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Biotechnological Valorization of Brewer's Spent Grain from Old Bread and Barley Malt: Fermentative Potential of *Saccharomyces cerevisiae*

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Abstract

Brewer's spent grain (BSG), the most abundant by-product from breweries, is mainly discarded or used as animal feed. However, to increase the brewing sustainability, biotechnological utilization of BSG is a much preferred solution. This study examined the fermentation of BSG, composed of old wheat bread and barley malt, by metabolic activity of *Saccharomyces cerevisiae* on both hydrolyzed and non-hydrolyzed media. Enzymatic hydrolysis with Viscozyme® W FG for 6 h was selected as the most effective and was used in the further research step to prepare the hydrolyzed BSG-based medium. Both media supported almost uniform yeast growth (numbers of *S. cerevisiae* cells was about 8 $\log_{10}$ CFU/g) in an acidic environment (pH value was about 5), but fermentation of hydrolyzed BSG resulted in 20% higher sugar consumption and 10% higher total titratable acidity. These findings underscore the potential of enzymatic pretreatment to improve fermentation performance. The adaptability of *S. cerevisiae* and the fermentability of both substrates suggest promising potential for scalable BSG valorization strategies in circular food systems.

Keywords: bakery leftovers; brewer's spent grain; enzymatic hydrolysis; fermentation; conventional yeast; brewing sustainability

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1. Introduction

Beer, as the main product of the brewing industry, demonstrates remarkable variability, even with minor modifications in its production process. Changes in mashing temperature and time, fermentation temperature and duration, or adjustments in raw materials, such as hop variety and timing of hop additions, can significantly influence the final sensory and chemical profile of beer [1]. A particularly promising avenue for waste valorization is the reuse of old bread, a commonly discarded food product. Across the globe, large quantities

of bread are wasted daily, leading to serious environmental impacts. Rather than being discarded, this leftover bread can be repurposed in food processing [2], especially within the brewing industry. Specifically, Martin-Lobera et al. (2022) highlighted the value of bread as an ingredient in craft ale beer production, illustrating how it can enhance both the sustainability of the brewing process and the sensory qualities of beer [3]. This not only helps reduce food waste but also supports a more sustainable production process, aligning with circular economy principles.

The most important by-product of the brewing industry is brewer's spent grain (BSG) [4]. Its composition can vary depending on the quality of barley or other grains used in beer production, as well as other factors such as harvest time, malting and mashing conditions, and the quality of unmalted raw materials [5]. The growing focus on sustainability is also evident in the utilization of BSG in biotechnological processes. Enzymatic treatments with commercial preparations (e.g., cellulases, xylanases) and cultivation of fungal strains selectively degrade BSG components while preserving valuable nutrients for fermentation [6]. Studies have explored the use of BSG as a substrate for fermentation by microorganisms like the conventional yeast *Saccharomyces cerevisiae*, which has proven effective in the production of citric acid [7], fungi like *Trametes versicolor*, which have shown potential for the production of polyphenols and the lignin-degrading enzyme laccase [8], as well as for non-conventional yeasts and lactic acid bacteria, which have shown potential in improving fermentation outcomes and product diversity [9]. Recent studies also demonstrate a high level of public interest in BSG-enriched foods, with a 2023 Uruguayan survey reporting that 86% of participants were willing to purchase such products [10]. These findings reinforce the importance of developing novel applications for BSG in the context of circular economy and sustainable food systems.

BSG is often undervalued by breweries and primarily used as animal feed or discarded as urban waste, imposing economic and environmental burdens on municipalities responsible for waste management [11]. Different preservation and valorization pathways have been evaluated to mitigate these impacts and increase BSG's use in human nutrition [12]. Recent research has demonstrated the financial and economic feasibility of establishing biorefineries to convert brewer's spent grain into special flour, particularly for craft beer breweries where waste volumes vary significantly [13].

S. cerevisiae is a common biocatalyst in the fermentation industry due to its ability to metabolize different substrates to produce a variety of valuable products [14]. In recent research, *S. cerevisiae* has been used for fermentation of kitchen waste [15], date palm waste [16], pineapple waste [17], watermelon waste [18], potato peels [19], sugarcane molasses [20] and bagasse [21], sugar beet molasses [22] and all intermediates of sugar beet processing [23], soybean molasses [24], industrial paper wastes [25], and other fractions of food waste and agro-industrial intermediates, by-products, and effluents. However, its potential for BSG fermentation has not been sufficiently examined.

This study aims to explore the utilization of BSG, a by-product from beer brewed with old bread and barley malt, as fermentation substrate for *S. cerevisiae*. Examination of the fermentative potential of *S. cerevisiae* on hydrolyzed and non-hydrolyzed BSG derived from old wheat bread and barley malt seeks to contribute to the development of more sustainable and resource-efficient brewing practices. The study evaluates key fermentation parameters, such as yeast cell number, reducing sugar content, and pH value total titratable acidity, offering insights into the potential of using these industrial by-products in fermentation to enhance sustainability and improve the quality of the final product.

