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Silica Wort Supplementation as an Alternative for Yeast Stress Relief on Corn Ethanol Production with Cell Recycling

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Abstract: In very high gravity (VHG) fermentation, yeast cells are subjected to a multitude of challenging conditions, including the osmotic pressure exerted by the high sugar content of the wort and the stress factors associated with the high ethanol concentrations present at the end of the fermentation cycle. The response of this biological system to abiotic stresses may be enhanced through biochemical and physiological routes. Silica may play a significant role in regulating the cellular homeostasis of yeast. Alternatively, it is expected that this outcome may be achieved through biochemical responses from the effects of vitamins on yeast cells and the physiological yeast route changing by the culture medium aeration. The objective of this study was to investigate the effects of adding 500 mg L^{-1} of silica on corn ethanol wort medium and the possibility of supplementing the same wort with vitamins alongside aeration (0.2 v v^{-1} min^{-1}) as an alternative resource to sustain the fermentation yield rather than adding silica in a fed-batch fermentation cycle with yeast recycling. Upon completion of the five fermentation cycles, yeast samples subjected to the treatment with the addition of silica exhibited a 3.1% higher fermentation yield in comparison to the results observed in the vitamins plus aeration medium bath. Even though greater biomass production (19.1 g L^{-1}) was observed through aerobic yeast behavior in vitaminized supplemented corn medium, the provided silica had a more beneficial effect on yeast stress relief for very high gravity fermentation in a corn hydrolyzed wort with cell recycling.

Keywords: fermentation; abiotic stress; yeast response; yeast recycling; *Saccharomyces cerevisiae*; silica; nutrition; aeration; biofuel; corn ethanol

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1. Introduction

Brazil is known for using sugarcane as its primary source of raw material for ethanol production. However, in recent years, there has been steady growth in the corn ethanol industry in the country [1,2]. This expansion is driven by Brazil's international commitments made at the 2015 Paris Climate Change Conference (COP21), which demand more efficient and sustainable bioenergy production in the national energy matrix [3]. To accommodate this increase in corn ethanol production, it will be essential to expand industrial facilities and enhance technologies to improve process efficiency [4].

In this scenario, the adoption of the emerging technology of very high gravity (VHG) fermentation could be an effective contribution to this policy. VHG fermentation has the capacity to increase process productivity and minimize effluent production. This technology aims to reduce process water requirements, thereby decreasing associated costs

such as distillation, effluent treatment, and others, representing a significant portion of the total energy costs, which in turn account for about 30% of the total production costs [5]. Additionally, VHG fermentation offers advantages over conventional approaches, such as higher alcohol yield, reduced labor requirements, lower capital costs, and minimized bacterial contamination [6,7].

VHG fermentation uses sugar concentrations exceeding 250 g L^{-1}, enabling the production of alcohol levels greater than 15% (v v^{-1}) [8]. However, during this process, yeast cells face various stressful factors. The high osmotic pressure caused by high sugar concentrations in the initial stages of fermentation, and the toxic effects of elevated ethanol concentrations are the main factors compromising yeast cell viability [9]. Consequently, these severe environmental stresses often result in fermentation stalling and the formation of more undesirable metabolic by-products [6]. In this context, enhancing resistance to stresses and reducing the formation of metabolic by-products would be beneficial to improve the performance of VHG fermentation yeast strains. To achieve this, the supplementation of VHG fermentation media with appropriate nutrients, which have a protective function, has been shown to be effective in mitigating the harmful effects of high osmotic pressure on yeast cells [10]. Studies have demonstrated that the addition of nutrients such as urea, yeast extract, and soy flour can result in improved cell viability, yeast growth rate, and ethanol production rate in VHG fermentation media [11]. Furthermore, the inclusion of silica in the fermentation medium has been shown to improve yeast fermentative performance in environments with high sugar and ethanol concentrations [12].

Considering the relevance of wort supplementation for VHG fermentation, the purpose of this study was to investigate the effects of adding silica at 500 mg L^{-1} and supplementing vitamins alongside aeration (0.2 v v^{-1} min^{-1}) to the wort used for conducting VHG fermentation in fed-batch mode with yeast recycling.

2. Results

2.1. Cell Viability

Yeast cells subjected to treatments T1, T2, and T3 initiated VHG fermentation with 88% cell viability (Table 1). However, at the end of the five fermentation cycles, a statistically significant difference ($p \leq 0.05$) in cell viability was observed compared to the initial viability of the assay. Yeast cells subjected to treatment T1 exhibited a cell viability of 85.1%, indicating a decrease of 3.4% compared to the initial viability of the assay. On the other hand, yeast cells subjected to treatment T2 showed an increase of 1.0% compared to the initial viability of the assay. However, yeast cells subjected to treatment T3 maintained cell viability at 88.4%, representing cell viability similar to the initial assay. These results indicate that aeration and medium supplementation with vitamins contributed to higher cell viability over the five fermentation cycles. Moreover, the addition of silica at 500 mg L^{-1} enabled the maintenance of initial cell viability at the end of the fermentation process.

When examining the effects of the treatments on yeast over the five fermentation cycles, statistically significant differences ($p \leq 0.05$) were observed. At the end of the five fermentation cycles, treatment T2 provided an increase in cell viability of 4.8% compared to T1 and 1.0% compared to T3. Meanwhile, T3 provided an increase in biomass production of 3.9% compared to T1. These findings suggest that aeration and medium supplementation with vitamins contributed to higher cell viability among all treatments, while the addition of silica at 500 mg L^{-1} helped protect yeast cells, resulting in higher cell viability than that observed in the treatment without supplementation (T1).

Table 1. Results of the fermentative parameters (cell viability, cell biomass, residual total reducing sugars, glycerol and alcohol content) of the yeast *Saccharomyces cerevisiae*, strain Thermosacc Dry®, submitted to treatments T1, T2 and T3.

