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Protective Effect of Silica on Adaptation of *saccharomyces cerevisiae,* Ethanol Red® for Very High Gravity Fermentation

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HIGHLIGHTS

- *Saccharomyces cerevisiae* yeast can gradually adapt to very high gravity fermentation.
- Supplementation of the medium with silica $^{(+A)}$ improves fermentative parameters.
- Silica $^{(+A)}$ has a protective effect on the yeasts *Saccharomyces cerevisiae.*
- Silica $^{(+A)}$ maintains the capacity to produce cellular biomass during fermentation.

Abstract: The Very High Gravity (VHG) fermentation is a technology that can lead to a reduction in waste generation, a reduction in energy consumption and GHG emissions and several technical, economic, and environmental advantages. Having, as a limiting factor, yeast tolerance to the most diverse stressors in the fermentation medium. To overcome this limitation, the aim of the work was to verify the potential protective effect of silica $^{(+A)}$ on *Saccharomyces cerevisiae* (Ethanol Red®) submitted to VHG fermentation. Initially, an adaptive test to VHG fermentation was carried out, with 5 cell recycles in musts from sugar cane syrup. Each recycle was subjected to the treatments, in quadruplicate: T1C (control) - Wort without silica supplementation; T2S100- Wort with supplementation of 100 mg L^{-1} of silica and T3S300- Wort with supplementation of 300 mg L^{-1} of silica. As a result, the T3S300 treatment in the adaptive test, showed viability of 77.5 to 81.55%; biomass production from 8.1 to 10.0 g L^{-1}; yield from 90.0 to 95.3% and productivity from 7.3 to 10.9 mL $L^{-1}h^{-1}$. In conclusion, the treatment of the wort with silica $^{(+A)}$ (100 and 300 mg L^{-1}) has an effect protector on yeast and may present positive responses in VHG fermentations.

Keywords: Cell recycling; Ethanol; Supplementation; Tolerance; Yeast strain.

INTRODUCTION

In December 2015, the Paris Agreement was approved, which is the first global legally linked climate change agreement. The agreement to increase countries' ability to deal with the impacts of climate change and mitigate greenhouse gas (GHG) emissions. Thus, the trend is to increase the participation of biofuels in the world energy matrix [1,2,3]. To contribute to this international policy, the Very High Gravity fermentation (VHG) is a technology that can contribute to the reduction of energy consumption and the generation of residues in the ethanol production process in plants [4]. VHG uses musts with more than 22g of dissolved solids / 100g L. The process presents several economic and environmental advantages; however, yeast has limitations to tolerate high gravity fermentation [5]. Throughout the fermentation process, yeast is exposed to the most diverse stressful conditions. However, the accumulation of ethanol and the production of organic acids provide the inhibition of cell growth. Although, such effects are intensified by the practice of cell recycling [6,7]. In cell recycling, yeast cells are subjected to acid treatment to control contaminants, which leads to loss of cell viability over the course of cycles [8]. This recycling step causes more intense stress to yeasts, in addition to those caused by the process itself [9,10]. Therefore, the addition of compounds that increase the cell's capacity to tolerate these stresses, such as Silica $^{(+A)}$ appears as an alternative to help yeast to withstand stressors.

Silica $^{(+A)}$ is found on the market as a micronized silicon dioxide mineral powder. It is a 100% natural, highly pure mineral, which is used to help potentiate the ionic exchanges between water and substances [11]. Composed of more than 98% silicon dioxide (SiO_2), Silica$^{(+A)}$ belongs to the silicate family. Silica itself is about 60% of the earth's crust; it is one of nature's most abundant minerals and is a large compound of sand, granite, and diatom cell walls [12]. Silica has been used to promote the growth of microorganisms in adverse conditions. Therefore, under stressful conditions, it is used to assist in the absorption of ammonia and CO_2 from microorganisms [13].