2. Materials and Methods

2.1. Collection of Raw Materials and Processing

American Pale Ale beer was brewed at the University of Mostar using a blend of 1/3 old wheat bread, aged for two days (Mlini d.o.o., Čapljina, Bosnia and Herzegovina), and 2/3 barley malt (SLAVONIJA-SLAD d.o.o., Nova Gradiška, Croatia), comprising 92.1% base malt, 3.9% Munich malt, and 3.9% caramel malt. The bread and malt were milled (Matmill Klassik Basis, Kirkel, Germany) and mixed with softened water (6 °dH) in a mashing system (Brew Monk, Brouwland, Beverlo, Belgium). The mashing process included five temperature rests: 47 °C for 10 min, 52 °C for 10 min, 65 °C for 50 min, 72 °C for 15 min, and 78 °C for 5 min. BSG generated during mashing was collected, frozen, and later used in this study. The moisture content of BSG was determined to be 32.4 ± 0.3%, measured by drying at 105 °C to constant weight according to AOAC Official Method 920 [26].

2.2. Microorganism and Culture Media

Saccharomyces cerevisiae was obtained from the Kaunas University of Technology (Kaunas, Lithuania) collection and was grown on yeast extract–peptone–dextrose (YPD) medium (Burlington, MA, USA). Before the analysis, yeast cells were collected from slants and transferred into 10 mL YPD medium and incubated for 24 h at 30 °C.

2.3. Enzymatic Hydrolysis and Fermentation of BSG

BSG was enzymatically hydrolyzed using commercial enzyme preparations from Novozymes A/S, Bagsværd, Denmark): Celluclast® 1.5L (cellulase) and Viscozyme® W FG (multi-enzyme complex containing β-glucanase, hemicellulase, and xylanase). BSG with an initial pH value of 6.05 ± 0.06 was mixed with distilled water at a ratio of 1:1.4 (*w*/*v*) and either Celluclast® 1.5 L (0.035 mL/g BSG) or Viscozyme® W FG (0.07 mL/g BSG). The enzymatic hydrolysis was conducted in a water bath at 50 °C with shaking (200 rpm) for 2 h and 6 h [27,28]. The samples with the highest reducing sugar content were selected for fermentation with *S. cerevisiae*, and as control for fermentation non-hydrolyzed BSG was used. In total, 1% of *S. cerevisiae* inoculum was introduced into the enzymatically hydrolyzed or non-hydrolyzed BSG and fermented 24 h at 30 °C under aerobic conditions.

2.4. Microbiological Analyses

Yeast count in BSG was performed by serial dilutions using a standard plate count technique according to ISO 21527-2:2008 [29]. After incubation at 30 °C for 24 h on YPD agar, colony-forming units (CFU) were counted and expressed as and $\log_{10}$ CFU/g.

2.5. pH Value and Total Titratable Acidity Analysis

The pH value and total titratable acidity (TTA) of BSG were assessed using a 10 g sample, which was homogenized in 90 mL of distilled water. The pH measurement was carried out directly with a pH meter (WinLab® Excellent Line, Clausthal-Zellerfeld, Germany). The TTA was determined based on the amount of 1 M NaOH solution needed to reach a pH of 8.5.

2.6. Determination of Reduced Sugar Content

The reducing sugar (RS) content was measured using the 3,5-dinitrosalicylic acid (DNS) assay according to Miller [30], with slight modifications. Briefly, 1 g of BSG was mixed with 100 mL of distilled water and left to stir for 10 min followed by centrifugation at 5000 rpm (Microcen 23, Ortoalresa, Madrid, Spain) for 15 min at room temperature. A total of 1 mL aliquot of the obtained supernatant was mixed with 1 mL of DNS reagent and heated at 95 °C for 5 min. The reaction mixture was then cooled and diluted with 6 mL of

distilled water. Absorbance was measured at 540 nm using a spectrophotometer (Genesys 10, Thermo Electron LED GmbH, Langenselbold, Germany). RS content was calculated based on a standard curve, which was generated using glucose solutions (1 mg/mL) diluted in distilled water to obtain final concentrations ranging from 0 to 1 mg/mL.

2.7. Statistical Analysis

All experiments were performed in triplicate, and the results were averaged and represented as mean ± standard deviation. The experimental results were processed using analysis of variance (two-way ANOVA was applied for evaluating hydrolysis success, and one-way ANOVA followed by Duncan's multiple range test was used for fermentation indicators). Statistical analysis was carried out at a significance level of $\alpha = 0.05$ using Statistica™ 14.0.0 software (TIBCO Software Inc., Palo Alto, CA, USA). For the graphical representation of the results, Box and Whisker plots (hydrolysis indicators) and column charts (fermentation indicators) were prepared.

3. Results

According to the aim of this research, two sets of experiments were conducted. First, the success of BSG hydrolysis with commercial enzyme preparations (Celluclast® 1.5 L and Viscozyme® W FG) was examined for different treatment times (2 h and 6 h). Further, fermentation of non-hydrolyzed and enzymatically hydrolyzed BSG (under previously selected conditions) was performed by *S. cerevisiae* to determine its fermentative potential. The results of both sets of experiments were statistically processed using appropriate tests, with a 95% confidence interval, and the obtained data were visualized for easier analysis and comparison.