Parameters	Treatments	Initial	1st Cycle	2nd Cycle	3rd Cycle	4th Cycle	5th Cycle
Cell viability (%)	T1	88.1 ^{Aa} (±0.4)	85.4 ^{BCc} (±0.4)	85.9 ^{Bb} (±0.4)	84.0 ^{Eb} (±0.4)	84.7 ^{Db} (±0.4)	85.1 ^{CDb} (±0.4)
	T2	88.3 ^{Ba} (±0.4)	88.2 ^{Ba} (±0.4)	88.3 ^{Ba} (±0.4)	87.5 ^{Ca} (±0.4)	88.7 ^{ABa} (±0.4)	89.2 ^{Aa} (±0.4)
	T3	88.2 ^{Aa} (±0.4)	87.1 ^{Cb} (±0.4)	88.0 ^{ABa} (±0.4)	87.6 ^{BCa} (±0.4)	88.1 ^{ABa} (±0.4)	88.4 ^{Aa} (±0.4)
Cell Biomass (g L^{-1})	T1	15.0 ^{Ea} (±0.2)	15.4 ^{Cc} (±0.2)	15.8 ^{Bc} (±0.2)	14.6 ^{Dc} (±0.2)	15.5 ^{Cc} (±0.2)	16.3 ^{Ac} (±0.2)
	T2	15.2 ^{Ea} (±0.2)	17.4 ^{Ca} (±0.2)	19.1 ^{Aa} (±0.2)	16.3 ^{Da} (±0.2)	18.4 ^{Ba} (±0.2)	19.1 ^{Aa} (±0.2)
	T3	15.0 ^{Ea} (±0.2)	15.9 ^{CDb} (±0.2)	16.3 ^{Bb} (±0.2)	15.6 ^{Db} (±0.2)	16.1 ^{BCb} (±0.2)	16.6 ^{Ab} (±0.2)
Residual total reducing sugars (g L^{-1})	T1	-	9.8 ^{Aa} (±0.2)	6.5 ^{Ba} (±0.2)	5.2 ^{Cb} (±0,2)	4.1 ^{Da} (±0.2)	3.7 ^{Da} (±0.2)
	T2	-	8.8 ^{Ab} (±0.2)	6.6 ^{Ba} (±0.2)	5.8 ^{Ca} (±0.2)	4.5 ^{CDa} (±0.2)	3.4 ^{Da} (±0.2)
	T3	-	8.5 ^{Ab} (±0.2)	5.1 ^{Bb} (±0.2)	4.7 ^{BCc} (±0.2)	4.5 ^{CDa} (±0.2)	3.6 ^{Da} (±0.2)
Glycerol (g L^{-1})	T1	0.0 ^{Da} (±0.0)	7.5 ^{Ca} (±0.2)	7.6 ^{Ca} (±0.2)	7.7 ^{Ca} (±0.2)	8.2 ^{Ba} (±0.2)	8.7 ^{Aa} (±0.2)
	T2	0.0 ^{Da} (±0.0)	6.8 ^{Cb} (±0.2)	6.9 ^{CBb} (±0.2)	7.3 ^{Bb} (±0.2)	7.9 ^{Aa} (±0.20)	8.2 ^{Ab} (±0.2)
	T3	0.0 ^{Ea} (±0.0)	6.5 ^{Db} (±0.2)	6.3 ^{Dc} (±0.49)	6.9 ^{Cb} (±0.55)	7.3 ^{Bb} (±0.2)	7.7 ^{Ac} (±0.2)
Alcohol content (v v^{-1})	T1	0.0 ^{Fa} (±0.0)	13.9 ^{Eb} (±0.2)	14.8 ^{Db} (±0.1)	15.2 ^{Cb} (±0.2)	15.7 ^{Ba} (±0.2)	16.2 ^{Aa} (±0.2)
	T2	0.0 ^{Fa} (±0.0)	13.4 ^{Ec} (±0.1)	13.7 ^{Dc} (±0.1)	14.6 ^{Cc} (±0.2)	15.4 ^{Ba} (±0.1)	15.8 ^{Ab} (±0.2)
	T3	0.0 ^{Da} (±0.0)	15.8 ^{Ca} (±0.2)	15.8 ^{Ba} (±0.2)	15.8 ^{Ba} (±0.2)	15.6 ^{Ba} (±0.2)	16.4 ^{Aa} (±0.2)

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 ($p \leq 0.05$).

2.2. Cellular Biomass

At the end of the five fermentation cycles, treatments T1, T2, and T3, respectively, resulted in an increase in cellular biomass production of 1.3 g L^{-1}, 4.1 g L^{-1}, and 1.6 g L^{-1} (dry weight). This represented an increase of 8.7%, 27.3%, and 10.7%, respectively, compared to their initial values. These results demonstrate that the high ethanol concentration in the medium did not prevent yeast cells from multiplying over the fermentation cycles.

When analyzing the effects of the treatments used on yeast over the five fermentation cycles, statistically significant differences ($p \leq 0.05$) were observed. At the end of the five fermentation cycles, treatment T2 provided an increase in biomass production of 17.2% compared to T1 and 15.1% compared to T3. Meanwhile, T3 provided an increase in biomass production of 1.8% compared to T1. Thus, aeration and medium supplementation with vitamins applied in the reactor favored higher cellular biomass formation. Additionally, the addition of silica at 500 mg L^{-1} to the fermentation medium helps protect the yeast cell, thus maintaining the ability to produce more cellular biomass than the control treatment during high-alcohol fermentation.

2.3. Total Reducing Sugar

Initially, all the wort to which the three treatments were applied initiated fermentations with the same concentrations of total reducing sugars. In all five fermentation cycles, the concentration of total reducing sugars present in the wort was 300 g L^{-1}. Over the five fermentation cycles, it was observed that as the number of fermentation cycles increased, the concentration of residual total reducing sugars decreased. This can be evidenced by comparing the concentrations of residual total reducing sugars obtained at the end of the first cycle with those of the fifth cycle. At the end of the fifth cycle, fermentations to which treatments T1, T2, and T3 were applied showed reductions in residual total reducing sugars of 62.2%, 61.4%, and 57.6%, respectively, compared to fermentations to which treatments T1, T2, and T3 were applied at the end of the first cycle. These results reveal that as the number of fermentation cycles increased, yeast utilized available sugar more efficiently, converting sugar into various products such as ethanol, cellular biomass, glycerol, and trehalose.