For these reasons, the purpose of the work was to verify the protective effect of electronically activated silica to yeasts in fermentation with high alcohol content, using *Saccharomyces cerevisiae*, strain Ethanol Red®, to increase the tolerance of these yeasts to alcoholic stress.

MATERIAL AND METHODS

Substrate

The wort was obtained from sugar cane syrup, from a plant in the region of Piracicaba / SP. Initially, the syrup was diluted with distilled water until it reached a concentration of total reducing sugar (TRS) of 261.2 g L^{-1} (standard wort). Then, the wort was placed in 5-liter glass containers and subjected to the clarification process, by adding 2.5 g L^{-1} of monobasic sodium phosphate ($NaH_2PO_4.H_2O$) and heating in an autoclave. This procedure consisted of sterilizing the material in an autoclave, under 121ºC and 1 atm, for 15 minutes. Then it was cooled at room temperature and in the sequence centrifuged under 4000 rpm for 10 min, using a refrigerated centrifuge model Sorvall ST 40R, Thermo Scientific®.

From the standard wort, musts were prepared for fermentations with the following concentrations of total reducing sugars (C1-182.2; C2 - 199.5; C3 - 221.2 and C4 - 241.1 and C5 -261.2 g L^{-1}TRS), being supplemented with 100 mg L^{-1} of urea. Then, these musts were autoclaved, under a temperature of 120 ºC, pressure of 1 atm, for 20 minutes, according to the methodology described by Braga [14], to later be enriched with silica $^{(+A)}$.

Treatments

The following treatments were evaluated: T1C: Control (0 mg L^{-1} of silica $^{(+A)}$); T2S100 (with the addition of 100 mg L^{-1} of silica $^{(+A)}$) T3S300 (with the addition of 300 mg L^{-1} of silica $^{(+A)}$) in fermentations with gradual increases in the initial concentration of sugars in the musts, being cell recycling carried out after each fermentation cycle. The treatments and fermentation cycles performed are shown in Table 1.

Table 1. Description of treatments performed regarding the concentration of silica in the fermentation cycles.

Treatments	Fermentation cycle	Total Reducing Sugars (g L^{-1})	Urea (mg L^{-1})
Control 0 mg L^{-1} (T1C)			
Silica $^{(+A)}$ 100 mg L^{-1} (T2S100)	1st cycle	182.2	100
Silica $^{(+A)}$ 300 mg L^{-1} (T3S300)			
Control (T1C)			
Silica $^{(+A)}$ 100 mg L^{-1} (T2S100)	2nd cycle	199.5	100
Silica $^{(+A)}$ 300 mg L^{-1} (T3S300)			
Control 0 mg L^{-1}(T1C)			
Silica $^{(+A)}$ 100 mg L^{-1} (T2S100)	3rd cycle	221.2	100
Silica $^{(+A)}$ 300 mg L^{-1} (T3S300)			
Control (T1C)			
Silica $^{(+A)}$ 100 mg L^{-1} (T2S100)	4th cycle	241.2	100
Silica $^{(+A)}$ 300 mg L^{-1} (T3S300)			
Control 0 mg L^{-1}(T1C)			
Silica $^{(+A)}$ 100 mg L^{-1} (T2S100)	5th cycle	261.2	100
Silica $^{(+A)}$ 300 mg L^{-1} (T3S300)			

Means of the same parameter followed by different capital letters, on the same line, differ statistically from each other through the Tukey test at the level of 5% significance ($p \leq 0.05$).

Means of the same parameter followed by different lowercase letters, in the same column, differ statistically from each other through the Tukey test at the level of 5% significance ($p \leq 0.05$).

Fermentation tests

Fermentations were carried out in 500 mL Erlenmeyers, containing 200 mL of previously prepared musts, as described in Table 1, and inoculated with the addition of 6 grams of yeast, *Saccharomyces cerevisiae*, Ethanol Red® (Lesaffre). The fermentations were carried out in a shaker, model Minitron, brand Infors HT®, under agitation of 120 rpm and temperature of 30 ℃ ± 1 ºC.