3.1. BSG Enzymatic Hydrolysis

The performance of hydrolysis of BSG, composed of old wheat bread and barley malt, was assessed based on the RS content in the hydrolysates obtained under the examined conditions. A sample without added enzyme (blank) was also tested as a control. The results of the hydrolysis experiments are given in Table 1.

Table 1. Reducing sugars content in BSG hydrolysates (mean ± standard deviation).

Enzyme	Hydrolysis Time (h)	Reducing Sugars Content (g/kg BSG)
Blank	2	23.40 ± 0.45
Blank	6	30.21 ± 1.36
Celluclast® 1.5 L	2	22.03 ± 2.19
Celluclast® 1.5 L	6	35.85 ± 0.26
Viscozyme® W FG	2	24.92 ± 0.67
Viscozyme® W FG	6	36.31 ± 1.49

The results summarized in Table 1 indicate that the success of BSG hydrolysis depends on both varied parameters. Therefore, the next step of the research involved statistical analysis of the experimental results to determine the significance of the influence of the examined factors on the selected response and to select the combination of enzyme and hydrolysis time for which the highest RS content in BSG hydrolysates was achieved. The results of the two-way ANOVA analysis for the effect of different enzymes and treatment time on the hydrolysis success indicator are provided in Table 2.

The two-way ANOVA summary results presented in Table 2 indicate that *p*-values for examined factors, i.e., used commercial enzyme preparations ($p = 0.000803$) and applied

treatment time ($p < 0.000001$), as well as their interaction ($p = 0.01416$) are much below 0.05, which is the critical value for a confidence interval of 95%. This means that examined enzymatic hydrolysis conditions have a statistically significant effect on the BSG hydrolysis success. In addition, the mean square values, given in the same table, indicate that the applied hydrolysis time has the greatest impact on the RS content in BSG hydrolysates. Statistical analysis also determined that the selection of enzyme and interaction of examined factors have an almost equally pronounced impact on the observed response.

Table 2. Two-way ANOVA results for the effect of different enzymes and treatment time on the performance of BSG hydrolysis.

Hydrolysis Success Indicator	Effect	SS	DF	MS	F-Ratio	*p*-Value
Reducing sugars content (g/kg BSG)	Enzyme	43.710	2	21.855	13.680	0.000803
	Hydrolysis time	512.459	1	512.459	320.764	<0.000001
	Enzyme and hydrolysis time	38.037	2	19.019	11.904	0.001416
	Error	19.171	12	1.598	-	-

SS—sum of squares; DF—degree of freedom; MS—mean square.

The results of the statistical analysis for the effect of used enzymes on the RS content in BSG hydrolysates are shown in Figure 1a. The graphically presented results indicate that the samples with the highest value of the analyzed parameter were obtained when Viscozyme® W FG was used to hydrolyze BSG, regardless of the applied treatment time. It is also evident that high values of RS content were detected in samples hydrolyzed with Celluclast® 1.5 L, while the RS content in blank samples may be the result of the action of enzymes in BSG that are activated during hydrolysis at the applied temperature.

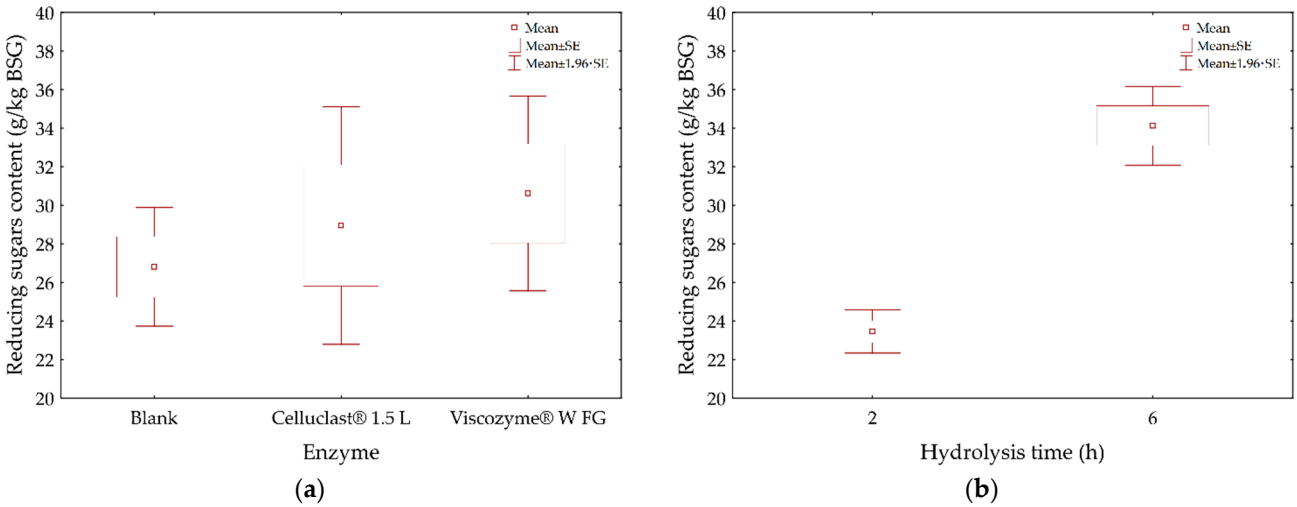

Figure 1. The effect of examined factors on reducing sugars content in BSG hydrolysates: (**a**) variation of commercial enzyme preparations; (**b**) variation of hydrolysis time.

