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Osmotic Stress Alleviation in *Saccharomyces cerevisiae* for High Ethanol Fermentations with Different Wort Substrates

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Abstract: High-gravity fermentation, used for ethanol production from sugarcane, corn, and mixed substrates, offers several benefits. Yeast, a rapidly multiplying unicellular microorganism, can be adapted for high sugar and ethanol tolerance on a lab scale. However, different substrates can enhance fermentation efficiency. Our study consisted of two experiments. In the first, we compared simple batch feeding with a fed-batch system for yeast selection in high-gravity fermentation. We ran eight cycles with increasing initial sugar contents (50 to 300 g L^{-1}). No significant differences were observed in the first seven cycles, but in the eighth, the fed-batch system showed lower glycerol and fructose contents and higher cell viability than the simple batch system. In the second experiment, we used the fed-batch system with 300 g L^{-1} from sugarcane, corn, and mixed wort. The results showed that mixed wort produced higher ethanol contents and greater fermentation efficiency compared to corn and sugarcane as substrates. In conclusion, our findings indicate that the fed-batch system is more suitable for high-gravity fermentation on a lab scale, and the combination of sugarcane juice and corn can enhance fermentation efficiency, paving the way for integrating these substrates in industrial ethanol production.

Keywords: bioenergy; corn ethanol; mixed wort; sugarcane ethanol; biofuel; high-gravity fermentation; *Saccharomyces*

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1. Introduction

In 2021, the world produced approximately 100 billion liters of ethanol, with 82% of it concentrated in Brazil (27%, mainly from sugarcane) and the United States (55%, primarily from corn) [1]. In Brazil, biofuel production is tending to increase due to the federal program RenovaBio. Under RenovaBio, biofuel producers receive a financial certificate known as a Carbon Credit (CBIO), equivalent to one ton of CO_2 emissions reduced through biofuel production. Fuel distributors are obligated to purchase CBIOs, and these certificates are also available to any interested investors [2,3]. In this context, while Brazilian sugarcane ethanol is considered sustainable with a significantly higher positive energy balance than corn ethanol [4,5], there is still room for adopting processes to further enhance its sustainability, even concerning the application and management of vinasse [6]. In Brazil, the production of ethanol from corn has also witnessed a considerable

increase in recent years, and alternatives to increase its sustainability are needed [7], such as the integration with sugarcane [8,9].

Vinasse is a brown liquid rich in minerals and organic matter that is the byproduct of the ethanol distillation process, which is considered the primary byproduct of ethanol production, as 10 to 15 L of vinasse is generated for every liter of ethanol produced [10]. Handling, transportation, and field application of vinasse is more costly [11,12], though the volume of vinasse generated depends on the ethanol content of the wine during distillation. Fermentation with higher fermentable sugar content can result in a wine with higher ethanol content, offering several advantages, such as reduced investments in reactor size, energy, and water, as well as improved sanitization conditions [13]. The resulting vinasse has a lower volume per ethanol unit, potentially reducing the volume of anaerobic digestion reactors and increasing methane production efficiency [14,15].

In Brazil, the fermentation of sugarcane juice for ethanol production typically lasts between 8 and 12 h. It usually starts with an initial soluble solids content of 16 °BRIX and results in a final ethanol content of approximately 8 to 10%. It is expected that if the wort has soluble solids around 32 °BRIX, the fermentation would last longer, and the final ethanol content would increase to around 16 to 18%, while the volume of vinasse generated would be reduced by half. However, fermenting with high fermentable sugar levels in an attempt to achieve elevated ethanol content can negatively impact yeast physiology and fermentation efficiency. High sugar levels can induce osmotic stress on yeast, leading to reduced cell viability and reproduction rates [16,17]. Additionally, it affects yeast physiology by closing the glycerol channel Fps1p, causing an accumulation of glycerol in yeast cells in an attempt to restore turgor pressure [18]. Additionally, the higher ethanol concentrations may also diminish yeast viability and population growth rates [19]. These effects collectively reduce fermentation efficiency and present barriers to the adoption of high-concentration fermentations.

The yeast (*Saccharomyces cerevisiae*) is a fungus capable of converting fermentable sugars, such as sucrose, glucose, and fructose, into ethanol. In the stoichiometric conversion of sugar to ethanol, one molecule of six-carbon sugar ($C_6H_{12}O_6$: 180 g mol^{-1}) generates two molecules of ethanol (C_2H_5OH: 2 × 46 g mol^{-1}), and two molecules of carbon dioxide (CO_2: 2 × 44 g mol^{-1}). This process is favored under lower oxygen conditions, as high oxygen stimulates yeast to reproduce and increase biomass [20,21]. Yeast is a single-celled microorganism that appears as round or oval structures under a microscope. They can range in size from 3 to 5 µm. Their rapid reproduction rates, high adaptability to environmental conditions, and ease of multiplication allow these microorganisms to be selected at the lab scale for application on an industrial scale [21–23].