After the fermentation was finished, the raw wine was centrifuged, under 3925 g, for 10 minutes, using a refrigerated centrifuge model Sorvall ST 40R, Thermo Scientific®, to separate the yeasts from the wine. After centrifugation, the supernatants (light fermented wine) were transferred to 200 mL plastic bottles, previously identified, and stored in a freezer, at a temperature of –20ºC ± 2, to be subsequently characterized through physical-chemical analysis. While, the sedimented material (yeasts) was used in the fermentative recycles.

Cell viability

Cell viability analysis was determined by differential staining of cells in 0.1% methylene blue solution, using a Neubauer chamber, optical microscope, Model E 200, Nikon® brand, considering the reading of living cells (transparent) and dead cells (blue), methodology described by Pierce [15].

Cell biomass

The determination of yeast cell biomass was performed shortly after the end of fermentation. The 500 mL samples of wine were subjected to centrifugation under 3925 g for 10 minutes. At the end of this operation, the volume of the supernatant was measured using a 500 mL graduated cylinder, so that, the volume of yeast cream was obtained by difference between the volume of wine and the volume of supernatant. From the

results of yeast cream volumes, yeast cell biomass concentrations (grams of dry matter per liter of wine) were determined according to the methodology described by Koshimizu and coauthors [16].

Determination of total sugars and glycerol

From 1 mL samples of must and wine, the concentrations of glucose, fructose, sucrose, and glycerol were determined. Samples of wines (25 times) and musts (500 times) were diluted and filtered in 0.45µm Durapore filter units [17]. After preparation, with the aid of an automatic injector, 0.25 µL of each sample was injected into an ion chromatograph, model IC 930, by Metrohm®. The chromatographic system used was chromatographic column, model MetrosepCarb 1 - 150 / 4.0; amperometric detector; prepared eluent solution was 200 mM sodium hydroxide and the flow rate was 1.0 mL min^{-1}. The column temperature was maintained at 35°C and the chromatographic run time was 9 minutes [17].

Determination of Alcohol Content

The determination of the alcohol content was carried out in the wort and wine samples. The analysis consisted of distilling 25 mL of light fermented wine sample, from each experimental unit, using an alcohol distiller, Model TE-010 (Tecnal brand). After this preparation, the distillate obtained by steam drag was analyzed for alcohol content in a densimeter model EDM 5000, brand Schimidt Haensch, at a controlled temperature of 20°C ± 0.1 °C [18].

Fermentative yield

The calculation of the fermentative yield was based on the stoichiometry of alcoholic fermentation, in which the yield of 100% occurs when there is formation of 51.11 g (64.75 mL) of ethanol from 100 g of total reducing sugars. Thus, the fermentative yield was calculated according to Camargo and Ushima [19].

Fermentation productivity

Productivity was calculated based on the alcoholic concentration of the wine at the end of the fermentation and the fermentation time [19].

Statistic

Statistical analyzes were performed using the R v program. 3.3.2 [20]. The experimental design used was a completely randomized design. Tukey's test was used to compare means at a 5% significance level ($p < 0.05$).

RESULTS

Cell viability

The average viability of yeast cells submitted to the conditions of the control treatment (T1C) increased over the 5 fermentative cycles. Since, in the 5th fermentative cycle, the average viability was 9.5% higher than the average at the beginning of the 1st fermentative cycle (after cellular rehydration) and 7% higher than at the end of the 1st cycle. It was also observed that, at the end of the 5th fermentative cycle, yeasts supplemented with 100 mg L^{-1} of silica $^{(+ A)}$ (T2S100) obtained cell viability 9.2% higher than at the beginning of the 1st fermentative cycle and 1.5 % higher than at the end of the 1st cycle. On the other hand, yeast cells supplemented with 300 mg L^{-1} of silica $^{(+ A)}$ (T3S300), at the end of the 5th fermentation cycle, showed average cell viability 15% higher than that observed at the beginning of the 1st fermentation cycle. However, they presented cell viability averages similar to those observed in the 2nd, 3rd and 4th cycles (Table 2).