A graphical representation of the statistical analysis of results for the effect of hydrolysis time on the RS content in BSG hydrolysates is given in Figure 1b. It is obvious that, regardless of the applied commercial enzyme preparation, samples with significantly higher RS content were achieved after a longer duration of pretreatment.

Further, the determined values of RS content in BSG hydrolysates were analyzed using Duncan's multiple range test to select the combination of commercial enzyme preparation and treatment time for which the most significant value of observed indicator was achieved.

It was found that four homogeneous groups of experimental results, differing in significance levels, have been formed, while the values with the highest statistical significance were determined in samples hydrolyzed for 6 h using both Celluclast® 1.5 L (35.85 ± 0.26 g/kg BSG) and Viscozyme® W FG (36.31 ± 1.49 g/kg BSG). The post hoc testing confirmed that there is no statistically significant difference between the RS content in BSG hydrolysates obtained using both enzymes for longer hydrolysis time (p = 0.667393).

3.2. BSG Fermentation

The success of fermentation of non-hydrolyzed and enzymatically hydrolyzed BSG by *S. cerevisiae* was estimated based on the number of yeast cells, pH value, RS content, and TTA, all determined in the media after 24 h of fermentation under the experimentally applied conditions. To evaluate yeast growth, the number of cells was also counted in both media immediately after inoculation, followed by the determination of other indicators to assess the contribution of fermentation to the change in their values. The results of the one-way ANOVA analysis for the effect of different BSG preparation procedures on the initial composition of the examined media and indicators of fermentation success are given in Tables 3 and 4, respectively.

Table 3. One-way ANOVA results for the effect of differently prepared BSG on the initial composition of the examined media.

Fermentation Success Indicator	Effect	SS	DF	MS	F-Ratio	*p*-Value
pH value (1)	BSG preparation	0.056	1	0.056	34.327	0.004236
	Error	0.007	4	0.002	-	-
Reducing sugars content (g/kg BSG)	BSG preparation	46.351	1	46.351	223.314	0.000117
	Error	0.30	4	0.208	-	-
Total titratable acidity (mL of 1 M NaOH)	BSG preparation	0.042	1	0.042	10.000	0.034109
	Error	0.017	4	0.004	-	-

SS—sum of squares; DF—degree of freedom; MS—mean square.

Table 4. One-way ANOVA results for the effect of differently prepared BSG on the success of fermentation by *S. cerevisiae*.

Fermentation Success Indicator	Effect	SS	DF	MS	F-Ratio	*p*-Value
Yeast cell number ($\log_{10}$ CFU/g)	BSG preparation	0.013	1	0.013	3.940	0.118142
	Error	0.013	4	0.003	-	-
pH value (1)	BSG preparation	0.132	1	0.132	226.314	0.000114
	Error	0.002	4	0.001	-	-
Reducing sugars content (g/kg BSG)	BSG preparation	15.779	1	15.779	5601.947	<0.000001
	Error	0.011	4	0.003	-	-
Total titratable acidity (mL of 1 M NaOH)	BSG preparation	0.427	1	0.427	9.143	0.039021
	Error	0.187	4	0.047	-	-

SS—sum of squares; DF—degree of freedom; MS—mean square.

The one-way ANOVA summary results presented in Tables 3 and 4 indicate that the p-values for pH value (p = 0.004236 for initial media and p = 0.000114 for fermented media), RS content (p = 0.000117 for initial media and p < 0.000001 for fermented media), and TTA (p = 0.034109 for initial media and p = 0.039021 for fermented media) are all below the critical value of 0.05. That means that utilized substrates have a statistically significant effect

on mentioned indicators of fermentation success. However, this does not apply to the yeast cell number determined in media after fermentation ($p = 0.118142$), since the p-value was greater than 0.05 (Table 4). This indicates that the examined BSG preparation procedures do not affect the growth of *S. cerevisiae* under the applied experimental conditions.

The results of the yeast cell number in non-hydrolyzed and enzymatically hydrolyzed BSG, determined after inoculation and fermentation, are shown in Figure 2. The graphically represented results show that media initially contained an almost identical number of *S. cerevisiae* cells (5.86 ± 0.01 $\log_{10}$ CFU/g for non-hydrolyzed BSG and 5.82 ± 0.07 $\log_{10}$ CFU/g for hydrolyzed BSG). In addition, the value of this parameter determined after fermentation also did not differ among the examined media (8.00 ± 0.03 $\log_{10}$ CFU/g for non-hydrolyzed BSG and 7.91 ± 0.08 $\log_{10}$ CFU/g for hydrolyzed BSG).