Lab-scale fermentations with increasing initial sugar content can serve as a valuable tool for the rapid selection of yeast strains tolerant to high ethanol levels and osmotic stress resulting from high fermentable sugar concentrations [24]. Nevertheless, lab-scale fermentations are typically performed in small reactors, such as Erlenmeyer flasks, where all substrate volumes are commonly added together with the yeast at the beginning of the fermentation in a simple batch system.

Expanding upon that, in this study, we conducted a first experiment (experiment 1) to compare how lab-scale continuous fed-batch (CF) systems affect fermentative parameters compared to a simple batch (SB) system. Our objective was to identify which system was the best to select yeast strains suitable for high-ethanol fermentations. Our hypothesis for this experiment was:

H1: *A fed-batch system would deliver sugar to the yeast gradually, thereby reducing osmotic stress at high initial sugar levels. This reduction in osmotic stress may lead to increased cell viability and improved fermentation efficiency, facilitating yeast adaptation.*

Furthermore, corn ethanol production in Brazil has seen significant growth in recent years [7]. In this context, various studies have proposed the integration of corn and

sugarcane to enhance ethanol production sustainability [8,9]. It also may be a business strategy to extend the industrial season through storage and processing of corn during sugarcane off-season in adapted brownfields sugarcane mills [25] or even a mechanism to increase the fermentation efficiency [8]

Sica et al. (2021) found that combining energy cane juice with corn improved fermentation efficiency compared to using only corn. Their findings suggested that the cane juice supplied minerals that enhanced yeast growth and fermentation efficiency [8]. However, their assessment was limited to an initial sugar content of 200 g L^{-1} in a simple batch system. Therefore, our study conducted a second experiment (experiment 2) with the objective to investigate how different substrates (corn, corn + sugarcane juice, sugarcane juice) influenced fermentation parameters at high sugar content (300 g L^{-1}) using a fed-batch system. Our hypothesis for experiment 2 was as follows:

H2: *Despite having the same sugar content, variations in substrate composition can influence yeast physiology, potentially affecting fermentation efficiency and other parameters.*

Therefore, in this study, we conducted two experiments. In the first experiment, we utilized the same substrate with increasing initial sugar contents to assess how different feeding systems affected fermentative parameters. Based on the results from experiment 1, we selected the fed-batch system to mitigate osmotic stress on the yeast and employed this system with different substrates with high initial sugar contents in experiment 2.

2. Results

2.1. Experiment 1

The ethanol content consistently increased at the end of each fermentation cycle. However, a significant difference in ethanol content between feeding systems was observed only in cycle 3 when the reactors were fed with syrup diluted to 150 g of fermentable sugars per L^{-1}. Cell viability remained relatively stable at around 85–90% for both treatments in all cycles, except for the last cycle, where the yeast viability in the simple batch (SB) feeding system dropped significantly to around 60% (Figure 1). No bacterial contamination was observed at the beginning or at the end of the fermentation.

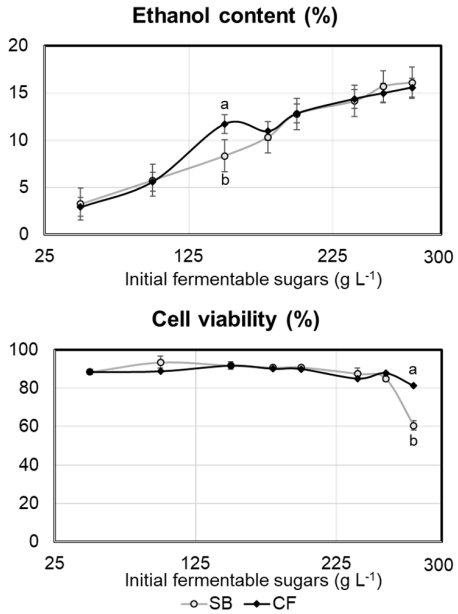

Figure 1. Ethanol content (%) and cell viability (%) at the end of the eight fermentation cycles with increasing initial fermentable sugars content (50, 100, 150, 180, 200, 240, 260, and 280 g of fermentable sugars L^{-1}) in experiment 1. Different letters indicate a significant difference between treatments in the same cycle (Student's test, <0.05).

Both glycerol and mannitol content showed an upward trend throughout the cycles. A significant difference between the treatments was observed only for glycerol in the last cycle, with SB having significantly higher levels than CF (Figure 2). Regarding residual sugars, no sucrose was detected in the centrifuged wine, and glucose levels remained relatively low for both treatments throughout the cycles. The fructose content began to increase after the sixth cycle (>240 g of fermentable sugars per L^{-1}), with CF exhibiting significantly higher residual fructose content in cycles 7 and 8 (Figure 3).