Analyzing the effect of the treatments used on fermentations over the five fermentation cycles, it was observed that at the end of the five fermentation cycles, there was no statistical

difference ($p \geq 0.05$) in the residual total reducing sugar of fermentations exposed to treatments T1, T2, and T3. This indicates that the treatments applied were not able to promote the complete conversion of sugar into products such as ethanol, cellular biomass, organic acids, glycerol, and trehalose.

2.4. Alcohol Content

Throughout the five fermentation cycles, it was observed that as the number of fermentation cycles increased, the alcohol content also increased. This can be evidenced by comparing the alcohol contents obtained at the end of the first cycle with those of the fifth cycle. At the end of the fifth cycle, fermentations subjected to treatments T1, T2, and T3 showed an increase in alcohol content of 16.5%, 17.9%, and 10.8%, respectively, compared to fermentations to which treatments T1, T2, and T3 were applied at the end of the first cycle. These results indicate that as the number of fermentation cycles increased, yeast utilized available sugar more efficiently, converting sugar into ethanol.

At the end of the five fermentation cycles, a statistically significant difference ($p \leq 0.05$) was observed in alcohol content. Fermentations subjected to treatment T2 showed a reduction in alcohol content of 2.5% compared to T1 and 3.6% compared to T3. However, there was no statistical difference ($p \geq 0.05$) in alcohol content between fermentations exposed to treatments T1 and T3. These data suggest that during the fermentation cycles, yeast subjected to treatment T2 utilized a portion of the available total reducing sugar to produce cellular biomass instead of using it for ethanol production. This is supported by the higher cellular biomass formation observed in yeast subjected to treatment T2 compared to yeast subjected to treatments T1 and T3.

2.5. Glycerol

During the five fermentation cycles, it was observed that as the number of fermentation cycles increased, glycerol production also increased. This trend can be identified by comparing the concentrations of glycerol produced at the end of the first cycle with those of the fifth cycle. At the end of the fifth cycle, fermentations subjected to treatments T1, T2, and T3 showed increases in glycerol concentrations of 16.0%, 20.6%, and 18.5%, respectively, compared to fermentations to which treatments T1, T2, and T3 were applied at the end of the first cycle. These data reveal that as the number of fermentation cycles increased, there was an increase in the concentration of glycerol produced, indicating an increase in the stress to which yeast were subjected. This correlation can be explained by the increase in alcohol production by yeast as the fermentation cycles progressed.

When assessing the impact of the treatments used on yeast over the five fermentation cycles, it was found that at the end of the fifth fermentation cycle, treatment T3 resulted in a reduction in glycerol production of 11.5% compared to T1 and 6.1% compared to T2. On the other hand, T2 resulted in a reduction in glycerol production of 5.7% compared to T1. This suggests that the addition of silica at 500 mg L^{-1} to the fermentation medium played an important role in minimizing the stressful effect on yeast cells, leading to a reduction in glycerol production.

2.6. Organic Acids

The organic acids analyzed during the five fermentation cycles were acetic acid, lactic acid, and succinic acid. Throughout the five fermentation cycles, the presence of lactic acid was not detected in fermentations subjected to treatments T1, T2, and T3. These results suggest that there was no contamination in the fermentations by lactic acid bacteria, indicating effective control of microbiological conditions during the fermentation process.

Over the five fermentation cycles, it was observed that as the number of fermentation cycles progressed, the concentrations of acetic acid and succinic acid decreased (Table 2). This trend can be identified by comparing the concentrations of acetic acid and succinic acid produced at the end of the first cycle with those of the fifth cycle. At the end of the fifth cycle, fermentations subjected to treatments T1, T2, and T3 showed reductions in acetic

acid concentrations of 52.0%, 63.1%, and 72.2%, respectively, compared to fermentations to which treatments T1, T2, and T3 were applied at the end of the first cycle. Similarly, at the end of the fifth cycle, fermentations subjected to treatments T1, T2, and T3 demonstrated reductions in succinic acid concentrations of 62.1%, 60.0%, and 58.3%, respectively, compared to the concentrations observed at the end of the first cycle under the same treatments. These findings reveal that in the initial cycles, yeast directed a portion of the total reducing sugar to produce organic acids. However, as the cycles progressed, yeast redirected this total reducing sugar to the production of other products, such as ethanol (mainly), glycerol, and trehalose, resulting in the decrease in acetic acid and succinic acid concentrations over time.

Table 2. Results of the fermentative parameters (trehalose, succinic acid, acetic acid, yield and productivity) of the yeast *Saccharomyces cerevisiae*, strain Thermosacc Dry®, submitted to treatments T1, T2 and T3.