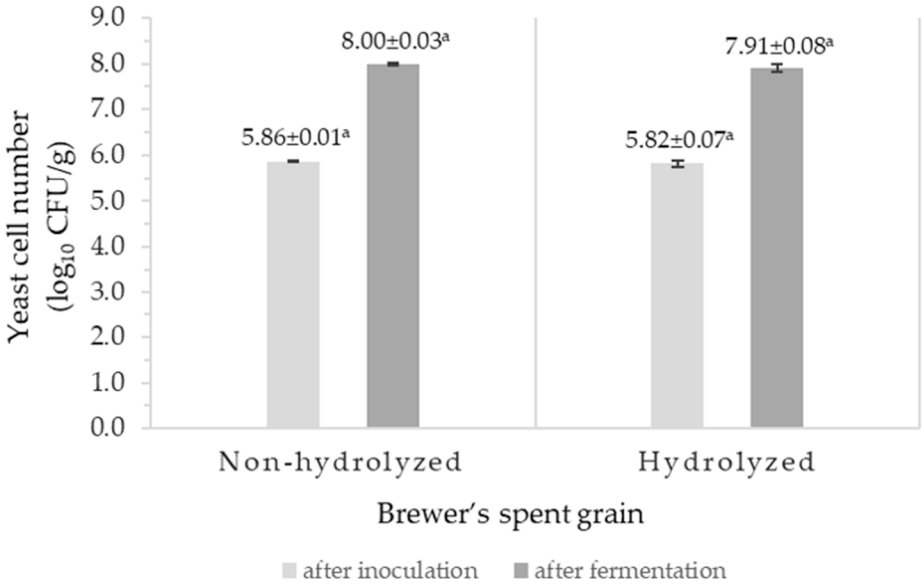

Figure 2. *S. cerevisiae* cell number in non-hydrolyzed and hydrolyzed BSG after inoculation and fermentation. Bars marked with the same letter for the same group of results are not significantly different at $\alpha = 0.05$ (according to Duncan's multiple range test).

A graphical representation of the pH values of non-hydrolyzed and enzymatically hydrolyzed BSG, measured after inoculation and fermentation by *S. cerevisiae*, is given in Figure 3. It is evident that the media at the beginning of the process were slightly acidic (initial pH value for non-hydrolyzed BSG was 6.05 ± 0.06 and 5.85 ± 0.01 for hydrolyzed BSG), while at the end of fermentation the acidity was more pronounced (pH value for non-hydrolyzed BSG was 5.04 ± 0.03 and 4.75 ± 0.02 for hydrolyzed BSG). In addition, post hoc testing determined that the hydrolyzed media had statistically significantly lower pH values both after inoculation ($p = 0.000364$) and after fermentation ($p = 0.000361$).

The results shown in Figure 4 represent the values of RS content in non-hydrolyzed and enzymatically hydrolyzed BSG, determined after inoculation and fermentation by *S. cerevisiae*. According to the graphically presented results, it is obvious that the hydrolyzed media initially contained more RS (18.63 ± 0.49 g/kg BSG) compared to non-hydrolyzed (13.07 ± 0.41 g/kg BSG) and that the same was determined at the end of fermentation (residual RS content in non-hydrolyzed BSG was 4.58 ± 0.04 g/kg BSG and 7.82 ± 0.06 g/kg BSG in hydrolyzed BSG). Also, a post hoc test confirmed a statistically significant difference between the RS content in initial and fermented media ($p = 0.000364$ for RS content in media after inoculation and $p = 0.000291$ for RS content in media after fermentation). Further analysis of the results showed that the *S. cerevisiae* metabolized about 8.50 g/kg BSG of sugar components from the non-

hydrolyzed BSG-based medium, resulting in a sugar conversion of about 65%. On the other hand, the consumed sugar in the hydrolyzed BSG-based medium was about 10.80 g/kg BSG, indicating that about 58% of the sugar components were converted during fermentation.

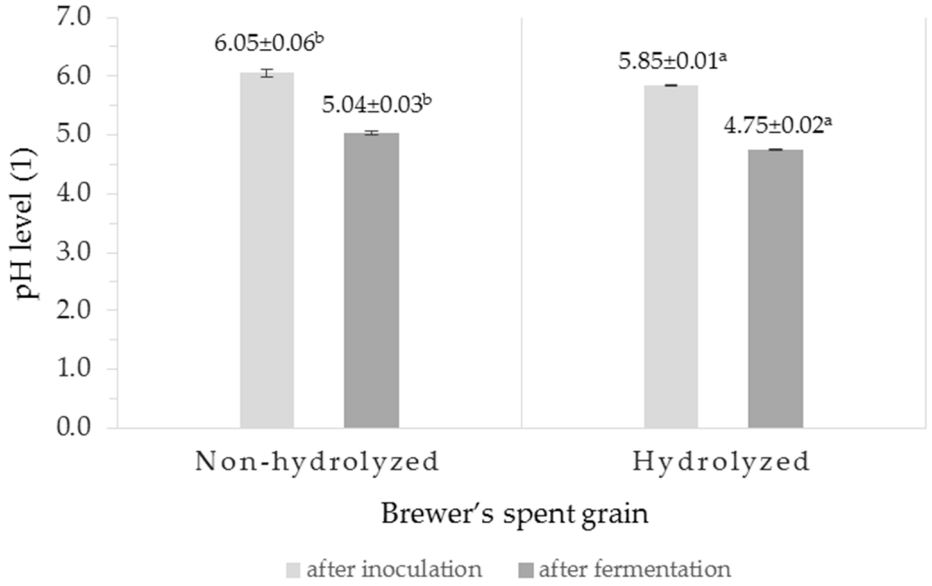

Figure 3. pH value of non-hydrolyzed and hydrolyzed BSG after inoculation and fermentation by *S. cerevisiae*. Bars marked with a different letter for the same group of results are significantly different at $\alpha = 0.05$ (according to Duncan's multiple range test).