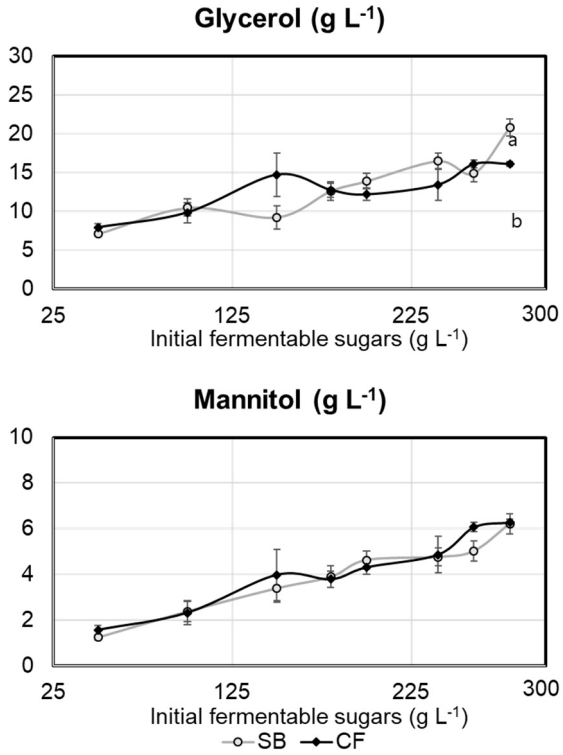

Figure 2. Glycerol (g L^{-1}) and mannitol (g L^{-1}) content at the end of the eight fermentation cycles with increasing initial fermentable sugars content (50, 100, 150, 180, 200, 240, 260, and 280 g of fermentable sugars L^{-1}) in experiment 1. Different letters indicate a significant difference between treatments in the same cycle (Student's test, <0.05).

2.2. Experiment 2

In experiment 2, no significant differences were observed in the final pH and total acidity of the wine among different substrates. However, for the corn (C) hydrolysate, the final yeast cell viability was significantly lower compared to the other treatments. No bacterial contamination was observed at the beginning or at the end of the fermentation (Table 1).

Table 1. Cell viability (%), total acidity (g L^{-1}), and pH of the wort at the beginning and the end of the fermentation in experiment 2 for different substrates. C: corn hydrolysate; C + S: corn mixed with sugarcane juice hydrolysate; S: concentrated sugarcane juice.

	Cell Viability		**Total Acidity**		**pH**	
Substrate *	**Initial**	**Final**	**Initial**	**Final**	**Initial**	**Final**
	(%)		**(g L^{-1})**			
C	87.7 ± 6	12.9 ± 1 b	2.44 ± 0.1	5.79 ± 0.4	5.59	4.44
C + S	87.6 ± 4	38.3 ± 2 a	2.32 ± 0.1	6.02 ± 0.4	5.51	4.49
S	87.2 ± 2	35.1 ± 4 a	1.84 ± 0.1	5.82 ± 0.5	5.30	4.53

Different letters indicate a significant difference between wort (Tukey HSD test, <0.05). * Wort and wine samples were collected and inoculated in a YEPD medium to assess bacterial contamination. No colony-forming units were detected at 10× dilution.

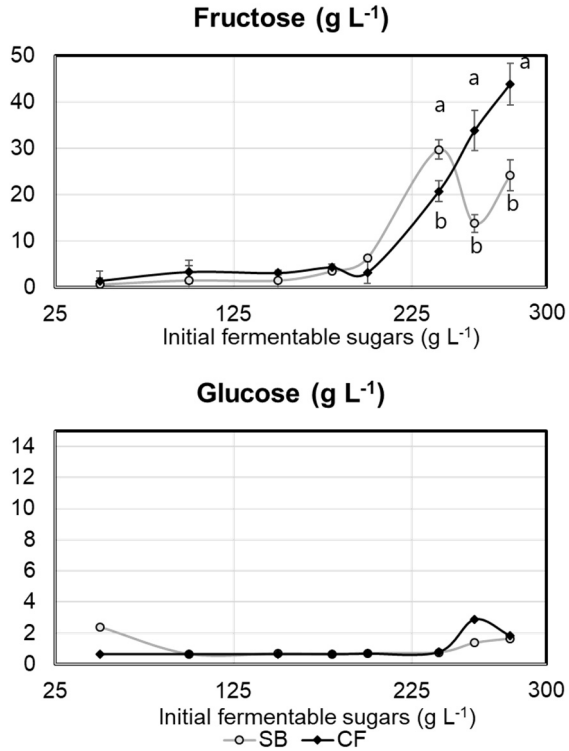

Figure 3. Residual fructose (g L^{-1}) and glucose (g L^{-1}) at the end of the eight fermentation cycles with increasing initial fermentable sugars content (50, 100, 150, 180, 200, 240, 260, and 280 g of fermentable sugars L^{-1}) in experiment 1. Different letters indicate a significant difference between treatments in the same cycle (Student's test, < 0.05).

The three worts' compositions have their chemical differences since corn (C) can provide only glucose as a monosaccharide. The others (S and S + C) also have fructose and sucrose, proportionally from the sugarcane syrup amount on wort preparation (see Section 4.2).

Both corn (C) and mixed wort (C + S) had significantly higher residual sugar content compared to the diluted sugarcane syrup (S). Additionally, the final ethanol content and productivity of the corn and sugarcane hydrolysate (C + S) were significantly higher than those of the corn hydrolysate (C) alone. Fermentation efficiency was also significantly higher for C + S compared to the other treatments (Table 2).