When the results of the cell viability of the yeasts of the Ethanol Red® strain submitted to different treatments were compared, it is observed that T3S300 (300 mg L^{-1} of silica $^{(+ A)}$) promoted an increase in the tolerance of maintaining viability of yeast cells under the above conditions compared to the other treatments T1C (0 mg L^{-1} of silica $^{(+ A)}$) and T2S100 (100 mg L^{-1} of silica $^{(+ A)}$). At the end of the 1st fermentation cycle, yeast cells submitted to T3S300 showed average cell viability of 77.5% (± 1.1). These were higher than the average cell viability of yeasts submitted to T1C (67.5% (± 1.2)) and T2S100 (72.7% (± 0.2)) ($p \leq 0.05$). At the end of the 5th fermentation cycle, the average cell viability of yeasts grown under T3S300 conditions remained greater than those observed in fermentations carried out under T1C and T2S100 conditions, 5.5% and 5.8% respectively.

Table 2. Results of the fermentative parameters of the yeast Ethanol Red, submitted to treatments.

Parameters	Treatments	Cell rehydration	1st cycle	2nd cycle	3rd cycle	4th cycle	5th cycle
Cell viability (%)	T1C	65.0 ^{Ca} (±1.0)	67.5 ^{Ba} (±1.3)	71.3^{Ab} (±1.3)	71.3^{Ac} (±1.3)	73.4^{Ab} (±1.2)	74.5^{Ab} (±1.5)
	T2S100	65.0 ^{Da} (±1.0)	72.7^{Cb} (±0.2)	73.8^{BCb} (±0.3)	75.0^{Bb} (±0.5)	78.3^{Aa} (±0.6)	74.2^{Bb} (±1.4)
	T3S300	65.0 ^{Da} (±1.0)	77.5^{Cc} (±1.1)	78.4^{BCa} (±0.6)	80.0^{ABa} (±1.5)	81.5^{Aa} (±1.0)	80.0^{ABa} (±1.1)
Cell Biomass (g L^{-1})	T1C	6.0 ^{Fa} (±0.1)	6.8^{Ec} (±0.2)	7.5^{Dc} (±0.2)	8.1^{Cc} (±0.2)	8.8^{Bc} (±0.2)	9.4^{Ab} (±0.2)
	T2S100	6.0 ^{Fa} (±0.1)	7.5^{Eb} (±0.2)	8.1^{Db} (±0.2)	8.8^{Cb} (±0.5)	9.4^{Bb} (±0.2)	10.0^{Aa} (±0.2)
	T3S300	6.0 ^{Ea} (±0.1)	8.1^{Da} (±0.2)	8.8^{Ca} (±0.2)	9.4^{Ba} (±0.2)	10.0^{Aa} (±0.2)	10.0^{Aa} (±0.2)
Residual total reducing sugars (g L^{-1})	T1C	-	0.06^{Aa} (±0.01)	0.05^{Aa} (±0.02)	0.18^{Aa} (±0,05)	0.14^{Aa} (±0.03)	0.04^{Aa} (±0.01)
	T2S100	-	0.09^{Aa} (±0.01)	0.07^{Aa} (±0.01)	0.22^{Aa} (±0.05)	0.02^{Aa} (±0.01)	0.03^{Aa} (±0.01)
	T3S300	-	0.02^{Aa} (±0.01)	0.05^{Aa} (±0.01)	0.06^{Aa} (±0.02)	0.03^{Aa} (±0.01)	0.01^{Aa} (±0.01)
Glycerol (g L^{-1})	T1C	0.0 ^{Ea} (±0.0)	11.24^{CDa} (±0.36)	10.60^{Da} (±0.50)	12.00^{BCa} (±0.27)	12.37^{ABa} (±0.55)	12.83^{Aa} (±0.40)
	T2S100	0.0 ^{Ca} (±0.0)	11.89^{Aa} (±0.58)	10.94^{Ba} (±0.43)	11.87^{Aa} (±0.31)	12.60^{Aa} (±0.30)	12.37^{Aa} (±0.39)