Parameters	Treatments	Initial	1st Cycle	2nd Cycle	3rd Cycle	4th Cycle	5th Cycle
Trehalose (g 100 g^{-1})	T1	11.1 ^{Fa} (±0.2)	13.1 ^{Ea} (±0.2)	15.1 ^{Da} (±0.2)	16.2 ^{Ca} (±0.2)	17.3 ^{Ba} (±0.2)	18.5 ^{Aa} (±0.2)
	T2	11.1 ^{Fa} (±0.2)	12.6 ^{Ea} (±0.1)	14.0 ^{Da} (±0.1)	14.9 ^{Ca} (±0.2)	15.9 ^{Ba} (±0.1)	16.6 ^{Ab} (±0.1)
	T3	11.1 ^{Da} (±0.2)	12.3 ^{Ea} (±0.1)	13.7 ^{Da} (±0.2)	14.5 ^{Ca} (±0.1)	15.4 ^{Ba} (±0.1)	16.3 ^{Ac} (±0.1)
Succinic acid (g L^{-1})	T1	0.0 ^{Fa} (±0.0)	2.9 ^{Aa} (±0.2)	2.4 ^{Ba} (±0.2)	1.9 ^{Ca} (±0.1)	1.3 ^{Da} (±0.1)	1.1 ^{Ea} (±0.1)
	T2	0.0 ^{Ea} (±0.0)	2.5 ^{Ab} (±0.1)	2.0 ^{Bb} (±0.1)	1.4 ^{Cb} (±0.1)	1.2 ^{Ca} (±0.1)	1.0 ^{Da} (±0.1)
	T3	0.0 ^{Fa} (±0.0)	2.4 ^{Ab} (±0.2)	1.6 ^{Bc} (±0.1)	1.4 ^{Cb} (±0.1)	1.2 ^{Da} (±0.1)	1.0 ^{Ea} (±0.1)
Acetic acid (g L^{-1})	T1	0.0 ^{Ea} (±0.0)	2.5 ^{Aa} (±0.1)	2.3 ^{Ba} (±0.1)	1.5 ^{Ca} (±0.1)	1.3 ^{Da} (±0.1)	1.2 ^{Da} (±0.1)
	T2	0.0 ^{Ca} (±0.0)	1.9 ^{Ab} (±0.1)	1.8 ^{Ab} (±0.1)	0.8 ^{Bb} (±0.1)	0.8 ^{Bb} (±0.1)	0.7 ^{Bb} (±0.2)
	T3	0.0 ^{Da} (±0.0)	1.8 ^{Ab} (±0.1)	1.7 ^{Ab} (±0.1)	0.8 ^{Bb} (±0.1)	0.7 ^{Bb} (±0.1)	0.5 ^{Cb} (±0.1)
Fermentative yield (%)	T1	-	71.6 ^{Eb} (±0.5)	76.2 ^{Db} (±0.5)	78.5 ^{Cb} (±0.5)	80.7 ^{Ba} (±0.5)	83.6 ^{Ab} (±0.3)
	T2	-	68.8 ^{Ec} (±0.5)	70.5 ^{Dc} (±0.5)	75.0 ^{Cc} (±0.5)	79.1 ^{Bb} (±0.5)	81.3 ^{Ac} (±0.5)
	T3	-	76.0 ^{Ca} (±0.5)	81.3 ^{Ba} (±0.5)	81.5 ^{Ba} (±0.5)	80.5 ^{Ba} (±0.5)	84.4 ^{Aa} (±0.4)
Fermentation productivity (mL L^{-1} h^{-1})	T1	-	2.1 ^{Cab} (±0.1)	2.2 ^{CBb} (±0.1)	2.3 ^{Bb} (±0.1)	2.3 ^{Ba} (±0.1)	2.4 ^{Aa} (±0.1)
	T2	-	2.0 ^{Cb} (±0.1)	2.0 ^{Cc} (±0.1)	2.2 ^{Bc} (±0.1)	2.3 ^{Ba} (±0.1)	2.4 ^{Aa} (±0.1)
	T3	-	2.2 ^{Ca} (±0.1)	2.4 ^{Aa} (±0.1)	2.4 ^{Aa} (±0.1)	2.3 ^{Ba} (±0.1)	2.4 ^{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 ($p \leq 0.05$).

At the end of the five fermentation cycles, a significant difference ($p \leq 0.05$) was observed in the production of acetic acid produced by yeast subjected to treatments T1, T2, and T3. However, there was no significant difference ($p \leq 0.05$) in the production of succinic acid produced by yeast subjected to treatments T1, T2, and T3. Thus, at the end of the five fermentation cycles, treatments T2 and T3 resulted in reductions in acetic acid concentrations of 41.7% and 58.3%, respectively, compared to treatment T1. However, there was no significant difference between treatments T2 and T3. These data reveal that both treatments with aeration and supplementation of the medium with vitamins and the treatment with the addition of silica at 500 mg L^{-1} helped to reduce the stress caused by the production of acetic acid in the fermentation process.

2.7. Trehalose

At the end of the five fermentation cycles, treatments T1, T2, and T3, respectively, resulted in increased trehalose production of 7.4 g 100 g^{-1}, 5.5 g 100 g^{-1}, and 5.2 g 100 g^{-1}. This represented an increase of 66.7%, 49.5%, and 46.8%, respectively, compared to their initial values. These results indicate that the yeast cells were subjected to stresses throughout the fermentation cycles, leading them to increase trehalose production as a strategy to mitigate these stresses.

When analyzing the effect of the treatments used on the yeast cells over the five fermentation cycles, statistically significant differences ($p \leq 0.05$) were observed. At the end of the five fermentation cycles, treatment T3 resulted in a reduction in trehalose production of 11.9% compared to T1 and 1.8% compared to T2. On the other hand, T2 resulted in a

reduction in trehalose production of 10.3% compared to T1. This reduction in trehalose production indicates that both the treatment with aeration and supplementation of the medium with vitamins and the treatment with the addition of silica at 500 mg L^{-1} helped to reduce the stress on the yeast cells throughout the fermentation cycles.

2.8. Fermentative Yield

During the five fermentation cycles, there was a trend of increased fermentative yield as the number of cycles increased. This trend can be observed by comparing the fermentative yields found at the end of the first cycle with those of the fifth cycle. At the end of the fifth cycle, fermentations subjected to treatments T1, T2, and T3 showed an increase in yield of 16.7%, 18.2%, and 11.0%, respectively, compared to fermentations under the same treatments at the end of the first cycle. These results show that as the fermentation cycles progressed, yeast cells converted total reducing sugar into ethanol more efficiently.

When examining the impact of the treatments used on the yield over the five fermentation cycles, statistically significant differences were found ($p \leq 0.05$). At the end of the fifth fermentation cycle, treatment T3 resulted in an increase in yield of 1.0% compared to T1 and 3.8% compared to T2. On the other hand, T1 resulted in an increase in yield of 2.8% compared to T2. These data suggest that aeration and supplementation of the medium with vitamins contributed to a lower conversion of sugar into alcohol. Meanwhile, the addition of silica at 500 mg L^{-1} increased the conversion of sugar into alcohol.

2.9. Fermentation Productivity

During the five fermentation cycles, there was a trend of increased fermentative productivity as the number of cycles increased. This trend can be observed by comparing the fermentative productivities found at the end of the first cycle with those of the fifth cycle. At the end of the fifth cycle, fermentations subjected to treatments T1, T2, and T3 showed an increase in productivity of 16.9%, 18.0%, and 11.4%, respectively, compared to fermentations under the same treatments at the end of the first cycle. However, when analyzing the effect of the treatments used on the fermentations over the five fermentation cycles, it was observed that at the end of the five fermentation cycles, there was no statistical difference ($p \geq 0.05$) in the fermentative productivity of fermentations exposed to treatments T1, T2, and T3. This suggests that yeast cells converted a greater amount of total reducing sugar into ethanol as the fermentation cycles progressed, regardless of the treatment applied.