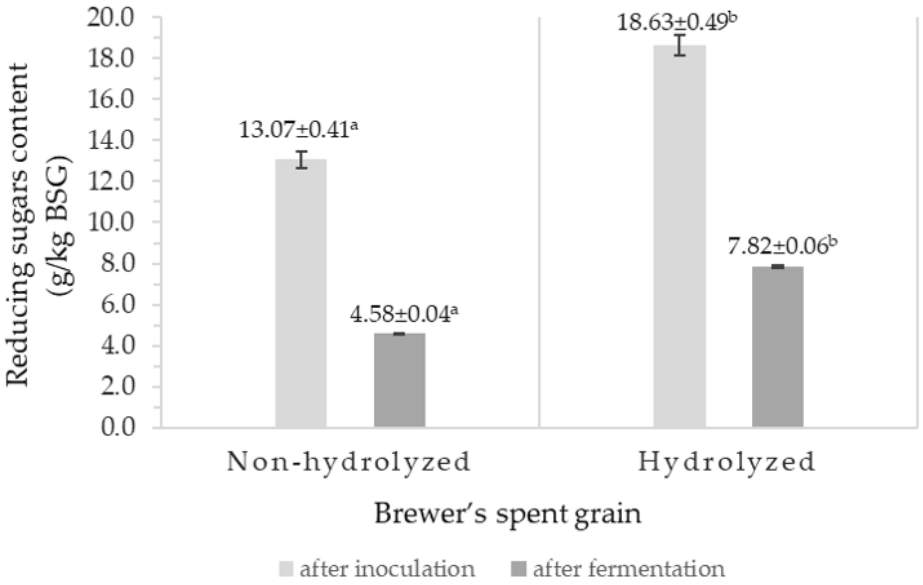

Figure 4. Reducing sugars content in non-hydrolyzed and hydrolyzed BSG after inoculation and fermentation by *S. cerevisiae*. Bars marked with a different letter for the same group of results are significantly different at $\alpha = 0.05$ (according to Duncan's multiple range test).

A graphical interpretation of the TTA values of non-hydrolyzed and enzymatically hydrolyzed BSG, after inoculation and fermentation by *S. cerevisiae*, is shown in Figure 5. Analysis of the experimental results revealed that examined BSG-based media initially contained similar (2.68 ± 0.08 mL of 1 M NaOH for non-hydrolyzed BSG and 2.85 ± 0.05 mL of 1 M NaOH for hydrolyzed BSG) but statistically significantly different TTA ($p = 0.034288$). It is also evident that the metabolic activity of the examined

yeast resulted in an increase in the acidity of both media (3.87 ± 0.23 mL of 1 M NaOH for non-hydrolyzed BSG and 4.40 ± 0.20 mL of 1 M NaOH for hydrolyzed BSG), which was statistically significantly higher in the medium based on the hydrolyzed substrate (p = 0.039190).

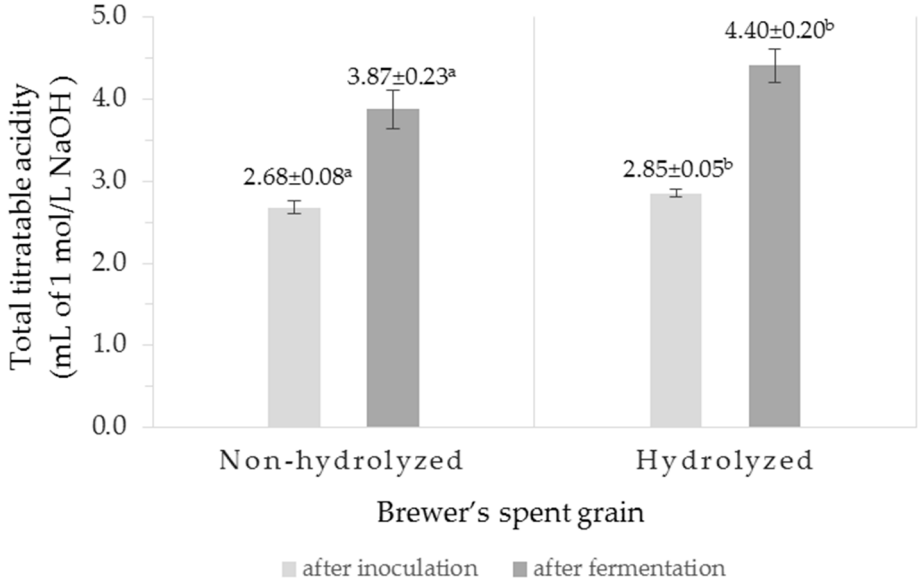

Figure 5. Total titratable acidity of non-hydrolyzed and hydrolyzed BSG after inoculation and fermentation by *S. cerevisiae*. Bars marked with a different letter for the same group of results are significantly different at α = 0.05 (according to Duncan's multiple range test).