	T3S300	0.0 ^{Da} (±0.0)	11.50^{BCa} (±0.38)	10.98^{Ca} (±0.49)	12.28^{ABa} (±0.55)	12.86^{Aa} (±0.39)	12.59^{Aa} (±0.36)
Alcohol content (v v^{-1})	T1C	0.0 ^{Fa} (±0.0)	9.7^{Eb} (±0.1)	11.5^{Db} (±0.3)	13.2^{Cb} (±0.1)	14.1^{Ba} (±0.1)	15.7^{Aa} (±0.2)
	T2S100	0.0 ^{Fa} (±0.0)	11.0^{Ea} (±0.2)	12.5^{Da} (±0.1)	14.1^{Ca} (±0.2)	14.4^{Ba} (±0.1)	15.9^{Aa} (±0.2)
	T3S300	0.0 ^{Fa} (±0.0)	11.0^{Ea} (±0.4)	12.8^{Da} (±0.1)	14.1^{Ca} (±0.2)	14.8^{Ba} (±0.2)	16.2^{Aa} (±0.1)
Yield (%)	T1C	-	82.3^{Cb} (±1.0)	86.2^{Bc} (±0.5)	89.1^{Ab} (±1.0)	86.7^{Bc} (±0.5)	88.5^{Ac} (±0.5)
	T2S100	-	93.0^{Ba} (±1.0)	93.7^{ABb} (±0.5)	94.9^{Aa} (±1.0)	88.1^{Cb} (±0.5)	89.4^{Cb} (±0.5)
	T3S300	-	92.8^{Ba} (±1.0)	95.3^{Aa} (±0.5)	94.6^{Aa} (±1.0)	90.0^{Ca} (±0.5)	90.6^{Ca} (±0.5)
Productivity (mL L^{-1} h^{-1})	T1C	-	6.5^{Cb} (±0.1)	8.2^{Bb} (±0.2)	9.5^{Ab} (±0.1)	9.4^{Ab} (±0.1)	9.3^{Aa} (±0.1)
	T2S100	-	7.3^{Da} (±0.2)	8.9^{Ca} (±0.1)	10.9^{Aa} (±0.1)	9.6^{Bab} (±0.3)	9.4^{Ba} (±0.3)
	T3S300	-	7.3^{Ea} (±0.3)	9.1^{Da} (±0.1)	10.9^{Ca} (±0.2)	9.8^{Ba} (±0.1)	9.5^{Aa} (±0.1)

Means of the same parameter followed by different capital letters, on the same line, differ statistically from each other through the Tukey test at the level of 5% significance ($p \leq 0.05$).

Means of the same parameter followed by different lowercase letters, in the same column, differ statistically from each other through the Tukey test at the level of 5% significance ($p \leq 0.05$).

Cell biomass

Analyzing the effect of the treatments used on yeasts over the first 4 fermentative cycles, it was possible to verify the increase in the average cell biomass value of yeasts submitted to T3S300 treatment, which was from 0.6 to 1.3% higher than those of yeasts submitted to T1C and T2S100. While, in the 5th fermentative cycle, yeasts grown under the T3S300 treatment conditions showed an increase of 0.6% in the formation of cell biomass in relation to those yeasts submitted to the T1C treatment. On the other hand, they showed no significant difference in relation to the T2S100 treatment. This means that the addition of silica $^{(+ A)}$ to the fermentation medium helps to protect the yeast cell, so it maintains the capacity to produce cellular biomass during fermentation with a high alcohol content.