3. Discussion

The results obtained in this study indicate that it was possible to carry out VHG fermentation in fed-batch mode using corn hydrolysate as a substrate, and it was also possible to employ a yeast recycling process over five consecutive cycles. To achieve this goal, the SHF (Separate Hydrolysis and Fermentation) process was performed. In this process, corn is ground and mixed with water to form the wort, which is then subjected to the cooking stage. This stage aims to gelatinize all the starch present, making the polymer more soluble and exposed to the action of enzymes during hydrolysis. Subsequently, the liquefaction stage occurs, converting starch into oligosaccharides, followed by saccharification, where oligosaccharides are converted into glucose. The resulting wort is transferred to fermenters where fermentation begins with the addition of yeast [13]. However, after fermentation, the yeast is not separated from the wine; instead, it is subjected together with the wine to distillation. This is because the wine has high viscosity and contains coarse particles, making it difficult to separate yeast from the wine [14,15]. Therefore, aiming to make the yeast recycling process viable, this study employed methods to remove particles from the corn hydrolysate, in order to prepare it for fermentation. For this purpose, a sequence of steps was adopted, including centrifugation, sieving, clarification, and vacuum filtration, with the aim of eliminating particulate matter present in the corn hydrolysate.

During VHG fermentation, yeast is subjected to adverse environmental conditions, including osmotic stress, ethanol toxicity, viscosity, and nutrient limitations [16]. Ethanol,

at high concentrations, can cause toxic effects on yeast, leading to alterations in its physiological state [17,18]. The effects of high ethanol concentration on yeast include cellular dehydration, increased permeability and ion fluidity, especially H^+, denaturation of crucial glycolytic enzymes such as pyruvate kinase and hexokinase, and increased intracellular reactive oxygen species (ROS) [19,20]. Thus, the exposure of yeast cells to high ethanol concentrations results in a reduction in cell biomass and a decrease in cell viability [5].

In the present study, yeast subjected to treatments T1, T2, and T3 did not show a decrease in cell biomass at the end of the five fermentation cycles. These results show that the high ethanol concentration in the medium did not prevent yeast cells from multiplying throughout the fermentation cycles. Furthermore, the viability of yeast subjected to these treatments remained high, remaining above 85.0% after the five fermentation cycles. This result was achieved due to the implementation of continuous feeding together with the supplementation of macronutrients and trace elements in the corn hydrolysate.

VHG fermentation is a technology that employs wort with a high sugar concentration. However, in VHG fermentations, yeast cells are subjected to high osmotic pressure from the substrate sugar at the beginning of the process if all the sugar is added at once [9]. However, fed-batch fermentation, in which nutrients are added incrementally to the fermenter throughout fermentation, reduces the osmotic stress caused by the substrate [21]. In addition, the continuous and gradual addition of nutrients can result in higher cell density during the exponential growth phase, which, in turn, improves product yields [22]. Adequate supplementation of nutrients to VHG fermentation media has been shown to be effective in mitigating the harmful effects of high osmotic pressure on yeast cells [11]. This adequate supplementation contributes to prolonging the yeast cells' logarithmic phase, resulting in higher ethanol productivity [5].

In the present study, as the number of fermentation cycles increased, yeast subjected to treatments T1, T2, and T3 used the available sugar more efficiently, increasing the amount of ethanol produced (from 10.8% to 17.9%). This phenomenon can be attributed to the adaptation capacity of the yeast *Saccharomyces cerevisiae* to adverse conditions, such as high sugar concentrations, high ethanol concentrations, and high temperatures [23,24]. However, as the fermentation cycles progressed and the ethanol concentration increased, an increase in glycerol concentration (16.0% to 20.6%) and trehalose production (46.8% to 66.7%) was also observed, suggesting an increase in stress faced by the yeast. In VHG fermentations, high ethanol concentrations cause greater stress to yeast, leading them to increase glycerol production as a strategy to optimize their survival and proliferation [16]. Glycerol plays a crucial role in maintaining the balance between the NAD^+/NADH ratio during cell growth, helping to regulate cellular respiration and protect the cell against oxidative damage. Another metabolite produced by yeast is trehalose, a storage carbohydrate that acts as a stress protector, preserving the integrity of the plasma membrane and stabilizing proteins [9]. The increase in intracellular trehalose accumulation is a response to environmental stress to which the yeast is exposed [25].

Throughout the five fermentation cycles, an increase in cell biomass production (8.7% to 27.3%) and ethanol concentration (10.8% to 17.9%) was observed; however, there was a decrease in acetic acid production (52.0% to 72.2%) and succinic acid (58.3% to 62.1%). This phenomenon can be attributed to the redirection of sugar by yeast for ethanol production at the expense of organic acids such as acetic acid and succinic acid. Organic acids can inhibit the growth of *Saccharomyces cerevisiae* and lead to intracellular acidification. Acetic acid, in particular, interferes with microbial cell homeostasis by entering the cell in its undissociated form and dissociating in the cytoplasm, resulting in pH reduction and acetate anion accumulation. This intracellular acidification lowers the pH, affecting the proton motive force for transport systems. As a result, a reduction in biomass concentration may occur [26]. Meanwhile, succinic acid, like acetic acid, contributes to disturbing yeast cell homeostasis, causing osmotic stress during alcoholic fermentation. However, succinic acid penetrates yeast cells weakly and slowly [27].

Throughout the five fermentation cycles, another monitored metabolite was lactic acid. However, no production of this acid was identified, indicating effective control of microbiological conditions during the fermentation process. This is because the yeast *Saccharomyces cerevisiae* cannot naturally synthesize lactic acid [28]. The presence of lactic acid bacteria in alcoholic fermentation can result in significant economic losses due to contamination. These bacteria have the potential to inhibit *Saccharomyces cerevisiae*, compromising the fermentation process and resulting in a reduction in ethanol production, either through the generation of lactic and acetic acids or through nutrient competition [29].