Table 2. Residual sugars, ethanol content and productivity, and fermentation efficiency in experiment 2 for different substrates. C: corn hydrolysate; C + S: corn mixed with sugarcane juice hydrolysate; S: concentrated sugarcane juice.

	Residual Sugars	Ethanol Content	Productivity	Efficiency
	(g L^{-1})	%	g L^{-1} h^{-1}	%
C	54.9 ± 0.1 a	12.25 ± 0.35 b	2.68 ± 0.08 b	89.8 ± 3.0 b
C + S	60.9 ± 0.2 a	14.87 ± 0.22 a	3.26 ± 0.05 a	96.8 ± 2.4 a
S	20.3 ± 0.2 b	14.01 ± 0.36 ab	3.07 ± 0.08 ab	88.4 ± 1.8 b

Different letters indicate a significant difference between wort (Tukey HSD test, <0.05).

3. Discussion

3.1. Wort Disinfection

In the sugarcane ethanol industry, *Lactobacillus* spp. is considered the primary contaminant affecting the fermentation process [26,27]. Bacteria-produced organic acids directly influence the medium's pH during fermentation, potentially disrupting sugar assimilation by the yeast, leading to incomplete sugar consumption [9] and reduced cell viability [28].

In both experiments conducted in this study, the wort was sterilized, and no contamination was observed at the beginning or end of the fermentation cycles. Therefore, the observed differences in fermentative parameters can be attributed to variations in the wort's physicochemical composition.

The wort disinfection process differed between the two experiments. In experiment 1, syrup underwent clarification, dilution, and autoclaving to reduce or eliminate bacterial contamination. While this method is commonly used in laboratory conditions, it can alter the wort composition by increasing sugar content due to water evaporation at higher temperatures [8,24]. In experiment 2, wort from different substrates was sterilized using ionizing radiation at 20 kGy. Ionizing radiation has been proposed as an alternative for wort disinfection in both laboratory-scale [9] and industrial-scale applications [29–31]. One of its primary advantages is that it does not affect the quality of the substrate [32]. In the industrial setting, it can also help reduce the consumption of antibiotics [9]. Recent studies have demonstrated its ability to remove contaminants in sugarcane wort [33] and control *Lactobacilli* in corn and corn mixed with sugarcane wort [9]. Therefore, the results of this study confirm the existing literature on the efficiency of ionizing radiation in controlling bacterial contamination in sugarcane, corn, and mixed wort on a laboratory scale.

3.2. Yeast Osmotic Stress

In this study, we hypothesized (H1) that to minimize osmotic stress and potential efficiency losses during fermentation when selecting yeast strains for high sugar and ethanol content fermentations, wort should be gradually fed to the yeast (fed-batch system). This method is commonly employed in 83% of Brazilian ethanol production units but is not always practiced frequently in lab-scale experiments [27]. Given the high concentrations of readily available sugars in the wort, we anticipated a significant osmotic differential within yeast cells in a simple batch system [34]. Such differentials have been known to potentially impact the quality of the fermentation process [35,36]. However, in experiment 1, this only occurred in the eighth cycle when the wort contained 280 g L^{-1} of sugar, resulting in a significant reduction in yeast cell viability and an increase in glycerol content in the wine. In experiment 1, we also observed that in the third cycle (150 g L^{-1} of sugar), the ethanol and glycerol contents for the fed batch were higher than in the fourth cycle (180 g L^{-1} of sugar). We speculate that this may indicate osmotic stress during the fourth cycle, leading to a reduction in fermentation efficiency [37]. Nevertheless, the yeast demonstrated adaptability, as evidenced by its ability to reach higher ethanol contents in the subsequent cycles.

Klein et al.'s (2017) results showed that *S. cerevisiae* undergoes metabolic adaptation in response to high sugar concentrations, a common feature of challenging environments. Glycerol synthesis serves as a critical mechanism for rapidly releasing carbohydrates to yeast under such conditions, alongside other stress-responsive compounds like trehalose [38]. Glycerol plays a vital role in cellular protection during osmotic stress conditions [39–41]. It also serves as an electron acceptor for the reoxidation of excess NADH, helping to maintain the cellular redox balance [42,43]. Throughout experiment 1, it was only in the last cycle that yeast began to exhibit an increase in glycerol production and a decrease in cell viability within the simple batch system, as compared to the fed-batch system. Surprisingly, this phenomenon did not have a discernible impact on ethanol production when comparing both treatments. In fact, an intriguing trend emerged in the last three cycles, where ethanol production appeared to stabilize at around 15%. Concurrently, there was a noticeable increase in residual fructose levels in the wine for both treatment groups.