At the end of the 5 fermentative cycles, yeast cell biomasses submitted to control treatments, silica $^{(+ A)}$ 100 mg L^{-1} treatment and silica $^{(+ A)}$ 300 mg L^{-1} treatment increased, respectively, by an average of 3.4 g L^{-1}, 4.0g L^{-1} and 4.0g L^{-1}. However, the T1C, T2S100 and T3S300 treatments showed an average increase in cell biomass from one fermentation cycle to the next, respectively, 9.4%, 11.0% and 11.3%. This indicates that possibly the concentration of ethanol in the medium did not prevent the yeast cells from multiplying, that is, by performing the adaptation of the yeast at high alcoholic levels, it was possible to minimize the effects of ethanol on the multiplication of the yeast Ethanol Red®.

Total reducing sugars

Initially, all musts to which the 3 treatments were applied started fermentations with the same concentrations of total reducing sugars. The initial concentrations of sugars present in the musts in each cycle were: 1st cycle 182.2 g L^{-1}; 2nd cycle 199, 5 g L^{-1}; 3rd cycle 211.2 g L^{-1}; 4th cycle 241.1 g L^{-1} and 5th cycle 261.2 g L^{-1} of TRS. At the end of each fermentation cycle, it was possible to verify that in all fermentations, all sugars available in the culture medium were consumed and converted into product. This indicates that, although the sugar concentration is conducive to osmotic stress under the yeast cells, they carry out fermentations until they consume all the sugars, transforming them into ethanol, glycerol, CO_2, and other compounds.

Glycerol

The average results obtained for glycerol, according to the variation between the 5 cycles, were: T1C - 11.24 to 12.83 (g L^{-1}), T2S100 - 10.94 to 12.60 (g L^{-1}), and T3S300 - 10.98 to 12.86 (g L^{-1}). This shows that as the concentrations of sugars in the substrates increased the Ethanol Red® strain were able to tolerate the stresses caused by the high concentration of sugar and ethanol in the medium. Investigating the effects of treatments on yeasts at each fermentation cycle it was possible to verify that there was no significant difference ($p \leq 0.05$) between treatments regarding glycerol formation. However, this result was not expected by the present study since silica $^{(+ A)}$ provided an increase in viability and cell biomass.

Ethanol content

The average results obtained for the concentration of ethanol, according to the variation between the 5 cycles, were: T1C - 9.7 to 15.7 (v v^{-1}), T2S100 - 11.0 to 15.9 (v v^{-1}), and T3S300 -11.0 to 16.2 (v v^{-1}). These results indicate that the high alcohol content throughout the fermentations, possibly, did not provide a toxic effect on the yeasts since they were adapted to the gradual increase of ethanol in the fermentation medium.

Fermentative yield

Yeasts grown under the conditions of the T2S100 and T3S300 treatments showed higher fermentative yield (0.9-10%) than the yeasts submitted to the T1C treatment over the 5 fermentative cycles. This shows that silica $^{(+ A)}$ acts in increasing cell viability and biomass, thus minimizing osmotic stress on yeast, which resulted in an increase in fermentative yield

However, the average yields of fermentations submitted to yeasts grown on the presence of silica $^{(+A)}$ showed statistically significant differences ($p \leq 0.05$) between the concentrations used during the 2nd, 4th and 5th fermentative cycles. The treatment with silica $^{(+ A)}$ 300 m L^{-1} provided the highest fermentative yield than treatment with silica $^{(+ A)}$ 100 m L^{-1}. This indicates that the concentration of silica $^{(+ A)}$ added in the process probably influences the fermentative yield.