In the present study, yeast subjected to treatments T1, T2, and T3 were able to convert a larger amount of total reducing sugar into ethanol as the fermentation cycles progressed, resulting in increased yield (11.0% to 18.2%) and fermentative productivity (11.4% to 18.0%). These findings corroborate with a previous study that also investigated fermentation with cell recycling, using flocculent yeast for ethanol production. In this research, the cell recycling strategy consistently demonstrated an increase in both yield and fermentative productivity over the fermentation cycles [30]. This occurs because cell reuse allows for higher cell density and more efficient substrate utilization, contributing to optimizing fermentation processes and increasing ethanol production [31].

Analyzing the effects of treatments, it was observed that treatment T2, which involved aeration and vitamin supplementation in the fermentation medium, showed superior results in terms of cell viability (89.2%) and cell biomass production (19.1%) when compared with all other treatments over the five fermentation cycles ($p \leq 0.05$). These results suggest that yeast subjected to treatment T2 directed a portion of the available total reducing sugar to cell biomass production instead of converting it into ethanol. This is evidenced by the increase in cell biomass formation in yeast treated with T2 compared to those subjected to treatments T1 and T3 (17.2% and 15.1%). Therefore, aeration and vitamin supplementation may have contributed to a lower conversion of sugar into alcohol, favoring cell biomass production. Adequate aeration plays a crucial role in maintaining cell viability under high ethanol concentration conditions during fermentation. The presence of oxygen can increase in intracellular contents of saturated fatty acids, unsaturated fatty acids, total fatty acids, and ergosterol in yeast cells, improving the functionality of the cell membrane and resulting in higher counts of viable yeast cells [32]. Additionally, adequate nutrient supplementation is effective in mitigating the negative effects of high osmotic pressure on yeast cells, improving cell viability, growth rate, and ethanol production under high sugar fermentation conditions [11].

Vitamins play a crucial role in the optimal performance of yeast in high-sugar fermentations. Adding a mixture of vitamins to the growth medium, along with other nutrients, can improve cell viability, yeast biomass production, yield, and fermentation productivity under high ethanol conditions [22,33]. Alfenore and colleagues [34] using *S. cerevisiae* strain CBS8066, observed an increase in final ethanol content up to 19.0% (v/v) in 45 h with a vitamin feeding strategy in fed-batch fermentation. On the other hand, Deesuth and colleagues [23] using *S. cerevisiae* NP 01 achieved a final ethanol content of 16.2% (v/v) in 60 h with nitrogen supplementation and an aeration rate of 0.31 v v^{-1} min^{-1}. Meanwhile, the present study using *S. cerevisiae* strain Thermosacc Dry® and similar conditions such as concentrations of vitamins and aeration (0.2 v v^{-1} min^{-1}) found ethanol content values of 15.8% (v/v) in 66.75 h. These differences in results can be attributed to the use of different yeast strains, as the effectiveness of supplementation and optimal aeration for ethanol production varies according to the yeast strain employed [32].

When analyzing the effects of treatments, it was observed that treatment T3, which involved supplementation of the fermentation medium with silica 500 mg L^{-1}, showed superior fermentative yield results (84.4%) when compared with all other treatments over the five fermentation cycles ($p \leq 0.05$). Additionally, it presented the highest reduction results in terms of trehalose (11.9% and 1.8%) and glycerol production (11.5% and 6.1%) when compared with treatments T1 and T2 over the five fermentation cycles ($p \leq 0.05$). These results indicate that the presence of silica 500 mg L^{-1} helped protect yeast cells from

ethanol-induced stress over the fermentation cycles, resulting in a higher conversion of sugar into ethanol. The inclusion of silica in the fermentation medium has been shown to improve yeast fermentative performance in environments with high sugar and ethanol concentrations, resulting in higher cell viability and biomass. This occurs because silica can be adsorbed onto the yeast cell surface, preventing water loss from the yeast cell. Therefore, silica may play a role in regulating cellular homeostasis, contributing to mitigating the effects of high sugar and ethanol concentrations during the fermentation process [12].

In this study, VHG fermentations were successfully conducted in a fed-batch mode, using yeast recycling and the addition of 500 mg L^{-1} over five consecutive cycles. At the end of these five fermentation cycles, yeast exhibited high cell viability at 89.2% and a 10.7% increase in cell biomass, indicating the yeast's potential for reuse in additional fermentation cycles. In contrast, previous studies by Douradinho and colleagues [4], who also performed VHG fermentations in a fed-batch mode with cell recycling, but without an osmoprotector, reported a decline in cell biomass and low cell viability of 19 to 23% at the end of the fifth fermentation cycle.

Furthermore, the present study highlighted that to increase fermentative parameters in VHG fermentations, it is necessary to combine three factors: fed-batch mode, nutrient supplementation, and the use of an osmoprotector. When examined individually, as shown by Phukoetphim and colleagues [32], various fed-batch schemes for VHG fermentation of sweet sorghum juice, involving different feeding times and rates, did not result in significant improvements in final ethanol concentration. Nutrient supplementation was essential for enhancing cell growth and ethanol production. Similarly, Chan-u-tit and colleagues [35] demonstrated that achieving high efficiency in ethanol production under VHG fermentation conditions required the supplementation of nutrients such as nitrogen and an osmoprotector such as glycine.

