al., 2007). Winkler et al. (1991) reported that there is a significant increase in the cellular trehalose content of yeasts exposed to temperature stress in media containing UDP-glucose and glucose-6-phosphate. Furthermore, Liu et al. (2016) reported that during a prolonged period of temperature stress, the enzymes responsible for trehalose synthesis are degraded, leading to reduced enzyme activity, thus ultimately reducing the cellular trehalose content. The increase in the trehalose content in response to stress noted in the wine yeast strains used in this study might have occurred via these mechanisms.

3.3 Correlation analyses results

In this study, the tolerance and response of yeasts to various temperature stresses were assessed via correlation analysis. The correlation among temperature, treatment

duration, Log N, and trehalose content for each strain at 30°C (optimum growth temperature) and at 45 °C, 50 °C, and 55 °C (temperature treatments) was determined using Pearson correlation. Table 3 presents the correlation coefficients (r). Accordingly, the r values representing the correlation between Log N and trehalose for strains 2, 7, 47, and S122 were 0.42, 0.39, 0.25, and 0.22, respectively; this correlation was significant only for strains 2 and 7 ($P < 0.05$). It was also observed that 18 % of the change in trehalose level in strain 2 and 15 % in strain 7 coded could be explained based on the decrease in the yeast count. Similar to the present study, Gomes et al. (2002) reported that the differences between the strains and the correlation between the cell survival rate and cellular trehalose content was significant for strain UFMG-A533 but not for strain UFMG-A1000. In the correlation analysis, the correlation between temperature x trehalose contents for all strains, except for strain 47, and temperature x Log N values for all strains was significant ($P < 0.05$). Although the highest cellular trehalose contents were noted for strain 47 based on the temperature treatments, no significant correlation was identified between the increase in cellular trehalose content and temperature increase ($P > 0.05$). The evaluation of the increase in the cellular trehalose content of the strains (Fig. 2) revealed that each unit increase in ambient temperature resulted in an increase in the cellular trehalose contents of 0.446, 0.309, and 0.217 mg/g dry weight for strains 2, 7, and S122, respectively. The assessment of the correlation between temperature x Log N revealed that the correlation was very strong and negative; thus, with increasing temperature, the count of each strain decreased (Table 3). The evaluation of the decrease in the yeast count showed yeast counts were 0.192, 0.195, 0.164, and 0.208 log cfu/mL for each unit increase in the ambient temperature in strains 2, 7, 47, and S122, respectively (Fig. 3). Accordingly, strains S122, 7, 2 and 47 had the highest yeast count at each unit increase in temperature stress in that order.

In line with the results of correlation analysis in the present study, Lewis et al. (1997) investigated the correlation between % survival rates and cellular trehalose contents under various stress factors in 14 wild *S. cerevisiae* strains using correlation analysis. They determined that the correlation between acetic acid stress and cellular trehalose content and that between salt stress and cellular trehalose content were significant with r values of 0.65 and 0.53, respectively.

For each strain, the correlation of Log N, ethanol concentration, and treatment duration with the trehalose content was analyzed using Pearson correlation. Table 3 presents the r values. For each of the strains 2, 7, and 47, a strong and significant correlation was determined between the cellular trehalose content x Log N, with r values of 0.62, 0.65, and 0.74, respectively, whereas the significance level of this correlation was lower for strain S122 than that of the other strains with the values of $r = 0.37$ ($P < 0.05$).

The correlation between the ethanol concentration and cellular trehalose content is shown in Fig. 4 and the correlation between the ethanol concentration and Log N is shown in Fig. 5 for each strain. For strains 2, 7, 47, and S122, each unit increase in the ethanol concentration resulted in an increase of 0.90, 0.79, 1.11, and 0.75 mg/g dry weight in the cellular trehalose content and a decrease of 0.28, 0.29, 0.28, and 0.29 in the yeast count. When the R^2 values calculated based on the correlation between the cellular trehalose content and Log N of strains 2, 7, 47, and S122 were assessed (Table 3), % changes of 0.39, 0.42, 0.55, and 0.14 were observed in the trehalose content of the strains, which may be explained based on the decrease in the yeast count. The data of the present study showed that the highest level of significance between the yeast count and cellular trehalose content was found for strain 47, whereas this correlation was the weakest for strain S122. However, Alexandre and Charpentier (1998) and Ribeiro et al. (1999) reported no correlation between the cellular trehalose content and survival rates of strains after ethanol treatment in their studies with *S. cerevisiae*.

4. Conclusions

This study evaluated the tolerance and response of four different wine strains of *S. cerevisiae* subjected to different temperature and ethanol stresses. The study findings revealed that 45 °C temperature and 14 % ethanol treatments resulted in the highest increase in the cellular trehalose content of yeast strains. Furthermore, there was a negative correlation between the yeast count and cellular trehalose content of strains across all temperature treatments, and this correlation was significant only for strains 2 and 7. In addition, 14 % ethanol treatment is the concentration with the highest strain inactivation rate, also inducing the highest increase in the cellular trehalose content. A significant and negative correlation was identified between the yeast count and cellular trehalose content of strains across all ethanol treatments. Each unit increase in the ethanol concentration resulted in similar decrease in the yeast count for all strains, and the cellular trehalose content increased the most in strain 47. The level of significance of the correlation between the yeast count and cellular trehalose content of strains after ethanol treatments was higher than that of the correlation obtained from the temperature treatment. Strains do not exhibit a clear trend in terms of decreasing yeast count and increasing cellular trehalose content due to stress exposure, and they may show different responses depending on the stress factor and treatment duration.

It is essential to explore the tolerance and response levels of yeasts to stress factors in order to accurately design the fermentation process involving these yeasts and to take necessary precautions. The identification of the yeast behavior in response to different stress factors allows for the selection a mild stress treatment, which may lead to a lower decrease in the yeast count but higher increase in the cellular metabolites content. The fermentation yield may be enhanced through the identification of the altered fermentation behaviors of yeasts in response to mild stress factors and the influence of these behavioral changes on the fermentation process.

5. Acknowledgments

This research was supported by the Scientific Research Project Fund of Sivas Cumhuriyet University (Grant no: M-632).

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