4. Discussion

In this section, i.e., in the following subsections, a discussion of the above results is provided. However, it is very important to consider the costs of the fermentation medium preparation because they significantly affect the overall production costs. From an economic standpoint, the use of non-hydrolyzed BSG may be more efficient, particularly in small-scale or resource-constrained settings, as it eliminates the need for additional enzyme input and associated processing costs. Although hydrolysis improves fermentation metrics such as titratable acidity and sugar availability, key for industrial applications focused on yield and consistency, the financial cost of enzymatic pretreatment may not always be justified. Therefore, the choice between hydrolyzed and non-hydrolyzed BSG should consider the specific goals of the production process, balancing cost-efficiency with performance. This dual approach highlights the potential of BSG valorization under different economic and technological conditions.

4.1. Success of BSG Enzymatic Hydrolysis

BSG valorization to obtain value-added ingredients or their utilization as substrates for microorganism cultivation has recently attracted considerable scientific interest [31]. To enhance the functional and technological properties of BSG, additional processing strategies are often employed, with enzymatic treatments and fermentation technologies being the most prominent approaches. BSG is predominantly composed of lignocellulosic material, including cellulose, non-cellulosic polysaccharides such as arabinoxylans, and lignin [32]. Due to the complex lignocellulosic structure of BSG, disrupting the polymer matrix to release simple sugars is essential for its effective utilization. Pretreatment techniques are therefore applied to induce structural modifications that facilitate the depolymerization of polysaccharides into monomeric sugars, thereby improving the efficiency of subsequent enzymatic hydrolysis [33]. In this context, enzymatic hydrolysis was performed using com-

mercial carbohydrase preparations, namely Celluclast® 1.5 L (cellulase) and Viscozyme® W FG (a multi-enzyme complex containing β-glucanase, hemicellulase, and xylanase) at different treatment times. The enzymatic hydrolysis process enables the cleavage of long-chain biopolymers into smaller molecular units, enhancing the solubilization and accessibility of BSG components [34]. In the present study, the highest concentration of RS was obtained when BSG was hydrolyzed using Viscozyme® W FG for 6 h (Table 1, Figure 1). However, hydrolysis using Celluclast® 1.5 L under the same conditions did not yield a statistically significantly lower RS. This indicates that the application of commercial enzyme blends proved to be particularly effective for the hydrolysis of BSG, confirming trends reported in previous studies. Wagner et al. [33] demonstrated that enzymatic pretreatment of BSG with enzyme cocktails significantly increased sugar yields. Similarly, Leijdekkers et al. [35] reported a 50% yield of soluble oligo- and polysaccharides from sugar beet pulp following enzymatic saccharification with Viscozyme® L at 45 °C over 12 h. Comparable increases in saccharide concentrations after enzymatic treatment with Viscozyme® L were also observed in studies with berry pomaces [36]. In addition, Plaza et al. [37] found that hydrolysis of BSG using Celluclast® 1.5 L under optimized conditions resulted in significant monosaccharide release, with 47.0 g/L glucose and 16.8 g/L xylose obtained. Similar enzymatic hydrolysis trends were reported by Bakari et al. [38], who showed that Viscozyme® L was more effective than Celluclast® 1.5 L in promoting saccharification of sweet sorghum stalks. Although BSG hydrolysis using Viscozyme® W FG and Celluclast® 1.5 L proved to be similarly efficient at the applied temperature, BSG enzymatically hydrolyzed with Viscozyme® W FG for 6 h was selected for *S. cerevisae* fermentation because this commercial enzyme has more advantages compared to Celluclast® 1.5 L as declared by the manufacturer (higher capacity and gluten recovery, improved starch purity, lower energy consumption and starch-in-fiber loss).

4.2. Number of Yeast Cells in Fermented Media

The number of *S. cerevisiae* cells was counted in both enzymatically hydrolyzed and non-hydrolyzed BSG, immediately after inoculation and after 24 h of fermentation (Figure 2). Initial yeast cell numbers were uniform in both substrates. This similarity indicates that the inoculation was consistent across conditions and that differences in fermentation success indicators are not due to uneven inoculation.

After 24 h of fermentation, an intensive increase in cell numbers was observed in both media, indicating that BSG, whether hydrolyzed or not, supports the proliferation of *S. cerevisiae* and that the BSG preparation procedure has no effect on yeast growth during fermentation under the applied experimental conditions. Interestingly, the final yeast cell number was also uniform in both media, suggesting that enzymatic pretreatment of the substrate did not markedly enhance yeast proliferation within this timeframe. This outcome may be attributed to the relatively simple nutrient requirements of *S. cerevisiae*, which is capable of utilizing a wide range of carbon sources [39]. The enzymatic pathways involved in the utilization of these carbon sources are well understood, with many enzymes being synthesized only when their corresponding substrates are available [40].