3.3. Effects of High Ethanol Content on Fermentation Efficiency

In a study conducted by Jones et al. (1994), they carried out a very high gravity fermentation using wort prepared from syrup with a fermentable sugar content of 27.1%, which is similar to the levels used in the eighth cycle of this study. During their fermentation process, they observed a sharp decrease in cell viability, leading to incomplete fermentation, resembling the high residual sugar levels observed in this study. Despite achieving an

ethanol content of 13.3% (v/v) in the wine, their yield was as low as 74% due to the incomplete conversion of sugars into ethanol. Subsequently, Jones et al. (1994) conducted further experiments using the same syrup but with a dilution to fermentable sugars of 12.2%. Interestingly, they attained a significantly higher fermentation yield of 94%. However, the alcohol content in the wine was reduced to 7.7% (v/v). In the absence of ethanol-induced stress, the fermentation continued until all available sugars were exhausted [44].

These findings align with our results from experiment 1, suggesting that yeast activity was reduced when the fermentable sugar content exceeded 240 g L^{-1}. This increase in ethanol content to around 15% had a detrimental impact on yeast performance in converting sugars to ethanol, resulting in elevated residual fructose levels. These high residual fructose and low glucose results are consistent with the fact that, upon hydrolysis, sucrose provides both glucose and fructose monomers to the solution, but yeast tends to prioritize glucose metabolism over fructose [45].

The primary effects of ethanol toxicity are centered on the yeast cellular membrane, specifically the lipid bilayers. Recent studies using lipidomics have helped uncover the mechanisms behind alcohol tolerance in yeast. These studies indicate that yeasts with higher tolerance to high ethanol concentrations have elevated levels of phosphatidylcholine species, which have demonstrated the ability to stabilize model membrane bilayers in the presence of high ethanol levels. In contrast, strains that cannot complete fermentation at high ethanol concentrations, resulting in high residual sugar levels, tend to have high levels of phosphatidylinositol [46]. Thus, the species of organic phosphorus synthesized and allocated in the cellular membrane lipids bilayers are crucial to determining the cell's ability to tolerate high ethanol content.

Jones et al. (1994) attributed the interruption of the fermentation process to insufficient phosphorus levels in the wort, which measured 286 mg L^{-1}. To test their hypothesis, they increased the phosphorus content to 814 mg L^{-1} by adding diammonium phosphate to the undiluted broth with a fermentable sugar content of 27.1%. This adjustment resulted in the maintenance of cell viability and an increase in yield, confirming their hypothesis [44]. Therefore, in order to reach successful results in the selection of yeast for high ethanol fermentations, supplementation with nutrients may be needed.

3.4. Effects of Substrate on Fermentation Efficiency and Future Perspectives

Recently, driven primarily by the low prices of raw materials, corn ethanol production in Brazil has been on the rise and is expected to continue increasing in the years to come [7]. In contrast, ethanol production from sugarcane in Brazil has a long-standing tradition [47]. Consequently, various studies have proposed integrating sugarcane and energy cane into the corn ethanol production system in Brazil, thereby adapting the conventional corn ethanol production methods commonly employed in the United States [8,9,48].

The energy cane, for example, emerges as a viable option for providing the necessary bagasse biomass to meet industrial plant energy requirements [49,50]. Additionally, the juice extracted from energy cane, which tends to be less concentrated than sugarcane juice [50], offers an opportunity to replace water in the dilution of corn during the ethanol production process [8,9]. In this study, the substitution of water during the hydrolysis step results in a water savings of approximately 122 mL for every 78 g of corn processed. When applied at an industrial scale, this volume assumes significant proportions, equating to approximately 1.56 m^3 of water conserved for every 1 ton of corn allocated for ethanol production. Considering average agro-industrial yields [51], this translates to a potential saving of about 3.9 L of water for every liter of ethanol produced.

In this research, we observed a significant increase in fermentation efficiency for the treatment involving mixed wort (corn + sugarcane juice). These findings align with those of Sica et al. (2021), who reported a 5% increase in fermentation efficiency when corn was mixed with energy cane juice compared to using corn alone. The improved efficiency was primarily attributed to the higher levels of phosphate and nitrate present in the medium, provided by the addition of energy cane juice [8]. Indeed, the supplementation

of micronutrients and macronutrients can increase fermentation efficiency, as demonstrated by Poisot et al. (2011) [52].

In contrast, the corn (C) treatment in our study exhibited the lowest final cell viability, resulting in a final ethanol content of 12.3%, which was significantly lower than that achieved with the mixed wort. Consequently, it also displayed a lower fermentation efficiency. As discussed earlier in experiment 1, yeast efficiency tended to decrease when the ethanol content reached around 15%, a threshold similar to that achieved in the C + S treatment in experiment 2. However, for the treatment involving only corn (C), cell viability plummeted, and ethanol production ceased at 12.3%.

As suggested by Sica et al. (2021) and corroborated by Jones et al. (1994), the additional nutrients, such as phosphates, provided by the sugarcane juice may have enhanced yeast tolerance to ethanol, resulting in significantly higher ethanol content. Nevertheless, this enhancement was insufficient to sustain further fermentation, as evidenced by the C + S treatment, which still exhibited a considerably high residual sugar content [8,44].

Nutrient supplementation is crucial to achieving high ethanol content in worts containing a mixture of sugarcane juice and corn hydrolysates [53]. The yeast's tolerance to ethanol may also depend on the availability of nutrients in each of the musts, as yeast cells have an increased demand for microelements when under stress, as mineral ions play an essential role in maintaining pH, osmotic stability, nutrient transport, and serving as cofactors in fermentation reactions [54].