Fermentation productivity

Fermentative productivities in T1C tests ranged from 6.5 to 9.5 mL L^{-1} h^{-1} in T2S100 ranged from 7.3 to 10.9 mL L^{-1} h^{-1} and T3S100 ranged from 7.3 to 10.9 mL L^{-1} h-1. Evaluating the effects of treatments on yeast cells throughout each fermentation cycle, it was possible to observe that, in yeasts submitted to T2S100 and T3S300 treatments, they showed higher fermentative productivity than yeasts submitted to T1C treatment over the first four cycles fermentative. This shows that silica $^{(+A)}$ acted to minimize osmotic stress on yeast cells. However, in the 5th cycle, there were no statistical differences ($p \leq 0.05$) in the fermentative productivity between the three treatments.

DISCUSSION

The results obtained in the present work indicate that the yeast strain Ethanol Red®, presented physiological capacity to adapt to the cultivation conditions, in simple batch, with the gradual increase of the sugar content in the must from 182.2 to 261.2 g L^{-1} of total reducing sugars (TRS), during the 5 fermentative cycles. This demonstrates the genetic potential of the strain to carry out fermentations under conditions of high concentrations of sugar in the wort and ethanol in the wine. According to Benitez and coauthors [21], each yeast strain of the *Saccharomyces cerevisiae* species has specific genetic capacity for sensitivity or tolerance to high concentrations of sugars and ethanol in the cultivation substrate. Whereas, during stress conditions, yeasts through the expression of several genes encode proteins involved in various cellular processes [21, 22].

Under the stressful conditions of the environment, the yeast strain produces glycerol in to protect the cell against cell lysis [23, 24]. However, the accumulation of glycerol is dependent on the intensity of stress that the yeast is subjected to. In this way, the yeast detects and assesses the condition of stress by adjusting the expression of genes to neutralize the toxic effects [25]. During alcoholic fermentations it is estimated that 5-8% of the sugars metabolized by yeast are converted into glycerol [6]. Thus, starting from the total consumption of sugars during the fermentation process, the musts of the cycles (1st to 5th) with 182.2; 199.5; 221.2; 241.1 and 261.2 g L^{-1} of TRS would have, respectively, the glycerol production estimated in the range of 9.1 to 14.6 g L^{-1}; 10.0 to 16.0 g L^{-1}; 11.1 to 17.7 g L^{-1}; 12 to 19.3 g L^{-1}; 13.1 to 20.9 g L^{-1}. Thus, it is possible to observe that the values from the 1st to the 4th cycle of all treatments are in the range described by [6]. While, in the 5th cycle, the results of glycerol obtained in all treatments were lower than expected by the literature. One hypothesis for this situation is the fact that the yeasts have been gradually adapted to the addition of sugars in the wort and consequently the yeast did not need to increase the production of glycerol to adapt to the medium with high alcohol content. Once, exposure to osmotic stress causes yeast to increase glycerol production in to minimize this toxic effect on the cell [26].

In Very High Gravity (VHG) fermentations yeasts are subjected to several stressors, such as nutritional deficiency, high temperatures, osmotic stress due to sugars, salts, acidity, bacterial contamination, and a high concentration of ethanol. Once the main factor that provides osmotic stress in yeast is a high initial concentration of sugar and salts in the must, and later a high concentration of ethanol produced by the yeast itself [27, 28]. Since ethanol is one of the main cell membrane denaturing agents, the mode of action of ethanol involves a series of consequences that affect various cellular functions [29, 30]. Ethanol can alter the integrity of the yeast cell membrane, cause conformational changes in proteins, cause an increase in reactive oxygen species (ROS), consequently, promoting an increase in the permeability and fluidity of some ions (mainly H^+), consequently, induce the dissipation of the electrochemical gradient across the plasma membrane, decreasing the intracellular pH. In addition to promoting the denaturation and inhibition of the main enzymes of the glycolytic pathway (mainly, pyruvate kinase and hexokinase), causing a reduction in the metabolic activity in yeast. In addition, intracellular ethanol can also cause damage to DNA and lipids [31, 32, 33, 34, 35]. Thus, high concentrations of ethanol in the fermentation medium can lead to inhibition of cell growth and reduced yeast cell viability [36].