4.3. pH Value of Fermented Media

The pH values of the media, measured after inoculation and 24 h of fermentation by *S. cerevisiae*, indicate a notable difference depending on the substrate utilized (Figure 3). It is obvious that media with an initial lower pH value are obtained by enzymatic hydrolysis of BSG. Further, when the examined yeast strain was cultivated in this medium, the measured final pH was lower compared to the fermentation of non-hydrolyzed BSG. While both substrates support yeast growth, as confirmed by cell count results (Figure 2),

the more pronounced acidification in the hydrolyzed sample reflects the influence of initial substrate composition on fermentation dynamics. This difference suggests that hydrolysis, in addition to lowering the pH, may release more fermentable sugars and readily available nutrients, thereby enhancing yeast metabolic activity and leading to the production of organic acids or other acidic metabolites, contributing to the lower value of this parameter [41]. In contrast, the more complex structural matrix of non-hydrolyzed BSG likely limits substrate accessibility, resulting in reduced metabolic activity and consequently lower acid production. However, this also highlights a common challenge in utilizing BSG as a fermentation substrate: its recalcitrant composition often necessitates advanced pretreatment methods to improve nutrient availability and fermentation efficiency [42].

4.4. Reducing Sugars Content in Fermented Media

The RS content in the media, determined after inoculation and fermentation by *S. cerevisiae*, varied significantly depending on the substrate used (Figure 4). Fermentation of enzymatically hydrolyzed BSG resulted in a notably higher RS content compared to the fermentation of non-hydrolyzed BSG, as detected at the beginning of the process. This difference can be directly attributed to the enzymatic pretreatment, which breaks down complex carbohydrates into simpler sugars such as glucose and maltose, thereby increasing the initial availability of fermentable substrates. The key role of nutrient availability, particularly sugar concentration, in fermentation performance and the formation of volatile compounds has been well documented in pure cultures of *S. cerevisiae*. The initial sugar content not only influences yeast growth but also determines the final ethanol yield and fermentation duration [43,44]. It is also important to note that a higher residual RS content does not indicate poor fermentation performance, as sugar consumption in the hydrolyzed BSG-based medium was 20% higher. On the other hand, the observed difference in sugar conversion can be explained by the higher initial RS content in the hydrolyzed medium. Although enzymatic hydrolysis increased the total amount of fermentable sugars, this may have led to partial inhibition or metabolic saturation in *S. cerevisiae*, thereby reducing the relative conversion efficiency. These results indicate that while enzymatic pretreatment enhances RS availability, it does not necessarily lead to proportionally improved sugar conversion. This underscores the importance of considering both the quantity and composition of fermentable sugars when optimizing fermentation of BSG-based media.

4.5. Total Titratable Acidity of Fermented Media

The TTA values, determined in the media after inoculation and fermentation by *S. cerevisiae*, indicate an observable difference between both the examined substrates (Figure 5). Enzymatically hydrolyzed BSG had slightly higher TTA than the non-hydrolyzed BSG, with this difference being more pronounced at the end of the process. These results align with the findings of Lalić et al. (2025), who reported higher TTA values for hydrolyzed BSG compared to non-hydrolyzed BSG, suggesting similar trends in acid production across different studies [9].

5. Conclusions

The results demonstrate that *Saccharomyces cerevisiae* effectively ferments both hydrolyzed and non-hydrolyzed BSG-based media, confirming its adaptability to these substrates. The comparable yeast growth and decrease in pH value under both conditions suggest that non-hydrolyzed BSG could serve as a cost-effective and more sustainable substrate—particularly attractive for small-scale or resource-limited operations where enzymatic pretreatment may not be feasible.

The observed reduction in reducing sugar content during fermentation indicates effective utilization of fermentable sugars by *S. cerevisiae*. The blank sample exhibited approximately an 85% reduction, while the hydrolyzed sample showed around a 78.5% reduction. The slightly lower percentage decrease in the hydrolyzed sample can be attributed to its higher initial sugar concentration, resulting in a larger residual sugar amount after fermentation. These results suggest that, although the proportional reduction was lower, sugar metabolism was active in both cases.

Notably, the higher reducing sugar consumption in absolute terms and enhanced titratable acidity in the hydrolyzed BSG indicate that enzymatic pretreatment significantly improves fermentation efficiency. This is particularly relevant for industrial applications prioritizing faster fermentation rates, higher metabolite production, or consistent quality control. The scalability of such processes will depend on balancing the operational cost of hydrolysis with the gains in productivity and product quality.

These findings highlight a practical trade-off: while hydrolysis enhances efficiency, non-hydrolyzed BSG offers simplicity and lower processing costs. Future work should explore process optimization, including enzyme dosing and fermentation duration, to assess feasibility for larger-scale bioprocessing and biorefinery integration.

It is important to note that this study represents an initial step in examining *S. cerevisiae* as a biocatalyst for the fermentation of BSG obtained from mixed origins, such as old bread and barley malt—an area that has previously been underexplored. Future research will expand this work by comparing *S. cerevisiae* with non-conventional yeast strains and investigating co-cultures with lactic acid bacteria, with a focus on detailed metabolite profiling, aroma development, and nutritional enhancement

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Abbreviations

The following abbreviations are used in this manuscript:

BSG	Brewer's Spent Grain
RS	Reducing Sugar
TTA	Total Titratable Acidity

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