4. Material and Methods

4.1. Chemicals and Reagents

The wort used was derived from corn hydrolysis, which underwent grinding in a hammer mill, Tramontina® (Porto Alegre, Brazil), model TRE40, to obtain fragments with a particle size smaller than 2 mm, using a sieve. The hydrolysis process began with the cooking of the particulate corn suspension at a temperature of 86 °C. For this, 1 kg of particulate corn was added to every 2 L of water with pH adjusted to 5.8 using a 0.01 N H_2SO_4 solution. Then, at a temperature of 86 °C, Liquozyme® (Novozymes, Copenhagen, Denmark) α-amylase enzyme (EC3.2.1.1) was added at a concentration of 0.025% w/w of particulate corn, and the mixture was kept under constant agitation for 150 min (liquefaction). After the liquefaction of corn starch was completed, the system was cooled until the temperature stabilized at 65 °C. At this point, the pH of the mixture was adjusted to 5.0. Under these conditions, Spirizyme® (Novozymes, Copenhagen, Denmark) glucoamylase enzyme (EC3.2.1.3) was added at 0.056% w/w of particulate corn, and the mixture was kept under constant agitation for 150 min (saccharification). To carry out (VHG) fermentation in fed-batch mode using corn hydrolyzate as a substrate and employing yeast recycling, the Separate Hydrolysis and Fermentation (SHF) process was implemented. Following the complete hydrolysis of starch, the residual particulates in the must were removed, resulting in a particulate-free corn hydrolyzate. To remove the particles, the material was centrifuged at 3925× g for 20 min using a refrigerated centrifuge, Thermo Scientific® (Waltham, MA, USA) model Sorvall ST 40R [36]. Then, the corn hydrolysate was sieved with a sieve with a mesh size of 0.037 mm and clarified using monobasic sodium phosphate according to the process described by the authors [37]. After clarification, the material was subjected to vacuum filtration with the aid of a vacuum pump and then filtered through 47 mm nylon membranes with pore sizes of 14 µm, 8 µm, and 0.45 µm. After the removal of particles, the corn hydrolysate was transferred to an aluminum pot and heated on a stove until it reached a total reducing sugar (TRS) concentration of 300 g L^{-1}. Subsequently, the corn hydrolysate was supplemented with macronutrients, including 0.8 g L^{-1} of urea,

3 g L^{-1} of KH_2PO_4, and 0.5 g L^{-1} of $MgSO_4 \cdot 7H_2O$. In addition to these nutrients, the medium was supplemented with the following trace elements: 4.5 mg L^{-1} of $ZnSO_4 \cdot 7H_2O$; 0.3 mg L^{-1} of $CoCl_2 \cdot 6H_2O$; 1 mg L^{-1} of $MnCl_2 \cdot 4H_2O$; 0.3 mg L^{-1} of $CuSO_4 \cdot 5H_2O$; 4.5 mg L^{-1} of $CaCl_2 \cdot 2H_2O$; 3 mg L^{-1} of $FeSO_4 \cdot 7H_2O$; 0.4 mg L^{-1} of $NaMoO_4 \cdot 2H_2O$; 1 mg L^{-1} of H_3BO_3, and 0.1 mg L^{-1} of KI. Subsequently, the wort was sterilized in an autoclave at a temperature of 121 °C for 20 min and at 1 atm [38].

4.2. Treatments

During the very high gravity (VHG) fermentation, three treatments were tested, each with five replications per fermentation cycle. These treatments were designed to assess the effects of adding 500 mg L^{-1} of silica and supplementing with vitamins, alongside aeration at a rate of 0.2 v v^{-1} min^{-1}, on yeast performance during VHG fermentation across five fermentation cycles, as outlined in Table 3.

Table 3. Treatments tested in VHG fermentation.

	Treatments
T1	VHG fermentation in the absence of aeration injection (0 v v^{-1} min^{-1}) and absence of vitamin supplementation in the medium.
T2	VHG fermentation in the presence of aeration (0.2 v v^{-1} min^{-1}) and vitamin supplementation: 5 mg L^{-1} thiamine (B1), 5 mg L^{-1} nicotinic acid (B3), 5 mg L^{-1} pantothenic acid (B5), 5 mg L^{-1} pyridoxine (B6), and 1 mg L^{-1} para-aminobenzoic acid in the medium.
T3	VHG fermentation in the absence of aeration injection (0 v v^{-1} min^{-1}) and supplementation with 500 mg L^{-1} of silica in the medium.

4.3. VHG Fermentation in Fed-Batch Mode

To initiate the first fermentation cycle, 15 g L^{-1} (dry weight) of *Saccharomyces cerevisiae*, Thermosacc Dry® strain from Lallemand®, located in Piracicaba, Brazil, was inoculated into 1 L Erlenmeyer flasks containing 75 mL of corn hydrolysate with a total reducing sugar concentration of 300 g L^{-1}. These flasks were maintained at a constant temperature of 30 °C, pH 5.0, and agitation of 180 rpm. After an initial 3 h period in simple batch mode, feeding of the reactor began using a peristaltic pump (Figure 1). This involved pumping 425 mL of corn hydrolysate, also containing a total reducing sugar concentration of 300 g L^{-1}, into the reactor at a rate of 6.67 mL per minute, with this feeding process continuing for 63.75 h. The fed-batch fermentations were conducted across all treatments (T1, T2, and T3) throughout five fermentation cycles, with each total fermentation process lasting 66.75 h. To aerate the fermentation medium, compressed air was used, as described in Table 3, with the amount of aeration being injected determined by using a digital flow meter, Siargo® (Santa Clara, CA, USA), model Mf5706, (0–10 L).

Upon completion of each fermentation cycle, the resulting crude wines were centrifuged at 3925× *g* for 10 min using a refrigerated centrifuge, Thermo Scientific® (Waltham, MA, USA) model Sorvall ST 40R. This process effectively separated the yeast from the wine. Following centrifugation, the clarified fermented wine was transferred to labeled 500 mL plastic bottles and stored at −20 °C ± 2 °C for subsequent physicochemical analysis. Meanwhile, the sedimented yeast material was reserved for use in subsequent new fermentation cycles. In total, five fermentation cycles were carried out, with each treatment replicated five times. The fermentative cycles were conducted to evaluate the yeast's resilience and ability to withstand the stress associated with repeated fermentation cycles.