For the reasons explained about the cytotoxicity of ethanol, to minimize its effects on yeast cells, it was investigated and observed that some nutritional adjuncts added to the fermentation medium, could act as protein-lipid complexes, particulate materials and osmoprotectors, reducing osmotic stress under the yeast cell, so that they increased cell viability and growth. The increase in yeast cell viability in medium with high sugar concentration and activated silica supplementation obtained in the present study corroborate with the results reported in the literature [28, 37, 38, 39, 40].

A possible explanation for why electronically activated silica may have increased cell viability and cell biomass may be related to its interaction with yeast. This is because the yeast cell wall is composed of a double layer of polysaccharides, formed by insoluble alkaline β-glucans, soluble alkaline β-glucans, manoproteins and chitin. However, the composition of the cell wall is subject to considerable variations according to the growing conditions [41]. The different polarities and the hydrophilic or hydrophobic nature of the polymers on this wall define the yeast's ability to retain or adsorb different molecules [42]. It is known that yeast cells bind to different molecules, such as mycotoxins and metal ions, through complex binding structures on the surface of the cell wall [43]. Brasser and coauthors [44] reported that silicate materials can be adsorbed on the cell surface and interact with ions, so that it can alter the cell's affinity for these ions by significantly altering the growth and cell viability of microorganisms. Weinzierl and coauthors [45], when they added the tetraethyl orthosilicate compound to the substrate, they verified its adhesion to the cell wall surface of the yeast *Saccharomyces cerevisiae*.

Thus, it is inferred that a possible explanation for these observations was that the silica $^{(+A)}$ may have acted in the regulation of cell homeostasis, contributing to reduce the effects caused by high concentrations of sugars at the beginning of fermentations and high concentrations of ethanol at the end of the fermentation process. According to Siderius and Mager [46], the exposure of yeast cells to high osmolarity in the medium, can provide cells with a decrease in intracellular water, leading to loss of turgor and, consequently, reduction of cell growth.

However, there is a lack of information in the literature on the effect of activated silica $^{(+A)}$ on yeasts. Although, there are studies showing the action of activated silica in other species. Decaux [47] reports that the addition of silica $^{(+A)}$ to pigs provides an increase in the ionic potential of water in the digestive system, increasing the absorption of calcium and phosphorus. While Tran and coauthors [48] found that the addition of silica $^{(+A)}$ in the diet of poultry increased the absorption of NH_4 and reduced the volatility of NH_3. In studies with microorganisms, Umamaheswari and coauthors [13] found that compounds containing silicon can promote the growth of *Pseudomonas stutzeri* under oligotrophic conditions, since silicon compounds can assist in the absorption of ammonia and CO_2 from the atmosphere, thus allowing bacteria to fix CO_2, using energy obtained from the oxidation of ammonium. Therefore, it is possible to infer that silica when adsorbed on the yeast cell wall may have helped in the assimilation of nitrogen sources present in the fermentation medium. In fact, in Very High Gravity fermentation, yeast requires nitrogen sources available in the medium to increase the efficiency of cell multiplication [49, 50]. However, the mechanism of action of activated silica $^{(+A)}$ in living organisms is not yet fully understood.

CONCLUSION

The silica $^{(+A)}$ added to the culture medium, in the concentrations of 100 and 300 mg L^{-1} of the product, has a protective effect on the yeasts *Saccharomyces cerevisiae*, strain Ethanol Red® to the stress promoted by the high concentrations of TRS in the wort and content alcoholic. This reduces the osmotic stress caused by fermentations with high levels of sugar in the wort and high concentrations of alcohol in the wine. This can be verified by the fact that in fermentations with high levels of sugar with silica $^{(+A)}$ supplementation, yeasts present increases in cell viability; production of biomass; fermentation yield and productivity.

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