In our experiment 2, we supplied the worts with 600 mg L^{-1} of urea, following the recommendation of Kovalchuk et al. (2017) [55]. The addition of nitrogen sources can boost the population of physiologically active yeast cells, increasing the chances of success in fermentations with worts containing high sugar concentrations [54]. Moreover, yeast's capacity for biosynthesis and biomass formation is closely linked to the availability of nitrogen in the medium [56]. In conditions of ethanol stress, the assimilation of nitrogenous sources is significantly impacted, as the accumulation of ethanol strongly inhibits the amino acid transport pathway [54].

However, we did not supplement with phosphorus sources, which could have increased the yeast tolerance to high ethanol content and allowed for an increased consumption of residual sugars, reaching higher ethanol content. The concentration of ethanol in the medium, therefore, enters as an aggravating factor to the nutritional condition of the yeast cells and, consequently, to the performance of the fermentation. In addition to the aforementioned effects, ethanol also denatures and inhibits glycolytic enzymes and can lead to the formation of mutant cells [19]. Such a condition reduces the regenerative and fermentative activity of yeasts [54].

4. Materials and Methods

This study consisted of two experiments. The first experiment aimed to assess how two different lab-scale feeding systems impacted yeast fermentation parameters during different fermentation cycles with increasing initial fermentable sugar content. Based on the findings from this first experiment, we selected the fed-batch system to investigate how different substrates affect various fermentation parameters under high initial sugar content conditions. Table 3 provides a brief overview of both experiments, while the subsequent sections offer a more detailed description of the experimental design and analyses.

The syrup utilized in this study was obtained from the Sugar and Ethanol Mill Granelli, situated in the municipality of Charqueada, São Paulo, Brazil. Upon collection, its fermentable sugar content was measured, and it was subsequently diluted to achieve the desired sugar content based on those measurements. To remove impurities, the diluted syrup underwent clarification using monobasic sodium phosphate, following the procedure detailed by Sica et al. (2021) [8]. The corn used in both experiments was purchased from a local store, composed of 71% starch, and ground by hammer milling (Marconi Laboratory Equipment, Piracicaba, Sao Paulo, Brazil) to obtain a fine powder (<2 mm).

Table 3. Brief description of the treatments and overview of experiment 1 and experiment 2.

Experiment 1	
Treatments:	Simple batch (SB) and continuous fed batch (CF)
Yeast:	*Saccharomyces cerevisiae*, strain: C22 mycofer
Substrate:	Diluted sugarcane syrup
Cycles:	8 cycles with increasing initial fermentable sugars: 50, 100, 150, 180, 200, 240, 260, 280 g L^{-1}
Experiment 2	
Treatments (substrates):	Different substrates: Corn hydrolysate (C), corn plus sugarcane juice hydrolysate (C + S), and sugarcane juice (S)
Yeast:	*Saccharomyces cerevisiae*, strain: Thermosacc
Feeding System:	Fed batch for first 135 min (1 mL min^{-1}), based on the results of experiment 1

4.1. Experiment 1 Setup

This experiment was carried out with eight consecutive fermentation cycles with an increasing initial fermentable sugar content. The syrup had its total fermentable sugar content determined and was properly diluted, aiming for the following content for each cycle: 50, 100, 150, 180, 200, 240, 260, and 280 g of fermentable sugars L^{-1} (Table 3). After dilution, the substrates were autoclaved at 125 °C with 1 kg-force per cm^{-2} of pressure (kgf/cm^2) for 25 min to be sterilized.

Fermentation was conducted in triplicates in Erlenmeyer flasks with a total working volume of 300 mL of substrate. In the first cycle, 9 g of yeast (strain: Mycoferm C22, in dried powder form, from the Italian company EVERINTEC) was added to the reactor (30 g L^{-1}). The dry yeast was inoculated in the first cycle, performed with low sugar content (50 g L^{-1}), also aiming to rehydrate and reactivate the yeast cells. The initial number of cells was 10^9 mL^{-1}. After 10 h of fermentation, the Erlenmeyer flasks were weighted every 2 h to estimate the amount of CO_2 released and estimate the sugar consumption. Based on that estimation, the time to finish the fermentation was determined. At the end of each cycle, the total volume was centrifuged at 5000 rpm (2719 g) for 10 min. The resulting supernatant (wine) was stored and analyzed for residual sugars, glycerol, mannitol, and ethanol content. The centrifuged solids, which included yeast, were thoroughly mixed and recycled to provide yeast for the subsequent cycle. This process was repeated for all fermentation cycles and is represented in Figure 4(left).

In this experiment, we employed two treatments. In one treatment, all 300 mL of the substrate were directly mixed with the yeast at the beginning of the fermentation, constituting a simple batch system (SB). In the other treatment, the yeast was mixed with 30 mL of the substrate at the beginning of the fermentation, while the remaining 270 mL was provided using a fed-batch system (as shown in Figure 4, left) at a rate of 2 mL per minute for 135 min.