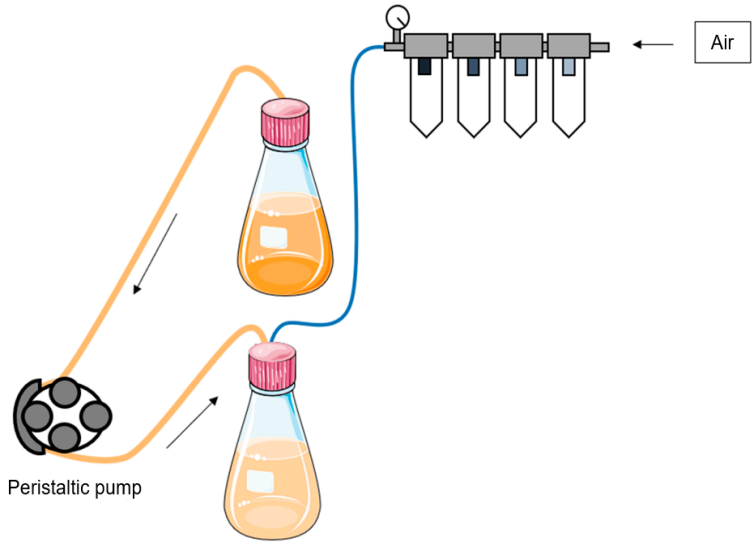

Figure 1. Illustration of the fermentative assay in the present study.

4.4. Cell Viability of Yeast

Cell viability was assessed through differential staining with 0.1% methylene blue solution using a Neubauer chamber and a Nikon® (Melville, NY, USA), model E 200 optical microscope at 400× magnification. Live cells appeared transparent, while dead cells were stained blue, following the method described by Pierce [39].

4.5. Yeast Biomass

Yeast cell biomass determination was performed immediately after fermentation. Samples of 500 mL of wine were centrifuged at 3925× *g* for 10 min. At the end of this operation, the volume of the supernatant was measured using a 500 mL graduated cylinder, so that the volume of the yeast cream was obtained by the difference between the wine volume and the supernatant volume. From the results of the yeast cream volumes, the concentrations of yeast cell biomass (grams of dry matter per liter of wine) were determined according to the methodology described by Koshimizu and colleagues [40].

4.6. Total Reducing Sugars and Glycerol Determination

The concentration of total reducing sugar and glycerol was determined from 1 mL samples of wort and wine. The samples were diluted (10–500×) and filtered using 0.45 µm filter units. Thus, 0.25 µL of the sample was injected into an ion chromatograph, Metrohm® (Riverview, FL, USA) model IC 930, using the following chromatographic system: chromatographic column, Metrosep Carb 1—150/4.0 model; amperometric detector; eluent solution prepared with 200 mM sodium hydroxide and a flow rate of 1.0 mL min^{-1}. The column temperature was maintained at 35 °C, and the chromatographic run time was 9 min [41].

4.7. Alcohol Content Determination

The alcohol content was determined in wine samples using yeast-free wine samples. These samples were distilled in an alcohol distiller, Tecnal® (Piracicaba, Brazil), model TE-010, followed by measurement of the alcohol content using a densitometer 5000, Schmidt Haensch® (Berlin, Germany) model EDM [42].

4.8. Organic Acids

Supernatant from wine samples, obtained after yeast removal by centrifugation, was analyzed for succinic acid, lactic acid, and acetic acid. For this, 100 µL of the sample was diluted with 900 µL of ultrapure water and filtered using 0.45 µm filter units. The samples were injected into an HPLC system with an HPX-87H ion exchange column (Bio-Rad®

located in Hercules, CA, USA) at 60 °C, a mobile phase of 5 mM H_2SO_4, and a flow rate of 0.6 mL min^{-1} [43].

4.9. Trehalose

Trehalose was extracted from yeast cells cooled in ice bath and washed with 2 mL of 0.5 M trichloroacetic acid. For trehalose quantification, 0.3 mL of the sample was mixed with 3 mL of Antrona solution and incubated in a water bath at 100 °C for 10 min. After incubation, the samples were cooled to room temperature and transferred to a quartz cuvette for absorbance measurement at 620 nm using a spectrophotometer Shimadzu® (Columbia, MD, USA) model UV mini-1240 [44,45].

4.10. Fermentative Yield

Fermentation yield was calculated based on the stoichiometry of alcoholic fermentation, where 100% yield corresponds to the formation of 51.11 g (64.75 mL) of ethanol from 100 g of total reducing sugars (TRS). Thus, the fermentation yield (*FY*) was calculated according to Equation (1) [46].

$$FY = \frac{g\ of\ ethanol\ in\ wine}{g\ of\ TRS\ in\ the\ wort \times 0.5111} \times 100 \quad (1)$$

4.11. Fermentation Productivity

Fermentation productivity was calculated based on the alcohol concentration in the wine at the end of fermentation and the fermentation time. Fermentation productivity (*FP*) was calculated according to Equation (2) [46].

$$FP = \frac{Ethanol\ concentration\ in\ the\ wine\ \left(\mathrm{g\ L^{-1}}\right)}{Fermentation\ time(\mathrm{h})} \quad (2)$$

4.12. Statistical Analysis

Statistical analyses were performed using R software version 3.3.2 [47]. The experimental design employed was the mixed linear model. Tukey's test was used for mean comparison at a significance level of 95% ($p < 0.05$).

5. Conclusions

In conclusion, this study demonstrated the feasibility of conducting very high gravity fermentation in fed-batch mode, implementing yeast recycling over five consecutive cycles. During this process, it was observed that yeast subjected to treatments T1, T2, and T3 were able to utilize available sugar more efficiently as the number of fermentation cycles increased, resulting in an increase in ethanol production ranging from 10.8% to 17.9%. However, as the fermentation cycles progressed and ethanol concentration increased, there was also an increase in glycerol concentration, ranging from 16.0% to 20.6%, and in trehalose production, ranging from 46.8% to 66.7%, suggesting an increase in stress faced by the yeast.

Furthermore, it was noted that treatment T2, which involved aeration and vitamin supplementation in the fermentation medium, diverted a portion of the total available reducing sugars to increase cell biomass production by 27.3%, instead of converting it into ethanol. This resulted in a lower fermentative yield (81.3%). Meanwhile, treatment T3, which involved supplementation of the fermentation medium with silica 500 mg L^{-1}, contributed to protecting yeast cells from ethanol-induced stress over the five fermentation cycles. This protection resulted in high cell viability rates, reaching 89.2%, and a superior fermentative yield of 84.4%.

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