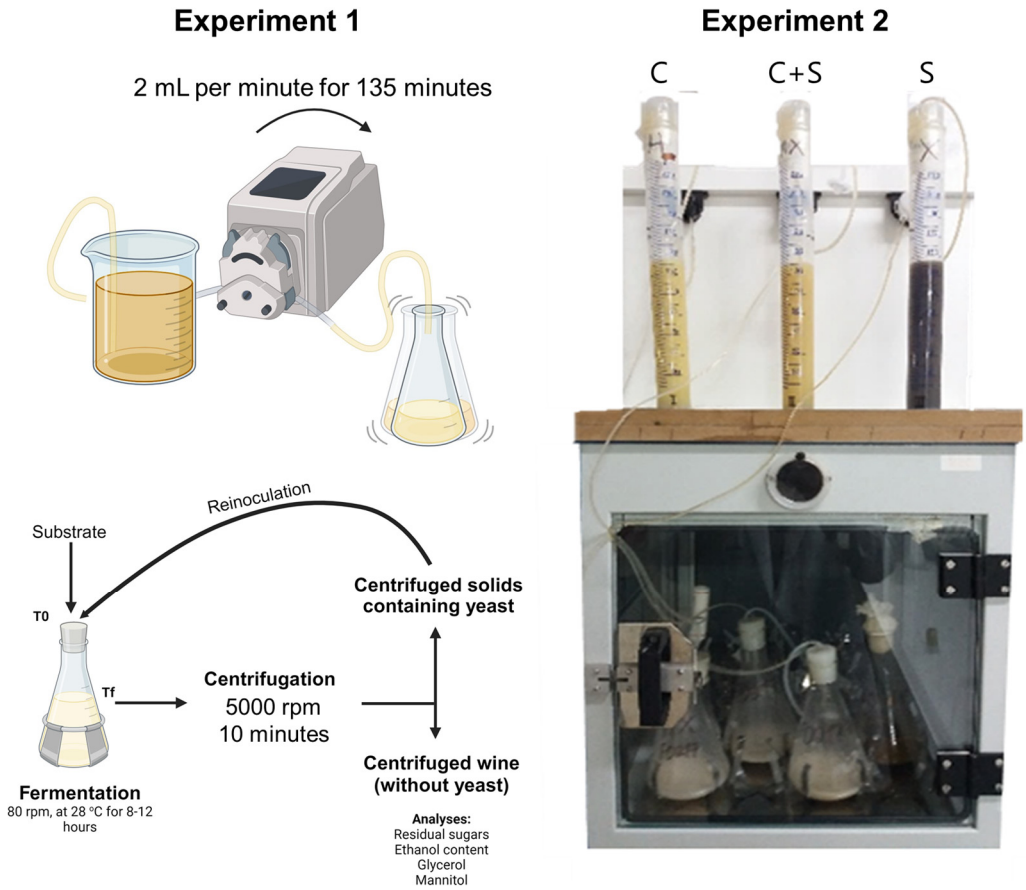

Figure 4. Schematic representation of the batch-feeding and recycling system used in experiment 1 (**left**) and the adaptation for the batch-feeding used in experiment 2 (**right**) for each block with different substrates. C: corn hydrolysate; C + S: corn mixed with sugarcane juice hydrolysate; S: concentrated sugarcane juice.

4.2. Experiment 2 Setup

The treatments and experimental setup used in experiment 2 are briefly demonstrated in Table 3 and Figure 4(right). In this experiment, we assessed how different wort compositions affected fermentative parameters. The syrup was diluted to 300 g of fermentable sugars L^{-1}, consisting of the treatment with only sugarcane-derived substrate (S). The corn (C) and corn plus sugarcane mixed (C + S) wort were hydrolyzed before the fermentation. For the corn wort, 639 g of corn was added to one liter of distilled water. For the mixed wort (C + S), 639 g of corn was added to one liter of diluted syrup (58 g fermentable sugars L^{-1}).

The distilled water and diluted syrup were preheated to 55 °C and already contained 80 mg of Liquozyme® α-amylase enzyme (Novozymes, Copenhagen, Denmark). After adding the corn particulates, the system was heated for approximately 40 min until the temperature stabilized at 88 °C. Upon reaching 88 °C, the mixture received an additional 80 mg of the same Liquozyme® α-amylase enzyme and was maintained under constant temperature stirring at 80 rpm for 150 min.

Once the corn starch liquefaction was complete, the system was cooled until the temperature stabilized at 65 °C. At this point, the pH of the mixture was 5.0, with no need to be adjusted. Under these conditions, 224 mg of Spirizyme® glucoamylase enzyme (Novozymes, Copenhagen, Denmark) was added, and it was maintained under constant temperature and stirred at 80 rpm for the saccharification. After the saccharification stage was concluded, the mixture, consisting of corn particles and sugar solution, was centrifuged using a Thermo Scientific® (Waltham, MA, USA) horizontal centrifuge, model Sorvall ST40R, at a rotation speed of 10,000 rpm (3924 g), at a temperature of 5 °C, for

10 min. The sugar solutions obtained as supernatant from centrifugation were then filtered through a 210 µm sieve and used to prepare the worts by diluting it to approximately 300 g of fermentable sugars L^{-1}. For each wort (C, C + S, and S), nitrogen was added by 600 mg L^{-1} of urea. To be sterilized, the worts were irradiated in an electron accelerator at a dose of 20 kGy, as described by Silva et al. (2023) [9].

The fermentation was carried out in Erlenmeyers with a volume of 180 mL with 5.4 g of yeast (3% w/v). First, the wort (10 mL) with twenty milliliters (20 mL) of sterilized distilled water was added to the reactors. This mixture is composed of a diluted solution designed to rehydrate the dehydrated yeast cells. Rehydration occurred under a constant temperature of 39 °C ± 0.2 for fifteen minutes, following the method described by Kraus, Scopp, and Chen (1981) [57]. After the rehydration period, the yeast cells in the reactors were fed with their respective wort for 135 min, maintaining constant agitation at 120 rpm and a temperature of 33 °C ± 0.2. The reactor received a total of 150 mL of wort, delivered via peristaltic duct devices connecting the wort reservoir to the alcoholic fermentation reactor (Figure 1, right). The experiment consisted of a single cycle of alcoholic fermentation without yeast recovery and was organized into five blocks, each containing one replicate of the three treatments in a randomized block experimental design.

4.3. Analytical Procedures

Yeast viability was assessed through the differential staining of living cells using a 0.1% methylene blue solution. Dead, live, and viable cells were counted in a Neubauer chamber under light microscopy, following the methodology described by [8,58,59].

For the determination of ethanol content, 25 mL of the supernatant of centrifuged samples were collected from each experimental unit at the end of the fermentation. These samples were distilled using a micro-distiller MA 012/1 (Marconi, Piracicaba, Sao Paulo, Brazil). After distillation, analysis was performed using the Schmidt Haensch Digital Densimeter EDM 4000.

In experiment 1, the supernatant of the centrifuged wine was also analyzed for sucrose (not detected), fructose, glucose (residual sugars), glycerol, and mannitol content using a 930 Compact IC Flex ion chromatograph (Metrohm) equipped with an amperometric detector and a Metrospec Carb 1 column. For the analysis, a 100 mmol L^{-1} NaOH solution was used as the eluent, flowing at a rate of 1 mL min^{-1}, and the temperature was maintained at 35 °C.

The determination of total acidity in the centrifuged wine from experiment 2 was carried out using the methodology proposed by Amerine and Ough (1981) [60]. Therefore, 20 mL of homogenized sample was transferred to an Erlenmeyer flask, added with 50 mL of deionized water and 2–3 drops of 1% phenolphthalein indicator solution (m/v), and then titrated until color changes (pH 8.2)—with a slightly pink turning point. From the consumed volume of 0.1 N NaOH, the total acidity was then determined, with the result expressed in grams of acetic acid per liter.

In experiment 2, the ethanol productivity was calculated as the amount of ethanol produced (in grams) per liter of wort per hour of fermentation. The fermentation efficiency is defined as the percentage ratio between practical and theoretical yields, considering the stoichiometric ethanol production per 100 g of six carbon sugars, according to Gay-Lussac (51.11 g of ethanol/100 g of sugars), as previously described by Sica et al. (2021) [8].

Bacterial and total mesophilic counts were conducted using the serial dilution and pour plate technique on the Plate Count Agar (PCA) medium. The plating procedures followed the protocols described by Oliveira et al. (1996) [59]. Serial dilutions were prepared in test tubes containing 9 mL of deionized water supplemented with 0.1% (w/v) peptone and sterilized. Petri dishes were incubated in a Marconi® oven (model MA415) at 30 ± 0.5 °C for 48 h to allow microbial growth, followed by colony counting. In counts aimed at determining the number of bacterial colonies exclusively, cycloheximide (Actidione®, Sigma-Aldrich; Darmstadt, Germany) was added to the culture media at a concentration of 10 mg L^{-1} to inhibit yeast growth.

4.4. Statistical Analysis

The statistical analyses were performed using the IBM SPSS 28.0 software. In experiment 1, one-way ANOVA was performed for all the parameters, and Student's *t*-test (<0.05) was used to compare the means of the feeding systems in each cycle. In experiment 2, one-way ANOVA was performed for all the parameters, and the differences among means for different substrates were assessed with the Tukey HSD test (<0.05).

5. Conclusions

This study highlighted key findings in yeast fermentation for biofuel production. The osmotic stress caused by high initial fermentable sugar content can be alleviated by gradual wort feeding; however, high ethanol levels may reduce yeast viability and stop fermentation at around 15% ethanol content. To overcome this, we suggest the supplementation of the wort with different nutrients, such as nitrogen and phosphorus, as our results confirmed that mixing corn with sugarcane juice enhances fermentation efficiency due to increased nutrient availability to the yeast. Therefore, these insights offer valuable guidance for optimizing biofuel production processes, especially in the context of integrating different feedstocks and improving fermentation outcomes.

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